Resistance

Virulence of *Erwinia amylovora* Strains to *Malus* sp. Novole Plants Grown in Vitro and in the Greenhouse

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ABSTRACT

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A rapid, efficient method to determine the virulence of strains of *Erwinia amylovora* to *Malus* sp. Novole has been developed and evaluated. The method uses plantlets of Novole propagated in vitro. Plantlets are inoculated by cutting one or more leaves with scissors dipped in a suspension of *E. amylovora* (5×10^7 colony-forming units per milliliter). Fourteen days later, those plantlets inoculated with strain E4001A (virulent to Novole) showed typical fire blight symptoms including systemic necrosis and watersoaking; plantlets inoculated with strain Ea 273 (avirulent to Novole but virulent on most other apple cultivars) showed no systemic fire blight symptoms. When 39 strains of *E. amylovora* were evaluated for virulence to Novole, there was a significant association between data obtained from the plantlet assay and from inoculation of greenhouse-grown Novole plants. The plantlet assay was used to evaluate the virulence of 142 field strains from North America, Europe, and Egypt. Twelve strains from the eastern and central United States and Canada were virulent to Novole. Although there was a good correlation of symptom development in plantlets and greenhouse-grown plants inoculated with a standard virulent and avirulent strain, the growth of these *E. amylovora* strains after inoculation differed in the plant materials grown in vitro and in the greenhouse. In greenhouse-grown Novole plants, populations of both virulent and avirulent strains decreased 6 hr after inoculation. Between 6 and 72 hr after inoculation the virulent strain increased by 10^2, whereas the avirulent strain increased very little. In in vitro plantlets, there was no decline in population after inoculation; instead cells of both virulent and avirulent strains increased by 10^4 and 10^2, respectively, between 0 and 96 hr after inoculation.

Race specific virulence in strains of the fire blight pathogen, *Erwinia amylovora* (Burr.) Winslow et al, has been described recently (20,21). *E. amylovora* strain Ea 273 is pathogenic on most apple cultivars but causes little or no disease on the apple cultivars Quinte, Ottawa 523, Novole, and *Malus* × *robusta* No. 5, whereas strain E4001A (referred to as Ea 266 in 20,21) is differentially virulent to these cultivars (1,20,21).

Research on the virulence of *E. amylovora* has been hampered by the heterozygous genotypes of apple cultivars; genetically uniform plant material can be obtained only by asexual propagation. Traditional methods of asexual propagation (grafting and budding) are slow and require large amounts of field or greenhouse space. Micropropagated plants of apple grown in vitro (plantlets) (12–14,24,28,29) might facilitate research on the virulence of *E. amylovora* as well as on the nature of resistance to this pathogen. Although such material is genetically uniform, its physiology and anatomy differ from plants grown in the field or in the greenhouse (19,23), which may cause differences in pathogenesis by *E. amylovora*.

The purpose of this study was to develop and evaluate a rapid assay that uses apple plantlets for determining the virulence of strains of *E. amylovora* to specific apple cultivars. The validity of the assay was established with plantlets of the apple rootstock Novole (3), which were inoculated with two strains of *E. amylovora* of known virulence to Novole. The assay then was used to screen 142 field strains of *E. amylovora* from North America, Europe, and Egypt for virulence to Novole. The process of pathogenesis in plantlets grown in vitro and in greenhouse-grown plants was compared by studying the growth of *E. amylovora* in the two plant materials.

MATERIALS AND METHODS

Bacterial strains and inoculum. Strain Ea 273 of *E. amylovora* is pathogenic on most apple cultivars but is avirulent on the apple rootstock Novole (*M.* × *sublobata* P.I. 286613) (9,21). Strain E4001A is differentially virulent to Novole (21). Strain E4001A

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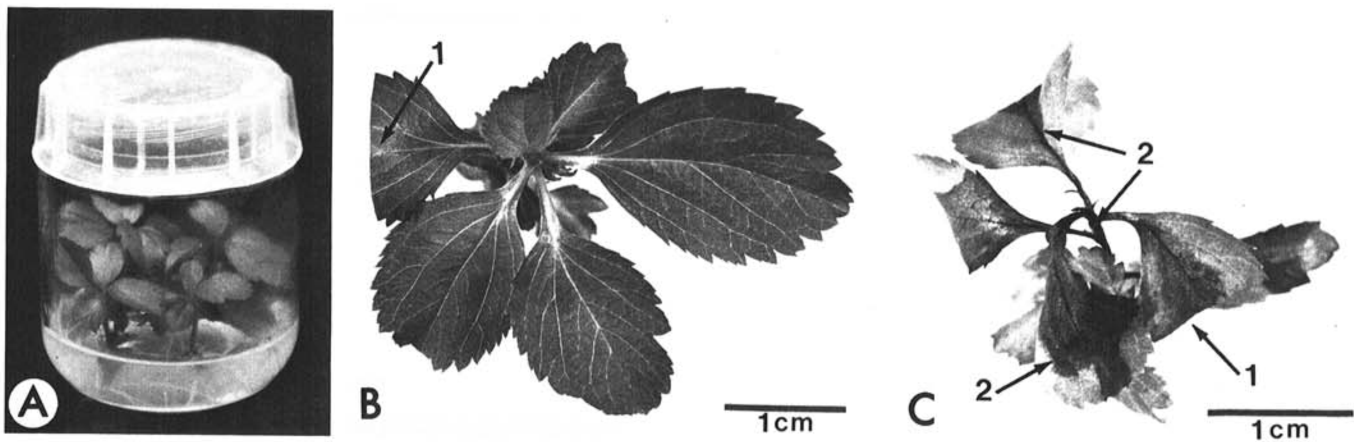

Fig. 1. A, In vitro plantlets of *Malus* sp. Novole propagated in baby food jar (diameter = 55 mm). **B,** Plantlets inoculated with *Erwinia amylovora* strain Ea 273. **C,** Inoculated with strain E4001A. 1, cut inoculated leaves. 2, systemic spread of necrosis to uninoculated leaves.

TABLE 1. Composition of media used for proliferation and rooting of *Malus* sp. Novole in vitro

Component	Proliferation medium	Rooting medium
NH_4NO_3	20.6 mM	10.3 mM
KNO_3	18.8 mM	9.4 mM
$CaCl_2 \cdot 2H_2O$	3.0 mM	1.5 mM
$MgSO_4 \cdot 7H_2O$	1.5 mM	0.75 mM
KH_2PO_4	1.2 mM	0.6 mM
$FeSO_4 \cdot 7H_2O$	0.1 mM	0.05 mM
H_3BO_3	0.1 mM	0.05 mM
$MnSO_4 \cdot H_2O$	0.1 mM	0.05 mM
Na_2EDTA	0.1 mM	0.05 mM
$ZnSO_4 \cdot 7H_2O$	30.0 μM	15.0 μM
KI	5.0 μM	2.5 μM
$Na_2MoO_4 \cdot 2H_2O$	1.0 μM	0.5 μM
$CoCl_2 \cdot 6H_2O$	0.1 μM	0.05 μM
$CuSO_4 \cdot 5H_2O$	0.1 μM	0.05 μM
Biotin	0.16 μM	0.08 μM
Glycine	2.7 μM	1.3 μM
myo-Inositol	560.0 μM	280.0 μM
Nicotinic acid	4.1 μM	2.1 μM
Pyridoxine HCl	1.3 μM	0.7 μM
Thiamine HCl	1.2 μM	0.6 μM
Sucrose	87.6 mM	43.8 mM
Benzyladenine	4.4 μM	...
Indolebutyric acid	4.9 μM	0.3 μM
Gibberellic acid	2.3 μM	...

Nal^r Rif^r and Ea 273 Nal^r Rif^r are naladixic acid- and rifampicin-resistant mutants of E4001A and Ea 273, respectively, that were selected by the method of Liang et al (17). The virulence of strains E4001A Nal^r Rif^r and Ea 273 Nal^r Rif^r does not differ significantly from E4001A and Ea 273, respectively, on the apple cultivars Delicious, Idared, McIntosh, and Quinte (J. L. Norelli, *unpublished data*). Strains E4001A, E4001A Nal^r Rif^r, Ea 273, and Ea 273 Nal^r Rif^r are maintained in the Cornell University Collection of Phytopathogenic Bacteria as strains 0071, 2182, 0273, and 2348, respectively.

Field strains tested for virulence to Novole were strains of *E. amylovora* received from various researchers in North America and Europe. They are maintained in the Cornell University Collection of Phytopathogenic Bacteria. The number of strains tested from specific locations were: California, 10; Illinois, 3; Maryland, 2; Michigan, 11; Minnesota, 1; Missouri, 6; Nebraska, 1; New York, 43; South Carolina, 2; Washington, 7; Alberta, 3; British Columbia, 1; Ontario, 3; Saskatchewan, 1; Belgium, 1; France, 12; Federal Republic of Germany, 11; The Netherlands, 6; Poland, 1; United Kingdom, 7; Egypt, 5; and unknown origin, 5.

Broth cultures used for inoculation were started from single colonies of freshly revived lyophilized cultures grown on nutrient broth-yeast extract-glucose agar. Inoculum consisted of 18-hr-old shake cultures grown in Kado 523 broth (15) at 28 C. The concentrations of strains E4001A and Ea 273 inoculum were equalized by adjusting the turbidity of the suspensions to *A* 0.83 and 0.65, respectively, at 620 nm, which corresponded to approximately 2×10^9 colony-forming units (cfu)/ml. The desired inoculum concentration then was obtained by diluting with chilled sterile 0.05 M potassium phosphate buffer, pH 6.5. Inoculum of field strains was produced by diluting 18-hr broth shake cultures 50-fold in buffer to yield $0.5–1 \times 10^8$ cfu/ml.

Plantlet assay. Plantlets of Novole were propagated in vitro by using the methods and media of Zimmerman and Broome (29) (Fig. 1A). The concentrations of plant hormones, vitamins, and mineral salts were adjusted for optimal growth of Novole (Table 1). The pH of the media was adjusted to 5.6 and it contained 0.75% agar. The containers used for tissue culture media were 120-ml baby food jars fitted with B-Caps (Magenta Corp., Chicago, IL). Shoot-tip cultures first were grown on a medium that stimulated the proliferation of shoots from axillary buds (Table 1). After shoot tip multiplication, six shoot tips were transferred to each jar of rooting medium (Table 1). All cultures were maintained at 23–27 C under fluorescent lights (photosynthetic photon flux density = 40 $\mu E\ m^{-2} sec^{-1}$) with a 16-hr photoperiod.

Three to five weeks after transfer to rooting medium, Novole cultures that had developed roots were inoculated with *E. amylovora*. Inoculation was accomplished by dipping a pair of scissors in a suspension of *E. amylovora* and then using the contaminated scissors to cut at least one leaf of each plantlet in a jar (Fig. 1B). Scissors were redipped in inoculum after each cut. Unless otherwise noted, inoculum concentration was about 5×10^7 cfu of *E. amylovora* per milliliter. The inoculated plantlets were incubated in a controlled environment chamber at 25 C under a combination of incandescent and fluorescent lights (photosynthetic photon flux density = 70 $\mu E\ m^{-2} sec^{-1}$) with a 16-hr photoperiod. Unless otherwise noted, disease development was evaluated 14 days after inoculation.

Plantlets were considered diseased if the stem and uninoculated leaves appeared necrotic (Fig. 1C). Plantlets with necrosis on only the inoculated leaf and petiole were not considered diseased. In most experiments, the results of inoculation were evaluated qualitatively; if jars contained one or more diseased plants, the strain used for inoculation was evaluated as virulent.

Effect of inoculum concentration and time of incubation on symptom development in the plantlet assay. Novole plantlets were inoculated with strain E4001A at inoculum concentrations of 10^3, 10^5, 10^7, and 10^9 cfu/ml and with strain Ea 273 at 10^9 cfu/ml. Plantlets in 10 jars were inoculated with the cut-leaf technique described above with each strain-inoculum concentration combination, and the proportion of symptomatic plantlets per jar was recorded 3, 7, 10, and 14 days after inoculation.

Greenhouse assay. The virulence of 39 strains of *E. amylovora* to Novole was determined in the greenhouse. Novole plants were

produced by micropropagation, as described above. Plantlets with 0.5–1.5 cm of root growth were transferred to greenhouse soil mix in a covered minigreenhouse (E. C. Geiger, Harleysville, PA), and the top of the container was gradually raised and removed over a 4–6-wk period. Plants were then transplanted to 7.7-cm pots and trained to single shoots by methods previously described (1).

Plants with vigorously growing shoots 15–30 cm long were inoculated by inserting a 0.46-mm-diameter (26-gauge) hypodermic needle through the stem just above the youngest unfolded leaf, and sufficient inoculum was introduced to fill the wound and leave visible drops at both ends of the wound. Inocula were produced in 18-hr broth shake cultures, as described above, and contained 10^9–10^{10} cfu/ml. Five plants were inoculated with each strain of *E. amylovora*. Greenhouse temperatures were 15–33 C during the period of disease development. The lengths of visible fire blight lesions, as determined by the formation of a determinate margin between diseased and healthy tissue, and of total shoot growth were recorded after all lesions had ceased to extend. The mean proportion of the shoot length that became necrotic was calculated.

The Kruskal-Wallis ranking procedure (16) was used to test the relationship between virulence determinations in the in vitro plantlet and the greenhouse assays. Strains of *E. amylovora* were tested in the plantlet assay by inoculating plantlets in two jars, as described above, in each of two separate tests. Strains were assigned to three groups based on the evaluation of their virulence in all four, one to three, or none of the four tissue culture jars. The null hypothesis tested was that there was no association between the three plantlet assay groups and the ranking of strains in the greenhouse assay.

Determination of populations of *E. amylovora* in inoculated plant material. Plantlets grown in vitro and plants grown in the greenhouse were inoculated by the cut leaf technique described above, with strains E4001A Nal^r Rif^r and Ea 273 Nal^r Rif^r, and sampled for populations of *E. amylovora* at time intervals after inoculation. One whole plantlet was chosen at random from each jar and shoots of greenhouse-grown plants were harvested two nodes below the point of inoculation. With both types of plant material, populations were determined in five replicate samples at each time interval. To determine bacterial populations in inoculated leaves and in uninoculated parts of plantlets, cut leaves were removed from plantlets with a sterile scalpel at the junction of the petiole and the stem and treated separately.

Plant material was homogenized in 1 ml of sterile phosphate buffer, described above, with a sterile pestle and mortar. An additional 9 ml of buffer was added and 0.1-ml amounts of serial buffer dilutions of the suspension were spread in petri dishes containing LB medium (18) amended with 40 μg of naladixic acid per milliliter. Dishes were incubated at 28 C for 2 days before colonies were counted.

Differences between the growth of strain E4001A Nal^r Rif^r and Ea 273 Nal^r Rif^r in each type of plant material were analyzed by a general linear regression model. Data were fitted to the regression model from 96 to 336 hr after inoculation for the plantlet assay, and from 72 to 240 hr after inoculation for the greenhouse assay. The regression model was then used to test if the slopes of the lines for each strain were different, if the y intercepts (population level) were different, and if there was evidence for an upward or downward trend in population.

RESULTS

Effect of inoculum concentration and time of incubation on symptom development in plantlet assay. The development of necrosis on Novole plantlets inoculated with strain E4001A of *E. amylovora* differed from those inoculated with strain Ea 273 (Fig. 2). Two weeks after inoculation, no systemic necrosis had developed on plantlets inoculated with 10^9 cfu/ml of the avirulent strain (Ea 273), but necrosis was apparent on the inoculated leaves and petioles of some of the plantlets. Plantlets inoculated with the virulent strain (E4001A), showed typical fire blight symptoms beginning 5 days after inoculation, but not all of the plantlets became symptomatic. Based on these results, an inoculum concentration of 10^7–10^8 cfu/ml and an incubation time of 14 days were chosen as standard conditions to differentiate the virulence of strains of *E. amylovora* to Novole in the plantlet assay.

Comparison of virulence as determined in the greenhouse and plantlet assay. The virulence of 39 field strains of *E. amylovora* to Novole as determined in the in vitro plantlet assay was compared with the virulence of the same strains in the greenhouse assay (Table 2). Seven strains that were consistently evaluated as virulent (four of four jars) in the plantlet assay also were evaluated as virulent to Novole in the greenhouse assay. Similarly, all 21 strains that were consistently evaluated as avirulent (zero of four jars) in the plantlet assay were evaluated as avirulent to Novole in the greenhouse assay. For 11 strains, the evaluation of virulence to Novole in the plantlet assay was inconsistent (one to three of four jars). Some of these strains were evaluated as virulent in the greenhouse assay and some were evaluated as avirulent (Table 2). The Kruskal-Wallis ranking procedure indicated that there was a significant association between the evaluation of a strain's virulence in the plantlet assay and the virulence of the strain to greenhouse-grown Novole plants (X^2 = 18.1; critical X^2 value: X^2 0.99 (df = 2) = 9.21).

Evaluation of virulence to Novole among field strains. One hundred and forty-two field strains of *E. amylovora* were evaluated for virulence to Novole using the in vitro plantlet assay. Twelve strains were consistently evaluated as virulent (four of four jars) (Table 3), 23 strains gave variable results (one to three of four jars), and 107 strains were consistently evaluated as avirulent in the plantlet assay. The 107 strains that were avirulent to Novole in the plantlet assay were all pathogenic on immature pear fruits.

Growth of *E. amylovora* in greenhouse-grown plants and in vitro plantlets. During the first 6 hr after inoculation of greenhouse-grown Novole plants, the number of colony-forming units of both the virulent strain (E4001A Nal^r Rif^r) and the avirulent strain (Ea 273 Nal^r Rif^r) recovered from shoots decreased 1–1.5 orders of magnitude (Fig. 3). After 6 hr, the population of the avirulent strain (Ea 273 Nal^r Rif^r) stabilized and remained relatively unchanged until 240 hr after inoculation; after 336 hr the number of colony-forming units recovered decreased another 1.5 orders of magnitude. In contrast, the number of colony-forming units of the virulent strain (E4001A Nal^r Rif^r) recovered increased 2.5 orders of magnitude from 6 to 72 hr after inoculation and then remained relatively unchanged through 336 hr after inoculation (Fig. 3). Symptoms were first visible in greenhouse-grown Novole plants inoculated with the virulent strain (E4001A Nal^r Rif^r) 120–168 hr after inoculation; no symptoms developed in plants inoculated with the avirulent strain (Ea 273 Nal^r Rif^r). Regression analysis of the population data from 72 to 240 hr after inoculation

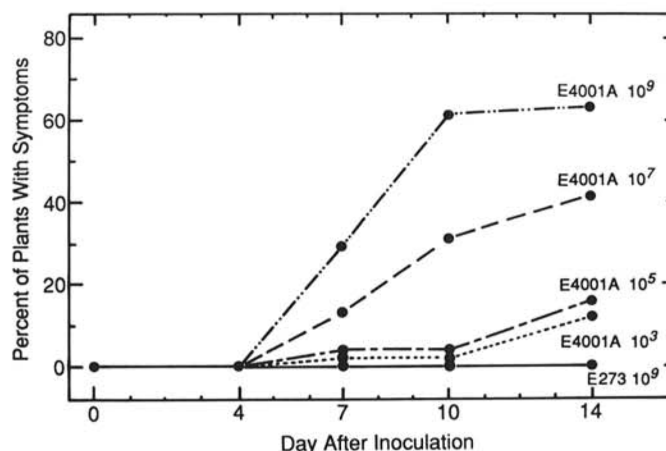

Fig. 2. Development of fire blight symptoms in Novole plantlets grown in vitro as a function of inoculum concentration and time of incubation. Plantlets were inoculated by cutting one or more leaves with scissors contaminated with *Erwinia amylovora* strains E4001A and Ea 273. The number after strain designation indicates inoculum concentration (cfu/ml).

indicated that the number of colony-forming units recovered per plant was significantly greater for the virulent strain (E4001A Nal^r Rif^r) than for the avirulent strain (Ea 273 Nal^r Rif^r) ($t = 8.66$, $df = 37$; critical t value: $t\,0.99\,(df = 37) = 2.71$). There was no evidence of an upward or downward trend in the population with either strain during this period of time.

In contrast to greenhouse-grown plants, the number of colony-forming units recovered from in vitro plantlets inoculated with either strain did not decrease in the first 6 hr after inoculation (Fig. 4). Rather, the population of both strains increased several orders of magnitude during the first 96 hr after inoculation (Fig. 4). After 96 hr, the populations of both strains remained relatively unchanged through 336 hr after inoculation. However, the number of colony-forming units of the virulent strain (E4001A Nal^r Rif^r) recovered was approximately two orders of magnitude greater than the avirulent strain (Ea 273 Nal^r Rif^r) at 144, 216, and 336 hr after inoculation (Fig. 4). Symptoms were first visible in plantlets

TABLE 2. Virulence of 39 field strains of *Erwinia amylovora* to Novole as determined by a greenhouse plant and an in vitro plantlet assay

Strain obtained from[w]	Strain	Virulence in greenhouse assay[x]	Virulence in *in vitro* plantlet assay[y]
DFR	MSU115	1.00 a[z]	4
WGB	E4001A	1.00 a	4
RNG	77b	0.99 ab	4
WGB	E4003P	0.89 abc	1
DFR	MSU112	0.80 abc	1
AKV	EA 101	0.80 abc	4
SMR	PEAR#1	0.78 abc	3
WGB	E2002A	0.77 bc	4
WGB	E7002M	0.76 c	3
DFR	MSU137	0.75 cd	1
SVB	Ea 222	0.70 cd	4
RNG	E9	0.53 de	4
SVB	Ea 404	0.40 e	2
SVB	Ea 224	0.01 f	0
SVB	Ea 226	0.01 f	0
SMR	APPLE#1	0.01 f	0
WGB	E7004M	0.01 f	1
DFR	MSU116	0.01 f	0
SVB	Ea 415	0.00 f	0
SVB	Ea 414	0.00 f	0
WZ	EA 6/75	0.00 f	0
JPP	1360-2	0.00 f	0
JPP	1314-1	0.00 f	0
JPP	CNPB1368	0.00 f	0
SVB	Ea 314	0.00 f	2
RNG	102b	0.00 f	1
RNG	28	0.00 f	0
EB	T	0.00 f	0
SVB	Ea 273	0.00 f	0
RPC	1286	0.00 f	0
SVB	Ea 225	0.00 f	0
SVB	Ea 221	0.00 f	0
SVB	Ea 220	0.00 f	1
WHB	EA 144	0.00 f	0
WHB	EA 17$8	0.00 f	0
WHB	Ea 15$13	0.00 f	0
SVB	Ea 201	0.00 f	1
SVT	Y33	0.00 f	0
SVT	SJ2	0.00 f	0

[w] AKV = Anne K. Vidaver, DFR = David F. Ritchie, EB = Eve Billing, JPP = Jean-Pierre Paulin, RNG = Robert N. Goodman, RPC = Ronald P. Covey Jr., SMR = Steven M. Ries, SVB = Steven V. Beer, SVT = Sherman V. Thomson, WGB = W. Gordon Bonn, WHB = Walter H. Burkholder, WZ = Wolfgang Zeller.

[x] Virulence is the severity of fire blight resulting from greenhouse inoculation. Values are expressed as the proportion of the shoot length that became necrotic and represent means of five replicates.

[y] Virulence is expressed as the number of jars of four that contained plantlets that developed necrosis following inoculation with *E. amylovora*.

[z] Strains followed by the same letter did not differ significantly at the $P = 0.05$ level according to Waller and Duncan's Bayesian *K*-ratio LSD rule (27).

TABLE 3. Strains of *Erwinia amylovora* evaluated as virulent to Novole in an in vitro plantlet assay

Strain obtained from[a]	Strain	Origin	
		Host	Location
AKV	EA 101	apple	Nebraska
DFR	MSU110	apple cv. Jonathan	Michigan
DFR	MSU115	apple cv. Jonathan	Michigan
DFR	MSU122	apple cv. Jonathan	Michigan
DFR	MSU133	apple cv. Jonathan	Michigan
HLK	EANC	pear cv. Bartlett	Maryland
RNG	E9		Missouri
RNG	77b		Missouri
SVB	Ea 222	apple cv. 20 Ounce	New York
SVB	Ea 384	apple cv. Malling 9	New York
WGB	E2002A	apple cv. Jonathan	Ontario
WGB	E4001A	apple cv. R. I. Greening	Ontario

[a] AKV = Anne K. Vidaver, DFR = David F. Ritchie, HLK = Harry L. Keil, RNG = Robert N. Goodman, SVB = Steven V. Beer, WGB = W. Gordon Bonn.

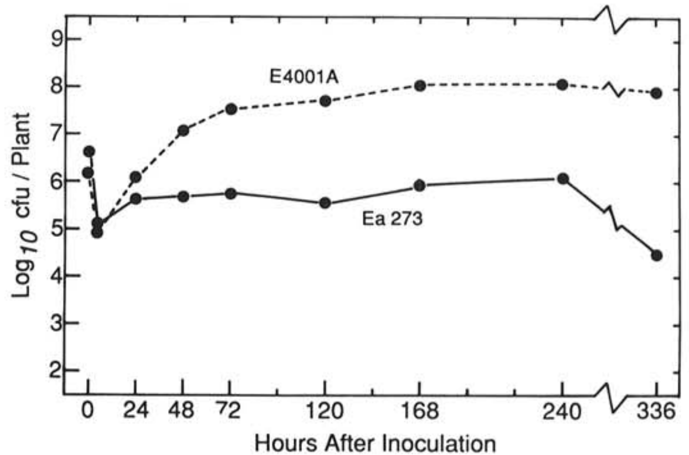

Fig. 3. Populations of *Erwinia amylovora* recovered from greenhouse-grown Novole plants after inoculation with strains E4001A Nal^r Rif^r (E4001A) and Ea 273 Nal^r Rif^r (Ea 273). Populations are expressed as the mean of the $\log_{10}$ of the colony-forming units (cfu) recovered per shoot.

TABLE 4. Populations of *Erwinia amylovora* recovered from inoculated leaves, uninoculated parts of plantlets, and whole Novole plantlets following inoculation with strains E4001A Nal^r Rif^r (E4001A) and Ea 273 Nal^r Rif^r (Ea 273) in vitro

Strain	Time after inoculation (hr)	Population recovered from		
		Inoculated leaves	Uninoculated plantlet-parts	Plantlet total
E4001A	0	3.45 (0.17)	0.06 (0.13)	3.45 (0.17)
E4001A	6	3.61 (0.62)	2.55 (1.61)	3.80 (0.63)
E4001A	24	6.38 (0.19)	4.58 (0.57)	6.40 (0.18)
E4001A	48	7.01 (0.72)	6.09 (0.51)	7.20 (0.43)
E4001A	72	7.58 (0.26)	6.92 (0.75)	7.77 (0.31)
E4001A	120	7.35 (0.37)	7.64 (0.70)	7.88 (0.49)
E4001A	168	6.52 (2.01)	6.76 (1.61)	7.41 (1.25)
E4001A	240	7.46 (1.05)	7.69 (2.10)	8.12 (1.42)
E4001A	336	7.17 (1.17)	7.93 (1.90)	8.12 (1.57)
Ea 273	0	3.03 (0.42)	2.87 (1.70)	3.60 (0.61)
Ea 273	6	3.81 (1.16)	3.16 (0.33)	4.01 (1.02)
Ea 273	24	4.36 (2.48)	4.63 (0.80)	5.18 (1.05)
Ea 273	48	5.82 (0.98)	5.18 (0.54)	6.13 (0.62)
Ea 273	72	6.16 (0.62)	3.94 (2.29)	6.19 (0.63)
Ea 273	120	6.09 (0.51)	5.33 (1.47)	6.27 (0.64)
Ea273	168	5.79 (0.49)	5.17 (0.65)	5.94 (0.49)
Ea 273	240	5.50 (1.61)	4.25 (0.97)	5.74 (1.47)
Ea 273	336	5.02 (1.06)	3.86 (0.32)	5.09 (1.00)

[a] Populations are expressed as the mean of the $\log_{10}$ of the colony-forming units recovered and are followed by the standard deviation of the mean in parenthesis.

inoculated with the virulent strain (E4001A Nal^r Rif^r) 120–240 hr after inoculation; systemic necrosis was not apparent on plantlets inoculated with the avirulent strain (Ea 273 Nal^r Rif^r). General linear regression analysis of the population data from 96 to 336 hr after inoculation indicated that the number of colony-forming units recoverable per plantlet was significantly greater for the virulent strain (E4001A Nal^r Rif^r) than for the avirulent strain (Ea 273 Nal^r Rif^r) (t = 17.69, df = 37). There was no evidence of an upward or downward trend in the population with either strain during this period of time.

When Novole plantlets were inoculated with greater numbers of bacteria, such that the number of colony-forming units recovered per plantlet immediately after inoculation was approximately 10^6, a similar trend was observed. The population of both the avirulent strain (Ea 273 Nal^r Rif^r) and the virulent strain (E4001A Nal^r Rif^r) increased one to two orders of magnitude in the first 24 hr after inoculation to 5×10^7 and 10^8 cfu recovered per plantlet, respectively; however, the number of colony-forming units of the avirulent strain (Ea 273 Nal^r Rif^r) recovered decreased after 48 hr. The number of colony-forming units of the avirulent strain (Ea 273 Nal^r Rif^r) recovered per plantlet at 216 and 336 hr after inoculation was approximately 10^6 as compared to 10^8 with the virulent strain (E4001A Nal^r Rif^r).

Separate determinations of populations of *E. amylovora* in inoculated leaves and other uninoculated parts of plantlets indicated that the distribution of bacteria in or on plantlets was similar whether plantlets were inoculated with the virulent strain (E4001A Nal^r Rif^r) or the avirulent strain (Ea 273 Nal^r Rif^r) (Table 4). When plantlets were inoculated with the virulent strain (E4001A Nal^r Rif^r) there was no significant difference ($P = 0.05$) in the number of colony-forming units recovered from inoculated leaves and uninoculated parts of plantlets from 48 to 336 hr after inoculation (Table 4). Although plantlets inoculated with the avirulent strain (Ea 273 Nal^r Rif^r) had lower numbers of bacteria, there was no significant difference ($P = 0.05$) in the number of colony-forming units recovered from inoculated leaves or uninoculated parts of plantlets at any of the sampling times (Table 4).

DISCUSSION

The virulence of strains E4001A and Ea 273 of *E. amylovora* to Novole was distinguished in a plantlet assay based on the use of in vitro propagated plantlets. Although the growth of these two strains of *E. amylovora* in in vitro plant material differed from that observed in greenhouse-grown material, symptoms, and respective virulence reactions caused by the strains appeared similar in both plant materials. The plantlet assay was also useful for the rapid detection of differential virulence to Novole with other strains of *E. amylovora*.

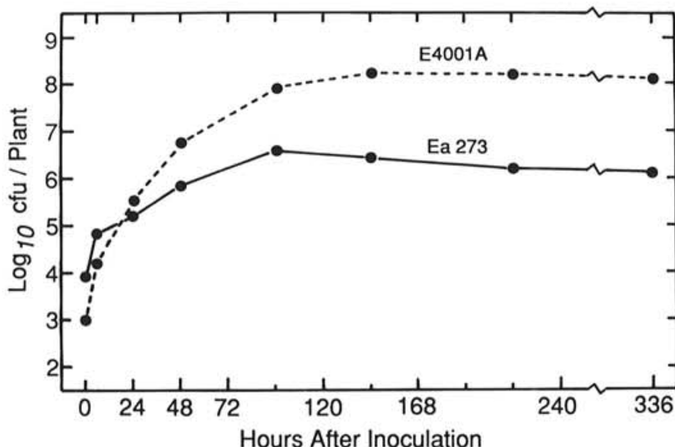

Fig. 4. Populations of *Erwinia amylovora* recovered from Novole plantlets grown in vitro after inoculation with strains E4001A Nal^r Rif^r (E4001A) and Ea 273 Nal^r Rif^r (Ea 273). Populations are expressed as the mean of the $\log_{10}$ of the colony-forming units (cfu) recovered per plantlet.

Most tissue-culture systems developed for the study of host-pathogen interactions and the selection of disease resistance have involved the use of cell or protoplast cultures (4). These cultures facilitate evaluating large numbers of individuals and direct observations of cell metabolism can be made (6,7,11). However, the use of organized plant tissue in vitro, as we describe, has proven useful in the study of the virulence of *E. amylovora*. Similar methods appear useful for the selection of genotypes of both apple and pear for resistance to *E. amylovora* (5,26).

Novole was chosen for the plantlet assay because it is the differential host that grows best in in vitro culture. Although Novole does not show the largest differential response of the *Malus* spp. cultivars tested in the greenhouse (21), its differential response is clear on in vitro material and on vigorously growing single-shoot plants grown in the greenhouse. However, Novole appears to have a high level of background resistance to *E. amylovora*, and usually strain E4001A causes little disease on grafted greenhouse or orchard trees (21).

Besides inoculum concentration, the amount of disease development in the plantlet assay depends on root development, incubation temperature, and the quantity of incident light during incubation (J. L. Norelli, *unpublished data*). Disease development was greater at an incubation temperature of 25 C than at 27 or 28 C. Perhaps this effect was caused by temperatures greater than ambient that may occur within the illuminated culture jars. Disease development was favored by less incident light and the use of nonrooted plant material. Disease symptoms were observed on Novole inoculated with strain Ea 273 of *E. amylovora* when nonrooted plant material, or inoculum levels greater than 10^9 cfu/ml, or low levels of incident light during incubation were used. Nonrooted plant material routinely was removed from culture jars before inoculation with *E. amylovora*.

The differential virulence of strains Ea 273 and E4001A to Novole was much less distinct when shoot tips growing on proliferation medium (Table 1) were inoculated (J. L. Norelli, *unpublished data*). In studies of the colonization of tobacco callus cultures by *Phytophthora parasitica* var. *nicotianae*, higher concentrations of some cytokinins reduced the resistance response of callus derived from resistant cultivars (10). Perhaps the relatively high concentration of benzyladenine in proliferation medium increased the susceptibility of Novole to infection by strain Ea 273. However, the effect of cytokinin on the infection of Novole by *E. amylovora* was not part of this study.

The virulence of strains Ea 273, E4001A, and most field strains was clearly differentiated in the in vitro plantlet assay; however, 16% of the field strains evaluated produced variable results in the in vitro plantlet assay. Some strains that gave a variable response in the in vitro plantlet assay were virulent on greenhouse-grown Novole, while others were avirulent (Table 2). The variable response of some field strains in the in vitro plantlet assay could be due to an intermediate virulence phenotype.

Although the field strains were collected from throughout North America and Europe, virulence to Novole occurred only in strains that originated in the eastern and central North America, except for one strain from Alberta, Canada. Similarly, strains inconsistently evaluated as virulent (one to three of four jars) to Novole in the plantlet assay also originated from those regions. The occurrence of differential virulence to Novole among strains of *E. amylovora* appeared, with the exception of the strain from western Canada, to be geographically limited to the putative center of origin of *E. amylovora* (25). In general, strains of *E. amylovora* that are virulent to Novole were isolated from highly susceptible hosts and, as previously noted (20), there was no association between host cultivar of origin and differential virulence to Novole.

The growth of strain E4001A of *E. amylovora* in greenhouse-grown Novole plants after cut-leaf inoculation (Fig. 3) was similar to the observed growth of *E. amylovora* in pear blossoms after inoculation of nectaries (2). In contrast, the number of colony-forming units of both strains recovered from in vitro plantlets did not decrease 6 hr after inoculation, and the avirulent strain (Ea 273

Nal^r Rif^r) grew for the first 96 hr after inoculation (Fig. 4). The differences in the growth of *E. amylovora* in the two types of plant material may be due to differences in the ability of the plant material to support saprophytic growth of the bacteria. The relative humidity in closed culture containers probably is near 100%. In addition, plant material grown in vitro may have more nutrients available to support bacterial growth. The growth of *E. amylovora* observed in in vitro plant material was similar to the observed growth of *Pseudomonas syringae* pathovars on leaves of greenhouse-grown host plants (8,22).

The distribution of bacteria on or in inoculated leaves and uninoculated parts of plantlets was similar on plantlets inoculated with the virulent strain (E4001A Nal^r Rif^r) and the avirulent (Ea 273 Nal^r Rif^r) strain 48 hr after inoculation (Table 4). Population determinations did not distinguish between bacteria growing epiphytically on plantlet surfaces or endophytically within plantlets. Because of the fine texture and porosity of plantlet tissues grown in vitro, washing or surface sterilization was not effective in distinguishing these two populations. The recovery of large numbers of the avirulent strain (Ea 273 Nal^r Rif^r) from uninoculated parts of plantlets, immediately after inoculation, was due presumably to contamination during the inoculation procedure (Table 4). These bacteria may have multiplied epiphytically on plantlet surfaces. Because plantlets inoculated with the virulent strain (E4001A Nal^r Rif^r) developed systemic necrosis on uninoculated parts of plantlets, a large portion of the bacteria recovered from these plantlets were assumed to be endophytic.

The plantlet assay described here is more economical in terms of time, space, and labor than assays that are conducted in the greenhouse. In addition, the in vitro assay can be conducted throughout the year. The plantlet assay should facilitate the evaluation of many strains of *E. amylovora* for virulence to Novole and studies of the molecular genetics of differential virulence.

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