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TECHNICAL ADVANCE

Arabidopsis thaliana Cells: A Model to Evaluate the Virulence of *Pectobacterium carotovorum*

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Pectobacterium carotovorum **are economically important plant pathogens that cause plant soft rot. These enterobacteria display high diversity world-wide. Their pathogenesis depends on production and secretion of virulence factors such as plant cell wall–degrading enzymes, type III effectors, a necrosis-inducing protein, and a secreted virulence factor from** *Xanthomonas* **spp., which are tightly regulated by quorum sensing.** *Pectobacterium carotovorum* **also present pathogen-associated molecular patterns that could participate in their pathogenicity. In this study, by using suspension cells of** *Arabidopsis thaliana,* **we correlate plant cell death and pectate lyase activities during coinfection with different** *P. carotovorum* **strains. When comparing soft rot symptoms induced on potato slices with pectate lyase activities and plant cell death observed during coculture with** *Arabidopsis thaliana* **cells, the order of strain virulence was found to be the same. Therefore,** *Arabidopsis thaliana* **cells could be an alternative tool to evaluate rapidly and efficiently the virulence of different** *P. carotovorum* **strains.**

Pectobacterium carotovorum (formerly *Erwinia carotovora* subsp. *carotovora*) are gram-negative, enteric bacteria that are economically important plant pathogens causing plant soft rot (Perombelon 2002; Toth et al. 2006). Production and secretion of virulence factors are key steps to the pathogenesis of these bacteria. Their primary virulence characteristic is the coordinated production of large amounts of multiple secreted plant cell wall–degrading enzymes (PCWDE). By cleaving structural polymers in the primary cell wall and middle lamella, PCWDE facilitate pathogen colonization and promote exploitation of the environment for nutrients released from killed cells (Toth et al. 2006). The development of maceration symptoms leads to the breakdown of plant tissues and the onset of soft rot disease (Barras et al. 1994; Collmer 1983; Hugouvieux-Cotte-Pattat et al. 1996). In addition to these PCWDE, *P. caro-*

tovorum also produce other secreted virulence factors, including type III effectors (Holeva et al. 2004; Kariola et al. 2003), a necrosis-inducing protein (Mattinen et al. 2004; Pemberton et al. 2005), and a secreted virulence factor from *Xanthomonas* spp. (Corbett et al. 2005). In *P. carotovorum*, production of PCWDE and the other secreted virulence factors is tightly regulated by *N*-acyl homoserine lactone (AHL) quorum sensing (QS) (Laasik et al. 2006; Toth et al. 2006). QS is a mechanism of cell to cell communication in which a bacterial population coordinately regulates gene expression in response to cell density by the production and detection of the AHL signal (Barnard and Salmon 2007; Perombelon 2002; Toth et al. 2006). *Pectobacterium carotovorum,* as all gram-negative bacteria, also exhibit pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides, the major PAMPs of enterobacteria, which could be potential pathogenic determinants (Toth et al. 2006) independent of QS.

Despite this general scheme, *P. carotovorum* strains isolated from host plants world-wide are surprisingly diverse (Duarte et al. 2004; Gross et al. 1991; Seo et al. 2002; Sledi et al. 2000; Yap et al. 2004). Many studies have analyzed the genetic diversity of *Pectobacterium* species (Aittamaa et al. 2008; Helias et al. 2004; Mäki-Vlakama and Karjalainen 2008; Parent et al. 1996; Sledi et al. 2000); however, the analysis of virulence diversity remains difficult for these bacteria. The objective of this work was to evaluate the virulence of different *P. carotovorum* strains by using *Arabidopsis thaliana* suspension cells.

RESULTS AND DISCUSSION

Pectobacterium carotovorum are known to trigger defense responses and cell death in *A. thaliana* (Kariola et al. 2003). To assess the virulence of *P. carotovorum*, we measured *A. thaliana* cell death by using cultured suspension cells, a convenient material for studying physiological events induced by pathogens or molecules derived from pathogens (Atkinson et al. 1986; Bouizgarne et al. 2006; Errakhi et al. 2008; Reboutier and Bouteau 2008; van Loon et al. 2008). Coculture of *A. thaliana* cells with 10^8 CFU of *P. carotovorum* 132C per milliliter led to a biphasic increase in plant cell death, i.e., a rapid but limited increase during the first 4 h, followed by a large exponential increase leading to death of all *A. thaliana* cells after 10 h (Fig. 1A). No significant increase in cell death was observed for control cultures with plant cells alone or those infected with heat-killed bacteria at a rate of 10^8 CFU·ml^{-1}
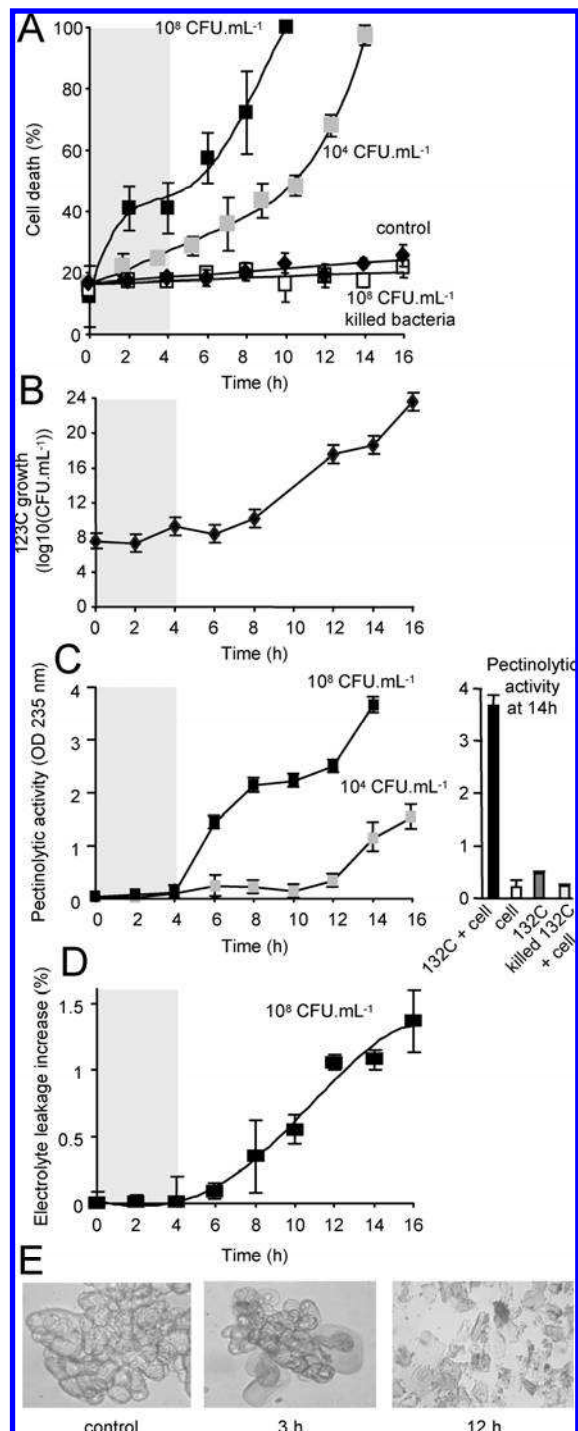
Fig. 1. **A,** Time course of *Arabidopsis thaliana* cell death after inoculation with *Pectobacterium carotovorum* 132C at rates of either 10^8 or 10^4 CFU·ml⁻¹. Controls were made with plant cells alone or cells infected with heat-killed bacteria (10^8 CFU·ml⁻¹). **B,** Time course of the growth of strain 132C (10^8 CFU·ml⁻¹) in Gamborg medium without plant cells. **C,** Time course of pectinolytic activities (measured by oligogalacturonate accumulation) in the culture medium after innoculation of *A. thaliana* cells with strain 132C at either 10^8 or 10^4 CFU·ml⁻¹. Pectinolytic activities measured in culture medium after 14 h of coculture with *P. carotovorum* at 10^8 CFU·ml⁻¹ compared with strain 132C alone, plant cells alone, and plant cells infected with heat-killed bacteria (10^8 CFU·ml⁻¹). **D,** Time course of electrolyte leakage increase in the culture medium during coculture of *A. thaliana* cell after inoculation with strain 132C at 10^8 CFU·ml⁻¹. **E,** Light micrographs of *A. thaliana* cells stained with Evans blue before (control) and 3 and 12 h after inoculation with *P. carotovorum* at 10^8 CFU·ml⁻¹. Data represent the mean of three independent replicates and error bars correspond to standard errors.

(Fig. 1A). Bacterial growth in the plant cell medium (10^8 CFU·ml⁻¹ without *A. thaliana* cells; Fig. 1B) was found to be delayed and began after 6 h. This indicated that the observed plant cell death was dependent on events induced by the coculture and could not be ascribed to a simple increase of bacteria in the culture medium. When the coculture was made with strain 132C at 10^4 CFU·ml⁻¹, cell death increased slightly from the first hours and the exponential increase in cell death was delayed after 10 h of coculture (Fig. 1A). *P. carotovorum* is characterized by its ability to produce high levels of PCWDE, especially pectate lyase, which are primarily responsible for the degradation of plant cell-wall components leading to cell death and tissue maceration (Barras et al. 1994; Collmer 1983; Hugouvieux-Cotte-Pattat et al. 1996). An increase in pectate lyase activities, measured by oligogalacturonate accumulation, was effectively detected in the coculture medium about 4 h after the beginning of coculture with strain 132C at 10^8 CFU·ml⁻¹ and this was delayed to 10 h with a rate of 10^4 CFU·ml⁻¹ (Fig. 1C). The kinetics of the exponential cell death and pectate lyase activities appear to be correlated (Fig. 1A and C), as expected from previous data highlighting the predominant role of PCWDE secretion in *P. carotovorum* virulence (Toth et al. 2006). A 14-h culture of strain 132C alone showed low pectinolytic activities, and this was also observed in controls containing plant cells alone or cells infected with heat-killed bacteria (Fig. 1C, inlet). Such data indicate that

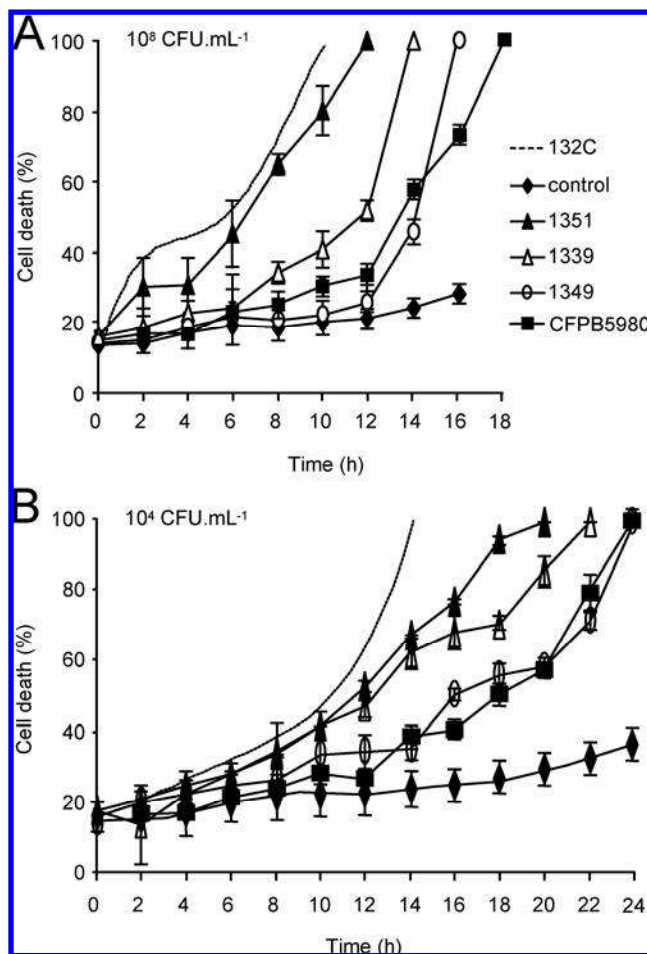**Fig. 2.** Time course of *Arabidopsis thaliana* cell death during coculture after inoculation with *Pectobacterium carotovorum* strains 1351, 1339, 1349 and CFPB5980 at rates of either **A,** 10^8 or **B,** 10^4 CFU·ml⁻¹. Dashed lines with no symbols correspond to the data of strain 132C. Data represent the mean of three independent replicates and error bars correspond to standard errors.

PCWDE secretion is linked to the coculture conditions. Previously, it has been shown that production and secretion of PCDWE is activated in the presence of pectin and plant extracts (Barras et al. 1994). An increase in electrolyte leakage, measured as an increase in conductivity of the culture medium, could also be recorded during coculture. This increase in conductivity showed the same kinetics as the change in pectate lyase activities (Fig. 1C and D), and it could be explained by cell breakage as revealed by Evans blue (EB) staining, since cell integrity was not preserved when pectate lyase activities increased (Fig. 1E). These data clearly demonstrate that *P. carotovorum* recognized *A. thaliana* suspension cells as host cells, which allowed the virulence process leading to cell death to be induced.

Upon infection of the suspension cells with *P. carotovorum* 132C at rates of 10^8 or 10^4 CFU·ml^{-1}, a small initial increase (of about 30%) in plant cell death could be seen before the exponential cell-death phase. This initial cell death could not be ascribed to PCWDE secretion, since dead cells only displayed plasmolysis and not cell-wall degradation (Fig. 1E). Moreover, this cell death occurred before pectate lyase activities were detectable (before 4 or 10 h for 10^8 or 10^4 CFU·ml^{-1}, respectively; Fig. 1A and C). Coculture of cells with heat-killed bacteria did not show any induction of this early and limited cell-death phase. This suggests the involvement of one or more factors that are either inactivated by the heat treatment or require active metabolism or bacterial growth. An advantage of this method is to allow parallel and easy measurement of cell death, ion leakage, and enzyme activities in a time-dependent manner. Thus, *Arabidopsis thaliana* suspension cells are an interesting model to discriminate between the effects of different virulence factors and, more generally, to analyze the virulence of *P. carotovorum*.

The virulence of different *P. carotovorum* strains (Supplemental Table 1) was compared with strain 132C by measuring the extent of cell death of *A. thaliana* suspension cells cocultured with these strains. When infected with 10^8 CFU·ml^{-1}, exponential cell death occurred at different times, while an early induced cell death was detected only with strains 132C and 1351 (Fig. 2A). In this way, strain virulence was found to be as follows: strain 132C > 1351 > 1339 > 1349 = CFPB5980. The same sequence was observed after infection of *A. thaliana* cells with each strain at the rate of 10^8 CFU·ml^{-1}, with cell death being delayed as expected for a QS regulation of the virulence (Fig. 2B). We further analyzed the pectate lyase activities of the culture medium upon coculture of *A. thaliana* cells with each *P. carotovorum* strain. As for strain 132C (Fig. 1C), pectate lyase activities were detected after a few hours of coculture (Fig. 3A). The appearance of these activities was correlated with the timing of the exponential cell death (Fig. 2A), thereby allowing us to propose the same sequence of virulence for the different strains: 132C > 1351 > 1339 > 1349 = CFPB5980. Cell electrolyte leakage during coculture with different strains was also delayed, but it was not perfectly synchronized with either exponential cell death or the increase in pectate lyase activities (Fig. 3B), although it gave the same sequence of virulence. Our comparisons indicate that all the strains are not able to induce an early limited cell death and that the timing of PCWDE secretion and subsequent large-scale cell death are variable and strain dependent. Furthermore, this method allows an easy comparison from a relatively large number of samples.

To evaluate the reliability of the data observed using *A. thaliana* suspension cells, the virulence of the different strains was evaluated with potato slices. Twelve potato slices (cv. Agatha) were infected with *P. carotovorum* strains, 132C, 1349, 1359, 1351, and CFPB5980 at 10^2, 10^4, or 10^8 CFU·ml^{-1}. The mean soft-rot diameters were measured after 24 h (Fig. 4). Infection of potato slices at the rate of 10^2 CFU·ml^{-1} showed that the strains fell into the same order when comparing their

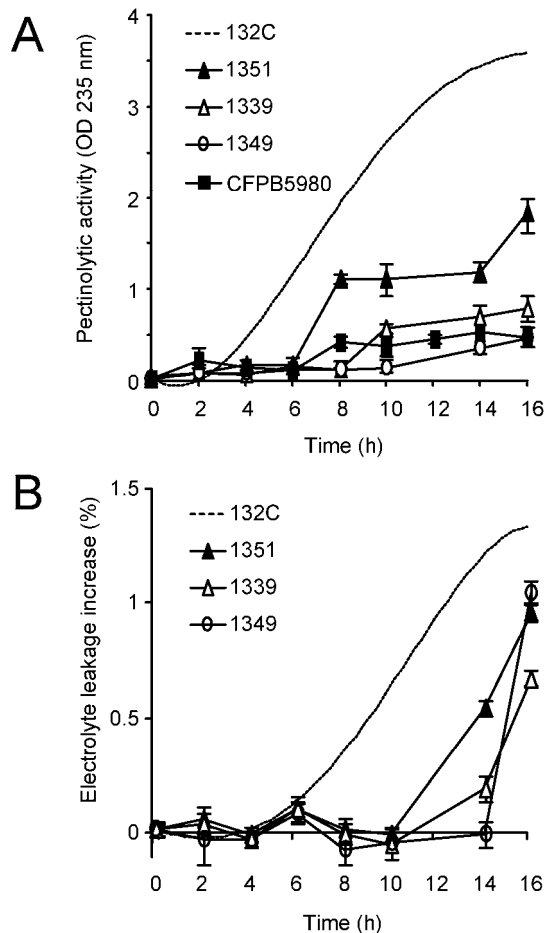**Fig. 3.** **A,** Time course of pectinoltytic activities measured in the culture medium during coculture of *Arabidopsis thaliana* cell after inoculation with various *Pectobacterium carotovorum* strains at the rate of 10^8 CFU·ml^{-1}. **B,** Time course of electrolyte leakage increase in the culture medium during coculture of *A. thaliana* cell after inoculation with various *P. carotovorum* strains at 10^8 CFU·ml^{-1}. Dashed lines with no symbols correspond to the data of strain 132C. Data represent the mean of three independent replicates and error bars correspond to standard errors.
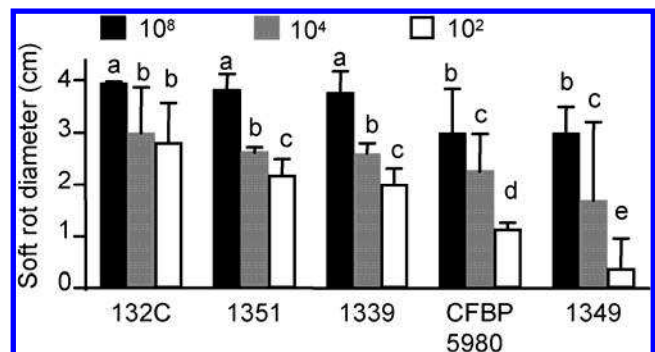

Fig. 4. Mean values of soft-rot diameter caused by the different *Pectobacterium carotovorum* strains at 10^2, 10^4, and 10^8 CFU·ml^{-1}. Control with sterile distillated water did not induce symptoms. Triplicate potato slices in the same petri dishes were carried out as repeated treatments for each test, error bars correspond to standard errors. For each treatment, variable bar values with the same letter are not significantly different according to Duncan's multiple range, $P \leq 0.05$.

action on cell death of *A. thaliana*–cultured cells, pectate lyase activities, and virulence on potato slices, i.e., 132C > 1351 > 1339 > 1349 = CFPB5980. Even if the differences between strains were not significant for infection at 10^8 and 10^4 CFU·ml^{-1}, the general trends suggest the same sequence of virulence for the different strains (Fig. 4). Therefore, the cell-death data observed in *A. thaliana* cells are in accordance with the soft-rot symptoms found with infected potato slices. This cell death is representative of the bacterial virulence and, interestingly allows a precise evaluation of the virulence of different strains.

In conclusion, *A. thaliana* suspension cells are a reliable model to undertake a rapid testing of virulence diversity. Our data highlight the use of *A. thaliana* suspension cells as a tool to dissect the different events involved in *P. carotovorum* pathogenicity and to evaluate efficiently and rapidly the virulence of different strains.

MATERIALS AND METHODS

Bacterial material.

Different strains of *P. carotovorum*, formerly *E. carotovora* subsp. *carotovora*, previously characterized by the Moroccan team, were used in this study and compared with the reference strain CFPB5980. All strains display the biochemical characteristics of *P. carotovorum*. PCR amplifications of total genomic DNA from all strains were positive with the primers Y1 and Y2, specific for *Pectobacterium* species (Darrasse et al. 1994) (amplified fragment of 434 bp), and they were negative with primers P143 and P145, specific for Dickeya chrysanthemi (El Hassouni et al. 1999). Strains were stored at –80°C. When required, each bacterial strain was cultured on LPGA agar medium (5 g yeast extract, 5 g peptone, 10 g glucose, and 16 g agar per liter, pH 7.2). Dead bacteria used for control were heat-killed at 100°C for 1 h.

Plant material.

Arabidopsis thaliana L. (ecotype Columbia) suspension cells were grown at 24 ± 2°C, under continuous white light (40 μE m^{-2} s^{-1}), with rotation shaking at 120 rpm in a 1-liter round-bottom flask containing 300 ml of Gamborg culture medium, pH 5.8. Cells were subcultured weekly by a 10-fold dilution in fresh medium (Bouizgarne et al. 2006; Errakhi et al. 2008).

Quantification of cell death.

Cell viability was assayed using the vital dye Evans Blue (EB) (Errakhi et al. 2008). *A. thaliana* cell suspensions (500 μl) infected with different bacterial suspensions during different time periods were incubated for 10 min in EB to a final concentration of 0.001% in 0.1 M phosphate-buffer, pH 7. Cells that accumulated EB were considered dead. A total of 500 cells were counted for each infection.

Measurement of electrolyte leakage.

Electrolyte leakage was measured from the clear supernatant of cultured cells infected with bacterial suspensions containing bacteria at 10^8 CFU·ml^{-1} that were incubated at 24 ± 2°C, under continuous white light (40 μE m^{-2} s^{-1}), with rotation shaking in flasks containing 10 ml of solution. Conductivity was measured every 2 h with a SevenMulticonductimeter (Mettler Toledo, Viroflay, France).

Pectate lyase assays.

Pectate lyase activities were measured by monitoring the unsaturated oligogalacturonates accumulated after the enzymatic cleavage of 0.5% (wt/vol) polygalacturonate (PGA). The assay was performed in 750 μl of substrate solution (0.5% PGA),

200 μl of clear supernatant of each treatment, 37.5 μl of 5 × 10^{-4} M CaCl$_2$, and 512.5 μl of 0.1 M Tris-HCl, pH 8.5. After incubation for 1 h at 30°C, the reaction was stopped by heating at 100°C for 20 min. Products resulting from sodium polypectate degradation were followed at 235 nm (Dinu et al. 2007; Pissavin et al. 1998).

Pathogenicity test on potato slices.

Fresh potatoes (cv. Agatha) were washed and chopped into equal pieces with a sterile borer. *P. carotovorum* strains 132C, 1349, 1359, 1351, and CFPB5980 were cultured, and the concentrations were adjusted to 10^2, 10^4, or 10^8 CFU·ml^{-1}. Twelve potato slices were inoculated for each concentration. They were incubated at 30°C for 24 h, and the maceration areas were quantified.

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