Physical, Enzymic, and Chemical Factors Affecting Viability and Germination of Oospores of *Phytophthora megasperma* f. sp. *medicaginis*

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This research was part of a Ph.D. dissertation, University of California, Riverside, by the senior author.
Accepted for publication 21 November 1985.

ABSTRACT

El-Hamalawi, Z. A., and Erwin, D. C. 1986. Physical, enzymic, and chemical factors affecting viability and germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis*. Phytopathology 76:503-507.

Oospores of *Phytophthora megasperma* f. sp. *medicaginis* were evaluated for viability (activated, dormant, or dead) by using tetrazolium bromide, a vital stain, and concomitantly other samples were evaluated for germination in aqueous media. The increase in percentage of activated oospores resulting from different treatments was positively correlated with the increase in the percentage of germination ($r = 0.985$). Conversely, the percentage of oospores that were dormant or dead was negatively correlated ($r = -0.922$) with germination. Pretreatments of 40-day-old oospores with 0.05% $KMnO_4$ for 10 min markedly increased oospore activation and germination from 23 to 78% in water and to 84% in root exudate and also killed all contaminating mycelial fragments. Heat shock of oospores at 33 C prior to germination also increased oospore activation and germination. Desiccation and treatment of oospores with 0.5% $KMnO_4$ for 10 min was lethal. Freezing (−15 C) was not lethal, but it reduced viability and germination of oospores. Some, but not all, of the contaminating hyphal fragments were killed by freezing. Sodium periodate at 0.05% had no effect on viability and germination, but at 1.0% oospore viability and germination decreased. Viability and germination of oospores pretreated with either a mixture of β-glucuronidase and aryl sulfatase or cellulase alone did not differ significantly from the untreated control; however, prolonged treatment (48 hr) with the mixture decreased oospore germination. The phytoalexin, medicarpin, added to the growth medium in which oospores were produced or used as a pregermination treatment of oospores, reduced their viability and germination. The percentage of germination was consistently two to three times as great in alfalfa root exudate as in distilled water. Germination of oospores followed by production of a germ sporangium occurred only in root exudate and not in water.

Additional key words: medicarpin, root exudate, tetrazolium.

Germination of oospores and their role in variability of several species of *Phytophthora* has been reviewed (17,21). Factors affecting germination of oospores in vitro has been of scientific interest because they are important survival propagules and a source of genetic variation (21,29).

Treatments of oospores with a commercial snail gut enzyme (β-glucuronidase and aryl sulfatase) enhanced oospore germination of *P. megasperma* Dreschs. f. sp. *medicaginis* (19) and *P. cactorum* (2) but did not increase oospore germination of *P. megasperma* f. sp. *glycinea* (16). A short pretreatment of oospores with heat (15,16,18) stimulated oospore germination. Root exudate increased the percentage of germination of *P. m.* f. sp. *medicaginis* (9) and *P. m.* f. sp. *glycinea* (12,23). The combination of desiccation, rehydration, high temperature, and short exposure to dilute $KMnO_4$ stimulated synchronous germination of oospores of *Pythium aphanidermatum* (18).

Dormancy, activation, or death of oospores has been detected prior to germination by the use of tetrazolium salts as vital stains (26). The differential staining is due to the reduction of the tetrazolium salt in the spore by a cellular dehydrogenase enzyme which forms a colored formazan product (10,13). Since oospores may be dormant, this method is supplementary to actual germination tests to determine viability (26).

In this investigation, the effects of several physical, enzymic, and chemical factors on viability and germination of oospores of *P. m.* f. sp. *medicaginis* were determined. Part of these results have been previously reported briefly (7) and in greater detail in a thesis (6).

MATERIALS AND METHODS

Oospore germination and cultural conditions. *P. m.* f. sp. *medicaginis* P1057, a single-zoospore isolate, was obtained from roots of alfalfa in California. Carrot broth (15 ml per 90-mm-diameter petri dish) medium was inoculated with minced mycelium and incubated at 24 C in the dark for 40 days. Carrot broth consisted of 200 g of fresh carrots blended in 1 L of double-glass-distilled water and filtered through four layers of cheesecloth. Water used in all experiments was distilled twice, boiled, and autoclaved before use and will be referred to hereafter as water.

The method used to separate oospores from mycelium was modified from that of Förster et al (9) by omission of the enzymes (Glusulase; Calbiochem-Behring, La Jolla, CA) (activity: 8.6 IU of β-glucuronidase and 1.7 IU of aryl sulfatase per milliliter) pretreatment and the sucrose density gradient centrifugation. Mycelial mats containing oospores from three petri dish cultures were comminuted in sterile water on ice for 5 min with a Sorvall Omnimixer (Ivan Sorvall Inc., Newton, CT) operating at medium speed, sonicated (Braun-Sonic 1510, S. Braun Instruments, South San Francisco, CA) on ice for 4 min at 100 W, and concentrated and washed at least three times by centrifugation at 1,200 *g*. Oospores were free of mycelial debris and media and were stored in water at 3 C for up to 2 wk. Freedom from viable mycelium was checked by plating samples of oospore suspensions on V8 juice agar. Oospores were pelleted by centrifugation for 2 min at 1,200 *g* prior to various treatments.

Staining and germination of oospores. A 0.1% solution of 3-(4,5-dimethylthiazolyl-2)2,5-diphenyl-2H-tetrazolium bromide (tetrazolium bromide) (Aldrich Chemical Co., Milwaukee, WI) in sterile water was used for viability tests. A suspension of oospores was mixed with the tetrazolium bromide solution (1:1, v/v) and incubated in sterile microbeakers (22 mm in diameter) containing about 200 oospores each for 48 hr at 36 C. Viability was classified by light microscopy at ×100 magnification: dormant oospores were rose-colored, activated oospores were blue, and nonviable oospores were black or unstained (26). Oospores tested for viability were not used in germination tests.

To determine germinability, oospores were incubated in 2 ml of the test solution in petri dishes (35 mm in diameter) under blue light at 800 $\mu W \cdot cm^{-2}$ (Westinghouse F15T8-B fluorescent bulb) (9) at 24

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C for 5–10 days. Although germination occurred under laboratory light, blue light was a favorable and readily reproducible regime for germination (H. Förster, O. K. Ribeiro, and D. C. Erwin, *unpublished*). Germination in aqueous media was determined microscopically after 5–7 days at ×100 magnification. Morphology of germinated oospores was similar to that previously described (9). Since preliminary comparisons showed that germination was always at least two to four times higher in aqueous media than on 1.5% water agar, all germination tests were conducted in aqueous media. Treatments in each viability and germination experiment were replicated three times and experiments were repeated one to three times.

Preparation of alfalfa root exudate. Alfalfa seeds of the Phytophthora root rot-susceptible cultivar Moapa 69 (M69) were surface sterilized by exposure to chlorine gas for 24 hr (27). The seeds were germinated aseptically on 1.5% Difco nutrient agar (Difco Laboratories, Detroit, MI) and incubated for 24 hr in the dark. One hundred uniform seedlings with roots protruding through holes in sterilized aluminum foil containers were floated in sterile water (40 ml) in deep petri dishes (90-mm diameter). Plants were incubated under fluorescent lamps (two 40 W Vitalite Power-Twist bulbs, Durotest Corp., North Bergen, NJ) with a spectral peak at 550 nm and intensity of 1,200 $\mu W \cdot cm^{-2}$ (12 hr light/12 hr dark). After 1 day, the seed coats were removed, and the water was replaced. Root exudate was collected after 4 days, replaced with sterile water, and collected again after 4 days; the two samples were pooled and verified for sterility by streaking samples on Difco nutrient agar or incubation in Difco Eugon nutrient broth. Contaminated exudates were discarded. Sterile exudates were centrifuged at 1,200 *g* to remove particulate material and stored at −5 C. Root exudate was passed through a Nalgene filter (0.2 μM) (Nalgene Sybron Corp., Rochester, NY) before use.

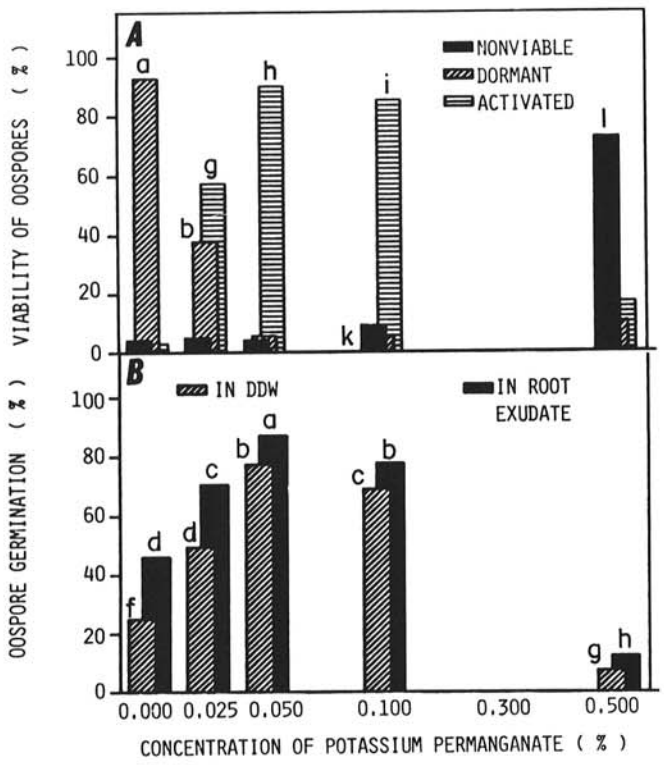

Fig. 1. A, Viability and **B,** germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis* treated with potassium permanganate ($KMnO_4$). Oospores, suspended in 10 ml $KMnO_4$ for 10 min at room temperature, washed by centrifugation, were incubated in sterile water and in alfalfa root exudate under blue light at 24 C for 10 days. DDW = double distilled water. Each germination value is the mean of three replicates (450 oospores per replicate). Viability of different samples of oospores was measured after treatment with tetrazolium bromide (three replicates, 200 oospores per replicate). Germination in water and in root exudate were correlated with activation ($r = 0.985$ and 0.986). Activation was correlated with dosage of $KMnO_4$ up to 0.1% ($r = 0.810$). Activation was negatively correlated with dormancy ($r = -0.999$).

Treatment of oospores with chemicals. Oospores were pelleted by centrifugation for 2 min at 1,200 *g*, and treated for various times in 20 ml of 5% β-glucuronidase (8.6 IU/ml) and aryl sulfatase (1.7 IU/ml) in 0.1 M sodium phosphate buffer (pH 6.0) or in 20 ml of 2.0% (w/v) cellulase (22) (Glusulase; Calbiochem-Behring, La Jolla, CA) in 0.1 M sodium phosphate buffer (pH 6.0) and incubated at 27 C in the dark. After different times of treatment, aliquots of oospores were removed and washed with water several times by centrifugation at 1,200 *g*.

Oospores were suspended in 10 ml of fresh $KMnO_4$ (0.025–0.50%) or $NaIO_4$ (3) (0.025–2.0%), agitated for 10 min on a Vortex mixer (Deluxe Mixer, Scientific Products, Evanston, IL), and washed several times by centrifugation. The effect of $KMnO_4$ on mycelial growth from mycelial fragments was determined by culturing treated fragments on V-8 agar.

The phytoalexin, medicarpin, was extracted from alfalfa seedlings, infected with *P. m.* f. sp. *medicaginis* by the method of Vaziri et al (28). Medicarpin in ethanol was added to autoclaved carrot broth to bring the final concentration to 100 μg/ml. Oospores were produced in unamended carrot broth, 1% ethanol-carrot broth (solvent control), and medicarpin-ethanol carrot broth, harvested after 40 days at 24 C, and tested for viability and germinability. Oospores from unamended carrot broth were incubated in medicarpin solution (100 μg/ml in 1% ethanol) for 24 hr at 5 C, washed by centrifugation, and tested for viability and germination.

Heat, desiccation, lyophilization, and freezing treatments of oospores. Oospores were heat treated at 24, 27, 30, 33, 36, and 39 C for 5 hr, cooled to 6 C, and concentrated by centrifugation for 2 min at 1,200 *g*. Physical treatments of carrot broth cultures containing oospores (40 days old) involved freezing (−15 C) for different times prior to thawing at room temperature (21 C), lyophilization or desiccation in a hood by continuous air flow at 21 C for 4 days. Oospores in the dried cultures were rehydrated, blended, and freed of mycelium as described previously. Oospores from all treatments were harvested and tested for viability and germinability.

RESULTS

Sonication effects on oospore germination. Sonication of an oospore-mycelium suspension for 3–4 min at 100 W, or 1.5 min at 150–250 W, destroyed mycelial fragments but did reduce oospore germination. At lower sonication intensities or at shorter time intervals, some hyphal fragments were still viable and produced colonies on V-8 agar. Based on these data, the oospore-mycelium suspension was sonicated for 4 min at 100 W on ice to prevent heating.

Determination of viability by tetrazolium bromide. The color reaction of oospores, assayed in parallel with germination tests, was correlated with germinability (Figs. 1 and 2). The percentage of activated oospores (blue) were positively correlated with germination (Fig. 1, $r = 0.985$ and Fig. 2, $r = 0.926$); whereas the percentage of dormant (rose) plus nonviable (black or unstained) oospores were negatively correlated with germination (Fig. 1, $r = -0.999$ and Fig. 2, $r = -0.900$).

Effect of chemicals on viability and germination. Pretreatment of oospores for 12 hr with the enzyme mixture did not affect the percentage of germination (49%) or activation (tetrazolium bromide test) but treatment for 48 hr decreased germination to 20% (Fig. 3). Increased time of exposure (0–4 hr) to cellulase increased the percentage of activated oospores from 3 to 21% and decreased the percentage of dormant oospores from 84 to 74% (activation and dormancy were negatively correlated $r = -0.999$). Germination in alfalfa root exudate was increased from 46 to 57% ($P = 0.01$) (Fig. 4).

Treatment with $KMnO_4$ (0.05%) for 10 min increased the percentage of activated oospores (from 3 to 90%), decreased the percentage of dormant oospores (from 93 to 6%) and increased the percentage of oospores that germinated in water (from 25 to 77%). In root exudate, germination increased from 46.0 to 87.0% (Fig. 1).

In water, germination was entirely by germ hyphae but in root exudate 80% of the total germinated by germ sporangia. $KMnO_4$ at 0.5% killed most of the oospores. Mycelial fragments from oospore suspensions treated with $KMnO_4$ at all dosages did not grow when plated on V-8 juice agar.

$NaIO_4$ at dosages of 0, 0.25, and 0.5% did not affect either viability or germination of oospores, but at 1.0% it reduced germination from 46 to 19% (significant $P = 0.01$) in root exudate. At 1.5% $NaIO_4$, nearly 80% of the oospores became nonviable (tetrazolium bromide test) and only 5% germinated. At 2% $NaIO_4$, no oospores germinated and 100% were nonviable ($P = 0.01$).

Germination in root exudate of oospores grown in medicarpin-ethanol-amended medium (26 ± 2.6%) was significantly less than germination of oospores grown in ethanol-amended medium (46 ± 2.6%) or in unamended medium (51 ± 2.0%). The percentage of dormant oospores from the medicarpin-amended medium (53.0 ± 2.6%) was less than from ethanol-amended medium (93.3 ± 1.2%) or from nonamended medium (95.7 ± 1.2%).

Treatment of oospores with medicarpin in 1% ethanol decreased the percentage of dormant oospores slightly from 95.7 ± 1.2% to 83 ± 2.6%, but 1% ethanol had no effect (93.3 ± 1.2%). Germination of medicarpin-treated oospores was 11.0 ± 1.8% in water and 23.0 ± 1.7% in root exudate; germination of ethanol-treated oospores was 19 ± 1.7% in water and 46 ± 2.6% in root exudate; germination of untreated oospores (control) was 22.0 ± 1.7% in water and 51.0 ± 2.0% in root exudate.

Effect of physical factors on viability and germination. An increase in pregermination incubation temperatures of 24–33 C significantly increased the percentage of activated oospores from 1% at 24 C to 75% at 33 C while the percentage of dormant spores decreased to 30%. The percentages of activated and dormant oospores were negatively correlated ($r = -0.922$). Germination in root exudate was correspondingly increased by pretreatment at 33 C from 41% (control at 24 C) to 89%. Temperatures of 36 and 39 C increased the percentage of nonviable oospores from about 5% to over 80%. Temperatures over 36 C decreased germination markedly (Fig. 2).

Desiccation decreased the percentage of dormant oospores significantly from 96% for the undesiccated control to 29.0%. Germination in root exudate was also decreased from 47 to 18%. Lyophilization reduced viability and germination to 0.

Freezing (−15 C) for 24 hr did not affect the percentage of dormant oospores, but longer periods of freezing (96 hr) increased the percentage of nonviable oospores from 0 to over 80% (Fig. 5). The increase in percentage of nonviable oospores was correlated with time of freezing ($r = 0.950$) and negatively correlated with percentages of dormant oospores ($r = -0.940$). As freezing time increased, oospore germination in root exudate decreased from 47% at 0 hr to 8% after 96 hr of freezing. Mycelial fragments that germinated from a 1-ml sample of an oospore-mycelium suspension on V-8 agar were decreased from 62 per plate at 0 time, to 48 at 24 hr, to 27 at 48 hr, to 13 at 72 hr, and to 5 at 96 hr. In no case did freezing kill all mycelial fragments. Differences were highly significant ($P = 0.01$).

Germ sporangia formation in root exudate. In water, germination occurred only by germ hyphae, but in root exudate over half of the oospores consistently germinated by germ sporangia (Figs. 3–5). These data were typical of all experiments reported here and for many others which are not presented (6).

DISCUSSION

This is the first report of the stimulatory effect of $KMnO_4$ on oospore germination of *Phytophthora*. Pregermination treatment

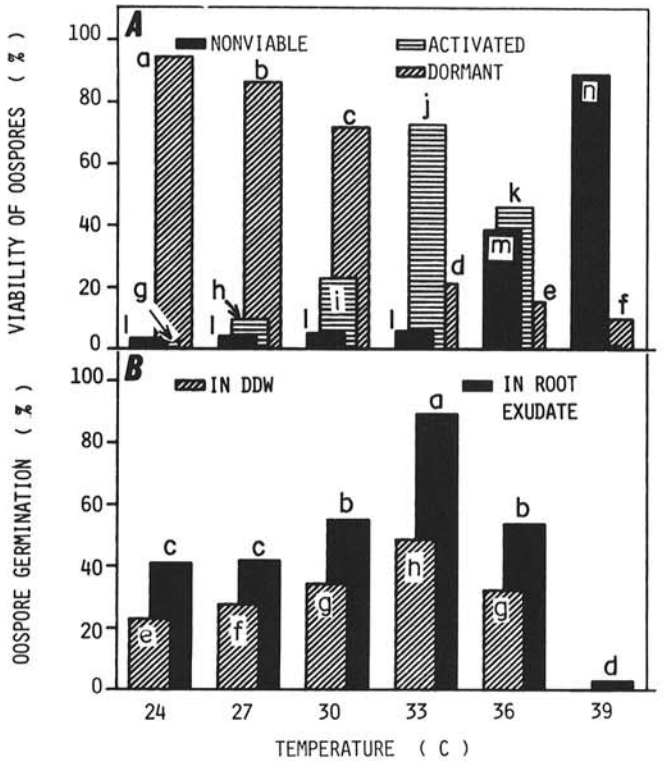

Fig. 2. A, Viability and **B,** germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis* pretreated at several temperatures. Oospores were incubated for 5 hr at 24–39 C before incubation in sterile water and in alfalfa root exudate under blue light at 24 C for 10 days. DDW = double distilled water. Each germination value is the mean of three replicates (450 oospores per replicate). Viability of different samples of oospores was measured after treatment with tetrazolium bromide (three replicates, 200 oospores per replicate). Germination in water and root exudate were correlated with activation ($r = 0.927$ and 0.928). Activation was negatively correlated with dormancy ($r = -0.922$).

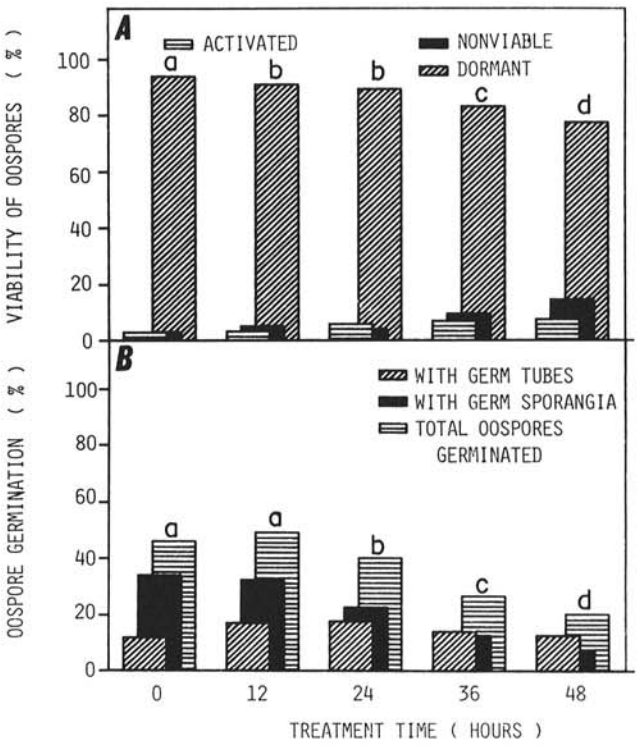

Fig. 3. A, Viability and **B,** germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis* treated with commercial snail gut enzyme. Oospores were incubated in a 5% mixture of β-glucuronidase and aryl sulfatase in 0.1 M phosphate buffer (pH 6.0) at 27 C before incubation in sterile alfalfa root exudate under blue light at 24 C for 10 days. Each value is the mean of three replicates (450 oospores per replicate). Germination percentages in sterile water were about 50% lower. Viability of different samples of oospores was measured after treatment with tetrazolium bromide (three replicates, 200 oospores per replicate).

of oospores with $KMnO_4$ at 0.05% markedly increased activation and stimulated germination in water to nearly that in root exudate. Since mycelial fragments were killed by $KMnO_4$, this should be an efficient method of obtaining activated, germinable oospores free of viable mycelium. Ruben et al (18) reported that $KMnO_4$ stimulated germination of predesiccated, but not undesiccated, oospores of *Pythium aphanidermatum*. The mechanism for the activating effect of $KMnO_4$ treatment is not known, but it may involve the chemical oxidation of wall components such as lipids (18).

Pretreatment of oospores at a temperature higher or lower than ambient often enhances germination (17). In our studies, pretreatment of the oospores at 33 C before germination increased the percentage of activation and also germination. This effect has been noted in several reports (5,15,16). Sussman (25) suggested that heat treatment may cause conformational changes in constituent proteins and lipids. Heat activation of spores of *Dictyostelium discoideum* spores was considered to be related to induced changes in regulatory proteins located in mitochondrial membranes (4) or to immediate structural alterations in the plasma membrane and an increase in osmolarity and permeability (11).

Results of this work confirms those of Förster et al (9) who reported that the percentage of oospore germination of *P. m.* f. sp. *medicaginis* was significantly greater in alfalfa root exudate than in water and also that oospores germinated by germ sporangia only in root exudate and not in water. The rapid increase in zoospore inoculum from this postgermination stage could have ecological and epidemiological significance.

The data on detection of dormancy, activation, and death (nonviability) of oospores by the tetrazolium bromide-oxidative enzyme-induced color reaction confirmed results of previous work (26) and showed that activation and germination of oospores were correlated. Conversely, the number of dormant or nonviable oospores detected was correlated with a decrease in germination. Thus, a low percentage of oospore germination is not necessarily due to lack of viability. Activating treatments, such as $KMnO_4$ or heat shock at 33 C, induced a shift in the proportion of dormant oospores to activated which was correlated with an increase in germination. Freezing oospores for 24 hr decreased the percentage of activated oospores, slightly increased the percentage which was dormant, and decreased the percentage of germination. Freezing for longer periods of time (96 hr) killed over 80% of the oospores but did not kill all contaminating mycelial fragments. These results are similar to the report by Sauve and Mitchell (20) for *Pythium* spp. Other workers assumed that freezing killed mycelial fragments but did not affect viability of the oospores (8,14).

Oospores of different species and even isolates of the same species of *Pythium* or *Phytophthora* often react differently to the same enzyme treatment (17). Oospore germination of *P. m.* f. sp. *medicaginis* (19) and *P. cactorum* (2) was enhanced by pretreatment with β-glucuronidase and aryl sulfatase. In our study, however, they did not increase the percentage of germination or increase activation of oospores of *P. m.* f. sp. *medicaginis* in either alfalfa root exudate or water (Fig. 3). Reduction in oospore germination by prolonged treatment with the mixture for 48 hr was also reported previously (9). Since there was no advantage in pretreating oospores with the enzyme, and nutrients released by cell walls might be a source of variability in our experiments, this treatment was not used. The reason our results differ from those of Salvatore et al (19), in which the enzymes increased germination markedly from about 5 to 60%, is not evident but could be associated with our use of a higher concentration (5%) than they (1–2%) (19) and 0.1 M phosphate buffer. However, our treatment with 5% enzyme mixture for 24 hr did not inhibit germination.

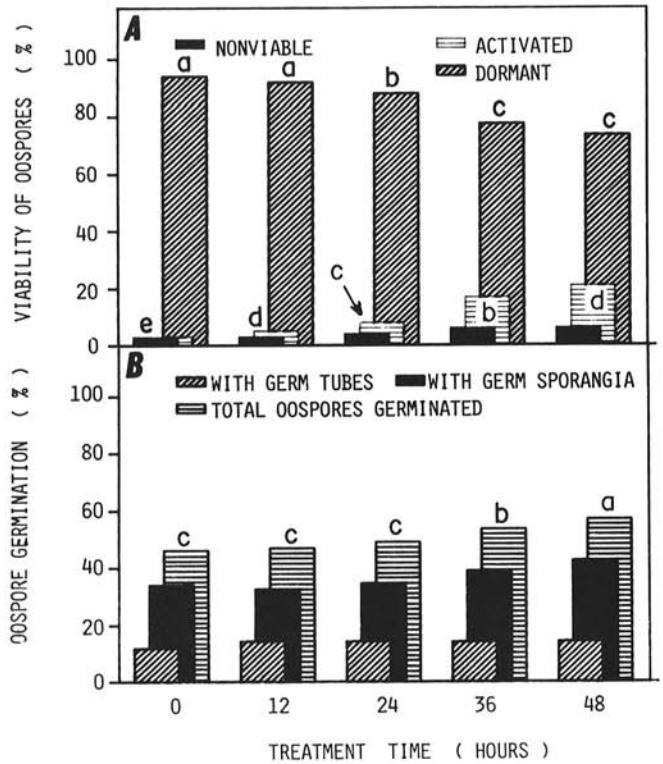

Fig. 4. A, Viability and **B,** germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis* treated with cellulase. Oospores were incubated in 2% cellulase in 0.1 M phosphate buffer (pH 6.0) at 27 C before incubation in sterile alfalfa root exudate under blue light at 24 C for 10 days. Each germination value is the mean of three replicates (450 oospores per replicate). Germination percentages in sterile water were about 50% lower. Germination was correlated with time of treatment ($r = 0.972$). Viability of different samples of oospores was measured with tetrazolium bromide (three replicates, 200 oospores per replicate). Activation was negatively correlated with dormancy ($r = -0.999$).

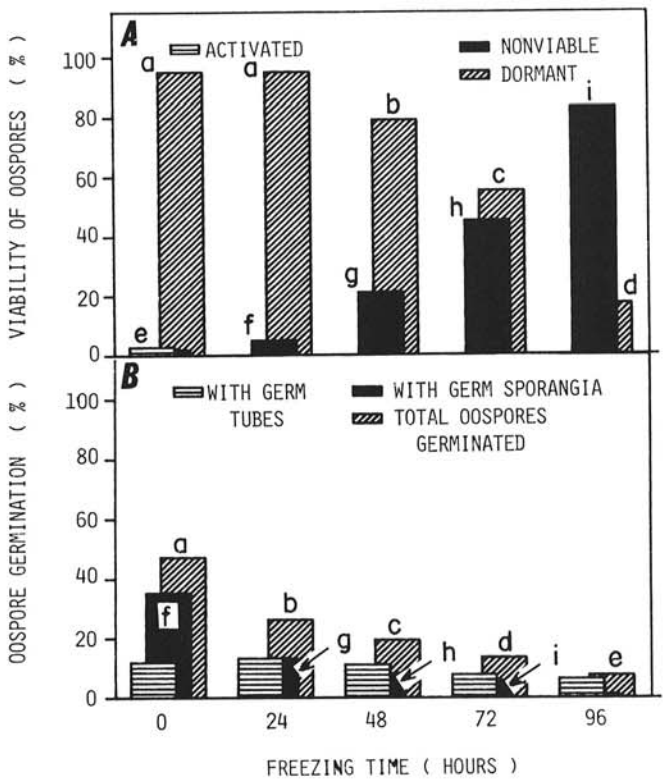

Fig. 5. A, Viability and **B,** germination of oospores of *Phytophthora megasperma* f. sp. *medicaginis* after different times of freezing. Cultures in carrot broth (40 days old) were frozen and stored for different periods of time at −15 C. Cultures were thawed and stored at room temperature and oospores were purified of mycelium before incubation in alfalfa root exudate under blue light at 24 C for 10 days. Each germination value is the mean of three replicates with 450 oospores per replicate. Oospore viability of different samples was measured with tetrazolium bromide (three replicates, 200 oospores per replicate).

Pretreatment of oospores of *P. m.* f. sp. *medicaginis* with 2% cellulase slightly increased germination and activation with increased time of exposure. The data presented suggest that a longer period of treatment might have increased oospore germination to a greater extent. Suave and Mitchell (29) reported that cellulase and hemicellulase increased oospore germination of *Pythium myriotylum*. The mechanism of cellulase enzyme enhancement of oospore germination is not known.

Although medicarpin in a growth medium stimulated an increase in numbers of oospores (28) the viability and germinability of oospores from medicarpin-amended media was significantly less than from the untreated control. Medicarpin treatment of oospores also decreased the viability and germinability of oospores. Thus, medicarpin not only inhibits mycelial growth (28) but also negatively affects oospore viability and germination.

Desiccation of oospores of *P. m.* f. sp. *medicaginis* decreased the viability and germination significantly; however, desiccation had a stimulatory effect on oospore germination of *Pythium aphanidermatum* (18). Drying oospores of *Pythium* sp. on agar disks increased subsequent germination (1).

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