SADAMCAP, a Technique for Quantifying Populations of *Phytophthora cactorum* in Apple Orchard Soils

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ABSTRACT

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SADAMCAP (Soil Air-Dried And Moistened Chilled And Plated), proved a sensitive and repeatable technique for assessing *Phytophthora cactorum* populations in apple orchard soils. Air-dried soil in petri plates was moistened and incubated under lights at 22°C for 96 h and then flooded with excess water and chilled at 6°C for 2 h. Plates were sloped and the water was drained, vortexed for 30 s, left at ambient temperature

for 10 min, and then diluted samples were plated on PARP media and colonies counted after 20, 28, and 40 to 44 h. Detailed studies of each step in the process highlighted the importance of consistency in experimental procedures. Factors such as the time drained water samples were vortexed and how long they remained at ambient temperature prior to plating had, respectively, up to a 3.5- and 7-fold effect on the colony number. Incubation of soil under lights was essential to recover high numbers of *P. cactorum* colonies.

Additional keyword: oospore.

Studies of the ecology and control of *Phytophthora cactorum* (Lebert & Cohn) Schröt. have been hampered by a lack of accurate and reliable quantification techniques. Since the late 1950's, most techniques for estimating *Phytophthora* populations have involved dilution/baiting methods using fruits, seedlings, or cotyledons (20,21). However, these techniques often have low sensitivity and high variability, fail to discriminate among different types of infective propagules, and are tedious and time-consuming. Some *Phytophthora* spp. have been enumerated successfully using soil dilution plating methods with selective media containing pimaricin and hymexazol (12,18,19). However, *P. cactorum* is inhibited by hymexazol (11,22). Without this chemical, background contamination by *Pythium* and *Mortierella* spp. is frequently a serious problem, thus reducing the usefulness of direct plating techniques for studies of *P. cactorum* populations (8).

Rahimian and Mitchell (15) modified Jeffers' extended baiting technique (10) to quantify *P. cactorum* in ginseng soils. Their technique involved sequential soil drying, moistening, flooding, draining, reflooding, chilling, and finally plating of the resultant zoospore suspension on a selective medium. Apparently, these steps enhance the germination of dormant oospores, which often germinate poorly on agar media (21), and also eliminate some common fungal contaminants, which allows plating on only partially selective media. However, our early attempts to use Rahimian and Mitchell's method to quantify *P. cactorum* populations in apple orchard soils provided highly variable results, and subsequent tests showed this technique to be extremely sensitive to minor changes in experimental procedures. Therefore, the objective of this work was to identify the sources of such experimental variability and, thereby, modify Rahimian and Mitchell's technique (15) to provide a reliable and repeatable method for quantifying *P. cactorum* propagules in apple orchard soils.

MATERIALS AND METHODS

After numerous exploratory experiments, a number of modifications to Rahimian and Mitchell's technique (15) were made. The new procedure, which we termed SADAMCAP (Soil Air-Dried And Moistened Chilled And Plated), involved sieving and air-drying of the soil, followed by moist incubation under lights for 4 days, and then flooding and chilling for 2 h. Next, the floodwater was drained, collected, vigorously mixed, incubated at room temperature for 10 min, and plated on PARP selective agar medium (5 ml of agar/9-cm diameter plate) (11). Previous experiments (I. J. Horner, *unpublished data*) showed PARP to be a more effective medium than either $PARPH_5$ (11), $PARPH_{50}$ (11), $V8_{10}B_{10}H_5P_5R_{25}$ (8), or PBNC (16). Colonies of *P. cactorum* were marked and counted after 22 h, and checked after 28 and 44 to 48 h. If confirmation of identity was desired, plates were incubated a further 4 to 7 days in the light to allow formation of sporangia, or subcultures were taken and examined further. The SADAMCAP steps are more thoroughly described in Table 1.

Influence of individual SADAMCAP variables. Once the basic SADAMCAP technique was developed, it was used in a series of experiments to determine the influence of each variable in the procedure and to identify that variable's optimum level for enumerating soil populations of *P. cactorum*. The soils examined were Odessa, Ovid, and Lima silt loams, collected in late summer from three research apple orchards in Geneva, NY. The soil was kept refrigerated for 0 to 5 months and used as required. To isolate the influence of individual variables, the standard SADAMCAP procedure was employed (Table 1), with only the variable under investigation altered. A minimum of eight replicate soil plates per treatment was used, and each experiment was conducted two to four times. Unless otherwise stated, for each repeat of an experiment a portion from one of the three stored soils was passed through a 2.36-mm sieve, air-dried, and thoroughly mixed prior to weighing 10-g subsamples into 9-cm diameter glass petri plates and randomly assigning to treatments. Data were recorded as the number of *P. cactorum* colonies per plate, and transformed using natural logarithms prior to analysis. Data from all of the

repeated experiments for a given variable were pooled and analyzed by the General Linear Model using Minitab Release 9 (Minitab Inc., State College, PA). For most of the variables tested, there were no significant treatment × experiment interactions. At each time such interactions were statistically significant, the mean square and F values were very small when compared to those for treatment effects, and in no instance affected trends in the data or the ranking of various treatments. Thus, pooling of data was considered acceptable.

Air-dry time (step 3). Soil samples were initially air-dried at 22°C for 0, 1, 2, 3, 4, 5, 7, 10, 14, 21, 35, 42, 56, 84, 100, or 175 days, prior to the subsequent steps. For repeat experiments, dry times of 0, 1, 3, 10, 35, 100, and 175 days were tested. To determine the weight of soil required for the "0 dry time" treatment, a 10-g sample from the test soil was oven-dried for 1 h, water volume calculated, and a correction made for the difference between air-dried and oven-dried soil (shown previously to approximate 2%). Three additional soils stored dry at room temperature for 3¹/₂ years were also tested.

Moist incubation regime (steps 5 and 6). To determine the effect of soil moisture during incubation, water volumes of either 2.7, 3, 3.3, 3.6, 4, 4.5, 5, 7, 9, 11, or 15 ml were added to each 10-g dried soil sample. Matric potentials of the soil water in these various treatments were estimated by interpolation from water retention curves, which were determined for each test soil by measuring the wet and dry weights of a 1.5-cm layer of sieved soil after 24-h equilibration in sintered glass Büchner funnels at various heights above a water reservoir (5). In these experiments, water volumes added in the subsequent flood/chill step were adjusted to give a total added volume of 16.3 ml in each treatment. In separate experiments (when the standard 3.8 ml of distilled water was added), temperature effects were examined by incubating for 96 h at 16, 18, 20, 22, and 24°C, and moist incubation times of 1, 12, 24, 32, 36, 40, 44, 48, 60, 72, 84, 96, 108, and 120 h were tested at 22°C.

Flood/chill (step 7). Added floodwater volumes of 7.5, 10, 12.5, 15, and 20 ml were tested. Each of these volumes resulted in saturation of the soil and the presence of free water on the surface. In separate experiments, floodwater at temperatures of either 22 or 6°C was added to plates prior to incubating at 6°C for either 20 min or 2 h. In additional experiments examining variables following the addition of 22°C floodwater, incubation temperatures (2-h duration) were tested at 2°C intervals from 2 to 20°C, as were chill (6°C) incubation durations of 1, 15, 20, 30, and 45 min, and 1, 2, 4, 8, and 16 h.

Draining (step 8). To determine if the method of draining plates affected the result, a pipette was used to drain the water either from level plates as described by Rahimian and Mitchell (15) or from the pool at the bottom edge of a plate that was sloped by placing one edge of the opened plate on its lid. In separate experiments, plates were either sloped and drained immediately after completing the chill incubation or transferred to the laboratory bench (22°C) for 10 min before sloping and draining. In other experiments, plates were either sloped for 60 s prior to drawing off water or sloped and immediately drained. Gentle agitation of soil plates for 5 s before immediate sloping and draining was compared to plates left unshaken.

Vortexing (step 9). Vortexing and shaking of the drained water/spore suspension for times of 0, 10, 30 s, 1, 2, 5, or 8 min was tested, with either immediate plating after vortexing or a period of ambient incubation between vortexing and plating. In the latter instance, the vortex time plus the period of ambient incubation totaled 10 min. Similar experiments with 0- to 8-min vortexing followed by immediate plating were carried out in a cold room (4°C). In separate experiments, the effect of vortexing for 30 s at either the start or end of the 10-min ambient incubation period was investigated.

Ambient incubation time (step 10). The time (0.5, 1, 1.5, 2, 3, 5, 7.5, 10, 15, and 20 min) that the drained water/spore suspen-

sion remained in test tubes at ambient laboratory temperature (22°C) prior to plating was investigated. Times included the standard 30-s vortexing at the start of ambient incubation. Similar experiments were also performed in a cold room with an ambient temperature of 4°C.

Dilution (step 11). The effect of subsampling and diluting the drained water/spore suspension prior to plating was tested by directly plating 0.5 ml of the suspension, and then immediately sampling another 0.5 ml from the same tube and mixing it with 2 ml of distilled water prior to plating 0.5 ml of this diluted sample.

Light. To investigate the effect of light at various steps in the SADAMCAP procedure, soil was collected at night, passed through a 4-mm sieve, and mixed in a totally dark room before assigning samples to treatments (Table 2). Samples were then resieved (2.36-mm mesh) under the light regime appropriate to their treatment. Soil samples kept in darkness throughout the SADAMCAP procedure were compared to those kept in continuous light, those exposed to light only during the 96-h moist incubation, and those exposed only during handling steps (sieving, weighing, and moistening). Exposure to light during the handling steps was less than 10 min at each step, and the source was fluorescent tubes ranging in intensity from 8 to 14 μmol m⁻²sec⁻¹. For treatments exposed to light during the drying or 96-h moist incubation steps, the light level (under fluorescent tubes) was approximately 10 μmol m⁻²sec⁻¹.

Repeated cycles. To determine the retention of viable *P. cactorum* propagules in orchard soils following repeated cycles of

TABLE 1. Summary of the SADAMCAP protocol for enumerating soil populations of *Phytophthora cactorum*

Step	Activity	Procedure
1	Collect soil	Remove litter, obtain core to 10-cm depth
2	Sieve soil	Pass soil through 2- to 2.5-mm sieve
3	Air-dry	Air-dry soil in light at room temperature, 2 to 4 days
4	Mix, plate	Thoroughly mix soil, add 10 g of soil to 9-cm diameter glass petri plate
5	Moisten	Moisten with 3.8 ml of distilled water
6	Incubate	Incubate covered plates in light at 22°C for 96 h
7	Flood/chill	Add 12.5 ml of distilled water (22°C) to plate, chill at 6°C for 2 h
8	Drain water	Slope plate on lid, immediately remove water from pool at bottom edge of plate, place in glass tube
9	Vortex/shake	Shake and/or vortex tube for 30 s
10	Ambient incubation	Incubate tubes at room temperature (≈ 22°C) for 10 min
11	Subsample/ dilute[a]/plate	Shake 5 s, remove 0.5 ml, add to 2 ml of distilled water in second tube, shake ≈ 2 s, add 0.5 ml to PARP plate, spread with bent glass rod
12	Incubate, record	Incubate at 20 to 22°C, mark and count colonies after ≈ 22, 28, and 44 h

[a] Dilution step can be varied depending on population density in test soil.

TABLE 2. Enumeration of *Phytophthora cactorum* populations in apple orchard soils as affected by exposure to light during various stages of the SADAMCAP procedure

Period of light exposure[a]	*P. cactorum* colonies[b]
None (dark all steps)	0.4 (0.3 to 0.6)[c]
Handling only (steps 2, 4, and 5)	17.6 (15.2 to 20.5)
Handling and drying only (steps 2 to 5, inclusive)	21.7 (18.7 to 25.2)
Moist incubation only (step 6)	84.7 (73.3 to 97.9)
All following collection (steps 2 to 12)	79.4 (68.7 to 91.7)

[a] Steps during the SADAMCAP procedure (as described in Table 1) during which soil samples were exposed to fluorescent light. Soil was collected at night and processed in a completely dark room at all times other than those indicated.

[b] Mean number of colonies from 20 plates per treatment (bulked data from two runs of the experiment × 10 replicate plates per treatment in each run).

[c] 95% confidence interval.

wetting and drying, individual 10-g samples were air-dried in their petri dishes following completion of the SADAMCAP procedure, and then were remoistened, flooded, and enumerated (steps 5 through 12) again. This cycle was terminated after 34
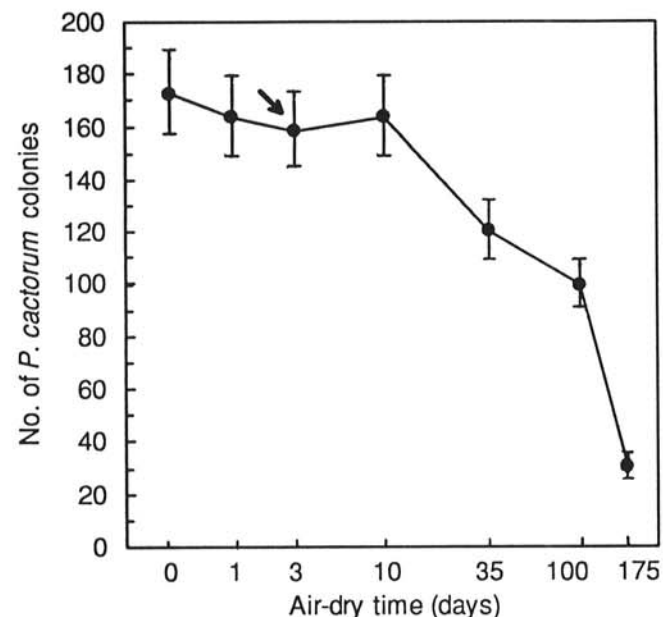

Fig. 1. Effect of air-dry time (SADAMCAP step 3) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Soil was air-dried for the indicated times and then moistened, incubated, flooded, and chilled prior to sampling an aliquot of the floodwater and plating on PARP selective medium (Table 1). Data points are mean colony counts pooled from two experiments, each with eight replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the air-dry time selected for the final SADAMCAP technique.

consecutive, sequential repetitions of the procedure in three experiments and extended to 50 sequential repetitions in two additional experiments.

Technique comparison. The SADAMCAP procedure outlined in Table 1 was compared with the serial dilution end point method (SDEP), the soil dilution plating method (SDP) on selective media, and Rahimian and Mitchell's (15) technique (RMT). Soil collected from beneath apple trees was sieved and thoroughly mixed before assigning to treatments. The experiment was repeated four times. The first two runs used an Odessa silt loam, and the third and fourth used Ovid and Canandaigua silt loams, respectively.

For RMT, 4 ml of distilled water was added to 10 g of air-dried soil. Samples were incubated under fluorescent lights at 20°C for 48 h and then flooded with 10 ml of distilled water for 48 h. Excess water (≈ 5.6 ml) was removed, replaced with 10 ml of chilled (8°C) water, and plates were incubated at 8°C for 2 h. Excess water was then drained and vortexed in a tube for 2 min before plating undiluted 0.5-ml samples on PARP plates. Care was taken to be very consistent with vortexing and handling time. Colonies were counted 20, 28, and 44 h later. For both SADAMCAP and RMT, eight replicate soil plates were used for each experiment. In order to compare the efficiency of the two methods, colony counts per plate were converted to propagules per gram of soil based upon the total weight of air-dried soil, the total volume of water at the time of sampling, and the volume of water sampled.

For SDEP, ten 20-g subsamples of each bulked soil were diluted with pasteurized soil (steamed for 60 min at 100°C) from the same orchard, using the split cup method of Tsao (20). Two-fold dilutions from $^1/_2$ to $^1/_{1,024}$ were flooded and baited with green cotyledons from germinated apple seeds (five cotyledons per sample). Five of the dilution series were flooded and baited immediately at 22°C, and the other five were air-dried for 2 days, and then moistened (7.5 ml of water/20 g of soil) and incubated under lights at 22°C for 4 days prior to flooding and baiting (10). Cotyledon margins were checked microscopically for the pres-
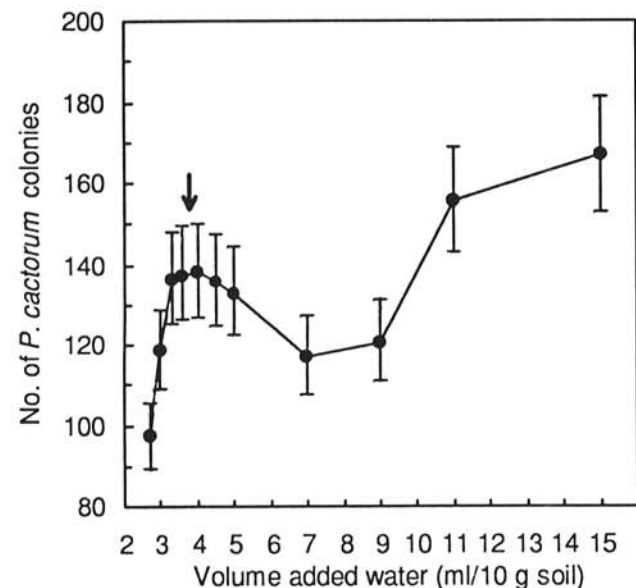

Fig. 2. Effect of water volume added prior to moist incubation (SADAMCAP step 5) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Plates containing 10 g of air-dried soil were moistened with the indicated volume of distilled water and then incubated, flooded, and chilled prior to sampling the floodwater and plating on PARP selective medium (Table 1). Data points are mean colony counts pooled from two experiments, each with eight replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the water volume selected for the final SADAMCAP technique.
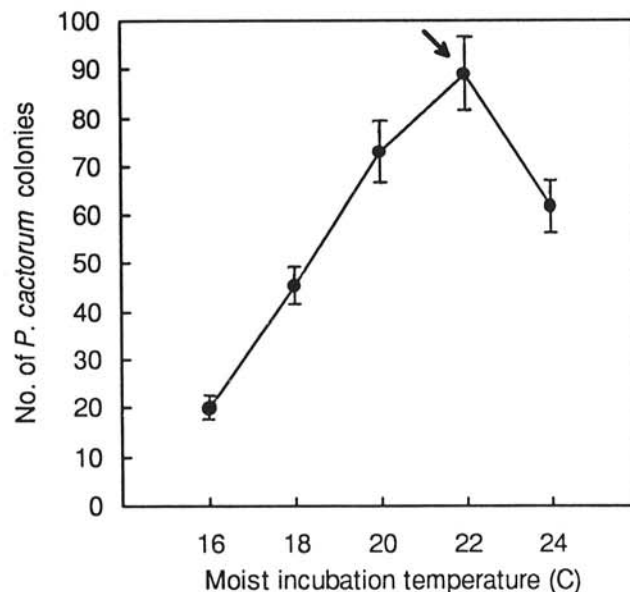

Fig. 3. Effect of moist incubation temperature (SADAMCAP step 6) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Plates of air-dried soil were moistened and incubated in the light for 96 h at the indicated temperature. Subsequently, plates were flooded, chilled at 6°C for 2 h, and then floodwater was drained, vortexed, incubated for 10 min, and a standard aliquot plated on PARP selective medium. Data points are mean colony counts pooled from four experiments, each with eight replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the moist incubation temperature selected for the final SADAMCAP technique.

ence of *P. cactorum* sporangia 4 days after baiting; samples without sporangia were incubated for a further 2 to 3 days and then rechecked.

For SDP, 2-g samples of the sieved, bulked soil were added to each of five 200-ml flasks containing 40 ml of 0.1% water agar and agitated for 2 h (8,11). Subsamples of the slurries were then diluted 10- and 100-fold with additional 0.1% water agar, and 2.0-ml aliquots were spread onto plates of the following selective media: PARP, PARPH$_5$ (11), and V8$_{10}$B$_{10}$H$_5$P$_5$R$_{25}$ (8). After 4 days of incubation under lights at 22°C, plates were gently rinsed to remove soil and *P. cactorum* colonies were observed and marked.

RESULTS

Influence of individual SADAMCAP variables. *Air-dry time (step 3).* Air-drying soil for up to 10 days had no significant effect on the number of *P. cactorum* colonies obtained (Fig. 1). Beyond 10 days of drying there was a steady decline in the measured population; after 5, 14, and 25 weeks of drying, measured populations were approximately 80, 60, and 10%, respectively, of those following 0 to 10 days of drying. When air-dried soil was stored at room temperature (20 to 24°C) for 3½ years, *P. cactorum* was still detectable in low numbers (average 2.3, 8.4, and 43.1 colonies/plate for three different soils, respectively, when dilution in step 11 was omitted).

Moist incubation regime (steps 5 and 6). With water volumes for moist incubation below 3.0 ml/10 g of soil, soil plates tended to dry out during the incubation period and colony counts were low. When soil was moistened with 3.3 to 4.5 ml of water/10 g of soil, relatively constant and high colony counts were obtained (Fig. 2). At higher water contents (5 to 9 ml/10 g of soil), there was a decline in the colony count (compared to that at 3.3 to 4.5 ml). In plates flooded with excess water (above 11 ml/10 g of soil), numbers increased sharply from those obtained from 5 to 9 ml. At the standard SADAMCAP water level of 3.8 ml/10 g of

soil, matric potentials of water in the test soils ranged from –2.0 to –4.0 kPa. At water levels of 4.5 and 2.7 ml/10 g of soil, matric potentials were approximately –1.0 and –15 to –20 kPa, respectively.

When a standard 96-h moist incubation regime was tested at various temperatures, *P. cactorum* colony counts increased progressively from 16 to 22°C and then declined at 24°C (Fig. 3). At the optimum temperature of 22°C, moist incubation periods of ≤ 32 h yielded no *P. cactorum* colonies, and few colonies were detected after 36- to 48-h periods. Colony counts increased progressively as moist incubation periods extended beyond 48 h, reaching a maximum at 96- to 120-h duration (Fig. 4). In other experiments, when moist incubation periods were extended beyond 120 h, some soil plates began to dry out and colony numbers declined (data not shown).

Flood/chill (step 7). Adding prechilled (6°C) rather than ambient (22°C) water to the soil plate prior to chilling for 2 h had no significant effect on the measured population ($P = 0.724$), with average colony counts of 74.4 and 76.9 for prechilled and ambient water, respectively. However, if the chill incubation period was shortened, colony counts were substantially less when prechilled rather than ambient floodwater was used; after a 20-min flood/chill period, an average of 35.7 colonies per plate was obtained with prechilled water, compared to 80.8 colonies with ambient water.

With the standard regime of ambient floodwater and 2-h subsequent incubation, temperatures ranging from 4 to 14°C resulted in maximal detection of *P. cactorum*, with an average of 105 to 115 colonies per plate (Fig. 5). Numbers declined outside this range, with colony counts at 2 and 20°C approximately 50 and 15% of this level, respectively.

Increasing the volume of floodwater beyond 7.5 ml proportionately lowered the subsequent colony counts per plate. This value was 142.0 when 7.5 ml was added, but was reduced to 99.6, 76.6, 53.9, and 31.0 when 10, 12.5, 15, and 20 ml of floodwater was added, respectively.
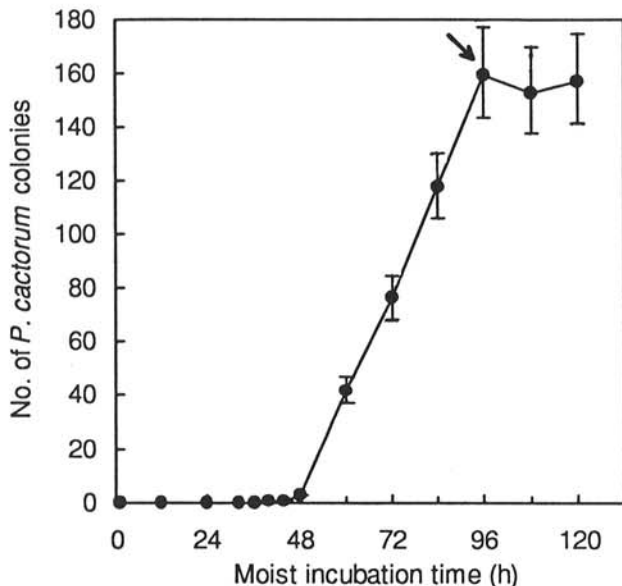

Fig. 4. Effect of moist incubation time (SADAMCAP step 6) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Plates of air-dried soil were moistened and incubated in the light at 22°C for the indicated time. Subsequently, plates were flooded, chilled at 6°C for 2 h, and then the floodwater was drained, vortexed, incubated for 10 min, and a standard aliquot plated on PARP selective medium. Data points are mean colony counts pooled from three experiments, each with eight replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the moist incubation time selected for the final SADAMCAP technique.
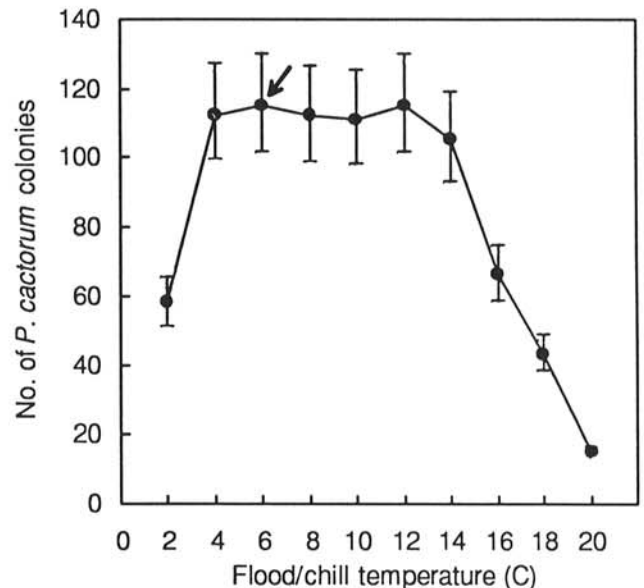
Fig. 5. Effect of flood/chill incubation temperature (SADAMCAP step 7) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Plates of air-dried soil were moistened and incubated in the light for 96 h, and then flooded and chilled at the indicated temperature for 2 h. Floodwater was then drained, vortexed, incubated for 10 min, and a standard aliquot plated on PARP selective medium. Data points are mean colony counts pooled from three experiments, each with eight replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the flood/chill incubation temperature selected for the final SADAMCAP technique.

When the duration of the chilled flooding period was varied, virtually no colonies (< 0.1 per plate) were detected after only 1 min of flooding, and only modest numbers after 15 min. Colony numbers then increased with increased flooding time, reaching a maximum after approximately 1 h. There was no significant difference ($P = 0.283$) in colony numbers for chilled flooding durations of 1 to 8 h (Fig. 6).

Draining (step 8). Sloping the soil plates and draining the water from the pool at the bottom edge resulted in significantly ($P < 0.001$) more colonies than if plates were kept level and water drained from the center of the plate (mean 105.0 colonies per plate for sloped versus 56.2 for level). In addition, draining water from the center of the plate was more difficult, more soil was drawn into the pipette, and more contaminating fungal colonies subsequently grew on agar plates.

Significantly ($P < 0.001$) more colonies were obtained when plates were sloped and drained immediately after removal from the 6°C incubator, relative to leaving plates at room temperature for 10 min prior to sloping and draining (mean values of 127.6 and 92.7 colonies per plate, respectively). Gentle agitation of soil plates prior to sloping and draining had no significant effect on the colony count ($P = 0.955$), but it made agar plates almost impossible to score without first rinsing off the excess soil debris. Draining immediately after plates were sloped resulted in significantly ($P < 0.001$) more colonies than when plates were sloped for 60 s prior to draining (159.8 and 107.6 colonies per plate, respectively).

Vortexing (step 9). When the drained water/spore suspension was vortexed and subsequently incubated at 22°C to provide a total 10-min treatment period, there was a substantial response to even short vortexing periods, with mean colony counts increasing by 259% after a 10-s vortex and by 346% after a 30-s vortex when compared to the no-vortex treatment (Fig. 7). There was no further increase in colony numbers if vortexing periods were extended beyond 30 s. However, the response to vortexing was less

acute if samples were plated immediately thereafter, i.e., without the subsequent incubation period. Given this regime, the mean colony number from the no-vortex treatment was very low, increased only slightly after a 1-min vortex, remained < 25% that of the incubated regime after a 2-min vortex, but was indistinguishable from the incubated regime after 5- or 8-min vortexing periods (Fig. 7). When samples were plated immediately after vortexing in a cold room (4°C), colony numbers increased with increasing vortex time, but remained about 20% of those obtained at 22°C (data not shown). In additional experiments, a 30-s vortex at the start of a 10-min ambient incubation period resulted in a significantly ($P < 0.001$) higher recovery of *P. cactorum* than vortexing at the end of the period (125.7 versus 43.6 colonies per plate, respectively).

Ambient incubation (step 10). The time that the drained water/spore suspension remained in test tubes at room temperature (22°C) prior to plating profoundly affected the number of colonies obtained (Fig. 8A). Samples plated within the first 2 min of draining soil plates and vortexing for 30 s yielded very low numbers of colonies, followed by a steady rise to a plateau after 7.5 min in the test tubes. No significant differences ($P = 0.539$) were observed between incubation times of 7.5 to 15 min. Colony counts from samples plated after 10 min at ambient temperature were approximately seven times higher than those plated immediately after the 30-s vortexing. At 4°C, colony numbers remained low even after 40-min incubation, and relatively high numbers were obtained only after tubes were subsequently exposed to room temperature for an additional 10 min (Fig. 8B).

The response to vortexing and subsequent ambient incubation for 10 min was additive. Vortexing for 30 s followed by 10-min ambient incubation resulted in a 15-fold increase in numbers compared to the no-vortex and no-ambient incubation treatment.

Subsampling and dilution (step 11). Subsampling and fivefold dilution of the drained water/spore suspension prior to plating had no significant effect on the calculated colony numbers ($P = 0.961$),
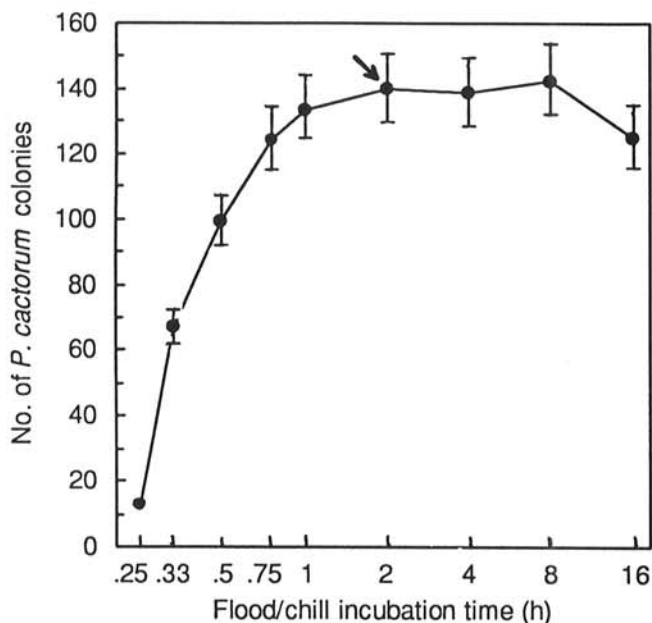

Fig. 6. Effect of flood/chill incubation time (SADAMCAP step 7) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Plates of air-dried soil were moistened and incubated in the light for 96 h, and then flooded and chilled at 6°C for the time indicated. Floodwater was then drained, vortexed, incubated for 10 min, and a standard aliquot plated on PARP selective medium. Data points are mean colony counts pooled from four experiments, each with eight replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the flood/chill incubation time selected for the final SADAMCAP technique.
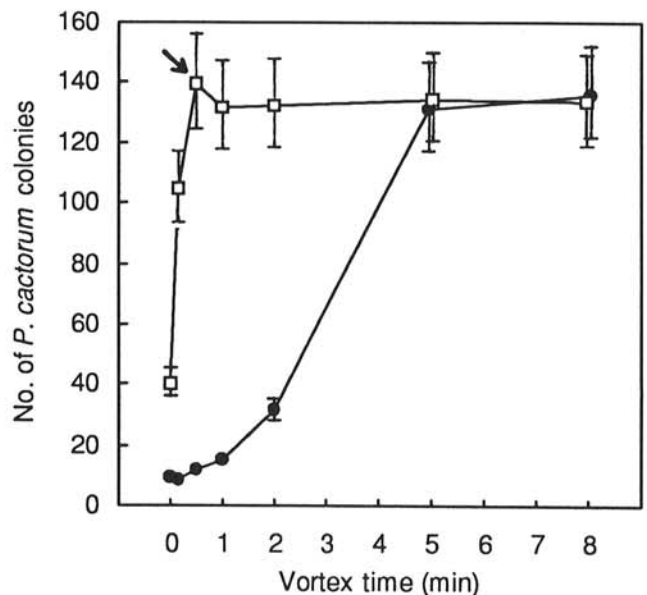
Fig. 7. Effect of vortex time (SADAMCAP step 9) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Plates of air-dried soil were moistened and incubated in the light for 96 h, flooded, chilled at 6°C for 2 h, and then floodwater was drained and vortexed for the time indicated. Solid circles (•) indicate samples that were plated on PARP selective medium immediately following vortexing. Hollow squares (□) indicate samples that were incubated at room temperature (22°C) following vortexing to provide a total 10-min treatment period prior to plating on PARP. Data points are mean colony counts pooled from two experiments, each with eight replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the vortex treatment selected for the final SADAMCAP technique.

i.e., diluted samples had approximately ¹/₅ the colonies of undiluted samples. Scoring of plates was simpler following dilution, because of a reduced number of contaminants.

Light. The response to light was extreme (Table 2). If the entire SADAMCAP procedure (prior to plating on agar) was performed in darkness, virtually no colonies were obtained. In contrast, if samples were kept in the light for all steps following collection, relatively high colony numbers resulted. However, exposure to

light only during the 96-h moist incubation period resulted in virtually the same colony numbers as when samples were kept in light throughout the SADAMCAP procedure. Brief (< 10 min) exposures to light during each of the handling steps (sieving, moistening, and flooding) substantially increased colony numbers relative to the dark treatment, but resulted in a mean colony number only 21 to 22% of those in treatments receiving light during moist incubation. Additional exposure to light during the 2- to 4-day drying period did not significantly increase colony numbers beyond those obtained when samples were illuminated during the handling steps only. Incubating agar plates in either light or dark had no effect on the colony number (data not shown).

Repeated cycles. Low levels of *P. cactorum* could still be obtained after 34 sequential wetting and drying cycles on the same 10-g soil samples. When the cycle was extended in two experiments (20 soil plates total) to 50 repetitions, recovery of *P. cactorum* continued at a very low level (< 0.1 colonies per plate). However, approximately 40% of the total colonies were obtained in the first SADAMCAP sequence (Fig. 9). When soil plates were left dry on the laboratory bench for 18 months following 50 repetitive sequences, five of the 20 plates produced one or more colonies when soils were once again moistened and flooded as before.

Technique comparison. The SDP method yielded very few *P. cactorum* colonies and major problems with contaminating fungi were encountered, even on media containing 5 µg/ml of hymexazol. The SDEP method was effective at detecting *P. cactorum* colonies at dilutions as high as ¹/₅₁₂, although the dilution end points (DEP) of detection varied among the replicate dilution series of the same soil (Table 3). For soil 1, *P. cactorum* colonies were detected at much greater dilutions after extended baiting, that is, when samples were baited after air-drying and a period of moist incubation (DEP from 64 to 256), rather than baited immediately after collection (DEP from 1 to 8). Therefore, soils 2, 3, and 4 were tested using only extended baiting.
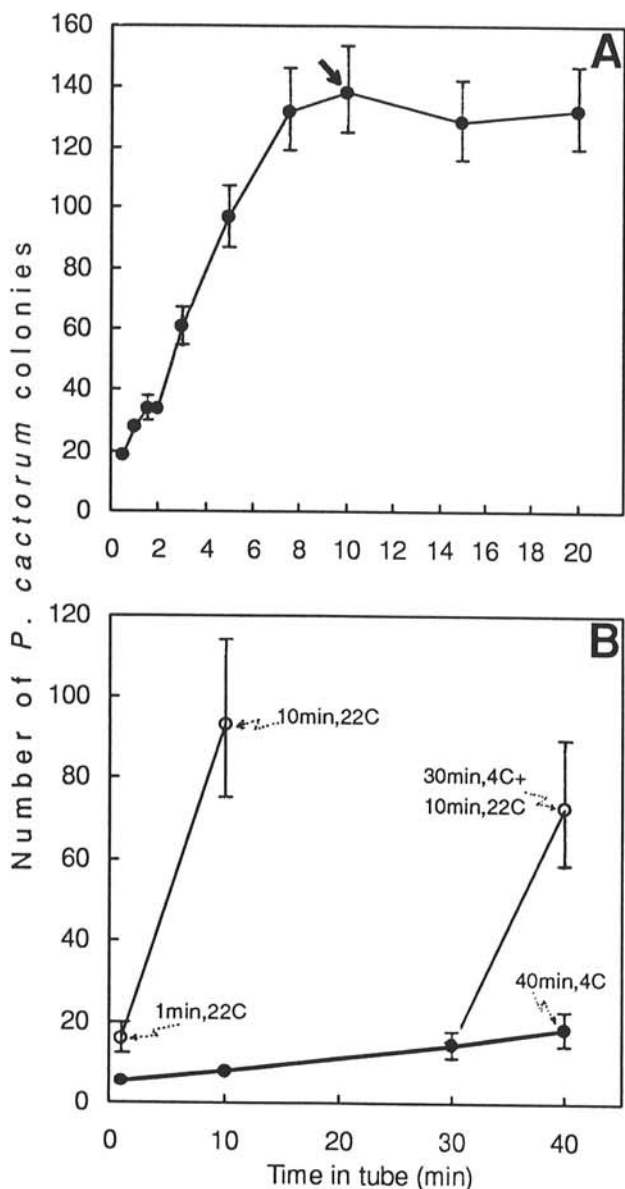

Fig. 8. Effect of the time drained water/spore suspensions remained in tubes (SADAMCAP step 10) on enumeration of the *Phytophthora cactorum* population in apple orchard soils. Plates of air-dried soil were moistened and incubated in the light for 96 h, and then flooded and chilled at 6°C for 2 h. A, At 22°C, floodwater was drained, vortexed for 30 s, incubated to provide the total treatment time indicated, and then standard aliquots were plated on PARP selective medium. B, In a 4°C cold room, floodwater was drained, vortexed for 30 s, and then incubated to provide the total incubation times indicated. In one set of treatments, tubes were incubated at 4°C for 30 min then transferred to 22°C for an additional 10 min (30min,4C+10min,22C). As a check, two treatments were vortexed for 30 s and incubated for 1 or 10 min at room temperature (1min,22C and 10min,22C, respectively). Standard aliquots were then plated on PARP selective medium. Data points are mean colony counts pooled from three or two experiments for A and B, respectively, each with eight replicate soil plates per treatment. Experiments in A and B were conducted independently. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals. The arrow indicates the time in tube at room temperature selected for the final SADAMCAP technique.
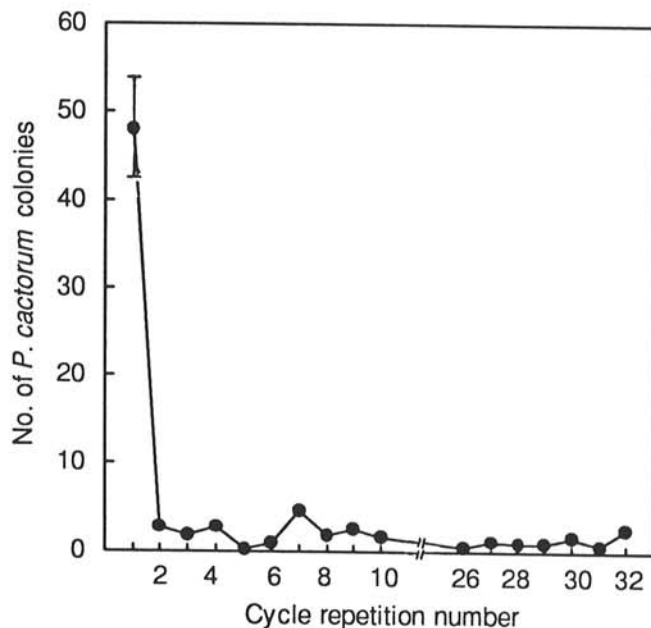

Fig. 9. Detection of *Phytophthora cactorum* from apple orchard soils after repeated wetting and drying cycles. Plates of air-dried soil were moistened and incubated in the light for 96 h, flooded, chilled at 6°C for 2 h, and then a standard aliquot of floodwater was drained and plated on PARP selective medium. Soil remaining in plates was again air-dried, moistened, flooded, chilled, drained, and a sample of the water plated. This cycle was repeated 32 times with the same soil samples, and *P. cactorum* colonies enumerated on each occasion. Data points are mean colony counts pooled from five experiments, each with 10 replicate soil plates per treatment. Data were transformed using natural logarithms prior to analysis. Bars represent 95% confidence intervals.

TABLE 3. Quantification of *Phytophthora cactorum* in four apple orchard soils using the serial dilution end point method

Soil[a]		Dilution factor											DEPs[b]
		1	2	4	8	16	32	64	128	256	512	1024	
1	No. positive plates (/5)[c]	5	5	5	5	5	5	5	3	1	1	...	64, 64, 128, 256, 256[d]
	No. positive baits (/25)[e]	25	24	25	25	24	21	20	8	1	1	...	
2	No. positive plates (/5)	5	5	5	5	5	5	3	2	2	0	...	32, 32, 128, 128[d], 256
	No. positive baits (/25)	25	25	25	25	25	22	12	5	6	0	...	
3	No. positive plates (/5)	5	5	5	5	5	5	5	5	4	2	...	256, 256, 256, 256[d], 512+
	No. positive baits (/25)	25	25	25	25	25	23	20	20	9	9	...	
4	No. positive plates (/5)	5	5	5	5	5	5	5	3	1	3	0	64, 128, 256[d], 256[d], 512
	No. positive baits (/25)	25	24	25	25	25	18	17	4	2	5	0	

[a] All samples were air-dried, moistened, and incubated in the light at 22°C for 4 days, and then flooded and baited with five apple cotyledons per plate (20 g of soil per plate). Infections were recorded microscopically after 5 days. Five replicate dilution series were prepared (using steamed soil) for each bulked and mixed soil.

[b] DEP = dilution end point (Disease Potential Index, Tsao [20]); i.e., reciprocal of the highest soil dilution to have one or more infected baits. Indices are for each of the five dilution series for each soil.

[c] Number of plates (out of five) with one or more cotyledon baits infected with *P. cactorum*.

[d] DEP reduced one level, due to a negative plate at a lesser dilution than the last positive.

[e] Total number of cotyledon baits (out of 25) infected with *P. cactorum*.

TABLE 4. Comparison of SADAMCAP with Rahimian and Mitchell's (15) technique for quantification of *Phytophthora cactorum* in four apple orchard soils

Technique	Soil[b]	*P. cactorum* colonies/plate[a]			CFU/g[d]
		Mean	Range	CV[c]	
SADAMCAP[e]	1	35.3	26 to 46	19.2	539.3
RMT[f]	1	59.5	29 to 84	34.1	207.1
SADAMCAP	2	37.6	29 to 44	12.9	575.7
RMT	2	37.9	21 to 52	33.9	131.8
SADAMCAP	3	39.9	28 to 52	20.7	610.1
RMT	3	27.4	14 to 38	28.2	95.3
SADAMCAP	4	7.5	5 to 10	25.7	114.8
RMT	4	5.5	2 to 12	68.0	19.1

[a] Number of *P. cactorum* colonies on PARP agar plates, eight replications/soil.

[b] Bulked and mixed soils.

[c] Coefficient of variability.

[d] Colony forming units per gram of air-dried soil.

[e] SADAMCAP technique as outlined in Table 1, including fivefold dilution.

[f] RMT = Rahimian and Mitchell's technique (15). Minor modifications were made to provide consistency, as described in Materials and Methods. Samples were not diluted.

SADAMCAP was more sensitive than RMT, providing calculated propagule densities that were 2.6 to 6.4 times higher (Table 4). SADAMCAP also provided more repeatable measurements, as indicated by the much smaller range of data for replicate samples of the same soil, and lower coefficients of variability than with RMT.

DISCUSSION

The results of this study dramatically illustrate the importance of consistency in experimental technique when attempting to quantify soil populations of *P. cactorum*. Some procedural factors that were expected to be of minor importance proved to have a substantial effect on the measured population, illustrating that small inconsistencies in these steps could result in highly variable and misleading data. The two factors that most profoundly affected results were the time that drained water/spore suspensions were incubated on the laboratory bench prior to plating (step 10), and the timing and duration of vortexing (step 9). The dramatic increase in *P. cactorum* colony numbers that developed as incubation time was extended from 0 to 10 min indicated the importance of this step. Plating of samples at arbitrary times within the first 7 min of ambient incubation would lead to considerable variability in results, and probably accounted for most of the large inconsistencies we found in preliminary experiments using RMT.

Vortexing of the samples also had a major effect on the colony number, particularly if vortexing was done at the start rather than at the end of the 10-min ambient incubation period. Rahimian and Mitchell (15) recommended vortexing samples for 2 to 3 min prior to plating, but our experiments showed that this did little to increase the colony count unless vortexing was accompanied by an ambient incubation period of at least 5 to 7 min. When such incubation was provided, vortexing beyond 30 s was unnecessary as counts were not further increased. Colony numbers obtained after 5- to 8-min vortexing and no subsequent incubation were comparable to colony numbers following a standard 30-s vortex and 10-min incubation, as indicated by the convergence of the two lines in Figure 7. Incubation at room temperature is inherent in the extended vortexing and would account for this convergence.

Colonies on agar plates were scored at a much earlier stage in our study than previously reported by other workers (8,15). After 20- to 24-h incubation, most *P. cactorum* colonies were merely a few fine hyphae, with colony diameters about 2 to 3 mm. Nevertheless, with experience it was possible to identify *P. cactorum* colonies at this stage with a high level of accuracy, with subsequent checks and corrections after about 28- and 40- to 48-h incubation. More than 99% of colonies identified as *P. cactorum* at 48 h were later confirmed as such. Other *Phytophthora* spp. (e.g., *P. citricola* Sawada) were rarely observed and could usually be distinguished on the original plate at 48 h. By scoring plates at such an early stage, dense populations of *P. cactorum* colonies could be enumerated before they coalesced or were overgrown by *Pythium* spp. and other fast-growing contaminants, although these were rarely a problem.

The observed responses to variables at different steps in the process gave some insight into the physiology and ecology of *P. cactorum*, particularly since the experiments were done with naturally infested field soil. Presumably, SADAMCAP provided a measure of only oospore (and perhaps chlamydospore) populations of *P. cactorum*, since these are the only propagules likely to survive the air-drying step (10,13,17). Colony-forming propagules (confirmed to be zoospores in limited microscopic examinations) were not detected in plated floodwater unless a minimum moist incubation period of 36 h (optimum 96 h) was provided beforehand, suggesting that they originated from the germination of such dormant structures during the moist incubation period rather than from sporangia or mycelium that had survived the air-drying process. Viable sporangia of *P. cactorum* and other *Phytophthora* spp. have been shown to release zoospores within 12 min to several hours in flooded soils (5,7,23). Maximum colony counts at a moist incubation temperature of 22°C were consistent with previous studies of temperature optima for oospore germi-

nation (1), sporangial formation (14), and detection using extended baiting (10).

The strong positive response to light during the moist incubation period was presumably related to the light requirement for optimal oospore germination, a phenomenon noted in agar cultures by many workers (1,2,3). That our results were obtained using naturally infested field soil suggested that the germination response to light was more than a laboratory artifact, and may have ecological significance. Similarly, Meyer and Schönbeck (14) noted that oospores of *P. cactorum* germinated only at the soil surface where light was available, and Banihashemi and Mitchell (2) found increased infection of safflower seedlings by this fungus when the soil was exposed to light rather than kept in darkness.

A higher rate of oospore germination, sporangial formation, and subsequent bait colonization has been documented for a number of *Phytophthora* spp., when soil was preincubated under moist rather than saturated conditions (5,9,10,17). Similarly, we obtained relatively high colony counts after soils were incubated moist (−10 to −1 kPa) rather than saturated (0 kPa) for 96 h. However, when water volumes were further increased so that soil was flooded during the 96-h moist incubation, subsequent colony numbers increased. An explanation for this bimodal response to soil water volumes (Fig. 2) may lie in the complex interactions between requirements for oxygen and water (5,7) and, in particular, differences in water and oxygen requirements for sporangial formation on the soil surface and in particles buried in the soil (5,6,7,14,17), summarized by Gisi (7; Fig. 1). Whatever the reason, high water volumes that produced flooding during the moist incubation period were avoided in the final SADAMCAP technique, in part because of slightly higher variability noted at these volumes, but mainly to prevent premature zoosporogenesis and asynchronous release of zoospores. For similar reasons, the 48-h flood incubation of RMT was eliminated in SADAMCAP, and the moist incubation period extended to 96 h. In this way, sporangia produced from germinated oospores could be maintained to undergo synchronous zoosporogenesis only when flooded and chilled for 2 h in SADAMCAP step 7. Browne and Mircetich (4) found that *P. cactorum* sporangia do not undergo zoosporogenesis at −2.0 kPa matric potential, roughly equivalent to SADAMCAP's moist incubation.

Results of the chill temperature experiments (Fig. 4) suggested that in naturally infested soils *P. cactorum* sporangia undergo zoosporogenesis over a wide range of temperatures, with an optimum from 4 to 14°C, and a minimum and maximum of ≤ 2 and ≥ 20°C, respectively. This differed from the findings of Rahimian and Mitchell (15) working with pure cultures, who found a clear peak in zoospore production at 8°C, with much lower levels at 4 and 12°C. It was not clear at exactly what point during the SADAMCAP process the bulk of zoospore differentiation, release, and encystment occur. Rahimian and Mitchell (15) implied that zoospores were released during the flood/chill step and, subsequently, encysted when the drained suspension was vortexed. However, in this study, the sevenfold increase in the colony number when the water/spore suspensions were incubated in glass tubes at room temperature for 10 min after vortexing (Fig. 8), and the corresponding lack of increase when flooded soil plates were incubated at ambient temperature for 10 min prior to draining, collectively suggested that the deciduous sporangia of *P. cactorum* may be recovered during the draining process, with subsequent releases of zoospores while in the tube.

For most of the variables tested, the levels that resulted in optimal colony numbers, when possible near the middle of a plateau region, were selected for the final SADAMCAP protocol. This approach should minimize the effects of minor variations in experimental techniques. In comparative tests, SADAMCAP proved superior to other quantification techniques. Although the SDEP method was able to detect *P. cactorum* at relatively high levels, populations as indicated by the DEP were not always consistent

with replicate dilution series from the same bulked soil. SDEP also proved very tedious and time-consuming, and would be impractical for studies involving large numbers of samples. Both RMT and SADAMCAP took substantially less time per sample than SDEP. As a result of cumulative fine-tuning modifications to RMT (e.g., incubation temperatures, water volumes, draining methods, and early scoring), together with some major changes (elimination of the 48-h flood incubation and, most importantly, the incubation of drained samples at room temperature for 10 min between vortexing and plating), SADAMCAP was a vastly improved technique for measuring *P. cactorum* populations. It proved superior to RMT both in terms of sensitivity (allowing dilution of most samples, with the subsequent diminution of contaminants and simplification of scoring on agar plates) and precision, giving consistent results with only small variations between replicate samples of the same bulked soil.

Attempts to translate "colonies per plate" to "propagules per gram of soil" are somewhat arbitrary and rely on a number of assumptions concerning the biology of oospores, sporangia, and zoospores. Thus, SADAMCAP should ideally be used to provide measurements of relative *P. cactorum* populations under different conditions or treatments. We found it to be an effective tool for assessing soil populations of *P. cactorum* in a wide range of apple and strawberry soils (I. J. Horner and W. F. Wilcox, *unpublished data*), and believe that, with consistent application of the technique, SADAMCAP should prove useful for various studies on the ecology of *P. cactorum* and the diseases it causes.

LITERATURE CITED

1. Banihashemi, Z., and Mitchell, J. E. 1976. Factors affecting oospore germination in *Phytophthora cactorum*, the incitant of apple collar rot. Phytopathology 66:443-448.
2. Banihashemi, Z., and Mitchell, J. E. 1981. Photobiology of oospores of *Phytophthora cactorum*. (Abstr.) Phytopathology 71:201.
3. Berg, L. A., and Gallegly, M. E. 1966. Effect of light on oospore germination in species of *Phytophthora*. (Abstr.) Phytopathology 56:583.
4. Browne, G. T., and Mircetich, S. M. 1988. Effects of flood duration on the development of *Phytophthora* root and crown rots of apple. Phytopathology 78:846-851.
5. Duniway, J. M. 1979. Water relations of water molds. Annu. Rev. Phytopathol. 17:431-460.
6. Gisi, U. 1975. Investigations on the soil phase of *Phytophthora cactorum* (Leb. et Cohn) Schroet. with fluorescent optical direct observation. Z. Pflanzenkrankh. Pflanzenschutz 82:355-377.
7. Gisi, U. 1983. Biophysical aspects of the development of *Phytophthora*. Pages 109-119 in: *Phytophthora*. Its Biology, Taxonomy, Ecology and Control. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. American Phytopathological Society, St. Paul, MN.
8. Harris, D. C., and Bielenin, A. 1986. Evaluation of selective media and bait methods for estimating *Phytophthora cactorum* in apple orchard soils. Plant Pathol. 35:565-574.
9. Ioannou, N., and Grogan, R. G. 1984. Water requirements for sporangium formation by *Phytophthora parasitica* in relation to bioassay in soil. Plant Dis. 68:1043-1048.
10. Jeffers, S. N., and Aldwinckle, H. S. 1987. Enhancing detection of *Phytophthora cactorum* in naturally infested soil. Phytopathology 77:1475-1482.
11. Jeffers, S. N., and Martin, S. B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Dis. 70:1038-1043.
12. Lutz, A. L., and Menge, J. A. 1991. Population fluctuations and the numbers and types of propagules of *Phytophthora parasitica* that occur in irrigated citrus groves. Plant Dis. 75:173-179.
13. McIntosh, D. L. 1972. Effects of soil water suction, soil temperature, carbon and nitrogen amendments, and host rootlets on survival in soil of zoospores of *Phytophthora cactorum*. Can. J. Bot. 50:269-272.
14. Meyer, D., and Schönbeck, F. 1975. Investigations on the development of *Phytophthora cactorum* (Leb. et Cohn) Schroet. in soil. Z. Pflanzenkrankh. Pflanzenschutz 82:337-354.
15. Rahimian, M. K., and Mitchell, J. E. 1988. Detection and quantification of *Phytophthora cactorum* in naturally infested soils. Phytopathology 78:949-952.
16. Schmitthenner, A. F. 1973. Isolation and identification methods for *Phytophthora* and *Pythium*. Pages 94-110 in: Proc. Woody Ornamental

Dis. Workshop. University of Missouri, Columbia.

17. Sneh, B., and McIntosh, D. L. 1974. Studies on the behavior and survival of *Phytophthora cactorum*. Can. J. Bot. 52:795-802.

18. Timmer, L. W., Sandler, H. A., Graham, J. H., and Zitko, S. E. 1988. Sampling citrus orchards in Florida to estimate populations of *Phytophthora parasitica*. Phytopathology 78:940-944.

19. Timmer, L. W., Zitko, S. E., Sandler, H. A., and Graham, J. H. 1989. Seasonal and spatial analysis of populations of *Phytophthora parasitica* in citrus orchards in Florida. Plant Dis. 73:810-813.

20. Tsao, P. H. 1960. A serial dilution end-point method for estimating disease potentials of citrus phytophthoras in soil. Phytopathology 50:717-724.

21. Tsao, P. H. 1983. Factors affecting isolation and quantitation of *Phytophthora* from soil. Pages 219-236 in: *Phytophthora*. Its Biology, Taxonomy, Ecology and Control. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. American Phytopathological Society, St. Paul, MN.

22. Tsao, P. H., and Guy, S. O. 1977. Inhibition of *Mortierella* and *Pythium* in a *Phytophthora*-isolation medium containing hymexazol. Phytopathology 67:796-801.

23. Wilcox, W. F., and Mircetich, S. M. 1985. Influence of soil water matric potential on the development of Phytophthora root and crown rots of Mahaleb cherry. Phytopathology 75:648-653.