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Research Article

QUANTIFICATION OF PEROXIREDOXINS IN TEOSINTE *ZEA DIPLOPERENNIS*

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ABSTRACT

The expression of peroxiredoxins in teosinte *Zea diploperennis* was analyzed as the first step to establish its relationship with its mechanism of antioxidant response to conditions of abiotic stress and infection with the most common plant pathogen to which it is susceptible (*Ustilago maydis*). The enzymatic activity was evaluated by a spectrophotometric method in which the residual amount of H_2O_2 is measured by its ability to produce a purple complex which absorbs at 480 nm. In saline stress, *Zea diploperennis* showed a high reduction of H_2O_2 with NaCl above $CdCl_2$ and healthy. Heat-stressed coleoptiles had higher activity than healthy coleoptile and those that were stressed at 4°C while in infected coleoptile activity was slightly higher than healthy coleoptile. The results obtained are novel since no results of this type have been reported in any variety of teosinte. The levels of H_2O_2 reduction that the samples exhibit are directly proportional to the concentration of peroxiredoxins shown under the different conditions. These values reflect the behavior of the seedlings towards the adverse conditions to which they were subjected, as well as describe some of their antioxidant mechanisms. As teosinte is the direct ancestor of maize, these values could be of primary utility in evaluating and defining new teosinte resistance mechanisms that could be used in maize.

INTRODUCTION

Aerobic metabolism uses molecular oxygen as an electron acceptor in energy producing oxidations that allow the impressive complexity of higher organisms, but the reduction of O_2 to water implies several drawbacks (Barranco, 2006). Molecular oxygen may be partially reduced to form the superoxide anion (O_2^-). This, although not very reactive on its own, is the precursor for the formation of peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and, in the presence of transition metals, H_2O_2 and O_2^- anion are responsible for the synthesis of hydroxyl radicals (OH^-) (Turrens, 2004). When the reduction is not complete, the inevitable consequence of aerobic metabolism is the formation of reactive oxygen species (ROS) (Barranco, 2006). These intermediate products have a high and diverse reactivity and due to the presence of unpaired electrons in their valence layer are less stable than O_2 or water (Foyer and Noctor, 2013).

Under normal conditions, all these ROS as well as reactive nitrogen species (RNS) are maintained at relatively constant intracellular concentrations by various enzymes and low molecular weight antioxidants, including superoxide dismutase (SOD), glutathione peroxidases, catalases and especially the peroxiredoxins. In addition, ROS are used as second messengers in intracellular signaling cascades (Poole and Nelson, 2008), so a rupture of this equilibrium would lead to a state of oxidative stress that can be deleterious and even deadly to the cell since ROS and RNS can damage various components of living cells such as unsaturated lipids, proteins or nucleic acids (Turrens, 2004).

Peroxiredoxins (Prx) are peroxidase enzymes that catalyze the transfer of electrons from sulfhydryl groups to peroxides (Baier and Dietz, 1996) phylogenetically forming a group of enzymes that exert their catalytic function through the detoxification of peroxides toxic to the cell (Chae *et al*, 1993; Storrz *et al*, 1989 and Lim *et al*, 1998). Although the substrate specificity of plant Prx has not been studied in detail, they have a broad substrate specificity, reducing both H_2O_2 and long chain and short chain alkylhydroperoxides, phospholiproperoxides and peroxynitrite (Barranco, 2006).

According to its taxonomic and morphological characteristics, teosinte is currently considered as the wild and direct predecessor of maize (*Zea mays* spp. *mays*) (Piperno *et al*., 2009). There are six species of teosintes grouped in two sections: Luxuriantes (*Zea perennis*, *Zea diploperennis*, *Zea*

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luxurians) and Zea (*Zea Huehuetenangensis*, *Zea mexicana*, *Zea parviglumis*) (Doebley and Iltis, 1980). *Zea parviglumis* is the closest to maize (Espíndola, 2010). All of them can produce fully fertile viable hybrids but some morphological features are under the control of multiple genes and quantitative inheritance (Beadle, 1977). This kinship has allowed to study some limiting aspects that modify its development, such as the attack of certain pathogens and the mechanisms of defense to them, the dwarfism, the adaptation to drought and the oxidative stress.

Although maize shows a high genetic overlap with its direct ancestor (*Zea parviglumis*) and other annual teosintes, maize and its ancestors differ in their resistance phenotypes with the teosintes being more susceptible to damages (Iltis and Doebley, 1980). Maize has lost part of its direct defenses during selective breeding involving the process of domestication (Bellota *et al*., 2013), a characteristic more evident in modern varieties than in creole (Rosenthal and Dirzo, 1997). Therefore, in terms of the resistance traits of plants, the perennial teosinte *Zea diploperennis* may be more resistant than the annual *Zea parviglumis* being the latter closer to maize (Dávila *et al*., 2013).

MATERIALS AND METHODS

The activity of peroxiredoxins was analyzed by a spectrophotometric method in which the residual amount of hydroperoxide is measured by its ability to produce a purple complex when reacted with a mixture of ferrous ammonium sulphate and potassium thiocyanate absorbing at 480 nm,for this purpose, a standard curve with known amounts of hydroperoxide was previously prepared in order to determine the residual concentración. This assay is based on the ability of Prx to use dithiothreitol (DTT) as an electron donor to reduce hydroperoxides. We carried out the test described by Thurman *et al*., (1972) with the modifications of Barranco (2006).

As a mechanism of reduction of the Prx, 4 mM DTT was used, with which the enzymes were previously incubated. The peroxidase activity was quantified as a function of the concentration of protein extracted with 25 mM Tris HCl buffer (pH 7.5), 5 mM MgCl 2, 5 mM 2-mercaptoethanol, 0.5 mM Na 2 EDTA and a comparative analysis of activity between the fractions subjected to saline stress, thermal stress and infection by a plant pathogen.

In order to reduce the disulfide bonds formed between the catalytic cysteine residues the assay was started with a preincubation mixture at 37°C containing in a volume of 300 µl: 50 µM Tris HCl (pH 8.0), 4 mM DTT and different concentrations of the extract containing peroxidase enzymes. After 10 min 300 µl of 3% H_2O_2 was added and incubated again for 10 min at 37°C. The reaction was stopped by adding 150 µl of 10% (w/v) trichloroacetic acid solution. The protein precipitate was removed by brief centrifugation and 200 µl of 10 mM ammonium ferrous sulfate and 100 µl of potassium thiocyanate 2.5 M were added to the reaction mixture; they reacted with the remaining hydroperoxide to form a purple complex. The peroxide concentration was determined spectrophotometrically at 480 nm using known amounts of peroxide as standard.

The *Zea diploperennis* teosinte seeds used were provided by the National Institute of Forestry, Agriculture and Livestock Research (INIFAP) of Mexico.

RESULTS

The enzyme activity in the germinated grain, without coleoptile, was analyzed under the same extraction conditions and compared with the raw coleoptile extract of teosinte *Zea diploperennis* (CTZD). In Figure 1, there is almost no reduction of H_2O_2 in the germinated teosinte grain sample, without seedling, whereas the crude extract of CTZD presents a high reduction of H_2O_2 reaching values of 130 nmoles.

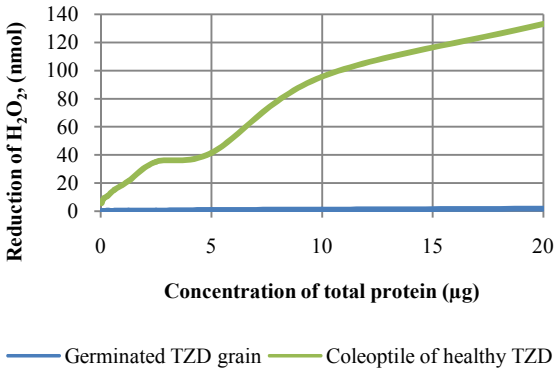

Figure 1 Comparison of peroxidase activities of healthy CTZD and germinated grain.

CTZD were subjected to saline stress with NaCl (50 mM) and $CdCl_2$ (10 µM) for 72 hours. Figure 2 shows the peroxidase activities of the treated CTZD extract fractions, under the same extraction conditions. The NaCl-stressed sample exhibits a high reduction of peroxide reaching values up to 1600 nmoles with respect to the sample treated with $CdCl_2$ (which only reduces to about 300 nmoles). The healthy sample reaches the lowest values with respect to the applied treatments, preserving the habitual behavior reducing to 140 nmoles in the highest concentration of protein.

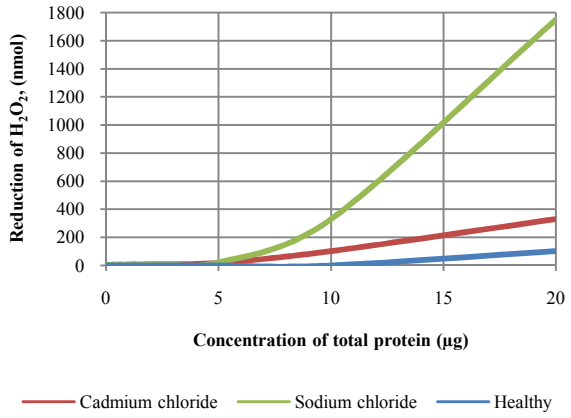

Figure 2 Activity of peroxidases in healthy and stressed CTZD with $CdCl_2$ (10 µM) and with NaCl (50 mM).

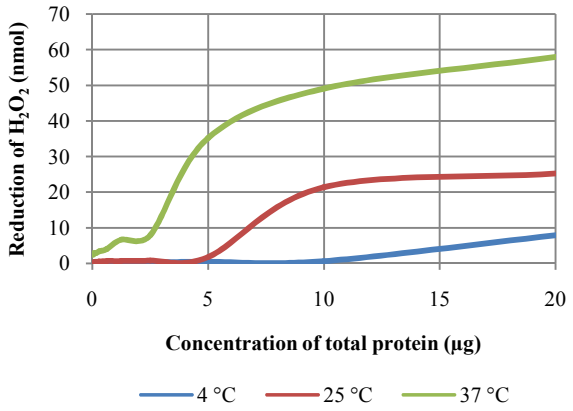

Figure 3 Activity of peroxidases in CTZD at different temperatures: 4, 25 and 37 ºC.

In the same way as in saline stresses, healthy CTZD were subjected to temperature stress. They were incubated at 4 and 37°C and the peroxidase activities were compared with that presented by crude extract of healthy CTZD preserved at 25°C. Figure 3 shows the peroxidase activities of samples stressed by thermal factors. The samples have a similar behavior, but it is possible to observe some differences: the heat-stressed CTZD at 37ºC shows higher enzymatic activity of the three, the enzyme activity in healthy CTZD is visualized average between the two treatments; Seedlings stressed at 4°C have the lowest peroxide reduction and, moreover, this is only evident at protein concentrations greater than 10 µg. Infection of teosinte coleoptilos Zea *diploperennis* was induced with *Ustilago maydis* and the reduction of hydrogen peroxide was evaluated 4 days after inoculation. The enzymatic activities can be visualized in Figure 4, in which the peroxidase activities obtained for healthy and fungal infected CTZD are compared.

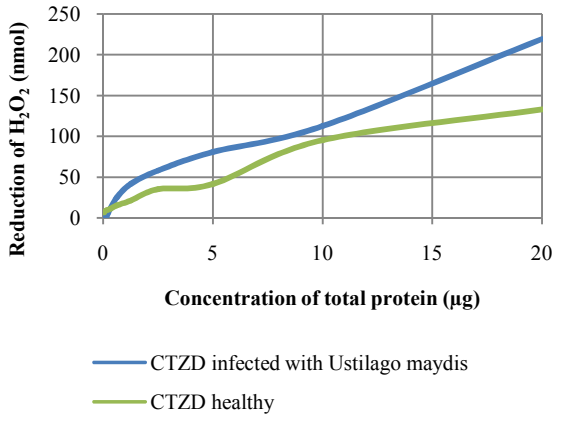

Figure 4 Activity of peroxidases in healthy and infected CTZD with *Ustilago maydis*.

The enzymatic activity of coleoptile of teosinte *Zea diploperennis* infected with *Ustilago maydis* is slightly higher with respect to peroxidase in healthy coleoptile. An important observation is that the reduction of peroxide becomes higher at concentrations greater than 10 µg being higher in the sample of CTZD infected with respect to healthy.

CONCLUSION

The high reduction of hydrogen peroxide by coleoptile of teosinte *Zea diploperennis* sane and the almost null reduction in seed indicate that the expression of peroxidases is more related to germination processes as established by Haslekas *et al*., (2015) than with the latency in teosinte seeds. Regarding stress treatments, the activity of peroxidase at 4 °C is lower than in healthy coleoptile, whereas the greater activity is reached with the stress at 37 °C, which allows us to admit that the expression of peroxiredoxins has a closer relationship with high temperatures, an aspect that is related to climates exclusively dry or very little wet, in which *Zea diploperennis* grows wild. In stress with salts, it is relevant that the enzyme activity increases proportionally to the protein concentration, and that the treatment with the two chlorides raises the synthesis of peroxidases in comparison with healthy coleoptilo, where it is smaller, which indicates that to these conditions this species responds by producing antioxidant enzymes that would form part of its mechanism of response to those characteristics typical of certain types of soils. Likewise, coleoptile infected with the fungus has a slightly higher enzymatic activity, but this is still not significant.

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