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Constitutive expression of pathogenesis-related proteins and antioxydant enzyme activities triggers maize resistance towards *Fusarium verticillioides*

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ABSTRACT

Fusarium verticillioides is a fungal pathogen of maize that causes ear rot and contaminates the grains with fumonisin mycotoxins. Breeding for resistance to *Fusarium* emerged as the most economic and environmentally safe strategy; therefore the discovery of resistant sources and effective molecular markers are a priority. Ears of resistant (CO441 and CO433) and susceptible (CO354 and CO389) maize lines were inoculated with *F. verticillioides* and the expression of pathogenesis-related (*PR*) genes (*PR1*, *PR5*, *PRm3*, *PRm6*) and genes that protect from oxidative stress (*peroxidase*, *catalase*, *superoxide dismutase* and *ascorbate peroxidase*) were evaluated in the kernels at 72 h post inoculation. In addition, the oxidation level and the enzymatic activity of ascorbate-glutathione cycle, catalase, superoxide dismutase and cytosolic and wall peroxidases were investigated. The uninoculated kernels of the resistant lines showed higher gene expression and enzymatic activities, highlighting the key role of constitutive resistance in limiting pathogen attack. In contrast, the susceptible lines activated defensive genes only after pathogen inoculation, resulting in increased levels of H_2O_2 and lipid peroxidation, as well as lower enzymatic activities. The constitutive defenses observed in this study from seed could be profitably exploited to develop markers to speed up conventional breeding programs in the selection of resistant genotypes.

1. Introduction

Ear rots and mycotoxin contamination of the kernels frequently reduce the quality and yield of an economically important and widely grown crop as maize (*Zea mays* L.). *Fusarium verticillioides* (Sacc.) Nirenberg is one of the most common fungal species associated with maize in temperate regions and produces fumonisins, considered carcinogenic mycotoxins (Santiago et al., 2015). Fumonisins are able to disrupt the metabolism of sphingolipids, important signaling molecules in animal and plants (Williams et al., 2007). *F. verticillioides* enters the ear through silks or through wounds due to insect injury and mechanical damage and causes ear rot in the tip or in scattered kernels (Logrieco et al., 2002; Munkvold, 2003a). The complexity of this pathosystem lies partially in the lifestyle of pathogen, which is both parasite and saprophyte and it can be transmitted from seed to plant as a symptomless intercellular endophyte (Bacon et al., 2008; Munkvold, 2003a). The process of *F. verticillioides* infection and mycotoxin accumulation is influenced by environmental conditions, but also by host resistance and biochemical composition of kernel, including moisture, development stage and lipid composition (Battilani et al., 2008; Maschietto et al., 2015; Sagaram et al., 2006; Woloshuk and Shim, 2013).

Agronomic practices for fumonisin content reduction are often ineffective and breeding for resistance to *Fusarium* species (particularly *F. verticillioides*) emerged as the most economic and environmentally safe strategy (Munkvold, 2003b). Quantitative Trait Locus (QTL) mapping studies in maize indicated that *Fusarium* resistance and fumonisin contamination are quantitative traits determined by small effect polygenes with moderate to high heritability (Ding et al., 2008; Robertson-Hoyt et al., 2006; Zila et al., 2013, 2014). Genetic resistance to *Fusarium* ear rot and fumonisin accumulation has been identified in maize lines and hybrids (Clements et al., 2004; Henry et al., 2009; Kleinschmidt et al., 2005; Löffler et al., 2010; Lanubile et al., 2011; Pascale et al., 2002; Santiago et al., 2013), but the complexity of these polygenic traits

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have so far hampered the development of resistant maize genotypes with high agronomic performance (Bush et al., 2004; Eller et al., 2008; Zila et al., 2013). Breeding for resistance will benefit from the discovery of efficient markers for phenotyping and QTLs consistent across populations, but also by a more extensive comprehension of the genetic basis underlying maize-*F. verticillioides* interaction.

Plant resistance to pathogen attack is polygenic, involving a hierarchy of genes that produce proteins and metabolites, either constitutive or induced post infection, including the synthesis and accumulation of reactive oxygen species (ROS), phytoalexins and pathogenesis-related (PR) proteins (Almagro et al., 2009; Torres, 2010). The success of infection depends on the number and abundance of these defense related products and the speed with which defense responses are mounted.

The oxidative burst is one of the main event associated to biotic stimuli. The accumulation of ROS, in particular hydrogen peroxide, suppresses pathogen entrance or induces host cell death or hypersensitive response to contain the pathogen (Baxter et al., 2014; Torres, 2010; Wirthmueller et al., 2013). The amount of ROS depends on the type and amount of enzymatic and non-enzymatic scavenging molecules that include: superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD) and the antioxidants ascorbate (ASC) and glutathione (GSH) (Mittler et al., 2004). ASC levels and ASC-GSH cycle components are tightly linked to plant tolerance to biotic and abiotic stresses (Locato et al., 2013; Paciolla et al., 2004, 2008).

In comparison to vegetative tissues, deeper and continuing efforts are still needed to unravel the mechanisms of qualitative and quantitative resistance in maize kernels against *F. verticillioides* infection. Candidate genes for host resistance towards this pathogen were searched in the last years thanks to the employment of maize mutants (Christensen et al., 2013, 2014; Gao et al., 2007) and genotypes showing contrasting level of resistance (Lanubile et al., 2010, 2012a,b, 2014; Maschietto et al., 2015). Both constitutive and induced resistance were proven to be involved in maize kernel defense against *F. verticillioides* infection, including the expression of PR proteins, lipoxygenases, ribosome-inactivating proteins (RIPs), WRKY and other transcription factors, jasmonate and ethylene signaling-related genes, genes related to primary and secondary metabolism, antioxidant enzyme activities and proteins involved in protein synthesis, folding and stabilization (Bravo et al., 2003; Campos-Bermudez et al., 2013; Christensen et al., 2013, 2014; Gao et al., 2007; Guo et al., 1997; Lanubile et al., 2014; Murillo et al., 1999; Zila et al., 2013, 2014). Host resistance was associated to a relatively high constitutive gene expression of defense-related genes in the resistant (CO441) kernels, whereas these genes were induced by pathogen attack in the susceptible (CO354) genotype starting from 48 h post inoculation (hpi) (Lanubile et al., 2010, 2012a,b, 2014). Moreover the resistant (CO433) maize genotype showed an earlier and enhanced expression of genes of the LOX pathway in comparison to the susceptible (CO354) line after *F. verticillioides* inoculation (Maschietto et al., 2015). Although hundreds of genes were detected in the studies reported above, the defense bases are still unclear, probably due to the complex nature of the pathosystem.

In addition to the previous findings reported above, this work extended the evaluation of the molecular and biochemical responses against *F. verticillioides* infection to other two maize genotypes, showing elevated levels of resistance (CO433) and susceptibility (CO389) to the pathogen. The study assessed the expression profile of selected defense-related genes (*PR* genes and genes involved in protection from oxidative stress) and the activity of the ASC-GSH cycle enzymes and the cell oxidative status with the purpose of correlating molecular and biochemical data. This study validated the results previously observed, confirming the hypothesis that the resistant genotypes have constitutive high levels of biochemical barriers before inoculation, providing a basal defense system to the pathogen. The candidate genes and biomarkers validated in this study could be applied to speed up conventional breeding programs for *Fusarium* resistance.

2. Materials and methods

2.1. Plant material and F. verticillioides inoculation assay

Four maize genotypes with contrasting phenotypes for resistance to *Fusarium* ear rot were used in this study: the resistant lines CO441 and CO433 and the susceptible lines CO354 and CO389, as previously reported (Lanubile et al., 2010; Maschietto et al., 2015; Reid et al., 2009). All lines were developed by the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada (Ottawa, Canada) and were maintained by sibling at the Department of Sustainable Crop Production in Piacenza, Italy. Seeds from each line were planted in pots (40 cm diameter, 35 cm height) and 10 plants were grown up. Before inoculation, pots were transferred to an environmentally controlled greenhouse with day-time and night-time conditions of 28 °C and 20 °C temperatures, respectively, and a light regime of 16 h using lamps, as described in Lanubile et al., 2015. *F. verticillioides* inoculation was performed using the isolate ITEM 1744 (Institute of Sciences of Food Production, National Research Council, Bari, Italy), a high fumonisin producer strain, cultured as previously described by Lanubile et al. (2010, 2012a). Maize ears were inoculated at 15 days after hand-pollination (DAP) using a side-needle inoculator, as reported by Lanubile et al. (2015). For the detection of *F. verticillioides*, real-time RT-PCR expression analysis, and enzymatic assays, seeds adjacent to the inoculated kernels were collected at 72 hpi, in the area around the point of inoculation, to evaluate fungal growth and colonization and to avoid mechanical damage due to needle-prick (Lanubile et al., 2013, 2014). Control seeds were sampled at the same inoculation time listed above and considered as uninoculated. Three pools of kernels for the 72 hpi time-point were prepared, where each pool derived from the mixing of kernels coming from three different maize ears.

2.2. RNA isolation and real-time RT-PCR expression analysis

Maize kernels of CO441, CO433, CO354 and CO389 genotypes were ground in liquid nitrogen with mortar and pestle and total RNA was extracted from 2.5 g of seeds using the TRIzol protocol (Invitrogen, Carlsbad, CA, USA) and purified with the RNA Clean-up protocol (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The amount and the quality of the total RNA were estimated by fluorometric assay (Qubit, Invitrogen) as well as by agarose gel electrophoresis.

Real-time RT-PCR experiments were performed on kernels collected at 72 hpi using the 2 x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the CFX-96 device (Bio-Rad). cDNA synthesis and relative quantitative analysis by real-time RT-PCR followed the previous method described by Lanubile et al. (2015). Briefly, 20 ng of single strand cDNA were used for real-time RT-PCR at the following conditions: 95 °C for 3 min and 40 cycles at 95 °C 10 s, 60 °C for 25 s (Lanubile et al., 2015). A melting curve analysis was performed and three technical replicates of each biological replicate were employed (Lanubile et al., 2015). The gene-specific primers for *PR1*, *PR5*, *PRm3*, *PRm6*, *POD*, *CAT*, *SOD* and *APX* are reported in Supplementary Table S1. Primers were designed possibly within consecutive exons, separated by an intron, using Primer3 software. Relative quantification of maize genes was normalized to the housekeeping gene *β-actin* (Supplementary Table S1) and FC val-

ues in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). To quantify the fungal growth, the copy number of *calmodulin* transcript was detected using real-time RT-PCR in inoculated kernels collected at 72 hpi. The primer pairs were designed within a conserved region positioned between nucleotides 13 and 162 of the *calmodulin* sequence of *F. verticillioides* (NCBI GenBank Accession No. HQ412321.1). The real-time RT-PCR thermal cycling conditions were the same as reported above. The *calmodulin* number of copies is related to ng of cDNA obtained from kernel tissues and determined based on the equation of the linear regression according to the technical manual (Bio-Rad). Fungal cDNA (20 ng) was serially diluted [1:1, 1:5, $1:5^2$, $1:5^3$, $1:5^4$, $1:5^5$] in sterile water and 20 ng of each kernel cDNA sample was compared to the dilution standard curve to determine fungal cDNA copy number.

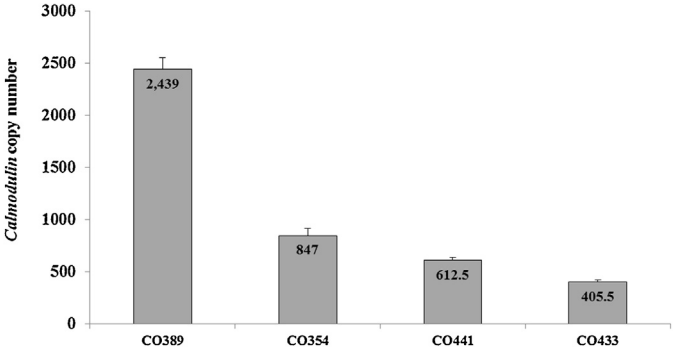

Fig. 1. Copy number of the *calmodulin* transcripts in kernels of susceptible (CO389 and CO354) and resistant (CO441 and CO433) maize lines at 72 h after *F. verticillioides* inoculation. Vertical bars indicate ± standard deviation.

2.3. Determination of enzymatic activities

Control or inoculated maize kernels of CO441, CO433, CO354 and CO389 genotypes were ground with a pestle and mortar at 4 °C in 50 mM Tris-HCl pH 7.8 containing 0.3 mM mannitol, 1 mM EDTA, and 0.05% (w/v) cysteine (buffer A) in a 1:3 ratio (w/v). The homogenate was centrifuged at 1000*g* for 5 min. The supernatant was re-centrifuged for 20 min at 25,000*g*. The resulting supernatant, assayed as cytosolic fraction, was desalted by dialysis against 50 mM Tris-HCl, pH 7.8 and used for spectrophotometric analysis. For wall POD analysis, the pellet resulting from the first centrifugation step was resuspended in buffer A plus 1% Triton X100 and centrifuged at 1000*g* for 5 min. The pellet was washed three times in buffer A, centrifuged as described above and then resuspended and incubated for 20 min in 1 M of NaCl and centrifuged for 20 min at 20,000*g*. The supernatant obtained (cell wall preparation) did not show glucose-6-phosphate dehydrogenase activity, as assayed according to Löhr and Waller (1974). The activities of all enzymes analyzed i.e. cytosolic APX (EC 1.11.1.11), CAT (EC 1.11.1.6), cytosolic (c) and wall (w) PODs (EC 1.11.1.7), SOD (EC 1.15.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.6.4.2) were tested according to Paciolla et al. (2008) and Mastropasqua et al. (2012). For APX, 1 U = 1 nmol of ascorbate oxidized min^{-1}; for CAT, 1 U = 1 nmol of hydrogen peroxide (H_2O_2) dismutated min^{-1}; for SOD, 1 U = the activity of enzyme required to inhibit the reduction rate of nitro blue tetrazolium (NBT) by 50% at 25 °C; for MDHAR, 1U = 1 nmol of NADH oxidized min^{-1}; for DHAR, 1 U = 1 nmol of dehydroascorbate (DHA) reduced min^{-1}; for GR, 1 U = 1 nmol of NADPH oxidized min^{-1}; for cytosolic POD, 1U = 1 nmol of 4-methoxy nafthol (MN) oxidized min^{-1}; for PODw, 1 U = 1 nmol of coniferyl alcohol oxidized min^{-1}. The protein content was determined according to Bradford (1976), using bovine serum albumin as a standard. Four independent experiments were conducted with at least five replications.

2.4. Determination of ASC and GSH pool content

Control and inoculated kernels were homogenized with two volumes of cold 5% (w/v) metaphosphoric acid in a porcelain mortar. The homogenate was centrifuged for 15 min at 20,000*g* and the supernatant was collected for analysis of ASC, DHA, GSH and GSSH according to Zhang and Kirkham (1996). Four independent experiments were conducted with at least five replications.

2.5. H_2O_2 and lipid peroxidation measurements

The H_2O_2 level in the kernels was evaluated according to Lee and Lee (2000). For lipid peroxidation, the kernels were ground with four volumes of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10,000*g* for 10 min. One milliliter of the supernatant was diluted with 1 mL of 20% trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The level of lipid peroxidation was measured in terms of the malondialdehyde (MDA) content determined by the thiobarbituric acid reaction as described by Zhang and Kirkham (1996). Four independent experiments were conducted with at least five replications.

2.6. Statistical analyses

The reported values for enzymatic activities and non-enzymatic assays are the average of at least five replications from four independent experiments. Two-factor ANOVA was performed on the observed means of the enzyme activity/compound content, considering genotypes and treatments (control and inoculated samples) as fixed factors to test the significance ($P < 0.05$) of genotypes, treatments and their interactions. One-factor ANOVA, followed by Tukey's HSD test ($P < 0.05$), was performed on the observed means of the enzyme activity/compound content within each treatment to set significant differences among genotypes. Differences among control and inoculated samples within the same genotype were performed using the Student's test and were considered to be significant at $P < 0.05$.

3. Results

3.1. Quantification of fungal growth in maize genotypes with contrasting resistance to F. verticillioides

Absolute quantification of the fungal *calmodulin* transcripts by real-time RT-PCR was carried out in this study, in order to evaluate the growth of *F. verticillioides* at 72 hpi in maize kernels of two resistant and two susceptible genotypes. We observed that in the inoculated kernels of the resistant genotypes (CO433 and CO441) the total *calmodulin* copy number was about three times lower compared to the susceptible ones (CO389 and CO354) (1018 vs. 3286, respectively), as indicated in Fig. 1. The CO433 appeared to be the most resistant genotype with a *calmodulin* copy number of 405, whereas the fungus grew more rapidly in CO389 kernels, where six times higher levels of transcript copy number (2439) were measured, indicating the more elevated degree of susceptibility of this genotype.

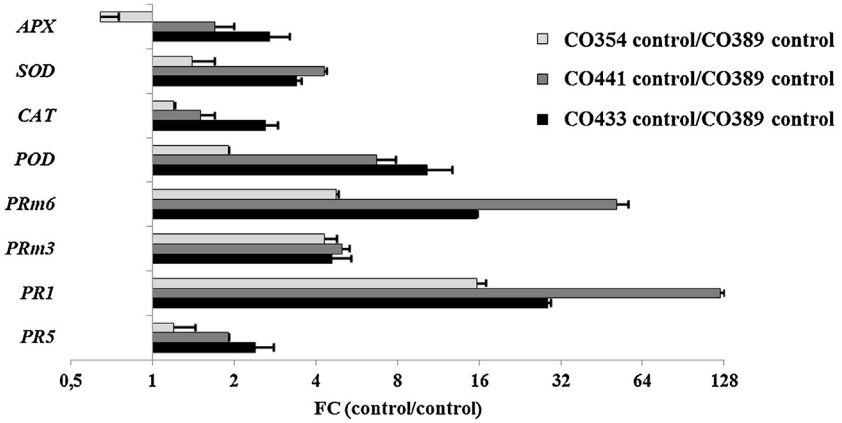

Fig. 2. Fold change (FC) of differentially expressed genes *PR5*, *PR1*, *PRm3*, *PRm6*, *Peroxidase* (*POD*), *Catalase* (*CAT*), *Superoxide dismutase* (*SOD*), *Ascorbate peroxidase* (*APX*), comparing uninoculated control kernels of CO354, CO441 and CO433 genotypes with uninoculated control kernels of the susceptible CO389 genotype. Vertical bars indicate ± standard deviation.

3.2. Differential expression of PR and oxidative stress-related genes among genotypes before and after F. verticillioides inoculation

Expression profiles of four *PR* genes (*PR5*, *PR1*, *PRm3* and *PRm6*) and four genes involved in response to the oxidative stress (*POD*, *CAT*, *SOD* and *APX*) were analyzed by real-time RT-PCR. In order to explore constitutive differences among the resistant and susceptible genotypes, fold changes (FCs) were measured by comparing uninoculated control CO433, CO441 and CO354 samples with the uninoculated control plants of the CO389 genotype (Fig. 2). Interestingly, we observed enhanced expression values for all tested genes in the two resistant genotypes (CO433 and CO441) before fungal inoculation. The resistant lines clearly showed higher FCs for *PR1*, *PRm6* and *POD* transcripts compared to CO389 control samples, with values ranging from 124 to 6.7, and from 28.6 to 10.3 for the CO441 and CO433 genotypes, respectively. Constitutive higher expression values were also measured for the remaining genes in control samples of the resistant genotypes, although the levels of transcripts were lower. Concerning the comparison between the two susceptible genotypes (CO354/CO389), less marked differences were detected and significant FCs were observed only for *PRm6, PRm3* and *PR1* genes (Fig. 2). These findings suggest that the resistance to *F. verticillioides* infection in the resistant genotypes could be potentially related to the highest constitutive expression of defense/stress-related genes, not found for the susceptible genotypes.

Gene expression changes induced after *F. verticillioides* inoculation were also investigated at 72 hpi in the four maize genotypes and shown in Fig. 3 as FCs in relation to uninoculated plants. The up-regulation of all assayed genes occurred in the resistant (CO433 and CO441) and susceptible (CO354 and CO389) genotypes, excluding *SOD* which was significantly down-regulated in CO433 (FC = 0.31). Among the *PR* genes, the most highly induced was *PR1* with an induction up to 1789-fold in the susceptible CO389 genotype. A similar trend was detected for *PRm6* gene, showing a strong increase up to 1,409.5-fold in the same genotype. Genes involved in the oxidative-stress reactions were induced with lower intensity and only *CAT* and *POD* were significantly up-regulated in all genotypes. Among them, *POD* gene reached the highest levels of induction, with values ranging from 1.9 (CO433, resistant) to 31.5 (CO389, susceptible), whereas *CAT* FC values ranged from 2.2 (CO433) to 5.5 (CO389).

The comparison of the induction levels among genotypes highlighted that, remarkably, the maximal levels of expression were reached by the susceptible CO354 and CO389 lines (Fig. 3). The strongest induction was observed in CO389 kernels for all tested genes, followed by CO354, while in contrast *F. verticillioides* inoculation elicited weaker alterations in the resistant CO441 and CO433 genotypes. The greatest differences were measured for *PR1* gene, whose expression increased of about 24 times in CO389 compared to the resistant CO433 genotype. *PRm3*, *POD* and *PRm6* genes were similarly up-regulated, showing transcript levels about 20, 17 and 13 times more elevated, respectively, comparing the same two genotypes.

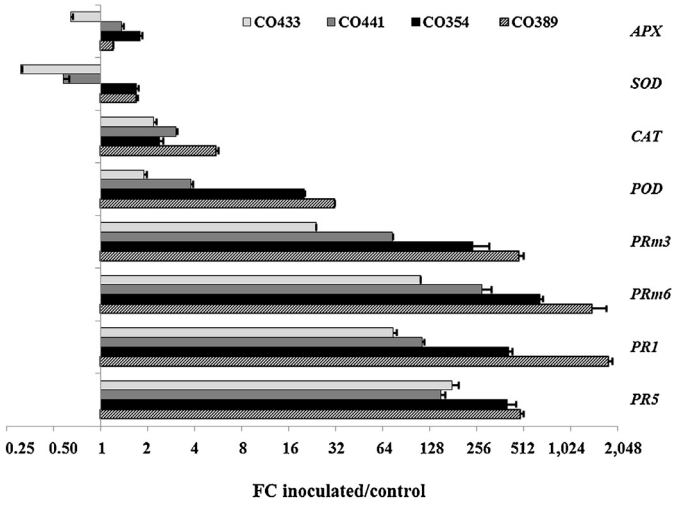

Fig. 3. Fold change (FC) of differentially expressed genes *PR5*, *PR1*, *PRm3*, *PRm6*, *Peroxidase* (*POD*), *Catalase* (*CAT*), *Superoxide dismutase* (*SOD*), *Ascorbate peroxidase* (*APX*), in kernels of susceptible (CO389 and CO354) and resistant (CO441 and CO433) maize lines at 72 h after *F. verticillioides* inoculation. Vertical bars indicate ± standard deviation.

In general, pathogen infection caused lower values of FC in the resistant genotypes (CO441 and CO433) at 72 hpi compared to the susceptible ones (Fig. 3), probably due to the highest constitutive levels of gene expression observed before inoculation (Fig. 2).

3.3. Enzyme activities and products related to oxidative stress in control and inoculated kernels

The enzymatic and not-enzymatic components of ascorbate-glutathione cycle, including ASC-GSH contents, APX, MDHAR, DHAR, GR, and other antioxidant systems (SOD, CAT, PODc and PODw) involved in the ROS detoxification and oxidative burst, were

analysed in the kernels of the four maize genotypes, comparing uninoculated and inoculated samples at 72 hpi with *F. verticillioides*.

Two-factor analysis of variance (ANOVA) revealed significant ($P < 0.001$) differences between the four genotypes in the observed means for all tested enzyme activities and compounds, with SOD as the only exception (Supplementary Table S2). Moreover, *F. verticillioides* inoculation led to a significant ($P < 0.001$) change in the means for all compounds and enzyme activities and the interaction between genotype and treatment was significant ($P < 0.001$) in all cases, excluding the MDA and oxidized glutathione (GSSG) contents (Supplementary Table S2).

The trend of the components of ASC-GSH cycle is shown in Fig. 4. According to one-factor ANOVA ($P < 0.05$), no significant differences in the analysed components were observed between the susceptible maize lines (CO389 and CO354) before inoculation, except for GSH and GR that were significantly higher in CO354 (Fig. 4C–D). In contrast, significant constitutive difference was found among the resistant lines (CO441 and CO433) in all the studied parameters. Particularly, CO441 showed significantly higher MDHAR and DHAR enzymatic activities and GSH content, while CO433 higher ASC and GSSG contents and GR enzymatic activity (Fig. 4A–F). Interestingly, all components of the ascorbate-glutathione cycle, except for the GSSG content, were significantly enhanced in the control of the resistant lines in comparison to the susceptible ones. After inoculation, the MDHAR activity significantly decreased (Student's *t*-test, $P < 0.05$) in both susceptible and resistant lines, with the highest decrement in the latter (Fig. 4A). A different trend was shown by DHAR, which reconverts DHA to ASC, similarly to MDHAR: a significant increase and decrease occurred in susceptible and resistant lines, respectively (Fig. 4B). GR, the enzyme of conversion of GSSG into GSH, significantly increased post inoculation in the resistant genotypes, while no significant change was observed in the susceptible lines (Fig. 4C). The contents of ASC (Fig. 4F) and GSH were significantly higher after inoculation in all lines, although with greater induction for the susceptible ones and particularly for CO389 (Fig. 4D). The content of GSSG, the oxidized form of GSH, did not show changes after inoculation in the susceptible lines, while a significant increase in both resistant genotypes was observed as compared to the respective controls (Fig. 4E). In general, the enzymes MDHAR, DHAR and GR showed higher constitutive activity in the resistant lines in comparison to the susceptible controls, corresponding also to the highest constitutive contents of GSH and ASC. After *F. verticillioides* inoculation a significant decrement of MDHAR and DHAR enzymatic activity was observed in all four lines, whilst a significant increment of GR activity was detected only for the resistant genotypes.

Dehydroascorbate (DHA) content and activity of APX were very low or undetectable in our experimental design (data not shown). CAT, PODc and PODw and SOD are also enzymes involved in the control of H_2O_2 level in plant cell. In uninoculated kernels, the specific activity of these enzymes was significantly (one-factor ANOVA, $P < 0.05$) higher in the CO441 and CO433 resistant lines than the susceptible CO389 and CO354 (Fig. 4G–I). Before fungal inoculation, similar activity was observed between the susceptible genotypes for CAT, PODc and PODw (Fig. 4G–I), while different activity was detected between the two resistant lines for CAT and PODw. The highest CAT and PODw activity was revealed in CO441 and CO433 genotypes, respectively. After inoculation with *F. verticillioides*, all three enzyme activities increased significantly in comparison to controls and reached greater values in the resistant lines (Fig. 4G–I). Particularly, the increase of PODw was higher for the resistant lines in CO433 and for the susceptible lines in CO354 (Fig. 4I). For SOD enzyme activity, except in CO441 where no change was observed, the inoculation with pathogen caused a significant reduction of its activity in the other lines with the highest decrease in CO389 genotype as compared to the respective controls (Fig. 4J). On the other hand, in uninoculated kernels similar constitutive levels for SOD activity were observed in CO354, CO441 and CO433, while the highest level of this enzyme was measured in CO389 line.

The basal level of H_2O_2 (Fig. 5A) and MDA (Fig. 5B) contents was significantly higher in the resistant lines than the susceptible ones (one-factor ANOVA, $P < 0.05$). The pathogen inoculation caused significant (Student's *t*-test, $P < 0.05$) enhanced levels of H_2O_2 and lipid peroxidation in the susceptible genotypes in respect to controls, whilst no significant changes occurred in the resistant genotypes (Fig. 5A-B).

4. Discussion

In this study we reported the key role of the constitutive host resistance in the outcome of *F. verticillioides*-maize interaction. The analyzed resistant CO433 and CO441 lines showed higher expressions of genes related to oxidative stress protection in comparison to the susceptible CO389 line in the uninoculated controls. Even higher differences with the CO389 expression levels were found in the resistant controls for the *PR* genes (FC up to 124 times for *PR1* in CO441). Regarding the changes occurring in the kernels after inoculation, surprisingly, gene expression was higher in the susceptible lines compared to the resistant ones for all tested genes. However, smaller differences in the induction of genes of the antioxidant cycle were detected between resistant and susceptible lines, and *APX* and *SOD* were even not affected by inoculation in the resistant lines. An earlier activation of these genes or the exploitation of the constitutively present proteins can explain the lack of induction in the resistant lines after *F. verticillioides* inoculation. *PR* genes were strongly induced by pathogen inoculation in all genotypes and the highest FCs (inoculated/control) were associated with these genes. In particular, induction was greater in the CO389 line, which was highly colonized by fungal growth. It is possible that in the susceptible lines, especially in CO389, *PR* transcripts were delayed in comparison to resistant genotypes.

In this investigation, the highest constitutive levels of *PR* transcripts were observed for *PR1* and *PRm6* in the resistant genotypes. Maize *PRms* genes were found to be expressed in seed tissues before *F. verticillioides* mycelium was visible, confirming that tissues display defense mechanisms before colonization (Shu et al., 2014). After infection by *F. verticillioides* and other fungal pathogens, PR1 proteins accumulate in maize seedlings primarily in those cell types that are in contact with the pathogen and, as a second barrier, in papillae at inner parts of the infected tissue (Murillo et al., 1999). The role of PR proteins in several plant-pathogen interactions was also demonstrated by overexpressing *PR1*, *PR5* and *PRm3* genes to enhance resistance to *F. graminearum* (Chen et al., 1999; Makandar et al., 2006). Moreover, constitutively overexpression of maize *PRms* showed a broad-spectrum resistance to infection by bacterial and fungal pathogens, including *F. verticillioides*, displaying a basal expression level of endogenous defense genes and stronger and quicker defense responses during pathogen infection (Gómez-Ariza et al., 2007).

Regarding the enzymatic activity and concentration of the components related to the oxidative stress, significant differences were detected among the genotypes, with SOD as the only exception. In general, the level of transcripts related to oxidative stress fit with their enzymatic activities: higher enzymatic activities were found in the control and inoculated kernels of the resistant lines, confirming that the late activation of defense responses in the susceptible lines resulted in a delay in protein synthesis.

Enzymatic activity and concentration of most of the ASC-GSH pathway components were significantly affected by *F. verticillioides* inoculation, in comparison to the respective controls. ASC-GSH cycle is involved in stress tolerance in plant since key part of the

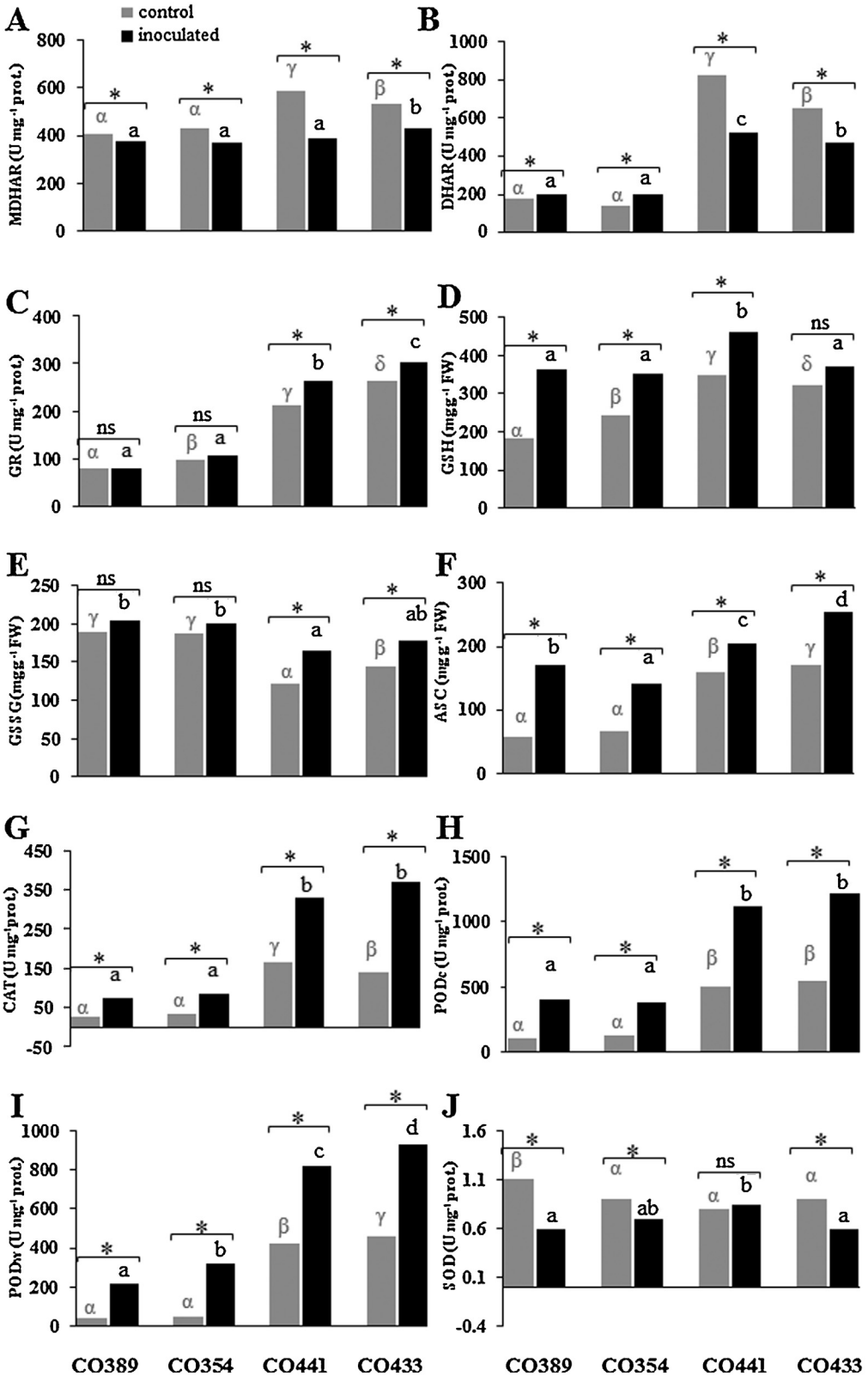

Fig. 4. Monodehydroascorbate reductase (MDHAR, A), dehydroascorbate reductase (DHAR, B), and glutathione reductase (GR, C) activities, reduced glutathione (GSH, D), oxidized glutathione (GSSG, E) and ascorbate (ASC, F) contents, catalase (CAT, G), cytosolic peroxidase (PODc, H), cell wall peroxidase (PODw, I) and superoxide dismutase (SOD, J) activities in kernels of susceptible (CO389 and CO354) and resistant (CO441 and CO433) maize lines before (control) and 72 h after *F. verticillioides* inoculation. Values represent the mean of at least five replications from four independent experiments. Values represent the mean of at least five replications from four independent experiments. Same letters over the histograms state no significant differences between means of control genotypes (greek letters) and inoculated genotypes (latin letters), as resulting from Tukey's HSD test ($P < 0.05$).* indicate significant differences among control and inoculated means within the same genotype, according to Student's test ($P < 0.05$).

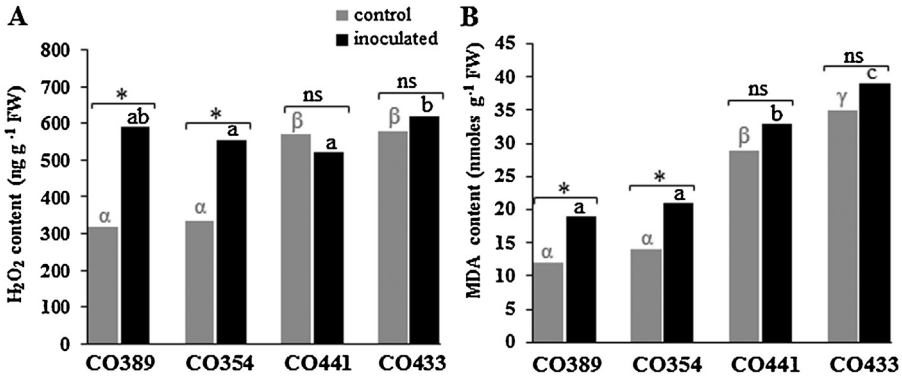

Fig. 5. Hydrogen peroxide (H_2O_2, A) and malondialdehyde (MDA, B) contents in kernels of susceptible (CO389 and CO354) and resistant (CO441 and CO433) maize lines before (control) and 72 h after *F. verticillioides* inoculation. Values represent the mean of at least five replications from four independent experiments. Same letters over the histograms state no significant differences between means of control genotypes (greek letters) and inoculated genotypes (latin letters), as resulting from Tukey's HSD test ($P < 0.05$).* indicate significant differences among control and inoculated means within the same genotype, according to Student's test ($P < 0.05$).

network of pathways for ROS scavenging in plants (Anjum et al., 2010; Mittler 2002). The enzymes MDHAR, DHAR and GR maintain the cellular pool of antioxidant ASC and GSH in their reduced state (Mittler et al., 2004), and, interestingly, they showed higher constitutive activity in the resistant lines in comparison to the susceptible controls, corresponding also to the highest constitutive contents of GSH and ASC (reduced forms). Moreover, *F. verticillioides* inoculation led to a significant decrement of MDHAR and DHAR enzymatic activity in all four lines, whilst a significant increment of GR activity was detected only for the resistant genotypes. This enzymatic pattern highlighted that the main maize reconversion pathway related to *F. verticillioides* detoxification did not involve the ascorbate, but the glutathione. The latter, in reduced form, has an essential role in many crucial biochemical reactions, as preventing the compound oxidation and the perturbations in any of step of thiol metabolism during penetration of fungi and possible diffusion of their mycotoxins. After pathogen inoculation, a different scenario is depicted in the susceptible lines where to the GSH content increment did not correspond a decrease of GSSG and an increase in GR activity, suggesting a *de novo* GSH synthesis due to a possible imbalance of the redox state in the cytosol (Foyer and Noctor 2005).

SOD, APX, CAT and POD are key enzymes to control the level of ROS, such as H_2O_2 and superoxide anion, *in planta*. Interestingly, SOD is the only component of the ROS-scavenging systems that did not show a significant difference in constitutive activity among controls of resistant (CO441 and CO433) and susceptible (CO354) lines, whilst significant higher levels were associated to CO389. Moreover at 72 hpi SOD activity significantly decreased in all genotypes (excluding CO441), and, in contrast, H_2O_2 contents significantly increased only in the susceptible genotypes, causing an oxidative burst in these genotypes. Therefore, other processes such as photorespiration, might contribute to the H_2O_2 production (Noctor et al., 2002). The resistant CO441 line showed a similar trend of SOD activity in seedlings inoculated with *F. verticillioides* (Lanubile et al., 2012b) and kernels inoculated with *F. proliferatum, F. subglutinans* and *Aspergillus flavus* (Lanubile et al., 2015), as well as in sheath tissue of wheat infected by *F. proliferatum* (Kwon and Anderson 2001), suggesting that SOD activation occurs at earlier stages of pathogen infection prior to 72 hpi.

The resistant genotypes showed a constitutive higher level of CAT, PODc and PODw, which significantly increased after inoculation, contributing to the efficient H_2O_2 scavenging. The high constitutive and induced PODw activity could be important in the resistant genotypes for the regulation of cell wall cross-links, providing higher stiffening to wall and acting as physical barrier against fungal pathogens (Paciolla et al., 2008).

In this sense, the oxidative burst detected in the susceptible genotypes may be due to a delayed activation of the CAT and POD enzyme activities, both induced by inoculation and constitutively less active in the controls. The oxidative burst, observed in the susceptible lines, could favor necrotrophic phase of *F. verticillioides* infection. Babaeizad et al. (2009) showed that overexpression of a gene encoding a cell death suppressor, BAX inhibitor-1, retarded *F. graminearum* (hemibiotroph) colonization of barley seedlings. Moreover, a higher catalase activity was associated to the resistance of maize roots to *F. verticillioides*-colonization (Kumar et al., 2009).

According to our study, the constitutive higher antioxidant content appeared to be crucial in maize kernels in preparation of pathogen attack. As a confirmation of our findings, enzyme activities involved in protection of plant tissues against oxidative damage and ROS detoxification, as represented by CAT, SOD and glutathione transferase were not constitutive and only induced upon *F. verticillioides* inoculation in susceptible maize germinating embryos (Campo et al., 2004). Additionally, resistant maize rachis to *A. flavus* inoculation showed higher levels of proteins involved in oxidative stress protection in comparison to a susceptible line already in the controls (Pechanova et al., 2011). Constitutive or wound–inducible defenses were more likely the driving force for the greater kernel resistance of CO441 in comparison to a susceptible line, since a reinforcement of PR proteins, chitinases, peroxidases, xylanase and proteinase inhibitors, already present in the mock-treated kernels, was observed at 48 hpi with *F. graminearum* (Mohammadi et al., 2011).

This study demonstrated that the seed constitutive defenses could be profitably exploited to develop useful markers for breeding. Resistant genotypes to *F. verticillioides* could be selected based on the expression levels of genes related to protection from the oxidative stress and *PR* genes. Therefore, the employment of markers developed from candidate genes linked to the resistance could be used for speeding marker assisted selection of resistant maize genotypes. The inbred lines chosen in this study showed analogous levels of resistance to *F. graminearum* and *F. verticillioides* inoculation (Reid et al., 2009). Moreover, the candidate genes tested in this study discriminated efficiently the susceptibility and resistance responses of the CO354 and CO441 lines, respectively, after inoculation with *F. proliferatum, F. subglutinans* and *Aspergillus flavus* (Lanubile et al., 2015). Further investigation of maize overexpressing *PR1* and *PRm6*, the best *PR* genes candidate for resistance in our study, could confirm their roles in *F. verticillioides* and broad-spectrum resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2016.06.006.

References

Almagro, L., Gómez Ros, L.V., Belchi-Navarro, S., Bru, R., Ros Barceló, A., Pedreño, M.A., 2009. Class III peroxidases in plant defense reactions. J. Exp. Bot. 60, 377–390.

Anjum, N.A., Umar, S., Chan, M.T., 2010. Ascorbate-Glutathione Pathway and Stress Tolerance in Plants. Springer Dordrecht, The Netherlands, pp. 1–429.

Babaeizad, V., Imani, J., Kogel, K.H., Eichmann, R., Hückelhoven, R., 2009. Overexpression of the cell death regulator BAX inhibitor-1 in barley confers reduced or enhanced susceptibility to distinct fungal pathogens. Theor. Appl. Genet. 1183, 455–463.

Bacon, C.W., Glenn, E., Yates, I.E., 2008. Fusarium verticillioides: managing the endophytic association with maize for reduced fumonisins accumulation. Toxin Rev. 27, 411–446.

Battilani, P., Pietri, A., Barbano, C., Scandolara, A., Bertuzzi, T., Marocco, A., 2008. Logistic regression modeling of cropping systems to predict fumonisin contamination in maize. J. Agric. Food Chem. 56, 10433–10438.

Baxter, A., Mittler, R., Suzuki, N., 2014. ROS as key players in plant stress signalling. J. Exp. Bot. 65, 1229–1240.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bravo, J.M., Campo, S., Murillo, I., Coca, M., San Segundo, B., 2003. Fungus- and wound-induced accumulation of mRNA containing a class II chitinase of the pathogenesis-related protein 4 (PR-4) family of maize. Plant Mol. Biol. 52, 745–759.

Bush, B.J., Carson, M.L., Cubeta, M., Hagler, W.M., Payne, G., 2004. Infection and fumonisin production by *Fusarium verticillioides* in developing maize kernels. Phytopathology 94, 88–93.

Campo, S., Carrascar, M., Coca, M., Abian, J., San Segundo, B., 2004. The defense response of germinating maize embryos against fungal infection: a proteomics approach. Proteomics 4, 383–396.

Campos-Bermudez, V., Fauguel, C.M., Tronconi, M., Casati, P., Presello, D., Andreo, C.S., 2013. Transcriptional and metabolic changes associated to the infection by *Fusarium verticillioides* in maize inbreds with contrasting ear rot resistance. PLoS One 8, e61580.

Chen, W.P., Chen, P.D., Liu, D.J., Kynast, R., Friebe, B., Velazhahan, R., Muthukrishnan, S., Gill, B.S., 1999. Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene. Theor. Appl. Genet. 99, 755–760.

Christensen, S.A., Nemchenko, A., Borrego, E., Murray, I., Sobhy, I.S., Bosak, L., Deblasio, S., Erb, M., Robert, C.A., Vaughn, K.A., Herrfurth, C., Tumlinson, J., Feussner, I., Jackson, D., Turlings, T.C., Engelberth, J., Nansen, C., Meeley, R., Kolomiets, M.V., 2013. The maize lipoxygenase, ZmLOX10, mediates green leaf volatile, jasmonate and herbivore-induced plant volatile production for defense against insect attack. Plant J. 74, 59–73.

Christensen, S.A., Nemchenko, A., Park, Y., Borrego, E., Huang, P., Schmelz, E.A., Kunze, S., Feussner, I., Yalpani, N., Meeley, R., Kolomiets, M.V., 2014. The novel monocot-specific 9-lipoxygenase ZmLOX12 is required to mount an effective jasmonate-mediated defense against *Fusarium verticillioides* in maize. Mol. Plant Microbe Interact. 27, 1263–1276.

Clements, M.J., Maragos, C.A., Pataky, J.K., White, D.G., 2004. Sources of resistance to fumonisin accumulation in grain and Fusarium ear and kernel rot of corn. Phytopathology 94, 251–260.

Ding, J.Q., Wang, X.M., Chander, S., Yan, J.E., Li, J.S., 2008. QTL mapping of resistance to Fusarium ear rot using a RIL population in maize. Mol. Breed. 22, 395–403.

Eller, M., Holland, J., Payne, G., 2008. Breeding for improved resistance to fumonisin contamination in maize. Toxin Rev. 27, 371–389.

Foyer, C.H., Noctor, A., 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28, 1056–1071.

Gómez-Ariza, J., Campo, S., Rufat, M., Messeguer, J., Segundo, B.S., Coca, M., 2007. Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. Mol. Plant Microbe Interact. 20, 832–842.

Gao, X., Shim, W.B., Göbel, C., Kunze, S., Feussner, I., Meeley, R., Balint-Kurti, P., Kolomiets, M., 2007. Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. Mol. Plant Microbe Interact. 20, 922–933.

Guo, B.Z., Chen, Z.Y., Brown, R.L., Lax, A.R., Cleveland, T.E., Russin, J.S., Mehta, A.D., Selitrennikoff, C.P., Widstrom, N.W., 1997. Germination induces accumulation of specific proteins and antifungal activities in corn kernels. Phytopathology 87, 1174–1178.

Henry, W.B., Williams, W.P., Windham, G.L., Hawkins, L.K., 2009. Evaluation of maize inbred lines for resistance to *Aspergillus* and *Fusarium* ear rot and mycotoxin accumulation. Agron J. 101, 1219–1226.

Kleinschmidt, C.E., Clements, M.J., Maragos, C.M., Pataky, J.K., White, D.G., 2005. Evaluation of food-grade dent corn hybrids for severity of Fusarium ear rot and fumonisin accumulation in grain. Plant Dis. 89, 291–297.

Kumar, M., Yadav, V., Tuteja, N., Kumar, A., 2009. Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. Microbiology 155, 780–790.

Kwon, S.I., Anderson, A.J., 2001. Differential production of superoxide dismutase and catalase isozymes during infection of wheat by a *Fusarium proliferatum*-like fungal isolate. Physiol. Mol. Plant Pathol. 58, 73–81.

Löffler, M., Kessel, B., Ouzunova, M., Miedaner, T., 2010. Population parameters for resistance to *Fusarium graminearum* and *Fusarium verticillioides* ear rot among large sets of early, mid-late and late maturing European maize (*Zea mays* L.) inbred lines. Theor. Appl. Genet. 120, 1053–1062.

Löhr, G.W., Waller, H.D., 1974. Glucose-6-phospate dehydrogenase. In: Methods of Enzymatic Analysis. Academic Press, New York, pp. 636–643.

Lanubile, A., Pasini, L., Marocco, A., 2010. Differential gene expression in kernels and silks of maize lines with contrasting levels of ear rot resistance after *Fusarium verticillioides* infection. J. Plant Physiol. 167, 1398–1406.

Lanubile, A., Pasini, L., Lo Pinto, M., Battilani, P., Prandini, A., Marocco, A., 2011. Evaluation of broad spectrum sources of resistance to *Fusarium verticillioides* and advanced maize breeding lines. World Mycotoxin J. 1, 43–51.

Lanubile, A., Bernardi, J., Battilani, P., Logrieco, A., Marocco, A., 2012a. Resistant and susceptible maize genotypes activate different transcriptional responses against *Fusarium verticillioides*. Physiol. Mol. Plant Pathol. 77, 52–59.

Lanubile, A., Bernardi, J., Marocco, A., Logrieco, A., Paciolla, C., 2012b. Differential activation of defense genes and enzymes in maize genotypes with contrasting levels of resistance to *Fusarium verticillioides*. Environ. Exp. Bot. 78, 39–46.

Lanubile, A., Logrieco, A., Battilani, P., Proctor, R.H., Marocco, A., 2013. Transcriptional changes in developing maize kernels in response to fumonisin-producing and nonproducing strains of *Fusarium verticillioides*. Plant Sci. 210, 183–192.

Lanubile, A., Ferrarini, A., Maschietto, V., Delledonne, M., Marocco, A., Bellin, D., 2014. Functional genomic analysis of constitutive and inducible defense responses to *Fusarium verticillioides* infection in maize genotypes with contrasting ear rot resistance. BMC Genomics 15, 710.

Lanubile, A., Maschietto, V., De Leonardis, S., Battilani, P., Paciolla, C., Marocco, A., 2015. Defense responses to mycotoxin-producing fungi *Fusarium proliferatum* F. subglutinans, and *Aspergillus flavus* in kernels of susceptible and resistant maize genotypes. Mol. Plant Microbe Interact. 28, 546–557.

Lee, D.H., Lee, C.B., 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. Plant Sci. 159, 75–85.

Locato, V., Cimini, S., De Gara, L., 2013. Strategies to increase vitamin C in plants: from plant defense perspective to food biofortification. Front. Plant Sci. 4, 152.

Logrieco, A., Mulè, G., Moretti, A., Bottalico, A., 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. Eur. J. Plant Pathol. 108, 597–609.

Makandar, R., Essig, J.S., Schapaugh, M., Trick, H.N., Shah, J., 2006. Genetically engineered resistance to *Fusarium* head blight in wheat by expression of *Arabidopsis* NPR1. Mol. Plant Microbe Interact. 19, 123–129.

Maschietto, V., Marocco, A., Malachova, A., Lanubile, A., 2015. Resistance to *Fusarium verticillioides* and fumonisin accumulation inmaize inbred lines involves an earlier and enhanced expression of lipoxygenase (LOX) genes. J. Plant Physiol. 188, 9–18.

Mastropasqua, L., Borraccino, G., Bianco, L., Paciolla, C., 2012. Light qualities and dose influence ascorbate pool size in detached oat leaves. Plant Sci. 183, 57–64.

Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 10, 490–498.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.

Mohammadi, M., Anoop, V., Gleddie, S., Harris, L.J., 2011. Proteomic profiling of two maize inbreds during early *Gibberella* ear rot infection. Proteomics 11, 3675–3684.

Munkvold, G.P., 2003a. Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. Eur. J. Plant Pathol. 109, 705–713.

Munkvold, G.P., 2003b. Cultural and genetic approaches to managing mycotoxins in maize. Annu. Rev. Phytopathol. 41, 99–116.

Murillo, I., Cavallarin, L., San Segundo, B., 1999. Cytology of infection of maize seedlings by *Fusarium moniliforme* and immunolocalization of the pathogenesis-related PRms protein. Phytopathology 89, 737–747.

Noctor, G., Veljovic-jovanovis, S., Driscoll, S., Novitskaya, L., Foyer, C.H., 2002. Drought and oxidative load in the leaves of C3 plants: a predominant role for photorespiration? Ann. Bot. 89, 841–850.

Paciolla, C., Dipierro, N., Mulè, G., Logrieco, A., Dipierro, S., 2004. The mycotoxins beauvericin and T-2 induce cell death and alteration to the ascorbate metabolism in tomato protoplasts. Physiol. Mol. Plant Pathol. 65, 49–56.

Paciolla, C., Ippolito, M.P., Logrieco, A., Dipierro, N., Mulè, G., Dipierro, S., 2008. A different trend of antioxidant defense responses makes tomato plants less

susceptible to beauvericin than to T-2 mycotoxin phytotoxicity. Physiol. Mol. Plant Pathol. 72, 3–9.
Pascale, M., Visconti, A., Chelkowski, J., 2002. Ear rot susceptibility and mycotoxin contamination of maize hybrids inoculated with *Fusarium* species under field conditions. Eur. J. Plant Pathol. 108, 645–651.
Pechanova, O., Pechan, T., Williams, W.P., Luthe, D.S., 2011. Proteomic analysis of the maize rachis: potential roles of constitutive and induced proteins in resistance to *Aspergillus flavus* infection and aflatoxin accumulation. Proteomics 11, 114–127.
Reid, L.M., Zhu, X., Parker, A., Yan, W., 2009. Increased resistance to *Ustilago zeae* and *Fusarium verticilliodes* in maize inbred lines bred for *Fusarium graminearum* resistance. Euphytica 165, 567–578.
Robertson-Hoyt, L.A., Jines, M.P., Balint-Kurti, P.J., Kleinschmidt, C.E., White, D.G., Payne, G.A., Maragos, C.M., Molnar, T.L., Holland, J.B., 2006. QTL mapping for *Fusarium* ear rot and fumonisin contamination resistance in two maize populations. Crop Sci. 46, 1734–1743.
Sagaram, U.S., Kolomiets, M., Shim, W.B., 2006. Regulation of fumonisin biosynthesis in *Fusarium verticillioides*-maize system. Plant Pathol. J. 22, 203–210.
Santiago, R., Cao, A., Malvar, R.A., Reid, L.M., Butrón, A., 2013. Assessment of corn resistance to fumonisin accumulation in a broad collection of inbred lines. Field Crop Res. 149, 193–202.
Santiago, R., Cao, A., Butrón, A., 2015. Genetic factors involved in fumonisin accumulation in maize kernels and their implications in maize agronomic management and breeding. Toxins 7, 3267–3296.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative $C_{(T)}$ method. Nat. Protoc. 3, 1101–1118.
Shu, X., Livingston, D.P., Franks, R.G., Boston, R.S., Woloshuk, C.P., Payne, G., 2014. Tissue-specific gene expression in maize seeds during colonization by *Aspergillus flavus* and *Fusarium verticillioides*. Mol. Plant Pathol. 16, 662–674.
Torres, M.A., 2010. ROS in biotic interactions. Physiol. Plant. 138, 414–429.
Williams, L.D., Glenn, A.E., Zimeri, A.M., Bacon, C.W., Smith, M.A., Riley, R.T., 2007. Fumonisin disruption of ceramide biosynthesis in maize roots and the effects on plant development and *Fusarium verticillioides*-induced seedling disease. J. Agric. Food Chem. 55, 2937–2946.
Wirthmueller, L., Maqbool, A., Banfield, M.J., 2013. On the front line: structural insights into plant-pathogen interactions. Nat. Rev. Microbiol. 11, 761–776.
Woloshuk, C.P., Shim, W.B., 2013. Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. FEMS Microbiol. Rev. 37, 94–109.
Zhang, J., Kirkham, B., 1996. Antioxidant responses to drought in sunflower and sorghum seedlings. New Phytol. 132, 361–373.
Zila, C.T., Samayoa, L.F., Santiago, R., Butron, A., Holland, J.B., 2013. A genome-wide association study reveals genes associated with *Fusarium* ear rot resistance in a maize core diversity panel. G3 Genes Genomes Genetics 3, 2095–2104.
Zila, C.T., Ogut, F., Romay, M.C., Gardner, C., Buckler, E.S., Holland, J.B., 2014. Genome-wide association study of *Fusarium* ear rot disease in the U.S.A. maize inbred line collection. BMC Plant Biol. 14, 372.