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Changes in leaf gas exchange, chlorophyll *a* fluorescence, and antioxidants in maize leaves infected by *Exserohilum turcicum*

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Abstract

Considering the importance of northern leaf blight, caused by *Exserohilum turcicum*, to decrease maize yield, this study evaluated electrolyte leakage, content of H₂O₂, malondialdehyde (MDA), chlorophyll (Chl) *a*, Chl *b*, and carotenoids (CAR), and activities of superoxide dismutase, catalase, ascorbate peroxidase, peroxidase, glutathione reductase, glutathione-S-transferase, and glutathione peroxidase, as well as expressions of superoxide dismutase (*sod*), catalase (*cat 1*), ascorbate peroxidase (*apx 2*), peroxidase (*pox*), glutathione reductase (*gr 1*), and glutathione-S-transferase (*gst 23*) genes, and leaf gas exchange and Chl *a* fluorescence parameters in maize leaves infected with *E. turcicum*. The content of H₂O₂ and MDA was high at 15 and 20 d after inoculation (dai) and electrolyte leakage was high from 10 dai onward in infected leaves. Net assimilation rate, stomatal conductance to water vapour, transpiration rate, internal CO₂ concentration, maximum quantum yield of photosystem (PS) II, and effective quantum yield of PS II significantly decreased as the disease developed. Quantum yield of regulated energy dissipation significantly increased at 10 and 15 dai but decreased at 20 dai whereas the quantum yield of non-regulated energy dissipation significantly increased at 15 and 20 dai. The content of Chl *a*, Chl *b*, and CAR decreased in infected leaves. The activities of the enzymes increased, and the relative expressions of the genes *sod*, *cat 1*, *apx 2*, *pox*, *gr 1*, and *gst 23* were higher in infected leaves compared to non-infected ones mainly from 10 dai onward. In conclusion, photosynthesis in infected leaves was dramatically affected and a late involvement of the antioxidant metabolism was not sufficiently helpful to counteract the deleterious effects of fungal infection.

Additional key words: carotenoids, gene expression, malondialdehyde, necrotrophic fungus, northern leaf blight, photosynthetic rate, stomatal conductance, transpiration rate.

Introduction

Northern leaf blight (NLB), caused by the fungus *Exserohilum turcicum* (Pass.) Leonard and Suggs (previously named *Setosphaeria turcica*), is a widespread disease in all maize areas and causes significant yield losses (Adipala *et al.* 1993). The NLB symptoms first appear on older leaves as elliptical lesions with necrotic centers and chlorotic halos (Munkvold and White 2016). Plants exposed to many types of stress, such as the infection by

pathogens, are able to produce reactive oxygen species (ROS), *e.g.*, hydrogen peroxide (H₂O₂), superoxide anion radical (O₂⁻), and hydroxyl free radical (OH⁻), to counter deleterious effects caused by these pathogens (Medhy 1994). The excessive production of ROS can damage the host cell by causing lipid peroxidation of cell membranes and oxidation of proteins (Lamb and Dixon 1997, Arora *et al.* 2002, Mittler 2002). The mitigation of oxidative damage caused by ROS is mediated through an antioxidant scavenging system utilized by plants, which

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Abbreviations: A - net assimilation rate; APX - ascorbate peroxidase; CAR - carotenoids; CAT - catalase; c_i - internal CO₂ concentration; Chl - chlorophyll; dai - days after inoculation; E - transpiration rate; EL - electrolyte leakage; F_v/F_m - maximum quantum yield of photosystem II; F_v'/F_m' - effective quantum yield of photosystem II; GR - glutathione reductase; gs - stomatal conductance; GST - glutathione-S-transferase; MDA - malondialdehyde; NLB - northern leaf blight; NPQ - quantum yield of regulated energy dissipation; PI - plant inoculation; POX - peroxidase; PS - photosystem; ROS - reactive oxygen species; SOD - superoxide dismutase; ST - sampling time; Y(NO) - quantum yield of non-regulated energy dissipation.

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involves the activation of antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione-S-transferase (GST), glutathione reductase (GR), peroxidase (POX), and superoxide dismutase (SOD) (Mittler 2002). An oxidation-reduction reaction of the O_2^- to produce H_2O_2 is carried out by SOD, which is further detoxified by the action of CAT, POX, APX, GPX, and GR (Mittler 2002, Apel and Hirt 2004). High H_2O_2 content is associated with an increased expression of the genes *cat 1*, *cat 2*, and *gst* in maize plants (Polidoros and Scandalios 1999).

Several studies have shown physiological disorders in plants infected by pathogens primarily at the photosynthetic level (Tatagiba *et al.* 2016, Rios *et al.* 2017, 2018). The image analysis of chlorophyll (Chl) *a* fluorescence has been used in studies investigating physiological changes in plants exposed to abiotic and biotic stresses (Lei *et al.* 2017, Yadav *et al.* 2017). It is a non-destructive technique, very sensitive and capable of providing a pre-symptomatic diagnosis based on changes in the photosynthetic apparatus in the leaves infected with pathogens (Gorbe and Calatayud 2012).

Considering the limited information on the maize-*E. turcicum* interaction at the biochemical and physiological levels, this study aimed to fill this gap by investigating the alterations of photosynthetic performance (leaf gas exchange and Chl *a* fluorescence imaging) and antioxidant systems (enzyme activities, gene expressions, and determination of ROS content) in maize leaves during the fungal infection process.

Materials and methods

Plant growth: Seeds of the maize (*Zea mays* L.) cv. P-1630 Hx (susceptible to *Exserohilum turcicum*) were sown in plastic pots containing 2 kg of moistened substrate composed of a 1:1:1 mixture of pine bark, peat, and expanded Vermiculite, amended with 1.63 g of calcium phosphate. Plants were kept in a greenhouse with day/night temperatures of $25 \pm 3 / 21 \pm 2$ °C, a relative humidity of 75 ± 5 %, a 12-h photoperiod, and an irradiance of $975 \mu\text{mol m}^{-2} \text{s}^{-1}$. They were fertilized weekly with 50 cm³ of a nutrient solution composed of 192 mg dm⁻³ KCl, 104.42 mg dm⁻³ K₂SO₄, 150.35 mg dm⁻³ MgSO₄, 61 mg dm⁻³ CH₄N₂O, 0.27 mg dm⁻³ NH₄MO₇O₂₄, 1.61 mg dm⁻³ H₃BO₃, 6.67 mg dm⁻³ ZnSO₄, 1.74 mg dm⁻³ CuSO₄, 4.10 mg dm⁻³ MnCl₂, 4.08 mg dm⁻³ FeSO₄, and 5.58 mg dm⁻³ ethylenediaminetetraacetic acid disodium salt.

Inoculation: Maize plants were inoculated with a monospore isolate (UFV-DFP Et22) of *E. turcicum*. For the production of the inoculum used in the experiments, the following procedure was performed: The fungus was grown in Petri dishes containing a lactose-casein hydrolysate medium (Malca and Ullstrup 1962) kept in an incubator (25 °C, a 16-h photoperiod, an irradiance of $975 \mu\text{mol m}^{-2} \text{s}^{-1}$, and a relative humidity of 75 ± 5 %) for 10 d. Plants were inoculated with a conidial suspension

(1×10^4 conidia cm⁻³) at 30 d after emergence (plants had five fully expanded leaves) using a VL Airbrush atomizer (Aibrush Company® USA). Gelatin (1 %, m/v) was added to the suspension to aid the adhesion of conidia to leaf blades. At 8 d after plant inoculation, a total of 200 leaf fragments (≈ 0.25 cm²) containing necrotic lesions were collected, disinfected in a solution of 70 % (v/v) ethanol and 2 % (m/v) sodium hypochlorite, and transferred to Petri dishes (five leaf fragments per dish) containing lactose casein hydrolysate medium. Petri dishes were transferred to an incubator for 10 d. After this period, a total of 1 cm³ of sterile distilled water was added to each Petri dish and fungal mycelia were carefully disrupted using a camel-hair brush within a laminar flow chamber to induce fungal sporulation. After this procedure, Petri dishes were transferred to an incubator with lamps emitting near ultraviolet radiation (320 - 400 nm; for 12 h and 12 h of dark) for 5 d. Plants with five fully expanded leaves were inoculated with a conidial suspension of *E. turcicum* as described above and then kept in a growth chamber (a temperature of 25 ± 2 °C and a relative humidity of 90 ± 5 %) under the dark for the first 12 h after inoculation to support fungal penetration. Thereafter, plants were transferred to a greenhouse (a temperature of a 25 ± 2 °C, a relative humidity of 75 ± 5 %, and maximum natural irradiance at plant canopy of $\approx 975 \mu\text{mol m}^{-2} \text{s}^{-1}$) for the duration of the experiments.

Severity of NLB was evaluated on the third fully expanded leaf, from base to top, of inoculated plants using a set of standard area diagrams (Vieira *et al.* 2014) at 5, 10, 15, and 20 days after inoculation (dai).

Determination of electrolyte leakage: A total of 20 leaf discs (8-mm in diameter) were collected from the third leaf from non-inoculated and inoculated plants at 5, 10, 15, and 20 dai. Leaf discs were placed in glass vials containing 60 cm³ of distilled water before being washed in deionized water. Electrolyte leakage (EL) was determined following the procedures of Lima *et al.* (2002).

Determination of photosynthetic pigments: Five leaf discs (8 mm in diameter) were weighed and placed in glass vials with 5 cm³ of dimethylsulfoxide and after 24 h at 25 °C, the absorbance of the extract was read at 470, 649, and 665 nm using dimethylsulfoxide as a blank (Wellburn *et al.* 1994, Santos *et al.* 2008).

Biochemical assays: A bulk sample formed by the second, third, and fourth fully expanded leaves was collected at 5, 10, 15, and 20 dai. Leaves were also collected from non-inoculated plants at the same sampling times. Leaves were collected in liquid nitrogen and stored at -80 °C.

For determination of H_2O_2 content, 0.2 g of leaf tissue was macerated in liquid nitrogen in a mortar and pestle to obtain a fine powder, which was homogenized in 2 cm³ of a solution of 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine. The homogenate was centrifuged at 10 000 g and 4 °C for 15 min, and 0.1 cm³ of the supernatant reacted with a mixture containing 100 μM ammonium ferrous sulphate, 25 mM sulfuric acid, 250 μM

xylanol orange, and 100 mM sorbitol (Gay and Gerbicki 2000, Kuo and Kao 2003) in a final volume of 2 cm³. Absorbance was determined at 560 nm after keeping the samples in darkness for 30 min. The controls for the reagent and leaf extracts were prepared under the same conditions and their absorbances subtracted from the sample. The H₂O₂ content was determined based on a standard curve of H₂O₂ (*Sigma-Aldrich*, São Paulo, Brazil).

For determination of malondialdehyde (MDA) content, 0.2 g of the fine leaf tissue powder was homogenized in 2 cm³ of a 1 % (m/v) trichloroacetic acid solution. The homogenate was centrifuged at 12 000 g and 4 °C for 15 min and 0.5 cm³ of the supernatant was mixed with 1.5 cm³ of a 0.5 % (m/v) thiobarbituric acid solution prepared in 20 % (m/v) trichloroacetic acid and incubated in a water bath at 95 °C for 30 min (Cakmak and Horst 1991). Thereafter, the reaction was quenched in an ice bath with following centrifugation at 9 000 g for 10 min. The specific absorbance of the supernatant was determined at 532 nm. Non-specific absorbance was measured at 600 nm and subtracted from the value of the specific absorbance. The coefficient of absorbance of 155 mM⁻¹ cm⁻¹ was used to calculate the MDA content (Heath and Packer 1968).

For determination of enzyme activities, 0.3 g of the leaf tissue fine powder was homogenized in 2 cm³ of potassium phosphate buffer 0.5 M (pH 6.8) containing 0.1 M ethylenediaminetetraacetic acid disodium salt, 0.1 % (v/v) *Triton X-100*, 3 mM DL-dithiothreitol, and 1 % (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12 000 g and 4 °C for 15 min and the supernatant was divided into aliquots, which were used for determinations of activities of APX (EC 1.11.1.11), CAT (EC 1.11.1.6), GPX (EC 1.11.1.9), GR (EC 1.8.1.7), GST (EC 2.5.1.18), POX (EC 1.11.1.7), SOD (EC 1.15.1.1) as previously described (Bermúdez-Cardona *et al.* 2015a, Tatagiba *et al.* 2016, Rios *et al.* 2017). Protein content was measured using the Bradford assay with bovine serum albumin as a standard (Bradford 1976).

RNA extraction and real time quantitative PCR:

Leaf samples were ground in a mortar and pestle with liquid nitrogen and the powder obtained was used for RNA extraction by using an *RNAqueous*[®] kit (*Applied Biosystems*, Foster City, USA). Total RNA concentration, quality, and purity were determined (Cruz *et al.* 2015, Debona *et al.* 2017). Then RNA was treated with RNase free DNase (*Promega*, São Paulo, Brazil) and quantified and evaluated for its integrity (Cruz *et al.* 2015, Debona *et al.* 2017). First-strand cDNA was synthesized using 1 µg of total RNA, *M-MLV* reverse transcriptase (*Invitrogen*, São Paulo, Brazil), and a primer oligo (dT) 12-18 (*Sigma-Aldrich*, São Paulo, Brazil) according to the manufacturer's instructions. The genes analyzed, the sequences of designed primers, and the sequences of real-time quantitative PCR primers are listed in Table 1 (Suppl.). Primer sequences for the amplification of the *glutathione-S-transferase* (*gst 23*), and *catalase* (*cat 1*) genes were obtained according to Hermes *et al.* (2013). Primer sequences for the genes *superoxide dismutase* (*sod*), *peroxidase* (*pox*), *ascorbate peroxidase* (*apx 2*),

and *glutathione reductase* (*gr 1*) were confirmed based on sequences deposited in *GenBank* (<http://www.ncbi.nlm.nih.gov/>). Relative standard curves for each target gene using serially diluted templates were generated to calculate amplification efficiencies. Relative quantification of gene transcripts was carried out using the method 2^{-ΔΔCt} (Livak and Schmittgen 2001). The accumulation of transcripts for each gene was normalized using the expression of the constitutive gene *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), which is constitutively expressed upon pathogen inoculation. A mixture of 10 mm³ of 2 × *SYBR-Green Master* PCR mix (*Applied Biosystems*, São Paulo, Brazil), 0.4 mm³ of primers, 5 mm³ of cDNA (20 ng), and 4.6 mm³ of water were used to determine the expression of *cat* and *apx* genes. A reaction mixture containing 10 mm³ of 2 × *SYBR-Green Master* PCR mix, 1.2 mm³ of primers, 5 mm³ of cDNA (20 ng), and 3.8 mm³ of water was used to determine the expressions of the *gr 1*, *sod*, and *gst 23* genes. Real-time PCR reaction to determine the expression of the *pox* gene was composed of 10 mm³ of 2 × *SYBR-Green Master* PCR mix (*Applied Biosystems*), 1.6 mm³ of primers, 5 mm³ of cDNA (20 ng), and 3.4 mm³ of water. The amplification conditions were two stages of 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 and 60 °C, respectively, for 30 s each. A dissociation curve was observed after amplification to ensure that only a single amplicon was produced in each reaction. The *CFX96* real-time PCR detection system (*Bio-Rad*, São Paulo, Brazil) was used to amplify the specific regions of targeted genes and for the real-time detection of amplicon production.

Leaf gas exchange parameters: A portable open-flow gas exchange system (*LI-6400XT*, *LI-COR*, Lincoln, NE, USA) was used to obtain the values of gas exchange parameters such as net carbon assimilation rate (A), stomatal conductance to water vapor (g_s), transpiration rate (E), and internal CO₂ concentration (c_i) on the fourth leaf from base of non-inoculated and inoculated plants at 5, 10, 15 and 20 dai. Evaluations were performed from 10:00 to 12:00 a.m. under an artificial irradiance of 1200 µmol m⁻² s⁻¹ and a CO₂ concentration of 400 µmol mol⁻¹.

Chlorophyll *a* fluorescence imaging: The images and fluorescence parameters of Chl *a*, maximum quantum yield of photosystem (PS) II (F_v/F_m), effective quantum yield of PS II (F_v'/F_m'), quantum yield of regulated energy dissipation (NPQ), and quantum yield of non-regulated energy dissipation Y(NO) were obtained at 5, 10, 15, and 20 dai using an *IMAGING-PAM* fluorometer (a *Maxi* version) and the *Imaging Win* software (Heinz Walz, Effeltrich, Germany). In order to obtain images at a resolution of 640 × 480 pixels, the fourth leaf was used to obtain the images of Chl *a* fluorescence following the procedures previously described (Debona *et al.* 2014, Silveira *et al.* 2015, Bermúdez-Cardona *et al.* 2015b, Tatagiba *et al.* 2015, Bispo *et al.* 2016, Rios *et al.* 2017, Rios *et al.* 2018).

Experimental design and statistical analysis: Two

experiments (Exps. 1 and 2) consisting of two treatments (non-inoculated and inoculated plants), were arranged in a completely randomized design with four repetitions to evaluate NLB severity and to obtain leaf samples for biochemical analysis. Two other experiments (Exps. 3 and 4) were performed to evaluate leaf gas exchange and Chl *a* fluorescence parameters and to obtain leaf samples to determine the content of photosynthetic pigments. They consisted of two treatments (non-inoculated and inoculated plants) arranged in a completely randomized design with four repetitions. Each experimental unit corresponded to a plastic pot containing two plants. Data from NLB severity and from biochemical variables from Exps. 1 and 2 and leaf gas exchange and Chl *a* fluorescence parameters and content of photosynthetic pigments from Exps. 3 and 4 were

analyzed using the *MIXED* procedure of the *SAS* software (v. 8.02, *SAS Institute*, Cary, NC, USA) to determine if data from these experiments could be combined (Moore and Dixon 2015). For all variables evaluated in Exps 1 to 4, *ANOVA* was considered for a 2×4 factorial experiment consisting of non-inoculated and inoculated plants - plant inoculation (PI) and four sampling times (STs). Means from the treatments were compared with the *F* test using *SAS*. Data from each variable and parameter were correlated among them using the Pearson correlation.

Results

Disease severity on the leaves of maize plants increased by 2, 5, 15, and 37 %, respectively, at 8, 12, 16, and 20 dai. The effect of PI was significant on EL, content of H₂O₂ and MDA while the effect of ST and interaction PI $\times$ ST were significant only for EL (Table 2 Suppl.). In the leaves of inoculated plants, there were significant increases (22 and 25 %) of H₂O₂ content (Fig. 1A) and of 30 and 21 % of MDA content (Fig. 1B) at 15 and 20 dai, respectively, in comparison to non-inoculated ones. Also, there were significant increases of 47, 37, and 45 % of EL at 10, 15, and 20 dai, respectively (Fig. 1C).

The effect of PI and ST were significant for all leaf gas exchange parameters evaluated, and interaction PI $\times$ ST was significant only for A and *g_s* (Table 3 Suppl.). In the leaves of inoculated plants, there were significant decreases of A (3, 20, 40, and 59 %), of *g_s* (33, 45, 55, and 87 %), of E (34, 41, 33, and 52 %), and of *c_i* (7, 10, 6, and 12 %) at 5, 10, 15, and 20 dai, respectively, in comparison to non-inoculated ones (Fig. 2A-D).

The effects of PI and ST as well as of interaction PI $\times$ ST were significant for *F_v/F_m*, *F_v'/F_m'*, NPQ, and Y(NO) (Table 3 Suppl.). In the inoculated plants, the *F_v/F_m* was significantly reduced by 4, 4, and 12 %, respectively, at 10, 15, and 20 dai in comparison to non-inoculated ones (Fig. 3A). The *F_v'/F_m'* was significantly reduced by 18 and 25 % at 15 and 20 dai, respectively (Fig. 3B). The NPQ significantly increased by 22 and 24 % at 10 and 15 dai, respectively, and decreased by 8 % at 20 dai (Fig. 3C). The Y(NO) significantly increased by 18 and 37 %, respectively, at 15 and 20 dai (Fig. 3D). Changes in the images of Chl *a* fluorescence were noticed at 5 dai and became more evident at 15 and 20 dai as indicated by dark areas, which corresponded to necrotic lesions on leaves. In the necrotic lesions, there were decreases in the *F_v/F_m* and *F_v'/F_m'* and increases in the NPQ at 10 and 15 dai, and in the Y(NO) at 15 and 20 dai (Fig. 4 Suppl.).

The effects of PI and ST as well as of interaction PI $\times$ ST were significant for the content of Chl *a*, Chl *b*, and CAR (Table 3 Suppl.). In the inoculated plants, there were significant decreases in Chl *a* content (48 and 58 %), in Chl *b* content (51 and 58 %), and in CAR content (34 and 51 %) at 15 and 20 dai, respectively, in comparison to non-inoculated ones (Fig. 4A-C).

The effect PI was significant for SOD, CAT, APX, POX, GR, GPX, and GST activities and the effect of ST was not significant only for GST activity. The interaction PI $\times$ ST

Fig. 1. Content of H₂O₂ (A) and malondialdehyde (MDA; B) and electrolyte leakage (EL; C) in the leaves of maize plants non-inoculated or inoculated with *Exserohilum turcicum*. Means $\pm$ SEs, *n* = 4, * - means are significantly different based on the *F* test (*P* $\leq$ 0.05).

Fig. 2. Net CO_2 assimilation rate, A (A), stomatal conductance to water vapour, g_s (B), transpiration rate, E (C), and internal CO_2 concentration, c_i (D) determined in the leaves of maize plants non-inoculated or inoculated with *Exserohilum turcicum*. Means $\pm$ SEs, $n = 4$, * - means are significantly different based on the F test ($P \leq 0.05$).

Fig. 3. Maximum quantum yield of photosystem II, F_v/F_m (A), effective quantum yield of photosystem II, F_v/F_m' (B), quantum yield of regulated energy dissipation, NPQ (C), and quantum yield of non-regulated energy dissipation, $Y(NO)$ (D) determined in the leaves of maize plants non-inoculated or inoculated with *Exserohilum turcicum*. Means $\pm$ SEs, $n = 4$, * - means are significantly different based on the F test ($P \leq 0.05$).

Fig. 4. Content of chlorophyll (Chl) *a* (A), Chl *b* (B), and carotenoids, CAR (C) in the leaves of maize plants non-inoculated or inoculated with *Exserohilum turcicum*. Means $\pm$ SEs $n = 4$, * - eans are significantly different based on the *F* test ($P \leq 0.05$).

was significant only for APX activity (Table 2 Suppl.). There were significant increases of 60, 54, and 51 % for SOD activity (Fig. 5A), of 43, 65, and 69 % for APX activity (Fig. 5C), of 68, 58, and 49 % for POX activity (Fig. 5D), and of 42, 25, and 49 % for GR activity (Fig. 5E), respectively, at 10, 15, and 20 dai in inoculated plants in comparison to non-inoculated ones. There were significant increases of 34, 33, and 41 % of CAT activity (Fig. 5B) at 5, 10, and 20 dai, respectively. Only at 20 dai, there were significant increases of 58 and 39 % for GST and GPX activities, respectively (Fig. 5F,G).

The expressions of all genes were higher in the inoculated plants when compared to the non-inoculated ones. For the inoculated plants, there were increases in *sod* expression (1.03-, 1.23-, 1.97- and 6.57-fold) at 5, 10, 15, and 20 dai, respectively (Fig. 6A), in *cat 1* expression (1.84-, 4.70-, 4.01-, and 8.11-fold) at 5, 10, 15, and 20 dai, respectively (Fig. 6B), in *apx 2* expression (1.06-, 1.38-, 1.86-, and 3.03-fold) at 5, 10, 15, and 20 dai, respectively

(Fig. 6C), in *pox* expression (2.88-, 8.03-, 5.7-, and 6.60 fold) at 5, 10, 15, and 20 dai, respectively (Fig. 6D), in *gr 1* expression (1.04-, 1.52-, 1.93-, and 4.17-fold) at 5, 10, 15, and 20 dai, respectively (Fig. 6E), and in *gst 23* expression (6.01-, 4.53-, 16.08-, and 22.08-fold) at 5, 10, 15, and 20 dai, respectively (Fig. 6F), in comparison to non-inoculated ones.

All the leaf gas exchange and Chl *a* fluorescence parameters with the exception of $Y(\text{NO})$, and Chl *a*, Chl *b*, and CAR content were negatively correlated with severity of NLB. All the leaf gas exchange parameters were positively correlated with each other, with Chl *a*, Chl *b*, and CAR content, and with F_v/F_m and F_v'/F_m' , but negatively correlated with $Y(\text{NO})$. Non-photochemical quenching was negatively correlated with A , g_s , and c_i , and positively with E . The F_v/F_m was positively correlated with F_v'/F_m' and with the content of Chl *a*, Chl *b*, and CAR, and negatively with NPQ and $Y(\text{NO})$. The F_v'/F_m' was positively correlated with the content of Chl *a*, Chl *b*, and CAR, and negatively with NPQ and $Y(\text{NO})$. The NPQ was negatively correlated with $Y(\text{NO})$, ETR, as well as with the content of Chl *a*, Chl *b*, and CAR (Table 4 Suppl.). Activity of SOD was positively correlated with activities of the other enzymes. Activity of CAT was negatively correlated with APX activity but positively with the other enzymes. Activity of APX was negatively correlated with GPX activity and positively with the others enzymes. Activity of POX was negatively correlated only with GST. Activity of GR was positively correlated with the other enzymes. Activity of GST was negatively correlated with activity of GPX. Disease severity was positively correlated with activities of all the enzymes with the exception of GST and GPX. Content of H_2O_2 was positively correlated with NLB severity and with SOD, CAT, and POX activities, and negatively with APX, GR, GST, and GPX activities. Content of MDA was positively correlated with NLB severity, GST, GPX, and H_2O_2 content. Severity of NLB, all enzyme activities, and MDA and H_2O_2 content were positively correlated with EL (Table 5 Suppl.).

Discussion

This study brings novel insights regarding the changes in photosynthesis and on the antioxidant metabolism of maize leaves during the *E. turcicum* infection process. The A , g_s , E , and c_i decreased in infected leaves before the appearance of the disease symptoms, which were noticed after 8 dai. Zhang *et al.* (2014) in a comparative proteomic study carried out to investigate molecular mechanisms underlying the defense responses of a maize resistant line A619 Ht2 against infection by race 13 of *E. turcicum* verified that at 3 dai, some defense-related proteins (e.g., SOD and polyamine oxidases) are up-regulated whereas the expression of proteins related to photosynthesis and metabolism are down-regulated. Most of the parameters of Chl *a* fluorescence began to show alterations of their values in the infected leaves at 10 dai although the changes in their images occurred from 5 dai. This finding confirms the potential of using

Fig. 5. Activities of superoxide dismutase, SOD (A), catalase, CAT (B), ascorbate peroxidase, APX (C), peroxidase, POX (D), glutathione reductase, GR (E), glutathione-S-transferase, GST (F), and glutathione peroxidase, GPX (E) in the leaves of maize plants non-inoculated or inoculated with *Exserohilum turcicum*. One unit (U) of SOD activity is defined as the amount of the enzyme necessary to inhibit nitroblue tetrazolium photobleaching by 50 %. Means $\pm$ SEs, $n = 4$, * - means are significantly different based on the F test ($P \leq 0.05$).

the Chl *a* fluorescence imaging as a tool for providing information regarding changes in physiological processes related to photosynthetic performance of plants before the occurrence of clear disease symptoms (Silveira *et al.* 2015, Tatagiba *et al.* 2015, Bermúdez-Cardona *et al.* 2015a). For many host-pathogen interactions, a decrease in A is linked

with low g_s and high c_i (Lopes and Berger 2001, Tatagiba *et al.* 2015, Bermúdez-Cardona *et al.* 2015a). In this scenario, photosynthetic restrictions can be attributed to limitation in CO_2 fixation at the biochemical level and not only to a reduction on CO_2 influx due to stomatal closure (Alves *et al.* 2011, Debona *et al.* 2014). Interestingly, in

Fig. 6 Relative expressions of genes encoding superoxide dismutase (*sod*) (A), catalase (*cat 1*) (B), ascorbate peroxidase (*apx 2*) (C), peroxidase (*pox*) (D), glutathione reductase (*gr 1*) (E), and glutathione-S-transferase (*gst 23*) (F) as determined by reverse transcription quantitative PCR in the leaves of maize plants non-inoculated or inoculated with *Exserohilum turcicum* and determined at 5, 10, 15, and 20 days after inoculation. Gene expressions were normalized based on the expression of the constitutive gene *glyceraldehyde-3-phosphate dehydrogenase* and are shown as relative expressions to the corresponding values of non-inoculated leaves. Means $\pm$ SEs, $n = 4$.

the present study, the reverse association was observed for the infected maize leaves. Stomatal limitations may be contributed to lower A since it was accompanied by a decreases in both g_s and c_i indicating a discrete influx of CO_2 into the infected leaf tissue, which may have contributed to impairment photosynthesis. Similar results were reported for mango-*Ceratocystis fimbriata* interaction where a less CO_2 influx in leaves is observed due to fungal infection on stem, which is accompanied by reductions in c_i/c_a ratio (Bispo *et al.* 2016). Stomatal closure of infected maize leaves results in a lower E and corroborates with the findings from maize *Stenocarpella macrospora* interaction (Bermúdez-Cardona *et al.* 2015b). According to Bermúdez-Cardona *et al.* (2015b), dried leaves caused by *S. macrospora* infection contribute to stomatal closure in order to avoid the excessive water loss

resulting, therefore, in lower E values. This can also be the case of maize - *E. turcicum* interaction since a common feature for disease symptoms is wilting leaves especially at the advanced stages of fungal infection.

Photosynthetic pigments, especially Chls, are intrinsically linked to photosynthesis since they are responsible for light absorption and the subsequent transfer of energy to the reaction centers where the photochemical phase of photosynthesis takes place (Taiz and Zeiger 2006). In the present study, a decrease of more than 50 % of the content of Chl *a*, Chl *b*, and CAR dramatically contributed to a decrease in photosynthesis in the infected leaves mainly from 15 to 20 dai. It is known that *E. turcicum* produces host toxins and non-host selective toxins, which, besides causing damage to the cell membranes, also inhibit the synthesis of Chls in maize cultivars susceptible to NLB (Bashan and Levy 1992, Chauhan *et al.* 1997). Thus, the production of host toxin and non-host selective toxins by *E. turcicum* may have contributed to reduction of the content of photosynthetic pigments contributing, therefore, to a lower A especially at advanced stages of fungal infection. The Chl *a* fluorescence image has allowed noticing changes in photosynthesis in plants infected by different pathogens (Scholes and Rolf 2009). The analysis of Chl *a* fluorescence image of wheat leaves infected by *Mycosphaerella graminicola*, a hemibiotrophic fungus, shows no changes in F_v/F_m , F_v'/F_m' , and NPQ during a biotrophic phase of infection indicating that this fungus is not able to cause any damage to the photosynthetic apparatus of its host or even impair the photosynthetic capacity of the infected plants (Scholes and Rolf 2009). There was damage to the photosynthetic apparatus of the infected maize leaves during the necrotrophic phase of *E. turcicum* (e.g., 15 dai) based on the significant decreases of F_v/F_m , F_v'/F_m' , and NPQ. In the present study, slight changes in the Chl *a* fluorescence parameters occurred in the infected leaves already at 5 dai, but at the early stage of fungal infection, a direct damage to the photosynthetic apparatus was not great. At 8 dai, when the first diseases symptoms were noticed, F_v/F_m began to decrease. This parameter represents the maximum quantum yield of PS II when the reaction centers are open (Baker 2008). Reductions in F_v'/F_m' were greater at advanced stages of *E. turcicum* infection when the disease symptoms became more evident. The F_v'/F_m' measures the proportion of radiation absorbed by Chls associated with PS II. This energy is actually used in the photochemical step of photosynthesis (Maxwell and Johnson 2000). Reductions in F_v'/F_m' in leaves infected by pathogens are associated with damage to the photosynthetic apparatus (Meyer *et al.* 2001). One alternative how to protect the photosynthetic apparatus is the release of heat, indicated by NPQ, which contributes to the regulation of energy involved in photosynthesis (Maxwell and Johnson 2000, Klughammer and Schreiber 2008). When the efficiency of photochemical processes increases, the quantum yield of non-photochemical processes decreases and *vice-versa* (Roháček *et al.* 2008). In general, plants exposed to different types of stress show an increase in NPQ indicating an inefficiency of the photochemical process that is reflected

in an increased release of energy as heat. In the present study, a significant increase in NPQ occurred from 10 to 15 dai indicating a reduction in photochemical efficiency. At advanced stages of *E. turcicum* infection, decreases in NPQ were accompanied by increases in Y(NO). A high Y(NO) is an indicator of inefficient both photochemical energy conversion and protective regulatory mechanisms the stressed plants (Klughammer and Schreiber 2008). Maize leaves infected with *S. macrospora* also show decreases in NPQ, which is accompanied by an increase in Y(NO) at advanced stages of fungal infection (Bermúdez-Cardona *et al.* 2015a).

The antioxidant metabolism was activated at advanced stages of fungal infection. A high H₂O₂ content in the infected leaves may serve as a signal to potentiate the expression of genes involved in the antioxidant metabolism and, consequently, the great activities of antioxidant enzymes resulted from their increased expression. An increase in EL and MDA content at advanced stages of fungal infection can be associated with the harmful effect of H₂O₂ to cell membranes. Also, an increase in cell membrane permeability can be associated with a high production of a host toxin by *E. turcicum* (Gao *et al.* 2000). Similarly, leaves of maize plants infected with *S. macrospora* show higher H₂O₂ and MDA content and an increase in EL (Bermúdez-Cardona *et al.* 2015a). In the present study, it is important to stress out that high EL and great content of H₂O₂ and MDA were correlated with NLB development.

The APX, CAT, GPX, and POX participate in the removal of H₂O₂ produced at fungal infection sites. Catalase operates predominantly in peroxisomes and mitochondria; APX removes H₂O₂ present in both cytosol and chloroplasts; POX acts in cell walls and vacuoles whereas GPX acts in cytosol, vacuoles, and cell walls (Sharma *et al.* 2012, Das and Roychoudhury, 2014). Both APX and POX showed an increase in their activities in the infected leaves from 10 dai corresponding, therefore, to a time where H₂O₂ content was high. According to Shimoni *et al.* (1991), POX activity before *E. turcicum* infection is high for resistant plants and increases over times for both susceptible and resistant plants after fungal infection. Indeed, three differential isoperoxidase bands were detected for plants from susceptible maize isolines after *E. turcicum* infection (Shimoni *et al.* 1991). Glutathione reductase participates in the ascorbate-glutathione cycle, and it is important in the generation of reduced glutathione, a compound that is involved in scavenging ROS (Das and Roychoudhury 2014). In comparison to GR, GST is also responsible for the generation of reduced glutathione, and in the present study, its activity was high at advanced stages of *E. turcicum* infection. The expression of the *gr 1* gene was up-regulated in the infected leaves mainly at 20 dai and maximum expressions of the *gst 23* gene were observed at 15 and 20 dai. The enhanced expressions of the genes and an increase in the activities of the enzymes involved in the antioxidant metabolism are consistent with the increase in the H₂O₂ content and corroborates with the fact that ROS are involved in the activation of host defense, and at the same time in the antioxidative metabolism of

stressed plants (Mittler *et al.* 2004, Scandalios 2005). Maize leaves exposed to low H₂O₂ concentrations show low transcriptions of the *cat 1*, *cat 3*, and *gst 1* genes (Polidoros and Scandalios 1999). Zhang *et al.* (2007) reported an increase in the expressions of the genes *cat 1*, *apx*, and *gr 1* and in the APX, CAT, GR, and SOD activities when maize leaves are exposed to H₂O₂. In the present study, a great H₂O₂ content, mainly at advanced stages of *E. turcicum* infection, was accompanied by an increase in both the expression of the genes and in the activity of enzymes involved in the antioxidative metabolism. By using the RNA-seq technique, Shi *et al.* (2018) found that several antioxidant-related genes are up-regulated whereas a PS II-related protein is down-regulated in resistant maize cultivars. Moreover, the genes related to the antioxidative metabolism are up-regulated only in plants from a susceptible cultivar (Shi *et al.* 2018).

Taking together, this study demonstrates that the infection of maize leaves by *E. turcicum* triggered biochemical and physiological impairments. Biochemical limitations as a consequence of *E. turcicum* infection dramatically impaired photosynthesis of maize leaves in association with a lower content of photosynthetic pigments. Although an increase in the expressions of genes and in the activities of enzymes involved in the antioxidative metabolism occurred in infected leaves, it was not sufficient to neutralize the deleterious effects of *E. turcicum* infection on maize leaves.

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