METHODS FOR ASSESSING VIABILITY AND MATURATION OF *APHANOMYCES COCHLIOIDES* OOSPORES

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Aphanomyces cochlioides (= *A. cochlioides* or *Aphanomyces*) is a well-documented pathogen of sugarbeet in southern Minnesota and the Red River Valley. The fungus causes stand loss and root rot of sugar beet seedlings and older plants. The "life cycle" of *A. cochlioides* includes two spore types – short-lived, motile zoospores and long-lived, dormant oospores (Figure 1). When soil is wet and warm, *A. cochlioides* becomes active because oospores are stimulated to germinate by exudates from sugarbeet roots. The oospore can directly infect the root or more commonly, produces a sporangium. Zoospores develop within the sporangium (which produces a few to 300 or more zoospores/sporangium), are exuded through an apical opening as a cluster, and then released to swim through soil water to sugarbeet roots. When a zoospore contacts a root, it encysts, germinates, and infects the root. Zoospores are delicate structures and live for up to 1 week but most die before they have a chance to infect roots.

After initial infection occurs, the fungus grows as microscopic threadlike structures (mycelium) that infiltrate the root and kill surrounding tissue (Figure 1). Infection eventually results in root rot and/or plant death. If wet and warm soil conditions persist, zoospores are produced on diseased roots and released into surrounding soil where they can re-infect the root and be transported via flowing water to other parts

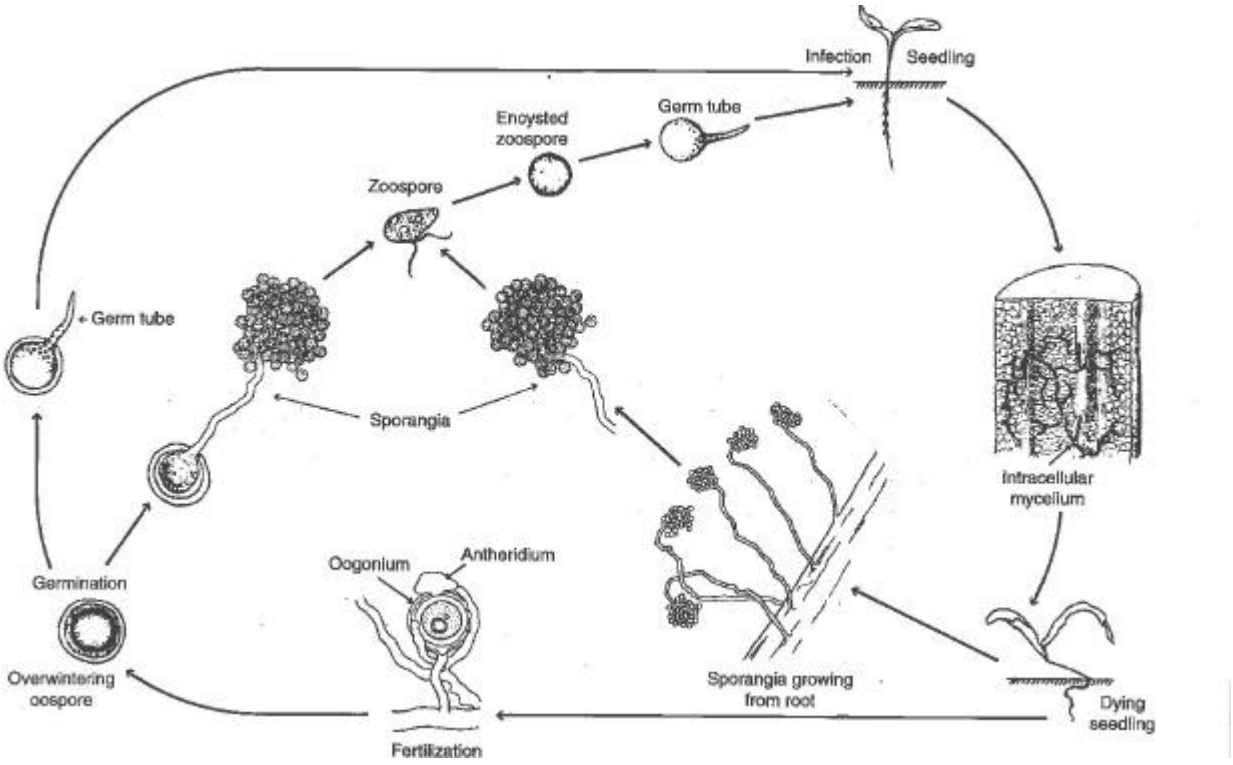

Figure 1. Life cycle of *Aphanomyces cochlioides* on sugarbeet.

of the field and infect other plants. As plants die, *A. cochlioides* rapidly produces oospores that are embedded in dead root tissues and are released as plant residues decompose. Consequently, oospore dispersal is largely passive, e.g., by tillage and other field activities; movement of contaminated soil on equipment and in tare soil; and also by high winds and flowing water that result in movement of contaminated soil. Overall, *A. cochlioides* spends only a short time as zoospores (up to 1 week) and most of the time, survives as dormant oospores. These thick-walled, survival structures are persistent in soil. Infested sugarbeet fields that are taken out of production and planted to beets 10 or 20 years later, still can suffer severe Aphanomyces root rot.

Although *A. cochlioides* exists predominantly as oospores, very little is known about them except in general terms. There are four stages of oospore development and activity (Figure 1) : 1.) formation of the antheridia and oogonia (sexual structures), 2.) fertilization of oogonia by antheridia, 3.) maturation of the fertilized oogonia into oospores, and 4.) germination of oospores. The first stage of oospore development has been well documented, but the other three stages have not been adequately described for *A. cochlioides.* Also, there are no known techniques to determine if an oospore of *A. cochlioides* is alive or dead, nor are data available on the effects of environmental stresses on the maturation or survival of oospores. Understanding the life cycle of plant pathogens, including how they survive, is fundamental to developing disease control strategies. For instance, a technique to ascertain if an oospore is alive or dead will be a valuable tool for identifying factors that induce oospore death. Such factors then can be exploited on a larger scale to reduce oospore populations in the field.

Viability of oospores of other related fungi, such as species of *Pythium* and *Phytophthora,* has been assessed either by visual assay or by bioassay. Visual assays have been done by microscopic examination after oospores are treated with vital stains such as tetrazolium bromide (stain rose or lavender if they are alive) or with 4M NaCl (plasmolysis occurs and the cytoplasm detaches from the cell wall if the oospore is alive). Another approach has been to first kill oospores (e.g., by immersion in boiling water) and then microscopically examine them for structural changes indicative of death. A difficulty with these visual methods has been in validating presumed oospore death by a bioassay (that is, loss of ability to cause disease). Bioassays (e.g., soil indexing, most probable number) to measure if oospores are living (cause disease) or dead (do not cause disease) have several limitations. They are labor intensive, require large numbers of oospores, and only a small percentage of living oospores germinate at any given time.

OBJECTIVES

The objectives of this research were to 1.) develop a simple, accurate, visual assay of oospore health (viable or dead oospores) and to 2.) validate results of the visual assay by determining if oospores assessed as dead can cause disease.

MATERIALS AND METHODS

Oospore production: Plastic pots (4 x 4 x 4 inches) were filled about 2.5 inches deep with a pasteurized greenhouse soil mix, planted with Crystal 205 (25 seeds/pot), and covered with 1 inch of soil. Pots were placed in a greenhouse with natural lighting for 16 hours at 73 ?F and 8 hours of dark at 61 ?F and watered as needed for 2 weeks. Seedlings then were removed and washed and hypocotyls (portion of root between seed and soil surface) were excised. Hypocotyls were surface-treated for 30 seconds in 0.5% sodium hypochlorite (bleach), rinsed twice with sterile distilled water (SDW), and placed in 7.5-inch diameter petri dishes (two hypocotyls/dish) containing a layer of SDW. Hypocotyls were inoculated with a culture of *A. cochlioides* and placed in the dark at 64-73 ?F until used in experiments. To extract oospores, 20 hypocotyls were placed in a 7 milliliter Tenbroeck tissue macerator filled with SDW and the plunger was depressed 12 times. The resulting oospore suspension was decanted and the process was repeated until all hypocotyls were macerated. Ages of oospores (i.e., 2 week old) given throughout the remainder of this paper are in reference to the time between inoculation of hypocotyls with *A. cochlioides* and when they were extracted.

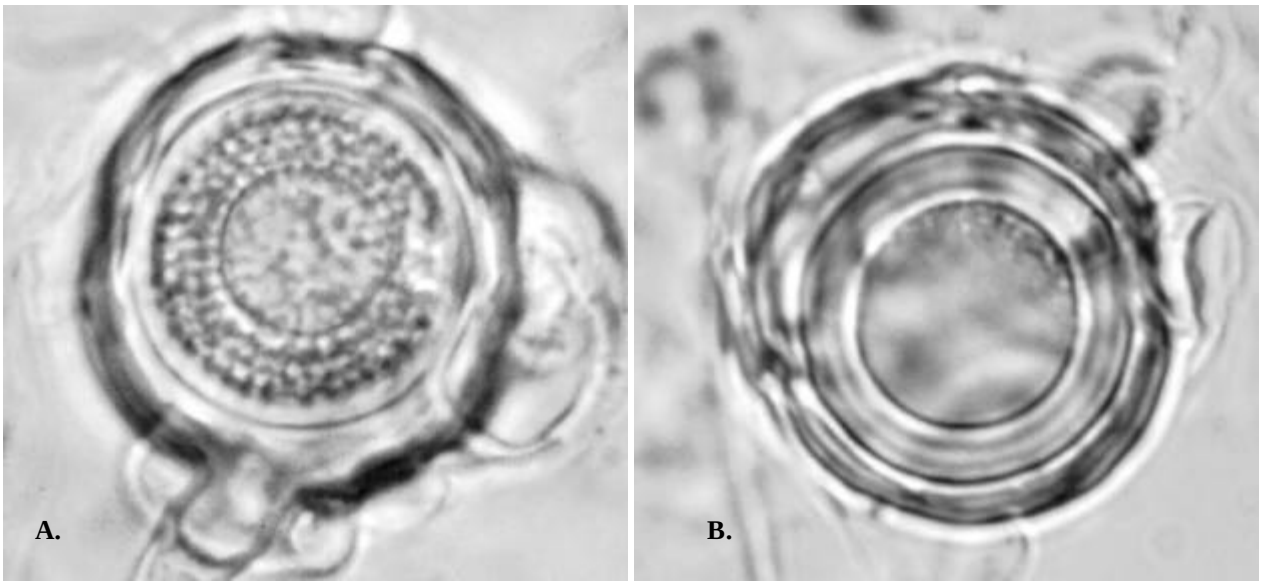

Figure 2. Microscopic view of oospores of *Aphanomyces cochlioides:* **A.)** densely organized uniform granular (DOUG) appearance of a viable oospore and **B.)** loosely organized nonuniform granular (LONG) appearance of a dead oospore.

Techniques to evaluate oospore death: Three aliquots were removed from a suspension of 8-week-old oospores and placed into individual glass vials. Oospore suspensions then were exposed to one of three lethal stresses to induce death: 35% ethanol for 24 hours, boiling water for 20 minutes, or a 0.5% bleach solution for 24 hours. Control oospores were untreated. The treated and control oospores then were maintained at 77 ?F for 24 hours and subsequently evaluated for viability by three tests including: 1.) plasmolysis in 4M NaCl for 2 hours, 2.) a tetrazolium bromide stain (0.1% MTT, pH 6.5, 95 ?F for 48 hours) and 3.) microscopic examination for densely organized, uniform granules (DOUG, typical appearance of living oospores, Figure 2A). For each lethal stress treatment and subsequent viability test, 150 oospores were assessed microscopically. The experiment was repeated with 10-week-old oospores.

Validation of visual assessment of oospore death by bioassay: After the above experiments were completed, it was found that treatment of oospores at 104 ?F for 48 hours resulted in a low percentage with the DOUG appearance typical of living oospores. Since heat treatment was gentler than boiling water or alcohol treatment, it was used to produce oospores for the bioassy to validate visual assessment of oospore death. Seven soils were prepared with an unpasteurized greenhouse soil mix that was inoculated with untreated 5-week-old oospores to bring the total number to 0, 5, 10, 15, 20, 25 or 30 oospores/cm^3 (cm^3 = about the amount of soil that fits into a small sewing thimble). About 86% of these oospores had been assessed as alive (DOUG) and the remaining 14% had an atypical appearance and were assumed dead (nonviable). Hence, numbers of viable oospores for this series of inoculated soils were about 0, 4, 8, 13, 17, 21, 25/cm^3. Another seven soils were prepared with an unpasteurized greenhouse soil mix that was inoculated with a mixture of untreated living oospores (DOUG) + heat-killed oospores to bring each soil to a total concentration of 30 oospores/cm^3. The heat-killed + DOUG oospores were mixed so final concentrations of DOUG oospores totaled 1, 3, 5, 9, 13, 17 or 22/cm^3 of soil.

For each of the 14 soil treatments, six pots were planted (as previously described in the oospore production section) and placed in a greenhouse with natural lighting at 73 ?F for 16 hours and in the dark at 61 ?F for 8 hours. After 7 days, pots were removed and placed in a growth chamber at 82 ?F, 70% humidity, and a 16-hour photoperiod; soil was kept moist to favor infection by *A. cochlioides*. Seedling stand counts were taken and dying seedlings were removed (to assay for pathogens) at 7 days after planting; this process was repeated every other day until 80% of the seedlings had died in at least one treatment. When the experiment was repeated, pots were placed in a growth chamber immediately after planting (73 ?F in the day, 61 ?F at night, 16 hour photoperiod). At 7 days after planting, temperature in the growth chamber was increased to 82 ?F to favor disease development.

Oospore maturation: Oospores appeared to be differentially stained by tetrazolium bromide based on age (youngest were stained, but older were not) rather than on viability, so an experiment was designed to determine if the stain measured maturation of oospores. Five hypocotyls of 2-week-old seedlings of Crystal 205 were inoculated as previously described. Segments, 3 millimeters long, were excised from each hypocotyl at 3, 5, 7, 9, 11, 14 and 28 days after inoculation and placed in 1 ml of 0.1% tetrazolium bromide (pH 6.5) at 95 ?F for 48 hours. Each segment then was microscopically examined at 400X to determine if oospores had stained a rose to lavender color. All oospores or oogonia within one randomly selected field of view were observed per hypocotyl segment and if they did not exceed 30 in number, an additional field of view was randomly selected and the combined results of the two views were recorded as a single observation. When the experiment was repeated, segments of hypocotyl were removed and stained at 4, 6, 8, 10, 12, and 14 days after inoculation and examined.

Statistical analysis: For the oospore bioassay, the proportion of dead seedlings out of total emerged for each pot was calculated for each observation day. From these proportions, an area under the disease progress curve (AUDPC, a value that sums rate and severity of disease development) was calculated for each pot. Because the data had a lower limit of zero and a biological upper limit (maximum AUDPC), data distribution at these limits was heavier than throughout the remainder of the data range. To remedy this, the final AUDPC values were divided by the maximum observed AUDPC value for the experiment and subjected to arcsine square root transformations. This transformation spreads out values at the upper and lower limits and normalizes the distribution of data. Transformed values were examined by regression analysis against the total number of living oospores (as determined by microscopic examination) for each treatment series. Slopes of the two resulting regression curves then were compared with an F-test.

RESULTS

Techniques to evaluate oospore death: There were no statistical differences between the first and second trials, so results were combined. Oospores treated with ethanol or boiling water, and the untreated control, that subsequently were exposed to plasmolysis with 4M NaCl, showed 3, 9, and 85% viability, respectively. Viability was confirmed by detachment of cytoplasm from the oospore wall; dead oospores showed no detachment. Oospores treated with ethanol or boiling water, and the untreated control, that subsequently were exposed to tetrazolium bromide, showed 15, 8, and 16% viability, respectively. Oospores counted as viable were those that stained rose or lavender; dead oospores were not stained. Treatment of oospores with bleach and then plasmolysis or tetrazolium bromide resulted in distortion of the spore wall and therefore, they were not assessed. For oospores treated with ethanol, boiling water, or bleach, and the untreated control, that subsequently were examined for densely organized uniform granules (DOUG, typical appearance of viable oospores, Figure 2A) viability was 0, 0, 11, and 84%, respectively. Oospores without the typical DOUG appearance lacked granules or contained loosely organized, nonuniform granules (LONG, Figure 2B) and were assumed to be dead.

Validation of visual assessment of oospore death by bioassay: Results of the two experiments were similar, so data are presented from one experiment. In the soil series where untreated DOUG oospores were added to soil so the total number of viable oospores was approximately 4, 8, 13, 17, 21, and 25/cm^3, the amount of disease ranged from a low AUDPC value of 2.0 to a high of 5.2 in soil with 4 and 21 viable oospores/cm^3, respectively (Figure 3). No seedlings died from Aphanomyces damping-off in the non-inoculated control. Regression analysis revealed a highly significant relationship (R^2= 0.48, P<0.001)

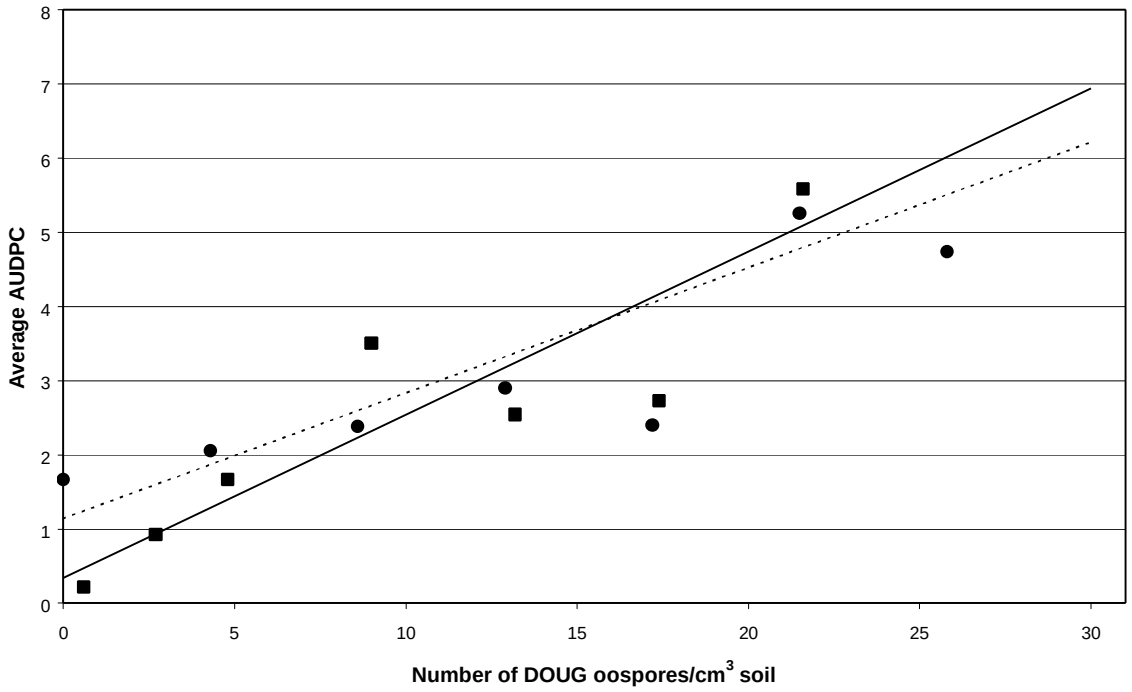

Figure 3. Regression lines showing the relationship between average area under the disease progress curve (AUDPC, data subjected to an arcsine square root transformation) and number of living oospores of *Aphanomyces cochlioides* (determined by microscopic examination of densely organized uniform granular appearance, DOUG) in two series of soil mixes with unpasteurized greenhouse soil. One series (——) was composed of seven soils inoculated with untreated DOUG oospores to bring the total number of viable oospores to 0, 4, 8, 13, 17, 21, and 25/cm^3. The other series (····) was composed of seven soils inoculated with a mixture of DOUG oospores + heat-killed oospores (loosely organized nonuniform granular appearance, LONG) to bring each soil to a total concentration of 30/cm^3 but oospores were mixed so the total number of DOUG oospores was 1, 3, 5, 9, 13, 17, or 22/cm^3.

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between disease severity and number of DOUG oospores (Figure 3). For the soil series where each of the seven mixtures contained living (DOUG) oospores + dead (LONG) oospores that totaled 30 oospores/g but represented an expected 1, 3, 5, 9, 13, 17, and 22 living (DOUG) oospores/cm^3 of soil, the amount of disease ranged from a low AUDPC value of 0.2 to a high of 5.6 in soil with 1 and 22 viable oospores/cm^3, respectively (Figure 3). Regression analysis showed a significant relationship (R^2 = 0.61, P<0.001) between disease severity and number of DOUG oospores. Comparison of slopes of regression lines showed no significant differences between disease in soils inoculated with DOUG oospores or with mixtures of DOUG + LONG oospores (P=0.27, Figure 3).

Most dying seedlings showed symptoms typical of Aphanomyces damping-off. In soil inoculated with the mixtures of DOUG + LONG oospores, an average of 6% and 7% of dying seedlings collected at the first and second stand counts, respectively, were infected by *Rhizoctonia solani* or *Pythium* species. In soil inoculated with DOUG oospores, an average of 26% of dying seedlings collected at the first stand count were infected by *R. solani* or *Pythium* species but isolation of these pathogens dropped to 5% by the second stand count.

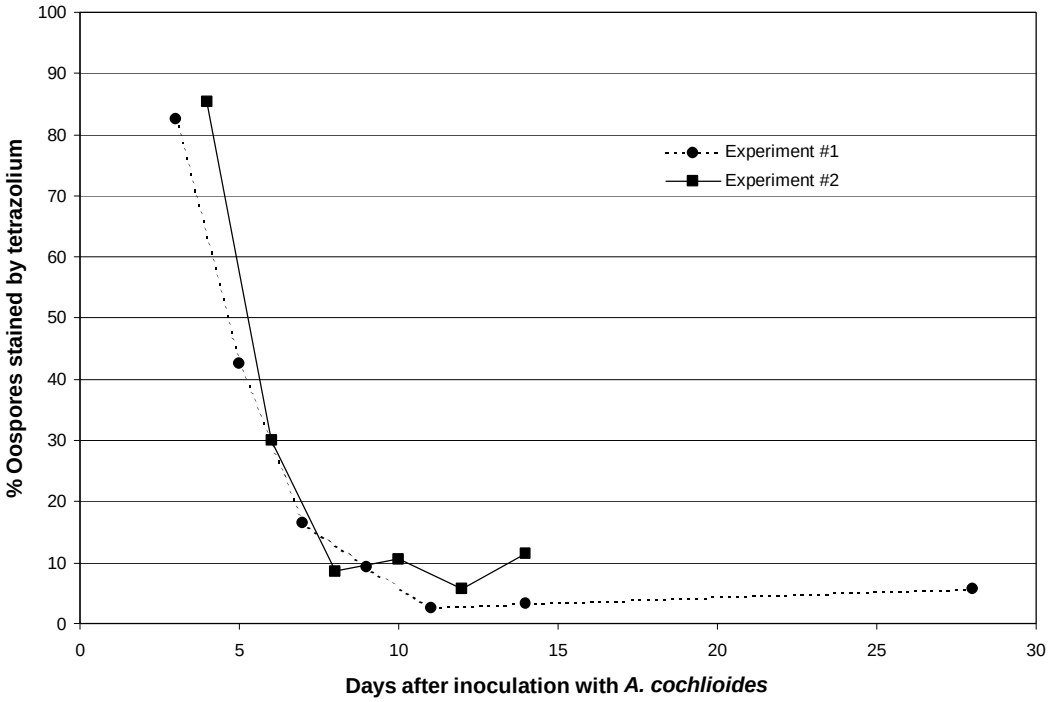

Figure 4. Percent oospores stained by tetrazolium bromide at intervals between 3 to 28 days after inoculation of hypocotyls with *Aphanomyces cochlioides* in experiment 1 and between 4 to 14 days in experiment 2. Each data point is an average based on microscopically examining 150 oospores.

Oospore maturation: An average of 82% of oospores/oogonia were stained by tetrazolium bromide 3 days after hypocotyls were inoculated with *A. cochlioides* in the first experiment (Figure 4). The percent of oospores/oogonia that were stained dropped rapidly thereafter, until 11 days after inoculation only 3% were stained. Similar results were obtained in the second experiment (Figure 4).

DISCUSSION

These results demonstrate that microscopic examination of oospores for a densely organized uniform granular (DOUG) appearance indicates viability while a loosely organized nonuniform granular (LONG) appearance indicates they are dead. Visual assessment of oospore viability was confirmed by bioassays of soil inoculated with a range of oospore concentrations composed of living (DOUG) + heat-killed (LONG) oospores compared to the same range of inoculum densities of DOUG oospores. Regression analysis showed a significant relationship between disease severity and number of DOUG oospores. Hence, similarities in AUDPCs in soils inoculated with comparable numbers of DOUG oospores, with and without heat-treated LONG oospores, provides strong evidence that LONG oospores are dead.

Different regression values, but similar slopes, were calculated for soils inoculated with DOUG oospores and with DOUG + LONG oospores. The difference in regression values is explained by a greater stand loss that initially occurred in soils inoculated with DOUG oospores compared to soils inoculated with DOUG + LONG oospores. About 26% of the dying seedlings collected at the first stand count in soils

inoculated with DOUG oospores were killed by *R. solani* and *Pythium* species compared to 7% in soils inoculated with the DOUG + LONG oospores. These pathogens usually are most active shortly after emergence. A single dying seedling early in the assay also provides a large addition of inoculum to a system. Since the AUDPC value is based on total disease and not just Aphanomyces damping-off, the disproportionate number of seedlings dying from other pathogens in the soils inoculated with DOUG oospores affected the regression value. We also observed less damping-off caused by *R. solani* and *Pythium* as concentrations of oospores, heat-treated and/or untreated, increased in soil.

Assessment of oospore viability by microscopic examination for the DOUG and LONG appearance is quick, easy, and more reliable than the alternative viability tests of plasmolysis or staining with tetrazolium bromide. Plasmolysis induced with 4 M NaCl provided very similar results to DOUG but was not as reliable in assessing oospore viability. Treatment of oospores with alcohol or boiling water (which should have killed all oospores) was determined at 3 and 9% viability by plasmolysis, respectively, but none of these oospores were assessed as viable by DOUG. Discrepancies in viability results between these two visual methods may be attributed to an artifact peculiar to plasmolysis when the presence of antheridia behind the oogonia/oospore is interpreted as a viable oospore. The DOUG method also has several other advantages over plasmolysis because it is non-destructive, reagents are not needed, and oospores can be viewed at any time.

Tetrazolium bromide did not ascertain viability or death of oospores as evidenced by a low percentage of oospores that stained pink or lavender when they were exposed to lethal stresses or not treated. The tetrazolium bromide stain likely is a measure of the permeability of the oospore wall and may reflect maturation or dormancy. Impermeability of the oospore wall to water and water-based stains is a common condition in other oospore-producing fungi such as species of *Pythium* or *Phytophthora.* A small but consistent number of oospores were stained by tetrazolium bromide, irrespective of age, which suggests an artifact or the possibility that oospores were in an active physiological condition. Perhaps this low percentage of oospores were primed and ready to germinate, which fits the observation that only a small percent of oospores germinate at any given time.

CONCLUSIONS

Examination of oospores of *A. cochlioides* for densely organized uniform granules (DOUG) is a quick, effective, reliable, and accurate measure of oospore viability. This procedure provides results similar to plasmolysis but has important advantages over the latter including clear visual designations, gentle treatment of oospores, and freedom from use of reagents. The tetrazolium bromide stain did not measure oospore viability but instead, provided information on the impermeability of the oospore to water and water-soluble substances. This impermeability relates to oospore maturity; stain permeability may indicate a predisposition to germination.

ACKNOWLEDGEMENTS

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