Evaluation of Tetrazolium Bromide as a Vital Stain for Fungal Oospores

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Journal Series Article 10429 from the Michigan Agricultural Experiment Station. This research was partially supported by a grant from the Allen H. Meyers Foundation and Cooperative Agreement 58-32U4-0-143 from the U.S. Department of Agriculture.

The authors thank J. L. Lockwood for discussion of the research presented here.

Accepted for publication 22 May 1983.

ABSTRACT

Sutherland, E. D., and Cohen, S. D. 1983. Evaluation of tetrazolium bromide as a vital stain for fungal oospores. Phytopathology 73:1532-1535.

Staining of fungal oospores with tetrazolium bromide (MTT) is proposed as a test for estimating oospore viability and as a technique for enhancing detection of oospores in root tissue. More than 80% of the oospores of *Phytophthora cactorum, P. megasperma* f. sp. *glycinea* races 1 and 3, and *Pythium aphanidermatum* stained a rose color with 0.1% MTT after 24 hr of incubation at 35 ± 2 C. Very few oospores of *Aphanomyces cochlioides, A. euteiches*, or *Pythium ultimum* stained under these same conditions. Staining of most fungal species was better at 35 ± 1 C than 23 ± 2 C. Oospore staining of *P. megasperma* f. sp. *glycinea* race 1 by MTT did not change significantly when the oospore concentration was 10^3, 10^4, or 10^5 oospores per milliliter, or when MTT concentration was 0.05, 0.5, or 5%. The number of oospores of *P. megasperma* f. sp. *glycinea* race 1 stained with MTT in phosphate buffer did not differ significantly from the number stained with MTT in water. Viability results as indicated by staining and germination were comparable when oospores were killed by autoclaving. When oospores of *P. megasperma* f. sp. *glycinea* race 1 were stored in sterile water for 1, 2, and 3 wk at 23 ± 2 C, oospore germination decreased, and the percentage of oospores which stained rose showed a corresponding decrease. Dormant spores stained rose and activated and germinated spores stained blue when MTT was added to germination plates. Oospores were detected easily in infected soybean root tissue stained with MTT.

Additional key words: oospore germination.

To study the survival of oospores of *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin [= *P. megasperma* Drechs. var. *sojae* Hildb.] that have been exposed to various environmental conditions, germination has been used as a measure of spore viability, but results from this method are complicated by the dormancy of the oospores (19). The use of vital stains, such as tetrazolium salts, offers an alternative to oospore germination for assessing viability. Nelson and Olsen (11) used tetrazolium chloride (TTC) to locate sporangia of *Synchytrium endobioticum* in potato tissue and verified the vital staining property of TTC by reducing both viability and staining of sporangia with heat and chemical treatments. Pathak et al (12) detected oospores of *Peronospora manshurica* in soybean seeds with TTC, and correlated the proportion of stained oospores with germination. Shetty et al (16) utilized TTC to locate oospores of *Sclerospora graminicola* on pearl millet seed. Workers (18) have questioned TTC as a vital oospore stain for *Sclerospora graminicola*, because oospore staining varied greatly with presoaking time, incubation time and temperature, and stain and oospore concentrations. Another tetrazolium compound, tetrazolium bromide, was used to test for viability of oospores of *Phytophthora infestans* in vitro (14), and was also recommended for other *Phytophthora* spp. (13). The chemistry of tetrazolium salts was reviewed by Altman (1). Briefly, the tetrazolium staining reaction occurs when the salt is reduced by a cellular dehydrogenase to form its colored formazan product (4,8).

The purpose of this study was to further investigate the use of tetrazolium bromide as a vital stain for oospores of several fungal species, to determine optimal staining conditions for oospores, and to describe the relationship of oospore staining to germination.

MATERIALS AND METHODS

Production and isolation of fungi. The fungal species used were *Aphanomyces cochlioides* Drechs. obtained from C. L. Schneider, Michigan State University, East Lansing; *A. euteiches* Drechs. isolate L30 from W. F. Pfender, University of Wisconsin, Madison; *Phytophthora cactorum* (Leb. and Cohn) Schroet. from J. E. Mitchell, University of Wisconsin, Madison; *P. megasperma* f. sp. *glycinea* races 1 (ATCC 44032) and 3 from A. F. Schmitthenner, Ohio Agricultural Research and Development Center, Wooster; and *Pythium aphanidermatum* (Edson) Fitzpatrick from R. D. Lumsden, USDA, Soilborne Diseases Laboratory, Beltsville, MD. *Pythium monospermum* Pringsheim, a hyperparasite of *P. megasperma* f. sp. *glycinea* (5), and *P. ultimum* Trow were isolated previously in our laboratory.

To produce oospores, *A. cochlioides* and *A. euteiches* were grown for 40 days in oatmeal broth (15), and *P. cactorum* and *P. megasperma* f. sp. *glycinea* were grown for 30 days in modified V-8 juice broth supplemented with cholesterol (2). *P. aphanidermatum, P. monospermum*, and *P. ultimum* were grown in clarified V-8 juice broth for 3 wk. Clarified V-8 juice was prepared by adding 10 g calcium carbonate to 1 L of V-8 juice (Campbell Soup Co., Camden, NJ 08101), heating the mixture to 80 C, and then centrifuging at 13,200 *g* for 10 min. One hundred milliliters of the supernatant plus 30 mg of cholesterol dissolved in 2 ml of 95% ethanol were added to 900 ml of distilled water.

Cultures of *A. cochlioides, A. euteiches, P. cactorum*, and *P. megasperma* f. sp. *glycinea* were frozen at −12 C for 1 hr to aid in separating the oospores from the mycelium. Mycelial mats of *P. aphanidermatum* were transferred to sterile petri dishes containing filter paper to dry and store the mats prior to oospore extraction. Oospores were dislodged from mycelia by comminution of the cultures suspended in water in a Servall Omni-mixer (Ivan Sorvall, Norwalk, CT 06856) at a rheostat setting at 70% of line voltage (117 v) with ten 5-sec pulses for *Aphanomyces* and *Pythium* spp., and at a rheostat setting of 40% of line voltage (117 v) for 10 min for *Phytophthora* spp. The fragments were reground in the Omni-mixer to release more oospores. The liquid containing the oospores was centrifuged for 15 sec at 3,100 *g*. The supernatant liquid was removed by suction, leaving the oospores concentrated in the pellet. Centrifugation was repeated until the oospore suspension was essentially free from hyphal fragments. Oospores were stored in sterile distilled water at 4 ± 2 C for up to 1 wk prior to use.

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Staining of oospores. A 0.1% solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Aldrich Chemical Company, Milwaukee, WI 53233) in sterile, distilled water was used in all viability tests. A 1:1 (v/v) mixture of MTT and a suspension of approximately 5×10^3 oospores, usually 0.5 ml of each, was prepared in sterile test tubes. Preparations were incubated for 48 hr at 35 ± 1 C except where indicated. Two replicates were prepared per treatment. Each replicate was sampled three times, with 100 oospores counted in each sample. All experiments were repeated at least once. The rose-colored oospores were counted and the oospores of the different species were compared for color values with color standards (3).

Germination of oospores. Oospore germination of *P. megasperma* f. sp. *glycinea* was assessed on soil films (7). One milliliter of the supernatant liquid from a 2% loam suspension was pipetted into a 60 × 15-mm glass petri dish and swirled to cover the bottom of the dish. The soil film was allowed to air-dry, and the dish was autoclaved for 1 hr. One milliliter of an oospore suspension (~10^4 spores per milliliter) was added to the petri dish. The dish was wrapped in Parafilm M (American Can Company, Greenwich, CT 06830) and incubated at 23 ± 1 C for 12 days. Germination of *P. aphanidermatum* was evaluated by placing 1 ml of an oospore suspension containing approximately 10^6 spores per milliliter on agar plates prepared as described by Mircetich (10). The percentage of germinated oospores was determined after 18 hr of incubation at 28 ± 1 C (9). Germination of *P. megasperma* f. sp. *glycinea* and *P. aphanidermatum* was assessed by counting 300 oospores for each of three replicates per treatment. Experiments were repeated at least once. Oospores of *P. aphanidermatum* were rated as germinated when the germ tubes were as long as the diameter of the oospore. Oospores of *P. megasperma* f. sp. *glycinea* were classified in three groups based on appearance as follows: dormant, thick-walled with finely granular cytoplasm; activated, thin-walled with coarsely granular cytoplasm; germinated, oospores with germ tubes at least as long as the diameter of the oospore, with or without sporangia (7).

RESULTS

Staining reaction of oospores. Oospores of species of *Aphanomyces, Phytophthora*, and *Pythium* became stained Spirea Red, China Rose, Rose Red, and Magenta Rose (names from the Horticultural Colour Chart of the British Colour Council [3]) after incubation at 35 ± 1 C for 2–4 days in MTT (Table 1). Color intensity varied slightly with different oospore preparations of each species, but was always within the limits of the specific colors. The intensity of Spirea Red varied within a given oospore preparation of *P. aphanidermatum*, while the oospores of the other species were evenly stained. Oospores that were neither rose colored nor clear (unstained) were black. Black oospores (usually <10% and often grossly distorted) were detected in all oomycete populations tested. More than 80% of the oospores of *P. cactorum, P. megasperma* f. sp. *glycinea* races 1 and 3, and *P. aphanidermatum* stained after 2 days of incubation in MTT at 35 ± 1 C (Table 1). Only a small percentage of oospores of *A. cochlioides* and *A. euteiches* (4%) or *P. ultimum* (30%) became stained under the conditions that were tested. None of the test organisms stained well at 23 ± 2 C except *P. monospermum*; 62% of the oospores stained during only 4 hr of incubation in MTT. Incubation of *P. monospermum* oospores at 35 ± 1 C did not enhance staining over that which occurred at 23 ± 2 C. Results were similar in other experiments.

MTT was added to oospores of each test fungus except *P. monospermum* and *P. megasperma* f. sp. *glycinea* race 3, and samples were incubated for 2, 8, 24, 48, and 72 hr to determine optimum incubation time. An average of 87% of the oospores of *P. megasperma* f. sp. *glycinea* race 1, and 79% of those of *P. aphanidermatum*, were stained after 8 hr (Fig. 1). Eighty-nine percent of the oospores of *P. cactorum* were stained after 24 hr. Staining of the oospores of *P. aphanidermatum* remained essentially the same after 24 hr.

Effect of solvent and MTT concentration on staining of oospores of *P. megasperma* f. sp. *glycinea* race 1. Oospores stained equally well when 0.1% MTT was prepared in filter-sterilized (Nalgene 0.45 μm nitrocellulose membrane) distilled water or 0.1 M phosphate buffer (pH 5.8). Eighty-five to 95% of the oospores of *P. megasperma* f. sp. *glycinea* stained in two experiments with no significant differences between water or phosphate buffer. However, the cytoplasm of oospores stained with MTT in buffer appeared slightly disorganized and pulled away from the oospore wall, whereas the cytoplasm of oospores stained with MTT in water was intact. Oospores stained with MTT in buffer took 24 hr longer to stain than did oospores in MTT in water.

Three different concentrations of MTT in water (0.05, 0.5, and 5%) did not affect the percentage of oospores stained in a suspension of 10^4 oospores per milliliter. In two experiments, 86% or more of the oospores of *P. megasperma* f. sp. *glycinea* were stained after 48 hr at 35 ± 1 C at all three concentrations. Likewise, oospores at concentrations of 10^3, 10^4, or 10^5/ml did not differ in the amount or rate of staining; more than 89% of the oospores of this species were stained at each of these oospore concentrations.

The relationship of germination and MTT staining of oospores. Once oospores of *P. megasperma* f. sp. *glycinea* race 1 were stained with MTT, they were rendered ungerminable. Therefore, oospores of *P. megasperma* f. sp. *glycinea* races 1 and 3, and *P.*

TABLE 1. Stain reactions of several oomycetous fungi when incubated with tetrazolium bromide (MTT) at 35 ± 1C

Fungus	Stained oospores (%)[a]	Time (days)
Aphanomyces cochlioides	4	3
Aphanomyces euteiches	4	3
Phytophthora cactorum	89	2
Phytophthora megasperma f. sp. *glycinea* race 1	85	2
Phytophthora megasperma f. sp. *glycinea* race 3	85	2
Pythium aphanidermatum	85	2
Pythium monospermum	55	2
Pythium ultimum	30	4

[a] Mean percentage of oospores stained in two replicate tubes.

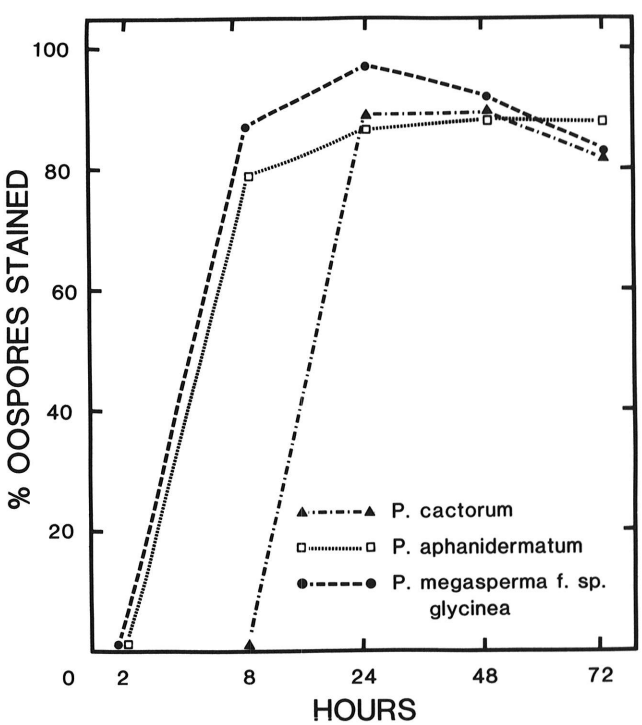

Fig. 1. Mean percentage of oospores of *Phytophthora cactorum, Phytophthora megasperma* f. sp. *glycinea* race 1, and *Pythium aphanidermatum* stained during 2–72 hr of incubation in a 0.1% solution of tetrazolium bromide (MTT) at 35 ± 1 C.

aphanidermatum were either stained with MTT in phosphate buffer or separately germinated on soil film or agar plates. Oospore germination was 32% for *P. megasperma* f. sp. *glycinea* race 1, 54% for race 3, and 56% for *P. aphanidermatum*, while staining of oospores after 48 hr at 35 ± 1 C ranged from 70–73% for all three organisms. Results were similar in two other experiments.

Stages in the germination of oospores of *P. megasperma* f. sp. *glycinea* race 1 could be distinguished by the addition of 1 ml of MTT to soil film plates in which oospores had been incubated for 2 wk. After 48 hr of 35 ± 1 C, the oospores were classified in the following categories: rose color = dormant; blue color = activated or germinated; black or clear = ungerminable oospores. Ninety-five percent of *P. megasperma* f. sp. *glycinea* race 1 oospores stained as dormant after harvest (Table 2). After 2 wk of incubation on soil film plates, the number of oospores stained as dormant declined to 59%, while the number of stained, activated, or germinated oospores increased to 37%. Little change was detected in the number of oospores assessed as ungerminable. Similar results were obtained in a second experiment.

The staining of oospores of *P. megasperma* f. sp. *glycinea* race 1 autoclaved at 121 C for 15 min were compared with that of untreated oospores. Eighty-one ± 1% of untreated oospores were stained after 48 hr at 35 ± 1 C, whereas 5 ± 1% of the autoclaved oospores were stained. Autoclaved oospores that did not stain a rose color were clear and did not germinate in soil film plates. Similar results were obtained in a second experiment.

TABLE 2. Detection of germination stages of oospores of *Phytophthora megasperma* f. sp. *glycinea* by tetrazolium bromide (MTT) staining before and after oospores were incubated on soil film germination plates for 2 wk

Germination stage	Color reaction	Oospores stained (%)[a]	
		Before incubation	After incubation
Dormant	Rose	95 ± 2	59 ± 9
Activated or germinated	Blue	1 ± 1	37 ± 11
Ungerminable	Black or clear	4 ± 1	4 ± 2

[a] Mean percentage of oospores stained ± standard error on four replicate soil film plates; 100 oospores were counted per plate.

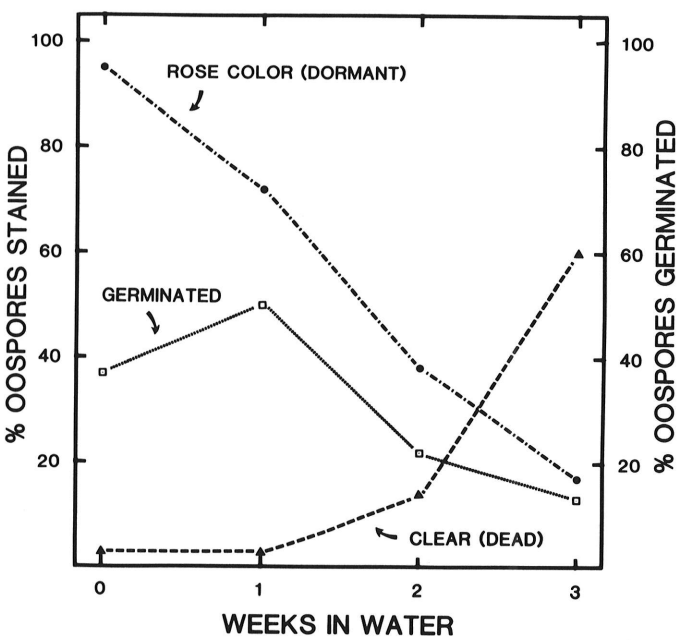

Fig. 2. Germination and tetrazolium bromide staining of oospores of *Phytophthora megasperma* f. sp. *glycinea* race 1 during storage in water at 23 ± 2 C for 3 wk. Oospores, 30 days of age, were either germinated on soil film plates or were incubated in stain and classified as follows: rose color = dormant; unstained = ungerminable or dead.

In a time-course study, 30-day-old oospores of *P. megasperma* f. sp. *glycinea* race 1 were stored in sterile distilled water at 23 ± 2 C, and either germinated on soil film plates or stained in MTT at 1 wk intervals for 3 wk. Oospore germination decreased from 37 to 13% after oospores were stored in sterile distilled water at 23 ± 2 C for 3 wk (Fig. 2). The percentage of oospores stained rose (dormant) with MTT after 48 hr at 35 ± 1 C also declined with time, decreasing from 95 to 17% after 3 wk. The percentage of unstained oospores (dead) increased from 3 to 60% over the same time period. Activated oospores as assessed in the stained preparations increased in frequency from 1% to 48% after 2 wk of storage at 23 ± 2 C, then declined to 23% after 3 wk. The number of oospores staining black was never greater than 1% during the 3 wk of sampling. Similar results were obtained in a second experiment.

Staining of oospores in soybean tissue. Roots of soybean (cultivar Hark) plants infected by *Phytophthora* were incubated at 35 ± 1 C in 0.1% MTT at an approximate ratio of 1 g tissue:1 ml stain. After 48 hr, roots were crushed on a microscope slide and viewed at ×150. All of the oospores of *P. megasperma* f. sp. *glycinea* race 1 stained in soybean root tissue. The root cells did not react intensively with MTT. The cell walls stained a light shade of purple, and oospores were easily detected in the tissue. Results were repeated in other tests.

DISCUSSION

A high percentage of oospores of *P. cactorum, P. megasperma* f. sp. *glycinea* races 1 and 3, and *P. aphanidermatum* stained with MTT, while few oospores of *Aphanomyces* spp. and *P. ultimum* stained under the same conditions (Table 1). The difficulty in staining oospores of *Aphanomyces* spp. and *P. ultimum* may have been due to a lack of stain penetration of the oogonial and oospore walls, or a delay in the enzymatic reduction within the oospore (11). A higher temperature, higher MTT concentration, a longer incubation time, or a combination of these conditions may allow for increased oospore staining of *A. euteiches, A. cochlioides*, or *P. ultimum*.

The color reaction of stained oospores varied slightly depending on the species tested, the oospore preparation, and possibly because of contamination of MTT with other tetrazolium salts. Difficulties encountered in determining the specific mode of action for individual tetrazolium salts has been, in part, due to contamination by other tetrazolium salts (1). The variation of color intensity in stained oospores of *P. aphanidermatum* may make the use of MTT unacceptable for this species.

Staining of oospores of *P. megasperma* f. sp. *glycinea* by MTT did not vary with oospore or stain concentration. However, staining of oospores of *Sclerospora graminicola* by TTC was influenced by oospore and stain concentrations (18). These differences may have been due to the test organisms, but may possibly be explained by the action of the tetrazolium compounds. The preparation of MTT and TTC in water or buffer did not affect oospore staining in our work or in that of Williams et al (18).

A low percentage of autoclaved *P. megasperma* f. sp. *glycinea* oospores stained rose after incubation in MTT. The reason for this staining is unknown. It is also not certain how to interpret the occurrence of black oospores within a given stained preparation. Many of the black oospores were deformed. However, the number of oospores of *Phytophthora* that stained black increased if they were kept in MTT several weeks at 23 or 35 C (*unpublished*). Sporangia of the oomycetes stained pink after 15 min at 23 C, and turned blue-black after 1 hr at this temperature or even sooner at 35 C. Perhaps overstaining of spores caused them to appear black.

Stainability could not be directly correlated with germinability when oospores were stained or germinated over a 3-wk period (Fig. 2). However, as the stainability of oospores of *P. megasperma* f. sp. *glycinea* decreased, there was a comparable decrease in oospore germination. Ishiguro and Ui (6) found that oospores of *Phytophthora vignae* 180 days of age did not germinate or stain in MTT as well as those 30 days of age. A higher percentage of oospores of *P. megasperma* f. sp. *glycinea* stained than germinated. While Pathak et al recorded more oospores of *Peronospora*

manshurica staining with TTC than germinating, the difference was not more than 16% (12). However, oospore germination of *P. manshurica* was not greater than 30%. An inherent difficulty in a study with oomycetes is that oospore dormancy impedes the assessment of germination. The use of MTT may alleviate this problem in part by allowing for easy detection of activated oospores.

Following the storage of oospores of *P. megasperma* f. sp. *glycinea* at 23 C for 2 wk, the number of activated oospores increased to 48%. These activated oospores failed to germinate, as reflected by the small percentage of germinated oospores at 3 wk (Fig. 2). It is suggested that either a critical amount of nutrients were leached from the spore through storage at 23 C, or external conditions were not conducive for germination.

Without further studies, MTT cannot be used solely for determining oospore viability. The stain, however, is valuable for viewing oospore germination and oospores in root tissue, or when studying hyperparasitism (17). MTT may prove to be a useful tool for studying the physiology of *P. aphanidermatum, P. cactorum, P. megasperma* f. sp. *glycinea*, and other pythiaceous fungi under defined conditions.

LITERATURE CITED

1. Altman, F. P. 1976. Tetrazolium salts and formazans. Prog. Histochem. Cytochem. 9:1-55.
2. Ayers, W. A., and Lumsden, R. D. 1975. Factors affecting production and germination of oospores of three *Pythium* species. Phytopathology 65:1094-1100.
3. British Colour Council. 1938. Horticultural Colour Chart. Published by the British Colour Council in collaboration with the Royal Horticultural Society. Printed 1938–1941 by H. Stone and Sons, Ltd., Banbury, England.
4. Gahan, P. B., and Kalinda, M. 1968. The use of tetrazolium salts in the histochemical demonstration of succinic dehydrogenase activity in plant tissues. Histochemie 14:81-88.
5. Humble, S. J., and Lockwood, J. L. 1981. Hyperparasitism of oospores of *Phytophthora megasperma* var. *sojae*. Soil Biol. Biochem. 13:355-360.
6. Ishiguro, K., and Ui, T. 1981. Factors influencing the germination of oospores of *Phytophthora vignae* Purss. Ann. Phytopathol. Soc. Jpn. 47:213-217 (in Japanese).
7. Jimenez, B., and Lockwood, J. L. 1982. Germination of the oospores of *Phytophthora megasperma* f. sp. *glycinea* in the presence of soil. Phytopathology 72:662-666.
8. Kalinda, M., and Palmer, J. M. 1968. The reduction of tetrazolium salts by plant mitochondria. Histochemie 14:366-374.
9. Lumsden, R. D., Ayers, W. A., and Dow, R. L. 1975. Differential isolation of *Pythium* species from soil by means of selective media, temperature, and pH. Can. J. Microbiol. 21:606-612.
10. Mircetich, S. M. 1971. The role of *Pythium* in feeder roots of diseased and symptomless peach trees and in orchard soils in peach tree decline. Phytopathology 61:357-360.
11. Nelson, G. A., and Olsen, O. A. 1967. Staining reactions of resting sporangia of Synchytrium endobioticum with a tetrazolium compound. Phytopathology 57:965-968.
12. Pathak, V. K., Mathur, S. K. B., and Neergaard, P. 1978. Detection of *Peronospora manshurica* (Naum.) Syd. in seeds of soybean, *Glycine max*. EPPO (Eur. Mediterr. Plant Prot. Organ.) Bull. 8:21-28.
13. Ribeiro, O. K. 1978. A sourcebook of the genus *Phytophthora*. J. Cramer: Lehre, Germany. 417 pp.
14. Ribeiro, O. K., Gallegly, M. E., and Young, R. J. 1971. Oospore production, viability, and germination in relation to establishment of F_2 cultures of *Phytophthora infestans*. (Abstr.) Phytopathology 61:907-908.
15. Schneider, C. L. 1978. Use of oospore inoculum of *Aphanomyces cochlioides* to initiate black root disease in sugarbeet seedlings. J. Am. Soc. Sugar Beet Technologists 20:55-62.
16. Shetty, H. S., Mathur, S. B., and Neergaard, P. 1980. *Sclerospora graminicola* in pearl millet seeds and its transmission. Trans. Br. Mycol. Soc. 74:127-134.
17. Sutherland, E. D. 1981. Hyperparasites of oospores of *Phytophthora megasperma* var. *sojae*: Host range, environmental parameters, and biological control. Ph.D. thesis, Michigan State University, East Lansing. 138 pp.
18. Williams, R. J., Pawar, M. N., and Huibers-Govaart, I. 1980. Factors affecting staining of *Sclerospora graminicola* oospores with triphenyl tetrazolium chloride. Phytopathology 70:1092-1096.
19. Zentmyer, G. A., and Erwin, D. C. 1970. Development and reproduction of *Phytophthora*. Phytopathology 60:1120-1127.