Natural variability in *Arabidopsis thaliana* germplasm response to *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

This work aimed to study the interaction between the model plant *Arabidopsis thaliana* and *Xanthomonas campestris* pv. *campestris* (Xcc), the pathogen responsible for black rot of crucifers. The response of 32 accessions of *A. thaliana* to the Brazilian isolate of Xcc CNPH 17 was evaluated. No immunity-like response was observed. "CS1308", "CS1566" and "CS1643" grown in continuous light were among the accessions that showed strongest resistance when inoculated with 5 x 10^6 CFU/mL. In contrast, "CS1194" and "CS1492" were among the most susceptible accessions. Similar results were obtained when plants were grown under short-day conditions. To quantify the differences in disease symptoms, total chlorophyll was extracted from contrasting accessions at different time points after inoculation. Chlorophyll levels from controls and Xcc inoculated plants showed a similar reduction in resistant accessions, whereas Xcc-inoculated susceptible accessions showed a greater reduction compared to controls. To test the specificity of resistance, accessions CS1308, CS1566, CS1643 and CS1438 (which showed partial resistance to CNPH 17), were inoculated with a more aggressive isolate of Xcc (CNPH 77) and *Ralstonia solanacearum.* Among the accessions tested, "CS1566" was the most resistant to Xcc CNPH 77 and also displayed resistance to *R. solanacearum.* Accessions CS1308, CS1566 and CS1643 were also inoculated with a high titer of Xcc CNPH 17 (5 x 10^8 CFU/mL). No collapse of tissue was observed up to 48 h after inoculation, indicating that a hypersensitive response is not involved in the resistance displayed by these accessions.

Additional keywords: susceptibility, resistance, black rot of crucifers, plant-pathogen interaction.

RESUMO

Variação natural na resposta a *Xanthomonas campestris* pv. *campestris* presente no germoplasma de *Arabidopsis thaliana*

Este trabalho visou a estudar a interação entre a planta modelo *Arabidopsis thaliana* e *Xanthomonas campestris* pv. *campestris* (Xcc), patógeno responsável pela podridão-negra das brássicas. A resposta de 32 ecótipos de *A. thaliana* ao isolado brasileiro de Xcc CNPH 17 foi avaliada. Reação do tipo imunidade não foi observada; entretanto, "CS1308", "CS1566" e "CS1643" cultivados em luz contínua estavam entre os acessos que demonstraram maior resistência quando inoculados com Xcc a uma concentração de 5 x 10^6 UFC/mL. Em contraste, "CS1194" e "CS1492" foram identificados entre os acessos mais suscetíveis. Resultados semelhantes foram obtidos quando as plantas foram cultivadas em dias curtos. Para quantificar as diferenças nos sintomas da doença, clorofila total foi extraída de acessos contrastantes em diferentes tempos após a inoculação. A quantidade de clorofila presente nos controles e nos acessos resistentes inoculados com Xcc mostraram um padrão de declínio semelhante, enquanto os acessos suscetíveis inoculados com Xcc mostraram queda mais acentuada que os controles. Para avaliar a especificadade da resistência, os acessos CS1308, CS1566, CS1643 e CS1438 (que demonstrou resistência parcial a Xcc CNPH 17), foram inoculados com um isolado mais agressivo de Xcc (CNPH 77) e com *Ralstonia solanacearum.* O acesso CS1566 mostrou-se o mais resistente ao isolado CNPH 77 de Xcc e também apresentou resistência a *R. solanacearum.* Em outro experimento, os acessos CS1308, CS1566 e CS1643 foram inoculados com uma alta concentração de Xcc CNPH 17 (5 x 10^8 CFU/mL). Nenhum colapso de tecido foi observado até 48 h após a inoculação, indicando que a hipersensibilidade não está envolvida na resposta de resistência apresentada pelos ecótipos estudados.

Palavras-chave adicionais: suscetibilidade, resistência, podridão negra das brássicas, interação planta-patógeno.

INTRODUCTION

The interaction between a plant and a pathogen can have different outcomes, ranging from the plant not being a host to full susceptibility to resistance. The identification and characterization of contrasting situations is the first step to understanding the biological and genetic basis for the outcome. Important advances have been made recently in understanding the molecular mechanisms of disease resistance, many of these using the model plant *Arabidopsis thaliana* (L.) Heynh. (Quirino & Bent, 2003).

Xanthomonas campestris pv. *campestris* (Pammel) Dowson (Xcc) is a Gram-negative bacterium and is responsible for black rot, considered the most important disease of crucifers throughout the world (Williams, 1980). Economically important plants that are affected by black rot include cabbage,

broccoli, cauliflower and kale. In Brazil black rot has also been a problem for cruciferous crops in different regions of the country (Azevedo *et al.*, 2002; Rodrigues Neto, 1995). Xcc enters the plant through hydathodes at the leaf margins causing V-shaped lesions or through stomata causing round lesions (Lopes & Quezado-Soares, 1997). Once inside the plant, Xcc colonizes the vascular system where it produces an extracellular polysaccharide known as xanthan, which can obstruct the xylem vessels causing tissue necrosis (Williams, 1980). The genome of Xcc has been completely sequenced by Brazilians (da Silva *et al*., 2002).

Arabidopsis thaliana is a crucifer and a host to Xcc (Simpson & Johnson, 1990). *Arabidopsis* is diploid and has a small genome of 125 Mbp, distributed in five pairs of chromosomes (Meinke *et al*., 1998), which was completely sequenced in 2000 (Initiative, 2000). This plant has a short life cycle, going from germination to the production of mature seeds in about six weeks. It can self-fertilize and has very prolific seed production. There are genetic and physical maps of all five chromosomes. *Arabidopsis* is also easily transformed. Furthermore, studies of gene function have greatly benefited from the development of knockout populations (Sussman *et al*., 2000). Recently, a number of laboratories have turned their attention to exploring the genetic variability found in *Arabidopsis* germplasm (Alonso-Blanco *et al*., 2005; Gassmann, 2005; Kover & Schaal, 2002).

Studies of *Arabidopsis* resistance to *Xanthomonas* have been pursued by different groups (Tsuji *et al.*, 1991; Lummerzheim *et al.* 1993; Buell & Somerville, 1995; Buell & Somerville, 1997; Godard *et al*., 2000). Each new study of the interaction between *Arabidopsis* and a different isolate of a pathogen may reveal new elements of host resistance and susceptibility. Furthermore, it is likely that the next breakthroughs in understanding the plant-pathogen interaction will come from pathosystems where both the plant and the pathogen have their genomes completely sequenced. *Arabidopsis* and Xcc are among the few pathosystems that fulfill this criterion. Here we report on initial studies about the genetic variability of the *Arabidopsis* response to a Brazilian isolate of Xcc.

MATERIALS AND METHODS

Origin of *Arabidopsis* accessions and growth conditions

Thirty-two accessions (Table 1) of *A. thaliana* were obtained from the *Arabidopsis* Biological Resource Center (Ohio, U.S.A.). Seeds were plated on MS ¼ medium (Sigma, M.O., U.S.A.), imbibed overnight at room-temperature and cold-treated for 2 days. Plates were transferred to light and one week after germination, seedlings were transplanted to pots, 7 cm in diameter, containing Plantmax Hortaliças HT substrate (Eucatex Agro, SP, Brazil). Plants were grown for 3-6 weeks under continuous light or short days (8 h light / 16 h dark), as specified in each experiment, with illumination from cool-white fluorescent lamps at approximately 100 μmol. m^{-2}. s^{-1}. Temperature was approximately 24 °C and relative humidity 50%. Plants were subirrigated with water as needed.

TABLE 1 - *Arabidopsis* accessions and their response to *Xanthomonas campestris* pv. *campestris* CNPH 17

Accession number	Accession name	Response to Xcc [1]
CS903	Kas-1	R
CS920	Em-D	PR
CS1020	Bu-8	S
CS1064	Can-0	S
CS1072	Chi-0	PR
CS1084	Co-1	PR
CS1093	Col-1	PR
CS1194	Gö-0	S
CS1198	Gr-1	PR
CS1298	La-0	PR
CS1308	Le-0	R
CS1354	Lz-0	PR
CS1438	Pa-1	PR
CS1466	Pla-4	PR
CS1492	Ri-0	PR
CS1540	Su-0	R
CS1566	Tu-0	R
CS1594	Wil-1	PR
CS1640	Tsu-1	PR
CS1643	Oy-1	R
CS2223	Ws-1	PR
CS3112	M7323S	PR
CS6100	Kelsterbach -1	PR
CS6175	Condara	PR
CS6181	Sn(5)-1	S
CS6604	Na-2	S
CS6674	Ct-1	S
CS6699	Es-0	PR
CS6922	Nd-1	PR
CS6930	Col-5 (gl1)	PR
CS8580	Cvi-1	PR
CS22353	Harvard square -7	PR

[1]R: resistant; PR: partially resistant; S: susceptible

Bacterial growth, plant inoculation and scoring of disease symptoms

The Xcc isolate CNPH 17 was obtained from a *Brassica oleracea* L. var. *acephala* field with black rot symptoms in Brazlândia, Federal District, Brazil, and the bacterial isolate CNPH 77 was obtained from a *Brassica oleracea* var. *capitata* in São José dos Pinhais, State of Paraná, Brazil. Each Xcc

isolate was kept in water at room temperature and streaked onto Nutrient-agar media plates. After incubation at 28 °C for two days, colony morphology was confirmed and colonies were re-plated on the same media. A bacterial mass was suspended in 10 mM $MgCl_2$ and the bacterial concentration was adjusted to approximately 5 x 10^6 CFU/mL for plant inoculation by making a 1:100 dilution of a bacterial suspension of 5 x 10^8 CFU/mL ($O.D._{600}$ = 0.3).

Plant inoculation of Xcc bacterial suspensions was performed with a syringe without the needle on the abaxial leaf surface. A scale of disease symptoms was used to evaluate plant response: 0- the half of the leaf inoculated did not show any disease symptoms; 1- rare lesions are present; 2- chlorosis symptoms present in approximately 10% of the inoculated area; 3- chlorosis present between 10% and 50% of the inoculated area; 4- presence of necrosis on most of the area inoculated but some regions remain photosynthetic and 5- the area inoculated is completely necrotic. For all experiments, *Brassica oleracea* var. *capitata* cv. Kenzan was used as a positive control for Xcc virulence.

The bacterial wilt tomato pathogen *Ralstonia solanacearum* (Smith) Yabuuchi *eta al.* isolate CNPH 221 (race 1, biovar 1) was obtained from a tomato field in Ponte Alta, Federal District, Brazil, and grown in modified TZC Kelman media (glycerol, 5 mL/L; bacterial peptone, 10 g/L; casein, 1g/L; bactoagar,15 g/L) for 2 days at 28 °C. A bacterial mass was re-suspended in sterile water and the bacterial concentration was adjusted to 5 x 10^8 CFU/mL ($O.D._{600}$=0.6). The soil at the base of the plants was perforated and 10 mL of bacterial suspension or sterile water was added. Wilt symptoms were scored at 10 and 15 days after inoculation according to the scale proposed by Yang & Ho (1998). Each accession was tested in at least two independent experiments with more than 10 plants. Tomato plants were also inoculated as positive controls for *R. solanacearum* virulence.

Statistical analysis

Data from experiments were analyzed with SAS (Statistical Analysis System, version 8.2). ANOVA was used to test whether there was statistically significant difference in disease severity symptoms among *Arabidopsis* accessions. To confirm these differences and define groups, the Tukey test of means was used. Experiments were grouped in an unbalanced dataset with four repetitions with 30 inoculated leaves for 32 accessions.

Some selected accessions identified as contrasting in the initial analysis were used in a smaller experiment to confirm results. Statistical analysis of this smaller experiment was carried out with an unbalanced dataset with two repetitions and 30 inoculated leaves for seven accessions.

Chlorophyll assays

Discs with a 7.9 mm diameter were collected from leaves inoculated with Xcc CNPH 17 at 5 x 10^6 CFU/mL or 10 mM $MgCl_2$ at 0, 2, 4 and 6 days after inoculation from a leaf region that had no lesions. Samples were frozen in liquid nitrogen and kept at -80 °C until chlorophyll extraction. Total chlorophyll levels (a+b) were determined photometrically using the method of Wintermans & DeMots (1965) for each plant using two leaf discs per plant. The mean chlorophyll level for three plants of each accession and the standard error were calculated.

RESULTS

Identification of resistant and susceptible accessions

Significant statistical difference was detected among all accessions tested (P<0.0001). According to the Tukey test of mean results, accessions were ranked and grouped as resistant, partially resistant and susceptible to Xcc (Table 1). The most resistant accessions were CS903, CS1308, CS1540, CS1566 and CS1643, which showed the fewest symptoms when challenged with Xcc. There was no statistical difference between accessions within this group in the Tukey test of means. The most susceptible accessions were CS1020, CS1064, CS1194, CS6181, CS6604 and CS6674, which showed the most symptoms when challenged with Xcc. Here again there was no statistical difference between accessions within this group. All accessions whose means of disease symptoms did not fall within the resistant and susceptible categories were grouped together as partially resistant. Immunity-like response was not observed in any of the accessions tested.

Based on the consistency of results and the number of seeds available, the resistant accessions CS1308, CS1566 and CS1643 and susceptible accessions CS1194 and CS1492 were chosen for further characterization. To allow direct comparison of the resistance/susceptibility phenotype, these selected accessions were grown in continuous light and scored for disease severity at five days after inoculation (Figure 1). To test if the changes in plant physiology associated with a different photoperiod had an impact on the phenotypes of resistance and susceptibility, experiments for these chosen accessions were repeated under short-day conditions. Similar results to those obtained in continuous light were obtained when plants were grown under short days (results not shown).

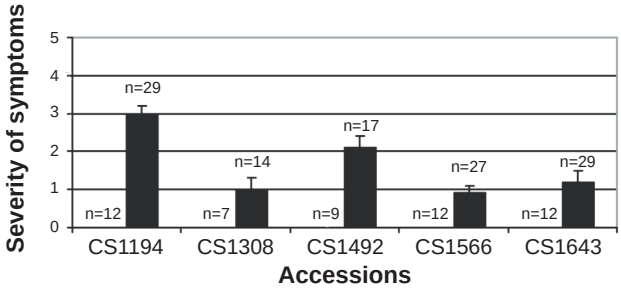

FIG. 1 - *Arabidopsis* accessions inoculated with buffer (left) or *Xanthomonas campestris* pv. *campestris* CNPH 17 (right) and scored for disease symptoms five days after inoculation. For each accession the number of leaves inoculated is shown (n). The vertical bars indicate the standard error. All leaves inoculated with buffer showed no symptoms of disease (0 on the scale) with a standard error of 0. According to groupings based on the Tukey test of means, accessions CS1308, CS1566 and CS1643 are resistant to Xcc, accession CS1194 is susceptible and CS1492 is partially resistant.

Chlorophyll measurements

In order to quantify differences in the degree of disease symptoms, total chlorophyll was extracted from contrasting accessions at different time points after inoculation. Chlorophyll levels from controls and inoculated plants showed a similar reduction in resistant accessions (Figure 2A-C). In susceptible accessions, inoculated plants showed a more pronounced reduction in chlorophyll levels than did control plants (Figure 2D-E).

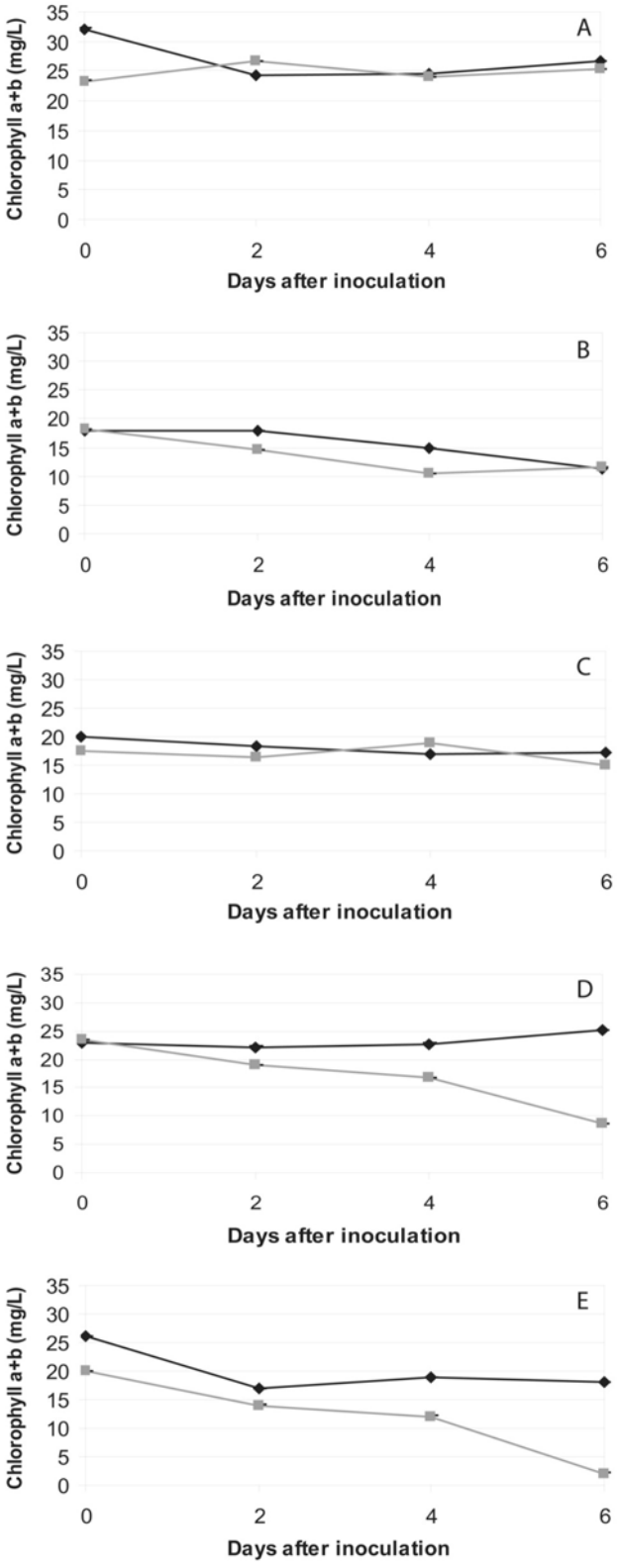

FIG. 2 – Mean value of total chlorophyll and standard error in accessions CS1308 (A), CS1566 (B), CS1643 (C), CS1194 (D) and CS1492 (E) at different time points after inoculation with buffer (---◆---) and *Xanthomonas campestris* pv. *campestris* CNPH 17 at 5 x 10^6 CFU/mL (---■---). Standard errors were ≤0.2.

Response of resistant accessions to a different Xcc isolate and to *R. solanacearum*

In order to test the response of resistant accessions to a different Xcc isolate, accessions CS1308, CS1566, CS1643 and CS1438 (which showed partial resistance to CNPH 17) were inoculated with the isolate CNPH 77 of Xcc (Figure 3). This isolate was more aggressive than CNPH 17 in a screening of cabbage genotypes at Embrapa Hortaliças, Brasília (Quezado-Duval, personal communication). All accessions developed disease symptoms, however, and accession CS1566 was significantly more resistant to this aggressive isolate of Xcc according to the Tukey test of means. Furthermore, the accessions resistant to isolate Xcc CNPH 17, which were CS1308, CS1566 and CS1643, were also inoculated with *Ralstonia solanacearum* (Rs CNPH 221) and were significantly more resistant (Table 2) to this pathogen according to the Tukey test of means.

Resistance to Xcc CNPH 17 does not involve a hypersensitive response

To test if the resistance observed in accessions CS1308, CS1566 and CS1643 involves a hypersensitive response, plants were grown in short day conditions and three leaves per plant of three to ten plants of each accession were inoculated with a high titer of bacteria (5 x 10^8 CFU/mL). Symptom development was monitored at 7, 24 and 48 h after inoculation. *Brassica oleracea* var. *capitata* cv. Kenzan was used as a control and developed disease symptoms as expected. However, no symptoms of hypersensitive response were observed in *Arabidopsis* plants. This experiment was repeated and the same results were obtained.

DISCUSSION

Despite the fact that *Arabidopsis* has been the subject of many studies over the last 20 years, most of these have focused on a few accessions such as Ler, Col-0 and Ws. For many aspects of plant development and particularly in plant-pathogen interaction studies, each accession can potentially behave differently due to specific differences in its genetic makeup. Many labs have realized that fact and are now starting to explore the natural variability present in the *Arabidopsis* germplasm. Our investigation of the *Arabidopsis* natural variation response to a Brazilian isolate of Xcc uncovered five accessions out of 32 that are resistant. About the same number of accessions were susceptible to Xcc, while most accessions tested displayed an intermediate phenotype here described as partially resistant.

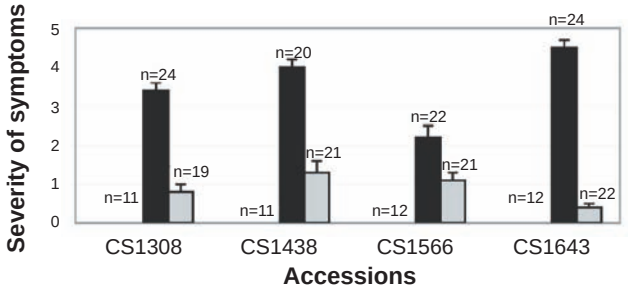

FIG. 3 - Severity of symptoms in *Arabidopsis* accessions, six days after inoculation with buffer (left column), *Xanthomonas campestris* pv. *campestris* CNPH 77 (middle column) and *Xanthomonas campestris* pv. *campestris* CNPH 17 (right column). For each accession the number of leaves inoculated is shown (n). Vertical bars are the standard error. All leaves inoculated with buffer showed no symptoms of disease (0 on the scale) with a standard error of 0. The Tukey test of means shows no significant difference in the response of "CS1643", "CS1438" and "CS1308" to Xcc CNPH 77; however, "CS1566" is statistically more resistant than the others.

TABLE 2 - Comparative analysis of the response of selected *Arabidopsis* accessions to *Xanthomonas campestris* pv. *campestris* (Xcc) and *Ralstonia solanacearum* (Rs)

Accession	Xcc CNPH 17	Xcc CNPH 77	Rs CNPH 221
CS1308	R	S	R
CS1566	R	R	R
CS1643	R	S	R
CS1194	S	-	S

R: resistant; S: susceptible; -: not determined

Xcc can enter the leaf by hydathodes or stomata (Lopes & Quezado-Soares, 1997). The latter case is associated with the presence of round lesions on the leaf blade. In electron microscopy studies of cabbage, Xcc could be found inside the xylem-conducting vessels and intercellular spaces (Bretschneider *et al*., 1989). Each time Xcc comes into contact with its host there is a potential opportunity for the plant to recognize that it is being attacked and trigger its defenses against the invading bacteria. Ideally, crop plants should have multiple resistance genes able to deter the pathogen at various entry points. In this study, a leaf infiltration method for assessing disease resistance was used to uncover resistances that will most likely be related to mesophyll cells and will perhaps be more effective against Xcc entry through stomata.

Thirty-two accessions were classified into three categories based on the Tukey test of means according to their degree of symptoms developed in response to Xcc CNPH 17. Based on the consistency of results and the number of seeds available, a few accessions with contrasting phenotypes were chosen for further characterization. The chlorophyll content of the resistant accessions (CS1308, CS1566 and CS1643) was measured and remained high after inoculation with the pathogen, while in susceptible accessions (CS1194 and CS1492) it declined sharply a few days after inoculation. A simple linear regression was performed and it was significant only for Xcc-treated plants in susceptible accessions (results not shown). Therefore, it is only in susceptible accessions that chlorophyll levels decline as a function of days after inoculation with Xcc. Because the measurement of chlorophyll is quantitative and not dependent on the subjective analysis of the observer, it was an ideal method to corroborate the results initially obtained

using a severity of symptoms scale.

Plant physiology can be affected by light conditions. For example, under long days *Arabidopsis* flowers earlier than under short days. To test if a different light condition would affect the resistant or susceptible phenotypes, plants were grown under short-day conditions (8h light / 16 h dark). The same results that were previously obtained under continuous light were observed in these conditions. Therefore, at least for the accessions under study, the light conditions did not have any impact on the outcome of the plant-pathogen interaction.

Inoculation of the accessions resistant to Xcc CNPH 17 with the isolate CNPH 77 showed that "CS1566" was the most resistant. Interestingly, this accession showed resistance to the soil pathogen *Ralstonia solanacearum*, which also causes a vascular disease. Currently the mechanism of resistance of accessions CS1308, CS1566 and CS1643 is unknown. However, experiments with a higher titer of Xcc indicate that a hypersensitive reaction is not involved. Crosses between resistant and susceptible accessions are being performed to generate populations for the genetic analysis of resistance. Furthermore, it is na ideal time to investigate basic aspects of plant-pathogen interaction using the Xcc/*Arabidopsis* pathosystem as a tool for postgenomic studies, and the study here reported sets the stage for such work using a Brazilian isolate of Xcc .

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