J. Phytopathology **149**, 659–664 (2001)

ISSN 0931-1785

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Oospore Germination and Formation by the Late Blight Pathogen *Phytophthora infestans in vitro* and under Field Conditions

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With one figure

Received March 5, 2001; accepted June 21, 2001

Keywords: late blight, oospores, potato, race-non-specific resistance

Abstract

The ability of the late blight pathogen *Phytophthora infestans* to form oospores in leaves of seven potato cultivars was examined at different incubation temperatures under controlled environmental conditions and under field conditions. At 10°C, the oospore formation in three intermediate-resistant cultivars all differed significantly from each other ($P < 0.05$), with the lowest amount formed in cv. Asterix. This latter cultivar did not form oospores at any other temperature. Under field conditions oospores were formed abundantly in a naturally infected field. A significant date by cultivar interaction showed that *P. infestans* increased the oospore formation in foliage by time in cvs Columbo, Hertha and Matilda, whereas no significant differences between dates were found for other cultivars. The genetic structure of *P. infestans* in the naturally infected field plot, where oospores formed abundantly, was studied by using amplified fragment length polymorphism and a high genetic diversity was revealed. Oospore germination from two Scandinavian (A1 and A2) *P. infestans* isolates was stimulated in visible light and in 1 : 2 and 1 : 10 soil extract. The effect of light and nutrients on oosporogenesis is discussed.

Introduction

The heterothallic pathogen *Phytophthora infestans* requires both mating types A1 and A2 to form the sexual oospore stage which may lead to recombination and increased variability. Self-fertile *P. infestans* strains forming oospores are sometimes present in the population, but usually to a much smaller extent (Pipe et al., 2000). The late blight pathogen as an asexual organism is essentially an obligate parasite and can therefore only survive between seasons in infected tubers. The thick-walled oospores, however, can survive from 0°C to at least −20°C (Fay and Fry, 1997; Medina and Platt, 1999); consequently, *P. infestans* may in this stage, be independent of its host and overwinter as a soil inoculum. In recent years several indications of infections from soil-borne oospores (Pittis and Shattock, 1994; Drenth et al., 1995; Andersson et al., 1998) have been observed. Light, temperature, and nutrition are examples of factors that influence germination of oospores of *Phytophthora* species (Ribeiro, 1983) but which are not well understood for *P. infestans*. Drenth et al. (1995) and Hanson and Shattock (1998) observed that *P. infestans* in leaf tissue formed more oospores in cultivars which were intermediate non-specific-resistant than in susceptible cultivars. However, Turkensteen et al., (2000) observed that the largest numbers of oospores were formed in cvs Bintje (susceptible) and Pimpernel (resistant) and the fewest in cv. Nicola (intermediate resistant). Thus, the correlation between race-non-specific resistance in the potato cultivar and the pathogen's competence to form oospores is unclear. The purpose of the present study was to investigate factors that influence oospore germination and oospore formation in foliage under various environmental conditions in mainly race-non-specific resistance cultivars used in Sweden. In addition to laboratory experiments performed under controlled environmental conditions, oospore formation in different cultivars was studied in a field where sexual recombination of *P. infestans* was suspected to have taken place in previous years.

Materials and Methods

Cultivars

Potted plants from certified tubers of *Solanum tuberosum* were grown 1997–98 from October to May in 2 l plastic pots in standardized soil (Enhetsjord K) mixed with sand 80 : 10 in a greenhouse maintained at a minimum

of 15°C. Natural light was supplemented by mercury lamps (Osram Power Stars HQI-E 400 W/DH, Plantarea Sth, Sweden) with a 16 h photoperiod at a light intensity of 15 W/m^2. Fully expanded leaflets from the third to fourth node were detached from 6- to 8-week-old plants. Potato cvs Bintje (3), Asterix (5), Ovatio (4) Sava (5) Timate (5), Matilda (7) and Appell (8) were used in the experiments. Figures within brackets show field resistance to late-blight disease ratings on a scale from 1 to 9, in which 1 is highly susceptible and 9 is highly resistant, based on a Swedish national list of recommended potato cultivars compiled in 1998 (Hagman, 1998). Cultivars with a score of 4–5 were considered to have intermediate resistance, whereas those with a lower or higher score were considered susceptible and resistant, respectively, to foliage late blight.

Isolates

Phytophthora infestans isolates A1 (T96 461) and A2 (T96 462) from the Norwegian Agricultural Board were used to produce oospores in leaflets of cv. Bintje for the leaf disc- and germination assay. They were isolated in Norway in 1996 and are known to readily form oospores when mated on agar or leaf discs. Zoosporangial inoculum was prepared from cultures grown on rye-A medium incubated for 2 weeks at 15°C in the dark. A1 and A2 sporangial suspension each of $1–5 \times 10^4$ spores/ml were mixed in sterile deionized water (SDW) and chilled for 2–3 h at 5°C to encourage zoospore release.

Leaf disc assay

Leaf discs (18 mm in diameter) from fully grown leaflets of the third to fourth node were floated abaxial side up in a 15-cm-diameter Petri dish containing distilled water (DW). Twenty leaf discs from each cultivar were inoculated on the abaxial side with one drop (20 μl) of zoosporangial suspension and then incubated for 10 days in a climate chamber at temperatures of 5, 10, 20, 25 and 30°C with a 16 h photoperiod and visible light, i.e. a spectral distribution of 550–650 nm (Philips TL40/29, AB Ninolab Sth, Sweden). Two leaf discs were randomly selected from each cultivar at 10 and 20°C with lesions covering at least half the leaf discs and with lesions covering less than half the leaf disc for temperatures 5, 25 and 30°C. The leaf discs were weighed, and ground with a pestle and mortar in 2 ml of DW. The mean number of oospores extracted per gram leaf disc was counted with a haemocytometer for each cultivar and experiment. All experiments except that with the 5°C incubation temperature were replicated at least twice and pooled. Experiments performed at 25 and 30°C gave similar results and were pooled. Data were analysed by a two-way ANOVA using SAS version 6.12 (SAS Institute Inc., 1995). The model included the factor cultivar and temperature and their interaction. To stabilize variances, values of counts were square-root transformed in the analysis and back-transformed for presentation. To test for differences between means, an LSD-test at a significance $P < 0.05$ was used.

Field plots

In 1998 a field in Uppsala (59°53′ N, 14°38′ E) Sweden, was planted with certified tubers of six potato cultivars carrying different levels of race-non-specific resistance and one cultivar with race-specific resistance. This field had been planted with potato crops in 1994, 1996 and 1997 that were infected with late blight. Each cultivar was replicated in four rows in a randomized design in three blocks. Each row contained about 40 plants. Most of the cultivars in the leaf disc assay were included but not all, owing to shortage of seed potatoes. In the field plot, the susceptible cv. Columbo (2) and race-specific-resistant cv. Hertha were added. During the late blight season, late blight was scored on a 1–9 scale where 1 is 0.1–1% leaf area blighted and 9 is 100% leaf area blighted, after Henfling (1982).

Oospore formation under natural conditions

Plants in the field were naturally infected by inoculum from the soil or the air. Two leaflets with multiple lesions from the mid-canopy were collected from each cultivar in each row (in all eight leaflets per cultivar) on 31 July, and 10 and 20 August. The sampling was carried out from plants in block A, having a late blight index that was as equal as possible (after Henfling, 1982). On August 20, cv. Bintje had 91–97% late blight and therefore sampling was discontinued. The leaflets were frozen at −20°C. An amount of 300 mg of tissue with lesions from four leaflets of each cultivar and sampling date, was chosen randomly from the sample, and analysed. The leaflets sample was weighed, and ground with a pestle and mortar in 2 ml of DW and oospores were determined as in the leaf disc assay. The total number of oospores extracted per gram lesion was calculated per cultivar. Square-root-transformed values of counts were analysed using a two-way ANOVA with the factors cultivar and time and their interaction. To test for differences between means, an LSD-test at a level of significance ($P < 0.05$) was used. Values were back-transformed for presentation.

Oospore germination assay

Soil (loamy soil) samples were taken from three places next to the field plot and thoroughly combined. A suspension with autoclaved extract from the natural soil was prepared as for *Phytophthora megasperma* (Sneh et al., 1981). Oospores were produced at 10°C in leaflets of cv. Bintje. Before extracting the oospores the leaflets were surface sterilized with 1% sodium hypochlorite and washed twice in SDW. The oospores were then extracted by treating the leaf tissue with an Ultra turrax (IKA Labortechnik, Staufen, Germany) with 8000 r.p.m. for 2 min in SDW. The suspension was successively force-filtered through an autoclaved 53 μm and 20 μm Nylon net using a water vacuum pump. Oospores collected on the 20 μm net were resuspended in 10 ml SDW; contaminating zoospores, sporangia and mycelia, were killed by incubating for 36 h in 18°C (dark) with 0.01 g Novozym and washed with SDW four times by centrifugation at 2000 ***g*** for 3 min. The

pellet was resuspended and adjusted in SDW to give 2.5–5 × 10^3 oospores/ml, stored at 5°C and used within 2 months.

The germination tests were performed in flat-bottom, 24-well, sterile tissue culture plates (Tissue Culture Cluster ^{24} 3524; Costar, Cambridge, MA, USA). One hundred microlitres of soil extract was diluted with SDW to v/w 1 : 1, 1 : 2, 1 : 10, and 1 : 100. The oospore suspension was supplemented with ampicillin (100 mg/l) and rifampicin (10 mg/l) to suppress bacterial contaminants. One hundred microlitres soil extract was mixed with 100 $\mu\lambda$ oospore suspension in each well giving 100–250 oospores/well. SDW was used as a control and each treatment was replicated four times. The tissue culture plates were incubated at 5, 10, 15, 20 and 25°C under a 18 h photoperiod. Oospores were observed day by day with a Nikon TMS divert inverted microscope. After 12 days of incubation, germination (%) was read from 100 oospores per treatment. Oospores which germinated formed one or more germ tubes from which sporangia sometimes developed.

Mating type

In order to study the mating type and the genetic diversity of the population of *P. infestans* in the field, leaflets with multiple lesions were sampled from cv. Bintje on 3, 7, 10 and 17 August in the field plot. Mycelial inoculum of *P. infestans* cultures, originating from field isolates of unknown mating type, were paired on rye-A agar known A1 (T96 461) and A2 (T96 462) mating types from the Norwegian Agricultural Board. Mating types of the field isolates where recorded after 7–15 days.

Amplified fragment length polymorphism analysis

Phytophthora infestans mycelium was cultured for 2 weeks in 9 cm Petri dishes containing 5 ml rye-A broth. The DNA was extracted according to Pipe and Shaw (1997). Amplified fragment length polymorphism (AFLP) reactions were performed essentially as described in the protocol from Perkin-Elmer/Applied Biosystems AFLP® (Foster City, CA, USA) plant mapping kit for small genomes which is based on the method of Vos et al. (1995), but uses non-radioactive fluorescent dyes to label the primers. The sequences of adaptors and primers used in this study are listed in Table 5. A single reaction was used to cleave DNA with EcoR1 and Mse1 and ligate adapters, followed by pre-selective amplification in 25 μl reactions containing 4 μl DNA sample as described in Samils et al. (2000). The selective amplification was carried out using both primer combination E19/M16 and E19/M40. Electrophoresis was performed at constant voltage (3000 V) for 2.5 h at 51°C using an automated DNA sequencer (Model 377; PE Applied Biosystems). GeneScan Analysis software (PE Applied Biosystem) was used to visualize and score digital profiles. DNA fragments were accurately sized, by means of the internal standard included in each lane. After initial analysis, samples were imported into Genotyper version 2,0 (PE Applied Biosystems). Initially, a potential marker was generated by the software, followed by manual selection of loci with clearly separated size ranges and overall high signals (fragments with scaled peak heights above 75), that could be unambiguously scored for all samples. Scoring was carried out by the software, with the presence of a fragment (marker allele) in a sample denoted as '1' and absence (null allele) as '0', resulting in a binary data matrix of the different AFLP multilocus phenotypes. Neighbour-joining analysis was conducted using PAUP* 4.0b2 (Swofford, 1999), based on a distance matrix (mean character difference). Branch support was estimated by 1000 bootstrap resampling replicates.

Results

Leaf disc assay

Ten days after inoculation, lesions formed to various degrees on all cultivars except in cultivar Appell. At the extreme temperatures 5, 25 and 30°C lesions formed to a lesser extent than at moderate temperatures. The number of oospores formed varied between repeated experiments but, 10 days after incubation, the ranking among cultivars was similar in all experiments. No oospores were formed at 5°C (Table 1). At 10°C, cv. Sava formed more oospores than any other cultivar, as shown by a significant cultivar by temperature interaction ($P = 0.0395$). At 10°C, the oospore formation in three intermediate-resistant cultivars all differed significantly from each other ($P < 0.05$), with the lowest amount formed in cv. Asterix, which did not form oopores at any other temperature. Cv. Ovatio formed three-fold more oospores at 20°C than at 10°C, whereas in contrast, cv.

Table 1
The presence of oospores per gram leaf discs of non-race-specific, resistant potato cultivars inoculated with *Phytophthora infestans* isolates T96.461 (A1) and T96.462 (A2)

Cultivar	Resistance[a]	5°C[b]	10°C[b]	20°C[b]	25° and 30°C[b]
Bintje	3	0	250 cd	833 ab	43 a
Ovatio	4	0	650 b	1933 a	9 a
Asterix	5	0	51 ed	0 b	0 a
Sava	5	0	1775 a	833 ab	0 a
Timate	5	0	400 cb	833 ab	530 b
Matilda	7	0	0 e	34 b	0 a
Appell	8	0	0 e	0 b	0 a

[a]Field resistance to late-blight disease ratings on a scale from 1 to 9, in which 1 is highly susceptible and 9 is highly resistant based on a Swedish national list of recommended potato (Hagman, 1998); [b]Means (from two to four experiments) of oospore numbers within a column with no lower-case letter in common are significantly different at $P \leq 0.5$.

Sava formed two-fold more oospores at 10°C than at 20°C. Only at 10°C did two non-specific intermediate-resistance cvs Ovatio and Sava form more oospores than the susceptible cv. Bintje. The resistant cv. Matilda only formed oospores at 20°C and in low amounts.

Field plot

On July 31, *P. infestans* had formed oospores only in cv. Bintje but oospores were later readily formed in all cultivars except cv. Appell, which was unaffected by late blight (Table 2). On 10 August, in the middle of the epidemic, a significant date by cv. interaction ($P = 0.0019$) showed that cvs Sava and Ovatio had formed three- and four-fold, respectively, more oospores than susceptible cvs Columbo but not Bintje. Oospore formation increased in cvs Columbo, Hertha and Matilda from 10–20 August ($P < 0.05$), whereas no significant differences between dates were found for other cultivars.

On 20 August, at the end of the epidemic, oospores were detected in 50–100% of the four leaflets examined in all cultivars that were attacked by late blight (Table 2). Even at this time point cv. Appell was almost unaffected by late blight. The differences in oospore formation between susceptible and intermediate-resistant cultivars had levelled out. Furthermore, the race-specific-resistant cv. Hertha formed as many oospores as the susceptible and intermediate, non-specific-resistant cvs. However, in 100% of examined leaflets the resistant cv. Matilda formed two-fold more oospores than cvs Columbo, Bintje and Sava. During the sampling period all cvs started to form oospores in this field when reaching a late blight index of 2, i.e. up to 10 small lesions per plant (Table 2).

Oospore germination assay

Oospores from the Scandinavia A1 (T96 461) and A2 (T96 462) mating germinated to an extent of 7% in SDW at 15°C and a 18 h light period (Table 3). Germination was clearly stimulated in visible light compared with continuous dark. This was specifically so if the oospores were incubated in 1 : 10 and 1 : 2 soil extract. Temperature was likewise an important factor and 10 and 15°C enhanced germination 20–50% in 1 : 10 soil extract in comparison with 5 and 20°C.

Mating type

Both A1 and A2 were found in the field but A2 was restricted to only one isolate from block A (Table 4).

AFLP analysis

Altogether the data matrix included 96 characters among which 55 (57%) were variable (polymorphic). Neighbour-joining analysis resulted in a tree in which the US isolates (US-6, US-1) and the Norwegian isolates (A1 T96 461, A2 T96 462) were well separated from the Swedish ones (Fig. 1). The field isolates revealed a high genetic diversity, but some isolates collected from the same block (Table 4) were clearly joined and indicated a close relationship. Two isolates (An1 and An2) had identical AFLP patterns and originated probably from the same infection or clone. A third sample from the

Table 2
The presence of oospores per gram lesion from leaflets of potato cultivars naturally infected by *Phytopthothora infestans* in the field sampled on two occasions

	Date of sampling: 10 August			Date of sampling: 20 August		
Cultivar	Late blight index[a]	% of leaflets with oospores	Oospores/g tissue[b]	Late blight index[a]	% of leaflets with oospores	Oospores/g tissue[b]
Columbo	2.4	25	825 dc	5.5	75	2500 b
Bintje	4.0	50	1260 abc	6.5	75	1275 b
Hertha	2.9	75	850 abc	4.6	50	2500 ab
Ovatio	2.5	50	3325 ab	3.8	75	2525 ab
Sava	2.8	100	2900 a	5.3	100	2100 b
Matilda	1.1	0	0 d	2.0	100	5625 a
Appell	1.0	0	0 d	1.0	0	0 c

[a]Late blight was scored on a 1–9 scale in which 1 is 0.1–1% blight and 9 is 100% blight, after Henfling (1982); [b]Means of oospore numbers in each column with no lower case letter in common are significanlty different at $P \leq 0.05$.

Table 3
Percentages oospore germination after 12 days incubation. The oospores were formed by two Scandinavian *Phytophthora infestans* isolates T96.461 (A1) and T96.462 (A2)

	Germination (%) in 18 h white light[a]				Germination (%) in continuous darkness[b]			
Treatment	5°C	10°C	15°C	20°C	5°C	10°C	15°C	20°C
Soil 1 : 1	2	6	8	0	0	2	2	2
Soil 1 : 2	6	10	10	4	0	1	1	2
Soil 1 : 10	12	16	14	8	2	3	1	2
Soil 1 : 100	2	6	5	4	2	3	4	3
SDW	2	5	7	5	3	6	3	4

[a]White light has a spectral distribution of a spectral distribution of 550–650 nm; [b]Germination was assessed as positive with one or more germ tubes from which sporangia sometimes developed.

Table 4
List of isolates used in the AFLP study

Isolate	Origin: Row	Block	Mating type	Date of isolation
An 1	3	C	A1	07–08–1998
An 2	3	C	A1	10–08–1998
An 3	17	B	A1	10–08–1998
An 4	3	C	A1	07–08–1998
An 6	8	A	A1	10–08–1998
An 11	23	A	A2	03–08–1998
An 12	23	A	A1	07–08–1998
An 13	8	C	A1	10–08–1998
An 14	17	B	A1	10–08–1998
An 16	17	B	A1	17–08–1998
A2 T96	Norway		A2	1996
A1 T96	Norway		A1	1996
US-1	USA		A1	
US-6	USA		A1	

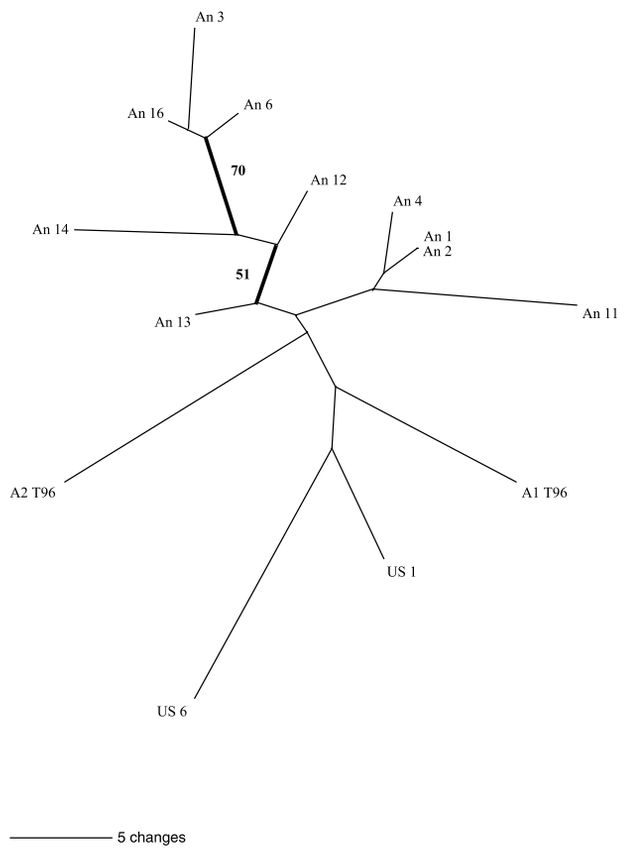

Fig. 1 Neighbour-joining tree obtained for isolates of *Phytophthora infestans*, based on AFLP markers. The tree is unrooted and significant bootstrap values (> 50%) are indicated

Table 5
Sequences of adaptors and primers used in the AFLP study

ECO 1 AD	5′to3′	CTC GTA GAC TGC GTA CC
ECO 2 AD	5′to3	AAT TGG TAC GCA GTC
MSE 1 AD	5′to3	GAC GAT GAG TCC TGA G
MSE 2 AD	5′to3	TAC TCA GGA CTC AT
EOO	5′to3	GAC TGC GTA CCA ATT C
MOO	5′to3	GAC GAT GAG TCC TGA GTA A
M16	5′to3	GAT GAG TCC TGA GTA ACC
M40	5′to3	GAT GAG TCC TGA GTA AAG C
E19	5′to3	GAC TGC GTA CCA ATT CGA

same block, An4, differed in only 5% of the characters. Another group of closely related samples was made up by An3, An6, and An16, differing in 4–8% of the characters.

Discussion

Germination of oospores of *Phytophthora* spp. is usually low and variable and does not exceed 10% (Ribeiro, 1983; Förster et al., 1983). This also applied to oospores formed by two Scandinavian isolates (A1 and A2) of *P. infestans*. There were, however, factors that caused slightly increased germination. We found, for instance, that germination of oospores from *P. infestans* when incubated in visible light was enhanced by soil extract diluted 1 : 2 and 1 : 10. Nutrients are important factors influencing oospore germination (Elliott, 1983; Erwin and Ribeiro, 1996). Exhaustion of nitrogen, for example, induces oospore germination whereas excessive glucose, fructose and sucrose inhibits germination of *Phytophthora* spp. oospores (Ribeiro, 1983). We suggest that the soil extract contained nutrients that stimulated the oospore germination in certain concentrations. Visible light and temperatures around 10–15°C also supported oospore germination. The long days in May–July with more than 18 h daylight and temperatures around 10°C in Scandinavian countries then give excellent conditions for oospore germination. Furthermore, in the last 4 years in Sweden there were outbreaks of late blight on new potatoes in early June from fields where oospores have been found in canopy of plants.

The formation of oospores in the cultivars that were tested during the present study did not fully follow the hypothesis stated by Drenth et al. (1995) of a positive correlation between race-non-specific resistance and oospore formation. It was shown, for instance, that intermediate-resistant cv. Asterix only formed oospores at 10°C and four-fold less oospores than susceptible cv. Bintje whereas other intermediate-resistant cultivars formed three-fold more. Further, resistant cv. Matilda formed in the field two- and four-fold more oospores than both susceptible and intermediate-resistant cvs. In contrast to the *in vitro* leaf disc assay in which cv. Matilda only formed a few oospores at 20°C, it

formed abundant oospores under field conditions. It seems likely that cv. Matilda was exposed to many more and variable strains of *P. infestans* in this field, as shown in the AFLP analysis, in contrast to only two strains in the leaf disc assay. Only in susceptible cv. Columbo, resistant cv. Matilda and race-specific cv. Hertha did oospore formation increase with time in the field. Together, these observations suggest that other factors in addition to level of race-non-specific resistance are important for oospore formation. Turkensteen et al. (2000), for instance, suggested that oospore formation may also be affected by factors other than the level of partial resistance, for instance, the sterol content of the host.

The abundant formation of oospores in the field was possible in spite of a low A1/A2 ratio, only 1 : 9. Turkensteen et al. (2000) reported that a ratio of 1 : 3 readily formed oospores in a commercial field. The abundant formation of oospores in the field as found in the present study, is also in contrast to findings by Cohen et al. (2000) who observed poor oospore formation in the field compared with *in vitro* formation. The present observations were made in a field where suspected sexual propagation has taken place for at least 3 years (Andersson et al., 1998), which might have formed a variable population with strains of *P. infestans* that easily mate. The present study also revealed a high genetic diversity of *P. infestans* and the different groups of isolates in the field may originate from different oospores. High genetic diversity of *P. infestans* populations has also been described from potato fields in other nordic countries such as Norway and Finland (Brurberg et al., 1999), indicating sexual reproduction.

It appears that oospore formation as well as oospore germination are dependent on visible light and temperatures and are possibly affected by different nutrients either from the host (cultivar) or the soil. It is well known for instance, that some carbon and nitrogen sources inhibit as well as stimulate oosporogenesis in *Phytophthora* spp. (Elliott, 1983; Erwin and Ribeiro, 1996).

Acknowledgements

We are grateful to Björn Andersson and Jannie Hagman, Department of Ecology and Crop Production Science, for help with certified tubers and arranging the field plots. Funding was provided by the Swedish Council for Forestry and Agricultural Research.

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