EFFECT OF OOMYCETE AND PLANT VARIATION ON ZOOSPORE COVER AND DISEASE SEVERITY

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SUMMARY

Four oomycetes (*Pythium aphanidermatum*, *Pythium dissimile*, *Pythium papillatum* and *Phytophthora nicotianae*) and three plants (Lucerne, sugarbeet and tomato) were used in order to study the effects of species diversity on zoospore encystment and pathogenicity. The plant intra-specific variation affected the number of *P. aphanidermatum* zoospores that encysted in the elongation zone of lucerne and tomato seedlings but not of sugarbeet. Differences in encystment were not reflected in disease severity with the exception of root reduction of lucerne seedlings. Differences in zoospore encystment between isolates of *P. aphanidermatum* and *Ph. nicotianae* were reflected to the severity of disease symptoms caused only on lucerne seedlings. The mixed inoculum experiment indicated that in most combinations the encystment density was intermediate between the observed for single oomycete inoculations. Disease symptoms of the mixed inocula varied and either resembled the symptoms caused by one oomycete or did not differ from the symptoms caused by single inoculations.

Key words: *Pythium, Phytophthora*, lucerne, intra-specific variation, mixed inoculum.

INTRODUCTION

Zoospore attraction towards plant roots was first reported by Goode (1956) and has since observed for many species of *Pythium* and *Phytophthora*. The significance of zoospores lies in their role as precise homing agents rather than as active dispersal agents because they have a limited capacity for dispersal unless they are transported in moving water (Deacon and Donaldson, 1993). During the pre-penetration phase, zoospores may be attracted to roots and swim around before coming to rest; the zoospores then encyst and adhere with precise orientation, so that the germ-tube emerges from a pre-determined point contiguous to the host and immediately initiates penetration (Jones *et al*., 1991).

Differential encystment of zoospores on plant roots suggests the involvement of surface-recognition phenomena such as specific differences in exudates (Hinch and Weste, 1979) and natural root-generated electric fields (van West *et al*., 2002). The relative importance of root-generated chemical and electrical cues to the accumulation and encystment of zoospores on root surface probably varies for different oomycete species and under different environmental conditions.

Pythium and *Phytophthora* are thought to be relatively non-specific in host-recognition, mainly because zoospores of several species can show similar tactic responses to both host and non-host roots (Carlile, 1983). Deacon (1988) suggested that the search for specificity has been wrongly directed as it may not occur in the motile phase but rather in the subsequent stages after the zoospores have reached their destinations. Furthermore, Van West *et al*., (2003) concluded that the zoospore phase is non-specific as far as host selection is concerned and the specificity occurs at the time of attempted penetration and invasion of the plant.

Major reviews on zoospore behaviour towards plant roots have concluded that there is a lack of comparative studies of several oomycetes, including parasites of different degree of specialisation and also saprophytic species, in relation to different plants, under standard conditions (Royle and Hickman, 1964; Ho and Hickman, 1970; Deacon and Donaldson, 1993).

The information on pathogenicity of many *Phytophthora* and particularly *Pythium* species is still limited, and restricted to species which cause epidemic diseases. The pathogenicity of *Pythium aphanidermatum* (Edson) Fitzp. has been reported on lucerne (Stanghellini and Burr 1973), sugarbeet (Stanghellini *et al*., 1982) and tomato (Mitchell, 1978). *Pythium dissimile* Vaartaja has been reported as moderately pathogenic only to wheat and oats (Vestberg, 1990). *Pythium papillatum* Matthews was originally isolated from soil (Plaats-Niterink, 1981) and there are no reports on its pathogenicity. *Phytophthora nicotianae* van Breda de Haan has a very wide host range and can cause seedling damping-off and root rot

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on tomato and lucerne (Blaker and Hewitt, 1987; Erwin and Ribeiro, 1996).

The aim of this study was to contribute into our knowledge of the effects of plant and oomycete intraspecific variation on zoospore encystment and disease severity. The inclusion of soil oomycetes that are saprophytes or minor pathogens, in mixed inoculations with proved plant pathogenic species was an attempt to investigate their role in zoospore encystment on plant roots and subsequent disease development.

MATERIALS AND METHODS

Plant growth. The plant species and varieties used in this study are shown in Table 1. Seeds were thoroughly rinsed under running tap water for 10 min and placed in a 70% ethyl alcohol solution for 2 min. Then, they were submerged into a disinfectant solution (sodium hypochlorite 2%, plus 2 drops of wetting agent Tween 20) for 15 min, in a sealed, sterile bottle and gently agitated. Under sterile conditions, the solution was decanted and seeds were washed with sterile distilled water several times. Aseptic seeds were placed in Petri dishes containing a 5 mm layer of water agar and sterile Whatman No.1 filter papers laid over the agar surface. Petri dishes were covered with their lids and placed in incubators (darkness, 20°C). Each healthy germinated seed, with root length almost 5 mm, was transferred to a test tube (1.5 × 15 cm) containing 5 ml of half-strength Hoagland's nutrient solution (Dhingra and Sinclair, 1985) and placed on a platform made from a Whatman No. 1 moist filter paper strip. The edges of the filter paper strip were long enough to extend near the tube bottom in order to maintain constant nutrient supply. The test tubes were sterile with plastic ventilated caps and supported in a black plastic plug tray. Plants were incubated using a 16-h photoperiod in a growth chamber. Light was provided by cool warm fluorescent lamps (40W, RS Components, UK), situated 60 cm above plants.

Photosynthetic photon flux density at leaf height was 180±30 μmols $m^{-2}s^{-1}$. The growth chamber was ventilated, the temperature was maintained at 25±2°C and the relative humidity at 70±10%. Plants were left in the incubators for 3 days before inoculation. Frequent randomization of the locations of cultures inside the incubators was applied to guard against a uniform or non-random environmental variable.

Zoospore production. Oomycete isolates were obtained from the Aquatic Phycomycetes Culture Collection, University of Reading, U.K. (APCC), and the Centraalbureau voor Schimmelcultures, in Netherlands (CBS). The isolates of *P. aphanidermatum*, were isolated from diseased tomato (CBS 634.70) and melon (APCC 4104b) plants. All other isolates of *P. dissimile* (APCC 4204a, 4204c, 4204e, 4204f), *P. papillatum* (APCC 4106a, 4106h, 4106j) and *Ph. nicotianae* (APCC 4600a, 4600b, NR17), were recovered from soil samples and their pathogenicity was unknown. All stock cultures were maintained on hemp seeds immersed in 50 ml of sterile distilled water, in 100 ml sealed flasks at 5°C, in the dark (Dick, 1965). Prior to use in experiments, cultures were removed from the stock solution and plated on 2% water agar for 3 days at 20°C, in the dark. With the aid of a scalpel, small orthogonal pieces (1 × 3 cm) were cut from the periphery of the colony, transferred to cleared V8 broth (Dhingra and Sinclair, 1985) and incubated at 25°C for 2 days, in darkness. Each colonized agar piece was placed in a sterile Petri dish and washed three times (30 min interval) with a mineral salts solution (Chen and Zentmyer, 1970). Finally, the sporangia bearing mycelia were washed with 3 changes of double glass distilled water, then incubated for 15 min at 8°C and returned to room temperature; zoospores were released in about 1 h. Zoospore concentration was determined using a haemocytometer.

Zoospore encystment. A sample of 30 plants for each plant - oomycete combination was selected randomly and a zoosporic suspension was added to each test tube containing a young plant, using a pipette with a long tip, taking care not to touch the plant that was supported on the filter paper bridge. After one hour of incubation, the col-

Table 1. Plant species and varieties used.

Scientific name	Common name	Variety	Supplier
Beta vulgaris L.	Sugarbeet	Saxon	Hilleshog (Norfolk, UK)
		Celt	Hilleshog (Norfolk, UK)
		Amethyst	Hilleshog (Norfolk, UK)
Medicago sativa L.	Lucerne	Vela	Dlf- Trifolium (Gloucestershire, UK)
		Europe	Elsoms Seeds Ltd (Lincolnshire, UK)
		Euver	Elsoms Seeds Ltd (Lincolnshire, UK)
Lycopersicon esculentum Mill.	Tomato	Moneymaker	Johnson Seeds (UK)
		Outdoor girl	Johnson Seeds (UK)
		Ailsa Graig	Johnson Seeds (UK)

onized roots were transferred to observation chambers for microscopic observation at x100 magnification. Zoospore cover was estimated as the percentage of the root elongation area covered with encysted zoospores in one field of view (0.25 mm of root length), at the centre of the area of root elongation.

Pathogenicity. A sample of 30 plants for each plant - oomycete combination was inoculated with a zoosporic inoculum at a certain concentration. Inoculated plants were left at the incubators for 10 days and then were removed from the test tubes. The root length and root discoloration were selected as criteria for disease assessment. Plants were spread over a white plastic surface and the length of their primary root was measured to the nearest millimetre using a ruler. The root length of inoculated plants was expressed as percentage of root reduction compared to control non-inoculated plants of the same species. Root discoloration was visually estimated and expressed as the percentage of the root system that showed browning or water soaking symptoms.

Experimental design and data analysis. Our study consisted of three experiments: a) All plant species and varieties were inoculated with a zoosporic suspension of *P. aphanidermatum* (CBS 634.70), at 10^4 zoospores ml^{-1}. b) Lucerne 'Vela' seedlings were inoculated with all isolates of *P. aphanidermatum*, *P. dissimile*, *P. papillatum* and *Ph. nicotianae*. c) Lucerne 'Vela' seedlings were inoculated with zoosporic mixtures of *P. aphanidermatum* (CBS 634.70), *P. dissimile* (APCC 4204f), *P. papillatum* (APCC 4106a) and *Ph. nicotianae* (APCC NR17) at a final concentration of 10^4 zoospores ml^{-1} (5,000 zoospores ml^{-1} per species) and compared with single species inoculations at 10^4 zoospores ml^{-1} and 5,000 zoospores ml^{-1}.

Experiments were designed as completely randomized. Treatments were compared in pairs using the *t*-test. All tests for significance were conducted at the $p \leq 0.05$ level.

RESULTS

Plant varieties. The percentage of root elongation area of lucerne seedlings covered with zoospores of *P. aphanidermatum* was positively related to root reduction but not to root discoloration rates (Table 2). Zoospores encysted in similar densities on the elongation zone of all sugarbeet varieties. However, the variety 'Amethyst' showed significantly lower root reduction and discoloration rates. Zoospore cover was significantly higher in the elongation zone of the tomato variety 'Ailsa Graig' compared to 'Moneymaker' but this difference was not reflected in disease symptoms.

Oomycete isolates. Isolates of all oomycetes showed significant differences in their encystment responses towards roots of lucerne (Table 3). The *P. aphanidermatum* isolate 4104b showed significantly lower zoospore root cover, root discoloration and root reduction compared to the CBS 634.7 isolate. The isolate 4204c of *P. dissimile* showed the highest rates of encystment and root discoloration. *P. papillatum* zoospores encysted poorly on the roots of lucerne and caused low rates of root reduction and discoloration. The significantly lower encystment of *Ph. nicotianae* isolate 4600b was related to lower rates of root reduction and discoloration compared to isolates 4600a and NR17.

Mixed inoculum. When a mix of equal zoosporic suspensions of *P. aphanidermatum* and *P. dissimile* was applied to the roots of lucerne seedlings, the encysted zoospores covered almost 50% of the elongation zone, a value between the cover rates observed for single species inoculations (Table 4). The root reduction caused by the mixed inoculum was similar to the rates caused by single species while the root discoloration rate of mixed inoculum was similar to that caused by *P. aphanidermatum*.

The mixed inoculum of *P. aphanidermatum* and *P. papillatum* resulted in a mean cyst cover and disease

Table 2. Zoospore cover of the root elongation zone and disease symptoms caused to plant varieties after inoculation with *P. aphanidermatum* (isolate: CBS 634.70). Means within one plant species followed by different letters are significantly different at $p \leq 0.05$ (n=30).

Plant	Variety	Zoospore cover (%) [a]	Root reduction (%)	Root discoloration (%)
Lucerne	Europe	50±8 a	39±5 a	37±4 a
	Euver	94±9 b	48±6 b	45±5 b
	Vela	100±0 c	58±6 c	38±4 a
Sugarbeet	Amethyst	100±0 a	20±4 a	59±6 a
	Celt	98±2 a	38±5 b	85±8 b
	Saxon	100±0 a	39±5 b	84±9 b
Tomato	Ailsa Graig	100±0 a	48±6 a	80±7 a
	Moneymaker	89±9 b	48±6 a	78±7 a
	Outdoor Girl	85±7 b	53±7 b	63±5 b

[a] Mean value ± standard deviations.

Table 3. Zoospore cover of the root elongation zone and disease symptoms of lucerne cv Vela seedlings after inoculation with isolates of *Pythium* and *Phytophthora* species. Means within one oomycete species followed by different letters are significantly different at p≤0.05 (n=30).

Oomycete	Isolate	Zoospore cover (%) [a]	Root reduction (%)	Root discoloration (%)
P. aphanidermatum	APCC 4104b	90±4 a	51±5 a	27±4 a
	CBS 634.70	100±0 b	58±6 b	38±4 b
P. dissimile	APCC 4204a	23±6 a	28±5 a	6±5 ab
	APCC 4204c	42±7 c	48±7 b	25±5 c
	APCC 4204e	25±6 a	46±7 b	8±4 b
	APCC 4204f	33±7 b	52±5 c	4±5 a
P. papillatum	APCC 4106a	7±4 b	17±5 a	1±3 a
	APCC 4106h	9±4 b	12±4 b	2±4 a
	APCC 4106j	2±4 a	8±4 c	1±3 a
Ph. nicotianae	APCC 4600a	48±8 b	59±6 d	47±6 b
	APCC 4600b	24±4 a	46±5 a	24±5 a
	APCC NR17	55±5 c	55±5 b	43±6 c

[a] Mean value ± standard deviations.

Table 4. Zoospore cover of the root elongation zone and disease symptoms of lucerne cv Vela seedlings after inoculation with *P. aphanidermatum* (CBS 634.70), *P. dissimile* (APCC 4204f), *P. papillatum* (APCC 4106a) and *Ph. nicotianae* (APCC NR17) and their mixtures. Means within one mixture followed by different letters are significantly different at p≤0.05 (n=30).

Oomycete inoculum	Zoospore concentrations (spore ml^{-1})	Zoospore cover (%) [a]	Root reduction (%)	Root discoloration (%)
P. aphanidermatum	5,000	96±6 d	48±4 a	30±4 b
P. aphanidermatum	10,000	100±0 e	58±6 c	38±4 c
P. aphanidermatum + *P. dissimile*	5,000+5,000	50±5 c	55±7 bc	33±6 b
P. dissimile	5,000	21±4 a	47±5 a	5±4 a
P. dissimile	10,000	33±7 b	52±5 b	4±5 a
P. aphanidermatum	5,000	96±6 d	48±4 c	30±4 c
P. aphanidermatum	10,000	100±0 e	58±6 d	38±4 d
P. aphanidermatum + *P. papillatum*	5,000+5,000	22±5 c	41±6 b	18±5 b
P. papillatum	5,000	2±4 a	14±6 a	1±3 a
P. papillatum	10,000	7±4 b	17±5 a	1±3 a
P. aphanidermatum	5,000	96±6 d	48±4 a	30±4 a
P. aphanidermatum	10,000	100±0 e	58±6 c	38±4 bc
P. aphanidermatum + *Ph. nicotianae*	5,000+5,000	42±6 b	47±6 a	36±6 ab
Ph. nicotianae	5,000	29±4 a	42±5 b	34±6 ab
Ph. nicotianae	10,000	55±5 c	55±5 c	43±6 c
P. dissimile	5,000	21±4 d	47±5 b	5±4 b
P. dissimile	10,000	33±7 e	52±5 b	4±5 b
P. dissimile + *P. papillatum*	5,000+5,000	12±6 c	47±4 b	5±4 b
P. papillatum	5,000	2±4 a	14±6 a	1±3 a
P. papillatum	10,000	7±4 b	17±5 a	1±3 a
P. dissimile	5,000	21±4 a	47±5 b	5±4 a
P. dissimile	10,000	33±7 b	52±5 cd	4±5 a
P. dissimile + *Ph. nicotianae*	5,000+5,000	25±7 a	51±4 c	25±6 b
Ph. nicotianae	5,000	29±6 b	42±5 a	34±6 c
Ph. nicotianae	10,000	55±5 c	55±5 d	43±6 d
P. papillatum	5,000	2±4 a	14±6 a	1±3 a
P. papillatum	10,000	7±4 b	17±5 a	1±3 a
P. papillatum + *Ph. nicotianae*	5,000+5,000	15±4 c	17±4 a	18±4 b
Ph. nicotianae	5,000	29±6 d	42±5 b	34±6 c
Ph. nicotianae	10,000	55±5 e	55±5 c	43±6 d

[a] Mean value ± standard deviations.

severity significantly lower from *P. aphanidermatum* alone and significantly higher from the encystment of *P. papillatum*. The root area of seedlings covered with cysts of the mixed inoculum of *P. aphanidermatum* and *Ph. nicotianae* was significantly lower from the cyst cover of single inoculations with *P. aphanidermatum* and *Ph. nicotianae* at 10^4 zoospores ml^{-1}. The root reduction and root discoloration rates caused by the mixed inoculum of *P. aphanidermatum* and *Ph. nicotianae* resembled the values caused by single inoculations.

The mixed inoculation with *P. dissimile* and *P. papillatum* resulted in a cyst cover of the elongation zone of lucerne significantly lower to that of *P. dissimile* alone. Root discoloration rates were very low for single and mixed inoculations while the root reduction caused by the mixed inoculum was not significantly different from the values of inoculation only with *P. dissimile*.

The cyst cover of the elongation zone inoculated with a mix of *P. dissimile* and *Ph. nicotianae* was significantly lower from the cyst cover observed for single inoculation with *Ph. nicotianae* and *P. dissimile* at 10^4 zoospores ml^{-1}. The root reduction caused by the mixed inoculum was similar to that caused by *P. dissimile*, while the rate of root browning was between the values observed for single species inoculations.

The addition of *P. papillatum* in the inoculum with *Ph. nicotianae* reduced the zoospore cover and disease severity symptoms observed for *Ph. nicotianae* alone.

DISCUSSION

The plant intra-specific variation affected the number of *P. aphanidermatum* zoospores that encysted in the elongation zone of lucerne and tomato seedlings but not of sugar beet.

Differences in encystment were not reflected in disease severity with the exception of root reduction of Lucerne seedlings. The susceptibility of the selected cultivars towards the selected oomycete isolates was tested for the first time. When *Phytophthora megasperma* was inoculated to lucerne seedlings, zoospores were attracted more to the roots of susceptible compared to resistant varieties (Chi and Sabo, 1978) while no difference was found in the encystment (Irwin, 1976). Zentmyer (1961) was the first to notice differences in zoospore behaviour around roots of varieties that differ in resistance and similar results have been reported for blueberry (Milholland, 1975). However, most other reports show that zoospores of *Pythium* and *Phytophthora* species were attracted and encysted similarly to roots of susceptible and resistant seedlings of plants such as avocado (Ho and Zentmyer, 1977), soybean (Beagle-Ristaino and Rissler, 1983), chickpea (Dale and Irwin, 1991), tomato (Blaker and Hewitt, 1987) and *Rubus* (Laun and Zinkernagel, 1997). The above inconclusive results suggest that plant species probably have a certain capacity for the induction of zoospore encystment and its exploitation depends on the encystment potential or infectivity of each oomycete species.

Differences in zoospore encystment between isolates of *P. aphanidermatum* and *Ph. nicotianae* were reflected to the severity of disease symptoms caused only on lucerne seedlings. Most related studies have shown that the intra-specific variation of *Phytophthora* and *Pythium* species does not affect zoospore responses towards plant roots (Ho and Hickman, 1967; Mehrotra, 1970; Milholland, 1975; Ho and Zentmyer, 1977; Miller and Maxwell, 1982; Halsall and Williams, 1984; Deacon and Mitchell, 1985; Palloix *et al.*, 1988) although some studies have shown differential responses (Halsall, 1976; Mitchell and Deacon, 1987; Goldberg *et al.*, 1989). It seems that zoospore behaviour in the rhizosphere might be largely non-host-specific, but at least some oomycetes can show differential encystment on host and non-host roots (Donaldson and Deacon, 1993).

The mixed inoculum experiment indicated that in most combinations the encystment density was intermediate between the observed for single oomycete inoculations. The results suggest that zoospores of different species probably compete for a restricted number of available sites for encystment on the root. For example, *P. papillatum* zoospores probably occupied some of the sites suitable for the accumulation and encystment of *P. aphanidermatum* zoospores. Disease symptoms of the mixed inocula varied and either resembled the symptoms caused by one oomycete or did not differ from the symptoms caused by single inoculations. Previous research has shown that interactions of different root pathogens with *Pythium* species can affect plant growth (Rao *et al.*, 1978). In general, pathogenicity of mixed inocula appears to be governed by the most virulent species. In tomato plants inoculated with a mixture of *P. aphanidermatum* and *P. dissotocum*, symptom development and disease severity resembled those caused by the more virulent pathogen at selected temperature (Goldberg *et al.*, 1989). Severity of root rot of sugarcane caused by a highly virulent isolate of *P. arrhenomanes* was unaffected by combinations with other less virulent *Pythium* species (Lee and Hoy, 1992). The composition of the population of oomycete species in the root varies and because is an important determinant of disease severity a better knowledge of the population ecology of *Pythium* and *Phytophthora* is needed in order to gain a better understanding of root diseases.

This comparative investigation has drawn attention to the possible imperfections of single host and parasite experimental designs. The interaction between *Pythium* and *Phytophthora* species with potential hosts has been shown to be complex. The role of antagonistic bacteria, arbuscular mycorrhizal fungi (Vigo *et al.*, 2000) and mycoparasitic fungi or oomycetes (Wulff *et al.*, 1998) on

the biocontrol of *Phytophthora* and *Pythium* diseases has been investigated and shown promising results. However, the involvement of saprophytes members of the community is likely to be a factor often omitted in field studies.

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