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ORIGINAL ARTICLE

Prospecting of pathogen-derived elicitors for the control of tomato bacterial spot

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Abstract

Plants can recognize molecules derived from pathogens and trigger systemic acquired resistance (SAR). In phytopathogenic bacteria, elicitors are constituent components of cellular structures, such as flagellin. We sought to select structural components of *Xanthomonas* spp. incompatible with tomato, aiming to control bacterial spot (*Xanthomonas perforans*). Initially, cell suspensions from 11 *Xanthomonas* spp. isolates were infiltrated into the leaves to assess their ability to cause a hypersensitivity response (HR) and the incompatible ones had their flagellin purified. The flagellin of the isolates were first applied at different concentrations, via infiltration and spraying. The pathogen, *X. perforans*, was inoculated after 24 h, to assess whether there would be any harmful reaction. No harmful reaction was observed in any treatment. Then, a second experiment was conducted to assess the severity of all isolates, at a concentration of 8.35 μg · ml^{-1}, via spraying, infiltration, and soil. The greatest reduction in Area Under the Disease Progress Curve (AUDPC) was observed in the treatment with XapRR, applied via spraying. Thus, prospecting for elicitors is the first step in developing a product for agricultural use. The flagellin elicitor of XapRR is promising and capable of producing these molecules on a large scale.

Keywords: hypersensitivity response, incompatibility, phytopathogenic bacteria, *Xanthomonas euvesicatoria* pv. *perforans*

Introduction

The tomato crop is significantly representative in the Brazilian economy and is subject to several diseases caused by different pathogens that limit its productivity. Bacteria are among the most important pathogenic agents that affect crops of economic importance in agriculture in Brazil, causing significant losses (Araújo *et al*. 2003). The control of bacterial spot in tomatoes is difficult and the chemical method is not very effective, even though it is still widely used in agriculture. It may trigger consequences for the environment, men and animals (Bettiol and Morandi 2009). The continuous application of pesticides can also affect the development of resistant isolates, making certain modes of action no longer efficient for some populations (Fialho 2004). As sustainable alternatives for the management of bacterial spot, biological control, integrated management, resistant species, and resistance induction stand out (Burketova *et al*. 2015).

The use of activating natural defenses of plants has been constantly studied, as it is a systemic, natural method of protection which is persistent and of a broad spectrum (Pascholati and Toffano 2007). Defense mechanisms of a plant are genetically controlled and must occur at the right time after host-pathogen contact. Plants can become more resistant to pathogens after the activation of these genes, thus triggering resistance induction (Araújo and Menezes 2009).

Plants have the ability to recognize molecules derived from pathogens and can trigger systemic acquired resistance (SAR), which promotes a non-specific response to infections by pathogens once the plant is in an induction state (Gao *et al*. 2015). The phenomenon characterized by the triggering of the metabolic routes responsible for the plant to respond to infection is called priming. This phenomenon can be elicited by a determined pattern of molecules present in a range of pathogenic microorganisms that make them capable of being recognized by the host (Conrath 2011).

In phytopathogenic bacteria, several elicitors are constitutive components, present in cellular structures, such as the flagellin (Desaki *et al*. 2006; Newman *et al*. 2013; Proietti *et al*. 2014). These elicitors, derived from phytopathogenic bacteria, present the molecular patterns capable of being recognized by plant cell receptors (Ranf 2016). Bacteria of the genus *Xanthomonas* are characteristically phytopathogens that have a well-defined host range, related to the species or to the pathovar to which they belong (Jacques *et al*. 2016). This well-defined host range means that, when the elicitors of the incompatible pathogen are recognized by receptors in plants, two possible biochemical and physical resistance responses are triggered: hypersensitivity response (HR) and SAR (Zhou *et al*. 2003; Mandani and Scholthof 2013). This interaction allows these elicitors to trigger a systemic, non-specific response, with practical application in the control of phytopathogenic bacteria. Thus, the search for elicitors with molecular patterns associated with pathogens (PAMPs) in *Xanthomonas* species and pathovars incompatible with certain hosts, may constitute a source of prospecting for elicitors capable of triggering SAR. This work, therefore, is based on the fact that bacteria of the genus *Xanthomonas*, which are incompatible with tomato, constitute a source of SAR elicitors capable of triggering a response that results in effective reduction of the severity of bacterial spot, with prospects for practical use.

Conventional measures of bacterial spot management are mainly based on applications of copper products, which are often inefficient, due to the occurrence of strains of bacteria resistant to copper in production areas (Griffini *et al.* 2017; Strayer-Scherer *et al*. 2018). Therefore, the identification and development of products formulated with elicitors of the most diverse nature have been increasingly in demand, especially for diseases that have few effective control strategies (Iriti and Varoni 2017). It is worth mentioning that the prospecting of elicitors is the first step towards the development of a product for agricultural use. Through bioengineering techniques, it is possible to produce these molecules on a large scale. Considering the non-specific nature of SAR, the results of this work may also offer an alternative to control other tomato diseases in the future. Therefore, this work aimed to obtain elicitors with molecular patterns associated with pathogens capable of controlling the bacterial spot of tomatoes through resistance induction.

Materials and Methods

The assays were initially conducted with 11 pre-selected isolates from municipalities of the states of São Paulo, Roraima and Pará, Brazil, obtained from different species and pathovars of *Xanthomonas* incompatible with tomato, and pathogenic to other plant species (Table 1). Base fertilization was carried out on NPK plants formulation 8-28-16, with 40 g of fertilizer for each 30 l of soil and correction was made with 30 g of lime for 30 l of soil. The experiments were carried out at the Laboratory of Environmental Microbiology, Laboratory of Genetics, and respective greenhouses at Embrapa Meio Ambiente, in Jaguariúna-SP.

Selection of *Xanthomonas* isolates as sources of elicitors

To select isolates capable of inciting resistance reaction in tomato and potential elicitor providers, HR experiment was conducted, where cell suspensions were grown in 523 medium (Kado and Heskett 1970) for 48 h and the bacterial concentration was adjusted to Abs_{540} = 0.2. To verify the incompatibility reaction to this host, tomato plants cv. Santa Cruz Kada, with four fully expanded compound leaves, had three of its leaflets infiltrated with the suspension, using a hypodermic syringe. The hypersensitivity response was evaluated after 24 h and possible changes in its evolution were recorded for 5 days after the infiltration. The isolates that triggered necrotic reactions were registered and selected to obtain the crude extract of flagellin.

Obtaining flagellin

Flagellin extracts were obtained from cultures grown for 48 h in medium 523 (Kado and Heskett 1970) at 27°C. The cells were suspended in potassium phosphate buffer (20 mM, pH 7.4) and centrifuged (5700 g, 5 min.). The pellet was resuspended in the same buffer and homogenized in a tissue crusher to release the flagellin. Cells and cell fragments were removed by centrifugation (23,400 g, 1 h, 4°C). The supernatant was then lyophilized to obtain the flagellin, which were kept at –20°C until the time of use (Meziane *et al*. 2005).

Table 1. *Xanthomonas* isolates

Isolates	Codes	Place	Host	Collection	Provider	References
Xanthomonas axonopodis pv. *passiflorae*	XapSP	Botucatu-SP	*Passiflora edulis*	Embrapa Meio Ambiente	Bernardo de Almeida Halfeld Vieira	Halfeld-Vieira *et al*. 2015
Xanthomonas citri pv. *malvacearum*	CNPA 329	Acreúna-GO	*Gossypium hirsutum*	Embrapa Algodão	Wirton Macedo Coutinho	Braga 2016
Xanthomonas citri pv. *malvacearum*	CNPA 321	Chapadão do Sul-MS	*Gossypium hirsutum*	Embrapa Algodão	Wirton Macedo Coutinho	Braga 2016
Xanthomonas axonopodis pv. *passiflorae*	XapRR	Roraima	*Passiflora edulis*	Embrapa Roraima	Daniel Augusto Schurt	Halfeld-Vieira *et al*. 2015
Xanthomonas campestris pv. *campestris*	CPATU Xcc5	Ananindeua-PA	*Brassica oleracea*	Embrapa Amazônia Oriental	Alessandra Keiko Nakasone	Freire 2016
Xanthomonas axonopodis pv. *passiflorae*	XapPA	Igarapé-Açu-PA	*Passiflora edulis*	Embrapa Amazônia Oriental	Alessandra Keiko Nakasone	Halfeld-Vieira *et al*. 2015
Xanthomonas axonopodis pv. *manihotis*	CPATU XAM 29	Igarapé Açu-PA	Mandioca	Embrapa Amazônia Oriental	Alessandra Keiko Nakasone	Ishida *et al*. 2016
Xanthomonas axonopodis pv. *manihotis*	CPATU XANS 10	Pará	*Manihot esculenta*	Embrapa Amazônia Oriental	Alessandra Keiko Nakasone	Ishida *et al*. 2016
Xanthomonas axonopodis pv. *passiflorae*	CPATU P.A.4.3	Pará	*Passiflora edulis*	Embrapa Amazônia Oriental	Alessandra Keiko Nakasone	Oliveira *et al*. 2011
Xanthomonas axonopodis pv. *manihotis*	CPATU XANS 17	Pará	*Manihot esculenta*	Embrapa Amazônia Oriental	Alessandra Keiko Nakasone	Ishida *et al*. 2016
Xanthomonas axonopodis pv. *passiflorae*	CPATU P.A. 20	Pará	*Passiflora edulis*	Embrapa Amazônia Oriental	Alessandra Keiko Nakasone	Oliveira *et al*. 2011
Xanthomonas perforans	*X. perforans*	Goiás	*Solanum lycopersicum*	IF Goiano-campus Morrinhos	Nadson de Carvalho Pontes	Mates *et al*. 2019

Evaluation of different concentrations and modes of application of flagellin elicitors to induce resistance in tomato

The flagellin obtained from the isolates were suspended in ultra-pure water at a final concentration of 10^8 cfu · ml^{-1}, about 8.35 μg · ml^{-1} (Meziane *et al*. 2005). The concentrations used were: 2.08 μg · ml^{-1} (1/4D), 4.17 μg · ml^{-1} (1/2D), 8.35 μg · ml^{-1} (1D) and 16.7 μg · ml^{-1} (2D). The suspensions were applied via one leaf on the aerial part of the leaf with a manual spray and through leaf infiltration, using a hypodermic syringe. The suspensions were infiltrated into one leaflet per tomato plant.

Within 6 days it was observed whether the flagellin caused damage to the plants. On the 7th day, *X. perforans* (Abs_{540} = 0.2) was inoculated and possible reactions of the plants were evaluated at 5, 12, and 16 DAI. The experimental design was completely randomized. There were 11 treatments, 10 of which were isolated from bacteria, and one control (water). There were 2 application routes (application via leaf and application via infiltration) with four repetitions. Each repetition was represented by a pot with a plant.

Severity assessments of tomato bacterial spot as a function of the flagellin as an elicitor

To assess the ability of flagellin to elicit resistance response, freeze-dried flagellin were suspended in ultrapure water at a final concentration of 10^8 cfu · ml^{-1}, corresponding to about 8.35 μg · ml^{-1} (Meziane *et al*. 2005). To evaluate the control capacity by means of leaf dispensing, the suspensions were applied to the aerial part with a manual sprayer. For soil evaluation, by dispensing the elicitor in the roots, 10 ml per plant were added to the suspensions, next to the plants' root collar. For control evaluation through infiltration in the leaf, the suspensions were infiltrated into one leaflet per tomato plant.

After 24 h, the suspension of *X. perforans* (Abs_{540} = = 0.2)was inoculated on the leaves of the plants, which were kept in a humid chamber for 24 h. As a control, only water applied via spraying, soil, and infiltration, was used. Severity was estimated by using a diagrammatic scale (Mello *et al*. 1997) on days 3, 7, 13, 20, 23, and 27 after inoculation of the pathogen.

The experimental design was completely randomized, in a factorial scheme, consisting of 10 sus-

pensions of flagellin obtained from *Xanthomonas* isolates. There were three routes of application (application via soil, spraying, and infiltration) and one control as an additional treatment (water), with seven repetitions. Each repetition was represented by a pot with a plant. The control had each repetition represented by the average values of three plants.

The results of the AUDPC were analyzed using the GLM proc of the SAS software version 9, comparing the means using the Fisher-LSD test at 5% significance. Comparisons of each interaction with the control were made using the Dunnett test at 5% significance.

Results

Hypersensitivity response in response to cell infiltration of *Xanthomonas* spp.

Of the 12 *Xanthomonas* isolates tested, 10 showed HR reaction, which were classified as weak, intermediate, or strong 24 h after inoculation (Table 2). HR results in the rapid and localized death of a limited number of cells that surround the site of infection. This interrupts the growth and development of the pathogen in plant tissue and is one of the most important defense mechanisms of plants. When an elicitor is recognized by a receptor on the plant, signal transduction pathways lead to the activation of defense mechanisms, expression of resistance genes and HR, indicating that this reaction is more a symptom of incompatibility than a primary determinant of disease resistance (Pascholati 1995).

The results show incompatible interactions between tomato and some isolates of *Xanthomonas* spp.

Table 2. Hypersensitivity reaction (HR) in tomato leaves 24 h after infiltration with cell suspension of *Xanthomonas* spp.

Isolates	HR		
	weak reaction	intermediate reaction	strong reaction
XapSP			X
CNPA 329	X		
CNPA 321	X		
XapRR			X
CPATU Xcc5		X	
XapPA		X	
CPATU XAM 29		X	
CPATU XANS10		X	
CPATU P.A.4.3		X	
CPATU XANS17	X		
CPATU P.A.20	N	N	N
Xanthomonas perforans	N	N	N

X – HR reaction; N – no HR reaction

and differences in intensity of reactions. Although all reactions are typical of the HR response, the intensity of the response in the 24-hour period can vary, with more intense reactions of cell death in some cases than in others. We suggest that this difference occurs due to the nature and availability of recognition factors that each isolate of *Xanthomonas* spp. has and that can potentially be used as elicitors of resistance induction. Given their ability to induce HR and, therefore, the ability to present recognition factors that triggered an incompatible reaction by the plant, the 10 isolates, XapSP, CNPA 329, CNPA 321, XapRR, CPATU Xcc5, XapPA, CPATU XAM 29, CPATU XANS 10, CPATU PA4.3, and CPATU XANS 17, were selected to obtain the flagellin and to evaluate them as potential providers of elicitors.

Evaluation of the ability of flagellin to promote the control of tomato bacterial spot

Within 6 days after infiltrating or spraying the flagellin of the isolates, no damage was observed in the tomato plants. After inoculation of *X. perforans*, on the 7th day, slight yellowing only around the infiltrated area was observed in all flagellin treatments.

Severity of tomato bacterial spot as a function of the flagellum as an elicitor

In the evaluation due to the flagellin as an elicitor, considering the AUDPC, except for the treatment with XapSP via infiltration, all the other treatments of the 10 isolates differed significantly from the control (Table 3). In application via infiltration, only XapRR, CPATU P.A.4.3 and CPATU Xcc5 reduced AUDPC by 21, 26, and 39%, respectively. Via spraying, XapSP, CPATU PA4.3, CPATU Xcc5 and XapRR reduced AUDPC by 30, 33, 36, and 43%, respectively and via soil, XapRR, CPATU PA4.3 and CPATU Xcc5 reduced AUDPC by 32, 36, and 40%, respectively.

Discussion

Resistance induction (IR) has several latent defense mechanisms in plants that are activated after treatment with biotic or abiotic agents (Bonaldo *et al.* 2005). Resistance induction can be a local protection, occurring only in adjacent tissues treated with the inducing agent, or it may be systemic, manifesting itself at a distance from the inductor's application site (Moraes 1992). In this work, the reduction of disease intensity with flagellin applied in places far from the infection sites, which highlights the nature of the elicitation of induced resistance, since these

Table 3. Intensity of tomato bacterial spot (AUDPC) after treatment via infiltration, spraying and soil of the flagellin of XapSP, XapRR, CPATU PA4.3, CNPA 321, CNPA 329, CPATU XANS 17, CPATU XANS 10, CPATU Xcc5 and CPATU XANS 29 isolates, followed by inoculation of the pathogen *Xanthomonas perforans*, after 24 h. The control treatment was represented by distilled water

Treatments	Application mode		
	infiltration	spraying	soil
XapSP	93.86 EFb	67.00 Dc*	162.36 Ba*
XapRR	75.50 FGa*	54.50 Dc*	64.64 Fb*
CPATUP.A.4.3	70.43 FGa*	64.14 Db*	60.86 Fb*
CNPA 321	155.29 ABa	114.69 Cab*	102.57 Eb
CNPA 329	115.61 DE*	102.93 Cc*	125.87 Da*
XapPA	130.00 DC*	132.68 Bab*	142.29 Ca*
CPATU XANS 17	172.00 Aa*	145.93 Bb*	151.71 BC*
CPATU XANS 10	158.64 AB*	172.36 Aa*	154.14 BC*
CPATU Xcc5	58.43 Ga*	60.86 Da*	56.94 Fa*
CPATU XANS 29	138.43 BC*	110.29 Cc*	185.21 Aa*
Control		96.19	

Averages followed by the same uppercase letter in the column direction or by lowercase letter in the direction of the line do not differ by the Fisher--LSD test; *treatment differs from control by Dunnett Test ($p < 0.05$)

components significantly reduced the intensity of bacterial spot for flagellin of several isolates. The results show however, that the elicitors answers are effective according to the place of application of the elicitor. For the most effective treatments, the flagellin of XapRR applied via spray presented the best percentages of bacterial spot control.

The contingency of *X. perforans* due to the induction of resistance triggered by flagellin may have caused HR on a smaller scale, without the visualization of damage to a large extent, corroborating previous studies by Farahani and Taghavi (2017). Possibly, the role of flagellin in the induction of basal defenses and to a lesser extent of RH allows the plant to trigger resistance without extensive tissue collapse adjacent to the infection sites.

Systematic acquired resistance induction also involves a process called priming or preconditioning (Fu and Dong 2013). This process leaves the plant sensitized and enables cells to increase the capacity for a rapid and effective activation of cellular defense responses, which are induced only after contact with the challenging pathogen. It has been reported that, once induced, the immune memory configured by SAR can last for weeks or months (Luna *et al*. 2012).

It is expected that the plant induced with eliciting agents will show changes in its metabolism. However, when compared to a plant induced with the same elicitor and later challenged with a pathogen, changes in metabolism are more intense than in the plant only challenged with a pathogen or only induced with an elicitor. This demonstrates that the plant is better able to respond to infection, since the presence of the pathogen, after induction, alters the magnitude of biochemical events, and promotes the triggering of other mechanisms. Plants, not induced and inoculated with the pathogen, have a lesser magnitude of these biochemical events.

Thus, the contribution of flagellin elicitors of isolates in the control of bacterial spot was to anticipate the response of the plant against the pathogen, leading to a delay in the development of the disease, with a consequent reduction in its intensity. In addition, the time needed to make recurrent applications of the elicitor must be considered, as well as the dispensation of these bio-inputs by other application technologies. Therefore, the interval between applications, different ways of applying elicitors, and their implications for productivity should be explored in the future.

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