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Protein expression profiling associated to biotic stress in maize.

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Protein expression profiling associated to biotic stress in maize

Plant biotic stresses are caused by pathogens, parasites, predators and their outcome results from interaction between, host, pathogen and environment. Pathogens attack could be due by several organisms as fungi, bacteria, viruses oomycetes, nematodes and insects that cause specific and systemic response. Two of the major pests affecting maize crop in Lombardy region are *Fusarium verticillioides* fungus and the *Diabrotica virgifera virgifera* LeConte insect. The etiology of two diseases is strictly interdependent, plant fungus infection is often deriving after kernel injuries by wounding or insect attack.

F. veritillioides infection typically occurs on kernels, with a high level of fungal infection and micotoxin contamination. In resistant inbred maize genotype, kernel showed significant decrease of infection incidence, with limited amounts of total fumonisin content and reduced fungal growth.

We attempt to identify protein involved in *Fusarium* resistance by protein profile resistant and susceptible lines. We found only two protein peaks at 5.79 and 14.96 Kda that were commonly expressed in the susceptible lines but no specific protein of interest were present in kernel from resistance lines. Only the susceptible line CO354, showed variation in the Late Embryogenesis Abundant LEA3 protein, previously described as protein associated to fungal resistance in maize.

D. virgifera attack is prevalentely due by direct larvae infestation and damage to the maize root system. Plants respond to insect infestation by emitting volatile compounds among which the sequiterpene (E)-β-caryophillene that attracts natural enthomopathogenic nematodes. This volatile compound has been found in response to herbivore damage in several wild relatives of maize and in cultivated maize lines from European breeding programs but not in most lines from the North American breeding program. In order to understand mechanisms involved in defence response, maize lines that differ in (E)-β-caryophillene synthase expression were characterized by protein profiling and *tps23* gene expression. We found that maize responds to methyl jasmonate treatment and *D. virgifera* infestation similarly by inducing the *tps23* gene, but the jasmonate mediated response differs quantitative and temporary in different maize lines. Methyl jasmonate treatment induce also the expression of other three proteins i.e. actin-depolymerizing factor 3 ADF3, nucleotide pyrophosphatase/phosphodiesterase

NPP and anionic peroxidase probably involved in maize herbivore insect response.

Proteomic characterization of maize lines differing in response to pathogen attack may be a useful approach to better understand mechanisms involved in plant pathogen response and to find new markers associated to biotic stress response. The potential uses of these biomarkers in assisted breeding program however remain still under investigation.

Variazione del profilo proteico associato alla risposta a stress biotici in Mais.

Per stress biotico s'intende l'effetto generato sulle piante dall'attacco da parte di un organismo vivente, sia esso fungo, batterio, virus, nematode, insetto o animale erbivoro. Il risultato di tale effetto dipende in maniera significativa dai meccanismi d'interazione che intercorrono tra l'ospite, il patogeno e da svariate componenti ambientali. In Lombardia il mais è particolarmente soggetto all'attacco da parte del fungo *Fusarium verticillioides* e dell'insetto *Diabrotica virgifera virgifera* LeConte. In campo il ciclo d'infezione del fungo può essere influenzato da un'interazione tra i due organismi: la forma adulta dell'insetto può causare danni alla granella aumentando così la contaminazione della stessa da spore fungine.

F. veritillioides si sviluppa prevalentemente a livello della granella che mostra una comparsa di muffe e una contaminazione in micotossine. Nelle varietà resistenti si ha invece un minor livello d'infezione con una bassa presenza di fumonisine.

Varietà di mais aventi diversa suscettibilità all'infezione fungina sono state caratterizzate attraverso l'acquisizione di profili proteici allo scopo di trovare proteine potenzialmente associabili alla resistenza verso *Fusarium.* Dall'analisi della granella in varietà di mais che mostravano una diversa resistenza al fungo sono stati ottenuti solo due picchi proteici a 5.79 e 14.96 Kda esclusivi per le varietà suscettibili, mentre le linee resistenti non hanno mostrato l'espressione di picchi d'interesse. Nella linea suscettibile CO354 è stato in ogni modo possibile identificare una variazione specifica nella proteina Late Embryogenesis Abundant LEA3, proteina già in precedenza associata alla resistenza ad infezione fungina in mais.

D.virgifera allo stadio larvale attacca il sistema radicale del mais. La pianta risponde con l'emissione di diversi composti volatili tra cui un sequiterpene (E)-β-caryophillene, che ha la funzione di attrarre dei nematodi dannosi per le larve stesse. E' stato inoltre osservato che questo composto viene emesso in risposta all'attacco da parte di insetti solo in alcune linee di mais ancestrali e in linee ottenute da programmi di selezione Europei; mentre la maggior parte delle linee Nord Americane sembrano avere perso questo meccanismo di difesa.

Linee di mais aventi una diversa espressione in (E)-β-caryophyllene sintetasi sono state caratterizzate sia attraverso una profilazione proteica che analizzando l'espressione del gene *tps23*, valutando così i possibili meccanismi coinvolti nella risposta verso il patogeno. Abbiamo riscontrato che il mais, sia dopo infestazione da larve di *D. virgifera* che dopo trattamento con metil jasmonato, risponde inducendo l'espressione del gene *tps23*. Il tipo di risposta mediata dall'attivazione del pathways del Jasmonato comunque è risultata diversa a livello quantitativo e temporaneo nelle diverse linee analizzate. Il trattamento con il metil jasmonato induce l'espressione anche di altre tre proteine: fattore depolimerizzante l'actina 3 ADF3, nucleotide pirofosfatase/fosfodiesterase NPP e una perossidasi anionica probabilmente implicate nella risposta a danni da insetti erbivori in mais.

La valutazione delle variazioni dei profili proteici espressi in linee di mais che mostrano una diversa risposta verso stress biotici, è stata utile per indagare alcuni aspetti inerenti all'attivazione dei meccanismi di difesa della pianta e ci ha permeso di ottenere dei marcatori associati allo stress biotico. Il loro potenziale utilizzo in programmi di selezione assistita in campo rimane comunque ancora da essere pienamente valutato.

Preamble

I started my Ph.D. project, working exclusively on maize *Fusarium* resistance, by protein expression profiling between *F.verticillioides* resistant and susceptible corn lines. However after the first year of experiments and the approval of MDF (Maize *Diabrotica Fusarium)* project founded by Regione Lombardia, focused on the development of new inbreed lines with enhance resistance to two of the major pest diseases *Fusarium* and *Diabrotica virgifera*, I expand my Ph.D. work also on maize *D. virgifera* resistance.

This was very fruitfully because during my first year I develop all the protocols that were then used to develope both tasks of the project. Then a similar approach consisting of protein proling by SELDI-ToF and RP-HPLC fractionation coupled to SDS-PAGE detection was employ for the analyses of proteins from kernel and root to study *F. verticillioides* and *D. virgifera* resistance respectively.

This approach was useful to find in both cases different proteins possibly related to the diseases and also understand some mechanisms related to maize pathogen response.

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Introduction

1 Fusarium verticillioides maize infection

1.1 Etiology and diversity of *Fusarium* species

Fungal species of the genus *Fusarium* are of major importance worldwide because of their ability to infected plants and contaminate foods with toxins. After first description of this genus by Link in 1809, an association of mycotoxicosis with the symptoms of Fusarium head blight (FHB) on wheat (*Triticum aestivum* L.) was reported from Russia in 1923 (Donunin 1926). Later, it became clear that FHB is not caused by a single species but by a species complex. In Northwestern Europe the main causative agents of fusariosis described are *Fusarium graminearum, Fusarium culmorum*, *Fusarium avenaceum, Gibberella avenacea*, and *Fusarium poae* (Parry et al. 1995, Bottalico and Perrone 2002).

Fusarium taxonomy is very complicated due to the characteristic of the fungi. Many species, populations within species, in the genus *Fusarium* exhibits a high degree of variation with respect to morphological and physiological characteristics; thus gives the ability of *Fusarium* species to colonize diverse ecological niches, with effects often related to the plant species and geographic areas of infection (Nelson et al. 1994). *Gibberella moniliformis* (*Fusarium vertcillioides*) is the casual agent (facultative endophyte) of ear rot in most maize-growing areas of Southern Europe, but it can also cause disease in other crop species. The developmental stage on which the fungus produces sexual spores is called the teleomorph (or perfect stage), the other, asexual, stage is called the anamorph (or imperfect stage); *Gibberella moniliformis* anamorph is known as *Fusarium verticillioides*. In maize, the fungus can develop at two levels:

- in the saprophytic phase the fungus grows by using nutrients from soil; on this stage the fungus is spread "horizontally", and infection is introduced from outside, through corn silk or injured tissue.
- in the endophytic phase the fungus has already infected the plant tissue and grows in the biotrophic association. It can be transmitted "vertically", or, through seed stage; that remains the reservoir from which infection and toxin biosynthesis takes place in each generation of plants (Yates et al. 1997).

1.2 Maize infection and transmission

Fusarium may infect different crops: wheat, barley, oats, rye, corn and forage grasses, however, the most relevant affected species are wheat, barley and corn. Concerning maize infection, *Fusarium* species cause two distinct diseases, both of which can result in mycotoxin contamination of maize grain:

- Gibberella ear rot or 'red ear rot' is caused by a single specie of *Fusarium graminearum* (teleomorph *Gibberella zeae*) that develops as a red or pink mold covering a large proportion of the ear.
- Fusarium ear rot is the most common fungal disease on corn ears and it typical due to infection by several species *F. verticillioides* (syn. *F. moniliforme*), *F. proliferatum* or *F. subglutinans*. Historically, *F. moniliforme* has been reported as the most common pathogen causing Fusarium ear rot; currently, its synonym *F. verticillioides* is considered the predominant species. It typically occurs on kernels or on physically injured kernels and consists of a white or light pink mold.

Fungus dispersion and maize infection is strictly correlated with the kind of agronomic practices. Currently, maize is typically grown in a short rotation with little overall crop diversity. As a result, maize plant residues remain in or on the soil during the winter and are the primary source of inoculum for infections of maize kernels. *Fusarium* species survive periods between host crops as mycelium or by producing specific structure; in particular *F. graminearum* produces clanydospores whereas *F. verticilloides* produces thickened hyphae. After spreading *Fusarium* spores can directly colonize the kernel by different mechanism; in direct silks infection the fungus can directly reach the silks by splashing through wind dispersal spores, and plant are highly susceptible during the first six days after silk emergence or kernel can be directed infected in particular after injuries by wound or insects. Fungus can also systemically colonize the entire maize plant and in this way can be transmitted from seed to plant to kernel but current seed production practices reduced the incidence of seedborn *Fusarium* infection (Munkvold 2003). Fusarium ear rots is the most economically significant disease in Europe; despite the use of good agronomical practice and the fact that maize seeds planted are treated with fungicides , disease problem continues to develop affecting both yield and quality of grain crop.

Infection by *Fusarium* spp. result not only in yield reduction but also in contamination with mycotoxins.

1.3 Micotoxine Production

Mycotoxins are classified as secondary metabolites, since their biosynthesis is not required for fungal growth and reproduction. Mycotoxin produced by *Fusarium* species are related to three major classes trichothecenes, zearalenones and fumonisin.

Trichothecenes

The trichothecenes are a very large family of chemically related toxins produced by *Fusarium* infection. Their chemical structure is based on a tetracyclic sesquiterpene nucleus which is characterized by an oxane ring, a double bond in the 9,10 position, and a stable epoxide ring in the 12,13 position; this structure and in particular the epoxy group is related to the toxic properties of these mycotoxins (Gentili et al. 2007). Trichothecenes are classified into four groups depending on their functional groups, denoted A, B, C, and D (Fig. 1). The A and B categories are the most important and are prevalently found in cereals products.

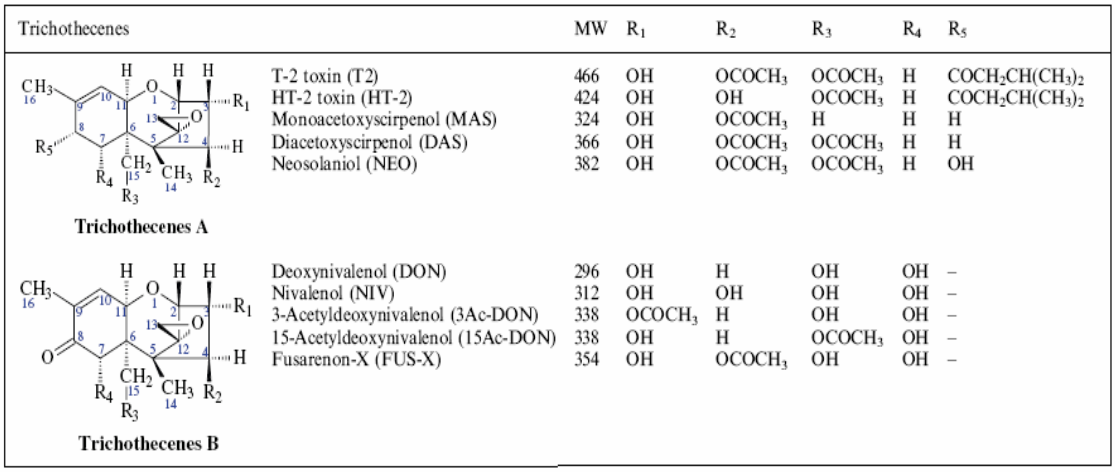
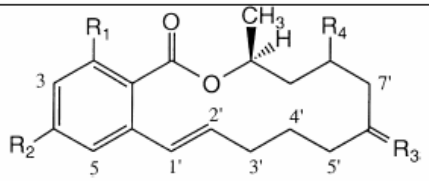


Figure 1. (from Gentili, 2007) General structure of Trichothecenes A, B.

Zearalenones

Zearalenones (ZEAs) are estrogenic mycotoxins that were firstly isolated from *G. zeae*. The structure of zearalenone consists of a resorcinol moiety fused to a 14-member macrocyclic lactone ring (Fig. 2). The structure is flexible and that allows to zearalenone to adopt a conformation able to bind to the mammalian estrogen receptor, even if with lower affinity than the natural estrogens (Shier et al. 2001).

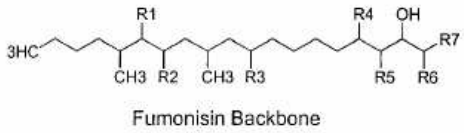


Sample	R_1	R_2	$1',2'$	R_3	R_4
α-Zearalenol	OH	OH	trans	H,α-OH	H
α-Zearalanol	OH	OH	dihydro	H,α-OH	H
2,4-O-dimethyl-8-hydroxyzearalenone	OCH_3	OCH_3	trans	O	OH
β-Zearalanol	OH	OH	dihydro	H,β-OH	H
6-amino-zearalenone	OH	OH	trans	H,NH_2	H
Zearalanone	OH	OH	dihydro	O	H
6′-acetyl-β-zearalanol	OH	OH	dihydro	H,β-OAc	H
Zearalenone	OH	OH	trans	O	H
2,4-dideoxy-zearalene	H	H	dihydro	H_2	H
cis-Zearalenone	OH	OH	cis	O	H
Zearalane	OH	OH	dihydro	H_2	H
4-O-methyl-zearalenone	OH	OCH_3	trans	O	H
β-Zearalenol	OH	OH	trans	H,β-OH	H
6′-acetyl-β-zearalenol	OH	OH	trans	H,β-Oac	H
2-O-methyl-zearalenone	OCH_3	OH	trans	O	H
2,4-O-dimethyl-zearalenone	OCH_3	OCH_3	trans	O	H
4-acetyl-zearalenone	OH	OAc	trans	O	H

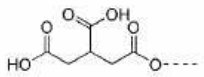
Figure 2. (From Shier 2001) General structure of Zearealenones

Fumonisins

Fumonisins (FBs) are mycotoxins produced mainly by *Fusarium verticillioides* and *F. proliferatum*. Fumonisins are structural similarities to sphingoid bases backbone and from a chemical viewpoint are polyhydroxylated alkyl amines, esterified on carbon 14 and 15 with two tricarballylic acid molecules (Fig. 3) (Rheeder et al. 2002).



Fumonisin Backbone



Tricarballylic Acid (TCA)

Analog	Side chains to fumonisin backbone[a]						
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
FBK_1	=O	TCA	OH	OH	H	NH_2	CH_3
FB_1	TCA	TCA	OH	OH	H	NH_2	CH_3
Iso-FB_1	TCA	TCA	OH	H	OH	NH_2	CH_3
$PHFB_{1a}$	TCA	OH	OH	OH	H	NH_2	CH_3
$PHFB_{1b}$	OH	TCA	OH	OH	H	NH_2	CH_3
HFB_1	OH	OH	OH	OH	H	NH_2	CH_3
FB_2	TCA	TCA	H	OH	H	NH_2	CH_3
FB_3	TCA	TCA	OH	H	H	NH_2	CH_3
FB_4	TCA	TCA	H	H	H	NH_2	CH_3
FB_5[c]							

Figure 3. (Adapted from Rheeder 2002). General structure of Fumonisin.

1.4 Toxicity and legislation

Crop mycotoxin contamination is strictly related to the type of infection and in particular to the *Fusarium* species that colonize the plant (Tab. 1).
Overall, the most frequently detected *Fusarium* toxin in cereal-based products in Europe was DON (57%), followed by fumonisins (47%), ZEA (32%), and T2-toxin (20%), (SCOOP 2003, Desjardins 2006).

***Fusarium* species**	**Mycotoxins[a]**
F. acuminatum	**T-2[b], HT-2, DAS, MAS**, MON, NEO
F. avenaceum	MON
F. chlamydosporum	MON
F. crookwellense	**NIV, FUS**, ZEA, ZOH
F. culmorum	**DON/NIV[c]**, ZEA, **FUS**, ZOH, **AcDON**
F. equiseti	**DAS**, ZEA, ZOH, **NIV, DAcDON, MAS, FUS**
F. graminearum	**DON/ NIV**, ZEA, **FUS, AcDON, DAcDON/ DAcNIV**
F. heterosporum	ZEA, ZOH
F. moniliforme	FB1
F. oxysporum	MON
F. poae	**DAS, MAS, NIV, FUS, T2, HT2**, NEO
F. proliferatum	FB1, BEA, MON, FUP
F. sambucinum	**DAS, T2**, NEO, ZEA, **MAS**
F. semitecum	ZEA
F. sporotrichoides	**T2, HT2**, NEO, **MAS, DAS**
F. subglutinans	BEA, MON, FUP
F. tricinctum	MON
F. venenatum	**DAS**

[a] AcDON: Monoacetyl-deoxynivalenol (3-AcDON, 15-AcDON); BEA: Beauvericin; DAcDON: Diacetyl-deoxynivalenol (3-AcDON, 15-AcDON); DAS: Diacetoxyscirpenol; DON: Deoxynivalenol; ENS: Enniatins; FUS: Fusarenone-X (4-Acetyl-NIV); FB1: Fumonisin B1; FUP: Fusarproliferin; HT2: HT2-toxin; MAS: Monoacetoxyscirpenol; MON: Moniliformin; NEO: Neosolaniol; NIV: Nivalenol; T2: T2-toxin; ZEA: Zearalenone; ZOH: zearalenols (α and β isomers).
[b] Trichothecenes are given in bold letters.
[c] Either DON or NIV is produced.

Table 1. (From Desjardins 2006) *Fusarium* species occurring in Europe and mycotoxins produced.

Several in vitro and in vivo (animals and humans) studies were conducted to evaluate potential toxic effect after feed/food mycotoxin contamination. Trichothecenes caused adverse effects in monogastric animals as nausea, vomiting, diarreha and immunosuppression. Trichothecenes effects were due by protein, DNA, and RNA inhibition synthesis, damage at cellular membranes, and apoptosis induction in lymphatic and haematopoietic tissues. Others in vitro (Cetin and Bullerman 2005) and vivo in pigs (Smith et al. 1997) studies reported adverse effects including health symptoms range from nausea and vomiting to retardation of growth, degeneration of immune, neural, and reproductive systems.
ZEAs have estrogenic effects in animal systems since they can bind to estrogen receptors (Minervini et al. 2001) Adverse effects of zearalenones were most commonly associated with the level of contamination of corn. In swine production, high levels of zearalenone caused constant estrus, pseudopregnancy, and infertility (Mirocha et al. 1971). At zearalenone concentrations much higher than commonly present in feeds, were also observed teratogenicity (Ruddick et al. 1976) and mutagenicity (Pfohl-Leszkowicz et al. 1995) effects. Fumonisins are known to be the cause of leukoencephalomalacia in equine, pulmonary edema in swine, and promote cancer in mice. Fumonisins additionally produced mild to fatal toxicity in liver, kidney and heart in horses, pigs, cattle. FBs cytotoxicity effects were observed in turkey and in broiler chicks lymphocytes and macrophages, and in rabbit kidney RK13 cells (Yazar and Omurtag 2008). In order to protect consumers from health risks associated with the intake of these mycotoxins, due to their adverse effect on eukaryotic, limits in the DONs, ZEAs and fumonisins contents tolerated in marketable cereals were laid down by national and European laws (Tab. 2),.

'2.4	**Deoxynivalenol** ([17])	ppb
2.4.1	Unprocessed cereals ([18]) ([19]) other than durum wheat, oats and maize	1 250
2.4.2	Unprocessed durum wheat and oats ([18]) ([19])	1 750
2.4.3	Unprocessed maize ([18]), with the exception of unprocessed maize intended to be processed by wet milling (*)	1 750 ([20])
2.4.4	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 2.4.7, 2.4.8 and 2.4.9	750
2.4.5	Pasta (dry) ([22])	750
2.4.6	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	500

2.5	**Zearalenone** ([17])	ppb
2.5.1	Unprocessed cereals ([18]) ([19]) other than maize	100
2.5.2	Unprocessed maize ([18]) with the exception of unprocessed maize intended to be processed by wet milling (*)	350 ([20])
2.5.3	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 2.5.6, 2.5.7, 2.5.8, 2.5.9 and 2.5.10	75
2.5.4	Refined maize oil	400 ([20])
2.5.5	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50

2.6	**Fumonisins**	ppb
2.6.1	Unprocessed maize ([18]), with the exception of unprocessed maize intended to be processed by wet milling (*)	4 000 ([23])
2.6.2	Maize intended for direct human consumption, maize-based foods for direct human consumption, with the exception of foodstuffs listed in 2.6.3 and 2.6.4	1 000 ([23])
2.6.3	Maize-based breakfast cereals and maize-based snacks	800 ([23])
2.6.4	Processed maize-based foods and baby foods for infants and young children ([3]) ([7])	200 ([23])

Table 2: Adapted from Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels (µg/ Kg; ppb) for certain contaminants in foodstuffs as regards micotoxins in maize and maize products

1.5 Plant-fungus-environmental Interaction and Resistance

The level of fungus contamination and micotoxin accumulation in maize results from different factor and their interaction referring to as “disease triangle” that combine the host susceptibility, the pathogen effect, and the conducive environment (Fig. 4).

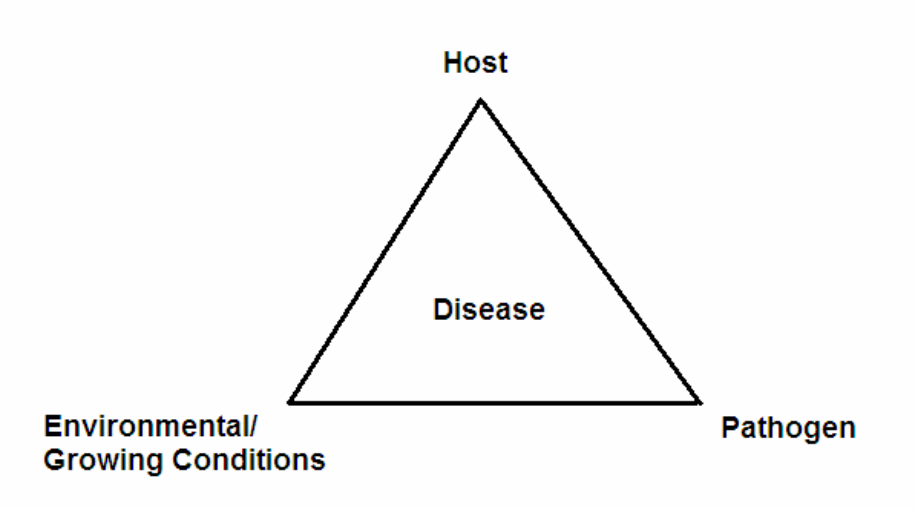


Figure 4. Disease triangle

The complexity plant/fungus interaction is also related to environmental condition that can influenced the transmission and level of infections and fumonisins contamination (Marin et al. 2010). In addition to maize genetic resistance and fungal strain toxicity, environmental conditions are relevant for fungus infection. Pedoclimatic conditions as relative humidity, air temperature, and rainfall could influence the dynamics of sporulation on maize residues and kernels. For each *Fusarium* species, spores production occurred between 5°C and 45°C. *F. verticillioides* grows well between 22,5 to 27.5°C and different fungus especially isolate from warm summer region can have the optimal growth temperature at 30°C. In general the sporulation linearly increase with temperature between 17 and 30 °C. The level of humidity can influence the sporulation where the number of moist days in the previous two weeks has positive effect on spores number. On the contrary, rainfall can also influence the level of contamination by reducing the number of spores probably by the dispersal and wash-off of conidia (Rossi et al.

2009). The fungus aggressiveness is another parameters that can affect the level of infection and crop contamination. In wheat, it is well known, that *Fusarium* isolate aggressiveness has a heritable component, which has an inheritance governed by several genes (Cumagun and Miedaner 2004). However first evidence of different effect of *F. verticillioides* isolate on maize infection were initially restricted to stalk infection in greenhouse (Jardine and Leslie 1999). Recently was investigated the aggressiveness and mycotoxin production of different isolates from *F. graminearum* and *F. verticillioides* on susceptible and resistance maize inbreed lines. However, differential effects both on kernel contamination and mycotoxins production were clearly appreciable when different isolate infected susceptible rather than resistance lines (Miedaner et al. 2010). In addiction *F. verticilloides* isolate could exhibit considerable heterogeneity in fumosins level during in field kernel contamination (Facao et al. 2011). The genetic diversity among elite maize inbreeds resulted the most important components to diminish harvest production of maize losses due to *Fusarium* disease and to diminish micotoxins contamination. Genetic resistance to *Fusarium* infection is polygenic with moderate/high heritability, although there are not evidences of a complete resistance, it exists a strong correlation between genotype effects on susceptibility to ear rot and fumonisins content. In general, genotypes with greater potential to resist ear rot also tend to have greater potential to avoid contamination by fumonisin (Robertson et al. 2006). New inbreed lines were created by hand impollination of resistant and susceptible inbreed lines for the production of parental F1, F2, F3 and backcross generation with reciprocals; resistance to the fungus was then evaluated by inoculation with *Fusarium.* Incidence of symptomatic and asympotomatyc infection of kernels by *Fusarium* was determined from inoculated and uninoculated ears to determine factors associated with the resistance (Headrick and Pataky 1991; Clemente et al. 2004). By using these approaches considerable improvements in genetic resistance have been achieved, that were also supported by repeated field and greenhouse-based screening and marker platforms that allow breeders to more fully implement MAS (Mark assisted Selection) (Anderson 2007). Although resistance to ear rot has high hereditability, other factors as environmental condition and difficulty in artificial inoculation affect the potential to find gene associated with resistance. QTL mapping experiment performed to evaluate potential loci associated with *Fusarium* resistance evidenced some traits associated with Fungus resistance

(Ding et al. 2008). Recently microarray experiment evaluated the differential gene expression of particular protein probably associated with Fungus response (Lanubile et al. 2010). Nevertheless, the plant factors, or genes deputed to *Fusarium* response in maize were completely understood.

1.6 Resistance and RAPs proteins

Fusarium resistance was studied in model plants as *Arabidobsis thaliana,* which ecotypes differ in their susceptibility to *Fusarium* infection. Starting from two different ecotypes and analysis of a cross between them were found six dominant resistance to *Fusarium Oxysporium* (RFO) Loci, one of this Loci RFO1 was identical to a previously named Arabidopsis gene WAKL22 (Wall-Associated Kinase-Like Kinase 22) (Diener and Ausubel 2005). This gene encoded for a family of protein kinases associated with cell wall that was previously described to be involved in plant pathogens response (He et al. 1998). Evidence of protein related to biotic stress response are present also in different crop species, e.g. barley produced a chymotrypsin/subtilisin (CI) inhibitors and an amylase/subtilisin inhibitor (BASI) that inhibit Fusarium proteinase (Pekkarinen and Jones 2003), wheat produce a Fusarium-induced stress protein (FISP) (Mittra et al. 2004). In maize proteins related to fungus resistance are well documented for *Aspergillus flavus* infection. A maize kernel pathogenesis-related protein (PR10) was seen highly expressed in *A. flavus* resistant but not in the susceptible genotype. The PR-10 overexpressed in *Escherichia coli* exhibited a ribonucleolytic and antifungal activities (Chen et al. 2006). Many proteins were identified as potential candidate in *Aspergillus flavus* resistance and they were defined as resistance associated protein (RAPs) (Tab. 3). (Brown 2010):

RAPs
Antifungals
Zeamatin
Trypsin inhibitor 14kDa (TI)
Ribosome inactivating protein (RIP)
β-1,3-glucanase
Pathogenesis-related protein 10 (PR10)
Stress-related
Aldose Reductase (ALD)
Water stress inducible protein (WSI)
Anionic peroxidase
Small heat shock protein 16.9/17.2 kDa
Glyoxalase I (GLX I)
Peroxiredoxin 1 (PER1)
Storage
Globulin I
Globulin II
Cupin domain containing protein (Zmcup)
Late embryogenesis abundant protein (LEA III)
LEA 14
Other
Serine/threonine protein kinase
Translation initiation factor 5A

Table 3. **From Brown 2010. RAPs identified through comparative proteomics.**

In maize, different proteins were found to be associated to *Fusarium* resistance. The Zea mays b-32 ribosome-inactivating protein inhibits growth of *Fusarium verticillioides* in vitro (Lanzanova et al. 2009). Another hydrophobic protein at 19.7 KDa purified from corn kernels inhibit the amylase activity of *Fusarium verticillioides* (Figueira et al. 2003). Campo *et al.* identified several proteins that were differentially expressed in germinated maize in response to *F. verticillioides* infection (Campo et al. 2004). It was also observed a correlation with the thickness of kernel pericarp and aleurone layers and the resistance to ear rot caused by *Fusarium* (Hoenish and Davis 1994).

Proteins associated to fungus resistance are often related to different plant response pathways as defence, stress response and development. Thus, indicating that plants have evolved a variety of different mechanisms to cope with the constant threat by phytopathogenic microorganisms. Plants have developed physical barriers and antimicrobial compounds that are preformed in advance of

pathogenic attack. After infection by pathogens, these constitutive defences are supported by inducible mechanisms: cell walls can be reinforced, hypersensitive cell death is triggered to isolate the pathogens from the healthy part of the plant and antimicrobial compounds, such as phytoalexins pathogenesis-related PR proteins are then produced. Molecular mechanisms governing the *F. vertcillioides* resistance however remain controversial even thought could consist of two components: (1) contrast to initial penetration and (2) resistance to growth inhibition and spreading through host tissue.

2 *Diabrotica virgifera virgifera* Le Conte

2.1 Biology and ecology of *Diabrotica virgifera*

The western corn rootworm (WCR) *Diabrotica virgifera* was first collected from *Cucurbita foetidissima* and described by John Lawrence Le Conte in 1868. Only after several years it was recognize as a pest after the description by Gilette that noted a strong correlation between the presence of the insect and corn root damage and allegation in Colorado from 1909 to 1911 (Gillette 1912). As a consequence of a strong monoculture, the WCR became the major "man made-pest" of maize in the U.S.A (Metcalf 1986). Annual cost and maize production losses due to corn rootworm, including WCR and north corn rootworm NCR are the largest expenditure for insect management in the U.S.A. (Sappington et al. 2006). In Europe *Diabrotica virgifera* was first observed in Serbia in 1992; in Italy was found in Veneto region in 1998 near Marco Polo airport of Venezia than in Lombardia in 2000 and in Piemonte in 2001. Maize is grown on large scale in many EU members, mainly silage and grain maize for animal feed and human food production. The fact that maize is the most profitable agriculture crop for farmers in addiction with the continuous maize cultivation (monoculture) make the *D. virgifera* pest be considered as one of the most serious threat to EU agriculture, the insect has been listed as a regulated harmful organism with quarantine status.

Insect biological cycle consists of one generation of WCR occurring per year, this under moderate climate conditions (Fig. 5). The insects overwinter in the egg stage in the soil and larvae emerge in the spring begin feeding on the root. Larvae undergo three developmental stages and then after the pupal metamorphosis adults individuals start feeding of corn silks and pollens (Berger 2001). Insects at beetle stage are very mobile and could be able to migrate long distances actively by flying or passively by terrestrial nautical or air transportation. Corn rootworms undergo four developmental stages: egg, larvae, pupa, adults.

- Eggs are yellow or whitish coloured and 0,65 x 0,45 mm in size; they are laid in the top 10 cm of soil. Eggs hatch after a diapausal period that need low temperature. Eggs developmental rates is influenced by the

temperature, in general much higher is the temperature less is the time in eggs hatching (Schaafsma et al. 1991).

- Larvae consisting of immature stage are whitish in colour with a brown head and anal plate. They measure 2 to 10 mm in length depending of which of three developmental stages (instars). Larvae occupy plant roots and soils around roots and they can easily adapt to different condition in the first 15 cm of soil. In our region (Pianura Padana) they are found from May until first days of July. They attack and feed on roots starting from May and June but the damage and allegation is clearly visible from June to July. Rate of larval development is temperature dependent. Average neonate to adults emergence times vary from 21 to 45 days at 30 or 18°C respectively (Jackson and Elliott 1988). Larvae have an optimal growth condition between 21 and 30° C; larvae with greatest head capsule result in higher adults survival. However in maize fields, survival from egg to adults range from 6 to 11% (Pierce and Gray 2007).
- Pupa generate from the third instars larvae that construct an earthen soil cocoon and remain inactive until maturation in adults.
- Adults vary in size depending on the sex of the beetles: in general, the males are 4,4- 6,6 mm and females 4,2- 6,8 mm in length. In general, both have yellow with three black stripes on forewings (elytra); however, females stripes are well distinct while in males stripes are less separate where the wing covers meet and they are much dark coloured. Antennae are also different in both sexes, in males are longer than beetle body instead in females are 3/4 of the body length. *Diabrotica virgifera* beeltes begin their flights onto maize from the second decade of June until September or October depending of the climate condition and the main flight periods of WCR are early morning and late afternoon. The presence of adults in field is much relevant in July during maize bloom due the high presence of pollen. *D. virgifera* beetles can live about 60 days (Branson and Johnson 1973) and one female within its lifespan is able to deposit over 1000 eggs however adults longevity and the number of deposited eggs mainly depend on several factors as food availability and quality, photoperiod and weather condition. Recently it has been reported a high variability on the fecundity and longevity of *Diabrotica virgifera* both with different laboratory and in field condition (Beres and Sionek 2010).

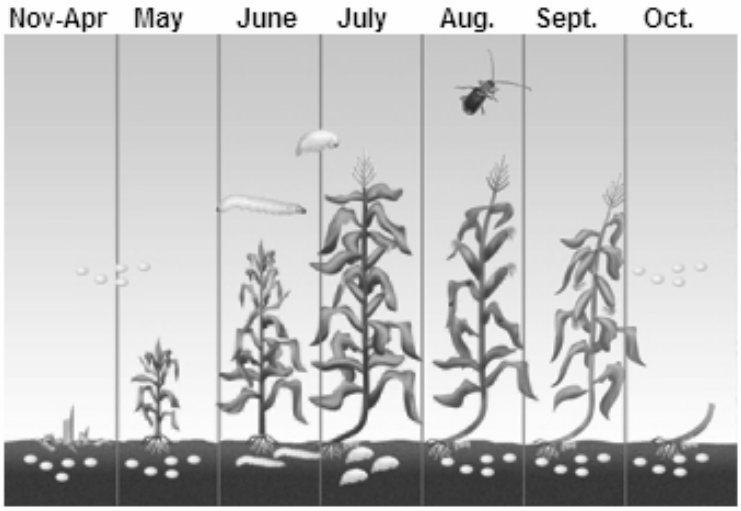


Figure 5. ***D. virgifera*** **life cycle**

2.2 Maize attack and management

D. virgifera were initially considered to exclusively feed and growth on maize but successively were reported their presence also in other cultivars mainly associated with Poaceae. This could be also due by the high capabilities of adaptation of this specie. In particular, in the past corn was annually rotated with soybean to limit rootworm larval damage, because WCR adults laid only in cornfields. Therefore, rootworm larvae from eggs laid in corn the previous year could not survive on soybean roots and eggs were absent after soybeans rotation. In 1995 was then reported rootworm larval damage in corn planted after soybeans with a high increase of WCR adults found in both corn and soybeans adults. This was probably caused by continuous rotation of corn and soybeans and pyrethroide insecticides that had forced WCR in strains more adaptable to soybeans field survival (Barna et al. 2000). Maize remains the favourite food source and it is necessary to WCR to produce eggs. Laboratory studies demonstrated that the soybean plant is a poor food for adult WCR. WCR that were fed only soybean plants produced no eggs, weighed less, and died before their siblings, which were fed an artificial diet or one of corn silks, tassels, foliage, and immature ears (Mabry and Spencer 2003). *D. virgifera* have a life cycle strictly contingent upon maize cultivar and especially in Italy where corn is cultivated as a monoculture could heavily damage and affect harvest yield. Adults are not monophagous,

because they also feed on plants classified under several other families, like Cucurbitaceae (Tallamy et al. 2005). In maize adults feed on every part of the plant above ground, of leaves before flowering and than of pollen and silk; in particular their feeding on newly emerging silks prior to pollination is most serious, resulting in sparsely filled ears. Serial reduction in the number of ripening kernels from the tip of the ear to the base or kernel damage can often be observed at harvest. The larvae are oriented by the carbon dioxide (between 2 to 100 mmol/mol) by respiring roots. They feed on, and tunnel inside, the root system of the plant giving the most serious problem in crop production. In maize symptoms of larval infestation of the roots often become visible when plant are 20-50 cm tall. Plants grow poorly, become stunted and yellow but can survive and produce grain; however, the damage inflicted causes reduces amount of nutrient for the plant and consequently a low yield. The behaviour and ecology including many factors as larval and adult movement oviposition alternate host use and chemical ecology are important in *D. virgifera* management. The difficulty of *D. virgifera* control is related to insect plasticity that in years has been able to adapt to agronomic changes, i.e. initially success to *D. virgifera* management by using annual crop rotation were been vain by the selection for behaviour resistance to this cultural control. The understanding of WCR biology is indispensable to integrate pest management and insect resistance management and efficiently threat this pest.

2.2 Maize Diabrotica resistance

The use of good agriculture practices, when possible avoiding monocultures system, remain the most effective methods to limit *D. virgifera* infestation. In last years, many attempts has been made to limit *D. virgifera* damages, but the use of existing tolerance and resistant maize inbreed against WCR still gives objectionable results. Also the recent use in U.S.A. of transgenic maize cultivar that express the *Bacillus thurigensis* Bt gene that codify for Cry3Bb1 insecticidal protein (Vaughn et. al 2005) didn't always demonstrate the same level of protection against *D. virgifera* larval attack. For most European Union country Bt maize is not approved commercially and the development of maize hybrids with native resistance to *D. virgifera* are still strongly needed. Introduction of resistance hybrids was also strongly recommended by the fact that some type of

pesticides i.e. Clothianidin (nicotinoide) that were largely use in pest management last years were also responsible for the persistent bee mortality in Europe.
Plants can develop both constitutive and inducible mechanism to protect themselves against pathogens. Two main defences strategies against herbivore attack depend on a resistance mechanism by limiting the pest attack and a tolerance mechanism by limiting the impact on plant fitness. Certain maize inbreed lines showed tolerance to *D. virgifera* feeding that was associated with larger root system and grater secondary root development (Owens et al. 1974). Lately it has been reported a strong association between the production of so-called herbivore-induced plant volatiles (HIPV) and the biotic resistance response. Recently maize plant was reported to emit volatile compound to protect against *D. virgifera* attack. Maize roots in response to feeding by *D. virgifera* larvae release a particular sequiterpene (E)-β-caryophyllene that recruit natural enthomopathogenic nematodes as defence mechanism (Rasmann et al. 2005).
The direct interaction between the plants and nematodes as defence mechanism was clearly demonstrated, and the use of entomopathogenic nematodes is a potential non-chemical approach to control the larvae of the invasive western corn rootworm in Europe (Toepfer et al. 2010).
(E)-β-caryophyllene synthesis and emission by plant in response to *D. virgifera* seem to be lost during the domestication. Most North American lines do not express the (E)-β-caryophyllene synthase gene (terpene synthase 23 TPS23), whereas Europe lines and wild ancestor, teosinte, readily response to *D. virgifera* attach by TPS23 induction (Köllner et al. 2008). Since most of the commercial maize inbreed lines are deriving from American maize varieties the TPS23 restoration by using European lines in inbreed program might help to increase maize resistance against this agronomically important pests.

2.3 Terpenes and (E)-β-caryophyllene synthase

Terpenes are producted by plant for many different purposes as plant growth and development (e.g. giberellin phytohormones) and as regulatory mechanism in interaction between plant and environment. Both volatile and non-volatile terpenes have attraction function for pollinators and predators and are often related to stress response, in protection, mediating thermotolerance and direct defense against microbes and insect. All terpenes are derived from the allylic

prenyl diphosphates (IPP) and dimethylallyl diphosphate (DMAPP), that were transformed by prenyltransferases in geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) derivate and finally converted the action of terpene synthase activities (Tholl 2006).
In particular a farnesyl diphosphate precursor through a complex cyclization reaction is converted by sequiterpene synthase enzyme Class I in (E)-β-caryophyllene (Fig. 6).

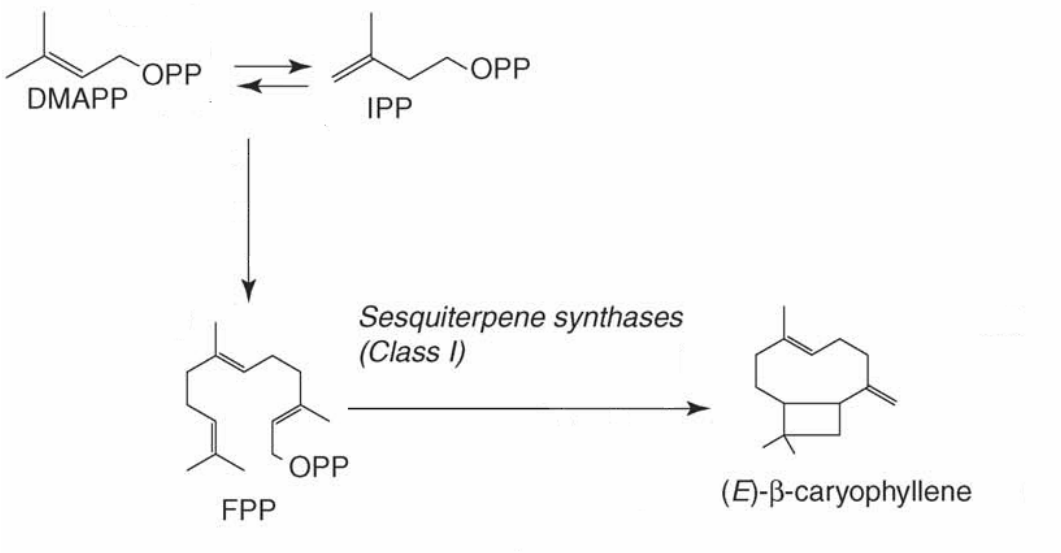


Figura 6. Synthesis of (E)-β-caryophyllene. Dimethylallyl diphosphate (DMAPP) is fused with 2 isopentenyl diphosphate (IPP)units in farnesyl diphosphate (FPP) derivate that are converted by sequiterpene synthase in (E)-β-caryophyllene.

In maize the sequiterpene synthases (Class I) family includes different enzymes TPS1, TPS4, TPS5, and TPS10, which contribute to the overall terpene composition of the aboveground plant parts (Schnee et al. 2002, Schnee et al. 2006). (E)-β-caryophyllene is synthesized in the roots and aerial part of plant by terpene synthase TPS23 (Köllner et al. 2008).

3 Jasmonic acid

3.1Jasmonate pathways

In 1962 a jasmonic acid methyl ester (MeJA) derivate was isolated for the first time from the essential oil of Jasminum grandiflorum (Demole et al. 1962). Twenty years later was described a senescence-promoting effect of JA and it was defined as a growth inhibitor (Ueda and Kato 1980, Dathe et al. 1981). Functional analysis and the mode of action of jasmonic acid (JA) or MeJA were described by their direct application to barley which affected protein pattern changing in leaves (Weidhase et al. 1987a, b). JA signalling pathways was also implicate in many developmental processes as root growth, pollen production (Crelman and Mullet 1997), and in biotic stress response to insects and pathogen mediating different defensive response against herbivore and pathogen parasitic plant or wounding reaction (McConn et al. 1997). JA and its methyl ester (methyl jasmonate MeJA) are compounds derived from linoleic acid (LA) that belongs to lipid-derivate signalling. JA and MeJa are deriving from phospholipids constituting the cell membrane. The activation of specific membrane receptor induce the phospholipids hydrolysis by phospholipases enzyme producing phosphoinositol bisphosphate (PIP2), inositol triphosphate (IP3) and phosphatidic acid (PA), that are well known intermediate in Ca^{2+} signalling and stress responses (Bargmann and Munnik 2006). The aliphatic part released consists of polyunsaturated fatty acids (PUFAs) that are sources of different signals. Class of PUFAs named oxylipins are converted in secondary messengers that include jasmonates. Oxilipins derived from a linolenic acid (18:3) (α-LeA) are released from chloroplast membranes after the activation of different hydrolase enzyme e.g. phospholipase A1, phospholipase A2, patatin-like acyl hydrolases, DAD-like lipases and SAG (senescence-associated gene) 101-like acyl hydrolases (Dormann 2005). JA pathways was seen to be activated by wounding response through the activation of phospholipase A2 enzyme (Narvàez-Vàsquez et al. 1999) and in developmental processes by DAD-like phospholipase A1 that is alter in a mutant with anther deficiency (Ishiguro et al. 2001). The receptor that leads to phospholipase activations and induce the JA pathways, however, is still unknown. Stress responses induce the α-LeA release by the action of different lipase at membrane level, and the synthesis of JA and its derivates through the action of

different enzyme, and in first instance of lipoxygenases 13 (13-LOX) (Wasternack 2007) (Fig. 7).

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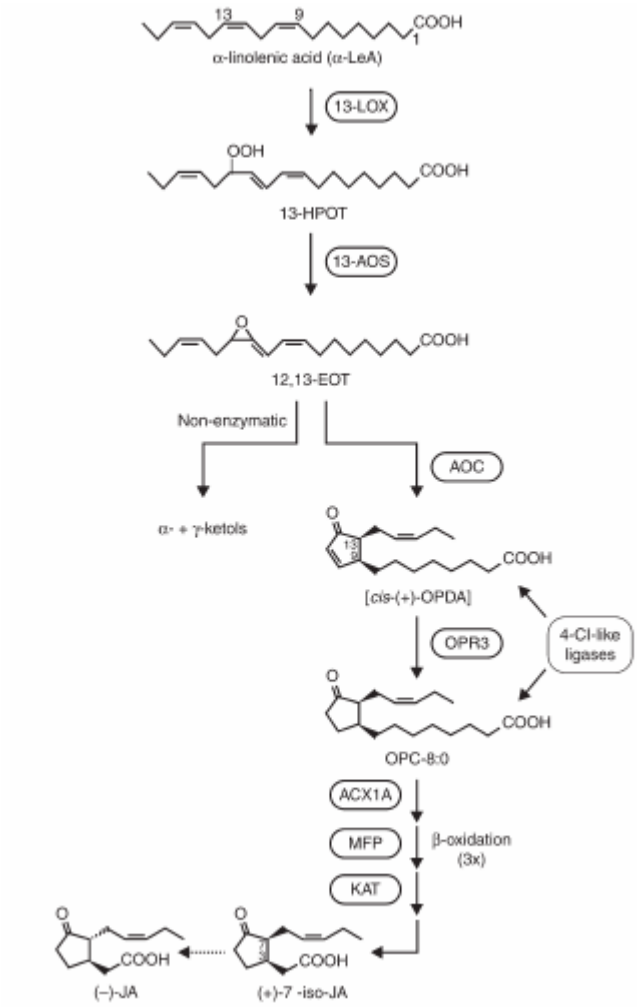


Figure 7 Adapted from Wasternack 2007 the 13-LOX pathways and JA synthesis

The activation of 13-LOX pathways induce the synthesis of all JA derivate including MeJA, hydroxyjasmonates (HOJAs), jasmonoyl-isoleucine (JA-Ile), 12-hydroxy jasmonoyl-isoleucine (12-HOJA-Ile), and 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile). These compounds mediated the spatial and temporal action in the plant systemic response with specific mechanism. JA-Ile is the biologically active form of jasmonate (Staswick and Tiryaki 2004). 12-HOJA-Ile has recently been reported to be wound induced in tomato leaves (Guranowski

et al. 2007). Both, wounding and herbivore damage activate the JAs pathway for a plant systemic response. Kinetic of JA response after wounding was determined and it, was seen to increase within 2-5 min after wounding and to accumulate in distal plant organ at least 3 cm/min in velocity (Glausser et al. 2008). The systemic response to herbivore attack is mediated by the the release of a systemic peptide signal named systemin that activate the JA pathways signalling (Ryan and Pearce 2003).

3.2 Jasmonate gene response

Jasmonic acid and its biologically active derivatives named jasmonates (JAs) that are synthesized in response to different signals, regulate some aspects concerning the plant development and biotic and abiotic stress challenges such as pathogens and pest response, wounding, exposure to ozone and water deficit (Fig. 8).

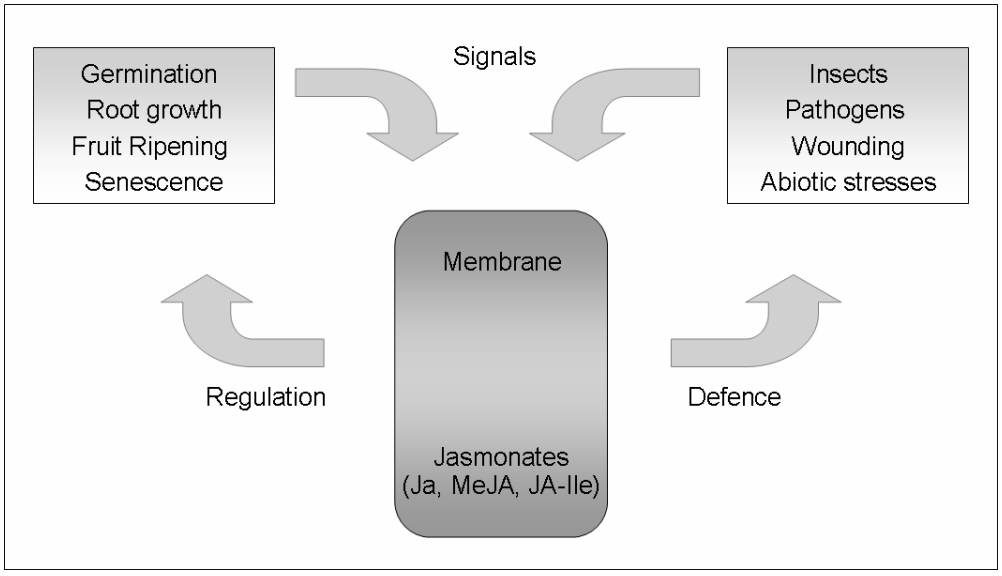


Figure 8. Scheme of factors involved in Jasmonates pathways activation and response.

Several regulator factors, involved in JAs response were discovered by analyzing *Arabidobsis* mutants. The coronative insensitive 1 COI1 protein was seen to be essential in the JA signalling since it was required for all JA-dependent response (Xie et al. 1998). With the cloning of JA-insensitive Arabidobsis mutant jin1 was found that the direct interaction with the transcription factor MYC2 was a key

component of the JA-signalling pathways. MYC2 binds to the G -box and derived sequences in the promoter of JA-induced genes and differentially regulates different branches of the JA pathway (Lorenzo et al. 2004). Lately was discover by transcription profiling analyses a Jasmonate Zim domain JAZ protein family that interact with COI1 and MYC2 as repressor of JA mediated gene transcription (Chini et al. 2007). Current models indicate that JAZ protein represses JA signalling through the direct interaction with the transcription factor MYC2. After different stimuli, JA levels increase and stimulate JAZ to bind to COI1. COI1 is a 66 KDa protein, bearing an N-terminal region containing an F-box motif and 16 leucin-rich repeats. It belongs to a multi-protein complex that promote the JAZ degradation via the ubiquitin/26S proteosoma pathway, thereby releasing MYC2 from repression. Finally, MYC2 induced the transcription and synthesis of the JAZ protein that ensure the formation of the repressor complex with MYC2 by turning off the JA response. Thus, provide a negative feedback typical of a hormone pulsed response (Chico et al. 2008) (Fig. 9).

JA response activates early genes that codify for different signals that induce the synthesis of different protein involved in the defence response or in the plant development. In particular a microarray-based screening of *Arabidopsis* rosette leaves treated with 100 µM methyl jasmonate, showed several genes belong to different classes that were up and down regulated e.g. defence, stress response, senescence, cell wall modification (Jung et al. 2007). MeJA treatment is also effective in different Poaceae crop species e.g. in wheat induced accumulation of β-1,3- glucanase and thaumatin-like PR-proteins (Jayaraj et al. 2004) or the synthesis of different defence compound in both aerial parts and roots (Moraes et al. 2007). In maize was recently observed that Jasmonate induce the expression of maize insect resistance 1-cysteine protease (Mir1-CP) (Ankala et al. 2009). Evidence that jasmonate mediate response is also involved in soil-borne pathogens defence was recently observed in sugarcane roots. After the exogenous application of MeJA were observed variation in the gene expression of different genes involved in the biotic and abiotic stress defence e.g. chalcone synthase, pathogenesis-related (PR) protein 5, PR-protein 10, benzothiadiazole-induced protein, glycine-rich and proline-rich protein, glutathione-S-transferase, ferredoxin, DNA repair protein etc.

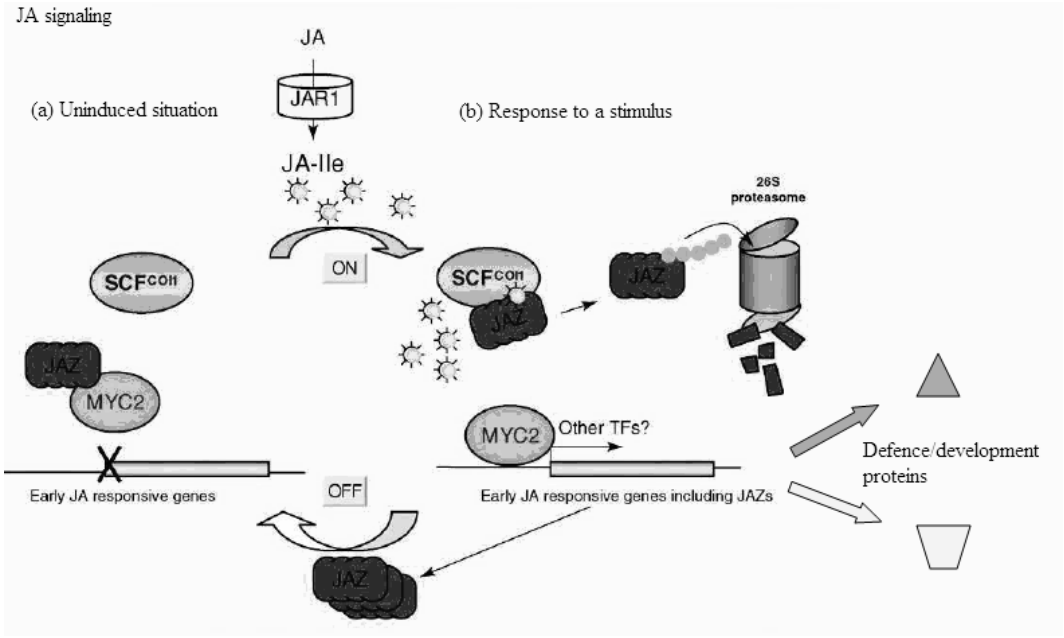


Figure 9. Adapted from Chico 2008. JA responses on and off. Model for the negative feedback loop regulation of JA responses in Arabidopsis. (a) Uninduced situation, b) response to stimulus

3.3 MeJA and TPS gene

A new type of defence mechanism in response to insect attack, termed indirect defence, was firstly described in maize. Corn seedlings fed upon by caterpillars released terpenoids volatile compounds to respond to herbivore damages. The release of these volatiles compound increased significantly in presence of oral secretions from the caterpillars rather than only artificially damage (Turling et al. 1990). The sequiterpenes volatile release of (E)-β-farnesene, (E)-α-bergamotene and (E)- β-caryophyllene were stimulate also by direct application of volicitin (N-(17-hydroxilinolenoyl)-L-glutamine) and jasmonic acid (Schmelz et al. 2001). Evidences that the expression of different terpene synthase TPS were induced by JA treatment were observed in different species e.g. *Medicago truncatula* (Gomez et al. 2005, Arimura et al. 2008), *Lycopersicon esculentum* (van Shie et al. 2007). Recently in maize was reported the expression of TPS1 gene in leaves and roots after JA treatment (Feng et al. 2010).

4 Scope of the projects

This Ph.D. project is part of the MDF (Maize *Diabrotica Fusarium)* project founded by Regione Lombardia, that is focused on the development of new inbreed lines with enhance resistance to two of the major pest diseases, *Fusarium* and *Diabrotica virgifera*.

Within the MDF network, this Ph.D. project aims to unravel the mechanisms related to plant biotic stress response by using different proteomic approaches. Indeed regulation and expression of specific proteins is commonly thought to be related to plant pathogen resistance and the use of innovative and high throughput proteomics technique is necessary to understand different aspects concerning host and pest interaction.

The first part of this Ph.D. work explored the proteomic profile of kernel from resistance and susceptible corn lines in order to evaluate variation at protein level associated to *Fusarium* resistance.

Then in the second part, it was investigated the defence response to *Diabrotica virgifera* through the synthesis of (E)-β-caryophyllene in the maize root system. We monitored the expression of the corresponding *tps23* gene and the protein profiling variation, after indirect (methyl jasmonate treatment) and direct (*Diabrotica virgifera* infestation) stimulation in different maize inbreeds.

Applications coming out of this project are various. First, we are looking of potential biomarkers associated to *Fusarium* resistance in order to evaluate their potential use in association studies within disease resistance in parental F1, F2, F3 and backcross generations. Secondly, it brings further knowledge about the TPS23 mediate response in maize, to get new solution to improve the efficacy of *D. virgifera* pest management and control.

Materials and methods

1 Plant materials and treatments

1.1 Maize lines (*Fusarium*)

Kernel was obtained from maize genotypes with contrasting phenotypes for tolerance to Fusarium ear rot. The tolerant lines were CO441, CO433, CO430, whereas the susceptible lines were CO354, GA209, T202 and a F1 inbreed CO441 x CO354. All the lines were kindly provided by AAFC (Agriculture and Agri-Food Canada) and Prof. MaroccoUniversità Cattolica di Piacenza.

1.2 Maize lines (*D.virgifera*)

Experiments were conducted from different maize genotypes B97, NC358, F2, B73, Oh43 and Mo17 with different expression of (E)-β-caryophillene synthase (Köllner et al. 2008). Plants were growth in a climate controlled chamber with 16 h light photoperiod 400 µE 400-700nm a temperature cycle of 22/18 °C (day/night) and 65% room humidity. Caryopses were sown on filter paper saturated with distilled water and incubated in dark at 26°C. For hydroponic experiments: seed after germination were transplanted into 5L plastic tanks (18 seedling per tank) containing a complete nutrient solution (Nocito et al. 2002). Plants were treated after fifteen days (15-30 cm high, three to four expanded leaves) by: direct wounding, salycilic acid (SA) 500 µM and methyl jasmonate (MeJA) at different concentration from 10 µM to 100µM. For Diabrotica experiments seed after germination were transferred in soil. Fifteen to twenty old plants (30-35 cm high, five expanded leaves) were subjected to three-four (second and third instar) *D. v. virgifera* larvae (from CABI Bioscence) for two days. Root from untreated and treated plants were collected at different time freezed in liquid nitrogen and stored at -80°C.

2 Protein extraction

2.1 Kernel proteins (*Fusarium*)

Proteins from kernel were fractionated in different component (Osborne 1897 modified). Each protein fraction albumins, globulins zeins were quantified by the Bradford protein assays (Bradford 1976) according to the manufacturer's instructions. Quality of the extracts were evaluated by SDS-PAGE, using a 15% (v/v) acrylamide gel with prestained protein molecular weight markers (Cell Signaling Technology) and detected by Comassie blue staining.

Albumins

500 mg of kernel from different maize inbreed lines was finely ground, and washed o.n. with 1 ml of ice-cold acetone. After centrifugation pellet was dried out to solvent elimination. Albumins were extracted three times (1h, 4°C) in 800ul of (1mM Phenylmethanesulfonyl fluoride PMSF) solution. The soluble fractions were collected together and evaluated for protein concentration. Albulin fraction was filtered 0.22 µm and stored -80°C.

Globullins

Pellet from the albumin extraction was extracted in a high salt buffer. Globulins were extracted three times (1h, 4°C) in 800µl of (NaCl 0.5 M, 1 mM PMSF) buffer. The soluble fractions were collected together and evaluated for protein concentration. Globulin fraction was filtered 0.22 µm and stored -80°C.

Zeins

500 mg of fine kernel powder was washed with 300ul of hexane at room temperature for 10 min. The suspension was centrifuged (12.000 g 4°C for 5') and the pellet was air dried over night. The pellet was extracted with 1 ml of buffer solution (Acetonitrile 60% v/v, 25mM ammonium hydroxide and 10mM DTT

Dithiothreitol) with gentle agitation 1 h at 60°C. After centrifugation (600 g RT for 10') the surnatant consisting of zein fraction was collected.

2.2 Root proteins (*D.virgifera*)

200 mg of roots were pulverized in a mortar with liquid nitrogen and extracted in 1 ml of Trizol (Invitrogen) according to the manufacturer's instructions. 1 ml of Trizol extract was centrifuged (12.000 g for 10' at 4°C). The supernatant was then transferred in a new vial, added of 200 µl of chloroform, vortexed and gently agitated for 10' at room temperature. The mixture was then centrifuged at 14.000 g for 10 min. The upper aqueous phase was than used for RNA extraction while the organic phase was used for protein extraction. DNA was precipitated from organic solution by adding 300 µl of ethanol. After gently mix for 5' at room temperature the suspension was centrifuged (2000 g for 5' at 4°C). The surnatant was transferred in a new vial, added of 1ml of isopropanol and gently agitated for 10 minute at room temperature for protein precipitation. The suspension was centrifuged (12000 g for 10' at 4°C) and the protein pellet was the washed four times with 1 ml of ethanol; for each washing step centrifuged (8000 g for 5' at 4°C). The pellet after complete removal of ethanol was then solubilized in 300 µl of acid acetic 50%. Proteins were quantified using the BCA assays according to the manufacturer's instructions.

3 Surface-Enhanced Laser Desorption/Ionization Time of Flight SELDI-ToF

Protein extracts from kernel and root were loaded in triplicate using different proteinchip surfaces (Gold not derived, CM10 cationic exchange, H50 Hydrophobic, Q10 anionic exchange, IMAC metal affinity). For all Proteinchip surfaces were used specific protocols.

3.1 Protein loaded on different Chips array

Gold array

Three micrograms of proteins were directly spotted on Gold array and 1 µl of matrix (half saturated sinapinic acid dissolved in 50% Acetonitrile (ACN)/0.5% TFA) was added in twice.

NP20 array

Chips were pre-activated with 5 µl of deionized water for 2 min, RT at 500 rpm. Five micrograms of proteins were directly spotted on NP20 array and dried. One µl of matrix (half-saturated sinapinic acid dissolved in 50% ACN/0.5% TFA) was added in twice.

CM10 array

Chips were pre-activated with 150 µl specific binding buffer (low-stringency buffer 0.1 M sodium acetate pH 4) for 5', RT at 500 rpm. Twenty micrograms of proteins were diluted up to 100 µl of binding buffer and incubated for 45 minutes at 500 rpm. After incubation, the chips were washed with 150 µl binding buffer for three times and 150 µl with deionized water twice. At the end, 1 µl of matrix (half-saturated sinapinic acid dissolved in 50% ACN/0.5% TFA) was added in twice.

H50 array

Chips were pre-activated with 150 µl of 50% ACN solution for 5' RT at 500 rpm. Twenty micrograms of proteins were diluted up to 100 µl of binding buffer (10% ACN/0.1% TFA) and incubated for 45 minutes at 250 rpm. After incubation, the chips were washed with 150 µl binding buffer for three times and with 150 µl deionized water twice. At the end, 1 ul of matrix (half-saturated sinapinic acid dissolved in 50% ACN/0.5% TFA) was added in twice.

Q10 array

Chips were pre-activated with 150 µl specific binding buffer (0.1 M Tris-HCl pH 8) for 5 min, RT at 500 rpm. Twenty micrograms of proteins were diluted up to 100 µl of binding buffer and incubated for 45 minutes at 500 rpm. After incubation, the chips were washed with 150 µl binding buffer for three times and 150 µl with deionized water twice. At the end, 1 µl of matrix (half-saturated sinapinic acid dissolved in 50% ACN/0.5% TFA) was added in twice.

IMAC array

Chips were pre-activated with 50 µl of 0.1 M of copper sulphate for 10 min, RT at 500 rpm and washed with 150 µl of deionized water three times. Chips were then added with neutralization buffer (0.1M sodium acetate pH4), at 500 rpm for 5' and washed with 150 µl of deionized water twice. Chips were equilibrated with 150 µl specific binding buffer (0.1 M sodium phosphate, o.5 M sodium chloride pH 7) for 5', RT at 500 rpm. Twenty micrograms of proteins were diluted up to 100 µl of binding buffer and incubated for 45 minutes at 500 rpm. After incubation, the chips were washed with 150 µl binding buffer for three times and 150 µl with deionized water twice. At the end, 1 µl of matrix (half-saturated sinapinic acid dissolved in 50% ACN/0.5% TFA) was added in twice.

3.2 Instrument condition and data analyses

All chips arrays were analyzed with mass spectrometer SELDI-ToF (Biorad). Each spot was analyzed with separate setting of laser and focus mass to allow optimal detection of spectral regions between 2– 15 kDa and 15– 30 kDa. SELDI-ToF spectras were generated by averaging 900 laser shots and calibrated with all-in-one Protein standard II (hirudin BHVK 7.0 kDa; bovine cytochrome C 12.2 kDa; equine myoglobin 17 kDa; bovine carbonic anhydrase 29 kDa; yeast enolase 46.7; bovine albumin 66.4 kDa; and bovine IgG proteins 147.3 kDa). Ciphergen Express software (version 3.5, Ciphergen Biosystems) was used for pre-processing data i.e. automatic peak detection after baseline subtraction and adjustment (S/N ratio > 5, cluster mass window 0.3% peak width). The program also allows basic statistical analysis, including hierarchical clustering, group scatter plots, P-values (t-test statistics).

4 Protein fractionation and SDS-PAGE analysis

4.1 Albumins fraction from Kernel (*Fusarium*)

100 µg of albumin fraction extracted from maize kernel was fractionated using reverse phase chromatography (Agilent mRP Hi-recovery protein columns 4.6 X 50mm) at 80°C by HPLC (Agilent 1200 Series) at 1 ml/min flow with 97 % buffer A, consisting of 0.1% TFA/water (v/v) and 3% buffer B consisting in 0.1% TFA/Acetonitrile(v/v). Proteins were eluted with a binary gradient 3-100% buffer B. The gradient used was: 5 minutes in 3% buffer B, 5.01-14 min 3-16% buffer B, 14.01-17 min 16-24 % buffer B, 17.01-20 min 24-33% buffer B, 20.01-30 min 33-45% buffer B, 30.01-47 min 45-60 % buffer B, 47.01-50 min 45-100 % buffer B, the column was washed with 100% buffer B for 4 min and equilibrated with 100% buffer A for 10 minutes. The proteins were detected at 220nm and one minute fractions were collected giving a total of 16 fraction (1mL volume fractions). The fractions were dried under vacuum using a "speedvac" and solubilized in 15µL of Laemmli buffer (62.5 mM tris HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue). Each fraction was separated by SDS-PAGE, using a 15% (v/v) acrylamide gel and prestained protein molecular weight markers (Cell Signalling Technology) and detected by Comassie blue staining.

4.2 Root proteins (*D. virgifera*)

150 µg of protein extracted from root was fractionated using reverse phase chromatography (Agilent mRP Hi-recovery protein columns 4.6 X 50mm) at 80°C by HPLC (Agilent 1200 Series at 1 ml/min flow with 97 % buffer A, consisting of 0.1% TFA/water (v/v) and 3% buffer B consisting in 0.1% TFA/Acetonitrile(v/v). Proteins were eluted with a binary gradient 3-100% buffer B. The gradient used was: 5 minutes in 3% buffer B, 5.01-11 min 3-30% buffer B, 11.01-27 min 30-55 % buffer B, 27.01-33 min 55-100% buffer B, the column was washed with 100% buffer B for 4 min and equilibrated with 100% buffer A for 10 minutes. The proteins were detected at 220nm and one minute fractions were collected giving a total of 14 fractions (1mL volume fractions). The fractions were dried under vacuum using a "speedvac" and solubilized in 15µL of Laemmli

buffer (62.5 mM tris HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue). Each fraction was separated by SDS-PAGE, using a 15% (v/v) acrylamide gel and prestained protein molecular weight markers (Cell Signalling Technology) and detected by Comassie blue staining.

5 TPS23 transcript (*D.virgifera*)

5.1 RNA extraction and retrotranscription

200 mg of roots were pulverized in a mortar with liquid nitrogen and extracted in 1 ml of Trizol (Invitrogen) according to the manufacturer's instructions. 1 ml of Trizol extract was centrifuged (12.000 g for 10min at 4°C). The supernatant was then transferred in a new vial, added of 200 µl of chloroform, vortexed and gently agitated for 10' at room temperature. The mixture was then centrifuged at 14.000 g for 10'. RNA was precipitated from the upper aqueous phase with 0.5 ml of isopropanol, mixed and gently agitated for 10 minutes at room temperature. RNA was pelleted by centrifugation at 4°C for 10 minutes at maximum speed. The supernatant was removed and the pellet washed by adding 1 ml of 70% ethanol. Samples were centrifuged at 4°C for 5 minutes at 7500 x g. The supernatant was removed and pellets were dissolved in nuclease free water. Concentration and purity of extracted RNA were assessed using Nanodrop. In order to verify quality and integrity of RNA, samples were supplemented with RNA sample buffer (50% formamide, 1% MOPS, 7.5 % formaldehyde) and electrophoresed onto a standard 1.5% agarose gel at 60 V for 45 minutes.

5.2 Polymerase Chain Reaction (PCR)

Prior to reverse transcription, RNA samples were treated with Deoxyribonuclease I, amplification grade (Invitrogen – Cat.N. 18068-015) as instruction protocols, to remove possible genomic DNA contamination. The reaction was incubated at room temperature for 15 minutes, stopped with 1 µl of 25 Mm EDTA and incubated at 65°C for 10 minutes. DnaseI-treated RNA samples were retro-transcribed with SuperScript III Reverse Transcriptase (Invitrogen – Cat.N. 18080044), an engineered version of the Moloney Murine Leukemia Virus (M-MLV) with increased thermal stability. A typical retro-transcription mixture reaction was composed of 1µg DnaseI-treated RNA, 10mM dNTP mix, oligo (dT20) 2.5 µM (Invitrogen-Cat.N. 18418-012). Following incubation at 65°C for 5 minutes and chilling on ice for 1 minute the mixture was added of 200 Units SuperScript III RT, first strand buffer (1X in solution), 40 U of RNAse OUT (Invitrogen-Cat.N. 10777019) and DTT 5mM and incubated at 50°C for 1 hour to

increase specificity and achieve higher cDNA yield. SuperScript III RT was inactivated by heating at 70°C for 15 minutes. cDNA samples were diluted 1:4 and used as templates in PCR reaction.

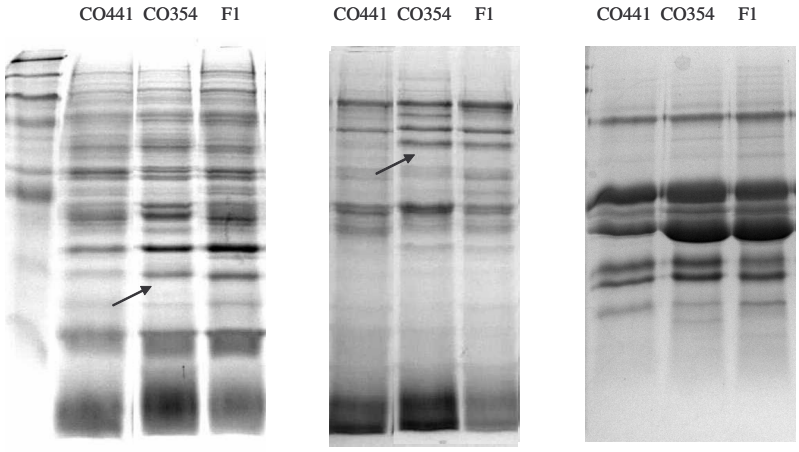
1 µl of template cDNA was amplified through a PCR reaction composed of 400nM forward and reverse primers, GoTaq MM colorless 2X (Promega-Cat.N.M7142). Reactions were incubated for 2 min at 94 °C, before being subjected to 30 cycles of amplification: 30 sec at 94 °C, 30 sec at annealing temperature 60°C and 1 min at 72 °C. A final period of 10 min at 72 °C was performed to promote complete elongation. . PCR reactions are loaded onto 1,5% agarose gel supplemented with Ethidium Bromide, and run for 1 hour at 50 V to get a higher resolution. The TPS23 primers TPS23 forward (5' CAG AAT GGC GTG ACA AGA AA 3') and TPS23 reverse (5 ' GCT CGA ACG ATT TTG GGA TA 3') were used to amplify 172 bp of (E)-beta-caryophillene synthase gene. The ZmTSA primers ZmTSA forward §(5' GTG TGG TTC AGA CGT GAT CG 3') and ZmTSA reverse (5' GCT TCA GGA TCG GGT TGT AA 3') were used to amplify 196 bp of tryptophan synthase as reference gene.

Results

1 Kernel protein characterization in Fusarium resistance/susceptible lines

1.1 SELDI-ToF profiling

The first part of the project consisted of kernel protein characterization from *Fusarium* resistant and susceptible maize lines. Different protein fractions were obtained by using a modified protocol from (Osborne 1897), that consist in the extraction of protein with different solubility in water (albumins), saline buffer (globulins) and organic phase (zeins). First experiments were performed starting from a resistant CO441 line, a susceptible CO354 line and their F1 CO441xCO354 inbreed. The different protein fractions were quantified to evaluate the extraction yield; quality of the extract was assessed by SDS-Page (Fig 10).



Maize Lines	Albumins mg/ml	Globulins mg/ml	Zeins mg/ml
CO441	1.03	1.00	0.77
CO354	1.23	1.07	0.73
F1(CO441xCO354	1.70	1.17	0.53

Figure 10. Protein quantification and SDS-Page analyses of different fraction obtained from CO441, CO354 lines and CO441 x CO354 F1 inbreed.

SDS-Page analyses of the two CO441 and CO354 lines showed difference in particular for two fractions albumins and globulins (arrows in Fig. 10) where some protein where highly expressed in the susceptible lines and in the inbreed

CO441xCO354 respectively. To better characterize and further understand kernel protein composition between the two lines different proteomic techniques as SELDI-ToF-MS and an HPLC fractionation and detection by SDS-PAGE were employed. SELDI-ToF characterization was initially optimized for each protein fraction by using all chip array available (CM10 cationic exchange, Q10 anionic exchange, H50 hydrophobic surface, IMAC metal affinity, NP20 normal phase, Gold w/o derivatization); however not all chip array gave good quality spectra and only some surfaces were selected to analyze the full set of sample. Albumins were analyzed on CM10, Gold and IMAC, globulins were analysed on CM10, NP20 and IMAC , and zeins on Gold and Q10 arrays and spectra where acquired in two mass ranges 2- 15 KDa and 15-30 KDa (Fig. 11, Fig. 12).

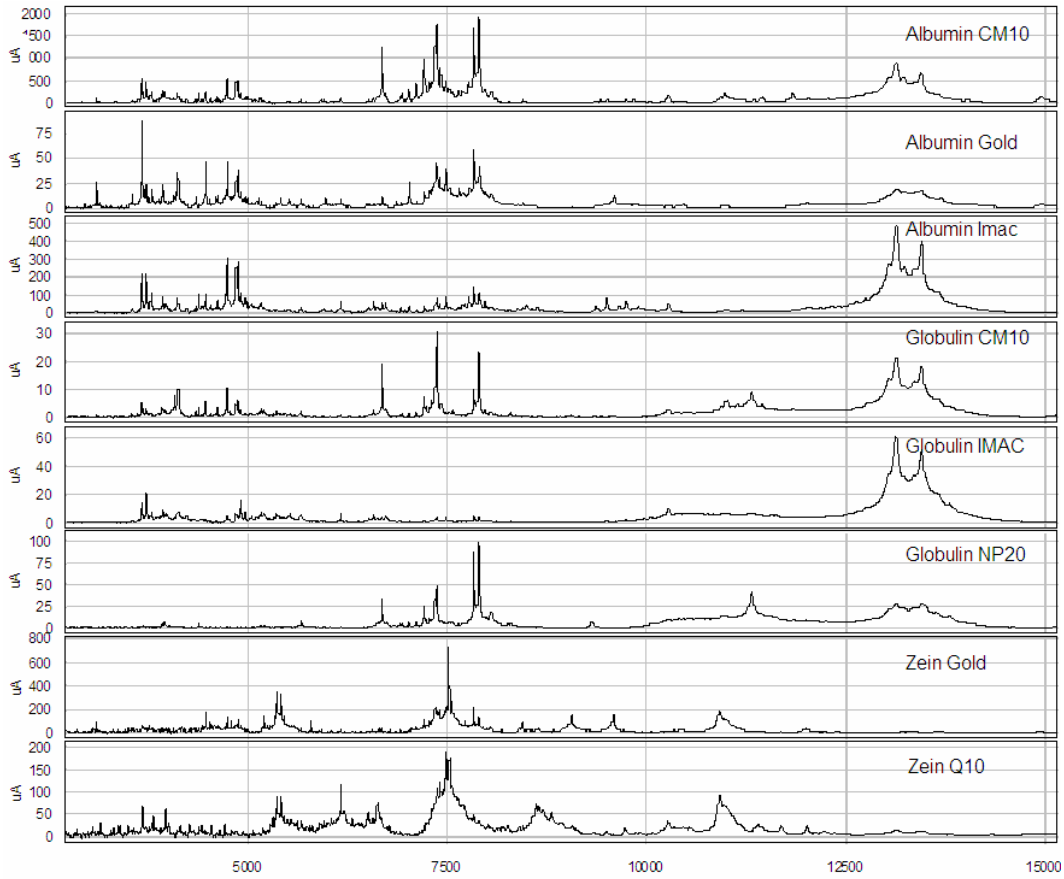


Figure 11. SELDI-ToF spectra (MW range from 2-15 KDa) of protein fraction from CO441 lines spotted on different arrays.

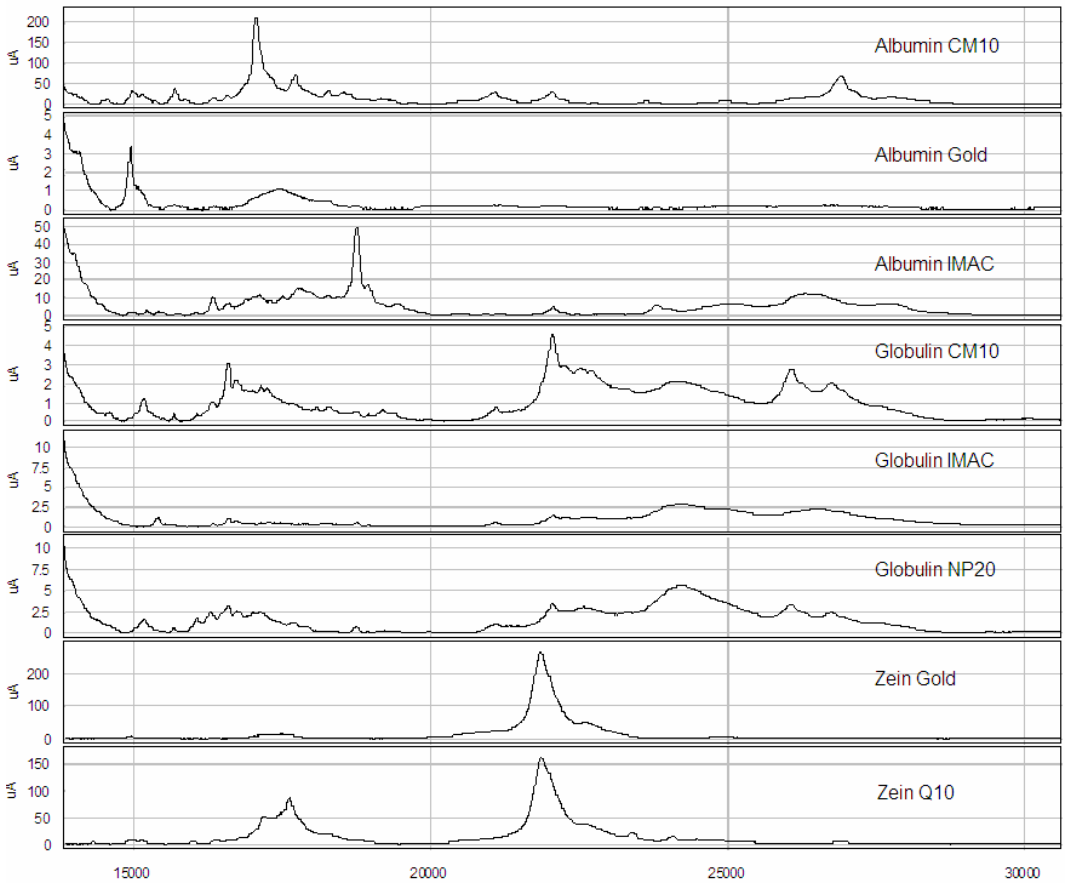


Figure 12. SELDI-ToF spectra (MW range from 15-30 KDa) of protein fraction from CO441 lines spotted on different arrays.

Sixteen spectra were acquired for each lines with a high number of peaks detected, notwithstanding several signals were observed to be redundant by their presence in different spectra belongs to different fraction or chip array. Efficacy of protein fractionation protocol was initially evaluated by comparing the SELDI-ToF spectras obtained by different protein extracts (albumins, globulins and zeins) on the same chip surface (Tab. 4).

Albumins vs globulins CM10

	CM10 (2-14 Kda)				CM10 (14-30 Kda)			
	albumins	globulins	common peaks	% Total	albumins	globulins	common peaks	% Total
CO441	42	26	18	26	21	21	3	7
CO354	42	37	21	27	19	25	5	11
F1	39	27	17	26	24	22	5	11

Albumins vs zeins Gold

	Gold (2-14 Kda)				Gold (14-30 Kda)			
	albumins	zeins	common peaks	% Total	albumins	Zeins	common peaks	% Total
CO441	47	42	15	17	8	13	2	10
CO354	48	42	12	13	11	13	2	8
F1	45	38	15	18	13	8	2	10

Table 4. Comparison of numbers of the peaks detected from different fractions albumins vs globulins and albumins vs zeins spotted on CM10 and Gold array respectively and analyzed by SELDI-ToF.

In particular albumins and globulins spectra comparison on the same CM10 array revealed about 25% of the protein in common between the two fractions in the range (2-14 KDa) and about 10% in the range (14-30 KDa). Albumins and zeins fraction showed instead about 15% in the range (2-14 KDa) and about 10% in the range (14-30 KDa) of peak in common. It is interesting to note that protein with a higher molecular weight were better separated during the extraction protocols with a less percentage of common peaks. Peaks detected by using different chip arrays also revealed common signal by using different surfaces, thus determined a decrease in the number of signals detected that were: 130, 125, 115 for albumins; 86, 110, 124 for globulins and 95, 87, 90 for zeins fraction for the CO441, CO354 and F1 lines respectively (Tab 5).

Albumins						
	CM10	Gold	IMAC		Total	
	Peak detected	Peak detected	Peak detected	Unique Peak	Common Peak	% Tot
CO441	69	54	67	92	38	29
CO354	66	58	65	82	43	34
F1	63	56	47	78	37	32
Globulins						
	CM10	NP20	IMAC		Total	
	Peak detected	Peak detected	Peak detected	Unique Peak	Common Peak	% Tot
CO441	53	30	45	55	31	36
CO354	64	51	52	70	40	36
F1	54	52	52	74	25	25
Zeins						
	Gold	Q10			Total	
	Peak detected	Peak detected		Unique Peak	Common peak	% Tot
CO441	55	48		87	8	8
CO354	57	41		76	11	13
F1	48	50		72	8	10

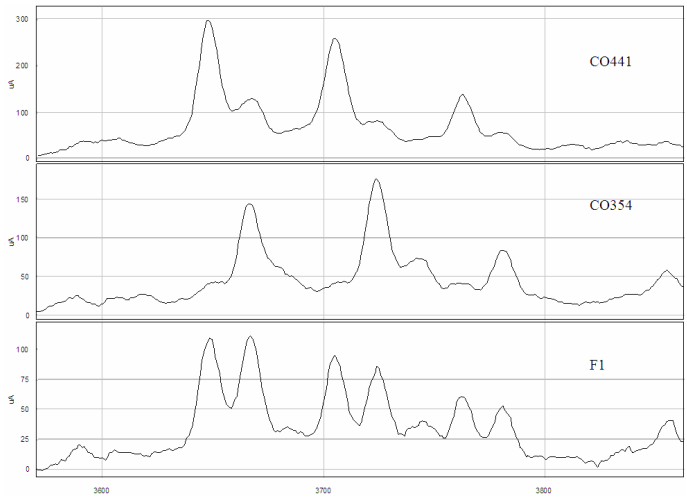
Table 5 Numbers of peaks detected by SELDI-ToF for different fraction albumins, globulins and zeins in the maize lines CO354, CO441 and F1 using different chip arrays CM10, Gold, IMAC, Q10

Differences in the kernel protein between resistant and susceptible lines were determined by processing the spectra acquired with Ciphergen software in order to obtain the differentially expressed peaks for each fraction and chip surfaces for CO441 and CO354. An example of the output from the Ciphergen Express software is shown in Table 6, where was reported for the albumins analyzed on CM10 array all the differentially expressed peaks and their significativity. Significant peaks (P-value < 0.05) were further filtered by manual detection and confirmed by their presence in F1 inbreed line (Fig. 13-A). A total of 64 peaks belongs to different fractions were seen to be differentially expressed between the two lines of which 39 for albumins, 21 for globulins and 13 for zeins respectively (Fig 13-B). CO441 and CO354 showed a high number of protein peaks that were differentially expressed between two lines. Finally other four lines (CO430, CO443, GA209, T202) with different susceptibility to *Fusarium* infection were profiled by SELDI-ToF to evaluate which protein peaks could potentially be associated to *Fusarium* resistance (Fig 14).

2-14 Kda		2-14 Kda		14-30 Kda	
m/z	p-value	m/z	p-value	m/z	p-value
3071	0,050	7633	0,050	14931	0,050
3293	0,050	7830	0,050	15026	0,050
3325	0,050	7859	0,050	15135	0,050
3536	0,050	7890	0,050	15700	0,050
3648	0,050	8080	0,050	16331	0,050
3667	0,050	8341	0,050	16570	0,050
3705	0,050	8529	0,050	16859	0,050
3724	0,050	8676	0,050	17040	0,050
3763	0,050	9366	0,050	17115	0,050
3781	0,050	9420	0,050	17704	0,050
3855	0,050	9577	0,050	18500	0,050
3919	0,050	9837	0,050	18616	0,050
3935	0,050	9997	0,050	18722	0,050
4107	0,050	10924	0,050	18909	0,050
4125	0,050	10981	0,050	19164	0,050
4364	0,050	11449	0,050	19382	0,050
4841	0,050	13217	0,050	21040	0,050
4989	0,050	13436	0,050	22003	0,050
5486	0,050	3522	0,127	22199	0,050
5655	0,050	4892	0,127	22397	0,050
5684	0,050	10270	0,127	22464	0,050
5914	0,050	11836	0,127	22690	0,050
6157	0,050	12630	0,127	24528	0,050
6188	0,050	3119	0,275	25088	0,050
6613	0,050	4859	0,275	26008	0,050
6676	0,050	5133	0,275	26650	0,050
6928	0,050	7330	0,275	26820	0,050
7016	0,050	9737	0,275	27004	0,050
7103	0,050	3912	0,513	27474	0,050
7203	0,050	7378	0,513	29845	0,050
7236	0,050	9500	0,513	14558	0,127
7274	0,050	13129	0,513	16952	0,127
7346	0,050	4457	0,827	18257	0,127
7362	0,050	4600	0,827	27627	0,127
7417	0,050	4729	0,827	19122	0,513
7479	0,050	8049	0,827	23726	0,513
7515	0,050			15671	0,827

Table 6 Output from the Ciphergen Express software for albumin fraction analyzed on CM10 array. Differentially peaks detected in two mass ranges (2-14 KDa and 14-30 KDa) differentially expressed between CO441 and CO354 lines. m/z represented the ratio between mass and charge for each peak detected and p-value the significativity of the differential peak observed,

A



B

	Peak detected	Differential peak p<0.05	Differential peak confirmed	Total differential peak
Albumins				
CM10	110	85	21	39
Gold	98	89	15	
IMAC	109	86	16	
Globulins				
CM10	93	68	7	21
NP20	70	39	8	
IMAC	80	61	10	
Zeins				
Gold	82	55	10	13
Q10	77	37	3	
Albumins + Globulins + Zeins				64

Figure 13 A) Example of differentially peaks expressed by the two parental lines (CO441 and CO354) and by the F1 inbreed, B) Table of differentially peak detected between the CO441 and CO354 lines and confirmed in the F1 lines.

The protein profiling showed a high level of polymorphism between different lines that limit the biomarker validation using the chipergen software. To asses the potential differential peaks expressed between the resistant and susceptible lines, all spectra were manually compared by analyzing the 64 signals peaks previously obtained by the CO441 and CO354 in the CO430, CO443, GA209 and T202.

From 64 peaks differentially peaks expressed between the CO354 and CO441 lines only two peaks at 14.96 and 5.79 were seen over-expressed in the susceptible lines (Fig 14).

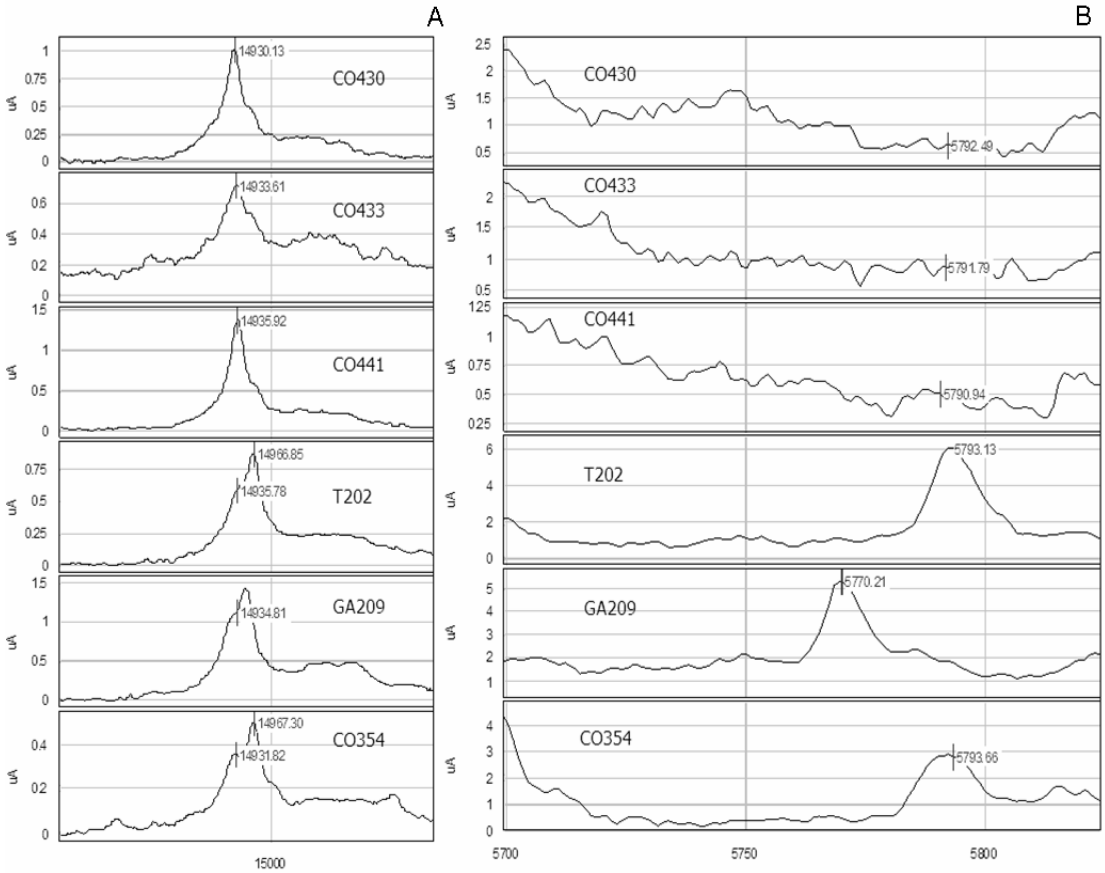


Figure 14. Protein profiling of CO430, CO433, CO441, T202, GA209, Co354 (top-down). A) Differential peaks detected at 14.955 KDa in the susceptible lines from albumins fraction analyzed on Gold array.B) Differential peaks detected at 5.793 KDa in the susceptible lines from globulins fraction analyzed on IMAC array.

1.2 HPLC-SDS-PAGE characterization

The albumin fraction from the two parental CO354 and CO441 lines and their inbreed were also characterize by using another approach. Protein extracts were separated by ReversePhase-chromatography and each fraction was then detected by SDS-PAGE. This method differs to 2D-Page techniques by the fact that in the first dimension protein were separated by their hydrophobic index rather than isoelectric point (Fig. 15). Each fraction was then analyzed in SDS-Page (Fig. 16).

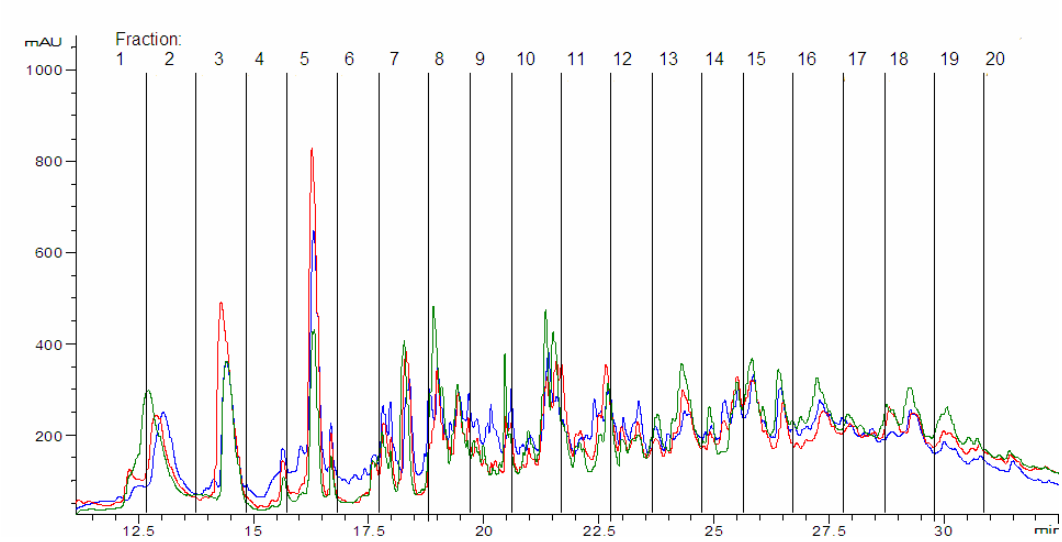


Figure 15 RP-HPLC profile of albumin fraction from different lines: CO354 in green, CO441 in blue and F1(CO354xCO441) in red.

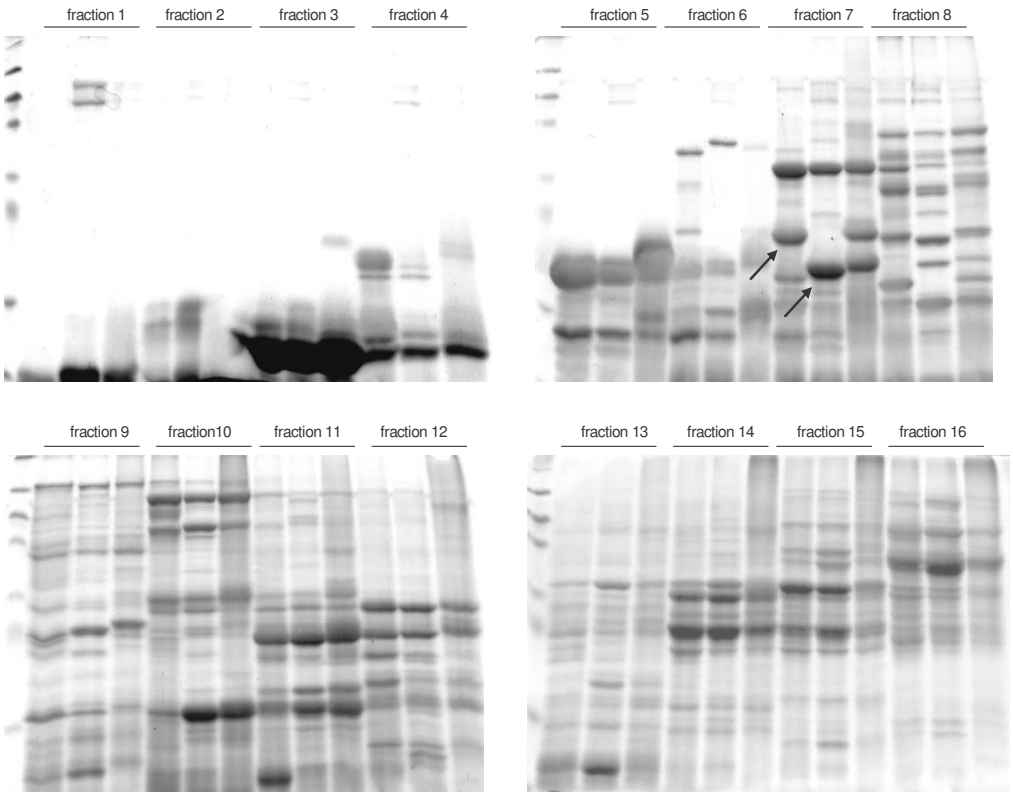


Figure 16 SDS-PAGE analysis of fractions collected from albumins extract separated on RP-HPLC. For each fraction in order: CO441, CO354, CO441XCO354 lines.

SDS-PAGE analyses revealed different protein that were differentially expressed in the resistant and susceptible line (as example arrow in Fig 16), that were confirmed by the contemporary presence in the inbreed line. The differentially expressed protein was identified as late embryogenesis abundant protein, group 3 LEA 3. Other resistant CO430, CO443 and susceptible GA209, T202 lines were than analyzed by using the same techniques to evaluate protein related to resistance. However, the comparison between the CO441 and CO354 lines and other lines with different susceptibility to *Fusarium* infection didn't show proteins that were present exclusively in resistant or susceptible lines possibly related to the fungus resistance (Fig 17).

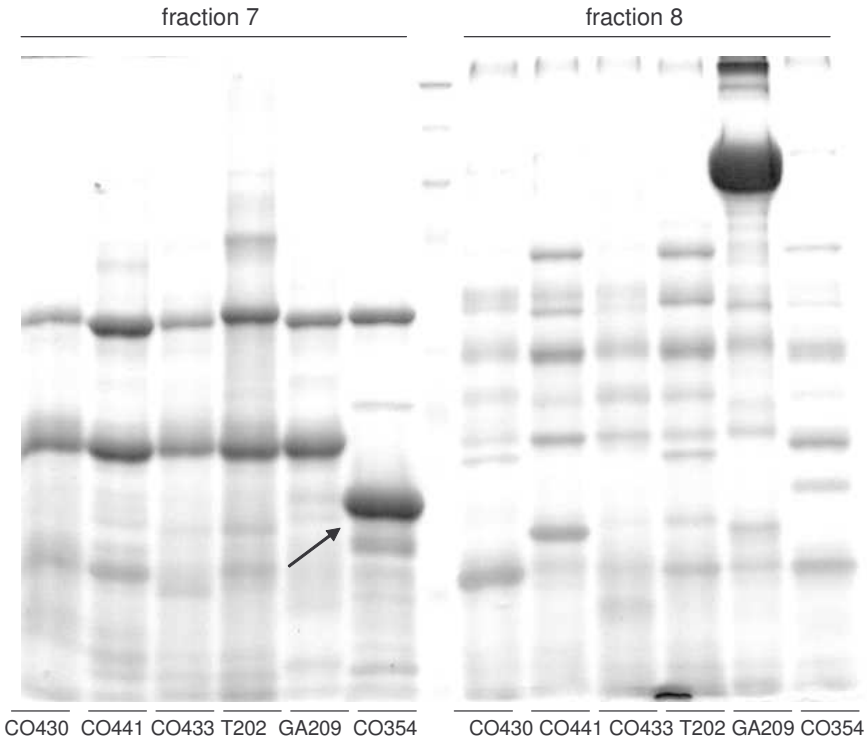


Figura 17 SDS-PAGE analysis of fractions seven and eight collected from albumins extract of different lines separated on RP-HPLC.

2 Protein characterization and TPS23 expression in maize roots associated to *D.virgifera* resistance

The second part of the Ph.D. project consisted in the characterization of maize lines having different expression in Terpene Synthase 23 TPS23, an enzyme involved in (E)-beta-caryophillene production and associated to *Diabrotica virgifera* resistance. In order to understand the molecular mechanism involved in maize *D. virgifera* defence; different maize lines, that were grown in hydroponic condition or in soil under different treatment, were characterized by protein profiling and for TPS23 expression at root level.

2.1 Hydroponic: Which mechanisms are involved in Biotic stress response in Maize?

Maize biotic stress response was studied in the B97 line growth in hydroponic condition. Plants were treated by wounding after mechanical root damage or in presence of different compounds. 100µM Methyl Jasmonate (MeJa) and 500µM Salicilic Acid (SA) added to the nutrient solution. Triplicate samples at different time-course point 0 h, 4 h, 8, 24, 48, 96 were collected and analyzed for protein expression profiling by SELDI-ToF and TPS23 expression by PCR (Fig. 18).

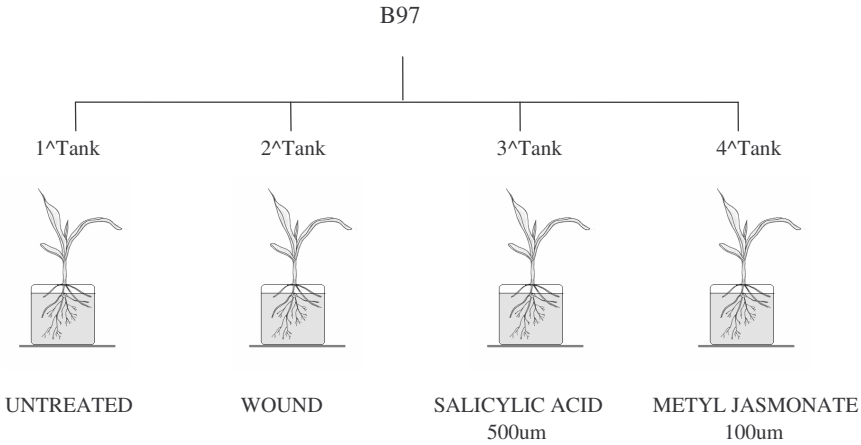


Figure 18. Hydroponic experiment with B97 maize line. Plants were treated by wounding, with 100µM MeJa and 500µM SA.

Plants grown in hydroponic system and treated in different condition showed phenotype alteration, clearly visible after 48 h. After wounding plants showed more branched roots, MeJA retarded root development and SA drastically alter the root system with roots very thin that tend to jellify (Fig. 19).

Figure 19 Phenotype of B97 Zea mays line growth in hydroponic system and treated in different condition.

SELDI ToF-MS showed different protein profiling changing for MeJA and SA treatment, corresponding to phenotype alterations (Fig. 20). Both chemical compounds altered the protein peak patterns starting from 4 h after treatment, when no plant phenotypes changing were appreciable. However plants responded differently to both treatment with specific protein peaks expression: MeJA induced a peak at 7141 Da while SA two peaks at 4780 and 7412 Da; probably indicating different mechanism of action in response of two compounds. Phenotype alterations were observed after 8h and became more visible at 48 and 96 hours. However if MeJA only retard roots development, SA profoundly altered the root system.

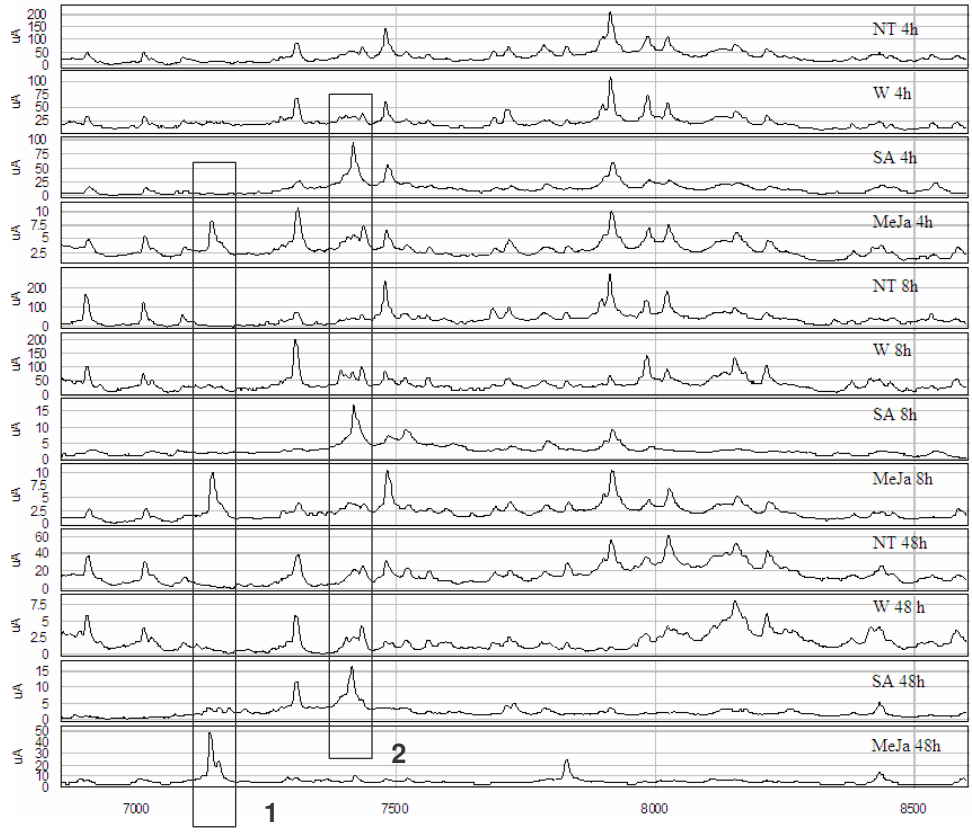


Figura 20 SELDI-ToF protein profiling of B97 line under MeJa, Sa or Wound treatments for 4h, 8h and 48h.

As observed for phenotype alteration, protein profiling in a time course experiment showed many changes with a high number of differential peaks expressed by B97 line after treatment (Tab. 7). SA induced a rapid variation with thirteen differential peaks after 8 hours while MeJA showed only one peak at the same time point. In the late time-course experiment, after 48 hours of SA and MEJA treatment 35 and 28 peaks were respectively over-expressed, of which thirteen were in common. Plants responded for each treatments with the expression of unique protein peaks; but also with some common response probably due to the prolonged exposure to both compounds.

MeJA						SA					
4 h		8 h		48 h		4 h		8 h		48 h	
UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN	UP	DOWN
7141		**7141**		3474	4872	4780		4578	5411	4984	5413
				3562	5669	7412		5217	**6303**	5218	6304
				5808	5758			**7141**	8021	5449	6327
				6893	**6304**			7413	**9055**	6117	6638
				7007	**9058**				**9458**	**7143**	6808
				7143	**9461**				10552	**7159**	6834
				7159	**10006**				10622	7178	**6893**
				7208	11333				18270	**7208**	6906
				7292	11431				19987	**7292**	7016
				7367						7308	**9058**
				7422						**7367**	9151
				7787						7414	**9461**
				7796						**7422**	**10006**
				7829						**7787**	10557
				8432						**7796**	10625
				8594						9615	10784
				14276						11729	14978
				14385							18288
				14606							

Table 7 SELDI-ToF peaks differentially expressed by B97 lines under MeJA and SA treatment after 4, 8 and 48 hours.

(E)-beta-caryophyllene synthase level was determined after each treatment in order to evaluate which type of stresses could give a response similar *D. virgifera* attack in plant. At each time point the RNA was collected, and after retro-transcripion amplified by PCR using specific primers for TPS23 and primers for Zm-TSA (tryptophan synthase α subunit) as reference gene. Roots from B97 maize plant under standard hydroponic condition did not express the TPS23 gene. After wounding or SA treatment TPS23 level did not increase, however starting from 4 hour (E)-β-caryophyllene synthase was strongly induced, and the gene was highly expressed from 8 to 48 hour with MeJA treatment (Fig. 21).

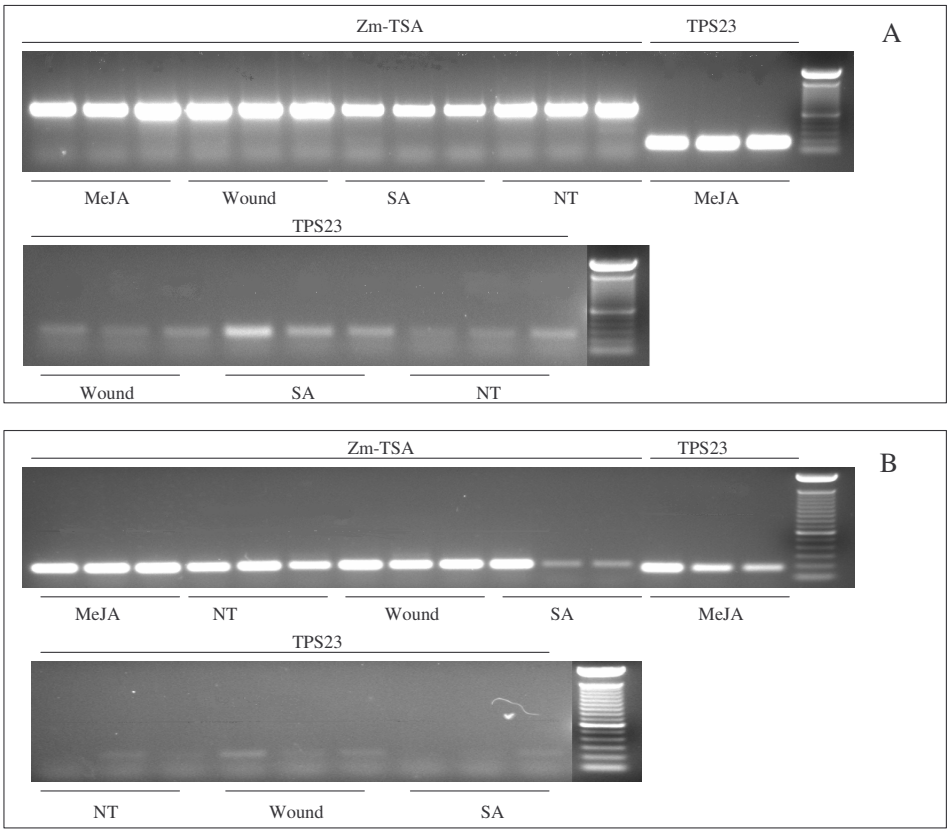


Figure 21 TPS23 and Zm-TSA (housekeeping) gene expression detected by PCR in B97 line under different treatments (NT not treated, MeJa methyl jasmonate, SA salicylic acid and W wound . A) after 8h of treatment, B) after 48h of treatment.

2.2 Hydroponic: How Diabrotica resistant and susceptible lines respond to MeJA

First experiments indicated that maize responded to MeJa by inducing specific mechanism and through a TPS23 response. In order to evaluate that *D. virgifera* defence mechanism could be mediated by JAs pathways; we tested other maize lines with different susceptibility to *D. virgifera*. Six maize lines of which three B97, NC358 and F2 TPS23 positive and three Mo17, Oh43 and B73 negative for TPS23 expression were treated with 100µM MeJA for 24 and 48 hours and evaluated for SELDI-ToF and RP-HPLC/SDS-PAGE protein profiling, and *tps23* gene expression.

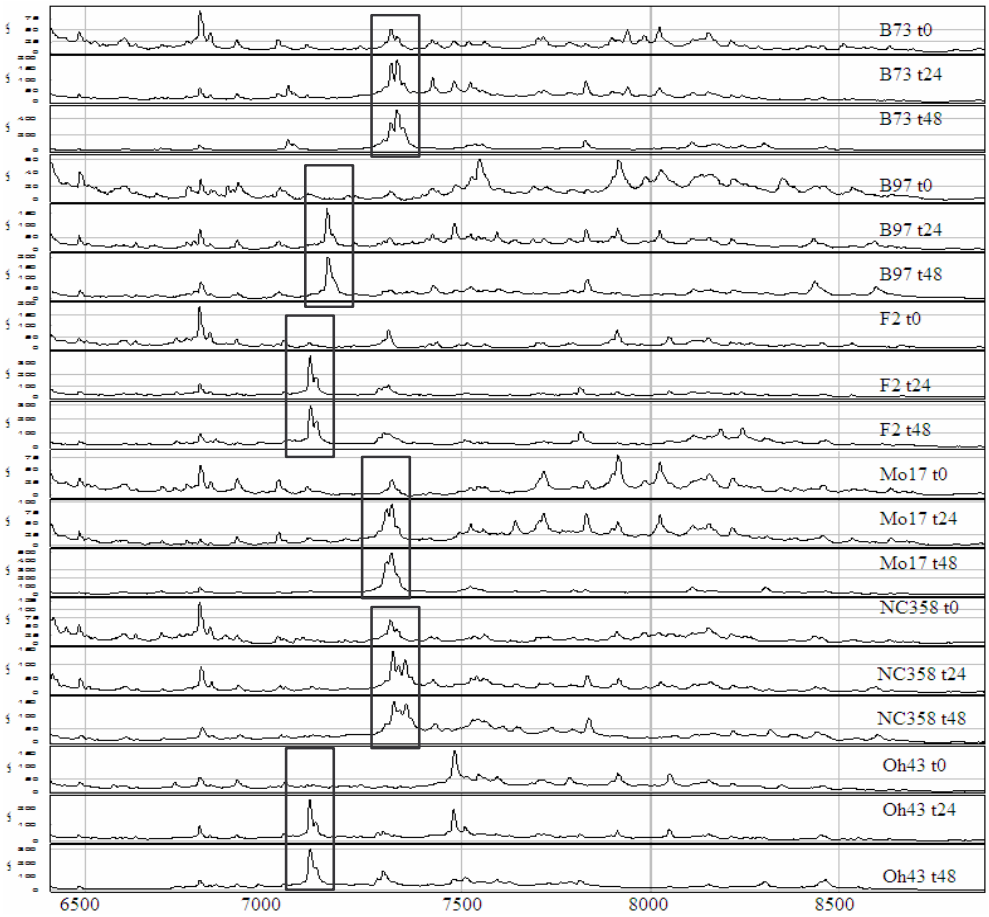


Figure 22. SELDI-ToF protein profiling of different maize lines after 14, 48 h methyl jasmonate treatment.

The protein profiling confirmed previously results, with the B97 lines that expressed a peak at 7140 Da during MeJA treatment. All other maize lines showed protein profiling variation due to MeJA treatment (Fig.22). Each line expressed specific differentially protein peaks, without common up or down regulated peaks (Tab. 8). The number of differential peaks increase from 24 to 48 hour, indicating a strong effect during the time course of treatment. Each line showed differentially protein peaks with similar molecular weights but that differ about hundreds of Dalton. These peaks in the same range mass could represent same polymorphic proteins, consistent with a similar response to MeJA e in all the line tested.

B97				NC358				F2			
24 h		48 h		24 h		48 h		24 h		48 h	
up	down	up	down	up	down	up	down	up	down	up	down
7140		7140	18268	7340		7339		7092		7093	12224
7590		7827		7824		7823		7108		7108	
7827		8593		14356		8304		7809		7809	
14258		14260				8589				8179	
14370		14370				14243				8236	
		14470				14356				10196	
						14449					

B73				Mo17				Oh43			
24 h		48 h		24 h		48 h		24 h		48 h	
up	down	up	down	up	down	up	down	up	down	up	down
7035		7036	6297	7309		7298	18268	7095		7095	10508
7307		7308	9146	14256		7310	19958	14457		7289	18261
7324		7326	9456	14372		7519				8299	
7823		7532	9567			8108				14250	
		7824	12223			8302				14326	
		8107	18257			14257				14459	
		8238	19354			14375				14576	
		8299	21236			14594					
		8460									
		13352									

Table 8 SELDI-ToF peaks differentially expressed by different maize lines under methyl jasmonate treatment after 24 and 48 hours.

RP-HPLC/SDS-PAGE protein profiling confirmed that after 48h of MeJA treatment different proteins were commonly up-regulated for all the line tested (Fig. 23).

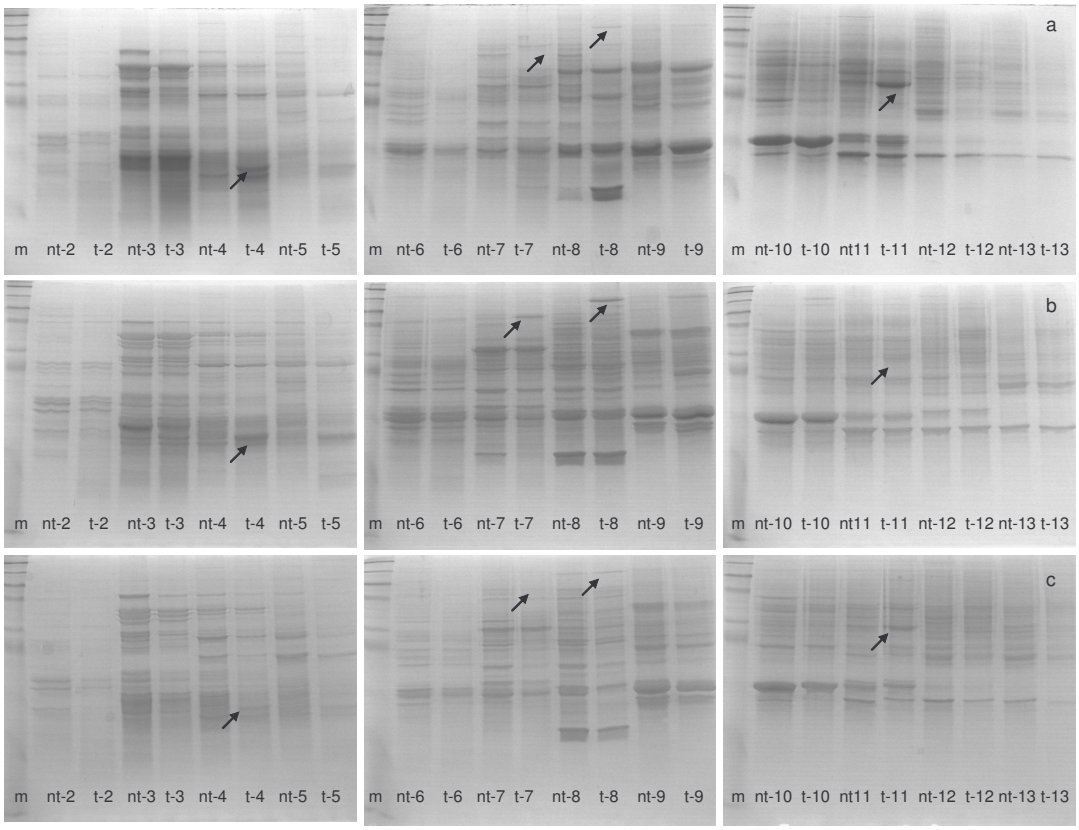


Figure 23 RP-HPLC/SDS-PAGE protein profiling of a)B97, b)NC358 and c)Mo17, after 48h methyl jasmonate treatment. For each SDS line in black were reported: m (marker) treatment (t, treated; nt, not treated)-RP-HPLC fraction (2,13).

These bands were excise and were identified as: actin-depolymerizing factor 3 (Fig. 24), a fragment of hypothetical protein LOC100191759 (Fig. 25), hypothetical protein LOC100191759 (Fig. 26), hypothetical protein LOC100194034 (Fig 27).

MATRIX SCIENCE Mascot Search Results

Protein View

Match to: **gi|162459533** Score: **531**
actin-depolymerizing factor 3 [Zea mays]
Found in search of BandaAGel1.mgf

Nominal mass (M_r): **16004**; Calculated pI value: **5.46**
NCBI BLAST search of gi|162459533 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Zea mays
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|17366520 from Zea mays
gi|1419370 from Zea mays
gi|194692910 from Zea mays
gi|195605882 from Zea mays
gi|195618220 from Zea mays
gi|195625550 from Zea mays
gi|195652823 from Zea mays
gi|238013380 from Zea mays
gi|238015232 from Zea mays

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **82%**

Matched peptides shown in **Bold Red**

```
     1 MANARSGVAV NDECMLKFGE LQSKRLHRFI TFKMDDKFKE IVVDQVGDRA
    51 TSYDDFTNSL PENDCRYAIY DFDFVTAEDV QKSRIFYILW SPSSAKVKSK
   101 MLYASSNQKF KSGLNGIQVE LQATDASEIS LDEIKDRAR
```

Figure 24 Mascot search Results for actin-depolymerizing factor 3.

MATRIX SCIENCE Mascot Search Results

Protein View

Match to: **gi|212274709** Score: **949**
hypothetical protein LOC100191759 [Zea mays]
Found in search of BandaBGel2.mgf

Nominal mass (M_r): **70778**; Calculated pI value: **5.53**
NCBI BLAST search of gi|212274709 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Zea mays
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|194689760 from Zea mays
gi|194690590 from Zea mays

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **38%**

Matched peptides shown in **Bold Red**

```
     1 MGREAPAMAV ALLAALVASA AMFMLAGTAS ASPAEGIQPL SKIAVHRATV
    51 EMQPSAYVRA TPSLLGEQGE DIEWVTVKFG WKEPSEDDWI GVFSPSEFNS
   101 SATCPNPWPA EEPYLCTAPI KYQFANYSAN YIYWGKGSIR LQLINQRSDF
   151 SFALFTGGLS NPRLIAVSEP ISFKNPKAPV FPRLAQGTSH DEMIVTWISG
   201 YAIDEAYPFV EWGALVAGGV RHTARAPAGT LTFNRGSMCG EPARTVGWRD
   251 PGFIHTAFLR DLWPNKEYHY RIGHELPDGS VVWGKPYSFR APPSPGQPSL
   301 QRVIVFGDMG KAERDGSNEY AAYQPGSLNT TDALIADLDN YDIVFHIGDM
   351 PYANGYISQW DQFTAQVAPI TARKPYMVGS GNHERDWPDT AAFWDVMDSG
   401 GECGVPAETY YYYPAENRAN FWYKVDYGMF RFCVGDSEHD WRVGTPQYDF
   451 IEHCLSTVDR KHQPWLIFAT HRVLGYSSNA WYAGEGSFEE PEGRENLQRL
   501 WQKYRVDIAF FGHVHNYERT CPMYQSQCMT SEKTHYSGTM NGTIFVVAGG
   551 GGCHLSSYTT AIPKWSIYRD YDFGFVKLTA FNHSSLLFEY KKSSDSKVYD
   601 SFTIDRDYRD VLRCVHDSCF PTTLAT
```

Figure 25 Mascot search Results for hypothetical protein LOC100191759 fragment.

Mascot Search Results

Protein View

Match to: **gi|212274709** Score: **1274**
hypothetical protein LOC100191759 [Zea mays]
Found in search of BandaCGel2.mgf

Nominal mass (M_r): **70778**; Calculated pI value: **5.53**
NCBI BLAST search of gi|212274709 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Zea mays
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|194689760 from Zea mays
gi|194690590 from Zea mays

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **47%**

Matched peptides shown in **Bold Red**

```
    1 MGREAPAMAV ALLAALVASA AMFMLAGTAS ASPAEGIQPL SKIAVHRATV
   51 EMQPSAYVRA TPSLLGEQGE DTEWVTVKFG WKEPSEDDWI GVFSPSEFNS
  101 SATCPNPWPA EEPYLCTAPI KYQFANYSAN YIYWGKGSIR LQLINQRSDF
  151 SFALFTGGLS NPRLIAVSEP ISFKNPKAPV FPRLAQGTSH DEMTVTWISG
  201 YAIDEAYPFV EWGALVAGGV RHTARAPAGT LTFNRGSMCG EPARIVGWRD
  251 PGFIHTAFLR DLWPNKEYHY RIGHELPDGS VVWGKPYSFR APPSPGQPSL
  301 QRVIVFGDMG KAERDGSNEY AAYQPGSLNT IDALIADLDN YDIVFHIGDM
  351 PYANGYISQW DQFTAQVAPI TARKPYMVGS GNHERDWPDT AAFWDVMDSG
  401 GECGVPAETY YYYPAENRAN FWYKVDYGMF RFCVGDSEHD WRVGTPQYDF
  451 IEHCLSTVDR KHQPWLIFAT HRVLGYSSNA WYAGEGSFEE PEGRENLQRL
  501 WQKYRVDIAF FGHVHNYERT CPMYQSQCMT SEKIHYSGIM NGTIFVVAGG
  551 GGCHLSSYTT AIPKWSIYRD YDFGFVKLIA FNHSSLLFEY KKSSDSKVYD
  601 SFTIDRDYRD VLRCVHDSCF PITLAT
```

Figure 26 Mascot search Results for hypothetical protein LOC100191759.

Mascot Search Results

Protein View

Match to: **gi|212722794** Score: **907**
hypothetical protein LOC100194034 [Zea mays]
Found in search of BandaDGel3.mgf

Nominal mass (M_r): **38041**; Calculated pI value: **6.22**
NCBI BLAST search of gi|212722794 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Zea mays
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|194694754 from Zea mays

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Gln->pyro-Glu (N-term Q),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **61%**

Matched peptides shown in **Bold Red**

```
    1 MKCKMASSSR VVAAAAVLVA FCAAALSSAT VTVNEPIVNG LSWSFYDASC
   51 PSVEGIVRWH VADALRRDIG IAAGLIRIFF HDCFPQGCDA SVLLSGSNSE
  101 QKQGPNQTLR PEALKLIDDI RAAVHAACGP KVSCADITTL ATRDAVVASG
  151 GPFFEVPLGR RDGLSPASSD QVFTLPGPDF DVPTLLAAFK NRSLDTADLV
  201 ALSGAHTVGR GHCSSFTSRL PPNADDGTMD PAFRRTLAAK CAKDASAAQV
  251 LDVRTPNAFD NKYYFDLIAK QGLFKSDQGL INDQTTKRAA TRFALNQAAF
  301 FDQFARSMVK MSQMDVLTGN AGEVRLNCAV RNAARVVSAD QLETAAGDEG
  351 LAADA
```

Figure 27 Mascot search Results for hypothetical protein LOC100194034.

Finally, all maize lines were tested to evaluate the TPS23 gene expression after MeJA treatment (Fig. 28). Unexpected the (E)-beta-caryophyllene synthase levels were high for all lines tested, that was in contrast with previous data (Köllner et al. 2008) describing B73, Mo17 and Oh43 as lines in which (E)-β-caryophyllene defence signal was lost during breeding. Notwithstanding the data indicating that these lines were susceptible to *D. virgifera* has been obtained by direct attack of the larvae in plant in soil.

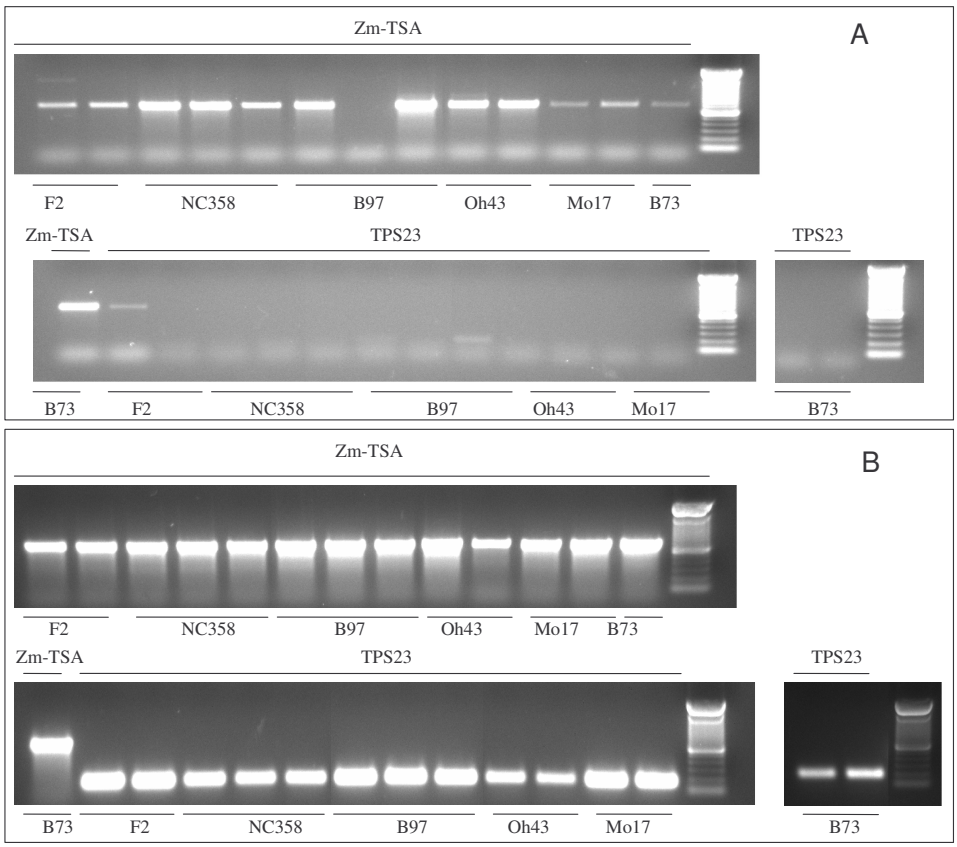


Figure 28 TPS23 and Zm-TSA (housekeeping) gene expression in different maize lines under methyl jasmonate treatment. A) Not treated, B) after 24h of treatment.

2.3 Diabrotica in vitro experiment: Does Diabrotica resistant and susceptible lines express TPS23 gene?

Data from previous experiments indicated that all lines tested response to MeJA treatment by direct synthesis of specific protein (by SELDI-ToF and RP-HPLC/SDS-PAGE) and expressing *tps23* gene (PCR). However in previous experiment (Köllner et al. 2008), the three susceptible lines (B73, Mo17 and Oh43), was defined unable to express the (E)-beta-caryophyllene synthase. To verify this hypothesis all the lines were tested for direct damage by Diabrotica larvae using the protocol previously described (Köllner et al. 2008). Plants were growth in soil and infested with Diabrotica larvae in order to evaluate the protein profiling variation and the TPS23 transcription level. After 48h from Diabrotica larvae infestation no relevant variations in the protein profiling were observed for all the line tested. The *tps23* gene however was amplified in different lines, B97, B73, NC358, F2, Mo17 with different intensities (Fig. 29).

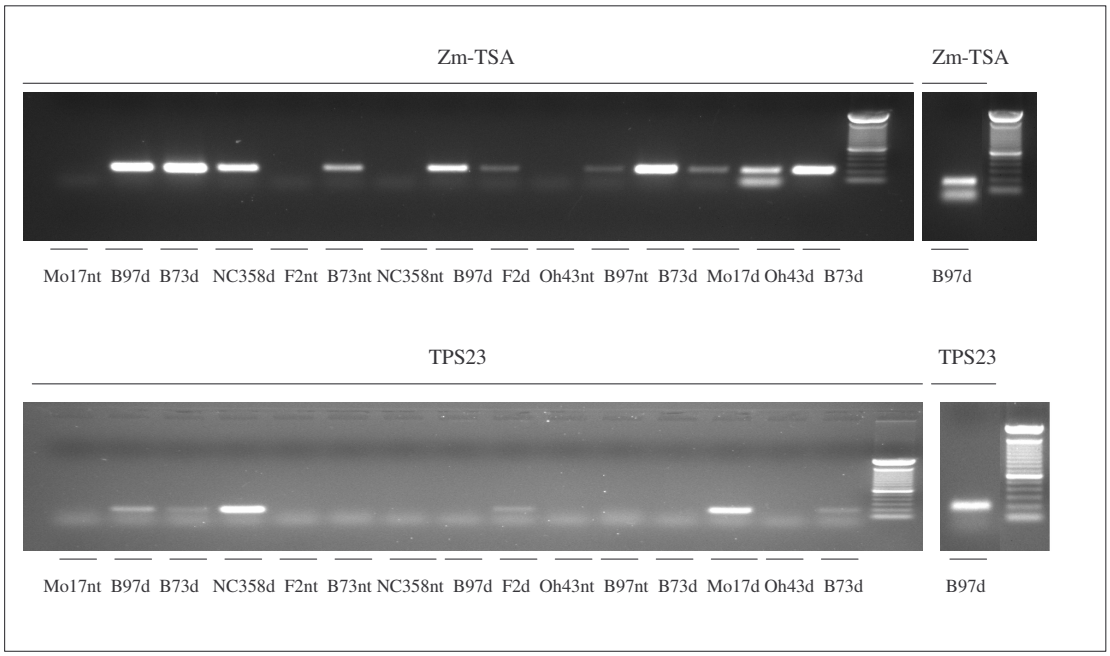


Figure 29 TPS23 and Zm-TSA (housekeeping) gene expression in different maize lines after 48 h *D. virgifera* larvae infestation.

2.4 Hydroponic: Does maize plant respond quantitatively to MeJA?

TS23 expression after *D. virgifera* larvae attack varied in intensities between different lines. In order to evaluated that TPS23 expression in the line tested could respond quantitatively to MeJA, we treated plant in hydroponic experiment with different amount of MeJA and for different time of exposure. Plants were treated with 10 µM MeJa for 24 hour or 100 µM MeJa for 2 hour and collected after 24 hours. SELDI-ToF profiling revealed variation in the protein expression consistent with previous results in which lines were treated with 100 µM MeJa for 24 and 48 hours (Tab. 8). The milder treatment showed low alteration in the protein profiling, however with the most significant peaks for each lines (B73, 7042 Da; B97, 7143 Da; F2, 7099 Da; Mo17, 7311 Da; 7097, Oh43; 7345, NC358), that vary in intensities compared to the untreated and previous treatment (Fig 30). The peak intensities seem to be related to the intensities of the treatment.

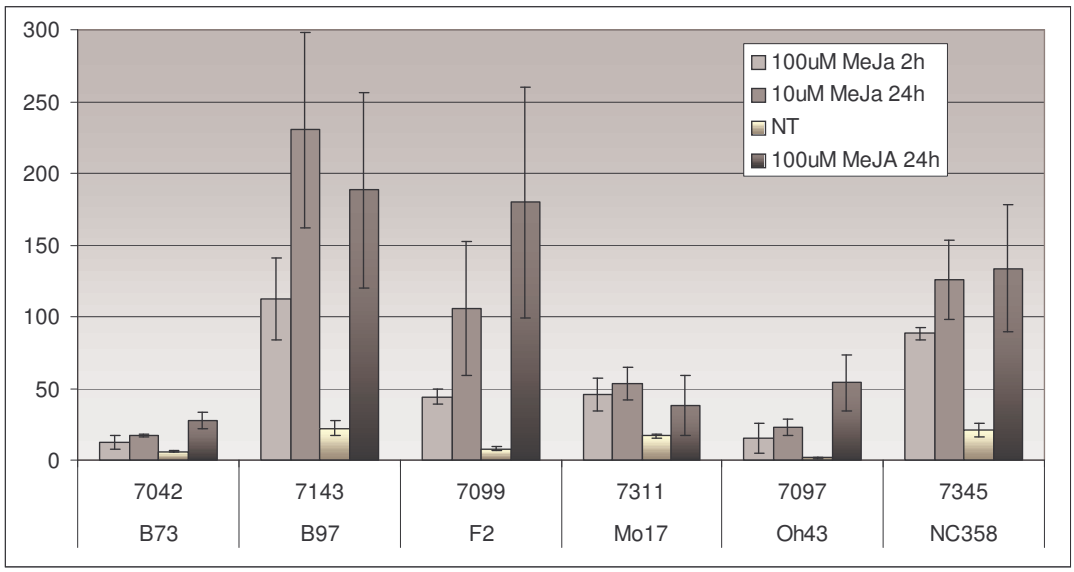


Figura 30 Intensities of differentially peaks expressed by different maize lines after different treatments with methyl jasmonate. In x-assis are reported the m/z of the peaks and the corresponding lines.

TPS23 expression in different lines was in accordance with proteomic profiling variation that resulted in a semi-quantitative response in function of the severity of

the treatment. In particular two lines Oh43 and B73 showed a lower TPS23 expression after 10 µM MeJa for 24 hours and 100 µM MeJa for 2 hours treatment (Fig. 31).

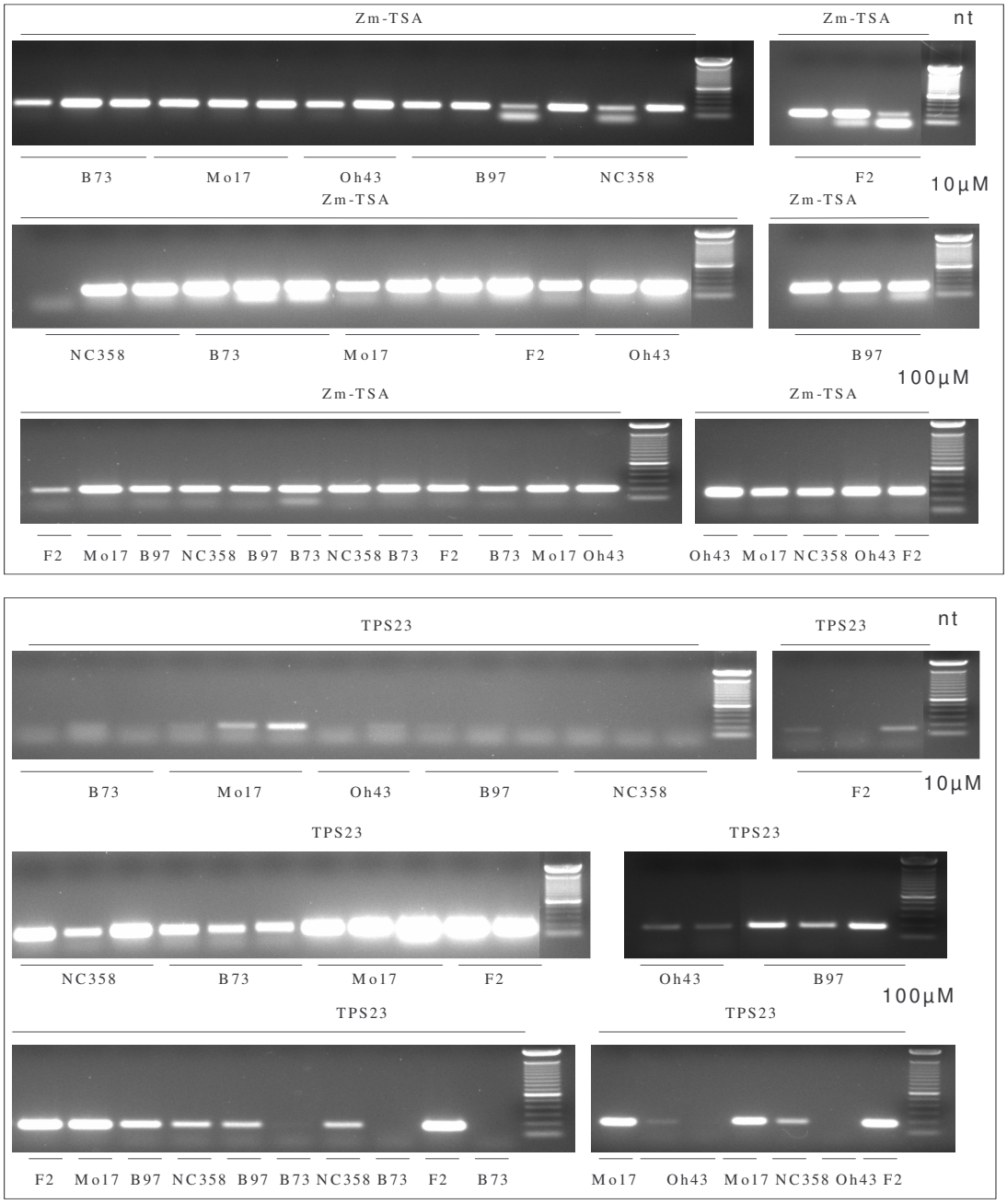
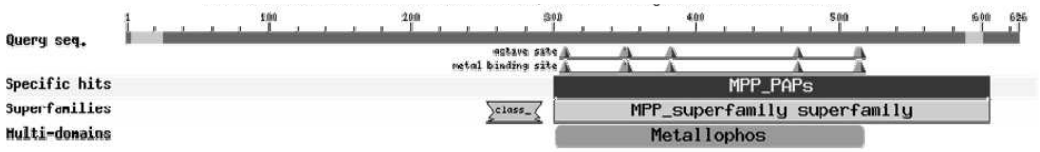


Figure 31 TPS23 expression in different maize lines after methyl jasmonate MeJa treatment (nt-not treated, 10 µM-10 µM MeJa for 24 hours, 100-100 µM MeJa for 2 hours)

2.5 Identification of hypothetical protein

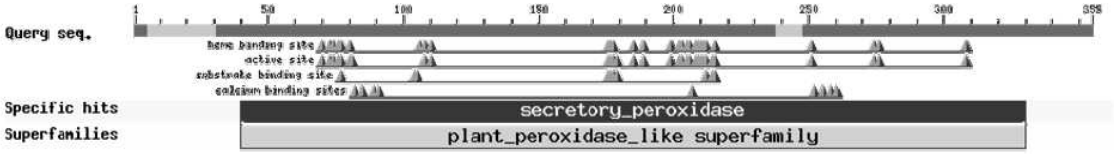
Maize lines expressed different proteins after MeJa induction by RP-HPLC protein profiling. Only one of them was identified directly as actin-depolymerizing factor 3. The other two proteins the hypothetical protein LOC100191759 and hypothetical protein LOC100194034 were blasted against NCBI database in order to find similarity with other known proteins sequence.
Protein blast of hypothetical protein LOC100191759 revealed similarity with the enzyme Nucleotide Pyrophosphatase/Phosphodiesterase NPP and Purple acid Phosphatase PAP (Fig 32).



Accession	Description	Max score	Total score	Query coverage
gi|212274709|NP_001130657.1	hypothetical protein LOC100191759 [Zea mays] >gi|194689760|gb|ACF78964.1| unknown [Zea mays] >gi|194690590|gb|ACF79379.1| unknown [Zea mays]	1254	1254	95%
gi|242079829|XP_002444683.1	hypothetical protein SORBIDRAFT_07g025980 [Sorghum bicolor] >gi|241941033|gb|EES14178.1| hypothetical protein SORBIDRAFT_07g025980 [Sorghum bicolor]	1171	1171	95%
gi|242082099|XP_002445818.1	hypothetical protein SORBIDRAFT_07g026270 [Sorghum bicolor] >gi|241942168|gb|EES15313.1| hypothetical protein SORBIDRAFT_07g026270 [Sorghum bicolor]	1151	1151	95%
gi|326505368|BAK03071.1	predicted protein [Hordeum vulgare subsp. vulgare]	1039	1039	95%
gi|326506570|BAJ91326.1	predicted protein [Hordeum vulgare subsp. vulgare]	1039	1039	95%
gi|326528861|BAJ97452.1	predicted protein [Hordeum vulgare subsp. vulgare]	1036	1036	95%
gi|115477469|NP_001062330.1	Os08g0531000 [Oryza sativa Japonica Group] >gi|42407885|dbj|BAD09026.1| putative diphosphonucleotide phosphatase [Oryza sativa Japonica Group] >gi|42407987|dbj|BAD09125.1| putative diphosphonucleotide phosphatase [Oryza sativa Japonica Group] >gi|111036652|dbj|BAF02354.1| nucleotide pyrophosphatase [Oryza sativa Japonica Group] >gi|113624299|dbj|BAF24244.1| Os08g0531000 [Oryza sativa Japonica Group] >gi|215704117|dbj|BAG92957.1| unnamed protein product [Oryza sativa Japonica Group] >gi|218201506|gb|EEC83933.1| hypothetical protein OsI_30014 [Oryza sativa Indica Group] >gi|222640919|gb|EEE69051.1| hypothetical protein OsJ_28052 [Oryza sativa Japonica Group]	1032	1032	95%
gi|224122002|XP_002318726.1	predicted protein [Populus trichocarpa] >gi|222859399|gb|EEE96946.1| predicted protein [Populus trichocarpa]	875	875	94%
gi|304421408|ADM32503.1	purple acid phosphatases [Glycine max]	874	874	94%
gi|212722374|NP_001132102.1	hypothetical protein LOC100193518 [Zea mays] >gi|194693428|gb|ACF80798.1| unknown [Zea mays]	871	871	95%
gi|242072590|XP_002446231.1	hypothetical protein SORBIDRAFT_06g006920 [Sorghum bicolor] >gi|241937414|gb|EES10559.1| hypothetical protein SORBIDRAFT_06g006920 [Sorghum bicolor]	870	870	95%
gi|195614828|ACG29244.1	nucleotide pyrophosphatase/phosphodiesterase [Zea mays]	869	869	95%

Figure 32 Protein blast of hypothetical protein LOC100191759

The hypothetical protein LOC100194034 showed similarity with anionic peroxidase (Fig 33).



Accession	Description	Max score	Total score	Query coverage
gi|212722794|NP_001132567.1	hypothetical protein LOC100194034 [Zea mays] >gi|194694754|gb|ACF81461.1| unknown [Zea mays]	623	623	100%
gi|162462940|NP_001105489.1	anionic peroxidase [Zea mays] >gi|2224846|emb|CAA74203.1| anionic peroxidase [Zea mays] >gi|167882598|gb|ACA05817.1| anionic peroxidase [Zea mays] >gi|167882600|gb|ACA05818.1| anionic peroxidase [Zea mays] >gi|167882602|gb|ACA05819.1| anionic peroxidase [Zea mays]	541	541	91%
gi|167882604|ACA05820.1	anionic peroxidase [Zea mays] >gi|167882606|gb|ACA05821.1| anionic peroxidase [Zea mays] >gi|167882608|gb|ACA05822.1| anionic peroxidase [Zea mays]	540	540	91%
gi|194694944|ACF81556.1	unknown [Zea mays]	540	540	91%
gi|242077768|XP_002448820.1	hypothetical protein SORBIDRAFT_06g033840 [Sorghum bicolor] >gi|241940003|gb|EES13148.1| hypothetical protein SORBIDRAFT_06g033840 [Sorghum bicolor]	491	491	90%
gi|115461474|NP_001054337.1	Os04g0688100 [Oryza sativa Japonica Group] >gi|38345506|emb|CAE01785.2| OSJNBa0039K24.4 [Oryza sativa Japonica Group] >gi|113565908|dbj|BAF16251.1| Os04g0688100 [Oryza sativa Japonica Group] >gi|215717130|dbj|BAG95493.1| unnamed protein product [Oryza sativa Japonica Group] >gi|215740826|dbj|BAG96982.1| unnamed protein product [Oryza sativa Japonica Group] >gi|218195870|gb|EEC78297.1| hypothetical protein OsI_18017 [Oryza sativa Indica Group] >gi|222629822|gb|EEE61954.1| hypothetical protein OsJ_16715 [Oryza sativa Japonica Group]	458	458	91%
gi|55700985|CAH69301.1	TPA: class III peroxidase 59 precursor [Oryza sativa Japonica Group]	455	455	91%
gi|2425101|AAB81720.1	cationic peroxidase [Oryza sativa]	441	441	84%
gi|5777629|CAB53490.1	CAA303717.1 protein [Oryza sativa]	434	434	91%

Figure 33 Protein blast of hypothetical protein LOC100194034

Discussion

1 *Fusarium*

Mechanism involved in kernel *Fusarium* infection and fumonisin accumulation are still unknown but it's clear that they depend by several factor: environmental condition such as temperature and nutrient availability that can influence moisture content and maize kernel development (Sagaram et al. 2006), genetic factor characterizing resistant and susceptible maize lines and Fungus isolate characteristic (Loffler et al. 2010). Contaminated ears maize produce infected kernel with micotoxins, but pathogen and plant interaction are still unknown. Maize lines with different genotypes respond differently to *Fusarium* infection and result in different degree of micotoxins contamination. In this Ph.D. project, maize lines with different susceptibility to fungus infection were protein profiled in order to evaluate the correlation between *Fusarium* resistance and differentially proteins expressed in the kernel.

Two lines a resistant CO441 and a susceptible CO354 were firstly analyzed by using different proteomic approaches as SELDI-ToF and protein fractionation by RP-HPLC coupled to SDS-PAGE profiling. SELDI-ToF-MS can detect multiple protein variation simultaneously with high sensitivity and specificity. The high-throughput nature and its feasibility in experimental procedures made this technology useful for biomarker discovery (Issaq et al. 2002, De bock et al. 2010). This technology was exclusively employed in fields strictly related to human and medical sciences. Only few works reported experiments in plants much more related to callus culture or transgenic line characterization (El-Gendy et al. 2001 and Badri et al. 2009). Recently SELDI-ToF protein profiling variation was observed in seed from *Oryza S.* that express different level of phytic acid demonstrate that this technology was effective for biomarkers discovery and protein characterization in different crop lines (Emami et al. 2010). SELDI-ToF protein profiling of different kernel fraction (albumins, globulins and zeins) using different chip arrays was performed to maximize the number of protein peak detectable for the biomarker discovery. About three hundred protein signals were detected for each line (Tab. 5), with from 10 to 25 % of the signals that were redundant in different fraction (Tab. 4). These data confirmed that protein fractionation by differential extraction methods is consistent with an enrichment rather than a complete separation in different protein components. Proteins are

distributed in different fraction depending of their solubility in the extraction solution. The two lines CO441 and CO354 were than compared by using the Ciphergen software (Cruz-Marcelo et al. 2008) to evaluate the differentially expressed peaks. A total of 64 peaks belongs to different fractions were seen to be differentially expressed between the two lines of which 39 belongs to albumins, 21 to globulins and 13 to zeins respectively. Each differential signal was confirmed by the presence in spectra from the CO441 x CO354 F1 inbreed (Fig 13A). Although SDS-PAGE profiling did not show many differences in the protein expressed by the two parental lines (Fig. 10) (Ghafoor 2008, Raj et al 2011), the SELDI-ToF analysis reveals a high level of polymorphism between the two lines. Thus was consistent with their pedigree: CO441 is a short-season corn inbred line derived from Jacques 7700 x CO298 while CO354 was developed from the commercial hybrid Asgrow RX777 (Reid et al. 2009). Other resistant C430, CO443 and susceptible GA209, T202 lines were profiled by using the same combination of protein extract and chip surface in order to confirm if the potential candidate biomarker were differentially expressed also in the other four lines. Protein profiling revealed a high degree of peak polymorphism between all the lines tested that avoid the use of specific software (Chipergen Express Client 3.5). However only two peaks at 14.96 and 5.79 KDa from the 64 signals previously found, were exclusively present in the susceptible lines CO354, GA209 and T202. We found the expression of differential protein peaks in the susceptible lines, that was in contrast with previous results in which mechanisms related to resistance were often associated with the expression of particular protein in the resistant lines (Chen et al. 2006, Chen et al 2011).

SELDI-TOF mass spectrometry is especially suited to detect changes in relative peak intensities of peptides and small proteins with mass comprise between 3 to 30KDa; profiling of higher molecular weight protein is usually performed by other techniques as 2D-PAGE. Maize protein from endosperm tissue was seen to change in response to abiotic/biotic stress by 2D-PAGE profiling (Riccardi et al. 1998, Chen et al. 2002, Fasoli et al. 2009).

In this Ph.D. work, we characterized high molecular weight protein by using an alternative method consisting of a RP-HPLC separation in the first dimension and protein detection by SDS-PAGE for each fraction (Du pont et al. 2005). Profiling of the albumin fraction showed variation in the protein expressed between CO441 and CO354 lines. In particular fraction seven obtained from the RP-HPLC

separation showed a protein that migrates at lower molecular weight for the CO354 lines. Thus was confirmed by the presence of both bands in the F1 inbreed that was consistent with a protein sequence variant that codify for protein that differ in their MW (Fig 16). Both bands were excise for protein identification that resulted in the LEA 3 late embryogenesis abundant protein, group 3. LEA protein belongs to proteins that are synthesize in the late embryogenesis phase and are accumulated during seed .dehydration (Galau et al. 1987). Amongst the several groups of LEA proteins, those belonging to group 3 are predicted to play a role in sequestering ions that are concentrated during cellular dehydration. Recently LEA 3 protein was seen over-expressed in maize endosperm belongs to a resistant line to aflatoxin contamination by *Aspergillus flavus* compared to susceptible genotypes and classified as additional resistance-associated proteins (RAPs) (Tab. 3) (Chen et al. 2002, Chen et al. 2007).

Other four lines two resistant CO430 and CO443 and two susceptible GA209 and T202 were profiled by RP-HPLC-SDS-PAGE. *Fusarium* resistant and susceptible lines did not show common proteins that were differentially expressed between the two groups (susceptible and resistant). In addition, the corresponding fraction that showed a shift of LEA 3 protein to lower MW for the CO354 line, did not present the same variation for the other two susceptible lines (Fig. 17). The CO354 line presented an exclusive variation indicating a specific alteration that could influence the susceptibility to *Fusarium* infection, or could be relate to its intrinsic polymorphism.

2 Diabrotica

Grasses, especially rice and maize, respond to insects attack by activating different defence mechanisms acting in parallel to limit the damage. Many of these defence mechanisms are based on plant secondary metabolites or defensive proteins that directly affect the herbivore insect due to their toxic effects or deterring properties. The second part of the Ph.D. thesis explored some of the mechanisms involved in *Zea Mays* response to *Diabrotica virgifera* attacks.

2.1 TPS23 induction

Zea Mays responds to *D. virgifera* larvae infestation by activating an indirect defence consisting of the expression of Terpene Synthase 23 *tps23* gene. The herbivore damage in maize root induces the TPS23 expression with the consequent synthesis of (E)-β-caryophyllene, a volatile defence signal that attracts natural enemies, entomopathogenic nematodes below ground (Rasman et al. 2005, Schnee et al. 2006). (E)-β-caryophyllene and other volatiles defence signals are compounds that are synthesize by different plant species in response to different stresses. These compounds have a high level of specificity in function of the biotic stress and are release by the aerial or below-surface part of the maize plant after direct infestation with insects. In particular the terpene blends produced from leaves, sheaths and roots after herbivore damage were prevalently formed by the expression at least of three sequiterpene synthase TPS1, TPS10, and TPS23 (Köllner et al. 2004), however *tps10* gene was prevalently expressed in leaves in response to insect aerial attack while *tps23* in roots after insect larvae damage. A strictly correlation between the level of TPS23 expression in root and *D. virgifera* response in maize, was reported by Köllner *et al* that hypothesized that North American maize lines in which the (E)-β-caryophyllene expression was lost during the breeding process were more susceptibility to *D. virgifera* attack (Köllner et al. 2008). In maize TPS23 expression was observed to vary only after *D. virgifera* larvae direct root feeding, however in other species i.e. *Oryza Sativa* TPS23 expression was observed to be induced also by biochemical treatment with Methyl Jasmonate MeJa (Cheng et al. 2007). Moreover other *tps* genes was seen to be induced by MeJA in maize, where plant growth in fine river sand and

irrigated with a defined nutrient solution expressed the *tps1* gene after MeJa treatment (Feng et al. 2010). Many papers reported that in plants herbivore attack elicits the rapid accumulation of jasmonic acid (JA) which results from the activation of constitutively expressed biosynthetic enzymes (Ton et al. 2007, Browse and Howe 2008). Molecular mechanisms controlling the activation of JA biosynthesis remain largely unknown however new researches have elucidated some of the early regulatory components involved in this process. In *Zea Mays* different biotic stresses as wounding or insect feeding mediate their response by activating methyl jasmonate pathways. Both insect attack and application of herbivore oral secretion to wounded leaves stimulates the emission of volatile organic compounds (VOCs) above the mechanical damage alone (Shmeltz et al. 2003). In B73 maize line was recently seen that elicitors treatment with *N*-(17-hydroxylinolenoyl)-L-glutamine FAC termed voliticin and related glutamine- and glutamate-containing FACs present in the insect oral secretion elicited rapid increase of ethylene and jasmonates levels and production and release of VOCs (Shmelz et al. 2009).

In the second part of the Ph.D. work, mechanisms involved in caryophyllene synthase response in different maize lines were investigated. Maize plant were differently stressed by chemical and physical treatments and *D. virgifera* larvae infestation to evaluate the TPS23 expression and protein profiling changing in roots. The TPS23 response was initially assessed in hydroponic experiments that mimiced biotic stresses. A B97 lines that expressed the TPS23 gene after Diabrotica larvae infestation (Köllner et al. 2008) was treated in presence of different compounds as methyl jasmonate and salicylic acid or by wounding (Fig. 18). TPS23 gene expression was evaluated by PCR amplification, that showed a strongly induction by MeJa treatment (Fig. 21). Partially expression of TPS23 after wounding was seen also after 4 h after wounding (data not shown). These results suggested for the first time that TPS23 gene is induced by the activation of JA pathways, that is consistent with previous results where maize plant sprayed with Jasmonic acid JA on the aerial part reported the synthesis of different chemical attract compounds among which (E)-b-caryophyllene (Ozawa et al. 2008). In agreement with previous results, SA treatment instead did not induce the caryophyllene production (Shmelz et al. 2009).

B97 line showed different plant structural phenotypic alteration after 48 h of MeJa and SA treatment, that are consistent with protein profile changing that reveal

differently expressed protein for both compounds during the time course of the treatment (Fig. 19-20). Variation in protein profiling was already observed from 4h after treatment, with the presence of specific protein peak depending on the compound used in the treatment: a signal at 7141 Da that was induced by MeJA while two signals at 4780 and 7412 Da that were induced by SA. During late time course of experiment both treatments increase considerably the number of differentially peaks expressed with several common peaks for both treatments (Tab. 7). Although after 48 hour of treatment plants presented specific alteration in the phenotype and typical variation in the protein profile, several peaks were commonly expressed by both treatments. The prolonged exposure to biotic stresses could lead to activate different response patterns with synergistic effects on regulation of some genes (Salzman et al. 2005).

First results indicate that maize responds to biotic stresses with specific mechanism. The MeJa pathway is involved in the *tps23* induction, gene expressed in response to *Diabrotica* larvae attack (Fig. 34).

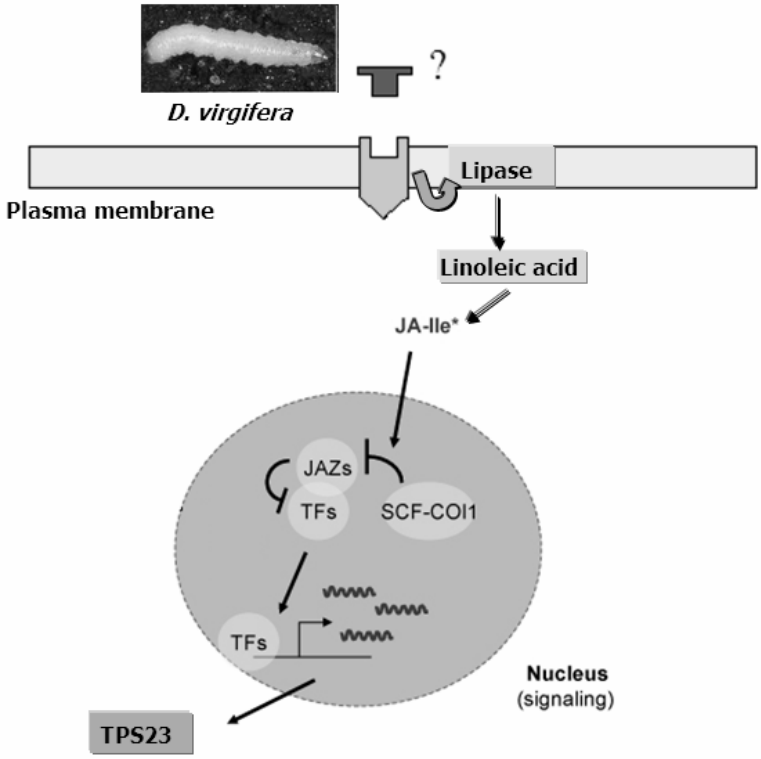


Figure 34 Hypothetical mechanism 1: TPS23 induction by *D. virgifera* is mediated by MeJA pathways. (JA jasmonoyl-isoleucine, JAZ Jasmonate Zim domain protein, SCF-COI1 coronative insensitive 1 complex, TFs Transcription Factors).

To verify this hypothesis other maize lines with different expression in *tps23* gene were treated with MeJA in hydroponic condition. In contrast with previous results (Köllner et al 2008) where northern blot experiments revealed the expression of the β-caryophyllene synthase after Diabrotica larvae feeding only in three lines the north American maize B97 and NC358 and the European F2, all line tested expressed the *tps23* gene after MeJA treatment (Fig. 28). These results were confirmed also by analysing the protein profile variation. All the lines presented differentially expressed protein peaks (Fig. 22 Tab. 8) in response to MeJA with specific peak variations for each line. Probably these peaks represented the same proteins set with variation in molecular weight ascribable to polymorphism in different lines.

The divergence between our and previous reported results (Köllner et al. 2008) should be explained by considering that *tps 23* gene is also induced through the MeJa pathways, however *Diabrotica* direct response is probably mediated by specific mechanism through the activation of different pathways (Fig. 35).

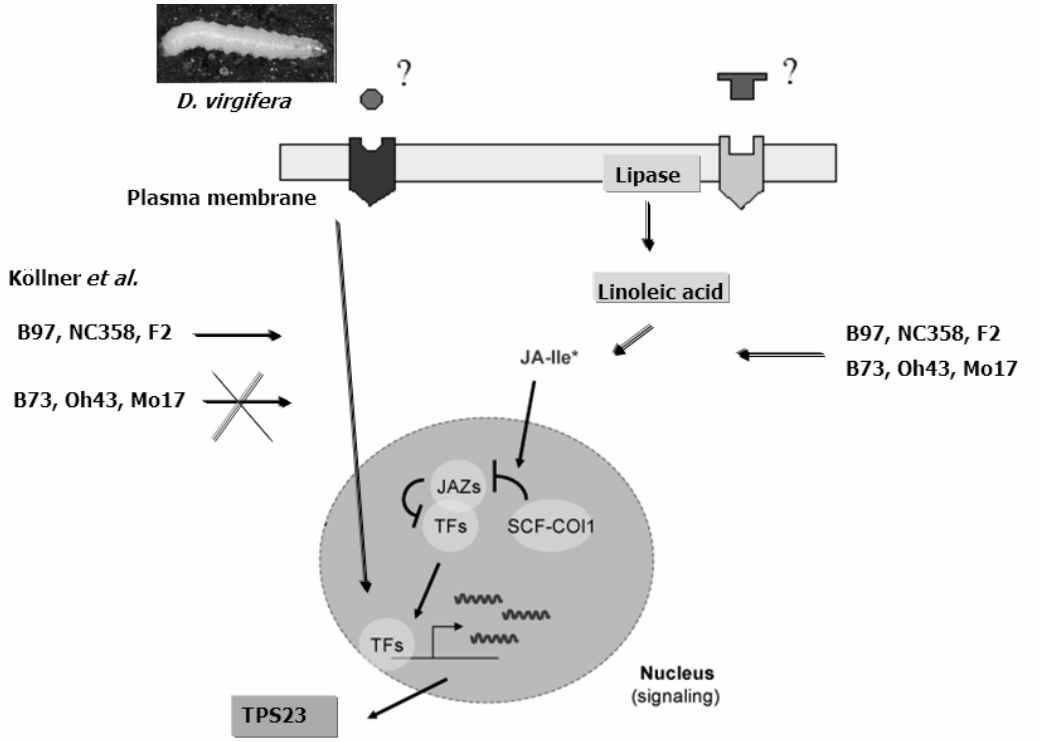


Figure 35 Hypothetical mechanism 2: TPS23 is directly induce by *D. virgifera* (only for B97, NC358 and F2 lines) and is also induced by MeJA pathways (for all the lines). (JA jasmonoyl-isoleucine, JAZ Jasmonate Zim domain protein, SCF-COI1 coronative insensitive 1 complex, TFs Transcription Factors).

However when experiments by direct infestation with *D. virgifera* larvae by using protocols previously adopted by Köllner *et al* were performed, TPS23 was seen to be expressed in almost any line tested. In contrast with previous results B73 and Mo17 lines expressed TPS23 indicating that *tps23* defensive trait wasn't lost during the breeding of North American maize lines. Discrepancy with previous results could be explained by the method used in the TPS23 detection (PCR amplification of retrotranscript vs northern blot), that is consistent with the higher level of sensitive in the PCR method (Dean et al. 2002). *D. virgifera* larvae damage induced a lower level of TPS23 transcript compare to MeJa treatment, which was also confirmed by analyzing protein profiling variation that did not show significant variation in protein peaks for each line (data not shown).

We also observed that the physiological response to *D. virgifera* infestation, induced a different expression in TPS23 in different lines. To verify that the semi-quantitative response could be effectively mediated by JAs response, all lines were treated in hydroponic condition with different amount of MeJa and time of exposure: 10 µM MeJa for 24 hour or 100 µM MeJa for 2 hour and both collected after 24 hours. Both treatments showed that each line responded differently to MeJA, indicating that different lines were differently sensitive to MeJa induction.

In particular, Oh43 and B73 were less sensitive to MeJA induction, while B97, NC358, F2 and Mo17 presented a high level of TPS23 transcript even with marked differences between lines (Fig. 31). In agreement with TPS23 expression level, protein profiling revealed a quantitative response to MeJa with previously differentially expressed protein peaks detected, that showed variation in intensities proportional to the level of treatment but quite similar for each lines (Fig. 30). Therefore, we can assume that (E)-β-caryophyllene synthase expression is probably mediated by JA pathways through a proper semi-quantitative response related to the level of herbivore damage response for each line. Terpene synthase expression has already seen to vary among maize varieties, where the transcript level of *tps 1* gene was differentially pronounced after herbivore induction (Schen et al. 2002). Quantitative variation in JA inducible gene was already observed in different species as *Arabidopsis* (Shan et al. 2009) and *Zea* (Norastehnia et al. 2006). Recently a stress response as wounding was seen to be spatially and temporary regulated by JAs signalling (Glauser et al. 2008).

JA-signaling pathway integrates the action of different signals in a multidimensional network that is regulated in special distribution and timing, whose output define the perception and response to different stimulus. Different cultivars and breeding lines could detect and adapt to environmental changes as biotic stress through quick transcriptional reprogramming. We evaluate that different maize lines could activate the JAs pathways with different efficacy influencing the level of expression of particularly proteins and defence genes. The expression of TPS23 seems to vary depending on both factors, efficiency in Jas pathways and variation of gene expression for each lines.

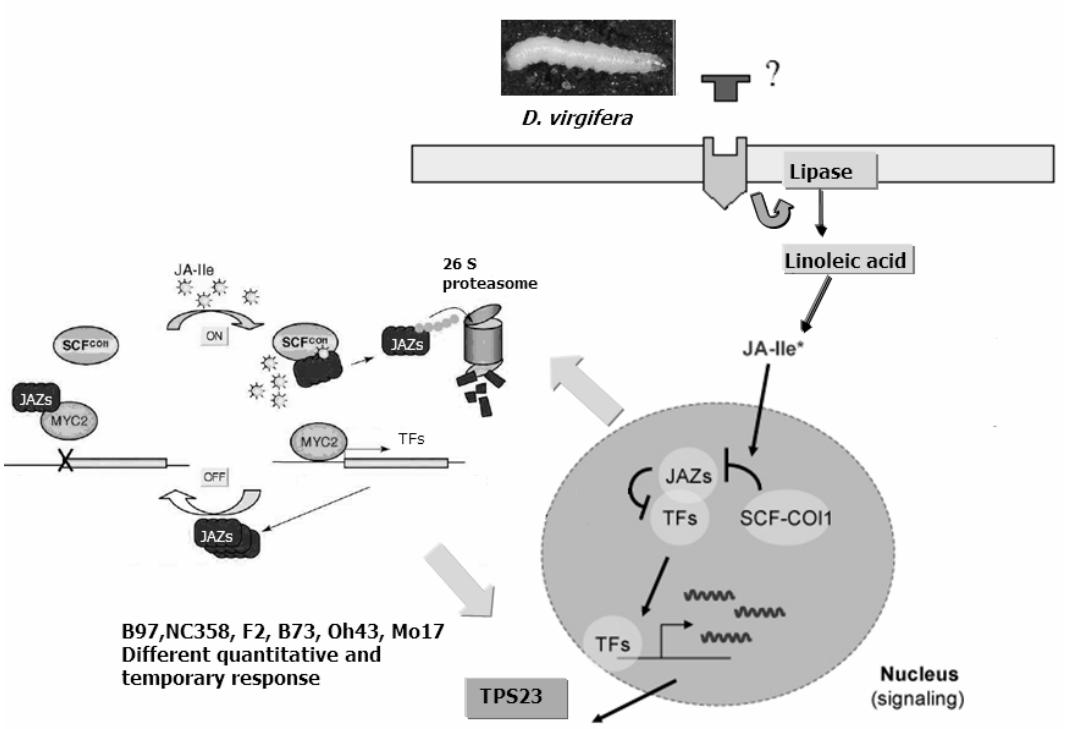


Figure 36 Hypothetical mechanism 3: TPS23 response after *D. vigifera* infestation is mediate by JA pathway. However different maize line presented different quantitative and temporary response to biotic stresses. (JA jasmonoyl-isoleucine, JAZ Jasmonate Zim domain protein, SCF-COI1 coronative insensitive 1 complex, TFs Transcription Factors).

2.2 Protein expression

Methyl Jasmonate hydroponic treatment in maize induces the expression of different proteins. The number of protein peaks differentially expressed detected by SELDI-ToF increased during the time course of experiment for all the lines tested (Table 8). RP-HPLC fractionation and SDS-PAGE detection of B97, Mo17, and NC358 lines showed common proteins bands that were up-regulated after 48 h of treatment. From trypsin digestion and LC-MS/MS analysis of the four bands, were identified three proteins: the actin-depolymerizing factor 3, a fragment and the intact form of hypothetical protein LOC100191759 and hypothetical protein LOC100194034.

The maize actin-depolymerizing factor, ZmADF3 controls the actin-based motile processes and redistributes to the growing tip of elongating in root hairs (Jang et al. 1997). Actin-Depolymerizing Factor (ADF) family regulated dynamic actin cytoskeleton rearrangements, which sense environmental changes and modulate the cytoskeleton through various biochemical activities (Hussey et al. 2006; Staiger and Blanchoin 2006). This class of protein responds to intracellular and extracellular signals and causes actin reorganization. In rice the levels of an actin depolymerizing factor in leaves, was changed by drought and osmotic stresses, but not cold or salt stresses, or abscisic acid treatment (Ali and Komatsu 2006).

Linking of ADFs with plant defences was reported in barley where HvADF3 in epidermal cells was shown to compromise penetration resistance to powdery mildew fungi (Miklis et al., 2007). Lately loss of AtADF4 confers on Arabidopsis enhanced susceptibility to P. syringae expressing AvrPph (Tian et al. 2009).

The hypothetical protein LOC100191759 showed a high similarity with the enzymes Nucleotide Pyrophosphatase/Phosphodiesterase (NPPs) and Purple Acid Phosphatase PAPs. NPPs are glycosylate protein that are located in the membrane system and can accumulate in vacuoles, or are secreted from the cells (Hiraga et al. 2001, Nanjo et al. 2006). They are deputed prevalently in the release of nucleoside 5′-monophosphates from nucleotides and their derivates and seem to play a crucial role in diverting carbon flux from starch and cell wall polysaccharide biosynthesis to other metabolic pathways (Baroja-Fernández et al. 2000). PAP are enzymes that catalyze the hydrolysis of phosphate (Pi) from

phosphate monoesters, and their function seems to be involved in the release, transport, and recycling of Pi, a crucial macronutrient for cellular metabolism and bioenergetics. These enzymes contain a metal center Fe(III)-Me(II), where Me(II) can be Zn, Mn, or Fe. Plants contain two major groups of PAPs: small PAPs, 35–40 kDa monomeric proteins homologous to mammalian enzymes, and large PAPs, 110–130 kDa homodimeric proteins, with or without a disulfide bridge between the two subunits (Olkzac et al 2003).

Finally, the hypothetical protein LOC100194034 showed similarity with anionic peroxidase. Classical secretory plant peroxidases are heme-containing enzymes that are implicated in different functions. Peroxidases are involved in cell wall modifications like lignification, suberisation, and cross-linking of hydroxyproline-rich glycoproteins and polysaccharides (Mika et al. 2004). They are implicated in phytohormone metabolism, senescence, and in several biosynthetic pathways (Dìaz et al. 2004), and probably play a role as producers of reactive oxygen species, and regulators of H_2O_2 signalling (Mika et al. 2004).

In different crop plant, peroxidase enzyme responds to stress-related process like plant–pathogen interactions and wound healing. Peroxidases are implicated in the stress-induced reinforcement of the cell wall in rice (Hilaire et al 2001), the production of phenolic phytoalexins in barley (Kristensen 2004) and a cationic peroxidase was seen to mediate resistances against pathogens and plant parasites in maize (Dowd and Johnson 2005). Recently the same anionic peroxidase from our findings was observed associated to insect resistance in maize and was suggested to be potentially use in marker assisted program (Dowd et al. 2010).

Conclusion

The aim of this Ph.D. project was to study the mechanisms associated to biotic stresses (*Fusarium* infection and *Diabrotica virgifera* infestation) in maize. Different lines were protein profiled and characterized for the expression of particular protein involved in pathogen plant response.

Different proteins in maize were found to be associated to *Fusarium* resistance. Analysis of kernel protein composition revealed a high degree of polymorphism with a high diversity in proteins expressed between *Fusarium* susceptible and resistance lines. Research of common biomarker in resistance lines did not result in any differentially expressed peaks. In contrast, susceptible lines show some common protein peaks potentially related to susceptibility of infection. We identify a variation in a Late Embryogenesis Abundant protein type 3 LEA 3, only for the susceptibility line CO354 consistent with previous results. This finding suggest that maize *Fusarium* response could be probably due by peculiar mechanism for each lines tested.

Diabrotica virgifera resistance was associated to E-β-caryophyllene emission in root after herbivore damage. This volatile compound has been found in response to herbivore damage in several wild relatives of maize and in cultivated maize lines from European breeding, but it is absent from maize lines originating from North American breeding programs. In contrast with previous results we found that the β-caryophyllene synthase TPS23 enzyme is induced in root after *D. virgifera* infestation and Methyl Jasmonate MeJa treatment in all the line tested, belong to both breeding programs. Taking into accounts all the results obtained we hypothesized that *tps23* gene is induced by activating the Jasmonates response through a proper semi-quantitative response for each line. Thus is confirmed also by the fact that different lines responded to MeJa treatment with specific protein profiling alteration that varied in a quantitative manner. Therefore, after a strong induction of JAs pathway we found other three proteins the actin-depolymerizing factor 3 ADF3, a Nucleotide Pyrophosphatase/Phosphodiesterase NPP and an anionic peroxidase which expression was probably involved in herbivore insect response mediated by JAs.

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