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Effect of metalaxyl on formation and germination of oospores of *Phytophthora infestans*

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Oospores of *Phytophthora infestans* were produced in potato leaf discs floating on metalaxyl solution (100 μg mL^{-1} a.i.) and inoculated with all combinations of two metalaxyl-sensitive and two -resistant parental isolates. Numbers of oospores produced varied between different matings, depending on parents, in the absence of the fungicide and when metalaxyl was added 0, 7, 14 and 21 days after inoculation. Oospores were not produced when metalaxyl was added at the time of inoculation (0 days) when either one or both parents were sensitive to metalaxyl. In two of three such matings further oospore formation was arrested when metalaxyl was added either 7 or 14 days after inoculation. Oospores extracted from leaf discs 14, 21 and 28 days after inoculation were assessed for germination on water agar after 21 days. Germination of oospores from water control treatments varied between 6 and 30% depending on the cross. Germination was significantly reduced in oospores of metalaxyl-sensitive parents extracted 28 days after inoculation of leaf discs treated with metalaxyl 0, 7 and 14 days after inoculation compared with the 21-day treatment. Minimal differences in germination were observed for oospores from the mating of resistant parents irrespective of metalaxyl treatment, although germination was generally low, not exceeding 8·5%.

Introduction

Following recent migrations of *Phytophthora infestans* over the past 20 years (Fry & Goodwin, 1995) both A1 and A2 mating type isolates and phenylamide-sensitive and -resistant phenotypes occur in late-blight-affected potato and tomato crops worldwide. Despite the continuing occurrence of phenylamide-resistant phenotypes of *P. infestans* (Shattock & Day, 1996), compounds such as metalaxyl, sold in prepack mixtures with protectant compounds, e.g. mancozeb, continue to be used as a part of potato late-blight control programmes (Bradshaw & Vaughan, 1996).

Together, A1 and A2 mating type isolates produce oospores *in planta* (Gallegly & Galindo, 1958; Frinking *et al*., 1987; Pittis & Shattock, 1994; Drenth *et al*., 1995). The importance of oospores in the population dynamics of *P. infestans* is currently under investigation, particularly their role in perennation, infection and source of variation.

The effects of phenylamides and other fungicides on oospore formation, survival and infection potential in oomycetes have been reviewed by Cohen & Coffey (1986). The production of oospores by various *Phytophthora* spp. sensitive to low concentrations of the phenylamide metalaxyl is mainly due to direct effects on mycelial growth (Cohen & Coffey, 1986). Where diseases originate from soil-borne oosporic inoculum, fungicides, including metalaxyl, have provided effective control. In these cases oospores may be directly sensitive to fungicides such as fosetyl-Al, as for *P.citricola* and *P.cinnamomi* (Coffey *et al*., 1984; Cohen & Coffey, 1986), or fungicides may act directly on germ tubes as described by Duncan (1985) for metalaxyl on *P. fragariae*.

Effects are reported here of metalaxyl on oospore formation *in planta* and subsequent germination *in vitro* involving matings between A1 and A2 isolates of *P. infestans* sensitive and resistant to metalaxyl.

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Materials and methods

Isolates of *P. infestans* and inoculation

Four isolates of *P. infestans* were chosen for this study on the basis of their ability to form oospores and on their sensitivity to metalaxyl (Table 1).

Isolates were maintained on rye-A agar (Caten & Jinks, 1968) until required, when they were passaged through leaves of the late-blight susceptible potato cultivar Home Guard. Inoculum was prepared by adding 10 mL sterile distilled water (SDW) to 7–8-day-old colonies growing on rye-A agar and transferring the suspension of sporangia to universal bottles. Sporangia

Table 1 Parental isolates of *Phytophthora infestans* collected in 1992 and 1993 in the United Kingdom

Isolate	Mating type	Metalaxyl phenotype	Source
93.15.1	A1	Sensitive	Potato leaves
93.17.1	A1	Resistant	Potato tuber
92.5	A2	Sensitive	Potato leaves
93.100	A2	Resistant	?

were allowed to sediment over 10 min, and the supernatant was removed and replaced with fresh SDW (to dilute any nutrients derived from the rye-A agar). After a further 10 min, the supernatant was removed again and the remaining sporangial suspension (approximately 1–5×10^4 sporangia per mL) was used to inoculate leaves of cv. Home Guard in plastic salad boxes (Ashwood Plastics Ltd, Levon Road, London, E14 0LN,UK), which contained a double layer of blue paper tissue above a damp J-cloth (retail suppliers). Boxes were incubated at 10°C for 2 h to promote the release of zoospores and were then transferred to a growth room maintained at 18°C, with a 16-h photoperiod of light supplied by eight 1·2 m 36 W white fluorescent tubes, which provided 120–140 μmoles m^{-2} s^{-2} of photosynthetically active radiation. Spore suspensions were prepared after 7 days by washing leaves bearing sporulating lesions with 10 mL SDW into sterile universal bottles. The spore suspension was adjusted to approximately 5×10^4 sporangia per mL.

Potato plants

Tubers of cv. Home Guard, obtained from commercial suppliers, were placed in trays in the light at room temperature for 1 month to encourage sprouting. These were planted at approximately 15 cm depth in 5-L pots of John Innes no. 1 compost and grown in a glasshouse at a minimum of 18°C in the day and 16°C at night on a bench with a capillary-mat watering system. The natural light supply was supplemented by 400-W high pressure sodium lamps with a 16-h photoperiod. Nutrients were supplied by watering weekly with 1:1:1 N:P:K fertilizer (Vitax Ltd, Selby Place, Skelmersdale, Lancs. WN8 8EF, UK) at the recommended rate. Seven to eight weeks after planting, leaves for experimental work were detached from the mid-third of the stem.

Oospore production

Potato leaf discs of cv. Home Guard (14 mm diameter), floating on SDW in 25-well repli-dishes, were inoculated with 25 μL sporangial suspensions of either A1 or A2 mating type isolates or 50 μL of A1 and A2 mating type isolates together (25 μL of each mating type) of the chosen *P. infestans* isolates and incubated at 18°C with a 16-h photoperiod. Metalaxyl (96% a.i. wettable powder) was added to the SDW on which the leaf discs were floating 0, 7, 14 or 21 days after inoculation to give a final concentration of 100 μL mL^{-1} a.i. Five replicates were prepared for each treatment together with a water control. Mean numbers of oospores were counted within the leaf discs at 7-day intervals by blending leaf tissue in 5 mL SDW using a Fisons glass grinder (Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, Leics LE11 0RG,UK).

The mean number of oospores extracted was determined by examining three 30-μL samples from each of five discs per treatment. For each mating, mean oospore numbers formed in discs inoculated with A1 and A2 at each of the time intervals of metalaxyl addition was calculated and analysed by ANOVA. For each mating, mean oospore numbers for each day of metalaxyl addition after inoculation were then compared statistically for each sampling date using the LSD test.

Oospore germination

Oospores extracted from leaf discs for counting at 7-day intervals were assessed for germination. Oospores extracted from metalaxyl-treated leaf discs were placed in sterile universal bottles and spore suspensions were surface-sterilized by adding an equal volume of 60 μg mL^{-1} mercuric chloride. After 5 min oospores were immediately collected on 20-μm pore nylon filters (Lockertex, LockerWire Weavers Ltd, PO Box 161, Warrington, Cheshire, WA1 2SU,UK) and washed with several changes of SDW to remove the mercuric chloride. Oospores were resuspended in 10 mL SDW containing 0·01 g NovoZym 234 (Novo Biolabs, Nova Enzyme Products Ltd, Farnham, Surrey, UK) which lysed any hyphal fragments and sporangia in the suspension (Pittis & Shattock, 1994). After a maximum of 36 h incubation with NovoZym 234 at 18°C oospores were collected on 20-μm pore nylon filters, washed with 100 mL SDW and resuspended in SDW to give a concentration of $5–10\times10^3$ oospores per mL.

Oospore germination was assessed by pipetting 0·5 mL of this oospore suspension onto 9 mL soft water agar containing rifamycin (6·25 μg mL^{-1}), ampicillin (6·25 μg mL^{-1}) and nystatin (12·5 μg mL^{-1}) to suppress any contamination. Petri-dishes were sealed with Parafilm (BDH Merck. Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leics. LE17 4XN, UK) and incubated at 18°C with a 16-h photoperiod (Whittaker *et al.*, 1991; Pittis & Shattock, 1994).

Germination of oospores extracted 14, 21 and 28 days after inoculation was assessed for all metalaxyl treatments 21 days later and based on the presence or absence of germ tubes usually with a terminal germ sporangium. For each mating, mean germination of oospores formed in discs inoculated with A1 and A2 at each of the time intervals of metalaxyl addition was calculated and analysed by ANOVA. For each cross, mean oospore germination for each day of metalaxyl addition after inoculation were then compared statistically for each sampling date using the LSD test.

Results

Effects of metalaxyl on oospore formation

No oospores were detected in leaf discs until 14 days after inoculation. No oospores were detected at any stage in leaf tissue inoculated with only one parental isolate. In the absence of metalaxyl, at day 21, there were differences (Fig. 1a–d) in the mean number of oospores of *P. infestans* produced between different

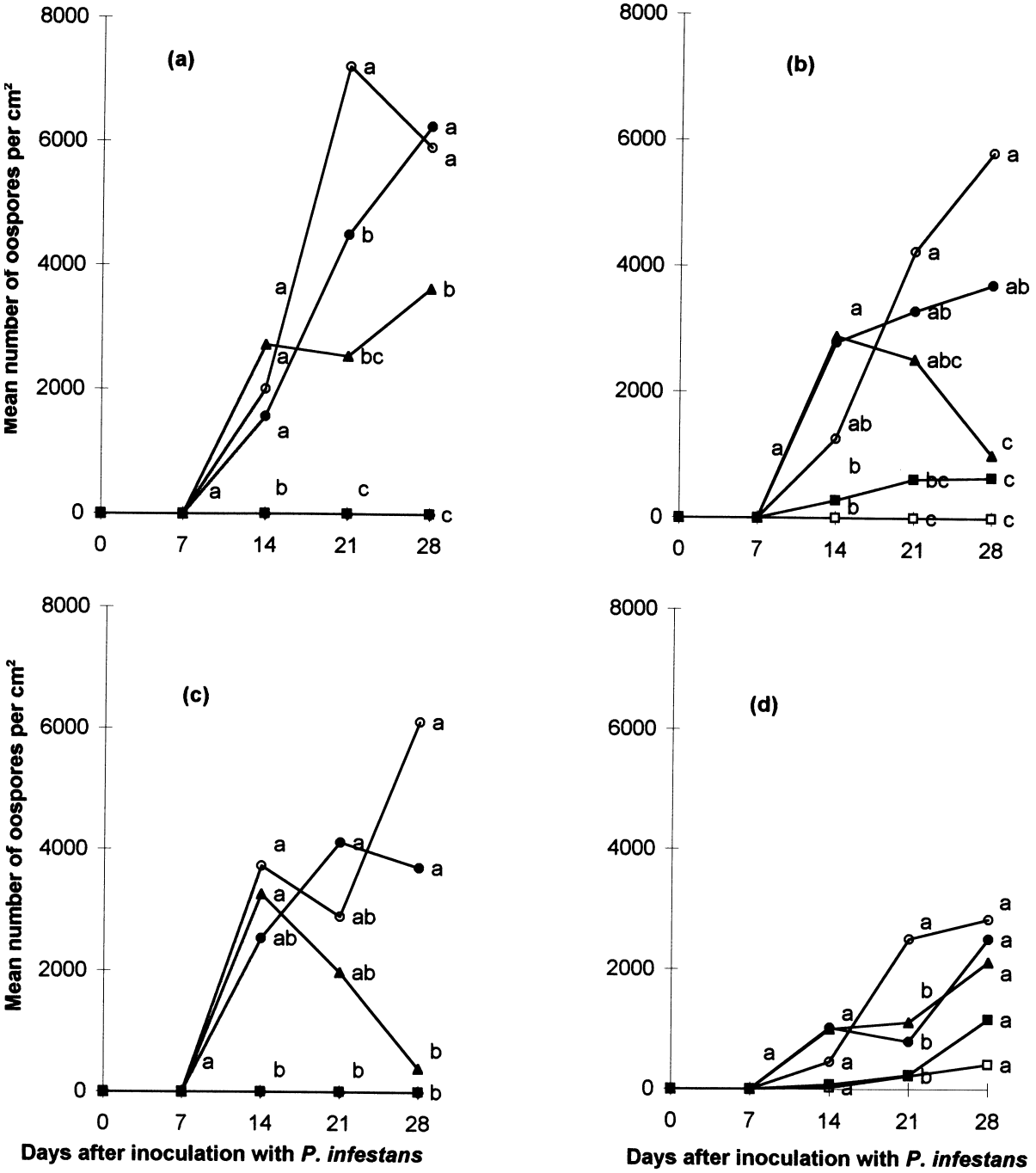

Figure 1 Number of oospores produced *in planta* over 28 days in matings between various metalaxyl-sensitive (MS) and -resistant (MR) isolates of *P.infestans* in the presence of metalaxyl (100 μg mL^{-1} a.i.). Cross (a) 93.15.1 (A1, MS)×92.5 (A2, MS), (b) 93.15.1 (A1, MS)×93.100 (A2, MR), (c) 93.17.1 (A1, MR)×92.5 (A2, MS), (d) 93.17.1 (A1, MR)×93.100 (A2, MR). Inoculated leaf discs were treated with metalaxyl after: □, day 0; ■, day 7; ▲, day 14; ●, day 21; ○, water control. Within each mating, for each day after inoculation, means (number of oospores per cm^2) with no letter in common are significantly different at $P<0{\cdot}05$.

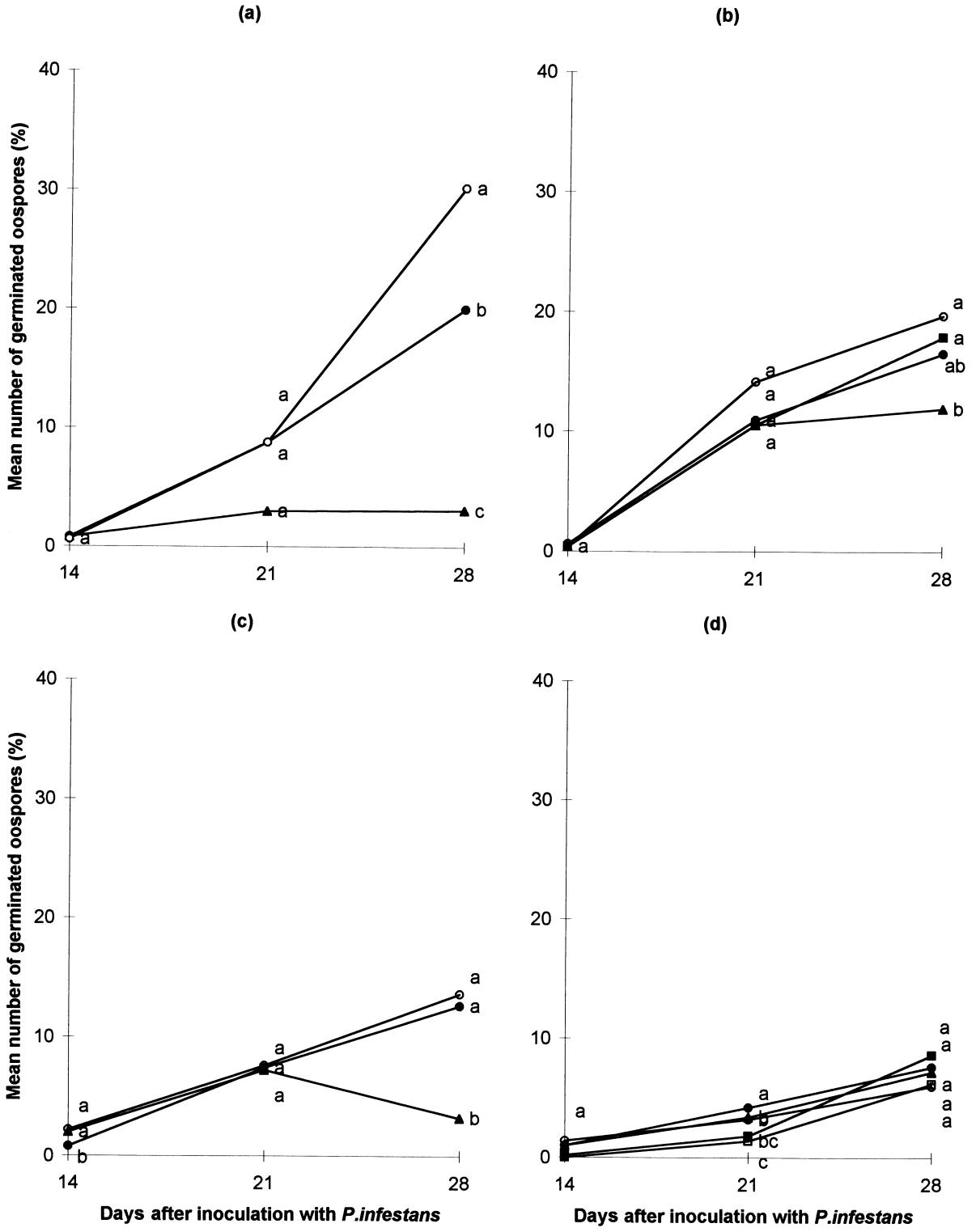

Figure 2 Germination of oospores on water agar after extraction from leaf discs 14, 21 and 28 days after inoculation with various metalaxyl-sensitive (MS) and -resistant (MR) isolates of *P.infestans* in the presence of metalaxyl (100 μg mL^{-1} a.i.). Mating (a) 93.15.1 (A1, MS)×92.5 (A2, MS), (b) 93.15.1 (A1, MS)×93.100 (A2, MR), (c) 93.17.1 (A1, MR)×92.5 (A2, MS), (d) 93.17.1 (A1, MR)×93.100 (A2, MR). Inoculated leaf discs were treated with metalaxyl after: □, day 0; ■, day 7; ▲, day 14; ●, day 21; ○, water control. Within each cross, for each day after inoculation, means (percentage of germinated oospores) with no letter in common are significantly different at $P<0{\cdot}05$.

parents. The lowest number (2508 oospores per cm^2 leaf) was recorded in mating 93.17.1 (resistant, A1)×93.100 (resistant, A2) (Fig. 1d), and the highest (7227 oospores per cm^2 leaf) in mating 93.15.1 (sensitive, A1)×92.5 (sensitive, A2) (Fig. 1a).

No oospores were observed in leaf discs inoculated with either one or both of the parental isolates being sensitive to metalaxyl when the fungicide was added at the time of inoculation (Fig. 1a–c). Adding metalaxyl, 7 and 14 days after inoculation, to matings 93.15.1 (sensitive, A1)×92.5 (sensitive, A2) and 93.17.1 (resistant, A1)×92.5 (sensitive, A2) may have been expected to arrest any further oospore formation (Fig. 1a,c). However, no oospores were formed at all in either mating when metalaxyl was added 7 days after inoculation, and when added 14 days after inoculation formation appears only to have been slowed and not stopped in mating 93.15.1 (sensitive, A1)×92.5 (sensitive, A2) (Fig. 1a) and a marked decline in number of oospores retrieved from samples was observed in cross 93.17.1 (resistant, A1)×92.5 (sensitive, A2). Mating 93.15.1 (sensitive, A1)×93.100 (resistant A2) showed an increase in oospore number over time when metalaxyl was added on either day 7 or 21, with a decline in oospores retrieved after addition of metalaxyl on day 14 (Fig. 1b).

The addition of metalaxyl to cross 93.17.1 (resistant, A1)×93.100 (resistant, A2) did not appear to inhibit oospore production completely. In all cases oospores were retrieved from leaf discs treated with metalaxyl 0, 7, 14 or 21 days after inoculation. There was no significant difference in number of oospores produced compared with the water controls except on day 21 (Fig. 1d). However, numbers of oospores were lower on addition of metalaxyl close to the day of inoculation than when metalaxyl was added later.

Oospore germination

Germination of oospores spread on soft water agar plates for 21 days at 18°C was only observed in those extracted from leaf discs 14, 21 and 28 days after inoculation. Significant differences ($P < 0{\cdot}05$) in oospore germination were noted between different crosses, with a range from 6·0% to 30·2% for those extracted 28 days after inoculation and produced in the absence of metalaxyl (Fig. 2a–d).

Oospores from the mating 93.15.1 (sensitive, A1)×92.5 (sensitive, A2), extracted from leaf discs 28 days after inoculation and to which metalaxyl had been added 14 days earlier, showed significantly lower germination than those either treated with metalaxyl 21 days after inoculation or in the control treatment without metalaxyl (Fig. 2a). This was also observed where one parent was sensitive to metalaxyl (93.15.1 (sensitive, A1)×93.100 (resistant, A2) and 93.17.1 (resistant, A2)×92.5 (sensitive, A2)), although the differences were less marked (Fig. 2b,c).

By contrast, oospores formed between resistant parents, to which metalaxyl had been added at different stages during oospore formation, showed minimal differences between treatments (Fig. 2d) except where metalaxyl had been added at inoculation or after 7 days, producing a significantly ($P < 0{\cdot}05$) lower percentage germination of oospores than when metalaxyl was absent (water control). There was no significant difference ($P < 0{\cdot}05$) between the percentage germination of oospores after 28 days for those treated with metalaxyl after 14 or 21 days. However, the mean percentage germination for oospores from this cross extracted from discs 21 days after inoculation was low for all treatments, ranging from only 1–7·6% (Fig. 2d).

Discussion

Depending upon the sensitivity of parental isolates to metalaxyl, the fungicide affected oosporogenesis of *P. infestans* in leaf tissue in three ways. Firstly, the simultaneous application of fungicide appeared to prevent infection and establishment by metalaxyl-sensitive isolates of the host. Secondly, metalaxyl reduced the numbers of oospores produced in some crosses. Thirdly, oospore germination was also affected.

When leaf discs were floated on metalaxyl solutions at the same time as inoculation (day 0), oospores were observed only in discs that had been inoculated with both metalaxyl-resistant parents 93.17.1 and 93.100 (Fig. 1a–d).

When metalaxyl was added 7, 14 or 21 days after inoculation of leaf discs with *P. infestans* in which one or both parental isolates were metalaxyl-sensitive, the number of oospores was reduced compared with the number in the absence of metalaxyl (Fig. 1a–c). This reduction in numbers was not observed in a mating where both parents were resistant to metalaxyl (Fig. 1d). Presumably, the effect that metalaxyl has on reducing mycelial growth (Schwinn & Margot, 1991) affected the ability of existing colonizing mycelium of sensitive isolates to interact and so to form oospores. Some oospores, however, were formed between isolates 93.15.1 (A1, metalaxyl-sensitive)×93.100 (A2, metalaxyl-resistant) when metalaxyl was added 7 days after inoculation (Fig. 1b). These may have been formed earlier or could possibly be oospores resulting from selfing of the metalaxyl-resistant parent (Shattock *et al.*, 1986; Whittaker *et al.*, 1991).

The presence of metalaxyl during oospore formation reduced the ability of extracted oospores to germinate *in vitro*. In matings where at least one parent was sensitive to metalaxyl, germination of oospores was reduced if metalaxyl was added as late as 14 days after inoculation. The fungicide may have affected the maturation of oospores which has been shown to be a requirement for successful germination of oospores of some *Phytophthora* species (Jiang *et al.*, 1989). No such reduction was seen when both parents were resistant to metalaxyl.

If viable oospores are produced in the presence of metalaxyl when one parent is sensitive then hybrid

progeny of intermediate sensitivity to metalaxyl are expected. These could still grow, albeit more slowly in the presence of metalaxyl, and could also, in pairings of like phenotypes, give rise to metalaxyl-sensitive, intermediate, and resistant progeny (Shattock, 1988). Oospores produced from metalaxyl-resistant parents would be expected to give rise to resistant progeny.

Despite the widespread occurrence of metalaxyl-resistant isolates in the field (Gisi & Cohen, 1996), metalaxyl is still used in prepack mixtures with dithiocarbamates such as mancozeb to control late-blight (Bradshaw & Vaughan, 1996). Other fungicides known to reduce intercellular mycelial growth, to which resistance has not been found, may be expected to reduce oospore formation by preventing hyphae of mixed mating type from interacting, but this depends on their systemicity. Sterols are required for oospore formation (Elliott, 1994) and so propamocarb hydrochloride, which is systemic and thought to interfere with sterol metabolism in *P. infestans*, may affect oospore formation. Various fungicides have been implicated in the switching of mating type in species of *Phytophthora*. Ko *et al*. (1986) reported that chloroneb and truban caused switching of mating type in *P. parasitica* and Ko (1994) described A2 phenotypes appearing in *in vitro* A1 mating type cultures of *P. infestans*. However, oospores were not observed in leaf discs inoculated with only A1 or A2 isolates.

The effect of fungicide applied to oospores that have already been formed was not studied here. Studies by Duncan (1985) on oospores of *P. fragariae*, and by Stasz & Martin (1988) on oospores of *Pythium ultimum*, indicated that metalaxyl, fosetyl-Al, captafol, dichlofluanid, captan, fenaminosulf, methyl bromide and thiram reduced germination of active (thin-walled) oospores, but not inactive (thick-walled) oospores. Thus, fully formed and physiologically dormant oospores may remain viable and germinate later in the absence of the fungicide. For this reason phenylamide treatment of micropropagated and elite nursery stock of soft fruit has been discouraged because symptoms of various oomycetous pathogens may be suppressed even though viable inocula (e.g. oospores) remain unaffected (Williamson *et al.*, 1997). Fungicides that reduce the germination of sporangia of *P. infestans* should be tested to see if they affect oospore germination. These include phosphites such as fosetyl-Al (tested by Duncan (1985) on *P. fragariae*) and those currently used against late-blight namely the dithiocarbamates, phthalonitriles, organotins, propamocarb HCl, fluazinam and the newly developed strobilurins.

In addition to the above considerations, the spatial and temporal activity of a fungicide within a crop will be of importance in determining any interaction with oosporogenesis and subsequent germination. Oospores may already be present in the plant or in the soil at the time of fungicide treatment. Systemic fungicides against late-blight are, with the exception of fosetyl-Al, all acropetal in movement, and may not reach oospores in previously formed stem lesions on the lower parts of plants. Dropleg sprayers (Ligertwood & Hinds, 1995), which can direct fungicides to stem bases and soil, as well as the top of the leaf canopy, could target such sites.

This study aimed to investigate the effect of metalaxyl on the formation and subsequent germination of oospores of *P. infestans*. It also highlighted the differences in oospore production and germination between different parental matings. Aside from any interaction with metalaxyl, the most oospores were formed in the mating 93.15.1 (sensitive, A1)×92.5 (sensitive, A2) and the fewest with 93.17.1 (resistant, A1)×93.100 (resistant, A2). Similarly, the highest percentage germination was achieved by oospores produced by the mating 93.15.1 (sensitive, A1)×92.5 (sensitive, A2) and the lowest by oospores produced by 93.17.1 (resistant, A1)×93.100 (resistant, A2). This apparent difference in fecundity observed between oospores produced in different crosses, as well as any interaction with metalaxyl, would be expected to affect the formation and viability of any oospores formed in the field.

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