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Oligoglucans as Elicitors of an Enzymatic Antioxidant System in Zucchini Squash (*Cucurbita pepo* L.) Seedlings at Low Temperature

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Abstract: Problem statement: Non-controlled low temperatures affect physiological and biochemical processes leading to Reactive Oxygen Species (ROS) accumulation in plants, which normally are stabilized by the antioxidant system. Superoxide Dismutase (SOD), Ascorbate Peroxidase (APX) and Catalase (CAT) are the most important ROS-detoxification enzymes in stressed plants. **Approach:** The activity of these enzymes were examined in response to fungal Oligoglucans (OG) in cold-stressed cotyledons of *Cucurbita pepo* L. Cold stress condition was settled in zucchini cotyledons using relative Ion Leakage (IL) and H_2O_2 accumulation. Then, OG were obtained from *Trichoderma harzianum* cell-wall by enzymatic and chemical hydrolysis. These elicitors were purified by size-exclusion and anion-exchange chromatography and tested in cold-stressed cotyledons. **Results:** No significant changes on H_2O_2 content and IL were observed between treated and control cold-stressed cotyledons, during the first 7 days of storage at 5°C. Afterwards H_2O_2 values increased greatly after cold-stressed. Then, cold stress conditions were established (7 d, 5°C). The APX and CAT activities remained stable, but not SOD activity, which decreased about 42% in control cotyledons. When a chemical OG mixture (CM) was applied to cold-stressed cotyledons, APX and CAT activity levels increased. The SOD activity was only increased by an enzymatic OG mixture (EM). The APX activity level was increased in cold-stressed cotyledons using OG size-exclusion chromatography fractions from both CM and EM; however, changes in CAT activity were only possible using EM fractions and no changes were detected in SOD activity. The highest CAT activity levels were triggered using OG anion-exchange chromatography sub-fractions from both CM and EM fractions (120 and 119 % of enzymatic activity, respectively). **Conclusion:** The oxidative stress can be counteracted by the triggering of APX and CAT activity in cold-stressed zucchini cotyledons mediated by *T. harzianum* cell-wall OG.

Key words: Antioxidant response, cold stress, fungal oligoglucans, Reactive Oxygen Species (ROS), Superoxide Dismutase (SOD), Ascorbate Peroxidase (APX), Catalase (CAT), Oligoglucans (OG), *Cucurbita pepo* L

INTRODUCTION

Plant development is challenged by many environmentally and physiologically adverse conditions that produce Reactive Oxygen Species (ROS) (Quan *et al*., 2008). An excess of these compounds affects plant growth and development resulting in low crop yields (Wahid *et al*., 2007). Generally, if plants are in non-stress conditions, ROS production and scavenging processes are in equilibrium; however, this condition can be easily perturbed by extreme temperatures and several abiotic and biotic factors (Del Río *et al*., 2006). For example, ROS accumulation begins in chloroplasts and mitochondria of plants, such as wheat, cucumber, corn, *Arabidopsis thaliana* and rice, due to low temperature condition (Dat *et al*., 2000; Al-Taisan, 2010). To decrease the damage of ROS in biomolecular targets (proteins, nucleic acids and lipids), plants employ a complex antioxidant system that helps them restore cellular redox homeostasis. This antioxidant system is divided in two principal sub-systems (Halliwell *et al*., 2006), the non-enzymatic (glutathione, ascorbate, tocopherols and phenolic compounds) and the enzymatic [Superoxide dismutase, SOD (EC. 1.15.1.1); Catalase, CAT (EC. 1.11.1.6); Ascorbate peroxidase,

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APX (EC. 1.11.1.7)]. However, the protective role offered by basal plant antioxidants does not always respond accurately under stress conditions; therefore, plants need to enhance several defensive responses to counteract this problem.

Many treatments have been successfully applied with the aim to elicit plant antioxidant systems, such as genetic manipulation of crops, conditioning thermal treatments or triggering of plant defense systems by exogenous elicitation (Falcón-Rodríguez *et al.,* 2009; Islas-Osuna *et al.,* 2010). Each has shown specific limitations due to sample heterogeneity or the different experimental strategies followed. There are many advantages to using Oligoglucans (OG) from fungal cell-walls as triggering molecules or elicitors; these molecules can be exploited to reduce the effects of oxidative stress (Nita-Lazar *et al*., 2004; Shinya *et al*., 2006). Several fungal OG have been tested under both *in vivo* and *in vitro* conditions and have shown an important relationship between the plant antioxidant system and their ability to induce changes in the activities of antioxidant enzymes (Chivasa *et al*., 2006; Zheng *et al*., 2008). Thus, in this study we examined if the application of *T. harzianum* OG may reduce damage in plants, triggering the activity of the enzymatic antioxidant system in cold-stressed *Cucurbita pepo* L. (zucchini) cotyledons.

MATERIALS AND METHODS

Plant material and stress conditions: Zucchini seedlings (*Cucurbita pepo* L. cv. "Raven") were cultured in plastic bags (10×15×5 cm) with peat moss, with a 16 h light photoperiod (100 µmol m^{-2} sec^{-1}, photosynthetic proton flux density) at 25 ± 3°C and a relative humidity of 80% for 10 day. Subsequently, zucchini seedlings were subjected to low temperature experimental conditions involving a dark refrigeration chamber at 5°C during the entire time period (0-15 day). All chemical reagents were purchased from Sigma Co. (St. Louis, MO., USA).

Ion Leakage (IL) and H_2O_2 content: Membrane damage was measured by a conductivity method (Friedman and Rot, 2006). Briefly, 1 g of zucchini cotyledons from squash seedlings were cut in small pieces (1 cm^2) and were immediately incubated with 20 mL of a 0.3 M D-mannitol solution. After 2 h of light mixing, an initial IL value (*vi*) was measured with an EC-METER 1481-61 Conductivity Meter (Cole-Palmer Instruments Co.). Subsequently, each sample was subjected to thermal treatment (121°C, 30 min) and was allowed to warm to room temperature while being lightly mixed (approximately 2 h). A final IL value (*vf*) was measured as previously described. The total IL value was derived from the following equation: [(*vi*/*vf*) ×100]. Hydrogen peroxide content was determined using the colorimetric method (Cervilla *et al*., 2007), where zucchini cotyledons were ground in extraction buffer [50 mM K_2HPO_4•$3H_2O$ (pH 6.8), 1 % (v/w) polyvinyl polypyrrolidone (PVPP)]. Samples were clarified by centrifugation (Sorvall RC, SM-24 rotor) for 20 min (8,000×g) at 25°C. An aliquot (1 mL) of the extracted solution was mixed with 0.3 mL of 0.1% titanium dioxide in 20 % (v/v) H_2SO_4 and the mixture was then centrifuged for 30 min (9,500×g). The intensity of the yellow color of the supernatant, as a consequence of the chemical reaction between H_2O_2 and titanium dioxide in acidic medium, was measured at 415 nm. The H_2O_2 concentration was calculated from a standard curve plotted within the range of 1-10 mM H_2O_2.

Fungal cell-wall preparation: First, spores of *Trichoderma harzianum* maintained on PDA slant (potato dextrose agar, Difco Co.) were inoculated (10^8 spores mL^{-1}) in 2.5 L PDB medium (potato dextrose broth, Difco, Co.) in an orbital shaker (150 rpm) at 27°C. After 10 d of incubation, the mycelium was harvested by vacuum filtration. The collected mycelium was washed twice with ten volumes of distillated water and was homogenized three times with ten volumes of chloroform:methanol (v/v) for further vacuum filtration. Finally, the recovered material was homogenized with five volumes of acetone, vacuum filtrated and then dried at room temperature. This cell-wall was used to isolate OG mixtures by chemical and enzymatic hydrolysis.

Isolation of OG mixtures: The chemical OG mixture (CM) hydrolyzed from fungal cell-walls was extracted (Nita-Lazar *et al*., 2004). Briefly, fungal cell-wall powder (1 g) was constantly mixed in 1 M NaOH (100 mL) at 65°C for 2 h and the obtained extract was neutralized with 50% acetic acid and was clarified by centrifugation (Sorvall RC, SM-14 rotor) for 10 min (10,000 rpm) at 25°C. The enzymatic OG mixture (EM) was obtained using Glucanex^{TM} (20 mg g^{-1}; Sigma Co.) and this enzymatic hydrolysis was conducted at 27°C over 6 h with constant mixing (Okinaka *et al*., 1995). The enzymatic reaction was stopped by autoclaving at 121°C for 30 min.

Biological assays using OG mixtures: Each chemical and enzymatic OG mixture was tested in fifteen cold-stressed zucchini plants using a double spray method

(500 ppm, 15 mL), with the first spray at the initial time (0 day) and the second at the final time of the treatment (7 day). Controls consisted of water-sprayed plants grown under ideal (25°C, steady-state activity level, the negative control) or stressed (5°C, positive control) temperature conditions. Sampling was performed 2 h after spraying OG, at which point zucchini cotyledons were cut from the seedlings and immediately frozen with liquid nitrogen and stored at -40°C until the leaf extract was obtained.

Changes in SOD activity (Wu *et al*., 2008) were expressed in units mg^{-1} protein, where one unit of SOD was defined as the amount of enzyme capable of reducing nitroblue tetrazolium chloride (NBT) by 50 %. The leaf tissue (1 g) plus 5 % (w/w) PVPP was homogenized in 2 mL of cold extraction buffer [50 mM K_2HPO_4•$3H_2O$ (pH 7.5), 1 mM EDTA] and was subsequently filtered and centrifuged for 15 min (12,000 x g) at 4°C. The recovery enzyme extract was kept in cold temperature (10°C) during SOD activity determination, which was monitored at 560 nm. One milliliter of the reaction mixtures was used, containing 0.1 M Na_2HPO_4•$7H_2O$ (pH 7.8), 0.01 M L-methionine, 0.025 % (v/v) Triton X-100, 0.11 mM EDTA, 57 µM NBT, 1.3 µM riboflavin and 50 µg of enzyme extract. Reaction mixtures were illuminated for 10 min with 30-W white light lamps (General Electric Co.). Non-illuminated mixtures were used to correct background absorbance.

Enzymatic activity of CAT (Aebi, 1984) was determined by following the consumption of H_2O_2 at 240 nm for 3 min at 30°C. The leaf tissue (1 g) was homogenized in 10 mL of cold extraction buffer [50 mM K_2HPO_4•$3H_2O$ (pH 7.8), 0.5 % (v/v) Triton X-100, 0.5 mM EDTA, 1 % (w/v) PVPP]. The homogenate was filtered and centrifuged for 20 min (18,000×g) at 4°C. The recovery enzyme extract was stored at 10°C during CAT activity determination. The reaction mixture (0.5 mL) consisted of 50 mM K_2HPO_4•$3H_2O$ (pH 7.0), 50 mM H_2O_2 and 25 µL of enzyme extract. The H_2O_2 molar extinction coefficient (28 mM^{-1} cm^{-1}) was used to calculate CAT activity, which was expressed in mmols of reduced H_2O_2 min^{-1} mg^{-1} protein.

Enzymatic activity of APX (Nakano and Asada, 1987) was determined by monitoring changes at 290 nm for 3 min at 25°C. The leaf tissue (1 g) was homogenized in 10 mL of cold extraction buffer [50 mM K_2HPO_4•$3H_2O$ (pH 7.8), 5 mM L-ascorbic acid, 1 mM EDTA, 1 % (w/v) PVPP]. The enzyme extract was obtained as previously described for the CAT enzyme extract. The reaction mixture (0.5 mL) consisted of 50 mM K_2HPO_4•$3H_2O$ (pH 7.0), 5 mM L-ascorbic acid, 0.1 M H_2O_2 and 50 µL of enzyme extract. The ascorbate molar extinction coefficient (2.8 mM^{-1} cm^{-1}) was used to calculate APX activity, which was expressed in mmols of oxidized ascorbate min^{-1} mg^{-1} protein. For all enzymatic extracts, total protein was measured using a colorimetric method (Bradford, 1976) with the Bio-Rad Protein Assay reagent and bovine serum albumin as a standard.

Biological assays using OG SEC fractions: Both OG mixtures (2 mg lyophilized powder mL^{-1} of each mixture) were fractionated by size-exclusion chromatography (SEC; Biogel P-6, column 2.6×50 cm, 6-kDa cut-off exclusion limit) previously equilibrated with distilled H_20 at a volumetric flow of 1 mL min^{-1}. Collected fractions were assayed for hexose content (Bailey, 1958) versus a D-glucose standard. These fractions were pooled into three main chemical (C1, C2 and C3) or enzymatic (E1, E2 and E3) fractions according to their retention time and were lyophilized before being applied to cold-stressed zucchini cotyledons using a double spray method (500 ppm, 15 mL each). Changes in the enzymatic antioxidant system activities were evaluated as described earlier for the OG mixtures.

Biological assays using OG AEC sub-fractions: The main chemical or enzymatic fractions obtained by SEC, which showed the most effective ability to trigger the activity of the enzymatic antioxidant system with respect to control cotyledons, were chosen. These fractions were sub-fractionated using anion-exchange chromatography (AEC; Q-sepharose fast flow, column 2.5×45 cm) with a linear gradient of 0-0.3 M HCO_2NH_4 (pH 6.5) in 1 M NaCl for 2 h and a volumetric flow of 2 mL min^{-1}. Collected fractions were assayed for hexose content (Bailey, 1958) versus a D-glucose standard and were pooled into three sub-fractions (1, 2 and 3) for each chemical or enzymatic OG fraction according to their elution time. Changes in enzymatic antioxidant activities were evaluated as described earlier for both OG mixtures and their fractions.

Statistical analysis: All enzymatic assays were repeated twice and compiled data were submitted to an ANOVA test using NCSS 2001 software (Kaysville, Utah, USA). To evaluate significant differences between OG, a Duncan test ($p \leq 0.05$) was conducted.

RESULTS

Establishment of cold stress condition: Control (25°C) zucchini cotyledons showed an IL value below

20% during the entire experimental time. For cold-stressed cotyledons, no changes in an IL values were appreciable during the first 7 day; however, after this time, gradual increments in damage of the cell membrane integrity were evident (Fig. 1A). The H_2O_2 content showed a similar trend in cold-stressed cotyledons, showing a high level of peroxide after 7 d of cold stress at 5°C (Fig. 1B). In both cases, when squash seedlings were transferred to a warmer environment (25°C) during 7 day for the promotion of the cell homeostasis recovery, only cold-stressed cotyledons for less than 7 day reached values below the maximum level allowed for these assays (IL of 50% and 10 mmols H_2O_2 g^{-1} FW). Afterwards no recovery of cell homeostasis was observed.

Changes in SOD activity: To determine the potential biological activities of OG mixtures, these samples were assayed in cold-stressed zucchini cotyledons. SOD activity showed a decrease (42%) induced by cold stress with respect to basal SOD activity (Fig. 2). Only the enzymatic OG mixture (EM) had a significant effect ($p \leq 0.05$) in the enhancement of SOD activity (Fig. 2A).

The chemical OG mixture (CM) showed the same effect as the control cotyledons (Fig. 2B). In fact, the reduced SOD activity in cold-stressed cotyledons was increased by applying CM. Once evaluated the EM and CM triggering effects in the zucchini squash enzymatic antioxidant system, both chemical (C1, C2 and C3) and enzymatic (E1, E2 and E3) OG fractions were obtained by SEC and were applied to cold-stressed cotyledons. None of these fractions was capable of triggering SOD activity (Fig. 2). Every SEC OG fraction showed a similar effect to control cotyledons. The biological activity of OG mixtures diminished when SEC OG fractions were tested (Fig. 2). No triggering activity was detected when sub-fractions obtained by AEC were applied to cold-stressed cotyledons (data not shown).

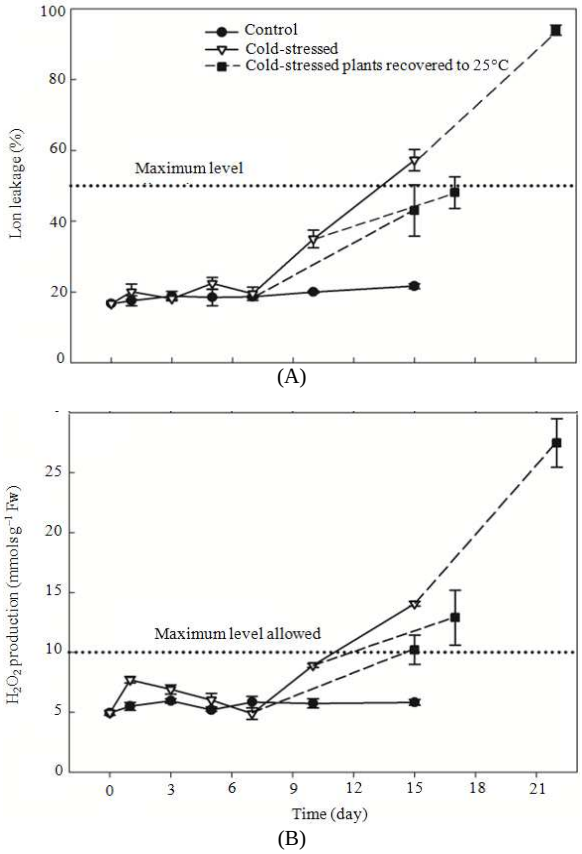

Fig. 1: Rate of ion leakage (A) and H_2O_2 production (B) in cold-stressed zucchini cotyledons (5°C). Results are mean ± SD of at least three replications

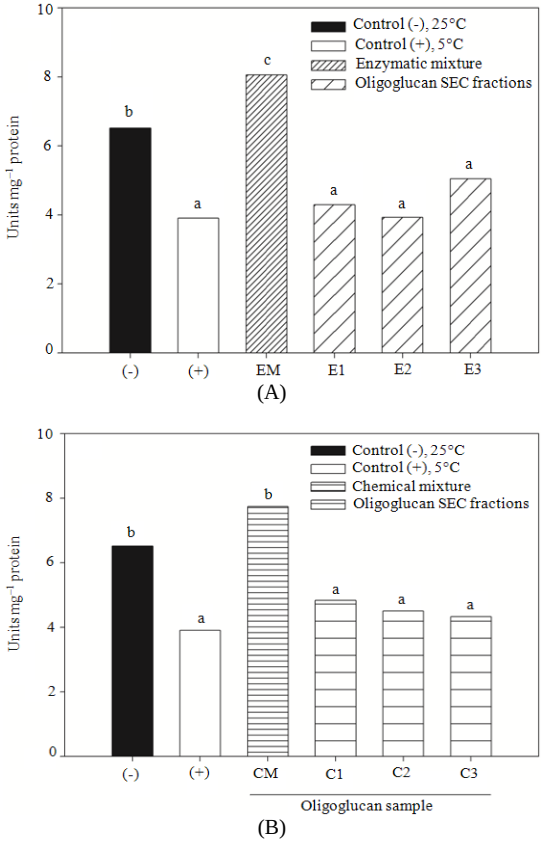

Fig. 2: Changes in SOD activity in cold-stressed zucchini cotyledons elicited with enzymatic (A) and chemical (B) oligoglucan mixtures. Both mixtures include their respective SEC fractions. Different letters indicate significant differences ($p \leq 0.05$) between values. Controls (-, +) correspond to non-elicited plants

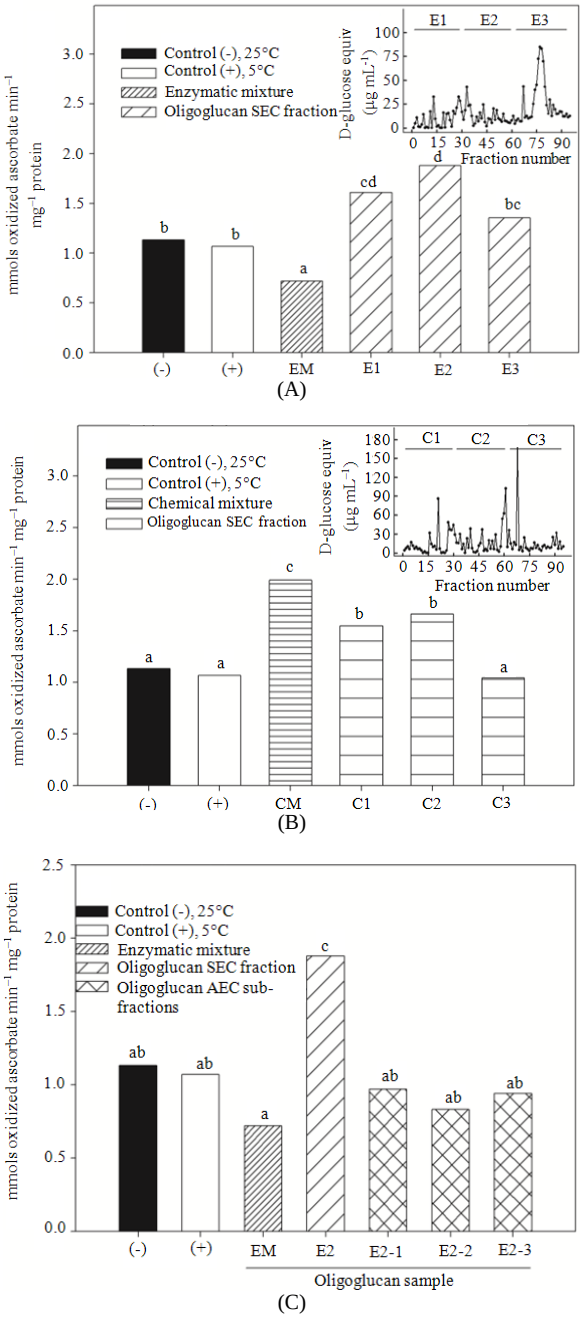

Fig. 3: Changes in APX activity in cold-stressed cotyledons elicited with enzymatic (A) and chemical (B) oligoglucan mixtures. Both mixtures include their respective SEC fractions and their AEC sub-fractions (only C). Different letters indicate significant differences ($p \leq 0.05$) between values. Controls (-, +) correspond to non-elicited plants. Inserts represent D-glucose contents from pooled enzymatic or chemical SEC fractions (A and B)

Changes in APX activity: The CM was the most effective treatment for triggering APX activity in cold-stressed cotyledons (Fig. 3B), whereas EM-elicited cotyledons showed the least APX activity level (36% less than control cotyledons). Several fractions from chemical and enzymatic OG SEC displayed the ability to trigger APX activity when applied to cold-stressed cotyledons. All of the enzymatic SEC fractions induced increments in APX activity with respect to control cotyledons ($p \leq 0.05$) mainly the E2 fraction (7% more than controls) followed by E1 and E3 (Fig. 3A). Only the C1 and C2 fractions induced increments with APX activity above control cotyledons but below the APX activity level triggered by CM (Fig. 3B). Due to the high induction of APX activity achieved by fraction E2, this fraction was further purified by AEC; sub-fractions E2-1, E2-2 and E2-3 were obtained and applied to cold-stressed cotyledons. No increase in APX activity was detected by effects of these enzymatic AEC sub-fractions (Fig. 3C).

Changes in CAT activity: The chemical OG mixture (CM) was more effective than EM for CAT activity enhancement in cold-stressed zucchini cotyledons (Fig. 4B). No apparent enhancing effect was attributed to EM, but when enzymatic SEC fractions were tested in cold-stressed cotyledons, these tissues showed high CAT activity levels. The main triggering effect was attributed to E1 (55.6% more than controls) followed by the E3 fraction (Fig. 4A). The E1 fraction was subjected to fractionation using AEC and then E1 sub-fractions were applied to cold-stressed cotyledons. The CAT activity levels were above control cotyledons and the greatest effect was achieved by the E1-1 sub-fraction (119% more than control plants, $p \leq 0.05$) followed by E1-2 and E1-3 (Fig. 4C). The same pattern was obtained in the CAT activity by the E3 sub-fractions (data no shown). In spite of the fact that CM was the most powerful OG mixture for triggering CAT activity, when this mixture was fractionated using SEC, no elicitor activity was detected by these chemical fractions. In contrast, when the chemical AEC sub-fractions were tested, the highest CAT activity level (120% more than control plants) was reached by the C3-1 OG sub-fraction (Fig. 4D).

DISCUSSION

World-crop production has been constantly influenced by detrimental effects of low temperature; this situation has been considered a serious problem because crop yields fell around fifty percent for several crops (Wahid *et al*., 2007).

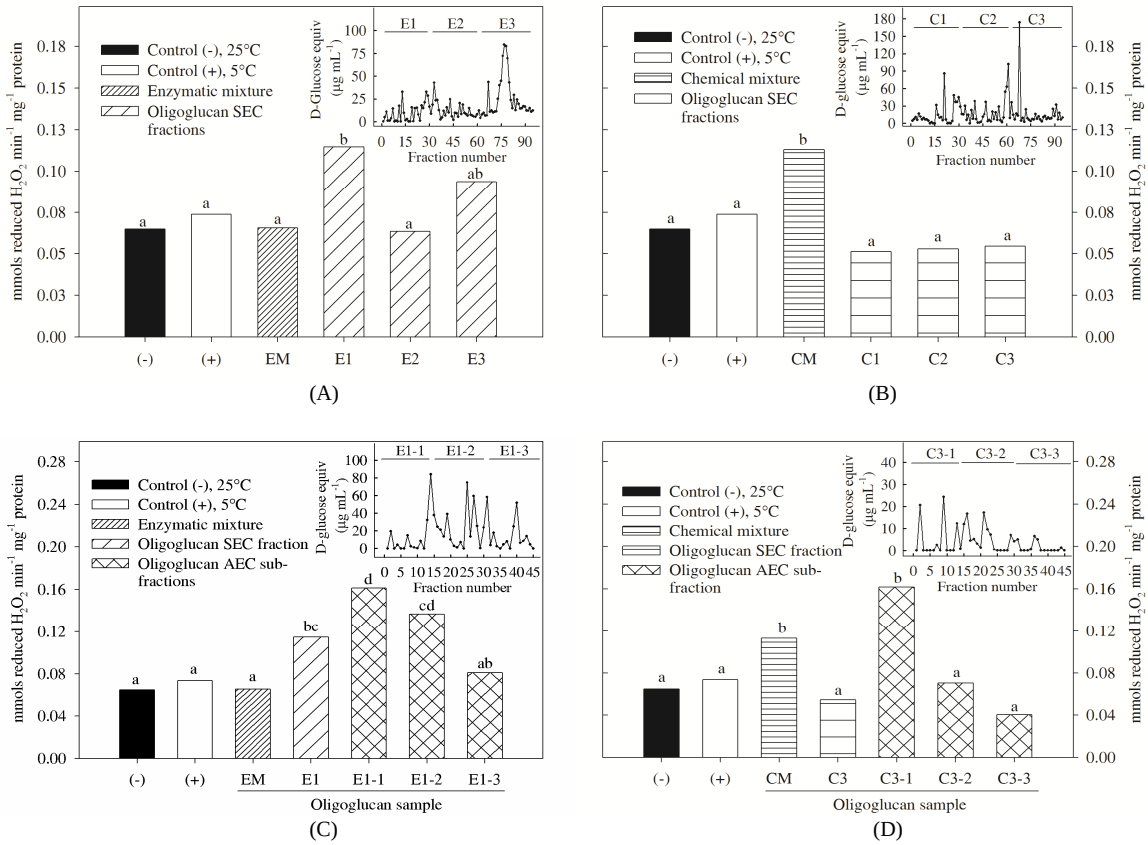

Fig. 4: Changes in CAT activity in cold-stressed cotyledons elicited with enzymatic (A) and chemical (B) oligoglucan mixtures. Both mixtures include their respective SEC fractions and their AEC sub-fractions (only C and D). Different letters indicate significant differences ($p \leq 0.05$) between values. Controls (-, +) correspond to non-elicited plants. Inserts represent D-glucose contents from pooled enzymatic or chemical SEC fractions (A and B) or AEC sub-fractions (C and D)

Important physiologic and metabolic plant processes are interrupted by cold-stressed conditions. Protein aggregation and denaturalization in chloroplasts and mitochondria, destruction of membrane lipids, production of toxic compounds and ROS overproduction (Howarth, 2005) are the most common responses of plant cells as a consequence of this type of thermal stress.

Zucchini cotyledons were subjected to cold-stressed treatment (5°C) to evaluate changes in the activity of antioxidant enzymes, such as SOD, APX and CAT, using fungal OG isolated by chemical or enzymatic hydrolysis from *T. harzianum* cell-walls. The IL and H_2O_2 production were the parameters used to indicate the level of damage in zucchini squash seedlings. When seedlings that had been cold-stressed at least for 7 day were brought to 25°C, cellular homeostasis recovery was observed as indicated by a decrease in an IL and H_2O_2 production. When seedlings were cold-stressed for longer than 7 day, the plasma membrane integrity, measured as IL, decayed severely and there was no cellular recovery. Values of IL above 50 % reflect serious membrane damage in cold-stressed cucumber fruit (4°C, 7 day) (Deell *et al*., 2000). At this level of membrane damage, important biochemical functions, such as the electron transport chain, the fixing of CO_2 or the electrolyte exchange between sub-cellular compartments, are irreversibly affected. Cold-stressed zucchini cotyledons showed an excessive IL value when stress exceeded 7 day.

Overproduction of ROS is an evident consequence of cold-stressed plants because the internal membrane damage directly affects mitochondrial respiratory activity and chloroplast CO_2 fixing is reduced (Esfandiari *et al*., 2007). Indeed, H_2O_2 levels in cold-stressed zucchini cotyledons increased significantly

after 7 d. This result reveals a typical response of low temperature treatments in stressed plants, such as cucumber, maize (Dat *et al*., 2000) and Gramineae grasses (Al-Taisan, 2010). Zucchini cotyledons showed a linear correlation between H_2O_2 accumulation and membrane damage measured as IL, contrary to the same parameters evaluated in barley plants infected with powdery mildew (Harrach *et al*., 2008). Even when biotic stress induced by phytopathogens varies in length and intensity, abiotic stress, such as low temperature, results in similar intracellular damage. Disturbances between environmental conditions and plant fitness are uncontrollable consequences that appear under field conditions and it has been one of the reasons for the plant vulnerability to extreme temperatures and crop yields reduction. The recovery of health using a fungal OG application could be achieved by triggering plant defense responses, which would avoid important economic and agricultural loses.

Once the low temperature conditions in zucchini cotyledons were established, different OG samples were applied for triggering enzymatic antioxidant activity. With respect to changes in enzymatic activities, cold-stressed condition by itself reduced SOD activity in zucchini cotyledons. Similar results in SOD activity have been previously reported in several cold-stressed rice lines (Rozkowicz *et al*., 2003). However, cold-stressed pre-flowering tobacco cv. "Petit Havana" plants (5°C for 4 day) showed an unaltered SOD activity level (Gechev *et al*., 2003). Increments in SOD activity levels were found in cold-stressed barley seedlings (2°C for 1 day) (Radyuk *et al*., 2009). Contrary to these contrasting results, under our experimental conditions, the low SOD activity level in cold-stressed zucchini cotyledons was reverted by application of OG mixtures, suggesting that the antioxidant system in cold-stressed zucchini cotyledons could be reinforced. These elicitors could have been recognized by non-specific OG receptors due to their complex chemical and structural composition, affecting the SOD activity. However, when the OG mixtures were subjected to SEC or AEC, no triggering was detected in cold-stressed cotyledons. We expected that the more purified the OG samples, the stronger the biological responses, but this was not observed regarding SOD activity. A different response was observed with the enzymes that detoxify H_2O_2 in cold-stressed zucchini cotyledons. These results showed a selective biological response to fungal OG derived from *T. harzianum* cell-walls.

Contrary to the effect on SOD activity, no changes in APX were detected in cold-stressed cotyledons, which is in accordance with the APX activity level in cold-stressed pre-flowering tobacco plants (Gechev *et al*., 2003). However, the fungal OG mixtures evaluated in this work display a well-differentiated effect in cold-stressed cotyledons. Clear differences between enzymatic and chemical OG mixtures (see inserts in Fig. 3A and B) may affect the recognizing process by zucchini cotyledons and, thus, display different APX activity levels. Some of the induced effects found in these experiments are an initial step in determining the complex role that these fungal elicitors play in up- or down-regulating the enzymatic antioxidant system activity in plants. First, differences in the effects of the fungal OG mixtures were evident under cold-stressed conditions. Moreover, significantly enhancements of APX and CAT activity levels were obtained by applying CM. These responses could be attributed to structural differences between *T. harzianum* OG mixtures and their accurate biological recognition by the host cell (Shinya *et al*., 2006, Falcón-Rodríguez *et al.,* 2009). Because the isolation procedure for both CM and EC were performed using a random or site-specific β-1,3 and β-1,6 degradation of *T. harzianum* cell-wall, respectively, the remaining OG had different structural and chemical characteristics that greatly influence their possible biological recognition and activity (Mithofer *et al*., 2001). In cold-stressed zucchini cotyledons, both chemical and enzymatic OG SEC fractions triggered APX activity. An elicitor isolated from the cell-walls of *Fusarium* spp. up-regulated the gene expression of several antioxidant enzymes in an *Arabidopsis thaliana* suspension cell culture (Chivasa *et al*., 2006), but many APX genes were simultaneously down-regulated. This fact is in disagreement with the global effects induced by *T. harzianum* OG in zucchini cotyledons, where both CAT and APX activity levels increased.

The most conclusive fact that OG mixtures have significant effects on the enhancement of the antioxidant system of cold-stressed zucchini cotyledons is the high CAT activity levels, similar to the effect in the enhancement of APX activity. A similar tendency was found in cold-stressed barley seedlings (Radyuk *et al*., 2009). Significant increments of CAT activity were obtained with E1 and E3 OG SEC fractions, which could be helpful to the plant antioxidant enzymatic system when dealing with unfavorable environmental conditions. The recognition of specific structural carbohydrates from these SEC fractions and AEC sub-fractions showed that OG with different molecular weights have the ability to increase CAT activity significantly (see inserts in Fig. 4). Biosynthesis of CAT is inhibited by cold stress (Hertwig *et al*., 1992); however, the fungal elicitors evaluated herein restored the CAT enzymatic levels in cold-stressed cotyledons.

The limited reports about the OG receptors, mainly involving soybean tissues, indicate that these elicitors are recognized by putative fungal glucan binding proteins anchored in the plasmatic membranes. In fact, the high specificity of these putative receptors reached nanomolar levels even when synthetic OG were evaluated (Fliegmann *et al*., 2005). A few reports reveal that the most used fungal OG from *Phytophthora* spp., have direct effects on the antioxidant system of potato (Mizuno *et al*., 2005) and soybean plants and cell cultures (Mithofer *et al*., 2001). The differential effects found between these antioxidant enzymatic activities may be due to differential and specific recognition capabilities of OG putative receptors (Shinya *et al*., 2006). The efficiency in biological recognition by specific membrane receptor could be affected by the structural and chemical composition of the fungal OG as a result of their isolation procedure.

In spite of the broad range of elicitation ability from different OG or even other types of oligosaccharides, there is not a rule that the same biological activity will be displayed upon different testing conditions in a selected model plant or tissues, such as suspension cell cultures, leaves, roots, seeds or fruits. Under stress conditions, squash seedlings must deal with the overproduction of ROS. The excessive accumulation of ROS could be the reason for transient inhibition of scavenging enzymes such as SOD. In cold-stressed zucchini cotyledons, peroxide production still continues along with other ROS enzymatic sources, such as NADPH oxidases, amino oxidase and flavin oxidases (De Gara *et al*., 2003).

CONCLUSION

The oxidative stress caused by ROS overproduction can be counteracted by triggering APX and CAT enzymatic activities in cold-stressed zucchini cotyledons, mediated by *T. harzianum* OG exogenous elicitation. It would be of great interest to investigate whether chemical and structural properties of these OG modify the antioxidant system activity in stressed plants. The results of this study could have important implications in restoring the adverse environmental effects in plants during their development, especially under non-controlled low temperatures.

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