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Physiological changes in tomato leaves arising from *Xanthomonas gardneri* infection

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ABSTRACT

Bacterial spot disease, which is caused by *Xanthomonas gardneri*, is one of the most important diseases affecting tomato production, and it can lead to significant losses in yield. Nevertheless, the tomato responses to this bacterial disease are not well studied. The present study aimed to evaluate the biochemical and physiological changes in the leaves of tomato plants (cv. Santa Clara) after infection by *X. gardneri*. Gas exchange and chlorophyll *a* fluorescence parameters, as well as the concentrations of photosynthetic pigments, were measured. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), non-specific peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione reductase (GR), as well as the concentrations of metabolites involved in oxidative stress responses (superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA)), were also evaluated. In plants inoculated with *X. gardneri*, the gas exchange and chlorophyll *a* fluorescence parameters, as well as the concentrations of photosynthetic pigments, were reduced. In general, during bacterial infection, the activity of SOD, CAT, APX, POX, GPX and GR was significantly higher in inoculated plants than in non-inoculated plants. The concentrations of $O_2^{\bullet -}$, H_2O_2 and MDA were also higher in the inoculated plants. Considering the extensive damage on leaf tissue caused by *X. gardneri* infection, the reactive oxygen species scavenging systems were apparently insufficient to reduce the increased levels of reactive oxygen species or the disease symptoms.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most profitable crops cultivated worldwide, and diseases contribute substantially to decreased yields [1]. Among the diseases affecting tomato plantations, bacterial spot disease, which is caused by bacteria from the genus *Xanthomonas*, is the most devastating, causing more than 50% of yield losses [1,2]. Currently, *X. euvesicatoria* (group A), *X. vesicatoria* (group B), *X. perforans* (group C) and *X. gardneri* (group D) are present in virtually all tomato-producing areas and are reported to be the causal agents of bacterial spot disease [3].

In Brazilian tomato fields, *X. gardneri* is the most prevalent bacterial species [4]. The bacterium is able to attack all aerial parts of the tomato plant, such as the leaves, stems, fruits and flowers, and the symptoms are circular brown lesions that appear water-soaked, especially under warm and moist conditions [3]. Lesions smaller than 3 mm in diameter easily coalesce when the plants are grown in locations with high relative humidity and with temperatures ranging from 24 to 30 °C [1]. The main control strategies to manage bacterial spot disease are the use of certified pathogen-free seeds and disease-free transplants, crop rotation, resistant cultivars and the application of copper-based products [1–3].

Plants that are exposed to biotic stress conditions, such as pathogen attack, may overproduce reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\bullet -}$) and the hydroxyl free radical (OH^-), which are highly reactive and toxic [5,6]. Excessive ROS can trigger damage to proteins, lipids, carbohydrates and DNA, ultimately resulting in oxidative stress and often leading to cell death [7–9]. To mitigate this damage, plants have developed mechanisms for ROS removal involving the production of a range of enzymes (*e.g.*, superoxide dismutase and catalase) and non-enzymatic components in a highly orchestrated antioxidant system [9]. Among these enzymes, superoxide dismutase (SOD) acts first and promotes the anion dismutation of $O_2^{\bullet -}$ to H_2O_2, which is further detoxified by a range of other enzymes such as non-specific peroxidases [9,10].

Photosynthesis may be regarded as a component of the

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integrated plant system, and it is a physiological process that is sensitive to several types of abiotic and biotic stresses [11,12]. Photosynthesis is the key physiological process affected by foliar pathogens, and its proper assessment can provide crucial insights into the mechanisms underlying a given host–pathogen interaction [11,13–15]. The technical evaluation of chlorophyll *a* fluorescence is a very useful tool for monitoring the photochemical activity of photosynthesis, and imaging techniques can be used to map changes in key parameters associated with photosynthesis in the affected leaves [16,17]. Particularly when combined with gas exchange measurements, these assessments can provide a detailed spatiotemporal analysis of how an infected leaf may respond to infection by a given pathogen [16,17]. The maximum photosystem (PS) II photochemical quantum efficiency, which is often assessed using the variable-to-maximum Chl *a* fluorescence ratio, has been widely used to compare healthy and infected leaf tissues [17].

Because the resistance of plants to a wide variety of stress conditions, including pathogen attack, has been associated with an increase in antioxidant activity in the leaves, it was hypothesized that infecting tomato leaves with *X. gardneri* would stimulate the tomato antioxidant system as a primary defense response. Thus, considering the aforementioned observations, this study aimed to provide an in-depth analysis of the antioxidative metabolism and photosynthetic performance of tomato leaves challenged with *X. gardneri* by combining chlorophyll *a* fluorescence imaging with measurements of gas exchange and photosynthetic pigment pools.

2. Materials and methods

2.1. Plant cultivation

Tomato seeds from the Santa Clara cultivar (Isla Sementes, São Paulo, Brazil), which is susceptible to *X. gardneri*, were surface-sterilized in 10% (v/v) NaOCl for 2 min, rinsed in sterile water for 3 min and germinated on distilled water-soaked germtest paper in a germination chamber (MA-835/2106UR; Marconi, São Paulo, Brazil) at 25 °C for 6 days. The seeds were sown in plastic pots containing 2 kg of Tropstrato® substrate (Vida Verde, Mogi Mirim, São Paulo, Brazil), which was composed of a 1:1:1 mixture of pine bark, peat and expanded vermiculite. A total of 1.63 g of monobasic calcium phosphate was added to each plastic pot. Five seeds were sown per pot. Five days after seedling emergence, each pot was thinned to one seedling. Fifteen days after sowing, the plants received 50 mL of nutrient solution containing 192 mg L^{-1} KCl, 104.42 mg L^{-1} K_2SO_4, 150.35 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 61 mg L^{-1} CH_4N_2O, 0.27 mg L^{-1} $NH_4MO_7O_{24} \cdot 4H_2O$, 1.61 mg L^{-1} H_3BO_3, 6.67 mg L^{-1} $ZnSO_4$, 1.74 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 4.10 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 4.08 mg L^{-1} $FeSO_4 \cdot 7H_2O$ and 5.58 mg L^{-1} disodium EDTA. This solution was applied weekly. The plants were kept in a greenhouse during the experiments and were watered as needed.

2.2. Inoculum production and inoculation procedure

The *X. gardneri* isolate (UFV-DFP *Xg*012) was obtained from the culture collection of the Laboratory of Phytobacteriology, Plant Pathology Department, Federal University of Viçosa, Minas Gerais State, Brazil. The isolate UFV-DFP *Xg*012 (previously preserved in a freezer at −80 °C) was grown in Kado's medium 523 [18] for 48 h at 28 °C. A 0.85% saline solution was added to the Petri dishes, and the resulting bacterial suspension was measured with a spectrophotometer (Analyser®) at 540 nm (an optical density of 0.3 was equal to 5×10^8 CFU mL^{-1}) and adjusted prior to inoculation. The plants were inoculated using an atomizer VL Airbrush (Paasche Airbrush Co., Chicago, IL) 38 days after sowing, when they had 5 to 6 fully expanded leaves. After inoculation, the plants were kept in a moist chamber at 25 °C for 24 h and were then transferred to a greenhouse at 25 ± 3 °C with a relative humidity of 75 ± 5%; they remained in the greenhouse until the end of the experiments.

2.3. Assessment of bacterial spot severity

The third and fourth leaves, from the base to the top, of each plant per replication of each treatment were marked and collected to evaluate bacterial spot severity at 3, 6, 9 and 18 days after inoculation (dai). The collected leaves were scanned at 300 dpi resolution and the obtained images were processed using QUANT software [19,20] to obtain the severity (chlorosis and necrosis symptoms) values.

2.4. Determination of enzyme activity

For all biochemical analyses, leaf samples were collected from the second, third and fourth fully expanded leaves (counting from the base to the top) of non-inoculated and inoculated plants at 3, 6, 9 and 18 dai. The samples were quickly frozen in liquid nitrogen during sampling and then stored at −80 °C for further analysis. The analysis of the enzymatic activity of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), non-specific peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione peroxidase (GPX, EC 1.11.1.9) and glutathione reductase (GR, EC 1.8.1.7) was performed as described by Ref. [21], with some modifications.

2.5. Determination of $O_2^{\bullet -}$, H_2O_2 and MDA concentrations

The $O_2^{\bullet -}$ concentration was determined based on the methodologies proposed by Refs. [22,23], with some modifications. Samples of 200 mg of leaf tissue were ground in liquid nitrogen, and the resulting powder was homogenized in 2 mL of 100 mM sodium phosphate buffer (pH 7.2) containing 1 mM diethyldithiocarbamate to inhibit SOD activity. The homogenate was centrifuged at 22,000 g for 20 min at 4 °C. The supernatant (300 μL) was added to 700 μL of a reaction medium of 100 mM sodium phosphate buffer (pH 7.2) containing 1 mM diethyldithiocarbamate and 0.25 mM nitro-blue tetrazolium. The $O_2^{\bullet -}$ anion was measured by its ability to reduce nitro blue tetrazolium chloride. The concentrations of H_2O_2 and MDA were determined as described by Ref. [21] with some modifications.

2.6. Analysis of photosynthetic pigments

The concentrations of chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*) and carotenoids were determined using dimethyl sulfoxide (DMSO) as a solvent [24]. Five disks (10 mm in diameter) were punched from the second, third and fourth leaves (counting from the base to the top of each plant) at 3, 6, 9, 12 and 15 dai. The disks were immersed in glass tubes containing 6 mL of DMSO solution saturated with 5 g L^{-1} calcium carbonate [25] and kept in the dark for 24 h. The absorbance of each extract was read at 480, 649 and 665 nm using a calcium carbonate-saturated solution of DMSO as a blank.

2.7. Leaf gas exchange parameters

The net carbon assimilation rate (A), stomatal conductance to water vapor (g_s), transpiration rate (E) and internal-to-ambient CO_2 concentration ratio (C_i/C_a) were measured at 3, 6, 12 and 15 dai in the third and fourth fully expanded leaves (counting from the base to the top) of each plant. These leaves were used in all analyses. The measurements were performed from approximately 0800 to