Technical Note

Expression of the peroxidase enzyme in hybrid *Saccharum* sp. plants inoculated with *Xanthomonas albilineans* Ashby (Dowson)

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Abstract

In this study the expression of the peroxidase enzyme (POX) in two sugarcane cultivars (hybrid *Saccharum* sp.) and the correlation with the response of each one to the invasion of the pathogen *Xanthomonas albilineans*, its causative agent, were evaluated. The evaluated cultivars were Mayarí 55-14 (tolerant) and Cuba 85-102 (susceptible); they were both inoculated at five months of age with the bacteria, and the controls, with sodium phosphate buffer. A completely randomized design, with three replications was used. Variance analysis (ANOVA) was performed on the data, through the statistical package Statgraphics plus version 5.1. In both cultivars the POX specific activity increased after the inoculation with the bacteria; in My55-14 it was higher with regards to C85-102 since two hours. The response to the pathogen infection was detected faster in the tolerant cultivar (2 hours) than in the susceptible one (4 hours). It is concluded that the peroxidase activity is involved in the defense processes of sugarcane against the bacteria *X. albilineans*; hence it can be used, because of its importance, in the identification of resistant genotypes to this pathogen, in the framework of the breeding programs of this plant, in order to guarantee obtaining higher quality biomass, for the sugar industry as well as for animal feeding.

Keywords: enzymatic activity, pathogen organisms, resistance to disease

Introduction

Hybrid *Saccharum* sp. is one of the crops used in livestock feeding, due to its forage value, to the fact that its biomass production volume is higher than that of other grasses and the fact that harvest is done in the dry season (Hernández, 2004), when there is higher feed scarcity for the animals.

One of the factors that influence remarkably the yield and, thus, the quality of sugarcane biomass is the noticeable incidence of pests. The leaf scald disease, caused by the bacteria *Xanthomonas albilineans* Ashby (Dowson), in its acute phase, has caused losses higher than 90 %, due to the reduction in the juice quality and quantity (Huerta-Lara *et al*., 2009).

Among the biochemical changes that occur in sugarcane as consequence of the invasion by *X. albilineans* the synthesis of molecules, such as polysaccharides (Blanch *et al*., 2008; Legaz *et al*., 2011); phenolic compounds; and several enzymatic systems, for example peroxidases (Santiago *et al*., 2009), which act in a combined way to prevent the dissemination of the pathogen in the plant, stand out.

Plant peroxidases (E.C.1.11.1.17), frequently known as class III peroxidases, are monomeric enzymes which participate in a wide range of physiological processes, such as: lignification, suberization, auxin metabolism, cell wall protein binding, salt tolerance, water stress (Ajithkumar and Panneerselvam, 2014) and defense against the attack of pathogens (Van Loon *et al*., 2006). They appear in plant tissues after infection by pathogens, and their expression in higher plants can be induced by bacteria (Legaz *et al*., 2011), fungi (Machado-Assefh *et al*., 2013) and viruses (Quistián and Valadez, 2011). Hence the identification of biochemical as well as molecular markers can be of great value in varietal identification (Arellano-Litardo *et al*., 2012) and in the characterization of genotypes in a breeding program, because it facilitates the selection for resistance to certain pathogens (Sharma *et al*. 2012).

In Cuba several studies have been conducted about the sugarcane-*X. albilineans* interaction for the evaluation of varietal performance; however, there is little information about the biochemical changes that occur in the plant as consequence of the pathogen invasion, specifically those that are related to the oxidative stress; as well as about the relation that could exist between them and the tolerance of a cultivar to disease.

Taking into consideration the importance of this crop in the production of sugar and other derivatives

as well as in animal feeding, the objective of this study was to evaluate the expression of the enzyme peroxidase in two sugarcane cultivars and the correlation with the response of each one to the invasion of the pathogen *X. albilineans*, its causative agent.

Materials and Methods

Obtainment and characterization of *X. albilineans*. The bacteria *X. albilineans* was isolated from plants with symptoms of leaf scald disease in the susceptible cultivar Lousiana 55-5 (L55-5), planted in areas of the Provincial Sugarcane Research Station Antonio Mesa (EPICA for its initials in Spanish), in the Jovellanos municipality –Matanzas, Cuba.

For such purpose plant extracts were obtained that were sown in Wilbrink medium and incubated at 28 ºC during 5 days, and the pertinent biochemical and serological tests (following the methodology used by Matos, 2002) were performed on the resulting colonies, to determine the presence of the bacteria *X. albilineans* and the serovar to which they belong. For the description of the morphological characteristics, the indicators growth time, color and size of the colonies were taken into consideration. The serological identification was done through the latex agglutination technique (Peralta *et al*., 1997). The pathogenicity of the colonies was tested by artificial inoculation in healthy sugarcane plants, cultivar L55-5, which were six weeks old. The inoculated plants were daily inspected and at 15 days the re-isolation of the pathogen was performed in Wilbrink cultivation method, as previously described.

The bacterial suspension used as inoculant was obtained from the cultivation of the colonies in Wilbrink liquid medium, during five days and incubated at 37 ºC. The cells were centrifuged (5 000 rpm per min. at 4 ºC), were washed three times with sterile distilled water to eliminate the components of the Wilbrink medium and were re-suspended in the sodium phosphate buffer (10 mmol L^{-1} at pH 7,5).

Sowing of the experiment. In the seed bank of the EPICA, plant cuttings without symptoms from cultivars My55-14 and C85-102, which had been previously reported as tolerant and susceptible to the disease, respectively, were collected (Matos, 2002). The cuttings were fragmented in pieces that had one bud, to which hydrothermal treatment was applied at 51 ºC during one hour, and were later planted in1 x 3 m beds, which had as substrate sterile soil and organic matter, in areas of the EPICA nursery. The trial was sown in November, in a completely randomized design with three replications.

The means of the climate conditions during the experimental stage were the following: temperature-20,2 ºC; humidity-78 %; rainfall- 9,5 mm; sun hours-7,6 h.

Inoculation of the sugarcane plants and sampling. Four treatments were used: plants of each cultivar that were inoculated with the bacterial suspension of *X. albilineans*, and with the sodium phosphate buffer (10 mmol L^{-1}, basis solution that was used to prepare that suspension, with pH 7,5), these last ones considered as controls.

The method used for the inoculation of the sugarcane plants, with and without the bacteria, was the infiltration with syringe, according to Díaz (2000). The infiltration was performed in the young leaves of the five-month old plants.

Random samples of leaves from different inoculated plants of each variety and of the controls were taken, before the infection (t = 0), and after the infection at 2, 4, 6, 24, 48, 72 hours; it was taken into consideration that the bacteria has slow growth and the disease symptoms are observed days after the infection, according to the report by Matos (2002).

The samples were taken in duplicate according to the above-mentioned times, were submerged in liquid nitrogen, and were later preserved at -20 ºC, until their processing in the laboratory.

Obtaining the extracts. One gram of leaves without the central nervure was weighed, macerated and homogenized with 2 mL of sodium phosphate buffer (0,1 mmol L^{-1}, at pH 7,5). It was centrifuged at 7 000 rpm and 4 ºC during 15 minutes. The supernatant obtained was used to make determinations of the enzymatic activity. The protein extract was stored at -20 ºC until its use.

Determination of the peroxidase activity. The peroxidase activity was determined by the continuous method. As substrates guaiacol (0,018 M) and hydrogen peroxide (30 %, adjusting the $A_{240\ nm}$ to 0,4 against distilled water) were used. The oxidation rate of guaiacol by the enzyme in the presence of hydrogen peroxide was determined, during five minutes, and the absorbance at 436 mm was measured in an Ultrospec 2100 pro UV/visible spectrophotometer. A unit of enzymatic activity was defined as the quantity of enzyme that can catalyze

the production of 1 µmoL per minute per milliliter of enz^{-1}.

Meanwhile, the protein concentration was analyzed through the method proposed by Lowry *et al*. (1951), and the absorbance reading was done at 750 nm in an Ultrospec 2100 pro UV/visible spectrophotometer, for which a pattern curve of bovine serum albumin (BSA) was elabrated from a mother solution of 1 mg mL^{-1}. The specific activity of the peroxidase enzyme was determined according to the expression:

Specific activity = enzymatic activity / concentration of proteins

Data processing. The data were processed through a simple classification variance analysis (ANOVA), and the means were compared by Duncan's (1955) test for 5 % significance, after verifying that they fulfilled the variance normality –Kolmogorov-Smirnov goodness of fit test– and homogeneity –Bartlett's test–. The indicators that did not fulfill the above-mentioned assumptions were compared through Kruskal-Wallis test and the means were contrasted by the Student-Newman-Keuls (SNK) multiple range test for 5 % significance. All this was done with the statistical package Statgraphics plus version 5.1 on Windows®.

Results and Discussion

The *X. albilineans* strains obtained from plants with symptoms of leaf scald in the cultivar L55-5 coincided with the characteristics reported in literature for the genus and species, which was corroborated through the above-described morphological, biochemical and serological evaluations (fig. 1).

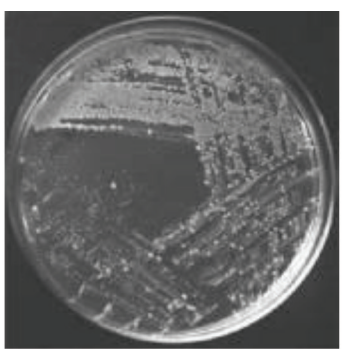


Figure 1. Typical colonies of the bacteria *X. albilineans.*

In the testing of the isolated strain pathogenicity the symptoms of leaf scald in the cultivar L55-5 could be reproduced, which proved the pathogenic capacity of the obtained isolates. In all the cases the bacteria *X. albilineans* was re-isolated and through the latex agglutination it was confirmed that the isolates belonged to previously reported serovar I (Matos, 2002).

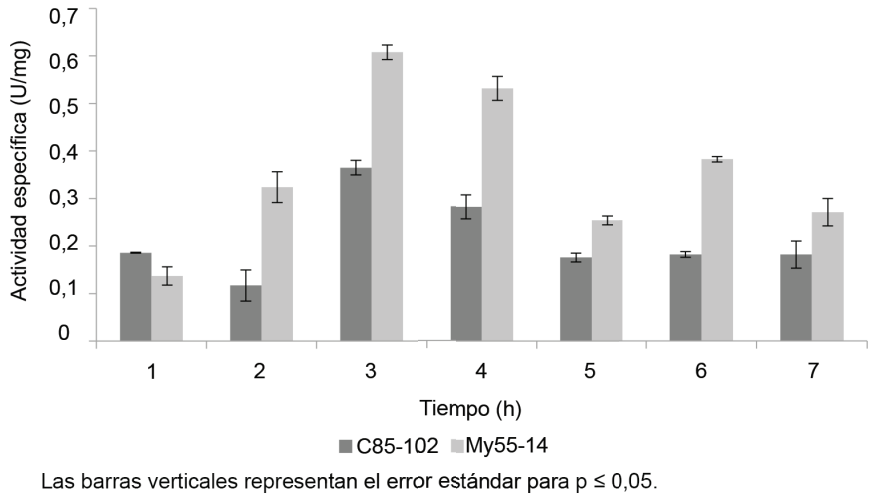
The phytopathogenic bacteria, at the moment of contact with the plant, trigger a wide group of reactions against the host which contribute to the success of the final invasion (Ryan *et al*., 2011). Among the mechanisms induced by the plant against the infection is the oxidative burst, whose objective is to stop the infection and the synthesis of compounds involved in the restriction of the invasive microorganism to the penetration area (Shama *et al*., 2012), such as, for example, the enzyme peroxidase, which has been evaluated in infected tissues, generally after damage or infection with increases in its activity (Machado-Assefh *et al*., 2013).

Figure 2 shows the results of the POX activity in the studied cultivars. Both reached constitutive values of the enzyme, represented by the control (t = 0); these basal values correspond to the metabolic activity of the plant under normal conditions. In the tolerant cultivar My 55-14 the POX activity started to increase after inoculation, for which the highest values of specific activity were reached at 4 hours, which coincides with the expected result, because the tolerant cultivar is capable of having a higher response to the pathogen invasion (Santiago *et al*., 2009).

In the susceptible cultivar C85-102, contrarily, the POX activity decreased 2 hours after inoculation with regards to the value of the activity of the enzyme at t = 0. At 4 hours the highest increase of the specific activity of the enzyme was observed, compared with the previous time and the later ones, although it was lower in the tolerant cultivar.

In general, the POX activity in the infected plants experienced an increase as response to biotic stress, which indicates that the machinery responsible for the elimination of H_2O_2 did not suffer any damage as consequence of the stress. On the other hand, the tolerant cultivar showed a more intense and earlier activation after infection than the susceptible one. Such results indicate that the accumulation of this catalyst is not restricted only to tolerant plants, but it can also be detected in susceptible interactions; nevertheless, in these cases, they can differ regarding the time of activity emergence and intensity (Bülow *et al*., 2004).

The study of the enzymatic activity also allowed a certain capacity of H_2O_2 detoxification



Las barras verticales representan el error estándar para p ≤ 0,05.

Figura 2. Actividad específica de la enzima peroxidasa en hojas de los cultivares de caña de azúcar evaluados, después de la inoculación con la bacteria X. albilineans.

to be observed in the infected plants with regards to the control plants. The increase of the specific activity coincides with the pathogen penetration process, which lasts between 4 and 36 hours after inoculation; as well as with the high coordination among the different signalization pathways in sugarcane, as response to different stress types (Damaj *et al*., 2005).

Table 1 shows the POX activity values depending on the cultivar and treatment.

In both cultivars there was an increase of the POX activity after inoculation with the pathogen; nevertheless, the tolerant cultivar My 55-14 significantly differed with regards to the control and the susceptible cultivar. It is possible that such differences can explain the resistance to the disease in these cultivars.

The results proved the importance of the enzyme peroxidase in the defensive response of the plant, by playing its protective role in the elimination of oxygen reactive species in leaf extracts (Diaz *et al*., 2010); as well as its usefulness for the selection of the degree of tolerance of sugarcane cultivars to leaf scald.

Other authors have reported similar results about the expression of the enzyme peroxidase and disease tolerance. Santiago *et al*. (2009) correlated the specific POX activity and other enzymatic systems in leaf discs of two sugarcane cultivars: My55-14 (tolerant) and L55-5 (susceptible), with different elicitor fractions obtained from *X. albilineans*. The tolerant cultivar My55-14 showed a high POX activity, compared with L55-5. All the elicitor fractions increased the enzyme activity with regards to the control, possibly to reinforce the cell walls.

Machado-Assefh *et al.* (2013) reported analogous results, when evaluating the peroxidase activity and its possible relation to the resistance or susceptibility of sugarcane cultivars to the brown rust caused by *Puccinia melanocephala* H. Sydow & P. Sydow, which increased after inoculation in both cultivars, but with higher speed in the resistant one. The differences in the enzyme activity were found between cultivars and also between the

Table 1. Results of the specific POX activity.

Cultivar	Treatment	Mean	SD
My 55-14	Control	0,246[b]	0,072
	Inoculated	0,470[a]	0,258
C85-102	Control	0,172[b]	0,035
	Inoculated	0,254[b]	0,134

The values show the mean for $p \leq 0,05$.

inoculated plants and the controls. This indicates that the increase of the peroxidase activity and gene expression could be related to the resistance to phytopathogens, and has been reported by different authors in plants subject to biotic stress: *Solanum lycopersicum* L. (Cerón, 2000), *Cicer arietinum* L. (García-Limones *et al*., 2002), *Capsicum annuum* L. (Do *et al*., 2003), *Bouteloua dactyloides* (Nutt.) Columbus (Gulsen *et al*., 2010) and *Panicum virgatum* L. (Saathoff *et al*., 2013).

The H_2O_2 produced by the plant, as defense response in the plant-pathogen interactions, induces the expression of genes related to the plant defenses, the pathways associated to the generation of the oxidative cascade (Sofo *et al*., 2015) and other endogenous cell protecting systems of the host, all of which limits the size of the lesion (Montoliu, 2010). The increase of the peroxidase activity, as consequence of the increase of H_2O_2 in the plants, could act as a catalyst in the polymerization of phenolic compounds for the formation of lignin and suberin, which acts as barrier to block the dissemination of the pathogen in the plant (Santiago *et al*., 2009).

From the above-described results, it is concluded that the evaluation of the peroxidase activity can be used, due to its importance, in the identification of sugarcane genotypes resistant to leaf scald, in the framework of breeding programs, in order to guarantee obtaining higher quality biomass, for the sugarcane industry as well as for animal feeding. Thus, it is recommended to evaluate this enzymatic system in the selection of sugarcane cultivars against this disease.

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