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CO_2 Assimilation of Bean Plants (*Phaseolus vulgaris* L.) in Response to Defense Activators to *Xanthomonas axonopodis* pv. Phaseoli and its Variant Fuscans

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A B S T R A C T

Common bacterial blight is one of the most important diseases in bean crop. Currently, the use of beneficial microorganisms to protect agricultural crops is booming, however, the control efficiency achieved with its application is even lower than conventional methods, which suggests more studies to understand the site and mechanism of protection. The present work was carried out with the purpose of evaluating the effect of the phytohormones jasmonic and salicylic acid, and of the beneficial *microorganisms Trichoderma asperellum* and *Bacillus pumilus* as foliar treatments for the protection against the bacterial common blight disease in bean plants. The phytopathogenic bacteria that cause this disease were isolated and identified through biochemical tests. The effect of these bacteria and the treatments on the assimilation of CO_2, stomatal conductance and opening of bean plants were evaluated. Isolation of the bacteria *Xanthomonas axonopodis* pv. phaseoli(*Xap*) and *Xanthomonas axonopodis* pv. phaseoli var. fuscans(*Xapf*) was achieved. The analysis of variance of the evaluated variables indicated a significant difference due to the effect of phytopathogenic bacteria and treatments. The plants inoculated with the *Xapf* bacteria showed greater severity of the disease. The plants treated with jasmonic acid presented higher tolerance to the causal agents with respect to the rest of the treatments and reduced the assimilation of CO_2. No statistically significant difference was found between the rest of the treatments and the control.

Introduction

Systemic resistance acquired and induced are natural phenomena that occur in plants in which there is an increase in tolerance to diseases. This increase is achieved through biotic and abiotic stimuli, and is also achieved with the exogenous application of some organic and inorganic compounds, beneficial microorganisms, as well as phytohormones and metabolites produced by plants (Martínez-Medina *et al.,* 2017; Pieterse *et al.,* 2014). Many plant species respond to the contact with different phytopathogens, such responses as the formation of structural barriers (Traw y Bergelson, 2003), are part of the first plant defense line. Although the multiplication of pathogens is usually specific to particular organs, their entry frequently takes place at distant sites due to the wide presence of natural openings or wounds caused in plants (Melotto *et al.,* 2008).

Phytopathogenic bacteria, unlike other pathogenic microorganisms, have the ability to enter through natural openings, such as stomata, wounds on plants, or transmitted by seed (Dutta *et al.,* 2014). The mechanism of opening and closing stomata plays an important role in restricting the entry of phytopathogens. However, this mechanism directly impacts the water metabolism of the plant, which is reflected in the stomatal conductance and photosynthetic rate (Su *et al.,* 2017; Melotto *et al.,* 2008). Melotto *et al.,* (2006) showed that stomata have different stomatal opening and closing patterns in response to contact with microbial pathogens. A key observation they showed was that these openings close in response to some bacteria. They also pointed out that pathogens have developed the ability to stimulate the reopening of stomata to facilitate their invasion into the leaves. Certain phytopathogenic bacteria such as *Pseudomonas syringae* have served as a useful model for studying the interactions of pathogenic microorganisms with plants (Xin *et al.,* 2018). However, until recently, studies were outlined to decipher the mechanisms used by other bacterial pathogens to adapt to their plant hosts, particularly those of the genus *Xanthomonas* (Denancé*et al.,* 2016). This genus is made up of 27 species which cause diseases to more than 400 plant species, among which there are many crops of high economic importance. These bacteria are characterized by having extracellular polysaccharides (such as xanthan) that serve as a virulence factor (Dow *et al.,* 2003). Bean (*Phaseolus vulgaris* L.) as an economically important crop are hosts of the species *Xanthomonas axonopodis*pv. phaseoli and *Xanthomonas axonopodis*pv. phaseolivar. fuscans which cause symptoms of leaf blight, being the second most virulent species that causes losses of up to 38% in the crop yield (Wallen and Jackson, 1975). It is likely that these bacteria similarly modify the stomatal opening of bean plants, thus it is important to understand the mechanism of opening and closing of stomata, as a primary defense line of plants mediated by defense inducers.

On the other hand, for plant protection, different beneficial microorganisms, such as the natural antagonists *Trichoderma* spp. and various growth promoting bacteria such as *Bacillus* spp. (Kumar *et al.,* 2015).

Likewise, several organic and synthetic compounds have been used to induce an increase response in the defensive capacity of plants. However, the exogenous application of standard substances that are part of the main defense pathways, salicylic acid for the "Acquired Systemic Resistance" and jasmonic acid for the "Systemic Resistance Induced", are valuable as experimental controls in the induction tests. According to the above, the present work was carried out with the purpose of recording the stomatal conductance and

opening and photosynthetic rate of bean plants (*Phaseolus vulgaris* L.) in response to treatment with the beneficial microorganisms *Trichoderma asperellum* and *Bacillus pumilus*, of the phytohormones salicylic acid and jasmonic acid, and inoculation with *X. a.* pv. *phaseoli* and *X. a.* pv. phaseolivar. fuscans; causal agents of the common bacterial blight".

Materials and Methods

Isolation of bacterial pathogens

The leaves of bean var. "Pinto Nacional" plants were collected at field, with symptoms of common bacterial blight (CBB), to which the epidermis was disinfected in a 2% sodium hypochlorite solution for two minutes. From the disinfected leaves, small portions of 1 cm^2 were taken from the margin of the leaf ticks to macerate them in sterile distilled water contained in a test tube.

From the resulting suspension of the mash, serial dilutions were made up to 10-3 of which 100 µL were taken to place it in TB semi-selective medium TB for *Xanthomonas* (10 mL of Tween 80, 10 g of Bacto Peptone, 18 g of Bacto agar, 10 g of KBr, 0.250 g of $CaCl_2$, 0.2 g of H_3BO_3, 75 mg of Cycloheximide, 25 mg of Cephalexin, 6 mg of 5-Fluorouracil, and 1000 mL of distilled water) (Schaad *et al.*, 2001).

The identification of the isolations was made by observing the morphological characteristics such as cell shape, motility, and spore formation, and by biochemical tests in which gram staining was observed, growth in TB medium, pigmentation of the medium, striation of the medium, starch hydrolysis, gelatin liquefaction, levan production, indole synthesis, aerobiosis, nitrate reduction, and fermentation of carbon compounds (glucose) (Abo-Elyousr, 2006; Almeida *et al.*, 2014).

Preparation of treatments and inoculation of bacteria

The treatments applied were: salicylic acid (SA) 2 mM (Sigma-Aldrich), jasmonic acid (JA) 0.5 mM (Sigma-Aldrich), *Trichoderma asperellum* (Ta) (10^5 spores/ml), *Bacilluspumilus* (B) (10^5 CFU/(ml), and control (only distilled water). The beneficial microorganisms used in this study were previously identified (Castillo-Reyes *et al.*, 2015, 557; Osorio-Hernández, 4596). Bacterial treatments and inoculations were applied in bean seedlings at 15 days after emergence. The inoculation of the isolated phytopathogenic bacteria, identified as two pathogens of *Xanthomonas axonopodis* (*Xap* and *Xapf*, Table 1), was performed 24 hours after the application of the treatments, which were collected from the growth medium based on nutrient agar. Two suspensions were formed with the colonies, which were adjusted to a concentration of 10^8 CFU/ml by nephelometry according to the McFarland scale. The inoculation was done by spraying 10 mL of the suspensions to each of the seedlings. After the inoculation, the plants were placed in a greenhouse at a temperature of 20 to 30°C with a humidity that ranged between 60 to 80% and were observed daily until the onset of symptoms.

Incidence and severity of the disease

The incidence and severity of the disease was recorded 21 days after the inoculation of phytopathogenic bacteria. The incidence registry was determined considering the percentage of leaves with symptoms of the disease per plant. The severity was determined on a scale of 1 to 9 according to Ogenga-Latigo *et al.*, (1993). The scales were:

1 = 0%, 2 = 1%, 3 = 5%, 4 = 10%, 5 = 20%, 6 = 25%, 7 = 50%, 8 = 75%, and 9 = 85%.

The experiment was conducted under a completely randomized design with 3 plants per experimental unit and 3 replicates per treatment, and a means test was performed with Tukey ($\alpha \leq 0.05$).

Measurement of stomatal conductance and aperture and photosynthetic rate

Stomatal conductance was quantified with a foliar porometer (SC-1, Decagon Devices, Pullman, WA) 21 days after inoculation. In order to measure the stomatal opening, epidermal impressions of the leaves were taken. The leaf impressions were obtained with PVC glue applied with a brush on the abaxial epidermis of the leaves on which was placed a transparent adhesive tape that was fixed on a slide for observation in a compound microscope. The stomatal opening was obtained by measuring the opening of the central stomata of 3 visual fields per leaf at 40x magnification with a compound microscope with integrated digital camera and the measurement was made with the AxioVision software Rel. 4.8. The photosynthetic rate was measured 22 days after the bacterial inoculations with a foliar CO_2 assimilation measurement equipment (LI-COR 6400). The readings of the stomatal conductance and opening and photosynthetic rate were made with 3 replications per treatment and 3 plants per experimental unit.

The data were subjected to an analysis of variance and were conducted under a factorial design in a completely randomized arrangement and a means test was performed with Tukey ($\alpha \leq 0.05$). The graphic outputs show the means with vertical bars that denote the standard deviation. In cases where no significant difference was observed, orthogonal contrasts were performed using the GLM procedure with the statistical software Statistical Analysis System (SAS Institute, 2002).

Results and Discussion

Isolation of bacterial pathogens

Two isolates were obtained that presented the formation of yellow colonies but with differences in the pigmentation of the TB culture medium, which visually corresponded to the bacteria *Xanthomonas axonopodis* pv. phaseoli (*Xap*) and *Xanthomonas axonopodis* pv. phaseoli var. fuscans (*Xapf*). This led us to carry out the identification through morphological and biochemical tests. The characteristics are listed in Table 1.

Both isolates showed the same biochemical properties, but with a difference in the pigmentation of the culture medium of the *isolate* 2. By means of the pathogenicity tests it was observed that said *isolate* caused the typical symptoms of the BCB but with greater severity of the disease, unlike the *isolated* 1. The greater severity of CBB disease has been observed in plants affected by the bacterial agent *Xapf* in comparison to *Xap* (Almedia *et al.*, 2014). It should be noted that both isolates showed the typical yellow color of the colonies 48 hours later on the plates with medium KB or TB (Figure 1). After 96 hours, the striation of the medium and the pigmentation of isolate 2 which is characteristic for *Xapf* was observed (Francisco *et al.*, 2013). Due to the morphological, biochemical and pathogenic characteristics of the bacterial isolates, it was concluded that the isolated phytopathogenic bacteria corresponded to *Xanthomonas axonopodis* pv. phaseoli and *X. axonopodis* pv. phaseoli var. fuscans.

Incidence and severity of the disease

No significant difference was observed in the incidence of the disease caused by the bacteria *Xap* and *Xapf*, which showed an average of 62% (data not shown). However,

the ANVA of the severity showed that the plants inoculated with *Xap* and *Xapf* presented an average severity scale of 3.95 and 4.92, equivalent to an affectation of between 5% and 10% of damaged leaf area, respectively. The plants treated with jasmonic acid showed lower severity of the disease by the bacteria compared to the rest of the treatments (Figure 2).

Stomatal conductance and opening and photosynthetic rate

The ANVA in factorial arrangement of the evaluated variables showed significant difference derived from the phytopathogenic bacteria and from the treatments as sources of variation, without presenting an interactive effect between these factors. Under this design it was possible to appreciate that the phytopathogenic bacteria caused a decrease in the assimilation of CO_2 of the bean plants; effect comparable to that obtained with the stomatal conductance and opening variables. The means test of the effect of the phytopathogenic bacteria on the assimilation of CO_2 showed a statistically significant reduction of 39% and 59% by the *Xap* and *Xapf* bacteria, respectively compared to the control (Table 2). The stomata of the plants inoculated with the bacteria showed a partial occlusion of the order of 24.5% and 25.3% respectively with respect to the control (Table 3).

Table.1 Morphological and biochemical characteristics of bacterial isolates

Test	Bacterial isolate	
	Xap (isolate 1)	*Xapf* (isolate 2)
Cell form (Bar)	+	+
Fluorescent pigments	-	-
Motility	+	+
Spore formation	-	-
Gram stain	-	-
Growth in KB, TB, and YDC medium	+	+
Pigmentation of TB medium	-	+Brown diffusible
Hydrolysis in starch	+	+
Hydrolysis of gelatin	-	-
Hydrolysis of Tween 80	+	+
Striation of the medium	+	+
Gelatin liquefaction	+	+
Levan production	+	+
Indolsynthesis	+	+
Aerobiosis	+	+
Nitratereduction	-	-
Pathogenicity test	+	+
The tests were confirmed by duplicate; (+) positive and (-) negative. *Xap* = *Xanthomonas axonopodis* pv. phaseoli. *Xapf* = *Xanthomonas axonoodis* pv. phaseolivar. fuscans.		

Table.2 CO_2 assimilation of bean plants (*Phaseolus vulgaris* L.) in response to the causal agents of bacterial blight and defense activators

	Control			*Xap*			*Xapf*			Mean of the treatments
	----------µmol CO_2 m^{-2} s^{-1}----------									
Treatments										
Control (To)	11.95 a	±	0.78	7.68 a	±	0.98	5.15 a	±	0.15	11.01 ab
Salicylicacid (Sa)	12.00 a	±	1.00	7.63 a	±	2.10	5.78 a	±	0.95	11.29 ab
Jasmonicacid (Ja)	13.45 a	±	1.83	9.78 a	±	2.40	6.60 a	±	1.08	13.26 a
***T. asperellum*(Ta)**	13.35 a	±	1.61	7.08 a	±	2.84	5.98 a	±	1.43	11.73 a
***B. pumilus*(Bp)**	9.78 a	±	3.31	6.40 a	±	0.93	4.15 a	±	1.01	9.03 b
Causal agent										
	16.14 a			10.28 b			7.37 c			
Contrasts										
(Ja) vs (To, Sa, *Ta*, and *Bp*)										**

Means with the same letter do not present statistical difference (tukey test, p ≤ 0.05).
***Xap* = *Xanthomonas axonopodis*pv. phaseoli.**
***Xapf* = *Xanthomonas axonoodis*pv. phaseolivar. fuscans.**
**** = highly significant difference.**

Table.3 Stomatal opening of bean plants (*Phaseolus vulgaris* L.) in response to the causal agents of bacterial common blight and defense activators

	Control			*Xap*			*Xapf*			Mean of the treatments
	----------µm ----------									
Treatments										
Control (To)	0.86 a	±	0.31	0.71 a	±	0.13	1.22 a	±	0.28	1.24 ab
Salicylic acid (Sa)	1.21 a	±	0.40	0.90 a	±	0.69	0.69 a	±	0.14	1.24 ab
Jasmonicacid (Ja)	1.67 a	±	0.91	1.14 a	±	0.49	1.33 a	±	0.72	1.84 a
***T. asperellum* (Ta)**	1.62 a	±	0.54	1.30 a	±	0.50	0.82 a	±	0.26	1.66 a
***B. pumilus*(Bp)**	0.90 a	±	0.11	0.68 a	±	0.09	0.62 a	±	0.27	0.98 b
Causal agent										
	1.67 a			1.26 ab			1.25 b			

Means with the same letter do not present statistical difference (tukey test, p ≤ 0.05).
***Xap* = *Xanthomonas axonopodis*pv. phaseoli.**
***Xapf* = *Xanthomonas axonoodis*pv. phaseolivar. fuscans.**

Table.4 Stomatal conductance of bean plants (*Phaseolus vulgaris* L.) in response to the causal agents of bacterial common blight and defense activators

	Control			*Xap*			*Xapf*			Mean of the treatments
	----------mmol m^{-2} s^{-1}----------									
Treatments										
Control (To)	51.65 b	±	11.11	43.95 a	±	17.81	53.03 a	±	8.97	66.05 a
Salicylicacid (Sa)	54.67 b	±	12.70	51.23 a	±	29.78	48.40 a	±	13.29	68.57 a
Jasmonicacid (Ja)	73.77 a	±	1.89	66.63 a	±	21.52	49.03 a	±	10.35	84.18 a
***T. asperellum*(Ta)**	53.50 b	±	5.00	44.78 a	±	20.12	48.35 a	±	12.87	65.16 a
***B. pumilus*(Bp)**	59.40 ab	±	11.56	46.55 a	±	22.20	45.10 a	±	16.91	67.13 a
Causal agent										
	78.13a			67.50a			65.04a			
Contrasts										
(Ja) vs (To, Sa, *Ta*, and *Bp*)										**
(Control) vs (*Xap*)										Ns
(Control) vs (*Xapf*)										*

Means with the same letter do not present statistical difference (tukey test, p ≤ 0.05).
***Xap* = *Xanthomonas axonopodis* pv. phaseoli.**
***Xapf* = *Xanthomonas axonoodis* pv. phaseolivar. fuscans.**
Ns = not significant.
*** = significant difference.**
**** = highly significant difference.**

Fig.1 A. Reproduction of the symptoms of the disease by *Xanthomonas axonopodis pv. phaseoli (Xap)* 21 days after spray on plants. B. Bacterial growth 24 hr after sowing at TB medium (note the start of pigmentation of the TB medium and halo blight on leaf). C. Growth of the bacteria Xap and *Xanthomona axonopodis pv. phaseolivar. fuscans (Xapf) in Tween B (TB) medium 48* hours after sowing

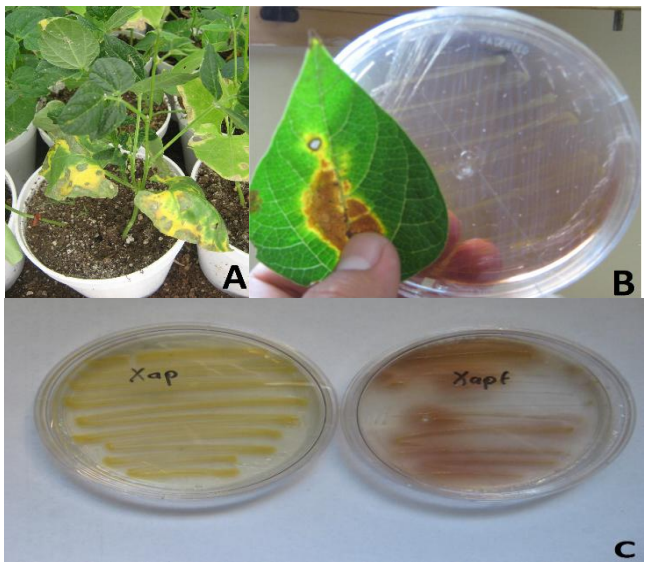
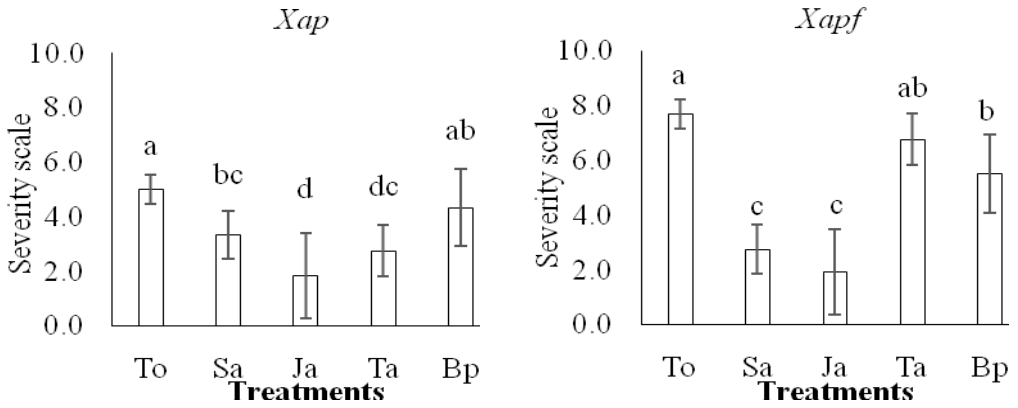


Fig.2 Severity of the disease in bean plants (*Phaseolus vulgaris* L.) caused by *Xanthomonas axonopodis pv. phaseoli (Xap) and Xanthomonas axonopodispv. phaseoli var. fuscans (Xapf).* Means with the same letter do not show statistical difference (Tukey test, $p \leq 0.05$). To: control; Sa: Salicylic acid; Ja: Jasmonic acid; Ta: *Trichoderma asperellum*; Bp: *Bacillus pumilus*



No statistical difference was observed by these sources of variation in the stomatal conductance variable under the factorial arrangement, however, the analysis in orthogonal contrasts showed significant difference between the means of the *Xapf* bacterium and the control (Table 4).

The treatments, as a source of variation in the ANVA conducted in factorial arrangement, showed that the plants treated with jasmonic acid (Ja) significantly maintained a high assimilation of CO_2 in comparison to the rest of the treatments, even when they presented the bacterial disease. This result was similar to the one obtained in the stomatal opening variable, however, the same tendency was appreciated in the stomatal conductance variable only under the analysis in orthogonal contrasts. Under the same analysis, the means test allowed to appreciate that the plants treated with *B. pumilus* maintained a low assimilation of CO_2 and stomatal opening in comparison to the rest of the treatments, not being statistically different in the stomatal conductance.

The results obtained show that the bacteria *X. axonopodis*pv. phaseoli var. fuscans more severely affects bean plants compared to pathogen *X. a.* pv. *phaseoli*. This severity of the disease affects the photosynthetic activity and stomatal opening and conductance. It should be noted that the plants treated with jasmonic acid showed lower severity of the disease caused by both phytopathogenic bacteria and a high photosynthetic rate compared to the rest of the treatments.

Stomata are natural openings that have been recognized as the greatest entry point for bacterial pathogens (Zeng *et al.*, 2010). Therefore, stomatal closure would be the first response of plants to contact with these microbial pathogens. It should be noted that the bacterium *Xapf* is known to be the most virulent causative agent of common bacterial blight in bean plants (Mutlu*et al.*, 2008). This virulence is attributed, among others, to the nature of the extracellular polysaccharide that protects it from environmental factors (Büttner and Bonas, 2010), thus this study suggests the effect of virulence in the stomatal closure and conductance and therefore in the photosynthetic rate.

On the other hand, it is known that plant defense is regulated by the signaling

pathways of jasmonic acid and/or salicylic acid against most phytopathogens (Zhu *et al.*, 2014). In this work it was observed that the exogenous application of jasmonic acid at 0.5 mM stimulated a greater tolerance of bean plants to the causal agents of the bacterial common blight in comparison to the application of salicylic acid. It has been observed that these pathways are incidentally activated in plants by beneficial microorganisms such as antagonistic fungi and rhizobacteria (Samaniego *et al.*, 2017). The microorganisms *Trichoderma asperellum* and *Bacillus pumilus* used in this work increased the resistance of the plants to the phytopathogenic bacteria, however, the superiority of the phytohormones jasmonic acid and salicylic acid were more remarkable. The fact that the plants treated with *B. pumilus* showed a lower photosynthetic rate in comparison to the rest of the treatments draws attention. Similar works have shown that when the foliar part of the plants comes in contact with the beneficial microorganisms, they also respond with a stomatal closure (Melotto *et al.*, 2006), this would explain the reduction in the assimilation of CO_2.

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