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Comparison of Media for Recovery of *Verticillium dahliae* from Soil

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ABSTRACT

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Polygalacturonic acid (PGA) is an important constituent of Sorensen's NP-10 medium (NP-10) for estimating the population density of *Verticillium dahliae* in soil. Different types of PGA are available, but not all of them favor the growth of *V. dahliae*. Unavailability of PGA sodium salt from orange (P-1879) has created an unprecedented problem for the quantification of microsclerotia (MS) of *V. dahliae* in soil. The PGA from orange (P-3889) that is now available does not support the growth of *V. dahliae*. Therefore, experiments were conducted to optimize the use of NP-10 prepared with P-3889 and various concentrations of NaOH. NP-10 with P-3889 amended with eight concentrations of NaOH were compared with NP-10 prepared from PGA sodium salt from orange (P-1879, now discontinued) and citrus (P-3850) along with cellophane and Na-pectate media for recovery of MS from soil and growth of *V. dahliae* on the media. Seven soils were assayed for MS, and eight isolates of *V. dahliae* were evaluated for growth and production of MS. Concentrations of NaOH >0.035N and <0.02N in NP-10 with P-3889 reduced mycelial growth, microsclerotial production, and recovery of MS from soils. Similarly, NP-10 with P-3850 alone, cellophane, and Na-pectate media had significantly reduced growth on media and recovery of *V. dahliae* from soils. The NP-10 with P-3889 and 0.025N NaOH consistently yielded numbers of *V. dahliae* MS from soil samples and supported the growth and production of MS similar to the NP-10 with P-1879. The medium developed in this study can serve as a direct replacement for the original NP-10 that was developed nearly three decades ago, an important component of which is no longer available.

Verticillium dahliae Kleb. is a ubiquitous soilborne fungus causing wilt diseases on many economically important crops (2,13,14,17). The pathogen forms multicelled, melanized, resting bodies called microsclerotia (MS), which persist in the soil for many years (14,20). MS in soil are the primary source of inoculum for *V. dahliae* infections in host plants (21). Incidence of Verticillium wilt in herbaceous hosts is generally proportional to the number of MS in soil (11,12,19), but can vary considerably depending on the cultivar and crop (5,6,8,21). For example, in cauliflower, 4 MS g^{-1} of dry soil can cause 16% wilt incidence but 10 MS g^{-1} of soil causes 50% wilt (21). In tomato, 0.5 MS g^{-1} of soil causes 50% wilt incidence but 6 MS g^{-1} of soil causes 100% wilt (5). In strawberry, however, as little as 0.3 MS g^{-1} of soil causes 5% wilt and just 2 MS g^{-1} of soil can cause 100% wilt (6). Therefore, accurate quantification of MS in the soil is essential to make planting decisions, to predict the risk of disease in a given field, and to manage Verticillium wilt in commercial fields.

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Three techniques have been employed widely to quantify MS from soil: wet sieving, soil dilution plating, and soil impaction plating using an Anderson sampler (10). Among these techniques, the Anderson sampler technique is considered to be the least biased method (3,18). With this technique, a known mass of soil is uniformly spread on a petri-plate with Sorensen's NP-10 semiselective medium (15) using the Anderson sampler. After a 3-week incubation in the dark, microsclerotial colonies are enumerated under a stereoscope.

Polygalacturonic acid (PGA) was identified as an important constituent in the semiselective medium for the growth of *V. dahliae* MS nearly three decades ago. Until recently, sodium salt of PGA from orange (P-1879) (Sigma-Aldrich, St. Louis) had been optimized for use in Sorensen's modified NP-10 medium (15) and has been the most widely used medium for soil assays of *V. dahliae*. About 3 years ago, Sigma-Aldrich discontinued this product and this has rendered accurate quantification of MS in soil using NP-10 medium difficult. One type of PGA that is currently available from Sigma-Aldrich is a PGA-sodium salt from citrus (P-3850). In preliminary studies, the use of this product in Sorensen's NP-10 medium greatly underestimated the number of MS present in soil and drastically reduced the production of MS. Another type of PGA that is available from Sigma-Aldrich is derived from orange (P-3889); however, in preliminary studies, NP-10 medium prepared with this PGA did not support the growth of *V. dahliae*. The pH of NP-10 with P-3889 was significantly lower than NP-10 with P-1879; therefore, we decided to amend the medium with various concentrations of NaOH to optimize the NP-10 for the growth and recovery of *V. dahliae*.

A number of substrate-specific methods have been developed and tested to estimate the MS recovery from soils. Among these methods, cellophane (1) and Na-pectate (3) media are considered effective, but never have been compared with the NP-10 medium for growth of *V. dahliae* and recovery of MS. The objectives of this study were to optimize NP-10 prepared with P-3889 using various concentrations of NaOH and compare this medium with currently available media for growth and production of MS by isolates of *V. dahliae* from various hosts, and to compare the media for soil assays of *V. dahliae* MS. A preliminary report has been published (9).

MATERIALS AND METHODS

Media preparation. Sorensen's NP-10 semiselective medium (15) was prepared with PGA-sodium salt from orange (P-1879; molecular weight 4,000 to 6,000), PGA-sodium salt from citrus (P-3850; molecular weight of pectin purified to make P-3850 is 50,000 to 150,000), or PGA from orange (P-3889; molecular weight 4,000 to 6,000) amended with NaOH. The NP-10 medium prepared with P-3889 was amended with concentrations of 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.045, and 0.055 N NaOH (Table 1). Each NP-10 medium consisted of two parts autoclaved separately. In the first part, 5 g of PGA (if PGA used was P-3889, then various amounts of NaOH were added to give the concentrations of NaOH listed above) in 500 ml of distilled water was autoclaved at 121°C for 15 min and cooled to 50°C. In the second part, 15 g of Bacto-Agar (0140-01; Difco Laboratories, Detroit), 1 g of KNO_3 (P-8394; Sigma-Aldrich), 1 g of KH_2PO_4 (P-5379; Sigma-Aldrich), 0.5 g of KCl (P-4504; Sigma-Aldrich), 0.5 g of $MgSO_4 \cdot 7H_2O$ (M-1880; Sigma-Aldrich), 0.5 ml of Tergitol (NP-10; Sigma-Aldrich), and 500 ml of distilled water (with a magnetic stir bar) were autoclaved at 121°C for 15 min and cooled to 50°C. After the second part equilibrated to 50°C, chloramphenicol (C-0378; Sigma-Aldrich), streptomycin sulfate (S-6501; Sigma-Aldrich), and chlorotetracycline

HCl (C-4881; Sigma-Aldrich) at 50 mg liter^{-1} each were added. When the antibiotics were completely dissolved, the two parts were mixed and placed on a magnetic stirrer. Each medium was dispensed (15 ml per dish) into 90-mm petri dishes immediately.

In addition to the above media, cellophane (1) and Na-pectate (3) media were used for soil assays of *V. dahliae* MS (Table 1). The pH of all media was measured using a pH meter (Fisher Scientific, Santa Clara, CA) when the media temperature ranged between 39 and 45°C after each batch was prepared. Each repetition was considered as a replication in the analysis of variance to determine pH differences between the media using the general linear model (GLM) procedure in SAS (SAS Institute, Cary, NC). Fisher's protected least significant difference (LSD) test was used to compare mean pH between the different media.

Mycelial growth and production of MS by isolates of *V. dahliae*. Isolates of *V. dahliae* from eight hosts (Table 2) were evaluated on all the media described above (Table 1). Single-spore isolations were made; cultures were purified and maintained on potato-dextrose agar (PDA) slants at 4°C. Three plates of each medium were inoculated centrally with a 0.4-cm-diameter agar disk from the advancing margins of 2-week-old cultures of each isolate on PDA. All plates were incubated for 4 weeks at room temperature (23 ± 1°C). Colony diameters on all plates and the diameter of the culture with MS were measured at 7, 14, 21, and 28 days of incubation. The experiment was conducted three times. Repeated measures analysis of variance was used to test radial growth of *V. dahliae* colonies and the portion of the medium with MS in cultures (SAS release 8.0; SAS Institute). Means and corresponding standard errors were computed for each treatment and sampling time.

Length and width of MS on various media. The optimum range of NaOH concentration in NP-10 medium containing P-3889 that supported the growth of *V. dahliae* identical to the NP-10 medium containing P-1879 was determined in the above experiment. The morphology of *V. dahliae* MS from several isolates were compared on A, B, E, G, K, and M media (Table 1). Only three isolates were selected for length and width of MS measurement based on their growth and uniformity of MS formation in NP-10 with P-1879. To demonstrate that isolates from different crops have consistent growth on these media, cultures of *V. dahliae* isolates from chili pepper (VdCa.36), lettuce (VdLs.17), and oilseed rape (VdBno.188) were produced on each of the above media as described above. The experiment was conducted twice. Each time, 10 agar plugs (4-mm-diameter) from each of the medium–isolate combinations were placed on a slide and spread with a cover slip. Measurements of the length and width of MS were made on 100 MS from 28-day-old cultures on each medium under a stereoscope at ×100. The length and width of MS data were analyzed using the SAS GLM procedure (SAS Institute). Data within each medium–isolate combination were pooled from the two experiments because there was no experiment–isolate interaction. Means and standard error of the mean were computed.

Production of MS. The variation in the number of MS produced on different semiselective media (A, B, E, G, K, and M; Table 1) was determined in three isolates (chili pepper (VdCa.36), lettuce (VdLs.17), and oilseed rape (VdBno.188)) of *V. dahliae* (Table 2). These isolates were selected based on their growth in the above experiment. Agar plugs (4-mm-diameter) from 28-day-old cultures of each medium–isolate combination were placed on a slide and uniformly spread with a cover slip. The number of MS was counted in five microscopic fields with a stereoscope at ×100 and, for each isolate, 20 agar plugs per medium were examined and each agar plug was considered as a replication. The experiment was repeated once. Analysis of variance was done using the GLM procedure of SAS to test the effects of semiselective media on MS production. Means and standard errors of the mean were computed.

Comparison of media for assay of *V. dahliae* MS in field soil. Ten soil samples each were collected to a depth of 10 cm from seven different commercial lettuce and strawberry fields in the Salinas Valley with moderate to high incidence of Verticillium wilt on the respective crops (Table 1). The 10 soil samples from each field were bulked for assay of individual field soils and air-dried on greenhouse benches. The air-dried soil was passed through a 425-µm (40-mesh) sieve. Sieved soil (10 g) from each sample was placed in a screw-capped plastic vial and 2.5 ml of methionine (M-9500; Sigma-Aldrich; 0.0075 g ml^{-1} of distilled H_2O) solution was dispensed into the vial. The vials then were incubated at 30°C for a week and air-dried at room

Table 2. *Verticillium dahliae* isolates used in this study with their hosts and geographical location

Isolate number	Original host	Source
VdCs.80	Artichoke (*Cynara scolymus* L.)	California
VdBoc.74	Cabbage (*Brassica oleracia* var. *capitata* L.)	California
VdBob.70	Cauliflower (*Brassica oleracia* var. *botrytis* L.)	California
VdCa.36	Chili pepper (*Capsicum annuum* L.)	California
VdLs.17	Lettuce (*Lactuca sativa* L.)	California
VdMp.89	Mint (*Mentha* × *piperita* L.)	Oregon
VdBno.188	Oilseed rape (*Brassica napus* L.)	Germany
VdFca.1	Strawberry (*Fragaria* × *ananassa* Duchesne)	California

Table 1. Effects of media on the quantification of microsclerotia from different soils

		Number of microsclerotia (MS) per gram of dry soil						
Media[a]	Media pH	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6	Soil 7
A	4.81	25	149	138	411	343	573	714
B	7.49	4	14	112	166	108	158	141
C	2.63	1	0	3	1	2	0	0
D	2.91	1	2	4	2	9	11	12
E	3.16	2	45	41	38	89	92	110
F	3.86	3	34	64	162	215	348	287
G	5.03	26	154	158	374	425	576	654
H	5.68	27	125	139	366	369	628	635
I	6.99	16	130	103	319	350	494	555
J	7.67	12	77	105	313	302	527	506
K	8.41	11	53	96	272	267	457	470
L	7.57	23	83	75	223	184	384	380
M	4.87	1	48	64	132	159	225	177
LSD	0.19	10	47	60	131	135	147	164

[a] Media are: A, polygalacturonic acid (PGA) (P-1879); B, PGA (P-3850); C, PGA (P-3889); D, P-3889 + 0.01 N NaOH; E, P-3889 +0.015 N NaOH; F, P-3889 + 0.02 N NaOH; G, P-3889 + 0.025 N NaOH; H, P-3889 + 0.03 N NaOH; I, P-3889 + 0.035 N NaOH; J, P-3889 + 0.045 N NaOH; K, P-3889 + 0.055 N NaOH; L, cellophane; and M, Na-pectate. PGA was added to the Sorensen's NP-10 medium (15); Na-pectate and cellophane were added to the media as describe in Butterfield and DeVay (3) and Ashworth et al. (1). LSD = least significant difference ($P \leq 0.05$).

temperature (23 ± 1°C) for a week. All air-dried soil from the vial then was poured into a mortar and pulverized gently to break the clods. The pulverized soil was plated onto five plates each, using the Anderson sampler technique on 13 different media (Table 1). The plates were incubated for 3 weeks in the dark at room temperature (23 ± 1°C). Following incubation, the surface of each plate was gently washed under a stream of water to remove soil. The numbers of microsclerotial colonies of *V. dahliae* on each plate were counted under a stereoscope at ×10 to ×20. The density of MS was expressed as the number of propagules per gram of dry soil. The experiment was conducted four times and served as replications. Analysis of variance was conducted for MS recovery from the soils in different media using GLM in SAS. Fisher's protected LSD test was used to compare mean MS recovery on different media.

Germination of the recovered MS from two soils also was tested on the cellophane and Na-pectate media, and NP-10 containing P-1879, P-3850, or P-3889 amended with 0.025 N NaOH. Single colonies of *V. dahliae* MS from soils 4 and 5 (Table 2) from corresponding media were transferred to 10 plates of each medium and incubated at room temperature. The MS were considered germinated if a new colony of *V. dahliae* developed on the plated media. The number of plates yielding *V. dahliae* colonies was expressed as a percentage of the 10 plates examined. The experiment was conducted three times. Data were log transformed prior to analysis to normalize variance. Analysis of variance was conducted and Fisher's protected LSD was used to compare germination of MS on the selective media.

Effects of the volume of soil plated on MS recovery. Even though the amount of soil for plating in the Anderson sampler technique was standardized several decades ago (3), because of the contemporary changes in the composition of NP-10 medium, the assay procedure had to be standardized anew. Therefore, the influence of plated soil volume on the efficiency of MS recovery in *V. dahliae*-infested soil was determined. Ten soil samples collected from two commercial lettuce fields in the central coast of California were pooled and air-dried in paper bags for 2 to 3 weeks in a greenhouse. The soils were processed as described above and each soil sample was plated onto five plates of medium G (Table 1) at the rate of one scoop (33 mg), two scoops (67 mg), three scoops (100 mg), four scoops (133 mg), five scoops (166 mg), and six scoops (200 mg) per plate.

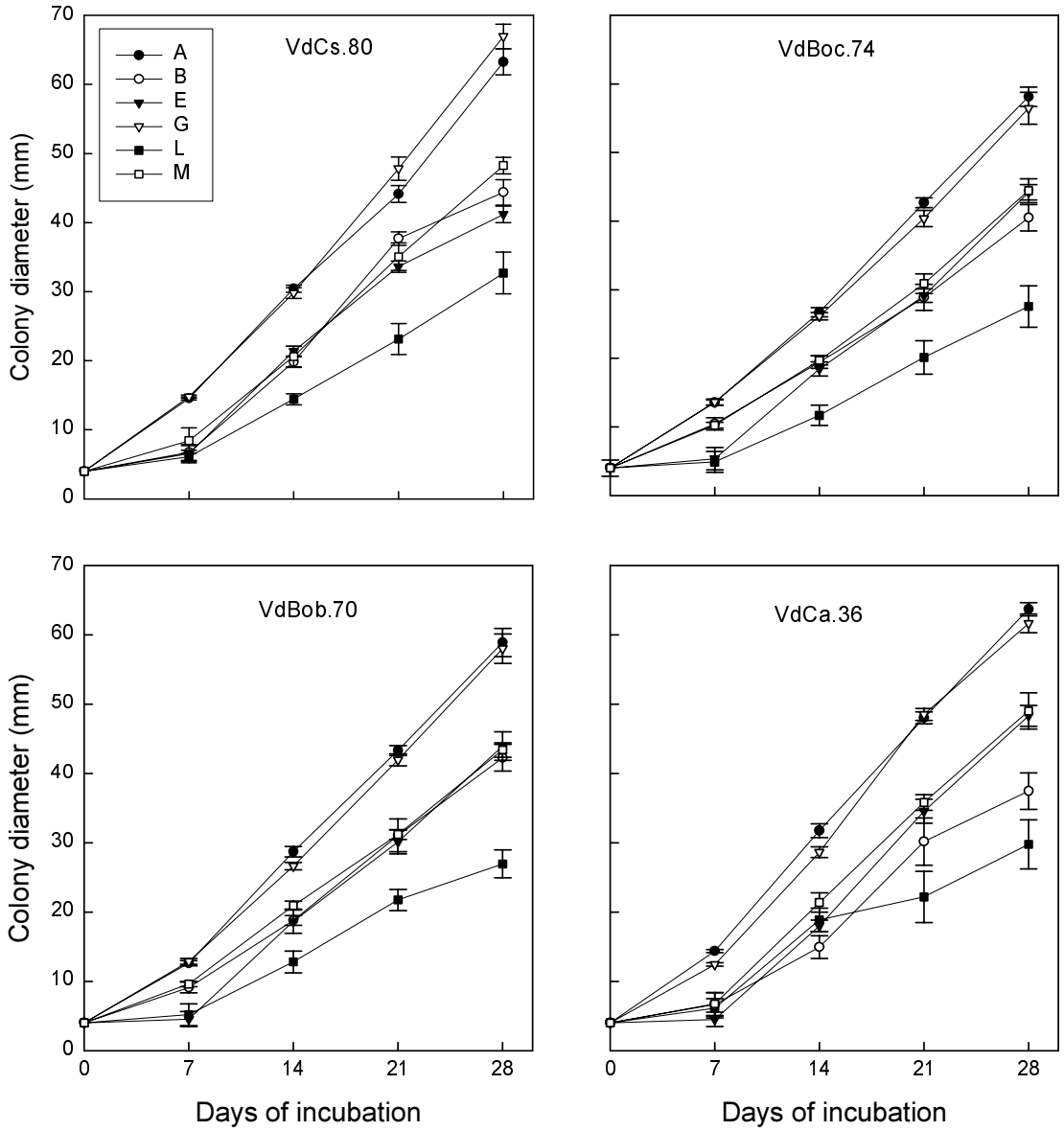

Fig. 1. Radial growth of *Verticillium dahliae* isolates from artichoke (VdCs.80), cabbage (VdBoc.74), cauliflower (VdBob.70), and chili pepper (VdCa.36) in different media after various days of incubation. The media are A, NP-10 with P-1879; B, NP-10 with P-3850; E, NP-10 with P-3889 and 0.015 N NaOH; G, NP-10 with P-3889 and 0.025 N NaOH; L, cellophane; and M, Na-pectate. Vertical bars represent the standard errors of the mean.

The plates were incubated for 3 weeks in the dark at room temperature (23 ± 1°C). Following incubation, the surface of each plate was washed gently under a stream of water to remove soil. The number of microsclerotial colonies of *V. dahliae* on each plate was counted under a stereoscope at ×10 or ×20. The density of MS was expressed as the number of propagules per gram of dry soil. The experiment was conducted four times; each experiment served as a replication. Analysis of variance was performed on the data to determine if soil volume affected recovery of MS. Means and standard errors were computed by pooling the data from the four experiments, because the experiment–treatment interaction was not significant.

RESULTS

pH of the media. The pH of NP-10 medium containing P-3889 was the lowest, cellophane or NP-10 containing P-3850 was the highest, and Na-pectate medium and NP-10 containing P-1879 were intermediate (Table 1). The NP-10 medium containing P-3889 amended with increasing concentrations of NaOH incrementally increased the pH and reached 8.41 with the highest concentration of NaOH included in the study (D to K in Table 1). The pH in NP-10 containing P-3889 amended with 0.025 N NaOH was 5.03, which was very close to the pH in NP-10 medium containing P-1879 (original PGA; A and G in Table 1).

Effect of media on mycelial growth. The NP-10 medium containing P-3889 alone did not support the growth of any *V. dahliae* isolates. However, mycelial growth was observed in all NP-10 media containing P-3889 and amended with different concentrations of NaOH. The mycelial growth progressively increased as the concentration of NaOH in the medium increased from 0.01 to 0.025 N, but then declined with further increase in the concentration of NaOH (*data not shown*). Colony diameters of all isolates of *V. dahliae* in NP-10 with P-3889 and 0.025 N NaOH were identical to that on NP-10 added with P-1879 ($P > 0.05$) and were significantly greater than on all other tested media (Figs. 1 and 2). At the end of the 4-week incubation, colony diameters of all *V. dahliae* isolates in these two media were identical and were significantly greater than in all other media.

Colony diameters of all isolates except VdMp.89 were smallest in the cellophane medium (Figs. 1 and 2). Overall colony diameters of the oilseed rape (VdBno.188) isolate was smaller than all other isolates on all media tested.

Number of MS. The number of MS produced by three isolates of *V. dahliae* in

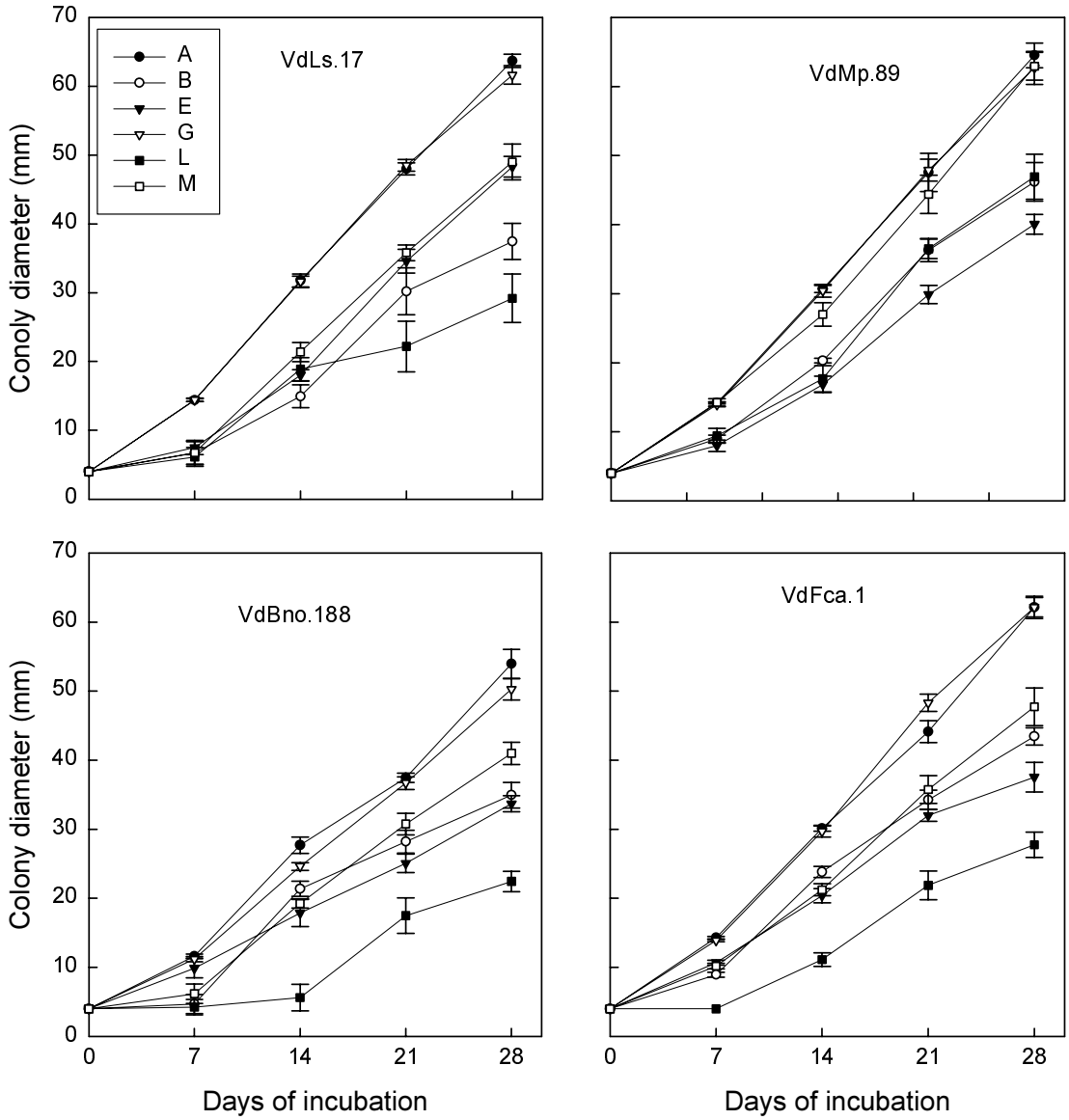

Fig. 2. Radial growth of *Verticillium dahliae* isolates from lettuce (VdLs.17), mint (VdMp.89), oilseed rape (VdBno.188), and strawberry (VdFca.1) in different media after various days of incubation. The media are A, NP-10 with P-1879; B, NP-10 with P-3850; E, NP-10 with P-3889 and 0.015 N NaOH; G, NP-10 with P-3889 and 0.025 N NaOH; L, cellophane; and M, Na-pectate. Vertical bars represent standard errors of the mean.

4-mm-diameter agar plugs of different media is summarized in Figure 3. All isolates produced MS in all media tested except for VdLs.17, which failed to produce MS on NP-10 with P-3889 and either 0.015 or 0.055 N NaOH (Fig. 3). The latter two media also supported the fewest number of MS by the other two isolates tested. In all media tested, isolate VdCa.36 produced higher numbers of MS than isolates VdLs.17 and VdBno.188. The numbers of MS produced by all three isolates in the NP-10 with either P-1879 or P-3889 and 0.025 N NaOH were nearly identical. The numbers of MS in these two media were significantly greater than in all other media except for VdLs.17 on Na-pectate medium.

Effect of media on microsclerotial length and width. The length and width of MS produced by isolate VdBno.188 were significantly greater than those produced by VdCa.36 and VdLs.17 on all media (Fig. 4). Both length and width of MS were not significantly different in NP-10 medium containing P-1879 and NP-10 with P-3889 and 0.025 N NaOH, but were significantly greater than in all other media.

Recovery of MS from soils. No microsclerotial colonies were observed on NP-10 medium containing P-3889 alone in three tested soils. In the other four soils, less than 1% of microsclerotial colonies were recovered. The recovery of MS of *V. dahliae* from soils on NP-10 medium containing P-3889 improved with the addition of NaOH to the medium, and progressively increased as the concentration of NaOH increased from 0.01 to 0.03 N, but then declined with further increase in the concentration of NaOH (Table 1). However, the numbers of MS recovered in the NP-10 medium containing P-3889 amended with 0.025 to 0.045 N NaOH were not significantly different (Table 1) in five soils. The recovery of MS from soils on the NP-10 medium containing P-3889 and 0.025 N NaOH was identical to that of NP-10 containing P-1879 in all soils (Table 1). The NP-10 medium containing P-3850, Na-pectate, and cellophane media supported the recovery of *V. dahliae* MS but at significantly lower numbers than NP-10 containing either P-1879 or P-3889 with 0.025 to 0.045 N NaOH (Table 1). Recovery of MS from soils by NP-10 containing P-3850 or Na-pectate was 9 to 81% and 4 to 46%, respectively, relative to NP-10 containing P-1879. However, the recovery by NP-10 containing P-3889 with 0.025 N NaOH was 91 to 123% of that in NP-10 containing P-1879 (Table 1).

Similarly, the germination of MS recovered from soils 4 and 5 also was highest on NP-10 containing P-1879 (63 and 60% for soils 4 and 5, respectively) or P-3889 with 0.025 N NaOH (57 and 63% for soils 4 and 5, respectively). Germination was least on the cellophane medium (17 and 13% for soils 4 and 5, respectively), and intermediate on Na-Pectate (48 and 30% for soils 4 and 5, respectively) and NP-10 containing P-3850 (37 and 33% for soils 4 and 5, respectively).

Effect of amount of soil plated on recovery of MS. The number of MS per gram of soil was not proportional to the amount of the soil plated on NP-10 medium in both tested soils (Fig. 5). In soil A, significantly lower numbers of MS were recovered when six scoops (200 mg) of soil were plated on each plate than at all other soil volumes plated. However, in soil B, the numbers of MS per gram of soil were significantly lower when one scoop (33 mg) of soil was plated. The numbers of MS increased when up to three scoops (100 mg) of soil was plated on each plate and declined thereafter in both tested soils (Fig. 5).

DISCUSSION

Soil assays of *V. dahliae* MS are an integral part of any laboratory working on the detection, epidemiology, and control of this pathogen on the myriad crops that it infects. The mainstay of soil assays in a majority of the laboratories has been the NP-10 semiselective medium for the recovery of microsclerotial colonies of *V. dahliae* (15). The critical component in the NP-10 semiselective medium has been the sodium salt of PGA (P-1879), hitherto supplied by Sigma-Aldrich. Discontinuation of this product by the company has necessitated the identification of alternative sources of PGA for efficient recovery of *V. dahliae* MS from soil. As demonstrated in this study, the numbers of MS recovered from different soils, the size of MS of different isolates, and the growth rates of these isolates on NP-10 with P-3889 and 0.025 N NaOH were identical to those obtained on NP-10 with P-1879. Thus, NP-10 with P-3889 and 0.025 N NaOH is an ideal alternative to NP-10 with P-1879.

NP-10 with P-3889 alone did not allow the recovery of MS from the soil and did not support the growth of *V. dahliae* isolates tested and, therefore, was unsuitable for soil assays for *V. dahliae*. However, NP-10 with P-3889 and concentrations of NaOH from 0.015 to 0.025 N improved the recovery of MS from soil and mycelial growth on the medium. Furthermore, the size of the MS was identical to that observed on NP-10 with P-1879, which is no longer available. Concentration of NaOH either below or above this range in the NP-10 containing P-3889 decreased the growth of *V. dahliae* and the recovery of MS from soils. It is likely that the pH of the media regulated by NaOH may have influenced the growth and recovery of *V. dahliae*. Increased pH in these media with high concentrations of NaOH also may have prevented the growth and recovery of *V. dahliae* as demonstrated previously on *V. marguandi* (16) and *V. dahliae* (19). A pH < 6.0 yielded optimum mycelial growth of

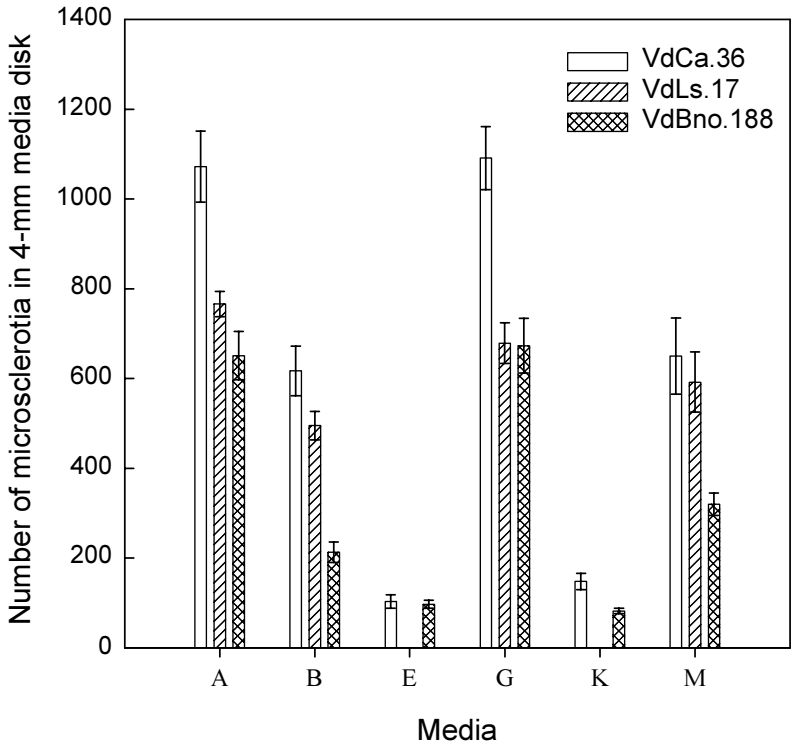

Fig. 3. Number of microsclerotia (in 4-mm agar plug) of *Verticillium dahliae* isolates from chili pepper (VdCa.36), lettuce (VdLs.17), and oilseed rape (VdBno.188) on different media: A, NP-10 with P-1879; B, NP-10 with P-3850; E, NP-10 with P-3889 and 0.015 N NaOH; G, NP-10 with P-3889 and 0.025 N NaOH; K, NP-10 with P-3889 and 0.055 N NaOH; and M, Na-pectate. Vertical bars represent the standard errors of the mean.

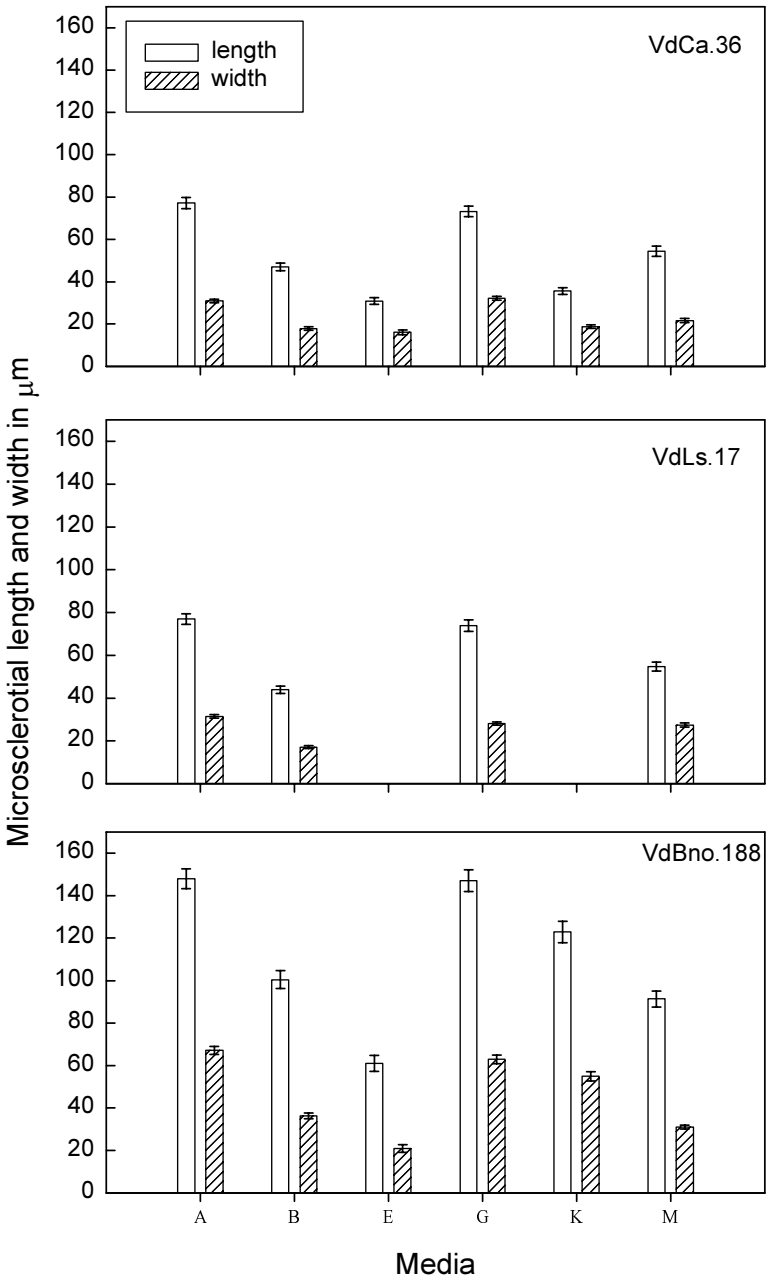

Fig. 4. Length and width of microsclerotia of *Verticillium dahliae* isolates from chili pepper (VdCa.36), lettuce (VdLs.17), and oilseed rape (VdBno.188) on different media: A, NP-10 with 1879; B, NP-10 with P-3850; E, NP-10 with P-3889 and 0.015 N NaOH; G, NP-10 with P-3889 and 0.025 N NaOH; K, NP-10 with P-3889 and 0.055 N NaOH; and M, Na-pectate Vertical bars represent the standard errors of the mean.

V. marguandi (16). In our study, suitable pH for mycelial growth of *V. dahliae* ranged from 4.81 to 5.03. Even though the pH of NP-10 with P-3889 alone was 2.63, growth of *V. dahliae* was inhibited, suggesting that addition of NaOH in the medium is essential to increase the pH of the media to an optimum level for the growth of *V. dahliae*. It also is possible that the Na^+ ion itself may have been toxic to the pathogen at higher concentrations of NaOH. The high concentration of NaOH (0.03 to 0.055 N) in the NP-10 media also changes the color of the media from light to dark brown, which may reduce the contrast between MS colonies and the media, making it difficult to obtain precise counts of MS colonies.

The NP-10 containing P-3850 with no NaOH allowed only partial recovery of the number of MS present in the soil relative to the NP-10 containing P-1879 (original PGA) and, thus, grossly underestimated the number of MS present in the soil. Butterfield and DeVay (3) observed 75% reduction in the number of MS from soil assays when the PGA used was from a different source. Both cellophane and Na-pectate media also reduced the recovery of MS from the seven tested soils between 12 to 91% and 54 to 96%, respectively, relative to P-1879 in the NP-10 medium, suggesting the requirement for NP-10 with either P-1879 or P-3889 and 0.025 N NaOH for soil assays of *V. dahliae* MS.

Mycelial growth was slower on the cellophane medium than on NP-10 with P-1879 or P-3889 and 0.025 N NaOH. Microsclerotial recovery from the soils by the cellophane medium was significantly higher than on Na-pectate medium in all soils but lower than on the NP-10 with P-1879 or P-3889 and 0.025 N in six soils. However, in one soil (soil 1, Table 1) with the lowest number of MS in our study, recovery of MS on cellophane medium was not significantly different from that on NP-10 containing P-1879, suggesting that cellophane medium could be useful in soils containing low densities of *V. dahliae*. One advantage of the cellophane medium, however, is its ability to distinguish *V. dahliae* colonies from *V. tricorpus* (1). However, cellulolytic microorganisms outgrew *V. dahliae* colonies and led to an underestimation of the number of MS. In contrast, growth of other fungal colonies on the NP-10 and Na-pectate media was slow and restricted; therefore, MS colonies were easily detectable under the microscope. Different types of cellophane have the potential to affect the growth and recovery of *V. dahliae* from soils. We tested three kinds of cellophane, such as cellophane (1), gel dryer cellophane membrane (Bio-Rad Laboratories, Hercules, CA), and an unknown type of cellophane. Neither the growth of different isolates of *V. dahliae* nor the recovery of MS from the soil was affected by the types of cellophane tested (*data not shown*), indicating that the type of cellophane may not be a major factor determining the growth and recovery of *V. dahliae*.

The amount of soil plated on the media can influence the accurate estimation of *V. dahliae* MS. Distributing 33 mg of soil in each plate failed to provide an accurate count of *V. dahliae* MS in soil. A small error in spreading this small amount of soil on plates may cause a large variation in the estimation of MS in both low- and high-density *V. dahliae* MS in soil. In contrast, a large amount of soil (200 mg) plated on the medium increased the number of the MS colonies to levels where an accurate counting of the number of colonies was difficult. Plating 100 mg of soil per plate was optimal for an accurate estimation of MS colonies, and this was consistent with the conclusions of the study by Butterfield and DeVay (3).

Microsclerotia of *Verticillium* spp. germinate repeatedly in soil (4). Nearly 60% of the MS recovered from the soil assayed on NP-10 with P-1879 or P-3889 and 0.025 N NaOH, further confirming the ability of NP-10 with P-3889 and 0.025 N NaOH to replace NP-10 with P-1879. Huisman and Ashworth (7) reported no significant reduction in germination between Na-pectate and cellophane media. In this

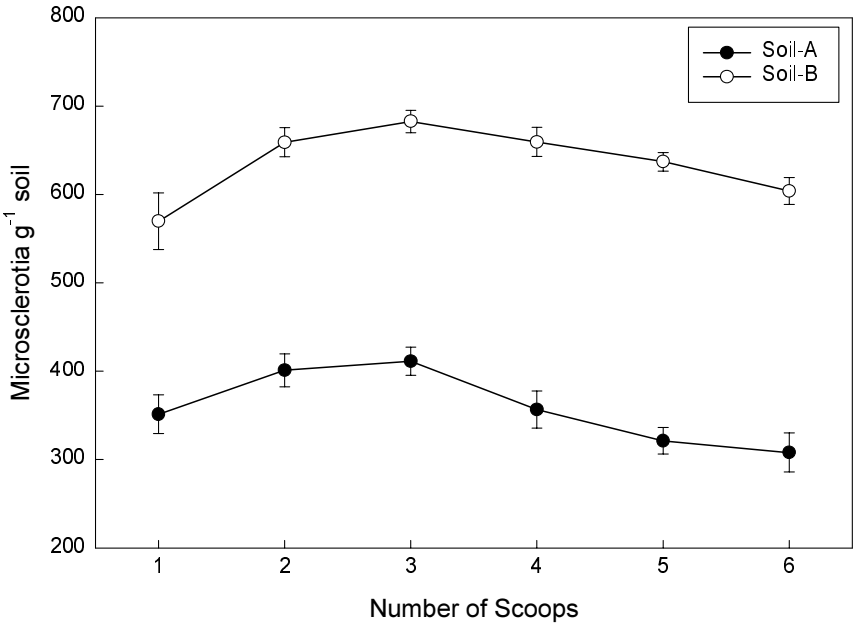

Fig. 5. Relationship between the amount of soil plated using the Anderson sampler technique and the number of *Verticillium dahliae* microsclerotia recovered in two soils with different levels of infestation by the pathogen. Vertical bars represent the standard errors of the mean.

study, however, germination of MS was significantly lower in the cellophane medium relative to that in Na-pectate medium.

Our results demonstrate that the PGA from orange (P-3889) amended with 0.025 N NaOH in the NP-10 is a suitable alternative to P-1879 (the erstwhile constituent of NP-10 semiselective medium). The pH, recovery of MS from soils, size of MS and their germination, and the growth of *V. dahliae* on these media were similar. Compared with the NP-10 with P-1879 or P-3889 amended with 0.025 N NaOH, *V. dahliae* grew more slowly on the NP-10 with P-3850 and on the cellophane medium for all isolates. Recovery of MS of *V. dahliae* was higher on the NP-10 with P-1879 or P-3889 amended with 0.025 N NaOH than on NP-10 with P-3850 or Na-pectate medium for all tested soils.

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