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Changes in the Quantity of Phenolic Compounds in Peppers (*Capsicum Annuum* L.) Sprinkled with Elicitors Under Cold Stress

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Abstract: Pepper is one important cultivated peppers in Mexico to commercial and industrialized profiles, however, grow and development are affected by environmental changing, especially low temperatures that cause important culture damage. In these words, it was developed a defensive mechanism against abiotic stress activated by inductor agents. The peppers seedling defensive response was evaluated by spectroscopy quantitative analysis using phenolic compounds. There was a relationship between elicitor's concentration and chilling tolerance, and the best protection was obtained from plants treated with 6.7 mM salicylic acid after 24 hours. Our finding that salicylic acid could be used as a seed treatment to prevent crop losses in pepper due to chilling stress may have significant practical applications.

Keywords: pepper, elicitor, chilling temperature, abiotic stress.

INTRODUCTION

Pepper (*Capsicum annuum* L.), a member of the *Solanaceae* family, is a very important crop, its fruits being the second worldwide consumable vegetables and excellent sources of many essential nutrients for humans, especially vitamin C, phenolic compounds, β-carotene and calcium. Additionally, some pepper cultivars contain significant quantities of capsaicinoids, a group of pungent phenolic-derived compounds with strong physiological and pharmacological properties[1]. Thus, the growing global demand of pepper fruits implies several strategies to increase crop production and fruit quality or promoting the investigation to improve the

plant resistance to environmental stresses. Plants are frequently exposed to different environmental stresses, which can be both biotic and/or abiotic. These stresses cause biochemical alterations as generation of hydrogen peroxide (H_2O_2) resulting in an early response of the plant defense mechanism, the oxidative burst, the generation of reactive oxygen species (ROS) [2, 3]. However, this signaling cascade can also be activated by the use of elicitors, stable molecules that induce an immune defense response in plants [4].

Low temperature is an environmental factor that has a significant influence in plant growth affecting photosynthesis, uptake of water and nutrients, among others. Many economically significant crops, such as cotton, maize, pepper, rice, soybean, tomato, some tropical fruits and subtropical fruits are low temperature sensitive, which affects their production and quality[5]. Thus, it has been shown that low temperature regulates the expression of many genes [5,6], and there are biochemical changes that affect the level of a number of proteins, lipids and metabolites. These include the synthesis of low molecular cryoprotective sugars (proline and raffinose), antifreeze proteins, dehydrins, ROS scavenging enzymes and soluble antioxidants[5,7-10].

Consequently, the optimum growth temperature in pepper is between 25 and 30 °C, in such a way that temperature changes affect a variety of physiological functions and morphological development. When temperature decreases below 15 °C, pepper growth is reduced, and bloom and fruit production stop[11]. Low temperature affects pepper vegetative development and reproduction by disturbing the function of the flower female organs and the number of viable pollen grains per flower[12-14].

Taking into account the important agronomical relevance of pepper[15], the main goal of this work was to study the antioxidant metabolism, phenolic compounds specifically in this plant species under low temperature conditions, since this environmental stress considerably affects pepper growth. The results obtained showed that in pepper plants low temperature increases oxidative stress during the first 24 h but after this period, plants seem to recover by an acclimation of their metabolism, which involves important changes in their cellular antioxidant and redox state.

METHODS

Seedlings of peppers (*Capsicum annuum* L.) of approximately 20 cm high to which were applied with a treatment by spraying with salicylic acid (0.1, 6.7 and 10 mM), and hydrogen peroxide (6, 14 and 18 mM). Subsequently they were subjected to abiotic stress at low temperatures for 90 minutes at 10-12 °C and 90 minutes at 0 °C. Methanolic extractions were performed immediately after being subjected to stress conditions, as well as 24, 48 and 72 hours after treatment.

Methanolic Extraction: The extraction was performed according to the methodology described by [16]. A frozen sample (1 g) was placed in a 50 mL flask and mixed with 10 mL of methanol (J.T. Baker). The flask was protected from light and shaken (LABNET model S1000) at 40 rpm for 24 h at 25 °C after incubation, the samples were centrifuged at 4000g for 10 min.

Quantification of Condensed Tannins: Condensed tannins expressed as milligrams of (+)-catechin equivalents per gram of sample were quantified according to the methodology described by [17] in microplate. Briefly, 200 L of vanillin reagent (0.5% vanillin, 4% HCl in methanol) was added to 50 L of methanolic extract and placed in a 96-well plate; each sample was tested in triplicate. Condensed tannins were quantified at 495 and 540 nm in a microplate reader (Thermo Scientific Multiskan Go model 51119300) using (+)-catechin (up to 0.2 mg/mL) as a reference standard (Sigma-Aldrich). To correct for potential interference

from natural pigments in sweet pepper, a blank sample was prepared by subjecting the original extract to the same conditions of reaction without the vanillin reagent.

Flavonoids Content: The spectrophotometric assay for the of flavonoid content determination was quantified according to the methodology described by [18]. Briefly, the method consisted of mixing 50 L of the methanolic extract with 180 L of distilled water and 20 L of a solution of 10 g/L 2-aminoethyldiphenylborate in a 96-well plate. The absorbance of the solution was monitored at 404 nm with a microplate reader (Thermo Scientific Multiskan Go model 51119300). A rutin standard (Sigma-Aldrich) was prepared in 80% methanol. Extract absorption was compared with that of a rutin standard curve (0-50 g mL^{-1}). Flavonoid content was expressed as milligrams of rutin equivalent per gram of sample.

RESULTS

Complex network of biochemical processes in plants, there are routes that provide functionality defense compounds, including phenolic compounds are, Table 1 shows the concentrations of condensed tannins and flavonoids in each of the treatments, the concentrations of these compounds increases after treatment at low temperatures to flavonoids and condensed tannins in the first 24 hours, subsequent hours decreased at 48 hours, and in most cases showed a slight increase at 72 hours for the condensed tannins. The treatment that remained condensed tannins production during different periods were shown in the concentration of salicylic acid 6.7 mM, and hydrogen peroxide concentration 18 mM. The salicylic acid treatment maintained the production to flavonoids were shown in the concentration of 6.7 mM and all hydrogen peroxide treatments showed a loss of at least half of the initial concentration.

However it should be noted that work by[19], indicate that the salicylic acid is inductor thermo-tolerance of seedlings mustard protecting corn plants against stress by low temperature, as shown in Figure 1 the 6.7 mM salicylic acid concentration induces increased production of condensed tannins after 48 hours of treatment with cold and in Figure 3 at a concentration of 10 mM is observed a better response in the concentration of flavonoids in the first 24 hours.

One of the fastest plant responses after recognition of the pathogen is called "oxidative burst", consisting of the production of reactive oxygen intermediates, mainly oxygen radicals and hydrogen peroxide. The increase in production of hydrogen peroxide also results in environmental stresses such as excessive irradiation, drought and cold. Work by[20] in the banana cultivar Calcutta 4, which is resistant to black Sigatoka, the presence of reactive oxygen species occurs in the first 72 hours after infection. Figure 2 showed a significant increase in the tannin concentration at 72 hours after treatment at low temperatures induced by hydrogen peroxide 18 mM, likewise in Figure 4 showed that treatment with hydrogen peroxide promotes increased production of flavonoids.

During the period of time in which pepper plants were exposed to low temperature an overall induction of enzymatic and non-enzymatic antioxidant systems was observed. Catalase and ascorbate peroxidase, involved in the direct removal of H_2O_2, were induced after 24 h. In other plant species, the response of the antioxidative systems to low temperature stress depends of how low the temperature and how long the treatment are. For example, in rice, low temperature induces a rapid increase in ascorbate peroxidase activity and then gradual increases of superoxide dismutase activities what suggests differential regulations of these enzymes; conversely, catalase activity was not significantly affected[21]. In rice seedling exposed to heat (42 °C for 24 h) before low temperature (5 °C for 7 d). Plants did not develop chilling injury and a higher level

of ascorbate peroxidase activity and expression was found. In our case, the H_2O_2 content did not show any significant change by low temperature treatment, which could suggest a participation of this metabolite in signaling processes without inducing cell damages. In pepper plants, the phenotypic symptoms observed during the first and second treatments were accompanied by a rise of lipid peroxidation, which are considered biochemical markers of both oxidative stresses[22, 23].

Thus, in leaves it was observed that low temperature produces lipid oxidation in vascular tissues, what is in good agreement with the leaf flaccidity observed during the first 48 h of exposure to low temperature. The oxidative stress observed by low temperature treatment in leaves of pepper plants during the first and second treatment is in agreement with previous studies reported in maize plants[24, 25].

Table 1: Quantitative analysis in condensed tannins and flavonoids.

Treatment	Condenses Tannins (mg (+)-catechin equivalents per gram of sample)				Flavonoids (mg rutin equivalent per gram of sample)			
	Time hours							
	Immediately	24	48	72	Immediately	24	48	72
Control (without cold)	10.738^{c}				8.748^{a}			
Control (with cold)	61.868^{a}	53.432^{a}	17.366^{ab}	19.131^{bc}	5.774^{ab}	8.786^{ab}	3.447^{abc}	3.081^{bc}
Salicylic Ácid 0.1 mM	55.541^{a}	49.731^{a}	12.417^{b}	12.417^{cd}	4.455^{bcd}	7.088^{a}	3.197^{ab}	3.169^{cd}
Salicylic Ácid 6.7 mM	57.177^{a}	48.612^{a}	20.852^{a}	26.921^{ab}	4.999^{abc}	8.199^{a}	3.268^{bcd}	3.122^{ab}
Salicylic Ácid 10 mM	54.035^{a}	51.151^{a}	13.708^{b}	14.310^{cd}	3.449^{d}	8.288^{a}	3.219^{a}	3.150^{cd}
Hydrogen peroxide 6 mM	39.531^{b}	34.538^{a}	14.138^{b}	15.817^{bcd}	6.543^{a}	9.010^{bc}	2.253^{d}	2.253^{bcd}
Hydrogen peroxide 14 mM	43.964^{b}	33.462^{a}	16.548^{ab}	3.852^{d}	5.264^{abc}	10.137^{bc}	2.419^{bcd}	2.124^{d}
Hydrogen peroxide 18 mM	37.207^{b}	34.797^{a}	16.720^{ab}	31.870^{a}	3.569^{cd}	10.574^{c}	2.021^{abc}	2.217^{a}

Significant difference (P<0.05) is expressed by the different letters in the same row

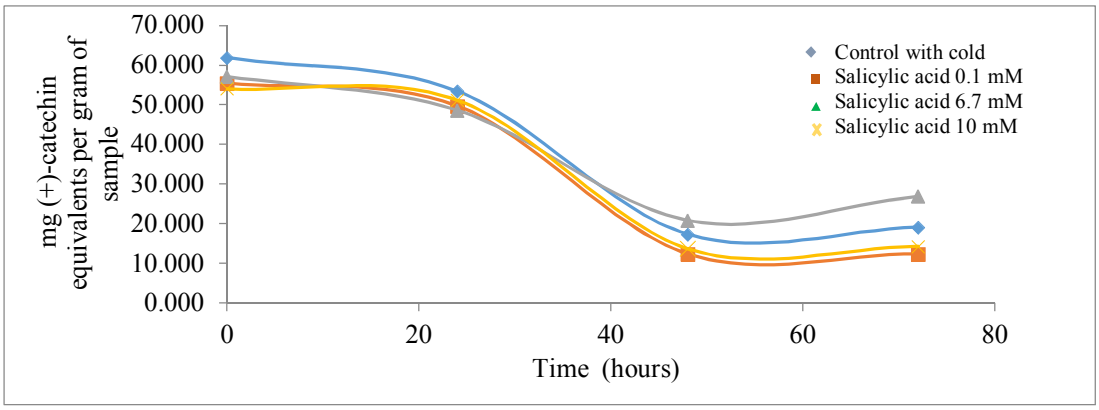

Figure 1: Analysis of condensed tannins in salicylic acid treatments.

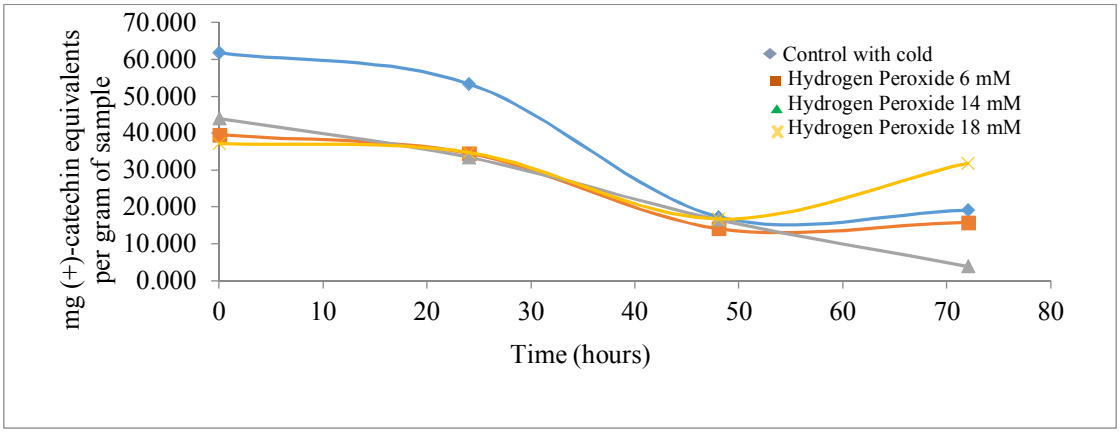

Figure 2: Analysis of condensed tannins in hydrogen peroxide treatments.

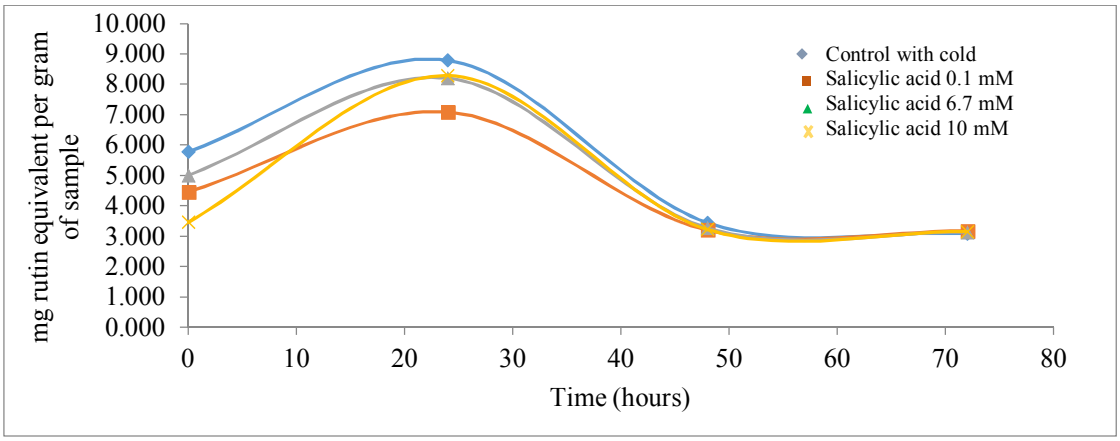

Figure 3: Analysis of flavonoids in salicylic acid treatments.

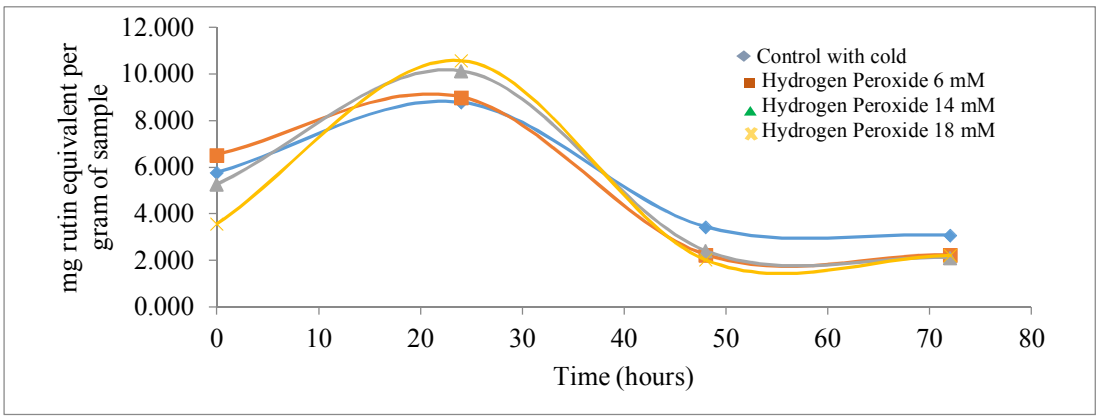

Figure 4: Analysis of flavonoids in hydrogen peroxide treatments.

CONCLUSION

The result of the present study revealed that pre-treatment with salicylic acid and hydrogen peroxide by foliar spray, were effective in inducing chilling tolerance in pepper seedlings. Foliar spray provided better protection against chilling stress. There was a curvilinear relationship between elicitor's concentration and chilling tolerance, and the best protection was obtained from plants pretreated with 6.7 mM salicylic acid after 24 hours. Our finding that salicylic acid could be used as a seed treatment to prevent crop losses in pepper due to chilling stress may have significant practical applications.

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