Rapid Evaluation of Pathogenicity in *Pseudomonas syringae* pv. *syringae* with a Lilac Tissue Culture Bioassay and Syringomycin DNA Probes

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ABSTRACT

Scheck, H. J., Canfield, M. L., Pscheidt, J. W., and Moore, L. W. 1997. Rapid evaluation of pathogenicity in *Pseudomonas syringae* pv. *syringae* with a lilac tissue culture bioassay and syringomycin DNA probes. *Plant Dis.* 81:905-910.

Losses from diseases caused by *Pseudomonas syringae* pv. *syringae* occur on a large number of deciduous woody plants in commercial nurseries in the Pacific Northwest. Bioassays for pathogenicity are one step in the identification of *P. syringae* pv. *syringae* and are usually performed on the host of isolation; however, woody plants can take months to develop symptoms. A bioassay with highly susceptible lilac (*Syringa vulgaris* 'Sensation') tissue culture plantlets evaluated pathogenicity in strains of *P. syringae* pv. *syringae* isolated from 25 species of deciduous woody plants. DNA colony hybridization with the *syrB* probe for a syringomycin synthetase gene and the *syrD* probe for a syringomycin export gene was also evaluated as a method for identifying pathogens. Of 552 strains provisionally identified as *P. syringae* pv. *syringae*, 59% were pathogenic in the bioassay and hybridized with the *syr* probes, while 19% were non-pathogenic and did not hybridize with the *syr* probes, giving 78% agreement between the two methods. Nine percent of strains were pathogenic in the bioassay but did not hybridize with the *syr* probes, and 13% were not pathogenic in the bioassay but did hybridize with the *syr* probes. These methods detected pathogenic strains of *P. syringae* pv. *syringae* isolated from diverse woody plants in 5 to 16 days.

Pseudomonas syringae pv. *syringae* infects a wide range of deciduous woody plants in Pacific Northwest nurseries, causing tip dieback, bud and flower blast, spots and blisters on fruit, stem canker, and leaf blight (6,35). Annual losses in woody plant nurseries in Oregon from *P. syringae* pv. *syringae* are estimated at \$8 million (40). Because *P. syringae* pv. *syringae* survives as an epiphyte on many woody and herbaceous plants (26), its association with diseased tissue cannot be equated to pathogenicity. Tests of the pathogenicity of strains of *P. syringae* pv. *syringae*, isolated either as epiphytes or from diseased plant tissue on the wide variety of potential hosts grown in commercial woody plant nurseries, would require extensive resources, and symptom development could take months (29). A rapid method to evaluate patho-

genicity of strains of *P. syringae* pv. *syringae* is crucial for the timely application of chemical sprays for disease control.

The traditional method of diagnosing diseases incited by *P. syringae* pv. *syringae* involves isolation of the suspected pathogen in pure culture and characterization by a combination of physiological and biochemical tests. The Levan production, Oxidase activity, Potato soft rot, Arginine dihydrolase activity, and Tobacco hypersensitive response (LOPAT) tests (24,25) are used to discriminate *P. syringae* from other species of fluorescent pseudomonads. The Gelatin liquefaction, Aesculin hydrolysis, Tyrosinase activity, and utilization of Tartrate (GATTA) tests (22) are used to separate pathovar *syringae* from other pathovars of *P. syringae*. More rapid identification of *P. syringae* pv. *syringae* has been attempted by measuring the ice nucleation activity (INA) of strains (1,16,20,26,38), by bacteriophage typing (3,4,16), and by serology (14,30,34). Because these methods do not directly assess pathogenicity, Young (49) proposed a protocol of 12 nutritional tests, an assay for syringomycin, and a lilac pathogenicity test to identify *P. syringae* pv. *syringae*.

There is no standard method for evaluating pathogenicity in *P. syringae* pv. *syringae*. Inoculations of immature pear and sweet cherry fruit (10,16,36), leaves and twigs of apricot, peach, nectarine, and plum, etiolated pear, apple and peach hypocotyls (10), immature yellow tomato

fruit (5,6), bean pods (23), lemon fruit and pea stems (30), lilac petioles (49), red maple stems (28) and pear blossoms (46,48) have all been tested. The results of all these methods were variability in symptom expression and low correlation of results from bioassays with physiological or biochemical tests.

Presumably, strains identified as *P. syringae* pv. *syringae* are pathogenic on lilac (*Syringa* sp.) or share hosts in common with pathogenic strains isolated from lilac (49). Maintaining lilac plants with sufficient young, susceptible leaves or flowers for pathogenicity testing is problematic. Yessad et al. (48) showed in vitro micro-cuttings of pear (*Pyrus communis*) were superior to pear seedlings or immature pear fruit for detecting pathogenic strains of *P. syringae* pv. *syringae*. Because lilac can be maintained in a juvenile state in aseptic tissue culture, it has advantages in a bioassay for testing strains of *P. syringae* pv. *syringae* for pathogenicity.

Pathogenic strains of *P. syringae* pv. *syringae* may not be equally virulent on any given host (6,10,16,48). DNA hybridization with labeled probes can be a sensitive and accurate method of identifying phytopathogenic bacteria without involving a host plant. Radio-labeled probes have been made to cryptic DNA sequences and used to identify pathogenic strains of *Erwinia amylovora* (11), *P. syringae* pv. *tomato* (9), *Xanthomonas campestris* pv. *phaseoli* (13), and *Clavibacter michiganense* subsp. *michiganense* (44). Probes for genes required for phaseolotoxin production in *P. syringae* pv. *phaseolicola* (39) and coronatine production in *P. syringae* pv. *tomato* (8) have been highly reliable for identifying pathogens in vitro and in planta.

Syringomycin is a necrotizing phyto-toxin with antimicrobial activity produced by most strains of *P. syringae* pv. *syringae* and not by other pathovars of *P. syringae* (51). *SyrB*, a syringomycin synthetase gene, and *syrD*, a syringomycin export gene, have been cloned and used as radio-labeled probes (36). Using probes for syringomycin genes is a more direct route for evaluating the toxigenic potential of strains than the standard bioassay, which measures growth inhibition of the syringomycin-sensitive fungus *Geotrichum candidum* Link (42). The *syr* sequences were shown to be highly specific to syringomycin-producing strains of *P. syrin-*

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Published as technical paper no. 11081 of the Oregon State University Agricultural Experiment Station.

Use of trade names in this article does not imply endorsement by Oregon State University of the products named or criticism of similar products not mentioned.

Accepted for publication 23 April 1997.

Publication no. D-1997-0602-03R
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gae pv. *syringae* isolated from diverse hosts including lilac, citrus, bean, rose, wheat, sugar beet, stone fruit, and pome fruit (36). The *syr* probes had no homology to *P. viridiflava* nor to *P. syringae* pvs. *phaseolicola*, *tabaci*, *tagetis*, *tomato*, *morsprunorum* or *pisi*. Thus, syringomycin genes should be useful for identifying pathogenic *P. syringae* pv. *syringae* isolated from woody plants.

The first objective of this research was to evaluate lilac (*Syringa vulgaris*) as a possible universal suspect in pathogenicity tests with strains of *P. syringae* pv. *syringae* isolated from diverse woody plants. The second objective was to test digoxigenin-labeled *syrB* and *syrD* DNA probes for syringomycin genes as a method to identify pathogens without a bioassay. Results of these two methods were compared with traditional physiological and nutritional tests for identifying *P. syringae*

pv. *syringae*. A preliminary report has been published (7).

MATERIALS AND METHODS

Isolation and characterization of strains. Deciduous woody plants with cankers, bud and blossom blights, leaf spots, and shoot diebacks were collected from 44 commercial nurseries and seven landscape plantings in the Willamette Valley, Oregon, March through May of 1992 and 1993. Isolation and purification of these strains has been described (40). Strains were provisionally identified as *P. syringae* pv. *syringae* based on blue-green fluorescence on King's Medium B (KB) (19) under UV at 350 nm and a negative test result for Gram's stain (38) and cytochrome oxidase activity (21). Bacteria were preserved at -80°C in sterile Luria-Bertani broth (31) amended with 20% sterile glycerol. Before each assay, strains

were recovered from frozen storage by streaking onto KB and incubating for 48 h at 28°C .

Fluorescent, oxidase-negative, gram-negative strains were further characterized by two determinative schemes: LOPAT tests (20,25) and GATTa tests (22). All strains were tested three times for LOPAT and GATTa characters.

INA of strains was determined by the method of Vali as modified by Lindow et al. (26). Cells were suspended to a concentration of approximately 1×10^8 CFU/ml ($\text{OD}_{590} = 0.3$) in sterile distilled water (SDW). Ten drops (10 μl each) of cell suspension were pipetted onto a paraffin-coated sheet of aluminum foil floating on a 70% ethanol solution maintained at -5°C (Exacal 300 bath, Neslab Instruments Inc., Portsmouth, NH). The number of drops that froze within 10 min was recorded; strains with eight or more frozen drops were considered INA. All strains were tested three times.

Tissue cultured plantlets. *S. vulgaris* 'Sensation' stock plants were obtained from a commercial tissue culture nursery (Microplant Nurseries, Gervais, OR). Plantlets were aseptically cultured in 200 ml glass jars with plastic lids on 35 ml of solid lilac tissue culture medium (Mura-shige and Skoog salts [33] 4.3 g, 6-benzylamino-purine 2 mg, thiamine 0.4 mg, myo-Inositol 100 mg, sucrose 30 g, agar 6.5 g, distilled water 1 liter, pH 5.2, all chemicals from Sigma Chemical Co., St. Louis). Plantlets were grown at 25°C with a 16 h photoperiod under 40W fluorescent bulbs in a constant temperature room and sub-cultured every 3 to 4 weeks.

Bioassay for pathogenicity. Bacteria were harvested from KB plates and suspended in SDW to a concentration of approximately 1×10^4 CFU/ml ($\text{OD}_{590} = 0.20$). Plantlets were submerged and gently swirled in the bacterial suspension for 30 sec and returned to their jars. Inoculated plantlets in the jars were exposed to a temperature of -5°C for 10 min to simulate a frost event, and then incubated at 25°C with a 16 h photoperiod. Symptom development on plantlets was observed after 14 days. Highly virulent *P. syringae* pv. *syringae* strain PAH5/92, isolated from diseased lilac (40), was used as a positive control; non-pathogenic *P. syringae* pv. *syringae* strain JL2000, isolated from a corn leaf surface (27), and water-inoculated plantlets were used as negative controls. Each strain was tested on three plantlets. Random stock plantlets were sampled periodically for contaminating bacteria, yeast, or fungi by macerating plantlets in SDW and streaking loopfuls of the resulting aqueous solution onto KB. Plates were incubated at 28°C for 7 days.

DNA-DNA colony hybridizations. Syringomycin probes were cloned from *P. syringae* pv. *syringae* strain B301D isolated from diseased pear (16). Probe p91

Table 1. Plant sources of 552 strains of *Pseudomonas syringae* pv. *syringae* and the results of pathogenicity tests with a lilac tissue-culture bioassay and DNA colony hybridizations with probes p91 (*syrB*) and p9 (*syrD*) for syringomycin genes

Family, genus, and species ^a	Common name	Pathogenicity tests (No. of strains ^b)			
		Bio+ CH+	Bio– CH–	Bio+ CH–	Bio– CH+
Aceraceae					
<i>Acer palmatum</i> Thunb.	Japanese maple	34	45	21	24
<i>A. platanoides</i> L.	Norway maple	1	4	0	0
<i>A. rubrum</i> L.	Red maple	6	4	4	3
<i>A. saccharum</i> Marsh.	Sugar maple	1	0	0	0
<i>A. truncatum</i> Bunge.	Shantung maple	3	0	1	1
Berberidaceae					
<i>Berberis aquifolium</i> Pursh.	Oregon grape	2	0	0	0
Caprifoliaceae					
<i>Viburnum dentatum</i> L.	Arrow-wood	0	4	2	5
Celastraceae					
<i>Euonymus alatus</i> (Thunb.) Siebold	Spindle tree	3	0	0	0
Ericaceae					
<i>Vaccinium corymbosum</i> L.	Highbush blueberry	3	12	1	1
Hamamelidaceae					
<i>Liquidambar styraciflua</i> L.	Sweet gum	1	3	0	1
Hydrangeaceae					
<i>Philadelphus coronarius</i> L.	Mock orange	7	2	1	1
<i>Magnolia grandiflora</i> L.	Southern magnolia	2	9	1	2
Oleaceae					
<i>Forsythia viridissima</i> Lind.	Golden bells	11	2	2	2
<i>Syringa amurensis</i> Rupr.	Amur lilac	1	0	0	2
<i>S. x chinensis</i> Willd.	Chinese lilac	9	0	0	3
<i>S. x persica</i> L.	Persian lilac	4	1	0	2
<i>S. vulgaris</i> L.	Common lilac	216	9	8	17
Rosaceae					
<i>Prunus armeniaca</i> L.	Apricot	4	1	2	1
<i>P. avium</i> (L.) L.	Sweet cherry	5	0	0	1
<i>P. laurocerasis</i> L. cv. Otto Leuken	Cherry laurel	2	1	0	1
<i>P. serrulata</i> Lindl.	Japanese flowering cherry	1	1	2	2
<i>Pyrus communis</i> L.	Common pear	2	2	0	2
<i>P. pyrifolia</i> (Burm f.) Nakai	Asian pear	5	5	0	0
<i>Sorbus aucuparia</i> L.	Mountain ash	2	0	0	2
Tiliaceae					
<i>Tilia cordata</i> Mill.	European linden	0	2	2	0
Totals		325	107	47	73

^a Latin binomials follow those in *Scientific and Common Names of 7000 Vascular Plants in the United States*. 1995. L. Brako, A. Rossman, and D. Farr, American Phytopathological Society, St. Paul, MN.

^b Pathogenic in lilac bioassay = Bio+, non-pathogenic in lilac bioassay = Bio-, positive in colony hybridizations with *syr* probes = CH+, negative in colony hybridizations with *syr* probes = CH-.

for a syringomycin synthetase gene contains a 1,107-bp *SalI* internal fragment of the *sydB* gene cloned into pUC18 and maintained in *Escherichia coli* DH5 α . Probe p9 for a syringomycin export gene is a 510-bp *EcoRI* fragment containing 500-bp of the 5'-end of the *sydD* gene, plus 10-bp of upstream DNA cloned into pUC19 and maintained in *E. coli* DH5 α (36). DNA fragments were labeled with digoxigenin-11-dUTP (Genius Kit; Boehringer Mannheim Biochemicals, Indianapolis, IN) as directed by the manufacturer.

The *sydB* and *sydD* probes were used in DNA colony hybridizations to detect syringomycin synthetase and export genes in strains of *P. syringae* pv. *syringae* isolated from diseased woody plants. For hybridizations, individual bacterial colonies were grown on KB for 48 h at 28°C. Sterilized, non-charged nylon membranes (Qiagen Inc., Chatsworth, CA) were laid on 250 ml of autoclaved, GY agar (modified from Keane et al. [18]: L-glutamic acid 2 g, yeast extract 1 g, KH₂PO₄ 0.2 g, MgSO₄ 0.5 g, NaCl 0.2 g, agar 15 g, distilled water 1 liter, pH 7.0; all chemicals from Sigma Chemical Co.) in 23- by 30-cm Pyrex cake pans. Individual bacterial colonies were aseptically transferred onto the membranes and incubated for 24 h at 28°C. Cells were lysed by placing the membranes on Whatman 3-mm filter paper saturated with 10% sodium dodecyl sulfate (SDS) for 30 min. The released DNA was denatured for 30 min on paper saturated with 0.5 M NaOH-1.5 M NaCl and neutralized for 30 min on paper saturated with 0.5 M Tris-HCl (pH 8.0)-1.5 M NaCl. Membranes were dried for 30 min at 20°C, then baked for 1 h at 80°C under vacuum. Cell debris was removed by washing 1 h at 68°C in 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM

EDTA and 0.1% SDS. Pre-hybridization and hybridization solution consisted of 5 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl-0.015 M sodium citrate), 1% blocking reagent (Boehringer Mannheim Biochemicals), 0.1% N-lauroyl sarcosine, and 0.2% SDS. Pre-hybridization was performed for a minimum of 1 h at 68°C. Hybridization with 10 ng/ml of digoxigenin-labeled probe was for a minimum of 12 h at 68°C. Post-hybridization washes were two 30-min washes at 20°C in 2 $\times$ SSC-0.1% SDS and two 60-min washes at 55°C in 0.1 $\times$ SSC-0.1% SDS. Membranes were incubated with Anti-digoxigenin (Fab) conjugated to alkaline phosphatase, and DNA-DNA hybridization was detected colorimetrically with X-phosphate and NBT (Boehringer Mannheim Biochemicals). Strains that hybridized to the probe were dark purple/brown following colorimetric detection, while the strains that did not hybridize remained unstained. Strain B301D was included as a positive control and W4N77, a non-syringomycin producing *P. syringae* pv. *syringae* strain isolated as an epiphyte on pear, was included as a negative control (36). All colony hybridizations were repeated.

RESULTS

Isolation and characterization of strains. Five-hundred fifty-two strains of fluorescent, oxidase negative, gram-negative bacteria isolated from diseased tissues of 25 species of woody plants (Table 1) were tested with standard physiological and biochemical diagnostic methods. In LOPAT tests, *P. syringae* strains were expected to be levan positive, oxidase negative, potato soft-rot negative, arginine dihydrolase negative, and tobacco HR positive. Of the 552 strains tested, 73%

were positive for levan, none caused potato soft-rot, 81% were arginine dihydrolase negative, and 79% were positive for tobacco HR (Table 2). Fifty-three percent of strains matched the LOPAT phenotype for *P. syringae*, while 32% differed in one test, 10% differed in two tests and 5% differed in three tests.

In GATTa tests, *P. syringae* pv. *syringae* strains should liquefy gelatin and hydrolyze aesculin but have no tyrosinase activity and be unable to utilize tartrate. Of the 552 strains tested, 90% liquefied gelatin, 75% hydrolyzed aesculin, 94% lacked tyrosinase activity, and 89% were unable to utilize tartrate (Table 2). Fifty-nine percent of strains matched the GATTa phenotype for *P. syringae* pv. *syringae*, while 30% differed in one test, 10% differed in two tests, and 1% differed in three tests. When the two schemes were combined, 42% of the 552 strains matched the expected results for *P. syringae* pv. *syringae*. Fifty-nine percent of all strains were INA, and 73% of the strains that conformed to the LOPAT/GATTa profile for *P. syringae* pv. *syringae* were INA.

Pathogenicity bioassay. Of the 552 strains tested, 67% caused bacterial blight symptoms in the tissue-cultured lilac bioassay (Table 1). Disease symptoms on the lilac plantlets included water soaked lesions on leaves, vein and petiole necrosis, and tip dieback. Symptoms appeared within as little as 2 days after inoculation, and plantlets were completely necrotic after 14 days. Plantlets inoculated with non-pathogenic strain W4N77 or water only showed no disease symptoms after 14 days and no damage from the 10-min exposure to freezing temperatures. No contaminant microorganisms were recovered from the stock plantlets, and *P. syringae*

Table 2. Physiological and biochemical characterization of fluorescent, gram-negative bacteria recovered from diseased woody plants

% Strains (n = 552)											
Phenotype	Total	Bio+ ^a	Bio-	CH+	CH-	Phenotype	Total	Bio+	Bio-	CH+	CH-
L+	73	52	21	55	18	L-	27	15	12	15	12
O-	100	67	33	72	28	O+	0	0	0	0	0
P-	100	67	33	72	28	P+	0	0	0	0	0
A-	81	60	21	61	20	A+	19	7	12	9	10
T _(HR) +	79	57	22	61	17	T _(HR) -	21	11	10	10	12
L+O-P-A-T _(HR) + ^b	53	42	11	45	8						
G+	90	62	28	64	26	G-	10	6	4	6	4
A _(e) +	75	53	22	54	21	A _(e) -	25	15	10	16	9
T-	94	65	29	67	27	T+	6	3	3	3	3
Ta-	89	60	29	63	26	Ta+	11	8	3	7	4
G+A _(e) +T-Ta- ^c	59	44	15	45	14						
L+O-P-A-T _(HR) + & G+A _(e) +T-Ta-	42	34	8	37	5						
INA+	59	44	15	47	12	INA-	41	24	17	24	17
	Matched all tests	Differed in 1 test	Differed in 2 tests	Differed in 3 tests							
LOPAT _(HR)	53	32	10	5							
GA _(e) TTa	59	30	10	1							

^a Bio = lilac bioassay, CH = colony hybridization with *sydB* probes, L = levan produced on 5% wt/vol sucrose agar, O = oxidase activity with Kovac's reagent, P = potato soft-rot, A = arginine dihydrolase activity, T_(HR) = tobacco hypersensitive response, G = gelatin liquefaction, A_(e) = aesculin hydrolysis, T = tyrosinase activity, Ta = tartrate utilization, INA = ice nucleation active.

^b Expected phenotype for *Pseudomonas syringae* in LOPAT_(HR) tests: L + O - P - A - T_(HR) +.

^c Expected phenotype for *Pseudomonas syringae* pv. *syringae* in GA_(e)TTa tests: G + A_(e) + T - Ta-.

pv. *syringae* was not able to grow on the lilac tissue-culture medium.

The proportion of strains pathogenic in the lilac bioassay varied according to the host from which they were initially isolated (Table 1). Of the 272 strains isolated from diseased lilacs, 88% were pathogenic in the bioassay, while only 47% of the 152 strains isolated from diseased maples were pathogenic. Strains from other woody plants were divided about evenly between pathogens and non-pathogens, with the notable exceptions of highbush blueberry and southern magnolia, which had a high proportion of non-pathogenic strains.

DNA colony hybridizations. Of the 552 strains tested, 72% hybridized with the *syr* gene probes (Table 1). None of the strains hybridized with just one of the *syr* probes. As in the bioassay, some woody hosts yielded a higher percentage of strains that hybridized to the probes than did others (Table 1). Of the strains isolated from lilacs, 93% hybridized with the probes while only 48% of the strains isolated from maples hybridized with the probes. The

strains from the remaining plants were divided almost evenly between those that hybridized with the probes and those that did not. Notable exceptions again were highbush blueberry and southern magnolia, which had a high proportion of strains that did not hybridize with the probes.

Comparison of lilac bioassay and colony hybridizations with *syr* gene probes. Results of the lilac bioassay were in 78% agreement with results of the colony hybridizations. Fifty-nine percent of the strains were positive and 19% of the strains were negative by both methods (Fig. 1). Of the remaining 22% of strains, 9% were pathogenic in the bioassay but did not hybridize with the *syr* probes, and 13% were non-pathogenic in the bioassay but hybridized with the *syr* probes. A higher percentage of strains that matched the established physiological and nutritional phenotypes for *P. syringae* pv. *syringae* were pathogenic in the lilac bioassay (88%) and hybridized with the probes (93%) than did strains with alternate phenotypes. Results were obtained from the

lilac bioassay in 16 days, provided that lilac stock plantlets were available. Colony hybridizations took 5 days.

Predictive value of diagnostic tools.

Predictive values describing the likely meaning or correctness of test results were calculated for each of the diagnostic tools used in identifying strains pathogenic on lilac (Table 3). Predictive value of a positive result describes the fraction of positive results which are true positives, while the predictive value of a negative result describes the fraction of negative results which are true negatives (52). All diagnostic tools had a higher positive predictive value than negative predictive value, thus false positives were less frequent than false negatives. The *syr* probes had the highest predictive values for *P. syringae* pv. *syringae* pathogenic on lilac (82% positive, 67% negative). The LOPAT tests were second-highest (79% positive, 49% negative), followed by the GATTa tests and INA, which were equal (75% positive, 44% negative). The lowest predictive values were from fluorescent, gram-negative, oxidase negative bacteria (67% positive, 0% negative).

DISCUSSION

The lilac tissue culture bioassay and DNA colony hybridization with the *syrB* and *syrD* probes for syringomycin genes provide two methods for rapid evaluation of pathogenicity in strains of *P. syringae* pv. *syringae* isolated from woody plants. Lilac plantlets produced uniform disease symptoms and were readily maintained in culture. Because none of the strains hybridized with only one of the probes, both *syrB* and *syrD* appear to be essential to syringomycin production, and either *syr* probe could be used exclusively. There was 78% agreement between the results of the lilac bioassay and hybridization with the *syr* probes, lending confidence that the two methods give similar results.

The LOPAT and GATTa testing schemes were developed to separate *P. syringae* pv. *syringae* from other *Pseudomonas* species and *P. syringae* pathovars, but we found many strains that were pathogenic in the bioassay and hybridized with the *syr* probes that did not match the predicted results. If used exclusively, the LOPAT and GATTa tests would have underestimated the number of pathogens in our collection of strains by 30%.

These determinative schemes did reveal considerable phenotypic diversity in our collection of presumptive *P. syringae* pv. *syringae* strains. Seven percent of strains were oxidase negative, arginine dihydrolase active, and pathogenic on lilac. The only phytopathogenic, fluorescent pseudomonad reported to have arginine dihydrolase activity is *P. marginalis* (45); however, *P. marginalis* is oxidase positive (24). Most previous studies have automatically excluded strains with arginine dihydrolase activity as potential pathogens and some

Fig. 1. Comparison of results of pathogenicity testing of 552 *Pseudomonas syringae* pv. *syringae* strains from a lilac tissue culture bioassay and DNA colony hybridization with probes p91 (*syrB*) and p9 (*syrD*) for syringomycin genes. Pathogenic in lilac bioassay = Bio+, non-pathogenic in lilac bioassay = Bio-, positive in colony hybridizations with *syr* probes = CH+, negative in colony hybridizations with *syr* probes = CH-.

could have been overlooked. Tobacco hypersensitivity was once believed to be strictly correlated with pathogenicity, but HR-negative pathogens have been identified previously (1,6,16,22). In this study, 11% of the HR-negative strains were pathogenic on lilac and thus we conclude that this test should not be used exclusively to identify pathogens. None of our strains caused potato soft-rot, so this test was of no benefit in distinguishing between pathogens and non-pathogens.

INA is a common phenotype of pathogenic *P. syringae* pv. *syringae* (1,16,26,38) and in our study, INA was as well-correlated with pathogenicity as were the GATTa tests. INA could be used as a preliminary screen, but in prior studies it has not been a reliable estimator of pathogenicity in *P. syringae* pv. *syringae* isolated from woody plants. Only half of the INA *P. syringae* strains isolated by Gross et al. (16) from fruit trees were pathogenic on immature pear and sweet cherry fruit. Fifty-eight percent of the strains isolated by Baca et al. (1) from woody ornamental plants were INA, yet 69% were pathogenic on immature tomato fruit.

In previously studies, between 25 and 85% of the strains provisionally identified as *P. syringae* pv. *syringae* by physiological and biochemical tests were pathogenic in bioassays (1,5,6,10,16,22,23,28,30,37,38,41,46,49,50). In our study, the predictive values (positive or negative) of the LOPAT, GATTa, and INA tests for *P. syringae* pv. *syringae* pathogenic on lilac were between 44 and 79%. When these tests lead to false negative results, concluding that a strain is not pathogenic when it actually is, growers may decide that no chemical disease control measures are needed and experience higher losses. Conversely, if all fluorescent, oxidase-negative, gram-negative bacteria isolated from woody plants are assumed to be pathogenic *P. syringae* pv. *syringae*, growers may decide to apply chemical bactericides when their use is not warranted.

Our lilac bioassay directly identified pathogens. Using tissue-cultured lilacs eliminates many variables from pathogenicity testing. No competing microflora was detected on or in the lilac plantlets. The microclimate inside the tissue-culture jars was uniform and conducive to disease development. Previous pathogenicity bioassays on lilac required bacteria to be di-

rectly injected into the petioles (49). Briefly subjecting the inoculated plantlets to -5°C mimicked a spring frost event and provided consistent and uniform infection of leaves, petioles and stems.

The percentages of pathogens detected in previous bioassays were especially low when the bioassay host was different than the original host. Although it is common for *P. syringae* pv. *syringae* strains isolated from diseased tissues to be pathogenic on other hosts, they were usually not as virulent on these other hosts as on their original host (12,40,43). Eighty-eight percent of our strains isolated from lilacs were pathogenic in the bioassay. Of hosts from which at least five strains were tested, more than the average 67% of strains isolated from mock orange, golden bells, apricot, and sweet cherry were pathogenic on lilac. Fewer than 67% of the strains isolated from Japanese and Norway maples, highbush blueberry, sweet gum, southern magnolia, common pear, and Asian pear were pathogenic on lilac. The strains that were non-pathogenic on lilac might be epiphytes that are non-pathogens on any host, or else they could have a host range that does not include lilac, making it problematic to classify them as *P. syringae* pv. *syringae*.

P. syringae pv. *syringae* is the only bacterium known to produce syringomycin, and colony hybridization with the *syr* genes is a rapid and specific method to identify toxigenic strains. Seventy-two percent of all our strains hybridized with the *syrB* and *syrD* gene probes for syringomycin. This is fewer than the nearly 90% of *P. syringae* pv. *syringae* strains pathogenic on immature cherry fruit that hybridized with the *syr* probes reported by Quigley and Gross (36). Ninety-three percent of strains isolated from lilac hybridized with the probes, while other plant sources (for example, highbush blueberry and maples) yielded lower percentages of probe-positive strains. The large numbers of non-pathogenic, non-toxigenic strains isolated from diseased tissues that cannot be distinguished from pathogens by standard protocols is one of the confounding factors in diagnosing *Pseudomonas*-incited diseases of woody plants (1,6,28,29).

Although the probes for syringomycin had the highest predictive values for identifying pathogens of any of the diagnostic tools in this study, 9% of strains were pathogenic in the bioassay but did not hy-

bridize with the *syr* probes. These strains may produce a different necrotizing toxin, such as syringopeptin (2), or may not rely on toxin production for pathogenesis. One study with peach seedlings reported that 25% of the canker-causing *P. syringae* pv. *syringae* strains did not produce syringomycin (34). Mutants rendered defective in syringomycin production were still able to cause small necrotic lesions on immature cherry fruits with an estimated 50% decrease in virulence (32,47). Thus syringomycin appears to be an important virulence factor, but not an obligate requirement, for pathogenicity in some assays and for some strains. The high proportion of syringomycin-producing strains in natural populations suggests that there is strong selective pressure for toxin production (15).

Thirteen percent of our strains were not pathogenic in the bioassay but hybridized with the probes for syringomycin. Non-pathogenic, syringomycin-producing strains of *P. syringae* pv. *syringae* have been isolated from maize (17) and from pome and stone fruit trees (16). Quigley and Gross (36) speculated that toxin production in these strains might be an altered phenotype selected through extensive laboratory culture. *SyrB* and *syrD* sequences were found in eight strains of *P. syringae* pv. *syringae* that were non-pathogenic on immature cherry fruit, most of which produced only low levels of syringomycin when grown on selected media. Strains that produce low levels of syringomycin would be probe-positive, even if they were very weakly pathogenic and unable to parasitize the lilac plantlets in our bioassay.

The majority of our strains, isolated from a wide variety of woody plants, were pathogenic on lilac and hybridized with the probes for syringomycin, allowing for rapid evaluation of pathogenicity in strains provisionally identified as *P. syringae* pv. *syringae*. Digoxigenin-labeling produced relatively inexpensive, stable, and sensitive *syr* probes that could be used in diagnostic labs without facilities for handling radioactivity. For routine diagnosis, a large number of suspected pathogens could be tested very rapidly for syringomycin genes by colony hybridization, and a smaller subset of those strains could be tested for pathogenicity on lilac. The syringomycin probes should be tested in squash or dot-blot hybridizations for their capacity to identify pathogenic strains of *P. syringae* pv. *syringae* in plant tissue. If successful, growers could submit plant samples, receive a diagnosis, and commence disease management practices in less than a week.

ACKNOWLEDGMENTS

This project was supported in part by the Oregon Association of Nurseriesmen and the Center for Applied Agricultural Research. We thank Dr. D. C. Gross for providing the probes for syringomycin and reviewing the manuscript, Dr. V. O. Stockwell for reviewing the manuscript, and Dr. J. M. Young for helpful comments.

Table 3. Predictive values of diagnostic tools for *Pseudomonas syringae* pv. *syringae* pathogenic on tissue-cultured lilacs

Diagnostic tool	Positive predictive value (%)	Negative predictive value (%)
Gram-negative, oxidase negative bacterium, fluorescent of KB	67	0
LOPAT _(HR)	79	49
GA _(e) TTa	75	44
Ice nucleation active	75	44
Colony hybridization with <i>syr</i> probes	82	67

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