

Extraction from plant tissue and germination in soil of *Phytophthora infestans* oospores

P.J. VAN BEKKUM, G.J.T. KESSEL, S.L.G.E. BURGERS,
M.G. FÖRCH AND W.G. FLIER

Plant Research International, P.O. Box 16, 6700 AA Wageningen, the Netherlands

Summary

Existing methods were modified and optimized to extract oospores of *Phytophthora infestans* from small (1-5 leaflets) and larger quantities (10-35 gram of dry weight) of potato leaf tissue. Recovery of extraction of oospores from small quantities was 96.7%. Extracted oospores were used to study germination of oospores in soil as influenced by time, soil type, soil moisture and soil temperature. Oospores were mixed with quartz sand as an inert medium to facilitate simple recovery. After incubation oospores were recovered and germination and viability were determined. Viability was determined using tetrazolium bromide. Oospore viability was not affected by any of the treatments during the duration of the experiment. Germination in soil, quantified as the formation of primary sporangia was found to be a slow process making quantitative analysis difficult. Trends for effects of soil moisture, temperature and incubation period were observed. The soil types included in the experiments did not have a statistically significant effect on oospore germination.

For practical potato late blight management purposes on oospore infested soils, it must be assumed that oospore germination is possible throughout most of the growing season. This results in a continuous presence of sporangia in the soil as an extra soil borne source of inoculum. This additional soil borne source of inoculum within the crop must be taken into account for effective potato late blight management.

Keywords

Late blight, potato, epidemiology, viability, temperature, soil moisture.

Introduction

In the Netherlands, surveys in 2000, 2001, 2002, 2003, 2004 and 2005 carried out within the framework of the growers (LTO Nederland) initiative "MasterPlan Phytophthora" and the Umbrella Plan Phytophthora indicate that oospores are becoming an increasingly important source of inoculum, especially in the North Eastern starch potato growing area (Turkensteen *et al.*, 2000; Kessel *et al.*, 2001). Consequently, contemporary management strategies for controlling potato late blight may have to be adapted to minimise the impact of oospores on late blight epidemiology.

Conventional potato late blight management basically applies protective fungicides when the protection level of the crop is low and an infection event is predicted. At least theoretically, this should

protect the crop from infection events originating from airborne inoculum produced outside the crop. Infection events originating from a soil borne source of inoculum inside the crop, such as oospores, may well occur under a different range of climatic conditions. Quantitative insight into oospore germination, as related to soil type and abiotic conditions, would help to predict critical periods with respect to oospore related infections. This knowledge would aid further refinement of potato late blight management strategies and decision support systems. Quantitatively however, germination of *P. infestans* oospores is poorly understood and the techniques available to quantitatively study oospore germination are not adapted for use in soil with the potato - *P. infestans* pathosystem.

The purpose of the present study was to adapt and optimise available methods for extraction of oospores from various plant tissues for use with the *P. infestans* - potato pathosystem. Furthermore we aimed to develop experimental techniques allowing incubation of oospores in soil followed by simple recovery and quantification of germinated oospores from soil. The final objective was to quantitatively study viability and germination of *P. infestans* oospores as influenced by time, soil type, temperature and soil moisture.

Materials and methods

Isolate selection and culturing

To optimise oospore production, nine combinations of A1 and A2 parental strains were tested for their capacity to produce large quantities of oospores in leaflets of potato cultivar Bintje. Three *P. infestans* A1 isolates (SN001a, IPO99001 and IPO98014) were mated to three *P. infestans* A2 isolates (US8, PIC96002 and IPO82001) in all nine possible combinations. Details on the isolates are given in Table 1.

Sporangial suspensions of all *P. infestans* isolates were prepared in tap water from potato leaflets, cultivar Bintje, showing abundant sporulation and adjusted to a concentration of 1×10^4 sporangia/ml. Mixed A1/A2 sporangial suspensions of all nine parental combinations were prepared by mixing the appropriate sporangial suspensions in a 1:1 ratio. Detached potato leaflets of cultivar Bintje were placed in 9 cm Petri dishes containing 1% water agar (WA), one leaflet per Petri dish, lower side up. Three Petri dishes were included for each parental combination. Leaflets were spray inoculated, using a spraying nozzle at a pressure of 0.5 kg m^{-2} , covering the leaflets with tiny droplets of the appropriate sporangial suspension. Petri dishes containing inoculated leaflets were placed in plastic trays lined with wet filter paper. Trays were placed in transparent polyethylene bags and incubated for at least 10 days using an incubation regime of 24h at 15°C in the dark followed by 24 hours at 15°C including a light period of 16h (12 Wm^{-2}), followed by 11°C and a light period of 16h/day (12 Wm^{-2}) for the rest of the incubation period. During incubation, leaflets were regularly sprayed with tap water to prevent dehydration.

Following incubation, oospore densities in the inoculated leaflets were determined using a microscope at 10×10 magnification. Four leaf areas of 1 mm^2 for each of three leaflets per parental combination were examined.

Extraction of oospores from leaf tissue.

The oospore extraction protocol used was modified from a protocol described by Van der Gaag & Frinking (1996) for extraction of *Peronospora viciae* oospores from pea tissue. The total leaf area of small samples (1 – 5 leaflets) was determined using an interactive digitizer (Minimop, Kontron, Oberkochen, Germany). Leaflets were homogenised in 5 ml crushed ice and 5 ml tap water (4°C) using an Ultra Turrax mixer (T25 basic, IKA Labortechnik, Germany) at 24000 rpm for 90 seconds. The resulting suspension was cooled to 4°C and homogenised a second time at 24000 rpm for 90 seconds. Cellulase (C8001, Duchefa, Haarlem, The Netherlands) and macerase (M8002, Duchefa, Haarlem, The Netherlands) were added to a final concentration of 0.5 mg ml^{-1} each to degrade

leaf tissue. Suspensions were incubated on an orbital shaker (SM25, Edmund Bühler, Tübingen, Germany) at room temperature for 2 h at 100 rpm followed by sonication (Branson 2510, Branson ultrasonics corporation, Danbury, USA) for 2 x 5 minutes. Following sonication, samples were again incubated on the orbital shaker at room temperature for 2 days at 100 rpm. The resulting suspensions were washed on a set of sieves (75 and 20 µm), using tap water, to remove enzymes and particles smaller than 20 µm. The residue on the 20 µm sieve was transferred to a 50 ml centrifuge tube and spun down for 3 minutes at 5000 g. The volume was reduced to 5 ml by removing supernatant. The oospore concentration in the remaining suspension was determined using a microscope at 10 x 10 magnification and a Fuchs-Rosenthal haemocytometer. The resulting suspensions can be air dried and stored at room temperature for future use in experiments as a highly concentrated source of *P. infestans* oospores.

For larger quantities of leaf tissue (10–35 g dry weight) the procedure described above was modified as follows: Stems were removed. The remaining leaflets were weighed and washed in water to remove sand. Washed samples were homogenized using a blender (Waring commercial, model 38BL40) in 50 ml crushed ice and 100 ml tap water (4°C) for 60 seconds at low speed followed by 30 seconds at high speed. The resulting suspensions were transferred to 500 ml Duran bottles and treated with cellulase and macerase as described above. Suspensions were then washed on a 250 µm, 125 µm, 75 µm and 20 µm sieve set. Residue on the 20 µm sieve was transferred to a 500 ml Duran bottle and left to settle over night before reducing the volume to 100 ml.

The recovery of the extraction protocol for small leaf samples was determined for three leaf samples of 1380 mm², 815 mm² and 1403 mm² respectively. Residue fractions on the 75 µm sieve, on the 20 µm sieve (oospore yield) and the residue passing the 20 µm sieve were collected separately and checked for presence of oospores.

Table 1. Characteristics of *Phytophthora infestans* isolates used in the oospore production experiment.

<i>P. infestans</i> isolate	Isolated from	Country	Year of collection	Mating type	Haplo type ¹
SN001A	Black nightshade (<i>Solanum nigrum</i>)	The Netherlands	1999	A1	IA
IPO99001	Commercial starch potato crop	The Netherlands	1998	A1	IA
IPO98014	Commercial starch potato crop	The Netherlands	1998	A1	IA
US8	Commercial potato crop	USA		A2	IA
PIC96002	Commercial potato crop	Mexico	1996	A2	IA
IPO82001	Commercial potato crop	Belgium	1982	A2	IA

¹: Mitochondrial Haplotype (Griffith and Shaw, 1998)

Oospore viability and germination

Oospore viability and germination was quantitatively monitored in a replicated experiment in which *P. infestans* oospores were incubated for three weeks in four types of soil at four constant temperatures and two levels of soil moisture. Bulk samples of a sandy soil, clay soil, a peaty soil and quartz sand, representing the three dominant soil types of the major Dutch potato growing areas and a reference soil respectively, were adjusted to field capacity (pF = 2). Characteristics of these soils are given in Table 2. Eight plastic containers (11x7.5x4.5 cm) were filled with each of these soils, resulting in a total of 32 containers. Saturated soils were produced by adding water to four out of the eight containers per soil type until a thin layer of water was permanently present on top of the soil.

Air-dried oospore-containing leaf residue from a bulk sample, produced as described above, was re-suspended in tap water, thoroughly mixed and adjusted to a concentration of 5x10⁴ oospores per ml. A volume of 10 ml oospore suspension was added to 200 g of dry sieved sterile quartz sand only containing particles > 125 µm, and thoroughly mixed. Approximately 1.5 g of this oospore

containing quartz sand was placed in each of 192 small polyester gauze bags (10 x 5 cm, 15 µm mesh, Lampe technical textiles b.v. Sneek, The Netherlands). Six bags were buried in each container after which the containers were covered with aluminium foil which was taped to the containers to prevent dehydration. Containers were incubated for three weeks in the dark at 5°C, 10°C, 15°C or 20°C. One container was used for each combination of soil type, soil moisture and incubation temperature. Initial oospore viability and germination was assessed from the oospore containing quartz sand bulk sample. During incubation, two randomly chosen gauze bags were removed from each of the containers after one, two and three weeks. Oospore viability and germination in these samples was determined using the following method:

Oospore containing quartz sand from the gauze bags was transferred to 5 ml water in a 50 ml centrifuge tube and carefully inverted ten times. The suspension was left to settle for approximately 10 seconds before the supernatant was transferred to a 10 ml centrifuge tube. 5 ml of tap water was added to the pellet in the 50 ml centrifuge tube and the procedure was repeated. The combined supernatant was spun down at 1500 g for 3 minutes. The resulting supernatant was carefully discarded until only 1 ml was left in the centrifuge tube. This residue, including the oospores, was transferred to a 1.5 ml eppendorf vial. Oospore viability was determined using tetrazolium bromide (MTT, Sigma M-2128) according to Jiang & Erwin (1990). A volume of 150 µl 0.1% MTT in 0.01 M phosphate buffer (pH=6.2) was added to each oospore-containing eppendorf vial which was then incubated for 2 days at 35°C in the dark. Following incubation, viability and germination were quantified in 50 µl aliquots using a microscope at 10x10 magnification. Viability was assessed using the colour reactions described by Erwin & Ribeiro (1996). Oospores were considered germinated when a germ tube and sporangium was attached to the oospore. At least 60 oospores were examined per aliquot.

Table 2. Soil characteristics for the soil types used in the oospore viability and germination experiment.

	Sandy soil	Clay	Peaty soil	Quartz sand
pH-KCL ¹	7.3	7.1	4.8	6.3
Organic matter ² (%)	3.0	3.4	20.2	0.5
CaCO ₃ (%)	0.6	2.2	0.1	0.1
Silt 0 – 16 (%)	8.6	64.1	6.9	0.1
Total sand 16 – 2000 (%)	87.8	30.4	72.8	99.3

1: - log (H⁺) in suspension

2: g/100g dry matter

Data analysis

Oospore viability and germination were determined as percentages (counts with a known maximum) and are therefore expected to follow a binomial variance function. The experimental design was a split-plot design where temperature was randomized over incubators, soil and moisture were randomized over containers and the sampling time was randomized over de bags within the container. This resulted in different levels of variances for the different treatments. For oospore viability the percentages measured were in the range 20-80%. Therefore it's reasonable to adopt a normal distribution and an analysis of variance was performed taking the split-plot design into account.

Oospore germination resulted in very low percentages (range 0-1%) of germination. In a GLMM-model (Generalized Linear Mixed Model) the binomial distribution and the different levels of variance of the data can be taken into account simultaneously. The Wald test can be used to test the significance of the treatment model term as it is added into the model. Although some results were obtained, the IRREML-procedure (Iterated Re-weighted REsidual Maximum Likelihood model) did not converge for the germination data due to the many zero's and very low percentages in the data. Therefore, an analysis of variance (ANOVA) was done on the germination data as well, leaving the treatments with only zero-data (all data obtained at 5°C and all data obtained after 1 week incubation) out of

the analysis to avoid a very small mean square (MS)-residual. The results of this analysis agreed with the Wald test of the IRREML analysis. Statistical analyses were performed using GenStat release 8.1 (GenStat, 2005).

Results

Selection of isolates and production of oospores

Five out of nine parental crosses (US8 x SN001A, US8 x IPO98014, PIC96002 x SN001A, IPO82001 x IPO99001 and IPO82001 x IPO98014) tested did not produce oospores in leaflets of cultivar Bintje. Crosses IPO99001 x US8, IPO99001 x PIC96002, and SN001a x IPO82001 produced oospores in one out of three inoculated leaflets. Cross IPO98014 x PIC96002 proved to be the most reliable with oospores in 2 out of 3 leaflets. In addition, the latter cross produced the highest oospore densities in potato leaf tissue.

P. infestans isolates IPO98014 and PIC96002 were therefore selected to produce oospores for the survival experiment using the methods described above.

Extraction of oospores from potato leaf tissue.

The oospore extraction procedure for small quantities of leaf tissue was found to be efficient. In the recovery experiment, 1338, 413 and 2063 oospores were found in the residues left behind on the 75 µm sieve for the 1380 mm², 815 mm² and 1403 mm² leaf samples respectively. The residues on the 20 µm sieve yielded 49500, 22690 and 38365 oospores respectively. The residue fractions passing the 20 µm sieve did not contain oospores for any of the samples. Average recovery was thus calculated as 96.7%. The recovery of oospores in large tissue samples could not be investigated. High numbers of oospores were recovered on the 20 µm sieve but the large amount of debris trapped on the 75 µm sieve made it impossible to reliably quantify oospores on the 75 µm sieve. The high dilution factor necessary to discern oospores from remaining leaf debris proved too high to reliably detect oospores. However, it can be safely assumed that the loss of oospores is significantly higher than for the extraction method described for small quantities leaf tissue. Consequently, this extraction method is only useful to extract large quantities of oospores in a production step for storage and/or later experimentation.

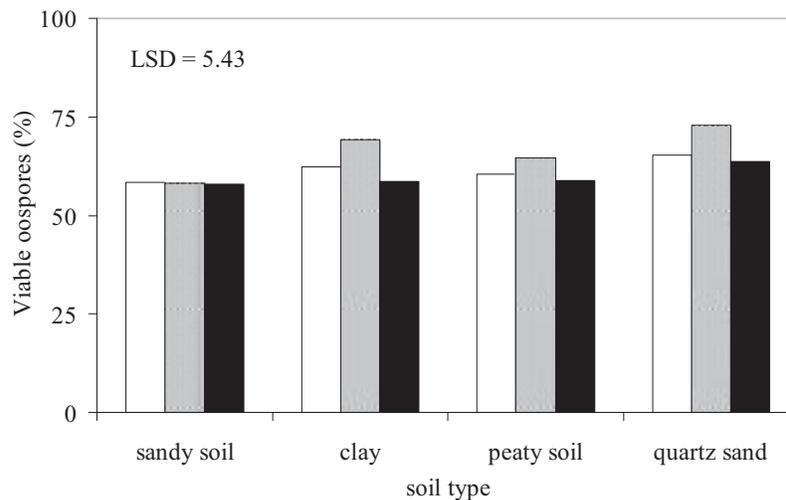


Figure 1. Effect of the incubation period (one week: white bars, two weeks: hatched bars and three weeks: black bars) on the viability of *P. infestans* oospores during incubation in three different soil types: a sandy soil, clay, a peaty soil and quartz sand, representing the three dominant soil types of the major Dutch potato growing areas and a reference soil.

Oospore viability and germination

The average initial oospore viability at the start of the experiments, as determined from the bulk samples, was 65%. Oospore viability was analysed using ANOVA. Significant effects for “incubation period” and the “soil x moisture” interaction were found. Temperature treatments incorporated in the current experiments did not significantly affect oospore viability. The effect of incubation period on oospore viability during this experiment is, although statistically significant, quantitatively negligible. Oospore viability remained more or less constant during the experiment (Figure 1). With respect to the “soil x moisture” interaction, generally, oospore viability is lower in saturated soils than in soils at field capacity for all three soils representing the Dutch potato growing areas. The reverse is true for quartz sand, causing a statistical interaction between the effects for soil and moisture (Figure 2).

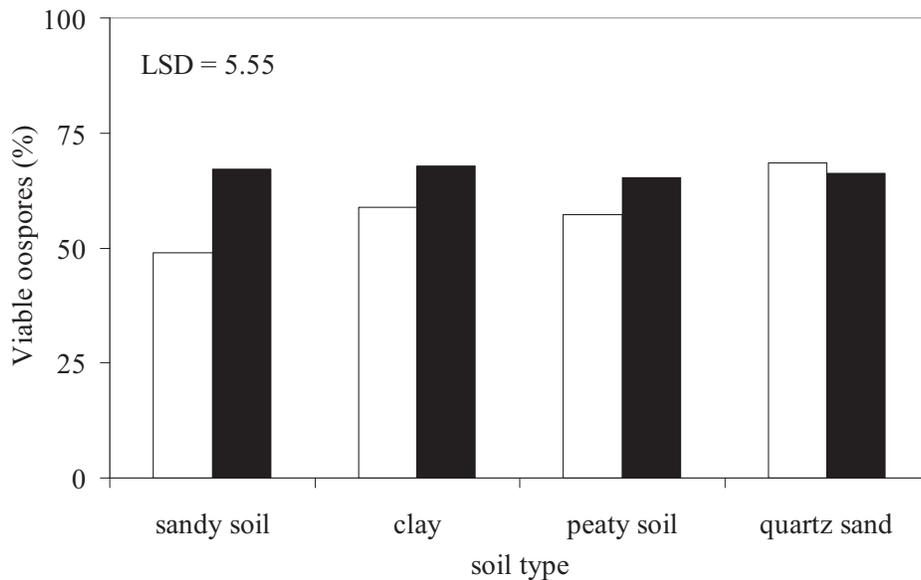


Figure 2. Effect of soil type and soil moisture level on viability of *P. infestans* oospores. Oospores were incubated in a sandy soil, clay, a peaty soil and quartz sand under saturated conditions (white bars) or at field capacity (black bars). Viability was determined using tetrazolium bromide.

No germinated oospores were found at the start of the experiments. Oospore germination was analysed using ANOVA. Germination did not occur in any of the treatments at 5°C or during the first week of incubation. To avoid very small MS-residues, ANOVA was performed excluding data from these two treatments. Three significant two-way interactions involving temperature, moisture and incubation time, and one, nearly significant, three-way interaction between these factors was found. Soil type was the only factor not significantly affecting oospore germination. In general, oospores germinate slowly, with percentages lower than 1% after three weeks incubation (Figure 3, Figure 4 & Figure 5). This makes it difficult to draw conclusions, despite the fact that statistical significance was reached for three two-way factorial interactions. Based on these significant two-way interactions, some trends may be discerned:

Although soil type did not significantly affect oospore germination, average germination in the peaty soil (0.111 %) was highest followed by quartz sand (0.098%), the sandy soil (0.058%) and clay soil (0.037%).

With respect to the temperature x moisture interaction, germination levels seem to increase with increasing temperatures for soils at field capacity but less so for saturated soils causing the interaction between the two factors (Figure 3).

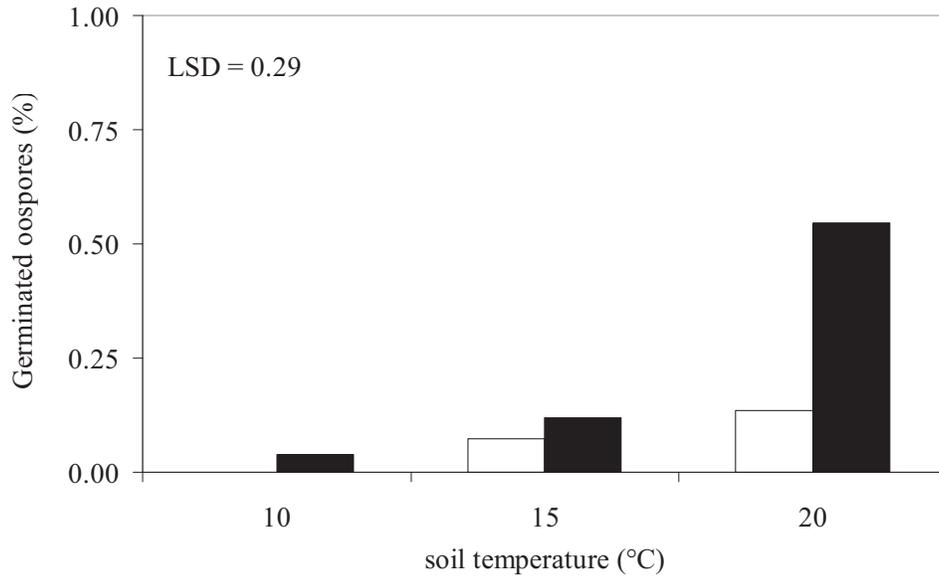


Figure 3. Effect of temperature and soil moisture level (white bars for saturated soils, black bars for soils at field capacity) on germination of *P. infestans* oospore

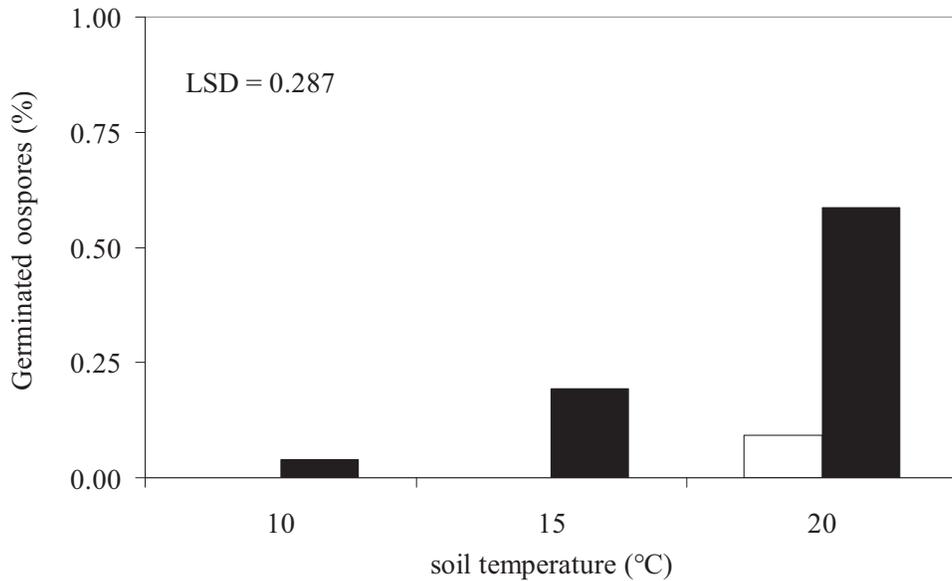


Figure 4. Effect of temperature and incubation period (two weeks: white bars and three weeks: black bars) on germination of *P. infestans* oospores. No germination was found at 5°C and after 1 week of incubation at all temperatures and soil types. Formation of a primary sporangium was used as the criterion for germination.

Posters

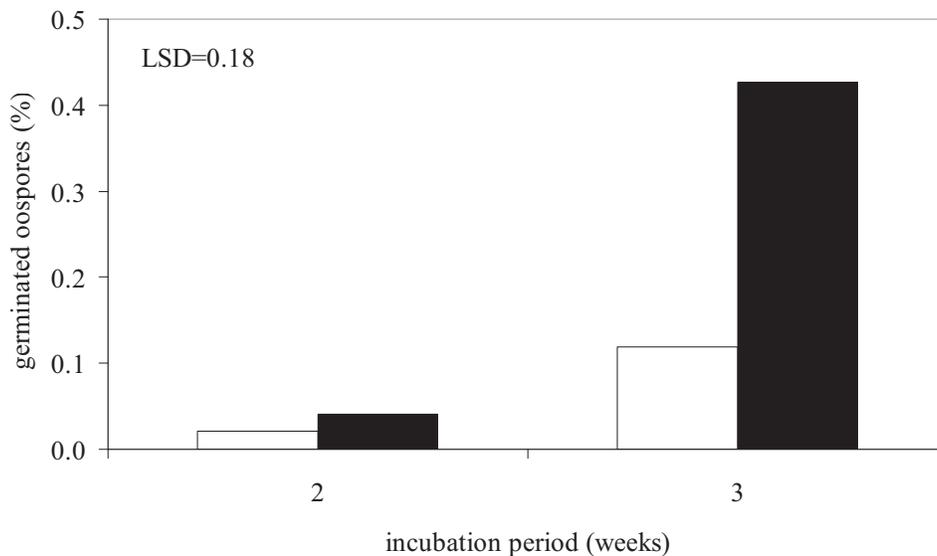


Figure 5. Effect of incubation period and soil moisture level (white bars for saturated soils, black bars for soils at field capacity) on germination of *P. infestans* oospores.

From Figure 3 and Figure 4, it can be concluded that the oospore germination rate increases with temperature. However, only at the highest temperature (20°C), germination is found already after two weeks incubation. At 10°C and 15 °C it took at least three weeks before oospore germination was detected. This contrast is likely to cause the factorial temperature x incubation period interaction. A similar explanation can be given for the incubation period x soil moisture interaction (Figure 5). In general, the oospore germination rate is higher at field capacity than under saturated conditions. This effect is however much less pronounced after two weeks incubation than after three weeks incubation causing the statistical interaction between both factors.

Discussion

Since the 1990's, evidence has been mounting that new introductions of *P. infestans* into Western Europe included both the A1 and A2 mating type. As a consequence, the Western European potato industry now faces a new *P. infestans* population which is reproducing sexually and is more aggressive than the old population (Drenth *et al.*, 1994; Drenth *et al.*, 1995; Flier & Turkensteen, 1999). For practical control purposes this implies that: 1) epidemic progress is faster and 2) oospores have to be taken into account as an additional, soil borne, source of (primary) inoculum. To adapt current control strategies to the presence of oospores as an extra soil borne source of inoculum, the population dynamics of soil borne oospore populations and factors driving survival and germination have to be studied. The work described in this paper was carried out with a dual purpose: to adapt and optimize methods facilitating quantitative research into *P. infestans* oospore ecology and to study *P. infestans* oospore germination as influenced by time, soil type, temperature and soil moisture.

As reported earlier by e.g. Cohen (2000) and Flier (2001), current results confirm that not all *P. infestans* A1 – A2 combinations produce oospores. Four out of nine A1 – A2 combinations tested produced oospores in potato leaflets but only one A1 – A2 combination (IPO98014 x PIC96002)

resulted in high oospore densities in two out of three crosses. Similar to the present findings, Flier (2001) also found that isolates differ in their average capability to form oospores when engaged in compatible matings. In both studies, specific parental combinations produced more oospores than other parental combinations. Cohen (1997) demonstrated that availability of free water is necessary for oospore production and that oospores were formed abundantly when leaflets were floating on water. In our experiment the availability of water was less than used by Cohen (1997) and may have been less than required for optimal oospore production, possibly explaining the relatively low oospore densities in the current experiment.

Both methods described to extract *P. infestans* oospores from small and larger leaf samples basically concentrate the available oospores in as little remaining leaf tissue as possible. For this purpose the leaf sample is comminuted, enzymatically degraded and sieved to remove as much of the leaf tissue as possible whilst at the same time retaining as many of the oospores as possible. The extraction procedure developed for small leaf samples is more time consuming than the procedure developed for large leaf samples but results in cleaner samples for microscopical oospore quantification.

Protocols were adapted from original protocols to extract *Peronospora viciae* oospores from pea (*Pisum sativum*, L.) by Van der Gaag & Frinking (1996). Major differences between the pea and potato extraction protocol for leaf material include two 90 sec. blending steps at 24000 rpm instead of one 5 min. step at an undefined high speed in the original protocol and a much longer enzymatic degradation step of over two days instead of 2 hours in the original protocol. These adaptations were done to accommodate for *P. infestans* oospore sensitivity to temperatures > 25°C (Drenth *et al.*, 1995) and for the relatively hard potato leaf tissue as compared to pea leaf tissue. Furthermore, surface sterilization of the plant tissue and antibiotics were not employed in the germination assays to mimic survival and (inhibition of) germination under natural conditions as much as possible.

Vice versa, adaptation of extraction techniques for use with other pathosystems is possible by adaptation of the duration of blending and enzymatic degradation. However, the temperature of the sample during blending has to be monitored and stabilized as much as possible since temperature directly influences oospore survival and germination (e.g. Ribeiro 1983; Drenth *et al.*, 1995; Fay & Fry, 1997). Thus, the above protocol was recently optimized to extract *Plasmopara viticola* oospores from grape leaves (van Bekkum, unpublished results).

Recovery of oospores from quartz sand containing only particles > 125 µm as described was found to be a simple but very effective method to quickly recover oospores from incubated gauze bags. This method potentially has a wide range of applications including experiments to shed light on factors stimulating or slowing down oospore germination by adding treatments with altered chemical (pH, CaCO₃) or biological (antagonists) properties of the otherwise inert quartz sand.

Oospore viability and germination was monitored during three weeks in a replicated factorial experiment including four constant temperatures, four soil types and two levels of soil moisture. Oospores were classified as germinated when a germ tube and sporangium were present. Presence of a germ tube by itself (Van der Gaag & Frinking, 1996) was not deemed sufficient because biologically, germination is not functionally completed when a germ tube is formed and practically, it was difficult to discriminate between adhering remaining leaf tissue and oospore germ tubes.

Oospore viability was not very much affected by any of the treatments during the experiment (Figure 1 and 2) as can be expected for soil borne long term survival structures. The average percentage viable oospores remained around 65% although viability in saturated soils seemed to be somewhat reduced. Apart from an effect on viability itself, incubation in water may have changed permeability of the oospore cell wall affecting the uptake of water and MTT and thus influencing the result of the viability test (Sutherland & Cohen, 1983).

Germination of oospores was found to be a slow process influenced by soil temperature and soil moisture. The germination rate increased with increasing temperatures (within the temperature range included) and too much available water (soil saturation) slowed germination down. The only factor not (statistically significant) affecting germination was soil type. The low percentages of germination found in the experiment make it however difficult to draw solid conclusions. Observations on germination in the experiments all fall within the 0 – 1% range. Purpose designed experiments could shed more light on some of the effects found in the current experiments but they are likely to be even more labour intensive.

When oospores are present in the soil, as a result of *P. infestans* infection of (a) previous crop(s), it can be assumed that during the growing season, apart from prolonged periods of hot and dry weather, oospores germinate continuously at a very low rate. Thus, newly formed sporangia are continuously being released to the soil at a low rate. The sporangial density in the soil thus depends on the oospore density in the soil, the oospore germination rate and the survival of sporangia under these conditions. In laboratory experiments sporangia have been reported to survive in unsterilized soil for periods up to 11 weeks (Lacey, 1965). Therefore, when oospores are expected, or known to be present in the soil, it has to be assumed that sporangia are also present in the soil during large parts of the growing season. Infections from this soil borne source of inoculum are therefore to be expected during each wet period fulfilling the requirements for puddle formation and/or splash dispersal together with circumstances favouring direct- or indirect germination of the sporangia. The foliage therefore has to be optimally protected by fungicides during each critical period with even less margin for error or delay than in the situation without oospores being present in the soil. When oospores, and thus sporangia, are in the soil during harvest, we should even consider the possibility of infection of tubers from this soil borne source of inoculum.

References

- Cohen Y, Farkash S, Reshit Z, Baider A, (1997). Oospore production of *Phytophthora infestans* in potato and tomato leaves. *Phytopathology* 87: 191-196
- Cohen Y, Farkash S, Baider A, Shaw DS (2000). Sprinkling Irrigation Enhances Production of Oospores of *Phytophthora infestans* in Field-Grown Crops of Potato. *Phytopathology* 90: 1105-1111
- Drenth A, Tas ICQ, Govers F (1994). DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. *European Journal of Plant Pathology* 100: 97-107
- Drenth A, Jansen EM, Govers F (1995). Formation and survival of oospores of *Phytophthora infestans* under natural conditions. *Plant Pathology* 44: 86-94
- Erwin DC, Ribeiro OK (1996). *Phytophthora Diseases Worldwide*. American Phytopathological Society Press, St Paul, MN
- Fay FC, Fry WE (1997). Effect of hot and cold temperatures on the survival of oospores produced by United strains of *Phytophthora infestans*. *American Potato Journal* 74: 315-323
- Flier WG, Turkensteen LJ (1999). Foliar aggressiveness of *Phytophthora infestans* in three potato growing regions in the Netherlands. *European Journal of Plant Pathology* 105: 381-388
- Flier WG, Grünwald NJ, Fry WE, Turkensteen LJ (2001). Formation, production and viability of oospores of *Phytophthora infestans* from potato and *Solanum demissum* in the Toluca Valley, central Mexico. *Mycological Research* 105 (8): 998-1006
- GenStat (2005). GenStat for Windows. Release 8.1, eight edition, VSN International Ltd., Oxford
- Griffith, GW, Shaw, DS (1998). Polymorphism in *Phytophthora infestans*: Four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. *Applied and environmental microbiology* 64 (10): 4007 – 4014

- Jiang J, Erwin DC (1990). Morphology, plasmolysis, and tetrazolium bromide stain as criteria for determining viability of *Phytophthora infestans*. *Mycologia* 82: 107-113
- Kessel GJT, Turkensteen LJ, Schepers HTAM, Van Bekkum PJ, Flier WG (2001). *P. infestans* oospores in the Netherlands: occurrence and effects of cultivars and fungicides. In: Schepers HTAM, Westerdijk CE, eds. Proceedings of the Sixth Workshop of an European Network for development of an integrated control strategy of potato late blight. Edinburgh, Scotland, September 2001. Applied Plant Research – Special Report no. 8. Wageningen, the Netherlands 203-209
- Lacey, J (1965). The infectivity of soils containing *Phytophthora infestans*. *Annual Applied Biology* 56: 363-380
- Ribeiro OK, 1983. Physiology of asexual sporulation and spore germination in *Phytophthora*. In: Erwin DC, Bartniciki-Garcia S, Tsao PH eds. *Phytophthora: its Biology, Taxonomy, Ecology, and Pathology*. St. Paul, MN: American Phytopathological Society 55-70.
- Sutherland ED, Cohen SD, 1983. Evaluation of Tetrazolium Bromide as a Vital Stain for Fungal Oospores. *Journal of Phytopathology* 73, 1532-1535
- Turkensteen LJ, Flier WG, Wanningen R, Mulder A, 2000. Production, survival and infectivity of oospores of *Phytophthora infestans*. *Plant Pathology* 49, 688-96.
- Van der Gaag DJ, Frinking HD (1996). Extraction from Plant Tissue and Germination of Oospores of *Peronospora viciae* f.sp. *pisi*. *Phytopathology* 144: 57-62

