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Enzymatic Activity in Tomato Fruits as a Response to Chemical Elicitors

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Abstract. The objective of this work was the evaluation of the peroxidase and catalase level of activity in the protein extract of tomato in response to different treatments with chemical elicitors in different stages of tomato development. The elicitor compounds chitosan at 0.1 % (w/v), 0.1 mM salicylic acid and 0.1 mM benzoic acid were sprayed on tomato fruits without applying them in the leaves and stems of the plant. The elicitors were sprayed at one of the following stages: fruit set, fruit growth and phase 3 of fruit ripening. The activity of the antioxidant enzymes catalase and peroxidase was determined in the phase 4 of fruit ripening, being found an increase in the activity of both enzymes upon applying the elicitors in certain stages of the fruit development. The chitosan applied during fruit growing, and the salicylic acid applied during fruit set increased the activity of catalase. The peroxidase activity increased significantly upon applying chitosan during fruit set and fruit growing, finding a smaller effect for the salicylic acid. Benzoic acid did not modify the peroxidase or catalase activity.

Key words: Benzoic acid, catalase, chitosan, peroxidase, salicylic acid, tomato.

Resumen. El objetivo de este trabajo fue la evaluación del nivel de la actividad de peroxidasa y catalasa en extracto proteínico de tomate en respuesta a los diferentes tratamientos con inductores químicos en diferentes estados de desarrollo del tomate. En forma exógena se aplicaron los inductores de tolerancia quitosano al 0.1 %, ácido salicílico 0.1 mM y ácido benzoico 0.1 mM en frutos de tomate sin aplicarlos en las hojas y tallos de la planta. Los compuestos inductores se aplicaron mediante aspersión a los racimos fructificados en alguna de las siguientes etapas: amarre, llenado y etapa 3 de la maduración del fruto. La actividad de las enzimas antioxidantes catalasa y peroxidasa se determinó en la etapa 4 de maduración, encontrándose aumento en la actividad de ambas enzimas al aplicar los inductores en ciertas etapas del crecimiento del fruto. El quitosano aplicado en el llenado de fruto y el ácido salicílico durante el amarre incrementaron la actividad de catalasa. La actividad de peroxidasa aumentó significativamente al aplicar quitosano en el amarre y llenado de fruto encontrando un efecto menor para el ácido salicílico. El ácido benzoico no modificó la actividad de catalasa o peroxidasa.

Palabras clave: Ácido benzoico, catalasa, quitosano, peroxidasa, ácido salicílico, tomate.

Introduction

Adaptation of plants to several types of stress depends upon a complex cellular sign system where reactive oxygen species (ROS), salicylates and cellulose and chitin oligomers [1, 2, 3] intervene. The presence of these elicitors activate the antioxidant and cellular defense systems against abiotic and biotic stress [4, 5], as well as fulfilling some development regulation functions [6] and tomato fruit ripening [7].

Chitosan (poly-N-acetyl-D-glucosamine), commercially prepared by alkaline deacetylation of chitin, seems to act as a stress tolerance inductor when directly applied to plant tissues, unchaining a hypersensible reaction and lignification [8], inducing lipid peroxidation, production of ROS, stoma closing and promoting the activation of defenses against pathogens [9]. In a previous work [10], the interpolyelectrolyte complexes of poly(acrylic acid)-chitosan were investigated as inductors of systemic resistance and the data showed that this treatment produced a higher resistance to the attack by *Fusarium oxysporum* and *Phytophthora capsici* pathogens in tomato seedlings. Indeed, the use of complexes showed a positive effect on the tomato seedling growth in the presence of *F.oxysporum* and *P.capsici* pathogens. On the other hand, in the absence of pathogens, the use of complexes increased the seedling weight.

Salicylic acid sprayed diminishes susceptibleness to pathogens harm and abiotic stress [11], increases fruit tolerance to cold conditions [12], and spans storage life [13]. It seems to act as a regulator over the oxidation/reduction balance of plant cells, inducing physiological, morphological, and adaptive responses in plants [5]. It participates in the activity of catalases and other enzymes which control EAO level [6] and mitochondrial oxydase [14].

Benzoic acid is another salicylate which, when applied to superior plants, modifies the growth, stress tolerance, anatomy and morphology of eatable and ornamental species [15].

Catalase (CAT) is an enzyme related to the cellular control of EAO level. Catalase catalyses the hydrogen peroxide dismutation in water and oxygen [16].

Peroxidase enzymes (PX) participate in hormone catabolism, phenol oxidation, polysaccharides and cell wall proteins intercrossing, lignin polymerization, fruit ripening, and defense against pathogens. During fruit ripening, and particularly during climacterium, peroxidase activity is increased along with the polygalacturonase and cellulase enzymes [17].

Even when different studies on the chitosan and salicylates effect on the vegetal tissues responses are found, less information about reproductive tissues response exists, especially in fruits. That is why the purpose of this work was to determine the level of enzymatic activity of peroxidase and

catalase induced by spraying chitosan, salicylic and benzoic acid in tomato fruits during several stages of their development.

Experimental

Methods and Materials

Tomato plants (*Lycopersicon esculentum* Mill.) from the "Rio Grande" variety (Petoseed) were used. The seeds were sowed on May 12, 2003 in a 200 cavities polystyrene tray, with peat moss TBK. The tray was placed in a floating bed of nutritious Douglas solution [18]. On the 14th of July, 80 seedlings were transplanted to 20 liter black polyethylene pots using PROMIX BX Canadian peat as a substrate. The pots were placed in a Colombian type greenhouse with passive ventilation, using the Bentley hydroponics technique [19]. A liter of Douglas fertilizing solution was applied every day until the start of flowering, 40 days after transplanting. Afterwards, 2 L by day were applied during flowering and fruit set, 65 days after transplanting, and 3 L by day during fruit growing and harvesting, 85 days after transplanting.

Chemical elicitors: salicylic and benzoic acid were supplied by ALQUIME and chitosan by Aldrich, its molecular weight was determined viscometrically in a solution of 0.2 M sodium acetate /0.3 M acetic acid at 30 °C by intrinsic viscosity and applying Mark-Houwink equation: $\eta = k \cdot M_v^\alpha$, where $k = 7.6 \times 10^{-2}$, $\alpha = 0.76$ [20] and was found equal to $M_v = 6.5 \times 10^4$. Deacetylation degree was determined by FTIR [21] and it was 83 %.

The applied concentrations of these compounds were as follows: chitosan at 0.1% (w/v) in acetic acid at 1 % (v/v), 0.1 mM salicylic and benzoic acid. Water was used as control.

Several floral racemes from different plants were marked 60 days after transplanting assuring at least 20 racemes per each of the 10 treatments. Elicitor spraying was carried out by means of aspersion in such a way that each raceme got only one spraying during a specific stage of its development. Treatments were: chitosan during fruit set stage, chitosan during fruit growing and chitosan during phase 3 of ripening. The same scheme was repeated for the salicylic and benzoic acid. The control was obtained when water was applied during fruit growing. Phase 3 was defined when at least 10 % and at most 30 % of fruit surface presented a change from green color to dark yellow, pink, red, or any combination of those colors [22].

All treated fruits were harvested when they arrived to phase 4 of ripening, when 30 % to 60 % of their surface showed a pink or red color. In such a stage, the highest values of antioxidant enzymes activity are expected [23]. The fruits were cut during the first hours in the morning to carry out the extraction and quantification of the enzymatic activity of catalase and peroxidase.

Extraction and determination of enzymatic activity

Catalase. Catalase extraction was made starting from 0.5 g of tomato pulp without skin in 5 mL of 100 mM buffer phosphates at pH 7, 50 mg of polyvinylpyrrolidone in a mortar previously cooled to 4 °C. The enzyme was obtained on the supernatant [24]. In order to determine the enzymatic activity of catalase, 5 mL of reaction mix was prepared in a test tube which contained: 15 μ L of 100 mM phosphate buffer at pH 6.8, 5 μ L of 100 μ M of H_2O_2 , 1 mL on the supernatant with the enzyme diluted 1:20, and distilled water to complete 5 mL.

The reaction mix was incubated for one minute at 25 °C. The reaction was stopped when 10 mL of H_2SO_4 at 2 % (v/v) was added. Residual H_2O_2 was titrated with a solution of 0.2 M $KMnO_4$, until a light purple color persisted for 15 minutes. A unit of catalase is defined as the quantity of enzyme necessary to decompose 1 μ M of H_2O_2 per minute at 25 °C [24].

Peroxidase. 0.5 g of peeled tomato pulp with 5 mL of 100 mM phosphate buffer at pH 6.8, in a mortar previously cooled at 4 °C were homogenized. The mix was centrifuged to 13000 rpm during 15 min at 4 °C. The on the surface containing the peroxidase enzyme is decanted and diluted to 1:20 degree. The enzymatic activity was determined with 6.25 μ L of 100 mM phosphate buffer at pH 6.8, 50 μ M of pyrogallol, 2.5 μ L of 50 μ M of H_2O_2 and 1 mL of enzyme extract diluted to 1:20, and distilled water to complete 5 mL.

The reaction mix (5 mL) was incubated for 1 minute at 25 °C, afterwards 0.5 mL of H_2SO_4 at 5 % (v/v) was added to stop the reaction. Purpurogallin concentration is measured to an absorbance of 445 nm. A unit of peroxidase is equal to 0.1 of absorbance [25].

In order to quantification the enzymatic activity, a totally random 4X3 experimental design was carried out with 3 replicates per treatment. The pulp of 3 different fruits was used as the experimental unity. The results of the determined variables were statistically analyzed by means of a variance analysis ($\alpha = 0.05$), using the SAS statistic software.

Results and Discussion

Catalase activity in tomato fruit

The results from the ANOVA statistical analysis indicated significant differences for the interaction treatments per application stage ($p < 0.05$). In effect, Figure 1 shows that chemical elicitors exert a different effect according to the stage in which they were applied. It was noticeable the increase in catalase activity in tomato fruit when chitosan was applied during the fruit growing, or salicylic acid during fruit set stage. On the other hand, the opposite happened when these compounds were applied during the phase 3 of ripening. Tasgin *et al.* [26] also found the salicylic acid

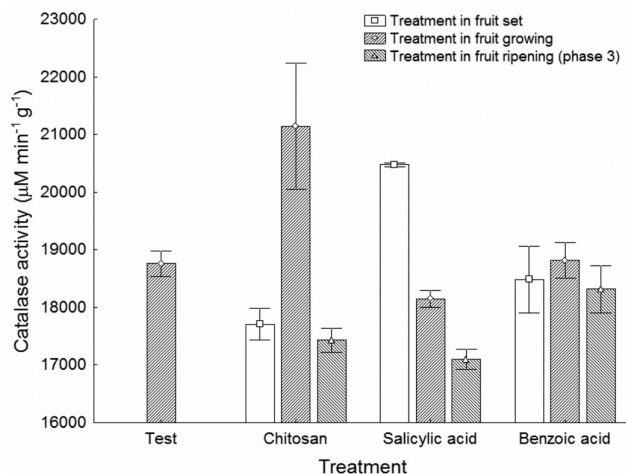


Fig. 1. Response of catalase enzymatic activity in tomato fruits treated with chemical elicitors: chitosan at 0.1 % (w/v), 0.1 mM salicylic acid and 0.1 mM benzoic acid.

applied to wheat plants altered the catalase activity in leaves, the opposite to what was observed on tomato fruit, that is, a reduction in catalase activity and an increase in peroxidase activity.

Salicylic and possibly benzoic acid are united in a specific manner to the enzymes containing iron such as catalases, aconitases and peroxidases [27]. Such a union modifies positively or negatively the enzymes activity, quickly changing the tolerance of plants and tissues to the oxidative stress [28]. Nevertheless, the results of these studies do not explain how catalase activity was modified on the phase 4 of ripening by the elicitors which were applied in earlier stages.

It is well known that the application of tolerance inductors modifies the metabolism and differential gene expression. These changes rule in the long term the tissue development [5]. Such an effect was observed when salicylic and benzoic acid were applied in without germinated seeds, giving seedlings more tolerant to low temperatures [29] and to salinity, even when such seedlings did not get any direct treatment. Our study showed that when applying chitosan or salicylic acid before fruit harvesting changed the catalase activity in fruits during the phase 4 of ripening. It can be thought that this effect was a consequence of the activity of some genes related to the cellular defense against stress [12].

The increase in the level of catalase was related to a greater tolerance to oxidative harm done by cold conditions [30]. The exogenous application of chitosan increased the tolerance to pathogens in unripe avocado fruit [31], but when applied to ripe tomato fruits diminished their firmness [32]; these responses somewhat opposite may be why some enzymatic responses, such as catalase, follow a particular pattern according to the stage where these elicitor compounds were applied.

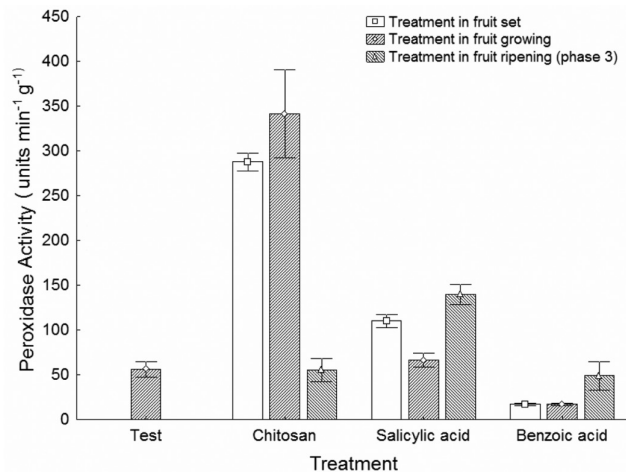


Fig. 2. Response of peroxidase enzymatic activity in tomato fruits treated with chemical elicitors: chitosan at 0.1 % (w/v), 0.1 mM salicylic acid and 0.1 mM benzoic acid.

Peroxidase activity in tomato fruit

The results from the ANOVA statistical analysis gave great differences between treatments ($p < 0.01$) as well as the interaction treatments x application stage ($p < 0.01$). As in catalase, it was observed that elicitors exerted a different effect on the peroxidase activity according to the application stage (Figure 2), although in this case chitosan produced the greatest differences against the control.

Plant peroxidase activity seems to be under the strict control depending on the development stage and the environmental stimulus [33]. Ali *et al.* [34] reported that peroxidase activity increased when higher temperatures were applied in *Phalaenopsis*. On the other hand, the activation of peroxidase is correlated to the defense responses of fruit in presence of pathogens [8]. A greater peroxidase activity with chitosan seems to indicate the effectiveness of this compound as an antioxidant system inductor of the plant.

Treatments where benzoic acid was applied did not show significant differences against the control. While treatments where salicylic acid was applied showed greater levels of activity as compared to benzoic acid, especially during the fruit set and harvest stages. This demonstrates the great capacity of salicylic acid of inducing physiological and adaptive responses in plants [35].

Conclusions

Exogenous application of chemical elicitors like chitosan and salicylic acid during different stages in fruit development notably increased the level of catalase and peroxidase enzymes activity in fruit tissue. Highest values of activation of the enzy-

matic activity were obtained when fruits were treated with chitosan at a concentration of 0.1 % (w/v) in the fruit growing stage.

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