Quest Journals
Journal of Research in Agriculture and Animal Science
Volume 11 ~ Issue 12 (2024) pp: 12-16
ISSN(Online) : 2321-9459
www.questjournals.org

Research Paper

Expression and activity of Ascorbate Peroxidase (APX) enzyme in Persian lime with HLB by the application of resistance elicitors

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ABSTRACT: *Persian lime is one of the most economically important citrus crops in Mexico, yet its productivity is significantly affected by diseases, with Huanglongbing (HLB) being the most devastating worldwide. Currently, no effective treatment exists to mitigate HLB. However, the exogenous application of resistance elicitors can reduce disease severity by inducing antioxidant response mechanisms in plants under biotic and abiotic stress. These biochemical responses include the production of reactive oxygen species (ROS), which trigger oxidative chain reactions neutralized by antioxidant defense systems. Key enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) play a role in activating defense-related gene expression. This study aimed to evaluate the gene expression and enzymatic activity of ascorbate peroxidase (APX) in Persian lime plants infected with HLB following treatment with resistance elicitors. Treatments included salicylic acid (SA), gamma-aminobutyric acid (GABA), and their combination (SA+GABA) at 10 mM via foliar application over eight weeks. Gene expression was analyzed through RT-PCR, and APX activity was quantified. Results indicated that SA induced the highest APX expression and enzymatic activity, followed by SA+GABA and GABA alone. The findings demonstrate that chemical elicitors enhance APX gene expression and enzymatic activity, contributing to the plant's defense against HLB.*

KEYWORDS:

R*eceived 01 Dec., 2024;* ***R****evised 08 Dec., 2024;* ***A****ccepted 10 Dec., 2024 © The author(s) 2024.*
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I. INTRODUCTION

HLB (Huanglongbing) is the most devastating disease affecting citrus crops worldwide [1]. There are limited options for controlling the disease once plants are infected with the causal agent, *Candidatus* Liberibacter asiaticus. One of the main issues caused by the infection is the over accumulation of reactive oxygen species (ROS), particularly hydrogen peroxide, resulting from an exacerbated defense response and the inhibition of the plant's detoxification systems [2,3].

Consequently, it has been recently proposed that HLB management could involve mechanisms that enhance the plant's antioxidant activity [2]. In this context, the use of chemical resistance elicitors may play a significant role, as one of their primary functions when applied exogenously is to increase antioxidant activity [4].

Since one of the antioxidant enzymes most affected by HLB is ascorbate peroxidase (APX) [3], increasing the expression of this enzyme is critical for managing HLB. Therefore, the objective of this study was to determine the effect of applying resistance elicitors, specifically salicylic acid (SA) and gamma-aminobutyric acid (GABA), on the expression of the gene encoding APX and its enzymatic activity in the foliar tissue of Persian lime plants showing HLB symptoms.

II. MATERIALS AND METHODS

Experimental Setup

The study was conducted in the greenhouse and phytosanitary diagnostic laboratory of INIFAP, Ixtacuaco Experimental Station, located in Javier Rojo Gómez, Tlapacoyan, Veracruz, Mexico, with geographical coordinates 20° 02' 36" N latitude and 97° 05' 52.5" W longitude, at an altitude of 112 masl.

Plant Preparation

Twenty Persian lime plants (*Citrus latifolia* Tanaka), 1.5 years old with 2 to 3 branches, grafted onto Citrumelo Swingle rootstock, were used. These plants, previously infected with buds carrying Huanglongbing (HLB), were transplanted into sterile sand. The sand was sieved, packed into 2 kg plastic bags, and sterilized in an autoclave. It was then placed in black planter bags (40 x 40 cm), filled with sterile sand, and adjusted to allow proper drainage.

Preparation of Resistance Elicitors

Four treatments using resistance elicitors were evaluated:

Salicylic acid (SA) at 10 mM.
Gamma-aminobutyric acid (GABA) at 10 mM.
Combination of SA and GABA at 10 mM.
Sterile water as a control.

Application of Elicitors

The elicitors were applied via spraying directly onto the adaxial and abaxial leaf surfaces for eight weeks. A manual sprayer was used to ensure uniform coverage. Leaves were collected for molecular analysis at the end of the treatment.

Nucleic Acid Extraction

Leaves from each plant under the established treatments were collected, processed in liquid nitrogen, and transported to the laboratory. DNA extraction was performed following the protocol described by Rodríguez et al. (2010). DNA quality was assessed via electrophoresis.

HLB Detection via Nested PCR

Nested PCR was performed using specific primers for *Candidatus* liberibacter asiaticus. The reaction conditions included a standard Master Mix with the following concentrations:

Buffer 5x.
$MgCl_2$ 25 mM.
dNTPs 10 mM.
External primers (OF and OR) and internal primers (IF and IR) at specific concentrations.
Taq polymerase 1 U/µL.

The thermocycler program included an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles of 95 °C for 30 seconds, 67 °C for 45 seconds, and 72 °C for 45 seconds, with a final extension at 72 °C for 10 minutes.

RNA Extraction and Analysis

Total RNA was extracted using Trizol Reagent® following the manufacturer's protocol with modifications. RNA quality was assessed via electrophoresis. Complementary DNA (cDNA) was synthesized from RNA via reverse transcription using a kit from Promega Corp., ensuring the removal of contaminant DNA.

Gene Expression Analysis

Gene expression was analyzed via RT-PCR using antioxidant genes as internal controls (CsACT, CsF-BOX, CsUPL-7, and CsAPX). Conditions and primers were based on previous studies (Mafra et al., 2012; Xu et al., 2008; Pitino et al., 2017).

Protein Extraction and Enzymatic Activity Evaluation

The activity of the enzyme ascorbate peroxidase (APX) was evaluated colorimetrically following the protocol provided by Sigma Aldrich Corp. Sample readings were taken at 570 nm to determine enzymatic activity, defined as the amount of enzyme reducing 1.0 µmol of H2O2 per minute at 37 °C.

III. RESULTS

Identification of *Candidatus* liberibacter asiaticus

Using nested PCR, it was determined that the plants used in the experiment were infected with the bacterium causing HLB, and thus were considered positive for the disease (Fig. 1).

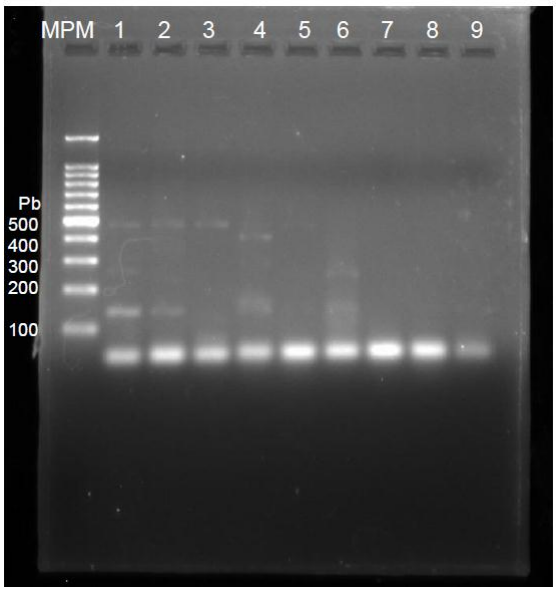

Figure 1. Molecular identification of *Candidatus* Liberibacter asiaticus in Persian lime plants.

Gene Expression Analysis

The RT-PCR results showed APX gene expression in treatments with salicylic acid (SA), gamma-aminobutyric acid (GABA), and a combination of SA and GABA (SA+GABA). The samples were visualized via 1.8% agarose gel electrophoresis in 1X TAE buffer.

Figure 2. Expression of the APX gene in trees infected with *Candidatus* liberibacter asiaticus after foliar application of treatments. Lanes: 1-2 (GABA), 3-4 (SA), 5-6 (SA+GABA), showing amplification at 240 base pairs (bp).

The inductive response of resistance elicitors to defense mechanisms, such as the activation of the APX antioxidant gene, was most pronounced with SA. Although GABA and SA+GABA also activated APX expression, their effects were less pronounced. The use of cDNA and APX gene insertion during PCR allowed amplification and visualization of this interaction, demonstrating how elicitors enhance systemic antioxidant mechanisms like APX.

Antioxidant Activity of APX

Standard Calibration Curve

A standard calibration curve for peroxidase activity was constructed to relate absorbance readings to enzymatic activity. Measurements were taken spectrophotometrically at 570 nm using positive HRP control and hydrogen peroxide substrate (1-5 nM).

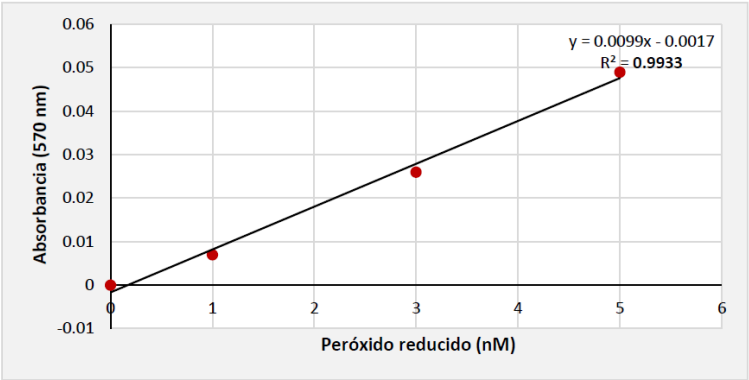

Figure 3. Standard curve for reduced peroxide quantification. Equation: y = 0.0099x - 0.0017, R^2 = 0.9933.

The high determination coefficient ($R^2 = 0.9933$) indicated a strong fit to the linear regression model. Enzymatic activity was calculated using absorbance data obtained at 0, 1, 3, and 5 minutes. Triplicate assays were performed for each treatment.

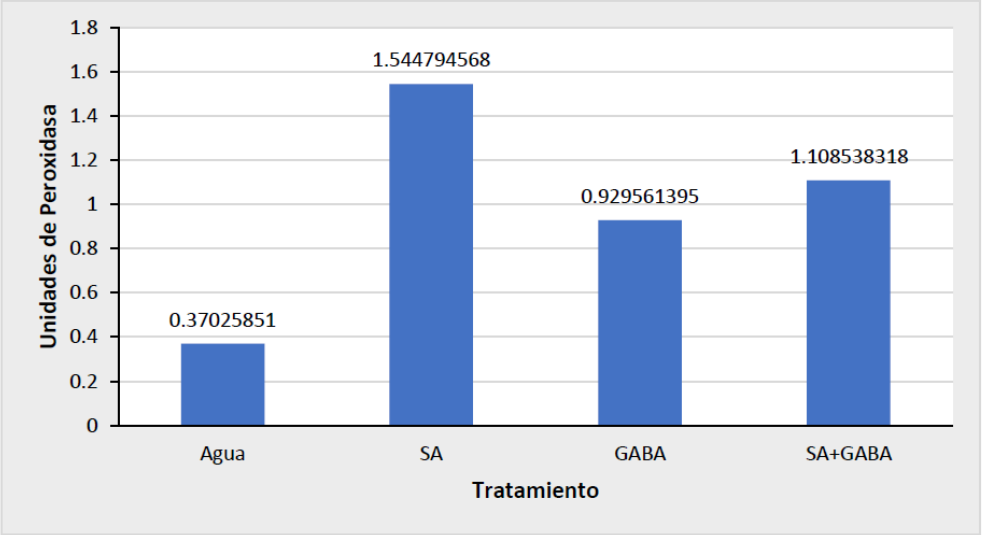

Figure 4. Ascorbate peroxidase activity under different treatments.

APX activity was positively affected by treatments, showing increased peroxidase units, which correlate with H2O2 reduction in infected plants. SA elicited the highest APX activity, followed by SA+GABA and GABA.

IV. DISCUSSION

The control of HLB in citrus focuses primarily on managing the vector *Diaphorina citri*, with limited information available on the pathogen *Candidatus* liberibacter asiaticus. The inability to culture the bacterium in vitro hampers detailed study, including its biochemical and biological roles, necessitating research into host-pathogen interactions.

The excessive accumulation of hydrogen peroxide caused by *Ca*Las results in toxicity, apoptosis, and plant death. Recent studies indicate that the pathogen suppresses antioxidant genes like CAT, APX, and SOD. This study demonstrated that resistance elicitors activate plant signaling molecules to induce defense hormones and antioxidant mechanisms, mitigating ROS effects. SA had the most significant impact on APX activity, highlighting its potential for managing oxidative stress in HLB-infected plants.

V. CONCLUSION

Our results show that the application of SA induces a higher expression of APX, which could be included in a management strategy for HLB disease in citrus crops.

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