Virulence on Citrus of *Pseudomonas syringae* Strains that Control Postharvest Green Mold of Citrus Fruit

J. L. Smilanick, Horticultural Crops Research Laboratory, 2021 South Peach Avenue, Fresno, CA 93727; **C. C. Gouin-Behe,** University of Florida/USDA-ARS, Beltsville, MD 20705; **D. A. Margosan** and **C. T. Bull,** Horticultural Crops Research Laboratory, 2021 South Peach Avenue, Fresno, CA 93727; and **B. E. Mackey,** Biometrics Unit, USDA-ARS, 800 Buchanan Street, Albany, CA 94710

ABSTRACT

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To determine if their commercial use would entail significant risk of introducing pathogens to citrus, *Pseudomonas syringae* strains (ESC-10 and ESC-11) used for postharvest biological control were compared with strain 485-10, which caused citrus black pit and blast. All three strains reduced postharvest green mold of lemons, caused by *Penicillium digitatum.* Properties of strains studied included tobacco hypersensitive reactions (HR), carbohydrate utilization (Biolog GN), cellular fatty acid profiles, and virulence. HR of ESC-10 and 485-10 were positive, while that of ESC-11 was negative. Carbohydrate utilization and cellular fatty acid analysis indicated ESC-10 was more closely related to 485-10 than was ESC-11. Shoots, leaves, and fruit of many cultivars were inoculated. Only strain 485-10 caused lesions on shoots and leaves, and large, sunken lesions on citrus fruit. ESC-10 caused darkening of wounds on fruit while water or ESC-11 did not. Only on lime fruit did all three strains cause lesions after the application of 10^8 CFU/ml, although those caused by ESC-10 and ESC-11 were very small compared with those of 485-10. Pathogenicity was detected and virulence was quantified faster by inoculation of lime fruit than by other methods. Strains ESC-10 and ESC-11 do not pose a significant threat to citrus fruit, foliage, or shoots of all varieties tested, except for Persian lime, in which small lesions were observed after the inoculation of wounds on lime fruit.

Wounds on citrus fruit are the primary infection court for green and blue molds of citrus, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *P. italicum* Wehmer, respectively (10). Some *Pseudomonas* species can be applied to citrus fruit after harvest to reduce the incidence of citrus green mold (17,24,30,35). Biological control agents, including *Pseudomonas syringae* strains, occupy wounds on citrus fruit and reduce the incidence of these diseases (31). However, because some *P. syringae* pv. *syringae* van Hall strains are pathogens of citrus and other plants (28), biological control strains should not be approved until their risk of virulence to many hosts has been determined. Furthermore, risk of injury to the fruit to which the bacteria are applied commercially should also be known. For example, Huang and co-workers (16) assessed the virulence of a *P. cepacia* strain used as an antagonist of citrus green mold. They reported that tissue around treated wounds on oranges became brown in color and soft rot symptoms developed after 5 to 7 days. Lesions of this size reduce the marketability of the fruit because they are a significant cosmetic quality deficiency.

Blast of citrus leaves and stems, and black pit of citrus fruit, are caused by some strains of *P. syringae* pv. *syringae* (34). Blast results in expanding lesions on citrus leaves and stems, which leads to defoliation of trees in severe cases. Black pit results in dark-colored, sunken blemishes on fruit, particularly limes and lemons (12), of up to 35 mm in diameter (33). On lemon fruit, black pit symptoms develop within 5 days, and a marked rise in fruit respiration occurs after 2 days (5). The disease is associated with cool, damp weather and physical injuries to the host caused by wind or hail (6). It occurs in Australia, Japan, South Africa, certain Mediterranean countries, central Asia, and, in the United States, in California but not Florida (34).

The purpose of this study was to determine the pathogenicity and virulence of two strains of *P. syringae* being developed as biological control agents, compared with a known pathogenic and virulent strain of *P. syringae,* on shoots and foliage of different citrus species grown in a greenhouse and on fruits of various citrus cultivars stored under conditions that simulate commercial postharvest practices. In order to estimate the risk that the biological control strains themselves could be significant pathogens to citrus, many varieties of citrus were employed in these studies. Part of this work was requested by the United States Environmental Protection Agency and a brief report of its findings was submitted to them (29); other studies were requested to estimate risk of ESC-10 and ESC-11 on hosts other than citrus. Strain ESC-11, then identified as L-59-66, did not cause symptoms on cucumber foliage or pear fruit from which it was originally isolated, and did not cause a hypersensitive reaction on tobacco (19).

MATERIALS AND METHODS

Characterization of bacterial strains. *P. syringae* strains ESC-10 and ESC-11 were isolated from healthy apple and pear leaves (18,19), respectively, and are being developed by EcoScience Corporation (East Brunswick, NJ) for the control of postharvest decay of citrus (31,36) and apples (20). Two commercial products, Bio-Save 10 and Bio-Save 11, containing wettable powder formulations of strains ESC-10 and ESC-11, respectively, were approved for postharvest use on fruit by the U.S. Environmental Protection Agency in 1995 (EPA reg. no. 64296-7), and were used in this study. *P. syringae* strain 485-10, isolated from citrus leaves exhibiting blast symptoms, was obtained from D. A. Cooksey (University of California, Riverside). In preliminary tests, strain 485-10 was similar in virulence to many other citrus blast and black pit strains evaluated. Cells of bacterial strains were stored at –80°C in a mixture of 0.2 ml of glycerol and 1.8 ml of dense nutrient broth culture until needed. The bacteria were cultured on nutrient agar for 4 days at 20°C before use in experiments. Cells were collected from plates in sterile distilled water (DW), centrifuged (10 min at 10,000 × *g*) and washed in sterile DW twice, and re-suspended in sterile DW. Cell densities were estimated by absorbance at 630 nm with a spectro-

Corresponding author: J. L. Smilanick
E-mail: jsmilanick@aol.com

Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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photometer. Total viable cells were verified by spreading dilutions of inoculum suspensions on nutrient agar plates and reported as CFU. For inoculation of leaves or shoots, cell suspensions of strains ESC-10 and ESC-11 were prepared with DW and a concentrated paste of cells grown in liquid culture (from EcoScience Corp.).

Strains were characterized by hypersensitive reaction (HR) on tobacco, hydrocarbon utilization capability, and cellular fatty acid analysis. The method of Klement (21) was used to determine HR. Strains were cultured in nutrient broth, suspended in sterile DW at a density of about 5×10^7 CFU/ml, then infiltrated into leaves of 2-month-old tobacco (*Nicotiana tabacum* L. cv. Daylight Sensation) plants. Leaf reactions were recorded after 24 h. Characterization of hydrocarbon substrate catabolism and fatty acid composition of the strains were determined by 24-h readings from the Biolog GN MicroPlate (Biolog, Inc., Hayward, CA) and MIDI Microbial Identification System (Microbial ID, Inc., Newark, DE), respectively. Culture and preparation of the strains, hydrocarbon substrates, gas chromatography conditions, data bases used for identification of strains, cluster analysis, and generation of dendrograms have been described previously by other workers (1,2).

Biological control of green mold. Bacterial strains were applied to freshly harvested lemons inoculated with *P. digitatum* spores to assess their ability to control postharvest green mold. Petri dishes of potato dextrose agar were inoculated with *P. digitatum* isolate M6R (from J. W. Eckert, University of California, Riverside) and incubated 2 weeks at 20°C. Spores were rubbed from the agar surface with a glass rod after a small volume of sterile 0.05% Triton X-100 was added. The spore suspension was passed through two layers of cheesecloth and diluted with sterile DW to an absorbance of 0.1 at 420 nm with a spectrophotometer. This density, approximately equivalent to 1×10^6 spores/ml, is recommended for evaluation of postharvest treatments to control green mold (9). Each lemon was inoculated once by dipping a stainless steel rod into the spore suspension and immediately making a puncture 2 mm deep and 1 mm wide in the rind. Treatments were applied within 15 min after inoculation. The bacteria were cultured 4 days at 25°C on nutrient agar, suspended in DW, and 0.5 ml containing 10^9 CFU/ml was applied to each fruit with an air-powered sprayer. For comparison, each test included water alone applied to run-off with an air-powered sprayer and a 1-min immersion in 1 mg of imazalil (Bromozil 50, Brogdex Co., Lindsay, CA) per liter. After 7 days incubation at 20°C, the number of decayed lemons was determined. Each treatment was applied to four replicates of 25 fruit each and the experiment was conducted twice.

Greenhouse conditions. Shoots and leaves of sweet orange (*Citrus sinensis* (L.) Osbeck) cultivars Hamlin, Pineapple, Valencia, and Washington Navel, grapefruit (*C.* × *paradisi* Macfady.) cultivars Marsh Seedless, Ruby Red, and Rio Red, lime (*C. aurantiifolia* (L.) Swingle) cultivars Persian and Bearss, lemon (*C. limon* (L.) N. L. Burm.) cultivars Eureka and Lisbon, and mandarin (*Citrus reticulata* Blanco) cultivar Sunburst were inoculated on young trees maintained in a greenhouse. In one test, shoots and leaves of Marsh grapefruit were inoculated on a tree growing outside in Fresno, CA. The experiments were conducted three times: test 1, in January 1994 at Fresno; test 2, in April 1994 at Beltsville, MD; and test 3, in June 1994 at Beltsville. Not all cultivars were represented in every test. Thirty days prior to inoculation, the production of fresh shoots on trees was enhanced by ample fertilization, watering, and relatively warm temperatures (15 to 25°C). Greenhouse temperatures in tests 1 and 3 were within the range reported as conducive to citrus blast (6) but temperatures in test 2 were higher, particularly for several days immediately after inoculation. The mean, minimum, and maximum (±SD) temperatures during test 1 in Fresno were 12.7 (±1.6), 8.5 (±1.5), and 19.2°C (±3.0), during test 2 in Beltsville were 22.2 (±2.5), 18.1 (±3.3), and 26.6°C (±3.8), and during test 3 in Beltsville were 22.8 (±3.4), 17.0 (±4.1), and 28.3°C (±5.2), respectively. Relative humidity was constantly high (85 to 99%) in test 1 and more variable (50 to 95%) in tests 2 and 3.

Leaf inoculation. Two methods of leaf inoculation were employed. The first method was used in tests 1, 2, and 3, while the second method was used in tests 1 and 2. For the first method, a pair of scissors was immersed briefly in water suspensions of 1×10^6, 1×10^7, or 1×10^8 CFU/ml of one of the three *P. syringae* strains or water, then immediately used to cut young leaves in half. Four sets of three leaves each were inoculated on each of three to five replicate trees of each cultivar. During the incubation period, the cut leaves were observed repeatedly and the date lesions initiated was recorded. After 1 month, symptoms on the leaves were rated on a 0 to 4 scale as follows: 0 = leaf healthy, cut healed; 1 = leaf healthy, cut tan to brown in color; 2 = necrosis at cut, some expansion of necrosis down midrib; 3 = necrosis at cut, extensive expansion of necrosis the length of the midrib; 4 = entire leaf necrotic. Leaves were inoculated by this method in all three greenhouse tests.

The second leaf inoculation method employed the application of 5 µl of water suspensions of one of the three *P. syringae* strains or water alone to leaves punctured in a four by four grid pattern with a 1-mm-diameter needle. The bacterial suspensions used contained 1×10^4, 1×10^5, 1×10^6, or 1×10^7 CFU/ml. In greenhouse test 1 (Fresno), a grid of inoculated punctures was prepared on two leaves each on three trees each of Lisbon and Eureka lemons, and Persian lime. In greenhouse test 2 (Beltsville), a similar grid was prepared on one leaf each on five trees of Hamlin orange, Valencia orange, Bearss lime, and Sunburst tangerine. During the incubation period, the inoculated punctures were observed repeatedly and the date lesions initiated was recorded. After 1 month, the symptoms were rated as follows: 0 = leaf healthy, puncture tan to brown in color; 1 = puncture dark brown in color with 0.5 to 1 cm halo; 2 = puncture dark brown in color, with a dark brown lesion >1 mm in diameter.

Shoot inoculation. Shoots of all citrus cultivars employed in greenhouse tests 1 and 2 were inoculated. Two incisions 2 cm in length and about 0.1 mm deep were made in stems about 5 and 7 cm below the shoot apex with a flame-sterilized scalpel. Four sets of two incisions each were made and inoculated on each of three to five replicate trees of all cultivars. Ten microliters of each of the three *P. syringae* strains adjusted to a concentration of 1×10^6 CFU/ml or water alone was applied to the incisions with a pipette. After inoculation, incisions 5 cm below the stem apex were wrapped with Parafilm to retard desiccation. The incisions 7 cm below the stem apex were not covered. During the incubation period, the exposed, inoculated incisions on shoots were observed repeatedly and the date lesions initiated was recorded. After 1 month, the Parafilm was removed and the symptoms of the covered incision were rated on a 1 to 3 scale as follows: 1 = incision unchanged, adjacent leaf tissue light colored; 2 = incision dark colored, but no expanding lesion present; and 3 = incision dark colored, with a dark-colored lesion 1 mm or more in length.

Fruit inoculations. Mature fruit of each Eureka lemon, Marsh grapefruit, Persian lime, and Washington Navel orange were harvested the day of the experiment, washed with household bleach diluted in water (0.525% NaOCl), and rinsed with sterile DW. On each fruit, 16 punctures 1 mm wide and 3 mm deep were made in a four by four grid pattern with a long stainless steel probe, and 10 µl of suspensions of each of the three *P. syringae* strains in water or water alone was applied with a pipette. The bacterial suspensions containing 1×10^6, 1×10^7, or 1×10^8 CFU/ml were applied to eight fruit per treatment. After inoculation, fruits were placed on plastic trays and incubated under humid conditions (relative humidity >90%) at 13°C. During incubation the fruit were observed repeatedly and the date lesions initiated was recorded. The diameter of the lesions was recorded 16 days after inoculation. The experiment was conducted twice.

Experimental design and statistical analysis. Green mold incidence data in biological control tests were analyzed with a two-way analysis of variance of arcsine-transformed percentages followed by Fisher's protected least significant difference test at $P = 0.05$ to separate the means. All greenhouse virulence assessment experiments employed a factorial design composed of variety × inoculation density × strain. Data that violated the assumption of homoscedasticity, a prerequisite for analysis of variance, were compensated for by two methods. When all the data within the replicates of a treatment were zero, these values were deleted before analysis. After analysis, those cells for which the variances were zero were compensated for by subtracting their degrees of freedom from the error term when confidence intervals on the means were calculated (8). Observations were analyzed with two- or three-way analysis of variance. When interactions were nonsignificant, inferences were directed to main effects, and comparisons were made by means and 95% confidence limits. The influence of inoculum density on the diameter of lesions on fruit was analyzed by linear regression (SAS Statistics, Inc., Cary, NC). Significant ($P \leq 0.05$) regressions describing this relationship and R^2 values were calculated.

RESULTS

Characterization of bacterial strains. Positive HR on tobacco leaves occurred within 24 h after infiltration with strains 485-10 and ESC-10, while no reaction occurred on leaves after infiltration with ESC-11. All strains were identified as *P. syringae* by both carbohydrate utilization tests and cellular fatty acid analysis, although pathovar conclusions differed and similarity coefficients were variable. Carbohydrate utilization tests identified strains ESC-10, ESC-11, and 485-10 as *P. syringae* pv. *syringae*, *P. syringae* pv. *aptata*, and *P. syringae* pv. *syringae* with similarity coefficients of 0.817, 0.515, and 0.439, respectively. Cellular fatty acid analysis

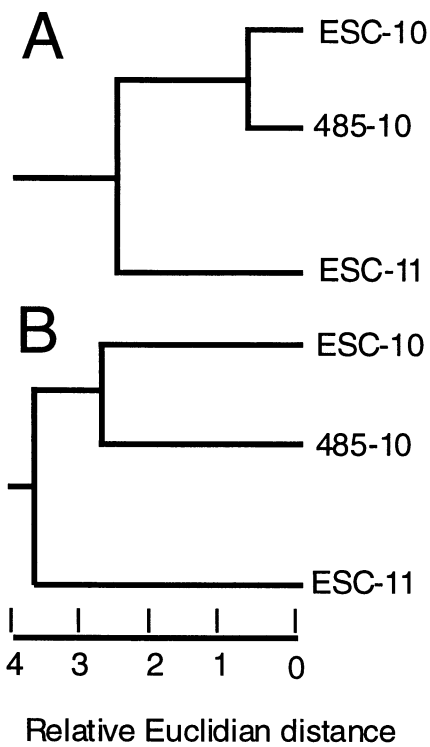

Fig. 1. Cluster analyses of *Pseudomonas syringae* strains determined by **(A)** differential utilization of 95 carbohydrate substrates on the Biolog GN MicroPlate or **(B)** cellular fatty acid composition based on the MIDI system.

Table 1. Lesion ratings of leaves of 12 citrus cultivars inoculated with *Pseudomonas syringae* strains at different densities or water and incubated 30 days in a greenhouse

		Leaf lesion rating after inoculation with CFU/ml[v]			
Cultivar	**Inoculum**	**1×10^6**	**1×10^7**	**1×10^8**	**Significance[w]**
Lisbon lemon[x]	Water	0	0	0	a
	ESC-11	0	0	0	a
	ESC-10	0	0	0	a
	485-10	1.0	0.7	2.0	b
Eureka lemon[x]	Water	0	0	0	a
	ESC-11	0	0	0	a
	ESC-10	0	0	0	a
	485-10	1.0	2.0	3.7	b
Bearss lime[x]	Water	0.1	0	0	a
	ESC-11	0.1	0.1	0.1	b
	ESC-10	0.3	0.5	0.8	c
	485-10	0.3	1.3	2.0	d
Persian lime[y]	Water	0	0	0	a
	ESC-11	0	0	0.1	b
	ESC-10	0.2	0.6	1.0	c
	485-10	0.6	1.0	1.8	d
Valencia orange[z]	Water	0	0	0	a
	ESC-11	0.1	0.2	0.2	b
	ESC-10	0.2	0.5	0.7	c
	485-10	0.9	0.8	1.7	d
Pineapple orange[y]	Water	0	0	0	a
	ESC-11	0	0	0.1	a
	ESC-10	0.4	0.9	1.3	b
	485-10	0.7	1.3	1.8	c
Hamlin orange[y]	Water	0	0	0	a
	ESC-11	0.1	0.3	0.3	b
	ESC-10	0.2	0.7	0.8	c
	485-10	0.3	1.3	1.9	d
Navel orange[z]	Water	0	0	0	a
	ESC-11	0.1	0.2	0.1	b
	ESC-10	0.2	0.4	0.5	c
	485-10	1.5	2.7	3.0	d
Marsh grapefruit[z]	Water	0	0	0	a
	ESC-11	0	0	0	a
	ESC-10	0.1	0.3	0.5	b
	485-10	0.2	0.8	1.3	c
Ruby Red grapefruit[y]	Water	0	0	0	a
	ESC-11	0	0	0	a
	ESC-10	0.3	0.8	1.3	b
	485-10	0.9	1.5	2.3	c
Rio Red grapefruit[x]	Water	0	0	0	a
	ESC-11	0	0	0	a
	ESC-10	0	0	0	a
	485-10	0.3	1.0	1.7	b
Sunburst tangerine[z]	Water	0	0	0	a
	ESC-11	0.1	0.1	0.2	a
	ESC-10	0.5	0.8	1.5	b
	485-10	1.1	1.8	2.6	c

[v] Leaf lesion ratings: 0 = leaf healthy, cut healed; 1 = leaf healthy, cut tan to brown in color; 2 = necrosis at cut, some expansion of necrosis down midrib; 3 = necrosis at cut, extensive expansion of necrosis the length of the midrib; 4 = entire leaf necrotic. Leaves were inoculated by cutting them with a pair of scissors dipped in a water suspension of *P. syringae* cells.

[w] Unlike letters indicate significant differences among inocula within each variety. Inocula separated by application of 95% confidence limits determined by inferences directed at inocula × inoculation density interactions following a three-way analysis of variance.

[x] Data from test 1 (Fresno, CA).

[y] Data from tests 2 and 3 (Beltsville, MD).

[z] Data the mean of all three tests.

identified strains ESC-10, ESC-11, and 485-10 as *P. syringae* pv. *morsprunorum*, *P. syringae* pv. *tagetes*, and *P. syringae* pv. *tomato* with similarity indices of 0.896, 0.967, and 0.938, respectively.

Relatedness among strains determined by carbohydrate utilization tests or cellular fatty acid analysis was similar (Fig. 1); ESC-10 and 485-10 were more similar to each other than to strain ESC-11. ESC-10 differed from 485-10 in the utilization of only two carbohydrate sources. Stain ESC-10 metabolized quinic acid while 485-10 did not, and 485-10 metabolized D-saccharic acid while ESC-10 did not. ESC-11 was able to metabolize many more carbohydrate sources than either ESC-10 or 485-10, including quinic acid, L-histidine, glucuronide, L-leucine, D-alanine, malonic acid, D-saccharic acid, and glycyl-L-glutamic acid. Cellular fatty acid and carbohydrate utilization profiles both indicated ESC-10 was more closely related to the known pathogen than was ESC-11, and subsequently ESC-10 was indeed more virulent to fruit and foliage than was ESC-11. However, despite the close similarity of ESC-10 to 485-10, it was much less virulent than 485-10.

Biological control of green mold. The percentage of lemons infected by green mold after application of water, imazalil, 485-10, ESC-10, or ESC-11, was 100.0, 1.5, 4.4, 10.5, and 61.0%, respectively. All were significantly different ($P = 0.05$) from each other. Large, dark-colored lesions, extending about 1 cm from the inoculation site, were present on many lemons treated with 485-10. Inoculated wounds treated with strains ESC-10 and ESC-11 were darker in color than those treated with water or imazalil.

Leaf inoculations. Lesions after scissor-cut inoculation of leaves ceased expansion and became clearly defined after about 3 weeks. When examined after 30 days, ESC-11 caused very small lesions on leaves on five of 12 citrus cultivars, ESC-10 caused small lesions on nine of 12 citrus cultivars, and 485-10 caused large lesions on every cultivar tested (Table 1). Mean lesion rating (±SD) of leaves 30 days after inoculation with water or ESC-11, ESC-10, or 485-10 applied at 10^8 CFU/ml was 0.0, 0.1 (±0.1), 0.7 (±0.5), and 2.2 (±0.7), respectively. On every cultivar, 485-10 was significantly more virulent than ESC-10 or ESC-11, and ESC-10 was significantly more virulent than ESC-11 on nine cultivars for which ESC-10 virulence ratings were greater than zero. Sites on leaves inoculated with ESC-10 and ESC-11 were darker in color than those treated with water.

Lesions around inoculated punctures on leaves developed slowly and were difficult to distinguish from healthy leaf tissue. Neither ESC-10 or ESC-11 caused necrosis or expanding lesions on leaves of the three cultivars (Lisbon lemon, Eureka lemon, and Persian lime) in test 1 nor the four cultivars (Hamlin orange, Valencia orange, Bearss lime, and Sunburst tangerine) in test 2. In test 1, strain 485-10 applied at 10^6 and 10^7 CFU/ml induced light-green–colored halos around punctures on the leaves of lemon and lime cultivars and expanding lesions, about 1 cm in size, on lime leaves. In test 2, no reaction was observed after all inoculations of the virulent reference strain.

Strain 485-10 caused a dark brown reaction or expanding lesions on inoculated shoots of all varieties in tests 1 and 3 but did not in test 2. Expanding lesions were first evident 2 weeks after inoculation on Navel orange, Eureka lemon, and Lisbon limon. ESC-10 and ESC-11 did not cause expanding lesions on shoots of any of 15 citrus cultivars tested after scalpel-cut inoculation and 30 days incubation.

Fruit inoculations. Lesions caused by 485-10 appeared about 4, 6, 9, and 12 days after inoculation on Persian limes, Eureka lemons, Marsh grapefruit, and Navel oranges, respectively. ESC-11 and ESC-10 did not cause any necrosis or expanding lesions after 16 days of storage at 13°C after inoculation of harvested fruit of Navel orange, Eureka lemon, or Marsh grapefruit (Table 2). On Persian lime, lesions developed after the application of all strains that significantly increased as inoculum density increased. Lesions were 2.8, 3.7, and 19.0 mm in diameter after application of 10^8 CFU/ml of ESC-11, ESC-10, and 485-10, respectively.

DISCUSSION

In this study we showed that the ability of strains ESC-10 and ESC-11 to partially control postharvest green mold was not associated with significant virulence to citrus plants or most fruit. They did not pose a significant threat to citrus fruit, foliage, or shoots of all the cultivars tested, except for Persian lime, where small lesions were observed after the inoculation of wounds on lime fruit. The known pathogenic strain 485-10 controlled green mold significantly better than ESC10 and ESC-11, but produced large lesions in most tests. Another finding of this work was that evaluation of the pathogenicity and virulence of strains was quantified in less than 1 week on lime fruit, and these results ranked the virulence of strains more quickly and clearly than did greenhouse tests with shoots or foliage that required 2 to 3 weeks for symptoms to develop.

The demand by regulators that nontarget host range studies be conducted is appropriate to minimize the risk of introducing pathogens or otherwise injurious microorganisms into the environment. Screening tests to avoid harmful strains should be a part of any effort to develop biological control antagonists, particularly pseudomonads from groups that contain pathogens. For example, although in this study strain 485-10 controlled green mold very well, it caused cosmetically unacceptable black pit symptoms on harvested fruit in every test and blast symptoms on shoots and foliage typical of this disease (5,7,12, 32,33) in most greenhouse tests. Like other plant pathogenic pseudomonads, *P. syringae* strains isolated from lesions can occasionally be virulent pathogens on other plants from which they were not isolated, although usually they are more virulent on their original host (28). Fawcett and coworkers (12) observed typical foliar citrus blast symptoms after inoculation with the citrus blast pathogen on all the citrus spe-

Table 2. Diameter of lesions on fruits of four citrus cultivars inoculated with three strains of *Pseudomonas syringae* at three densities or water and incubated at 13°C for 16 days

Cultivar	Inoculum	Fruit lesion diameter (mm) after inoculation with CFU/ml[y]			Regression[z]	R^2
		1×10^6	1×10^7	1×10^8		
Navel orange	Water	1.0	1.1	1.0	NS	
	ESC-11	1.0	1.0	1.0	NS	
	ESC-10	1.0	1.0	1.0	NS	
	485-10	3.8	5.6	6.0	$y = 1.5 (\log x) - 2.567$	0.88
Eureka lemon	Water	1.0	1.1	1.0	NS	
	ESC-11	1.0	1.2	1.1	NS	
	ESC-10	1.0	1.2	1.1	NS	
	485-10	5.4	7.2	8.4	$y = 1.5 (\log x) - 3.50$	0.99
Persian lime	Water	1.6	1.8	1.5	NS	
	ESC-11	1.5	2.5	2.8	$y = 0.65 (\log x) - 2.283$	0.91
	ESC-10	2.5	3.3	3.7	$y = 0.6 (\log x) - 1.033$	0.96
	485-10	15.0	17.0	19.0	$y = 2.0 (\log x) + 3.0$	0.99
March grapefruit	Water	1.0	1.0	1.0	NS	
	ESC-11	1.0	1.0	1.0	NS	
	ESC-10	1.0	1.0	1.0	NS	
	485-10	3.4	4.4	4.8	$y = 0.7 (\log x) - 0.7$	0.94

[y] Results from two tests in Fresno, CA. Each fruit inoculated by placing 10 µl of each suspension in 1 mm by 3 mm deep punctures.
[z] NS = regression not significant ($P = 0.05$).

cies evaluated (grapefruit, oranges, tangerine, and lemon) and two oak species (*Quercus wislezenii* and *Q. agrifolia*), but not cherry, apricot, pear, or almond. Later, Smith and Fawcett (32) inoculated *P. syringae* strains isolated from avocado, citrus, and lilac hosts with blast symptoms and from apricots with gummosis. With these, they inoculated the leaves, twigs, and fruit of 24 plants, including tomato, apple, and English walnut, and were able to observe symptoms on all. Pseudomonads isolated from lesions on cherry or plum foliage by Garrett and co-workers (13) produced very small, superficial lesions on lemon fruit after inoculation. They usually noted larger and more spreading lesions after a *P. syringae* strain was inoculated into the same host from which it was isolated.

HR on tobacco is used in lieu of the screening of many potential hosts to determine the pathogenicity of pseudomonad strains (21). The positive HR of strain 485-10 correctly predicted its pathogenicity, while the HR of strains ESC-10 and ESC-11, positive and negative, respectively, were less predictive of their reactions on citrus. Although smaller necrotic areas were observed after ESC-11 inoculation on citrus than with ESC-10, both were weakly virulent pathogens on lime fruit, demonstrating that HR alone did not conclusively predict pathogenicity when we applied a very rigorous definition of pathogenicity (capable of producing lesions of any size). A positive HR on tobacco indicates a pseudomonad is a pathogen and a positive reaction is a common attribute of all *P. syringae* pathovars in classical identification schemes (11). Exceptions to these criteria have been reported. Some pseudomonads identified as *P. syringae* were HR negative on tobacco and nonpathogens on several hosts (4,23,27), while others that were HR positive on tobacco were also nonpathogens on many hosts (4,14). The ability of *P. syringae* to elicit HR on tobacco is controlled by *hrp* genes that are needed for HR on nonhost plants and pathogenicity on host plants (25). Huang and co-workers (15) hypothesized that HR-negative strains arise due to mutations in one of several *hrp* genes. Only rarely are HR-negative strains pathogens; HR more often overestimates the frequency of virulent pathogens than it underestimates them, because avirulent strains are also HR positive (22). Therefore, although HR results cannot be used alone to predict the risk of injury to plants, HR should be employed to partially estimate this risk; strains with a negative HR on tobacco would have less risk of injury to plants, while those with a positive HR on tobacco would warrant more extensive evaluation of their virulence on many hosts.

A brown color developed in the tissue of leaves after all inoculations with 485-10, most inoculations with ESC-10, and many inoculations with ESC-11. In an evaluation of 216 *P. syringae* strains collected worldwide, Burkowicz and Rudolph (3) observed light brown reactions associated with virulent *P. syringae* strains on cherry fruit and dark brown reactions following inoculation with nonvirulent strains; they speculated that the dark brown reactions indicate a more vigorous plant defense reaction, since defensive reactions involve the oxidation of phenolic compounds. Huang and co-workers (16) reported that only a strain of *P. cepacia* that prevented citrus green mold caused browning, while a strain that promoted the disease did not. The consequences of this brown reaction caused by ESC-10 and ESC-11 on the visual quality of the fruit in the present study are probably of minor importance under commercial conditions. The relatively large, fresh wounds employed in this study may not simulate many of those inflicted during typical harvest and subsequent handling. Under commercial conditions, delays of 1 day or longer are common between harvest, when most injuries occur, and the application of postharvest treatments in packinghouses. Mercier and Wilson (26) reported dry wounds on apple fruit were more difficult to colonize with the antagonistic yeast *Candida oleophila* than moist, fresh wounds. Further testing of the commercial formulations of ESC-10 and ESC-11 (Bio-Save 10 and Bio-Save 11, respectively) is needed under commercial conditions, including assessment of biological control efficacy, population dynamics of these antagonists on the fruit, and observations of the quality of treated fruit.

An important element in the selection of biological control antagonists is the necessity to avoid strains that may injure either the plants they are applied on to protect, or other plants of economic importance. Although many studies have been conducted, the determinants of virulence remain incompletely elucidated and empirical tests such as the one we report here should continue to be required to establish the pathogenicity and virulence of pseudomonad strains before their commercial use can be recommended.

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