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Lipid mediated cross-kingdom communication among hosts and pathogens

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A thesis submitted for the degree of

Doctor of Philosophy

in Environmental and Evolutionary Biology

October 2017

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Collection of articles

Review

Fatty acids, sphingolipids and oxylipins: structural and signaling components

A lipidomic approach to evaluate the host pathogen interaction

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Which are the main biological functions of lipids? Lipids have a structural role in forming cell membranes and play a role in energy storage. Current knowledge indicates that the lipids have an additional role in cell signaling. When a fungal pathogen contacts the host, the cell surfaces can exchange signals, among which, several are lipids. Lipids occur in fungi not only as major constituents of the membrane system, but also as cell wall components, as storage material in abundant and readily observed lipid bodies, and in some cases, as extracellular products. Fungi contain a various set of lipids, including fatty acids, oxylipins, sphingolipids, phospholipids, glycolipids and sterols. We are going to examine some fungal lipid classes involved in the pathogenic interaction with the host. These signals can confer different information. In some cases can trigger host immune responses; conversely, they may function as virulence factors altering the normal homeostasis of the host or even causing the death.

Introduction

Lipids are classified thereby as being sparingly soluble in water but readily soluble in organic solvents. Structures of the more common lipids can be divided into two categories: structures based on long-chain fatty acid and their derivatives and structure derived from an isoprene unit (Fahy et al., 2009). Fatty acid (FA) either can be in the free form (FFA) or linked to other molecules, most commonly to glycerol. Fatty acid may be fully saturated and may be linear, branched, or contain alicyclic rings. Unsaturated fatty acids occur as frequently as the saturated ones; they may contain several double bond, though 1 or 2 is the most usual. A wide variety of fatty acids with a second oxygen-containing functional group generates what are termed oxylipins. FFAs do not usually accumulate intracellularly because of their toxic effects: they can bind to and inactivate many enzymes and other proteins. FA may form complex with various alcohols and amines, glycerol being the prime example of the former. The fatty acids can be esterified with glycerol and with sugar such as glucose.

One distinctive group of lipids, the sphingolipids, is based on long-chain, polyhydroxy amines (sphingoid bases). Other structural variations include homology and branching of the carbon skeleton. N-Acylation of the 2-amino group of the bases by a fatty acid produces compounds collectively known as ceramides. The ceramides in turn can be further derivatized through the 1-hydroxy group, leading to families of phospholipids and glycolipids comparable with, but less abundant than, those derived from diacylglycerols. There are also several glycosylated sphingolipids.

Second lipid group is that of terpenoids. They are characterized by the repetition of the isoprene unit incorporated in the basic molecular skeleton.

Lipids are the major constituent of fungi plasma membrane and intracellular membranes, but they also have a biological function in energy storage, signal transduction, and stress response. The role of lipids in host-fungus interactions has strongly increased since the availability of highly sensitive analytical technologies, including gas chromatography and high-pressure liquid chromatography coupled to mass spectrometry (Han et al., 2012).

This review aims to give an overview of the status of our knowledge on the involvement of lipids in host-pathogen interaction, indicating that lipids play a key role in infection processes.

Crucial lipids: fatty acids

Fatty acid biosynthesis. Long-chain fatty acids are an important metabolic energy source and the building blocks of membrane lipids. They can be incorporated into phospholipids or serve as an energy reservoir in triacylglycerols (TG) and steryl esters

(SE) stored in lipid droplets. Fatty acids synthesis (Figure 1) in *Saccharomyces cerevisiae* is initiated by the acetyl-CoA carboxylase Aac1 and continued by the cytosolic multi-enzyme fatty acid synthase complex consisting of Fas1p and Fas2p, yielding acyl-CoA with an acyl chain length of 16 or 18 carbon atoms (Tehlivets et al., 2007). Fatty acid elongation up to 26 carbon atoms in contrast to *de novo* fatty acid synthesis takes place in the endoplasmatic reticulum (ER) presumably due to the hydrophobic nature of the very long chain fatty acids (VLCFA) of C_{20}-C_{26} carbon atoms that may be inserted directly into ceramide by the ceramide synthase. The major VLCFA species in yeast is C_{26}, predominantly present in an amide linkage of the ceramide backbone of sphingolipids (Raffaele et al., 2009). *De novo* synthesis uses acetyl-CoA as primers, while the elongation requires malonyl-CoA, which is provided by Aac1. *S. cerevisiae* encodes different fatty acid elongation enzymes, the *elo1* gene and his paralog *elo2* encode enzymes involved in lengthening fatty acids from 14 to 16 carbons (Toke et al., 1996), *ELO3* is involved in the formation of VLCFA (Oh et al., 1997). Membrane fluidity has an important physical property, which can depend by the ratio of saturated versus unsaturated fatty acids (Los et al., 2004), unsaturated fatty acids are the most abundant acyl group in the membrane glycerolipids and consist of a wide range of C_{14}-C_{26} species with one to six double bonds. In *S. cerevisiae* approximately 70-80% of their total glycerolipid acyl chains consist of the monounsaturated fatty acids. The Δ9-fatty acid desaturase Ole1p synthesizes monounsaturated fatty acid (i.e. oleic acid C18:1), recently it was shown that Sct1p, a glycerol-3-phosphate acyltransferase catalysing the production of lyso-phosphatidic acid, competes with Ole1p for the saturation (De Smet et al., 2012). The monounsaturated fatty acids can be desaturated further by the action of other desaturases producing PUFA (Polyunsaturated Fatty Acids). PUFAs are a family of lipids that contain more than one double bond in their backbone. PUFAs are biosynthesized via an extension of the saturated-fatty acid pathway, in which stearate C18:0 is converted to oleate C18:1 and then to linoleate C18:2, which is the central precursor for the ω-6 and ω-3 series.

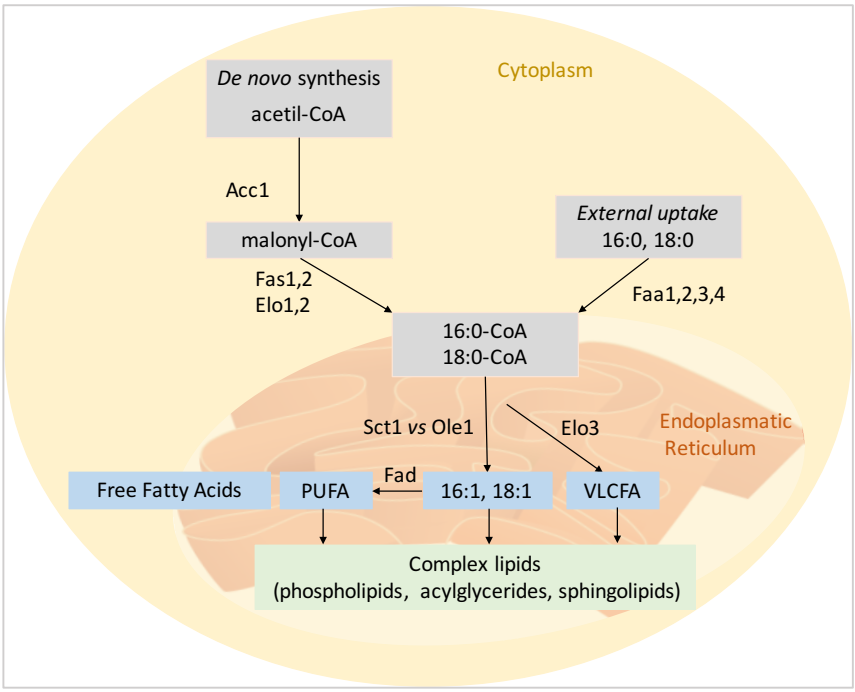

Figure1. Fungal fatty acid metabolism. Fatty acids are derived from de novo synthesis and external uptake. The free fatty acids can be incorporated into complex membrane lipids. Enzymes catalyzing these processes are described in the text.

Fatty acid in host-pathogen interaction. FA are not only membrane molecules, the free fatty acid (FFA), can also function as transcriptional regulators and signaling molecules and can be involved in post-translational modification of proteins.

The fungi can grow over a range of physiological and nutritional conditions that require different adaptations of membrane lipids. The expression of the crucial gene *ole1* is regulated and responds to a number of different stimuli, including a feedback by the FFAs. *ole1* expression is regulated by the import of long chain fatty acids from the environment, the supplemented species

(i.e. the monounsaturated palmitoleic acid C16:1 and oleic acid C18:1) increase in membrane lipids. The linoleic acid C18:2 added was incorporated into microsomal lipids but after several generation of growth replaces the monounsaturated fatty acids 16:1 and 18:1 produced by Ole1p. The enzyme Ole1p is repressed when the cells were grown in presence of monounsaturated fatty acids or PUFAs (Bossie et al., 1989). *Ole1* mRNA levels are regulated at transcriptional (Choi et al., 1996) and post-transcriptional level (Gonzalez et al., 1996). The addition of unsaturated fatty acids to the growth medium decreases the desaturase activity (Bossie et al., 1989). The transcription of the *ole1* gene can be repressed by *cis*-unsaturated fatty acids products of Ole1p and polyunsaturated species (McDonough et al., 1992; Fujiwara et al., 1999). It should be noted that only the fatty acids with the *cis* conformation maintain the repression of the gene. When the fungus contacts the host, an alteration of the fatty acid composition occurs. This alteration can be caused by a re-modulation of the fatty acid uptake. A second way to modify the fatty acid composition is by modulating the activity or expression of enzymes involved in fatty acid synthesis. It was demonstrated that the fatty acids can influence the growth of the fungi. *In vitro* assays indicate that the unsaturated fatty acid, linoleic 18:2 and linolenic 18:3 acids, reduce the mycelial growth in several fungi species, suggesting an antifungal activity (Agoramoorthy et al., 2007).

The fatty acids can be imported from the environment through a simple diffusion or an active transport, and they can be catabolized after the conversion to a coenzyme A (coA) derivate. The extracellular fatty acid transport across the plasma membranes occurs through transporters and receptors (Daum et al., 1998). Several enzymes can mediate the transport and the activation: Fat1p, Faa1p, Faa2p, Faa3p and Faa4p. These enzymes can be localized not only to the plasma membrane but also to ER, lipid droplets and peroxisomes (Di Russo et al., 1999). Fat1p can be involved in the maintenance of VLCFAs homeostasis; Fat1p appears to be involved to fatty acid transport and very long chain acyl-CoA synthetase activity, therefore it is involved in both the transmembrane movement of fatty acids and in the turnover of very long chain fatty acids (Zou et al., 2002; Black & Di Russo, 2007). The exogenous fatty acids are activated by the acyl-CoA synthetases (Faa1-4p) (Johnsos et al., 1994). *Faa1* and *faa4* genes encode acyl-CoA synthetases required for activation of imported exogenous fatty acids. Faa1p exhibits a preference for fatty acids C_{12}-C_{16} and can import sphingoid chain bases (Narita et al., 2016). Faa2p and Faa3p can activate only the fatty acids synthesized within the cell (Johnson et al., 1994). Faa2p has been localized to the matrix side of peroxisomal membranes and it is mainly active toward the fatty acids with 9-13 carbon atoms, while Faa3p prefers fatty acids with 16-18 carbons (Knoll et al., 1994).

Membrane lipids and lipid-derived molecules from the plant or the fungal organism play important roles during the infection process. Lipid-derived molecules are crucial for intracellular signaling in the plant as well as in the fungal cell. The free fatty acids derived from the action of some enzyme (see below), and are the precursors for oxylipins (Siebers et al., 2016).

A major portion of the intracellular lipids of fungi reside in the membrane elements, some lipids, however, tend to accumulate as droplets or globules in the cytoplasm. The fatty acids may be part of more complex lipids, as acylglycerides, phospholipids and sphingolipids (that will be detailed subsequently).

The expression of several genes encoding enzymes of lipid metabolism is up-regulated after infection of plant cells, resulting in the synthesis, modification, or re-allocation of lipid-derived molecules. Lipid-modifying enzymes are essential regulators of the spatial and temporal production of lipid metabolites involved in signaling and membrane proliferation for the establishment of intracellular compartments or compositional changes of lipid bilayers.

Acylglycerides are the ester of fatty acids link to the glycerol, this class of lipids includes monoacyl, diacyl- and triacyl-esters; since fatty acids are the major carbon source of energy in living system triacylglycerol are the major constituent of the lipid droplets. The esterases are a class of hydrolase which catalyse the hydrolysis of triglycerides to glycerol and free fatty acids. Several works indicate that the secreted lipases, that release the fatty acids, might serve as virulence factors in plant pathogenic fungi (Commenil et al., 1998; Voigt et al., 2005).

Phospholipids (PL) contain two fatty acids esterified to the *sn-1* and *sn-2* positions of a glycerol backbone, and a polar head group attached to the *sn-3* position. The phospholipid class comprises phosphatidic acid (PA), phosphatidylserine (PS),

phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI). Each phospholipid class includes many molecular species due to a large number of fatty acids varying in chain length and degree of desaturation. Phospholipids are predominantly synthesized in the ER.

Phospholipases and phospholipid-derived molecules are involved in signaling and plant immunity during plant–pathogen interactions (Arisz et al., 2009; Pleskot et al., 2013). Phospholipases can catalyze the conversion of phospholipids into fatty acids free form, they can function as a modulator of many signal transduction pathways.

It was seen that changes in phospholipid content and phospholipase activities during host–pathogen interactions elicit the activation of defense and resistance response to the necrotrophic pathogen (Zhao et al., 2013). Different sites of phospholipid hydrolysis are noted as shown in figure 2**.**

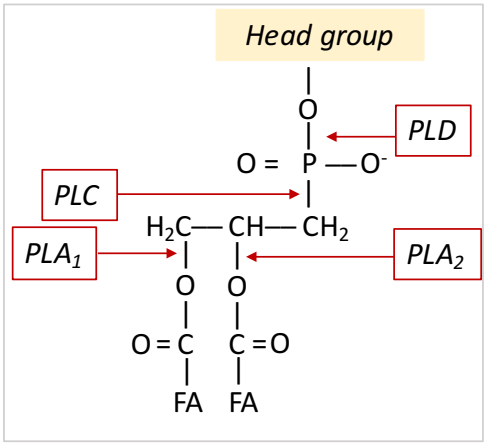

Figure 2. Sites of phospholipid hydrolysis by PLD, PLC, PLA_1, PLA_1.

The phospholipase D (PLD) catalyze the hydrolysis of PC to generate choline and phosphatidic acid, the phospholipase C (PLC) produces two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which mediate the activation of protein kinase C and intracellular calcium release, respectively. The phospholipases A1 (PLA1) and A2 (PLA2) can remove acyl groups from both sn-1 and sn-2 positions yielding to the free fatty acids and lysophospholipids formation.

Plant PLD and PLA1-2 release the fatty acids during the host-pathogen interaction, the fatty acids are the substrates for the formation of the plant oxylipins implicated in the defense (Blée et al., 2002).

Furthermore, exist a link between oxidative burst and PLA activity, it was demonstrated that extracts of the pathogenic fungus *Verticillium dahliae* induce PLA activity and ROS production in soybean cells (Chandra et al., 1996).

Fatty acids can be produced by *de novo* synthesis or are released from complex lipids by the enzymes indicated above, the fatty acid can be converted into bioactive lipid mediators, as the oxylipins (see below). Changes in the oleic acid C18:1 levels result in alterations of the defense responses (Kachroo et al., 2005), also the trienoic acids (Hexadecatrienoic acid C16:3 and α-linolenic acid C18:3) are involved in defense responses against avirulent bacterial pathogens (Yaeno et al., 2004).

We can say that the knowledge of the fatty acid biosynthesis and the study of their metabolism is useful in understanding the lipid signals that mediate the plant-pathogen interaction.

Oxidised fatty acids: the oxylipins

Oxylipin biosynthesis. Oxylipins constitute a large family of oxidized fatty acids and metabolites derived therefrom. These bioactive lipids are abundant in mammals (Funk et al., 2001) plants, bacteria and fungi.

The plant oxylipins have been studied more in-depth and can provide valuable insights into the description of the biosynthesis of the fungal oxylipins. These compounds can regulate developmental processes and mediate responses to biotic and abiotic stresses (Howe et al., 2009). Plant-oxylipins include fatty acid hydroperoxides, hydroxyl-, epoxy-, keto- and oxo- fatty acids,

and epoxy alcohols, divinyl-ethers, volatile alcohols or aldehydes, and jasmonic acid (JA) and its corresponding derivatives (Mosblech et al., 2009). These compounds can be formed through two routes: by non- enzymatic or enzymatic processes. The biological systems are subjected continually to autoxidation and the double bond of fatty acids are particularly susceptible of modifications by reactive oxygen species. In the higher plants, linoleic acid C18:2 and α-linolenic acid C18:3 represent the most abundant polyunsaturated fatty acids (PUFA). PUFAs easily react with O_2 generating hydroperoxides into membrane bound lipids, in turn affecting membrane fluidity (Girotti 1998). Plants have to develop a broad range of defense responses to cope with the pathogenic infections. The oxidative burst, a rapid and transient production of reactive oxygen species (ROS), is one of the earliest observable aspects of a plant's defense strategy. The term ROS is used to describe the products of the sequential reduction of molecular oxygen as shown in the scheme:

$$\begin{array}{ccccccccc} & & e^- & & e^- & & e^- & & e^- \\ O_2 & \rightarrow & \bullet O_2^- & \rightarrow & H_2O_2 & \rightarrow & \bullet OH & \rightarrow & H_2O_2 \\ & & & & 2H^+ & & H^+ & & H^+ \end{array}$$

The superoxide radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet$OH) are the species predominantly detected in plant-pathogen interaction (Wojtaszek, 1997). The ROS are able to oxidase the PUFAs on the plasma membrane, and biologically active oxylipins are (Grun et al. 2007, Mosblech et al., 2009). To limit these oxidation reactions, thus reducing potentially damaging effects, cells have evolved a wide battery of antioxidant defense systems (Reverberi et al., 2012).

We describe some examples to understand the activity. The superoxide radical ($O_2\bullet$) can behave either as a reducing agent (often reducing Fe^{3+} to Fe^{2+}) or as a weak oxidizing agent as in its interaction with ascorbic acid. Superoxide radicals are detoxified partially in cells to hydrogen peroxide through the enzyme superoxide dismutase that is then detoxified by a group of heme-containing protein, the catalases, to water and oxygen. Hydrogen peroxide (H_2O_2) is detoxified through the actions of both superoxide dismutase and ascorbate peroxidase (Asada et al., 1999). Hydrogen peroxide is a weak oxidizing agent but in the presence of cellular Fe^{2+} or other transition metals, forms hydroxyl radicals ($\bullet$OH), which lacking charge, are able to enter the interior of cell membranes and are the most reactive and damaging of the reactive oxygen species. The decomposition of hydrogen peroxide by Fe^{2+} and some Fe^{3+} complexes is referred to as Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\cdot}OH$). All these compounds alter the oxidant/antioxidant balance which plays a critical role in lipid peroxidation. Autoxidation is the direct reaction of molecular oxygen with organic compounds; the lipid oxidation involves a free radical mechanism consisting of initiation, propagation and termination steps, frequently overlapping (Halliwell et al., 2005). The relative rates of oxidation of C18-series reveal that more double bonds C=C corresponding to more reactivity, C18:3 is more instable of C18:2, which in turn is more instable of C18:1 (Frankel, 2014).

In plants, the formation of non-enzymatic oxylipins also takes place in response to pathogen attack and levels of esterified hydroxyl-fatty acids increase significantly in esterified lipids, up to 30 times more abundant than free oxylipins suggesting that they are predominantly generated in membranes from which they can be released (Grun et al., 2007).

The enzymatic routes (Figure 3) act primarily on linoleic acid C18:2 and α-linolenic acid C18:3. The biosynthetic oxylipin pathway starts with an initial peroxidation reaction of a polyunsaturated fatty acid (PUFA) that is catalyzed by lipoxygenases (LOXs). LOXs are non-heme iron-containing enzymes that catalyze the stereospecific and regiospecific oxidation of PUFA containing a 1Z,4Z-pentadiene system (Andreou and Feussner, 2009). The hydroperoxy fatty acid can be converted to different products by enzymes belong to the family of cytochrome P450 enzymes (Pinot, F and Beisson 2011). Some work suggests that LOXs utilize non-esterified fatty acids and are capable of acting on esterified acyl chains in triacylglycerols or phospholipids (Andreou and Feussner, 2009). Plant oxylipins are produced by distinct plant LOX isozymes that preferentially introduce molecular oxygen into linoleic and linolenic acids either at C-9 (9-LOX) or at C-13 (13-LOX) of the hydrocarbon backbone of the fatty acid.

Oxylipins in host-pathogen interaction. The structural similarity of plant and fungal oxylipins has given rise to a hypothesis that they are important molecules in cross-kingdom communication based (Tsitsigiannis & Keller, 2007). The Linoleate Diol Synthase (LDS), which catalyzes the enzymatic conversion of linoleic acid into dihydroxy-linoleate was the first oxylipin biosynthetic enzyme that was biochemically characterized from the fungus, in particular from *Gaeumannomyces graminis* (Su et al., 1996). Based on sequence homology studies, LDS of *G. graminis* led to the discovery of three oxylipin biosynthetic genes in *Aspergillus nidulans* named Ppo: psi (precocious sexual inducer)-producing oxygenases (Tsitsigiannis et al., 2005 *Microbiology*) and in *Aspergillus fumigatus* (Tsitsigiannis et al., 2005 *Infection and immunity*). PpoA, PpoB and PpoC are able to regulate reproduction, dispersal of spores and mycotoxins production (Tsitsigiannis et al., 2004 a; Tsitsigiannis et al., 2004 b*;* Brodhagen & Keller 2006; Tsitsigiannis & Keller 2006). Three different LDS isoforms were identified in *Fusarium verticillioides* too (Scala et al., 2013); the deletion of the one of these LDS generates a mutant that produces more mycotoxins, more conidia and resulted more virulent in maize ears (Scala et al., 2014).

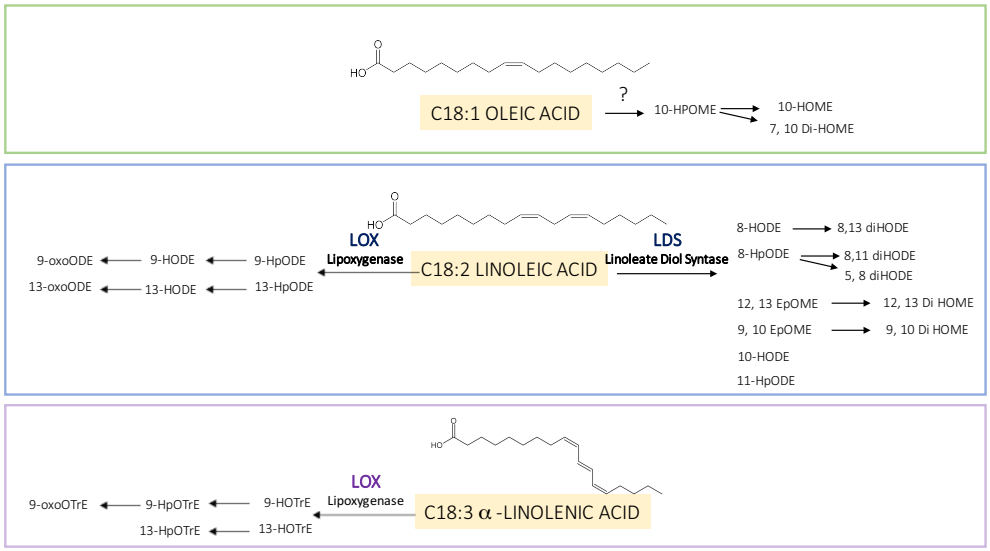

Figure 3. Fungal oxylipins formation by enzymatic route. Principal substrates of oxylipins (oleic, linoleic and α-linolenic acid) and relative hydroperoxides.

In fungi, the oxylipins modulate sexual and asexual sporulation, coordinate the quorum sensing and regulate the density-dependent sporulation (Brown et al., 2009). The density can determine the colonization of the host and the consequent mycotoxins production. Also in yeast, in human pathogen *C. albicans*, the oxidised farnesol regulates the quorum sensing and the biofilm formation (Langford et al., 2009). Even within prokaryotes, lipid molecules regulate some crucial event of the life cycle as the virulence and the biofilm formation (Martínez & Campos-Gómez, 2016).

The oxylipins are among the molecules capable of signaling; they can drive signals between different organism, and studies demonstrate that the oxylipins can be sensed by mean of G-protein coupled receptors (Tag et al., 2000). Christensen & Kolomiets in 2011 proposed a hypothetical model of oxylipin-mediated signal communication between plants and fungi. They suggest that the: "*fungal lipases could be secreted into plant cells where fatty acid substrates are cleaved and processed by fungal secreted lipoxygenases and/or plant lipoxygenases for oxylipin production. Plant-produced oxylipins are perceived and exploited by fungi to regulate GPCR-, PkaA-, and Ppo-mediated growth, sporogenesis, and mycotoxin production. Host manipulation through oxylipin mimicry (i.e. fungal oxylipins binding to host GPCRs and secretion of JA-Ile-like coronatine into host cells) is also implicated*". The model is still valid and the missing dots are less and less.

Sphingolipids: signaling mediators and membrane component

Sphingolipid biosynthesis. A class of lipids gaining new prominence in both mammalian and fungal research is the sphingolipid family. In mammals, these lipids are involved in several processes, like cell proliferation, apoptosis, and stress response. In recent years, the role of sphingolipids in pathogenic fungi was amply studied. The main themes are the sphingolipids involvement in signaling, growth and virulence. Sphingolipids play an important role in the regulation of the balance between the pathogen and the host. ER is the site of sphingolipid biosynthesis (Figure 4), and they can be transported to other organelles especially to the plasma membrane. The structure of sphingolipids consists of a long-chain base (LCB) or sphingoid base and a saturated fatty acid that is amide-linked to the LCB to form a ceramide (Cer). The Ceramide synthase (CerS) is the enzyme that catalyses the amide bond, exist different isoform on the base of the fatty acids length. Another variability factor is the grade of hydroxylation of FAs and LCBs (Breslow et al., 2010; Merril et al., 2011). Complex sphingolipids are the glucosylceramides (GlcCer) and phosphoinositol ceramides (IPC), they are produced by the respective synthase (GlcCerS and IPCS).

Referring to the model organism *S. cerevisiae de novo* synthesis of sphingolipids starts with condensation of a serine and a fatty acyl-CoA, normally palmitoyl-CoA or stearyl-CoA, catalysed by the serine palmitoyl-transferase (SPT) complex (Buede et al., 1991; Nagiec et al., 1994) generates 3-ketodihydrosphingosine, and the reductase Tsc10p catalyses the formation of dihydrosphingosine (DHS) (Beeler et al., 1998). DHS can be hydroxylated by Sur2p to another LCB, the phytosphingosine (PHS) (Bae et al., 2004).

The association between the LCB and VLCFA is catalysed by another ceramide synthase complex formed of Lag1p, Lac1p and Lip1p (D'mello et al., 1994; Valée et al., 2005), leading to dihydroceramide (DHCer) and phytoceramide (PHCer). Complex yeast ceramides provide the addiction of head groups; the IPC synthase Aur1 synthesise IPC (Hashida-Okado et al., 1996). Two ER's proteins Orm1p and Orm2p that bind to SPT and inhibit its activity (Han et al., 2010) regulate the biosynthesis of the sphingolipids.

The catabolism of sphingolipids can be regulated by the phospholipase C encoded by *ISC1*; this enzyme hydrolysed the ceramides DHCer and PHCer by phospholipids. The ceramides are reduced in FFA and LCB by Ydc1p and its paralog Ypc1 (Mao et al., 2000). The free LCBs can be phosphorylated by Lcb4 and Lcb5 leading to DHS-P and PHS-P (Funato et al., 2003).

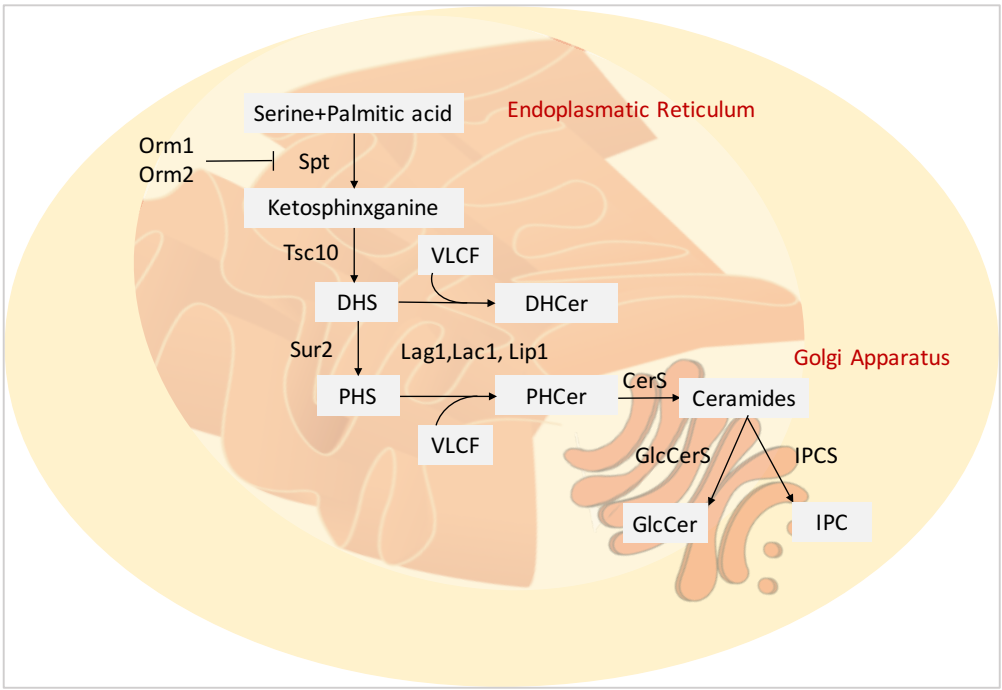

Figure 4. Fungal sphingolipid synthesis. Enzymes catalyzing these processes are described in the text.

Sphingolipids in host-pathogen interaction. Sphingolipids play an important role in the regulation of the delicate balance between the pathogen and the host. Pathogens can produce sphingolipid species that are not present in the host (Heung et al., 2006). The fungal GlcCer presents C-9 methyl group and it is very important for the pathogenesis potentially inducing different plant defence mechanisms (Ramamoorthy et al., 2007). GclCers sprayed on rice leaves function as elicitors and induce the pathogenesis related (PR) protein synthesis (Umemura et al., 2002).

Some plant necrotrophic pathogens can regulate the CerS through the mycotoxins production. *Alternaria alternata* f. sp. *lycopersici*, tomato's pathogen, produce the AAL toxin (Abbas et al., 1995). This toxin is structurally similar to sphingosine and inhibits the CerS activity accumulating LCB into cell sap (Shier et al., 1995); the accumulation of LCBs triggers PCD in tomato (Spassieva et al., 2002). The fumonisins (FBs) are fungal toxins produced by the *Fusarium* genus, in particular by *F. verticillioides* and *F. proliferatum*; FBs are able to inhibit the CerS activity due to the structure similar to sphingosine (Sydenham et al., 1990; Thiel et al., 1991). The two fungal toxins, AAL and FB, are therefore able to induce PCD inhibiting the CerS, and this mechanism can be seen as a virulence strategy of necrotrophic pathogens suggesting a close link between sphingolipid metabolism and plant PCD.

The sphingolipids are important molecules because are the main component of the lipid rafts (LRs).

In the eukaryotic plasma membranes, these structures are rich in sterols and sphingolipids, in mammal, the main sterol is the cholesterol. In fungal LRs, the main components are the glycol-sphingolipids and ergosterol, these areas are implicated in the secretory pathway. In yeast, it was demonstrated the existence of functional lipid rafts composed of glycosphingolipids and the ergosterol (Bagnat et al., 2000). The LRs play important role in the dynamic processes, including protein sorting, cell polarity, and signal transduction. In the budding yeast *S. cerevisiae* LRs are detected with filipin staining on the hyphal tips of the cells induced with mating pheromone (Bagnat et al., 2002; Proszynski et al., 2006). The LRs at the tips of pheromone-induced cells plays an important role in cell polarization and proper localization of components needed for cell and membrane fusion during mating. LRs have also been reported at sites of polarized morphogenesis in *Cryptococcus neoformans* and *Aspergillus nidulans* (Alvarez et al., 2007). These domains may be formed by the clustering of lipid rafts and may therefore function as organizing centers in the membrane.

It was reported that the sphingolipids, in particular the ceramides, can stabilize the LR formation (Massey et al.,2001), this may have important physiological consequences, the ability of ceramide to participate in raft formation, stability, organization is important in signaling, as proposed in several studies (Grassmé et al., 2001, Cremesti et al., 2001).

Conclusion

The knowledge of lipid-mediated signaling in fungi is still fragmented. Nevertheless, the area of lipid-mediated signal communication is a new and fast growing field with implications in quorum sensing in bacteria, microbe niche interactions, insect–plant communication and mammalian disease defenses. Fatty acid biosynthesis and their release phospholipase-mediated regulate the balance between the free fatty acids and the complex lipids, this mechanism is closely linked to the pathogenesis induction and defense responses. Fatty acids can be converted in the modulators of many signal transduction pathways. They can be oxidised and form oxylipins able to mediate the cross-talk between a host and a pathogen. We have seen that also the sphingolipids are crucial in cell signaling, furthermore they can reshape the membrane, forming the lipid rafts that perceive stimuli and trigger response. Thus, lipids play a role that goes further beyond the mere constituents of membranes. Moreover, even their role within the membrane is not "static" by highly dynamic contributing, for instance, to the instant and transient assembly of the intriguing Lipid Rafts. Their chameleonic nature made these molecules fabulous drivers of information exchange. In relation to this, lipids act as shapers of the host-pathogen interaction, allowing to each contenders to develop fine-tuned strategies of defense or virulence.

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1. Sphingolipids in *Fusarium verticillioides – Zea mays* interaction

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The sphingolipidome, i.e. the whole ensemble of sphingolipids, goes through perturbation during the interaction between the caryopses of *Zea mays* and the mycotoxigenic fungus *Fusarium verticillioides*. Fumonisins, mycotoxins produced by *Fusarium verticillioides*, should inhibit the activity of ceramide synthase in plant host cell, leading to sphingoid bases accumulation and complex sphingolipids depletion. Sphingoid bases accumulation should trigger, in turn, programmed cell death (PCD) activating MPK6-Salycilic acid pathway, while complex sphingolipids depletion could cause cell death because of membrane and vesicle impairment. Maize sphingolipidome could be perturbed also in the early phases of infection, when the cell membrane could go through a rearrangement leading to the formation of defense related lipid rafts enriched in complex sphingolipids.
The level of variation of several sphingolipids and salicylic acid was evaluated, by MRM approach in LC-MS/MS, in maize ears artificially infected with *F. verticillioides* at different times post inoculation. We compared this metabolic profile with the expression of genes related to PCD onset and execution. We hypothesize that *F. verticillioides* could jeopardize the sphingolipids metabolism of the wounded caryopses of *Z. mays* for switching its pathogenic growth from endophytic to necrotrophic.

Introduction

Sphingolipids are a wide group of compounds, having both structural and signaling roles in every eukaryote. The common backbones shared by all sphingolipids are long-chain bases, even called sphingoid bases: they are hydroxylated hydrocarbon chains with an amino group on carbon two. LCBs, eventually hydroxylated or unsaturated, are building blocks of sphingolipids together with fatty acyl-CoA. They are substrates of Ceramide Synthases (CerS), which catalyze amide bond formation between sphingoid base amine group and fatty acid carboxylic group. These N-acyl-derivatives are called generally ceramides (*Merrill et al. 2005, Merrill et al., 2011*). There are different isoforms of CerS preferring very long chain fatty acids (VLCFA), i.e. fatty acids from 20 up 36 carbons chain length, or long chain fatty acids. Another factor affecting sphingolipids variability is, in addition to fatty acids length, their hydroxylation degree: they can be α-hydroxylated or Δ9 unsaturated (*Markham et al. 2013; Merrill et al., 2011*).
Ceramides are backbones of more complex sphingolipids such as glucosylceramides (GluCers) or glycosil-inositol-phospho-ceramides (GIPCs). GIPCs and GluCers are aconstitutive element of plant plasma membrane and endo-membranes and have a pivotal role in defining membrane microdomains known as "lipid rafts" (*Malinsky et al., 2013; Simon-Plas et al., 2011*). There is an intimate connection between the unbalance of sphingolipid metabolism and the onset of PCD in plants disease caused by necrotrophic pathogens (*Wolpert et al. 2002*). Sphinganine analog mycotoxins – SAM (SAM) such as AAL toxin and fumonisins inhibit the host ceramide synthases causing LCB accumulation and cell death in *Arabidopsis* cell (*Stone et al., 2000*). The mutant strain of *F. verticillioides* being deficient for FUM1 polyketide synthase gene are not virulent (*Sanchez-Rangel et al., 2012*). Fumonisin-induced PCD in *Arabidopsis* is triggered by increased LCB levels (*Shi et al., 2007*) and reveals association with ROS generation, callose and phenolic compounds deposition, phytoalexins production and generally with pathogenesis-related genes expression (*Nishimura et al., 2003; Stone et al., 2000*). It requires ethylene, jasmonate and salicylic acid (SA) dependent signaling pathways (*Asai et al., 2000; Nishimura et al., 2003; Sanchez-Rangel et al., 2015*). SA synthesis is modulated by MPK6, a crucial molecular hub for different signaling pathways (*Bartels et al., 2009*). MPK6 is involved in PAMP-triggered

immunity (PTI) (*Pitzsche et al., 2009; Zhang et al., 2007*), in effector-triggered immunity (ETI) (*Menke et al., 2004*) and, very interestingly, it is probably downstream to long chain bases in SA-dependent PCD provoked by ceramide synthases inhibitors such as fumonisins (*Sanchez-Rangel et al., 2015*). A recent work in *Arabidopsis* demonstrates that fumonisin FB1 or sphinganine administration increased the activity of MPK6; while in the MPK6-deficient mutant, neither FB1 nor exogenous sphinganine can induce PCD. It is not clear up today if sphingoid bases modify MPK6 directly or not (*Saucedo-Garcìa et al., 2011*). Another fundamental feature of fumonisins is the activation of the SA-dependent PCD inducing hydrolytic enzymes. Among these, vacuolar processing enzyme (VPE) and caspase-like proteins are active (*Kuroyanagi et al., 2005; Li et al., 2008*). As regards last ones, a special mention is for metacaspases. Several metacaspases were characterized in *Arabidopsis* and there are increasing researchers about monocots. Particularly, TaMCA4 type II wheat metacaspase was found to give resistance against the biotrophic pathogen *Puccinia striiformis* f.sp. *tritici* (*Wang et al., 2012*).

To summarize, it seems clear that toxin perturbation of sphingolipid metabolism leads to a SA signaling dependent PCD, but it is necessary to understand what molecules are definitively responsible for it. Contrasting results are reported about recognizing the main role as PCD inducer for sphinganine or phytosphingosine. Some studies suggest the first one (*Saucedo-Garcìa et al., 2011*), some other the second (*Shi et al., 2007*). Surely, the increase of endogenous LCBs is related generally to AAL toxin and Fumonisins (especially FB1) derived PCD, consistently with the action mechanism of such sphinganine-like toxins (*Akamatu et al., 1997 Brandwagt et al., 2002, Gechev et al., 2004, Islam et al., 2012*).

PCD is not triggered only by LCBs, but by ceramides too. *Arabidopsis* deficient mutant for ceramide kinase *acd5* showed ceramide accumulation leading to PCD, while phosphate ceramides could suppress it (*Liang et al., 2003*).

Another factor to be considered in ceramide-triggered PCD is fatty acids length. It seems that VLCFA ceramides are involved in vesicle trafficking and lipid rafts formation. VLCFA sphingolipids define both membrane microdomains, in which typical pathogenic response protein take place, and specific cargo vesicle membranes, which definitely concur to microdomains formation (*Berkey et al., 2012, Markham et al., 2011*). Consequently, their depletion can provoke plasma membrane organization impairment and finally cell death (*Markham et al., 2011*).

Over-expression of a specific ceramide synthase (i.e. LOH2) in *Arabidopsis* leads to d18/16:0 accumulation and triggering of SA accumulation and PCD (Siebers et al. 2016). Pathogen-specific sphingolipids similarly to plant ones may elicit a immune response in the host. This is the case of cerebrosides, classified as glycosphingolipids, from *Magnaporthe oryzae*. Cerebrosides treatment in rice leaves causes phytoalexins accumulation, HR, increased resistance to subsequent infection with compatible pathogens. As intact molecules, their unglycosylated cognate ceramides and long chain bases have been found to induce an immune response (*Umemura et al., 2000; Umemura et al., 2004).* Anyway, the underlying mechanism has to be clarified. It is likely that PCD may be driven by long-chain bases "perception" as previously seen for toxin-induced sphingolipid metabolism perturbation; nonetheless, another fascinating hypothesis should be proved, i.e. the possibility that exogenous sphingolipid could act as PAMP (*Kurusu et al., 2011*). Concerning this, the presence in the host of a receptor for fungal sphingolipids remained elusive.

Taking in account this whole background, we tried to define a model describing the host-pathogen interaction between *Zea mays* (maize) and *Fusarium verticillioides*. Our *ex vivo* experimental system, reproducing naturally occurring field infection, consisted in artificially infected maize ears. Before evaluating sphingolipidome perturbation in infected maize along disease progression, we proceeded in maize and *F. verticillioides* sphingolipidome characterization, in order to find species specific compounds or molecules showing abundance variation along the different infection steps. At the same time, we tried to shed light on maize PCD-related genes expression during the interaction with *F. verticillioides*.

RESULTS

Maize and *F. verticillioides* sphingolipidome characterization

Extracted sphingolipids were separated and characterized by LC-MS/MS, as described in Materials and Methods section. For each compound class fragmentation spectra have been chosen. After fragmentation, in positive ionization, sphingoid bases lose water moieties. Parental ion (or molecular ion) [M+H$^+$] should be the less abundant ion, while di-dehydrate one [M+H$^+$-2H2O] the most present. Saturated C18 LCBs, i.e. sphinganine and phytosphingosine, can exhibit a typical 60 m/z fragment indicative of a trimethyl-ammonium moiety (**Supplementary figure 1**).

As regards ceramides and phytoceramides, their molecular ion undergoes water losses as well as sphingoid bases. Furthermore, their fragmentation pattern clearly includes cognate sphingoid base fragments with their associated water losses, and eventually the amide bounded fatty acid ion (**Supplementary Figure 1**). Glucosyl-ceramides harbor an additional glucose moiety constituting a neutral loss, hence not detectable as positive ion. The sphingolipidome profile presented significant differences between *Z. mays* and *F. verticillioides*, referring to two implemented MRM methods. The first MRM method includes some glucosyl d18:2 ceramides, various types of ceramides and fungal d19:2 glucosyl-ceramides, the second one phytoceramides and dehydro-phytoceramides.

In **Supplementary Figure 2 (A and B)**, non-infected maize kernels and *F. verticillioides* mycelia overlaid total ion chromatograms, are shown. Just at glance, without the need of further plot magnification, it is possible to note different profiles. This is because some compounds are detected only in *F. verticillioides* (m/z 506, 680, 710, 736, 754, 822), some other only in maize (m/z 536, 562, 564, 566, 596, 696, 698, 724, 770, 780, 808).

Specifically, in **Supplementary Tables 1-6** all characterized compounds are listed and grouped according to their detection into the sole maize, in *F. verticillioides* or shared by maize and *F. verticillioides.*

Sphingolipids level evaluation in infected maize

Sphingolipidome perturbation is observed among maize-specific and maize-fungus shared sphingolipids. Firstly, we evaluated LCBs level along infection, calculating infected /control samples ratios, and we kept proceeding the same way for all characterized compounds. The LCBs are down-represented at 2 dpi (days post inoculation) in particular the levels of d18:2, d18:1, d18:0 and t18:0. Following infection, LCB levels increased: at 4 dpi all LCBs are over-represented. At the same time point, d18:0 and d18:2 reached their maximum, while top level of d18:1 and t18:0 is reached at 7 dpi. Afterwards they tend to decrease but remaining over-represented, except for d18:0 (**Figure 1**).

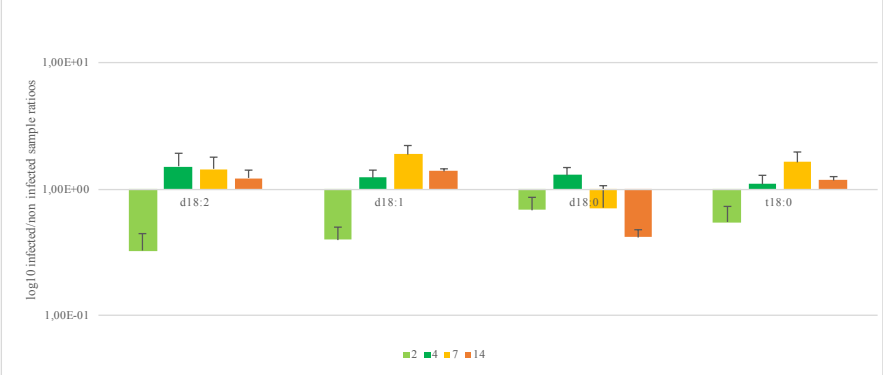
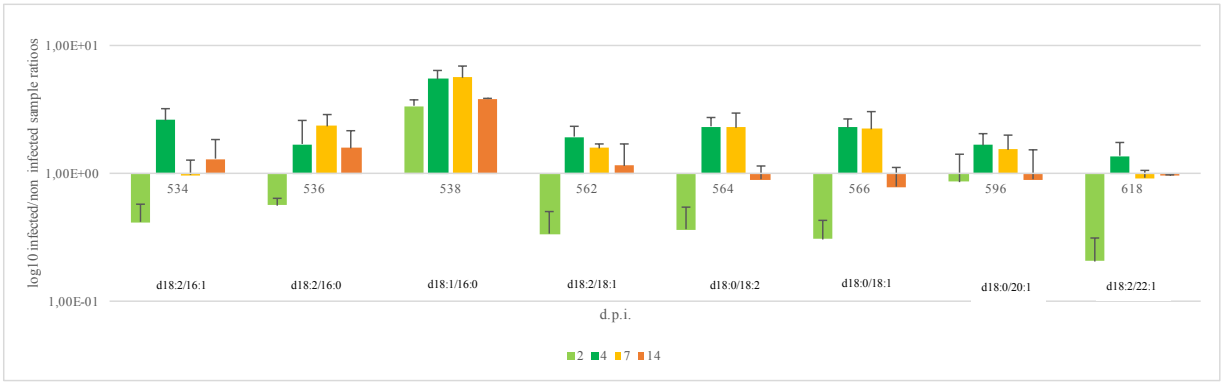


Figure 1. Long chain bases level variation at different days post inoculation. Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

As regards the rest of sphingolipidome, i.e. sphingolipids *stricto sensu*, we observed different trends. d18:2 and d18:0 ceramides apparently had a trend similar to LCB, with a slight down-representation at 2 dpi and a maximum amount achieved between

and 14 dpi (**Figure 2A**). The sole d18:1-ceramide considered is the d18:1/16:0. Notably, this ceramide is over-represented into the infected samples at 4-7 dpi (**Figure 2B**).

A)



B)

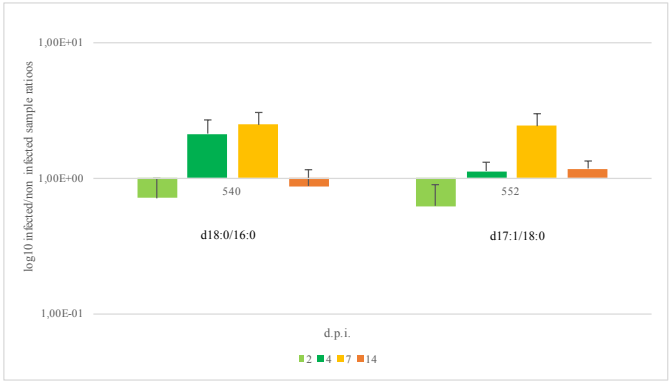


Figure 2A and B. Ceramides level variation at different days post inoculation. Plots A refers to ceramides characterized only in maize; B) ceramides characterized both in maize and *Fv10027*; Y axis: log10 of infected/non infected sample ratios. X axis: time points, i.e. days post inoculation. Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

Similarly d18:1/16:0, glucosyl-ceramides are constantly over-represented from 2 to 7 dpi and then decrease with a compound-specific level of significance at 14 dpi (**Figure 3**).

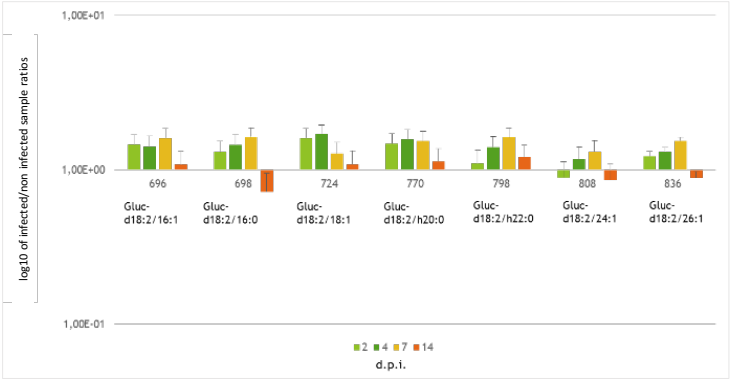
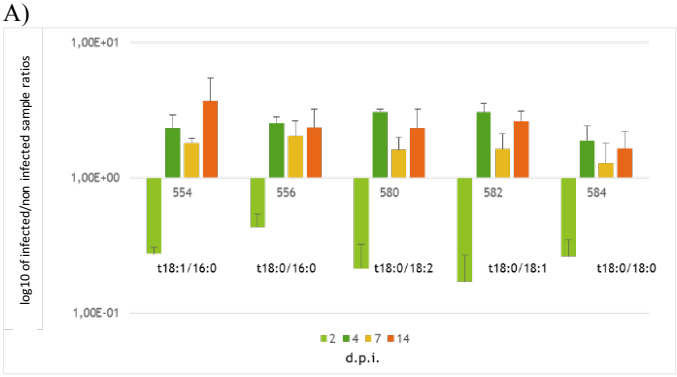


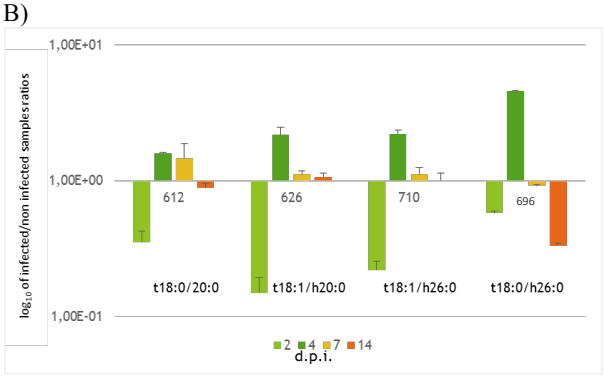
Figure 3. Glucosyl Ceramides level variation at different days post inoculation. Each compound of this group was characterized only in maize. Y axis: log10 of infected/non infected sample ratios. X axis: time points, i.e. days post inoculation. Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

Phytoceramides and dehydrophytoceramides cluster into two different trends; first one including m/z from 554 to 584 (**Figure 4A**), the second one from 612 to 696 (**Figure 4B**). The first group includes sphingolipids harboring long chain fatty acids (LCFA), as palmitic, stearic, linoleic, linolenic, whereas the second group compounds include into their skeleton very long chain fatty acids (VLCFA) such as nervonic acid. Both classes were under-represented at 2 dpi, but while the first group showed a following over- representation persisting for the rest of infection period, the second group exhibited a maximum at 4 dpi followed by a marked decrease leading to an under-representation at 14 dpi (**Figure 4 A-B**). Compounds with m/z 656, 684 and 698 were previously identified as up-regulated in naturally infected maize hybrids (**Figure 4C**; Dall'Asta *et al.*, 2014).

A)



B)



C)

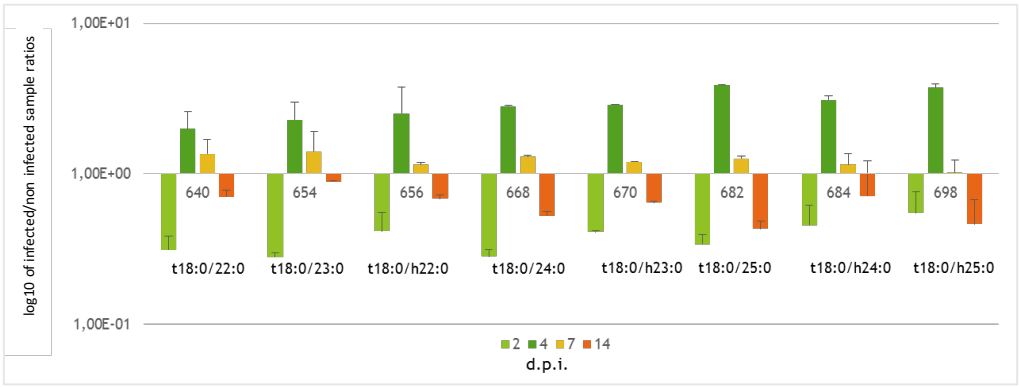


Figure 4 A-C. Phytoceramides and DehydroPhyoCeramides level variation at different days post inoculation. A) Sphingolipids harboring long chain fatty acids, 554 (t18:1/16:0) is the only dehydrophytoceramide of this group. B) Sphingolipids harbouring very long chain fatty acids (VLCFA). Compounds shown in A and B have been characterized only in maize. **C**) Compounds characterized both in maize and *in F. verticillioides*. Y axis: log10 of infected/non infected sample ratios. X axis: time points, i.e. days post inoculation. Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE)

Fumonisin production analysis

The total amount of fumonisins B (B1+B2+B3) production in maize ears during *F. verticillioides* infection was quantified by LC-MS/MS. **Figure 5** showed an increasing trend in FBs total production along the whole time course of infection in Fv10027-infected maize ears. No significant amount of FBs was detected into mock.

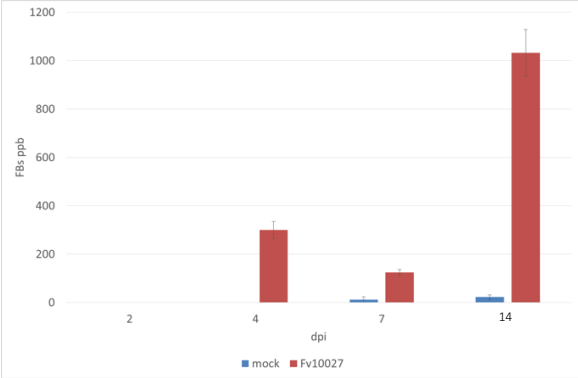
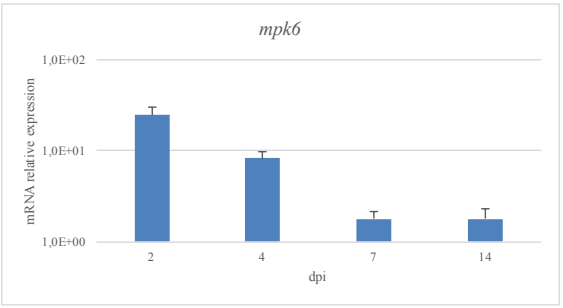


Figure 5. Fumonisins B (B1+B2+B3) synthesis in maize-infected ears at different time post infection (2-15; dpi). Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

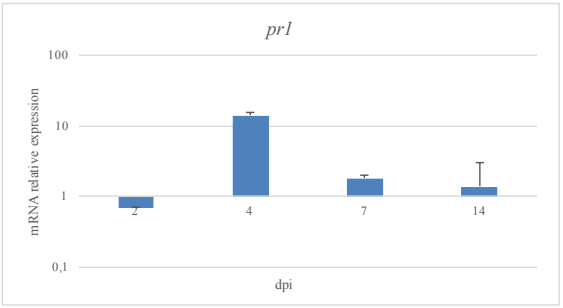
Gene expression analysis and SA quantification

To evaluate the respond of the maize infection we have analyzed some genes involved in PCD, the chosen genes are *mpk6*, *pr1* and *mcaII*. As previously discussed, *Arabidopsis* MPK6 is a crucial molecular hub for different signaling pathways as well as PTI, ETI and LCB-induced PCD. MPK6 is supposed to induce Salicylic Acid synthesis, which in turn is supposed to be necessary to induce the transcription of *pr1* and *mcaII* genes. The first one encodes a defense antifungal protein, the second a type II metacaspase, hydrolytic enzyme involved in PCD triggering. Gene expression plots were shown in **Figure 6 A-C**. Infected kernels up-regulated all three genes in comparison with uninfected ones. Notably, *mpk6* was early induced soon after fungal inoculation (2dpi) whereas the SA-dependent genes, *pr1* and *mcaII*, achieved the maximum at 4 dpi (**Figs. 6 A-C**).

A)



B)



C)

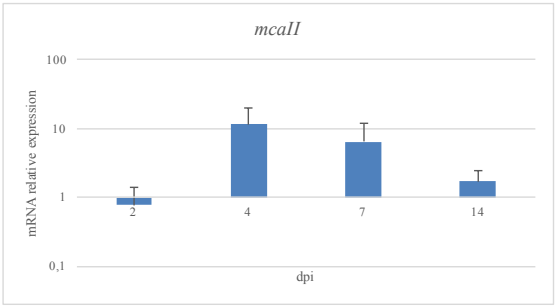


Figure 6 A-C. mRNA relative expression of *mpk6*, *pr1* and *mcaII* calculated as $2^{-\Delta\Delta ct}$. It was used α-actin of *Zea mays* as housekeeping gene and the ct values of infected samples were normalized on mock (uninfected). Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

Salicylic acid (SA) was quantified by LC-MS/MS. In **figure 7**, it is reported the ratio between the values of SA into Fv10027 infected maize kernels *versus* SA values into uninfected mock (expressed as log10). SA amount (ppb) into mock and Fv10027-

infected kernels is reported in **Supplementary Fig. 3**. SA increased into infected samples soon after 4 dpi and the trend kept growing up to 14 dpi (**Fig. 7**).

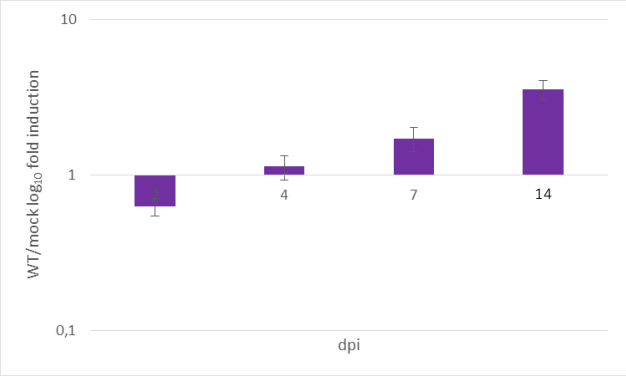


Figure 7. Salicylic acid fold induction (expressed as log10) into Fv10027 infected maize kernels *versus* uninfected mock at different time after infection (2-14 dpi). Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

Localization of *Fusarium verticillioides* in maize during seed colonization and consequences on seed structure

All kernels inoculated with *F. verticillioides* resulted externally infected; fungal mycelium was clearly observed outside kernels after 6 days of incubation and became more evident after 12 days of incubation (**Figure 8 A, B**). In untreated kernels, no fungal growth on kernel surface was observed during the experiment.

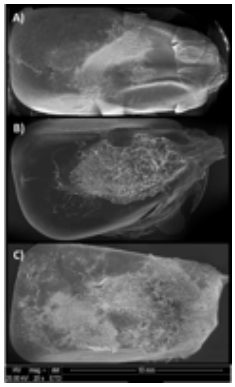


Figure 8 A-C. *Fusarium verticillioides* mycelium on maize kernel after 6 (A), 12 (B) and 15 (C) days of incubation at 25°C in a high humidity chamber (Scanning electron microscope, 20x).

Fusarium verticillioides mycelium resulted always present in the internal section of artificially inoculated kernels, since 6 days of incubation, and the biomass increased gradually up to 14 days (**Figure 9 A-F**). In untreated kernels (control), no fungal growth in the inner part of seeds was observed, even after 14 days of incubation. In both samples (uninfected and Fv10027-infected) the external layer probably collapsed and detached from endosperm since, as evidenced even in mock, the tissues acquired a *pro*-apoptotic phenotype (*Sabelli 2012*). This phenomenon resulted anticipated in infected samples (white arrows in **Fig. 9 A, D**). Concerning the viability of embryo, apparently this is preserved normally in uninfected samples, where the coleoptile and the radicle were developing (white arrows in **Fig. 9 A-C**); in Fv10027-infected samples indeed, symptoms of embryo degeneration and/or abnormal development were already visible at 6 dpi and became more evident at 12 dpi. At 15 dpi, the embryo of infected samples has completely collapsed (**Fig. 9 F**).

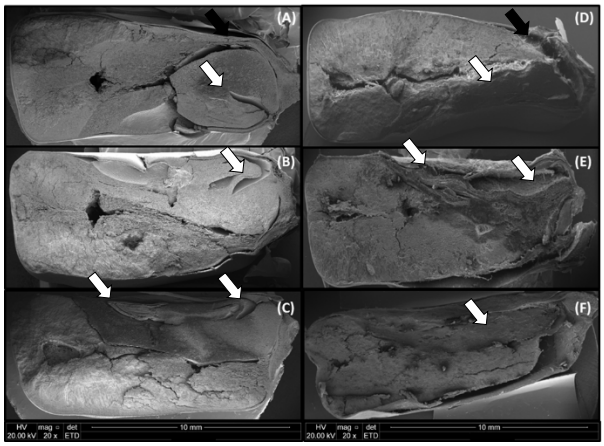


Figure 9 A-F. Section of maize kernels, untreated (A) and artificially inoculated (D) with *F. verticillioides* (inoculum 10^6 spores/mL) after 6 days, 12 days (B and E, respectively) and 15 days of incubation (C and F, respectively) at 25°C (Scanning electron microscope, 20x).

DISCUSSION

F. verticillioides can be considered both a parasite and a saprophyte of maize and it is normally found as an intercellular endophyte, frequently symptomless. This balanced relationship can change into a disease depending on both biotic and abiotic factors (*Bacon et al., 2008*). Maize infection takes place through silks during flowering as main pathway (*Leslie and Summerell 2006*) and it can be facilitated by pest damages since insect favor *F. verticillioides* dissemination and infection (*Sobek and Munkvold, 1999*). The visual results of ear infection vary; some kernels are symptomless while others are obviously damaged and infected. Within the kernels, *F. verticillioides* might locate in the pedicel of kernels as a very small amount of hyphae but in caryopses hugely contaminated with fumonisins, the pathogen is usually found as an extensive mass of sporulating hyphae, which colonizes most of the internal section of the kernel, including the embryo. The nature of the systemic kernel infection is such that seed germination is not necessarily affected, but rather seedling vigor and growth can be reduced (*Baconi and Nelson, 1994; Duncan and Howard, 2010*). The nature of the interaction with seed embryo is still unclear. Notably, no clear assessment on the fate of maize embryo after *F. verticillioides* invasion of kernel tissues is available.

According to us, in certain conditions such as kernel damages induced by insects or simulated by artificial wounding, the pathogen may trigger programmed cell death into kernel tissues, thus prompting a necrotrophic growth at the expense of embryonic lipid-rich tissue. To achieve this program, *F. verticillioides* would jeopardize the sphingolipid metabolism of maize kernels, forcing the host to accumulate LCBs and LCFA-ceramides such as d18:1/16:0. Indeed, it has been already suggested that FB1 may lead to ceramide synthase inhibition with a consequent accumulation of LCBs into plant tissues (*Abbas et al., 1994; Williams et al., 2007*). In our study, LCB levels (d18:0, d18:1 and d18:2) increase following kernel infection already at 4 dpi, that is when FBs are detected at significant values. LCBs level increase along *F. verticillioides* infection is consistent with previous literature describing a correlation between fumonisin level and ceramide synthase inhibition. According to such data, LCBs achieve their top level just before the maximum production of Fumonisins. These data confirm for the first time at *in planta* level using a real infection (instead of the amendment of fumonisin *per se*) the correlation between fumonisins and LCB increase in maize kernels. Nevertheless, we cannot assess with the dataset that this increase is actually caused by cerS inhibition. It is clear that LCBs are accumulated during *F. verticillioides* infection of maize caryopses. Since *F. verticillioides* spent part of its infection cycle as an endophyte and subsequently that at the end of kernels colonization could affect embryo viability and/or efficiency, behaving more as a necrotrophic pathogen than an opportunistic one, we may hypothesize that fumonisin accumulation could pave the way for this change in the infection *modus operandi.* Other fungi, such as *Parastagonospora nodorum* uses necrotrophic effectors such as SnToxA for eliciting PCD in wheat leaves and switch from an epiphytic to an endonecrotrophic growth (DuFall *et al.*, 2012). Thus, we hypothesized that *F. verticillioides* uses fumonisin for hampering

sphingolipid metabolism and accumulating LCB. In turn, accumulation of LCB (*Barkey et al., 2012*) and of specific ceramides (i.e. d18/16; *Siebers et al., 2016*) may trigger defense and PCD processes. In *Arabidopsis* MPK6, as already discussed, is a MAP Kinase known to be an LCB-downstream Salicylic acid synthesis inductor. Saucedo Garcia *et al*. demonstrated the capability of free LCBs and FB1 to enhance MPK6 kinase activity trough *in vitro* assays (*Saucedo-Garcìa et al., 2011*). Maize homologous *MPK6* transcription levels arisen early (at 2dpi) indicating a putative availability of MPK6 enzyme to be modified by LCB accumulating parallel and following FBs production. In turn, MPK6 should be able to control SA biosynthesis and its related signaling pathway as stated elsewhere (*Bartels et al., 2009*; *Chai et al., 2014*).

In our system, following pathogen perception SA arise above the basal level (Fig. 7) following 4 dpi, i.e. early after *mpk6* upregulation (at 2 dpi, Fig. 6A) and co-occurring with maize enrichment with LCB and d18/16 ceramides (Fig. 1-2) as depicted in the scheme (**Figure 10**). As Glucosyl Ceramides are known to be mainly cell membrane elements, it is reasonable to "read" their increase (Fig. 3) as infection-driven cell membrane rearrangement, likely linked to lipid rafts formation. It would be interesting to verify this hypothesis through lipid rafts investigation approaches. Starting from 4 dpi, maize homologous *pr1* and *mcaII* seem instead to be best over expressed, with a following slight decrease. As their transcription is likely Salicylic Acid dependent, we can hypothesize that only at 4 dpi, SA level is sufficient for inducing downstream transcription. More important from 4 dpi, *F. verticillioides* starts to produce fumonisins. It is conceivable that FBs accumulation could significantly alter maize sphingolipidome as suggested by our results. Notably, VLCFA biosynthesis could be more affected by fumonisins contamination. Recent studies (*Markham et al. 2011*) demonstrated that, in *Arabidopsis*, different isoforms of CerSs possess differential preference on fatty acids substrate and, in particular, there are CerSs preferring VLCFA. The same study suggests that FB1 could selectively inhibit VLCFA sphingolipids synthesis. The authors indicate a putative role for VLCFA sphingolipids depletion in PCD triggering, related to the possible impairment of defense membrane protein vesicular transport. More recently, the deletion of VLCFA-producing cerS (LOH1 and LOH3) and the resulting up-regulation of LCFA-producing LOH2 triggers SA pathway and cell death (Siebers et al., 2016). In relation to this, fumonisins may impair the synthesis of VLCFA sphingolipids and enhance that of LCFA-Cer in our system leading to PCD, paving the way for the necrotrophic growth of *F. verticillioides* into maize caryopsis tissues as indicated in **figure 10** and suggested by embryo tissue degeneration starting from 6/12 dpi (Fig. 9 D-E).

In conclusion, we can remark a general sphingolipidome perturbation in *F. verticillioides*-infected maize kernels. Compounds whose level was observed to significantly vary are putative biomarkers of fungal infection. Some lipid species have been characterized only in the host or in the pathogen, and they are absent during the interaction, we can suggest a double interpretation: the first is that they are species-specific, the second is that these species could undergo a negative feedback caused by the co-presence of the plant and fungus. Implementation of MRM methods regarding naturally infected cobs would be desirable in order to detect *F. verticillioides* presence even at early stage of maize maturation even at its endophytic stage. If further proven that the fumonisins are able to alter the sphingolipidome leading to a change in the pathogen lifestyle (from endophytic to necrotrophic) and it explains the occurrence of symptoms in the case of infection starting from a wound artificially or naturally caused in maize kernels.

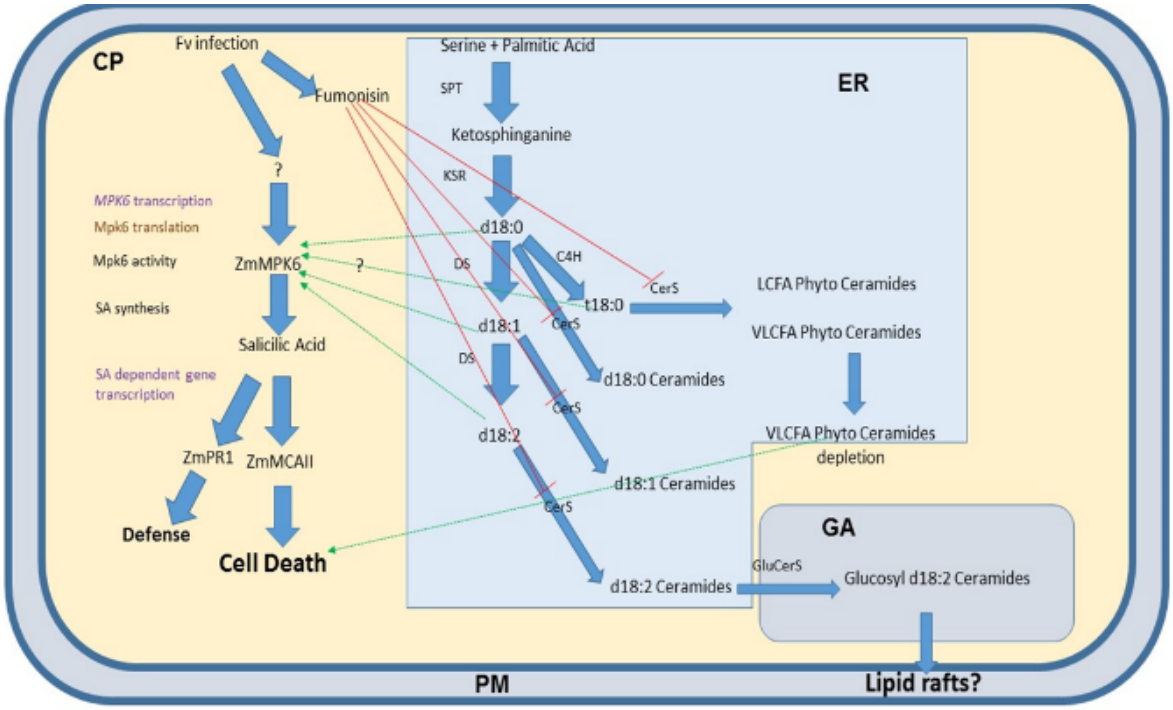


Figure 10. A scheme representing our hypothesis. At early stage of infection (2-4 dpi) the innate system of the plant perceives the pathogen and trigger a phosporilative cascade involving *mpk6*. At early stage (2 dpi) the whole sphingolipidome is severely affected with the notable exception of glc-cer that results enhanced. At 4 dpi, pathogen produced FBs starting to inhibit the production of VLCFA-Cer and lead accumulating LCB and LCFA among which the cell death inducer d18:16:0 (*Siebers et al., 2016*). In this hypothesis, MPK6 might be activated (Saucedo-Garcìa et *al.*, 2011) and stimulate SA synthesis and signaling pathway as indicated by *pr1* and *mcaII* mRNA enhancement. Co-occurrence of metacaspase-like activation and VLCFA accumulation may explain the severe degeneration of embryonic tissue into Fv10027-infected caryopses.

MATERIALS AND METHODS

Fusarium verticillioides strains and culture conditions

Fusarium verticillioides ITEM 10027 was isolated from maize kernels by Università Cattolica del Sacro Cuore (Prof. P. Battilani; code MPVP 294) and filed in Institute of Science and Food Production collection (ISPA-CNR, Bari, Italy; http://server.ispa.cnr.it/ITEM/Collection). Fv10027 wild type strain was cultured on Czapek-DOX medium amended with yeast extract (CDY):Yeast extract 0.5% w/v; Sucrose 3g/L; $NaNO_3$ 2g/L; KH_2PO_4 1g/L; $MgSO_4$ 0,4 g/L; KCl 0,5 g/L; $FeSO_4$ 0.016 g/L; $ZnSO_4$ 5 g/L; $NaMoO_4$ 1 g/L.

Before inoculation, media were sterilized in an autoclave at 121° C for twenty minutes. CDY cultures (50 mL) were inoculated with a suspension of 1 x 10^4 *F. verticillioides* conidia/mL, and incubated at 25° C in the dark on a rotary shaker. Mycelia were harvested 7 days post-inoculation, filtered, lyophilized and turned to powder in liquid nitrogen. All the culture media were provided by Sigma-Aldrich (St.Louis, MO, USA).

Artificially inoculated maize cobs and culture conditions

Wild type *F. verticillioides* strains were inoculated onto Potato Dextrose Agar (PDA) plates and incubated at 25°C in darkness for a week. 5mL of sterile water was added to each colony in order to prepare conidial suspensions, which after are adjusted to a 10^5 conidia/mL concentration. Commercial maize hybrids cobs were collected at dough stage and infected by the pin bar

technique. It consists in making wounds all around the middle area of the cob, without removing the husk, obtaining visible holes. Each wound was inoculated with 10^4 conidia. After inoculation cobs were placed in plastic bottles containing, as a nutritional source, 50 mL of Hoagland's solution (1 mL KH_2PO_4 1M, 5 mL KNO_3 1M, 5 mL $Ca(NO_3)_2$ x $4H_2O$ 1M, 2mL $MgSO_4$ x $7H_2O$ 1M and sterile water to 1L) at the bottom. Both inoculated and non-inoculated cobs were incubated at room temperature. Environmental conditions were conducive for *F. verticillioides* infection. Room temperature was stable at 23-25°C and there was natural light with natural day/night duration. Different incubations time were considered: 0, 2, 4, 7, 14 days after inoculation. Each thesis was managed in triplicate. After incubation, cobs were stored at -18°C until analysis. Cobs were hand de-husking. Kernels from each cob (i.e in triplicate for each incubation time) were lyophilized, ground and turned to powder.

Chemicals

All solvents used for sphingolipids extraction and HPLC/MS analysis were of HPLC/MS grade. Methanol (MeOH), isopropyl alcohol (iPrOH), ethyl acetate (EtOAc), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Formic acid (HCOOH) was purchased from Fluka (Buchs SG, Swiss).

Mycelia and maize sphingolipids extraction

Sphingolipids were extracted either from *F. verticillioides* or from infected and non-infected maize kernels. The internal reference standard added for the quantitative analysis was N-palmitoyl-d31-D-*erythro*-sphingosine (C16 D31 ceramide, MW 569.092 g mol^{-1}), at a final 1µM concentration. For both maize kernels and mycelia, the extraction was performed on 30 mg of lyophilized matrix previously turned to powder. They were vortex-mixed with 2 mL of extraction mixture (iPrOH: H_2O: EtOAc 1:1:3 v/v, with 0.0025% w/v of butylated hydroxytoluene to prevent peroxidation). After centrifugation, the ethyl acetate upper phase was collected in a clear tube and dried under nitrogen gas flux. The extraction was repeated on the initial matrix adding 1,2 mL of EtOAc and then vortex-mixing. After centrifugation, the upper phase was recovered and transferred to the collection tube together with the previously extracted fraction, and dried under nitrogen flux. The dried samples were reconstituted in 100 µL of MeOH and loaded onto the HPLC auto-sampler.

Sphingolipids analysis by LC-MS/MS

The equipment, chromatographic column and analysis software were all from Agilent Technologies (Santa Clara, CA, USA). Samples were analyzed by LC (HPLC 1200 series rapid resolution) coupled to a triple quadrupole MS (G6420 series triple quadrupole, QqQ; Agilent Technologies) equipped with an electrospray ionization source (ESI). The acquisition was in positive ion mode. Chromatographic separation was performed through a Zorbax SB-C8 rapid resolution HT 2.1 x 50 mm 1.8 µm 600 bar column (Agilent Technologies). The mobile phases consisted of A phase (water: B phase 40:60 v/v, containing ammonium formate 5mM), B phase (MeOH: ACN: iPrOH 70:20:10 v/v). The elution programme was as follows: 0-6 min 20% B, 6-14 min 98% B , 14-18 min 98% B, 18-20 min 100% B, 18-20 min 100% B, 20-22 min 100% B, 22-26 min 20% B, 26-28 min 20% B. The flow rate was 0.6 ml min^{-1}. The column was thermostated at 60°C. The injection volume was 5 µL. The injector needle was washed with the mobile phase in the wash port at the end of each HPLC run. Nitrogen was used as the nebulising and desolvation gas. Drying gas temperature was 350°C, its flow was 10 l min^{-1}, the nebulization pressure was 20 psi. Experiments of precursor ion and product ion to characterize sphingolipidome, and of MRM to quantify each compound were performed. The fragmentor voltage and collision energies were optimised for each compound. Among fragments, the most abundant and possibly compound-specific are chosen. These are denoted as main transition and qualifier ions. Each compound is identified through MRM experiments by associating a precursor ion to its specific fragment and chosen CE and fragmentor (see Supplementary Tables 1-6).

SA analysis by LC-MS/MS

SA is extracted from *Fusarium verticillioides* infected and non-infected maize kernels. The quantification was done by the addition of internal standard 1-Naphthaleneacetic acid (NAA MW 186.21 g mol^{-1}), at the final 5 μM concentration. An amount of 30 mg of lyophilized mycelium was ground under liquid nitrogen with mortar and pestle. SA was extracted with a method described previously with some modification (cit.). SA was extracted with 750 μL of MeOH:H_2O:HOAc (90:9:1, v/v/v), they were mixed and centrifugated for 1 min at 10.000 rpm. The supernatant was collected and the extraction was repeated. Pooled supernatants were dried under nitrogen gas flux. The dried samples were resuspended in 200 μL of 0.05% HOAc in H_2O-ACN (85:15, v/v). The analysis of SA was performed with the instrumentation, previously described, used for the sphingolipid analysis. The acquisition was in MRM negative ion mode $[M-H]^-$. Chromatographic separation was performed with a Zorbax ECLIPSE XDB-C18 rapid resolution HT 4.6 × 50 mm 1.8 μm p.s. column (Agilent Technologies), was used at ambient temperature and the injected volume was 10 μL. The mobile phases consisted of A: H_2O containing 0,05% HOAc, and B: ACN. The elution gradient was as follows: 0-3 min 15%B, 3-5 min 100% B, 5-6 min 100% B, 6-7 min 15% B, 7-8 min 15% B. The gradient was followed by 5 min for re-equilibration. The flow-rate was constant at 0,6 ml min-1. The injector needle was washed with the mobile phase in the wash at the end of each HPLC run. Nitrogen was used as the nebulizing and desolvation gas. The temperature and flow of the drying gas were 350°C and 10 l min–1, respectively, with a nebulization pressure of 20 psi. The capillary and cone voltage was 4000 V. The main transition and qualifier ions for SA is 137.2→92.9, and for the internal standard NAA is 245→180.8. We used the following parameters for the analysis: for the SA the fragmentor is 135 V and collision energy 20 eV, for the internal standard NAA the fragmentor is 100 V and the collision energy is 16 eV.

Maize RNA extraction and Gene expression analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) based protocol, with the only DEPC sterilized material. RNA was extracted as previously described upon different post-inoculation time infected and non-infected maize cob (see *Artificially inoculated maize cobs and culture conditions* paragraph in this section). The extraction was performed on 30 mg of lyophilized matrix previously turned to powder in liquid nitrogen, adding 1 mL of cold Trizol previously described (Scala et al., 2014). RNA was quantified trough Qubit 2.0, using Qubit® RNA Assay kit. The quantification protocol was performed as recommended in factory user manual (Invitrogen, Eugene, Oregon, USA). Starting from RNA, cDNA was synthesized using Tetro cDNA Synthesis kit; the protocol was performed as recommended in factory user manual (Bioline). Maize gene expression was evaluated by SYBR ®Green RT-PCR. Fold change variation of target genes was measured through $2^{-\Delta\Delta CT}$ method. mRNA levels at different days post inoculation were compared with time 0 levels. The reference gene used to calculate ΔCts was the housekeeping maize gene Zm α-actin. Primers couples employed for gene expression evaluation on cDNA and for genomic DNA amplification are listed in **Supplementary Table 7**. RT-PCR was performed by a Line GeneK (Bioer) Termocycler.

Scan electron microscope analysis

Control and infected kernels, at each incubation time, were dehydrated with subsequent 4 hours steps in ethanol-water solutions (60%, 70%, 80%, 90% and then absolute ethanol). Kernels were then spattered with gold and observed externally and internally, after a longitudinal cut, with a scanning electron microscope QUANTA SEG 250 ESEM (FEI, Hillsboro, Oregon, USA) in order to observe the distribution of fungal mycelium.

Statistical analysis

For lipidomic analysis, infected/non infected samples ratios were calculated on internal standard normalized peak areas. Gene expression and lipidomics data are presented as the mean value (± SE) of three independent measurements. For each thesis, we had biological duplicates. Data sets were compared using t Student test (n=3, $p<0.05$).

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Sphingolipids in *Fusarium verticillioides – Zea mays* interaction

Supplementary tables 1-6. MRM condition for LC-MS/MS analysis
Compounds characterized in:

maize
fusarium
both

Table 1. Sphingoid bases MRM conditions

analyte identification	precursor ion (amu)	main transition (amu)	transition identification	qualifier	qualifier identification	CE (eV)	Fragmentor (V)
d18:2	298	262	$[M+H-2H_2O]^+$	250	$[M+H-H_2O-HCOOH]^+$	20	80
d18:1	300	264	$[M+H-2H_2O]^+$	252	$[M+H-H_2O-HCOOH]^+$	20	80
d18:0	302	266	$[M+H-2H_2O]^+$	284	$[M+H-H_2O]^+$	20	80
t18:0	318	282	$[M+H-2H_2O]^+$	264	$[M+H-H_2O]^+$	22	80

Table 2. Ceramides MRM conditions

analyte identification	precursor ion (amu)	main transition (amu)	transition identification	qualifier	qualifier identification	CE (eV)	Fragmentor(V)
d17:1/18:0	552	250	[base-2H2O]+	268	[base-H2O]+	28	140
d18:2/14:1	506	262	[base-2H2O]+	280	[base-H2O]+	20	140
d18:2/16:1	534	262	[base-2H2O]+	280	[base-H2O]+	26	140
d18:2/16:0	536	262	[base-2H2O]+	280	[base-H2O]+	26	140
d18:2/18:1	562	262	[base-2H2O]+	280	[base-H2O]+	24	140
d18:2/22:1	618	262	[base-2H2O]+	280	[base-H2O]+	28	140
d18:1/16:0	538	264	[base-2H2O]+	282	[base-H2O]+	28	140
d18:1/h22:0	638	264	[base-2H2O]+	282	[base-H2O]+	28	140
d18:0/16:0	540	266	[base-2H2O]+	284	[base-H2O]+	26	140
d18:0/18:2	564	266	[base-2H2O]+	284	[base-H2O]+	26	140
d18:0/18:1	566	266	[base-2H2O]+	284	[base-H2O]+	30	140
d18:0/18:0	568	266	[base-2H2O]+	284	[base-H2O]+	26	140
d18:0/h17:0	570	266	[base-2H2O]+	284	[base-H2O]+	26	140
d18:0/20:0	596	266	[base-2H2O]+	284	[base-H2O]+	28	140
d18:0/24:0	652	266	[base-2H2O]+	284	[base-H2O]+	24	140

Table 3. PhytoCers MRM conditions

analyte identification	precursor ion (amu)	main transition (amu)	transition identification	qualifier	qualifier identification	CE (eV)	Fragmentor (V)
t18:0/16:0	556	282	[base-2H2O]+	300	[base-H2O]+	24	140
t18:0/h15:0	558	282	[base-2H2O]+	300	[base-H2O]+	26	140
t18:0/18:2	580	282	[base-2H2O]+	300	[base-H2O]+	30	140
t18:0/18:1	582	282	[base-2H2O]+	300	[base-H2O]+	30	140
t18:0/18:0	584	282	[base-2H2O]+	300	[base-H2O]+	32	140
t18:0/19:1	596	282	[base-2H2O]+	300	[base-H2O]+	28	140
t18:0/20:0	612	282	[base-2H2O]+	300	[base-H2O]+	28	140
t18:0/22:0	640	282	[base-2H2O]+	300	[base-H2O]+	26	140
t18:0/23:0	654	282	[base-2H2O]+	300	[base-H2O]+	24	140
t18:0/24:0	668	282	[base-2H2O]+	300	[base-H2O]+	24	140

t18:0/25:0	682	282	[base-2H2O]+	300	[base-H2O]+	28	140
t18/h22:0	656	282	[base-2H2O]+	300	[base-H2O]+	24	140
t18:0/h23:0	670	282	[base-2H2O]+	300	[base-H2O]+	28	140
t18:0/h24:0	684	282	[base-2H2O]+	300	[base-H2O]+	26	140
t18:0/26:0	696	282	[base-2H2O]+	300	[base-H2O]+	30	140
t18:0/h25:0	698	282	[base-2H2O]+	300	[base-H2O]+	26	140

Table 4. DehydroPhytoCers MRM conditions

analyte identification	precursor ion (amu)	main transition (amu)	transition identification	qualifier	qualifier identification	CE (eV)	Fragmentor (V)
t18:1/16:0	554	280	[base-2H2O]+	298	[base-H2O]+	28	140
t18:1/h20:0	626	280	[base-2H2O]+	298	[base-H2O]+	30	140
t18:1/h22:0	654	280	[base-2H2O]+	298	[base-H2O]+	26	140
t18:1/h24:0	682	280	[base-2H2O]+	298	[base-H2O]+	28	140
t18:1/h26:0	710	280	[base-2H2O]+	298	[base-H2O]+	28	140

Table 5. GluCers MRM conditions

analyte identification	precursor ion (amu)	main transition (amu)	transition identification	qualifier	qualifier identification	CE (eV)	Fragmentor (V)
Glc d18:2/16:1	696	262	[base-2H2O]+	280	[base-H2O]+	28	140
Glc d18:2/16:0	698	262	[base-2H2O]+	280	[base-H2O]+	28	140
Glc d18:2/18:1	724	262	[base-2H2O]+	280	[base-H2O]+	26	140
Glc d18:2/20:0	754	262	[base-2H2O]+	280	[base-H2O]+	30	140
Glc d18:2/h20:0	770	262	[base-2H2O]+	280	[base-H2O]+	28	140
Glc d18:2/h22:0	798	262	[base-2H2O]+	280	[base-H2O]+	28	140
glc d18:2/24:1	808	262	[base-2H2O]+	280	[base-H2O]+	30	140
glc d18:2/25:1	822	262	[base-2H2O]+	280	[base-H2O]+	30	140
glc d18:2/26:1	836	262	[base-2H2O]+	280	[base-H2O]+	30	140

Table 6. d19:2 GluCers MRM conditions

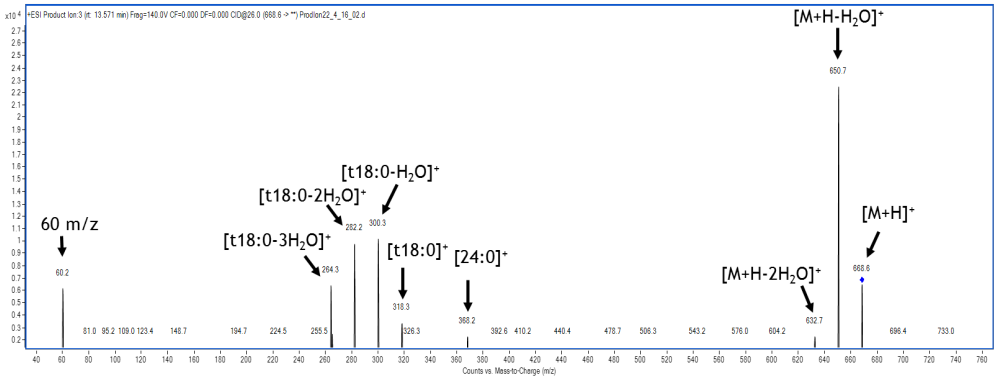
analyte identification	precursor ion (amu)	main transition (amu)	transition identification	qualifier	qualifier identification	CE (eV)	Fragmentor (V)
Glcd19:2/14:2	680	276	[base-2H2O]+	294	[base-H2O]+	26	140
Glcd19:2/16:1	710	276	[base-2H2O]+	294	[base-H2O]+	26	140
Glcd19:2/18:2	736	276	[base-2H2O]+	294	[base-H2O]+	26	140
Glcd19:2/19:0	754	276	[base-2H2O]+	294	[base-H2O]+	26	140
Glcd19:2/24:1	822	276	[base-2H2O]+	294	[base-H2O]+	26	140

Supplementary Table 7. List of primers used for cDNA amplification. The reference gene used to calculate ΔCts was the housekeeping maize gene α-actin

Gene	Accession No.	Sequence
mpk6	ZEAMMB73_145303	Fw GCATATCTTGCTCCCTCCGT Rev CGTCCAAAAGATGGCGGTTG
pr1	NM_001153579.1	Fw TGAACCAGCATGAAGCCACT Rev CAGAAGACGTTCTCCCCGAG
mcaII	ZEAMMB73_302091	Fw GCCTACCTGATGGCTGATGT Rev ATACAAAGTTCCCGCTGTGC
α-actin	DQ492681	Fw TCCTGACACTGAAGTACCCGATTG Rev CGTTGTAGAAGGTGTGATGCCAGTT

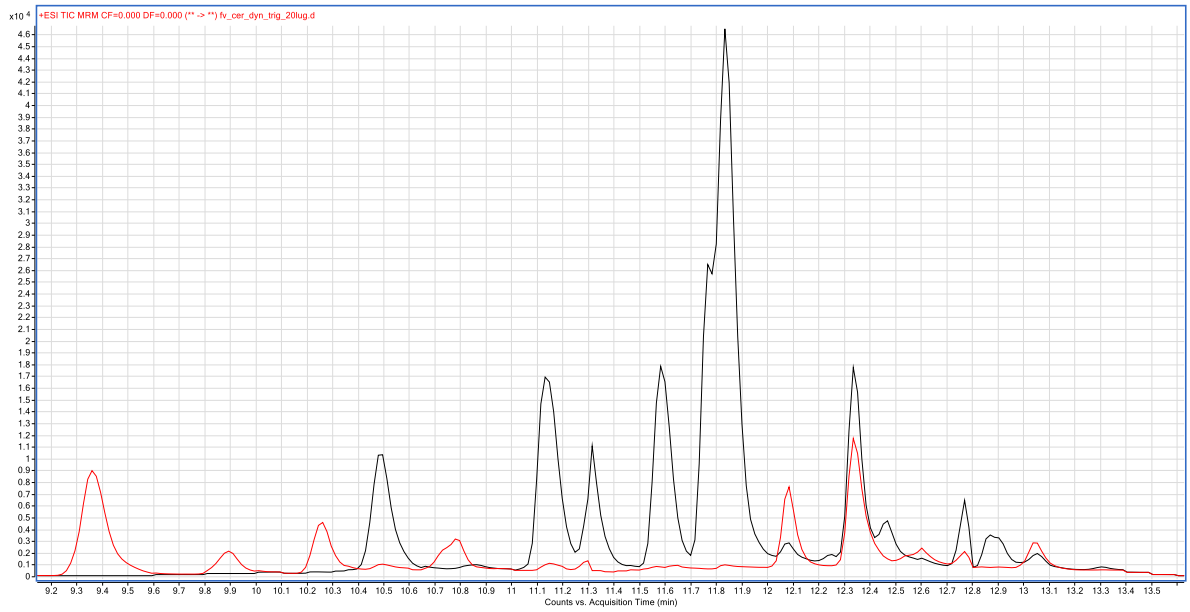
Sphingolipids in *Fusarium verticillioides – Zea mays* interaction

Supplementary Figure 1. Example of fragmentation pattern for a sphingolipid. Namely the phytoceramide with m/z 667.6 (t18:0) showed a clear fragmentation pattern with the 60 m/z fragment typical of sphingoid bases (*Sherer et al., 2010*).

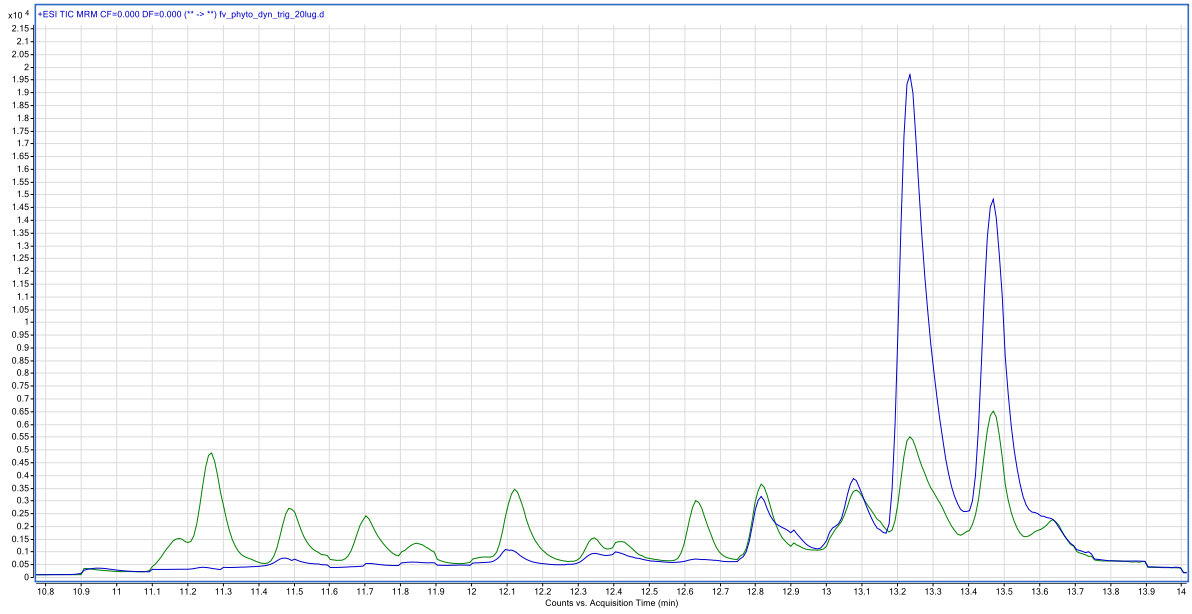


Sphingolipids in *Fusarium verticillioides – Zea mays* interaction

Supplementary Figure 2A Total ion chromatogram of ceramides/Glucosyl ceramides MRM method. *F. verticillioides* in red, maize in black

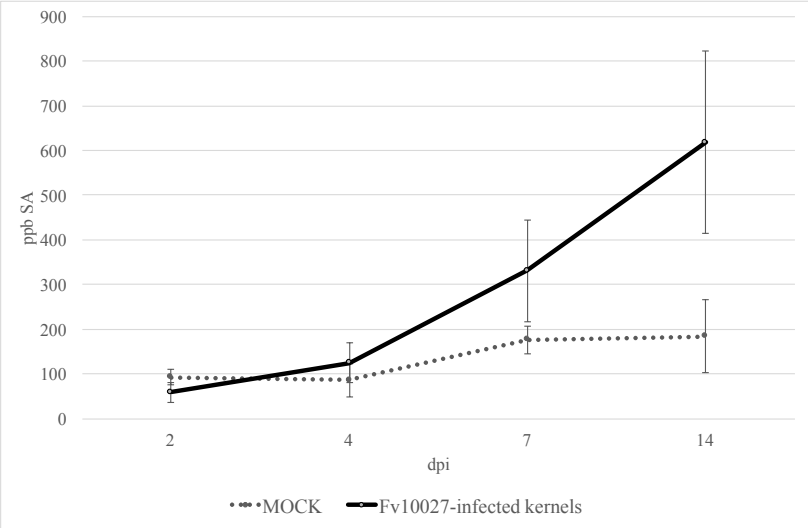


2B. Total ion chromatogram of PhytoCeramides/DehydroPhytoCeramides MRM method. *F. verticillioides* in blue, maize in green



Sphingolipids in *Fusarium verticillioides – Zea mays* interaction

Supplementary Figure 3. SA amount (ppb) into mock and Fv10027-infected kernels. SA increased into infected samples soon after 4 dpi and the trend kept growing up to 14 dpi. SA quantification was determined by LC-MS/MS. The calibration curve was prepared with SA, the concentration levels incremented from 0,1 μM to 10 μM. The standard NAA, with fixed concentration of 1μM , is added to the vials containing SA. The standard curve has a correlation value of 0,996. Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).



PPB SA		
DPI	MOCK	Fv10027-infected
2	92,46 ± 17,8	58,46 ± 22,05
4	85,72 ± 36,8	124,72 ± 44,10
7	175,76 ±31,4	330,85 ± 113,96
14	184,16 ± 82,0	618,43 ±203,67

Edizioni ETS Pisa, 2015
Journal of Plant Pathology (2015), **97** (1), 193-197

SHORT COMMUNICATION

2. ANALYSIS OF THE EXPRESSION OF GENES RELATED TO OXYLIPIN BIOSYNTHESIS IN *FUSARIUM VERTICILLIOIDES* AND MAIZE KERNELS DURING THEIR INTERACTION

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SUMMARY

Fusarium verticillioides is a pathogen that can cause ear and stalk rot in maize. Under suitable environmental conditions, this fungus produces large amount of fumonisins, a potential carcinogenic to humans and animals classified as IARC2B. Recent studies have shown that pathogen and host exchange an oxylipin-based cross talk during their interaction. This study was aimed at investigating whether environmental conditions, namely water activity (aw) at 0.90 and 20°C, which are the thresholds for *F. verticillioides* development, affect the maize/fungal oxylipin gene expression profile. Fungal Fv*lds1-3*, Fv*lox*, Zm*lox3* and the maize defense related Zm*PR1* genes significantly changed their expression after infection at 0.90 aw and 20°C. Moreover, the expression of maize genes peaked after that of fungal genes, indicating that, under these experimental conditions, plant and pathogen coordinate the oxylipin gene expression reciprocally. This kind of modulation of fungal and plant gene expression is suggestive of the "zig zag model" proposed for other plant-pathogen interaction systems.

Keywords: oxylipins, maize kernels, water activity, *F. verticillioides*.

Fusarium verticillioides (teleomorph *Gibberella moniliformis*) infections of maize can be either symptomless or cause a disease of ears, stalks and seedlings (Munkvold, 2003). The infection as such has a limited impact on the yield, but the production of fumonisins represents a relevant food and feed safety problem due to their effects on animal and human health. For this reason, the European Community and other countries (e.g. USA) have set limits for their presence in maize and derived products destined to humans (Directive 576/2007/EC) and recommendations for animal feed (Directive 1881/2006/EC).

Environmental conditions may strongly affect kernel maturation and fungal infection (Cao *et al*., 2013). The main factors influencing fungal growth and fumonisin production are water activity (aw) and temperature (Marin *et al.*, 2010; Sanchis *et al*., 2006). In general, an aw of 0.98- 0.99 and a temperature of 25°C greatly favours *F. verticillioides* growth (Marín *et al*., 2010; Samapundo *et al*., 2005) whereas a lower aw and temperature inhibits fungal growth and induces *FUM1* gene expression (Marin *et al.*, 2010; Jurado *et al*., 2008; Lazzaro *et al.*, 2013).

In a recent review, Picot *et al.* (2010) reported that ecophysiological factors, among which plant defense metabolites such as oxylipins, are important for regulating fumonisin production under laboratory conditions. Scala *et al*. (2013) showed that *F. verticillioides* linoleate diol synthase (*lds*) and lipoxygenases (*lox*) genes and their expression products (e.g. 8-10-HPODE/10-HODE

and 9/13-H(P)ODE, respectively) are upregulated during fungal growth in liquid media amended with cracked maize. These authors suggested that some compounds released by cracked maize could incite an elicitation of oxylipin-related pathways in *F. verticillioides*, inducing a modulation of fungal oxylipins. In turn, these modifications of the fungal oxylipin profile could affect the interaction with the host (Scala *et al*., 2013). Oxylipins are signal molecules able to affect fungal growth, specifically during the interaction with the host (Christensen and Kolomiets, 2011; Dall'Asta *et al*., 2014; Reverberi *et al*., 2010) and to regulate differentiation (conidiogenesis and sporogenesis) and secondary metabolites synthesis in different fungi (Tsitsigiannis and Keller, 2007; Scarpari *et al*., 2014). Moreover, oxylipins can play a key role in driving the fungal ability to colonize the host (Brodhagen *et al*., 2008; Christensen *et al*., 2012).

The defence-related functions of the plethora of metabolites produced by the 9-LOXs and other oxylipin enzymes, are poorly understood (Gao *et al*., 2009). In the field, *F. verticillioides* infecting the maize plant, encounters stressful conditions during kernel ripening, while water availability progressively decreases because of starch formation (Picot *et al*., 2010). Ageing process ongoing during seed ripening could lead to oxidative stress conditions (Muller *et al*., 2009; Picot *et al*., 2010) and formation of oxylipins. Notably, during kernel invasion water stress conditions (Cao *et al*., 2013), chemical composition (Reynoso *et al*., 2002; Dall'Asta *et al*., 2014), insect damages (Miller, 2001) may severely influence the pathogen exploitation of the host and, therefore, the plant-fungus signal transduction like oxylipin signals. Lipids and specifically 9-oxylipins produced and accumulated during maize kernels maturation (Picot *et al*., 2010; Dall'Asta *et al*., 2014) may trigger signal transduction as it occurs in other pathosystems (Gao *et al*., 2009; Reverberi *et al*., 2010). Plant lipids and their oxidized derivatives may re-shape the fungal lipidome by altering oxylipin synthesis at the transcriptional level (Scala *et al*., 2013; Scarpari *et al*., 2014). Oxylipin genes are crucial in regulating fungal physiology (Brown *et al*., 2009; Reverberi *et al*., 2010; Scarpari *et al*., 2014; Scala *et al*., 2013, 2014). In general, plant and fungi may adapt to different environmental conditions (e.g. drought, cold, heavy metals) by re-modulating the lipidome and specifically altering the unsaturation pattern and the oxylipin pathways (Maggi *et al*., 2013; Christensen and Kolomiets, 2011).

The aim of this work was to study the oxylipins gene expression of mature maize kernels and *F. verticillioides* during their interaction. Grains of a commercial maize hybrid were infected with *F. verticillioides* then incubated at 0.90 aw and 20°C, which are limiting thresholds for fungal growth but conditions under which *FUM1* is expressed (Jurado *et al*., 2008.). It was found that the oxylipin genes are expressed in both fungus and kernels during their interaction and an intriguing zig-zag "shaped" cross talk emerges.

Five kg of maize kernels of a hybrid (H3) were collected at maturity in a field located in Northern Italy and used in this study since, as shown by Mukherjee *et al*. (2011), detached mature maize kernels entertain an active cross talk with the pathogen.

Water was measured using AquaLab LITE (version 1.3, Decagon Devices, USA) equipment and adjusted to 0.90 aw by adding sterile distilled water to the grains, and their viability was estimated by germination at 20°C. These conditions were chosen as they are regarded as stressful for *F. verticillioides* invasion of maize kernels (Marin *et al.*, 2010; Jurado *et al*., 2008). Seed germinability was *ca.* 97%. Grains were partitioned in 100 g aliquots.

A *F. verticillioides* strain (ITEM 10027), grown on potato dextrose agar medium (PDA) was used for maize inoculation. The maize samples, previously washed as described by Christensen *et al*. (2012), were separated in two groups, one of which was inoculated with 1 ml of fungal spores at a concentration of 106 spores/ml, incubated at 20°C whereas the other was not inoculated, serving as control.

Samples were analysed 1-4-7-14-21-28 days post inoculation (dpi) for fungal and plant oxylipin genes expression. The fungal growth on maize kernels infected and not, was evaluated at 25°C and 0.99 aw, the reportedly optimal conditions (Marin *et al*., 2010), and at 0.90 aw and 20°C, the limiting conditions. The fungal growth was evaluated by qPCR (Scala *et al*., 2014), while total RNA extraction from 100 mg of freeze-dried maize kernels, reverse transcription and SYBR green cDNA amplification were carried out

as described by Scala *et al*. (2013). The expression of oxylipinsrelated genes linoleate diol synthase 1, 2 and 3 (*lds1*, *lds2*, *lds3*) and lipoxygenase (*lox*) were analyzed using the primers reported in Scala *et al.* (2013). The primer sequences for Zm*lox3*, Zm*PR1* and Zm*act* (α-actin) are reported in Brodhagen *et al*. (2008), Kong *et al*. (2013), and Picot *et al*. (2011), respectively. The relative expression of *F. verticillioides* genes *lox*, *lds1*, *lds2* and *lds3* and of maize genes Zm*lox3* and Zm*PR1* was analyzed from 1 up to 28 dpi in maize kernels under growth-limiting conditions, (20°C and aw of 0.90). The expression of genes of interest were normalized using fungal β-tubulin and maize α-actin as housekeeping genes (HKG).

Different authors have reported how the inter-kingdom oxylipin cross talk occurring between plant and pathogen may drive the fate of their interaction (Brown *et al*., 2009; Gao *et al*., 2009; Scarpari *et al*., 2014; Christensen and Kolomiets, 2011). Taking this into account, we tried to understand if 0.90 aw and 20°C, i.e. conditions that limit fungal growth and induce fumonisin synthesis, were able to modulate the expression of oxylipin-related genes during *F. verticillioides* exploitation of mature viable maize kernels.

The expression of Fv*lds1-3*, Fv*lox*, Zm*lox3* and the plant defense-related Zm*PR1* genes was evaluated in maize kernels from 1 up to 28dpi of *F. verticillioides* (Fig. 1). All the fungal oxylipin-related genes were upregulated during the interaction, starting before 4dpi, and achieving the maximum expression 28 dpi (except for Fv*lox*) (Fig. 1 a-d). Fungal linoleate diol synthase genes (*lds1*, *lds2* and *lds3*), whose products are involved in the synthesis of different di-HODE oxylipins, were all expressed in infected kernels, *lds2* being the most expressed 28dpi. The expression of Fv*lox* clearly peaked in the infected kernels at 4 and 14 dpi.

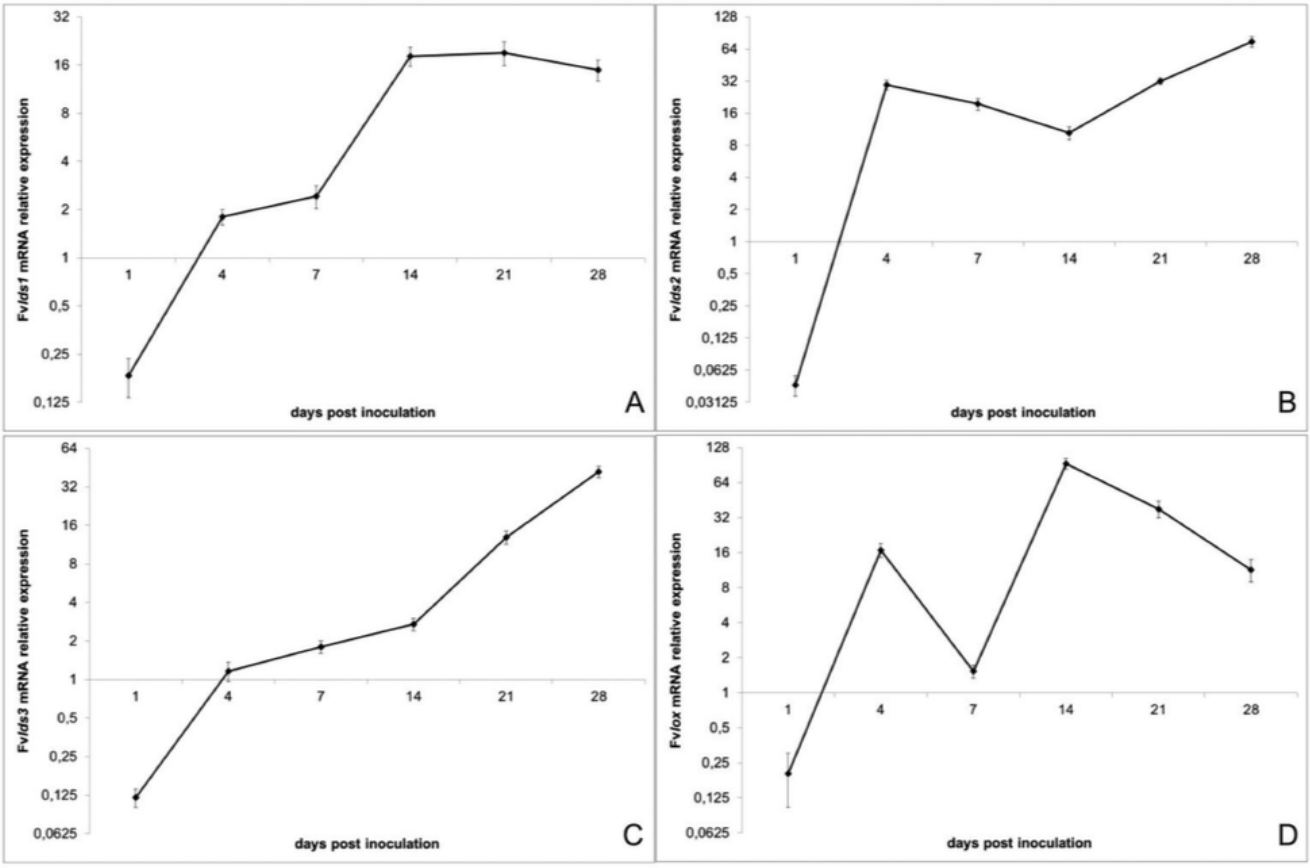

Fig. 1. mRNA relative expression of *lds1* (A), B *lds2* (B), *lds3* (C) and *lox* (D) genes of *F. verticillioides* inoculated on maize kernels and grown at 20°C and aw 0.90 at 1, 4, 7, 14, 21 and 28 days post inoculation. The expression was relative to that determined at inoculation (time 0). Results are the mean (± SE) of six replicates from two independent experiments.

qPCR assay showed that fungal growth was reduced at 0.90 aw and 20°C compared to optimal conditions, and the plateau was reached between 21-28 dpi (data not shown), confirming the results of Marin *et al*. (2010).

Zm*lox3* and Zm*PR1* are defense-related genes that maize can activate for reacting to pathogen invasion (Gao *et al*., 2009). These genes were more expressed in the inoculated – specifically at 7 and 21 dpi – than in the non-inoculated kernels (Fig. 2 a, b). The comparison of fungal and plant oxylipin genes expression pinpointed that fungal *lds* and *lox* genes were expressed since 4dpi and *lox* peaked at 4 and 14 dpi (Fig. 1 and 2, in comparisons). Coordinately, the plant Zm*lox3* and Zm*PR1* peaked at 7 and 21 dpi even after the fungal *lox* gene expression, occurring at 4 and 14 dpi.

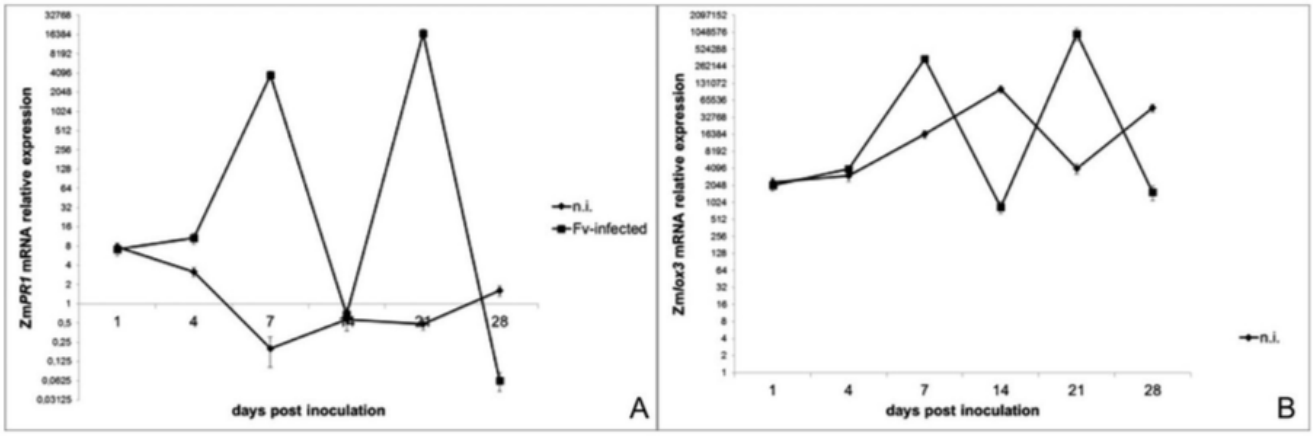

Fig. 2. mRNA relative expression of Zm*PR1* (A) and Zm*lox3* (B) genes of maize kernels that were inoculated or not (n.i.) with *F. verticillioides* and incubated at 20°C and aw 0.90 at 1, 4, 7, 14, 21 and 28 days post inoculation. The expression was relative to that determined at inoculation (time 0). Results are the mean (± SE) of six replicates from two independent experiments.

Apparently, the presence of *F. verticillioides* on the seeds affects the expression of the maize genes considered. *F. verticillioides* produces fumonisins in whose synthesis oxylipins play a role (Gao *et al*., 2009; Christensen and Kolomiets, 2011; Scala *et al*., 2014). In this study, we analysed oxylipin gene expression of *F. verticillioides* after the infection of viable maize kernels under stressful temperature and water activity. We showed that *F. verticillioides* expressed the oxylipin genes *lds* and *lox* in mature detached kernels with *lox* gene presenting a peak at 4 and 14 dpi. We also checked the expression of plant defense-related gene putatively under the control of salicylate/ethylene pathway (PR1), upon *F. verticillioides* infection. The results showed that fungal oxylipin-related genes switched on soon after kernels invasion and that fungal infection triggered plant genes expression. This behavior suggests an involvement of these genes in the reaction to pathogen invasion. Moreover, there is an apparent coordination between fungal oxylipins gene expression and Zm*PR1* as well as between fungal *lox* and Zm*lox3*. It was confirmed that Zm*lox3* has a role in the switch from tolerance to susceptibility into as previously described for *A. nidulans* and *A. flavus/Zea mays* interaction (Gao *et al*., 2009). Mature kernels try to activate their own defense during *F. verticillioides* infection and, in turn, the stimulation of these activities may re-shape the fungal oxylipins (Christensen and Kolomiets, 2011).

The trend of oxylipins-correlated gene expression during the interaction suggested a "zig-zag model" to explain the progression from PTI (pathogen-triggered immunity) to ETS (effector-triggered susceptibility) and to ETI (effector-triggered immunity), in plant-pathogen interactions (Jones and Dangl, 2006). In relation to this, fungal oxylipins try to reprogram pathogen-triggered immunity and in turn, the host reacts by producing its own oxylipins to interfere with pathogen invasion. Nevertheless, in some cases, these host oxylipins favour pathogen virulence, inducing a sort of effector-triggered susceptibility.

ACKNOWLEDGEMENTS

This study was supported by the project FIRB-RBFR- 08JKHI_003, MIUR.

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3. Fatty acid composition and metabolism in the maize pathogen *Fusarium verticillioides*

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Fatty acids (FA) are main components of cellular plasma membranes and important signal molecules, both in their *naïve* and modified or conjugated forms. Oxylipins, a family of oxygenated lipid-derived metabolites can be generated either enzymatically, through the action of enzymes such as lipoxygenases (LOX) or linoleate diol synthases (LDS), or non-enzymatically in oxidizing environments. *Lds1* deletion mutant shows that LDS1-derived oxylipins are involved in controlling growth, sporulation and mycotoxin production by the fungus, as well as in shaping the oxylipin production (Scala et al., 2013 and 2014). Here we show that *lds1*-defective mutant of the fungal pathogen *Fusarium verticillioides* present a striking down regulation of FA biosynthesis, mostly of PUFA, when grown in presence of their plant host. We hypothesize the existence of a tight co-regulation between fatty acid biosynthesis and oxylipin generation during pathogen-host interaction. Indeed, *in silico* searches retrieved the common calcineurin dependent response elements (CDREs) transcription factor binding motifs in promoter regions of both FA biosynthetic and oxylipin-transforming enzyme genes. To further study this hypothesis we performed targeted gene deletion of the transcription factor Crazy1 (Crz1), which is known to bind CDREs motifs (Thewes et al. 2014), and found that *crz1*Δ deletion mutants are impaired in fatty acid metabolism. Furthermore, Crz1 is also required to efficiently regulate conidial germination and mycelial growth upon membrane stress. These results indicate that an appropriate balance in fatty acid composition is in turn required for oxylipin synthesis and pathogen fitness.

Introduction

The term fatty acids (FA) refers to short or long chain molecules with an equal number of carbon atoms, without branching and acyclic (i.e. they do not have closed ring chains). When fatty acids are found in their free form, i.e. not bound to other chemical species, they are referred as free fatty acids (FFAs), major constituents of plant, animal, and fungal lipid pools.

Lipid composition determines the physical properties of biological membranes (Dowhan et al., 1997), where variation in FAs modulate membrane plasticity, an essential feature in all organisms to adapt to diverse environmental and cellular processes such as energy storage, signaling, transport and membrane fusion and fission (Dowhan et al., 1997; Aguilar and de Mendoza, 2006; Hagen et al., 2010).

FA are classified according to the presence or absence of double bonds. Saturated fatty acids present only single bonds in the carbon chain (palmitic acid C16:0, stearic acid C18:0), while unsaturated fatty acids present variable amounts of double bonds (Jill et al., 1997; Jump et al., 2002). Unsaturated fatty acids are further distinguished in monounsaturated fatty acids (MUFAs), when a single double bond is present (i.e. oleic acid C18: 1) or polyunsaturated fatty acids (PUFAs), when two or more double bonds are present (i.e. linoleic acid C18:2, α-linolenic acid C18:3). An increase in the number of double bonds, results in lower solubility in aqueous solutions and tri-dimensional structure alterations, conferring higher membrane (Singer et al., 2014).

FA biosynthesis, in the majority of organisms, culminates with the formation of C16 or C18 saturated fatty acids. Subsequently FA can be modified by various enzymes such as desaturases, which catalyse the carbon/carbon double bond formation and elongases, which add two carbon atoms to lengthen the carbon chain.

MUFAs and PUFA can undergo oxidation and conversion into oxylipins. This step can be mediated by an enzymatic or non-enzymatic reaction (i.e. through the activity of reactive oxygen species). Dioxygenase (DOX), lipoxygenase (LOX), and linoleate diol synthase (LDS) are the main enzymes involved in fungal oxylipin synthesis (Brodhun et al., 2011). Oxylipins are implicated in various fungal, mammal, plant and bacterial signaling processes, and represent a means of communication among the kingdoms (Fischer & Keller, 2016). Fungal oxylipins are involved in the regulation of growth, sexual and asexual spore differentiation, apoptosis, and pathogenicity (Tsitsigiannis et al., 2005). Oxylipins have also been identified as molecules capable of mediating the host-pathogen communication between plant and fungi (Reverberi et al., 2012). In plants oxylipins might promote both pathogen resistance or susceptibility as pathogenic fungi can use them to attack or evade plant defense systems (Tsitsigiannis et al., 2007; Gao et Kolomiets, 2009).

Previous studies described the role of lipids and specifically oxylipins within the interaction between *Fusarium verticillioides* (*Fv*) and *Zea mays* (cracked maize and maize ears). Scala and collaborators (2013), using cracked maize as constituent of culture media, underlined the relationship occurring between oxylipin and fumonisin synthesis. Scala and coworkers (2014) also described the role of the linoleate diol synthase gene *lds1* as a main determinant in linoleic acid-derived oxylipin formation and consequent control of mycelial growth and sporulation, and mycotoxin production.

It was noted that the *lds1Δ* mutant, during the growth, in maize presence, shows a synchronous regulation of the expression in the oxylipin synthesis genes, while the FFA amount was decreased. Starting from these observations we wondered if the genes linked to oxylipin biosynthesis were controlled by a common transcription factor. Therefore, we investigated if the desaturase pathway was linked to the oxylipin pathway.

Based on our own knowledge, we tried to describe some crucial steps within the process from which oxylipins are derived, i.e. the synthesis of their FA precursors by the enzymes that catalyze their formation, i.e. desaturases and elongases. It emerges a scenario within which FA synthesis is tightly synchronised with oxylipin synthesis. The main agent of this timely-coordination is apparently *Crazy1* (Crz1), a Calcineurin-Responsive Zinc finger 1 nuclear transcription factor. Crz1 was first identified in yeast as a calcineurin target (Stathopoulos et al., 1997). In fact, we identified putative consensus sequences CDRE (Calcineurin dependent response elements) within the promoter of desaturases, elongases and oxylipin-related genes. Thewes et al. 2014 describe Crz1-dependent genes, it emerged that they are involved in resistance to metal ions, development, integrity of the cell wall, virulence and resistance to drugs. The FFA and oxylipin profile of the strain of *Fv* lacking *crz1* generated within this study, apparently confirm our hypothesis.

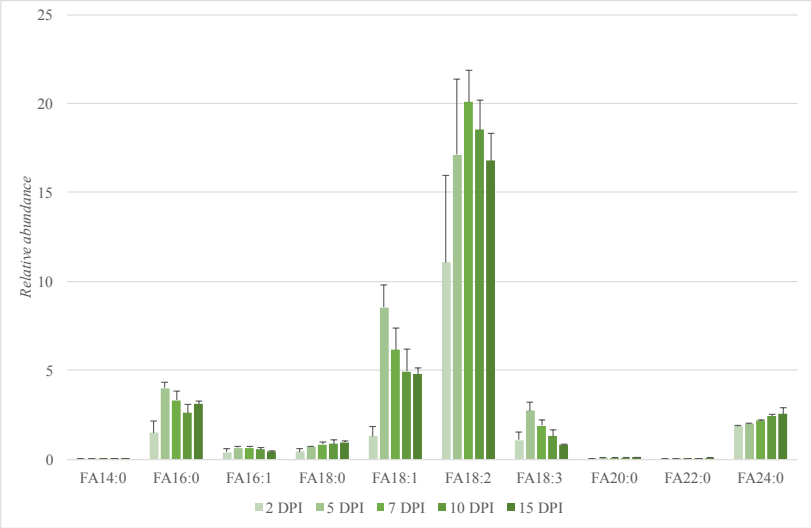
Results

Evaluation of free fatty acid composition during the interaction

Fatty acids alteration is observed in *Fv* mycelium grown in presence or in absence of cracked maize (CM) at different DPI (i.e. 2, 5, 7, 10, 15 days) in WT as well as in *lds1*Δ strains. Fatty acids analyzed are: myristic acid (14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3) and arachidic acid (C20:0), behenic acid (22:0) and lignoceric acid (24:0); our fatty acid metabolism model is subsequently build up on these substrates. We evaluate FFA level along the whole growth, in **figure 1A** are reported the peak areas of FFAs present in *Fv* grown with CM, the data are normalized on the internal standard C17:0, the margaric acid normally not present in the fungi. Results indicate that the palmitic acid (C16:0) and the FAs with 18 carbon atoms are the most abundant, in particular the linoleic acid (C18:2) and the oleic acid (C18:1).

In *lds1*Δ strain (**Figure 1B**) we observe a decrement of the FA content from 2 to 7 DPI. The the linoleic acid C18:2 is less present in the mutant strain during all the time points analized.

A)



B)

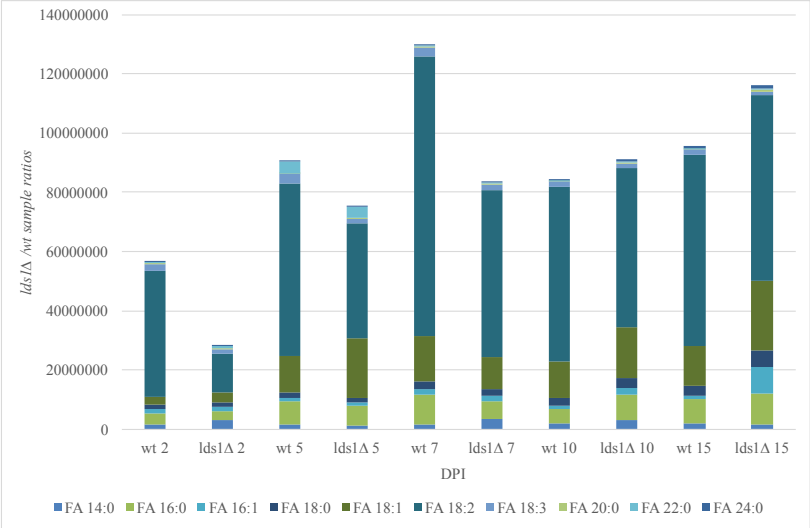
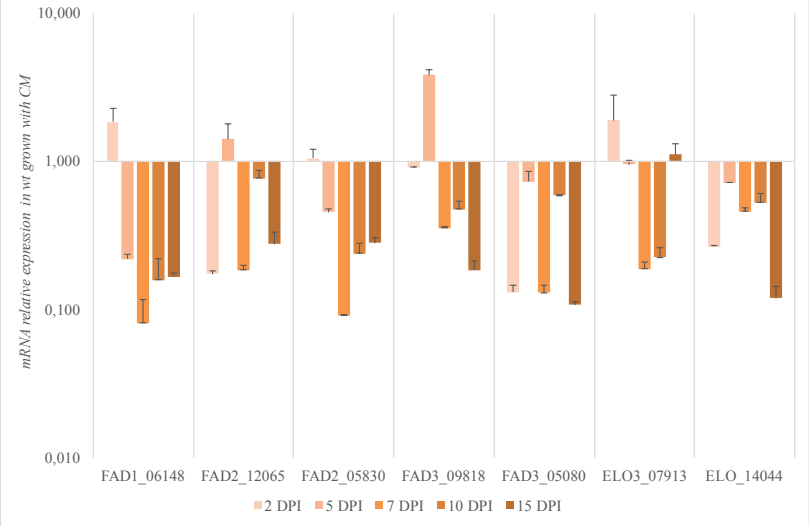


Figure 1. Fatty acids level variation at different days post inoculation. A) FAs were extracted from *F. verticillioides wt* grown with CM. Y axis: FA amount normalized on the internal standard C17:0 (relative abundance). X-axis: FA species and relative growth times, i.e days post inoculation (DPI), of *wt* strain. B) FAs were extracted from *F. verticillioides wt* and *lds1*Δ grown with CM; Y axis: *lds1*Δ/*wt* sample ratios. X-axis: strains and relative growth times, i.e days post inoculation (DPI). Results are the mean of the values derived from three biological experiment technically repeated three times (n=3; ± SE).

Fad and *elo* gene expression during the interaction

A RT-qPCR method was used to study expression of the five FA desaturases (FAD1_06148, FAD2_12065, FAD2_05830, FAD3_09818, FAD3_05080) and two elongases (ELO3_07913 and ELO_14044) during *Fv*-maize interaction; the mycelium was collected from 2 to 15 DPI (**Fig. 2A**).

A)



B)

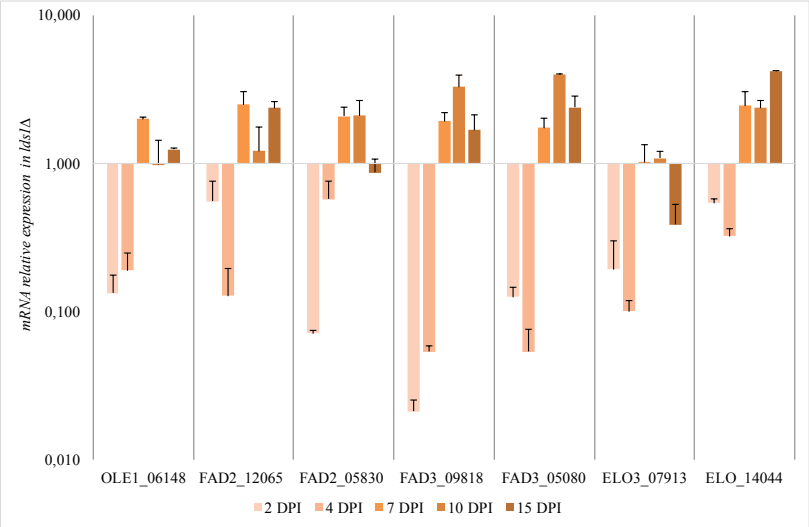


Figure 2. Relative expression of the fatty acid desaturase (*fad*) and elongase (*elo*) genes. A) *F. verticillioides* grown in presence (treated) or in absence (control) of CM. Y-axis: $\log_{10}$ of treated/control. B) *wt* and *lds1*Δ grown with CM. Y-axis: $\log_{10}$ of *lds1*Δ/*wt*. For both charts the X-axis: time points, *i.e.* days post inoculation. Relative mRNA expression was calculated as $2^{-\Delta\Delta ct}$ method using *F. verticillioides* β-tubulin as housekeeping gene. Results are the mean of the values derived from three biological experiment technically repeated three times (n=3; ± SE).

With the exception of early time points (2-5 DPI) the expression was significantly down-regulated for all the genes investigated. Gene expressions followed a similar pattern: a significant decrease from 2 to 15 DPI. Exception in this trend were reported at 2 DPI for *fad1* and *elo3* and at 5 DPI for *fad2_12065* and *fad3_09818*.

The same expression analysis was performed for the *lds1*Δ mutant. *Wt* and *lds1* were grown in presence of CM and we analyzed the desaturases and elongases expression (**Fig.2B**). The chart shows the expression of *lds1*Δ compared to *wt*, is possible to observe a coordinated down regulation of the expression of all the genes considered from 2 to 5 DPI.

Investigation on transcriptional factor (TF) crazy1

Transcriptional analysis was performed on the promoter region of all desaturases, elongases, lipoxygenases and linoleate diol synthases individuated in our *F. verticillioides* strain. Employing TomTom MEME analysis (http://meme-

suite.org/tools/tomtom), we identified four putative CDRE motifs in the following promoters: (1) 5'-CTCAGCCGC in *lds1* (FVEG_09294), (2) 5'-CTCAGCCGC in *lds*2 (FVEG_12540), (3) 5'-CTCCGCCAT in *lds*3 (FVEG_11670), (4) 5'-CACGGCCCC in *fad2* (FVEG_12065); the conserved nucleotides are underlined. *In silico* analysis, suggest that LDS-derived oxylipins and the linoleic acid formation (their precursor), deriving from the FAD2_12065 action, are under the control of the same transcriptional factor.

This analysis not underline CDREs sequence in the elongase and lipoxygenase promotors.

Our results indicated that Crz1 governs mRNA expression of the target genes; therefore, Crz1 is potentially involved in the metabolism of fatty acids and oxylipins in *F. verticillioides*.

Molecular profile of Fv_Crz1Δ

The transcription factor Crz1 is a major calcineurin effector in *Saccharomyces cerevisiae* and other fungi (Thewes et al., 2014). BLAST-P (by NCBI) analysis have identified that the putative gene FVEG_01476 of *F. verticillioides 7600* is an orthologue of *Saccharomyces cerevisiae CRZ1* (YNL027W). To test the actual involvement of Crz1 in the synthesis of linoleic acid and LDS-derived oxylipins (see previous paragraph), a homologous recombination for deleting *Crz1* locus was performed (**Supplementary Image 1**). The *Crz1*-deleted strains were PCR-screened for testing the homolog recombination and the presence of *Hph* presence (data not shown). The genomic organization of *Fv-Crz1Δ* was studied by Southern Blot hybridization using a probe specifically recognizing its 5'-UTR. The results indicated the proper deletion at the *Crz1* locus (**Supplementary Image 1**).

Preliminary analysis was performed for the *crz1* gene expression during the growth in CDY basal medium (**Fig. 3A**). The deletion of *lds1* generates a *crz1* down-regulation compared to *wt* (**Fig. 3B**).

A) B)

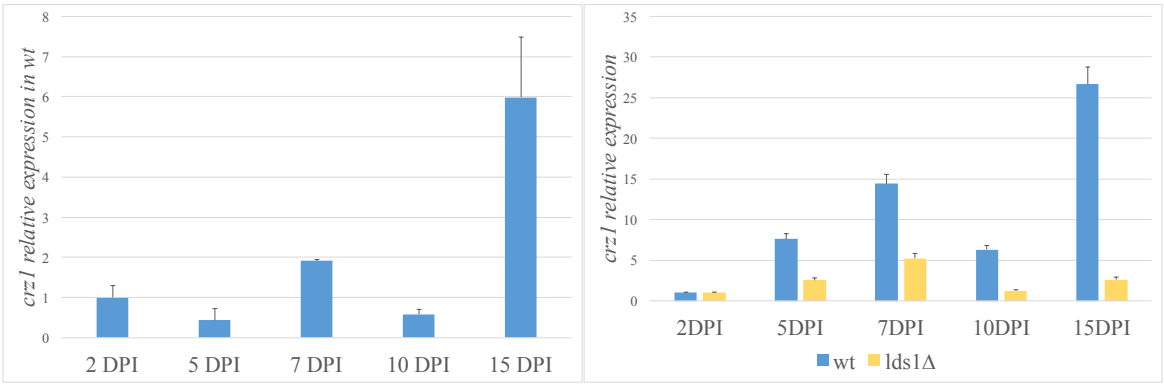


Figure 3. *crz1* expression during the growth. A) *wt* grown in CDY B) wt and *lds1*Δ grown in CDY. Relative mRNA expression was calculated as $2^{-\Delta\Delta ct}$ method using *F. verticillioides* β-tubulin as housekeeping gene. Results are the mean of the values derived from three biological experiment technically repeated three times (n=3; ± SE).

Phenotypic characterization of *crz1*Δ

To evaluate spore germination rates, 1×10^6 spores/mL of *Fv* were inoculated in minimal medium (MM) to emphasize the basal growth characteristic. The percentage of germinated spores was estimated at 25°C 13 h after inoculation (**Fig. 4**). The *wt* strain has higher germination rate compared to the mutant.

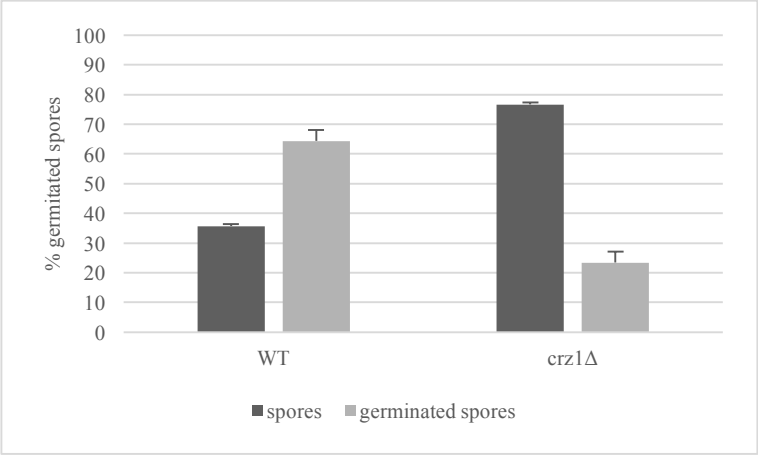


Figure 4. Quantification of conidia germination. Microconidia of the indicated strains were inoculated at a concentration of 10^6 conidia/ml in minimal medium. Data are presented as the mean from three experiments. n=100 conidia.

Phenotypic assay demonstrates that the *crz1*Δ is sensible to calcium. The mutant growth is inhibited at $CaCl_2$ 0,7 M concentration, as shows in **figure 5** the *wt* and the mutant were grown on MM plate for 4 days. The *wt* was able to grown and the colony reach a maximum diameter of 1,4 cm in the different spore concentrations whereas the *crz1*Δ was not able to growth the different spores concentration. The deletion predicts a lipid metabolism alteration, suggests and alteration in the membrane permeability, in fact the mutant strain not tolerates the anionic surfactant sodium dodecyl sulfate (SDS) (Woertz et al., 2004). The mutant not grown well with SDS 0.01%.

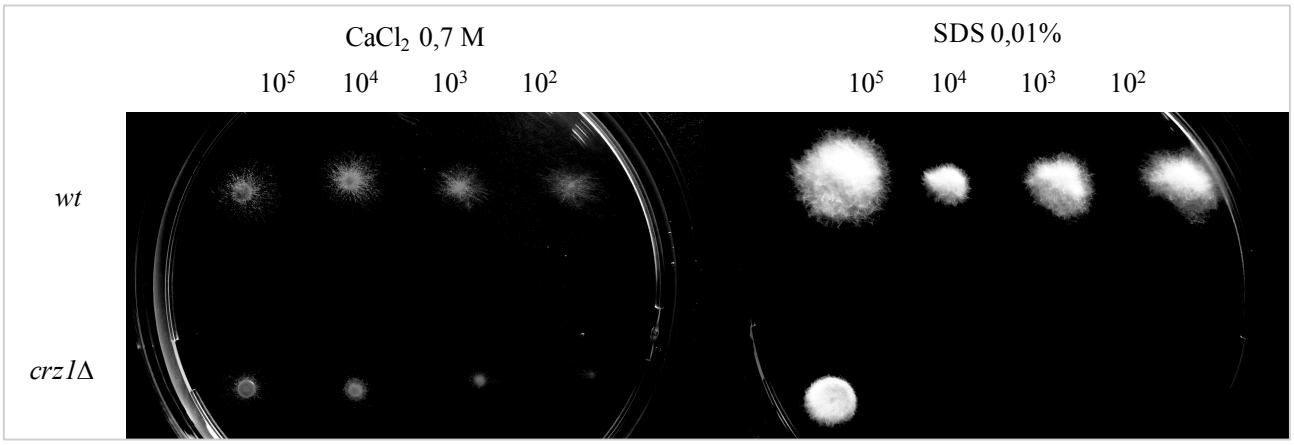


Figure 5. Stress response phenotypes of *F. verticillioides wt* and *crz1*Δ. Colony phenotypes of the indicated strains grown on MM (sucrose 30 g/L, KCl 0,5 g/L, $MgSO_4$ $7H_2O$ 0,5 g/L, KH_2PO_4 1 g/L, $NaNO_3$ 2 g/L, agar oxoid 20 g/L) medium in the presence of the indicated compounds. Plates were spot inoculated with the indicated number of microconidia, incubated for 4 days at 25°C and scanned.

The mutant grown under the medium surface (vegetative mycelium), while in *wt* strain is favored the growth of aerial mycelium (**Fig. 6**).

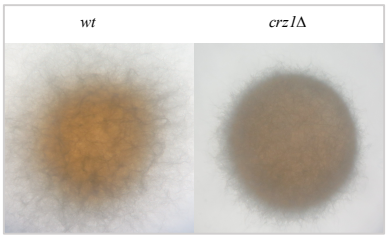


Figure 6. Mycelium growth of *F. verticillioides wt* and *crz1*Δ. Colony phenotypes of the indicated strains grown on MM (composition is indicated above) added of SDS 0,01%, the plates ware incubated for 4 days at 25°C and scanned.

Fad, *elo*, *lds* and lox gene expression in *crz1Δ* mutant

A RT-qPCR method was used to evaluate if the *crz1* deletion implies an alteration in the expression of genes involved in fatty acid metabolism: desaturases (FAD1_06148, FAD2_12065, FAD2_05830, FAD3_09818, FAD3_05080), elongases (ELO3_07913 and ELO_14044), linoleate diol synthases (LDS1_09294, LDS2_12540, LDS3_11670) and lipoxygenase (LOX_09897). The expression was analysed from 16 hours to 7 days of growth: a coordinated down-regulation in the early times (16 hours) appeared in the elongases, in *fad2* and in the oxylipin-related genes (**Fig. 6**). Nevertheless, this synchronization that led to an up-regulation of gene expression starting from 2 DPI, is apparent for *fad1, elo3* and the oxylipin genes (**Fig. 8**).

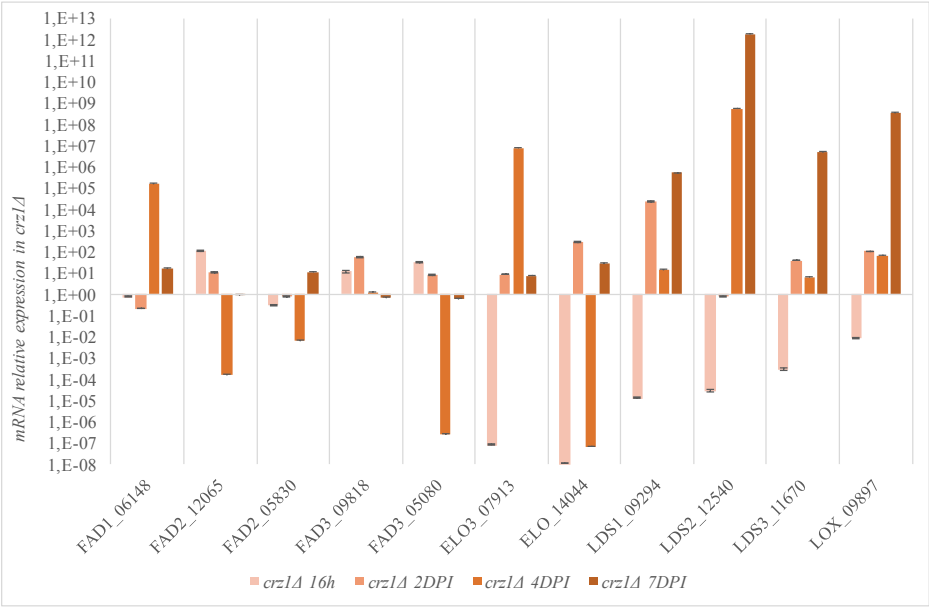


Figure 6. Relative expression of the fatty acid desaturase (*fad*) elongase (*elo*) and genes of oxylipins biosynthesis (*lds* and *lox*). *F. verticillioides wt* and *crz1Δ* grown in CDY medium. Relative mRNA expression was calculated as $2^{-\Delta\Delta ct}$ method using *F. verticillioides* β-tubulin as housekeeping gene and expression in WT as calibrator. Y-axis: $\log_{10}$ of *crz1Δ*/ *wt*. . Results are the mean of the values derived from three biological experiment technically repeated three times (n=3; ± SE).

Evaluation of fatty acid composition in *crz1Δ*

Fatty acids composition is observed in *Fv wt* and in *crz1Δ* mycelium grown at different DPI (i.e. 16h, 2, 5, 7 days) in the sole CDY medium to assess if basal differences in FFAs exist. The fatty acids analysis was performed as indicated above. We evaluate FFA level during the growth, the data reported were expressed as the ratio between peak areas of FAs present in *crz1Δ* and the FAs present in *wt*: a decrement of the FFAs considered in *crz1Δ*. We can observe a general reduction in FA content in the mutant strain (**Fig. 6**).

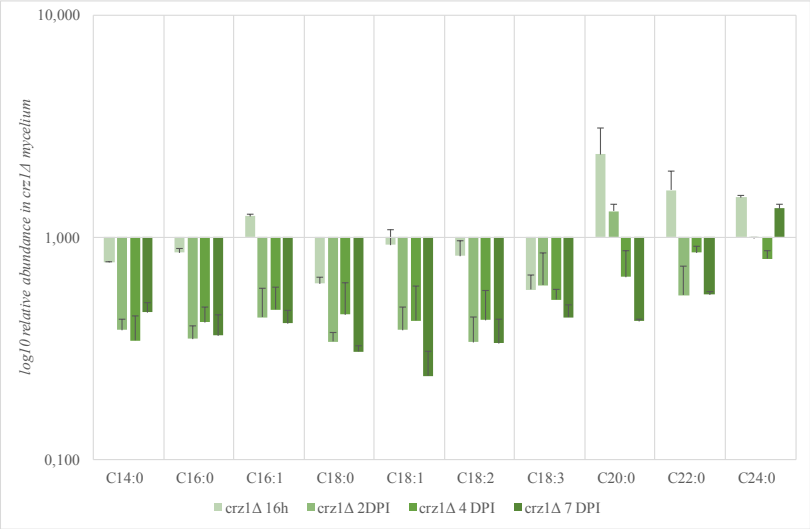


Figure 6. Fatty acids level variation at different days post inoculation. FAs were extracted from *Fv wt* and *crz1Δ* grown in CDY; Y axis: $\log_{10}$ of *crz1Δ*/*wt* samples ratios. X axis: time points, i.e. days post inoculation. . Results are the mean of the values derived from three biological experiment technically repeated three times (n=3; ± SE).

Oxylipin profile in *crz1Δ*

We quantify the amount of the oxylipins produced by *Fv wt* and *crz1Δ* strains grown in the sole CDY medium to assess if basal differences in oxylipin exists. Specifically, we analyzed the linoleic and α-linolenic-derived oxylipins. For the linoleic acid products we can differentiate between LDS and LOX-derived oxylipins, while on α-linolenic acid acts only LOX enzyme. It is not clear how Crz1 acts on the oxylipin biosynthesis, the total amount seems to be affected (**Fig. 7A**). In the in the **figure 7B** are represented the different oxylipins, the most affected are the linoleic acid-derived. At 16 hours post inoculation the 8-HOME and the 9-HpODE are less present in mutant strain, the first oxylipin derived from Lds1 action, the second one from the LOX activity. LOX acts on α-linolenic acid and generates the 9-HODE, an oxylipin very present during the growth, in particular in *wt* strain.

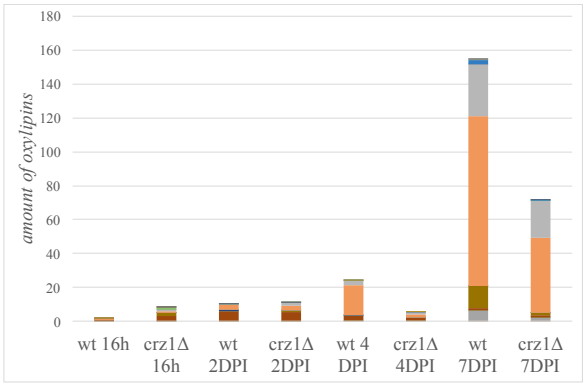


Figure 7A. MRM oxylipins quantification. *wt* and *crz1Δ* oxylipins extract from mycelium. FA amount normalized on the internal standard 9-HODEd_4.Y axis: relative amount of oxylipins. X axis: time points, i.e. days post inoculation. Results are the mean of the values derived from three biological experiment technically repeated three times (n=3; ± SE).

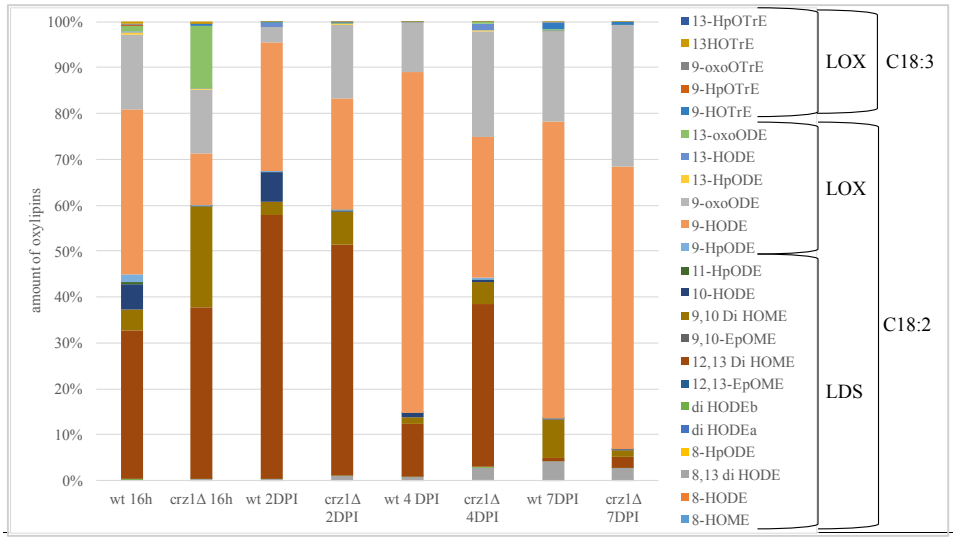


Figure 7B. MRM oxylipins quantification. *wt* and *crz1Δ* oxylipins extract from mycelium. FA amount normalized on the internal standard 9-HODEd_4.Y axis: percentage of oxylipin amount (max value 100%). X axis: time points, i.e. days post inoculation. Results are the mean of the values derived from three biological experiment technically repeated three times (n=3; ± SE).

Discussion

Scala et al., 2014 demonstrate that among the genes involved in the oxylipins synthesis in the presence of cracked maize, *lds1* (Linoleate Diol Synthase1) is the most expressed, and the knock-out for *lds1*affects growth, fumonisins synthesis and virulence in maize. In addition, the lipidomic profile of *lds1*Δ was affected since the expression of some oxylipin-genes (i.e. *lds2*, *lds3* and *lox*) underwent to a remodelling as well as the oxylipin component in general. From these indications, we have developed our line of investigation: on the one hand, through lipidomic approach we quantified the fatty acids present in *wt* as well as in *lds1*Δ. Secondly we studied the modulation of the expression of genes whose products are involved in the formation of both FFA and oxylipins in the WT and *lds1*Δ. At last, we looked for a possible transcription factor capable to synchronise the expression of such genes in *Fv*.

Fatty acids alteration is observed in *Fv wt* and *lds1*Δ. Under the induction of CM the fatty acids more present in *wt* the palmitic acid (C16:0) the FAs with 18 carbon atoms are the most abundant, in particular the linoleic acid (C18:2) and the oleic acid (C18:1) (**Fig. 1A**). The *lds1* deletion perturb the fatty acid content. The absence of *lds1*, the gene most transcript during the growth with CM, generates an imbalance: all the fatty acids are moderately over-present in *lds1*Δ, but we can observe a punctual decrease into the linoleic acid (C18:2) and the linolenic acid (C18:3) that result less present in the mutant respect to *wt* (**Fig. 1B**). Surprisingly we attend to an increment of all the fatty acids analyzed, confirming that the fatty acid metabolism is coordinated and that the role of Lds1 is important not only in the oxylipins formation but also in the fatty acid content.

The *fad*s and *elo*s expression reflects the growth rate of *Fv,* in Scala et al.,2013 it was reported that during the interaction with the maize kernels the mycelium growth was decreased; the whole fatty acid machinery is under-regulated (**Fig. 2A**), or rather, the gene that codify for enzymes involved in the fatty acids elongation and desaturation are less expressed, and these results are in line with the growth rate. How can we explain this effect apparently in contrast to lipidomic data? We hypothesise that when the fungus interacts with the host, *de novo* fatty acid synthesis is inhibited, favouring a lipids faster release from the plasma membrane and lipid droplets (Blümke et al., 2014).

In the *lds1*Δ mutant we can compare the *FADs* expression profile with the lipid profile of the unsaturated fatty acids produced by the desaturases themselves, there is a clear correlation due to the *F. verticillioides* transformation event. It emerges that in the mutant *lds1*Δ the *fad*s are down-regulated among 2 and 5 DPI (**Fig. 2B**), and this is reflected in a smaller amount of PUFA (C18:2 and C18:3) produced by these desaturases (**Fig.1B**). In other words, the deletion of the *lds1* gene represents a signal for

the *fads* to produce less substrates for the oxylipins, the lacking of *lds1* generates a mutant not ables to produce oxylipins. It should be stressed that these observations, most evident among 2-5 DPI are in agreement with the growth curve of *F. verticillioides* proposed in the work of Scala et al. 2013, where it is evident that strong growth of the mycelium among 2 and 5 DPI, on the other hand, in the later times the growth reaches a plateau.

During the trophophase (2-5 DPI) the fatty acid synthesis machinery, composed by elongases and desaturases, is down-regulated in the *lds1*-defective strain, suggesting a feedback mechanism ongoing between the oxylipin synthesis and the fatty acids metabolism.

From these dataset, it emerges a scenario in which the synthesis of oxylipins is tightly synchronized with the synthesis of their precursors, i.e. free fatty acids. Moving from this idea, we search for the possible presence of a transcription factor capable to regulate this delicate fatty acid balance. *In silico* analysis, suggest that *crz1* is the better candidate. We found consensus sequence in the promoter regions of one *fad2* and all the *lds* present in *Fv.* Previous work underlines also other regulation system, for example *fad1* expression is transcriptionally regulated by different stimuli: temperature, carbon source, presence of fatty acids in the cultured medium and oxygen levels (Martin et al., 2007), and the mRNA stability is controlled by two homodimeric proteins present on the membrane of ER, Spt23 and M2.

To study the transcription factor Crz1 we adopt a gene deletion approach. In general, *crz1Δ* strain appears less competitive than its *naïve* counterpart does. This phenotype has already reported in *C. albicans* (Onyewu et al., 2004) and *A. fumigatus* (Steinbach et al., 2006), in fact the sporulation and germination rate results affected in the respective *Crz1*-defective strains.

From gene espression analysis the obtained data show that Crz1 could be involved in the transcription machinery in the first time point analyzed, *i.e.* 2DPI. Our results are preliminary, in the basal medium, when the oxylipin machinery is not stimulated only the expression of *FADs* and *ELOs* , is affected by the deletion of *Crz1*, since this genes are involved in the fatty acid metabolism.

The quantification of the fatty acid show that the whole content is lower in *crz1Δ* than *wt.* The absence of the transcription factor generates a strong alteration, suggesting that Crz1 can influence different lipid pathways. Different works reported that the *crz1* deletion can affect the cell wall biogenesis and the lipid metabolism, in *S. cerevisiae* was seen that exist 116 Crz1 dependent genes (Yoshimoto et al., 2002), while in *C. neoformans* even 208 (Adler et al.,2011).

Only the long (LCFA) and the very long chain fatty acids (VLCFA) weakly increase during the growth, probably for their importance for the reshaping of the plasma membrane.

The current study provides some ideas for understanding the genetic components of the lipid metabolism in *F. verticillioides.* Apparently, the availability of fatty acids in the substrate allow *Fv* down-modulating FFA biosynthesis occurring through the canonical pathways (FAD, ELO). Nevertheless, some specific genes (in succession, ELO3_07913 at 2 DPI; FAD2_12065 at 5 DPI) and consequently some FFA are expressed and produced at precise time intervals. These compounds, such as linoleic acid (LA, accumulated at 7 DPI) can provide the substrate for oxylipin formation. In *lds1*Δ mutant, LA synthesis is greatly hampered suggesting a feedback control of its synthesis from LA-derived oxylipins. Namely, the lack of the product of LDS1 (e.g. 8,13-diHODE) negatively affects the synthesis of its precursor LA. Which is the mean for *Fv* to exert this control? Bioinformatic analysis of the promoter of *elo, fad* and *lds1* genes allow us to identify CDRE within them. CDRE are recognized by the calcium-dependent transcription factor CRZ1 (Thewes et al.,2014). *Crz1* deletion negatively affects FFAs. Is mostly clear that the deletion event contributing in reshaping fungal morphology and growth, as presented in the colony growth, the mutant is sensible to high $CaCl_2$ concentration (Crz1-calmodulin mudulator), and to the membrane permeability is alterated (tested with the SDS). In this strain, the coordination among the set of genes (FFA and oxylipins) failed, mostly due to a more profound alteration on the lipid metabolism.

The fatty acids at elevate concentration can be toxic, but can act as hormone-like molecules. Accurate analysis about the lipid signaling will allow better understanding the host-pathogen relationships suggested in the **figure 8**, but further investigations are required to fill the knowledge gap.

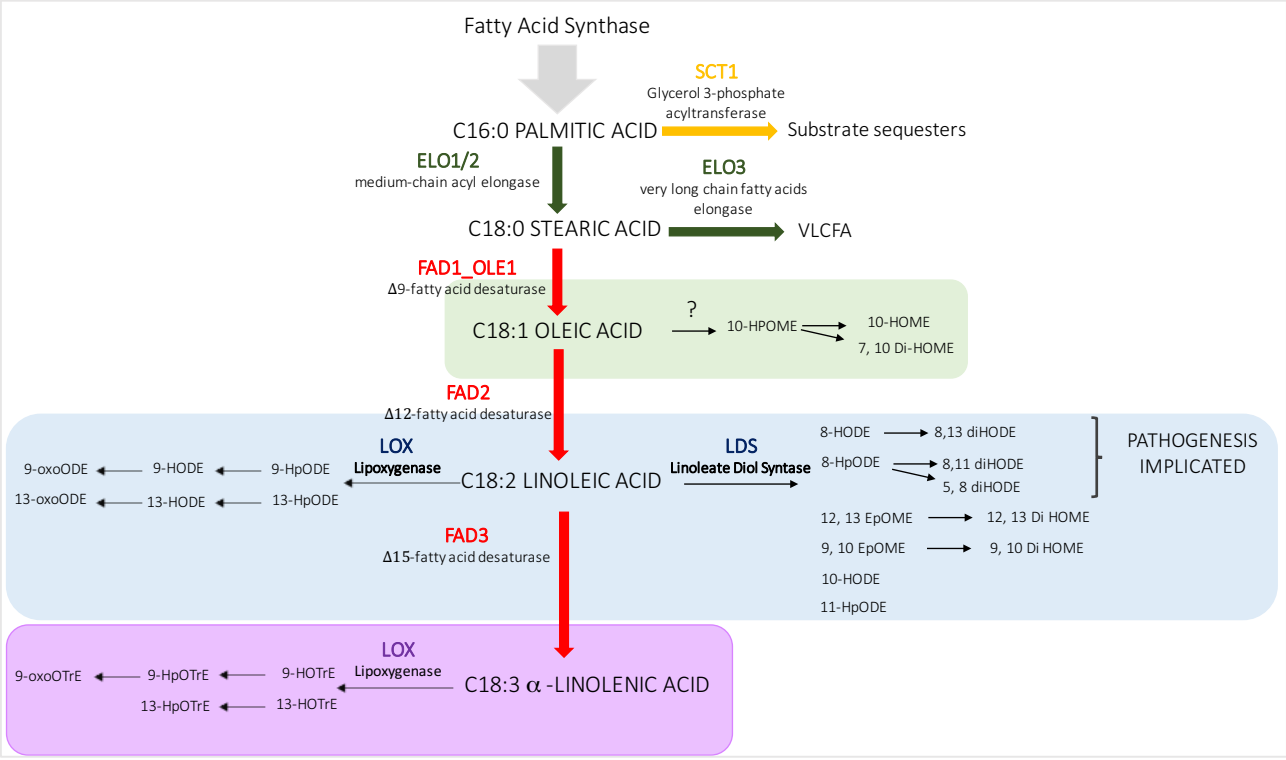


Figure 8. Fatty acid metabolism in *F. verticillioides*. The fatty acid synthase releases C16:0 which can follow different destinies. The C16:0 can be transformed in C18:0 through the ELO1/2 (medium chain acyl elongase) action, or can be sequestered by the glycerol 3-phosphate acyltransferase SCT1 (De Smet et al., 2012). C18:0 is the second hub, from which can act the ELO3 (very long chain fatty acid elongase) or the FAD1 (Δ9-fatty acid desaturase). C18:1 can be converted in potential oxylipins through an unknown system, or the FAD2 (Δ12-fatty acid desaturase) can produce the C18:2 PUFA. Three different destines are showed for the C18:2, it can be transformed in oxylipins through LDS (linoleate diol synthase) or LOX (lipoxygenase), in C18:3 for the FAD3 (Δ15-fatty acid desaturase) action. C18:3 PUFA can be converted in oxylipins by a LOX activity.

Materials and methods

Strains and media

F. verticillioides isolate ITEM 10027 was isolated from maize kernels by the Università Cattolica del Sacro Cuore (Professor P. Battilani; code MPVP 294) deposited in the collection of the Institute of Sciences of Food Production (ISPA-CNR, Bari, Italy; http://server.ispa.cnr.it/ ITEM/Collection) and stored at -80°C with glycerol as microconidial suspension. For microconidia production, cultures were grown in potato dextrose broth (PDB, Difco) at 25°C, with shaking at 150 rpm. For phenotypic analysis of colony growth, four spot of 2 µl water containing 2 10^5-2 10^4-2 10^3-2 10^2 freshly obtained microconidia were transferred to agar plates of minimal medium (MM) (Perez-Nadales et al., 2015). When needed, MM was supplemented with $CaCl_2$ (MW 110,98 g mol^{-1}) or SDS (Sodium dodecyl sulfate MW 288,372 g mol^{-1}) at the indicated concentrations. After the inoculation the plates are maintained in the dark at 25°C for 4 days.

For the germination assays freshly collected microconidia at the concentration 10^6 or 10^7 conidia/mL were inoculated in MM and incubated 13h at 25°C and 150 rpm. The number of total and germinated conidia was then counted with a microscope. At

least 100 events were examined for each isolate and each experiment was repeated at least three times. The number of germinated conidia was scored and expressed as a percentage of germinated over the total number of counted conidia.
For preliminary gene expression and lipid analysis *F. verticillioides* was grown from 2 to 15 days in CDY and CDYM, in witch it was added 2% of lyophilized maize kernels. We have added cracked maize (called CM in the text) for to simulate the fungus-maize interaction. The mycelium was collected at different days post inoculation (DPI) as reported in Scala et al. 2013. The work was conducted also in the *lds1*Δ. This mutant has already been characterized in the work of Scala et al. 2014.
For the *crz1Δ* analysis, the mutant and the *wild type* strains were grown from 16 hours to 7 days.

Nucleic acid manipulation

Total RNA and genomic DNA were extracted from *F. verticillioides* mycelium as described previously (Scala et al., 2013; Raeder et al., 1985). Quality and quantity of extracted nucleic acids were determined by running aliquots in ethidium bromide stained agarose gels and by spectrophotometric analysis in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), respectively. DNA and protein sequence databases were searched using the BLAST algorithm (NCBI).
For quantitative reverse transcription PCR (RT-qPCR) the total RNA was isolated from mycelia growth on CDY from 16h to 7 days. The c-DNA was transcribed with reverse transcriptase (SensiFAST™ cDNA Synthesis Kit, Bioline)
For PCR amplification, primers *crz1_01476, fad1_06148, fad2_1265, fad2_05830, fad3_09818, fad3_05080, lds1_09294, lds2_12540, lds3_11670, lox_09897* were used (see **Table 1 in the supplemental material**). All the samples were amplified during identical numbers of cycles. Controls included amplification of the house-keeping β-tubulin gene (see **Table 1 in the supplemental material**) for normalization of RT-qPCR. Gene expression was calculated by using the 2^{-ct} method. SYBR green (SensiMixTM SYBER No-ROX Kit (BIOLINE, Trento, Italy) qPCR amplification was performed in a Line Gene 9620 thermocycler (Bioer, Hangzhou, Zhejiang Province, China) as specified elsewhere (Scala et al., 2017).

Motif discovery

To perform motif discovery, we employed the degenerated CDRE motif sequences (MWMMGCCMC) of *S. cerevisiae* and the MEME suite was employed through use of the MEME webserver (Multiple Expectation maximization for Motif Elucidation). Candidate motifs were tested for enrichment in the upstream 1 kb promoter sequences of calcineurin-Crz1 putative regulated genes.

Gene deletion and gene complementation

Targeted gene replacement of the *crz1* gene in the wild-type was performed as reported previously (Lopez-Berges et al., 2010) using the split-marker method with the hygromycin resistance cassette. PCRs were routinely performed with the High Fidelity Template PCR system (Roche) using a thermal cycler (Bio-Rad). The amplified flanking sequences were PCR fused with partially overlapping truncated versions of the hygromycin B (HygB) resistance cassette. The primers used are indicated in the **Supplementary Table 1**. Transformants were purified by monoconidial isolation and analysed by PCR and Southern blot to verify homologous insertion of the construct (**Supplementary Image 1**). Southern blot analysis was carried out as previously reported (Di Pietro and Roncero, 1998) by using the non isotopic digoxigenin labeling kit (Roche Diagnostic).

Chemicals

All solvents used for fatty acids and oxylipins extraction and HPLC/MS analysis were of HPLC/MS grade. Methanol (MeOH), isopropyl alcohol (iPrOH), ethyl acetate (EtOAc), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Ammonium formate ($HCOONH_4$) was purchased in granular form from Fluka (Buchs SG, Switzerland).

Fatty acids and oxylipins extraction

Fatty acids and oxylipins were extracted from 20 mg of mycelium lyophilized. The internal reference standard (IST) added for the quantitative analysis was the margaric acid (C17:0, 270.45 g mol^{-1}) for the fatty acids and deuterated 9-hydroxy-10E,12Z-octadecadienoic acid (9-HODEd4, MW 300.5 g mol^{-1}) for the oxylipins analysis. Both ISTs at the final 2 μM concentration. The samples were vortex-mixed with 2 mL of extraction mixture (iPrOH: H_2O: EtOAc 1:1:3 v/v, with 0.0025% w/v of butylated hydroxytoluene, BHT, to prevent peroxidation). After centrifugation, the ethyl acetate upper phase was collected in a clear tube and dried under nitrogen gas flux. After centrifugation, the upper phase was recovered and transferred to the collection tube together with the previously extracted fraction, and dried under nitrogen flux. The dried samples were reconstituted in 100 μL of MeOH and loaded onto the HPLC auto-sampler.

Fatty acids analysis by LC-MS/MS

The equipment, chromatographic column and analysis software were all from Agilent Technologies (Santa Clara, CA, USA). Samples were analysed by LC (HPLC 1200 series rapid resolution) coupled to a triple quadrupole MS (G6420 series triple quadrupole, QqQ; Agilent Technologies) equipped with an electrospray ionization source (ESI). The acquisition was in negative ion polarity, and the characterization was in MS2 SCAN mode. Chromatographic separation was performed through a Zorbax SB-C8 rapid resolution HT 2.1 x 50 mm 1.8 μm 600 bar column (Agilent Technologies). The mobile phases consisted of A phase (water: B phase 80:20 v/v, containing ammonium formate 5mM), B phase (MeOH: iPrOH 95:5 v/v). The elution programme was as follows: 0-6 min 60% B, 6-30 min 99% B, 30-38 min 60% B.

The flow rate was 0.4 ml min^{-1}. The column was thermostated at 60°C. The injection volume was 2 μL. The injector needle was washed with the mobile phase in the wash port at the end of each HPLC run. Nitrogen was used as the nebulising and desolvation gas. Drying gas temperature was 350°C, its flow was 10 l min^{-1}, the nebulization pressure was 20 psi. The parameters used for the analysis of fatty acids are reported in the **Supplementary Table 2**.

Oxylipin analysis by LC-MS/MS

The equipment is the same utilized for the fatty acid analysis, the quantitative analysis was performed in MRM as reported in Ludovici et al. 2014.

Statistical analysis

For fatty acid and oxylipin analysis, *Fv* growth with LMK/*Fv* growth without LMK samples ratios were calculated on internal standard normalized peak areas. The same analysis was performed for the fatty acids and oxylipins present in the *wild type* and mutant. Gene expression and lipidomics data are presented as the mean value (± SE) of three independent measurements. For each thesis, we had biological triplicates. Data sets were compared using t Student test (n=3, $p<0.01$).

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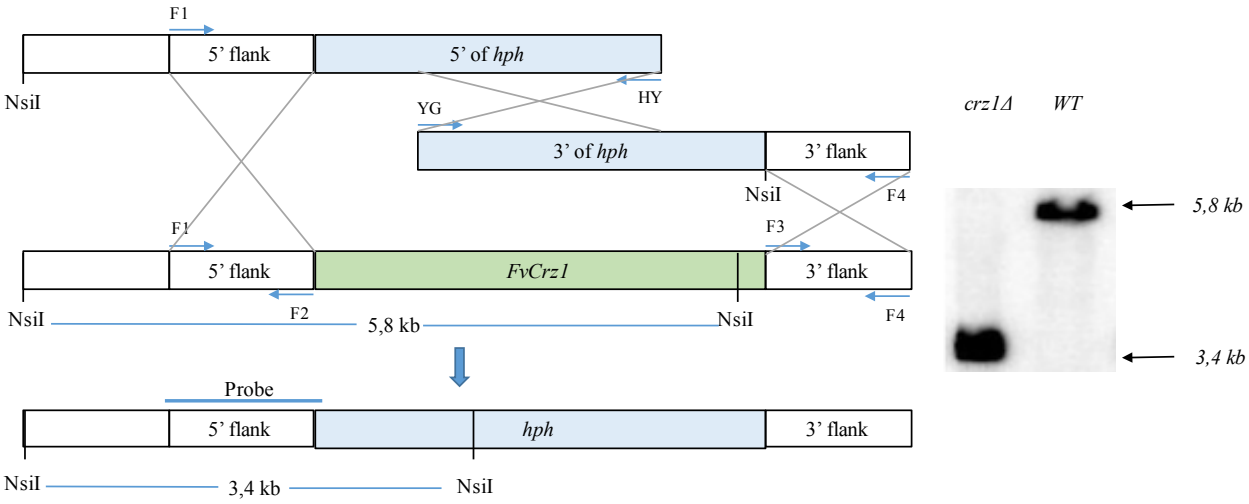
Fatty acid composition and metabolism in the maize pathogen *Fusarium verticillioides*

Supplementary Image 1. Southern Blot Hybridization. Representation of split marker technique performed for the transformation; design of Southern blot hybridization through the utilising of 5' flank probe. The restriction enzyme NsiI generates different profile of hybridization in *WT* (5,8 kb fragment) and *crz1*Δ (3,4 kb fragment).



Fatty acid composition and metabolism in the maize pathogen *Fusarium verticillioides*

Supplementary table1: A) List of primers used for cDNA amplification. The reference gene used to calculate ΔCts was the housekeeping fungal β-tubulin. B) Primers for knockout design, probe construction for Southern blot hybridization and PCR transformant screening.

A) Primers utilized for gene expression analyses	Sequence	Accession number
Crz1_Fw Crz1_Rev	AGCTCCAACACTTCGCTACC ACCAGGTTGTTGAGCATCGT	FVEG_01476
Fad1_Fw Fad1_Rev	AGAACCCTAAGCGACAAGGC ACGAAGCAAACACGGAGGA	FVEG_06148
Fad2_12065_Fw Fad2_12065_Rev	GGCCAAGTCTGAACCTGTGT TGCTGGGGATGTAGGTGAGA	FVEG_12065
Fad2_05830_Fw Fad2_05830_Rev	ACTCTTCTCATGCTCGTCCTTC CTGTTCTCGTACAGAGGGCT	FVEG_05830
Fad3_09818_Fw Fad3_09818_Rev	CCACCACCTATTCCCTCGTG TCTGTTGCCATCGGTGAAGT	FVEG_09818
Fad3_05080_Fw Fad3_05080_Rev	GAGACACGCCAGAAAGTCCT GTAAGCGCCTCCCTGGATAG	FVEG_05080
Lds1_Fw Lds1_Rev	GGACTCGCTGCGATCGTGTGG TCGCCCTTCTGGGCAATGGC	FVEG_09294
Lds2_Fw Lds2_Rev	AGACCCCCACCGAGGCCAAG CCACTGCCCAGCCTCCCAGA	FVEG_12540
Lds3_Fw Lds3_Rev	CGCACGATTCAGCTCGTGGC AGTATGGAGTTGGCGCGGCG	FVEG_11670
Lox_Fw Lox_Rev	ACGATTCCCAAAGACGAGCAAGTG AGGCCGATGTTGTGTCCTTGTTCA	FVEG_09897
β-Tub_Fw β-Tub_Rev	CTCTGCTCATTTCCAAGATCCGCG GTAGTTGAGGTCACCGTAGGAGG	FVEG_04081

B) Primers for knockout	Sequence
FvCrz1PromFor	TTGTGGGGTCGGATCGTGG
FvCrz1PromForNest	ACATGGAGGCACGCTAGAAG
Up5_crz1_fw	CATTTCCTGCCTTGTCCCCT
FvCrz1PromRev	ATGGAGGATGGGGAATGCTG
FvCrz1TerFor	TTGGGAGGAGTGCTTGGGTA
FVCrz1Rev2	TCATTCGTGGGTTTGACTTGG
Down3_crz1_Rev	AGTCAGTGCGGTGTTTTTGC
FvCrz1TerRevNest	AACACGTCCTGGTCTTGCATA
FvCrz1TerRev	AGAGCCAAGAGAGGACATGAA
HygG	CGTTGCAAGACCTGCCTGAA
HygY	GGATGCCTCCGCTCGAAGTA

Fatty acid composition and metabolism in the maize pathogen *Fusarium verticillioides*

Supplementary Table 2. Acquisition Method Info. Parameters utilised for fatty acids quantification.

Ion Source ESI

Scan Type MS2 Scan

Scan Segments

Start Mass	End Mass	Scan Time	Frag (V)	Cell Acc (V)	Polarity
100	500	1000	80	7	Negative

Fatty Acid	m/z	[M-H]	Retention time	
C14:0	228,4	227,4	2,9	Target
C16:1	254,4	253,4	3,6	Target
C16:0	256,4	255,4	5,9	Target
C17:0	270,5	269,5	8,4	Internal Standard
C18:0	284,5	283,5	10,3	Target
C18:1	282,5	281,5	7,3	Target
C18:2	280,5	279,5	5	Target
C18:3	278,4	277,4	3,3	Target
C20:0	312,6	311,5	13,1	Target
C22:0	340,6	399,6	14,9	Target
C24:0	368,6	367,6	16,3	Target

4. Fungal secreted oleic acid represents a signal for reinforcing the host-oriented growth in *Fusarium oxysporum*

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How can we explain the host-oriented growth of the pathogenic fungi? The key word is "chemotropism". *Fusarium oxysporum* is a pathogen of tomato, *inter alia*, and a model organism in the *Fusarium* genus. Recent studies demonstrated that *F. oxysporum* could orientate hyphal growth following gradients of different chemoattractants: sugars, amino acids, peptide pheromones, plant root exudates and lipids. For instance, the secreted class III peroxidase (POX), a family of heme-containing enzymes present in all land plants, may drive the hyphal growth (Turrà et al., 2015). The POX can be sensed by the co-presence of two membrane receptors, Ste2 and Ste3 that singularly perceive the α and *a* pheromone respectively. Among the signals able to drive the host-pathogen interaction, the lipid molecules can play an interesting role. In our experimental design, we analyzed the lipid signals generated following the growth of *F. oxysporum* in presence of plant peroxidase. We tested the *wild type* and two mutant strains *ste2Δ* and *ste3Δ* incapable to sense POX gradient.

It emerges that the peroxidase *stimuli* cause an alteration of the lipidomic asset in the mycelium as well as in the cultural filtrate. In particularly we found a high concentration of oleic acid in cultural filtrate secreted soon after the POX perception. Its accumulation occurs at a significantly different level in the *naïve* strain compared to the POX-insensitive mutants of *F. oxysporum*.

Introduction

Plant pathogen *F. oxysporum* can growth towards the root of one of its host: *Solanum lycopersicum*. The chemotropism explains the directional growth of hyphae exposed to a variety of chemoattractant signals. These can be divided into fungal-, nutrients- and host-related signals. The secreted class III peroxidases (POX), oxidoreductase heme-containing enzymes, represent a formidable signal able orienting the growth of hyphae toward the tomato roots (Turrà and Di Pietro, 2015). Peroxidases can be considered as bifunctional enzymes that can oxidize various substrates in the presence of H_2O_2, produce reactive oxygen species (ROS), as $\bullet O_2$, H_2O_2 and $\bullet OH$), and modify the cell wall (Passardi et al., 2004).

The chemotropic response to the POX gradient is supported by co-presence of Ste2 and Ste3, two transmembrane proteins normally able to perceive the α and a pheromone respectively (Unpublished data Vitale S.). The fungal pheromone signals have been studied during sexual development in *Saccharomyces cerevisiae*. When genetically distinct individuals of opposite sex are chemotropically attracted, two pheromones, a or α, that identify the mating type are secreted (Arkowitz et al., 2009). Yeast cells, if subjected to a gradient of opposite mating type pheromone, produce spatially oriented mating projections termed *shmoos*. The chemical gradient is recognized through the G-protein coupled receptors (GPCR) that indeed are Ste2 and Ste3.

The mitogen-activated protein kinase (MAPK) cascades is the pathway activated by Ste2 and Ste3. The MAPKs in general can mediate cellular response to environmental signals. In previous work, Segorbe et al. (2017) demonstrated that three MAPKs Fmk1, Mpk1 and Hog1, are involved in the stress response, development and virulence of *F. oxysporum.* In particular, Mpk1 and Hog1 regulate cellular adaptation to different types of stress, whereas Fmk1 and Mpk1 jointly contribute to reactive oxygen species (ROS) homeostasis. Ste2 and Ste3 activate Mpk1 and consequently, the same MAPK cascade mediates chemotropism driven by the POX (Turrà et al., 2015).

A close link among the POX, MAPKs and ROS actually occurs. These species participate to the adaptive response leading to the cell membrane re-shape during plant-fungus interaction (Passardi et al., 2004; Turrà et al., 2014; Reverberi et al., 2012). In this context, it emerged that the lipids are not just membrane building blocks being able to perceive the environment alteration

and activate several cellular responses. In general, lipids are required for the formation of membranes and are involved in cell signaling, energy supply, and cell death. The main block are the fatty acids and their composition, so the length and the unsaturation grade, can regulate the membrane fluidity (De Smet et al., 2012).

In our study emerged that a free fatty acid, in particular the oleic acid (C18:1), can intervene in the host-pathogen interaction and could be involved in the chemotropism. The oleic acid high concentration is toxic for the cell, and we presume that it can be converted in the deriving-oxylipins such as10S HpOME and 7S, 10S-DiHOME, 10S-HOME. The oxylipins are fatty acids oxidized produced either in enzymatic way as in presence of the ROS (Brodhun et al., 2011). These bioactive lipids are abundant in mammals (Funk et al., 2001), as well as in non-mammals, including flowering plants (Koo et al., 2009), mosses, algae, bacteria and fungi (Andreou et al., 2009). Oxylipins act as hormone-like molecules modulating cell responses such as development, reproduction and defense/virulence in plants as well as in pathogens (Reverberi et al., 2012). As reported in Fischer et al. 2016, oxylipins are intimately linked to basic fungal development by alterations in the ratio of sexual to asexual structures, spore shape, germination rate, quorum sensing and mycotoxin (e.g. secondary metabolite) synthesis. Thus, oxylipins are central to many host-pathogen interactions. In particularly over recent years emerged an involvement in microbe virulence of the oxylipins that derive from the oleic acid oxidation (Martínez & Campos-Gómez 2016).

Results

Oxylipins and fatty acids quantification after POX induction by LC-MS/MS analysis

Wt, *ste2*Δ and *ste3*Δ strains of *F. oxysporum* grown in a minimal medium amended or not with plant POX. The control condition is represented by the same strains grown in presence of boiled POX. To check the presence of lipid exo-signals the cultural filtrates (CF) of the different samples were extracted. MRM analysis detect several oxylipins, which resulted variable in amount and type within the different strains (**Figure 1**). In *wt* strain 12,13-EpOME, 12, 13 di HOME, 9, 10 EpOME and 9,10 di HOME, oxylipins that derive from the linoleic acid and LDS1 action, resulted particularly present following treatment; while in the mutant strains (unable to sense POX) we observed an increase of 8-series oxylipins (8-HODE, 8, 13 di HODE, 8-HpODE), 10-HODE and 11-HpODE, all derived from the linoleic acid and LDS1 activity. However, especially in the CF of the mutants 9-oxoODE, an oxylipin products of LOX on the linoleic acid, is over-produced.

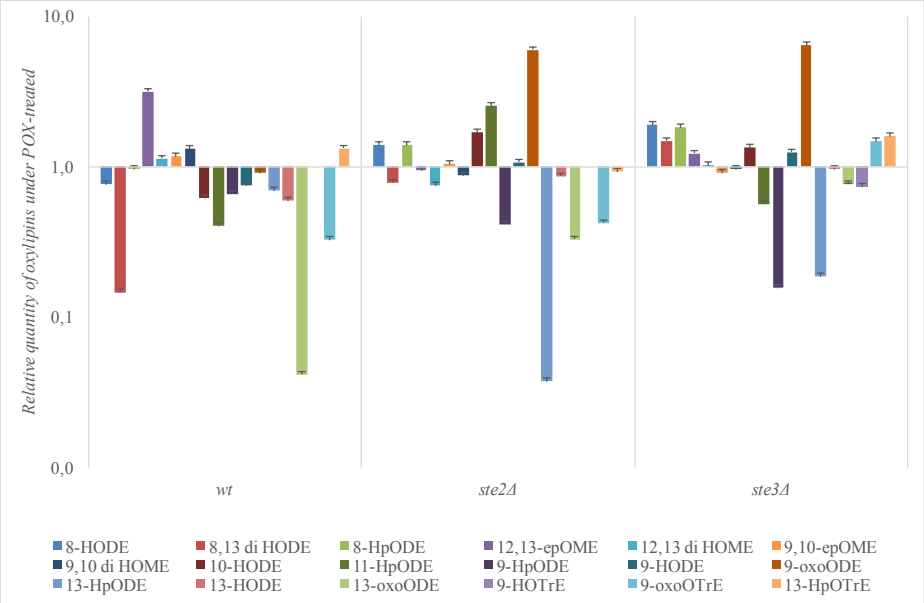


Figure 1. MRM oxylipin profile. The oxylipins were extracted from the cultural filtrate in which *wt*, *ste2*Δ and *ste3*Δ have been grown. The legend shows the 18 oxylipins analyzed. The oxylipin peak areas are normalised on the peak area of ISTD 9-HODEd_4. Y axis: treated: cultural filtrate+POX/ control: cultural filtrate+POX boiled. Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

Negative scan method was performed to analyze the fatty acid content in CF. We analyzed 11 fatty acids and we have found that in *wt* treated with POX, a high concentration of oleic acid was present (Figure 2a), while the mutant strains did not represent the same amount (Figure 2b).

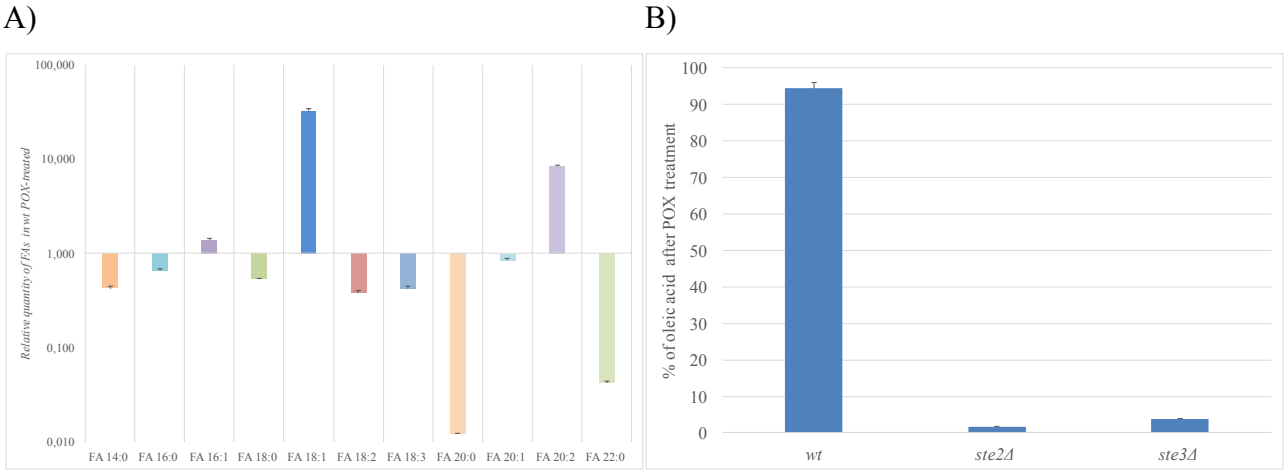
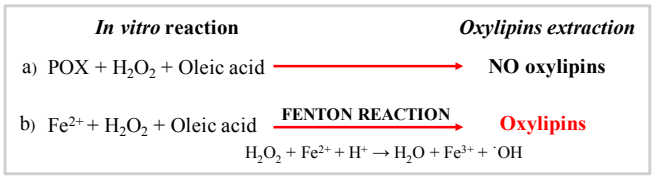


Figure 2. SCAN fatty acid content. A) 11 fatty acids are quantified in *wt* strain, the peak areas are normalised on the peak area of ISTD C17:0. Y axis: treated: cultural filtrate+POX/ control: cultural filtrate+POX boiled. B) oleic acid content in *wt*, *ste2*Δ and *ste3*Δ. Y axis: treated: cultural filtrate+POX/ control: cultural filtrate+POX boiled; the relative quantity is expressd in percentage. Results represented the mean of 6 values originated by two biological replicates technically repeated trice (n=3; ± SE).

Susceptibility of oleic acid to Ferrous Iron/Hydrogen Peroxide (Fenton-) mediated oxidation

The oleic acid is a substrate suitable for producing oxylipins. We tested if the POX was able to generate peroxides of the oleic acid. The peroxidation reaction for testing POX activity on the oleic acid is showed in figure 3a, after the incubation the oxylipins have been extracted and analyzed in LC-MS/MS. However, we did not found any oxylipins prevalently formed by this reaction (any value <LOD). The second reaction that we have tested, is a Fenton reaction, in which we have joined $FeSO_4$, that in solution release the cation ferrous, and the H_2O_2. Iron may stimulate peroxidation by hydroxyl radical (•OH) formation and may promote the decomposition of hydroperoxides. Fe^{2+} and H_2O_2 can generate •OH by means of the Fenton reaction. The hydroxyl radical •OH can abstract a hydrogen atom from methylene group of oleic acid to yield a carbon center radical. The oleic acid undergoes molecular rearrangement, and the center radical can then react with O_2 to form a hydroperoxides, *i.e.* oxylipins.

The lipids are re-extracted at different reaction times, that is immediately after joining reagents (t0), and following 30 and 60 minutes of incubation. The analysis was performed for the Fenton reaction and for two negative test, one without the H_2O_2 and the other without $FeSO_4$. LC-MS/MS analysis shows the formation of specific hydroperoxides (Figure 3c) only in the test containing oleic acid, $FeSO_4$ and H_2O_2. The negative SCAN method is able to discriminate the hydroperoxides by mass, and we found two putative oxylipins whose m/z ratio is 314,24 (10S HpOME and 7S, 10S-DiHOME), and one oxylipin identified with 298,25 m/z (10S-HOME). The nature of this oxylipins was confirmed by following the previously described MRM methods for the analysis of oxylipins. Therefore, we can conclude that the Fenton reaction was capable to generate hydroperoxides.



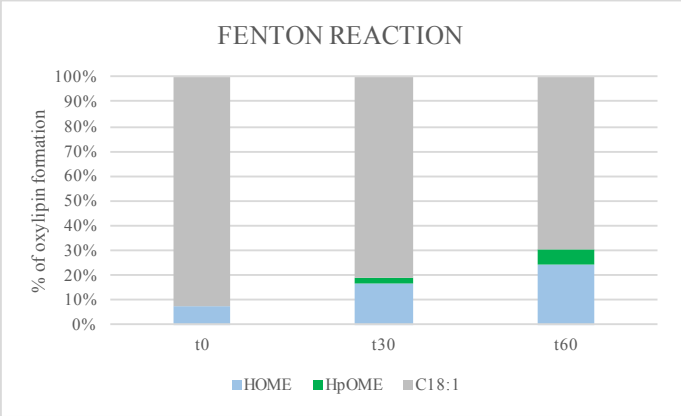


Figure 3. Oleic acid peroxidation. a) oleic acid 50 nM with H_2O_2 (10 mM) and HRP (0,07 U/mL) reaction not generates peroxidation products. b) oleic acid 50 nM with Fe^{2+} (0,42 mM) and H_2O_2 (0,73 M) reaction generates peroxidation products, *i.e.* oxylipins. c)The reaction was performed at three time points ($t_{0\ min}$, $t_{30\ min}$, $t_{60\ min}$) at 37°C. The oxylipins formation it was tested by LC-MS/MS, as reported in the chart. All experiments were performed at least twice.

Chemotropism assays

F. oxysporum exhibits directed growth towards the host plant, and it was observed that responds to the gradient of peroxidase activity exuded by tomato roots. *F. oxysporum* exposed to a gradient of commercial HRP produced significantly more germ tubes pointing towards the peroxidase source than towards the solvent control (Figure 4a), resulting in positive chemotropism (Turrà et al., 2015).

It was tested the chemotropism of *F. oxysporum* in response to the gradient of some lipid compounds. Notably, we tested the oleic acid and the oxylipins at different concentrations. Microconidia show a positive chemotropism towards the oleic acid, the concentration tested are 5 µM, 10 µM and 20 µM. The oxylipins tested are 9S-HpODE and 13S-HpODE at the same concentration utilised for the oleic acid (Vitale S. unpublished data). These compounds are able to produce a chemoattractant gradient (Figure 4b, c), in fact the microconidia are germinated and oriented towards the lipids.

As previously described the hydroperoxides of the oleic acid can be formed in presence of H_2O_2 and Fe^{2+}. We could confirm it through the utilizing of an *in vivo* system. Two relevant chemotropism assays were performed by using two deletion mutants: the first presented a deletion into the R subunit of the NOX enzymatic complex (*noxR*Δ mutant), leading to the lack of production of H_2O_2; in the second the gene that codifies for the Ferro-O2-oxidoreductase (*fet3*Δ mutant) was deleted. These two mutant are affected in two principal components of Fenton reaction (H_2O_2 and Fe^{2+}), so much so that did not respond to the chemotropism of the oleic acid (Figure 4d, e). In figure 4 f is reported the chemotropism index evaluated as reported in Turrà et al., 2015, it can be noticed that mutants did not respond to the gradient emitted by oleic acid.

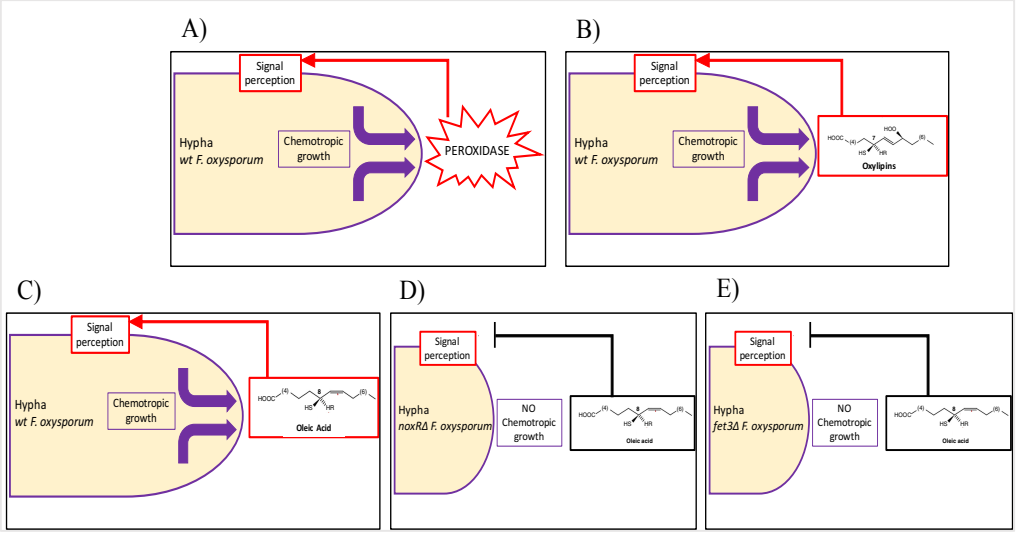


Figure 4. Chemotropism assays (A-E). A-C) *wt* microconidia in presence of POX, oxylipins and oleic acid respond positively to their compounds, and it's established a chemotropism towards these compounds; when we tested the germination of *noxR*Δ and *fet3*Δ microconidia (D and E) we haven't visualized germination toward the oleic acid gradient.

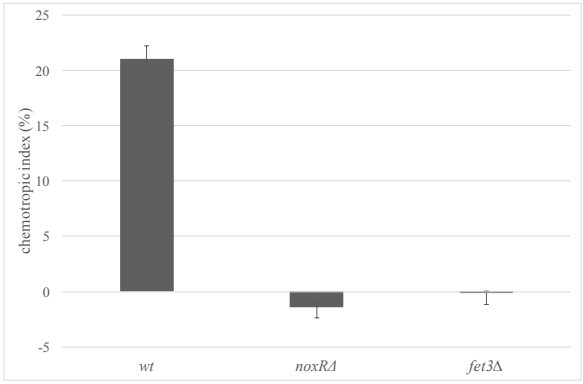


Figure 4F. Chemotropism assays. Chemotropism index derived from the oleic acid in *wt, noxR*Δ and *fet3*Δ. The *wt* responds positively to the gradient, while the mutants may to be unable to perceive the input. Data are presented as the mean from two experiments. *n* = 500 germ tubes.

Discussion

Fungi can react to external stimuli with responses occurring at different levels of cellular organization. The fungus, in our case a pathogen, must activate all the responses allowing the access to source of nutrition and growing in the direction of the host. This is the case of *F. oxysporum* that perceiving the exudate produced by tomato roots directing its growth toward these organs. There are so many perceptible signals, some of which are just nutritious, others can stimulate the growth and invasion of the pathogen, this is the case of plant peroxidase, capable of being perceived by *F. oxysporum* stimulating chemotropic growth. Through the chemotropic assay developed by Turrà et al. 2015, this interaction was simulated, i.e. the growth of spores in the presence of peroxidase. Peroxidases can therefore be considered as bifunctional enzymes that can oxidize various substrates in the presence of H_2O_2 but also produce ROS. Their involvement in several physiological and developmental processes can be detected in plants from germination to senescence (Passardi et al., 2004).

A closely link among peroxidase, H_2O_2 and lipids actually exists. Lipids, in addition being the mainly constituent of the membrane are also important signal of communication. This is the case of the oxylipins (Tsitsigiannis et al., 2017; Scala et al., 2013), oxidised fatty acids. In this study, we analysed the oxylipin content in the cultural filtrate of *F. oxysporum* grown in presence of peroxidase and identified that POX induction leads to an increment of the linoleic acid-derived oxylipins. Notably,

an increment of 8-HODE, 8,13 di HODE, 8-HpODE, 10-HODE and 11-HpODE occurred in POX-induced samples. Chemotropic tests with 9-HpODE and 13-HpODE were performed: *F. oxysporum* can grow toward these compounds suggesting that these two oxylipins may be present in the pool secreted by the host. Other oxylipins, such as those derived by oleic acid, can be present too. This aspect will be further characterized in future studies. In relation to this, we argue if other than already formed oxylipins, it could be present some precursor of these oxidized compounds such as linolenic, linoleic or oleic acid. Analyzing the cultural filtrate, we found almost high oleic acid concentration. Recent works suggest that the oxylipins derived from oleic acid can promote the biofilm formation and the virulence in *Pseudomonas aeruginosa* (Eriel Martínez & Javier Campos-Gómez, Nature 2016), in yeast, in human pathogen *C. albicans*, the oxidised farnesol regulates the quorum sensing and the biofilm formation (Langford et al., 2009). Nevertheless, in our interaction model we suggest that the oleic acid itself may represent a chemoattractant, even if our experiments suggest that its activity probably relate to the presence of other key factors that act during the plant pathogen interaction: the cation Fe^{2+} and hydrogen peroxide H_2O_2. In the presence of these reactants, the oleic acid can be converted in other reactive form through the hydroxyl radical formed during the reaction of Fenton such as in oleic acid-derived oxylipins. Thus, our hypothesis rely on the fact that more than oleic acid, the growth of the hyphae can be polarized by its derived oxylipins, which in turn, should be generated in a highly oxidising environment. In previous works it was reported that the oxylipins regulate cell growth (Nover et al., 2003).

To test the Fenton reaction in biological system, and show the involvement of H_2O_2 and Fe^{2+} in the hydroperoxidation of the oleic acid, we have assayed if two mutants in which were altered the release of these two elements.

The first mutant subject to the oleic acid gradient was the *noxR.* The recent recognition that isoforms of the cellular NADPH-dependent oxidases, collectively known as the NOX protein family, participate in a wide range of physiologic and pathophysiologic processes in both the animal and plant kingdoms, in presence of POX release H_2O_2 (Nauseef, 2014). To test the involvement of the iron it was tested *fet3*Δ. *S.* cerevisiae *fet3* encodes a multicopper oxidase required for ferrous iron uptake. (Askwith et al.,1994). The two mutant tested, *noxR* and *fet3,* not respond to the oleic acid gradient, suggesting the H_2O_2 and Fe^{2+} involvement in the oxylipins formation.

In figure 6, we explain the model of interaction that we have hypothesized. The first stimulus is represented by the catalytic activity of secreted class III peroxidase that induces the microconidia to germ towards the source of the compound, that *in vivo* would be the roots of the host tomato. The chemotropic response requires the co-presence of two transmembrane proteins Ste2 and Ste3 (unpublished data Vitale et al.), whose signaling cascade is Mpk1-mediated. The lipid profile, after the induction POX-mediated show an increment of oleic acid in the cultural filtrate. Apparently, the directional growth of the hyphae could be perpetuated by the release of oleic acid. Different studies report that during the plant-pathogen interaction the fatty acids are released from the membrane phospholipids through the phospholipases action **(**Upchurch 2008). The fatty acid in an oxidant environment can be transformed into oxylipins (10S HpOME, 7S, 10S-DiHOME and 10S-HOME). By our experimental evidences, we can suggest that the Fenton reaction actually occurs and it is specifically supported by the NOX complex and Fet3 protein on the tip of hypha. The H_2O_2 release by the NOX complex and the ferro-O_2-oxidoreductase presence may lead to the •OH formation by Fenton reaction. In turn, this radical specie can generate hydroperoxides of the fatty acid (Halliwell & Chirico, 1993). The oxylipins can convey the message, and perpetuate the growth towards the plant cells. We don't know how the fungus can sense the oxylipins gradient. Some studies suggest the existence of a putative G-protein able perceiving the oxylipins (Affeldt et al., 2012).

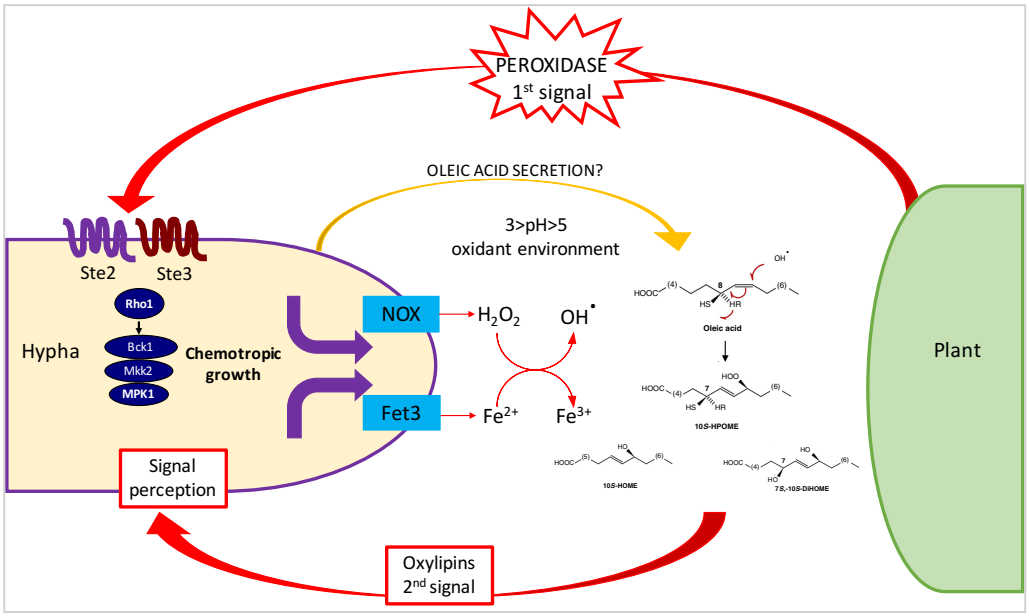


Figure 6. The interaction model oleic acid-mediated. The hyphal grow towards the plant cell is trigged by the peroxidase (first signal), the transmembrane proteins Ste2 and Ste3 perceive the signal and activate the Mpk1 signaling. The fungus releases oleic acid and it can be converted in hydroperoxides (*i.e.* oxylipins) in response to the hydroxyl radical formation (Fenton reaction) thank to the presence of NOX (NADPH oxidase) complex and Fet3 (Ferro-O2-oxidoreductase). The oxylipins (second signal) could perpetuate and magnify the signals for an oriented grow toward the root.

Materials and methods

Fungal strain culture and transformation. Fungal strains used in this study are listed in **Supplementary Table1**. All are derivatives of *F. oxysporum* f. sp. *lycopersici* isolate 4287 (FGSC 9935). Strain culture and storage were performed as described in Di Pietro et al. 2001. *F. oxysporum* gene data are available in the *Fusarium* Comparative Database under the following accession numbers: *ste2*, FOXG_10633; *ste3*, FOXG_02147; *noxR*, FOXG_11514; *fet3*, FOXG_07915.

F. oxysporum germs treated with peroxidase

F. oxysporum germs were grown in 250 mL of minimal medium MM (Di Pietro et al., 1998) supplemented with sodium glutamate (GluNa) 5mM at pH 7 at 1,6x10^6 sp/mL concentration. The germs were recovered and treated in 25 mL of MM+5 GluNa pH 7 with the commercial horseradish peroxidase POX (HRP Sigma) at the final concentration of 3,84 µM, for the control the POX was boiled for 25 minutes. The germs were incubated for 1 hours at 28°C and 90 rpm. It was recovered the media and germlings via filtration on 0,45 µM nylon filters.

Chemicals

All solvents used for fatty acids and oxylipins extraction and HPLC/MS analysis were of HPLC/MS grade. Methanol (MeOH), isopropyl alcohol (iPrOH), ethyl acetate (EtOAc), acetonitrile (ACN), hexane were purchased from Merck (Darmstadt, Germany). Ammonium formate (HCOONH4) was purchased in granular form from Fluka (Buchs SG, Switzerland).

Lipid extraction protocol

Lipids were extracted from the *F. oxysporum wt*, *ste2*Δ and *ste3*Δ treated with peroxidase and peroxidase boiled. The internal reference standard added for the quantitative analysis was the 9-hydroxy-10E,12Z-octadecadienoic acid (9-HODEd4, MW 300.5 g mol^{-1}) at a final 2µM concentration. The extraction was performed on 30 mL of lyophilized cultured filtrate. They were

vortex-mixed with 10 mL of extraction mixture (iPrOH: H_2O: EtOAc 1:1:3 v/v, with 0.0025% w/v of butylated hydroxytoluene, BHT, to prevent peroxidation). After centrifugation, the ethyl acetate upper phase was collected in a clear tube and dried under nitrogen gas flux. After centrifugation, the upper phase was recovered and transferred to the collection tube together with the previously extracted fraction, and dried under nitrogen flux. The dried samples were reconstituted in 100 µL of MeOH and loaded onto the HPLC auto-sampler.

Fatty acids analysis by LC-MS/MS

The equipment, chromatographic column and analysis software were all from Agilent Technologies (Santa Clara, CA, USA). Samples were analyzed by LC (HPLC 1200 series rapid resolution) coupled to a triple quadrupole MS (G6420 series triple quadrupole, QqQ; Agilent Technologies) equipped with an electrospray ionization source (ESI). The acquisition was in negative ion polarity, and the characterization was in MS2 SCAN mode. Chromatographic separation was performed through a Zorbax SB-C8 rapid resolution HT 2.1 x 50 mm 1.8 µm 600 bar column (Agilent Technologies). The mobile phases consisted of A phase (water: B phase 80:20 v/v, containing ammonium formate 5mM), B phase (MeOH: iPrOH 95:5 v/v). The elution programme was as follows: 0-6 min 60% B, 6-30 min 99% B, 30-38 min 60% B.

The flow rate was 0.4 ml min^{-1}. The column was thermostated at 60°C. The injection volume was 2 µL. The injector needle was washed with the mobile phase in the wash port at the end of each HPLC run. Nitrogen was used as the nebulising and desolvation gas. Drying gas temperature was 350°C, its flow was 10 l min^{-1}, the nebulization pressure was 20 psi. The parameters used for the analysis of fatty acids are reported in the **Supplementary Table 2**.

Oxylipin MRM analysis by LC-MS/MS

Oxylipins were extracted as described by Ludovici et al., 2014 with slight modifications. Samples were analyzed by liquid chromatography (HPLC 1200 series rapid resolution, Agilent Technologies, Santa Clara, CA, USA) coupled to triple quadrupole (G6410A series triple quadrupole, QqQ, Agilent Technologies, CA, USA) equipped with an electrospray ionization (ESI). Experiments in MRM in negative ion mode $[M-H]^-$.

Oleic acid oxidation system and oxylipin analysis by LC-MS/MS

Oleic acid peroxidation was performed with the following reagent: oleic acid (C18:1 MW 282.46, Sigma), HRP (Sigma), syringaldazine (MW 360,36, Sigma), H_2O_2 (30% w/w in H_2O, Sigma), DMSO (MW 78,13, Sigma), Potassium Phosphate Monobasic Anhydrous (KH_2PO_4 MW 136,09), Iron (II) Sulphate ($FeSO_4$ 151,908). The experimental design provides two typologies of reactions for testing the oxylipins formation (see the reaction table below).

1- POX assay

POX reaction	BLANK	CONTROL
Phosphate Buffer 14 mM	Phosphate Buffer 14 mM	Phosphate Buffer 14 mM
H_2O_2 10 mM	H_2O_2 10 mM	H_2O_2 10 mM
DMSO 20%	DMSO 20%	DMSO 20%
HRP 0,7 U	-	HRP 0,7 U
C18:1 50 nM	C18:1 50 nM	-
-	-	Syringaldazine 50 nM
H_2O up 6 mL	H_2O up 6 mL	H_2O up 6 mL

2 Fenton assay

Fenton	$BLANK_1$	$BLANK_2$
H_2O_2 0,73 M	-	H_2O_2 0,73 M
$FeSO_4$ 0,42 mM	$FeSO_4$	-
C18:1 50 nM	C18:1 50 nM	C18:1 50 nM
H_2O up 6 mL	H_2O up 6 mL	H_2O up 6 mL

Each reaction it was tested during the time (t_0, $t_{30\ min}$, $t_{60\ min}$) at 37°C. For the POX assay we have added the control reaction, with syringaldazine a substrate of the POX, for testing the enzyme activity in addition to the blank without the HRP. For the Fenton reaction we have tested two blanks, one without the H_2O_2 and the other without $FeSO_4$. At each time point we have drawn 1 mL, the reactions were stopped and the lipids were extracted adding 1 mL of extraction mixture (hexane:iPrOH 3:2 v/v, with 0.0025% w/v of BHT), it was added 9-$HODE_{d4}$ at the final concentration of 2 µM. The sample were vortex-mixed for three minutes and after centrifugation the hexane phase was collected in a clear tube and dried under nitrogen gas flux.The dried samples were reconstituted in 100 µL of MeOH and loaded onto the HPLC auto-sampler.

Oxylipins analysis it was performed with the method and parameters utilized for the fatty acids quantification. The acquisition was in negative ion polarity, and the characterization was in MS2 SCAN mode. We have searched for the following masses that correspond to the relative oleic acid-derived oxylipins with *m/z* 314,24 (10S-HpOME, 7S-10S-DiHOME) and *m/z* 298,25 (10S-HOME). Experiments of product ion to characterize oxylipins were performed. The fragmentor voltage and collision energies were optimised for each compound.

Quantification of fungal chemotropism

The fungal chemotropism was evaluated as reported in Turrà ae al., 2015. Tested compounds were: oleic acid sodium salt (MW 386,522) and the oxylipins (9-HpODE and 13-HpODE, MW 312,23), they are assayed at different concentration, starting from 5 µM to 40 µM. Sterile water or methanol were used as solvent controls. All experiments were performed at least twice. Statistical analysis was conducted using *t*-test.

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Fungal secreted oleic acid represents a signal for reinforcing the host-oriented growth in *Fusarium oxysporum*

Supplementary Table 1. Fungal strains used in this study.

Strain	Genotype	Gene Function	Reference
FGSC	wild type		Di Pietro et al., 2001
*ste2*Δ	ste2::HYG	GPCR	Turrà et al., 2015
*ste3*Δ	ste3::HYG	GPCR	Vitale Stefania et al. unpublished results
*noxR*Δ	noxR::HYG	$\bullet O_2$ and H_2O_2 generation	Nordzieke Daniela et al. unpublished results
*fet3*Δ	fet3::HYG	Ferro-O2-oxidoreductase	Vitale Stefania et al. unpublished results

Fungal secreted oleic acid represents a signal for reinforcing the host-oriented growth in *Fusarium oxysporum*

Supplementary Table 2. Acquisition Method Info. Parameters utilised for fatty acids quantification.

Ion Source ESI

Scan Type MS2 Scan

Scan Segments

Start Mass	End Mass	Scan Time	Frag (V)	Cell Acc (V)	Polarity
100	500	1000	80	7	Negative

Fatty Acid	m/z	$[M-H]^-$	Retention time	
C14:0	228,4	227,4	2,9	Target
C16:1	254,4	253,4	3,6	Target
C16:0	256,4	255,4	5,9	Target
C18:0	284,5	283,5	10,3	Target
C18:1	282,5	281,5	7,3	Target
C18:2	280,5	279,5	5	Target
C18:3	278,4	277,4	3,3	Target
C20:0	312,6	311,5	13,1	Target
C22:0	340,6	399,6	14,9	Target
C24:0	368,6	367,6	16,3	Target

Final Discussion and Conclusion

Fungi-plants communication: the lipid language

The lipid molecules mediate the inter-kingdom signaling during the plant-fungus interaction. Our knowledge on the role of lipids in plant–microbe interactions has strongly increased since the availability of highly sensitive analytical technologies, as the high-pressure liquid chromatography coupled to mass spectrometry. Our interest has been focused within the lipid profile of the pathogenic genus *Fusarium* during the interaction with host plants such as maize and tomato. Despite advancements in mechanical and chemical technology, crops continue to suffer annual losses of yield, with significant losses due to fungal diseases. In order to secure a growing demand for food supply, mechanisms moving plant-pathogen interactions must be elucidated to develop strategies of disease resistance and novel, environmentally sustainable prevention system. Lipids have gained appreciation over recent years for their role as mediators in the interaction between plant and fungi, contributing to both plant defense and fungal pathogenicity.

Lipids are a large group of structurally diverse molecules that have different metabolic involvement. One group of lipids, oxygenated lipids (oxylipins), are very interesting because are able to orchestrate a myriad of metabolic processes in both plant and fungi. The oxylipins can regulate the sporulation and the secondary metabolite production, as the mycotoxins, but also they can manipulate the plant metabolism and defense responses. One of the major triggers for the biosynthesis of oxylipins is the alteration of the cell redox status, closely linked to the burst of the reactive oxygen species (ROS) and lipid peroxidation processes. The term "oxylipin" was introduced for the first time from Gerwick in 1991, the oxylipins are produced through enzymatic as well as non-enzymatic oxygenation (Gerwick et al, 1991). Oxylipin biosynthesis may arise from fatty acids liberated from diverse cellular membranes by lipases action. The deletion of some genes implicated in the oxylipin synthesis generates a severe alteration of sporogenesis and secondary metabolism (Scala et al., 2014).

The oxylipins can define the initiation or the success of the interaction but they are not the only lipid molecules that can do this. The fungus is able to generate mycotoxins, secondary metabolites, capable of killing the host. *Fusarium verticillioides*, during the maize infection, produces a mycotoxin very toxic for the human health, the fumonisin B1 (FB1). The fumonisin can block the activity of ceramide synthase. This block may cause a cell plant suicide or as some cell compartments undergo to PCD (programmed cell death). In the article ***"Sphingolipids in Fusarium verticillioides–Zea mays interaction"*** (pag.18) we hypothesised that *F. verticillioides* uses fumonisin to hampering sphingolipid metabolism, to accumulating LCB and specific ceramides may trigger defence and PCD processes, as reported in figure 10 (pag. 27). This imbalance could activate a signal cascade that involves a phosphorilative process and the ormone production. Is the case of one *Zea mays* MAP Kinase involved in plant defences, it's indicated as MPK6, which it should be able to control salicylic acid (SA) levels after the pathogen's attack.

In turn, SA regulates at transcriptional level the expression of some defence-related genes such as the pathogenesis related protein (*pr1*), and some genes that codified for the metacaspases (*mcaII*) involved in the programmed cell death. In our experimental set, i.e. *Fv* infection of maize kernels, *mpk6* is up-regulated and in turn, SA levels arise. Consequentially, *pr1* and *mcaII* are up-regulated.

This host pathogen interaction follows two ecological route, or rather, *Fusarium verticillioides* initially defined hemibiotrophic, in the first stage of infection the fumonisin is not produce, but after some days the fungus starts to produce the toxin and leads the plant cells to the death, so we assist to a transition towards the necrotrophic features. During the hemibiotrophic infection the penetration hypha develops a vesicle that invades the the plant cell and after a limited time plant plasma membrane disintegration starts, leading to host cell death. This process is normally activated by the secretion of large amounts of cell-wall-degrading enzymes by the secondary hyphae, as reported in the work of Mendgen & Hahn (2002), it was seen in the case study of *Colletotrichum*. In our study we suggest that the toxins Fumonisin B1 (FB1) is ceramide synthesis inhibitors that disrupt sphingolipid metabolism resulting in host accumulation of free sphingoid bases (Abbas et al., 1994). Plants FB1-resistant fail

to generate ROS and are resistant to FB1-induced cell death due to attenuated accumulation of free bases. Therefore, the increase in free sphingoid base levels correlate with ROS and cell death (Shi et al., 2007). The results emerged from *"Sphingolipids in Fusarium verticillioides–Zea mays interaction"* (pag. 18) underlined the switch to the necrotrophic lifestyle of *Fusarium verticillioides.*

Another fumonisin effect observed during *F. verticillioides*-maize interaction is the accumulation of glucosyl-ceramides; these are known to be mainly cell membrane elements and it is reasonable to "read" their increase as an infection-driven cell membrane rearrangement, likely linked to lipid rafts (LRs) formation (Bagnat et al., 2000). LRs are very active areas rich in sterols, glucosyl-ceramides and proteins. These areas are specialized in perception of the environment and stimuli that it offers. The stimuli of chemical- or physical-nature may orientate the growth of the hypha towards the source; this phenomenon is defined "chemotropism" (Turrà & Di Pietro 2015). Chemotropic signaling in fungi can be stimulated by gradients of nutrients, peptide pheromones or plant signals and are perceived by cellular receptors and signaling cascades (Turrà & Di Pietro 2015; Vitale et al., 2017).

Among the plant signals, the peroxidase III, a heme-oxidase, may form a gradient able inducing the growth of *Fusarium oxysporum* towards the roots of the host *Solanum lycopersicum* (Turrà et al. 2015), this system was functional for the analysis of lipid exchange.

F. oxysporum may perceive the plant peroxidas through the sex pheromones receptors, Ste2 and Ste3, two G-protein coupled-receptors (GPCR). These transmembrane proteins are localized on the tip of hyphae, probably in the LRs. Ste2 and Ste3 activate a kinase-signal transduction through the Mpk1-route, and promote the host-oriented growth.

In the article **"*Fungal secreted oleic acid represents a signal for reinforcing the host-oriented growth in Fusarium oxysporum*"** (pag. 64) the lipidomic analysis of the surrounding environment has highlighted an accumulation of oleic acid, a fatty acid with 18 carbon atoms and one unsaturation. High concentrations of oleic acid are toxic for the cell, and we have hypothesized that it was converted in another substrate, more effective in terms of signaling. As documented, fatty acids, in addition to be cell structural components, e.g. triacylglicerides, sphingolipids and other, may act as signaling molecules when oxidized, as in the case of oxylipins (Tsitsigiannis & Keller, 2007).

We found that the reaction able oxidizing the oleic acid is a consequence of the "complex simplicity of nature"; we have previously reported that a spontaneous and energetically favorable reaction can generates these modulators of interaction (non-enzymatic synthesis of oxylipins; Halliwell & Gutteridge, 2015). During the plant-pathogen interaction we are witnessing to an increase of the oxygen species production (Nanda & Dunand, 2010), including the hydrogen peroxide, which in presence of reduced iron, component and cofactor present in the environment, leads to the Fenton reaction. This reaction generating the hydroxyl radical (•OH) is able to oxidize the oleic acid (Halliwell & Chirico, 1993). Through this spontaneous reaction, a series of oxylipins (e.g. 10-HOME) are generated and future studies will bring us to decipher their role during the plant-pathogen interaction. We here hypothesize that through GPGR-like protein the oxylipins are perceived from the hypha as a secondary stimulus; notably, this recognition could perpetuate and magnify the directional growth towards the plant. The modest amount of oleic acid found in the surrounding environment could be promoted by the action of phospholipases capable of releasing it from membrane complex lipids such as triacylglycerides and sphingolipids.

In **"*Fatty acid composition and metabolism in the maize pathogen Fusarium verticillioides*"** (pag. 46) was evaluated the close *liaison* between fatty acid and oxylipin biosynthesis.

This research is based on preliminary analysis conducted in two precedent works published by Scala and coworkers (2014) and in **"*Analysis of the expression of genes related to oxylipin biosynthesis in Fusarium verticillioides and maize kernels during their interaction"*** (pag. 40), presented in this thesis. It emerges that *F. verticillioides* in maize presence alter the oxylipin profile in an apparently coordinated manner. Therefore, we sought a common transcriptional factor capable of coordinating this synthesis. The bioinformatic analysis suggests a potential candidate. The transcription factor *crazy1* (Crz1) is potentially able

to control (through CDRE) the expression of some oxylipin-related genes as well as of other genes involved in the synthesis of their FA precursors in *F. verticillioides*.

It emerges a complex scenario in which oxylipins have a positive transcriptional feedback on the expression of some genes related to the synthesis of FA, so the genes for the desaturases, implicated in the double bonds formation, and the elongases, able to add two carbons to the chain, result influenced.

Notably, *lds1Δ* mutant strain is affected in oxylipins as well as in FFA synthesis (especially linoleic and linolenic acid). Our results suggest that this coordination is exerted, at least in the exponential growth phase (16-48 hour after inoculation), by the calcium-sensitive transcription factor Crz1. In fact, Crz1 possesses different responsive elements (i.e. CDRE) within the promoter of both oxylipin and FFA synthetic genes. Thus, we can suggest that under perception of environmental *stimuli* the calcium wave, typically originated upon signal perception (Thewes et al, 2012), may activate Crz1 that in turn early coordinate the synthesis of FFA and of their modifying enzymes, needed for producing hormonal like molecules such as oxylipins. The latter can act as true hormone-like compounds and control several aspect of fungal life: rate of growth, conidiogenesis, secondary metabolism and virulence (Tsitsigiannis and Keller, 2007; Reverberi et al., 2012; Scala et al., 2014).

In conclusion, the works focus on the link between lipid signaling and host-pathogen comunication, the data emerged are briefly shown below:

- During *Fusarium verticillioides- Zea mays* interaction the pathogen jeopardises the sphingolipid metabolism through the mycotoxyns production. Sphingolipid alteration and salycilic acid increase cause in the plant cell death, suggesting for the pathogen a switch from an endophytic to a necrotrophic state.
- Enviromental conditions influence fungal oxylipin gene expression profile and the fatty acid metabolism.
- The transcription factor Crz1, found in *Fusarium verticillioides*, is a novel regulator of lipid metabolism during the interaction with the plant host. It seems able to coordinate the linoleic acid production and the resulting oxylipins.
- Oleic acid can be considered a novel signaling molecule released fro the fungal pathogen *Fusarium oxysporum* to reinforce host-oriented growth.

Results are encouraging and allow us hypothesizing that the lipid signaling system may be maintained in different systems of plant-fungus interaction. However, the circle will close when all the actors will have their place in the scene.

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