OOSPORE POPULATIONS OF *PERONOSPORA VICIAE*: QUANTIFICATION, GERMINABILITY AND SURVIVAL

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OOSPORE POPULATIONS OF *PERONOSPORA VICIAE*: QUANTIFICATION, GERMINABILITY AND SURVIVAL

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Author's abstract

Peronospora viciae causes downy mildew on pea, field and broad bean. Two pathogens can be distinguished, *P. viciae* f.sp. *pisi* on pea (*Pisum sativum*) and *P. viciae* f.sp. *fabae* on field and broad bean (*Vicia faba*). These pathogens form resting spores, called oospores, in their host plant tissue. The oospores infest the soil after decomposition of the surrounding plant tissue and are the means by which the pathogens survive in the absence of host plants. In the present thesis aspects of the population biology of the oospores were investigated. Numbers of oospores were determined by extraction from plant tissue or soil. A vital stain test and a germination assay were used to assess viability and germinability of oospore populations. Monoconidial isolates of the two pathogens were able to produce oospores in monoculture and thus both pathogens are homothallic. *P. viciae* f.sp. *pisi* had a minimum temperature of about 10°C for oospore production and *P. viciae* f.sp. *fabae* below 5°C. Dry-stored oospores of *P. viciae* f.sp. *pisi* germinated in water and appeared independent of an exogenous chemical stimulus for germination. Germinability of these oospores increased with oospore age up to a certain point and was related to their infectivity in a bioassay. Dry-stored oospores of *P. viciae* f.sp. *fabae* did neither germinate in water nor did they infect seedlings in a bioassay. After incorporation in field soil, oospore populations of both pathogens declined rapidly, and small, probably non-random, proportions of the original oospore populations survived for more than a year. The ability of oospores of *P. viciae* f.sp. *pisi* to germinate in water declined rapidly after incorporation in soil. Low percentages of oospores extracted from soil germinated in water, but relatively high disease incidences were obtained after inoculation of pea seeds with these oospores which suggested that soil-exposed oospores become dependent on a chemical stimulus for germination. Oospores of *P. viciae* f.sp. *fabae* were infective after incubation in field soil for two years.

Voorwoord

Na bijna vier jaar als assistent in opleiding (AIO) op de vakgroep Fytopathologie rond te hebben gelopen rond ik mijn AIO-onderzoek af. Het oorspronkelijke onderwerp van het onderzoek was de temporele dynamiek van oösporenpopulaties van *Peronospora viciae* f.sp. *fabae*. Omdat in het eerste half jaar van het onderzoek, op een enkele kiembuis en geïnfecteerde plant na, geen enkel positief resultaat werd verkregen werd besloten het onderzoek uit te breiden met oösporen van *P. viciae* f.sp. *pisi*. Deze oösporen gedroegen zich aanvankelijk minder saai en consequent dan die van *P. viciae* f.sp. *fabae* en kiemingspercentages varieerden van 0 tot 85 in plaats van 0 tot 1. Het onderzoek heeft uiteindelijk geleid tot dit boekje over oösporen van beide pathogenen.

In dit voorwoord wil ik graag een aantal mensen bedanken die hebben bijgedragen aan de uitvoering van het onderzoek en de realisering van dit boekje.

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1 General introduction

1.1 Oospores of downy mildew fungi

General characteristics of downy mildew fungi

The term 'downy mildew' denotes the white or greyish fluff which appears at high relative humidities on plant parts infected by downy mildew fungi. Downy mildew fungi are members of the family of the Peronosporaceae, which belong to the fungal class of the Oomycetes. They are obligate biotrophic parasites which need living host tissue for growth and sporulation. Downy mildew fungi occur world-wide on a broad range of host plant spp. (Lebeda and Schwinn, 1994). Some of them are of high economic importance, being able to cause severe crop losses (Viennot-Bourgin, 1981; Lebeda and Schwinn, 1994). The economically most important pathogens were listed by Lebeda and Schwinn (1994).

The life cycle of downy mildew fungi comprises both an asexual and a sexual infection cycle. In the asexual infection cycle short living sporangia or conidia are formed, depending on the fungal species. Sporangia germinate indirectly by release of zoospores. Conidia germinate directly by forming germ tubes. Sporangia and conidia are formed on sporangiophores and conidiophores, respectively, which appear through the stomata on the outside of colonized host plant tissue at high relative humidities ('downy mildew'). They are dispersed by rain or wind and can be transmitted over long distances (Lebeda and Schwinn, 1994).

In the sexual infection cycle downy mildew fungi form oospores after meiosis and fusion of two haploid nuclei, as all Oomycetes do (Michelmore *et al.*, 1988) (Fig. 1.1). Oospores are thick-walled survival structures and are mostly deposited in soil with crop residues and usually remain close to their place of origin. They can be dispersed over longer distances adhered to bulbs, farming machineries, and seeds. Wind dispersal of oospores may also occur (Bock *et al.*, 1996).

Oospores

Oospores of downy mildew fungi may be a source of genetic recombinants of the fungus (Michelmore and Ingram, 1981) and may also be the means of survival during periods without living host tissue. Michelmore *et al.* (1988) gave an overview of the way of transmission of 19 fungal species causing downy mildew. In 15 of these species, oospores (may) contribute to the transmission of the pathogen. Oospores left in plant debris in the field can be infective when the surrounding plant tissue has decomposed. Oospores may infect below ground plant parts, as in *Peronospora viciae* (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven, the causal agent of downy mildew on pea (*Pisum sativum* L.) (Stegmark, 1994), or above ground plant parts as in *Peronospora tabacina* Adam, the causal agent of downy mildew on tobacco (*Nicotiana tabacum* L.), the tobacco blue mould (Borovskaya, 1968).

The relative importance of oospores in the transmission of the fungus varies among species. Palti and Rotem (1981) mentioned nine species in which soil-borne oospores are significantly involved in perennation. In *Peronosclerospora sorghi* (Weston & Uppal) C.G. Shaw, causing downy mildew on sorghum (*Sorghum bicolor*

(L.) Moench), and *Peronospora viciae* f.sp. *pisi*, for example, oospores are believed to be the major or exclusive source of primary infection. In other species oospores seem to be of minor importance. For example, *Pseudoperonospora humuli* (Miy. & Tak.) Wilson, causing downy mildew on hop (*Humulus lupulus* L.), survives by means of mycelium in perennial rootstocks of its host plant (Royle and Kremheller, 1981). Wild hosts may play a role as a refuge for downy mildew fungi during periods when the cultivated host is not grown (Renfro and Shankara Bhat, 1981).

Sexual system

Michelmore *et al.* (1988) reviewed the sexual system of 19 downy mildew fungi species. Some species appear to be homothallic, some heterothallic, while other species have both homothallic and heterothallic strains. In several downy mildew fungi the sexual system has not yet been studied.

Production of oospores

Oospore formation was reviewed by Populer (1981). Production of oospores may be stimulated by conditions unfavourable for conidial/sporangial formation, thus at temperatures too high or humidities too low for conidial/sporangial formation (Populer, 1981). *Plasmopara viticola* (Berk & Curt.) Berl. & De Toni, for example, the causal agent of downy mildew on vine (*Vitis vinifera* L.), forms oospores mostly in lesions on ageing leaves in the autumn. They are less frequently formed in the summer and then especially when the humidity is too low for asexual sporulation (Populer, 1981). In *Bremia lactucae* Regel, the causal agent of downy mildew on lettuce (*Lactuca sativa* L.), however, conidia and oospores are simultaneously formed at the same temperature range of 5-22°C (Michelmore, 1981). Apparently, conditions at which oospores of downy mildew fungi are formed cannot be generalized.

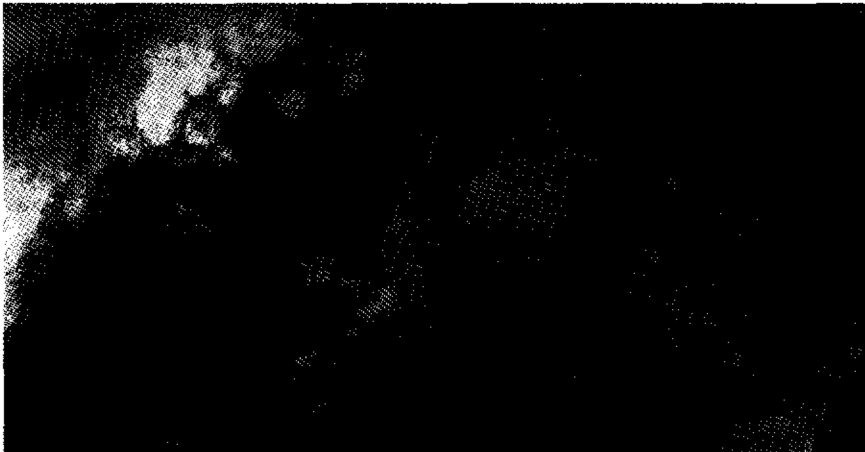

Fig. 1.1. Oospores of *Peronospora viciae* f.sp. *pisi* in leaf tissue of pea.

Survival of oospores and natural densities

Longevity of oospores of downy mildew fungi varies from one winter to over 10 years (Populer, 1981). Survival studies were mostly carried out on oospores in plant debris and not in soil. Although it is generally believed that oospores in soil play a major role in the carry over of several downy mildew diseases from one growing season to the next (Palti and Rotem, 1981), little research has been done on the survival of oospore populations in soil. Mathematical description of survival of soil-borne inoculum is limited (Gilligan, 1990; 1994), and no data on the shape of survival curves of oospore populations are known.

Published estimates of natural inoculum densities of oospores in soil are scarce. Pratt and Janke (1978) extracted oospores of *Peronosclerospora sorghi* from 21 naturally infested soils in Texas (US). They found oospore densities of 8 to 180 per gram of soil. Schuh *et al.* (1988) found oospore densities of the same species from 1 to 56 per gram of soil in one field in Texas. Numbers of extracted oospores of *Sclerospora graminicola* (Sacc.) Schroet., the causal agent of downy mildew on pearl millet (*Pennisetum glaucum* (L.) R.Br.), extracted from soil samples from 20 locations in Niger, varied from 26 to 768 oospores per gram soil (E. Gilijamse, pers. comm.) with a recovery of extraction estimated at 70%.

Germination of oospores

Oospore germination has been examined in several downy mildew fungi (Table 1.1). The conditions or treatments used in the studies to induce germination of oospores are described in this section.

Twenty-three to 33% of the oospores of *Bremia lactucae* germinated in the vicinity of seedlings and no or only a few germinated in distilled water (Morgan, 1978; 1983). Percentages germination did not significantly change when oospores were stored in tap water during a year.

Germination of oospores of *Peronosclerospora sorghi* was found at low levels, less than 1%, adjacent to roots of hosts and nonhosts (Pratt, 1978). No germination occurred with oospores freshly harvested in the field. Some oospores germinated after storage at 25°C for 6-10 months but they did not germinate after storage at 4°C. French and Schmitt (1980) studied oospore germination *in vitro*. Percentages germination varied greatly. The variation was partly ascribed to differences among replications in the degree of contamination with other micro-organisms. They found a positive effect of furfural on germination. Percentages germination in their studies did not exceed 20.

In Ireland, McKay (1957) conducted a long term study on the behaviour of oospores of *Peronospora destructor*. Germination of oospores was only observed after several years of weathering outside. Germination was erratic until oospores were almost 4 years old. Percentages germination increased from 1, after 4 years, to 5, after 7 years of storage, when oospores were incubated in water. When 0.01-0.02% potassium permanganate was added to the water percentages germination were 60 and 95 after 4 and 7 years, respectively.

Oospores of *Peronospora manshurica* germinated after washing for 1 week in running tap water (Dunleavy and Snyder, 1962). It was suggested that a germination inhibitor was present which was removed by washing. Pathak *et al.* (1978) studied

Table 1.1. Percentages germination reported for oospores of downy mildew fungi by several authors.

Pathogen	Host	Germ%	References
Bremia lactucae Regel	lettuce (*Lactuca sativa* L.)	23-33	Morgan (1978, 1983)
Peronosclerospora sorghi (Weston & Uppal) C.G. Shaw	sorghum (*Sorghum bicolor* (L.) Moench)	0-20	Pratt (1978), French and Schmitt (1980)
Peronospora destructor (Berk.) Casp. ex Berk.	onion (*Allium cepa* L.)	0-95	McKay (1957)
Peronospora manshurica (Naoum.) Syd. ex Gäum.	soybean (*Glycine max* (L.) Merrill	8-30	Pathak *et al.* (1978)
Peronospora parasitica (Pers. ex Fr.)Fr.	cabbage (*Brassica oleracea* L.)	0[a]	Frinking (pers. comm.)
	radish (*Raphanus sativus* L.)	42	Jang and Safeeulla (1990)
Peronospora tabacina Adam	tobacco (*Nicotiana tabacum* L.)	0-50	Von Pawlik (1961), Von Kröber (1969), Borovskaya (1968)
Peronospora viciae (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven	pea (*Pisum sativum* L.)	0-40	Geesteranus (1961), Von Heydendorff (1977), Clark (1989); Günther (1992)
Plasmopara viticola (Berk. & Curt.) Berl. & De Toni	grape vine (*Vitis vinifera* L.)	0-30	Tran Manh Sung *et al.*(1990), Cortesi and Zerbetto (1991), Siegfried and Bosshard (1991), Burruano *et al.*(1992)
Sclerophthora macrospora (Sacc.) Thirum., Shaw & Naras	maize (*Zea mays* L.)	9-23	Dernoeden and Jackson (1981)
Sclerospora graminicola (Sacc.) Schroet.	pearl millet (*Pennisetum glaucum* (L.) R.Br.)	0-68	Nene and Singh (1976)

[a] Infection of cabbage seedlings by oospores have been reported (LeBeau, 1945; Moss *et al.*, 1994; H.D. Frinking, pers. comm.).

germination of oospores from different origins; percentages germination ranged from 8 to 30.

In vitro germination of oospores of *Peronospora parasitica*, causing downy mildew on members of the genus *Brassica*, has not been observed although seedlings can be infected by oospores (LeBeau, 1945; Moss *et al.*, 1994; H. Frinking, pers. comm.). Jang and Safeeulla (1990) reported *in vitro* germination (42%) of oospores of *Peronospora parasitica* causing downy mildew on radish.

Oospores of *Peronospora tabacina* germinated poorly in several studies (Von Pawlik, 1961; Von Kröber, 1969). Infectivity of oospores was low and did not increase when stored in soil for more than four years (Von Kröber, 1969). Borovskaya (1968), however, found an increase in germination of the oospores after incubation in soil for two years, and more so after three and four years. The oospore wall became thinner during incubation in soil.

Oospore germination of *Peronospora viciae* f.sp. *pisi* was reported by several researchers. Geesteranus (1961) observed some germinated oospores. Von Heydendorff (1977) also found a few germinated oospores. Günther (1992) found percentages germination up to 40% on 2% water agar on which some free water was added. Oospore germination of *P. viciae* f.sp. *fabae*, causing downy mildew on Faba bean (*Vicia faba* L.) was observed by Jamoussi (1968) but he did not mention conditions nor percentages of germination.

Oospores of *Plasmopara viticola* become germinable about 4 to 5 months after formation. This period and the numbers of germinable oospores depend on soil humidity and temperature (Tran Manh Sung *et al.*, 1990; Cortesi and Zerbetto, 1991; Siegfried and Bosshard, 1991; Burruano *et al.*, 1992). No specific trigger seems to be required for germination. In these French and Italian studies, leaf discs containing oospores were incubated outside in plaster tubes or on glass slides. Percentages germination generally did not exceed 30 %.

Oospores of *Sclerophthora macrospora* were found to germinate in low percentages only. Addition of gibberellic acid, charcoal or rye seedling exudates appeared to increase percentages germination (Dernoeden and Jackson, 1981).

Germination of oospores of *Sclerospora graminicola* was observed by several researchers. Nene and Singh (1976) reviewed the reports on oospore germination of this pathogen, percentages germination varied from 0 to 68. Direct germination by germ tubes and indirect germination through zoosporangia were reported. Infectivity of oospores increased after weathering (Suryanarayana, 1962; Nene and Singh, 1976).

Generally, oospores of downy mildew fungi germinate poorly. Low germinability may be due to a large proportion of non-viable oospores or to the existence of dormancy. Dormancy may end after exposure to natural conditions or with ageing of the oospores. The studies of McKay (1957) and Borovskaya (1968) on oospores of *Peronospora destructor* and *Peronospora tabacina*, respectively, showed that a dormancy period of at least two years can exist.

Observations on oospore germination can easily be disturbed by growth of hyphae of mycoparasitic fungi from within oospores. Oospores can be parasitized by several fungi (Sneh *et al.*, 1977). Person and Lucas (1953) first believed to see germination of oospores of *Peronospora tabacina* by sporangia, but later reported that the sporangia actually originated from a chytrid species which had invaded the oospore (Person *et al.*, 1955). Pratt (1978) discussed the problems of 'false' germination of

Peronosclerospora sorghi oospores due to invasion of the oospores by filamentous fungi. 'True' germination could be distinguished from 'false' germination since 'true' germ tubes were thicker than the hyphae of the mycoparasites, and they were coenocytic.

Studies on the behaviour of the oospores are needed to gain more insight in the factors affecting germinability and survival of oospores of downy mildew fungi. Knowledge on the oospore behaviour can be valuable for genetical studies in which large numbers of infections by oospores are required to obtain a large sexual progeny, and for the control of those downy mildew fungi of which the oospores play an important role in the epidemiology of the downy mildew disease.

1.2 Behaviour of fungal spores

Terminology and definitions

In research on fungal spores several terms are used to indicate the state of a spore, the periods in which the state of a spore changes, and the processes which lead to another spore state. These terms have been defined and discussed in several reviews (Sussman, 1965; Sussman and Halvorson, 1966; Griffin, 1981). Some terms have been defined in various ways, which is often due to difficulties in the distinction of the states. In this section the problems and difficulties in terminology and definitions in fungal spore research are discussed and the definitions as applied to oospores in the present thesis are indicated.

Formation
Formation of a spore is 'the stages leading from active growth to quiescence' according to Griffin (1981). In the present thesis the end of spore formation is defined as the moment that a spore has reached its final morphological state.

Viability, dormancy, and germinability
The state of a fungal spore can be classified according to the scheme presented in Fig. 1.2. A spore is either viable or non-viable. Viable and viability have been defined as 'able to live' and 'the ability to live', respectively (Federation of British Plant Pathologists, 1973). But how can we measure the ability to live? Is it the survival time of a spore under given environmental conditions? Campbell and Madden (1990) have given a more operational definition of viability, 'the ability of a propagule to germinate (or hatch) and initiate vegetative or reproductive growth given proper environmental conditions'. However, the ability of a propagule to germinate at a certain time may not reflect its viability as it may be dormant (Fig. 1.2). Therefore, a viable spore is defined here as a spore which is able to germinate and initiate growth given proper environmental conditions or which is able to do so after a period of dormancy.

Dormancy has been defined by Sussman (1965) as 'any rest period or reversible interruption of the phenotypic development of an organism'. Sussman (1965) distinguished two types of dormancy: constitutive dormancy, 'a condition wherein development is delayed due to an innate property of the dormant stage such as a

barrier to the penetration of nutrients, a metabolic block, or the production of a self-inhibitor', and exogenous dormancy, 'a condition wherein development is delayed because of unfavorable chemical or physical conditions of the environment'. Cochrane (1974) argued to apply the term dormancy only to states of innate dormancy since exogenous dormancy refers to the environmental conditions inhibiting germination of germinable spores. Cochrane's point of view is taken over as it is agreed that dormancy is an intrinsic spore characteristic. Thus, the above mentioned definition of Sussman (1965) for constitutive dormancy, treating dormancy as an innate property of a spore, is used here as the definition for dormancy. In the present thesis, a dormant spore is defined as a viable spore that fails to germinate although it is exposed to proper environmental conditions, i.e. conditions that support germination.

In the present thesis germinability is defined as the ability to germinate given proper environmental conditions, and a germinable spore is a spore able to germinate given proper environmental conditions. In terms of a spore population viability and germinability are defined as the percentages of 'viable' and 'germinable' spores, respectively.

Maturation, after-ripening, and activation

The terms maturation, after-ripening, and activation are used to indicate the processes which lead to another state or to the germination of a fungal spore, or to indicate the periods in which these processes take place. These terms and their definitions will be discussed.

Maturation has been defined as 'the complex of changes associated with the development of the resting stage of dormant organisms or of the germinable stage in those without a dormant period' (Sussman, 1965). This definition makes a distinction between immaturity and dormancy. However, other authors include immaturity in dormancy (Gottlieb, 1950; Cooke and Whipps, 1993). Cochrane (1974) discussed whether immaturity should be included in dormancy or not. During maturation cytological changes occur, while in a dormant spore no cytological changes are discerned (Cochrane, 1974). In practice, it is difficult to distinguish between immature and dormant spores. Cochrane pointed out that a definition in which dormancy includes

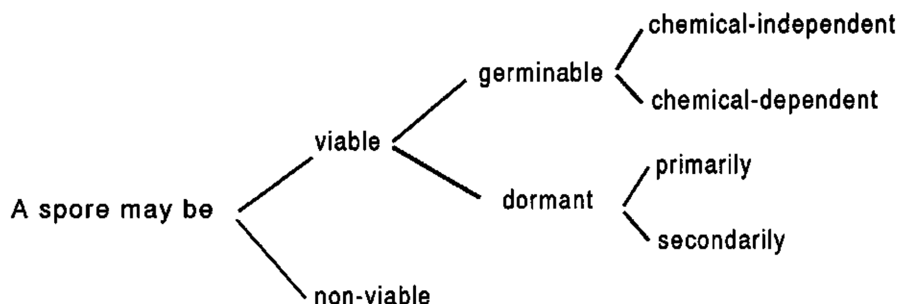

Fig. 1.2. The possible states of a fungal spore.

immaturity is more operational, while the other, discriminating between maturation and dormancy is more mechanistic and analytical. Because of these difficulties and the possibly incorrect use of 'maturation', Gottlieb (1950) is here followed, including immaturity in dormancy. Immaturity is then considered a special state of dormancy. The terms immaturity and maturation should only be used when they are supported by cytological observations.

After-ripening is 'that part of the dormant period during which the changes occur which lead to germination', and activation is 'the application of the environmental stimuli which induce after-ripening' as defined by Sussman (1965). The definitions of after-ripening and activation differ between authors. According to Sussman (1965), after-ripening includes the period during which changes occur leading to a germinable stage and the part of the germination process untill emergence of the germ tubes or release of the zoospores. Sussman and Halvorson (1966) defined activation as a treatment which induces germination, but they also used this term for treatments which break dormancy, such as heat-activation. According to the definitions of Griffin (1981) after-ripening is a period, part of the dormant period, in which the changes occur in a mature spore which lead to a germinable spore; activation shortens this period. No part of the germination process is included in this definition. Activation as used by several researchers on oospore germination refers to the initiation of germination: the dissolution of the inner oospore wall (Jimenez and Lockwood, 1982; Beakes and Bartnicki-Garcia, 1989; Jiang and Erwin, 1993).

Sussman (1965) stated that no clear distinction exists between dormant period and requirement for long incubation till emergence of the germ tube. But the time needed for the germination process is far less than the incubation time some spores need to become germinable. For example, oospores of *Plasmopara viticola* need a period of about 4 months after formation until they are germinable, whereas germination takes place within 6 to 10 days *in vitro* (Tran Manh Sung and Clerjeau, 1988). Germination is an irreversible process, while dormancy in some spores can be induced or terminated. Therefore, it is argued that attempts should be made to distinguish dormancy breaking treatments from germination inducing ones. It is proposed to use the term activation only for treatments or conditions which induce germination, thus strictly following the definition of Sussman and Halvorson (1966), and the term after-ripening only for that part of the dormancy period in which the spore becomes germinable.

Germination

Germination has been defined by Sussman (1965) as 'the first appearance of the stage which follows the spore or other propagule'. Sussman and Halvorson (1966) have defined germination in a similar way; they emphasized the irreversibility of the germination process. A more operational definition which also includes the processes prior to the formation of a germ tube or other structure has been given by Zadoks and Schein (1979). They defined germination as 'the process in which a dispersal unit, e.g. a fungal spore, under specific environmental conditions, assumes increased metabolic activity, resulting in the production of new structures, most often a germ tube'. Germination is considered an irreversible process, but it needs not necessarily to be completed as specific factors may be missing, for example to produce germ tubes (Jiang and Erwin, 1993). Germination may also be interrupted for some time when the

proper conditions are interrupted (Zadoks and Schein, 1979). In the present thesis the definition of Zadoks and Schein (1979) for germination is followed.

A behavioural model for fungal spores

Spores can be classified into two groups: memnospores which generally are long-living and remain close to their place of origin, and xenospores which are relatively short-living and dispersed over larger distances (Gregory, 1966; Cooke and Whipps, 1993). In this section a general model is proposed for the behaviour of memnospores. This dynamic model is not based on the underlying physiological mechanisms. Hence, the terms maturation and after-ripening, discussed in section 1.2.1, were not included in the model (Fig. 1.3).

Primarily dormant spores (Fig. 1.3). Primary dormancy is the dormancy which may occur directly after formation of the spore. Examples of fungal spores with a period of primary dormancy are the oospores of many Pythiaceous fungi. Germinability of *Phytophthora* species. generally increases with increasing age up to a certain point (Ribeiro, 1983). Oospores of several *Pythium* spp. are borne dormant (Stanghellini, 1974). Conditions which favour the conversion of dormant oospores to germinable ones vary among species. For example, oospores of *Pythium ultimum* Trow need to be incubated in water or moist soil to become germinable, whereas germinability of oospores of *P. aphanidermatum* (Edson) Fitzp. increases after air-drying (Ayers and Lumsden, 1975).

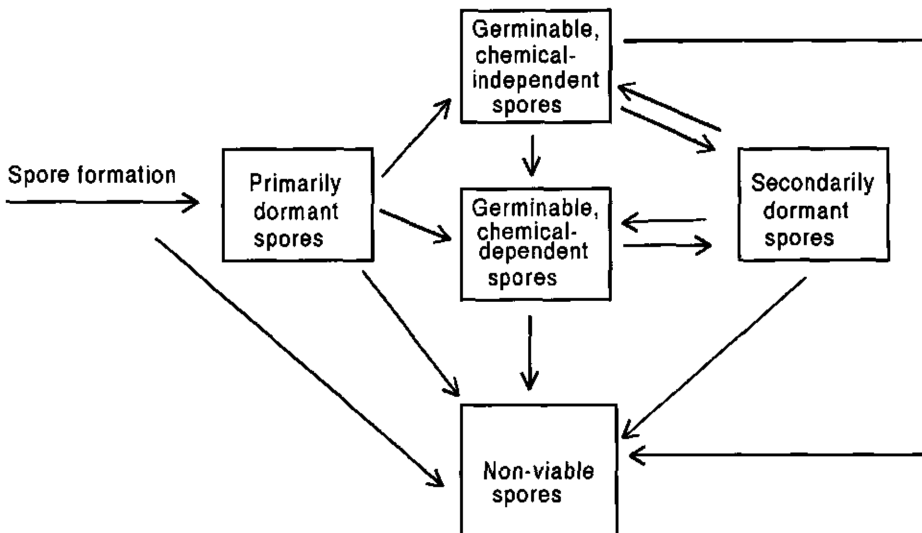

Fig. 1.3. Behavioural model for fungal spores expressed in states and state transitions.

Germinable spores (Fig. 1.3). For germination, some spores are independent of exogenous chemical stimuli, while others need a exogenous chemical stimulus. For example, percentages germination of oospores of several Oomycetes are strongly increased by the addition of nutrients (Förster *et al.*, 1983; El-Hamalawi and Erwin, 1986; Hord and Ristaino, 1991). Dependence of nutrients for germination can change over time. Spores of some fungi loose their ability to germinate without an exogenous nutrient source, because of a decrease in endogenous nutrient levels through respiration or exudation (Filonow and Lockwood, 1983; Bruehl, 1987; Hyakumachi and Lockwood, 1989). Jiang and Erwin (1993) showed that nutrient-dependency can also be induced by cold-treatments: oospores of *Phytophthora cactorum* (Lebert & Cohn) Schroeter hardly germinate in water after storage at 2°C, but cold-treated oospores do germinate after addition of nutrients to the medium. Fungal spores have been called nutrient-(in)dependent or energy-(in)dependent in their ability to germinate (Bruehl, 1987; Benson, 1994), but as a chemical stimulus may not necessarily act as an energy-source, spores are indicated more generally as chemical-(in)dependent for germination in the present thesis (Fig. 1.3). Chemical-dependency does not include the requirements for water and oxygen of a spore to germinate.

Secondarily dormant spores (Fig. 1.3). Secondary dormancy is the dormancy which may occur after a previous germinable state. Secondary dormancy is well-known in seeds of higher plants and it may also occur in fungal spores (Sussman, 1965; Sussman and Halvorson, 1966). Examples of fungal spores which can become dormant after a previous germinable state have been listed by Sussman and Halvorson (1966). Environmental factors may affect dormancy and spores may pass cycles from germinable to dormant and forth to germinable. The ecological meaning might be synchronization of spore germination and host plant growth and/or possibly a prolonged survival by a lower respiratory activity in the dormant state.

Non-viable spores (Fig. 1.3). After some time, spores will loose their viability and eventually die. Loss of viability may be caused (i) by stress due to unfavourable abiotic conditions of chemical or physical nature, (ii) by biotic factors such as parasitism, or (iii) viability may be lost after germination.

Methods to quantify viability and germinability of spore populations

Methods for assessing viability and germinability of spore populations, defined as the percentages of viable and germinable spores, respectively, are indispensible for studies on the state variables and state transitions of a spore population (Fig. 1.3). The use of germination assays and vital stain tests to assess the percentages viable and germinable spores, and to distinguish between primary and secondary dormancy are discussed in this section.

Viability

Viability of a spore population has often been assessed by the percentage of germinated spores in a germination assay. A germination assay may underestimate viability if dormancy in spores occurs (Fig. 1.4). If dormancy can be broken by certain treatments, e.g. heating, the percentage of viable spores can be estimated from the percentage germination after application of the dormancy-breaking treatments.

Additionally, and in cases dormancy cannot be broken, a vital stain test may be used. Vital stain tests are based on biochemical reactions which only occur in living tissue. Staining of spores does not necessarily imply that spores are viable. Spores may be alive and thus stain, but may not be able to germinate for example by the lack of sufficient energy reserves which cannot be compensated by exogenous energy sources. Therefore, percentages of stained spores might overestimate the percentage of viable spores (Fig. 1.4).

Germinability
Germinability of an oospore population can be assessed by the percentage germination obtained in a germination assay under optimum conditions. As one can never be sure that the chosen conditions are optimal, the germination assay might underestimate 'true' germinability (Fig. 1.4). For practical purposes, germinability is defined as the percentage germination under specified conditions within a specified time. The time course of the percentage of germinable spores will be referred to as the the germinability curve.

Primary and secondary dormancy
Primary and secondary dormancy (Fig. 1.3) can only be distinguished if the history of a spore population is known, unless characteristic features of the spores, for example cytological ones, enable distinction. An increase in germinability over time after formation of the spores will indicate the occurrence of a period of primary dormancy, a decrease may be explained by loss of viability or by the occurrence of secondary dormancy. A decrease followed by an increase will point to secondary dormancy.

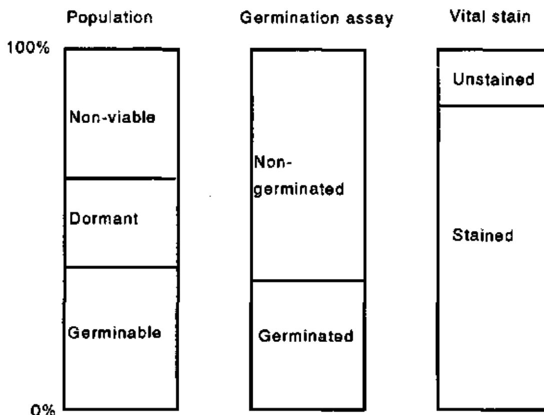

Fig. 1.4. Characterization of a fungal spore population consisting of germinable, dormant, and non-viable spores using a germination assay and a vital stain. The germination assay underestimates and the vital stain overestimates the percentage of viable (germinable and dormant) spores.

1.3 This thesis

Peronospora viciae (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven and *P. viciae* f.sp. *fabae* (Jacz. & Serg.) Boerema & *al.* are the causal agents of downy mildew on pea (*Pisum sativum* L.) and Faba bean (*Vicia fabae* L.), respectively (Boerema *et al.*, 1993). They are common diseases in pea and Faba bean growing areas (Dixon, 1981; Stegmark, 1994). Pea downy mildew has been reviewed several times (Von Heydendorff, 1977; Dixon, 1981; Günther, 1992) and recently by Stegmark (1994). Little has been published on Faba bean downy mildew, although high disease levels have been observed in recent years in England (A. Biddle, pers. comm.). The main characteristics of *P. viciae* will be briefly discussed.

P. viciae f.sp. *pisi* disperses by means of conidia, formed in the asexual infection cycle on infected host plant tissue over a temperature range of 4-24°C, with an optimum at 12-16°C, and a relative humidity above 90% (Pegg and Mence, 1970). In the colonized host plant tissue oospores are formed as a result of sexual reproduction. The oospores are important in the transmission of the fungus from one growing season of the host plant to the next (Stegmark, 1994). *P. viciae* f.sp. *fabae* has a life cycle similar to that of *P. viciae* f.sp. *pisi*. Oospores supposedly are the survival units between growing seasons of the host plant (Jamoussi, 1968; Dixon, 1981).

Much research has been done on the production, germination, and infectivity of conidia (Pegg and Mence, 1970; 1972; Mence and Pegg, 1971; Von Heydendorff, 1977; Singh *et al.*, 1988), but few quantitative studies on oospore populations of *P. viciae* and of downy mildew fungi in general have been conducted despite the important role of the oospores in the life cycle of several of these fungi.

The aim of the present thesis was to investigate the population biology of oospore populations of *P. viciae*. The research comprised the following aspects:

(i) development of methods to quantify oospore populations,
(ii) determination of the amount of oospores produced in plant tissue and the conditions under which oospores are produced,
(iii) determination of the physical and chemical conditions under which dry-stored oospores germinate *in vitro* and investigation of the relation between *in vitro* germinability and infectivity of dry-stored oospores,
(iv) determination of survival and germinability curves of oospores stored under dry conditions,
(v) determination of survival and germinability curves of oospore populations exposed to soil conditions and investigation of the relation between *in vitro* germinability and infectivity of soil-exposed oospores.

In Chapters 2-4, methods for the quantification of oospore populations are described and evaluated. In Chapters 5-7 and 8-10, studies on the production, germinability, infectivity, and survival of oospores of *P. viciae* f.sp. *pisi* and *P. viciae* f.sp. *fabae*, respectively are described.

QUANTIFICATION OF OOSPORE POPULATIONS OF *PERONOSPORA VICIAE*

2 The effect of pH on staining of oospores of *Peronospora viciae* with tetrazolium bromide. *D.J. van der Gaag. Mycologia 86 (1994): 454-457.*

3 Extraction from plant tissue and germination of oospores of *Peronospora viciae* f.sp. *pisi*. *D.J. van der Gaag and H.D. Frinking. Journal of Phytopathology 144 (1996): 57-62.*

4 Extraction of oospores of *Peronospora viciae* from soil. *D.J. van der Gaag and H.D. Frinking. (submitted).*

2 The effect of pH on staining of oospores of *Peronospora viciae* with tetrazolium bromide

Abstract

Tetrazolium bromide was tested as a vital stain for oospores of *Peronospora viciae* f.sp. *fabae* and *P. viciae* f.sp. *pisi* in phosphate buffers with a pH ranging from 6.0 to 8.0. Oospores stained mostly pink to red, seldom black. The number of black oospores increased with incubation time but the total number of pink to red and black oospores did not increase. The results suggested overstaining as the cause of black oospores. The number of stained, non-autoclaved oospores showed a small increase with increasing pH. The pH had a large effect on staining of oospores killed by autoclaving. Similar numbers of autoclaved and non-autoclaved oospores stained at pH 7.5 and 8.0 but few autoclaved oospores stained at pH lower than 7.0. When using tetrazolium bromide (0.1% solution) as a vital stain for oospores of *Peronospora viciae*, good staining results are obtained with a 48 h incubation at 35°C in solutions at pH 6.0 to 6.5.

Introduction

Peronospora viciae (Berk.) Casp., the causal agent of downy mildew on pea and Faba bean, survives by means of oospores in soil (Dixon, 1981). A method to asess the viability of oospore populations is needed to investigate the survival of the oospores. Since a germination assay may underestimate the number of viable oospores because of dormancy of oospores, a vital stain can be useful in addition or as analternative to assess viability of the oospores.

Tetrazolium salts have frequently been used as a vital stain for seeds (Kopooshian, 1968; MacKay, 1972), pollen (Binder *et al.*, 1974) and fungal spores, especially oospores (Nelson and Olsen, 1967; Shetty *et al.*, 1977; Pathak *et al.*, 1978; Sutherland and Cohen, 1983; Cohen, 1984; El-Hamalawi and Erwin, 1986; An and Hendrix, 1988; Bowers *et al.*, 1990; Jiang and Erwin, 1990). In living tissue tetrazolium is reduced to insoluble red formazan by dehydrogenase enzymes. Since dead tissue lacks active dehydrogenase enzymes it remains unstained (Kopooshian, 1968). Fungal spores staining pink or red after incubation in tetrazolium solutions are considered to be viable and unstained spores to be dead. However, small numbers of deliberately killed oospores were found to stain pink (Sutherland and Cohen, 1983). Black stained oospores were also observed and considered non-viable by El-Hamalawi and Erwin (1986), Bowers *et al.* (1990) and Jiang and Erwin (1990). Sutherland and Cohen (1983) explained black staining of oospores by overstaining because the fraction of black oospores increased with incubation time in tetrazolium bromide. An increase in black stained oospores with longer incubation time was also observed with endogonaceous spores by An and Hendrix (1988).

Temperature and pH affect staining results with tetrazolium salts (Kopooshian, 1968; MacKay, 1972; Altman, 1976). With oospores, good staining results were obtained with 48 h of incubation in tetrazolium solutions at 35 and 36°C (Sutherland and Cohen, 1983; Cohen, 1984; Jiang and Erwin, 1990). A pH of 6-7 is optimal for staining of seeds with tetrazolium bromide (MacKay, 1972), but the influence of pH on

staining of oospores has not been examined. Control of pH may be important for an accurate viability test. The purpose of this study was to examine the use of tetrazolium bromide as a vital stain for oospores of *Peronospora viciae*, and to determine optimal incubation time and pH for staining of oospores. Therefore, staining results of non-killed and deliberately killed oospores of *P. viciae* were compared within a pH range of 6.0 to 8.0.

Materials and methods

Extraction of oospores

Oospores of *Peronospora viciae* f.sp. *fabae* (Jacz. & Serg.) Boerema & *al.* and *P. viciae* f.sp. *pisi* (H. Sydow) Boerema & Verhoeven were extracted from infected leaves of *Vicia faba* L. cv. Metissa and *Pisum sativum* L. cv. Finale, respectively. Leaves were air dried at 20°C and ground in a Retsch grinding mill (0.5 mm sieve). Fragments were ground for five minutes in a Retsch laboratory planetary mill at a frequency of 480 min^{-1} with two steel balls (diameter 12 mm) in a 25 ml steel beaker (Retsch, Haan, Germany). Deionized water was added to the dust and the suspension was sonicated with intermittent operation for three minutes with a high intensity ultrasonic processor (375 Watt-model, Sonics & Materials Inc., Danbury, Connecticut). During the sonication process, the oospore suspension was immersed in an icebath to maintain oospore viability. After sonication the suspension was sieved through two sieves with pore sizes of 90 and 20 μm, respectively. The residue on the sieve with 20 μm pore sizes was suspended in deionized water. Oospore densities were determined by counting oospores in three 10-μl drops.

Staining of oospores

Tetrazolium bromide solutions of 0.1% (MTT, 3-(4,5-dimethylthiazol-2)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma Chemical Company, St. Louis, Missouri) were prepared in phosphate buffers of different pH. Buffer solutions were made by adding 10 mM KH_2PO_4 and 10 mM K_2HPO_4 solutions in different proportions. Two series of tetrazolium bromide solutions were made: one series with the pH ranging from 6.0 to 8.0 with increments of 0.5 pH units and the other with the pH ranging from 6.0 to 7.0 with increments of 0.2 pH units. The pH meter used was a Radiometer PHM83 (Copenhagen, Denmark).

Equal volumes (50 μl) of tetrazolium bromide solutions and oospore suspensions (10^4/ml) were mixed in test tubes and placed in the dark at 35°C. Non-living oospores were prepared by autoclaving oospore suspensions for 15 min at 121°C. Autoclaved oospore suspensions were first cooled for at least 2 h at room temperature before incubation in MTT.

Observations and data analysis

To find the optimum incubation time, four test tubes were prepared per pH and during 96 h each 24 h one tube was sampled and 200 oospores were observed. In a second experiment, the effect of pH on the numbers of stained oospores was determined. Therefore, one tube was prepared for each pH and after 48 h 200 oospores were counted from each tube. The response of staining to pH was analyzed by regression

analysis. The error variance was estimated from staining data of six samples of 200 oospores incubated for 48 h in independently made MTT solutions, three with a pH of 6.0 and three with a pH of 7.0.

Results and discussion

Stained oospores were mostly light pink to deep red. Black stained oospores were also observed. The percentage of black stained oospores increased with incubation time, but the total number of stained oospores (pink, red and black) did not increase after 48 h of incubation (Figs. 2.1, 2.2). More oospores stained black with increasing pH (Fig. 2.2). Black stained oospores of *P. viciae* f.sp. *fabae* were only observed after 96 h of incubation at pH 8.0. Twelve and 3 percent of non-autoclaved and autoclaved oospores, respectively, stained black. These results indicated that the black colour of oospores was caused by overstaining. Deep red stained oospores were often difficult to distinct from black stained oospores which also points to high staining intensities as the cause of black staining of oospores. The observations are in agreement with those of Sutherland and Cohen (1983) and An and Hendrix (1988) who also reported an increase in the number of black spores with increasing incubation time in MTT.

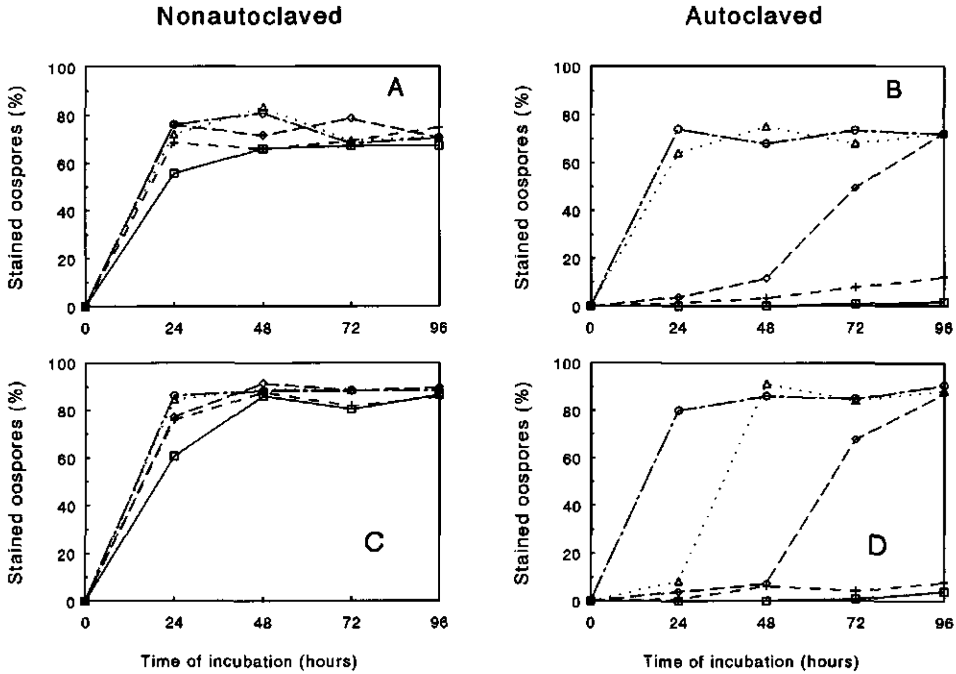

Fig. 2.1. Progress in percentages of non-autoclaved (A,C) and autoclaved (B,D) stained oospores (pink to red and black) of *Peronospora viciae* f.sp. *fabae* (A,B) and *P. viciae* f.sp. *pisi* (C,D) during 96 h of incubation in 0.1 % tetrazolium bromide solutions with pH ranging from 6.0 to 8.0. –▫– pH=6.0; –+– pH=6.5; –◇– pH=7.0; ··▿·· pH=7.5; –◆– pH=8.0.

Oospores which were visibly damaged (with inner and outer oospore wall broken), 10 to 15 % of non-autoclaved and autoclaved oospores of *P. viciae* f.sp. *fabae* and less than 5 % of the oospores of *P. viciae* f.sp. *pisi*, did not stain.

The number of stained, non-autoclaved oospores did not increase after 48 h of incubation at any pH (Fig. 2.1). For autoclaved oospores, the pH had a large effect on the progress of staining, and oospores stained earlier at higher pH (Fig. 2.1).

The pH had a small effect on staining of non-autoclaved oospores. The percentage of stained oospores showed a small increase at higher pH (Fig. 2.3). For non-autoclaved oospores the relation between the percentage of stained oospores and pH could be described by linear equations:

For *P. viciae* f.sp. *fabae*, $y = 43.64 + 5.60\,x$, $R^2 = 0.67$, $P = 0.088$,

for *P. viciae* f.sp. *pisi*, $y = 73.44 + 2.48\,x$, $R^2 = 0.80$, $P = 0.041$,

where y is percentage stained oospores, and x is the pH ($6.0 \leq x \leq 8.0$). The pH had a large effect on staining of oospores which had been killed by autoclaving: at pH 7.0 and lower, few oospores were stained after 48 h of incubation (Fig. 2.3) and the intensity of staining was mostly low. The relation between the percentage of stained oospores and pH could be described by a logistice model:

For *P. viciae* f.sp. *fabae*, $z' = -29.05 + 3.98\,x$, $R^2 = 0.96$, $P = 0.004$,

for *P. viciae* f.sp. *pisi*, $z' = -30.55 + 4.18\,x$, $R^2 = 0.90$, $P = 0.015$,

where z' is $\log(z/(1-z))$, z is the fraction stained oospores, and x is the pH ($6.0 \leq x \leq 8.0$).

The large difference between numbers of stained non-autoclaved and autoclaved oospores in the pH range 6.0 to 7.0 was confirmed by the independent MTT series with the pH ranging from 6.0 to 7.0 with increments of 0.2 pH units.

Staining of oospores deliberately killed by autoclaving indicates the presence of a reducing agent in autoclaved oospores. The reducing power of this agent decreased with decreasing pH, as was shown by a lower number of stained oospores at lower pH. The pH dependence of reduction of MTT could be described by a sigmoid-shaped

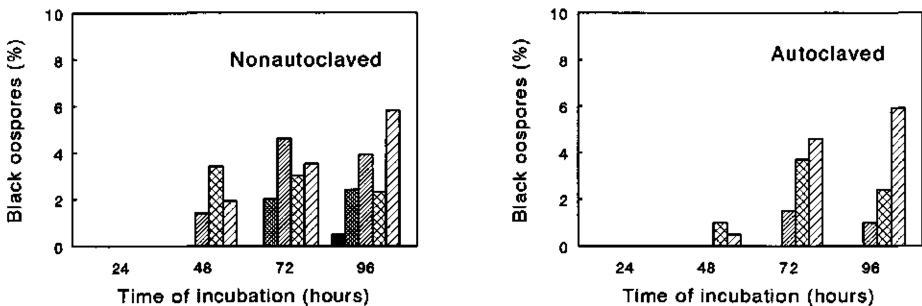

Fig. 2.2. Percentage of black stained oospores of *Peronospora viciae* f.sp. *pisi* in 0.1% tetrazolium bromide solutions with different pH. ■ pH=6.0; ▩ pH=6.5; ▨ pH=7.0; ▦ pH=7.5; ▧ pH=8.0.

curve, the logistic model. A sigmoid-shaped curve was also found for the non-enzymic reduction of MTT by reduced menadione versus pH by Hess and Pearse (1963).

The reducing agent present in autoclaved oospores probably disappears when oospores are dead for a longer time. However, when oospores were incubated in MTT 14 days after they had been killed by autoclaving similar percentages of autoclaved and non-autoclaved oospores stained at pH 7.5 and 8.0. Staining of autoclaved oospores in MTT has been reported before. Sutherland and Cohen (1983) found staining of 81 and 5 % of non-autoclaved and autoclaved oospores, respectively, of *Phytophthora megasperma* Drechs. f.sp. *glycinea* Kuan & Erwin, after incubation for 48 h in 0.1% tetrazolium bromide in distilled water.

A vital stain should make a clear distinction between dead and living tissue. Therefore, the percentage of living oospores of *Peronospora viciae* should be tested with tetrazolium bromide at a pH between 6.0 and 6.5, since only minor numbers of killed oospores stained at that pH.

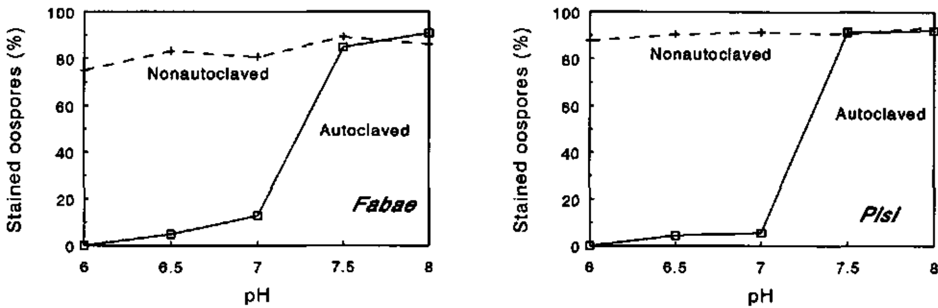

Fig. 2.3. Influence of pH (6.0-8.0) on staining of non-autoclaved and autoclaved oospores of *Peronospora viciae* f.sp. *fabae* and *P. viciae* f.sp. *pisi* in 0.1% tetrazolium bromide solutions, after 48 h of incubation. Data points are staining percentages of samples of 200 oospores; estimated standard deviation was 2.2 and 2.3 for *P. viciae* f.sp. *fabae* and *P. viciae* f.sp. *pisi*, respectively.

3 Extraction from plant tissue and germination of oospores of *Peronospora viciae* f.sp. *pisi*

Abstract

A protocol was developed to extract oospores of *Peronospora viciae* f.sp. *pisi* from plant tissue and control bacterial contamination in a germination assay. Oospores were extracted by comminuting infected leaves and pods in water, sonicating the suspension and sieving it through mesh sizes 53 and 20 μm, respectively. Extracted oospores were germinated in water. Germination of oospores was negatively influenced by addition of chloramphenicol and penicillin. A combination of 100 μg/ml ampicillin and 10 μg/ml rifampicin strongly inhibited bacterial growth at 10°C, and did not negatively affect germination of the oospores. Washing of oospores in water or 0.02% Tween-80, and sonication did not influence germination. Treating oospore suspensions with cellulase buffered at pH 4.6 for 2 h digested most plant tissue but did not influence germination. Incubation in 0.05 M acetate buffer, pH 4.6, delayed germination. Germination was unaffected when oospores were incubated in 0.05 M citrate buffer with a pH of 4.6.

Introduction

Like several other downy mildew fungi, *Peronospora viciae* (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven, which causes downy mildew on pea (*Pisum sativum* L.), survives by means of oospores in soil or plant debris in the absence of living host tissue (Dixon, 1981; Populer, 1981; Reiling, 1984). Oospores are produced in large numbers in colonized host tissue and remain on the field in crop debris after harvest. Oospores of *Peronospora viciae* f.sp. *pisi* may survive for several years (Olofsson, 1966), but no data are available on the dynamics of oospore populations and factors determining survival and germinability of oospores. High infection levels of pea seedlings have been obtained in oospore-infested soil (Ryan, 1971), but the oospore infection appeared to be highly variable (Stegmark, 1991). Variation in oospore infection can occur owing to differences in the numbers of living and germinable oospores. The percentage of living oospores can be assessed by using the vital stain tetrazolium bromide (Sutherland and Cohen, 1983; Van der Gaag, 1994), and the numbers of germinable oospores could be estimated in a germination assay.

To study the behaviour of oospores, standardized methods are required to extract and germinate oospores. Oospores in plant debris are usually contaminated by several microorganisms. These contaminants may affect germination and should be avoided in a germination assay to determine the percentages of germinable oospores. French and Schmitt (1980) observed a great variation in percentages germination of oospores of *Peronosclerospora sorghi* (Weston & Uppal) C.G. Shaw, which they attributed partly to the variation in contamination.

Antibiotics can be used to suppress bacterial growth in a germination assay but members of the Peronosporales are sensitive to many antibiotics (Tsao, 1983). Therfore, antibiotics should be tested before use. Methods used to extract fungal spores are among others maceration of plant tissue or mycelium by enzymes, washing and sonication (Sauve and Mitchell, 1977; Bruton and Craig, 1992). These methods may affect germination and should therefore be evaluated. No evaluation of the use of

different antibiotics in germination assays for oospores of downy mildew fungi nor of methods to extract oospores appears to have been published. Thus, the objective of this study was to develop a protocol for extraction of oospores of *P. viciae* f.sp. *pisi* from plant tissue and to develop a germination assay for the oospores. Effects of several antibiotics and of extraction methods on germination were investigated.

Materials and methods

Extraction of oospores

Oospores were extracted from freshly harvested or dried leaves and pods of the pea cv. Kelvedon Wonder. Plants were inoculated in the third or fourth leaf stage or in the flat pod stage, incubated at 15°C and 100% RH for 24 h, and then incubated at 20°C for oospore production. The isolate originated from a pea crop in Wageningen and was maintained asexually on seedlings in a growth chamber at 15°C. Before extraction, the presence of oospores was checked by light microscopy (×100). Leaves containing oospores were surface sterilized for 4 min in 1% sodium hypochlorite solution and rinsed for 1 min in running tap water. Oospores are often present on the outside of the pod endocarp and therefore, to avoid possible damage to oospores by sodium hypochlorite, pod walls were only rinsed in water. After rinsing, leaves (one to ten leaflets) or pod (one pod wall or less), were comminuted for 5 min in 130 ml purified water (Elgastat water purifier, option 4, Elga, High Wycombe, UK) and 20 ml ice in a commercial Waring blender at high speed. The resulting suspension, about 150 ml, was sonicated (375 W-model, Sonics & Materials, Danbury, CI, USA) 2 × 5 min with a 19 mm standard tip using a 80% duty cycle and output control 9 (the maximum power output is about 100 W). Before sonication the suspension was cooled to 10°C by placing it in an ice bath. During sonication, the temperature did not rise above 22°C. After sonication the suspension was sieved through mesh sizes 53 and 20 μm, respectively. The residue on the 20 μm sieve was suspended in sterile purified water (SPW).

Suspensions prepared from infected pod walls contained mainly oospores. Suspensions from leaves contained oospores and fragments of plant cell walls. To digest the plant material, the suspension was treated with cellulase (Onozuka R-10, Kinki Yaku MFG Co., Nishinomiya, Japan). Equal volumes of the suspension and a buffered solution of cellulase (4 mg/ml in 0.1 M acetate buffer, pH 4.6) were mixed together, resulting in a final concentration of 2 mg cellulase per ml in 0.05 M acetate buffer, and incubated for 2 h at 20°C. After washing the suspension four times in SPW by centrifugation at 2000 *g* for 3 min, it was practically free from plant fragments. The oospore concentration of the suspension was determined by counting oospores in two drops of 50 μl and adjusted to 1×10^3 oospores per ml. Oospore suspensions were used directly after preparation. For germination assays, oospores were incubated at 10°C in the dark. Oospores were classified as germinated when the germ tube was longer than the diameter of the oospore.

Antibiotics

Chloramphenicol (Calbiochem-Behring, La Jolla, CA, USA), vancomycin (Sigma V-2002, St. Louis, MO, USA), ampicillin (Merck, Darmstadt, Germany), rifampicin

(Sigma R-3501), and penicillin-G (Mycofarm, Delft, The Netherlands) were tested for effectiveness in bacterial control and their influence on oospore germination. Chloramphenicol was chosen for its broad antibacterial spectrum; vancomycin, ampicillin, rifampicin, and penicillin were chosen because they are suitable antibiotics in isolation of *Phytophthora* spp. (Tsao, 1983), which belong to the same fungal class, the Oomycetes, as *Peronospora* spp. Antibiotics were dissolved in purified water and then filter sterilized (0.20 μm, Schleicher & Schuell, Dassel, Germany). Germination assays were conducted by adding antibiotics, SPW, and 2 ml of an oospore suspension in sterile glass petri dishes (60 mm diameter) to give a final volume of 3 ml.

Concentrations of 10, 50, and 100 μg/ml chloramphenicol, 50 μg/ml vancomycin, 50 and 100 μg/ml ampicillin, 10 μg/ml rifampicin, 25 μg/ml penicillin, and combinations of 50 and 100 μg/ml ampicillin and 10 μg/ml rifampicin were tested. Except for chloramphenicol, each antibiotic was tested at least twice. As oospore samples vary in the degree of contamination, experiments were performed with oospores from samples differing in age and site of formation (leaves or pods). Each treatment was replicated four to six times and 200 oospores were observed per replicate on one or several days between 7 and 14 days of incubation (magnification ×100). Petri dishes were not opened during observations.

Because oospores were suspended in water, bacterial growth could not be quantified. To evaluate the effect of the antibiotics on bacterial growth, contamination in dishes amended with antibiotics was compared with that in control dishes and characterized in terms of hardly any, partial, or good (no visible or very few bacteria) control of bacteria.

Effect of washing

Oospores were extracted from an infected pod wall which was not rinsed before. Oospores were either incubated directly in 100 μg/ml ampicillin or first washed one or four times in SPW in sterile plastic centrifuge tubes. They were incubated in 100 μg/ml ampicillin. Oospores were washed by repeated centrifugation for 3 min at 2000 *g* in 10 ml SPW. In a second experiment oospores were washed three times in 0.02% Tween-80 and once in SPW. Each treatment was replicated six times and 200 oospores were observed 7 and 10 days after incubation.

Effect of sonication

The effect of sonication on germination was determined using oospores extracted from pod walls. The oospore suspension was equally distributed into 12 beakers and water was added to obtain a total volume of 150 ml in each one. Six randomly chosen beakers were sonicated as described above. Each suspension was then sieved separately through a 20-μm sieve and suspended in a small amount of SPW. Oospores were incubated in SPW amended with 100 μg/ml ampicillin. Percentages germination were determined 7 and 10 days after incubation by counting 200 oospores.

Effect of cellulase, acetate and citrate buffers

To determine the effect of the cellulase pretreatment on germination, oospores were incubated in a buffered cellulase solution as described above or incubated in the acetate buffer for 2 h. Oospores were washed and then isolated using a microsyringe (Terumo, Tokyo, Japan) and a dissecting microscope (×50). For this purpose, the oospore

suspension was spread on 1% water agar from which oospores could easily be removed by suction with the microsyringe and placed in a well of a sterile plastic tissue culture plate (No. 662160, Greiner B.V., Alphen a/d Rijn, the Netherlands) in 0.3 ml SPW amended with 100 μg/ml ampicillin. As a control, oospores were directly removed from the oospore suspension using the microsyringe. Six replications with 80 oospores each were used and germination was recorded over a 17 day period. This experiment was performed three times. In an additional experiment 0.05 M citrate buffer at pH 4.6 was used instead of acetate. This experiment experiment was conducted twice.

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 with either a *t*-test (two treatments) or a Tukey-test (three or more treatments). Data recorded on more than two days were analysed using multivariate repeated measures analysis of variance (Moser and Saxton, 1990). If time × treatment interaction was significant ($P \leq 0.05$), treatment means for each observation time were compared by a *t*-test. All analyses were performed using the Statistical Analysis System, version 6.04 (SAS Institute Inc., Cary, NC, USA).

Results

Morphology
Germination of oospores occurred both in presence and absence of the oogonium wall. The only form of germination was by means of germ tubes (Fig. 3.1), which became relatively long (up to 4 mm). Germ tubes were coenocytic and sometimes branched (Fig. 3.1B). In a few cases, an oospore developed two germ tubes (Fig. 3.1C). Protoplasm in germ tubes became fragmented, with most of the protoplasm at the tip of the germ tube (Figs. 3.1A-E). Occasionally, germ tubes showed one or several swellings, especially on the bottom of wells of the plastic tissue culture plates (Figs. 3.1D, E).

Antibiotics
Chloramphenicol inhibited oospore germination at concentrations of 10 μg/ml and higher (Table 3.1). Vancomycin (50 μg/ml) inhibited bacterial growth partially, but in some experiments oospore germination was highly variable among vancomycin amended dishes. This was due to very low percentages germination in some of the replicates. In one experiment for example in one dish only 3.5% of the oospores had germinated, with very short germ tubes after 14 days of incubation, while in the other five dishes oospore germination ranged from 15.0-22.2% with relatively long germ tubes.

Percentages germination in the control dishes, without antibiotics, were significantly lower than in dishes amended with ampicillin or were similar (Tukey's test, $P \leq 0.05$; Table 3.2). In two other independent experiments, percentages germination were significantly lower without ampicillin (*t*-test, $P=0.0086$; percentages germination in the control and the ampicillin amended dishes were 13.9 ± 2.4 and

27.3 ± 2.6, respectively) or no differences in germination were found (data not shown). Ampicillin partly controlled bacterial contamination. A lower percentage germination in the control than in the ampicillin amended dishes was associated with strong bacterial growth in the control. Percentages germination in 10 μg/ml rifampicin did not differ significantly from the control (Table 3.2). Percentages germination in dishes amended with both ampicillin and rifampicin was similar to dishes amended with ampicillin alone (Table 3.2). Bacterial growth was almost completely suppressed using a combination of 100 μg/ml ampicillin and 10 μg/ml rifampicin. A dose of 25 μg/ml penicillin inhibited germination (data not shown).

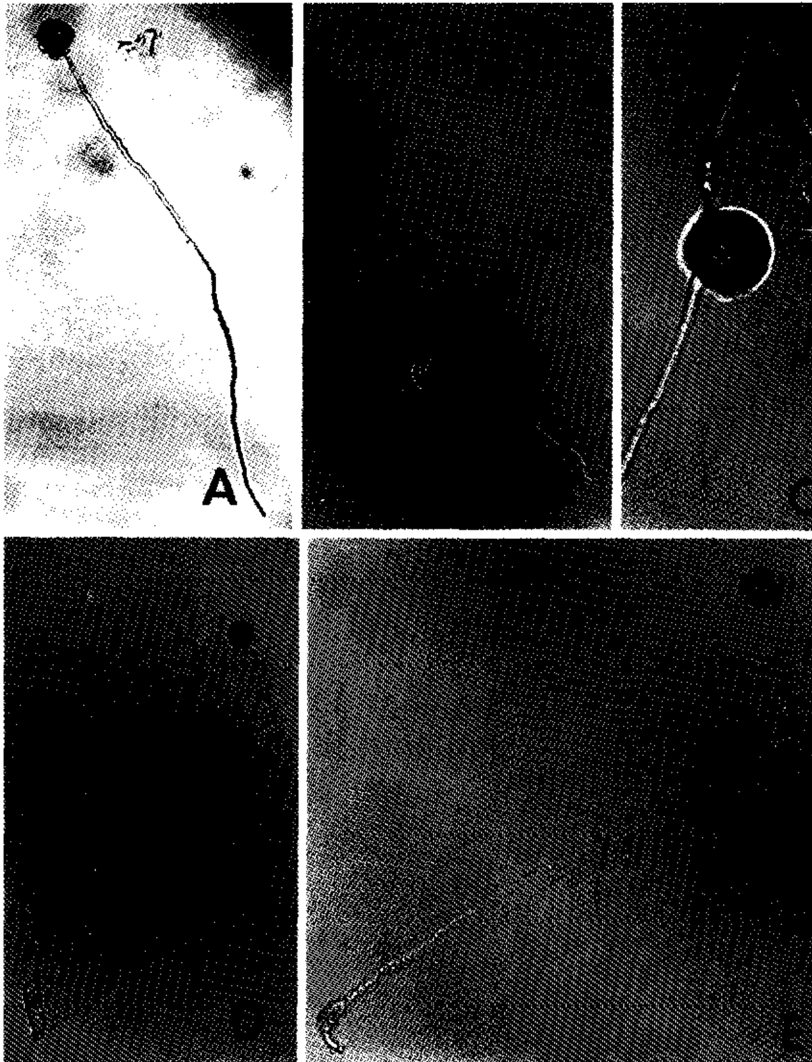

Fig. 3.1. Germinated oospores of *Peronospora viciae* f.sp. *pisi*. A) Unbranched germ tube. B) Branched germ tube (arrow) with fragmented cytoplasm. C) Two germ tubes emerging from one oospore. D) Germ tube with a swelling at the tip. E) Germ tubes with several swellings.

Table 3.1. Effect of chloramphenicol on germination of oospores of *Peronospora viciae* f.sp. *pisi*

Chloramphenicol (μg/ml)	Oospore germination (%)[y]
0	11.3 (±0.9)[z]
10	5.8 (±1.2)
50	1.4 (±0.4)
100	0

[y] Germination was recorded after 14 days of incubation.
[z] Values in parentheses are standard errors. Six replications.

Table 3.2. Effect of rifampicin and ampicillin on germination of oospores of *Peronospora viciae* f.sp. *pisi*

	Oospore germination (%)		
	Expt 1		Expt 2
Antibiotic[y]	Day 12	Day 14	Day 9
Control	0.8a[z]	1.2a	63.9ab
R10	2.8ab	3.6ab	52.3a
A50 + R10	1.8ab	4.6 b	55.9ab
A100 + R10	2.3ab	5.3 b	63.6ab
A50	3.6ab	5.7 b	62.4ab
A100	3.7 b	5.8 b	66.8 b

[y] R10 = 10 μg/ml rifampicin, A50 = 50 μg/ml ampicillin, and A100 = 100 μg/ml ampicillin.
[z] Values followed by different letters in each column are significant different according to Tukey's-test ($P \leq 0.05$).

Washing and sonication

Repeated washing of oospores in SPW did not affect germination (*F*-test, $P \leq 0.05$): 85.7, 81.9, and 82.9 ± 1.7 (mean ± pooled standard error) per cent of the oospores germinated after zero, one, and four times of washing, respectively. Washing oospores in 0.02% Tween-80 did not influence germination nor did sonication of oospores (*t*-test, $P \leq 0.05$; data not shown).

Cellulase, acetate and citrate buffers
Cellulase strongly reduced the amount of plant fragments in oospore suspensions. Degradation of plant tissue was almost complete after 2 h of incubation at pH 4.6. Cellulase did not affect germination of oospores, but the 2 h of incubation in 0.05 M acetate buffer (pH 4.6) delayed germination of oospores (Wilks' lambda for time × treatment significant at P=0.0014 on square-root transformed data). The total numbers of germinated oospores, however, did not differ from the control (Fig. 3.2A). Similar results were obtained in two other, independent experiments (Wilks' lambda for time × treatment significant at P=0.0017, and P=0.0002, respectively, on square root transformed data). When oospores were incubated in 0.05 M citrate buffer at pH 4.6 the progress in germination was unaffected (Fig. 3.2B).

Discussion

Morphology
In vitro germination of oospores of *P. viciae* f.sp. *pisi* has been reported by other workers. Geesteranus (1961) observed some germinated oospores with branched germ tubes. Von Heydendorff (1977) found a few germinated oospores on 2% Difco-agar, all with a single coenocytic germ tube. Günther (1992) found germination up to 40 % of oospores incubated on 2 % water-agar amended with water; the germtubes were single and coenocytic. The morphology of germination observed in this study was as described by Pratt (1978) and French and Schmitt (1980) for oospores of *Peronosclerospora sorghi*, although they did not mention swellings of the germ tube.

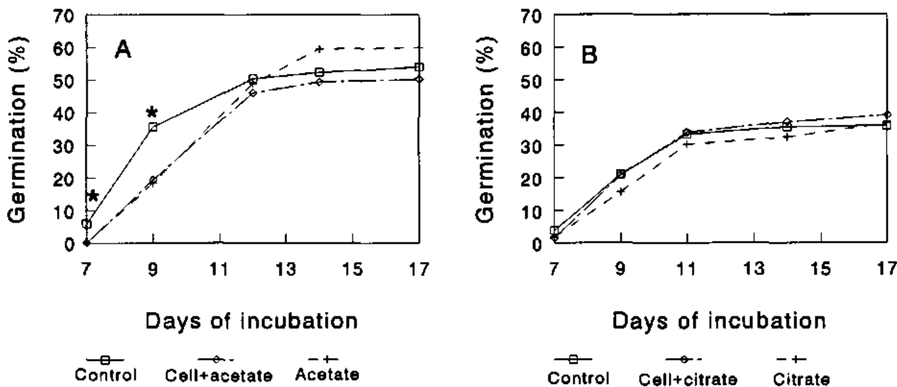

Fig. 3.2. Germination of oospores of *Peronospora viciae* f.sp. *pisi* after several treatments. Oospores were either placed directly in water (control), or first pretreated with 2 mg ml^{-1} cellulase in 0.05 M acetate buffer (A: cell+acetate) or 0.05 M citrate buffer (B: cell+citrate) for 2 h, or only incubated in the buffer for 2 h (A: acetate; B: citrate). An asterisk indicates a statistically significant difference between the control and treatments at a particular time (t-test, $P \leq 0.05$).

In the present study, swellings of the germ tube occurred especially on the bottom of the plastic wells of the culture plates on which germ tubes appeared to adhere (Fig. 3.1D, E). As germ tubes did not adhere to glass Petri dishes the adhesive nature of plastic may explain the difference in frequency of swellings formed on plastic and glass.

The swellings in the oospore germ tubes resembled the thickenings of conidial germlings of *P. viciae* f.sp. *pisi* which were formed at the junctions of leaf epidermal cells, described by Allard (1970). They were called 'secondary conidia', and were also observed *in vitro* by Von Heydendorff (1977). The terminal swelling of an oospore germ tube (Fig. 3.1D) may be an appressorium-like structure, as formed *in vivo* on the host before penetration. Appressoria of conidial germlings formed just before direct cuticular penetration of the host were described by Von Heydendorff (1977).

Antibiotics

Chloramphenicol, which inhibits protein synthesis (Franklin and Snow, 1989) strongly inhibited germination of oospores of *P. viciae* f.sp. *pisi* (Table 3.1). Chloramphenicol generally inhibits growth of fungi of the Peronosporales (Tsao, 1983), but exceptions are known. Germination of oospores of *Phytophthora megasperma* Drechs. f.sp. *medicaginis* was not inhibited in 10 μg/ml chloramphenicol (Förster *et al.*, 1983).

Vancomycin gave variable results. Vancomycin very likely did not affect germination directly, as germination was inhibited only in some replicates. If vancomycin inhibits germination, it should reduce germination in all replicates. Interaction with contaminants could be the reason for the strong inhibition of germination in some replicates. However, in view of the high variation in germination, vancomycin is not considered to be suitable for germination assays.

Results with ampicillin suggest that germination of oospores of *P. viciae* f.sp. *pisi* is inhibited by gram-negative bacteria, as ampicillin acts mainly against gram-negative bacteria (Franklin and Snow, 1989). In Petri dishes in which bacterial growth was uncontrolled, germ tubes lysed and were more difficult to observe than in dishes in which bacterial growth was inhibited by ampicillin and/or rifampicin. Suppression of bacterial growth is important for studies on the effects of physical and chemical factors on oospore germination. To control bacteria, a combination of 100 μg/ml ampicillin and 10 μg/ml rifampicin would be most suitable, as bacteria were best suppressed in media amended with both antibiotics and germination was not negatively affected by these antibiotics. Penicillin partly inhibited bacterial growth, but had a negative effect on germination, and so should not be used in a germination assay.

Washing, sonication and cellulase treatments

Washing oospores four times by repeated centrifugation did not affect germination. Dunleavy and Snyder (1962) reported germination of oospores of *Peronospora manshurica* (Naum.) Syd. after washing spores for 1 week in running tap water. They suggested that washing removed a germination inhibitor and, therefore, induced germination. The relatively high percentage germination (85.7) without washing indicates that an inhibitor may not be involved in oospore germination by *P. viciae* f.sp. *pisi*.

The sonication treatment increased the numbers of oospores freed from adhering plant debris and did not adversely affect germination. Oospores of *Pythium aphanidermatum* (Edson) Fitzp. and *P. myriotylum* Drechs. were also found to be resistant to sonication (Sauve and Mitchell 1977). Only at the high intensities of 60 and 80 % of the maximum intensity of a Biosonic III ultrasonic system for periods of 100 and 80 seconds, respectively, was germination negatively affected. Ultrasonic treatment can also be useful in decreasing contamination, as it can disperse microbes attached to spore walls and disrupt microorganisms (Oakley *et al.*, 1977, Tommerup and Kidby, 1980).

The cellulase treatment of the oospore suspensions degraded most of the plant fragments and did not affect oospore germination. Cellulase is often used to isolate oospores from mycelium in Pythiaceous fungi (Sauve and Mitchell, 1977). It is also helpful in extracting oospores from plant tissue. A significant effect of cellulase treatment on germination of oospores of *Phytophthora megasperma* f.sp. *medicaginis* was found by El-Hamalawi and Erwin (1986): oospore germination increased slightly with a longer period of cellulase treatment. In preliminary experiments, we observed a negative effect of cellulase on germination of oospores of *P. viciae* f.sp. *pisi* after an incubation period of 24 h. Cell walls of Oomycetes contain cellulose (Bold *et al.*, 1987). The fluorochrome Uvitex, binding to ß-glucans in cell walls (Butt *et al.*, 1989), did not stain the oospore wall, but did stain the oogonial wall and the thin inner oospore wall of germinated oospores of *P. viciae* f.sp. *pisi* (unpublished data). As cellulose is a ß-glucan (Bartnicki-Garcia and Wang, 1983), these observations suggest that the outer oospore wall does not contain cellulose. A long incubation period with cellulase may affect the inner oospore wall and germinability of the oospores. Therefore, when using cellulase to digest plant fragments, it is important to control cellulase activity by use of the appropriate incubation time, temperature and pH. We do not have an explanation for the delay in germination when oospores were incubated in acetate buffer for 2 h. However, the citrate buffer did not affect the progress of germination (Fig. 3.2B) and may, therefore, be preferable in cases in which the germination progress is being studied.

Percentages germination differed greatly among the experiments when oospores from different samples of the same isolate were used (Tables 3.1 and 3.2, Fig. 3.2). The variation can be attributed to differences in numbers of germinable oospores as contamination was suppressed by antibiotics and as the extraction methods did not affect percentages germination. Large variation in germination has also been found for oospores of *Phytophthora megasperma* (Drechs) var. *sojae* Hideb. (Sneh *et al.*, 1981) and *Phytophthora capsici* Leonian (Hord and Ristaino, 1991). Oospore age, conditions during formation, and environmental conditions in the period from formation to germination may all affect germinability.

The present study provides a protocol to extract oospores of *P. viciae* f.sp. *pisi*, and to control contamination in a germination assay. This protocol is indispensible for the study of oospore behaviour.

Conclusions

For germination assays, oospores of *Peronospora viciae* f.sp. *pisi* can be extracted from plant tissue by comminuting the tissue in a blender, followed by sonication, sieving, and incubating the suspension in 2 mg/ml cellulase at pH 4.6 for 2 h. If a

further separation of the oospores from the remaining plant fragments is desired, oospores can be separated using a microsyringe. To control bacterial contamination, a combination of 100 μg/ml ampicillin and 10 μg/ml rifampicin is recommended.

4 Extraction of oospores of *Peronospora viciae* from soil

Abstract

A method for the extraction of oospores of *Peronospora viciae* from soil is described. Approximately 75% of the oospores added to silty clay loam and loamy sand soil samples were recovered. Percentage recovery was independent of oospore density. The extraction method did not affect viability as determined by the tetrazolium bromide test and a germination assay and may therefore be used to study survival of oospores in soil. Numbers of oospores extracted from soil samples taken from seven fields which were naturally infested with oospores of *P. viciae* f.sp. *pisi* ranged from 2 to 21 oospores/g soil. Oospore density was not significantly correlated with disease incidence as determined by a bioassay.

Introduction

Several fungi which cause downy mildew diseases can survive in the absence of host plants by means of oospores in soil or plant debris. Such oospores act as a source of primary inoculum at the start of the growing season. Soil-borne infection by oospores often results in systemically colonized host plants (Frederiksen, 1980; Dixon, 1981; Palti and Rotem, 1981).

Methods for the quantification of oospores in soil samples are required to study density, spatial patterns and temporal dynamics of oospore populations in soil. As downy mildew fungi are obligate parasites the number of oospores can only be determined by physical extraction of the oospores from soil samples. Pratt and Janke (1978) have described a method for the extraction of oospores of *Peronosclerospora sorghi* (Weston & Uppal) C.G. Shaw from soil. The method was based on a separation of the silt fraction from other soil particles by washing and sedimentation of the soil and subsequent retention of oospores with the silt fraction. On average, 97% of the oospores were recovered. However, this method was laborious as extraction took at least 2 h per sample. Schuh *et al.* (1988) extracted oospores of *P. sorghi* from a sandy loam soil by repeated centrifugation. This extraction method required at least 3½ h per sample. The percentage recovery and precision of the method were not stated.

The purpose of the present study was to develop a rapid method to extract oospores of *Peronospora viciae* (Berk.) Casp. from soil samples and to determine the percentage recovery in relation to oospore density and soil texture. The effect of the extraction method on viability of oospores was investigated and the method was used to assess oospore density of seven field soils naturally infested with oospores.

Materials and methods

Addition of oospores to soil samples

Oospores of *Peronospora viciae* f.sp. *fabae* and *P. viciae* f.sp. *pisi* were extracted from diseased broad bean leaves (cv. Metissa) and pea shoots (cv. Kelvedon Wonder), respectively, by comminuting leaves for 5 min in tap water in a blender, followed by sonication (Van der Gaag and Frinking, 1996a). The resulting suspension was filtered

through two sieves with pore sizes of 75 and 20 μm, and the residue on the 20-μm sieve was resuspended in water. Concentration of oospores in the suspension was determined from the mean of five counts of each of five 50-μl subsamples which were taken during stirring of the suspension.

Two soil types, a silty clay loam (30% clay, 66% silt, 4% sand) and a loamy sand (6% clay, 7% silt, 87% sand) were used. The soils were collected from fields where neither pea nor Faba bean had been grown for at least 25 years. Soils were air dried at 20°C. To allow for a homogeneous mixture of soil and oospores, the silty clay loam soil was ground in a Retsch grinding mill to pass through openings 1 mm in diameter (Retsch, Haan, Germany) before addition of the oospores.

Five different amounts of oospore suspension containing approximately 83, 249, 498, 996, and 1993 oospores were each added to 5-g samples of the ground silty clay loam soil. In another experiment, three samples per oospore density were prepared. Three different amounts of oospore suspension containing approximately 251, 603, and 1172 oospores were added to 10-g samples of the loamy sand soil. Ten samples per oospore density were prepared. Artificially infested soil samples were thoroughly mixed.

Extraction of oospores from soil
Seventy ml of tap water was added to each soil sample in a 100 ml beaker. The soil suspension was mixed by hand using a stirring bar and subsequently sonicated for 5 min with a 19 mm standard tip using a 90% duty cycle with a maximum power output of 120 Watt (375 Watt-model, Sonics & Materials Inc., Danbury, Connecticut, USA). The suspension was filtered through two sieves with pore sizes of 75 and 20 μm. The residue on the 20 μm pore size sieve was resuspended in 15 ml tap water. About 0.1 ml of 1% Tween-80 was added and the suspension was stirred. The suspension was poured onto 40 ml of a 70% sucrose solution (2.33 g/ml, specific gravity = 1.35 g/ml) in a 80 ml glass centrifuge tube and centrifuged at 1000 *g* for 3 min. The supernatant was poured through a sieve with 20-μm pore size and the sucrose solution washed down with tap water. The residue on the sieve was resuspended in 10 ml water in a plastic centrifuge tube and centrifuged at 2000 *g* for 3 min to concentrate the oospores. After centrifugation the supernatant was carefully discarded by means of a pipette until 2 ml remained. The exact volume was determined and numbers of oospores were counted in each of five 50-μl subsamples using a light microscope at ×100 magnification. The mean number of oospores per ml was multiplied by the total volume of the suspension to give the number of oospores extracted.

The precision of the extraction method was assessed using three series of assays performed on 5-g samples of the silty clay loam soil to which approximately 246, 336, and 852 oospores had been added, respectively. The variance among the numbers of oospores added (σ^2_{added}) was determined and a variance components analysis was performed to estimate the variance among soil samples (σ^2_s) and 50-μl subsamples (σ^2) (Campbell and Nelson, 1986). The variance generated by the extraction method (σ^2_{ext}) was derived by the formula: $\sigma^2_{ext} = \sigma^2_s - \sigma^2_{added}$.

Effect of extraction on viability of oospores
The effect of the extraction method on percentage viability of oospores was investigated using the vital stain tetrazolium bromide (MTT). Oospores of *P. viciae*

f.sp. *fabae* were added to 5-g samples of the silty clay loam soil and subsequently extracted. Before sonication, soil suspensions were first cooled to 5°C by placing them in an ice-bath to avoid heat damage to the oospores. During sonication, the temperature did not rise above 25°C. Non-extracted (control) and extracted oospores were incubated in 0.1% MTT in 0.01 M phosphate buffer (pH 6.2) at 35°C (Van der Gaag, 1994). After 48 hours, the percentage red stained oospores was determined. Four replicate samples were used and observations were made on 100 oospores per replication. Two experiments using these procedures were conducted.

The effect of the extraction method on germinability of oospores was tested using oospores of *P. viciae* f.sp. *pisi* (Van der Gaag and Frinking, 1996a) because oospores of *P. viciae* f.sp. *fabae* germinate poorly (unpublished data). Eight samples were taken from an oospore suspension of which four were sonicated and centrifuged on a 70% sucrose solution as described above. For the germination assay, 80 to 100 oospores from each sample were placed in 0.3 ml sterile distilled water amended with 100 μg/ml ampicillin in a well of a sterile plastic culture plate, using a microsyringe and a dissecting microscope (Van der Gaag and Frinking, 1996a). Oospores were incubated at 10°C in the dark and germination was recorded after 12 days. Two experiments using these procedures were conducted.

Oospore density and infectivity of naturally infested soil

Fourteen to 20 portions of soil (approximately 400 ml each) were collected from the upper 15-cm of an 0.01-0.1 ha area in each of seven fields and these portions were combined to a composite sample in February 1996. Each field sampled had been cropped with pea in a recent year (1992 to 1995) at which downy mildew symptoms had been observed. As control, one field was sampled on which pea had not been grown for at least 20 years. Fields were located in Eastern England (UK): five, including the control, at the National Institute of Agricultural Botany (NIAB, Cambridge) and three near the Processors and Growers Research Organisation (PGRO, Peterborough).

Soil samples were air dried for one night at room temperature and subsequently sieved through a mesh size of 1 × 1 cm. Soil was thoroughly mixed and stored for 1 to 6 days at 7°C prior to planting of pea seeds. Four trays (12.5 × 17 × 6 cm) were filled with soil for each composite sample. Seeds of the highly susceptible cv. Kelvedon Wonder were surface-sterilized by incubation in 1% NaOCl for 10 min, washed three times in tap water and sown at a depth of about 2 cm. Three rows each of eight seeds were sown per tray. Soils were covered with an 1-cm layer of steam-sterilized sand to reduce evaporation. Trays were placed in a growth chamber at 10°C. Thirty-four days after sowing, when seedlings were at the third leaf stage, trays were covered with polyethyl bags to increase humidity and promote sporulation. The numbers of healthy and diseased (sporulating) seedlings were recorded one day later.

Oospores were extracted from four 10 g-subsamples taken from each composite soil sample. Each subsample was mixed in water in a blender for 3 min to grind large soil particles and oospores were subsequently extracted as described above. Extracted oospores were concentrated in about 0.5 ml of water and the numbers of oospores were counted in three aliquots of 0.050 ml each.

Results

Extraction of oospores from artificially infested soil samples

The relation between the numbers of oospores added to and recovered from samples of the silty clay loam and loamy sand soil was well described by simple linear regression equations with slopes of 0.78 and 0.76, respectively (R^2=0.99; 0.95), which indicated a recovery of about 75% independent of oospore density (Fig. 4.1). The variance generated by extraction with its sonication, sieving and centrifugation (σ^2_{ext}) was much higher than the variance among 50-μl subsamples (σ^2_{subs}), which indicated that an increase in the number of subsamples counted per sample would have a limited effect on the precision of the assay (Table 4.1).

The extraction method did not significantly affect the vital stain results with MTT. The percentage of stained oospores, averaged over two independent experiments, was 88.8 for control and 88.6 for extracted (pooled standard error 0.9; eight replications) oospores, respectively. The percentage of germinable oospores was not significantly affected by the extraction method, being 30.3 for control and 33.2 for extracted (pooled standard error 1.9; eight replications) oospores, respectively.

Oospore density and infectivity of naturally infested soil

Between 2 and 21 oospores/g soil were extracted from soils with a pea downy mildew history (Table 4.2). Seedling emergence in the bioassay was generally poor ranging from 20 to 70%, due to seedling diseases. Incidence of downy mildew ranged from 14 to 75% and was not significantly correlated with oospore density (r=0.67, *t*-test: P=0.010) (Table 4.2). No oospores were extracted from the control soil. Since 0.150 ml out of 0.5 ml suspension containing the extracted oospores was examined, the detection threshold was calculated as 0.150/0.5 × 1/0.75 = 4.4 oospores per 10-g subsample.

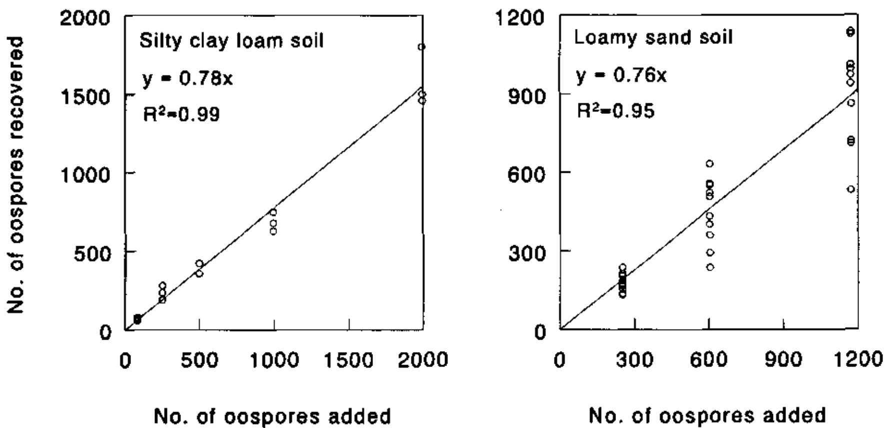

Fig. 4.1. Relation between numbers of oospores of *Peronospora viciae* recovered and numbers of oospores added. Oospores had been added to samples of 5 g of a silty clay loam soil and 10 g of a loamy sand soil after which they were extracted using the method described in the text.

Table 4.1. Mean numbers of oospores of *Peronospora viciae* recovered from a silty clay loam soil and estimates of variance due to processing of the soil samples (σ^2_{ext}), and counting five subsamples per sample ($\sigma^2/5$)

	Oospores added		Oospores recovered		Estimates of variance components		
N[a]	Mean	σ^2_{added}[b]	Mean	σ^2_t[c]	σ^2_{ext}	$\sigma^2/5$	CV
5	246	626	199	3661	2411	625	25
5	336	304	283	5985	3774	1907	22
8	852	9	646	12134	7420	4705	13

[a] Numbers of samples assayed.
[b] Variance among numbers of oospores added to soil samples.
[c] Variance among numbers of oospores recovered from soil samples.
[d] CV = coefficient of variation = $(\sigma_{ext}/\mu) \times 100$ (in %), μ is the mean number of oospores recovered.

Discussion

Percentage recovery of the extraction method was similar for the silty clay loam and the loamy sand soil samples (Fig. 4.1). Percentage recovery was also similar (recovery: 74%) for 10-g samples of a silt loam soil (26% clay, 52% silt, 22% sand) and for 10-g samples of the silty clay loam soil (recovery: 80%) obtained in other studies (unpublished data). These results indicate that the extraction method allows the comparison of oospore densities in soils with different textures.

The recovery of the extraction method, about 75%, is lower than the 97% obtained by a method for oospores of *Peronosclerospora sorghi* (Pratt and Janke, 1978). Their method required more than 2 h per sample to complete, while the method described in this paper requires only about 25 min (without counting the oospores). Moreover, our method resulted in suspensions practically free from soil particles. This makes it much easier to count oospores than in the method described by Pratt and Janke (1978) where oospores are retained with the silt particles especially in soils which have high silt fractions.

The extraction method described in the present paper has recently been modified (omitting the sonication treatment) and used to extract oospores of *S. graminicola* (Sacc.) Schroet. from samples of sandy soils (86-98 % sand) in Niger (Gilijamse *et al.*, 1997). In that study about 70% of the oospores were recovered. In soils with very high sand contents, soil particles are easily dispersed without sonication. However, in preliminary experiments using soils with a low sand content sonication was highly important for dispersal of particles to obtain a high percentage recovery. The exact percentage recovery may vary among laboratories and studies, depending on the equipment available, size of soil samples etc., and should be determined if necessary. From soils with very high organic matter content, for example peaty soil, percentage recovery may be low. Percentage recovery will be different when oospores are present

Table 4.2. Numbers of oospores of *Peronospora viciae* extracted and incidence of downy mildew in a bioassay in February 1996 of soil samples collected from seven naturally infested fields

Field	Oospores/g soil[a]	No of seedlings tested	Incidence of downy mildew (%)	Year of last pea crop
1	1.7 ± 0.4	35	17	1995
2	2.2 ± 0.4	19	32	1992
3	6.5 ± 1.8	22	23	1992
4	8.0 ± 2.0	43	14	1993
5	14.7 ± 1.8	40	48	1995
6	17.1 ± 1.9	62	21	1995
7	20.7 ± 3.2	36	75	1995
8 (control)	0	67	0	none[b]

[a] Mean number ± standard error of oospores/g soil extracted from four subsamples of 10 g each. Recovery was about 75% and detection level 0.44/g soil per subsample.
[b] Peas had not been grown for at least 20 years.

in plant debris in soil. In an experiment in which pea pod pieces were incorporated in soil, recovery from 20-gsubsamples was about 100% before and 69% after pod tissue had decomposed (Van der Gaag and Frinking, 1997c).

The extraction method did not affect viability of oospores and is, therefore, suitable to study survival of oospores in soil in combination with the tetrazolium bromide test and germination assay.

Density of *P. viciae* oospores in the naturally infested soil samples was generally lower than that found for oospores of *Peronosclerospora sorghi* in 18 field soils in Texas. From samples collected from these fields, 8 to 95 oospores/g soil were extracted with most samples exceeding 20/g soil (recovery: 97%) (Pratt and Janke, 1978). Much higher densities, 26 to 768/g soil (recovery: 70%), were found by Gilijamse *et al.* (1997) for oospores of *Sclerospora graminicola* in 20 fields in Niger. The differences in oospore density among downy mildew pathgens may be due to differences in the amount of oospores produced per unit area, and death and decomposition rate of the oospores in soil and/or in crop rotation. Pearl millet, the host plant of *Sclerospora graminicola*, is grown each year in Niger in the same fields, while pea is usually grown in a three to five year crop rotation in most of the fields sampled in this study.

The lack of a significant correlation between disease incidence and oospore density may be due to differences in percentage viable and/or dormant oospores present in the soil samples. Numbers of oospores/10 g soil were too low to estimate the percentage of living oospores using the tetrazolium bromide test. It is not known if dormancy of oospores of *P. viciae* f.sp. *pisi* occurs in soil. Differences in physical, chemical and/or biotic conditions between the soil samples may also account for the lack of significant correlation between downy mildew incidence and oospore density.

Schuh *et al.* (1988) found similar patterns of oospores of *Peronosclerospora sorghi* and diseased plants within a field. Pratt and Janke (1978), however, found no significant correlation between disease incidence and density of oospores of *P. sorghi* using samples from 18 fields.

Oospores must be morphologically distinguishable from other propagules for assessing the number of oospores/g soil. Since oospores from different *formae speciales* of the same species are not distinguishable, for example oospores of *P. viciae* f.sp. *pisi* and *P. viciae* f.sp. *fabae*, a bioassay may be necessary to determine which form is present. The cropping history of a field may suggest the origin of the oospores, although oospores of related pathogens of other host plant species, either wind-blown or carried-in, cannot be excluded.

PRODUCTION, GERMINABILITY AND SURVIVAL OF OOSPORE POPULATIONS OF *PERONOSPORA VICIAE* F.SP. *PISI*

5 Homothallism in *Peronospora viciae* f.sp. *pisi* and the effect of temperature on oospore production. *D.J. van der Gaag and H.D. Frinking. Plant Pathology 45 (1996): 990-996.*

6 Factors affecting germination and germinability of oospores of *Peronospora viciae* f.sp. *pisi in vitro. D.J. van der Gaag and H.D. Frinking. (submitted).*

7 Survival and germinability curves of oospore populations of *Peronospora viciae* f.sp. *pisi* in soil. *D.J. van der Gaag and H.D. Frinking. (submitted in adapted form).*

5 Homothallism in *Peronospora viciae* f.sp. *pisi* and the effect of temperature on oospore production

Abstract

Monoconidial cultures derived from seven *Peronospora viciae* f.sp. *pisi* isolates, obtained from different countries, were able to produce oospores. Apparently, these isolates were homothallic. Oospore production of one isolate was studied at 5, 10, 15, and 20°C in systemically colonized shoots, and in local lesions on leaflets, stem parts, and pods of the *Pisum sativum* cv. Kelvedon Wonder. The number of oospores produced per gram systemically colonized tissue increased with temperature. In lesions of leaflets and of stem parts, including tendrils, petioles and main stem, most oospores were produced at 20°C. At 10°C, a few oospores were found in stem parts but none in leaflet lesions. At 5°C, no oospores were formed at all. In pods, more oospores were produced at 15 and 20°C than at 10°C, but the effect of temperature on the numbers of oospores was smaller than in the other plant parts. Oospores formed at lower temperatures were larger than those formed at higher temperatures. At 20°C, similar oospore densities were found in leaflet lesions of three cultivars widely differing in resistance to downy mildew.

Introduction

Peronospora viciae (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven, the causal agent of downy mildew on pea (*Pisum sativum* L.), spreads by conidia formed on diseased plant parts. Oospores are formed in colonized host plant tissue. The oospores remain in the plant debris on the field after harvest of the crop. They play a role in the survival of the pathogen during host-free periods (Dixon, 1981; Populer, 1981; Stegmark, 1994).

The pathogen can cause different kinds of symptoms in a pea crop (Stegmark, 1994). (i) Seedlings or shoots can be systemically colonized, which causes stunted growth. Systemic growth of the fungus can result from infection by soil-borne oospores (Stegmark, 1994) or from conidial infection of the apical bud (Mence and Pegg, 1971). Systemic growth may also develop from lesions on leaflets (Taylor *et al.*, 1990). (ii) Local lesions on leaves and tendrils develop from infections by conidia. (iii) Lesions on pods usually result from conidial infections of young pods and seldom from systemic infection (Mence and Pegg, 1971).

Conidia are formed on colonized leaves and stems at relative humidities above 90% and at temperatures of 4-24°C (Pegg and Mence, 1970). Oospores can be formed in all the infected plant parts (Von Heydendorff, 1977), but despite their role as survival units in the life cycle of *P. viciae* f.sp. *pisi*, it is unknown to what extent oospore production is affected by environmental conditions. Neither it is known whether isolates of *P. viciae* f.sp. *pisi* are homothallic or not (Michelmore *et al.*, 1988). To predict the amount of infestation of soil with oospores after incorporation of the plant debris of the previous pea crop it is necessary to know at which conditions oospores are produced and whether the pathogen is homothallic or not.

In this study, the sexual system of isolates from different localities was determined and the effect of temperature on oospore production in different plant parts was studied. Oospore production in leaflet lesions of three cultivars was compared.

Materials and methods

Maintenance of isolates

Seven isolates of *Peronospora viciae* f.sp. *pisi* were obtained from five different countries. Three isolates were supplied by J.E. Thomas (National Institute of Agricultural Botany, Cambridge, UK). One isolate was provided by R.E. Falloon (New Zealand Institute for Crop & Food Research, Christchurch, New Zealand). One isolate was supplied by G. Enqjvist (Svalöf AB, Svalöf, Sweden). One by H. Schulz (Botanisk Afdeling Plantevaernscentret, Lyngby, Denmark), and one isolate (NL) was collected near Wageningen in the Netherlands. All isolates were obtained from oospore samples except the Swedish isolate (SW), which was obtained from conidia.

To obtain isolates from oospores, oospores were extracted as described later. One ml of an oospore suspension, containing 10^3 oospores, was pipetted onto a single pea seed (cv. Kelvedon Wonder) in previously steam-sterilized potting soil. Pots containing the inoculated seeds were placed in plastic bags to avoid contamination and to induce production of conidia (Stegmark, 1990). Pots were placed at 10°C. After about 4 weeks, conidia were produced on infected seedlings. Conidia were suspended in demineralized water by washing the sporulating leaves. The conidial suspension, adjusted to 10^5 conidia/ml, was sprayed onto seedlings (cv. Kelvedon Wonder) using a DeVilbiss atomizer. Preparation of conidial suspensions and inoculations were done in a separate room on a sterile bench.

Inoculated seedlings were incubated at 15°C in a saturated atmosphere for 20 h, and then maintained at 5 or 10°C. To avoid contamination, pots of seedlings were covered with cellophane which permits exchange of air but not of fungal spores.

Sexual system

Isolates were examined for their ability to produce oospores in seedling leaflets of the cv. Kelvedon Wonder at 20°C and 60-80% RH. Oospore formation was also examined of monoconidial cultures derived from each of the isolates. Monoconidial cultures were obtained by spreading a conidial suspension on 1% water agar. Single conidia were removed using a dissecting microscope at 50× magnification and a microsyringe. One conidium was placed on a small block of water agar and placed spore-side down on an excised leaflet on 1% water agar in a Petri dish. The dish was sealed and placed at 15°C in a growth chamber. As soon as sporulation began, about 14 days after inoculation, spores of the monoconidial isolate were used to inoculate seedlings. Inoculated seedlings were first incubated in a saturated atmosphere at 15°C for 20 h to promote infection, and then placed at 20°C to stimulate production of oospores. After 14 days, diseased leaflets were harvested, cleared in a saturated chloralhydrate solution for 24 h and examined for oospores by light microscopy (100× magnification). One to six monospore cultures were derived from each isolate. The Swedish isolate was propagated by single spore transfers for three generations and then examined for oospore production.

Effect of temperature on oospore production

Oospores in systemically colonized host tissue. The effect of temperature on oospore production was studied using a monoconidial culture of the Dutch isolate (NL) and the pea cv. Kelvedon Wonder. Pea seedlings were raised at 15°C in square plastic pots of 11 cm. Each pot contained four plants unless stated otherwise.

To quantify oospore formation in systemically colonized plant tissue, pea seedlings (third leaf stage) were inoculated by placing a drop of a conidial suspension (3×10^5 conidia/ml) into the apical bud using a Pasteur pipette. This inoculation method results in a high frequency of systemically colonized plants (Mence and Pegg, 1971). After incubation in a saturated atmosphere and 15°C for 20 h, the pots were randomly distributed over four growth chambers at 5, 10, 15, and 20°C (20 pots per temperature) at a light intensity of 18-26 W/m^2, 16 h per day. The RH was 60-80%. Twenty-one and 28 days after inoculation (d.a.i.), the systemically colonized plant parts were harvested from five pots, and oven-dried at 105°C. Plants incubated at 5 and 10°C were also harvested at 42 d.a.i.

Oospores in local lesions. To study oospore production in locally colonized leaflets and stem parts, pea seedlings (third leaf stage) were inoculated by spraying a conidial suspension containing 3×10^5 conidia per ml. After incubation at 15°C in a saturated atmosphere for 20 h, the pots were randomly distributed over the temperatures 5, 10, 15, and 20°C. Leaflets from the second and third leaf were harvested at 6, 10, 14, and 21 d.a.i. Five pots were sampled at each sampling time. Plants incubated at 10 and 5°C were also harvested at 35 and 56 d.a.i., respectively. The tendrils and petioles of the second and third leaves and the main stem between these leaves (referred to as 'stem parts') were taken together. The plant parts were cut off and immediately placed in paper bags in an oven at 105°C for 24 h. From 10 d.a.i., when lesions on the leaflets were visible at 15 and 20°C, the total and diseased leaflet area was estimated using a transparent dot counting strip with dots at 3 mm distances. The dot counting strip was placed on a leaflet and the number of dots covering the whole leaflet and the diseased area, respectively, were counted. Each dot represented 9 mm^2. Since leaflets generally became necrotic beginning about 14 d.a.i. at 15 and 20°C, the diseased leaflet area from plants to be sampled at 21 d.a.i. was estimated at 14 d.a.i.

Oospores in pods. To study oospore production in pods, plants were grown (three plants per pot) at 15 or 20°C until the flat pod stage. Pods were then inoculated by spraying a conidial suspension and incubated for 20 h at high humidity. After incubation, the pots were distributed over the four temperature regimes. Diseased pods were harvested when plants were in early senescence (stage 301; Knott, 1987) and air-dried at the same temperature at which they had been incubated for 10 days. Pods incubated at 5°C were examined for lesions about four months after inoculation. The number of oospores was determined in one cm^2 pod pieces cut from the diseased pod wall area. For each temperature five pods were examined (one cm^2 per pod). The experiment was conducted three times. To study the effect of temperature on oospore size, the diameter of 20 oospores from each of five pods was measured in two experiments at a 400× magnification.

Effect of host plant genotype on oospore production

Oospore production was studied in three cultivars with different levels of resistance: Kelvedon Wonder (highly susceptible), Finale (susceptible), and Cobri (incompletely resistant). On Cobri, lesions only develop on the youngest leaves present at inoculation. These lesions are surrounded by a narrow rings of necrotic tissue. To obtain plants of the same growth stage (third leaf stage), Kelvedon Wonder was sown two days earlier than Finale and Cobri. Seedlings were inoculated by spraying a conidial suspension and incubated at 15°C in a saturated atmosphere for 20 h. Then, the seedlings were placed at 20°C. Fourteen days after inoculation, diseased leaflets from second and third leaves of the cvs Kelvedon Wonder and Finale were harvested. Cobri had only lesions on leaflets of the fourth leaf, and these were harvested. The diseased leaflet area was estimated using the dot-counting method and the harvested leaflets were oven-dried.

Quantification of oospores

The number of oospores produced was assessed as described previously (Van der Gaag *et al.*, 1993). Briefly, the dried plant parts were weighed, comminuted in a blender for 5 min, sonicated, and finally sieved through mesh sizes of 90 and 20 μm, respectively. The residue on the sieve with 20 μm mesh sizes was resuspended in a quantity of about 15 ml of water, to be measured precisely after suspending. The number of oospores was counted in each of six droplets of 20 μl each. Droplets were taken with a micropipette from the suspension which was stirred for homogenization. When oospore densities were high (more than about 200 per 20 μl), the oospore suspension was diluted to about 250 ml. When the number of oospores still exceeded 200 oospores/20 μl, oospores were counted in six droplets of 10 μl. The total number of oospores extracted per cm^2 of diseased leaflet or pod wall area and per gram dry diseased tissue was calculated.

Data were tested for homoscedasticity and, when necessary, $\log_{10}$ transformed. Data on oospore production were studied by analysis of variance (ANOVA) and *F*-test using PROC GLM of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Temperature treatments, which had resulted in zero values only, were excluded from ANOVA. Treatment means were compared by *t*-tests. Data on oospore diameter were analyzed by regression.

Results

Sexual breeding system

All isolates examined formed oospores at 20°C. Monoconidial cultures derived from these isolates also produced oospores. After repeated single sporing of conidia of one isolate for three generations, oospores were still formed.

Effect of temperature on oospore production

In systemically colonized tissue highest oospore densities were found at 20°C (Table 5.1). At 10°C oospores were formed, but oospore densities were more than a tenth lower than at 15 and 20°C (Table 5.1). At 5°C no oospores were found.

Table 5.1. Oospore production of *Peronospora viciae* f.sp. *pisi* in systemically colonized shoots of *Pisum sativum* cv. Kelvedon Wonder at four temperatures

Days after	Temperature (°C)			
inoculation	5	10	15	20
21	n.e.[x]	4.03a[y]	6.64b	6.87c
28	0	5.44a	6.84b	6.98c
42	0	4.99	n.e.	n.e.
s.e.[z]		0.21	0.02	0.04

[x] n.e. = not examined.
[y] Log_{10} transformed values of numbers of oospores per gram dry weight. In each row, values followed by different letters are significantly different according to *t*-tests ($P<0.05$).
[z] Pooled standard error; five replications.

In local lesions, the first oospores were formed between 6 and 10 d.a.i. at 15 and 20°C (Fig. 5.1). In leaflet lesions, oospore densities were higher at 20° than at 15°C (*F*-test, P=0.0001) (Fig. 5.1A). In stem parts, more oospores were formed at 20 than at 15°C (*F*-test, P=0.0029) (Fig. 5.1B). At 5 and 10°C no oospores were found in local lesions of leaflets. At 10°, few oospores [44.3 ± 28.7 (mean ± s.e.) per plant] were found in locally infected stem parts. At 5°C no oospores were formed in stem parts.

In pods, significantly more oospores were formed at 15 and 20°C than at 10°C in two experiments; in one experiment no significant differences were found (Table 5.2). At 5°C no lesions were observed. The effect of temperature on oospore production in pods (Table 5.2) was smaller than in systemically colonized tissue (Table 5.1) or in locally infected leaves and stem parts (Fig. 5.1).

The diameter of the oospores increased with decreasing temperatures (Table 5.3). The relationship between oospore diameter and temperature could be described by a linear equation:

$y = 37.76 - 0.25x$, $R^2 = 0.53$, P=0.0020, n=15 (Expt I),

$y = 39.48 - 0.37x$, $R^2 = 0.60$, P=0.0007, n=15 (Expt II),

in which y is the mean diameter in μm of a sample of 20 oospores from an infected pod, and x ($10 \leq x \leq 20$) is the temperature in °C.

Effect of host plant genotype on oospore production

No significant differences were found in numbers of oospores per cm^2 in leaflet lesions of the three cultivars (Table 5.4).

Table 5.2. Oospore production of *Peronospora viciae* f.sp. *pisi* in pods of *Pisum sativum* cv. Kelvedon Wonder at different temperatures

	Expt I		Expt II		Expt III	
Temperature (°C)	No/cm^{2} [w]	No/g [x]	No/cm^2	No/g	No/cm^2	No/g
10	3.91a [y]	5.51a	4.30a	6.00a	4.33a	5.98a
15	4.77b	6.48b	4.96b	6.68b	4.55a	6.23a
20	5.00b	6.72b	4.84b	6.60b	4.38a	6.17a
s.e. [z]	0.23	0.22	0.15	0.15	0.16	0.15

[w] Log_{10} transformed values of numbers of oospores per cm^2 diseased pod wall.
[x] Log_{10} transformed values of numbers of oospores per gram dry weight.
[y] In each column, values followed by different letters are significantly different according to *t*-tests ($P < 0.05$).
[z] Pooled standard error; five replications.

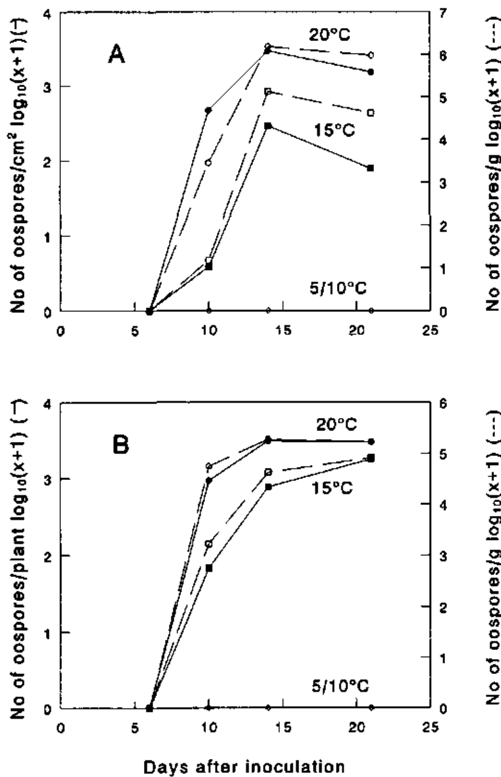

Fig. 5.1. Oospore production by *Peronospora viciae* f.sp. *pisi* in pea cv. Kelvedon Wonder at four temperatures. (A) Numbers of oospores in leaflet lesions. Pooled standard error was 0.23 for numbers/cm^2, and 0.42 for numbers/g dry weight; five replications per sampling day. (B) Numbers of oospores in locally infected stem parts. Pooled standard error was 0.24 for numbers per plant and 0.36 for number/g dry weight; five replications per sampling day. Drawn lines = $\log_{10}(x+1)$ of numbers of oospores/cm^2 or per plant. Broken lines = $\log_{10}(x+1)$ of numbers of oospores/g.

Table 5.3. The effect of temperature on the diameter of oospores of *Peronospora viciae* f.sp. *pisi* produced in pods of the pea cv. Kelvedon Wonder

	Oospore diameter			
	Expt I		Expt II	
Temperature[w]	Mean[x]	Range[y]	Mean	Range
10	35.4	22.3 - 41.5	36.1	24.0 - 42.5
15	33.9	22.3 - 41.5	33.4	23.8 - 38.8
20	32.9	22.0 - 41.3	32.5	25.0 - 38.8
s.e.[z]	0.5		0.6	

[w] Regression analysis showed that oospore diameter decreased significantly with increasing temperature (slopes of the regression lines were significantly different from zero; *t*-test, $P=0.0020$ and $P=0.0007$ for expts I and II, respectively).
[x] Mean of five samples, each sample consisting of 20 oospores.
[y] Range of 100 oospores.
[z] Pooled standard error; five replications.

Discussion

Sexual system
In some downy mildew pathogens, e.g. *Peronospora parasitica* (Pers. ex Fr.)Fr. (Sheriff and Lucas, 1989), both homothallic and heterothallic isolates have been found, while others appear to be either homothallic, e.g. *Peronosclerospora sorghi* (Weston & Uppal) C.G. Shaw, or heterothallic, e.g. *Sclerospora graminicola* (Sacc.) Schroet. (Michelmore *et al.*, 1988). If the pathogen is heterothallic, the host plant tissue mustbe colonized by both mating types to produce oospores. Thus, heterothallism inhibits oospore formation when only one mating type is present. If oospores are important in the survival and transmission of the pathogen, as they are believed to be for *P. viciae* f.sp. *pisi* (Dixon, 1981; Populer, 1981; Stegmark, 1994), homothallism may ensure oospore production and thus increase the probability of survival.

P. viciae f.sp. *pisi* forms oogonia and antheridia (Von Heydendorff, 1977; own observations), and we assume that fertilization occurs at the formation of oospores as demonstrated for *Bremia lactucae* Regel (Tommerup *et al.*, 1974) and *Peronospora parasitica* (Sansome and Sansome, 1974). Self-fertility of the seven isolates of *P. viciae* f.sp. *pisi* studied appeared to be a stable property as all monoconidial isolates derived were able to produce oospores. Two isolates were maintained for several months under growth chamber conditions by repeated conidial inoculation and were still able to produce oospores afterwards.

Previously, oospore formation by *P. viciae* f.sp. *pisi* has been reported for isolates in Germany and England (Pegg and Mence, 1970; Von Heydendorff, 1977). So, it appears that *P. viciae* f.sp. *pisi* is a predominantly homothallic pathogen.

Table 5.4. Oospore production of *Peronospora viciae* f.sp. *pisi* in leaves of three cultivars of *Pisum sativum* at 20°C

Cultivar	Level of resistance	Oospores[y]	
		No/cm^2	No/g
Kelvedon Wonder	Highly susceptible	2.51	5.60
Finale	Susceptible	2.26	4.52
Cobri	Incompletely resistant	2.91	5.28
s.e.[z]		0.32	0.60

[y] Log_{10} transformed values; no significant cultivar effect (*F*-test, $P>0.05$).
[z] Pooled standard error; six replications.

Effect of temperature on oospore production
Oospore production was favoured by higher temperatures in the range of 5-20°C studied (Fig. 5.1, Tables 5.1, 5.2). The results corresponded with field observations during June and July, 1991. In June, when the average weekly temperature ranged from 10.8°-13.7°C, oospores were not found in leaflet lesions of the cv. Finale, but in July, when the average daily temperature was above 15°C, oospores were observed in leaflet lesions.

Formation of oospores of downy mildew fungi may be stimulated by conditions unfavourable for formation of conidia/sporangia (Populer, 1981), e.g. at temperatures too high for asexual sporulation. In this study, as many or more oospores were produced at 20°C than at the lower temperatures, which indicates that the optimum temperature for oospore production is higher than for conidial production, which is between 12 and 16°C (Pegg and Mence, 1970). In the case of *P. viciae* f.sp. *fabae* (Jacz. & Serg.) Boerema *& al.*, however, oospores are formed abundantly in leaves at a temperature range of 5-20°C (Van der Gaag *et al.*, 1993). *Bremia lactucae* produces oospores at a range of 5-22°C (Michelmore, 1981), which is similar to that of its asexual sporulation (Crute and Dixon, 1981). Thus, conditions at which oospores of downy mildew fungi are formed cannot be generalized.

The minimum temperature for oospore formation varied among plant parts (Fig. 5.1; Tables 5.1, 5.2), which suggested that temperature also affects sexual sporulation indirectly by influencing host plant factors and/or vegetative growth of the fungus.

Oospores produced at lower temperatures were larger than those produced at higher temperatures (Table 5.3). A similar temperature effect on the size of conidia of *P. viciae* f.sp. *pisi* was found by Pegg and Mence (1970). Development of plants from temperate regions, such as *Pisum sativum*, accelerates faster with increasing temperature than growth resulting in the formation of smaller plants at higher temperatures than at lower temperatures (Brouwer and Kuiper, 1972). A similar phenomenon may explain the formation of smaller spores of *P. viciae* f.sp. *pisi* with increasing temperature. Larger oospores may contain more energy reserves, and therefore, may have a longer survival time, a hypothesis amenable to experimental testing.

Effect of host plant genotype on oospore production

Oospore densities in lesions of three genotypes with different levels of resistance to downy mildew were similar (Table 5.3). In the hemibiotrophic potato late blight pathogen *Phytophthora infestans* (Mont.) de Bary more oospores are formed in moderately resistant than in susceptible cultivars, which can be explained by a delay in the deterioration of infected plant tissue which results in extra time for oospore production (Estrada, 1967; Drenth *et al.*, 1995). Deterioration of leaf lesions caused by the biotrophic fungus *P. viciae* f.sp. *pisi*, however, began after about 14 days in the lower leaves of all the three cultivars. As the maximum numbers of oospores were reached at or before 14 days after inoculation (Fig. 5.1), leaf deterioriation will not be limiting for oospore production. Thus, in a pea crop, the number of oospores produced in a susceptible cultivar will be higher than in a more resistant one because of the higher disease severity in the susceptible cultivar.

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

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 percentages of germination. Light inhibited germination. Germ tubes began to appear after 4, 7, and 14 days of incubation at 15, 10, and 5°C, respectively. Percentages germination were highest and similar at 5 and 10°C. At 20°C germination was poor and at 25°C no germination was observed. When oospores were retrieved from membrane filters incubated on soil for six days, oospores in water at 10°C germinated within two days. On soil significantly less oospores germinated than in water. Germinability of oospores stored dark at 5 or 20°C at 30 or 76% RH was studied over a two-years period. Germinability increased during the first ca. 200 days of storage after which fluctuations were observed indicating the occurrence of secondary dormancy. Time courses of germinability were generally similar for oospores stored at the various temperatures and humidities. No effect of light on the time course of germinability was found when oospores were exposed to alternating light-dark periods or stored in continuous dark during 140 days. Germinability of the oospores in water was related to their infectivity 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, 1996b), are important in the survival of the pathogen during host-free periods (Dixon, 1981; Stegmark, 1994).

Host plant 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 a few germinated oospores only. Günther (1992) obtained percentages germination of 0 to 40 from oospores sampled in the field. Large variations in percentages germination were found by Clark (1989) and Van der Gaag and Frinking (1996a). 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 the temperature of storage (Pratt, 1978). After storage at 4°C no germination was observed, but after incubation at 25°C a few, 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 this study were (i) to determine the effects of host plant exudates and physical factors on 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

Oospore populations were used which had been produced by a monoconidial culture from an isolate of *Peronospora 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, 1996b). Oospore populations were either produced in pods or in locally or systemically infected seedlings at 20°C in a growth chamber (Van der Gaag and Frinking, 1996b). 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, RH 35-65% RH) in the dark, unless stated otherwise.

Extraction of oospores and germination assay

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

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, 1996a).

Seed exudates were obtained by surface-sterilization of pea seeds (cv. Kelvedon Wonder) following 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 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 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. Root exudates were tested for contamination in the same way as 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.

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 Wm^{-2} for 16 h per day at 5°C. Control dishes were packed in two layers of aluminium foil. Temperature in light exposed dishes was about 1°C higher than in dark incubated dishes. 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-days period.

The light and temperature experiments were repeated using oospores from other populations.

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 one mm. Soils were stored at 5°C before use.

Soil was placed in Petri dishes (16 cm ϕ), up to a height of about seven 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 a previous section. Oospores were isolated from

the suspension using a microsyringe and placed in water (Van der Gaag and Frinking, 1996a). Oospore germination was recorded after 4-16 days every second day.

Effects of oospore age and temperature on germinability

Oospores were produced in locally infected 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 at 5 or 20°C and at relative humidities of about 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 subsample of 0.02 g and incubated for germination every 3-9 weeks during two years, beginning on d.a.i. 26. 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 were observed per dish each time.

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 Wm^{-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 subsamples and the percentages germinable and living oospores were determined as described above at 1-6-week intervals during 20 weeks, beginning at d.a.i. 21. One hundred oospores from each sample were observed each time.

Relation between germinability and infectivity

The relation between germinability and infectivity of oospores was investigated. Germinability of oospores was assessed using the germination assay as described above. Infectivity was assessed in a bioassay. Plastic trays of 17 × 13 × 6 cm were filled with a layer of one cm steam-sterilized potting soil and four 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 two 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 one cm steam-sterilized sand was deposited 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 W/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 were used from samples of eight different populations with four replications per population in the germination test and the bioassay.

Table 6.1. Effect of host plant exudates on germination of oospores of *Peronospora viciae* f.sp. *pisi*

	Germination (%)[a]	
Medium	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] Exudates were tested on oospores from two populations.
[c] Values are means of four replications. One hundred oospores were observed per replication.

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 percentages 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
Percentages germination were 9.8 and 9.3 at densities of 100 and 1000 oospores ml^{-1}, respectively, and did not differ significantly (standard error 1.7, six replications). Percentages germination of oospores were either unaffected or decreased by addition of host plant exudates (Table 6.1).

Light and temperature
Light negatively affected germination (*t*-test, $P<0.05$) (Table 6.2). Temperature had large effects on time till appearance of the first germ tubes, and on total percentage germination. The germ tubes began to appear after about 4, 7, and 14 days of incubation at 15, 10, and 5°C, respectively (Fig. 6.1). Percentages germination were high and similar at 5 and 10°C. Germination curves at 5 and 10°C were similar when

Table 6.2. Effect of light on germination of oospores of *Peronospora viciae* f.sp. *pisi*

	Oospore germination[a] (%)	
	Expt 1	Expt 2
Dark	35.4 (1.9)[b]	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] Values are means of four replications. One hundred oospores were observed per replication. Values in parentheses are standard errors.

percentages germination were 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, $P<0.05$) (Fig. 6.1). At 20°C germination was generally poor (Fig. 6.1), and at 25°C oospores did not germinate.

Incubation on soil
Germination curves of oospores incubated in water and those retrieved from soil after six days were not significantly different (Fig. 6.2). On the loamy sand soil oospores did not germinate. On the silt loam soil some oospores germinated but significantly less than in water (Wilks' Lambda for time × treatment significant at $P=0.049$) (Fig. 6.2).

Oospore age and storage conditions
Oospores stored at 20°C and 76% RH rapidly lost their viability as shown by a decrease in percentages staining in MTT (Fig. 6.3). At day 292 6% of the oospores stained faintly. The percentage of living oospores of those stored at 5°C with low and

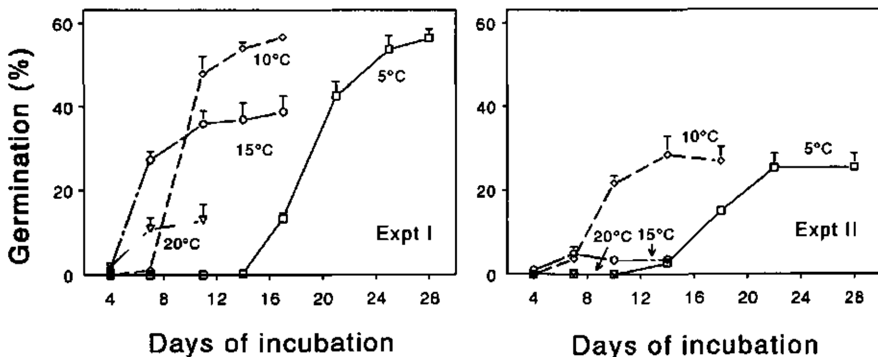

Fig. 6.1. Effect of temperature on germination of oospores of *Peronospora viciae* f.sp. *pisi* in two experiments using oospores from different populations. Values are means of four replications and 100 oospores were observed per replicate. Bars indicate standard errors.

high humidity and at 20°C with low humidity remained similar over time (Fig. 6.3A). Germinability of oospores increased with oospore age till about 200 days (Fig. 6.3B). Oospore germinability differed significantly between oospores stored at different conditions on some sampling dates (*t*-tests, $P<0.05$), but the effects were not consistent over time (Fig. 6.3B). A significant decrease in germinability was found between day 257 and 292 of oospores stored at 5°C, which was followed by an increase until day 390. At 20°C/30% RH and 5°C/30% RH, germinability decreased significantly after day 390 which was followed by an increase (Fig. 6.3B).

Percentages staining in MTT remained similar over time for oospores exposed to light/dark or dark conditions, and were on an average 94.6. Germinability of oospores developed similarly at light/dark and dark conditions (Fig. 6.4).

Germinability and infectivity
Seedling infection showed a linear response to germination for 100 oospores 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, $y = 1\text{-}0.71e^{(-4.8x)}$ ($3.6 \leq x \leq 76.2$), adequately described the data (Fig. 6.5).

Discussion

Oospore density and host plant exudates
Oospore densities of 100 to 1000 oospores can be used in a germination assay as the results were not affected using 100 or 1000 oospores per ml.

Germination of oospores *in vitro* was not increased by host plant exudates. In some additional experiments no stimulating effect was found for the amino acids

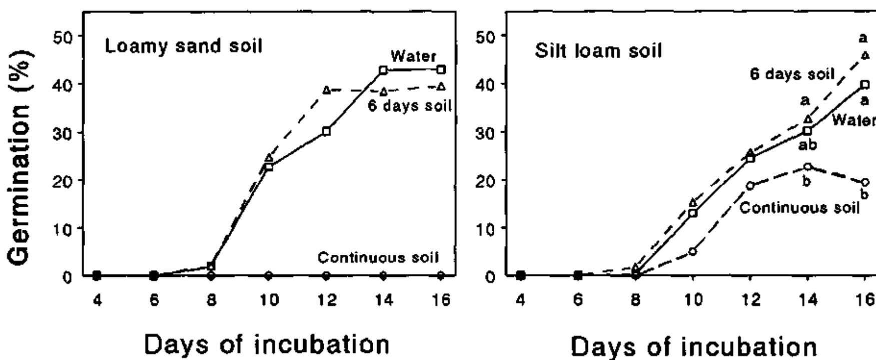

Fig. 6.2. Comparison of germination of oospores of *Peronospora viciae* f.sp. *pisi* in water, on soil, and in water after incubation for six days on soil. Oospores did not germinate on the loamy sand soil. Significant differences in percentages of germination are indicated by different letters for the silt loam soil (*t*-tests, $P<0.05$). Values are means of four replications with 100 oospores each.

glutamin and asparagin, nor for the sugars, glucose, fructose, and sucrose. Oospores of *P. viciae* f.sp. *pisi* contrast with oospores of several *Phytophthora* spp. (Förster et al., 1983; El-Hamalawi and Erwin, 1986; Hord and Ristaino, 1991) and *Bremia lactucae* (Morgan, 1978, 1983) where germination depends on or was increased by addition of host plant exudates or amino acids. Although oospores of *P. viciae* f.sp. *pisi* do not demonstrably depend on host plant factors for germination, it is hypothesized that under natural conditions germination is inhibited by soil fungistasis (Bruehl, 1987), and that host(plant) factors are needed to induce germination.

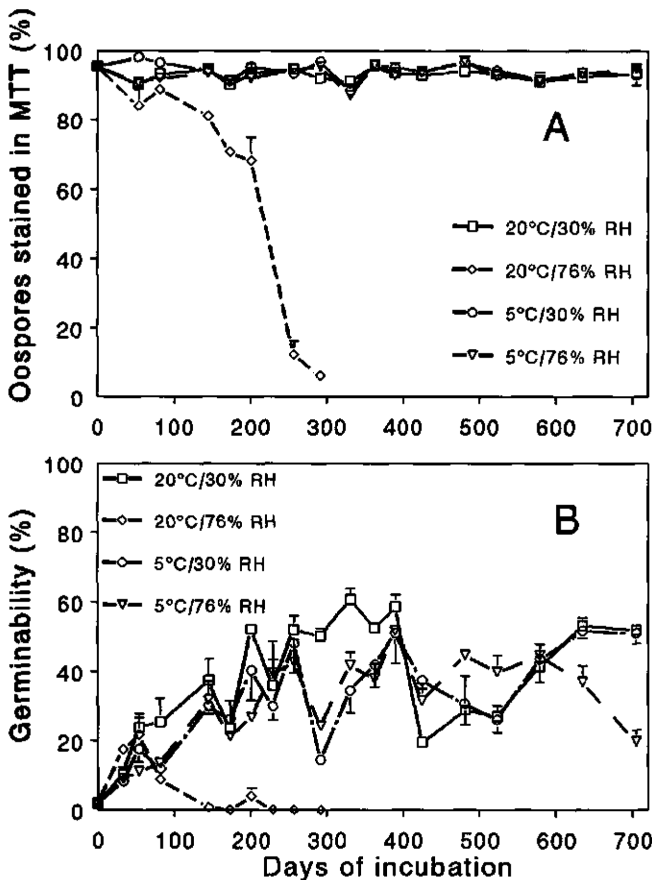

Fig. 6.3. Effect of incubation time and temperature and relative humidity during incubation on percentages of oospores of *Peronospora viciae* f.sp. *pisi* staining red in tetrazolium bromide (MTT) (A) and their germinability (B). Oospores produced in pea seedlings, inoculated with conidia, were stored at two temperatures and relative humidities from 26 days after inoculation. Values are means of three replications with 100 oospores each. Bars indicate standard errors.

Light

Light negatively affected germination of oospores of *P. viciae* f.sp. *pisi*. Their behaviour does not correspond with that of oospores of Pythiaceous fungi of which 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, the 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 germination of oospores of *P. viciae* f.sp. *pisi* contrasts with that of the conidia of which germination is unaffected by light (Pegg and Mence, 1970). Sensitivity to light may prevent oospores to germinate above soil which may be advantageous for survival.

Temperature

More oospores germinated at 5 and 10°C than at 15 and 20°C (Fig. 6.1). Apparently, a critical temperature for germination exists between 10 and 15°C. The relative percentages germination at 10, 15, and 20°C differed between the experiments using oospores from different populations (Fig. 6.1). In expt I about 1.5 times more oospores germinated at 10 than at 15°C, while in expt II about six times more oospores germinated at 10 than at 15°C. Thus, oospores may show differences in relative germinability at optimal and suboptimal temperatures. In additional experiments not reported here oospores from different populations showing almost similar germination percentages at 10°C, greatly differed in germinability at 15 or 20°C, thus corroborating the conclusion that among populations differences exist in temperature respons of germination.

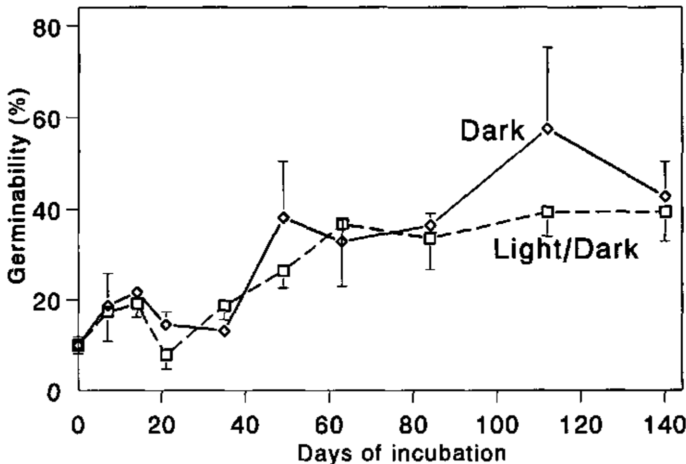

Fig. 6.4. Effect of incubation time and light on germinability of oospores of *Peronospora viciae* f.sp. *pisi*. Oospores were produced in pea seedlings inoculated with conidia and were stored in the dark or exposed to alternating dark light periods (16/8 h, 29 W/m^2) from 26 days after inoculation. Values are means of three replications with 100 oospores each. Bars indicate standard errors.

The observations of an earlier appearance of germ tubes at 15 and 20°C than at 5 and 10°C, and a higher percentage germination at 5 and 10°C than at 15 and 20°C suggest the occurrence of two different processes when dry-stored oospores are incubated in water. First, water is taken up by the spores before the second process, germination, can begin.

The low optimum temperature for germination (<15°C) corresponds with soil temperature at the start of the growing season in pea-grown areas. In a bioassay, higher infection percentages were obtained at 10 than at 14°C (unpublished data).

Soil

When oospores were incubated on wet soil, germination was inhibited or partly inhibited compared to incubation in water (Fig. 6.2). 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 (unpublished data). Apparently, oospores incubated under wet 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 they have been 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 germination inhibiting factors have been relieved or after a germination inducing signal has been received.

In soil, abiotic and/or biotic factors may be involved in 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 deposited on the MFs. Bacterial growth has been associated with

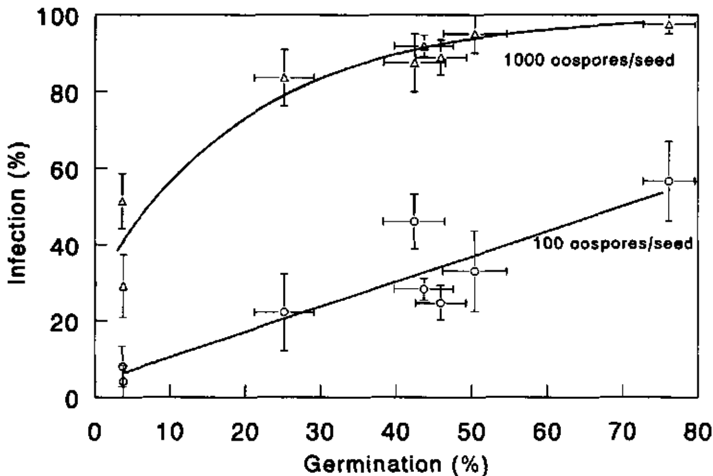

Fig. 6.5. Relation between seedling infection in a bioassay and percentage germination *in vitro* of oospores of *Peronospora viciae* f.sp. *pisi*. One hundred or 1000 oospores were added to a pea seed. Bars indicate standard errors.

lower percentages of germination *in vitro* (Van der Gaag and Frinking, 1996a), but a temperature of 10°C might have been too low to achieve a sufficient bacterial colonization of the MFs to be inhibitory for germination.

Oospore age and storage conditions

An increase in germinability of oospores with age was observed during about the first 200 days after formation. Increases in germinability with oospore age 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 lead to loss of dormancy and biochemical changes in dry seeds have been postulated (Roberts and Smith, 1977).

Significant decreases in germinability over time of oospores of *P. viciae* f.sp. *pisi* were observed. At 20°C/76% RH, the decrease can be explained by loss of viability since the percentages of living oospores also decreased as indicated by the MTT test (Fig. 6.3). Decreases observed with oospores stored at 5°C or 20°C/30% RH suggest the occurrence of secondary dormancy. This phenomenon may also explain why percentages 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 percentages germination observed in several studies (Clark, 1989; Günther, 1992; Van der Gaag and Frinking, 1996a). No clear periodicity in germinability was found during the two years of study. The causes of the variability in germination of oospores over time are unknown.

Temperature (5 or 20°C) and light (29 Wm^{-2}) did not affect the time course of germinability of dry-stored oospores to any great extent. Percentages germination of oospores stored at 5 or 20°C (30% RH) did not differ significantly on most sampling dates (Fig. 6.3). Under more natural conditions, in soil, the time course of germinability may be different from that under dry conditions, an hypothesis to be tested in future research.

Germinability and infectivity

A linear increase of percentage seedling infection with oospore germination was expected. Such a relationship was found when 100 oospores were added per seed. The intercept of the line (Fig. 6.5) did not significantly deviate from zero which suggests that percentages germination are not higher in the vicinity of the host plant than in water. This result corresponds with the results of the *in vitro* experiments where germination of oospores was not increased by the addition of host plant exudates. Apparently, oospores which do not germinate in water will not germinate in the infection court of the host either. 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).

At quantities of 1000 oospores, the ratio of diseased seedlings to oospore germination decreased. The results suggest competition among germinated oospores at high densities in soil. Germinated oospores may compete for a susceptible infection site.

The strong correlation between the percentage of oospores germinating *in vitro* and the percentage of diseased seedlings in the bioassay showed that results from the germination assay provide a good measure for infectivity of dry-stored oospores. In the present study only dry-stored oospores were used and the properties of oospores might change after incubation in soil for some time, which has to be addressed in future research. Besides germinability of the oospores, the number of infections in soil will depend on the physical, chemical, and biotic conditions of the soil. Soil texture and humidity for example have been shown to affect infection of sorghum plants by oospores of *Peronosclerospora sorghi* (Schuh *et al.*, 1987).

7 Survival and germinability curves of oospore populations of *Peronospora viciae* f.sp. *pisi* in soil

Abstract

Survival in soil and the effects of soil conditions on germinability of oospore populations of *Peronospora viciae* f.sp. *pisi* were investigated. The percentage survival was assessed using the vital stain tetrazolium bromide. Germinability was defined as the percentage of oospores which germinated in water. One to 3 weeks old oospores embedded in plant tissue were incorporated in a loamy sand, pF 2.1, or silt loam soil, pF 2.2, and incubated at 3, 10, or 20°C or stored dry at 20°C and 30% RH. The percentage surviving oospores in soil decreased rapidly after decomposition of the surrounding plant tissue at 10 and 20°C. After 29 weeks less than 10% of the oospores had survived. At 3°C, survival was 25% or more after 29 weeks. Germinability of the oospores was 3% at time of incorporation and had increased to 30% or more after 4 weeks in soil, except in the silt loam soil at 10 and 20°C. Increase in germinability of the dry-stored oospores was significantly later than that of the soil-incorporated oospores. In soil, the initial increase in germinability was followed by a decline after decomposition of the surrounding plant tissue. Survival and germinability of oospores under natural conditions were investigated by burying nylon nets containing oospore-infested soil in the field at 5 cm depth. Ground shoots of seedlings or pod pieces measuring 1 × 1 cm of pea plants containing 7 or 5 months old oospores, respectively, were incorporated in two loamy sand soils or a silt loam soil. Plant tissue in which the oospores were embedded had decomposed within 4 weeks and the percentage of surviving oospores decreased rapidly in each of the three soils, indicating that abiotic and/or biotic conditions non-specific for a particular soil were the major factor causing oospore death. In the various soils, the median survival time ranged from 1.3 to 2.5 weeks for oospores from the ground stem and leaf tissue and from 5.1 to 6.9 weeks for oospores from the pod pieces in the various soils. After 52 weeks, less than 6% of the original oospore populations had survived in each soil. A relatively low percentage survival of oospores from the pod pieces in one of the loamy sand soils was associated with a high percentage of oospores invaded by fungi. The lognormal model described survival curves better than the exponential or Weibull model, indicating that the probability of oospores to die in soil first increased and later decreased. Germinability of the oospores decreased rapidly from 62% at time of incorporation to less than 10% after 4 weeks and varied between 0 and 12% between 4 and 52 weeks after incorporation.

Introduction

Peronospora viciae (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven (Boerema *et al.*, 1993) is a biotrophic parasite which causes downy mildew on pea (*Pisum sativum* L.). It produces large numbers of oospores in infected leaves, stems, and pod walls of the host plant (Van der Gaag and Frinking, 1996b). After harvest, crop residues are ploughed in so that the oospores are mixed with the soil. The oospores are important as survival units during host-free periods and cause primary infections in following growing seasons of the pea crop (Dixon, 1981; Stegmark,

1994). The oospores possibly survive for up to 15 years in soil (Olofsson, 1966). In Sweden, soil-borne infections are common in six-year crop rotations of pea (Stegmark, 1994). The proportion of an initial oospore population which persists in soil for such long periods is not known.

A general hypothesis on survival of fungal propagules in soil states that a large proportion of the original population dies within a relatively short time while only a small proportion will survive for long periods (Griffin and Baker, 1991). Survival studies on oospores of Pythiaceous fungi indeed show a rapid decline of viable oospores soon after incorporation in soil (Harris, 1985; Bowers *et al.*, 1990; Johnson *et al.*, 1990). No studies on survival of oospores of Peronosporaceous fungi in soil have been found.

Survival of fungal propagules has been the subject of many studies but few attempts have been made to analyse propagule survival mathematically (Gilligan, 1990; 1994). The log transformation with and without a lag period before the onset of death and the log-probit transformation have been proposed to linearize survival curves for data analysis (Yarwood and Sylvester, 1959; Dimond and Horsfall, 1965; Baker, 1991). The log transformation assumes that survival time of an individual propagule is exponentially distributed which implies that the probability of a propagule to die is constant over time. The log-probit transformation assumes a lognormal distribution of survival time which means that the probability to die first increases and, almost as soon as the median is passed, decreases (Lee, 1980). These distributions do not account for a constantly increasing or decreasing probability of death which may occur for fungal propagules. The Weibull model can describe survival of propagules with a probability to die either increasing or decreasing over time (Lee, 1980).

The time course of germinability, the germinability curve, has been determined for oospores of *P. viciae* f.sp. *pisi* stored under air-dry conditions at constant temperature (Van der Gaag and Frinking, 1997b). Under those conditions, germinability of an oospore population, defined as the percentage germinable oospores in water, increased during the first few months of storage, after which it fluctuated around 50%. In soil, germinability of the oospores will supposedly be affected by the soil biotic and abiotic environment, and germinability curves in soil might be expected to differ from those of dry-stored oospores.

The objective of the present work was to quantify survival in soil and the effect of soil conditions on the germinability of oospores of *Peronospora viciae* f.sp. *pisi*, and the selection of appropriate models to describe survival curves. Survival and germinability of oospores in soil were studied in the laboratory at constant temperatures and in the field under natural conditions.

Materials and methods

Production of oospore populations

Oospores were produced by a monoconidial isolate of *Peronospora viciae* f.sp. *pisi* in systemically colonized seedlings or in pods of pea cv. Kelvedon Wonder in a growth chamber at 20°C (Van der Gaag and Frinking, 1996b). Diseased seedling tissue was harvested, air-dried and ground in a Retsch grinding mill to pass through openings 1 mm in diameter (Retsch, Haan, Germany). Diseased pods were harvested, air-dried

and pod walls were cut into pieces of about 1 × 1 cm. Oospore containing tissue was stored air-dry at room temperature until experiments began.

Temperature experiment

The effect of temperature on survival of oospores was determined using samples of a loamy sand soil (pH-KCl 7.4) and a silt loam soil (pH-KCl 7.4) from the upper 15 cm of two field plots in Wageningen, the Netherlands. Oospores produced in systemically colonized seedlings were 1-3 weeks old when incorporated in soil.

Soil was placed in small plastic pots (30 mm in diameter, 47 mm high) to a depth of 15 mm. On top of this soil layer, 0.032 g of ground tissue containing $1.5 \pm 0.2 \times 10^5$ oospores, was deposited and the whole was covered with a further layer of 15 mm soil. Pots were closed with plastic lids with a tiny perforation for gas exchange. Pots were weighed and placed at 3, 10, or 20°C. The loamy sand and silt loam soil had a pF of 2.1 and 2.2, respectively. This level of soil moisture was maintained by weighing pots every three weeks (20°C) or nine weeks (3°C, 10°C) and supplementing with water. Ground tissue with oospores from the same population was placed at 20°C and 30% RH.

At 2-7-week intervals, three pots per temperature were removed over a 29-week period to extract the oospores. Additionally, oospores were extracted from three subsamples, each of 0.01 g, of the dry-stored plant tissue on each sampling day (Van der Gaag and Frinking, 1996a).

Outdoor experiment: survival under natural conditions

Survival of oospores was studied in two loamy sand soils with pH-KCl of 4.3 and 4.9, respectively (LS4.3 and LS4.9), and a silt loam soil with pH-KCl 7.4 (SL7.4). Soil samples were collected from the upper 15 cm of field plots located in Wageningen and air-dried. The silt loam soil was ground in a Retsch grinding mill to pass through openings of 1 mm in diameter before oospores were mixed through out the soil. Two oospore populations were studied in this experiment, produced in either systemically colonized tissue (PS) or in pods (PP). Oospores were about 7 (PS) and 5 (PP) months old.

Twenty gram of air-dried soil was mixed with 0.025 g of the ground tissue containing $1.5 \pm 0.1 \times 10^5$ oospores, and placed in nylon nets. Two pod pieces of 1 × 1 cm each, containing $7.0 \pm 0.4 \times 10^4$ oospores, were incorporated in 20 g of soil in a nylon net.

On 12 September 1995, nets with oospore-infested soil were buried in the field plots in a regular grid of fifteen rows of 10 nets each at a depth of 5 cm. Within and between rows distances were 15 cm. The two oospore populations were randomly distributed over the grid and the positions of the nets were marked. Survival was studied over a period of one year. Three nets chosen at random from each of the two populations were removed from each plot on each sampling day.

Quantification of oospore populations

Oospores were extracted from each soil sample as previously described (Van der Gaag and Frinking, 1997a). Briefly, a soil suspension was prepared by mixing the soil sample in water in a blender for 3 min. After sonication, the suspension was sieved through sieves with mesh sizes of 75 and 20 μm, respectively. The residue on the

latter sieve was resuspended in about 10 ml water and centrifuged on a sucrose solution. The supernatant was poured through a sieve with 20-μm pore size and the sugar solution was washed down with water. The oospores thus extracted were resuspended in 10-50 ml water. The exact volume of the suspension was determined and the numbers of oospores were counted in each of three 20 μl-droplets which were taken during stirring of the suspension.

Oospores were extracted from plant tissue by comminuting the tissue in water in a blender and subsequently sonicating and sieving the suspension (Van der Gaag and Frinking, 1996a).

The percentage of living oospores was determined using the vital stain tetrazolium bromide, MTT (Sutherland and Cohen, 1983; Van der Gaag, 1994). Twohundred μl of the oospore suspension and 200 μl of a 0.1 % MTT solution in 20 mM phosphate buffer (pH 6.2) were mixed and incubated at 35°C for 72 h. The percentages of red, black and non-stained oospores were assessed by observation of 100 oospores per replication. Red stained oospores were assumed to be living, black-stained and non-stained oospores dead.

The percentage of oospores colonized by fungi in soil was investigated. Oospores extracted from soil were placed individually on water agar in Petri dishes (9 cm ϕ) using a microsyringe. Twelve oospores per dish were placed at marked positions. Three dishes were prepared per replication. The Petri dishes were sealed and placed at 20°C in the dark. Oospores were observed for outgrowth of hyphae after 24 and 48 h of incubation at 70× magnification.

Germinability of the oospores was assessed by addition of 100 or 200 μl of the oospore suspension to water amended with ampicillin (100 μg/ml) and rifampicin (10 μg/ml) in glass Petri dishes (Van der Gaag and Frinking, 1996a). Per replication, two dishes were prepared each containing 100-400 oospores. Petri dishes were incubated at 10°C and 50 oospores per Petri dish were observed after 14 days. Oospores were classified as germinated when the germ tube was longer than the diameter of the oospore.

Infectivity of oospores extracted from the silt loam soil (SL7.4) was investigated 52 weeks after incorporation. One hundred oospores were added per seed of the highly susceptible cv. Kelvedon Wonder as described by Van der Gaag and Frinking (1997b). Before inoculation, seeds were pregerminated by incubation between wet filter papers for 72 h. Fifteen pregerminated seeds were inoculated per replication and incubated at 10°C. Four weeks after inoculation, seedlings were covered with polyethyl sheets to increase humidity of the ambient air and stimulate sporulation of the fungus on diseased seedlings. The number of healthy and diseased seedlings were determined one day later.

Data analysis

The proportion of surviving oospores was computed according to:

$$P=[(N_{extr}P_{eMTT})/(N_{inc}P_{iMTT})][1/Rec],$$

where: N_{extr} is the number of oospores extracted and P_{eMTT} is the proportion of extracted oospores which stained red in MTT. N_{inc} is the initial number of oospores incorporated and P_{iMTT} is the proportion which stained red. *Rec* is the proportion of oospores extracted from soil; *Rec* was estimated before decomposition of oospore containing plant tissue by comparing numbers extracted on the first one or two

Table 7.1. Models[a] used in survival data analysis on oospores of *Peronospora viciae* f.sp. *pisi*

Model	Equation $S(t)$		Linearized form of $F(t)=1-S(t)$	
Exponential	$\exp(-\lambda(t-g))$	$t \geq g$	$\ln[1/(1-F)] = -\lambda g + \lambda t$	$t \geq g$
	1	$t < g$	0	$t < g$
Weibull	$\exp[-(\lambda t)^{\gamma}]$		$\log_{10}\{\ln[1/(1-F)]\} = \gamma\log_{10}t - \gamma\log_{10}(1/\lambda)$	
Lognormal	$1-G[(\log_{10}t-\mu)/\sigma]$		$G^{-1}(F) = [(\log_{10}t)/\sigma] - [\mu/\sigma]$	

[a] $S(t)$ is the survivorship function, $F(t)$ the cumulative distribution function and F is the fraction of the distribution below time t. G() is the cumulative distribution function of a standard normal variable and G^{-1} its inverse. g is the time within which no deaths occur. λ in the exponential model is the hazard rate. λ and γ in the Weibull model are the scale and shape parameters, respectively. μ and σ are the scale and shape parameters, respectively, in the lognormal model. Symbols for functions and parameters were adopted from Lee (1980).

sampling dates with the initial numbers incorporated into soil (when oospores were still embedded in plant tissue). *Rec* was estimated after decomposition of the tissue from data of one or two consecutive sampling dates after tissue had decomposed. No decomposition of oospores was assumed until these sampling dates.

Survival of oospores was analyzed by fitting the two-parameter exponential, Weibull, and lognormal models to the survival data. Linearized forms of the models were used in regression analysis to compare the models (Table 7.1). Predicted transformed data were backtransformed to calculate R^{*2}, the coefficient of determination between observed and predicted data to compare the models for goodness-of-fit (Campbell and Madden, 1990). Additionally, the residual plots were used as a criterium for the goodness-of-fit of the models (Campbell and Madden, 1990).

Parameters and their standard errors were estimated for the best fitting model using nonlinear regression analysis. Starting values for the parameters needed to compute their final values, were derived from the intercept and slope estimated in the linear regression analysis. Parameters estimated for each survival curve were compared by *t*-tests (Campbell and Madden, 1990).

Calculations were made using PROC REG for linear regression analysis and PROC NLIN with the DUD-method for nonlinear regression analysis in SAS version 6.04 for personal computers (SAS Institute Inc., Cary, NC).

Results

Temperature experiment

Prior to decomposition of the surrounding plant tissue, mean recoveries of oospores from soil were 100 and 97% for the loamy sand and silt loam soil, respectively. After decomposition of the surrounding tissue, recoveries decreased to 81 and 63%,

respectively, as estimated from the mean percentages of oospores recovered on the two consecutive sampling dates after decomposition of the plant tissue (Fig. 7.1).

At incorporation 92.7 ± 1.7 per cent (mean ± standard error) of the oospores stained red in MTT. This percentage did not change significantly over time for dry-stored oospores. The percentage of oospores extracted from soil which stained red in MTT decreased rapidly especially after the plant tissue had decomposed (Figs. 7.1, 7.2).

Dry-stored oospores did not stain black in MTT but black stained oospores were observed after four weeks of incubation in the silt loam soil at 20°C and after six weeks of incubation otherwise (Table 7.2). Often, black stained oospores were not stained over the whole area within the oospore wall whereas red stained oospores usually were. Black staining was highest among oospores extracted from the silt loam soil incubated at 20°C (Table 7.2).

The lognormal model described survival curves of oospores best. The coefficient of determination (R^{*2}) for agreement between observed and predicted values was generally highest for the lognormal model and the residual plots showed an acceptable pattern in each case (Table 7.3, Fig. 7.3). Estimates of the parameters of the lognormal model for each soil × temperature treatment were used to compare survival at the different temperatures in each soil (Table 7.4). The median time, the time at which 50% of the oospores had died, decreased significantly with increasing temperature within each soil (Table 7.4).

Germinability curves of the dry-stored oospores and soil-incubated oospores were different (Fig. 7.2). Percentages germination of dry-stored oospores first remained low, showed a peak at 12 weeks of incubation and gradually increased later on (Fig. 7.2). Germinability of soil-incubated oospores increased 2 weeks after incorporation and began to decrease 4-9 weeks after incorporation, with the exception of the silt loam soil at 10 and 20°C (Fig. 7.2). Many oospores which had been incubated at 20°C and to a lesser extent at 10°C in the silt loam soil showed outgrowth of hyphae other than *P. viciae* germ tubes in the germination assay.

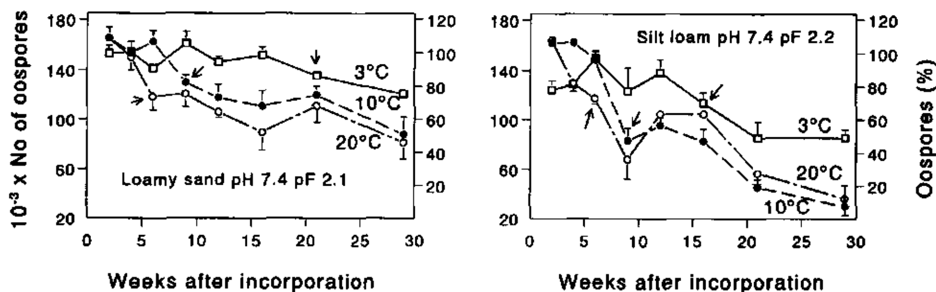

Fig. 7.1. The temperature experiment. Oospores of *Peronospora viciae* f.sp. *pisi* extracted from a loamy sand and silt loam soil in numbers (left-axis) and in percentages from the initial numbers of oospores incorporated (right-axis). Oospores had been incubated in soil at 3, 10 and 20°C. Initial number of oospores incorporated in soil was 1.5 ± 0.2 × 10^5. At incorporation oospores were embedded in plant tissue. Arrows point to the sampling time when plant tissue had decomposed and oospores were free in soil. Bars indicate standard errors.

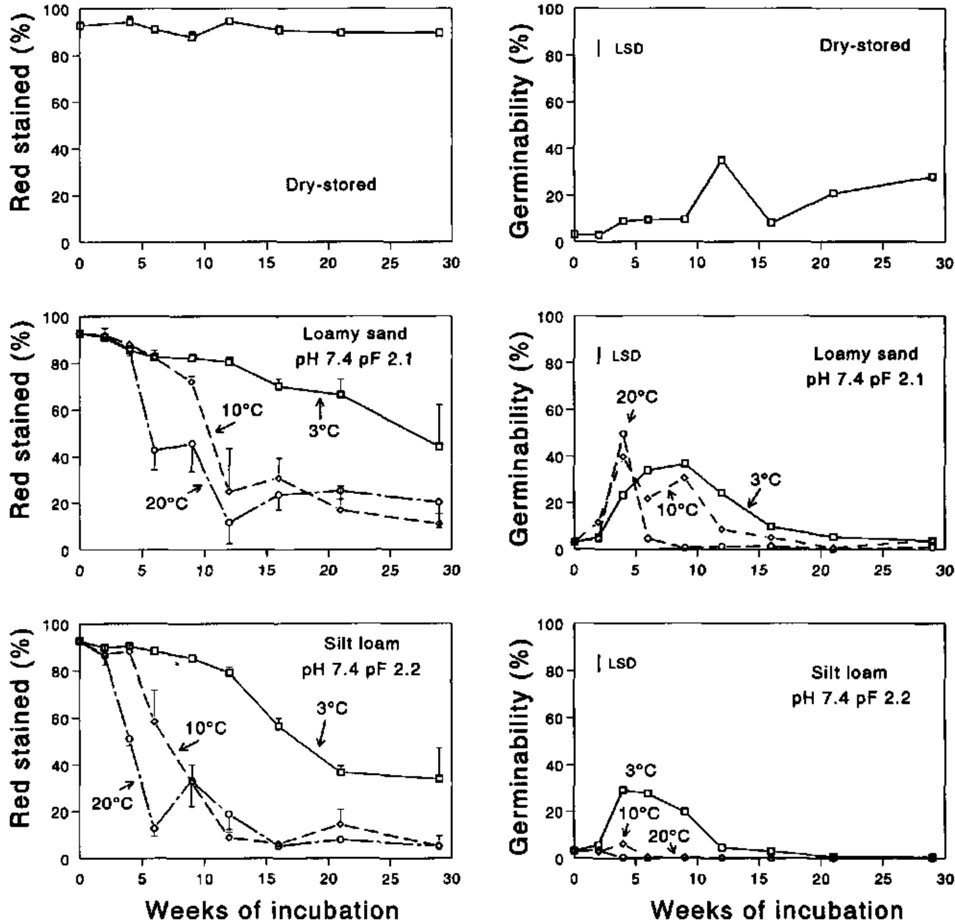

Fig. 7.2. The temperature experiment. Percentages of oospores which stained red in tetrazolium bromide (left graphs) and percentages of oospores which germinated in water (germinability, right graphs) after extraction from dry-stored plant tissue or soil. Oospores in plant tissue had been stored dry at 20°C and 30% RH or incubated in a loamy sand or silt loam soil at 3, 10, or 20°C. LSD: least significant difference, P=0.05.

Table 7.2. Percentages of oospores of *Peronospora viciae* f.sp. *pisi* which stained black in tetrazolium bromide after incubation in a loamy sand or silt loam soil at 3, 10, or 20°C for 2 to 29 weeks

		Temperature (°C)		
Soil	Week	3	10	20
Loamy sand	2	0.0 (0.0)[a]	0.0 (0.0)	0.0 (0.0)
pH-KCl 7.4	4	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
pF 2.1	6	2.3 (0.7)	4.1 (0.7)	2.2 (0.6)
	9	3.2 (1.2)	0.0 (0.0)	3.6 (0.6)
	12	9.0 (3.5)	14.9 (12.6)	6.0 (4.6)
	16	2.0 (0.6)	4.0 (2.5)	14.2 (4.9)
	21	3.3 (0.9)	8.7 (5.4)	17.8 (7.6)
	29	2.3 (0.4)	14.2 (3.5)	8.9 (2.6)
Silt loam	2	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
pH-KCl 7.4	4	0.0 (0.0)	0.0 (0.0)	17.7 (1.2)
pF 2.2	6	0.9 (0.5)	2.0 (0.6)	28.4 (12.0)
	9	1.7 (0.3)	1.5 (1.1)	23.3 (5.9)
	12	3.1 (0.1)	9.0 (3.5)	28.9 (7.2)
	16	3.2 (1.2)	5.2 (1.2)	27.5 (6.5)
	21	2.8 (0.6)	13.5 (6.7)	25.0 (11.6)
	29	2.9 (0.0)	23.2 (7.4)	42.3 (11.6)

[a] Values are means of three replications of 100 oospores each. In parentheses the standard error.

Outdoor experiment

Within two weeks after incorporation of oospores of population PS, the ground plant tissue had decomposed and oospores were fully exposed to soil conditions. Percentages of oospores extracted from soil were similar for the three soils studied. The recovery was approximately 66% (Fig. 7.4). Oospores from PP were still embedded in pod tissue after two weeks in soil. After four weeks the pod tissue had decomposed so far that it no longer contained oospores. Approximately 100 and 69% of PP oospores were extracted before and after decomposition of the pod pieces (= before and after four weeks) (Fig. 7.4).

At incorporation, 91.7 ± 1.9 and 95.3 ± 0.9% (mean ± standard error) of oospores from populations PS and PP stained red in MTT, respectively, and none of the oospores stained black. Percentages of oospores which stained red in MTT decreased rapidly after incorporation in soil. Generally, low percentages of oospores

Table 7.3. Summary of linear regression statistics used in the evaluation of three models describing survival curves of oospores of *Peronospora viciae* f.sp. *pisi* in a loamy sand or silt loam soil at 3, 10, or 20°C

Soil	Temperature (°C)	Model	R^2(%)[a]	R^{*2}(%)[b]	Residuals[c]
Loamy sand	3	Exponential	38.0	44.4	
pH-KCl 7.4		Weibull	47.4	55.4	+
pF 2.1		Lognormal	52.2	53.9	+
	10	Exponential	48.4	66.5	+
		Weibull	70.6	71.8	+
		Lognormal	73.0	79.3	+
	20	Exponential	37.7	68.3	+ $g<0$[d]
		Weibull	71.1	50.8	
		Lognormal	73.7	72.9	+
Silt loam	3	Exponential	65.8	52.9	
pH-KCl 7.4		Weibull	15.2	32.5	+
pF 2.2		Lognormal	27.0	51.5	+
	10	Exponential	78.4	81.2	+
		Weibull	80.0	31.9	
		Lognormal	86.8	87.2	+
	20	Exponential	77.8	75.1	+
		Weibull	64.5	30.4	
		Lognormal	80.6	67.0	+

[a] R^2 = coefficient of determination for agreement between observed and predicted transformed values.
[b] R^{*2} = coefficient of determination for agreement between observed and predicted values.
[c] + = the residual plot, the plot of studentized residuals against predicted values, has an acceptable pattern (Draper and Smith, 1981; Campbell and Madden, 1990).
[d] $g<0$ = estimate of the time within which no deaths occur < 0 (Table 7.1).

extracted from soil stained black, usually less than 5%, but high percentages were observed with PP oospores incubated in the loamy sand soil with a pH-KCl of 4.3 (Table 7.5).

In general, the lognormal model fitted the survival data best (Table 7.6). The two-parameter exponential model did not give a good description of the survival curves as negative values of g, the time within which no deaths occur, were obtained (Tables 7.1, 7.6). The one-parameter exponential model, with $g = 0$, did not give a good description either as the fitted curves of this model usually overestimated percentages of survival till about 25 weeks after which it underestimated survival.

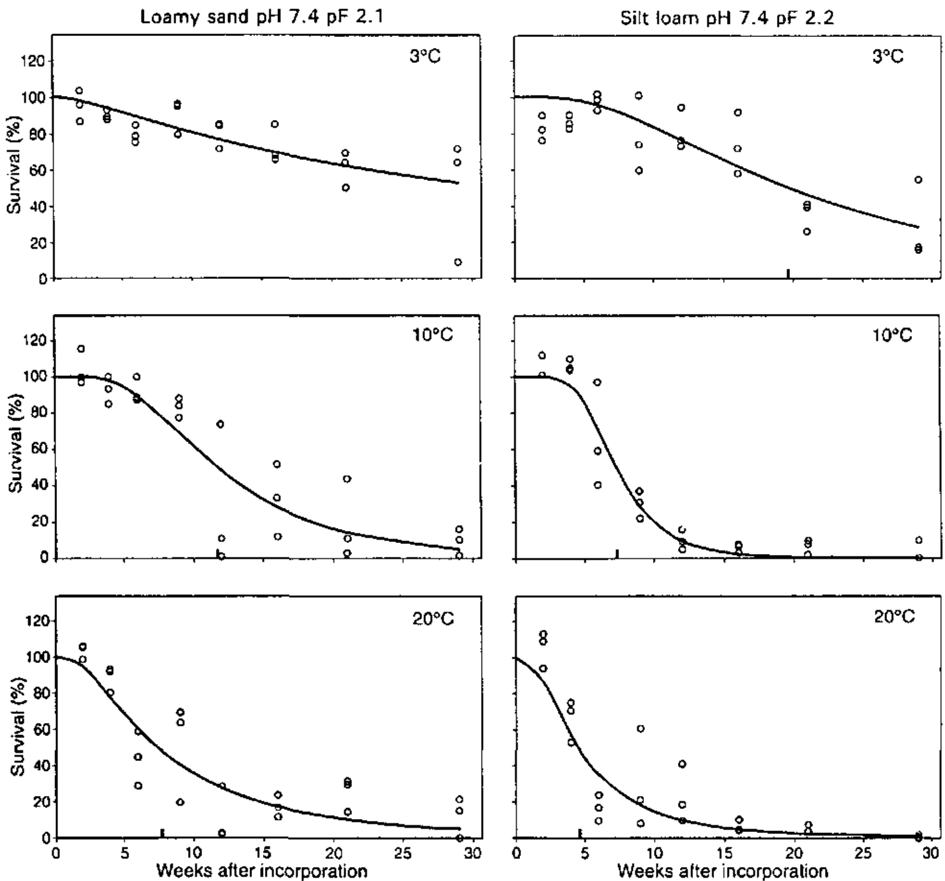

Fig. 7.3. The temperature experiment. Survival curves of oospores of *Peronospora viciae* f.sp. *pisi* incorporated in a loamy sand or silt loam soil and incubated at 3, 10, or 20°C. Fitted lines are lognormal survivorship functions. Median values are indicated by a vertical bar on the x-axis. Calculations are explained in the text (data analysis).

Percentages survivors decreased slower for oospores from pod tissue (PP) than from ground tissue (PS) during the first two to four weeks of incubation in soil and the median survival times were greater for PP than for PS (Fig. 7.6, Table 7.7). The shape parameters, σ's, of the fitted lognormal survival models were greater for PS oospores than for PP oospores. The median survival time of PP oospores was similar in the three soils, but σ was significantly smaller for PP oospores in LS4.3 than in SL7.4 (Table 7.7).

The percentage of oospores which showed outgrowth of fungal hyphae other than *P. viciae* f.sp. *pisi* germ tubes was highest for PP oospores extracted from the loamy sand soil with a pH-KCl of 4.3 (Table 7.8).

Germinabilities of PS and PP oospore populations at incorporation were 62.0 $\pm$ 7.2 and 61.1 $\pm$ 13.3 (mean $\pm$ standard error), respectively, and decreased strongly after incorporation in soil, varying between 0 and 20% (Fig. 7.5). Increases in germinability were observed between four and six weeks of incubation for oospores in the loamy sand soils and between week 25 and 34 for PP oospores in the silt loam soil. Germinability of living oospores as estimated by the ratio between the percentage germinated and red stained oospores ranged between 0 and 100%. On most sampling days the percentages germination were much lower than the percentages staining in MTT. At 52 weeks, the percentages of germinated and red stained oospores were the same (5%) for PP oospores from LS4.9 (Fig. 7.5).

Disease incidences in the bioassay were 12.3 $\pm$ 5.4 and 14.4 $\pm$ 1.0 % (mean $\pm$ standard error) after inoculation with oospores from PS and PP, respectively, extracted from the silt loam soil 52 weeks after incorporation.

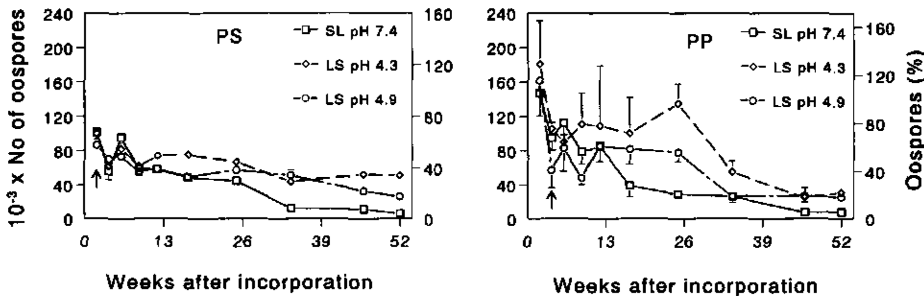

Fig. 7.4. The outdoor experiment. Oospores of *Peronospora viciae* f.sp. *pisi* extracted from soil in numbers (left-axis) and in percentages from the initial numbers of oospores incorporated (right-axis). Oospores from two populations (PS and PP) in plant tissue had been incorporated in a silt loam with pH-KCl 7.4 (SL pH 7.4), a loamy sand with pH-KCl 4.3 (LS pH 4.3), or a loamy sand with pH-KCl 4.9 (LS pH 4.9). Initial numbers of oospores incorporated in soil were 1.5 $\pm$ 0.1 $\times$ 10^5 for PS and 1.4 $\pm$ 0.6 $\times$ 10^5 for PP. At incorporation oospores were embedded in plant tissue. Arrows point to the sampling time when plant tissue had decomposed and oospores were free in soil. Bars indicate standard errors.

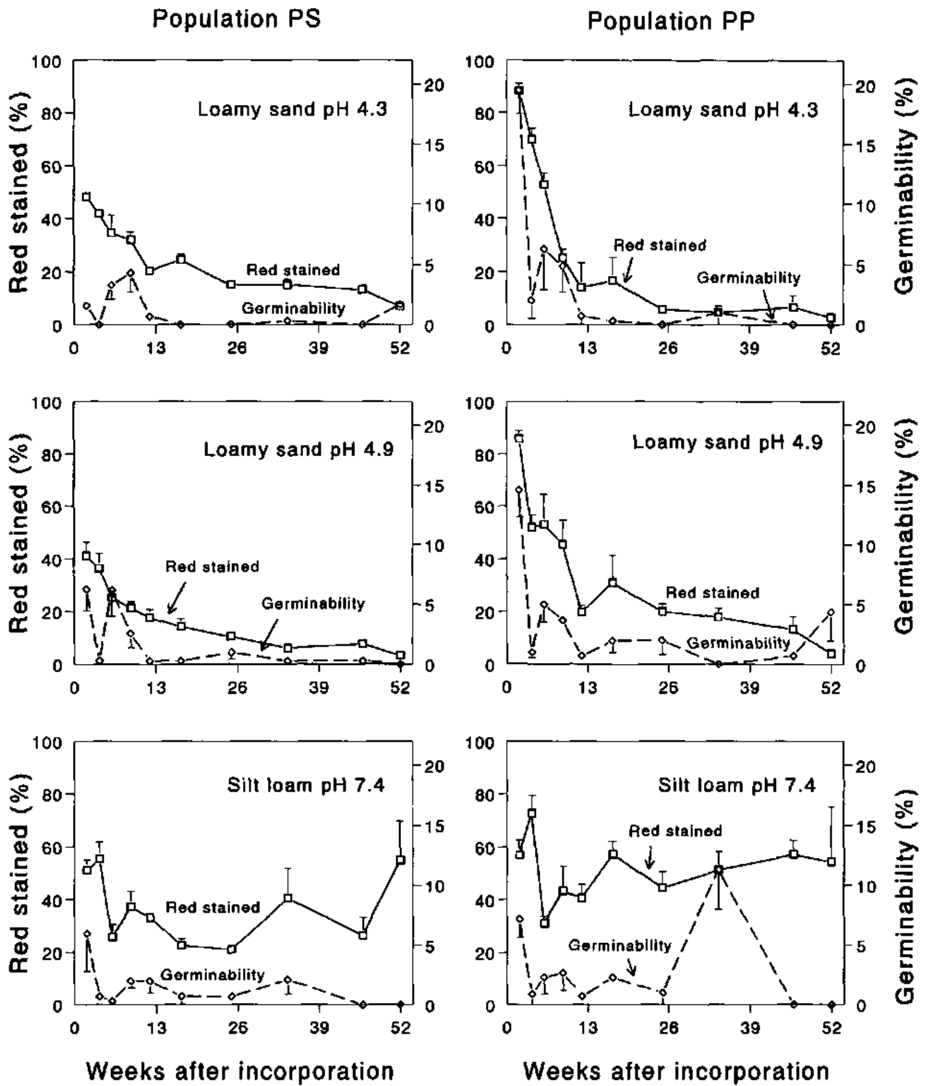

Fig. 7.5. The outdoor experiment. Percentages of oospores which stained red in tetrazolium bromide (left axis) and percentages of oospores which germinated in water (germinability, right axis) after extraction from soil. Oospores from populations PS and PP had been incorporated in three soils and exposed to natural conditions.

Table 7.4. Nonlinear regression estimates of parameters μ and σ of the lognormal model describing survival of oospores of *Peronospora viciae* f.sp. *pisi* in a loamy sand or silt loam soil at 3, 10, or 20°C

Temperature (°C)	Loamy sand soil			Silt loam soil		
	μ[a]	σ	median[b]	μ	σ	median
3	1.50c	0.57b	31.6c	1.29c	0.30ab	19.7c
10	1.07b	0.23a	11.7b	0.87b	0.16a	7.3b
20	0.87a	0.35ab	7.4a	0.66a	0.32b	4.6a

[a] Symbols are explained in Table 7.1. Values in each column followed by different letters are significantly different according to *t*-tests ($P \leq 0.05$).
[b] Median in weeks: 10^{μ}.

Discussion

Quantification of oospore populations
The recovery of oospores from soil was about 100% when oospores were embedded in host plant tissue in soil. After decomposition of the plant tissue, the recovery dropped to 63-81%, not much deviating from the 75% obtained in a previous study in which oospores were extracted from 10-g soil samples (Van der Gaag and Frinking, 1997a).

In the outdoor experiment, the numbers of oospores extracted from the silt loam soil decreased faster than those extracted from the loamy sand soils (Fig. 7.4). The difference can be explained by a difference in decomposition rate of dead oospores since higher percentages of oospores from the silt loam soil stained red in MTT than from the loamy sand soils (Fig. 7.5).

Results obtained in a previous study point to the occurrence of dormancy in oospores of *Peronospora viciae* f.sp. *pisi* (Van der Gaag and Frinking, 1997b). Therefore, a vital stain is needed to assess the percentage of viable oospores present as this will be underestimated by a germination assay. Tetrazolium bromide (MTT) has been used as a vital stain in several studies on oospores (Sutherland and Cohen, 1983; Bowers *et al.*, 1990). Bowers *et al.* (1990) used this stain in a study on survival of oospores of *Phytophthora capsici* Leonian in soil in which red stained oospores were considered to be viable and non-stained and black stained oospores non-viable. No reason was given for the black staining of oospores nor why they were considered non-viable. MTT was evaluated by Van der Gaag (1994) as a vital stain for oospores of *P. viciae*. In that study, low percentages of dry-stored oospores stained black in MTT-solutions, e.g. after 72 h of incubation 2% of the oospores stained black at pH 6.5 and no oospores stained black at pH 6.0. Black staining was suggested to be caused by overstaining of living oospores as the percentages increased with longer incubation periods and increasing pH values. At a higher pH oospores stained earlier than at a lower pH. In the present study, the high percentages of black stained oospores observed after 72 h of incubation in MTT at pH 6.2, were probably not due to overstaining of viable oospores. The percentages of oospores which stained black

Table 7.5. Percentages of oospores of *Peronospora viciae* f.sp. *pisi* from two populations which stained black in tetrazolium bromide after incubation in soil under natural conditions for 0 to 52 weeks

Oospore		Soil		
population	Wk[a]	Loamy sand pH 4.3	Loamy sand pH 4.9	Silt loam pH 7.4
PS	0	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)
	2	1.6 (0.6)	0.7 (0.3)	1.0 (1.0)
	4	1.3 (0.3)	2.3 (1.3)	0.7 (0.3)
	6	6.6 (2.4)	1.0 (0.6)	2.2 (1.1)
	9	5.5 (1.7)	1.0 (0.0)	2.0 (0.6)
	12	7.7 (0.9)	1.9 (1.5)	0.9 (0.5)
	17	6.7 (1.4)	3.0 (1.5)	0.7 (0.3)
	25	6.4 (1.2)	4.0 (1.2)	0.7 (0.3)
	34	4.0 (2.1)	2.0 (1.0)	3.9 (0.0)
	46	7.4 (3.3)	1.3 (0.7)	1.3 (0.9)
	52	4.7 (1.8)	1.0 (1.0)	2.3 (1.5)
PP	0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	2	1.6 (1.1)	1.6 (0.6)	3.9 (1.9)
	4	0.5 (0.5)	0.3 (0.3)	0.6 (0.3)
	6	2.9 (0.6)	1.3 (0.8)	1.9 (0.0)
	9	21.2 (10.6)	2.5 (0.8)	1.3 (0.3)
	12	51.1 (7.6)	20.7 (9.9)	1.5 (0.3)
	17	18.5 (2.3)	14.8 (8.6)	1.0 (0.6)
	25	41.9 (9.9)	4.0 (1.4)	1.0 (0.6)
	34	27.7 (2.5)	6.1 (3.3)	1.6 (0.3)
	46	25.4 (7.3)	9.0 (1.5)	2.7 (0.9)
	52	12.2 (2.1)	4.8 (2.4)	18.0 (16.5)

[a] Number of weeks after incorporation of oospores in soil.
[b] Values are means of three replications of 100 oospores each. In parentheses the standard error.

Table 7.6. Summary of linear regression statistics used to evaluate three models describing survival curves of two oospore populations of *Peronospora viciae* f.sp. *pisi* populations in three soils under natural conditions

Soil	Population	Model	R^2(%)[a]	R^{*2}(%)[b]	Residuals[c]	
Loamy sand	PS	Exponential	84.6	72.8	+	$g<0$[d]
pH-KCl 4.3		Weibull	87.6	86.7	+	
		Lognormal	88.2	87.8	+	
	PP	Exponential	81.0	73.1		$g<0$
		Weibull	63.6	67.5		
		Lognormal	84.6	90.7	+	
Loamy sand	PS	Exponential	88.0	75.1		$g<0$
pH-KCl 4.9		Weibull	90.4	88.5	+	
		Lognormal	89.8	88.2	+	
	PP	Exponential	84.4	52.1	+	$g<0$
		Weibull	40.3	<0		
		Lognormal	58.2	43.8	+	
Silt loam	PS	Exponential	89.0	78.7	+	$g<0$
pH-KCl 7.4		Weibull	92.1	91.5	+	
		Lognormal	92.4	92.2	+	
	PP	Exponential	76.0	69.0	+	$g<0$
		Weibull	58.2	54.9		
		Lognormal	74.4	72.2	+	

[a] R^2 = coefficient of determination for agreement between observed and predicted transformed values.
[b] R^{*2} = coefficient of determination for agreement between observed and predicted values.
[c] + = the residual plot, the plot of studentized residuals against predicted values, has an acceptable pattern (Draper and Smith, 1981; Campbell and Madden, 1990).
[d] $g<0$ = estimate of the time within which no deaths occur <0 (Table 7.1).

Table 7.7. Nonlinear regression estimates of parameters μ and σ of the lognormal model describing survival of oospores of *Peronospora viciae* f.sp. *pisi* from two populations (PS and PP) in three soils under natural conditions

	PS			PP		
Soil[a]	μ[b]	σ	median[c]	μ	σ	median
LS4.3	0.31b	0.89a	2.0b	0.81a	0.23a	6.5a
LS4.9	0.12a	0.80a	1.3a	0.71a	0.47ab	5.1a
SL7.4	0.40b	0.74a	2.5b	0.84a	0.52b	6.9a

[a] LS4.3 = Loamy sand, pH-KCl 4.3; LS4.9 = Loamy sand, pH-KCl 4.9; SL7.4 = Silt loam pH-KCl 7.4.
[b] Symbols are explained in Table 7.1. Values in each column followed by different letters are significantly different according to *t*-tests ($P \leq 0.05$).
[c] Median in weeks: 10^{μ}.

were low during the first weeks of incubation in each soil. They strongly increased in the silt loam soil at 10 and 20°C in the temperature experiment (Table 7.2), and in the loamy sand soil pH-KCl 4.3 in the outdoor experiment (Table 7.5). We hypothesize that the black staining of oospores in the present study was due to either invasion of living oospores by mycoparasites or by colonization of dead oospores in soil. A relatively high respiration rate of the invader may have caused the heavy staining reaction. High percentages of black stained oospores of PP from LS4.3 in week 9 to 34 corresponded with high percentages of oospores which showed hyphal outgrowth on water agar (Tables 7.7, 7.8). The observation that black stained oospores were often not stained over the whole area within the oospore wall suggests distortion of the oospore cytoplasm and, thus, non-viability. So, only red stained oospores were considered to be living in this study. Overestimation of the percentage of living oospores due to red staining of oospores which were invaded by microorganism cannot be excluded. Oospores infected by bacteria and/or other microorganisms are, however, expected to degrade rapidly and will no longer stain in MTT. They can be colonized by other microorganisms which may result in black staining in MTT.

Survival curves of oospore populations

Three models were chosen to account for a constant (exponential), increasing or decreasing (Weibull), or increasing followed by a decreasing (lognormal) probability of death. The exponential model describes monotone concave curves while the Weibull and lognormal can also account for S-shaped curves. S-shaped survival curves were obtained when the plant tissue in which the oospores were embedded had not decomposed before the first sampling time (two weeks after incorporation). This was the case in the temperature experiment and to a lesser extent with oospores in the pod pieces (PP) buried in the field.

During the one-year period of incubation in soil, the lognormal model generally described survival of the oospores best. This model fitted the data well both in the steep and flat parts of the curve. These parts have been referred to as the rapid or logarithmic death and long-term survival or residual phase of the survival curve,

respectively (Baker, 1991; Benson, 1994). Over periods longer than one year, the lognormal model may not adequately describe survival of oospores in the residual phase, and the fitted curves might over or underestimate survival on the long run.

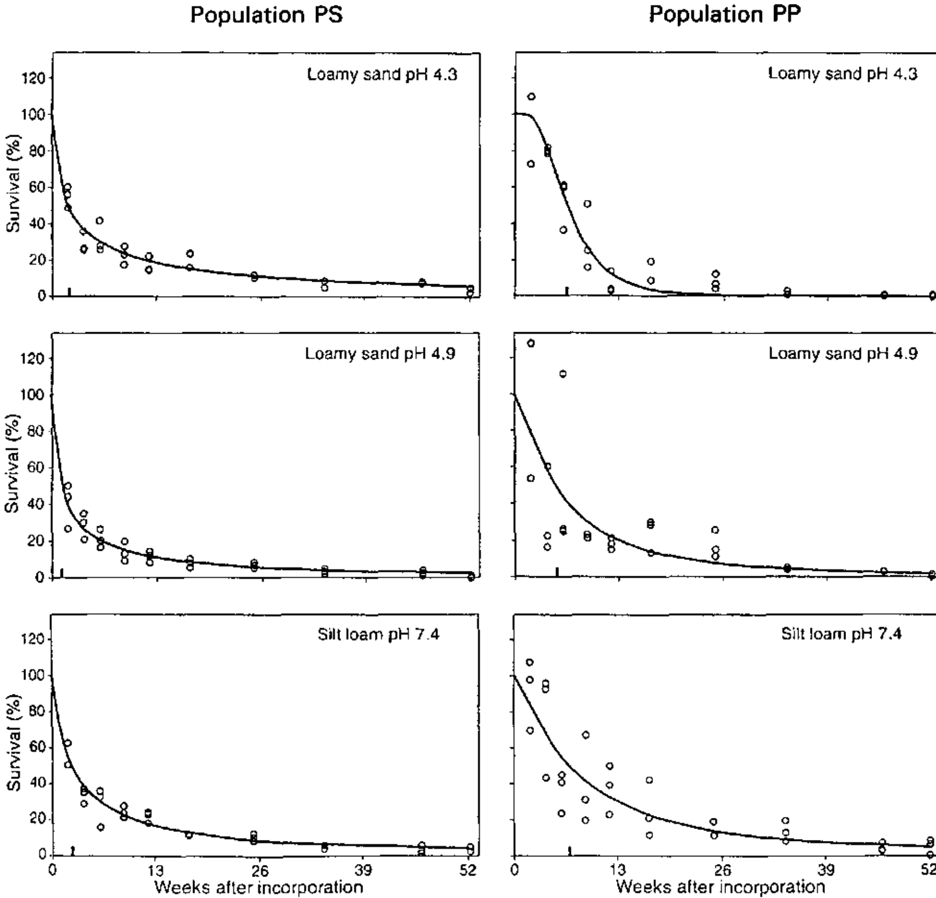

Fig. 7.6. The outdoor experiment. Survival curves of oospores of *Peronospora viciae* f.sp. *pisi* incorporated in three soils and exposed to natural conditions. Fitted lines are lognormal survivorship functions. Median values are indicated by a vertical bar on the x-axis. Calculations are explained in the text (data analysis).

Table 7.8. Percentages of oospores of *Peronospora viciae* f.sp. *pisi* from which hyphae developed belonging to microorganisms other than *P. viciae* after incubation in soil for 0 to 34 weeks

Oospore		Soil		
Population	Wk[a]	Loamy sand pH 4.3	Loamy sand pH 4.9	Silt loam pH 7.4
PS	0	0.0 (0.0)[b]	0.0 (0.0)	0.0 (0.0)
	2	4.8 (3.6)	15.2 (5.1)	2.9 (2.9)
	4	10.6 (6.2)	8.0 (2.3)	3.6 (1.8)
	6	16.4 (3.8)	4.9 (2.0)	11.4 (0.3)
	9	18.0 (2.5)	16.9 (5.2)	5.6 (0.7)
	12	21.0 (2.0)	15.3 (6.2)	10.8 (2.6)
	17	30.9 (5.7)	18.4 (1.3)	12.5 (3.0)
	25	32.2 (0.6)	33.5 (5.8)	14.4 (0.3)
	34	34.7 (5.5)	19.2 (4.7)	12.3 (2.3)
PP	0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	2	2.8 (1.6)	11.8 (2.7)	9.4 (1.0)
	4	1.9 (0.9)	6.5 (3.3)	3.6 (1.8)
	6	20.1 (5.4)	13.7 (1.2)	16.7 (3.4)
	9	33.4 (8.4)	13.5 (5.9)	14.1 (1.0)
	12	49.7 (4.3)	28.7 (13.1)	24.1 (0.8)
	17	50.8 (2.6)	26.3 (10.4)	21.0 (1.3)
	25	66.1 (3.4)	21.8 (5.8)	24.2 (3.0)
	34	64.5 (4.1)	23.1 (5.7)	19.6 (4.0)

[a] Number of weeks after incorporation of oospores soil.
[b] Values are means of three replications of 100 oospores each. In parentheses the standard error.

The lognormal model is characterized by two parameters, μ and σ, the scale and shape parameters, respectively. The greater the value of μ the greater the median survival time 10^{μ}. The greater the value of σ the more skewed the survival time distribution. The values for μ, and thus the median survival times were greater for PP than for PS oospores because protection of PP oospores by host plant tissue lasted longer than for PS oospores. However, the values for σ were smaller for PP than for PS oospores and percentages of oospores which had survived for 52 weeks in soil did not significantly differ between oospores from the two populations. Apparently, in the

long term, the host plant tissue initially surrounding the oospores does not increase the survival time of oospores in soil.

The marked decline in survival soon after incorporation in soil occurred in each soil at constant temperature (except at the low temperature of 3°C) as well as under natural conditions. Thus, soil abiotic and/or biotic conditions which are not specific for a particular soil are the major causal factors for oospore death in the steep part of the survival curves. We propose to call these non-specific soil conditions the primary causal factor of oospore death. These conditions possibly caused an increased respiration of the oospores after incorporation in soil leading to depletion of endogenous energy reserves and finally death of many oospores. In the flatter part of the survival curves the probability of oospores to die decreases and secondary, more soil-specific, factors, such as mycoparasitism may become of increasing importance in decreasing the numbers of oospores in soil. Oospore populations consisting originally of a large proportion of oospores not able to survive for long periods in soil, and of a small, non-random, proportion able survive for prolonged periods may explain the trend of a first increasing and later decreasing probability of oospore death. Survival data reported here correspond to the low percentages of survival in soil previously found for oospores of *P. viciae* f.sp. *fabae* and reported for members of the Pythiaceae (Harris, 1985; Bowers, 1990; Johnson *et al.*, 1990; Van der Gaag and Frinking, 1997d), and support the generally low survival of oospore populations in soil.

Parasitism of oospores

A high degree of parasitism of oospores probably contributed to the low percentages of survival in the silt loam soil at 10 or 20°C after 29 weeks. Many oospores showed outgrowth of hyphae other than *P. viciae* germ tubes in the germination assay. Possibly, the aggregation of the oospores in soil favoured a high degree of parasitism. In the field, comparatively few oospores from PP had survived in LS4.3 more than 26 weeks (Fig. 7.6). This low percentage survival was also indicated by the low value for the shape parameter σ (Table 7.7). The high level of aggregation of PP oospores, together with conditions favourable for oospore mycoparasites, possibly caused the low percentages of survival. The low soil pH may have stimulated fungi to grow and colonize oospores as inhibition of fungal growth by bacteria in soil usually decreases with decreasing pH (Rosenzweig and Stotzky, 1979). High percentages of black stained oospores and oospores showing outgrowth of invaders were found with PP in LS4.3 (Tables 7.5, 7.8).

Hyphal outgrowth from oospores as shown in the germination assay and quantified on water agar is probably not restricted to mycoparasites but may also originate from colonizers of dead oospores. Although samples were sonicated and rinsed with water in a sieve after centrifugation on sugar, other fungi may have adhered to the oospores and germinated on water agar. The oospore wall obscures the internal structure of the oospore and therefore the determination whether hyphae originated from within an oospore or not.

Germinability curves of oospore populations

A previous study demonstrated that germinability of oospores of *P. viciae* f.sp. *pisi* is low just after formation but increases during the first few months of dry storage at 5 and 20°C (Van der Gaag and Frinking, 1997b). In the temperature experiment

described in the present paper germinability of oospores embedded in plant tissue and incorporated in soil increased earlier than those stored dry (Fig. 7.2). Apparently, the period of primary dormancy, following the formation of oospores, is shortened under moist conditions. After decomposition of the surrounding tissue, germinability decreased (Figs. 7.1, 7.2). This decrease can be explained only in part by death of oospores since the ratio between percentage germination and percentage red stained oospores also decreased. Large fluctuations in germinability of dry-stored oospores (Fig. 7.2), have also been reported in a previous study (Van der Gaag and Frinking, 1997b). The underlying mechanism of this phenomenon is unknown.

What is the state of oospores which do stain red in MTT and thus are assumed to be alive, but do not germinate in water? Some of these oospores may no longer be able to germinate and consequently may be non-viable. Alternatively, some oospores may have lost their ability to germinate in pure water and become dependent on a chemical stimulus for germination. Germinability of dry-stored oospores in water has been shown to be directly related to the ability of the oospores to infect the host plant (Van der Gaag and Frinking, 1997b). In soil, however, oospores may enter a state in which they germinate only in the presence of a chemical stimulus exuded by the host plant. No germination was observed of oospores extracted from the silt loam soil after 52 weeks. However, oospores from the same silt loam soil samples did infect seedlings in the bioassay. The relatively high percentages diseased seedlings do support the idea that some oospores not germinating in water can still germinate in soil in the presence of the host plant. In a previous study, a percentage germination of 4 for dry-stored oospores in water was related to a disease incidence of about 6% (Van der Gaag and Frinking, 1997b), while in the present study the comparable figures were 0 and 13%.

Biotic factors in soil may decrease the ability of oospores to germinate in water. Germinability remained high or even increased (Figs. 7.2, 7.5) when oospores were embedded in plant tissue, protecting them from the biotic environment. Germinability decreased rapidly when the plant tissue had decomposed. Fradkin and Patrick (1985a) found that conidia of *Cochliobolus sativus* (Ito and Kurib.) Drechs. ex. Dastur in soil unprotected from bacterial colonization of their surface developed chemical-dependency for germination much more rapidly than conidia protected from bacterial colonization. Evidence for biotic effects on germinability of oospores of *P. viciae* f.sp. *pisi* were obtained in a small additional experiment (unpublished data) in which oospores, freed from plant tissue, were incorporated in sterilized and non-sterilized soil. Decreases in percentages of living oospores determined by the MTT-test were similar for oospores from the sterilized and the non-sterilized soil after one week. The percentages of germination in water, however, were significantly higher for oospores from the sterilized soil than from the non-sterilized soil.

Oospores not germinating in the germination assay might be in a state of secondary dormancy, i.e. neither able to germinate in water nor in the vicinity of the host plant, as suggested by the fluctuations in germinability observed over time (Fig. 7.5). The different states of germinability/dormancy which oospores may enter in soil, (i) germinability in pure water, (ii) chemical-dependent germinability, or (iii) secondary dormancy, need further investigation.

PRODUCTION, GERMINABILITY AND SURVIVAL OF OOSPORE POPULATIONS OF *PERONOSPORA VICIAE* F.SP. *FABAE*

8 Production of cospores by *Peronospora viciae* f.sp. *fabae*. *D.J. van der Gaag, H.D. Frinking and C.F. Geerds. Netherlands Journal of Plant Pathology 99 Suppl. 3 (1993): 83-91.*

9 Survival, germinability and infectivity of oospores of *Peronospora viciae* f.sp. *fabae*. *D.J. van der Gaag and H.D. Frinking. Journal of Phytopathology (accepted).*

10 The infection court of Faba bean seedlings for oospores of *Peronospora viciae* f.sp. *fabae* in soil. *D.J. van der Gaag and H.D. Frinking. (submitted).*

8 Production of oospores by *Peronospora viciae* f.sp. *fabae*

Abstract

Oospore production in *Vicia faba* cv. Metissa was quantified in the field after plants had been inoculated with a conidial suspension of a homothallic isolate of *Peronospora viciae* f.sp. *fabae*. Oospores were produced abundantly during the whole growing season from three weeks after inoculation on. Oospores were found in all plant parts above soil level, except in the seeds. Most oospores were found in the leaves. Less oospores were formed in leaves inoculated in an older stage than in those inoculated in a younger stage. Towards the end of the season, in August, the numbers of oospores in pods strongly increased.

Oospore production in leaves of three cultivars, Metissa, Toret and Maris Bead, was studied in growth chambers at 5, 10, 15, and 20°C at 16 h light. Oospores were formed earlier at higher temperatures than at lower temperatures. The ultimate numbers of oospores produced in leaves were highest at 10 and 15°C. Similar numbers of oospores were formed in leaves of cultivars Metissa and Toret. In leaves of cv. Maris Bead significant less oospores were produced than in leaves of cv. Metissa and cv. Toret. Total numbers of oospores produced were not related to the level of host plant resistance to downy mildew. The percentage of asexually sporulating leaf area, assessed in a resistance test, was largest in cv. Metissa and smallest in cv. Toret.

Introduction

Peronospora viciae (Berk.) Casp., the causal agent of downy mildew on pea (*Pisum sativum* L.), field and broad bean (*Vicia faba* L.), and other leguminous species (Mukerji, 1975) survives in the absence of susceptible hosts, like several other downy mildew fungi, with oospores in plant debris and soil (Dixon, 1981; Populer, 1981; Reiling, 1984). Oospores are formed as a result of sexual reproduction in colonized tissue of the host plant, and they are usually disseminated in the soil with crop residues. The number of oospores which come into the soil after the growing season is of great importance for survival of the pathogen.

Formation of oospores has been studied in different downy mildew species (Inaba and Hino, 1981; Michelmore, 1981; Populer, 1981). Formation of oospores by *P. viciae* in pea has been reported by several authors (Ramsey, 1931; Allard, 1970; Pegg and Mence, 1970; Von Heydendorff, 1977). In Faba bean oospores have been found in leaves, stems, and pods (Glasscock, 1963; Jamoussi, 1968). From literature it is not known whether *P. viciae* isolates are predominantly homothallic or heterothallic.

Environmental conditions affecting sexual sporulation have been studied for several downy mildews. In *Plasmopara viticola* (Populer, 1981) and in *Pseudoperonospora humuli* (Arens, 1930) conditions unfavourable for asexual sporulation appeared to stimulate sexual sporulation. Oospores were found in larger quantities at the end of the season when conditions became unfavourable for asexual sporulation. Inaba and Hino (1980) studied oospore formation of *Peronospora manshurica* at a temperature range of 5-25°C and found most oospores at 15 and 20°C. At 5 and 25°C they did not observe oospores.

Host plant genotype may affect sexual sporulation as well, but while the influence of host plant genotype on asexual sporulation in downy mildews has been subject of much research (Matthews, 1981; Stegmark, 1992), we are not aware of studies on the effect of host plant genotype on sexual sporulation.

Despite the important role of oospores in the disease cycle, sexual sporulation and the effect of environmental conditions and host plant genotype on the amount of oospores produced by *P. viciae* has never been quantified. In this study the production of oospores in Faba bean by *P. viciae* f.sp. *fabae* was quantified. Objectives of this study were: (i) to examine oospore production in different plant parts of field grown Faba bean, and (ii) to determine the effect of temperature on oospore production in Faba bean cultivars with different levels of resistance to downy mildew.

Materials and methods

Sexual nature of the isolate

All experiments were carried out with the same isolate of *P. viciae* (Berk.) Casp f.sp. *fabae* (Jacz. & Serg.) Boerema & *al* (unknown physiological race collected from a bean field in Wageningen, the Netherlands). The isolate was maintained by asexual reproduction in a growth chamber at 10°C. To determine the sexual nature of this isolate, single spore colonies were made as described by Sherriff and Lucas (1989). One ml of a conidial suspension (± 100 conidia per ml) was sprayed on 1% wateragar in a plastic Petri dish. Individual conidia were cut out with a small block of agar and placed spore-side down on leaves of Faba bean cultivar Metissa. Leaves were placed individually in Petri dishes containing 0.5% water agar with 80 μg/ml benzimidazole. Inoculated leaves were incubated for 24 hours at 10°C and subsequently placed at 15°C and 16 hours light. Oospore formation was studied 14 days after inoculation by fixation of leaf pieces during 15 minutes in boiling lactophenol:ethanol (1:2 v/v) with 0.03% trypan blue, and subsequently clearing them for 24 h in a nearly saturated solution of chloral hydrate (5:2 w/v) (modified procedure, Niks, 1986).

Field experiment

On 24 April and 8 May 1991, Faba bean cultivar Metissa was sown in a field on which Faba bean had not been grown for at least 25 years. On each date six plots of 12 x 12.75 m were sown with a between row distance of 50 cm and a within row distance of 9 cm.

On 27 May 16 plants of five plots of each sowing date were inoculated with a conidial suspension (10^4 conidia/ml) by means of a micro ulva sprayer (Micron sprayers LTD, Bromyard, England). At that time plants sown on 24 April and 8 May were in the third and second leaf stage, respectively (code 23 and 21, respectively; Kittlitz *et al*, 1984). Two plots were not inoculated and served as a control.

During the experiment air temperature was monitored by means of a hygrothermograph. Daily rainfall was measured with a rain gauge.

From one week after inoculation on, one plant from each field was harvested weekly (with an interception of one week in the beginning of June) till the end of July and then for the last time on 13 August 1991. Plants were taken to the laboratory and separated into various plant parts: leaves 1 to 3 (L1), leaves 4 to 6 (L2), leaf 7 and

higher (L3), stem, influorescence, and if present, seeds. The numbers of oospores in different plant parts were determined.

Growth chamber experiments

Plant material and inoculation. Three cultivars of Faba bean were chosen: Metissa (MT), Toret (TO), and Maris Bead (MB). For all experiments, four seeds were sown in plastic pots of 11 x 11 cm filled with steamed potting soil. Seedlings were depleted to three plants per pot after emergence. All plants were raised in a growth chamber at 20°C and 16 h light.

Plants were inoculated with a conidial suspension of *P. viciae* f.sp. *fabae* in deionized water (10^4 to 10^5 conidia/ml), in the second leaf stage (code 21, Kittlitz *et al.*, 1984). The inoculum was sprayed on the plants by means of a DeVilbiss atomizer till just before droplets run-off.

After inoculation plants were incubated under plastic cover for 24 h at 10°C temperature at 16 h light to stimulate infection. Then, the covers were removed and pots were distributed randomly over growth chambers with different temperatures: 5, 10, 15 and 20°C and 16 h light. Light intensity was 23 W/m^2.

Production of oospores. Oospore production in leaves was determined in two experiments, in 1991 and 1992, respectively. In 1991 (Exp I) at 5, 10, 15 and 20°C till 31 days after inoculation and in 1992 (Exp II) at 5 and 10°C till 59 days after inoculation. Exp II was carried out to study oospore production at 5°C over a longer period as only minor numbers of oospores were found at 5°C 31 days after inoculation in Exp I.

During the experiments leaves which aborted naturally were left on the soil surface. All leaves of the plants (including the aborted ones) in each pot were harvested at 10, 17, 24, and 31 days after inoculation in Exp I, and 17, 31, 45, and 59 days after inoculation in Exp II. In both experiments six pots of each cultivar were harvested and treated separately on each sampling date for all temperature treatments.

Resistance test. To determine the relative susceptibility of the three cultivars (Metissa, Toret, and Maris Bead), the percentage asexually sporulating leaf area was assessed at 5, 10 and 15°C. At 20°C no conidia were formed. Per temperature and cultivar, six pots each with three plants were inoculated. To induce sporulation a 100 % relative humidity was established by placing plastic covers over the plants two days before first sporulation was expected. This period was based on results of preliminary experiments. The percentage sporulating leaf area was estimated until secondary infections could sporulate (twice the latency period minus one day from time of inoculation on).

Extraction and counting of oospores

Directly after harvesting, plant parts were oven dried in paper bags for 48 h at 100°C and subsequently weighed. Dry plant material was soaked and pulverized in water, using a commercial Waring blendor. In order to loosen the oospores from the plant tissue the suspension was vibrated with intermittent operation during five minutes with a high intensity ultrasonic processor (375 Watt-model, Sonics & Materials, inc. Danbury, Connecticut, USA). After vibration the oospore suspension was filtered

through a set of sieves with pore sizes of 0.3 mm, 90 μm and 20 μm, respectively. The residue on the last sieve was resuspended in about 40-100 ml water and oospores were counted in three droplets of 50 μl using a precision pipet. The exact amount of suspension was recorded to determine the total number of oospores. When less than five oospores per droplet were found the suspension was settled down for 24 h and then carefully decanted until 20 ml was left. The number of oospores was again determined by counting oospores in three droplets of 50 μl. The number of oospores per plant part and the number of oospores per gram dry weight were computed.

Data analysis
The numbers of oospores were transformed with the $\log_{10}$ transformation ($\log_{10}(x+1)$). In the growth chamber experiment the numbers of oospores on the last assessment day were analyzed by means of a Multifactor Analysis of Variance using the software program Statgraphics version 4.0. Treatment means were compared with either a *t*-test (two treatments) or a Tukey-test (three or more treatments). Individual pots were considered as replications.

Results

Sexual nature of the isolate
Mycelium originating from single conidia produced oospores, which indicated the homothallic nature of the *P. viciae* f.sp. *fabae* isolate used.

Field experiment
The first oospores were found three weeks after inoculation. The number of oospores in leaves first increased and later decreased (Fig. 8.1A,B), because leaves dropped. Relatively few oospores were found in stems. In flowers of Faba bean oospores were also produced. Towards the end of the season numbers of oospores in the inflorescence strongly increased (Fig. 8.1C,D) due to oospore production in the pods. Oospores were not found in seeds.

Oospores were formed during cool and rainy weather in the first four weeks of the experiment and during relatively warm and dry weather in the second half of the season (Figs. 8.1 and 8.2).

Time course of the number of oospores was similar in corresponding plant parts of both growth stages of Faba bean, apart from a significant lower number of oospores in the first three leaves (L1) of plants inoculated in the third leaf stage (Fig. 8.1B) than in those of plants inoculated in the second leaf stage (Fig. 8.1A) (*t*-test, $P \leq 0.05$).

In leaves of the non-inoculated control plants oospores were found in relatively low numbers from six weeks after inoculation. This was due to secondary infections.

Growth chamber experiments
Production of oospores. At higher temperatures oospores were formed earlier than at lower temperatures (Fig. 8.3). At 5°C, only few oospores were found in Exp I. In Exp II, where oospore production was examined over a longer period, a clear increase in number of oospores was found on the last sampling date at 5°C (Fig. 8.3).

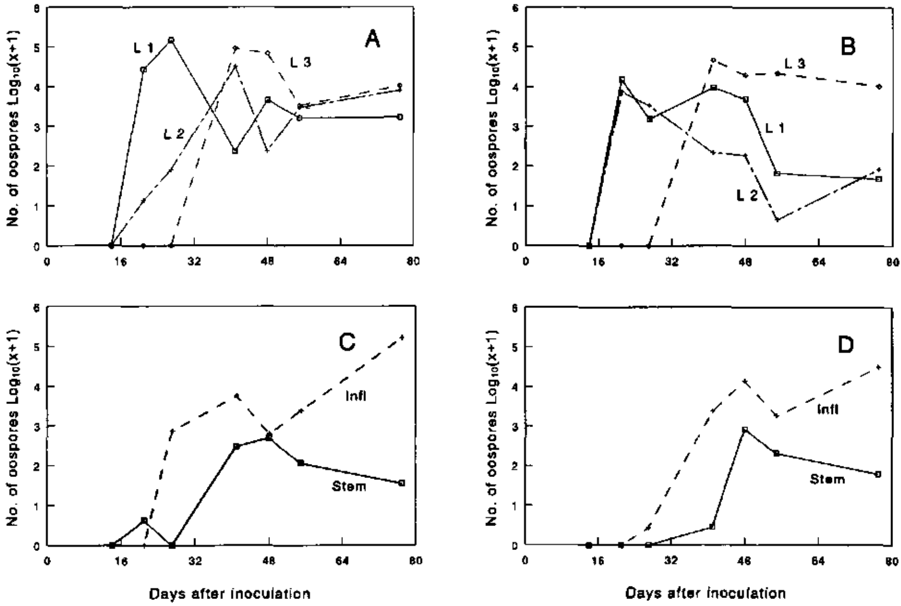

Fig. 8.1. Numbers of oospores of *Peronospora viciae* f.sp. *fabae* per plant in leaves (A,B), stem and inflorescence (C,D) of Faba bean in the field experiment. Plants of cultivar Metissa were inoculated with a conidial suspension in the second (A,C) and third leaf stage (B,D). L1 = leaves 1-3, L2 = leaves 4-6, L3 = leaf 7 and higher, Infl = inflorescence.

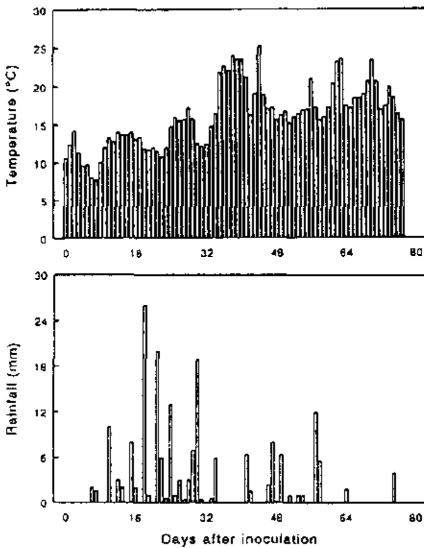

Fig. 8.2. Mean daily temperature and daily rainfall during the field experiment.

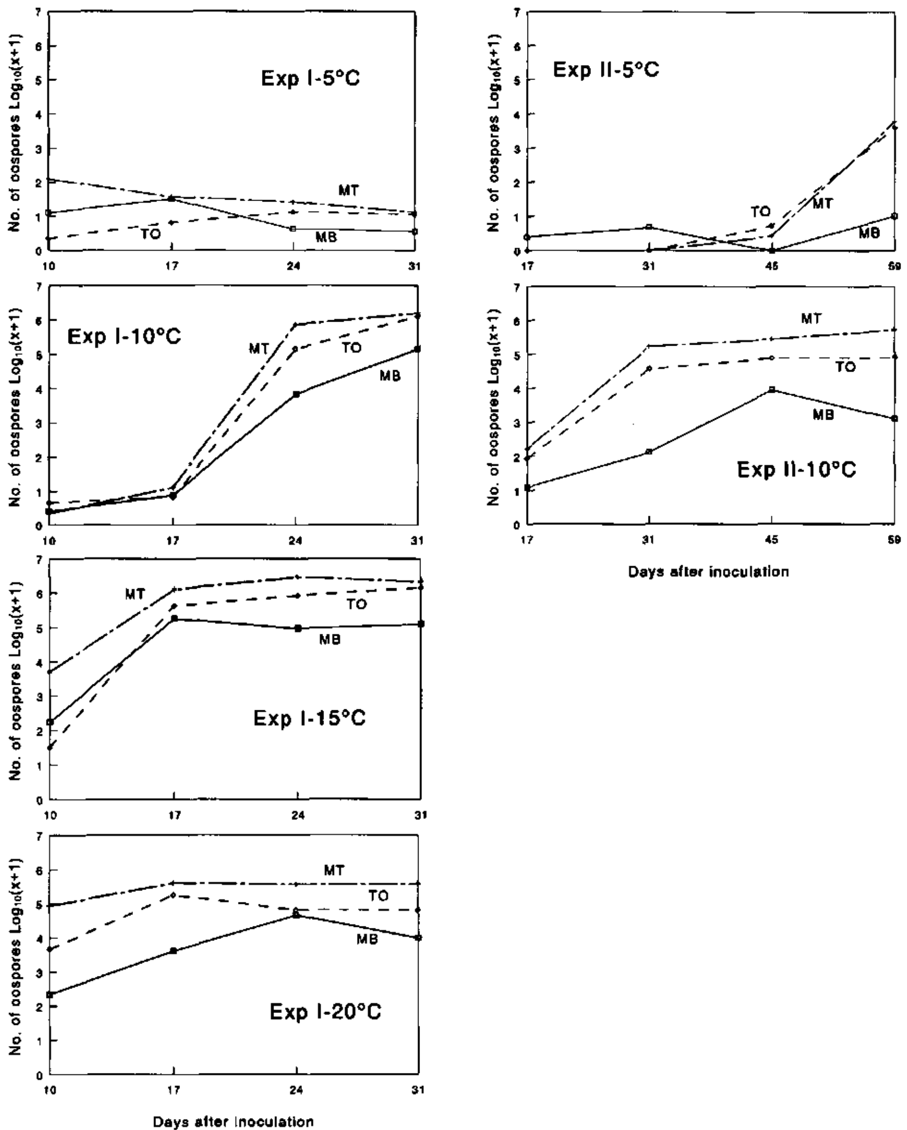

Fig. 8.3. Numbers of oospores of *Peronospora viciae* f.sp *fabae* per plant in leaves of three cultivars of Faba bean: Metissa (MT), Toret (TO), and Maris Bead (MB). Oospore production was examined in two experiments at four different temperatures, 5, 10, 15 and 20°C, in experiment one (Exp I), and at two temperatures, 5 and 10°C, in experiment two (Exp II). Plants were inoculated with a conidial suspension. Values are means of $\log_{10}$ transformed data of six pots with three plants each. Standard error of the mean number of oospores on the last sampling data was 0.29 and 0.42 for Exp I and Exp II, respectively.

Table 8.1. Mean number of oospores of *Peronospora viciae* f.sp. *fabae* per plant and per gram dry weight in leaves of Faba bean at different temperatures 31 days after inoculation. Entries are means of log_{10}-transformed values of three cultivars: Metissa, Toret and Maris Bead

Temperature	No. of oospores per plant	No. of oospores per gram dry weight
5	0.92a[1]	1.08a
10	5.81b	6.12b
15	5.85b	6.08b
20	4.80c	4.93c

[1] Values in a column followed by a different letter are significantly different according to the Tukey-test ($P \leq 0.05$).

No interaction between temperature and cultivar was found (P=0.66 (Exp I) and P=0.62 (Exp II)). Temperature had a significant effect on the numbers of oospores produced. Most oospores, in numbers per plant and numbers per gram dry weight, were extracted from leaves of plants grown at 10 and 15°C (Table 8.1). With increasing temperature plant developmental rate increased and infected leaves died earlier.

In both experiments less oospores (in numbers per plant and in numbers per gram dry weight) were produced in 'Maris Bead' than in 'Metissa' and 'Toret' (Tukey's test, $P \leq 0.05$). The number of oospores produced in leaves of 'Metissa' and 'Toret' did not differ significantly.

Resistance test. At the lower temperatures conidia were formed later than at the higher temperatures. The percentage of asexually sporulating leaf area increased with lower temperatures (Fig. 8.4). 'Metissa' was found to be the most susceptible and 'Toret' the most resistant cultivar as indicated by the percentage of sporulating leaf area (Fig. 8.4).

Discussion

Oospore production in the field

In the field oospores were formed during the whole growing season in all plant parts (Fig. 8.1). Large numbers of oospores were produced during periods when weather conditions were favourable for asexual sporulation as well as during periods when conditions were unfavourable for production of conidia (Figs. 8.1 and 8.2). Apparently *P. viciae* f.sp. *fabae* differs in its behaviour from that of *Plasmopara viticola* (Populer, 1981) and *Pseudoperonospora humuli* (Arens, 1930), where oospores were produced less frequently during periods which were favourable for asexual sporulation. Because *P. viciae* f.sp. *fabae* produces oospores at a large range of weather conditions, conditions favourable for disease development in the crop will lead to large numbers of oospores produced during the season.

Less oospores were found in the first three leaves (L1) of plants inoculated in the third leaf stage than plants inoculated in the second leaf stage. This was likely due to a lower number of infections and a restricted fungal growth in older leaves than in younger leaves (Mence and Pegg, 1971; Dickinson and Crute, 1974; Von Heydendorff, 1977).

The effect of temperature on oospore production

Oospores were formed by *P. viciae* f.sp. *fabae* at all temperatures studied, ranging from 5 to 20°C (Fig. 8.3). Oospore formation of *Bremia lactucae* was also observed at a wide range of temperatures (5-22°C) (Michelmore, 1981).

Oospores of *P. viciae* f.sp. *fabae* were formed earlier at higher than at lower temperatures. These results correspond with those found for *Peronospora manshurica* (Inaba and Hino, 1980), where oospores were also formed earlier at higher than at

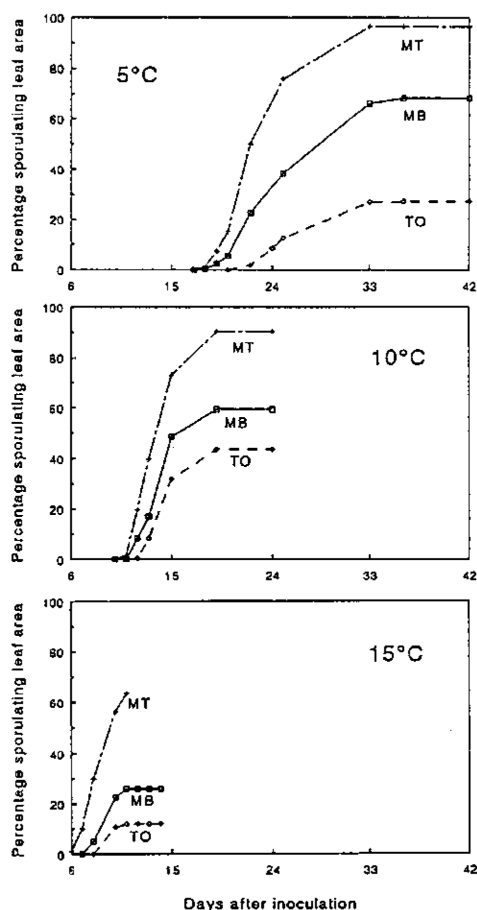

Fig. 8.4. Progress in percentage of asexually sporulating leaf area on three Faba bean cultivars, Metissa (MT), Toret (TO), and Maris Bead (MB). Plants were inoculated with a conidial suspension of *Peronospora viciae* f.sp. *fabae*.

lower temperatures. Most oospores of *P. viciae* f.sp. *fabae* were eventually produced at 10 and 15°C (Table 8.1). The percentage leaf area with conidial production increased with decreasing temperature (Fig. 8.4), which indicated that a larger leaf area is colonized by the fungus at lower temperatures than at higher temperatures. Therefore, similar numbers of oospores produced at 10 and 15°C may suggest a lower oospore density in colonized leaf tissue at 10°C than at 15°C.

Temperature influences the growth rate of the host plant tissue and therefore it may indirectly affect oospore production. With increasing temperature host plant tissue dies earlier and fungal growth will be inhibited earlier than at lower temperatures. The lower numbers of oospores found at 20°C than at 10 and 15°C may be explained by a higher rate of development of the host plant tissue, relative to the growth rate of the fungus at 20°C than at 10 and 15°C.

The effect of cultivar on oospore production

The numbers of oospores produced in leaves of the three cultivars were not correlated with the percentages asexually sporulating leaf area as determined in the resistance test. The numbers of oospores produced did not differ significantly between 'Metissa' and 'Toret', although the percentage diseased leaf area of 'Metissa' was larger than that of 'Toret' (Fig. 8.4). The percentage diseased leaf area of 'Maris Bead' was intermediate between those of 'Metissa' and 'Toret', but about 10 time less oospores were formed in leaves of 'Maris Bead' than in leaves of 'Metissa' and 'Toret'. Since the total leaf area of the three cultivars did not differ much, these results suggest that differences in oospore density can occur in leaf tissue of different Faba bean cultivars.

9 Survival, germinability and infectivity of oospores of *Peronospora viciae* f.sp. *fabae*

Abstract

A series of experiments was conducted to germinate oospores of *Peronospora viciae* f.sp. *fabae*. With rare exceptions, dry-stored oospores did not germinate in water nor did they infect Faba bean seedlings in soil. Long-term storage, pretreatment with $KMnO_4$ or addition of nutrients to the medium did not induce germination. Survival and infectivity of dry-stored oospores were compared to those of oospores incorporated in a silt loam and a loamy sand soil in the field during 21-22 months. Under dry conditions, the percentage of living oospores did not change as determined by the vital stain tetrazolium bromide. In soil, less than 2% of the oospores had survived after 21 months. Infectivity of oospores was determined by a bioassay 17 and 21 months after oospores had been incorporated in soil. Diseased seedlings were obtained after inoculation of Faba bean seeds with oospores extracted from soil but not with the dry-stored ones. Soil samples from two field plots naturally infested with oospores two and three years before the bioassay were infective. Oospores collected with diseased plant material on one of these plots and subsequently stored dry for three years were not infective. The results suggested that oospores need a period of natural weathering to become germinable and infective.

Introduction

Peronospora viciae (Berk.) Casp. f.sp. *fabae* (Jacz. & Serg.) Boerema *& al.* (Boerema *et al.*, 1993) is the causal agent of downy mildew on Faba bean (*Vicia faba* L.). The pathogen disperses by conidia formed on infected leaves at high humidity. Within the infected host tissue, the pathogen produces large numbers of oospores (Van der Gaag *et al.*, 1993). The oospores are believed to be the survival units in the absence of the host plant (Dixon, 1981). However, few data on survival and germination of the oospores are available. Jamoussi (1968) reported germination of oospores, but he did not mention germination conditions nor percentages. Soils infested with oospores of the downy mildew fungus on pea (*Pisum sativum* L.), *P. viciae* (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven can be infective for several years (Stegmark, 1994; Van der Gaag and Frinking, 1997a). However, no such data are available for oospores of *P. viciae* f.sp. *fabae*. Knowledge is needed on the survival and infectivity of the oospores to assess their importance in the life cycle of the pathogen. Therefore, a series of experiments was conducted to study survival, germinability and infectivity of oospores of *P. viciae* f.sp. *fabae*. Germinability and infectivity of dry-stored oospores were compared to those of oospores incorporated in soil.

Materials and methods

Germinability of dry-stored oospores

The isolate of *P. viciae* f.sp. *fabae* used in this study was collected from a bean field in Wageningen (the Netherlands) and was maintained asexually on Faba bean

Table 9.1. Experiments conducted to induce germination of oospores of *Peronospora viciae* f.sp. *fabae*

Pretreatment[a]	Germination medium[b]	Physical conditions[c]
-	water	-
-	water	16h light(10W/m^2)/8h dark
-	soil extract	-
-	root extract	-
-	Gibberellic acid (GA_3) 0.001/0.01/0.1/1 ppm	-
0.1% $KMnO_4$ for 15 min.	water	-
-	0.01% $KMnO_4$	-
-	water	5/10/15°C
-	water without antibiotics	-
Dry storage at 0°C for 1 yr, followed by 1.5 yrs at 5°C	water	-

[a] Unless stated otherwise, oospores extracted from Faba bean leaves stored dry at 18-22°C were incubated in the germination medium without any pretreatment.
[b] Unless stated otherwise, media were supplemented with 100 μg/ml ampicillin and 10 μg/ml rifampicin.
[c] Unless stated otherwise, oospores were incubated in the dark at 10°C.

seedlings (Van der Gaag *et al.*, 1993). Oospores were produced in leaves of the Faba bean cv. Metissa in growth chambers at 15 or 20°C (Van der Gaag *et al.*, 1993). Diseased leaves containing oospores were stored dry at room temperature (18-22°C) until use. Oospores were extracted from diseased leaves following Van der Gaag and Frinking (1996a). Briefly, leaves containing oospores were comminuted in a blender, sonicated, and finally sieved through mesh sizes of 53 and 20 μm, respectively. The residu on the lower sieve (mesh size 20 μm), containing the oospores, was resuspended in water.

Oospores were incubated in 3 ml of water supplemented with 100 μg/ml ampicillin and 10 μg/ml rifampicin to control bacterial growth in Petri dishes (6 cm ϕ). This combination of antibiotics has no negative effect on germination of oospores of *P. viciae* f.sp. *pisi* (Van der Gaag and Frinking, 1996a). Treatments tested for their effect on germination are listed in Table 9.1. The age of the oospores used in the germination experiments varied from about 1 months to 2.5 years. Two replications were used in each experiment and 100 oospores were observed per replication. Germination was recorded after 14 days. Oospores were classified as germinated when the germ tube was longer than the diameter of the oospore.

Infectivity of dry-stored oospores: bioassay
Seeds of the highly susceptible cv. Metissa were inoculated with oospores following Van der Gaag and Frinking (1997b). Briefly, seeds were surface-disinfested by immersion in 1% NaOCl for 10 min, followed by three rinses with tap water. Surface-disinfested seeds were placed with the flat side horizontally at a depth of 2 cm in a sandy soil (organic matter 0.3%, pH-KCl 7.0) in plastic trays of 17 × 13 × 6 cm. Two rows each of four seeds were sown per tray. One ml of an oospore suspension was gently pipetted onto each seed. Four densities were used: 10^2, 10^3, 10^4, and 10^5 oospores/ml. Two trays per inoculum density were prepared. Trays with inoculated seeds were placed in a growth chamber at 10°C. Twenty-eight days after inoculation, when seedlings were in second leaf stage, trays were covered with polyethyleen to increase humidity and promote conidial formation. The numbers of healthy and diseased (sporulating) seedlings were recorded one day later.

Survival and germinability of oospores incorporated in soil
Soil samples were collected from the upper 15 cm of two field plots and air-dried. Soils were a loamy sand soil (organic matter 2.6%, pH-KCl 4.3) and a silt loam soil (organic matter 2.4%; pH-KCl 7.4). Soils were air-dried and the silt loam soil was ground in a Retsch grinding mill using a 1 mm mesh size sieve (Retsch, Haan, Germany) to allow for a homogeneous mixture with oospores.

Oospores were produced in a growth chamber at 15°C in October 1994. Diseased leaves were harvested, air-dried, and ground to pass through openings 1 mm in diameter. The number of oospores per g leaf tissue was estimated (Van der Gaag *et al.*, 1993). A subsample of 0.2 g ground leaf tissue, containing 10^6 oospores, was mixed through 20 g of soil and placed in a nylon net. Twelve nets were prepared per soil type. Nets were buried in the field in November 1994 and December 1994 at 5 cm depth in the loamy sand and silt loam soil, respectively. Oospores from the same population were also stored dry at room conditions. Nets were sampled on six dates during 21-22 months (Table 9.2). Per sampling time oospores from one net of each soil and from 0.01 g dry-stored tissue were extracted.

Oospores were extracted from soil following Van der Gaag and Frinking (1997a). Briefly, a soil suspension was prepared by mixing the soil in a blender for 3 min followed by sonication of the suspension for 5 min. The soil suspension was then sieved through mesh sizes of 75 and 20 μm, respectively. The residue on the lower sieve (mesh size 20 μm) was resuspended in water and centrifuged on a sucrose solution (2.33 g/ml). The supernatant was poured through a sieve with 20 μm mesh size, and the sugar solution was washed down with tap water. The residue on the sieve, containing the extracted oospores, was resuspended in water. Recovery of the extraction method had been estimated at 66% for 20-g samples of both soils (Van der Gaag and Frinking, 1997c). Oospores were extracted from the plant tissue as described above.

The percentage of living oospores was determined using the vital stain tetrazolium bromide (MTT) (Sutherland and Cohen, 1983; Van der Gaag, 1994). Two hundred μl of the oospore suspension, containing 400-800 oospores was added to 200 μl of a 0.1% MTT solution. After 72 h of incubation at 35°C, 100 oospores were observed. Germinability of oospores in water was assessed by observation of 200 oospores in Petri dishes as described above.

In May and September 1996 three replicate nets were sampled per soil and oospores were extracted from each net. Oospores were also extracted from three subsamples of the dry stored oospore-containing plant tissue. The MTT and germination tests and a bioassay were performed as described above. In the bioassay, 10^3 oospores in one ml water were pipetted onto a seed. As a control one ml water was added per seed. Fifteen seeds were used per replication.

Density and infectivity of oospores in naturally infested soil

In March 1996 sixteen portions of soil (300-400 ml each) were collected from the upper 15 cm from each of two field plots and mixed per plot (plot sample). Plot I (8 × 8 m, silty clay loam soil, pH-KCl 7.2) had been cropped with Faba bean cv. Metissa in 1993 and plot II (3 × 3 m, silt loam soil, pH-KCl 7.4) in 1994. None of the plots had a previous cropping history with Faba bean and oospores of *P. viciae* could not be detected in soil sampled one month before Faba bean was sown in 1993 and 1994. The detection threshold of the extraction method is 0.4/g soil (Van der Gaag and Frinking, 1997a). Plants in both plots had been inoculated with a conidial suspension in second leaf stage using an automatic sprayer (Van der Gaag *et al.*, 1993). From plot I, oospores had been sampled by harvesting diseased plant tissue in August 1993 which had been stored dry at room conditions (dry-stored oospores). As a control, soil with a texture and pH similar to that of plot I was sampled from a field on which Faba bean had not been grown (fresh soil). Each of the three plot samples (soil from plot I and II, and the fresh soil) was mixed and large particles were removed by sieving the soil through openings of 1 cm in diameter.

Oospore density in each plot sample was determined by extraction of oospores from four subsamples of each of 10 g air-dried soil as described above. Oospores extracted were suspended in about 0.5 ml water. The exact volume of the suspension was determined and the number of oospores was counted in each of three droplets of 50 μl. Recovery from 10-g soil samples is about 75% (Van der Gaag and Frinking, 1997a).

A bioassay was performed to compare infectivity of the oospores in the plot samples with the dry-stored oospores. The plot samples were moistened till a pF of 2.0 and incubated at 10°C for five days before sowing. Dry-stored oospores were extracted from plant tissue as described above and mixed through five liter of the fresh-soil to obtain a density of about 100 oospores/g soil, after which the oospore-infested fresh soil (pF 2.0) was incubated at 10°C for two days. Soils were placed in plastic trays as described above (see bioassay). Three rows of five seeds each were sown at a depth of 2 cm. Soil was covered with a layer of 1 cm steam-sterilized sand to reduce evaporation. Infectivity of the dry-stored oospores was also tested by pipetting 500 oospores in 0.2 ml water onto each of 15 seeds placed in a steam-sterilized sandy soil as described above (see bioassay). Non-infested samples of the fresh and steam-sterilized sandy soil were the controls. Five trays were prepared per soil. Trays were covered with polyethyleen till emergence (10 days after sowing) to minimize evaporation and were gently watered weekly beginning 2 weeks after sowing. Five weeks after sowing, trays were covered with polyethyleen and the numbers of healthy and diseased (sporulating) seedlings were recorded one day later.

Results

Germinability and infectivity of dry-stored oospores
Germination of dry-stored oospores was observed in some rare occasions using oospores from different populations and ages varying from one month to 2.5 yrs. Percentages germination never exceeded one and none of the treatments listed in Table 9.1 stimulated germination. Omission of the antibiotics did not stimulate germination either. No infection was obtained in the bioassay with 10^2 to 10^5 oospores per seed.

Survival and germinability of oospores incorporated in soil
The percentage of surviving oospores decreased rapidly in soil and less than 2% of the oospores had survived after 21-22 months (Table 9.2). Germinability of the oospores was low. One per cent germination using the dry-stored oospores was obtained in March 1995. Low percentages germination were obtained with oospores extracted from the silt loam soil from March through September 1996, after incubation in this soil for 15 months or longer (Table 9.2). No germination was observed of oospores extracted from the loamy sand soil. In bioassays, infection of Faba bean seedlings was obtained using oospores extracted from the silt loam soil in May and September 1996 and of oospores extracted from the loamy sand soil in September 1996 (Table 9.2). No infection was obtained using oospores extracted from the dry-stored plant tissue.

Density and infectivity of oospores in naturally infested soil
Infection was obtained by sowing seeds in soil samples from plots which had been naturally infested with oospores (plots I and II, Table 9.3). No infection was obtained after inoculation with oospores sampled from diseased plant tissue on plot I and stored dry for three years. Sowing of seeds in the non-infested fresh soil and the steam-sterilized sandy soil did not give rise to diseased seedlings either.

Discussion

Great differences exist in germinability among oospores of downy mildew pathogens. Attempts to germinate oospores of several downy mildew fungi have been described in literature. Some of these attempts had no or limited success despite the application of various treatments (Populer, 1981). Little is known about the reasons for the poor germination of oospores. It may be due to a lack of stimuli for germination, occurrence of dormancy, or lack of viability. The percentage of germination of dry-stored oospores of *Peronospora viciae* f.sp. *pisi* usually varies between 20 and 60 after incubation in water, and more than 80% diseased pea seedlings were obtained by pipetting the oospores onto seeds or by mixing the oospores through potting soil (Ryan, 1971; Van der Gaag and Frinking, 1997b). In this study on *P. viciae* f.sp. *fabae* germination and infection were rarely obtained using dry-stored oospores. Sowing seeds in potting soil inoculated with oospores as Ryan (1971) did to infect pea seedlings, did not give rise to diseased Faba bean seedlings either. With some rare exceptions, dry-stored oospores of *P. viciae* f.sp. *fabae* did not appear to be germinable nor infective and this was not changed by ageing under dry condition.

Table 9.2. Survival, germinability and infectivity of oospores of *Peronospora viciae* f.sp. *fabae* stored dry and exposed to natural conditions in two soils

Sampling date	Incubation conditions[a]												
	Dry			Loamy sand soil pH-KCl 4.3					Silt loam soil pH-KCl 7.4				
	MTT[b]	Germ[c]	DI[d]	Extr.[e]	MTT	Germ	Surv[f]	DI	Extr.	MTT	Germ	Surv	DI
03-95	83	1.0		n.d.[g]	45	0			n.d.	79	0		
05-95	89	0		n.d.	31	0			n.d.	70	0		
09-95	90	0		4.8×10^5	20	0	16.2		6.8×10^4	74	0	8.5	
03-96	93	0		4.0×10^5	4	0	2.7		5.0×10^4	70	0.5	6.0	
05-96	92	0	0	3.0×10^5	4	0	2.0	0	6.2×10^4	17	1.3	1.8	14.0 ± 7.3
09-96	91	0	0	2.5×10^5	2	0	0.7	3.0 ± 3.0	3.6×10^4	21	0.3	1.3	6.4 ± 3.2

[a] Oospores were stored dry at room conditions or incorporated in the field in a loamy sand soil in November 1994, or in a silt loam soil in December 1994.

[b] Percentage of oospores stained red in tetrazolium bromide.

[c] Percentage germination in water after extraction from plant tissue (dry storage) or soil.

[d] Mean disease incidence (%) ± standard error after inoculation of Faba bean seeds with oospores.

[e] Numbers of oospores extracted. The initial number of oospores incorporated in soil was 10^6. Percentage recovery of the extraction method had been estimated on 66 for both soils in a previous study (Van der Gaag and Frinking, 1997c).

[f] Percentage survival as calculated by the formula: $[(N_{extr}P_{eMTT})/(N_{inc}P_{iMTT})][1/Rec]$; N_{extr} is the number of oospores extracted and P_{eMTT} the proportion of the extracted oospores which stained red in MTT, N_{inc} the number of oospores incorporated and P_{MTTi} the proportion which stained red in MTT. *Rec* is the proportion of oospores extracted from soil.

[g] Not determined.

Table 9.3. Numbers of oospores of *Peronospora viciae* f.sp. *fabae* extracted per g soil and disease incidence of downy mildew in bioassays (March 1996) using soil samples from two field plots which had been naturally infested with oospores in 1993 and 1994

Plot	Oospores/g soil[a]	Disease incidence (%)	Faba bean crop[b]
I	10.8 ± 1.7	16	1993
II	7.2 ± 1.1	13	1994
Control	0	0	No Faba bean crop

[a] Mean number of oospores extracted ± standard error, determined from 4 subsamples of 10 g each. Recovery is about 75% and detection threshold 0.4 oospores/g soil (Van der Gaag and Frinking, 1997a).
[b] Year in which the field was cropped with Faba bean in the last 25 years.

Oospores were incorporated in soil and their survival, germinability and infectivity were compared to those of dry-stored oospores. In soil, low percentages of oospores survived for more than a year (Table 9.2). Low percentages survival have also been found for oospores of *P. viciae* f.sp. *pisi* and for members of the Pythiaceae (Harris, 1985; Bowers *et al.*, 1990; Johnson *et al.*, 1990; Van der Gaag and Frinking, 1997c). Thus, a low percentage of survival of oospore populations in soil seems a general phenomenon.

Infection of Faba bean seedlings was obtained after inoculation with oospores of *P. viciae* f.sp. *fabae* which had been exposed to natural conditions in soil for 17 months or longer but not with dry-stored oospores (Table 9.2). A lower disease incidence was obtained with oospores extracted from the loamy sand than from the silt loam soil. Decomposition rate of dead oospores was lower in the loamy sand than in the silt loam soil as more oospores were extracted from the loamy sand soil of which a smaller proportion was living than from the silt loam soil (Table 9.2). Bacterial activity usually decreases with decreasing pH which can explain the difference in decomposition rate as the soil pH of the loamy sand was lower than that of the silt loam soil. In the bioassay, 10^3 oospores were inoculated onto each seed. Only a small proportion of these oospores was living. This proportion was four to ten times higher for oospores extracted from the silt loam than from the loamy sand soil, a difference which may explain the difference in disease incidence. An effect of soil pH and/or clay content on germinability of the oospores might be another explanation for the difference in disease incidence.

A difference between infectivity of dry-stored oospores and oospores exposed to natural conditions was also shown by the observation that naturally infested soil was infective while oospores sampled from this field and stored under dry conditions were not. The results suggest that oospores of *P. viciae* f.sp. *fabae* need a period of natural weathering before they are able to germinate and cause infections. Such a dormancy behaviour would resemble that of oospores of *Peronospora destructor* (Berk.) Casp. ex Berk., and *Peronospora tabacina* Adam which became germinable after four and two years exposure to natural conditions, respectively (McKay, 1957; Borovskaya, 1968). No germination was observed of oospores of *Peronospora destructor* that were kept in dried leaves under laboratory conditions (McKay, 1957). A possible mechanism of

dormancy may be impermeability of the wall of freshly formed oospores and a subsequent increase of permeability under natural conditions whereas no change occurs under dry conditions.

The present study does not specify the conditions needed by oospores of *P. viciae* f.sp. *fabae* to become germinable and infective. Probably, incubation in wet soil for some time is sufficient. In addition, natural fluctuations in temperature may be necessary. A study is needed to determine how long oospores have to stay in soil before they become infective. In a small additional experiment in which oospores were incubated in the silt loam soil at 0, 10, or 20°C (pF=2.0) for three or eight weeks no infection was obtained in a bioassay. As germination of oospores extracted from the silt loam soil was observed from 15 to 21 months after incorporation, oospores might need more than one season in soil to become germinable.

10 The infection court of Faba bean seedlings for oospores of *Peronospora viciae* f.sp. *fabae* in soil

Abstract

The infection court of Faba bean seedlings for oospores of *Peronospora viciae* f.sp. *fabae* in soil was determined. Soil naturally infested with oospores was placed as 3-cm thick layers at four different depths relative to Faba bean seeds. Seedlings with downy mildew were obtained only from seeds sown in the middle of a 3-cm layer of oospore-infested soil. No infection was obtained from oospore-infested soil placed more than 1.5 cm above or below seeds. Histological observations showed that the hypocotyl and the first part of the main root were the most probable sites of infection.

Introduction

Downy mildew on Faba bean (*Vicia faba* L.) is caused by *Peronospora viciae* (Berk.) Casp. f.sp. *fabae* (Jacz. & Serg.) Boerema *& al.* (Boerema *et al.*, 1993). The pathogen is assumed to persist between growing seasons of the host plant as oospores in soil (Jamoussi, 1968; Dixon, 1981). Oospores can survive in soil for at least two and a half years (Van der Gaag and Frinking, 1997d). However, the infection court i.e. the plant part susceptible to infection (Gilligan, 1990), for oospores is unknown.

The epicotyl, hypocotyl, and some root parts of pea seedlings are infection courts for oospores of *P. viciae* (Berk.) Casp. f.sp. *pisi* (H. Sydow) Boerema & Verhoeven. Placing potting soil infested with oospores above seeds or at sowing depth resulted in a disease incidence of 90 to 100%. Inoculum placed below sowing depth resulted in a lower disease incidence of 20 to 45% (Ryan, 1971).

The objective of this study was to determine the infection court of Faba bean seedlings for oospores of *P. viciae* f.sp. *fabae* in soil.

Materials and methods

Placement of inoculum

Soil was sampled from the upper 15 cm from a field plot with a history of Faba bean downy mildew on an experimental farm in Wageningen (the Netherlands). Soil with a similar texture but without a history of downy mildew was sampled from a field on the same experimental farm and served as control soil.

Soil was sieved through openings of 5 × 5 mm and thoroughly mixed. Oospore density was estimated by extraction of oospores from four subsamples of 10 g soil each, following Van der Gaag and Frinking (1997a). Briefly, a soil suspension was prepared by mixing and sonication. The suspension was sieved through mesh sizes of 75 and 20 μm, and the residue on the latter sieve was centrifugated on a sucrose solution. The method extracts about 75 % of the oospores and the detection threshold is about 0.4 oospore/g soil.

Water was added to the soil till a pF of 2.0 - 2.1 was reached and the soil was incubated in a plastic tray at 10°C for two days. Infested soil was placed at 3 cm layers in control soil at four different depths in pots 20 cm in diameter and 17 cm deep

(Fig. 10.1). In one treatment only control soil was used. Twenty seeds of the highly susceptible Faba bean cv. Metissa were sown per pot at a depth of 4.5 cm. A 1.5-cm layer of steam-sterilized sand was deposited on top of the soil to reduce water loss by evaporation. Each pot was placed on a dish, weighed and placed in a growth chamber at 10°C with 16 h light. Pots were weighted weekly and water was added to the dish until the initial weight was reached. Five weeks after sowing, when seedlings were in the second or third leaf stage, pots were covered with polyethyleen to increase humidity for induction of conidial formation. One day later the numbers of non-sporulating and sporulating seedlings were counted.

The experiment was performed twice using soil originating from plots on two different experimental farms. In Expt I, the soil was a silty clay loam soil (pH-KCl 7.2) which had been infested with oospores of *P. viciae* f.sp. *fabae* in 1993. In Expt II the soil was a silt loam soil (pH-KCl 7.4) which had been infested in 1994 (Van der Gaag and Frinking, 1997d). Six replicate pots were used per treatment in each experiment.

Data were analyzed using the nonparametric Kruskal-Wallis test. Computations were done with PROC NPAR1WAY of the Statistical Analytical System which provides a chi-square approximate test (SAS Institute, 1989).

Histological observations

Histological observations were made to determine whether the pathogen infects the epicotyl, hypocotyl/main root, and/or the cotyledons. A 1-cm segment of the epicotyl (diameter about 4 mm) just above the cotyledons and a 1-cm segment of the hypocotyl/main root (diameter 3 - 4 mm) just below the sites of the cotyledons were cut off. Each segment was cut longitudinally into four slices (Fig. 10.2). Petioles of the cotyledons, about 1.5 - 2 mm in length, were harvested and cut longitudinally into three slices. Slices were boiled in 0.3 mg/ml trypan blue in 96% ethanol for 15 min

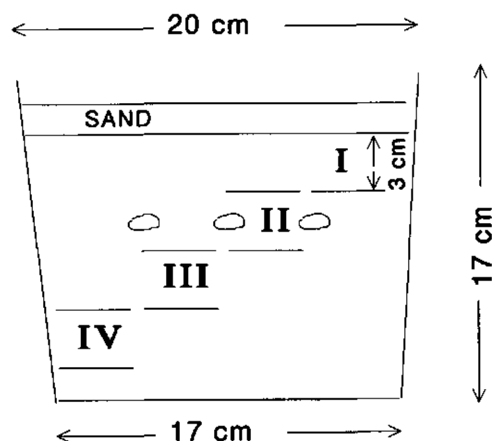

Fig. 10.1. Pot divided into four layers. A 3-cm thick layer of soil infested with oospores of *Peronospora viciae* f.sp. *fabae* was placed at four different depths (layers I-IV) relative to seeds to determine the infection court of faba bean seedlings for oospores.

and incubated in the staining solution for three days. Excessive stain was removed by incubation in an aqueous solution of chloralhydrate (2.5g/ml) for about 4 h. The slices were observed using a Zeiss light microscope (× 250 magnification) for presence of the fungus. Slices from epicotyl and hypocotyl/main root were examined transversally at both ends (Fig. 10.2). Slices from the petioles of the cotyledons were examined over the whole area. Ten seedlings with downy mildew symptoms were examined in this way.

Results and discussion

Thirty-nine ± 2 and 48 ± 12 oospores (mean ± standard error) were extracted from 10-g samples of soils used in Expts I and II, respectively. Estimated oospore densities were (1/0.75) × 39/10 = 5.2 and (1/0.75) × 48/10 = 6.4 per g soil (0.75 is the estimated fraction of oospores extracted).

The position of inoculum in soil had a significant effect on percentages of infection (Kruskal-Wallis test, Chi-square approximation, P=0.0005 and P=0.0001 for Expt I and II, respectively). No diseased seedlings were obtained by placing oospore-infested soil more than 1.5 cm below or above seeds. Percentages of seedlings with downy mildew emerging from seeds sown in a 3-cm layer of infested soil (layer II in Fig. 10.1) were 10.3 and 18.0 in Expts I and II, respectively.

Histological examination did not show any hyphae in the petioles of the cotyledons. Hyphae were observed at both sides of one or sometimes more slices from the epicotyl segment of each of the ten seedlings examined which indicated that the hypocotyl and the first part of the main root were the most probable sites of infection (Table 10.1). Presence of hyphae at both ends of the epicotyl-segment does not exclude the possibility of infection of the epicotyl from soil since infections of the same seedling above and below the cotyledons may have occurred. However, the relatively low percentages of seedlings with downy mildew in the experiments pointed to low numbers of multiple infections. Assuming a random distribution of the oospores

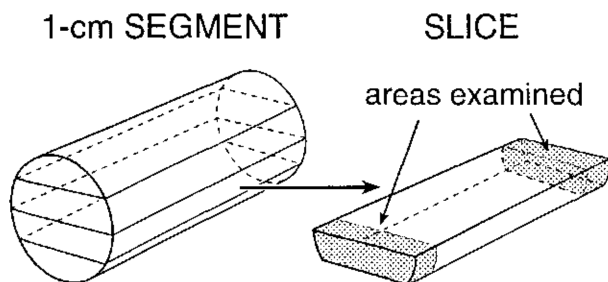

Fig. 10.2. A 1-cm segment from epicotyl or hypocotyl of oospore infected Faba bean seedlings was cut into four slices which were stained in trypan blue. Each slice was examined microscopically at both ends for the presence of hyphae of *Peronospora viciae* f.sp. *fabae*.

through the soils the mean number of infections (m) can be estimated from the proportion of diseased seedlings (x) as $m = \ln(1/(1-x))$ (Gregory, 1948). Using this equation the mean numbers of infections per seedling were estimated at 0.109 and 0.198 in Expts I and II, respectively, which are only slightly higher than the proportions (0.103 and 0.180) of mildewed seedlings.

Assuming that the infection court of the highly susceptible cv. Metissa used in this study is representative for the infection court of Faba bean genotypes in general, it can be concluded that oospore infections more than 1.5 cm below or above Faba bean seeds are unlikely to occur in soil. The infection court of Faba bean seedlings for oospores is much smaller than that of pea seedlings. Oospores of *P. viciae* f.sp. *pisi* can successfully infect the epicotyl but also root parts of pea seedlings down to about 6.5 cm below seed depth (Ryan, 1971). Highest percentages of infection were obtained when the inoculum was at or above seed depth. For other host plants of downy mildew fungi the extent of susceptible sites for oospore infection is not known precisely, but generally plant parts just emerging from a germinating seed are susceptible to oospore infection. Infection of lettuce and cabbage seedlings was obtained by sowing seeds directly on oospore containing tissue (Blok, 1981; Moss *et al.*, 1994). Coating sorghum seeds with powder containing oospores lead to infection (Schuh *et al.*, 1987).

Table 10.1. Presence/absence of hyphae of *Peronospora viciae* f.sp. *fabae* in the petioles of cotyledons and 1-cm segments from epicotyl and hypocotyl/main root of Faba bean seedlings, sown in oospore-infested soil, with downy mildew

		Presence/absence of hyphae		
Expt	Plant	Petioles of cotyledons	Epicotyl[a]	Hypocotyl/ main root[b]
I	1	-[c]	++[d]	+-
	2	-	++	++
	3	-	++	+-
	4	-	++	--
II	5	-	++	+-
	6	-	++	+-
	7	-	++	+-
	8	-	++	+-
	9	-	++	++
	10	-	++	+-

[a] Segment cut from the epicotyl directly above the cotyledons.
[b] Segment cut from the hypocotyl/main root directly below the cotyledons.
[c] - = no hyphae observed.
[d] -- = no hyphae observed at either side of the segment, +- = hyphae observed at one side of the segment, ++ = hyphae observed at both sides of the segment.

Some practical statements can be derived from this study. Screening Faba bean for downy mildew resistance using oospore-infested soil as inoculum, or isolation of the pathogen from soil will be most effective by sowing seeds in a layer of the infested soil. In order to assess the inoculum potential of a field for downy mildew in the next growing season, soil samples must preferably be taken at seed depth after soil tillage.

The small infection court of Faba bean seedlings and its short distance from the seed may provide possibilities for biological control. Biocontrol agents applied to seeds have to colonize or have to be transported with the elongating root tip only over a distance of about 2 cm. Moreover, the biocontrol agent might need to be effective for a short period only as resistance of host plant tissue to downy mildew fungi usually increases with age. Control of primary infections by oospores in soil will be significant, as primarily- infected plants usually die at an early stage which can result in crop losses. Reduction of the number of primarily-infected plants will reduce the amount of secondary inoculum, the conidia, in the field. This reduction will, however, only be significant if the amount of inoculum blown-in from other fields is relatively small.

11 General discussion

Quantification of the amount of oospores in plant tissue and soil

The aim of the research presented in this thesis was to investigate the population biology of oospores of the downy mildew fungus *Peronospora viciae*. The oospores are formed in host plant tissue and will eventually infest the soil under field conditions. Methods for the quantitative detection of the oospores in plant tissue and soil were indispensible to study their biology. Agar plating techniques cannot be used as *P. viciae* cannot be cultured *in vitro*. Therefore, direct enumeration methods had to be developed. Thus, the present study began with the development and evaluation of methods to quantify the amount of oospores in plant tissue and soil.

Methods were available to extract oospores of *P. viciae* from plant tissue, but the percentages of viable oospores present were not known. Since percentages germination of the oospores were usually low and inconsistent in earlier studies (Chapter 1), dormancy was supposed to occur in the oospores. Thus, the first objective was to find a suitable vital stain for assessing the percentage of viable oospores. In pilot experiments, fluorescein diacetate (FDA) was found unsuitable as it did not stain oospores which were assumed to be living. Tetrazolium bromide (MTT) had been used as a vital stain for oospores by other researchers. False positives, dead oospores staining in tetrazolium salts solutions, were reported (Sutherland and Cohen, 1983; Pittis and Shattock, 1994) and were also found in preliminary work on oospores of *P. viciae*. Therefore, staining of non-killed and killed (autoclaved) oospores were compared. It was shown that at pH of 6.0-6.5 only few killed oospores stained in MTT. A non-enzymatic reductor present in autoclaved oospores was suggested to be responsible for staining of killed oospores (Chapter 2). Several additional observations indicated that MTT was a reliable stain. Percentages of staining did not change over a period of almost two years when oospores were stored under dry conditions and percentage staining declined when oospores were stored under humid conditions. A decline in the percentage of oospores which stained in MTT went together with a decline in germinability at high humidity of the ambient air (Chapter 6). The high percentages of newly-formed and dry-stored oospores staining in MTT, usually more than 90%, did not suggest that false negatives, living oospores not staining in MTT, occurred or at least not in large numbers.

The second objective was a standardized germination assay. Control of bacteria was important as bacteria could negatively affect germination. Several antibiotics were tested for their effectiveness to inhibit bacterial growth and for their effect on germination. A combination of 100 μg/ml ampicillin and 10 μg/ml rifampicin inhibited bacterial growth and did not negatively affect germination. The method used to extract oospores from plant tissue did not affect germinability (Chapter 3).

The third objective was to find a method to extract oospores from soil. Two methods described in literature were considered (Pratt and Janke, 1978; Schuh *et al.*, 1988), but these were found either unsuitable for soils with high clay contents or too time consuming. The method described in this thesis was suitable for soils with both high and low clay contents and it was relatively fast, whereas viability of the oospores was not affected. The method requires a blender, centrifuge, and a sonicator. In soils with low clay contents the sonication process may be omitted without affecting the percentage recovery (Chapter 4).

Homothallism and production of oospores

Isolates of two pathogens studied, seven from *P. viciae* f.sp. *pisi* and one from *P. viciae* f.sp. *fabae*, were able to produce oospores in monoculture, and thus homothallic (Chapters 5 and 8).

Oospores were formed in all plant parts. A clear difference was found between the two pathogens. *P. viciae* f.sp. *pisi* had a minimum temperature of about 10°C for oospore formation while *P. viciae* f.sp. *fabae* still formed oospores at 5°C (Chapter 8). These studies on the effect of temperature on oospore production were conducted with only one isolate per pathogen. Differences in temperature mimimum for oospore formation among isolates might occur within each pathogen.

Homothallism may be an advantage for pathogens which depend on oospores to survive host-free periods since colonization of host tissue by two isolates of opposite mating types is no prerequisite for the formation of oospores. Apart from the minimum temperature for oospore formation of *P. viciae* f.sp. *pisi* no specific environmental conditions seemed to be required for formation of oospores. In the field oospores of *P. viciae* f.sp. *fabae* were formed abundantly during weeks in which conditions were favourable and also during weeks in which conditions were unfavourable for conidial formation (Chapter 8). These observations do not fit in the general hypothesis that oospores are formed especially during periods unfavourable for the formation of conidia or sporangia (Populer, 1981). Similarly, Michelmore (1981) could not confirm this hypothesis as formation of conidia and oospores by *Bremia lactucae* Regel occurred simultaneously over the same temperature range (5-22°C). For a plant pathogen such as *P. viciae*, which is probably fully dependent on oospores for its survival, homothallism and the ability to produce oospores under a broad range of conditions obviously is an advantage for survival.

Germination and germinability of dry-stored oospores

Peronospora viciae f.sp. *pisi*

Oospores of *P. viciae* could be stored under air-dry conditions at room temperature (ca. 20°C) for more than a year without loosing their viability. The effect of physical and chemical factors on germination of dry-stored oospores was studied for two reasons. One reason was to gain insight in the behaviour of the oospores and another was to determine optimum germination conditions in the germination assay.

Germination of dry-stored oospores was activated some days after they had been placed in water. High percentages germination were obtained in pure water, a suitable medium to test germinability of oospores. The effects of temperature, light, and chemical factors on germination were studied. Temperatures of 5-10°C appeared to be optimal whereas light inhibited germination (Chapter 6). Addition of host plant exudates did not increase percentages of germination. Germinability of oospores in water was related to their infectivity in a sandy soil. Germination of oospores of *P. viciae* f.sp. *pisi* deviated from that of oospores of many other Oomycetes in their inhibition by light and their nutrient-independency (Chapter 6).

Germinability of dry-stored oospores, indicated as the percentage germination in water in the dark at 10°C after 14 days, increased during the first few months of

storage but the time course of germinability, the germinability curve, differed largely among oospore populations used in different experiments. Decreases followed by increases in germinability, as observed in several experiments, pointed to the occurrence of secondary dormancy in dry-stored oospores (Chapters 6 and 7). No clear periodicity in germinability of oospores stored at constant temperature and RH could be observed and percentages germination were largely inpredictable (Chapter 6).

Peronospora viciae f.sp. *fabae*
Germination of dry-stored oospores of *P. viciae* f.sp. *fabae* was found in few occasions and in low percentages only (1% or less). Treatments successful to induce germination of (oo)spores of other fungi, such as addition of nutrients and $KMnO_4$, did not induce germination of oospores. Seedling infection was found in rare occasions after deposition of large numbers of dry-stored oospores onto seeds in soil. Long-term storage under dry conditions did not increase germinability and infectivity of oospores of *P. viciae* f.sp. *fabae* (Chapter 9).

Survival and germinability curves of oospore populations in soil

Peronospora viciae f.sp. *pisi*
Survival curves. Under dry conditions oospores may have a lifetime of many years. In soil under natural conditions, however, the percentage of surviving oospores decreased soon after they had been incorporated and, after one year, less than 6% had survived (Chapter 7). These results fitted a general hypothesis on survival of fungal propagules in soil which holds that a large proportion of an original population dies soon after incorporation in soil and only a small proportion survives for longer periods (Baker, 1991; Griffin and Baker, 1991). The rapid decline of an oospore population soon after incorporation occurred in each soil studied. The large variation of these soils in texture, pH, and/or cropping history suggested that conditions non-specific for a particular soil caused the rapid death of most oospores. These non-specific conditions were called the primary factor causing oospore death. An accelerated nutrient deprivation of the oospores by increased respiration rates after incorporation in soil may have lead to the rapid death of most oospores. Decline in viability of fungal propagules soon after incorporation in soil has been related to loss of endogenous carbon by increased respiration (Hyakumachi and Lockwood, 1989; Hyakumachi *et al.*, 1989; Mondal *et al.*, 1995;1996).

A small proportion of oospores, less than 10%, survived for a relatively long time and the probability of death decreased over time as demonstrated by the good fit of the lognormal model (Chapter 7). The oospores with prolonged survival must presumably have decreased their respiratory activity in such a way that they can utilize their energy-reserves over a long period of time. What causes this presumably decreased respiration? Certain soil conditions possibly lead to a decreased metabolic activity of some oospores. Oospores not incorporated in soil but stored at high humidity (20°C, 76% RH) survived less than a year. After 300 days of storage at these conditions, only 6% of the oospores stained faintly in tetrazolium bromide and, because of the low staining intensities, were assumed to be non-viable (Fig. 6.3, p. 58). Nutrient deprivation by respiration of the oospores presumably lead to the death of the

oospores. Future research should focus on the long-term survivors in soil to elucidate the soil factors which suppress or induce a low metabolic activity of oospores.

The long-term surviving oospores in the flatter part of the survival curve presumably have a low respiratory activity, and secondary factors such as mycoparasitism may become more important as causal factors of oospore death. Comparatively low percentages survival of oospores in a silt loam soil (pH-KCl 7.4) in a laboratory experiment and in a loamy sand soil (pH-KCl 4.3) in an outdoor experiment were observed. These low percentages of survival were possibly caused by a high degree of parasitism of the oospores in these soils (Chapter 7). Application of mycoparasites to plant debris prior to incorporation of the plant debris into soil might reduce soil-infestation with oospores and consequently downy mildew incidence in a next growing season of the host plant.

The large differences among oospores in survival time in soil may be an inherent characteristic of an oospore population based on genetic and/or non-genetic differences among oospores generated somehow during the process of formation. Variability in environmental conditions at the micro-scale in soil might also contribute to the large differences in survival time. Physical contact with clay minerals for example has been found to prolong survival of *Cochliobolus sativus* (Ito and Kurib.) Drechs. ex Dastur in soil (Fradkin and Patrick, 1985b).

Germinability curves. Germinability of oospore populations increased over time which demonstrated that oospores have a period of primary dormancy (Chapters 6 and 7). For oospores in soil (pF 2), embedded in plant tissue, this period of primary dormancy was shortened in comparison to dry conditions (Chapter 7). After decomposition of the surrounding plant tissue in soil, germinability of oospores in water decreased strongly (Chapter 7). Biotic factors possibly contributed to this decrease since germinability of oospores decreased more rapidly in non-sterilized soil than in sterilized soil while decreases in percentages of living oospores (percentages red stained oospores in MTT) were similar (Table 11.1; data not presented in a former chapter). Differences in germinability decreased over time which may be explained by recolonization of the sterilized soil as oospores extracted from plant tissue are not contaminant-free. Using contaminant-free oospores, thus incubation of oospores under complete sterile conditions, differences might be much more pronounced. Contaminant-free oospores can be produced in aseptically grown pea plants and these oospores can be incorporated in sterile and non-sterile soil to study the effect of biotic factors on their germinability. Physical separation from the soil biota can be achieved by incubation of oospores between membrane filters as Fradkin and Patrick (1985a) did to study germinability of conidia of *Cochliobolus sativus* in soil.

The fluctuations in germinability of soil-exposed oospores in water suggested the occurrence of secondary dormancy (Chapter 7). Development of chemical-dependency for germination was also suggested for oospores in soil. Some oospores may only germinate in the presence of certain compounds (e.g. nutrients) exuded by the host plant in soil since no oospores extracted from soil germinated in water whereas 13% of seedlings were infected in a bioassay using the same batch of oospores (Chapter 7). In an additional experiment not reported before, oospores from the populations PS (from ground seedling shoots) and PP (from pods) were extracted from the three soils (LS4.3, LS4.9, SL7.4) described in Chapter 7, 59 weeks after incorporation. The

Table 11.1. Percentages of living and germinable oospores of *Peronospora viciae* f.sp. *pisi* after incubation in non-sterilized and sterilized soil

Time (weeks)	Living/ Germinable (%)[a]	Soil: Loamy sand pH-KCl 4.3		Soil: Silt loam pH-KCl 7.4	
		Non-sterilized	Sterilized	Non-sterilized	Sterilized
1	Living[b]	70.1	87.1	57.7	58.0
	Germinable[c]	1.7a	14.5b	3.0a	16.0b
2	Living	47.7	53.0	39.9	53.6
	Germinable	0.3a	8.2b	1.0a	0.7a
4	Living	38.6	30.0	45.0	46.9
	Germinable	0.0a	0.7a	0.0a	5.4b

[a] The percentage of living oospores was determined using the vital stain tetrazolium bromide (Chapter 2) and the percentage of germinable oospore by germinating oospores in water (Chapter 7).

[b] Values are means of three replications. Per replication 100 oospores were observed. Previous sterilization of either soil did not affect survival of oospores (F-test, $P>0.05$).

[c] Values are means of three replications. Per replication 100 oospores were observed. Previous sterilization of either soil did affect germinability (F-test: sterilization × time significant at $P=0.0003$ and $P=0.0083$ for the loamy sand and silt loam soil, respectively). Significant differences in germinability are indicated by different letters for each time and soil (t-tests, $P<0.05$).

percentages of living and germinable (in water) oospores were determined, and a bioassay was conducted as described in Chapter 7. Infectivity of the oospores in the bioassay was not correlated with their ability to germinate in water (Spearman rank correlation coefficient, r_s, between disease incidence and percentage germination was -0.05, $P=0.84$, $n=18$), but was correlated with the percentage of living oospores (r_s = 0.85, $P=0.0001$, $n=18$) (Table 11.2; data not presented in a former chapter). High disease incidences were obtained after inoculation with oospores extracted from samples of the silt loam soil (SL7.4) but oospores from the same samples germinated poorly in water (Table 11.2). These results clearly support the idea that oospores which do not germinate in water after incubation in soil may germinate in the presence of the host plant in soil and are in some way chemical-dependent for germination. The properties of oospores incubated in soil differs from oospores stored under dry conditions as germinability of dry-stored oospores in water was related to their infectivity (Chapter 6). Attempts to germinate oospores extracted from soil in the vicinity of pea germlings on water agar failed which may suggest that the soil environment plays a significant role in the induction of germination of the oospores by the host plant. *In vivo* germination of the oospores needs attention in future research.

Table 11.2. Characteristics of oospores of *Peronospora viciae* f.sp. *pisi*, extracted from soil, expressed as percentages of red staining in tetrazolium bromide, of germination in water and of disease incidence in a bioassay

Soil[a]	Oospore population	Red staining (%)[b]	Germination (%)	Disease incidence (%)
LS4.3	PS	11.7 (3.3)[c]	3.6 (2.6)[c]	12.3 (6.4)[d]
	PP	2.0 (1.2)	0.0 (0.0)	0.0 (0.0)
LS4.9	PS	2.0 (1.0)	2.0 (1.0)	4.8 (4.8)
	PP	6.0 (2.8)	3.0 (2.1)	8.9 (4.4)
SL7.4	PS	82.8 (1.6)	0.0 (0.0)	48.1 (1.9)
	PP	85.7 (4.7)	0.7 (0.7)	65.1 (8.3)

[a] For descriptions of the soils and oospore populations see Chapter 7. Oospores had been exposed to natural conditions in soil for 59 weeks.

[b] For descriptions of the tetrazolium bromide (% red stained) and germination (% germination) tests, and the bioassay (disease incidence) see Chapter 7.

[c] Values are means of three replications of 100 oospores each. In parentheses the standard error.

[d] Values are means of three replications of 15 pea seedlings each. In parentheses the standard error.

A dormancy-germinability model for oospores of *P. viciae* f.sp. *pisi*, with primary and secondary dormancies and two germinable states, could explain the observations (Fig. 11.1). More experimental work is needed to test this hypothetical model of four oospore states and to determine the conditions for transition from one state to another.

Peronospora viciae f.sp. *fabae*

Reports on oospores of the downy mildew fungi *Peronospora destructor* (Berk.) Casp. ex Berk. and *Peronospora tabacina* Adam suggest that exposure of oospores to natural conditions is a prerequisite for germination, i.e. for the transition of the (primarily) dormant state to the germinable state (McKay, 1957; Borovskaya, 1968). Natural weathering of oospores of *P. viciae* f.sp. *fabae* might increase germinability and infectivity. Therefore, oospores were mixed through soil and their germinability and infectivity was compared to that of oospores stored under dry conditions (Chapter 9).

Percentages survival of oospores in soil were low and comparable to those found for oospores of *P. viciae* f.sp. *pisi*. After 22 months in soil about 1% of the oospores had survived.

After 15 months (including two winter seasons) of incubation in soil a few germinated oospores were observed in the germination assay. Oospores from the same samples were able to cause infection as shown in a bioassay. Two soils naturally infested with oospores were infective two and three years after infestation, respectively.

The experiments demonstrated that infectivity of oospores increased after incubation in soil under natural conditions. Neither the minimum duration for oospores in soil to become infective nor the time course of the infectivity of an oospore population is known today. A stay longer than one winter season in soil might be needed for oospores to become germinable and infective (Chapter 9). Knowledge of the time course of infectivity of the oospores is indispensable for assessment of the inoculum potential of a field for Faba bean downy mildew.

Host plant infection

The relation between percentage infection and germination showed a straight line passing through the origin, which indicated that one oospore of *P. viciae* f.sp. *pisi* was enough to cause disease (Van der Plank, 1975; Chapter 6). Indeed, it was possible to obtain infection by placing one germinated oospore on the hypocotyl of a young seedling (unpublished data).

The infection court of Faba bean seedlings for oospores was shown to be restricted to the hypocotyl and the upper part of the root over a length of about 2 cm measured from the cotyledons. The limited infection court and its location near the seed may provide possibilities for biological control of Faba bean downy mildew by application of biocontrol agents to seeds (Chapter 10). The infection court of pea seedlings extends over a larger part of the below ground plant parts, including the epicotyl, hypocotyl, and also root parts, as oospores were able to cause disease up to a depth of about 6 cm below the seeds (Chapter 10; Ryan, 1971). The extent of the infection court of the host plant must be known to determine at which depth soil should be sampled to assess the inoculum potential of a field.

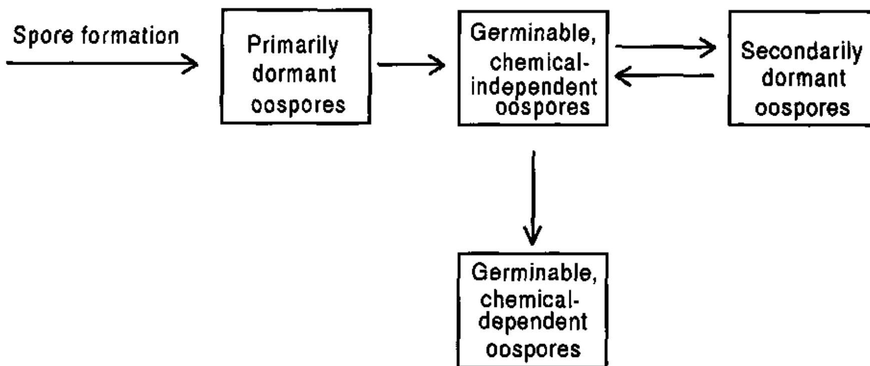

Fig. 11.1. A hypothetical model for the behaviour of oospores of *Peronospora viciae* f.sp. *pisi*, expressed in states and state transitions.

The role of oospores in the epidemiology of downy mildew

Oospores in soil are considered to be the only source of primary inoculum of *P. viciae* f.sp. *pisi* at the beginning of the growing season (Stegmark, 1994). Soil-borne oospores can give rise to high levels of disease (Ryan, 1971; Stegmark, 1994). In the present thesis, disease incidences of up to 75% were obtained by sowing seeds in soil samples collected from fields which had previously been cropped with pea in Eastern England (Chapter 4). Oospores have been found in seeds but pea seedlings grown from oospore-contaminated seeds did not develop downy mildew symptoms and seed-borne infection appears to be unimportant (Hagedorn, 1974; Stegmark, 1994). Short rotation periods resulting in an accumulation of oospores in soil, together with cool and wet weather, have been suggested to be key factors of severe downy mildew epidemics (Hagedorn, 1974).

Oospores of *P. viciae* f.sp. *fabae* in soil are also believed to be the primary source of inoculum at the beginning of the growing season in the spring (Jamoussi, 1968; Dixon, 1981). Soil samples collected from fields naturally infested with oospores of *P. viciae* f.sp. *fabae* in the Netherlands two or three years before collection of the samples were infective which indicates that soil-borne oospores can serve as primary inoculum (Chapters 9 and 10). In some areas, peas and beans are also grown as winter crops, called winter peas and winter beans, and these crops may serve as an alternative way for the downy mildew pathogens to survive winter periods. In Eastern England, however, no downy mildew symptoms have been observed on winter crops in autumn or early spring (A. Biddle, pers. comm.). Inoculum overwintering in or on green plants is, therefore, not believed to be an important primary source of inoculum for spring peas and spring beans in that region.

Concluding remarks

Some aspects of the population biology of oospores of two downy mildew fungi, *P. viciae* f.sp. *pisi* and *P. viciae* f.sp. *fabae*, were investigated. Methods were developed for the quantification of oospore populations of *P. viciae*. The isolates of the two pathogens examined were homothallic and produced large numbers of oospores in diseased host plant tissue. About 90% of these oospores were living and this percentage did not change under air-dry conditions during two years of study. A clear difference between oospores of the two *P. viciae* pathogens was observed in the dormancy-germinability patterns. Oospores of *P. viciae* f.sp. *pisi* became germinable, in water, when they were stored under dry conditions and this process was shown to be accelerated when oospores embedded in plant tissue were incorporated in soil. Oospores of *P. viciae* f.sp. *fabae* neither became germinable nor infective under dry conditions. They became infective under field conditions. No ecological interpretation could be given for the difference in dormancy-germinability patterns between the two *P. viciae* forms. Oospores of both pathogens supposedly have the same role as survival units of the pathogens during host plant-free periods, and the host plants, pea and broad(field)bean, are both annual crops grown with similar rotation periods (3-6 years). No conclusions about the dormancy-germinability patterns of oospores can be

derived from their role in the life cycle of the pathogen and the cropping pattern of the host plant.

After incorporation of oospores in soil, a large proportion died within a few months and a small proportion, less than 10%, survived for more than a year. The rapid death of a large proportion of an original spore population in soil and a prolonged survival of a small fraction is considered to be a general phenomenon for soil-borne infection units (Griffin and Baker, 1991). Many studies have been conducted on the behaviour of original populations of spores (or other infection units) of soil-borne pathogens, i.e. populations not reduced in size during exposure to natural conditions. Results obtained using spores from such populations may give limited information on the properties of spores exposed to natural conditions for two reasons, (i) a non-random sample from an original spore population probably survives for a prolonged period of time and constitutes the primary inoculum source in soil, and (ii) the soil environment can change the properties of the spores, for example the conditions at which a spore will germinate. The ideas of a changing composition of a fungal spore population and changes in the properties of the spores under natural conditions in soil should be addressed in future research.

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Summary

Peronospora viciae is a plant pathogenic fungus which causes downy mildew on pea (*Pisum sativum*), field and broad bean (*Vicia faba*). Two pathogens can be distinguished, *P. viciae* f.sp. *pisi* on pea and *P. viciae* f.sp. *fabae* on field and broad bean. These plant pathogens produce oospores in their host plant tissue. The oospores infest the soil after the surrounding plant tissue has decomposed and are the means by which the pathogens survive in the absence of the host plants. In the present thesis, studies on the population biology of the oospores are described. In Chapters 2-4, methods for the extraction of oospores from plant tissue and soil, a vital stain and a germination assay are described. Experimental studies on the production, germinability and survival of oospores of *P. viciae* f.sp. *pisi* on pea and *P. viciae* f.sp. *fabae* on field and broad bean are described in Chapters 5-7, 11 and 8-10, respectively.

Quantification of oospore populations of P. viciae *(Chapters 2-4)*

Chapter 2. Tetrazolium bromide was tested as a vital stain for oospores of *P. viciae* f.sp. *fabae* and *P. viciae* f.sp. *pisi* in phosphate buffers with a pH ranging from 6.0 to 8.0. Oospores stained mostly pink to red, seldom black. The number of black stained oospores increased with incubation time but the total number of pink to red and black stained oospores did not increase. The results suggest overstaining as the cause of black oospores. Staining of non-autoclaved oospores was compared to that of oospores killed by autoclaving. The number of stained, non-autoclaved oospores showed a small increase with increasing pH. The pH had a large effect on staining of oospores killed by autoclaving. Similar numbers of autoclaved and non-autoclaved oospores stained at pH 7.5 and 8.0 but few autoclaved oospores stained at pH lower than 7.0. When using tetrazolium bromide (0.1% solution) as a vital stain for oospores of *P. viciae*, good staining results were obtained with a 48 h incubation at 35°C in solutions at pH 6.0 to 6.5.

Chapter 3. A protocol was developed to extract oospores of *P. viciae* f.sp. *pisi* from plant tissue and to control bacterial contamination in a germination assay. Oospores were extracted by comminuting infected leaves and pods in water, sonicating the suspension and sieving it through mesh sizes 53 and 20 μm, respectively. Extracted oospores were germinated in water. Germination of oospores was negatively influenced by addition of chloramphenicol and penicillin. A combination of 100 μg/ml ampicillin and 10 μg/ml rifampicin strongly inhibited bacterial growth at 10°C, and did not negatively affect germination of the oospores. Washing of oospores in water or 0.02% Tween-80, and sonication did not influence germination. Treating oospore suspensions with cellulase buffered at pH 4.6 for 2 h digested most plant tissue but did not influence germination. Incubation in 0.05 M acetate buffer, pH 4.6, delayed germination. Germination was unaffected when oospores were incubated in 0.05 M citrate buffer with a pH of 4.6.

Chapter 4. A method for the extraction of oospores of *P. viciae* from soil was described and evaluated. Approximately 75% of the oospores added to silty clay loam and loamy sand soil samples were recovered. Percentage recovery was independent of oospore density. This extraction method did not affect viability as determined by the tetrazolium bromide test and a germination assay and may therefore be used to study

survival of oospores in soil. Numbers of oospores extracted from soil samples taken from seven fields which were naturally infested with oospores of *P. viciae* f.sp. *pisi* ranged from 2 to 21 oospores/g soil. Oospore density was not significantly correlated with disease incidence as determined by a bioassay.

Production, germinability and survival of oospore populations of Peronospora viciae f.sp. pisi (Chapters 5-7, 11)

Chapter 5. Monoconidial cultures derived from seven *P. viciae* f.sp. *pisi* isolates, obtained from different countries, were able to produce oospores. Apparently, these isolates were homothallic. Oospore production of one isolate was studied at 5, 10, 15, and 20°C in systemically colonized shoots, and in local lesions on leaflets, stem parts, and pods of the *Pisum sativum* cv. Kelvedon Wonder. The number of oospores produced per gram systemically colonized tissue increased with temperature. In lesions of leaflets and of stem parts, including tendrils, petioles and main stem, most oospores were produced at 20°C. At 10°C, a few oospores were found in stem parts but none in leaflet lesions. At 5°C, no oospores were formed at all. In pods, more oospores were produced at 15 and 20°C than at 10°C, but the effect of temperature on the numbers of oospores was smaller than in the other plant parts. Oospores formed at lower temperatures were larger than those formed at higher temperatures. At 20°C, similar oospore densities were found in leaflet lesions of three cultivars widely differing in resistance to downy mildew.

Chapter 6. The effects of host plant exudates, light and temperature on germination of oospores extracted from plant tissue were investigated. Seed and root exudates did not increase percentages of germination. Light inhibited germination. Germ tubes began to appear after 4, 7, and 14 days of incubation at 15, 10 and 5°C, respectively. Percentages germination were highest and similar at 5 and 10°C. At 20°C germination was poor and at 25°C no germination was observed. When oospores were retrieved from membrane filters incubated on soil for six days, oospores in water at 10°C germinated within two days. On soil significantly fewer oospores germinated than in water. Germinability of oospores stored dark at 5 or 20°C at 30 or 76% RH was studied over a two-years period. Germinability increased during the first ca. 200 days of storage after which fluctuations were observed indicating the occurrence of secondary dormancy. Time courses of germinability were generally similar for oospores stored at the various temperatures and humidities. No effect of light on the time course of germinability was found when oospores were exposed to alternating light-dark periods or stored in continuous dark during 140 days. Germinability of dry-stored oospores in water was related to their infectivity as determined by a bioassay.

Chapter 7. Survival in soil and the effects of soil conditions on germinability of oospore populations of *Peronospora viciae* f.sp. *pisi* were investigated. Percentage survival was determined by the vital stain tetrazolium bromide. Germinability was defined as the percentage of oospores which germinated in water. One to 3 weeks old oospores embedded in plant tissue were incorporated in a loamy sand, pF 2.1, or silt loam soil, pF 2.2, and incubated at 3, 10, or 20°C or stored dry at 20°C and 30% RH. The percentage of surviving oospores in soil decreased rapidly after decomposition of the surrounding plant tissue at 10 and 20°C. After 29 weeks less

than 10% of the oospores had survived. At 3°C, survival was 25% or more after 29 weeks. Germinability of the oospores was 3% at time of incorporation and had increased to 30% or more after 4 weeks in soil, except in the silt loam soil at 10 or 20°C. Increase in germinability of the dry-stored oospores was significantly later than that of the soil-incorporated oospores. In soil, the initial increase in germinability was followed by a decline after decomposition of the surrounding plant tissue. Survival and germinability of oospores under natural conditions were investigated by burying nylon nets containing oospore-infested soil in the field at 5 cm depth. Ground stem and leaf tissue or pod pieces measuring 1 × 1 cm of pea plants containing 7 or 5 months old oospores, respectively, were incorporated in two loamy sand soils or a silt loam soil. Plant tissue in which the oospores were embedded had decomposed within 4 weeks and the percentage of surviving oospores decreased rapidly in each of the three soils, indicating that abiotic and/or biotic conditions non-specific for a particular soil were the major factor causing oospore death. In the various soils, the median survival time ranged from 1.3 to 2.5 weeks for oospores from the ground stem and leaf tissue and from 5.1 to 6.9 weeks for oospores from the pod pieces in the various soils. After 52 weeks, less than 6% of the original oospore populations had survived in each soil. A relatively low percentage survival of oospores from the pod pieces in one of the loamy sand soils was associated with a high percentage of oospores invaded by fungi. The lognormal model described survival curves better than the exponential or Weibull model, indicating that the probability of oospores to die in soil first increased and later decreased. Germinability of the oospores decreased rapidly from 62% at time of incorporation to less than 10% after 4 weeks and varied between 0 and 12% between 4 and 52 weeks after incorporation.

Chapter 11. Germinability in water of oospores which had been incubated in soil was not correlated with their infectivity in a bioassay. High disease incidences were obtained after inoculation with oospores extracted from soil samples whereas oospores from the same samples germinated poorly in water.

Production, germinability and survival of oospore populations of Peronospora viciae f.sp. fabae (Chapters 8-10)

Chapter 8. Oospore production in *Vicia faba*, cv. Metissa, was quantified in the field after plants had been inoculated with a conidial suspension of a homothallic isolate of *P. viciae* f.sp. *fabae*. Oospores were produced abundantly during the whole growing season from three weeks after inoculation on. Oospores were found in all plant parts above soil level, except in the seeds. Most oospores were found in the leaves. Fewer oospores were formed in leaves inoculated in an older stage than in those inoculated in a younger stage. Towards the end of the season, in August, the numbers of oospores in pods strongly increased.

Oospore production in leaves of three cultivars, Metissa, Toret, and Maris Bead, was studied in growth chambers at 5, 10, 15 and 20°C at 16 h light. Oospores were formed earlier at higher temperatures than at lower temperatures. The ultimate numbers of oospores produced in leaves were highest at 10 and 15°C. Similar numbers of oospores were formed in leaves of cultivars Metissa and Toret. In leaves of cv. Maris Bead significant less oospores were produced than in leaves of cv. Metissa and

cv. Toret. Total numbers of oospores produced were not related to the level of host plant resistance to downy mildew. The percentage of asexually sporulating leaf area, assessed in a resistance test, was largest in cv. Metissa and smallest in cv. Toret.

Chapter 9. A series of experiments was conducted to germinate oospores. With rare exceptions, dry-stored oospores did not germinate in water nor infect Faba bean seedlings in soil. Long-term storage, pretreatment with $KMnO_4$ or addition of nutrients to the germination medium did not induce germination. Survival and infectivity of dry-stored oospores were compared to those of oospores incorporated in a silt loam and a loamy sand soil in the field during 21-22 months. Under dry conditions, the percentage of living oospores did not change as determined by the vital stain tetrazolium bromide. In soil, less than 2% of the oospores had survived after 21 months. Infectivity of oospores was determined by a bioassay 17 and 21 months after oospores had been incorporated in soil. Diseased seedlings were obtained after inoculation of Faba bean seeds with oospores extracted from soil but not with the dry-stored ones. Soil samples from two field plots naturally infested with oospores two and three years before the bioassay were infective. Oospores collected with diseased plant material on one of these plots and subsequently stored dry for three years were not infective. The results suggested that oospores need a period of natural weathering to become germinable and infective.

Chapter 10. The infection court of faba bean seedlings for oospores in soil was determined. Soil naturally infested with oospores was placed as 3-cm thick layers at four different depths relative to faba bean seeds. Seedlings with downy mildew were obtained only from seeds sown in the middle of a 3-cm layer of oospore-infested soil. No infection was obtained from oospore-infested soil placed more than 1.5 cm above or below seeds. Histological observations showed that the hypocotyl and the first part of the main root were the most probable sites of infection.

Main conclusions

The plant pathogenic fungus *P. viciae* produces large numbers of oospores in its host plant tissue. A small, probably non-random, proportion of these oospores will survive in soil for prolonged periods. Oospores of *P. viciae* f.sp. *pisi* initially are germinable in water but can loose this ability in soil, and become dependent on the host plant, *Pisum sativum*, for germination. Oospores of *P. viciae* f.sp. *fabae* initially are neither germinable in water nor able to infect the host plant, *Vicia faba*. These oospores become infective in soil under natural conditions.

Samenvatting

Peronospora viciae is een plantenpathogene schimmel die valse meeldauw veroorzaakt op erwten (*Pisum sativum*), tuin- en veldbonen (*Vicia faba*). Twee pathogenen kunnen worden onderscheiden, *P. viciae* f.sp. *pisi* op erwten and *P. viciae* f.sp. *fabae* op tuin- en veldbonen. Deze plantenpathogenen produceren rustsporen, oösporen, in de door hen geïnfecteerde delen van de waardplant. De oösporen besmetten de grond nadat het plantenweefsel waarin ze zijn ingebed is afgebroken. Door middel van deze oösporen overleven de pathogenen in afwezigheid van hun waardplanten. In dit proefschrift worden enkele studies naar de populatiebiologie van de oösporen beschreven. In de Hoofdstukken 2-4 worden een vitaliteitskleuring, een kiemtoets en methoden om oösporen uit plantenweefsel en grond te extraheren beschreven. Experimentele studies naar de productie, het kiemvermogen en de overleving van oösporen van *P. viciae* f.sp. *pisi* en *P. viciae* f.sp. *fabae* worden respectievelijk beschreven in de Hoofdstukken 5-7, 11 en 8-10.

Kwantificering van oösporenpopulaties van Peronospora viciae (Hoofdstukken 2-4)

Hoofdstuk 2. Tetrazolium bromide werd getest als een vitaliteitskleurstof voor oösporen van *P. viciae* f.sp. *fabae* and *P. viciae* f.sp. *pisi* in fosfaatbuffers met een pH-range van 6.0 tot 8.0. Oösporen kleurden meestal roze tot rood, zelden zwart. Oösporen die kleuren worden verondersteld levend te zijn. Het aantal zwart gekleurde oösporen nam toe met de incubatietijd, maar het totaal aantal roze tot rood en zwart gekleurde oösporen nam niet toe. Deze resultaten suggereren dat de zwartkleuring een gevolg is van overkleuring van levende oösporen. Kleuring van niet-geautoclaveerde oösporen werd vergeleken met kleuring van oösporen die door autoclavering gedood waren. Het aantal gekleurde niet-geautoclaveerde oösporen nam licht toe met de pH. De pH had een groot effect op kleuring van door autoclavering gedode oösporen. Vergelijkbare aantallen geautoclaveerde en niet-geautoclaveerde oösporen kleurden bij een pH van 7.5 en 8.0, maar weinig geautoclaveerde oösporen kleurden bij een pH lager dan 7.0. Indien tetrazolium bromide (0.1% oplossing) werd gebruikt als vitaliteitskleurstof voor oösporen van *P. viciae* werden goede kleuringsresultaten verkregen na 48 uur incubatie bij 35°C in oplossingen met een pH van 6.0 tot 6.5.

Hoofdstuk 3. Een protocol werd ontwikkeld om oösporen van *P. viciae* f.sp. *pisi* uit plantenweefsel te extraheren en om de ontwikkeling van bacteriën in een kiemtoets tegen te gaan. Oösporen werden geëxtraheerd door geïnfecteerde bladeren en peulen te vermalen in water en de suspensie te sonificeren en vervolgens te zeven door maaswijdten van respectievelijk 53 en 20 μm. De geëxtraheerde oösporen werden in water geïncubeerd om te kiemen. Kieming van oösporen werd negatief beïnvloed door toevoeging van chloramphenicol en penicilline. Een combinatie van 100 μg/ml ampicilline en 10 μg/ml rifampicine remde de bacteriële groei en had geen negatief effect op de kieming van de oösporen. Wassen van oösporen in water of in een 0.02% Tween-80 oplossing en sonificatie van oösporensuspensies hadden geen effect op de kieming. Door behandeling van oösporensuspensies met cellulase gedurende 2 uur bij een pH van 4.6 werd het meeste plantenweefsel afgebroken maar kieming van de oösporen werd niet beïnvloed. Incubatie in 0.05 M acetaat buffer, pH 4.6, vertraagde

de kieming van de oösporen. Kieming werd niet beïnvloed door incubatie in 0.05 M citraat buffer met een pH van 4.6.

Hoofdstuk 4. Een methode om oösporen van *P. viciae* uit grond te extraheren werd beschreven en geëvalueerd. Ongeveer 75% van de oösporen toegevoegd aan monsters van een klei- en zandgrond werd geëxtraheerd. Het extractiepercentage was onafhankelijk van de oösporendichtheid. De extractiemethode beïnvloedde de levensvatbaarheid van de oösporen niet, zoals die bepaald werd met de tetrazolium bromide test en een kiemtoets. De extractiemethode kan daarom gebruikt worden om de overleving van oösporen in grond te bestuderen. Het aantal oösporen geëxtraheerd uit grondmonsters afkomstig van zeven velden die op natuurlijke wijze met oösporen van *P. viciae* f.sp. *pisi* waren besmet varieerde van 2 tot 21 per g grond. De oösporendichtheid was niet gecorreleerd met ziekte-incidentie verkregen in een biotoets.

Productie, kiemvermogen en overleving van oösporenpopulaties van Peronospora viciae f.sp. pisi (Hoofdstukken 5-7, 11)

Hoofdstuk 5. Monoconidiale culturen verkregen uit zeven *P. viciae* f.sp. *pisi* isolaten, afkomstig uit verschillende landen, waren in staat om oösporen te produceren. De isolaten waren dus homothallisch. De productie van oösporen van één isolaat werd bestudeerd bij 5, 10, 15, en 20°C in systemisch geïnfecteerde scheuten, en in lokale lesies op blaadjes, stengeldelen, en peulen van de *Pisum sativum* cv. Kelvedon Wonder. Het aantal geproduceerde oösporen per gram systemisch geïnfecteerd plantenweefsel nam toe met de temperatuur. In lesies op blaadjes en stengeldelen, waaronder de bladranken, bladstelen en hoofdstengel, werden de meeste oösporen geproduceerd bij 20°C. Bij 10°C werden weinig oösporen in de stengeldelen en geen in lesies op blaadjes gevonden. Bij 5°C werden helemaal geen oösporen gevormd. In peulen werden meer oösporen geproduceerd bij 15 en 20°C dan bij 10°C, maar het effect van temperatuur op het aantal geproduceerde oösporen was kleiner dan in de andere plantendelen. Oösporen gevormd bij lagere temperaturen waren groter dan die gevormd bij hogere temperaturen. Bij 20°C werden vergelijkbare dichtheden gevonden aan oösporen in lesies op blaadjes van drie cultivars die sterk verschilden in de mate van resistentie tegen valse meeldauw.

Hoofdstuk 6. Het effect van waardplantexudaten, licht en temperatuur op de kieming van uit plantenweefsel geëxtraheerde oösporen van *P. viciae* f.sp. *pisi* werd onderzocht. Zaad- en wortelexudaten hadden geen positief effect op de kieming. Licht remde de kieming. De eerste kiembuizen verschenen na 4, 7 en 14 dagen bij respectievelijk 15, 10 en 5°C. Kiemingspercentages waren het hoogst bij 5 en 10°C. Bij 20°C kiemden weinig 1en bij 25°C geen oösporen. Na incubatie op membraanfilters op met water verzadigde grond gedurende 6 dagen kiemden de oösporen in water bij 10°C binnen twee dagen. Op grond kiemden significant minder oösporen dan in water. Het kiemvermogen van oösporen opgeslagen in het donker bij 5 of 20°C en 30 of 76% RV werd bestudeerd gedurende twee jaar. Het kiemvermogen van de oösporen nam toe gedurende de eerste ca. 200 dagen. Daarna werden fluctuaties waargenomen wat duidde op het voorkomen van secundaire kiemrust in oösporen. Het verloop van het kiemvermogen in de tijd was over het algemeen

overeenkomstig bij de verschillende temperaturen en luchtvochtigheden. Er werd geen effect van licht op het tijdsverloop van het kiemvermogen waargenomen wanneer oösporen gedurende 140 dagen werden blootgesteld aan afwisselend licht-donker perioden of aan een continu-donker periode. Het vermogen van luchtdroog-bewaarde oösporen om in water te kiemen was gecorreleerd met hun infectievermogen in een biotoets.

Hoofdstuk 7. Overleving in grond en het effect van grond op het kiemvermogen van oösporenpopulaties van *P. viciae* f.sp. *pisi* werden onderzocht. Het percentage levende oösporen werd bepaald met behulp van de vitaliteitskleuring tetrazoliumbromide. Het kiemvermogen werd gedefinieerd als het percentage oösporen dat kiemde in water. Eén tot drie weken oude oösporen ingebed in plantenweefsel werden geïncorporeerd in een zandgrond, pF 2.1, of een kleigrond, pF 2.2, en geïncubeerd bij 3, 10 of 20°C of droog opgeslagen bij 20°C en 30% RV. In grond bij 10 en 20°C nam het percentage overlevende oösporen snel af nadat het plantenweefsel waarin de oösporen waren ingebed was afgebroken. Na 29 weken had minder dan 10% van de oösporen overleefd. Bij 3°C had 25% of meer 29 weken in grond overleefd. Het kiemvermogen van de oösporen was 3% op het moment van incorporatie en was toegenomen tot 30% of meer na 4 weken in grond, behalve in de kleigrond bij 10 en 20°C. Het kiemvermogen van de droog opgeslagen oösporen nam significant later toe dan dat van de in grond geïncorporeerde oösporen. De initiële toename van het kiemvermogen van oösporen in grond werd gevolgd door een afname nadat het de oösporen omringende plantenweefsel was afgebroken. Overleving en kiemvermogen van oösporen onder natuurlijke condities werd onderzocht door nylon zakken gevuld met met oösporen-besmette grond op een diepte van 5 cm te begraven in het veld. Vermalen stengel- en bladweefsel of peulstukjes van 1 × 1 cm die respectievelijk 7 of 5 maanden oude oösporen bevatten, werden geïncorporeerd in twee zandgronden en een kleigrond. Het plantenweefsel dat de oösporen omringde was binnen 4 weken afgebroken en het percentage overlevende oösporen nam in alle drie de gronden snel af, wat aangaf dat abiotische en/of biotische condities niet specifiek voor een bepaalde grond de belangrijkste oorzakelijke factoren waren voor de dood van de oösporen. De mediaan van de overlevingsduur varieerde van 1,3 tot 2,5 weken voor oösporen afkomstig uit het vermalen plantenweefsel en van 5,1 tot 6,9 weken voor oösporen uit de peulstukjes in de verschillende gronden. Na 52 weken had minder dan 6% van de oösporen van de oorspronkelijke populaties overleefd in elke grond. Een relatief laag overlevingspercentage van de oösporen uit de peulstukjes in één van de zandgronden was gecorreleerd met een hoog percentage oösporen dat in die grond gekoloniseerd was door schimmels. Het lognormale model beschreef de overlevingscurven beter dan het exponentiële of het Weibull model. Dit gaf aan dat de sterftekans van oösporen eerst toenam en daarna afnam in grond. Het kiemvermogen van de oösporen nam snel af van 62%, op het moment van incorporatie, tot minder dan 10% na 4 weken en varieerde tussen de 0 en 12% tussen de 4 en 52 weken na incorporatie.

Hoofdstuk 11. Het vermogen van oösporen die in grond waren geïncubeerd om in water te kiemen was niet gecorreleerd met het infectievermogen van de oösporen. Hoge ziekte-incidenties werden verkregen na inoculatie met oösporen geëxtraheerd uit grondmonsters, terwijl oösporen uit dezelfde monsters slecht in water kiemden.

Productie, kiemvermogen en overleving van oösporen van Peronospora viciae f.sp. fabae (Hoofdstukken 8-10)

Hoofdstuk 8. Productie van oösporen in *Vicia faba* cv. Metissa onder veldomstandigheden werd gekwantificeerd nadat planten waren geïnoculeerd met een conidiënsuspensie van een homothallisch isolaat van *P. viciae* f.sp. *fabae*. Vanaf 3 weken na inoculatie werden gedurende het gehele groeiseizoen oösporen in grote hoeveelheden geproduceerd. Oösporen werden in alle plantendelen gevonden behalve in de zaden. De meeste oösporen werden gevonden in de bladeren. In bladeren die geïnoculeerd waren in een ouder stadium werden minder oösporen gevormd dan in bladeren die in een jonger stadium waren geïnoculeerd. Tegen het einde van het groeiseizoen, in augustus, nam het aantal oösporen in de peulen sterk toe.

Productie van oösporen van *P. viciae* f.sp. *fabae* in bladeren van drie cultivars, Metissa, Toret en Maris Bead, werd bestudeerd in klimaatkamers bij 5, 10, 15 en 20°C en 16 uur licht. Oösporen werden eerder gevormd bij hogere dan bij lagere temperaturen. Het totaal aantal in de bladeren geproduceerde oösporen was het hoogst bij 10 en 15°C. Vergelijkbare aantallen oösporen werden gevormd in bladeren van de cvs. Metissa en Toret. In bladeren van de cv. Maris Bead werden significant minder oösporen gevonden dan in bladeren van de cvs. Metissa en Toret. Het totaal aantal geproduceerde oösporen was niet gerelateerd aan de waardplantresistentie tegen valse meeldauw. Het percentage asexueel sporulerend bladoppervlak, zoals bepaald in een resistentie-toets, was het grootst in cv. Metissa en het kleinst in cv. Toret.

Hoofdstuk 9. Een reeks van experimenten werd uitgevoerd om kieming van oösporen van *P. viciae* f.sp. *fabae* te induceren. Op enkele uitzonderingen na kiemden droog-bewaarde oösporen niet in water noch konden ze tuinboonzaailingen infecteren. Noch het opslaan van de oösporen gedurende lange tijd, noch voorbehandeling van de oösporen met $KMnO_4$, noch toevoeging van nutriënten aan het kiemingsmedium kon kieming induceren. De overleving en het infectievermogen van droog bewaarde oösporen werd gedurende 21-22 maanden vergeleken met die van oösporen ingegraven in een klei- en een zandgrond in het veld. Bij droge bewaring nam het percentage levende oösporen niet af, zoals bepaald met de vitaliteitskleurstof tetrazolium bromide. In grond overleefden minder dan 2% van de oösporen 21 maanden. Het infectievermogen van de oösporen werd bepaald in een biotoets, 17 en 21 maanden nadat de oösporen waren ingegraven. Zieke zaailingen werden verkregen na inoculatie van tuinboonzaden met oösporen geëxtraheerd uit grond maar niet met droog-bewaarde oösporen. Grondmonsters afkomstig van twee velden die twee en drie jaar voor de biotoets op natuurlijke wijze wijze met oösporen waren besmet waren infectieus. Oösporen die verzameld waren in zieke plantendelen op een van deze velden en vervolgens droog bewaard gedurende drie jaar waren niet infectieus. De resultaten suggereerden dat oösporen bloot moeten staan aan natuurlijke omstandigheden voordat ze kunnen kiemen en infectieus zijn.

Hoofdstuk 10. De vatbare delen van tuinboonzaailingen in grond voor infectie door oösporen van *P. viciae* f.sp. *fabae* werden bepaald. Grond die op natuurlijke wijze met oösporen besmet was werd in laagjes van 3 cm op vier verschillende diepten ten op zichte van tuinboonzaden aangebracht. Zaailingen met valse meeldauw werden alleen verkregen uit zaden gezaaid in het midden van een met oösporen besmette 3-cm dikke laag grond. Geen infectie werd verkregen uit besmette grond die meer dan 1.5

cm boven of beneden de zaden was geplaatst. Histologisch onderzoek toonde aan dat het hypocotyl en het eerste deel van de hoofdwortel de meest waarschijnlijke plaatsen van infectie zijn.

De belangrijkste conclusies

De plantenpathogene schimmel *P. viciae* vormt grote aantallen oösporen in de door hem geïnfecteerde delen van zijn waardplanten. Van deze oösporen kan slechts een geringe, waarschijnlijk niet-random, fractie langere tijd in de grond overleven. Oösporen van *P. viciae* f.sp. *pisi* zijn aanvankelijk in staat om in water te kiemen, maar kunnen dit vermogen verliezen na incubatie in grond en dan afhankelijk worden van de waardplant, *Pisum sativum*, om te kiemen. Oösporen van *P. viciae* f.sp. *fabae* kunnen aanvankelijk niet in water kiemen noch de waardplant, *Vicia faba*, infecteren. Deze oösporen worden infectieus in grond onder natuurlijke omstandigheden.

Curriculum vitae

Dirk Jan van der Gaag werd geboren op 3 augustus 1968 in Rotterdam. Hij behaalde in juni 1986 het VWO-diploma en begon in september 1986 met de studie Plantenveredeling aan de Landbouwuniversiteit Wageningen (LUW). In september 1987 werd de propaedeuse 'met lof' behaald. De doctoraalfase omvatte twee afstudeervakken Resistentieveredeling en één afstudeervak Fytopathologie. Van mei 1991 tot en met juli 1991 bezocht hij het Department of Plant Pathology van de North Carolina State University in de Verenigde Staten waar hij onderzoek deed aan ruimtelijke verspreidingspatronen van plantenpathogene schimmels. In augustus 1992 werd het doctoraaldiploma 'met lof' behaald. Van september 1992 tot januari 1993 had hij tijdelijk een aanstelling als toegevoegd universitair docent op de vakgroep Wiskunde van de LUW en gaf hij werkcolleges Voortzetting Statistiek. In februari 1993 begon hij als assistent in opleiding (AIO) aan het onderzoek naar de populatiebiologie van oösporen van de valse meeldauwschimmels op erwt, veld- en tuinboon, zoals beschreven in dit proefschrift. In 1996 was hij secretaris van de organisatiecommissie van de 'Autumn School on soil physical factors and biotic interactions' van de C.T. de Wit onderzoekschool Productie Ecologie. Per 1 april 1997 zal hij als post-doc gaan werken aan biologische bestrijding van pathogene bodemschimmels op de Plant Pathology and Biological Control Unit van de Swedish University of Agricultural Sciences in Uppsala (Enheten för växtpatologi och biologisk bekämpning, Sveriges Lantbruksuniversitet).

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