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e-Xtra*

Defense Responses to Mycotoxin-Producing Fungi *Fusarium proliferatum, F. subglutinans*, and *Aspergillus flavus* in Kernels of Susceptible and Resistant Maize Genotypes

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Developing kernels of resistant and susceptible maize genotypes were inoculated with *Fusarium proliferatum*, *F. subglutinans*, and *Aspergillus flavus*. Selected defense systems were investigated using real-time reverse transcription-polymerase chain reaction to monitor the expression of pathogenesis-related (PR) genes (*PR1*, *PR5*, *PRm3*, *PRm6*) and genes protective from oxidative stress (*peroxidase*, *catalase*, *superoxide dismutase* and *ascorbate peroxidase*) at 72 h postinoculation. The study was also extended to the analysis of the ascorbate-glutathione cycle and catalase, superoxide dismutase, and cytosolic and wall peroxidases enzymes. Furthermore, the hydrogen peroxide and malondialdehyde contents were studied to evaluate the oxidation level. Higher gene expression and enzymatic activities were observed in uninoculated kernels of resistant line, conferring a major readiness to the pathogen attack. Moreover expression values of PR genes remained higher in the resistant line after inoculation, demonstrating a potentiated response to the pathogen invasions. In contrast, reactive oxygen species–scavenging genes were strongly induced in the susceptible line only after pathogen inoculation, although their enzymatic activity was higher in the resistant line. Our data provide an important basis for further investigation of defense gene functions in developing kernels in order to improve resistance to fungal pathogens. Maize genotypes with overexpressed resistance traits could be profitably utilized in breeding programs focused on resistance to pathogens and grain safety.

Among the fungal pathogens that affect maize worldwide and reduce yield and grain quality, *Aspergillus flavus* and some *Fusarium* species are of concern because they produce mycotoxins, secondary metabolites that seriously threaten animal and human health (IARC 1993). Fungal pathogens are basically classified in three main categories: biotrophs, necrotrophs, and hemibiotrophs (Oliver and Ipcho 2004).

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Aspergillus flavus possesses the characteristics of necrotrophic fungal pathogens (Kelley et al. 2012) and both infects plants in the field and colonizes harvested or stored plant products, producing the hepatotoxic and carcinogenic aflatoxins (IARC 1993). Host response to necrotrophs are complex and poorly known, involving quantitative resistance genes and jasmonic acid and ethylene–dependent signaling pathways (Glazebrook 2005; Hammond-Kosack and Parker 2003). Moreover hypersensitive response (HR), a localized reaction with cell death to limit biotrophic growth, seems to enhance host plant susceptibility and facilitate necrotrophic colonization (Govrin and Levine 2000). Due to the importance of improving plant resistance and reducing aflatoxin contamination, the fungal pathogenicity and the plant-pathogen interactions have been the object of numerous studies (Bhatnagar et al. 2006; Cleveland et al. 2004). Although commercial maize hybrids are generally susceptible to *A. flavus* (Kelley et al. 2012), it was demonstrated that maize lines show different levels of resistance to its infection and aflatoxin accumulation (Williams 2006; Williams and Windham 2006). These traits are controlled by quantitative resistance genes and several resistance-related quantitative trait loci (QTL) have been identified in maize (Brooks et al. 2005; Warburton et al. 2009). Maize resistance to *A. flavus* infection and aflatoxin accumulation is due to the inhibitory action of antifungal proteins, including β-1,3-glucanases, chitinases, trypsin inhibitors, pathogenesis-related (PR)10, ribosome inactivating proteins, zeamatin, and lectin-like protein from kernels, calli, embryo, endosperm, and silk (Baker et al. 2009; Chen et al. 1998, 2007; Kelley et al. 2012; Lozovaya et al. 1998; Neucere 1996; Nielsen et al. 2001; Peethambaran et al. 2010). Also supposed to improve kernel resistance are proteins linked to cell protection by oxidative stress damage, such as aldose reductase, glyoxalase I, small heat-shock proteins (HSPs), peroxiredoxin, cold-regulated protein, anionic peroxidase, and storage proteins (Chen et al. 2002; Guo et al. 1997). Their level or activity was shown to be higher in resistant genotypes, either before or after infection with *A. flavus*.

Fusarium species are widespread pathogens of maize in temperate and semitropical areas, including all European maize-growing areas (Logrieco et al. 2002). Hemibiotrophic *F. graminearum* and other *Fusarium* species infect living tissue as biotrophs but, after a latency period, they can cause the death of host tissues and, therefore, they become saprotrophs (Bacon and Yates 2006). Among *Fusarium* mycotoxins, fumonisins

produced by *F. verticillioides* and *F. proliferatum* are associated to cancer and severe diseases in humans and livestock and are the most frequently occurring class of mycotoxins found in maize kernels (Bennett and Klich 2003; Logrieco et al. 2003; Voss et al. 2007). In contrast to *A. flavus*, the toxigenic *Fusarium* species require a high moisture content in the substrate for growth and mycotoxin synthesis (>20%), so they represent a hazard in preharvested or freshly harvested plants that are drying (Logrieco et al. 2003). *F. verticillioides* and *F. proliferatum* are most frequently isolated from ears affected by *Fusarium* ear rot, followed by *F. subglutinans*, particularly in lower latitudes (Logrieco et al. 2003). Entry of *Fusarium* species into maize ears can occur by growth of mycelium down the silks to the kernels and rachis from spores germinating on the silks or through wounds on the husks caused by insects or birds (Munkvold 2003). Moreover they can behave as endophytes and systematically colonize the entire maize plant without causing symptoms, being transmitted from seed to plant and thereafter to kernels (Munkvold 2003). On the other hand, fumonisins, despite their phytotoxicity, are not necessary for tissue invasion by these fungi, since mutant atoxigenic *F. verticillioides* strains are still able to cause ear rot (Desjardins et al. 2002; Lanubile et al. 2013).

Efforts to enlarge the knowledge of genetic bases of *Fusarium* spp.–maize interactions mainly addressed the study of *F. verticillioides* and *F. graminearum* maize pathogens. *F. verticillioides* inoculation induced transcriptional changes in both susceptible and resistant maize kernels surrounding the inoculation site starting from 48 h postinoculation (hpi) (Lanubile et al. 2010; 2012a and b; 2014). The resistant host exhibited relatively high constitutive levels of defense-related gene expression, whereas the susceptible genotype required pathogen attack to induce defense-related genes. Interestingly, the expression of PR proteins, such as β-1,3-glucanases (PRm6) and chitinases (PRm3), appeared to be constitutive in maize embryos, suggesting an additional role in regulating the normal process of seed germination (Campo et al. 2004). PR5 proteins and chitinases, such as PRm3, were overexpressed in silks of resistant maize lines in response to *A. flavus* and *F. verticillioides* inoculations (Peethambaran et al. 2010; Sekhon et al. 2006). Mohammadi and coworkers (2011) demonstrated that *F. graminearum* infection led to the increase, at 48 hpi, of PR proteins, chitinases, peroxidase (POD) and xylanase, and proteinase inhibitors more strongly in the resistant CO441 line as compared with the susceptible B73 line. Moreover kernels of CO441 contained higher levels of these defense-related proteins after mock treatment, suggesting that these proteins may provide a basal defense against *F. verticillioides* infection.

Differences among cultivars in sensitivity to biotic and environmental stresses have been correlated with increased capability of ascorbate biosynthesis and activity of ascorbate-glutathione (ASC-GSH) cycle enzymes (Noctor and Foyer 1998; Paciolla et al. 2004, 2008). Indeed, pathogen interactions and abiotic stress factors can lead to one or both increased production or accumulation of reactive oxygen species (ROS) in plant tissue. The plants have evolved enzymatic and nonenzymatic defense systems to reduce and scavenge ROS, including ASC-GSH cycle, catalase (CAT), POD, and superoxide dismutase (SOD). When these ROS-scavenging systems fail, overaccumulation of ROS can trigger different signaling cascades, leading to cell death (Van Breusegem and Dat 2006).

It was demonstrated by field artificial inoculation tests that some of the QTL involved in resistance to ear rots caused by *F. verticillioides*, *F. proliferatum*, and *A. flavus* and mycotoxin contamination are identical or genetically linked (Robertson-Hoyt et al. 2007). For this reason, inbred lines resistant to *A. flavus* and *F. graminearum* ear rot could be used to select advanced breeding lines with increased resistance to *F. verticillioides* ear rot (Lanubile et al. 2011). Wisser and coworkers (2006) reported evidence that QTL to different diseases are clustered in the maize genome.

This study evaluates the expression profile of selected defense-related genes in resistant (CO441) and susceptible (CO354) maize kernels at 72 hpi with mycotoxin-producing strains of *F. proliferatum*, *F. subglutinans*, and *A. flavus*. The aim of this work is to observe the transcriptional regulation of genes belonging to both constitutive and induced defense responses to inoculation with hemibiotrophic pathogens (*Fusarium* spp.) and a necrotrophic pathogen (*A. flavus*). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was employed to monitor PR genes (*PR1*, *PR5*, *PRm3*, *PRm6*) and genes involved in protection from oxidative stress, such as *POD*, *CAT*, *SOD*, and *ascorbate peroxidase* (*APX*). Moreover, enzymatic and nonenzymatic assays of the components of the ASC-GSH cycle and indexes of cell oxidative status, such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), were investigated and correlated to the corresponding gene transcripts. The combination of these approaches allows a clearer view of the defense response of resistant and susceptible maize kernels in the metabolic perturbations induced by several fungal infections.

RESULTS

Plant defense responses after *F. subglutinans*, *F. proliferatum*, and *A. flavus* inoculations were probed in two maize genotypes with contrasting resistance to ear rot, the resistant CO441 and the susceptible CO354 maize lines. The response was assayed at 72 hpi based on the evidence obtained in previous experiments in which infection with *F. verticillioides* was monitored over a time course of 96 h by performing microarrays and RNA-Seq analysis. The highest activation of resistance mechanisms was observed at 72 hpi (Lanubile et al. 2012a, 2014).

Evaluation of fungal growth on CO441 and CO354 maize genotypes.

The growth of *F. proliferatum*, *F. subglutinans*, and *A. flavus* was assayed at 72 hpi, in maize kernels surrounding the inoculation point, by absolute quantification of the *calmodulin* transcript through real-time RT-PCR. For all assayed fungal species, the inoculated kernels of resistant maize line showed a statistically significant lower growth of pathogens, revealed by a lower copy number of the housekeeping gene (Fig. 1). In particular, the absolute copy number was 1.33 times higher in CO354, compared with CO441, after *F. proliferatum* inoculation, 1.54 times higher after *F. subglutinans* inoculation, and 1.70 times higher after *A. flavus* inoculation. *F. subglutinans* appeared to be the pathogen that grew more rapidly in maize kernels (2,050 vs. 3,163 in CO441 and CO354, respectively), followed by *F. proliferatum* (1,013 vs. 1,352), and *A. flavus* (482 vs. 818).

Regulation of PR and oxidative stress–related genes before and after fungal inoculations.

The expression levels of PR genes and genes protecting from oxidative stress were measured in real-time RT-PCR. The assayed genes consisted in *PR5*, *PRm6*, *PRm3*, *PR1*, *POD*, *CAT*, *SOD*, and *APX*. Their expression profile is reported in Figure 2 as fold change (FC) in transcript content of inoculated kernels over uninoculated controls. All assayed genes were up-regulated in both maize lines after fungal inoculations with all strains. Exceptions were observed for *POD* after *A. flavus* inoculation (FC = 0.9) (Fig. 2C) and for *APX* and *SOD* genes for all fungal inoculation in the resistant line (FC ≤ 2). Significant inductions of these genes were detected in the susceptible line (FC ranging from 2.7 to 3.8), except for *A. flavus* inoculation (FC < 2). Higher changes in expression (FC > 2) were associated with PR

transcripts rather than oxidative stress–related genes in both maize lines, with FC ranging from 35.0 to 3,587.5 for the former category and from 0.9 to 10.9 for the latter one. *PRm6* showed the highest induction values in CO354 line ranging from 3,417.1 with *F. proliferatum* inoculation to 3,587.5 with *A. flavus* inoculation. Among oxidative-stress genes, *CAT* was the most induced, with values ranging from 7.6 and 10.9 in the susceptible line after *F. proliferatum* and *F. subglutinans* inoculation, respectively (Fig. 2A and B). The susceptible line showed a stronger induction at 72 hpi for all genes in comparison with the resistant line. The greatest difference was found in the expression of *PRm6*, for which *F. proliferatum* inoculation elicited a reaction almost 70 times higher in the susceptible line than that in the resistant one (3,417.1 vs. 49.7, respectively) (Fig. 2A). *PR1* was another gene strongly induced in the susceptible line, with a FC 15 times greater in the case of *F. subglutinans* inoculation (639.2 vs. 41.9) and almost eight times greater after *F. proliferatum* inoculation (129.8 vs. 17.4) (Fig. 2A and B). Although a weak induction of *POD* occurred after *A. flavus* inoculation in the resistant line, the FC value was 10 times higher in the CO354 genotype (Fig. 2C).

Comparing the plant responses to different fungal inoculations, stronger changes in gene expression were generally featured after *F. subglutinans* treatment in both genotypes (Fig. 2B). Intriguingly, this pathogen recorded the most extended growth in maize kernels, as stated by absolute quantification of the *calmodulin* gene (Fig. 1). However, higher differences in gene induction between genotypes were observed after *F. proliferatum* and *A. flavus* inoculations, with the exception of *PR1*, for which a more elevated FC was detected in CO354 kernels inoculated with *F. subglutinans*.

In order to consider constitutive defense responses, the FC was measured by comparing control samples (CO441 control vs. CO354 control) and inoculated samples (CO441 72 hpi vs. CO354 72 hpi) (Fig. 3). Interestingly, in control samples, enhanced expression levels were measured for the assayed genes in the resistant line before the fungal inoculation. The differences in the expression results were greater for PR genes than for genes involved in oxidative stress, with values reaching 74.9 for *PRm6*, 16.7 for *PRm3*, 7.84 for *PR1*, and 3.90 for *PR5*. Overexpression was also detected for the remaining genes in control samples of the resistant line, albeit at lower levels, ranging from 2.4 for *POD* to 1.5 for *SOD*.

Transcriptional changes induced by pathogens were also investigated, by comparing resistant and susceptible inoculated kernels. In general, induction of PR genes was stronger in the resistant line (Fig. 3). Greater differences were found for *PRm6* in CO441 as compared with CO354, in which *A. flavus* inoculation induced its expression value up to 51.7 times and *F. subglutinans* and *F. proliferatum* up to 40.7 and 3.4, respectively. In contrast to PR genes, the expression of ROS-scavenging genes was unaffected by the fungal inoculations (*SOD*) or was greater in the susceptible line (*POD*, *APX*, *CAT*). Comparing plant defense responses elicited by the different fungal inoculations (Fig. 3), *F. proliferatum* causes a similar regulation of PR genes in the two genotypes, excluding the more induced *PR5* in the susceptible line (0.5). In comparison with other fungi, the inoculation with *F. proliferatum* induced lower FC values for ROS-scavenging genes in the resistant line, although *POD* was more down-regulated in response to *A. flavus*.

Regulation of the oxidative status in control and inoculated kernels.

The study of the differential plant defense responses was extended to proteins and products related to oxidative stress. The assays were conducted, on the same susceptible and resistant maize kernels, by comparing uninoculated control samples and inoculated samples at 72 hpi with *F. proliferatum*, *F. subglutinans*, and *A. flavus*. The enzymatic assays included enzymes involved in the oxidative burst and in ROS detoxification, i.e., SOD, APX, CAT, cytosolic and wall PODs (PODc and PODw, respectively), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). The amount of H_2O_2, the substrates or products of these enzymes, and the index of oxidative stress were measured contextually to MDA and GSH-ASC contents.

The specific activity of CAT in CO441 and CO354 kernels is shown in Figure 4A. In uninoculated kernels, CAT results were significantly ($P < 0.001$) different from the inoculated kernels in both genotypes and the resistant line showed considerably more CAT activity (166 vs. 35). After inoculation with all pathogens, CAT activity increased in kernels of both lines in comparison with controls, and the greatest differences occurred after *A. flavus* inoculation for CO441 line and after *F. subglutinans* for CO354 line. In the resistant line, CAT activity results were higher in absolute terms, although a greater increase occurred in the susceptible line.

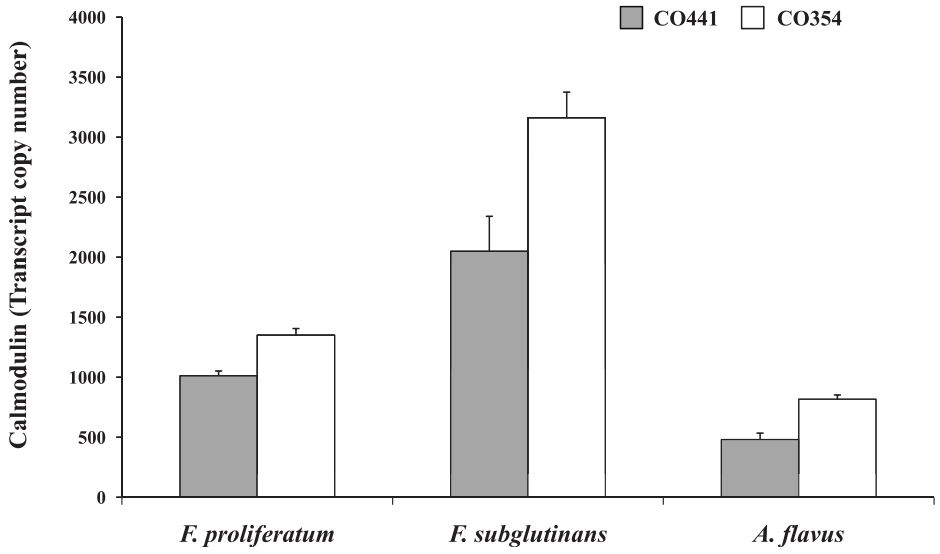

Fig. 1. Copy number of transcripts for the constitutive gene *calmodulin* in kernels of the resistant CO441 (gray boxes) and susceptible CO354 (white boxes) maize lines at 72 h after *Fusarium proliferatum*, *F. subglutinans* and *Aspergillus flavus* inoculation. Vertical bars indicate ± standard deviation.

Activity of APX, an enzyme considered as important as CAT in H_2O_2 removal in plant cells, was very low or unmeasurable in our experimental design (data not shown).

A significant increment ($P < 0.001$) of PODc activity occurred after pathogen inoculations in both genotypes in comparison with controls, similarly to CAT activity, but the greatest increases were detected after *F. subglutinans* treatment for both lines (Fig. 4B). As for CAT, PODc showed more activity in the resistant genotype, both for control kernels (509 vs. 129) and for inoculated kernels. Anyway, as seen previously, in inoculated CO354 kernels the activity increment was greater than CO441.

The specific activity of PODw is shown in Figure 4C. PODw activity in uninoculated CO441, as compared with CO354, was considerably higher (424 vs. 47). Fungal stress caused by enhanced PODw activity in both maize genotypes and controls was significantly different ($P < 0.001$) from inoculated samples for both genotypes. Particularly in CO354, the increase was higher than in CO441, although in the latter, the values of enzyme-specific activity were greater. *F.proliferatum* and *F. subglutinans* inoculations caused the highest differences when compared with the control in the resistant genotype. On the contrary, *A. flavus* treatment triggered the highest PODw activity in the susceptible line in comparison with the control.

The inoculations with all pathogens caused a significant ($P < 0.001$) reduction of SOD activity only in the susceptible line, in particular after *A. flavus* inoculation (Fig. 4D). No significant differences on SOD activity were found in the resistant line between the control and the inoculated samples and only *A. flavus* treatment appeared significantly different ($P < 0.05$) from the other fungal inoculations.

After inoculation with *F. proliferatum* and *F. subglutinans*, the H_2O_2 content significantly ($P < 0.001$) increased in susceptible line kernels, while no significant changes were observed for the CO441 genotype. *A. flavus* caused a significant ($P < 0.001$) enhanced level in both genotypes, although with higher content for the susceptible line (Fig. 5A).

The level of lipid peroxidation was determined as an indicator of the integrity of biological membranes. In resistant line CO441, no significant change in MDA content occurred in the inoculated kernels as compared with the control. On the other hand, a significant increase ($P < 0.001$) was observed in the kernels of the susceptible line upon all fungal inoculations (Fig. 5B).

The components of the ASC-GSH cycle showed a different trend at 72 hpi. The activity of enzymes of reconversion of DHA in ASC, namely MDHAR and DHAR, is shown in Figure 6A and B, respectively. As compared with line CO354, the uninoculated sample of CO441 showed higher activity of MDHAR (587 vs. 432), followed by a significantly decreased ($P < 0.001$) activity in both maize genotypes after inoculation. The same trend was observed for DHAR activity in control CO441 kernels compared with the CO354 line (820 vs. 143). At 72 hpi, DHAR activity was opposite in the two genotypes, showing a significant increase ($P < 0.001$) in the susceptible line and a significant decrease ($P < 0.001$) in the resistant line (Fig. 6B). As for MDHAR and DHAR enzymes, GR activity was higher in CO441 than in the CO354 control (213 vs. 98) (Fig. 6C). In CO441, all pathogens caused its significant increase ($P < 0.001$), whereas a significant change ($P < 0.05$) occurred in the susceptible line only after *A. flavus* inoculation.

The trend of nonenzymatic components of the ASC-GSH cycle is shown in Figure 7. The GSH content was lower in the control susceptible genotype compared with the resistant one (245 vs. 350) (Figure 7A). At 72 hpi, it significantly ($P < 0.001$) increased in CO354, while no significant change was detected in the resistant genotype. Oxidized glutathione (GSSG) was higher in the susceptible line than the resistant one; a significant increment ($P < 0.001$) followed after different fungal inoculations only in the resistant line (Fig. 7B). The ASC level was higher in the resistant line compared with CO354 before inoculation (159 vs. 67; Figure 7C), while at 72 hpi, no significant change and a significant increase ($P < 0.001$) were observed in CO441 and CO354, respectively, with the highest increment associated to *F. subglutinans* and *A. flavus* inoculations. DHA content was very low or unmeasurable in our experiment (data not shown).

According to two-factor analysis of variance (ANOVA), CO441 and CO354 genotypes results were significantly different ($P < 0.05$) in their means for all tested enzyme activity and compounds (Supplementary Table S3). Significant differences ($P < 0.05$) were also detected between the treatments (control and inoculated samples) and a significant interaction ($P < 0.01$) was determined between treatment and genotypes. Controls results were significantly different ($P < 0.05$) from all inoculated samples for all tested enzymes and compounds, with the exception

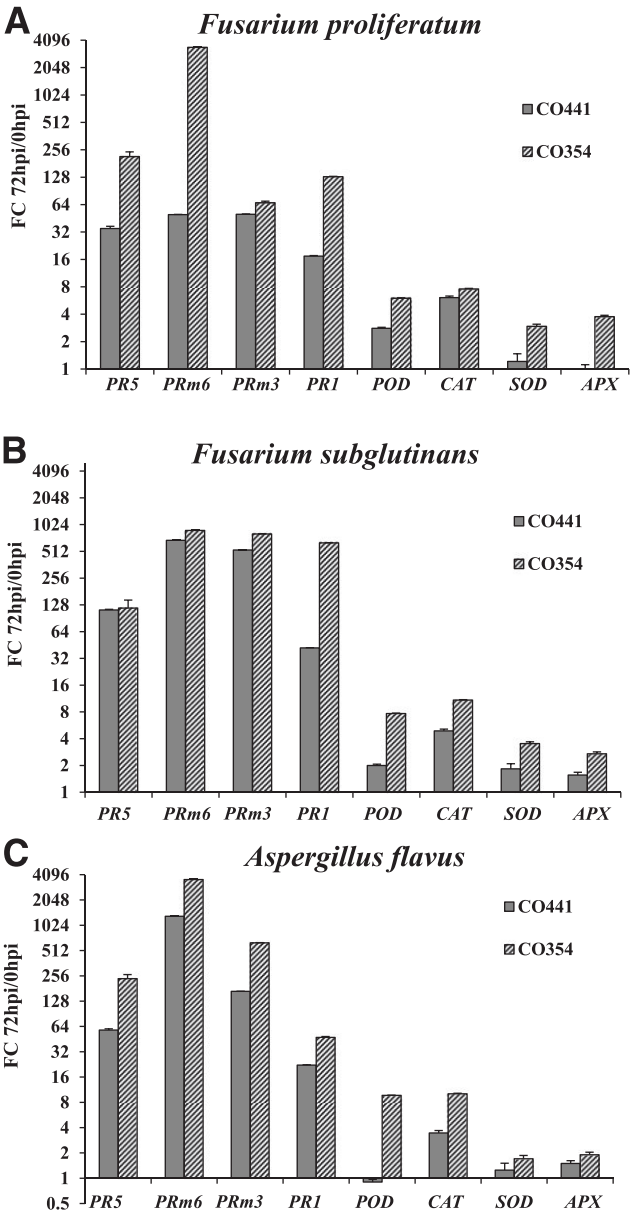

Fig. 2. Fold change (FC) of differentially expressed genes *PR5*, *PRm6*, *PRm3*, *PR1*, *peroxidase* (*POD*), *catalase* (*CAT*), *superoxide dismutase* (*SOD*), *ascorbate peroxidase* (*APX*) in kernels of resistant CO441 and susceptible CO354 genotypes at 72 h after **A,** *Fusarium proliferatum*, **B,** *F. subglutinans*, and **C,** *Aspergilllus flavus* inoculations. Vertical bars indicate ± standard deviation.

GSSG content, for which control results were significantly different ($P < 0.05$) only from *F. subglutinans* inoculation.

DISCUSSION

In order to extend our knowledge about the molecular and enzymatic changes produced by infection with some of the most important maize pathogens, such as *F. proliferatum*, *F. subglutinans*, and *A. flavus*, we analyzed the behavior of genes and enzymes involved in defense response at 72 hpi by real-time RT-PCR and enzymatic and nonenzymatic assays in resistant and susceptible maize inbreds. Although some of the mechanisms affecting disease resistance in earlier stages of infection were evaluated in previous works by microarrays and RNA-Seq technologies in the pathosystem *F. verticillioides*–maize (Lanubile et al. 2010; 2012a and b, 2013, 2014), this study for the first time extends the use of gene expression and enzymatic analysis to several mycotoxin-producing fungal species, in order to understand if mechanisms of resistance are common and inhibit the spread of different pathogens.

In this experiment, *PR1*, *PR5*, *PRm3*, and *PRm6* genes were strongly induced in inoculated kernels in the susceptible line. In all ways, the resistant line demonstrated an abundant transcription of PR genes independently from the inoculation and their expression remained high also at 72 hpi. Higher contents of these genes were found also in kernels of resistant line CO441, both in control samples and at 48 hpi with *F. verticillioides* (Lanubile et al. 2012b) and with *F. graminearum* inoculation (Mohammadi et al. 2011). Furthermore, our results were in agreement with those reported by Murillo et al. (1999), showing that seven fungi were able to induce pathogenesis-related maize seed proteins, suggesting that their accumulation could be a general response of germinating maize seeds to fungal infection.

On the other hand, the transcription of ROS-scavenging genes was higher in the susceptible line after inoculations, and differences between genotypes in the constitutive gene expression were less marked. In general, the transcription of these genes appeared induced by fungal inoculation in the susceptible line whereas, in the resistant line, higher constitutive defense genes allowed a ready response against pathogen attacks. Higher activities of ROS-scavenging enzymes in the uninoculated resistant controls correlated with gene constitutive expression levels of POD and CAT. Conversely, in inoculated samples, no correspondence was found between gene expression and enzymatic activities. Gene transcription was augmented in the susceptible line after fungal inoculations, but POD and CAT activities was greater in the resistant line. Protein level or enzyme activity do not always correlate with gene transcript abundance. There is growing evidence that, at least in eukaryotes, protein levels can change independently of the levels of the transcripts that encode them; in *Arabidopsis*, most enzyme activities changed less and much more slowly than transcripts, and the attenuation and delay varied from enzyme to enzyme (Greenbaum et al. 2003; Horak and Snyder 2002; Gibon et al. 2004; this shift may be attributed to either the intervention at the enzymatic level of unknown effectors, post-translational regulation that alters the kinetic properties, or both (Gibon et al. 2004). Moreover the enzyme assays do not distinguish among isoforms or forms that could be generated by alternative splicing of messenger RNA.

In plant cells, the steady-state level of H_2O_2 and superoxide anion (O_2^{-}) depends on SOD, APX, CAT, and POD activity. According to enzymatic assays, a small difference was detected in the SOD activity of controls of the two maize genotypes. Surprisingly, H_2O_2 content increased in the susceptible line upon fungal inoculation, although an inverse trend occurred in SOD activity. These findings suggest the presence of other sources of H_2O_2 production such as photorespiratory processes (Noctor et al. 2002). On the other hand, the stability of SOD activity in the resistant line confirmed the trend of this enzyme occurring in resistant seedlings of the same maize genotypes of kernels inoculated with *F. verticillioides* (Lanubile et al. 2012b) and in sheath tissue of wheat infected by *F. proliferatum* (Kwon and Anderson 2001). In opposition, SOD and POD activities increased rapidly in wheat in the early incubation period (6 hpi) after *F. graminearum* inoculation (Ding et al. 2011), suggesting that SOD activation occurs at earlier stages of pathogen infection prior to 72 hpi.

Interestingly, although *Fusarium* inoculations do not affect H_2O_2 content in the resistant line, the inoculation with *A. flavus* heavily increased the H_2O_2 content in both genotypes,

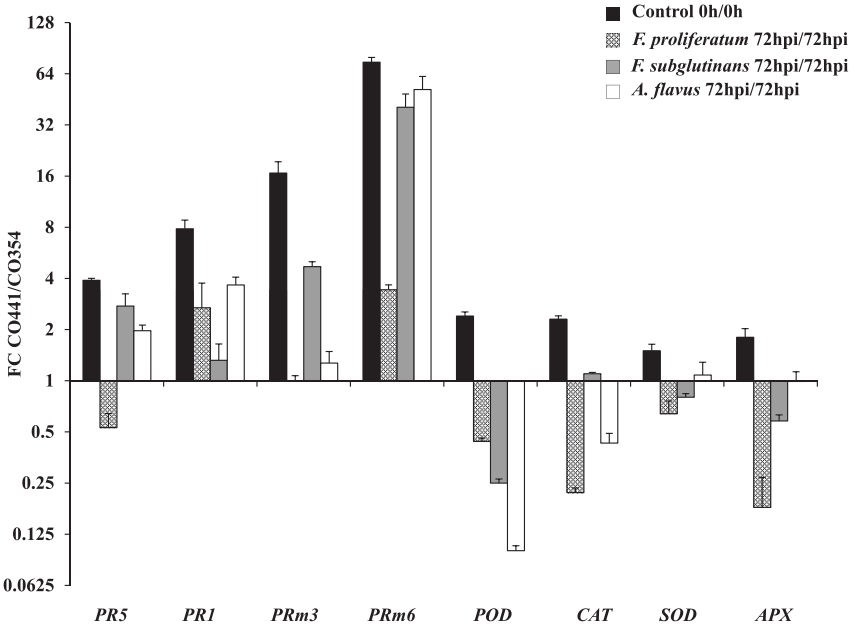

Fig. 3. Fold change (FC) of differentially expressed genes *PR5*, *PRm6*, *PRm3*, *PR1*, *peroxidase* (*POD*), *catalase* (*CAT*), *superoxide dismutase* (*SOD*), *ascorbate peroxidase* (*APX*) before (0 hpi) and after (72 hpi) inoculation with *Fusarium proliferatum*, *F. subglutinans*, and *Aspergillus flavus* in kernels of the resistant CO441 genotype compared with the susceptible CO354 genotype. Vertical bars indicate ± standard deviation.

especially in the susceptible one. Therefore, *A. flavus* inoculation seems to cause an oxidative burst in the plant cells. These findings can be justified by the hypothesis that the production of H_2O_2 may be somehow generated or induced in the plant by the fungus to favor the necrosis. Maybe this could derive from the necrotrophic nature of *A. flavus*, while *Fusarium* species could raise a slower response due to their initial biotrophic lifestyle. However, *A. flavus* is the pathogen that showed the lowest growth on maize kernels, based on the absolute quantification of *calmodulin* transcript. The oxidative burst can be efficiently circumscribed in the resistant line by the strong augmented CAT and POD activity, while in the susceptible line, H_2O_2 remained higher, maybe favoring *A. flavus* pathogenesis.

POD and CAT activities were augmented after inoculation of all considered fungi, especially in the resistant line. Moreover, POD and CAT were expressed at a higher constitutive level in the resistant line, since enhanced activity was detected already in the uninoculated kernels. Despite the very low activity of APX, considered an important enzyme in H_2O_2 removal in

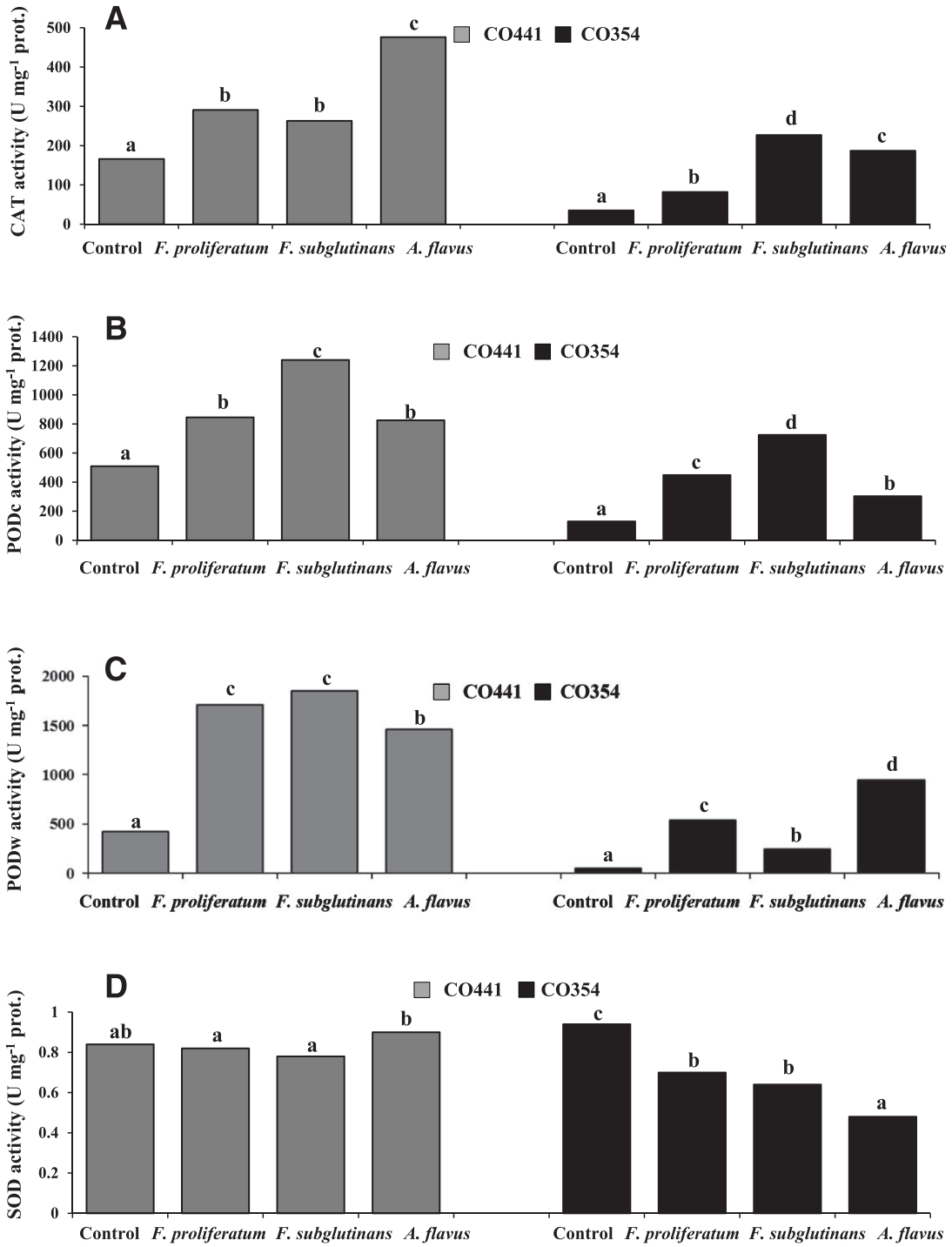

Fig. 4. A, Catalase (CAT), **B,** cytosolic peroxidase (PODc), **C,** cell-wall peroxidase (PODw), and **D,** superoxide dismutase (SOD) activities in kernels of resistant CO441 and susceptible CO354 maize genotypes before (control) and 72 h after *Fusarium proliferatum*, *F. subglutinans*, and *Aspergillus flavus* inoculation. Values represent the mean of at least five replications from four independent experiments. Same letters over the histograms state not significant differences between means of the treatments within the same genotype, as resulting from Tukey's honestly significant difference test ($P < 0.05$).

plant cells, the intervention at 72 h of CAT and soluble POD contributed to the detected low amount of H_2O_2. These findings confirm the resistance of immature maize embryos to *A. flavus* inoculation associated to higher catalase (Cat3) activity (Magbanua et al. 2007) and were consistent with higher CAT activity levels in resistant wheat plants after inoculation with *Puccinia recondita* (Ivanov et al. 2005) and in *F. verticillioides*–colonized maize roots (Kumar et al. 2009). On the other hand, the high accumulation of H_2O_2 by pathogens in the susceptible line together with a large decrease in SOD activity might indicate a lower cell H_2O_2 detoxification rather than higher production (Nemat Alla and Hassan 2006); the increase in CAT and POD activity was probably not sufficient to counteract the pathogen toxicity in this line. Indeed, the increase in MDA content in the susceptible line emphasizes an augmented lipid peroxidation of biological membranes favoring cell death. Scavenging of H_2O_2 can occur in plants by POD, which is induced in host plants upon pathogen infection, to limit the spread of infectious agents through the generation of extracellular ROS, the synthesis of phytoalexins, and the formation of physical barriers and remodeling of cell walls (Almagro et al. 2009). Greater wall POD activity observed before and upon inoculation in the resistant genotype might give greater strengthening and stiffening to cell walls along with providing a solid mechanical barrier against fungal invasion, as also reported by Paciolla et al. (2008).

The ASC-GSH pathway is involved in stress tolerance in plants, since key part of the network of reactions involving enzymes and metabolites with redox properties for the detoxification of ROS (Anjum et al. 2010). The cellular pools of antioxidant ASC and GSH are maintained in their reduced state by a set of enzymes, namely MDHAR, DHAR, and GR, capable of using NAD(P)H to regenerate reduced GSH or ASC (Mittler et al. 2004). Remarkably, reduced ASC and GSH were present at higher levels in the control of the resistant line, in which considerably greater activities of MDHAR, DHAR, and GR were ready to reconvert dehydroascorbate and GSSG to reduced forms. Nevertheless, the decrement of the enzymes of reconversion, MDHAR and DHAR, in the resistant genotype upon infection did not affect the ASC level, highlighting its probable marginal role in detoxification reaction. On the contrary, the increased GR allowed a useful reconversion of GSSG to the reduced form. The reduced GSH could prevent oxidation of enzymes of crucial metabolic pathways or maintain the reduced state of thiol proteins during one or both the fungal penetration or production and the diffusion of its mycotoxins. On the other hand, the strong postinoculation increment of reduced GSH and ASC in the susceptible line suggested that, in these kernels, metabolic perturbations resulting in alteration of cell redox status occurred and the increase in GSH content is not due to augmented GR activity but probably to a de novo GSH synthesis indicating an enhanced oxidation of the cytosol (Foyer and Noctor 2005).

This study highlights the fact that maize resistance can be attributed to the readiness of maize to face invader pathogens, thanks to a remarkable constitutive content of PR and ROS scavenging proteins, and its speed in building up PR proteins. The delay in the susceptible line in accumulation of PR proteins results in its infection, despite its robust response to fungal attack, also due to transcription of genes protecting from oxidative stress. Previously, in a parallel work, we analyzed the expression of PR and stress-related genes and performed biochemical assays on a large number of resistant and susceptible maize genotypes inoculated with *F. verticillioides*, in order to

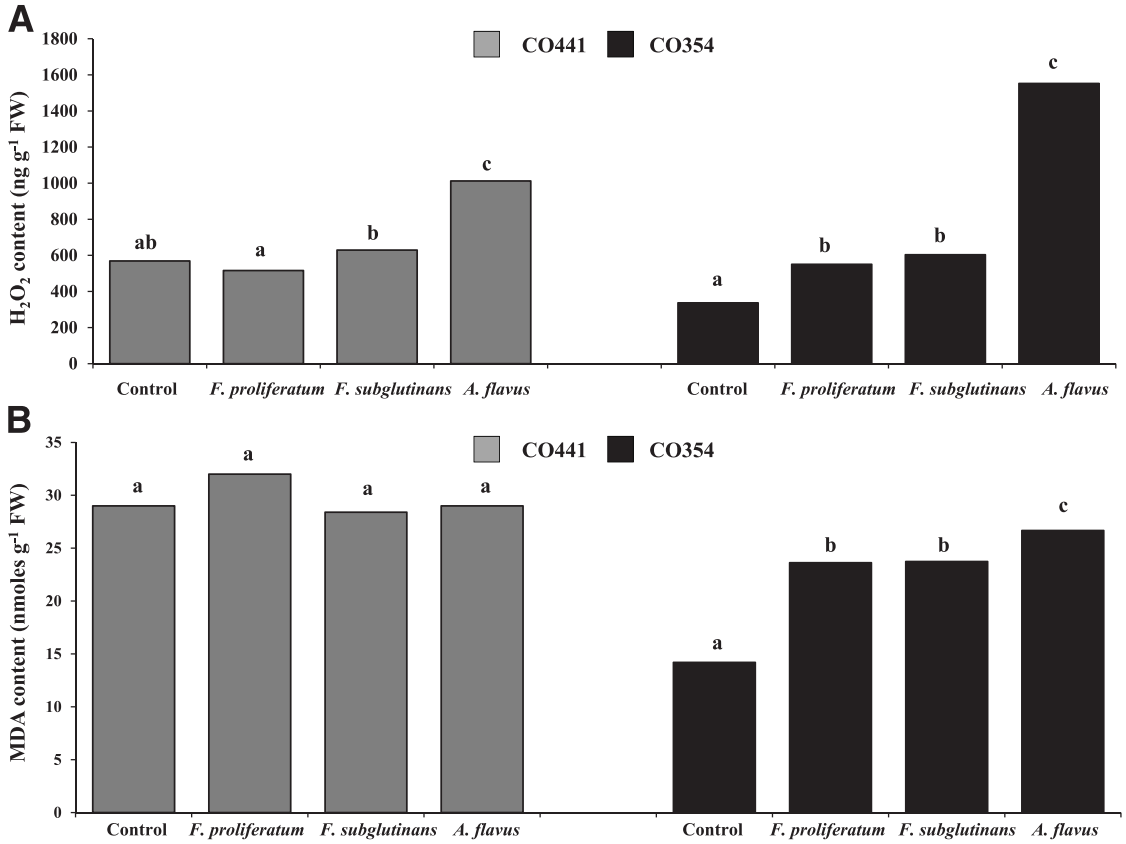

Fig. 5. A, Hydrogen peroxide (H_2O_2) and **B,** malondialdehyde (MDA) contents in kernels of resistant CO441 and susceptible CO354 maize genotypes before (control) and 72 h after *Fusarium proliferatum*, *F. subglutinans*, and *Aspergillus flavus* inoculation. Values represent the mean of at least five replications from four independent experiments. Same letters over the histograms state not significant differences between means of the treatments within the same genotype, as resulting from Tukey's honestly significant difference test ($P < 0.05$). FW, fresh weight.

confirm the data obtained with CO441 and CO354 lines. All genotypes were able to induce the expression of defense/stress related genes and the activation of biochemical pathways upon infection with *F. verticillioides*, and in particular the susceptible genotypes responded more intensely to pathogen infection. Furthermore all the resistant lines evaluated showed a basal defense to *F. verticillioides*, confirming our hypothesis that the resistant genotypes have already high levels of defense-related transcripts/enzymatic activities before inoculation, providing a basal defense system to pathogens.

Confirming our hypothesis, Pechanova et al. (2011) proved that developing resistant rachis from aflatoxin accumulation had higher levels of HSPs and detoxifying enzymes, including APX, DHAR, and SOD. A QTL against aflatoxin accumulation was discovered on maize chromosome 4, near the *cat3* map location (Brooks et al. 2005; Magbanua et al. 2007). It was proposed that *A. flavus* synthesizes aflatoxin in response to oxidative stress (Chen et al. 2004). Since heat and drought stress in the field can lead to the accumulation of ROS in ear tissue, only genotypes that can respond to these abiotic stresses and control ROS levels with antioxidants may, at the same time, prevent aflatoxin production. On the other hand, rachis from the susceptible line had higher levels of PR proteins, which are indicative of biotic stress response (Pechanova et al. 2011).

This work confirms what was previously seen in resistant and susceptible maize lines after *F. verticillioides* inoculation, demonstrating the overlap or community of genes and regulation pathways activated in response to other pathogens, such as *F. proliferatum, F. subglutinans*, and *A. flavus*. The selection of

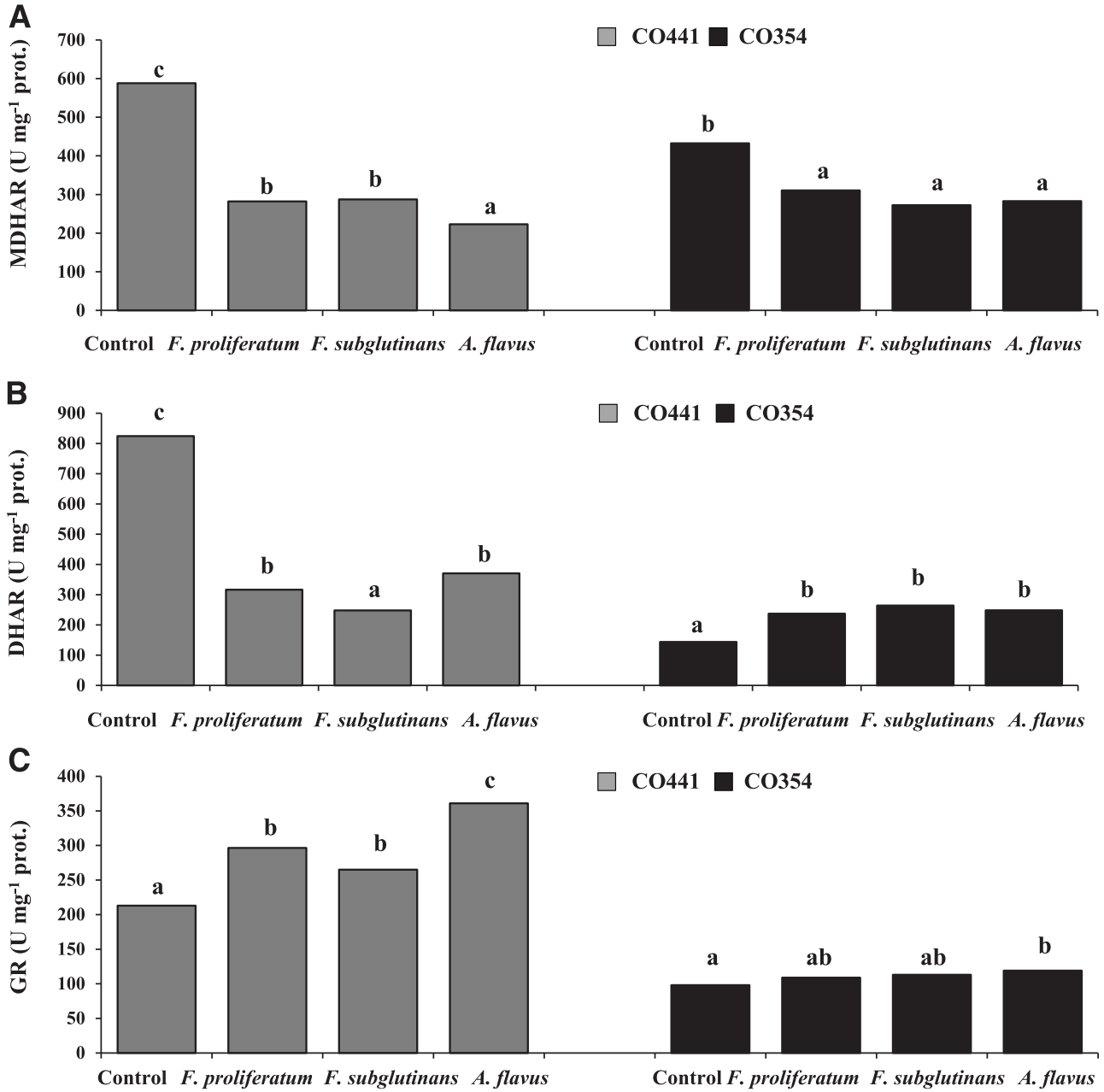

Fig. 6. A, Monodehydroascorbate reductase (MDHAR), **B,** dehydroascorbate reductase (DHAR), and **C,** glutathione reductase (GR) activities in kernels of resistant CO441 and susceptible CO354 maize genotypes before (control) and 72 h after *Fusarium proliferatum*, *F. subglutinans*, and *Aspergillus flavus* inoculation. Values represent the mean of at least five replications from four independent experiments. Same letters over the histograms state not significant differences between means of the treatments within the same genotype, as resulting from Tukey's honestly significant difference test ($P < 0.05$).

maize inbred lines and hybrids resistant to multiple ear rots therefore appears feasible and useful for the reduction of mycotoxin content in grains. The resistant maize genotypes could be profitably employed in breeding programs focused on the resistance to pathogens and grain safety.

MATERIALS AND METHODS

Maize inbred lines, growth conditions, fungal strains, and inoculation assays.

The maize inbred lines CO441 and CO354 were used as resistant and susceptible genotypes, as previously reported (Lanubile et al. 2010; 2011; 2012a and b; 2013; 2014). Both 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 Institute of Agronomy in Piacenza, Italy. Seeds of the two lines were planted in pots (40-cm diameter, 35-cm height) and 30 plants of each line were grown in an environmentally controlled greenhouse with day-time and night-time conditions of 28 and 20°C temperatures, respectively, and a 16-h light regime, using lamps at intensity of 500 µmol m^{-2} s^{-1} (Master TLD 58 W/830, Royal Philips Electronics, Eindhoven, The Netherlands). Plants were hand sib-pollinated.

The following fungal strains were used for inoculation assays: *F. proliferatum* ITEM 1719, producer of fumonisins, beauvericin

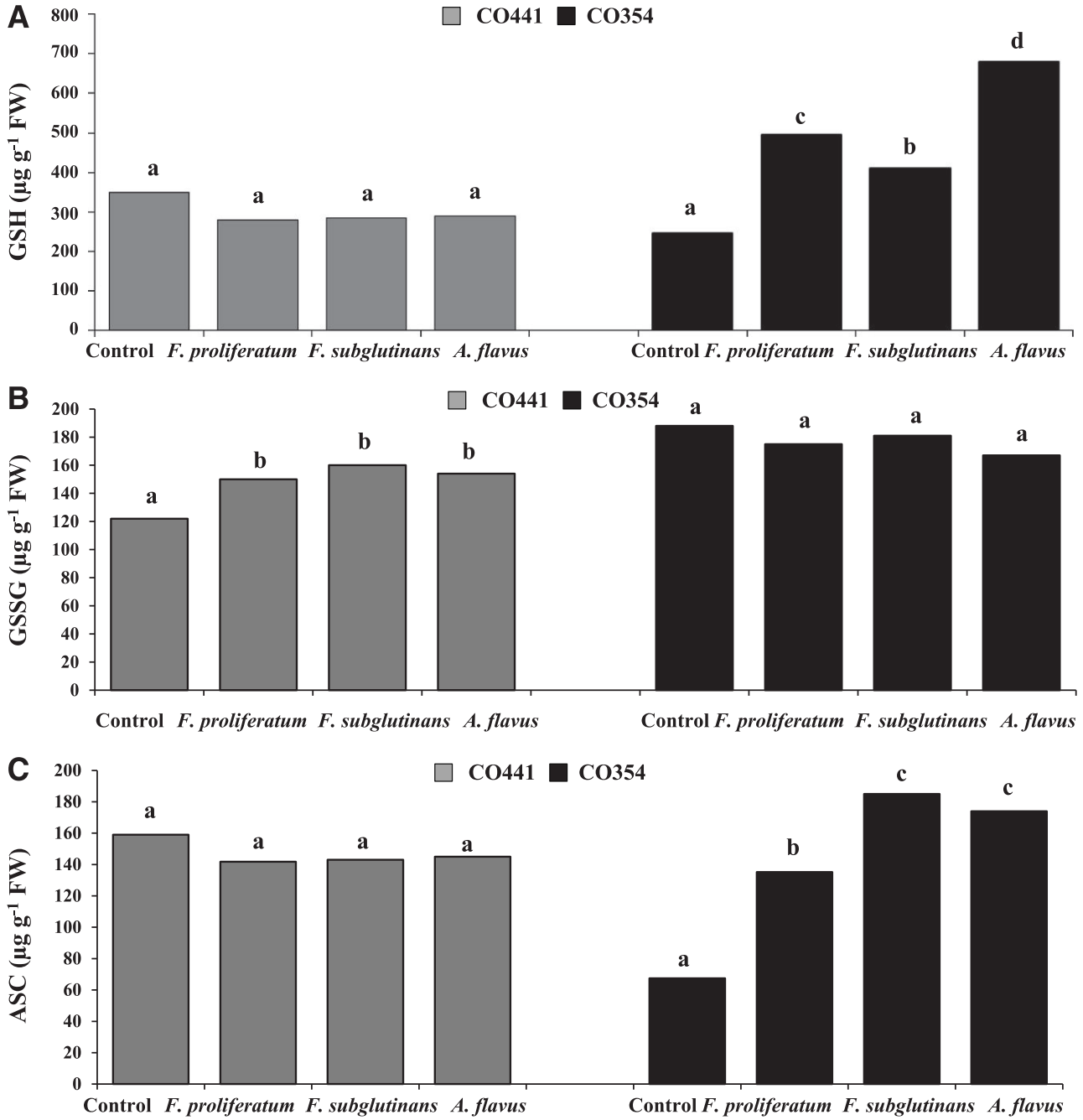

Fig. 7. A, Reduced glutathione (GSH), **B,** oxidized glutathione (GSSG) and **C,** ascorbate (ASC) contents in kernels of resistant CO441 and susceptible CO354 maize genotypes before (control) and 72 h after *Fusarium proliferatum*, *F. subglutinans*, and *Aspergillus flavus* inoculation. Values represent the mean of at least five replications from four independent experiments. Same letters over the histograms state not significant differences between means of the treatments within the same genotype, as resulting from Tukey's honestly significant difference test ($P < 0.05$). FW, fresh weight.

and fusaproliferin; *F. subglutinans* ITEM 1521, producer of beauvericin and fusaproliferin; and *A. flavus* ITEM 8096, producer of aflatoxins. All items were obtained from the Institute of Sciences of Food Production, National Research Council, Bari, Italy. Cultures were maintained on petri plates (9-cm diameter) in potato dextrose agar (infusion from potatoes, 200 g; dextrose, 15 g; agar, 20 g; H_2O to 1 liter) for *F. proliferatum* and *F. subglutinans* and in Czapek agar (sucrose, 30 g; $NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; Fe $SO_4 \cdot 7H_2O$, 0.01 g; K_2HPO_4, 1 g; $ZnSO_4 \cdot 7H_2O$, 0.001 g; $CuSO_4 \cdot 7H_2O$, 0.005 g; agar, 15 g; H_2O to 1 liter) for *A. flavus* and were incubated at 25°C with a 12-h photoperiod for 14 days. Conidia were collected by rinsing plates with sterile water, scraping the agar surface with a scalpel, and filtering the conidia suspension through sterile cloth. Spore suspension was adjusted to a final concentration of 3.5×10^6 conidia/ml based on microscopic counts using a Bürker chamber.

Maize ears were inoculated at 15 days after hand-pollination, using a side-needle inoculator. The inoculating device consists of three 250-mm-long needles mounted on a plastic handle. Pins were dipped in the conidial suspensions and the bar was pressed through the husks sideways and into the center of the ear, penetrating the kernels to a depth of 5 to 10 mm. For the detection of fungal strains, real-time RT-PCR expression analysis, and enzymatic assays, seeds adjacent to the inoculated kernels were collected at 72 hpi, to evaluate fungal growth and colonization and to avoid mechanical damage due to needle-prick (Lanubile et al. 2014). Control seeds were sampled at the same inoculation time listed above and were considered as uninoculated. Three pools of kernels for the 72-hpi time point were prepared, each pool derived from the mixing of kernels from three different maize ears.

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

The collected kernels were ground in liquid nitrogen with a pestle and mortar and total RNA was extracted from 2.5 g of seeds, using the TRIzol protocol (Invitrogen, Carlsbad, CA, U.S.A.). RNA was then purified with the RNA Clean-up protocol (Qiagen, Valencia, CA, U.S.A.), 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 seeds collected at 72 hpi, as reported before, using the 2× iQ SYBR green supermix (Bio-Rad, Hercules, CA, U.S.A.) and the CFX-96 device (Bio-Rad). A 1-µg sample of total RNA was used for cDNA synthesis, following the iScript cDNA synthesis kit protocol (Bio-Rad). Single-strand cDNA (20 ng), determined by fluorometric assay (Qubit), were used for real-time RT-PCR. Relative quantitative analysis was performed under the following conditions: 95°C for 3 min and 44 cycles at 95°C for 10 s, 60°C for 25 s. A melting curve analysis, ranging from 60 to 95°C, was used to identify different amplicons, including nonspecific products. Three technical replicates (within each biological replicate) were employed for each tested sample and the template-free negative controls. Gene-specific primers were designed within consecutive exons and separated by an intron, using Primer3 software, and their sequences are shown in Supplementary Table S1. Relative quantification was normalized to the housekeeping control gene *β-actin* and FC in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). Efficiency values for housekeeping and target genes are reported in Supplementary Table S2. To quantify the growth of *F. proliferatum*, *F. subglutinans*, and *A. flavus*, the copy number of *calmodulin* transcript was detected using real-time RT-PCR in seeds 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. proliferatum* and *F. subglutinans* (National Center for Biotechnology Information [NCBI] GenBank accession number HQ412321.1) and nucleotides 3 and 156 of the *calmodulin* sequence of *A. flavus* (NCBI GenBank accession number XM_002374071.1). The real-time RT-PCR thermal cycling conditions were reported as indicated above. The number of *calmodulin* 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) from *F. proliferatum* ITEM 1719, *F. subglutinans* ITEM 1521 and *A. flavus* ITEM 8096 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 with the dilution standard curve to determine fungal cDNA copy number.

Determination of enzymatic activities.

Maize kernels of CO441 and CO354 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% (wt/vol) cysteine (buffer A) in a 1:3 ratio (wt/vol). The homogenate was centrifuged at 1,000 × *g* for 5 min. The supernatant was recentrifuged 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 was 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 was centrifuged at 1,000 × *g* for 5 min. The pellet was washed three times in buffer A, was centrifuged as described above, and was then resuspended, 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), PODc and PODw (EC 1.11.1.7), SOD (EC 1.15.11), MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1), and 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 ASC oxidized per minute; for CAT, 1 U = 1 nmol of H_2O_2 dismutated per minute; for SOD, 1 U = the amount of enzyme required to inhibit the reduction rate of nitro blue tetrazolium by 50% at 25°C; for MDHAR, 1U = 1 nmol of NADH oxidized per minute; for DHAR, 1 U = 1 nmol of dehydroascorbate reduced per minute; for GR, 1 U = 1 nmol of NADPH oxidized per minute; for cytosolic POD, 1U = 1 nmol of 4-methoxy nafthol oxidized per minute; and for PODw, 1 U = 1 nmol of coniferyl alcohol oxidized per minute. The protein content was determined according to Bradford (1976), using bovine serum albumin as a standard.

Determination of ASC and GSH pool content.

Control or treated kernels were homogenized with two volumes of cold 5% (wt/vol) 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 and GSH pool content according to Zhang and Kirkham (1996).

Lipid peroxidation and H_2O_2 measurements.

For lipid peroxidation, kernels were ground with four volumes of 0.1% (wt/vol) 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% (wt/vol) thiobarbituric acid. The level of lipid peroxidation was measured in terms of the MDA content determined by the thiobarbituric acid reaction, as described by Zhang and Kirkham (1996).

H_2O_2 level was evaluated according to Lee and Lee (2000).

Statistical analyses.

The reported values for enzymatic activities and non-enzymatic assays are the average of at least five replications from four independent experiments. One-factor ANOVA was performed on the observed means of the enzyme activity or compound content for each genotype, and significance of the different treatments (control and inoculated samples) within each genotype was evaluated by Tukey's honestly significant difference (HSD) test ($P < 0.05$).

Two-factor ANOVA was performed on the observed means of the enzyme activity or compound content, considering genotypes and treatments as fixed factors to test the significance ($P < 0.05$) of genotypes, treatments, and their interactions. Tukey's HSD ($P < 0.05$) determined the significantly different treatments.

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