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Factors affecting germination of oospores of *Peronospora viciae* f.sp. *pisi in vitro*

Dirk Jan van der Gaag* and Herman D. Frinking
*Department of Phytopathology, Wageningen Agricultural University, P.O.Box 8025, 6700 EE Wageningen, the Netherlands; *Present address: Plant Pathology and Biological Control Unit, Swedish Univ. of Agric. Sci., P.O. Box 7044, S-750 07 Uppsala, Sweden*

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Abstract

The effects of host plant exudates, light and temperature on germination of oospores of *Peronospora viciae* f.sp. *pisi in vitro* were investigated. Seed and root exudates did not increase percentage germination, whereas light inhibited germination. The first germ tubes appeared after 4, 7, and 14 days of incubation at 15, 10 and 5 °C, respectively. The eventual level of germination was highest and had similar values at 5 and 10 °C. At 20 °C germination was poor and at 25 °C no germination was observed. Oospores placed on membrane filters were incubated on soil. When oospores were retrieved from the membrane filters after six days and placed in water at 10 °C, they germinated within 2 days. On soil significantly less oospores germinated than in water. Germinability of oospores stored in the dark at 5 or 20 °C at 30 or 76% RH was studied over a two-year period. Germinability generally increased over time, but fluctuations were observed indicating the occurrence of secondary dormancy. Time courses of germinability were generally similar for oospores stored at several temperatures and humidities. No effect of light on time course of germinability was found when oospores were exposed to alternating light-dark periods or stored in continuous dark for 140 days. Percentage germination observed in a germination assay was correlated with percentage infection determined in a bioassay.

Introduction

Peronospora viciae (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven (Boerema et al., 1993) is the causal agent of downy mildew on pea (*Pisum sativum* L.). The pathogen disperses by conidia formed on infected host tissue at high humidities (Dixon, 1981). Oospores, formed in the colonized plant parts (Van der Gaag and Frinking, 1996a), are important for the survival of the pathogen during host-free periods (Dixon, 1981; Stegmark, 1994).

Host infection using oospores as inoculum of *P. viciae* f.sp. *pisi* has been reported (Ryan, 1971; Stegmark, 1991), but germination of the oospores is poorly understood. Geesteranus (1961) and Von Heydendorff (1977) observed germination in only a few oospores. Günther (1992) obtained percentage germination of 0–40 in oospores taken from the field. Large variations in percentage germination were found by Clark (1989) and Van der Gaag and Frinking (1996b). The latter authors showed that germination was unaffected by the methods used to extract oospores from plant tissue, which indicated that germinability was an inherent characteristic of an oospore sample. They suggested that germinability of oospores may be affected by their age and by the conditions to which the oospores had been exposed.

Temperature treatments affect germinability of oospores of several *Phytophthora* species (Ribeiro, 1983). Germinability of oospores of the downy mildew fungus *Peronosclerospora sorghi* (Weston & Uppal) C.G. Shaw is influenced by storage temperature (Pratt, 1978). After storage at 4 °C no germination was observed, but after incubation at 25 °C less than one per cent of the oospores germinated. Germinability of oospores can be affected by light. With oospores of

Phytophthora capsici Leonian the germination process was not affected by light, but exposure to light during oospore formation decreased germinability (Hord and Ristaino, 1991).

The objectives of the study presented here were (*i*) to determine the effects of host plant exudates and physical factors on oospore germination, (*ii*) to study the effect of oospore age and storage conditions on germinability, and (*iii*) to study the relationship between germinability and infectivity of oospores of *P. viciae* f.sp. *pisi*.

Materials and methods

Production of oospore populations

The oospore populations used originated in a monoconidial culture from an isolate of *P. viciae* f.sp. *pisi*, originating from a pea field in Wageningen (isolate NL). In one experiment, oospores were derived from a monoconidial culture of an isolate from Eastern England, kindly provided by J.E. Thomas (National Institute of Agricultural Botany, Cambridge, UK) (isolate UK3). The cultures were maintained asexually on pea seedlings in a growth chamber (Van der Gaag and Frinking, 1996a). The oospores were produced either in pods or in locally or systemically infected seedlings grown at 20 °C (Van der Gaag and Frinking, 1996a). Seedlings or pods (cv. Kelvedon Wonder) were inoculated with a suspension of conidia, and diseased plant parts were harvested 2–3 weeks (seedlings) or 3–4 weeks (pods) after inoculation. The diseased parts were air-dried and stored in paper bags at room conditions (19–23 °C, 35–65% RH) in the dark.

Extraction of oospores and germination assay

Oospores were extracted from plant tissue as described by Van der Gaag and Frinking (1996b): plant tissue containing oospores was comminuted in a blender, sonicated, and sieved through mesh sizes of 53 and 20 μm. The residue on the 20-μm sieve containing the oospores was suspended in water. Oospores were germinated in water amended with 100 μg ml^{-1} ampicillin and 10 μg ml^{-1} rifampicin in sterile glass Petri dishes at 10 °C in the dark (Van der Gaag and Frinking, 1996b). In each experiment four replicate dishes (6 cm ϕ) were used per treatment. One hundred oospores were observed per replication. Observations were made 14 days after incubation of the oospores in the germination medium.

Effects of oospore density and host plant exudates on germination

The effect of oospore density on germination was evaluated by incubation of oospores in Petri dishes at densities of 100 and 1000 oospores ml^{-1}. Higher densities were not included as observations at densities much higher than 1000 ml^{-1} are not precise due to clumping of germ tubes which can reach lengths of several mm (Van der Gaag and Frinking, 1996b).

Seed exudates were obtained by surface-sterilization of pea seeds (cv. Kelvedon Wonder) following the methodology of El-Hamalawi and Erwin (1986), and placing 20 of these seeds in an Erlenmeyer flask in 20 ml SPW (sterile purified water). The flask was incubated at 20 °C in the dark for 42 h. To test whether seed exudates were contaminant-free, aliquots of 200 μl exudate were spread out over bouillon agar in Petri dishes (9 cm ϕ) and incubated at 25 °C. After 5 days, Petri dishes were observed for bacterial growth and contaminated exudates were discarded. Seed exudates were stored at –20 °C before use. Root exudates were obtained by placing 20 surface-sterilized seeds on bouillon agar at 20 °C. After three days, uncontaminated germinated seeds were placed in Petri dishes (9 cm ϕ) in 20 ml water at 20 °C in the dark. Root exudates were harvested 7 days later, and tested for contamination as for the seed exudates. Root exudates were stored at –20 °C before use.

Oospores were germinated in diluted seed or root exudates (1:10 or 1:100) since undiluted exudates showed a negative effect on germination in preliminary experiments. Exudates were tested on oospores from two populations each produced in systemically infected seedlings. Oospores of the populations, I and II, were 15 and 14 months old, respectively.

Effects of light and temperature on germination

The effect of light on germination was investigated by placing Petri dishes with oospores under Philips TLD lamps (18W/84) at a light intensity of 10 Js^{-1}m^{-2} for a 16 h per day at 5 °C. Control dishes were protected by two layers of aluminium foil. Temperature in the light exposed dishes was about 1 °C higher than those in the dark. Germination was recorded 25 days after incubation. The effect of temperature on germination was investigated by incubation of oospores in water at

5, 10, 15, 20, and 25 °C. Germination was observed over a 28-day period. The light experiment was performed twice using 4 month old oospores produced in pods (Expt I) and leaves (Expt II). The temperature experiment was performed twice using 3 (Expt I) and 2 (Expt II) month old oospores produced in pods.

Germination of oospores after incubation on soil

Samples from two soils were collected, a loamy sand soil (pH-KCl 4.3, organic matter 2.6%), and a silt loam soil (pH-KCl 7.4; organic matter 2.4%). Soil samples were air-dried. The silt loam soil was ground in a Retsch grinding mill (Retsch, Haan, Germany), and both soils were sieved through openings of 1 mm. Soils were stored at 5 °C before use. Soil was placed in Petri dishes (16 cm ϕ), to a height of approximately 7 mm. Water was added until saturation and the soil surface was smoothed. Soil samples thus prepared were incubated for five days at 10 °C in the dark to equilibrate.

Droplets from an oospore suspension were evenly deposited with a pipette on cellulose nitrate membrane filters (MFs, 12-μm pore sizes) placed on filter paper in which water was drawn (Pratt, 1978). MFs with the oospores were placed on the soil surface in the Petri dishes, which were sealed and placed at 10 °C. Four dishes were prepared in this way, each containing seven MFs (25 mm ϕ) with 1×10^3 oospores and one MF (47 mm ϕ) with 4×10^3 oospores. Oospores from the same suspension were also incubated in water in four replicate Petri dishes.

One membrane filter (25 mm ϕ) per dish was removed and placed on a glass slide for investigation of oospore germination on soil. A few drops of a 1 mg/ml Uvitex 2BT (Ciba Geigy) solution were added to each MF to stain germ tubes. Observations were made with a Zeiss fluorescence microscope, excitation filter BP 395–440 nm, barrier filter LP 470 nm. The 47 mm MF was removed six days after incubation and oospores were retrieved by comminuting the MF in a blender, sonicating the suspension and sieving as described in an earlier section. Oospores were isolated from the suspension using a microsyringe and placed in water (Van der Gaag and Frinking, 1996b). Oospore germination was recorded on alternate days from day 4 to 16.

Effects of oospore age and temperature on germinability

For this work the oospores were produced in local lesions on seedlings using isolate UK3. Seventeen days after plant tissue had been inoculated with conidia, diseased plant parts were harvested and air-dried at 20 °C in a growth chamber (RH 60–70%) for nine days. After this drying period, the debris was ground to pass through 1 mm openings, and the powder containing oospores was incubated as described below at 5 or 20 °C and at relative humidities of 30 or 76%. One gram of the powder was spread evenly in a Petri dish (6 cm ϕ) which in turn was placed in a larger Petri dish (16 cm ϕ). Humidity was controlled by placing two open dishes (6 cm ϕ) with glycerol (RH 30%) or a saturated NaCl solution (RH 76%) in the large dish. The Petri dish (16 cm ϕ) was sealed. Oospores were extracted from a sub-sample of 0.02 g and incubated for assessment of germination every 3-9 weeks over a two year-period, beginning 26 days after inoculation (d.a.i.). The percentage of living oospores was assessed using the tetrazolium bromide test (Sutherland and Cohen, 1983; Van der Gaag, 1994). Three replicate dishes per treatment were prepared and 100 oospores per dish were observed at each assessment date.

Effects of oospore age and light on germinability

Oospores were produced in systemically infected seedlings. Diseased plant parts were harvested 3 weeks after inoculation and divided into six samples. Three randomly chosen samples were incubated at a light intensity of 29 $Js^{-1}m^{-2}$ (Philips TL MF 40W/33 RS), 16 h per day. The other three samples were incubated in the dark (RH 60–70%). Oospores were extracted from sub-samples and the percentages of germinating and living oospores determined as described above at 1–6-week intervals during 20 weeks, beginning at d.a.i. 26. One hundred oospores from each sample were observed each time.

Relationship between germinability and infectivity

Germinability of oospores was assessed using the germination assay as described above. Infectivity was assessed in a bioassay. A layer of 1 cm steam-sterilized potting soil was placed in plastic trays ($17 \times 13 \times 6$ cm) followed by 4 cm of a steam-sterilized sandy soil (pH 7.0; organic matter 0.3%; pF 1.8). Surface-sterilized

seeds (10 min in 1% NaOCl, followed by three rinses with tap water) were placed at a depth of 2 cm in the sandy soil. Ten seeds per tray were placed in two rows of five seeds each. One ml of an oospore suspension was added to each seed, and seeds were covered with the sandy soil. Finally, a layer of 1 cm steam-sterilized sand was placed on top of the sandy soil to reduce water loss by evaporation. Trays were placed in a growth chamber at 10 °C and a light intensity of 23 $Js^{-1}m^2$ for 16h (RH 70–80%). Thirty days after inoculation, trays were covered with plastic to increase the humidity for induction of conidial formation. The numbers of healthy and diseased (sporulating) seedlings were counted 20 hours later. Oospores from samples of eight different populations were used in the germination test and the bioassay with four replications per population.

Data analysis

Data were tested for homoscedasticity and, if necessary, transformed (square root or arc-sine square root transformation) before analysis of variance (Gomez and Gomez, 1984). Treatment means were compared using *t*-tests. Data recorded on more than two days were analyzed using multivariate repeated measures analysis of variance (Moser and Saxton, 1990). If time × treatment interaction was significant ($P \leq 0.05$), treatment means per observation time were compared by *t*-tests. Infection and percentage germination were compared using regression methods. All analyses were performed using the Statistical Analysis System, version 6.04 (SAS Institute Inc., Cary. NC).

Results

Host plant exudates and oospore density

Percentage germination was 9.8 and 9.3 at densities of 100 and 1000 oospores ml^{-1}, respectively; these did not differ significantly (standard error 1.7, six replications). Germination of oospores was either unaffected or decreased by the addition of host plant exudates (Table 1).

Light and temperature

Light negatively affected germination (*t*-test, $P < 0.05$) (Table 2). Temperature had a major effect on the time of appearance of the first germ tubes, and on the eventual level of germination. First germ tubes appeared after about 4, 7, and 14 days of incubation at 15, 10, and 5 °C, respectively (Figure 1). The eventual level of germination was highest and had similar values at 5 and 10 °C (Figure 1). Germination curves at 5 and 10 °C were similar when percentage germination was plotted against degree-days. For calculation of degree-days the threshold temperature was set at 0 °C. Significantly lower percentages of oospores germinated at 15 than at 10 °C (*t*-test, $P<0.05$) (Figure 1). At 20 °C germination was generally poor (Figure 1), and at 25 °C no germination was observed.

Table 1. Effect of host plant exudates on germination of oospores of *Peronospora viciae* f.sp. *pisi*

Medium	Germination (%)[a]	
	Population I[b]	Population II
Purified water	44.3[c]	28.5
Seed exudates		
diluted 1:100	25.4*	17.5*
diluted 1:10	19.9*	17.9*
Root exudates		
diluted 1:100	37.8	26.8
diluted 1:10	32.1*	24.7
Standard error	2.8	3.0

[a] Values followed by an asterisk are significantly different from the control (purified water) (*t*-test, $P<0.05$).
[b] Oospores were used from two populations of 15 (Population I) and 14 months (Population II) old each produced in systemically infected pea seedlings.
[c] Values are means of four replications. One hundred oospores were observed per replication.

Table 2. Effect of light on germination of oospores of *Peronospora viciae* f.sp. *pisi*

Treatment	Oospore germination[a] (%)	
	Expt I[b]	Expt II
Dark	35.4 (1.9)[c]	51.2 (2.0)
Light/Dark (18/6h)	17.9 (1.9)	31.8 (2.0)

[a] Values in each column are significantly different according to the *t*-test ($P < 0.05$).
[b] In Expt I and Expt II oospores were 4 months old and originated from pea pods and leaves, respectively.
[c] Values are means of four replications. One hundred oospores were observed per replication. Values in parentheses are standard errors.

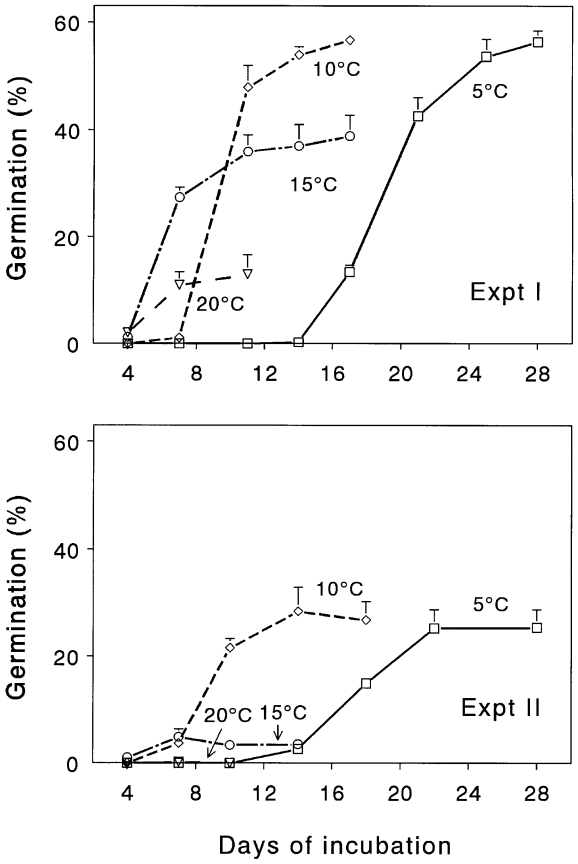

Figure 1. Effect of temperature on germination of oospores of *Peronospora viciae* f.sp. *pisi* in two experiments using 3 (Expt I) and 2 (Expt II) months old oospores produced in pods. Values are means of four replications and 100 oospores were observed per replicate. Bars indicate standard errors.

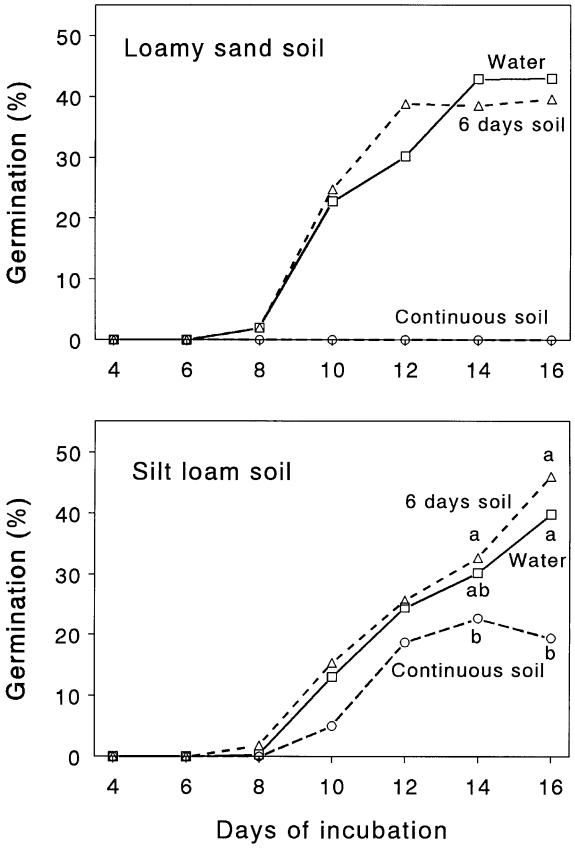

Figure 2. Comparison of germination of oospores of *Peronospora viciae* f.sp. *pisi* in water, on soil, and in water after incubation for six days on soil. Significant differences are indicated by different letters for the silt loam soil (*t*-tests, $P < 0.05$). Values are means of four replications with 100 oospores each.

Incubation on soil

Germination curves of oospores incubated in water and those retrieved from soil after six days were not significantly different (Figure 2). On the loamy sand soil, oospores did not germinate and significantly lower numbers of oospores germinated on the silt loam soil than in water (Wilks' Lambda for time × treatment significant at $P = 0.049$) (Figure 2).

Oospore age and storage conditions

Oospores stored at 20 °C and 76% RH rapidly lost their viability as shown by a decrease in the percentages staining in MTT (Figure 3). At day 292, 6% of the oospores stained faintly. The percentage of living oospores of those stored at 5 °C with low and high humidity and at 20 °C with low humidity remained similar over time (Figure 3A). Germinability of oospores increased with oospore age up to approximately 200 days (Figure 3B). Oospore germinability differed significantly between oospores stored under different conditions on some sampling dates (*t*-tests, $P < 0.05$), but the effects were not consistent over time (Figure 3B). Significant decreases in germinability were found between day 257 and 292 of oospores stored at 5 °C, followed by increases until day 390. At 20 °C/30% RH and 5 °C/30% RH, germinability decreased significantly after day 390 followed by an increase (Figure 3B). Percentage staining in MTT remained similar over time for oospores exposed to light/dark or dark conditions, and averaged 94.6. No significant difference in germinability of oospores in light/dark and dark conditions was found (Figure 4).

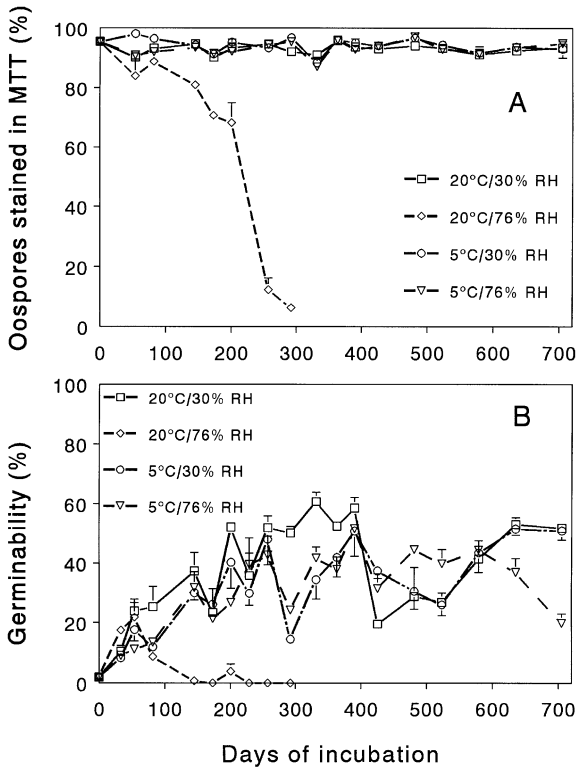

Figure 3. Effect of time, temperature and relative humidity during storage on percentages of oospores of *Peronospora viciae* f.sp. *pisi* staining red in tetrazolium bromide (MTT) (A) and their germinability (B). Oospores were produced in pea seedlings inoculated with conidia. Time is counted from 26 days after inoculation. Values are means of three replications with 100 oospores each. Bars indicate standard errors.

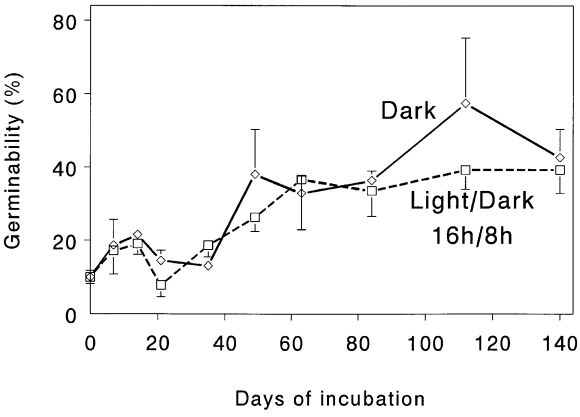

Figure 4. Effect of alternating light-dark periods (16/8 h, 29 $Js^{-1}m^{-2}$) on germinability of oospores of *Peronospora viciae* f.sp. *pisi*. Oospores were produced in pea seedlings inoculated with conidia. Time is counted from 26 days after inoculation. Values are means of three replications with 100 oospores each. Bars indicate standard errors.

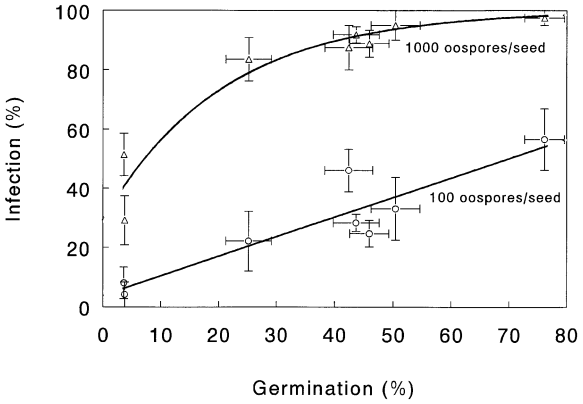

Figure 5. Relationship between seedling infection and germination of oospores of *Peronospora viciae* f.sp. *pisi* when 100 or 1000 oospores were added per pea seed. Bars indicate standard errors.

Germinability and infectivity

Seedling infection showed a linear relationship with germination when 100 oospores were added per seed: $y = 3.90 + 0.66x$ ($3.6 \leq x \leq 76.2$, $R^2 = 0.84$), where y is the percentage infection and x the percentage germination. The intercept did not significantly deviate from zero. At a density of 1000 oospores per seed, the asymptotic curve, $y = 1\text{-}0.71e^{(-4.8398x)}$ ($3.6 \leq x \leq 76.2$), described the data (Figure 5).

Discussion

This work demonstrates that densities of 100 to 1000 oospores ml^{-1} can be used in germination assays. Oospore germination *in vitro* was not increased by any host plant exudates. Oospores of *P. viciae* f.sp. *pisi* differ from oospores of several *Phytophthora* spp. (Hord and Ristaino, 1991; El-Hamalawi and Erwin, 1986; Förster et al., 1983) and *Bremia lactucae* (Morgan, 1978, 1983) where germination depended on or was increased by addition of host plant exudates. Although oospores of *P. viciae* f.sp. *pisi* do not demonstrably depend on host plant factors for germination, it is suggested that under natural conditions germination is inhibited by soil fungistasis (Bruehl, 1987), and (host)plant factors are needed for the induction of germination.

Light negatively affected germination of oospores. This observed effect does not correspond with that of oospores of Pythiaceous fungi where germination is generally stimulated by light (Ribeiro, 1983). Little

information is available on the effect of light on germination of oospores of downy mildew fungi (Peronosporaceous fungi). Shetty and Safeeulla (1980) observed that light did not affect germination of oospores of *Peronosclerospora sorghi*, but light conditions during incubation were not reported. The light sensitivity of the germination of oospores of *P. viciae* f.sp. *pisi* observed here contrasts with that of the conidia where germination is unaffected by light (Pegg and Mence, 1970). Sensitivity to light may prevent oospores from germinating above soil and this may be advantageous for survival.

A greater number of oospores germinated at 5 and 10 °C than at 15 and 20 °C (Figure 1). Apparently, a critical temperature for germination exists between 10 and 15 °C. The absolute and the relative levels of germination at 10, 15, and 20 °C differed between experiments using oospores from different populations. Thus, oospore populations may show differences in relative germinability at optimal and sub-optimal temperatures. The low optimum temperature for maximum germination (<15 °C) is relevant to soil temperatures at the start of the growing season in pea-growing areas. In a bioassay not reported here, higher percentage infection was obtained at 10 than at 14 °C.

When oospores were incubated on wet soil, germination was inhibited or partly inhibited compared to incubation in water (Figure 2). When the oospores were retrieved from soil after six days and incubated in water germ tubes appeared within two days. Germination of oospores incubated in soil under natural conditions has been observed within 24 h after extraction from the soil (Van der Gaag, data unpublished). Apparently, oospores incubated under moist conditions are able to germinate within a few days when conditions are favourable. A similar phenomenon has been reported for chlamydospores of some fungal species which germinate rapidly when kept moist but show a delay in germination when they have been stored dry (Baker and Cook, 1974). In moist soil, non-dormant oospores are probably ready to germinate after inhibiting factors have been removed or after an induction signal has been received.

In soil, abiotic and/or biotic factors may be involved in the inhibition of spore germination. On the loamy sand soil, the low pH, 4.3, might have been inhibitory for germination. The relatively high percentage germination on the silt loam soil might be explained by the fact that oospores were extracted from plant tissue and were almost contaminant-free when deposited on the MFs. Bacterial growth has been associated with lower germination percentages *in vitro* (Van der Gaag and Frinking, 1996b), but a temperature of 10 °C might have been too low for sufficient bacterial colonization of the MFs to be inhibitory to germination.

Increases in germinability with oospore age as observed here have also been found for oospores of many *Phytophthora* species (Ribeiro, 1983). Apparently, oospores of several Oomycetes have a period of primary dormancy after formation. Although activities of oospores are assumed to be low under dry conditions, oospores of *P. viciae* f.sp. *pisi* became germinable after some time under dry conditions. Dry storage of some plant seeds also leads to loss of dormancy and biochemical changes in dry seeds have been postulated (Roberts and Smith, 1977).

The significant decreases in germinability observed over time of oospores at 20 °C/76% RH can be explained by a loss of viability since the percentage of living oospores also decreased as indicated by the MTT test (Figure 3). Decreases observed with oospores stored at 5 °C or 20 °C/30% RH suggest the appearance of secondary dormancy. This phenomenon may also explain why the percentage germination remained relatively low; under dry conditions some oospores become germinable while others become dormant. The occurrence of primary and secondary dormancy can be an explanation of the large variations in percentage germination observed in several studies (Clark, 1989; Günther, 1992; Van der Gaag and Frinking, 1996b). The causes of the fluctuations in germinability of the oospores are unknown. No clear periodicity in germinability was found during the two years of this study. Temperature (5 or 20 °C) and light (29 $Js^{-1}m^{-2}$) did not affect the germinability of dry-stored oospores to any great extent.

A linear increase of seedling infection with oospore germination was expected. Such a relationship was found when 100 oospores were added per seed. The intercept of the line (Figure 5) did not significantly deviate from zero which suggests that percentage germination is not higher in the vicinity of the host plant than in water. This result corresponds with that of the *in vitro* experiments where germination of oospores was not increased by the addition of host plant exudates. Apparently, oospores which fail to germinate in water will not germinate in the infection court of the host. A straight line through the origin indicates that one oospore can succesfully infect a seedling and that oospores act independently (Van der Plank, 1975; Zadoks and Schein, 1979). The decrease in the

ratio of diseased seedlings to oospore germination at 1000 oospores/seed suggests competition for susceptible infection sites at high densities.

The present study shows that dry-stored oospores of *P. viciae* f.sp. *pisi* germinate independently of a chemical stimulus in water and that germinability of the oospores generally increases with oospore age up to a certain point. The strong correlation between germination *in vitro* and infection *in vivo* showed that results from the germination assay provide a good measure of the infectivity of the oospores. In the germination and bioassays presented in this study only dry-stored oospores were used; oospore behaviour might change after incubation in moist soil for some time and this should be addressed in future research.

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