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Role of Ammonia and Calcium in Lysis of Zoospores of *Phytophthora cactorum* by *Bacillus cereus* Strain UW85

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GILBERT, G. S., HANDELSMAN, J., AND PARKE, J. L. 1990. Role of ammonia and calcium in lysis of zoospores of *Phytophthora cactorum* by *Bacillus cereus* strain UW85. *Experimental Mycology* 14, 1–8. Cultures and cell-free culture filtrates of the biological control agent *Bacillus cereus* strain UW85 lysed zoospores of *Phytophthora cactorum in vitro*. Changes in the ionic composition of the growth medium caused by growth of UW85 account for the lytic activity. UW85 raised the pH, excreted ammonia, and removed calcium from the medium during growth and sporulation. Zoospores lysed when pCa^{2+}:pNH_3 was greater than 0.8. The lytic activity was produced in uninoculated growth medium by adding ammonium chloride and base to create a pCa^{2+}:pNH_3 ratio similar to that of UW85 culture filtrate.

INDEX DESCRIPTORS: *Phytophthora cactorum; Bacillus cereus;* ammonia; calcium; cation; zoospore; cyst; lysis.

Zoospores of *Phytophthora* de Bary have long been the focus of both developmental and ecological studies. Zoospores are motile asexual spores that lack a cell wall. After a period of motility, zoospores lose their flagella and form cell walls to become cysts, which can then germinate to form hyphae. Encystment is induced by physical factors as well as by a wide range of organic and inorganic compounds (Newhook *et al.*, 1981; Byrt *et al.*, 1982a, b; Irving and Grant, 1984; Grant *et al.*, 1985, 1986; Griffith *et al.*, 1988). At high concentrations many of these same compounds will damage the zoospores (Byrt *et al.*, 1982a, b; Grant *et al.*, 1986).

Culture filtrates of *Bacillus cereus* Franklin and Franklin strain UW85 lyse *Phytophthora* zoospores *in vitro*, and since UW85 is of interest because of its potential as a biological control agent (Handelsman *et al.*, 1988), we investigated the mechanism of zoospore lysis. The results presented here suggest that the high ratio of NH_3 to Ca^{2+} in UW85 culture filtrate is responsible for zoospore lysis.

MATERIALS AND METHODS

Cultures

Phytophthora cactorum strain 36C1. P. cactorum strain 36C was isolated from a rotting American ginseng root (*Panax quinquefolium* L.) from Blue River, Wisconsin (Grant County). A single zoospore from that isolation was used to infect a healthy ginseng plant, from which strain 36C1 was isolated.

Two milliliters containing approximately 10^5 zoospores ml^{-1} was spread onto V-8 juice agar Petri plates [200 ml V-8 juice (Campbell Soup Co., Camden, NJ),[1] 2 g $CaCO_3$, 15 g Bacto-agar (Difco, Detroit, MI), 800 ml H_2O] and incubated for 3 days at 22°C under continuous fluorescent light (25.7 $\mu E\ m^{-2}\ s^{-1}$). The medium plus mycelium was divided in half, and one portion was transferred to a separate Petri plate. Each half was flooded with 15 ml

[1] Trade and company names are included for the benefit of the reader and do not imply endorsement by the University of Wisconsin.

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sterile distilled water and returned to the same incubation conditions for 1 day, during which time sporangia formed. The sporangia from the two halves of the culture were gently dislodged with a rubber policeperson and decanted into a sterile plastic Petri plate to which the sporangia quickly attached. The liquid was replaced with 20 ml 4°C Type I water (Milli-Q, Bedford, MA). The sporangia were incubated at 22°C for 20 min, during which time zoospores were released, resulting in a concentration of about 10^5 zoospores ml^{-1}. Zoospores were separated from sporangia and mycelial fragments by passage through nylon mesh with 15-μm openings.

Bacillus cereus strain UW85. B. cereus strain UW85 was isolated from the rhizosphere of an alfalfa plant from the Arlington Experiment Station, University of Wisconsin (Columbia County, WI).

Two hundred fifty milliliters casamino acid broth (CB),[2] pH 5.2 [1%(w/v) casein, acid hydrolysate; 0.5%(w/v) glucose; 8.04 m*M* K_2HPO_4; 4.41 m*M* KH_2PO_4; 1.52 m*M* $(NH_4)_2SO_4$; 0.34 m*M* citric acid–trisodium salt dihydrate; 0.17 m*M* $MgSO_4 \cdot 7H_2O$] was inoculated with a loopful of bacteria from a CB agar slant culture, and incubated at 28°C on a shaker (66 cycles min^{-1}) for 6 days. The culture was then checked with a phase contrast microscope (400×) for release of spores. The spores and cells were removed from the broth by centrifugation at 30,000*g*, followed by filtration through a 0.22-μm nitrocellulose filter (Gelman Sciences, Ann Arbor, MI). The resulting culture filtrate (BcF) (pH 8.94) was stored at −20°C.

Chemicals

The chemicals used were from the following sources: $CaCl_2 \cdot 2H_2O$, KCl, NaCl, and $CaCO_3$ (Tested Reagent Grade, Amend, Irvington, NJ); $MgCl_2 \cdot 7H_2O$ and $Na_3C_6H_5O_7 \cdot 2H_2O$ (ACS, Columbus Chemical Industries, Columbus, WI); NaOH and NH_4Cl (Analytical Reagent, Mallinckrodt, Paris, KY); HCl (Technical Grade, Columbus Chemical Industries); $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, dipicolinic acid (pyridine-2,6-dicarboxylic acid), casein (acid hydrolysate, Type 1), and ascorbic acid (Sigma, St. Louis, MO); glacial acetic acid and acetone (Baker Analyzed Reagent, J. T. Baker, Phillipsburg, NJ); glutaraldehyde (practical grade, J. T. Baker).

Assays

Three experiments were performed. The first experiment tested the effect on zoospores of 10% CB or 10%BcF, with additional 0, 1, 2, 3, 4, or 5 m*M* $CaCl_2$, $MgCl_2$, NaCl, or KCl, at pH 4, 5, 6, 7, 8, or 9, in all possible combinations. The second experiment tested final concentrations of $CaCl_2$ at 0, 0.2, 0.4, 0.6, 0.8, and 1.0 m*M*, and the third tested the effect of final concentrations of NH_4Cl at 0, 0.1, 1, 5, 10, and 20 m*M*, each in the same combinations with CB, BcF, and pH as in the first experiment. The zoospore lysis assay was a modification of that used by Byrt *et al.* (1982a). The pH of BcF or CB was adjusted with 1 *M* NaOH to pH 9.0. These solutions were diluted to 20% with water or salt solutions at 2.22 times the desired final cation concentration. The solutions were then adjusted to the desired pH with 1 *M* NaOH or 1 *M* HCl, and 0.1-ml aliquots of each solution were placed in wells of microtiter assay plates (Microtest III, Falcon). A control treatment of water was included. Zoospores were prepared as described above, and 0.1 ml zoospore suspension (10^4 zoospores) was added to each well with an eight-channel multipipette (Titertek, Finland) to make up 50% of the final volume. The plates were incubated under fluorescent light for 1 h at 22°C. The zoospores

[2] Abbreviations used: BcF, cell-free UW85 culture filtrate; CB, casamino acid broth; DPA, dipicolinic acid; ICPES, inductively coupled plasma emission spectrophotometry;

were then fixed with 0.05 ml of 4% glutaraldehyde in water. Spores settled to the bottom of the wells, and were classified according to shape and the condition of the cytoplasm by observing with an inverted microscope (200× magnification).

The spores were counted as zoospores, cysts, germlings, and lysed cells. Spores that retained the characteristic shape of zoospores were considered motile (Fig. 1A). Flagella could usually be discerned on motile cells. Spores with a smooth, refractile cell wall and a well-organized cytoplasm were counted as cysts (Fig. 1B). Cysts with germ tubes at least as long as the cyst diameter were counted as germlings (Fig. 1C). Germlings exceeded 9% of the total only at pH 4, at which $CaCl_2$ was 1 m*M* or greater. Lysed cells included two morphologies: swollen cells with an apparently intact plasma membrane but with highly disrupted cytoplasm and no cell wall (Fig. 1D), and cells in which the integrity of the plasma membrane appeared to be lost, though the contents of the spores remained together (Fig. 1E).

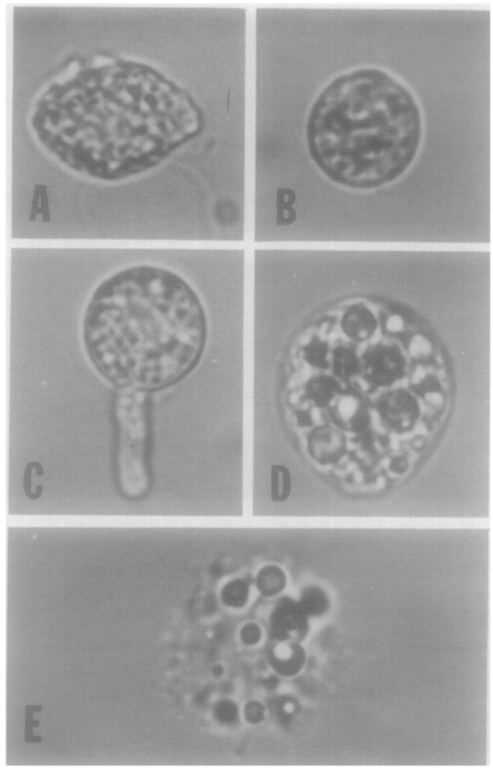

FIG. 1. Light micrographs of glutaraldehyde-fixed *Phytophthora cactorum* spore forms described in the text. (A) Zoospore, (B) cyst, (C) germling, (D, E) lysed cells. 1800×.

Each treatment was replicated in seven wells in the first experiment, and nine in the second and third experiments. Replicate wells were blocked over three time points for each experiment, with two or three replicates of each treatment in each block. The data from all blocks were combined for analysis.

To determine whether zoospores disintegrate after lysis, and therefore are not countable, the same number of zoospores was added to wells containing either glutaraldehyde or BcF ($n = 8$). In the glutaraldehyde treatment, 98% of spores were scored as zoospores, and in the BcF treatment 98% of spores were scored as lysed. All spores in nine contiguous photofields across the middle of each well were counted (1.45 mm^2 per well). The number of spores counted did not differ significantly between the two treatments ($P = 0.88$ by t test). This indicates that lysed spores remained intact and could be counted as efficiently as the motile spores.

To determine whether the lysed cells were viable we assessed their ability to germinate when provided with appropriate conditions. Zoospores in microtiter wells were treated with 1% BcF, and after 1 h the wells contained 21% cysts, 2% germlings, and 77% lysed cells (59% of the type represented in Fig. 1D, 18% of the type represented in Fig. 1E). Fifty microliters of 1% glucose in 5 m*M* $CaCl_2$ at pH 4 was added to the wells to stimulate cyst germination. After 2 h the spores were scored as 3% cysts, 15% germlings, and 82% lysed, indicating that the lysed cells did not germinate. Although these results strongly suggest that the lysed cells are not viable, we

cannot eliminate the unlikely possibility that they are alive but unable to germinate.

Statistical Analysis

Because the data are the results of three separate experiments, determination of a formal predictive regression model is statistically improper. The models presented are for descriptive purposes only. A protected LSD test (Snedecor and Cochran, 1980) was performed on the data presented in Fig. 2, both on all three experiments combined and on each separately, with the same results, except as noted. Means and standard errors for Fig. 2 were calculated from the data from all experiments, since the treatments were identical in each case. Mean values in all other graphs are for treatments in each independent experiment.

Chemical Analysis

The concentration of dipicolinic acid (DPA) in 100% BcF was determined according to the method of Janssen *et al.* (1958), except that the solutions were not autoclaved or centrifuged. The standard curve was determined with dipicolinic acid in CB and was linear over the range 60 to 600 μM [μM DPA = $(A_{440} - 0.01594)/0.001225$; $R^2 = 0.986$). The reference cell contained CB plus water. Absorption was measured on a Beckman DB-G spectrophotometer.

CB, BcF, and filtered zoospore suspension were analyzed for elemental composition by the Soil and Plant Analysis Laboratory, University of Wisconsin—Extension. P, K, Ca, Mg, S, and Na were analyzed by inductively coupled plasma emission spectrophotometry (ICPES). NH_4^+ and NO_3^- were analyzed by Kjeldahl distillation. Cl^- was determined by potentiometric titration (Schulte *et al.*, 1987).

The computer program PHREEQE (Parkhurst *et al.*, 1985) was used to thermodynamically predict the equilibrium concentrations of ion species in the various treatments. The data base was modified to calculate N-species distributions from NH_4^+ concentrations. Including citrate in the model did not change distributions of ions of interest by more than 3.8%, and generally less than 2%. Citrate and other organic compounds were not included in PHREEQE to estimate the ion speciations presented here.

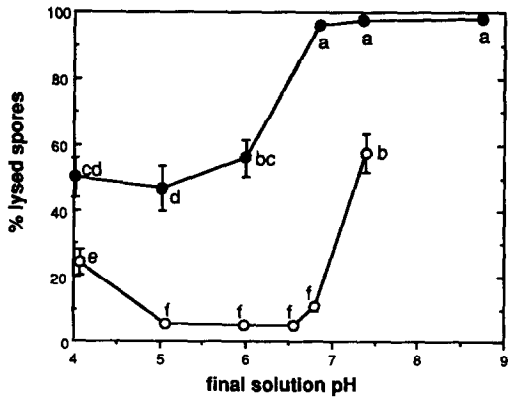

FIG. 2. Zoospores lysed by (○) 10% CB and (●) 10% BcF at various pH's. Each point represents the mean of three experiments ± SE ($n = 25$). Means with the same letter are not significantly different ($P = 0.05$).

RESULTS

Effect of pH

In three separate experiments, 20% CB or BcF in water was adjusted to pH 4, 5, 6, 7, 8, or 9, and mixed with an equal volume of zoospores. The relationship between the final solution pH and zoospore lysis is shown in Fig. 2. BcF (initial pH 8.9), the culture filtrate of UW85 grown in CB (initial pH 5.2), lysed *P. cactorum* zoospores *in vitro*, whereas CB did not. BcF-induced lysis was reduced below pH 7, and CB lysed zoospores at pH 4 and 9. However, BcF caused significantly more lysis than did CB at all levels of pH ($P = 0.05$) except pH 4 in the second experiment, indicating

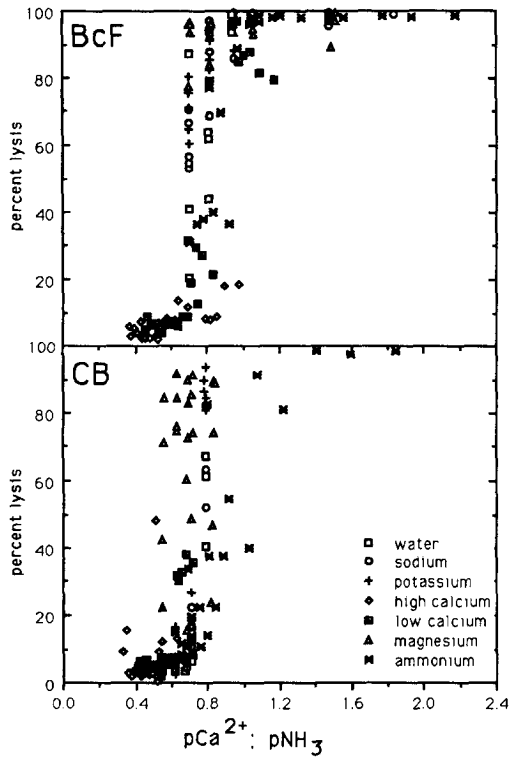

FIG. 3. Effect of pCa^{2+}:pNH_3 on zoospore lysis in 10% CB and 10% BcF, with the addition of various salts. Each point represents the mean of seven or nine replicates per treatment. pH 4 treatments are not shown.

that the high initial pH of BcF is not the only factor responsible for lysis.

Inorganic Effects

UW85 caused significant changes in the inorganic composition of CB. The contributions of the major elements and compounds by CB, BcF, and zoospore suspensions to 200-μl assay solutions are shown in Table 1. The distribution of ion species (i.e., free metals, ion pairs, solids) is influenced by both the total concentration of the elements and the pH of the solution, and different ion species of the same element have different biological activities. PHREEQE was used to model the equilibrium ion species distribution in the final solutions containing equal volumes of 10% CB or BcF and zoospore suspension, plus any added salts at each pH tested. Ca^{2+} is more available as a free metal at low pH than at high pH. Below pH 7 ammonia is present almost entirely as the protonated ammonium ion (NH_4^+), but as the pH rises above 7, deprotonated ammonia (NH_3) becomes more prevalent, and the concentrations of NH_3 and NH_4^+ are nearly equal at pH 9. Na^+ and K^+ are not affected by pH. The higher concentration of Na in BcF than in CB was not observed in any of three similar experiments.

Percentage zoospore lysis was plotted against the free ion concentrations for each ionic species independently and in various combinations. Lysis was correlated with the ratio of ammonia to free calcium, and was not correlated with any other individual ions or any combinations of ions. Percentage lysis versus pCa^{2+}:pNH_3 (where $pX = -\log$ molar concentration of X) provides the best model for all treatments in the three experiments in the pH range 5 to 9. The high percentage lysis observed in

TABLE 1
Micromoles of Each Element Contributed to a 200-μl Assay Volume by Each Solution, as Determined by ICPES, Potentiometric Titration, or Kjeldahl Analysis

	μmol per 200-μl assay							
Solution	Ca	Mg	Na	K	NH_4^+	S	P	Cl
CB	0.0199	0.0448	0.0194	0.0415	0.1498	0.0484	0.0261	0.0037
BcF	0.0001	0.0004	0.0810	0.0257	0.5930	0.0245	0.0070	0.0034
Zoospores	0.0042	0.0007	0.0213	0.0077	ND[a]	0.0008	ND	0.0183

[a] Not detected.

treatments at pH 4, particularly in 10% CB, do not fit this model well. This may be due to toxicity caused by the increased availability of Al^{3+} at pH 4 (Lindsay, 1979). The concentrations of Al^{3+} in CB and BcF were below the limits of detection by ICPES (<13 μM), and therefore could not be included in the PHREEQE models. All treatments at pH 4 were not included in the development of the model. Plots of percentage lysis versus pCa^{2+}:PNH_3 for all treatments except those at pH 4 are shown in Fig. 3. Less than 8% lysis was observed when zoospores were treated with water (pH 5.8).

Effect of Added Salts on Lysis

BcF treatments showed a distinct lytic threshold at pCa^{2+}:PNH_3 = 0.8. Adding calcium or acid to BcF lowered the pCa^{2+}:PNH_3 below 0.8, and very little lysis occurred. Above that threshold, nearly all the zoospores lysed (Fig. 3). The addition of NH_4Cl to CB at high pH conferred on CB lytic properties similar to those of BcF. Percentage lysis in CB treatments shows a similar lytic threshold at pCa^{2+}:PNH_3 = 0.8. The addition of $MgCl_2$ to CB also induced lysis.

DISCUSSION

We have shown that lysis of zoospores of *P. cactorum* by culture filtrates of *B. cereus* strain UW85 can be largely accounted for by the change in the ionic composition of the culture medium resulting from growth and sporulation of UW85. Filtrates of fully sporulated cultures of UW85 which had high zoospore lysis activity had a much higher pCa^{2+}:PNH_3 ratio than did the uninoculated culture medium, which had little zoospore lysis activity.

The ionic changes that we measured are consistent with the known effects of *Bacillus* on its culture medium. When *Bacillus* sporulates, it sequesters large amounts of calcium and some other di- and trivalent cations in its endospores with the chelator DPA (Rosen, 1982). *Bacillus* also produces large amounts of ammonium by metabolizing amino acids, and in the process, increases the pH of the medium. The analyses presented in Table 1 confirm that strain UW85 produced these changes in the medium during growth in CB. In addition, UW85 decreases the Mg^{2+} concentration of CB from approximately 24 to 2 μM. Zoospores lysed in the presence of millimolar concentrations of magnesium in CB, but apparently magnesium is not important in zoospore lysis by BcF.

The chemical analyses of CB and BcF in Table 1 measured the total concentrations of those elements in all their chemical species. The activity of the ions varies greatly depending on pH and the concentration of other chemical species. The computer program PHREEQE provided predictions of the equilibrium chemical species distributions for CB and BcF in the various treatments used in the experiments. The most significant pH-related changes in the ion species distribution are the decrease in Ca^{2+} and NH_4^+ activities and an increase in NH_3 activity with increasing pH. There are numerous reports in the literature on the toxicity of NH_3 to *Phytophthora* and other microorganisms (Gilpatrick, 1969; Schippers *et al.*, 1982; Rush and Lyda, 1982). Tsao and Oster (1981) demonstrated that NH_3, but not NH_4^+, is toxic to sporangia and chlamydospores of *Phytophthora* species, but Grant *et al.* (1986) showed that 3 mM NH_4^+ lyses zoospores. Grant *et al.* (1986) also reported that both divalent cation chelators and Mg^{2+} lyse *P. palmivora* (Butler) Butler zoospores, and that Ca^{2+} blocks zoospore lysis caused by several cations including Mg^{2+}, but not by NH_4^+. In the present study, lysis is most prevalent at high pH, when the activity of Ca^{2+} is lowest and NH_3 is highest, or when activity of Mg^{2+} is high. These results are consistent with previously published work on ionic conditions that damage zoospores.

Although zoospore lysis is strongly affected by pH, Fig. 2 indicates that the high pH of BcF is not sufficient to explain all of the lysis.

In the microtiter plate assay, zoospores remained either motile, encysted, or lysed. In nonlytic treatments, spores that did not remain motile formed cysts, whereas in lytic treatments, few cysts formed, creating an inverse relationship between the percentages of lysed and motile cells (data not shown).

Although we cannot dismiss the possibility that organic chelators alter the free calcium concentration in BcF, this seems unlikely for two reasons. First, since DPA is the most likely chelator to be found in the filtrate of any strain of *Bacillus* (Rosen, 1982), we investigated the potential impact of DPA on our model. We measured 72.76 μM DPA in the culture filtrate. Using the dissociation constants $K_1 = 10^{-2.07}$ and $K_2 = 10^{-4.53}$ and the stability constant $K_{CaDPA} = 10^{4.05}$, and assuming no competition for DPA^{2-} by other cations, we calculated that the amount of DPA present in 10% BcF could reduce the Ca^{2+} activity by 1.8 to 7.5%, depending on the pH (Chung *et al.*, 1971). This small change in the pCa^{2+} would not significantly affect the results of the experiment, suggesting that DPA does not play a significant role in zoospore lysis by culture filtrate.

Furthermore, in related experiments we addressed the potential importance of chelators on pCa^{2+} in CB and BcF at various pH's and $CaCl_2$ or NH_4Cl concentrations. We used ion selective electrodes to measure pCa^{2+} and pH, and determined the ammoniacal-N concentration colorimetrically. The NH_3 activity was calculated based on the pH, and lysis of zoospores induced by the solutions was plotted against pCa^{2+}:pNH_3, with results similar to those presented here (data not shown). This supports the assertion that the PHREEQE model accurately predicts the actual pCa^{2+}:pNH_3 of the solutions.

Future studies will address the role of zoospore lysis in the biocontrol activity of UW85. It remains to be determined whether UW85 can sufficiently alter the ionic environment of the rhizosphere to either lyse zoospores that reach the rhizosphere or cause them to swim away, thereby reducing the probability of infection. There are several examples in the literature of suppression of diseases caused by *Phytophthora* and other oomycetes by increasing soil ammonia levels with fertilizers (Gilpatrick, 1969; Tsao and Oster, 1981; Lewis and Lumsden, 1984). UW85 may suppress disease by this same mechanism, and may have the potential advantage of being present in the infection site. Understanding the role of zoospore lysis in biocontrol may help us to manipulate the agroecosystem to obtain better disease suppression.

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REFERENCES

Byrt, P. N., Irving, H. R., and Grant, B. R. 1982a. The effect of cations on zoospores of the fungus *Phytophthora cinnamomi*. *J. Gen. Microbiol.* 128: 1189–1198.

Byrt, P. N., Irving, H. R., and Grant, B. R. 1982b. The effect of organic compounds on the encystment, viability and germination of zoospores of *Phytophthora cinnamomi*. *J. Gen. Microbiol.* 128: 2343–2351.

Chung, L., Rajan, K. S., Merdinger, E., and Grecz, N. 1971. Coordinative binding of divalent cations with ligands related to bacterial spores. *Biophys. J.* 11: 469–482.

Duniway, J. M. 1983. Role of physical factors in the development of *Phytophthora* diseases. In *Phytophthora—Its Biology, Taxonomy, Ecology, and Pathology* (D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, Eds.), pp. 175–187. American Phytopathological Society, St. Paul, MN.

Gilpatrick, J. D. 1969. Role of ammonia in the control of avocado root rot with alfalfa meal soil amendment. *Phytopathology* 59: 973–978.

GRANT, B. R., GRIFFITH, J. M., AND IRVING, H. R. 1986. A model to explain ion-induced differentiation in zoospores of *Phytophthora palmivora*. *Exp. Mycol.* **10**: 89–98.

GRANT, B. R., IRVING, H. R., AND RADDA, M. 1985. The effect of pectin and related compounds on encystment and germination of *Phytophthora palmivora* zoospores. *J. Gen. Microbiol.* **131**: 669–676.

GRIFFITH, J. M., ISER, J. R., AND GRANT, B. R. 1988. Calcium control of differentiation in *Phytophthora palmivora*. *Arch. Microbiol.* **149**: 565–571.

HANDELSMAN, J., MESTER, E. H., AND RAFFEL, S. 1988. Mechanism of biocontrol of *Phytophthora* by *Bacillus cereus* UW85. In *Molecular Genetics of Plant–Microbe Interactions* (R. Palacios and D. P. S. Verma, Eds.), pp. 303–308. American Phytopathological Society, St. Paul, MN.

IRVING, H. R., AND GRANT, B. R. 1984. The effects of pectin and plant root surface carbohydrates on encystment and development of *Phytophthora cinnamomi* zoospores. *J. Gen. Microbiol.* **130**: 1015–1018.

JANSSEN, F. W., LUND, A. J., AND ANDERSON, L. E. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**: 26–27.

LEWIS, J. A., AND LUMSDEN, R. D. 1984. Reduction of pre-emergence damping-off of peas caused by *Pythium ultimum* with calcium oxide. *Canad. J. Plant Pathol.* **6**: 227–232.

LINDSAY, W. L. 1979. *Chemical Equilibria in Soils*. Wiley, New York.

NEWHOOK, F. J., YOUNG, B. R., ALLEN, S. D., AND ALLEN, R. N. 1981. Zoospore motility of *Phytophthora cinnamomi* in particulate substrates. *Phytopathol. Z.* **101**: 202–209.

PARKHURST, D. L., THORSTENSON, D. C., AND PLUMMER, L. N. 1985. *PHREEQE—A Computer Program for Geochemical Calculations*. U.S. Geological Survey, Water Resources Investigations 80-96, Reston, VA.

ROSEN, B. P. 1982. Calcium transport in microorganisms. In *Membrane Transport of Calcium* (E. Carafoli, Ed.), pp. 187–216. Academic Press, New York.

RUSH, C. M., AND LYDA, S. D. 1982. The effects of anhydrous ammonia on membrane stability of *Phymatotrichum omnivorum*. *Mycopathologia* **79**: 147–152.

SCHIPPERS, B., MEIJER, J. W., AND LIEM, J. I. 1982. Effect of ammonia and other soil volatiles on germination and growth of soil fungi. *Trans. Brit. Mycol. Soc.* **79**: 253–259.

SCHULTE, E. E., PETERS, J. B., AND HODGSON, P. R. 1987. *Wisconsin Procedures for Soil Testing*. Department of Soil Sciences, University of Wisconsin.

SNEDECOR, G. W., AND COCHRAN, W. G. 1980. *Statistical Methods*, 7th ed. Iowa State Univ. Press, Ames.

TSAO, P. H., AND OSTER, J. J. 1981. Relation of ammonia and nitrous acid to suppression of *Phytophthora* in soils amended with nitrogenous organic substances. *Phytopathology* **71**: 53–59.