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Temperature and Water Effects on the Viability of Alginate-Formulated Oospores of *Pythium Oligandrum*

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تأثير الحرارة والجهد المائي على حيوية الأبواغ البيضية لفطر
(بيثيم اوليجاندرم *Pythium oligandrum*) المحملة في كريات الألجنين

الملخص : إن انخفاض معدل إنبات الأبواغ البيضية يعيق كفاءة استخدام فطر بيثيم اوليجاندرم *Pythium oligandrum* كمقاوم حيوي ضد كائنات التربة الممرضة للنبات . ومن أجل تحسين درجة الاعتماد على لقاح الأبواغ البيضية لهذا الفطر في مجال المكافحة الحيوية لأمراض الجذور ، تم تحميل أبواغ الفطر البيضية في كريات من الجينات الكالسيوم (كريات الأبواغ) . عند حفظ كريات الأبواغ لخمس عزلات مختلفة من الفطر تحت درجة حرارة ٤°م وظروف تخزين جافة ، وجد أن الأبواغ المحملة تحافظ على حيويتها (معدل إنبات أكثر من ٩٩ % لكل مرحلة زمنية) خلال فترة ١٢ شهرا بغض النظر عن الوسط المزرعي الذي أنتجت فيه الأبواغ . وبالمقارنة فان كريات الأبواغ المخزنة تحت درجة ٢٥°م ونفس الظروف السابقة أبقت على حيويتها لمدة ٣ أشهر ثم انحدرت حيويتها إلى ٥٠ % في الأشهر التسعة التالية . إن أعلى إنبات (٩٨%) للأبواغ المحملة (كريات الأبواغ) حدثت تحت جهد أسموزي أعلى من -١,٣٣ ميجاباسكال أو تحت رطوبة نسبية أعلى من ٩٧% ودرجات حرارة ٢٥ و ٣٣°م . وقد تدنى الإنبات بشدة بانخفاض الجهد الأسموزي من -١,٣٣ إلى -٢,٥٩ ميجاباسكال وانخفاض الرطوبة النسبية من ٩٧ إلى ٩٤% مع توقف الإنبات تماما عند -٣,٥٩ ميجاباسكال و ٩٢ % رطوبة نسبية. أما تحت درجة ١٦°م فقد بلغ الإنبات ٦٠% فقط في المدى الأعلى للجهد الأسموزي والرطوبة النسبية ، ولم يحدث أي إنبات عند -١,٣٣ ميجاباسكال و ٩٥ % رطوبة نسبية . إن نمو الخيوط الفطرية كان مشابها في تحمله لنفس ظروف الرطوبة والحرارة ، كما أن النسبة المرتفعة لبقاء الأبواغ البيضية المحملة أثناء التخزين مترافقة مع نسبة الإنبات العالي في الظروف الجافة نسبيا تدل على إمكانية استخدام كريات الأبواغ كلقاح فعال عند استخدام فطر بيثيم اوليجاندرم *Pythium oligandrum* لمكافحة مسببات الأمراض في التربة أو أي من الخلائط المستخدمة بدلها تحت درجات الحرارة المرتفعة أو تحت ظروف البيوت المحمية .

ABSTRACT: The low germination rates of oospores hinder the use of *Pythium oligandrum* as a biocontrol agent of soilborne plant pathogens. To improve the reliability of oospore-inoculum of *P. oligandrum* for the biological control of root diseases, oospores were formulated in calcium alginate pellets (oospore-pellets). Oospore-pellets of five isolates of *P. oligandrum* stored at 4°C under dry conditions remained viable (≥ 99% germination for each time interval) over 12 months regardless of the culture medium used to produce the oospores. In contrast, oospore-pellets stored at 25°C under the same conditions maintained their viability for 3 months but declined to 50% viability in the following 9 months. Germination of oospore-pellets was at the highest (98%) at solute potentials (Ψ_s) higher than -1.33 MPa or relative humidities (RH) higher than 97% at 25° and 33°C. Germination declined sharply as Ψ_s decreased from -1.33 to -2.59 MPa and RH decreased from 97 to 94% with no germination occurring at -3.59 MPa and 92% RH. Only 60% germination was found at 16°C in the top range of Ψ_s and RH, and germination declined sharply as Ψ_s and RH were decreased, with no germination occurring at -1.33 MPa and 95% RH. Mycelial growth exhibited similar ranges of tolerance under the same moisture conditions and temperatures. High degrees of survival during storage accompanied by high percentages of germination under relatively dry conditions suggest that the oospore-pellet inoculum of *P. oligandrum* can be used for the control of pathogens in soil or a potting mix at high temperatures during a warm season or under greenhouse conditions.

Pythium oligandrum Drechsler is a potential biocontrol agent of several soilborne plant pathogenic fungi (Al-Hamadani *et al.*, 1983; Al-Rawahi, 1995; Martin & Hancock,1987; McQuilken *et al.*, 1990a; Vesely, 1979). It is a mycoparasitic Oomycete and a strong microbial competitor on the surfaces of seeds, protecting them from certain damping-off diseases (Al-Hamadani *et al.*, 1983; Martin & Hancock, 1987; Vesely, 1979). Although it is not a plant pathogen, there are reports that some

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isolates may have small deleterious effects on plant growth (Kilpatrick, 1968; Pieczarka & Abawi, 1978; Vesely, 1978). A major factor that has slowed progress in the use of *P. oligandrum* for the biocontrol of soilborne plant pathogens in agriculture is the low percentage of germination of its oospores, which are thick-walled, sexual reproductive structures. Oospores are the principal survival spores of *P. oligandrum* in soil and could be an ideal inoculum in biocontrol applications. However, oospores are constitutively dormant for 40 to 200 days (Drechsler, 1946), which results in variable and usually low germination rates and confounds their use as reliable inoculum units in biocontrol experimentation and applications.

Martin & Hancock (1987) reported 5% germination of oospores from 3 to 4 week-old corn meal agar (CMA) cultures, and McQuilken *et al.* (1990b) reported initial germination of 16 to 22% of oospores produced on molasses broth (MoB). However, germination declined to a low of 6% after four months of storage between 5 and 30°C (McQuilken *et al.*, 1990b). Similar observations have been recorded for several isolates of *P. oligandrum* grown on MoB or on a synthetic medium where germination of separate batches of oospores varied between 3 and 30% (Al-Rawahi, 1995). The problem of poor germination or inoculum quality was overcome by coating seeds with large quantities of oospores, which ensured mycelial colonization of seed surfaces by the biocontrol agent (Al-Hamadani *et al.*, 1883; Martin & Hancock, 1987). Although this tactic is successful in seed treatments, it is not practical for inoculum delivery to soil and roots where large amounts of inoculum are needed.

Formulating oospores into pellets could improve the inoculum quality and enhance the logistics of delivery. Formulated oospores could be applied as seed inoculants in a fashion similar to methods routinely used with legume-rhizobial inoculations, which also have been used successfully for several biocontrol agents of plant pathogens (Dandurand & Knudsen, 1993). Formulations incorporate the inoculum in a solid carrier to facilitate packaging, shipment, storage, and application. Cells or reproductive units of beneficial microbials are mass-packaged, and formulations should optimize conditions for their longevity as well as renew growth after application (Smith, 1992).

Several materials have been tested or used as carriers in the formulation of inocula of microbial biocontrol agents, including polyacrylamide, Laponite™, and alginate gels (Dandurand & Knudsen, 1993; Dommergues *et al.*, 1979; Fravel *et al.*, 1985; Knudsen *et al.*, 1991; Lewis & Papavizas, 1985; McQuilken *et al.*, 1992b; Walker & Connick, 1983). Sodium alginate possesses the desired features of a carrier and has been used widely for chemical and microbial herbicides. In an aqueous solution, it reacts with cations such as Ca^{2+} to form gels that solidify into dry, uniform sized, biodegradable pellets (Connick, 1982; Walker & Connick, 1983). It may also be used with bulking agents such as clay and osmoregulants such as polyethylene glycol (PEG). Mc Quilken *et al.* (1992b) reported that alginate oospore-pellets of *P. oligandrum* were less effective than oospore-coated seeds in preventing damping-off of cress (*Lepidium sativum*). However, there may be advantages in the use of oospore-pellets in soil treatments and as root inoculants. This study evaluated an alginate-made oospore formulation in relation to the longevity and germination of oospore-pellet inoculum over a range of moisture and temperature regimes.

Materials and Methods

FUNGI AND OOSPORE PRODUCTION: The following five isolates of *P. oligandrum* were used in this study: 76-13, isolated in association with bean roots, Salinas, CA; 86-21, ATCC 38472, isolated from a sugar beet field, CA; 87-6; 87-7, obtained from the collection of M. C. Middleton, University of California, Berkeley; and 91-4, isolated from a tomato root, CA. Stock cultures were stored on 25 ml hemp seed water or wheat leaf water in screw capped, 100-ml prescription bottles at room temperature (24±2°C) (Raabe *et al.*, 1973; Singleton, 1986). Fresh cultures were maintained on the following Difco agars: 5.25% potato-dextrose agar (PDA), 1.75% water agar (WA), or 1.7% corn meal agar (CMA).

Large quantities of oospores were obtained by growing each isolate of *P. oligandrum* on a liquid synthetic medium (minimal medium for *P. oligandrum* [MMPO]) or on a molasses broth (MoB) medium (Al-Rawahi, 1995; McQuilken *et al.*, 1990b). MoB (6%, v/v) was made from de-sulfured sugar cane molasses (Grandma's molasses™, Cadbury Beverages Inc.; Stamford, CT, USA). MMPO consisted on a per liter basis of D-glucose, 6 g; DL-asparagine, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 40 mg; cholesterol, 60 mg; KH_2PO_4, 0.8 g; K_2HPO_4, 0.15 g; thiamine -HCl, 75 μg; $FeSO_4 \cdot 7H_2O$, 1 mg; and 1 ml of a microelement solution. The microelement solution was composed of $Na_2MoO_4 \cdot 2H_2O$, 41 mg; $CuSO_4 \cdot 5H_2O$, 8 mg; $ZnSO_4 \cdot 7H_2O$, 88 mg; $MnSO_4 \cdot H_2O$,15 mg; and $Na_2B_4O_7$, 0.5 mg; in 100 ml of deionized, distilled H_2O. Each medium was dispensed into large test tubes (25 mm x 200 mm) fitted with autoclavable caps as 10 ml per tube and were autoclaved at 121°C and 0.1 MPa for 20 min. Each isolate of *P. oligandrum* was inoculated in at least 20 tubes using 10 mm-diameter disks from 4-day-old WA cultures. Test tubes were sealed in plastic bags and slanted at 20° angle to maximize surface area, and incubated at room temperature (24±2°C) for 6 weeks. At harvest,

contents of tubes were sonicated for 80 seconds continuously in an ice bath at 50% duty cycle (Branson Sonifier, Cell Disruptor 185). Such treatment disrupted about 99% of the mycelium without causing any obvious damage to oospores. Oospores were then washed with sterile distilled H_2O by centrifugation at 3,000*g* for 10 min. The washing process was repeated at least three times until all mycelial debris was removed. Concentrated oospore suspensions of each isolate were stored separately at 4°C until use.

FORMULATION METHODS: The apparatus used for the formulation of oospores was a modification of that described by Fravel *et al.* (1985) (Figure 1). It consisted of a sterile glass bottle (500-ml size) fitted at its bottom side with a tubular outlet to which a Y-adapter was connected. One end of the adapter was connected with a short polypropylene (Nalgene™, Nalge Co., Rochester, NY) tube that served to control pressure flow. The tube was fitted at its end with 0.45 μm membrane air filter to prevent air-borne contamination. The other branch was fitted with a series of Y-connections arranged dichotomously to permit the fitting of 8 pasteur pipettes (1 mm orifice diameter). Each branch was controlled by a valve to regulate suspension flow into 1 l size sterile beaker containing sterile 0.25 M $CaCl_2$. Tubes (7 mm diameter) and connections (6.25 mm size) were composed of autoclavable polypropylene and were autoclaved according to manufacturer instructions.

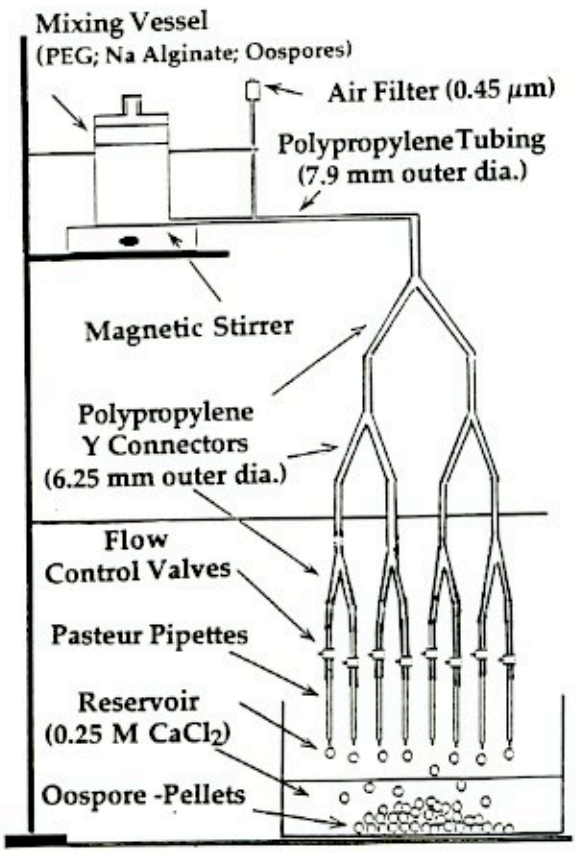

Figure 1. Apparatus used to formulate oospores of P. oligandrum into calcium-alginate pellets. Concentrated suspensions of oospores were mixed with aqueous solutions of polyethylene glycol 8000 (PEG) and sodium alginate before drops were added to 0.25 M $CaCl_2$.

Preliminary evaluations of several concentrations of sodium alginate (medium viscosity; Sigma Chemical Co., St. Louis, Mo.) showed that 10 mg ml^{-1} yielded the best results. Sodium alginate (2 g) was dissolved in 100 ml of sterile distilled H_2O over night at room temperature (24±2°C). Forty grams of PEG8000 (Sigma Chemical CO.) were included as osmoregulant, and dissolved in 95 ml of sterile d. H_2O to give a final concentration of 0.2 g ml^{-1} with a matric potential Ψ_m = - 0.526 MPa at 25°C (Michel, 1983). To initiate the formulation process, the sodium alginate solution and the osmoregulant solution were aseptically blended together in the mixing apparatus with the aid of a magnetic stirrer. Five ml of a suspension containing 3 x 10^6 oospores were added to 200 ml of the sodium alginate-osmoregulant solution and homogenized for 20 minutes. This was dripped into the calcium chloride solution. Thus the suspension was transformed into pellets (20-30 min), which were sieved using a double layer of sterile cheese cloth. Pellets were dried as single layer on wax paper in a laminar-flow hood at room temperature (24±2°C) and 40-50% RH for 24 h. This process was repeated for each isolate and medium. The dry weight of pellets obtained for each batch and isolate was determined, the diameter size was measured, and the average weight and expected number of oospores per pellet were calculated. The number of oospores per pellet was verified by examining microscopically thin sections of gelled droplets before drying into pellets (magnification 10x).

GERMINATION OF FORMULATED AND NON-FORMULATED OOSPORES: Two-day-old oospore pellets (i.e. formulated oospores, FO) and oospores harvested from MMPO or MoB cultures of the five isolates were tested for germination on CMA (1.7%, Difco). Thirty randomly selected pellets were seeded into duplicate plates of CMA (15 pellets/plate). Non-formulated oospores (NFO) were obtained from the same batches used for formulation and were also tested for germination. Duplicates of CMA were inoculated with 15 oospore-droplets (10 μl each) containing 600 oospores per droplet. Plates were incubated at 10, 16, 25 or 33°C for 48 h and examined for germination. Germination from oospore pellets and oospore droplets was recorded when germ tubes were at least 15 μm in length. Each pellet or droplet was considered to be a single unit in calculations.

GERMINATION OF OOSPORE-PELLETS UNDER DIFFERENT MOISTURE AND TEMPERATURE CONDITIONS: The effects of solute potential (Ψ_s) and temperature on oospore-pellet germination were determined on CMA (1.7%, Difco). Cultures were amended with seven concentrations of KCl to provide Ψ_s ranging from - 0.59 MPa (unamended CMA) to -5.5 MPa (Subbarao *et al.*, 1993). Verification of the Ψ_s of the KCl amendments was made at the end of each experiment using a thermocouple psychometer (model SC-10A, Decagon Devices, Inc., Pullman, WA). Aliquots of 20 ml of autoclaved media were deposited on petri plates (100 x 15 mm), and allowed to solidify without any condensation under laminar flow for 20 min. Plates were covered and incubated at 24±2°C for 3 days using conditions of moisture equilibrium. Three factors of variation were studied, namely: temperature with three treatments (16, 25, and 33°C), isolates of *P. oligandrum* with four treatments (76-13, 86-21, 87-6, and 91-4), and Ψ_s with seven treatments (-0.59, -0.70, -0.78, -1.33, -2.7, -3.6, and -5.5 MPa). Treatments were arranged in a multifactorial design and experiments were repeated using the same medium to avoid batch-to-batch variation in Ψ_s (Woods & Duniway, 1986). A total of 30 randomly selected pellets were seeded in three plates (10 pellets per plate) for each factorial treatment. Each pellet was considered as an experimental unit for each Ψ_s - temperature combination. To compare the germination of pellets with mycelial growth under similar osmotic conditions, five plates for each factorial treatment were inoculated with an 8 mm diameter disk of four-day-old WA cultures (76-13 and 91-4). Percentage germination and colony diameter were measured 72 h following inoculation.

The influence of relative humidity (RH) and temperature on oospore-pellet germination was assessed using a modification of the agar dish isopiestic equilibration technique described by Harris *et al.* (1970) and (Arauz & Sutton 1989). The agar-based humidity chambers consisted of petri plates (100 x 15 mm), the bottom of which was used to hold WA (2% Difco). This was amended with molal concentrations of NaCl at 44 ml per plate. A thin layer of CMA (about 10 ml per plate) was added to the lids of plates to yield a 5 mm air-gap between the two agar surfaces. The RH was related to NaCl molality using the data of Robinson & Stokes (1965). The following RH values were tested: 100, 99.6, 99.3, 98.6, 98.0, 95.0, 92.0, and 88.5% corresponding to: -0.46, -0.71,-1.13, -2.0, -2.76, -7.17, -10.3, and -15.5 MPa, respectively. Incubation temperatures of the same four isolates of *P. oligandrum* were 16, 25, and 33°C. Replication per factorial treatment involved 30 randomly selected pellets which were seeded into the CMA surface of duplicate humidity chambers (15 pellets per plate). The humidity chambers were conditioned in the incubation temperature for 24 h prior to inoculation and thereafter were sealed with parafilm. To evaluate mycelial growth, five humidity chambers per treatment were inoculated in the center of the CMA layer in lids with an 8 mm diameter disk of four-day-old WA cultures (76-13, 87-7, and 91-4). Percentage germination and colony diameter were determined 72 h following inoculation. These experiments were carried out twice.

LONGEVITY OF OOSPORE-PELLETS: Oospores of the five isolates of *P. oligandrum* in the form of calcium-alginate pellets were stored in sterile petri plates in the dark at 4 or 25°C. Plate containing pellets were wrapped with parafilm and each group of plates were sealed together in a plastic bag. To compare the viability of oospore-pellets, oospores of the five isolates were harvested from one batch of MMPO or MoB after six weeks of incubation and formulated into alginate pellets. Germination of samples of oospore-pellets was tested periodically over 12 months. In doing so, 30 randomly selected pellets for each isolate and storage condition were seeded into two CMA plates and incubated at 33°C for 48 h. Then the germination of oospore-pellets was measured. This experiment was repeated once.

STATISTICAL ANALYSIS: Data were analyzed with the CoStat™ software (Cohort Inc., Berkeley, CA). This involved chi-square, multiple regression and a three way analysis of variance (ANOVA) with the least significant difference of the Student-Newman-Keuls test.

Results

OOSPORE-PELLETS: The average weight of a dry pellet was 5 mg and its diameter was about 1 mm. Oospore densities ranged between 555 and 750 with a mean of 640 ± 40 oospores per pellet. The calculated oospore densities in pellets based on the original added inoculum were not significantly different from those counted in pellets using a microscope and a chi-square analysis. The yield was 23.7 ± 1.5 g air-dry pellets per 200 ml of the formulation suspension.

GERMINATION OF FORMULATED AND NON-FORMULATED OOSPORES: Percentage germination at 25 and 33°C for the five isolates of *P. oligandrum* was almost 100% (<1% variation) for both formulated and non-formulated oospore-droplets (FO and NFO, respectively). At 16°C, this percentage was reduced to 60 and 48% for FO and NFO, respectively, and no germination was observed at 10°C.

GERMINATION OF OOSPORE-PELLETS UNDER DIFFERENT MOISTURE AND TEMPERATURE

CONDITIONS: Since variation in oospore-pellet germination and mycelial growth between isolates of *P. oligandrum* were not statistically significant ($P > 0.01$), the influence of moisture and temperature was examined. Mycelial growth and germination of oospore-pellets of *P. oligandrum* were stimulated with increasing temperature but inhibited with reduced levels of water (Figures 2 and 3). Germination of oospore-pellets and mycelial growth were significantly affected by the multiple factors of temperature and Ψ_s according to the multiple regression analysis ($P<0.001$), $R^2 = 0.72$ for germination, and $R^2 = 0.68$ for growth).

Germination of oospore-pellets was at the highest (98%) at Ψ_s between -0.59 and -1.33 MPa at incubation temperatures of 25 and 33°C (Figure 2A). Percentage germination declined sharply at Ψ_s between -1.33 and -2.59 MPa, with no germination occurring at -3.59 MPa. On the other hand, 60% germination was observed at 16°C at high Ψ_s and germination declined sharply at low Ψ_s with no germination occurring at -1.33 MPa. Mycelial growth exhibited similar responses to Ψ_s, with separate growth patterns being observed at each temperature (Figure 2B). Mycelial growth of the four isolates of *P. oligandrum* was significantly higher ($P<0.01$) at 33°C than at 25 or 16°C over the graded range of Ψ_s, with no growth occurring at - 3.59 MPa (Figure 2B).

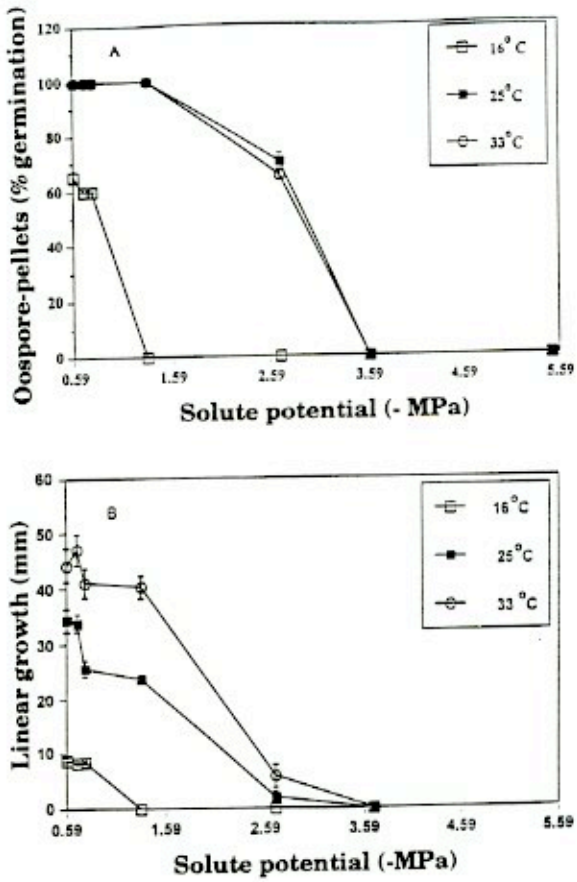

Figure 2. Effect of temperature and solute potential on oospore-pellet germination (A) and linear growth (B) of P. oligandrum after 72 h. Percentage germination was based on 240 pellets for each data point. There were no significant differences (LSD) at $P<0.01$. Growth data points are means of 20 replicates and the standard error of the mean is shown by the error bars.

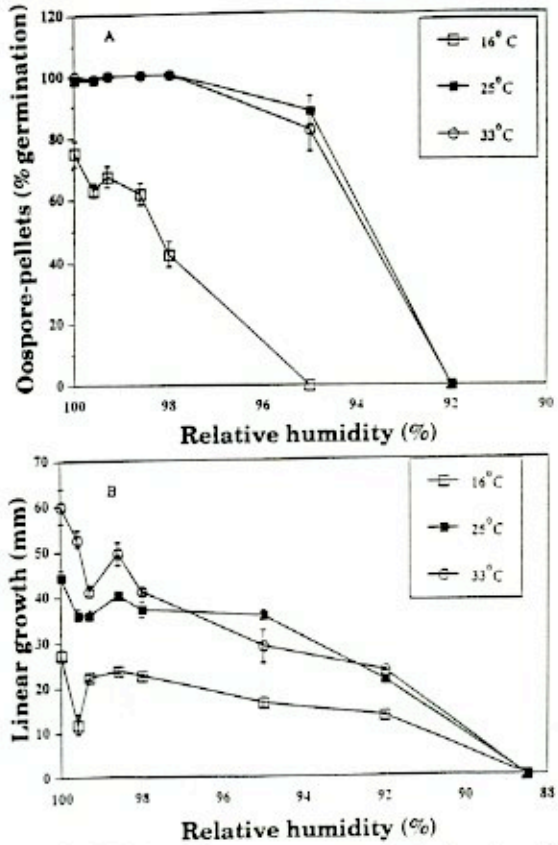

Figure 3. Effect of temperature and relative humidity on oospore-pellet germination (A) and linear growth (B) of P. oligandrum after 72h. Percentage germination was based on 240 pellets for each data point. There were no significant differences among the four isolates and between trials based on the least significant difference (LSD) at $P<0.01$. Growth data points are means of 20 replicates and the standard error of the mean is shown by the error bars.

The RH - temperature and the Ψ_s - temperature interactions were very similar with respect to oospore-pellet germination and mycelial growth of *P. oligandrum* (Figures 2 and 3). However, the water level tolerated for germination and growth was wider for RH, hence extending these physiological processes to 95 and 88.5% RH (= -7.1 and -15.5 MPa, respectively).

LONGEVITY OF OOSPORE-PELLETS: Oospore-pellets of five isolates of *P. oligandrum* stored at 4°C under dry conditions retained their viability (99% germination for each time interval) over a 12 month-period regardless of the medium (MMPO or MoB) used to produce the

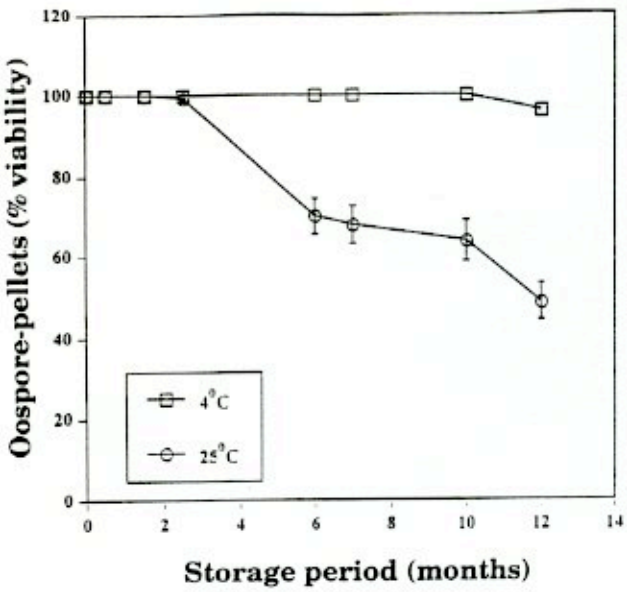

Figure 4. Germination of oospore-pellets of P. oligandrum as a function of storage time. Data points and the standard error of the mean (error bars) are given for five isolates of P. oligandrum. No significant differences between isolates or medium were observed based on the Student-Newman-Keuls test ($P<0.05$).

oospores (Figure 4). Oospore-pellets stored at 25°C remained fully viable for 3 months, but their viability declined gradually in the subsequent 9 months (Figure 4).

Discussion

Oospores of *P. oligandrum* mass-packed in calcium alginate pellets ensured high germination rates of the inoculum units. Oospore-pellets retained their ability to germinate over a wide range of moisture conditions and temperatures. The range of Ψ_s tolerated by this fungus for both mycelial growth and oospore-pellet germination was between 0 and -3.5 MPa at 25 and 33 °C. A similar range of Ψ_s tolerance was reported previously for the mycelial growth and individual oospore germination of *P. oligandrum* (McQuilken *et al.*, 1992a) and for other plant parasitic species of *Pythium* and *Phytophthora* (Adebayo & Harris, 1971; Duniway, 1979; Sommers *et al.*, 1970; Wilson & Griffin, 1975). Mass-packaging has the advantage of compensating for low and variable germination rates of individual oospores at low water potentials. For example, McQuilken *et al.* (1992a) reported germination of 19-26% of individual oospores at -0.5 MPa. However, we found that oospore-droplets (600 oospores per droplet) and oospore-pellets (640 ± 40 oospores per pellet) germinated 100% at -0.5 MPa. In addition, oospore-pellets germinated from 98 to 100% at Ψ_s from -0.5 to -1.33 MPa with germination rates being reduced about 40% at -2.59 MPa (Figure 2). Increased tolerance of oospore-pellet germination to moisture was also found in our studies on RH. These results show that oospore mass-packing in calcium alginate pellets offsets germination limitations associated with individual oospores (Drechsler, 1946). Obviously, the ability of oospore-pellets to maintain optimum germination over a wide range of temperatures and moisture conditions is important. This is particularly crucial when water potentials are lower than -1.5 MPa, i.e. the permanent wilting point of mesophytic higher plants (Slatyer, 1957). Extending the range of conditions for rhizosphere colonization by *P. oligandrum* should enhance its usefulness as a root protectant in soil.

Germination of oospore-pellets was lower under dry conditions at 16°C than at 25°C, a finding that could restrict the use of *P. oligandrum* as a biocontrol agent in warm soils. Planting into soils at 16°C or lower is common in temperate zones and many soilborne diseases are favored by cool soil temperatures. In preliminary investigations on protection of sugar beet seeds against damping-off by *P. ultimum*, it was found that some of the isolates were less effective at 14°C as compared to their action at 21 and 28°C (Shull, unpublished data). Vesely (1986) reported that certain isolates of this fungus were effective as biocontrol agents at soil temperatures ranging from 5 to 30°C. Therefore, identifying and selecting isolates that are adapted to low temperatures could negate this limitation. Even with this limitation, oospore-pellets of *P. oligandrum* could be used for biocontrol during warm seasons, in regions with warm soils, and under greenhouse conditions for the control of soilborne diseases that occur with warm temperatures.

Oospore-pellets should remain viable for long periods if they are stored under cool, dry conditions. Although the longevity or "shelf-life" of oospore-pellets should be tested thoroughly in settings apart from the laboratory, we found that oospore-pellets remained fully germinable when stored under refrigeration for a year (Figure 4). McQuilken *et al.* (1992b) also found that oospore inoculum of *P. oligandrum* coated onto seeds retained its biocontrol activity for a year. Unfortunately, studies on the viability of formulated propagules of several bacterial and fungal biocontrol agents were terminated after three, four or six months of storage of *Trichoderma harzianum* Rifai (Dandurand & Knudsen, 1993; Fravel *et al.*, 1985). This study clearly demonstrates that more attention should be paid to inoculum longevity as a critical feature for the biocontrol of plant disease under agricultural settings.

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TEMPERATURE AND WATER EFFECTS ON THE VIABILITY OF ALGINATE FORMULATED OOSPORES OF *PYTHIUM OLIGANDRUM*

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