Limitations of Tests Used to Detect Soft-Rotting Fluorescent Pseudomonads

I. J. Misaghi and C. R. Donndelinger

Department of Plant Pathology, University of Arizona, Tucson 85721.
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ABSTRACT

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Thirteen strains of soft-rotting, oxidase-positive, fluorescent pseudomonads were found to be highly variable in ability to macerate tissue slices of nine different plant species and to degrade polypectate and polygalacturonate. Some strains macerated tissue slices of only one or two plant species, while others exhibited a wider host range. Differences in macerating ability also were exhibited by some strains on three potato cultivars that were tested. In addition, some strains macerated stems, but not leaves, of head lettuce. All strains capable of causing moderate to extensive tissue maceration also degraded polypectate and polygalacturonate. However, the pattern and intensity of pectolytic activity of the strains varied in polypectate and polygalacturonate media. One strain that macerated potato and cucumber slightly (macerating index of 0.3–0.4 on a 4-point scale) did not degrade polypectate or polygalacturonate. Implications of these findings on the interpretations of the results of tests used to detect soft-rotting fluorescent pseudomonads are discussed.

Additional key words: polygalacturonase, *Pseudomonas fluorescens, P. marginalis, Solanum tuberosum*.

Soft-rotting, oxidase-positive, fluorescent pseudomonads (FP) are a ubiquitous group of bacteria capable of causing soft rot in a number of plants including carrots and cabbage (3), celery (29), onion (26), cucumber (15), cauliflower (4), lettuce (1,21), potato (7,22), chicory (8), fennel (28), leek (9), garlic (23), and hyacinth (12). Since soft-rotting strains cannot be distinguished from non-soft-rotting, non-plant pathogenic, oxidase-positive FP on the basis of any character other than soft-rotting activity (16,20,24,25,27), they are routinely identified on the basis of their ability to macerate potato tuber tissue (20,24,25) and/or to degrade pectate media (2,3,10). However, results of our preliminary observations showed that the popular potato soft-rot test is not reliable for detecting soft-rotting FP. For example, two strains of FP that did not macerate potato tuber tissue were capable of macerating tissue slices of other plants. The objective of this study was to assess the reliability of the use of potato tuber tissue and pectate media to detect soft-rotting FP.

MATERIALS AND METHODS

Bacterial strains. Of 13 pectolytic strains of FP used in the study, three were designated by the donors as *Pseudomonas marginalis* and one as *P. fluorescens*. Nine strains were obtained from soil samples collected from fields in southern Arizona (Table 1). Pectolytic strains were isolated from soil by using tissues from fresh vegetables obtained from local grocers. Potato (*Solanum tuberosum* L. 'Russet Burbank,' 'White Rose,' and 'Red LaSoda') tubers; onion (*Allium cepa* L.) bulbs; Chinese cabbage (*Brassica chinensis* L. 'Pak-choi') leaves; carrot (*Daucus carota* L. var. *sativa* DC) and garden red radish (*Raphanus sativus* L.) roots; celery (*Apium graveolens* L. var. *dulce* DC 'Florigreen') and head lettuce (*Lactuca sativa* L. var. *capitata*) stems; and cucumber (*Cucumis sativus* L.) and black zucchini squash (*Cucurbita pepo* L.) fruits were washed and surface sterilized by placing them in a 0.52% solution of sodium hypochlorite for 10 min. With the exception of Chinese cabbage, plant parts were cut with a sterilized knife into sections ~6 mm thick. Chinese cabbage leaves were cut into sections (~16 cm^2) prior to surface sterilization. Aseptic lettuce leaf sections (~16 cm^2) were collected from the center of heads that had been torn apart without cutting. Soil suspensions were prepared by vigorously shaking 1 g of soil in 10 ml of sterile water for a few minutes. One-tenth milliliter of soil suspensions was placed in the center of tissue sections, which were placed on moistened sterile filter papers in petri plates. Plates were kept at 25 C in plastic boxes lined with moistened paper towels and examined for tissue maceration after 3 days. To recover soft-rotting strains from rotted tissues a small amount of the tissue was added to about 5 ml of sterile water and shaken vigorously. Plates containing medium B (13) were seeded with 0.1 ml of various dilutions of the bacterial suspensions and incubated at 25 C. Discrete bacterial colonies were picked after 24 hr, and their purity was tested by repeated streaking on the medium.

Tissue maceration. Since the objective of the study was to evaluate the efficacy of the potato soft-rot test, tissue slices of potato tubers and of other plants (Table 1) were prepared and inoculated according to the commonly used standard procedures. Bacterial suspensions containing 10^8 colony-forming units (cfu) per milliliter were placed in the center of sterile tissue sections (prepared as described above) on moistened sterile filter papers in petri plates. Bacterial suspensions used for inoculation were obtained from 48-hr-old cultures on medium B and were standardized by using cell number-optical density (610 nm) curves. Tissue sections treated with sterile water served as controls. Plates were incubated in plastic boxes lined with moistened paper towels for 3 days at 25 C. Degree of tissue maceration (tissue firmness determined by stabbing with a dissecting needle) was judged as slight, moderate, extensive, and very extensive for average maceration indices of <1.0, 1.0–2.0, 2.0–3.0, and 3.0–4.0, respectively. Soft-rotting tests were repeated three times each with two to five replications. Except for the preliminary studies, all soft-rotting tests reported here were performed on plant materials obtained from one vendor. Plant materials were stored at appropriate storage temperatures during the course of the experiment.

Polypectate and polygalacturonate degradation. The abilities of the strains to degrade polypectate (pH 7.1) and polygalacturonate (pH 7.6) were tested on media described by Cuppels and Kelman (2) and Hsu and Vaughn (11), respectively. Polypectate culture plates were seeded by placing approximately 30 μl of each bacterial suspension containing 10^8 cfu/ml in the center of the plates. Polygalacturonate culture plates were inoculated by stabbing the medium with sterile toothpicks dipped into each bacterial suspension containing 10^8 cfu/ml. Culture plates were incubated at 25 C for 72 hr. Pectolytic activities of the strains on the polypectate medium were rated on a scale from 0 (no noticeable degradation) to

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TABLE 1. Macerating and pectolytic activity of oxidase-positive fluorescent pseudomonads on plant tissues and on pectate media

Strains[a]	Pectate degradation[b]		Tissue maceration[c]											
	Poly-galactu-ronate	Poly-pec-tate	Lettuce stem	Lettuce leaves	Zucchini squash	Cucumber	Chinese cabbage	Celery	Radish	Carrot	Onion	Potato cultivars: Red LaSoda	White Rose	Russet Burbank
M1	5.3	1.0	0.8	0.0	0.15	1.3	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.8
M2	14.2	1.0	1.8	1.5	0.5	1.8	0.0	0.0	0.6	0.0	0.0	2.0	0.5	2.3
M3	20.0	1.4	2.3	2.8	0.0	2.2	2.4	1.7	0.3	0.0	0.0	2.2	1.5	2.9
F1	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4
SR1	15.0	3.7	1.4	0.3	2.3	3.5	2.0	2.0	1.1	2.7	0.6	1.8	0.8	3.8
SR2	9.7	4.0	0.0	0.0	0.4	1.7	1.7	1.3	0.4	0.0	0.0	1.8	0.0	2.9
SR3	1.8	1.7	2.4	0.0	0.3	0.6	0.5	1.0	0.3	0.0	0.0	0.0	0.4	1.7
SR4	14.0	4.0	3.3	0.0	2.3	3.6	2.5	2.5	1.0	1.0	0.4	3.8	2.7	3.1
SR5	9.3	2.6	2.8	2.3	0.7	1.0	0.6	0.0	0.0	0.0	1.0	0.0	0.0	0.0
SR6	0.0	2.0	2.0	0.9	0.6	2.5	0.0	1.7	0.0	0.0	0.6	0.0	0.0	1.0
SR7	7.2	2.1	0.4	0.0	0.0	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SR8	6.2	4.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SR9	9.6	3.1	1.3	0.0	0.0	2.5	0.4	0.0	0.0	0.0	0.0	0.6	0.0	0.9

[a] Strain M1 received as *Pseudomonas marginalis* from D. C. Hildebrand, M2 as *P. marginalis* (UCPPB 546), M3 as *P. marginalis* (UCPPB 547) both from M. N. Schroth, F1 as *P. fluorescens* (ATCC-13525). Strains SR1-SR9 were recovered from soil samples from fields in southern Arizona.

[b] Tests for polypectate and polygalacturonate degradation were repeated three times each with two replications. Polypectate degradation values (average of six readings) are based on a scale from 0 (no noticeable polypectate degradation) to 4 (extensive degradation) observed after 3 days at 25 C. Polygalacturonate degradation values (average of six readings) were obtained by measuring the diameters (mm) of the clear zones which appeared after flooding the culture plates with 1.0 N HCl after 3 days at 25 C.

[c] Maceration tests were repeated three times each with two to five replications. Figures in the 12 columns on the right of the table represent tissue maceration index (averages of six to 15 readings) based on a scale from 0 (no maceration) to 4 (very extensive maceration) after 3 days of incubation at 25 C.The inoculation method is described in the text.

TABLE 2. Macerating indices of soft-rotting, oxidase-positive fluorescent pseudomonads on Russet Burbank potato tuber disks obtained in tests repeated at different times under identical conditions

Strain[a]	Maceration index
SR2	4,4,1,4,4,4,4,4,4,3,3,2,3,3,0,0
SR3	0.5,3,4,0.5,0.5,4,4,0.5,1,1,1,1,1,1,4,1
SR6	0,2,0,0,2,1.5,2,0,2,3,0,1

[a] Strains were recovered from soil samples collected from fields in southern Arizona. Each figure represents tissue maceration index based on a scale from 0 (no maceration) to 4 (extensive maceration) of one tuber disk in different tests. Inoculated and uninoculated tuber disks were incubated for 3 days at 25 C. The inoculation method is described in the text.

4 (extensive degradation). Activity on polygalacturonate medium was determined by measuring the diameter of the clear zones appearing after flooding the cultures with 1.0 N HCl. These tests were repeated three times each with two replications.

Biochemical and physiological tests. Tests for the presence of oxidase, arginine dihydrolase, lipase, and production of levan were done according to the procedures described earlier (20).

Hypersensitivity of tobacco. Attached leaves of 8-wk-old *Nicotiana glutinosa* L. were injected with bacterial suspensions (10^8 cfu/ml) taken from 24-hr-old cultures on medium B of all test strains. *P. syringae* pv. *syringae* was used as a hypersensitive-positive check and water served as control. Plants were maintained in a greenhouse at 25–27 C. Tissue collapse in injected leaf areas within 24 hr was considered a positive response (14). The test was repeated twice.

RESULTS

All 13 strains used in this study were oxidase-positive fluorescent pseudomonads similar to those placed in *P. fluorescens* biotype A group by Stanier et al (27) and to those placed in *P. marginalis* by Lelliott et al (16). All strains caused slight to extensive maceration of tissue of one or more plants, produced fluorescent pigments in medium B, reacted negatively in tobacco hypersensitivity tests, and were variable in levan and lipase tests.

Strains were highly variable for ability to macerate tissue slices from different plant species tested. For example, some strains (M3, SR1, and SR4) caused moderate to extensive maceration of tissue slices of 5 to 8 different species, while others (M1 and SR7) moderately macerated tissue slices from only one species (Table 1). Tissue selectivity also was observed on three potato cultivars tested and on lettuce leaves and stems. Two strains (M3 and SR4) caused moderate to very extensive maceration of all three potato cultivars. Three strains (M2, SR1, and SR2) macerated moderately to very extensively only two potato cultivars. Other strains were either slightly to moderately soft-rotting or non-soft-rotting on tuber tissue of one or two potato cultivars. Some of those strains that did not macerate potato macerated tissue slices from other plants. Three strains (SR3, SR4, and SR9) macerated moderately to very extensively stems, but not leaves, of lettuce.

Results of soft-rotting tests using a single bacterial strain on tissue from a single plant cultivar (obtained from one source) varied between tests (Table 2). The variability is surprising since the tests were repeated under identical conditions.

In less than 5% of the tests one or more tissue slices treated with sterile water as control rotted. Results of such tests were discarded.

The pattern and intensity of pectolytic activity of the strains varied in polypectate and polygalacturonate media (Table 1). All strains capable of causing moderate to very extensive tissue maceration also degraded polypectate. However, one strain (F1), which macerated Russet Burbank potato and cucumber slightly (maceration index 0.3–0.4), did not degrade either polypectate or polygalacturonate. Another strain (SR6), which macerated tissue slices from a few plants and degraded polypectate medium did not degrade polygalacturonate. Of the three strains that exhibited the greatest soft-rotting activity on tissue slices of different plants, two strains (SR1 and SR4) were strongly active on both polypectate and polygalacturonate. However, the third (M3) was highly active on polygalacturonate but only slightly active on polypectate.

DISCUSSION

Soft-rotting strains of FP are known to macerate tissues of different plants (1,3,4,7–9,12,15,21,23,28,29). Nevertheless, soft-rotting potential of strains of *Pseudomonas* and *Erwinia* is commonly determined on the basis of their ability to macerate a single cultivar of potato in a test known as the potato soft-rot test. Results of this study reveal the danger of using a single plant species such as potato in tests designed to detect soft-rotting FP. Our data (Table 1) show that while most of the pectolytic strains of FP may

be detected by the commonly used potato soft-rot test, some may remain undetected unless they are tested on other hosts. For example, strain SR2 and probably strain SR1 would not have been identified as soft-rotting had we used only White Rose potato as the substrate. Similarly a number of other strains would have been labeled as non-soft-rotting had we not tested them on a number of different plants. Therefore, it is important that the soft-rotting ability of FP strains be determined not only on different potato cultivars, but on other plants, particularly celery and lettuce stems, cucumber and zucchini squash fruits, and lettuce leaves.

Results of soft-rotting tests using a single bacterial strain on tissue slices from a single plant cultivar were variable even though tests were repeated under identical conditions (Table 2). However, the variability of the results was not so great as to prevent detection of soft-rotting strains or their host range if tests were repeated sufficiently. For example, every one of the 11 soft-rotting strains was detected as a soft-rotter when it was tested three times on each of the test plants. Moreover, strains that did not macerate tissue slices of certain plants in the first three repeating tests remained non-soft-rotting in five to seven additional tests. Judging from these results, it is recommended that soft-rotting tests designed to determine soft-rotting potential of strains be repeated sufficiently to avoid wrong conclusions.

The pattern and intensity of responses of strains were different in two pectate media. These results are not surprising because the response of pseudomonad strains on pectate media is known to be influenced by pH and the presence of different substrates in the media (10).

Since macerating activity of bacteria is influenced by a variety of environmental factors (5,17–19) soft-rotting tests should be conducted under strictly controlled conditions. Resistance to decay in potato tubers injected with viable *Erwinia carotovora* (5,19) or filter-sterilized pectic enzyme preparations from *E. carotovora* (19) increases with increase in oxygen level. Resistance to maceration in air is thought to be a host-mediated response (17,19,30). Maher and Kelman (19) have pointed out that the relevance of tissue slice bioassays for detection of soft-rotting strains of *E. carotovora* to in vivo conditions should be reevaluated. They point out that oxygen level, which influences the degree of maceration, is affected by water or buffer films on the tissue surfaces. The size and the thickness of tissue slices may also influence the outcome of the tests.

The basis for the observed differences in tissue selectivity of the soft-rotting strains is not known. Such selectivity may be mediated by difference in types and concentrations of nutrients in the tissue and the presence or absence in the tissue of certain inducers and suppressors of bacterial pectolytic enzymes. Zucker et al (31) found that vegetable extracts exert both repressive and synergistic effects on lyase synthesis in *Erwinia* and *Pseudomonas* species and suggested that host factors may play an important role in the ecology of bacterial soft-rot diseases.

The taxonomic status of soft-rotting, oxidase-positive FP, which generally are labeled as *Pseudomonas marginalis*, is not clearly understood. Except for pectolytic activity, which is obviously a variable character, members of the group cannot be distinguished from non-soft-rotting, nonpathogenic, oxidase-positive FP (*P. fluorescens, P. putida*) on the basis of nutritional tests as well as a number of important diagnostic tests (16,20,24,25,27). Utilizing the results of 146 nutritional and biochemical tests to assess percent similarity among 18 nomenspecies of FP, Misaghi and Grogan (20) found *P. marginalis* to be closely related to saprophytes, *P. fluorescens* (74%), *P. putida* (69%), and *P. aeruginosa* (70%) and distantly related to members of oxidase-negative plant pathogenic FP (33–46%). Similar results have been found by Sands et al (25).

The close relatedness of soft-rotting and non-rotting, oxidase-positive FP and the variability among members of the former group warrant reevaluation of the taxonomic status of *P. marginalis* (6). Until this is done *P. marginalis* should be retained to denote all soft-rotting oxidase-positive, arginine dihydrolase-positive FP as suggested by Cuppels and Kelman (3).

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