A Semiselective Medium for Detecting Epiphytic and Systemic Populations of *Pseudomonas savastanoi* from Oleander

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ABSTRACT

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A new medium, oleander knot agar (OKA), was developed for isolation of *Pseudomonas savastanoi* from oleander plants. OKA contained (in grams per liter) agar (14.0), L-serine (2.0), yeast extract (1.0), $NH_4H_2PO_4$ (0.5), $K_2HPO_4 \cdot 3H_2O$ (0.5), NaCl (5.0), $MgSO_4 \cdot 7H_2O$ (0.2), boric acid (1.0), vancomycin (0.15), cephalexin (0.05), bacitracin (0.05), ampicillin (0.015), novobiocin (0.02), and cycloheximide (100). All 39 strains of *P. savastanoi* from different geographical areas grew on OKA, and the quantitative recovery ranged from 5.7 to 93.6% (mean = 43.6%). OKA was more selective than other media tested, including BCBRVB, M-71, KBBC, MSP, proline agar, and PVF-1, and inhibited 89 to 96.7% of saprophytic bacterial strains recovered from oleander tissues. Forty-four percent of other plant-pathogenic bacteria tested did not grow on OKA, and 13% grew sparingly. OKA was used to detect epiphytic populations of *P. savastanoi* from galls, leaves, stems, and flowers and to detect systemic movement of the bacterium in oleander stems. The pathogen was present in wash water from 26 of 34 apparently healthy oleander plants. Thirty-one of these plants subsequently developed galls within 1 year. The bacterium was also capable of systemic movement up to 25 cm above and 20 cm below the site of inoculation.

Pseudomonas savastanoi induces tumorlike knots or galls on oleander (*Nerium oleander* L.), olive (*Olea europaea* L.), ash (*Fraxinus excelsior* L.), privet (*Ligustrum japonicum*), jasmine (*Jasminum* spp.), and a few other plants (1,2,5,6,13,14,16). Oleander knot disease causes significant losses at the nursery level because of regulations against shipment of infected plants. It is difficult to visually assess whether cuttings or mother block plants are infested with this pathogen because it can exist on the surface and, apparently, in the vascular system of the host plant without necessarily causing disease symptoms. If the pathogen is systemic, then attempts to control the disease by surface disinfestation of cuttings would not be effective. The key to control of the disease in nursery stock is, therefore, early detection in source plants and cuttings. However, prevention of the disease through the use of pathogen-free propagation materials is currently difficult because of inadequate methods of detecting low levels of bacteria in plant materials. Recovery of *P. savastanoi* on nonselective media is difficult, especially when its population is very low, because the bacterial colonies do not have a distinctive morphology.

Several semiselective media have been described for the isolation of pseudomonads from soil or plant materials. We tested a number of these media, including Difco pseudomonas isolation medium (8), BCBRVB (4), M-71 (9), KBBC (10), MSP (10), and proline agar (11), and found them unsuitable for supporting the growth of *P. savastanoi* strains or eliminating some of the other bacteria, especially those that share the same ecological niche with *P. savastanoi.* Changes made in the composition of these media by increasing or reducing the concentration of some of the key components, such as carbon source or antibiotics, did not improve their suitability. The PVF-1 medium developed for the isolation of *P. savastanoi* from olive tissue (15) was also unsatisfactory for the selective isolation of *P. savastanoi* from the oleander phylloplane because of the presence of other bacteria that occur in the same habitat and grow on this medium.

The purpose of this study was to develop and evaluate a new medium for isolation of *P. savastanoi* from naturally infested plant tissue and to use this medium in determining the systemic nature of the pathogen.

MATERIALS AND METHODS

Bacterial strains. Cultures of *P. savastanoi* included 36 strains isolated from oleander in this study, four strains from oleander (0185-8, 0693-10, 0693-11, and 1293-1) isolated previously by our laboratory, and oleander strains ATCC 13526, NCPPB 639, (National Collection of Plant Pathogenic Bacteria, Harpenden, England), and UCD B-62 (D. Gilchrist, University of California, Davis). Strains of other species and pathovars used in the study are listed in Table 1. Cultures were stored in 40% glycerol at −70°C or on plates of mannitol-glutamate-yeast extract agar (MGY) (7) or yeast extract-dextrose-calcium carbonate agar (YDC) (17) at 4°C for routine use.

Development of a semiselective agar medium. The basal medium was the salt solution of medium C of Dye (3) containing (in grams per liter) $MgSO_4 \cdot 7H_2O$ (0.2), $NH_4H_2PO_4$ (0.5), $K_2HPO_4 \cdot 3H_2O$ (0.5), and NaCl (5.0) supplemented with 0.1% yeast extract and 1.4% agar. A number of amino acids and sugars, three to six carbon alcohols and acids, Tween 20, and Tween 80 were tested as carbon sources at 2 g per liter. All carbon sources and inhibitory compounds were prepared as 10× stock solutions, filter sterilized (Millipore, 0.22 μm), and aseptically added, individually or in combination, to the autoclaved test medium before the plates were poured. Two strains of *P. savastanoi* (0185-8 and 0485-9), 20 representative strains of different bacterial genera

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(indicated with an asterisk in Table 1), and four unknown bacterial strains associated with oleander tissue were used for initial screening. In addition, the bacteria were screened for utilization of 95 carbon sources on Biolog GN microplates (Biolog, Hayward, CA).

After an appropriate carbon source had been determined, several inhibitory compounds were added to the medium at different concentrations to determine their ability to inhibit microbial contaminants associated with oleander tissue without reducing the recovery of strains of *P. savastanoi*.

Other media used for comparison were MG (7), MGY, PVF-1, and OKAm (OKA without antibiotics). All inoculated media were incubated at 28°C for 1 to 7 days before growth was recorded.

Isolation of *P. savastanoi* on OKA. Thirty-nine samples of oleander tissue (leaves, twigs, and flowers) with galls were collected from different geographical locations in southern California. The tissues were cut with a razor blade at a distance of 0.5 to 1.0 cm from the galls, and the oozing sap was either plated directly by touching the cut surfaces onto agar plates or collected in a Microfuge tube containing 0.5 ml of sterile distilled water. Galls were then excised from each sample and macerated in 0.5 to 1.0 ml of sterile distilled water. The macerates were kept at 4°C for 30 min. Serial dilutions were made from the gall macerates and the sap solutions, and 100-µl aliquots of each dilution were spread onto three plates each of MGY, PVF-1, OKA, and OKAm. All plates were incubated at 28°C for 2 to 7 days. Colonies morphologically similar to *P. savastanoi* were picked and further purified by streaking them onto MGY plates. The pathogenicity of 36 isolates recovered from the 39 plant samples was confirmed by inoculation of oleander plants. Each strain was also tested on Biolog GN microplates with known *P. savastanoi* isolates to determine the reliability of the Biolog system for rapid confirmation of the identity of strains isolated on OKA.

Relative plating efficiencies. Growth and recovery of *P. savastanoi* strains on the final OKA medium were determined and compared with those on a nonselective medium (MGY) by the dilution plate technique (12,18). The colonies on each plate were counted after 1 to 7 days of incubation at 28°C, and the percent recovery was calculated as the number of colony-forming units recovered on OKA × 100 divided by the number of colony-forming units recovered on MGY. OKAm and PVF-1 were used for comparison.

Evaluation of the selectivity of OKA. The efficiency of the OKA medium in inhibiting 89 strains of saprophytic and plant-pathogenic bacteria (Table 1) was determined by spotting 10 µl of a cell suspension of each strain on OKA plates and recording growth after 1 to 7 days at 28°C. Cell suspensions for each strain were prepared by suspending five 48-h-old colonies from MGY plates in 0.5 ml of sterile distilled water. MGY and PVF-1 were used for comparison.

Further evaluation of the selectivity of OKA was done in two tests with mixtures of *P. savastanoi* and other bacteria. In the first test, the 20 strains used for initial screening of selective media (indicated with an asterisk in Table 1) and three strains of *P. savastanoi* (0185-8, 0693-11, and UCD B-62) were individually streaked on MGY plates and incubated at 28°C for 48 h. Five single colonies of each strain were suspended in 0.5 ml of sterile distilled water, and 100 µl of each of the 20 strains were mixed together. This 2-ml mixture of bacteria was divided into three

TABLE 1. Growth of different bacteria on oleander knot agar (OKA) and PVF-1

Bacterial strain[a] Pathovars	PVF-1	OKA
Pseudomonas syringae		
aptata (NCPPB 871, CFBP 2042), *atropurpurea* (NCPPB 2397), 'cilantro' (0788-9, 0788-10, 0788-11), *coronafaciens* (NCPPB 600), *dysoxyli* (NCPPB 225), *glycinea* (NCPPB 2411), 'hibisci' (CFBP 2895), *japonica* (NCPPB 3093), *lachrymans* (1188-1), *morsprunorum* (NCPPB 560, NCPPB 2995, CFBP 2115, NCPPB 2724), *maculicola* (0788-18, 0888-2, 0888-3, 0190-2), *mori* (NCPPB 1413), 'oryzae' (NCPPB 3683), *panici* (NCPPB1 498), *papulans* (NCPPB 2848), *phaseolicola* (0285-1), *pisi* (NCPPB 2585), 'porri' (CFBP 2360, CFBP 1912), *sesami* (NCPPB 1016), *syringae* (0584-6*, 0485-10*), *tabaci* (NCPPB 1427), *theae* (NCPPB 2598), *tomato* (0893-14, 0893-15), *zizaniae* (CFBP 11040)	+	+
aptata (CFBP 2134), *atrofaciens* (NCPPB 2612), 'cilantro' (0790-2, 0790-3, 0788-14), *eriobotryae* (NCPPB 2331), *garcae* (NCPPB 2710), *helianthi* (CFBP 2149), 'porri' (NCPPB 3364), *tomato* (0683-23), *viburni* (NCPPB 1921)	+	Weak growth
aesculi (CFBP 2894), *apii* (0988-1, 0988-2, 0690-5), 'avallanae' (CFBP 10963), *berberidis* (NCPPB 2724), *ciccaronei* (NCPPB 2355), 'dendropanacis' (NCPPB 3464), *helianthi* (NCPPB 2640, CFBP 2043), *mellea* (NCPPB 2356), *mori* (NCPPB 1034), 'myricae' (NCPPB 3143), *persicae* (NCPPB 2761), *phaseolicola* (G-50[b]), 'philadelphi' (NCPPB 3257), 'photiniae' (CFBP 2899), *syringae* (W-1b[c]*), *tabaci* (0791-14[d]), *tagetis* (NCPPB 2488)	+	–
P. fluorescens (0292-1*)	+	+
P. marginalis (1192-7*)	+	+
P. putida (1192-6*)	+	+
P. corrugta (0692-2*)	+	–
Agrobacterium tumefaciens (C-58[c]*)	+	–
Escherichia coli (0683-1*)	+	–
Erwinia carotovora (0692-10*)	+	–
E. herbicola (0692-1*)	+	–
E. rhapantici (0788-13*)	+	–
E. amylovora (0692-3*)	–	–
Bacillus spp. (1192-8*)	–	–
Xanthomonas campestris		
alfalfae (1089-2), *campestris* (0186-1*, B-24[c]*), *phaseoli* (1282-9), *dieffenbachiae* (B-400[c]), *translucens* (0683-4*, B430[c]*, B433[c]), *vesicatoria* (5619-3[f]*, 0692-5[g]*)	–	–

[a] NCPPB = National Collection of Plant Pathogenic Bacteria, Harpenden, England, and CFBP = Collection Francaise de Bacteries Phytopathogenes, Institut National de la Recherche Agronomique, Angers, France. All cultures with a numerical designation only are from the collection of D. A. Cooksey. * = Culture was used in initial screening for medium OKA.

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parts, and to 90 µl of each part, 10 µl of a cell suspension of one strain of *P. savastanoi* was added (9:1 ratio). The final cell mixtures were serially diluted and plated in triplicate on MGY, OKA, OKAm, and PVF-1 agar plates with a sterile glass rod. In the second test, mixtures of 19 unidentified bacteria isolated from oleander tissue in association with *P. savastanoi* were mixed with cells of *P. savastanoi* in an approximate 9:1 ratio and plated as described above. The plates were incubated at 28°C for 2 to 7 days, and then the types and numbers of bacteria were recorded. Biolog GN microplates were also used for bacterial identification.

Detection of epiphytic *P. savastanoi*. Two groups of plants were selected for this study. Plants in group one were 5 to 10 years old and located at the University of California, Riverside. Plants in group two were 1 to 2 years old and were received from several nurseries in southern California. The second group was kept on a greenhouse bench in natural light at average temperatures of 30°C during the day and 20°C at night. Three types of samples were collected from each group: i) healthy leaves from plants without disease symptoms; ii) healthy leaves from naturally infected plants with galls on leaves, twigs, and flowers; and iii) diseased leaves (with at least one gall) from diseased plants. Each sample weighed 5 g and consisted of 10 to 15 leaves. Leaves were added to 25 ml of sterile saline (0.85% NaCl) containing two drops of Tween 20 in a 100-ml Erlenmeyer flask. Flasks were kept at 4°C for 3 h with occasional shaking. One milliliter was removed from each flask and serially diluted to 10^{-6} in saline. Aliquots (100 µl) of each dilution were plated in triplicate onto OKA. Internode segments of twigs, 1 to 2 cm long (15 segments per flask), were also tested in the same manner as the leaves. Other media used for comparison included YDC, MGY, OKAm, and PVF-1. All agar plates were incubated at 28°C for 1 to 7 days. Colonies that were tentatively considered to be *P. savastanoi* on the basis of their morphology were restreaked on MGY and further tested by Gram staining, oxidase production, and the Biolog test. The percentage of reduction in epiphytic bacteria was calculated as 100 – (number of colony-forming units recovered on test media × 100/number of colony-forming units recovered on MGY). The healthy plants were observed for 1 year from the date of sampling for the development of galls.

Infection and systemic movement of *P. savastanoi*. Two strains of *P. savastanoi* (0185-8 and 0693-11) were used in this study. Spontaneous rifampicin- and nalidixic acid-resistant mutants were obtained. For inoculation, the mutants were grown on MGY agar in the presence of both antibiotics for 2 days. Cell suspensions were prepared in sterile distilled water and adjusted to 10^6 CFU/ml. All plants were 1 to 2 years old. One group of plants was sprayed with the bacterial suspensions to run off. Another group of plants was inoculated by applying the 2-day-old cultures directly to wounds made in the bark of stems or the epidermis of leaves with an 18-gauge needle. Inoculation sites were either 5 to 10 cm above the soil level or at the upper third of the plants. Water inoculations were used as controls. Inoculated plants in each group were further divided into two subgroups. One subgroup was held on a greenhouse bench in natural light at 20 to 30°C and 70 to 80% relative humidity. The other subgroup was placed on a mist bench in the same greenhouse and misted for 5 s every 15 min. Plants were observed for the development of symptoms for up to 90 days after inoculation.

To test epiphytic survival of the bacteria, 5-g samples of leaves and twigs were collected, washed, and plated as described earlier. To confirm that the inoculated bacteria were the cause of gall formation, the galls were macerated and plated as before. To study the systemic movement of bacteria, branches or whole stems from the inoculated plants were removed and surface sterilized with 95% ethanol. They were then cut at 5-cm intervals, and the oozing sap was directly plated onto MGY and OKA supplemented with 50 µg of both rifampicin and nalidixic acid per milliliter.

RESULTS

Development of OKA. Strains of *P. savastanoi* either did not grow at all or grew poorly on the basal medium C of Dye with any carbon source unless it was supplemented with at least 0.1% yeast extract. When Casamino Acid was substituted for yeast extract, growth of *P. savastanoi* strains either did not improve or was noticeably reduced. Addition of a few biological dyes, including bromthymol blue, bromcresol purple, methylene blue, crystal violet, malachite green, neutral red, and phenol red at rates of 0.001 to 0.1 g per liter of the medium, did not give any unique and identifiable characteristics to colonies of *P. savastanoi*. Among all the compounds used as carbon sources, L-serine was found to be the most suitable. Of all the antibiotics tested, the combination of vancomycin, cephalexin, bacitracin, ampicillin, novobiocin, and boric acid at 150, 50, 50, 15, 20, and 1,000 g/ml of medium, respectively, was the best in supporting maximum growth of *P. savastanoi* strains but preventing or reducing the growth of the maximum number of other bacteria. Later experiments have suggested that sodium dodecyl sulfate (SDS) at 0.04% can be substituted for bacitracin, ampicillin, novobiocin, and cycloheximide without any noticeable change in the selectivity of the medium (*unpublished*). For the experiments described, however, the original formulation containing those antibiotics was used. OKA therefore consisted (in grams per liter) of agar (14.0), L-serine (2.0), yeast extract (1.0), $NH_4H_2PO_4$ (0.5), $K_2HPO_4 \cdot 3H_2O$ (0.5), NaCl (5.0), $MgSO_4 \cdot 7H_2O$ (0.2), boric acid (1.0), vancomycin (0.15), cephalexin (0.05), bacitracin (0.05), ampicillin (0.015), novobiocin (0.02), and cycloheximide (100).

All strains of *P. savastanoi* grew slowly on OKA medium. Colonies were visible after 3 to 4 days of incubation at 28°C. After 6 to 7 days, colonies were circular, smooth, milky white, slightly raised, and convex with entire margins (Fig. 1). Most strains produced colonies of two sizes, 0.5 to 1 and 2 to 3 mm in diameter, which occurred on the same plate (Fig. 2). The smaller colonies were more prevalent than the larger ones. More uniform colonies were later obtained on the modified OKA formulation with SDS (*unpublished*). All strains showed either no fluorescence or pale blue fluorescence under UV light after 6 to 7 days of incubation.

Isolation of *P. savastanoi* on OKA. *P. savastanoi* was recovered on OKA from almost all of the 39 diseased oleander samples in the first attempt. Isolation of *P. savastanoi* on OKAm or MGY was more difficult because of the presence of high numbers of saprophytic bacteria (Fig. 1) and had to be repeated two to six times with the same or different galls. Colonies of *P. savastanoi* on OKA were generally easy to count at a dilution of 10^{-1} because of the presence of few saprophytes. The identification of *P. savastanoi* was facilitated by the use of Biolog GN microplates. Five known isolates of *P. savastanoi* (ATCC 13526, UCD B-26, 0693-10, 0693-11, and 1293-1) and all 36 strains isolated in the present study were identified as *P. syringae* pv. *nerii* by the Biolog database, which uses that designation for oleander strains of *P. savastanoi*. Two other known strains of *P. savastanoi* were identified as *P. syringae* pv. *morsprunorum* (0185-8) and *P. syringae* pv. *tomato* (NCPPB 639). The similarity of all the strains to *P. syringae* pv. *nerii* in the Biolog database ranged from 43 to 78.2%.

Plant sap, when expressed from freshly cut tissues, gelled and quickly became practically insoluble in water. No bacteria were recovered from serial dilutions of the gelatinous material. On the other hand, different kinds of bacteria grew from the gelatinous sap when it was plated directly on MGY and OKAm and to some extent on PVF-1, but not on OKA. Of the 39 diseased oleander tissues originally sampled, *P. savastanoi* was recovered from only two sap samples plated on OKA, OKAm, and PVF-1.

Relative plating efficiencies. Recovery of the 43 strains of *P. savastanoi* on OKA was variable, ranging from 5.7 to 93.6%.

Plating efficiencies were increased significantly by elimination of the antibiotics from the medium (OKAm). This increased recovery ranged from 52.6 to 140.0%. The recovery on PVF-1 was somewhat higher and less variable than that on OKA, and its range was 11.3 to 83.3%. No correlation was observed between the recovery of the strains on OKA and PVF-1; a particular strain with a high recovery rate on OKA did not necessarily have a high recovery rate on PVF-1 and vice versa.

Evaluation of the selectivity of OKA. All bacterial strains grew on MGY as expected. The colony size and mass of bacteria that grew on PVF-1 were comparable to those on MGY but were greater than those on OKA. The rate of growth on PVF-1 was also faster than that on OKA. Of 89 strains tested, 39 (44%) did not grow on OKA and 11 (13%) grew only sparingly (Table 1). In contrast, 12 strains (14%) did not grow on PVF-1.

In the test in which *P. savastanoi* was mixed with 20 bacterial strains (indicated with an asterisk in Table 1), *P. savastanoi* was not detected on the plates of OKAm or MGY at any of the dilutions tested (0 to 10^{-7}) because of the profuse growth of the other bacteria. On PVF-1, dilutions of 0 to 10^{-5} also produced a lawn of growth, and at dilutions of 10^{-6} and 10^{-7}, no colonies of *P. savastanoi* were detected, even though other bacterial colonies were countable. On OKA plates, no bacteria grew at dilutions of 10^{-6} and 10^{-7}. At dilutions of 0 to 10^{-5}, two types of colonies were detected: the oxidase-positive, fast-growing, large colonies with pronounced fluorescent pigmentation were identified as *P.*

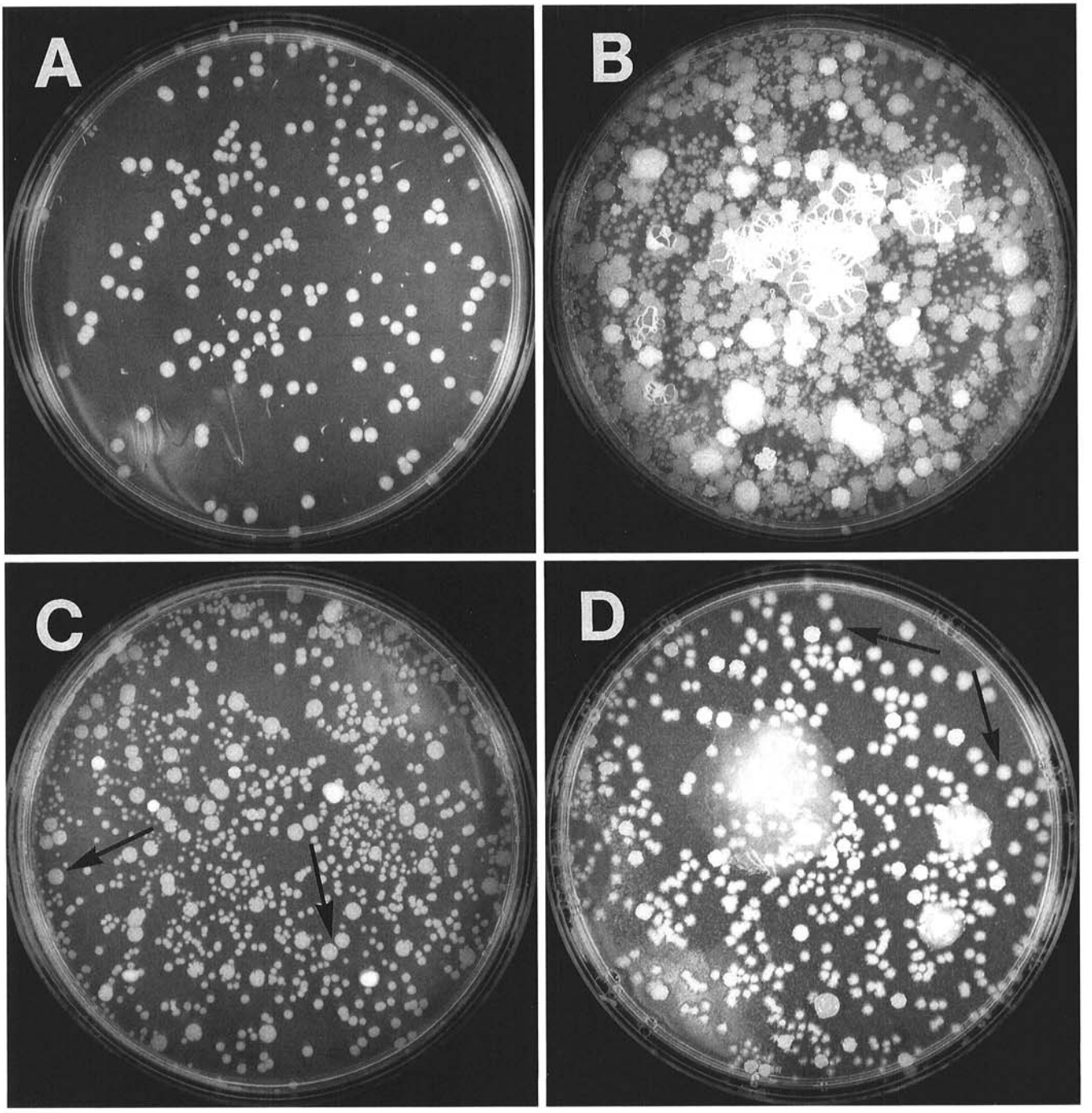

Fig. 1. Inhibition of growth of microorganisms from oleander tissues on **A,** oleander knot agar (OKA); **B,** mannitol-glutamate-yeast extract agar; **C,** OKA without antibiotics; and **D,** PVF-1. Bacteria are from wash water from naturally infected leaves plated at a dilution of 10^{-3} and incubated at 28°C for 3 (**B and D**) or 7 (**A and C**) days. Observed colonies of *Pseudomonas savastanoi* are indicated with arrows. *P. savastanoi* cells grew on OKA (**A**) in almost pure culture.

fluorescens or *P. putida*, and the colonies that grew slowly, were oxidase negative, and did not produce any pigment were *P. savastanoi*. In the second test, in which 19 unidentified bacteria from oleander plants were mixed with *P. savastanoi* strains, the results for MGY, OKAm, and PVF-1 were similar to those presented above. The inhibition of other bacteria on OKA was 89%. In addition to *P. savastanoi*, three other bacteria grew on OKA plates and could be easily identified on the basis of their Gram stains, oxidase reactions, motility, flagellation, and pigmentation. Two of these bacteria were tentatively identified as *Pseudomonas* spp., and the third was a gram-positive bacterium.

Detection of epiphytic *P. savastanoi*. The total number and types of epiphytic bacteria recovered on OKA were generally higher in samples taken from outdoor plants than in those taken from plants in the greenhouse. Similarly, more bacteria and bacterial types were recovered from diseased samples than from healthy samples. In addition to *P. savastanoi*, 19 other bacteria were recovered from all samples; eight of these were identified as pseudomonads. *P. savastanoi* was recovered from diseased samples on all media; however, the percent recovery was higher in samples from the greenhouse because of a lower number of saprophytic bacteria. The recovery of *P. savastanoi* from healthy samples was variable. In healthy tissues from diseased plants, the bacterium was recovered on OKA. Recovery percentages on other media were as follows: MGY, 10%; YDC, 10%; OKAm, 30%; and PVF-1, 50%. The most diverse results were obtained with samples collected from healthy plants. Of the 34 samples in this group, *P. savastanoi* was recovered from 3, 2, 7, 12, and 26 samples on MGY, YDC, OKAm, PVF-1, and OKA, respectively. The overall reduction in the number of saprophytes on OKAm, PVF-1, and OKA was 62, 71, and 96.7%, respectively. The inhibitory effect of OKA was greater than that observed in earlier experiments with mixed cultures. One year after the sampling date, 31 of the 34 apparently healthy plants had developed one or more galls. Even though the results of the tests with twig samples were similar to those observed for leaf samples, the number of bacteria recovered from twigs was almost 30% less than that from leaves.

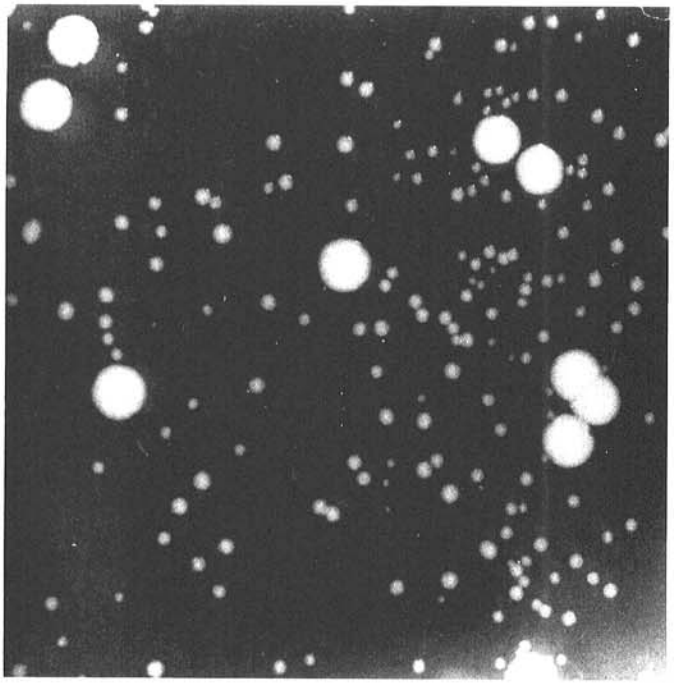

Fig. 2. Two sizes of *Pseudomonas savastanoi* colonies on oleander knot agar after 7 days of incubation at 28°C.

Infection and systemic movement of *P. savastanoi*. Of the two strains used in these experiments, *P. savastanoi* strain 0693-11 was more virulent than strain 0185-8. When plants were spray inoculated with strain 0693-11, galls 2 to 10 mm in diameter were formed on leaves and flower pods, mostly on the upper half of 67% of the plants. Galls were seldom seen on the lower half of the canopy or on the stems. Strain 0185-8 failed to cause any infection in spray inoculations.

In wound inoculations, gall formation was greater on new growth and younger tissues. Inoculations at the bases of the plant stems resulted in gall formation only for strain 0693-11, and the galls were restricted to the inoculation sites. In inoculations made on stems in the upper parts of the plants with strain 0693-11, galls formed at inoculation sites and up to 25 cm above and 20 cm below these sites. Vascular discoloration originating from the inoculation sites and extending 5 to 6 cm away from these sites was common. Strain 0185-8 produced 60% fewer and smaller (1 to 3 mm) galls than strain 0693-11 when inoculated in the upper parts of plants. Some galls also formed 2 to 4 cm above and below sites inoculated with strain 0185-8. Direct plating of sap from surface-sterilized stem sections indicated that strain 0693-11 moved within plants up to 25 cm above and 20 cm below its entry point, but strain 0185-8 was not recovered from stem sections above or below inoculation sites. Strain 0185-8 was recovered from all gall macerates, leaf washings, and direct platings of sap taken at inoculation sites in stems and leaves, indicating that it had survived in and on oleander plants but was not as virulent as strain 0693-11. Galls also formed on many leaves located above inoculation sites but not on leaves located below them for strain 0693-11. Almost all inoculations of strain 0693-11 made on leaves resulted in gall formation.

Plants maintained on the mist bench had fewer galls than plants in the other groups, but the galls were two to four times larger. In plants inoculated with strain 0693-11, galls were formed up to 10 cm above and 5 cm below inoculation points, and *P. savastanoi* was recovered from the sap up to 13 cm above and 15 cm below the inoculation points.

DISCUSSION

OKA is a medium that allows the recovery of *P. savastanoi* from different sources and from mixtures with other bacteria. PVF-1 agar was developed particularly for the isolation of *P. savastanoi* from olive, but we found it inferior to OKA for isolation from oleander. Even OKAm had better selectivity, in most cases, than PVF-1 and was more suitable than general media such as MGY and YDC for isolation of *P. savastanoi*. Surico and Lavermicocca (15) found that a relatively high number of yellow gram-negative bacteria could grow on PVF-1. We confirmed this finding and found that these fast-growing bacteria reduced the suitability of PVF-1 for isolation of *P. savastanoi*. OKA generally inhibited growth of 89 to 96.7% of the twig-, leaf-, and flower-associated saprophytic bacteria, depending on sample source. This variability is probably the result of environmental conditions and the physiological state of plants that affect the number and types of microorganisms in or on a particular plant tissue at a given time. Because of the elimination of such high percentages of saprophytic bacteria, gall macerates and wash liquid from oleander tissues can be concentrated before plating on OKA to detect very low levels of *P. savastanoi* in those tissues.

Surico and Lavermicocca (15) suggested that fairly young tissues or galls be used for the isolation of *P. savastanoi* because the presence of many different contaminants and a low viable population of the pathogen in the diseased tissues from old, cracked galls often made isolations very difficult. Our work agrees with these findings and furthermore shows that OKA is superior to PVF-1 when dealing with old galls because many saprophytes are eliminated on OKA.

One limitation reported in the use of PVF-1 is its relative toxicity toward some strains of *P. savastanoi*, preventing their growth (15). Although we did not observe a total elimination of any of the strains on OKA, we believe that it also has limitations. For example, colonies of *P. savastanoi* on OKA were of two sizes. There may have been a low level of toxicity of the antibiotics exerted upon some of the *P. savastanoi* cells, reducing their rate of growth and resulting in smaller colonies. The wide range in percent recovery of all the strains on OKA (5.7 to 97.6%) supports this suggestion and further indicates that this toxicity may result in the death of some of the cells, thus reducing the percent recovery. In work subsequent to that reported here, we used a modified formulation of OKA on which colonies of *P. savastanoi* were of more uniform size. In the modified OKA formulation, SDS was added at 0.04% and bacitracin, ampicillin, novobiocin, and cycloheximide were omitted without any noticeable change in the selectivity of the medium (*unpublished*).

A wide range of variability (43 to 78.2%) was observed among *P. savastanoi* strains in the Biolog test. Since Biolog microplates consist of 96 wells, each with a different compound as a carbon source, this variability can be attributed to the existence of nutritional diversity among strains of *P. savastanoi*. Strains were variable in the utilization of D- and L-alanine, L-asparagine, L-histidine, L-leucine, alaninamide, glucuronamide, propionic acid, and glycyl-L-glutamic acid. In spite of this variability, the Biolog assay was useful as a rapid confirmation of the identity of most strains of *P. savastanoi* recovered from oleander. Most strains were identified as *P. syringae* pv. *nerii*, which is the designation for oleander strains of *P. savastanoi* used in the Biolog database.

In conclusion, OKA is simple, easy to prepare, and inexpensive. We have stored OKA agar plates for at least 3 months at 4°C without a noticeable loss of selective properties. Therefore, it is suitable for use in detection, isolation, and epidemiological studies of *P. savastanoi* and detection of epiphytic and systemic infections for screening of propagative materials.

LITERATURE CITED

1. Bottalico, A., and Ercolani, G. L. 1971. *Pseudomonas savastanoi* (E. F. Smith) Stevens su ligustro giapponese in pugalia. Phytopathol. Mediterr. 10:132-135.
2. Brown, N. A. 1932. Canker of ash trees produced by a variety of the olive tubercule organism, *Bacterium savastanoi*. J. Agric. Res. 44:701-722.
3. Dye, D. W. 1968. A taxonomic study of the genus *Erwinia*. I. The "Amylovora" group. N.Z. J. Sci. 11:590-607.
4. Hildebrand, D. C, Schroth, M. N, and Sands, D. C. 1988. Gram-negative bacteria, *Pseudomonas*. Pages 60-80 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd ed. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN.
5. Janse, J. D. 1981. The bacterial disease of ash (*Fraxinus excelsior*) caused by *Pseudomonas syringae* subsp. *savastanoi* pv. *fraxini*. II. Etiology and taxonomic considerations. Eur. J. For. Pathol. 11:425-438.
6. Janse, J. D. 1982. *Pseudomonas syringae* subsp. *savastanoi* (ex Smith) subsp. nov., nom. rev., the bacterium causing excrescences on Oleaceae and *Nerium oleander* L. Int. J. Syst. Bacteriol. 32:166-169.
7. Keane, P. J., Kerr, A., and New, P. B. 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23:585-595.
8. Lane, A. L. 1984. Page 711 in: Difco Manual, 10th ed. Difco Laboratories, Detroit, MI.
9. Leben, C. 1972. The development of a selective medium for *Pseudomonas glycinea*. Phytopathology 62:674-676.
10. Mohan, S. K., and Schaad, N. W. 1987. An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola* in contaminated bean seed. Phytopathology 77:1390-1395.
11. Moustafa, F. A., Clark, G. A., and Whittenbury, R. 1970. Two partially selective media; one for *Pseudomonas morsprunorum*, *Pseudomonas syringae*, *Pseudomonas phaseolicola*, and *Pseudomonas tabaci*, and one for *Agrobacteria*. Phytopathol. Z. 67:342-344.
12. Schaad, N. W., and Forster, R. L. 1985. A semiselective agar medium for isolating *Xanthomonas campestris* pv. *translucens* from wheat seeds. Phytopathology 75:260-263.
13. Skerman, V. B. D., McGowan, V., and Sneath, P. H. A., eds. 1989. Approved Lists of Bacterial Names, amended ed. American Society for Microbiology, Washington, DC.
14. Smith, C. O. 1922. Pathogenicity of the olive knot organism on host related to the olive. (Abstr.) Phytopathology 12:106.
15. Surico, G., and Lavermicocca, P. 1989. A semiselective medium for the isolation of *Pseudomonas syringae* pv. *savastanoi*. Phytopathology 79:185-190.
16. Wilson, E. E., and Magie, A. R. 1963. Physiological, serological, and pathological evidence that *Pseudomonas tonelliana* is identical with *Pseudomonas savastanoi*. Phytopathology 53:653-659.
17. Wilson, E. E., Zeitoun, F. M., and Fredrickson, D. L. 1967. Bacterial phloem canker, a new disease of Persian walnut trees. Phytopathology 57:618-621.
18. Vidaver, A. K., and Davis, M. J. 1988. Gram-positive bacteria. Pages 104-113 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd ed. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN.