European Journal of Plant Pathology **108:** 547–553, 2002.

Protective effects against *Erwinia amylovora* induced by *hrp* mutants in the whole plant are only partially mimicked in cultivated cells

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Accepted 8 May 2002

Key words: apple, plant–bacteria interaction, cell death, protection

Abstract

A virulent strain of *Erwinia amylovora*, the causal agent of fire blight of *Maloideae*, and two of its non-virulent *hrp* mutants (a secretory and a regulatory mutant) were inoculated into apple cell suspensions either alone or in mixed inoculations. In single inoculations, death of 4- to 5-day-old apple cells occurred only when the concentration of the virulent strain of *E. amylovora* reached a threshold inoculum concentration of 10^4 CFU ml^{-1}, while high concentrations of the *hrp* mutants were unable to kill apple cells. When mixed inoculated with the virulent parental strain, both *hrp* mutants protected apple cells from death caused by the virulent strain. The protective effect was associated with a decrease in the population level of the virulent strain and it was dependent on the non-virulent to virulent inoculum concentration suggesting a competition between the non-virulent mutant and the virulent strain. However, no differential protective ability between the two types of mutants could be noticed, contrary to previous results obtained with apple seedlings or apple flowers in which the regulatory mutant was significantly more effective than the secretory mutant. In conclusion, inoculation of apple cell cultures with *E. amylovora* does not seem to be a model suitable for investigating mechanisms leading to protection.

Introduction

Fire blight caused by *Erwinia amylovora* is a disease of economic importance which affects apple and pear trees as well as ornamentals including *Crataegus*, *Cotoneaster* and *Pyracantha* (Vanneste, 2000).

Molecular studies of *E. amylovora* revealed the existence of an *hrp–dsp* cluster involved in pathogenicity (compatible interaction) and the hypersensitive reaction (incompatible interaction) (Steinberger and Beer, 1988; Barny et al., 1990; Bauer and Beer, 1991). Genes of this cluster encode (i) proteins involved in a type III secretory apparatus, (ii) secreted proteins (Harpin N, Harpin W, DspA) and (iii) regulatory proteins (HrpX, HrpY, HrpS and HrpL) (for review see Kim and Beer, 2000).

Under controlled conditions, non-virulent *hrp* mutants of *E. amylovora* protected apple seedlings and apple flowers from developing fire blight symptoms (Tharaud et al., 1997; Faize et al., 1999). In addition, *hrp* regulatory mutants appeared to have a significantly higher protective ability than secretory mutants in these two bioassays. The level of protection was dependent on the non-virulent to virulent inoculum concentration and was associated with a decrease in the population level of the virulent strain. Furthermore, accumulation of two families of enzymes involved in plant defense responses, phenylalanine ammonia lyase (PAL) and peroxidase (POD), was observed in leaves treated with a regulatory mutant, but not in leaves treated with a secretory mutant. However, the use of flowers or seedlings is not a convenient system for such studies. Indeed, flowers are not available all the year long and seedlings are genetically heterogeneous (open-pollination).

Suspension cultured cells have been suggested to be a suitable model for studying the interaction between bacteria and plants. For example tomato, tobacco and

soybean cells were used to analyze the attachment of *Agrobacterium tumefaciens* (Matthysse and Gurlitz, 1982; Neff and Binns, 1985). Apple and tobacco cells were used to characterize the hypersensitive and disease reactions caused by *Pseudomonas syringae* pv. *tabaci* and *E. amylovora* (Brisset and Paulin, 1992). Cell suspensions were also used to study plant–bacteria saprophytic interaction (Baker et al., 1991). Moreover, cell suspensions have been widely used to investigate early events following application of pathogen-derived molecules (Apostol et al., 1989; Blein et al., 1991; Vera-Estrella et al., 1992; Honée et al., 1998; Dorey et al., 1999) including Harpin N from *E. amylovora* (Baker et al., 1993; Popham et al., 1995).

In this paper, we examined whether apple cell suspension cultures could afford a reliable system for the study of early events associated with the interaction between non-virulent mutants of *E. amylovora*, the parental virulent strain and the host plant. First, we optimized some factors, such as the age of apple cells and the inoculum concentration of the pathogen, involved in the establishment of the interaction between *E. amylovora* and apple cell suspensions. Then we cultured the apple cells with both non-virulent and virulent strains of *E. amylovora* and showed that it was possible to reveal some characteristics of the protective effect with the *hrp* mutants of *E. amylovora* but not to reproduce all the aspects of the protection noticed on flowers and apple seedlings.

Materials and methods

Bacterial strains and culture conditions

Three isolates of *E. amylovora* were used; a wild-type virulent strain CFBP1430 (Paulin and Samson, 1973) and two of its transposon mutants, PMV6023, a non-virulent *hrp* secretory mutant (Gaudriault et al., 1997), and PMV6046, a non-virulent mutant altered in the regulatory functions of the *hrp* cluster (Wei and Beer, 1995; Tharaud et al., 1997). Bacteria were grown routinely for 24 h at 27 °C on King's medium B (KB, King et al., 1954), supplemented with chloramphenicol (20 μg ml^{-1}) for the mutants. For selective recovery in population dynamic studies a spontaneous mutant of CFBP1430 resistant to spectinomycin (100 μg ml^{-1}) designated CFBP1430spe^{r} and exhibiting the same level of virulence as the parental strain was used. For inoculum preparation, bacterial suspensions were washed in the assay medium (morpholinoethane sulfonic acid 5 mM + mannitol 3%, adjusted to pH 5.8 with KOH) by centrifugation at 10 000× *g* for 10 min then resuspended in the same assay medium to the required inoculum concentration, which was checked by plating serial dilutions onto KB. When necessary, bacterial suspensions were heat-killed in a water bath at 45 °C for 3 h.

Apple cell suspension culture

Apple cell suspensions obtained from leaf calli of the susceptible rootstock MM106 were grown in Murashige and Skoog medium (1962) with benzylaminopurine (0.5 mg ml^{-1}), naphthalene acetic acid (2 mg ml^{-1}), sucrose (3%), at 25 °C, in 250 ml Erlenmeyer flasks, on a rotary shaker (Infors, Bottmingen, Switzerland) (130 rpm) under continuous light (3500 lux). Apple cells were sub-cultured every 7 days: for this purpose, cells were filtered successively through two sieves (mesh size 500 and 50 μm), then cells and aggregates between 500 and 50 μm (*ca* 10 g of fresh weight) were suspended into 150 ml of fresh medium.

Preparation and inoculation of apple cell suspensions

The apple cell suspension assay was derived from the technique described by Brisset and Paulin (1992). Packed cell volumes (PCV) for cell suspensions were determined after centrifugation of an aliquot of 10 ml at 600× *g* for 15 min in graduated tubes. Cells were adjusted to a concentration of 10% PCV (about 10^6 cells ml^{-1}) by dilution in the assay medium and washed twice by centrifugation at 150× *g* for 3 min and resuspension in the assay medium, and incubated for 2 h on the rotary shaker used for their growth. Cell suspensions were finally dispensed into 12-welled plastic dishes (Nunc, Brand products) (1.8 ml per well). They were inoculated with 200 μl of bacterial suspension or of assay medium for the control.

For the study of the protective effect of the *hrp* mutants, two inoculation schemes derived from the method described by Tharaud et al. (1997) were used: sequential inoculation, in which the mutant strain was applied to the apple cell suspensions 2 h before the virulent strain, and simultaneous inoculation in which the mutant and the virulent strains were mixed and immediately applied to the apple cell suspensions. In both cases, 100 μl of each bacterial suspension were used.

Final bacterial inoculum concentrations used in these assays are given with the Results. The assay cultures were incubated for 48 h under the same conditions as used for the growth of cell suspensions. For each test, three replicates were inoculated and the experiments were performed at least twice. The protective effect was assessed through the percentage of apple cell mortality which was determined after 48 h of co-culture with fluorescein diacetate (0.1 mg ml^{-1}) (Widholm, 1972). 800 cells spreading over at least 5 microscopic fields were assessed for viability.

After 48 h, bacterial populations in each assay were assessed by sonicating the suspension (Branson Sonifier 450) for 10 s to detach bacteria bound to apple cells. After centrifugation (600× *g* for 15 min) supernatants were serial-diluted and plated onto KB medium supplemented with the appropriate antibiotic. This experiment was repeated three times.

Results

Effect of the age of apple cell cultures on their susceptibility to E. amylovora

The growth curve of apple cell suspensions subcultured in the fresh medium obtained through PCV assessment showed a 2-day latency phase, followed by an exponential phase of 3 days, and then a stationary phase (data not shown). When suspensions of 3- to 6-day-old apple cells were cultivated with virulent strain CFBP1430 (10^6 CFU ml^{-1}) the highest level of apple cell mortality (about 90%) was observed with 4- to 5-day-old apple cells (i.e. in their exponential phase) (Figure 1). Mortality was significantly reduced in co-cultures with 6-day-old apple cells (i.e. in their stationary phase).

Influence of the bacterial concentration on the apple cell mortality

Suspensions of 4-day-old apple cell were cultivated with increasing concentrations of the virulent strain CFBP1430 and of non-virulent mutants PMV6046 (regulatory mutant) and PMV6023 (secretory mutant). In cultures with strain CFBP1430, a minimal concentration of 10^4 CFU ml^{-1} was necessary to induce apple cell mortality (Figure 2). This appeared to be a significant threshold value, since the low level of apple cell mortality (20%) noticed with an inoculum concentration of 10^3 CFU ml^{-1} jumped to 90% for 10^4 CFU ml^{-1}

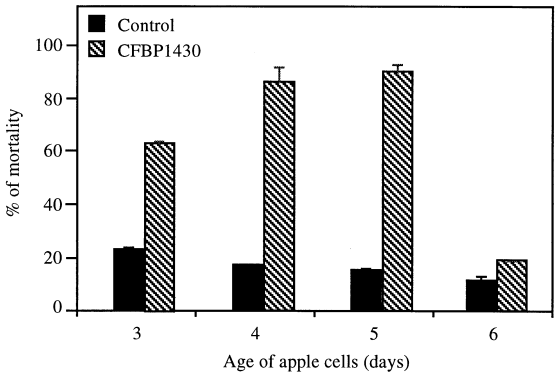

Figure 1. Susceptibility of 3- to 6-day-old apple cell cultures to *E. amylovora*. Mortality of apple cells was assessed 48 h after. They were cultured in an assay medium (control) and after inoculation with virulent strain CFBP1430 (10^6 CFU ml^{-1}). Means and standard deviations (5%) of three replicates.

and remained stable for higher bacterial concentrations (up to 10^7 CFU ml^{-1}). In the cultures with non-virulent mutants, no significant increase of apple cell mortality was observed, regardless of the mutant, even with the highest bacterial concentration (10^7 CFU ml^{-1}).

Protective effect of hrp *mutants*

When the assays were inoculated simultaneously with non-virulent to virulent cell concentrations of (CFU ml^{-1}) $10^5/10^4$ (Figure 3A) or $10^6/10^5$ (data not shown) the percentage of mortality of apple cells was low and not significantly different from the control. When the inoculum concentrations were $10^7/10^6$ (Figure 3B) or ($10^8/10^7$) (data not shown) the protective effect was not observed with either mutant: the percentage of apple cell death was high (95%) and not significantly different from that obtained with the virulent strain inoculated alone. When the non-virulent to virulent cell concentrations were $10^5/10^5$ (Figure 3C) no protective effect from the non-virulent strain was observed.

When the mutant strains were inoculated 2 h before the virulent strain with a non-virulent to virulent cell concentration of $10^5/10^4$ (Figure 4A) apple cell mortality was low compared to apple cells inoculated only with the virulent strain. The level of cell death for mutants alone was similar to that obtained in the control. With non-virulent to virulent inoculum concentrations of $10^8/10^7$ or $10^7/10^6$ no protection was observed (data not shown). When the PMV6046 mutant was inoculated 2 h after the virulent strain or

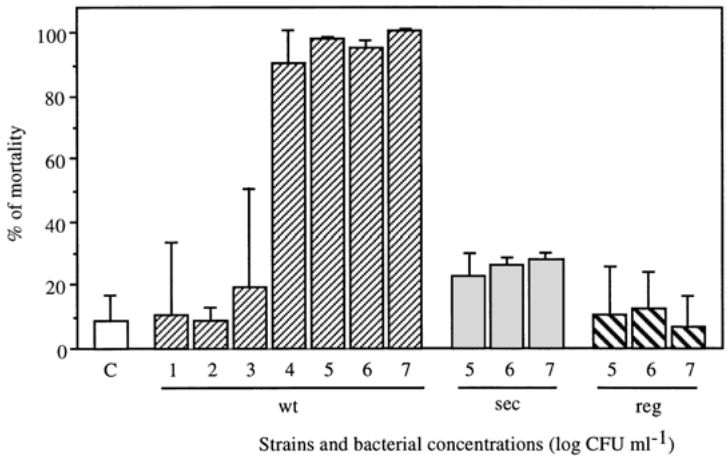

Figure 2. Influence of initial concentrations of three *E. amylovora* strains on apple cell mortality. Suspensions of 4-day-old apple cells were cultivated with increasing concentrations (log CFU ml^{-1}) of virulent strain CFBP1430 (wt) or of non-virulent secretory mutant PMV6023 (sec) and regulatory mutant PMV6046 (reg). Mortality of apple cells was assessed after 48 h inoculation with the bacteria. The control (C) consisted on non-inoculated cell suspensions. Means and standard deviations (5%) of three replicates.

was heat-killed (Figure 4B) no protective effect was observed.

Dynamics of bacterial populations in the co-cultures

Strains PMV6046 and CFBP1430spe^r were applied to 4-day-old apple cell suspensions either alone (single inoculation) or simultaneously at inoculum concentrations of $10^6/10^5$ (which led to a protective effect) or $10^5/10^5$ (which gave no significant protective effect), respectively (Table 1). Using single inoculation the virulent strain CFBP1430spe^r multiplied to reach 10^8 CFU ml^{-1} after 48 h. Populations of PMV6046 declined slightly when inoculated at 10^5 CFU ml^{-1} and increased slightly when inoculated at 10^6 CFU ml^{-1}. When PMV6046 was inoculated ahead of CFBP1430spe^r, populations of CFBP1430 decreased from 10^5 to 10^2 CFU ml^{-1} after 48 h while those for PMV6046 reached the same population level (10^6 CFU ml^{-1}) as when inoculated alone. In the situation of 'non-protection' the population level of the virulent CFBP1430spe^r increased from 10^5 to 10^8 CFU ml^{-1} after 48 h of co-culture. Similarly PMV6046 increased from 10^5 to 10^7 CFU ml^{-1}.

Discussion

This work aimed to investigate the reliability of apple cell suspensions as a tool in the study of the protective effect induced against *E. amylovora* by non-virulent mutants of the pathogen. We first showed that the susceptibility of apple cells to a virulent *E. amylovora* was dependent on their age. Indeed, cells in their exponential growth phase (4- to 5-day-old, in our conditions) were more susceptible to *E. amylovora* than those in their stationary phase. This result is in agreement with the observations of Koumba-Koumba and Macheix (1982) who showed that cultures of apple fruit cells were able to accumulate phenolic compounds during their latency and stationary phases. The low percentage of mortality of apple cells in their stationary phase might be due to the accumulation of such compounds, which are known to be involved in plant defense. In addition, the results revealed that the non-virulent *hrp* secretory or regulatory mutants were unable to kill apple cells, even when inoculated at high inoculum concentrations and that with the virulent strain a threshold inoculum concentration of 10^4 CFU ml^{-1} was necessary to induce a significant level of apple cell mortality.

Then we examined whether a protective effect against *E. amylovora* could be obtained with non-virulent mutants on apple cell suspension, as observed on apple seedlings or apple flowers (Tharaud et al., 1997; Faize et al., 1999). The results demonstrate the existence of such a protective effect in apple cell suspensions provided that some concentrations and ratios of bacterial inoculum are respected, as we previously reported with whole plant inoculations. The protective effect was observed for non-virulent to virulent

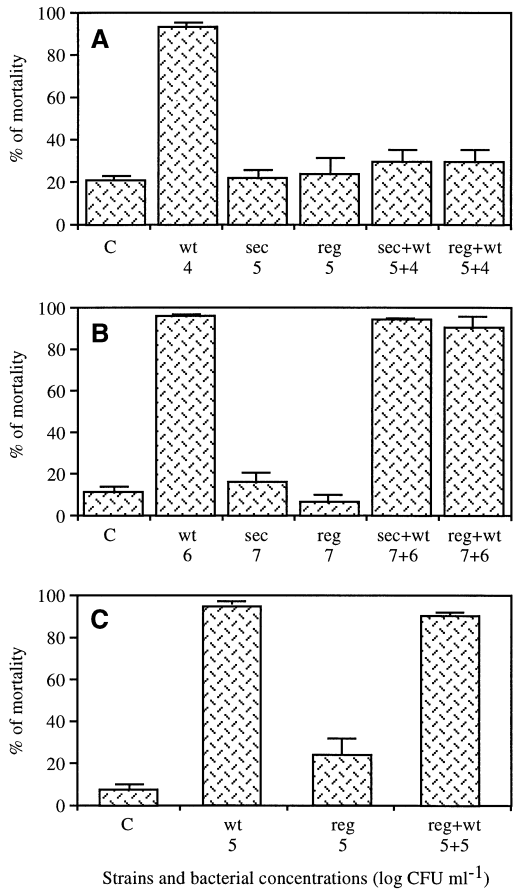

Figure 3. Reduction in mortality of apple cell suspensions when inoculated simultaneously with the non-virulent and virulent strains of *E. amylovora*. The non-virulent secretory PMV6023 (sec) or regulatory PMV6046 (reg) mutants and the virulent CFBP1430 (wt) strains were applied either alone or simultaneously to 4-day-old apple cells. The inoculum concentrations (log CFU ml^{-1}) were: (A) wt: 4; sec and reg: 5, (B) wt: 6; sec and reg: 7, and (C) wt: 5; reg: 5. Mortality of apple cells was assessed after 48 h inoculation with the bacteria. The control (C) consisted on non-inoculated cell suspensions. Means and standard deviations (5%) of three replicates.

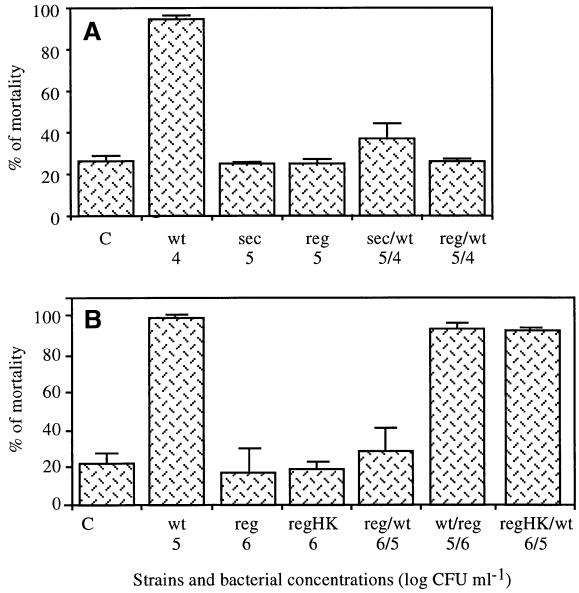

Figure 4. Reduction in mortality of apple cell suspensions when inoculated successively with the non-virulent and virulent strains of *E. amylovora*. (A) The non-virulent secretory PMV6023 (sec) or regulatory PMV6046 (reg) mutants were inoculated either alone or 2 h before the virulent CFBP1430 (wt) strain at the inoculum concentrations of (log CFU ml^{-1}): wt: 4; sec and reg: 5. (B) The regulatory PMV6046 (reg) mutant alive or heat-killed (reg HK) was inoculated either alone or 2 h before or after the virulent CFBP1430 (wt) at the inoculum concentrations (log CFU ml^{-1}) of wt: 5; reg and reg HK: 6. Mortality of apple cells was assessed after 48 h inoculation with the bacteria. The control (C) consisted on non-inoculated cell suspensions. Means and standard deviations (5%) of three replicates.

inoculum concentrations (CFU ml^{-1}) of $10^5/10^4$ or $10^6/10^5$, but not revealed with $10^7/10^6$ or $10^8/10^7$ nor with $10^5/10^5$. This phenomenon could be explained by a competition between cells of the non-virulent mutant and of the virulent strain for putative binding or infection sites on apple cells. Indeed, with the non-virulent to virulent inoculum concentrations of $10^5/10^4$ or $10^6/10^5$ the occupation of such sites by the mutant could prevent the contact of sufficient number of cells of the virulent strain to reach the threshold (10^4 CFU ml^{-1}) shown to be necessary to trigger apple cell mortality. On the contrary, the threshold might be reached with the non-virulent to virulent inoculum concentrations of $10^7/10^6$, $10^8/10^7$ as well as of $10^5/10^5$. The hypothetical necessity of an access to these putative sites is in agreement with our observation that the protective mutant must be alive for an effective control. It is also supported by our observation that when the mutant strain was inoculated 2 h after the virulent strain no protective effect was detected.

Conversely to bioassays using seedlings or flowers in which the regulatory *hrp* mutants were significantly more effective in the protection than the secretory *hrp* mutants, no differential protective ability between the two types of mutants was noticed in the assay with apple cell suspensions, whatever their timing of introduction. This discrepancy could be explained by the suppression in apple cell suspensions of some of the early steps of the plant–bacteria interaction, which could occur in the intercellular spaces of organized

Table 1. Bacterial populations cultured with apple cells

Strains*	Bacterial populations (log CFU ml^{-1})	
	Initial**	After 48 h**
Single inoculation		
None	0	0
CFBP1430spe^r	4.98 ± 0.05	7.95 ± 0.26
PMV6046	5.05 ± 0.12	4.67 ± 0.34
PMV6046	6.12 ± 0.14	6.44 ± 0.11
Simultaneous inoculation with protection		
PMV6046	6.17 ± 0.03	6.50 ± 0.05
+	+	+
CFBP1430spe^r	5.05 ± 0.11	2.20 ± 1.20
Simultaneous inoculation without protection		
PMV6046	5.14 ± 0.07	7.14 ± 0.60
+	+	+
CFBP1430spe^r	5.13 ± 0.12	7.93 ± 0.50

*The virulent CFBP1430spe^r and the non-virulent regulatory mutant PMV6046 were inoculated to 4-day-old apple cells either alone (single inoculation) or simultaneously at the inoculum concentrations leading to a situation of protection or to a situation of non-protection. **Means and standard deviations (5%) of three replicates.

tissues only, or by a higher ability of the regulatory mutants to reach infection sites when inoculated on apple seedlings or apple flowers.

The study of bacterial population dynamics in the co-cultures showed that the protective effect was associated with the decrease of the population level of the virulent strain. When there was no protection both the virulent strain and the non-virulent mutant multiplied. These results are in concordance with our previous results obtained with apple seedlings and apple flowers (Faize et al., 1999). They suggest that in this bioassay plant defense responses may be involved in the protective effect as shown on the whole plant. However, high spontaneous variability of PAL and POD activities in apple cell suspensions did not allow to obtain reliable results (data not shown).

Altogether the results led to the conclusion that apple cell suspensions cultured with non-virulent *E. amylovora* cells are protected from inoculation by the wild-type strain under certain conditions. This clearly mimicks events observed with other bioassays such as apple seedlings or apple blossoms inoculated in the glasshouse. Nevertheless, conversely to bioassays using whole plant inoculation, no difference between the protective ability of the secretory and the regulatory mutants could be noticed in the cultured cells bioassay, indicating that some aspects of the complex interaction between the three partners (non-virulent–virulent bacterial cells/susceptible apple cells) are not reproduced in this simplified bioassay. It raises the question of the experimental use of cultured plant cells for the complete investigation of plant–pathogenic bacteria interactions.

Aknowledgements

Thanks to Christelle Heintz for excellent technical assistance. This research was funded in part by EPR Pays de la Loire and DGER of the Ministère de l'Agriculture, France. Mohamed Faize was supported by a grant from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche, France.

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