Selective Isolation of *Pseudomonas cichorii* from Soil and from Leaves and Buds of *Dendranthema grandiflora*

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ABSTRACT

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Two semiselective media were developed for ecological studies on *Pseudomonas cichorii*. The first is PCM-1, a defined medium that contains fungal and bacterial inhibitors and L(+)-tartrate as the carbon source. Most nontarget microbes in soil and plants are sensitive to the inhibitors and cannot utilize L(+)-tartrate. The second, PCM-2, is a modification of King's medium B. It too contains antibiotics and L(+)-tartrate but also contains peptone. In a recovery efficiency study with 12 strains of *P. cichorii*, recovery on the two semiselective media ranged from 80 to 118% of that on King's medium B. Selective media developed for other fluorescent pseudomonads were less efficient than PCM-1 and PCM-2 for recovering *P. cichorii* and suppressing nontarget microbes. *P. cichorii* was isolated from 11 of 41 samples of healthy-appearing chrysanthemum buds at populations ranging from 1.0×10^3 to 1.1×10^5 cfu per 25 buds. Over a 20-wk period, *P. cichorii* was detected on 12, 19, and 27% of symptomless leaves of chrysanthemum cultivars Bright Golden Ann, Iceberg, and Mountain Peak, respectively.

Bacterial leaf spot of ornamental and vegetable crops incited by *Pseudomonas cichorii* (Swingle) Stapp may be destructive in Florida and in other areas where temperatures and rainfall are high (9). On florist's chrysanthemum (*Dendranthema grandiflora* Tzvelev., formerly *Chrysanthemum* × *morifolium* Ramat.), *P. cichorii* is responsible for a leaf spot (12), stem necrosis (7), and bud blight (12) and has caused significant losses to the cut-flower industry in Florida. The bacterium causes a leaf spot and flower blight of *Pelargonium* (4) and a leaf spot on *Gerbera jamesonii* H. Bolus ex Hook f. (13). The bacterium can also be of importance on foliage (3,20) and vegetable plants (5,19).

Understanding the ecology of *P. cichorii* is important for the maintenance of disease-free plants. This species has been isolated from soil free from plant debris, detected in association with roots (5), isolated from symptomless lettuce leaves 63 days after inoculation, and isolated from lettuce debris 115 days after incorporation in soil (1). It was important for us to characterize the association of *P. cichorii* with symptomless chrysanthemums.

A sensitive assay procedure is critical for a precise understanding of the ecology of a bacterial plant pathogen. Semiselective media aid in the detection of an organism among others in environments where general bacteriological media have little value. Several semiselective media have been developed for isolation of *P. cichorii* and/or other fluorescent pseudomonads (2,10,15,18). In a preliminary test, however, we found the medium of Uematsu et al (18), originally developed for *P. cichorii*, to be ineffective for isolation of a number of Florida strains of this species (J. B. Jones, *unpublished*). Therefore, the objective of our research was to develop effective semiselective media for isolation of *P. cichorii* from soil, leaves, and buds. One medium, PCM-1, was developed primarily for isolation from soil and leaves and a second, PCM-2, for isolation from leaves and buds.

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MATERIALS AND METHODS

Development of selective media. The approaches to development of selective media were to: 1) develop a minimal medium with a carbon source used by *P. cichorii* but few other microorganisms, 2) develop a complex medium modified from King's medium B (KMB) (11), and 3) add antimicrobial agents to the media to reduce the contaminating microorganisms with minimal effect on *P. cichorii*. For medium PCM-1, L(+)-tartrate was chosen as the carbon source because of limited utilization by other bacteria (6,14,16), and $(NH_4)_2SO_4$ was chosen as the nitrogen source (Table 1). In preliminary tests, L(+)-tartrate, used in combination with an inorganic nitrogen source, had reduced the number of contaminating organisms. Medium PCM-2, a derivative of KMB, was made more selective than KMB by replacing glycerol with L(+)-tartrate (Table 1); much of its selectivity, however, resulted from the addition of antimicrobial agents. Ampicillin, bacitracin, boric acid, cephalexin, cycloheximide, dodecyltrimethylammonium bromide, malachite green, novobiocin, potassium tellurite, sodium dodecyl sulfate, and vancomycin were screened singly and in various combinations, at concentrations ranging from 0.2 to 600 μg/ml, for their effects on *P. cichorii* and microorganisms derived from soil suspensions. None of these agents was lethal to *P. cichorii* at the rates used in the final media (Table 1), whereas many other agents tested were toxic alone or in combination or were not effective in eliminating contaminating bacteria and fungi. Antibiotics were dissolved in warm deionized water, except for cephalexin, which was dissolved in hot water. All antimicrobial agents and L(+)-tartrate were obtained from Sigma Chemical Co. (St. Louis, MO). The semiselective media were compared with each other and with several other selective media, including: PCSM, developed for *P. cichorii* (18); D4 and SR, developed for fluorescent pseudomonads (10,15); and PSM, developed for *P. syringae* pv. *papulans* and reported to be effective for one strain of *P. cichorii* (2).

Strains of *P. cichorii* used in these studies were isolated from diverse plant species (Table 2) and characterized as previously described (7). Cultures were maintained in tap water at room temperature, or in 15% glycerol at −70 C for long-term storage, and then grown for 24–48 hr on KMB.

Efficiency of recovery of *P. cichorii* on various media. Bacteria from cultures 24 hr old were suspended in deionized water, diluted to approximately 10^8 cfu/ml (estimated turbidimetrically), and diluted to 10^3 cfu/ml. Aliquots of 0.1 ml were spread uniformly over the agar surface in petri plates containing KMB, PCM-1, or PCM-2. The plates were incubated at 28 C for 3–5 days. Recovery efficiency was computed as (cfu on selective medium/cfu on KMB) × 100. The tests were performed twice for all strains.

The same procedure was used to determine the recovery efficiency of PCSM, PSM, SR, and D4 for different strains of *P. cichorii*. Cells of strains of

P. cichorii, P. viridiflava, and various pathovars of *P. syringae*, from cultures grown 24–48 hr on KMB, were suspended in sterile 0.015 M potassium phosphate buffer adjusted to ph 6.8. The suspensions were serially diluted to approximately 10^3 cfu/ml and tested on the media described above. The recovery efficiencies were subjected to the arcsin transformation and then to an analysis of variance.

Inhibition of nontarget organisms from soil and leaf samples. Five samples of soil and one of potting mix were collected from around growing chrysanthemum plants. A 1-g subsample was suspended in 10 ml of deionized water. Serial 10-fold dilutions were plated on PCM-1, PCM-2, and KMB. The total number of bacterial and fungal colonies on KMB was used as the basis for determining the effectiveness of the selective media in preventing development of nontarget organisms. Colonies suspected of being *P. cichorii* were tested for production of oxidase (8) and arginine dihydrolase (17) and for pathogenicity on chrysanthemum (tested by the pinprick method [9]). In a second test, three samples of recently matured chrysanthemum leaves (less than 10 g) and three samples of field soil (1 g) from beds of field-grown chrysanthemums were placed in separate 500-ml Erlenmeyer flasks containing 100 ml of sterile potassium phosphate buffer, pH 6.8. The flasks were shaken vigorously with a wrist-action shaker for 30 min. Serial 10-fold dilutions were made in the phosphate buffer. The washings and dilutions were plated on MB, PCM-1, PSM, SR, and D4. In a third test, PCSM and PCM-1 were compared for suppression of nontarget organisms from six samples of field soils as described above.

Detection of *P. cichorii* in buds and on leaves of chrysanthemum. Twenty-five symptomless buds from each of 41 field-grown chrysanthemum cultivars were ground with a mortar and pestle in 25 ml of sterile deionized water. Serial 10-fold dilutions were made and plated on KMB and PCM-2. Fluorescent bacteria suspected of being *P. cichorii* were characterized as described above.

The usefulness of medium PCM-1 for isolation of *P. cichorii* from leaves was tested with rooted cuttings of the chrysanthemum cultivars Bright Golden Ann, Iceberg, and Mountain Peak growing in field plots. The cuttings were first sampled and assayed for *P. cichorii* as described below, then inoculated immediately by being sprayed with 10^8 cfu/ml of *P. cichorii*, and finally planted in nonreplicated plots. Each plot (0.76 m^2) contained 36 cuttings of a single cultivar; spacing between plots was 0.3 m. The cuttings were covered with Saran cloth to provide 30% shade and kept moist for 14 days to enhance rooting. Twenty leaves of each cultivar were harvested on each of 11 days up to 17 wk after planting, placed in individual plastic bags in a cooler on ice, and transported to the laboratory. The leaves were placed individually into 125-ml Erlenmeyer flasks containing 10 ml of sterile phosphate buffer, pH 6.8, and shaken on a rotary shaker for 30 min. Samples of the resulting suspension were plated on PCM-1. The plates were incubated at 28 C for 3 days. Colonies that fluoresced under long-wavelength ultravi-

Table 1. Semiselective media for isolation of *Pseudomonas cichorii*

PCM-1		PCM-2	
L(+)-tartrate	2.0 g	L(+)-tartrate	2.0 g
$(NH_4)_2SO_4$	0.2 g	K_2HPO_4	1.5 g
KH_2PO_4	0.8 g	$MgSO_4 \cdot 7H_2O$	1.5 g
K_2HPO_4	0.8 g	Proteose peptone No. 3	20.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g	Tergitol 7	1.5 ml
Boric acid	0.25 g	Malachite green oxalate	10 mg
Sodium dodecyl sulfate	0.1 g	Adjust pH to 7.0, add:	
Adjust pH to 7.2, add:		Bacto agar	15.0 g
Bacto agar	15.0 g	Autoclave, cool to 50 C, add:	
Autoclave, cool to 50 C, add:		Cephalexin[x]	100 mg
Cephalexin[x]	20 mg	Novobiocin[y]	25 mg
Dodecyltrimethylammonium bromide	30 mg	Bacitracin[y]	400 mg
Potassium tellurite	0.2 mg	Vancomycin[y]	400 mg
Cycloheximide[y]	50 mg	Adjust volume to 1 L with distilled water	
Ampicillin[y]	5 mg		
Novobiocin[y]	4 mg		
Malachite green[z]	4 mg		
Adjust volume to 1 L with distilled water			

[x]Dissolved in 5 ml of hot sterile water and added to medium.
[y]Dissolved in 5 ml of warm sterile water and added to medium.
[z]Dissolved in 2 ml of ethanol and added to medium.

Table 2. Comparison of selective media for recovery of bacteria from suspensions of pure cultures of *Pseudomonas cichorii* and other fluorescent pseudomonads

Strain[w]	Source[x]	Recovery efficiency on medium[y]				
		PCM-1	PCSM	PSM	SR	D4
PC1	Geranium	78	29	9	0	0
PC2	Geranium	95	19	72	95	73
PC3	Geranium	24	34	0	0	15
PC4	Chrysanthemum	34	33	97	86	70
PC5	Chrysanthemum	84	67	91	94	90
PC6	Chrysanthemum	19	4	111	113	86
PC7	Chrysanthemum	113	3	95	58	18
PC8	Chrysanthemum	52	0	0	0	65
PC9	Chrysanthemum	107	28	55	22	74
PC10	Chrysanthemum	43	0	96	109	29
PC14	Chrysanthemum	75	37	ND	ND	ND
PC11	Celery	94	38	15	84	0
PC15	Celery	103	22	ND	ND	ND
PC12	Gerbera	88	12	ND	ND	ND
PC17	Gerbera	103	28	ND	ND	ND
PC19	Gerbera	65	60	81	62	0
PC20	Gerbera	124	27	ND	ND	ND
PC24	Gerbera	87	26	ND	ND	ND
PC16	Staghorn fern	90	180	ND	ND	ND
PC22	ATCC 10857	93	59	ND	ND	ND
PC23	Schefflera	95	15	ND	ND	ND
P. viridiflava (4)[z]		8	ND	ND	ND	ND
P. syringae pv. *hibisci* (2)		44	ND	ND	ND	ND
P. s. pv. *syringae* (3)		15	ND	ND	ND	ND
P. s. pv. *tabaci* (1)		0	ND	ND	ND	ND

[w]Strains originated from our laboratory or were supplied by A. Chase and J. Miller.
[x]Geranium = *Pelargonium* × *hortorum* Bailey, chrysanthemum = *Dendranthema grandiflora* Tzvelev., celery = *Apium graveolens* L. var. *dulce* (Mill.) Pers., gerbera = *Gerbera jamesonii* H. Bolus ex Hook. f., schefflera = *Schefflera arboricola* Hayata ex Kanehira., staghorn fern = *Platycerium* sp.
[y]Recovery efficiency = (cfu on selective medium)/(cfu on King's medium B) × 100, with each value representing the average recovery efficiency on three petri plates; ND = not determined. PCSM = selective for *P. cichorii* (18), PSM = selective for *P. syringae* pv. *papulans* (2), SR = selective for fluorescent pseudomonads (15), D4 = selective for pseudomonads (10).
[z]Number in parentheses = number of cultures tested.

olet light and were suspected of being *P. cichorii* were selected and tested as described previously.

RESULTS

Characteristics and recovery of *P. cichorii* on the two selective media. After 3 days, small, light blue, generally translucent, round colonies were present on PCM-1. Although strains varied in the appearance of colonies, all colonies fluoresced under long-wavelength ultraviolet light. Generally, the saprophytic fluorescent pseudomonads grew more rapidly, were opaque, and fluoresced more intensely than *P. cichorii* on this medium. On PCM-2, fluorescent, slightly raised, and fluidal colonies of *P. cichorii* developed within 48 hr. Occasionally, saprophytic bacteria produced similar colonies, which made the distinction difficult.

When 12 strains of *P. cichorii* were plated on PCM-1, PCM-2, and KMB, the recovery efficiencies were 119 on PCM-1 and and 102 on PCM-2. The average recovery efficiencies on PCM-1, PCSM, PSM, SR, and D4 for 21, 21, 12, 12, and 12 strains of *P. cichorii*, respectively, were 79, 27, 60, 60, and 43, respectively (Table 2). PCM-1 had a significantly higher recovery efficiency than PCSM and D4 but not SR or PSM. All strains tested grew on PCM-1, but recovery ranged from 19 to 124% of that on KMB (Table 2). Certain strains of *P. cichorii* were not able to grow on PSM, D4, SR, and PCSM; PCSM inhibited the multiplication of many strains. After 7 days of incubation on PCM-1 and PCSM, the average colonies had diameters of 1.74 and 0.87 mm, respectively, which were significantly different. When other fluorescent phytopathogenic pseudomonads were tested on PCM-1 (Table 2), recovery efficiency was low, ranging from 0 to 44% of that on KMB. Colonies of these strains were barely visible (less than 0.5 mm) on PCM-1 after 5 days.

Comparison of PCM-1, PCM-2, and other selective media for inhibition of nontarget organisms from soil, potting mix, and foliar samples. Compared with KMB, PCM-1 and PCM-2 reduced nontarget organisms to less than 10% of those isolated from potting mix and four of five soil samples. *P. cichorii* was isolated from only one of the soil samples using PCM-1 and was detected at 5×10^3 cfu per gram of soil.

When compared with four other selective media for reduction of nontarget organisms isolated from leaf and soil samples, PCM-1 was the only medium to significantly reduce the number of nontarget organisms more than the control in soil washings, whereas all selective media reduced nontarget organisms significantly in leaf washings (Table 3). The number of nontarget organisms growing on PCM-1 was 85.7–99.9% less than the number of those growing on KMB. In a comparison of PCM-1 and PCSM to KMB, nontarget organisms from six soils were reduced 89.8–99.8% and 76.4–99.9%, respectively.

Detection of *P. cichorii* in bud and leaf samples of chrysanthemum. *P. cichorii* was isolated from 25% of the bud samples (Table 4). The populations ranged from 1×10^3 to 1.1×10^5 cfu per 25 buds. This bacterium was detected on 12, 19, and 27% of leaves of Bright Golden Ann, Iceberg, and Mountain Peak, respectively (Table 5). The highest proportion of leaves with *P. cichorii* occurred in the third week, 1 wk after the shade cloth had been removed. The incidence of recovery was considerably lower during the remainder of the experiment. There did not appear to be a direct correlation of rainfall, measured at the plots, with incidence of *P. cichorii* (Table 5).

Table 3. Comparison of PCM-1 with four selective media for isolation of nontarget microorganisms from soil and leaf samples

Sample	Number of nontarget organisms on selective media[y]				
	KMB	PCM-1	PSM	D4	SR
Chrysanthemum leaves	1.3×10^6 a[z]	6.2×10^2 b	1.5×10^4 b	9.4×10^3 b	1.5×10^4 b
Soil	1.7×10^7 a	3.7×10^4 b	3.0×10^5 ab	4.2×10^5 ab	8.3×10^5 ab

[y] Values are numbers of contaminants per gram of tissue or soil and are the average of three samples, with each sample representing the average of three petri plates. KMB = King's medium B (11); PSM = selective for *Pseudomonas syringae* pv. *papulans* (2), D4 = selective for pseudomonads (10), SR = selective for fluorescent pseudomonads (15).
[z] Numbers in the same row followed by the same letter are not significantly different at $P = 0.05$.

Table 4. Recovery of *Pseudomonas cichorii* from apparently healthy buds of chrysanthemum cultivars

Cultivar[y]	No. samples yielding *P. cichorii*/ no. tested	Population level of *P. cichorii* on PCM-2[z]
Mountain Peak	1/1	2×10^3
Paragon	1/1	1.1×10^5
Balcome Perfection	1/2	5×10^3
Ice Capade	1/1	1×10^5
Omegon	1/1	1.4×10^2
Florida Marble	1/2	1×10^4
Jackpot	1/1	1×10^3
Surfine	1/1	5×10^3
Wildfire	1/1	2×10^3
Yellow Cloud	2/2	1.2×10^4
Total	11/13	

[y] Also tested but negative for isolation: Iceberg, Alert, Surf, Senorita, Nob Hill, Copper Ann, Dark Circus, Red Belair, Streamer, Jade, Flame Belair, Dolly, Blue Marble, Ballerina, Snow Purple, Coral Marble, Amber, Indio, White Marble, Pink Marble, Fortune, Beauregard, Goldstrike, Revere, Red Remarkable, Flaming Sun, Deep Hot Pink, Applause, Stardom, White Grandchild, and Intrepid Gold.
[z] Colony-forming units per 25 buds.

Table 5. Incidence of *Pseudomonas cichorii* on symptomless, recently matured leaves of three cultivars of chrysanthemum in the summer of 1987

Week[y]	Cultivar			Rain total (cm) for 7 days before sampling
	Mountain Peak	Bright Golden Ann	Iceberg	
1	0[z]	0	0	0.00
3	19	15	14	0.79
5	16	5	4	3.85
9	0	0	0	0.24
10	8	0	ND	0.00
11	6	6	11	0.71
12	0	0	3	0.25
13	1	1	7	0.30
14	0	0	0	0.14
15	9	0	0	2.75
16	2	0	0	1.02
17	4	2	3	6.12

[y] Sampling begun 6 June 1987.
[z] Number of leaves per 20 sampled from which *P. cichorii* was isolated; minimum detection level was 10 cells per gram of tissue. ND = not determined.

DISCUSSION

Two semiselective media, PCM-1 and PCM-2, were effective for isolating *P. cichorii* from plants and soil. Although the media are only moderately effective for separation of *P. cichorii* from other fluorescent pseudomonads, they did eliminate a high percentage of contaminants. Few fluorescent pseudomonads other than *P. cichorii* utilize L(+)-tartrate (6,14,16), which makes it a useful compound to use in medium PCM-1. In soils tested in our studies, however, there were a number of fluorescent pseudomonads that grew quite proficiently on PCM-1. Thus, fluorescent colonies that grow on PCM-1 cannot be presumed to be *P. cichorii*. Also, because colony characteristics vary between strains of *P. cichorii* growing on the PCM media, these characteristics are not reliable for readily distinguishing *P. cichorii* from other fluorescent pseudomonads.

The other semiselective media available for detection of fluorescent pseudomonads (2,10,15,18), although quite efficient for removal of potential competitors, were not as effective as PCM-1 or PCM-2 for recovery of *P. cichorii* or were toxic to certain strains of *P. cichorii*. PCSM (18) effectively eliminated nontarget microorganisms but was toxic to numerous strains of *P. cichorii*.

P. cichorii was recovered from a high percentage of bud and leaf samples. Thus, nonsymptomatic chrysanthemum tissue harbors the bacterium and may be a factor in the survival and the distribution of the bacterium to environs where *P. cichorii* was previously absent. The fact that the highest incidence of detection of the bacterium occurred after a period of high humidity is supportive of an important role moisture plays in the maintenance of the bacterium on the leaf surface (9).

Contamination problems that occur on KMB make its use for detecting *P. cichorii* in the natural habitat tedious. The reduction in nontarget bacteria by PCM-1 and PCM-2 has made population studies more practical. Both PCM media are recommended for isolation of *P. cichorii* (PCM-1 for leaves and soil and PCM-2 for buds) because both suppress nontarget organisms significantly while allowing growth of the target organism.

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