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In vitro somatic growth and reproduction of phenylamide-resistant and -sensitive isolates of *Phytophthora erythroseptica* from infected potato tubers in Idaho

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Pink rot of potato, most commonly caused by *Phytophthora erythroseptica*, is a major field and post-harvest problem in southern Idaho, USA, particularly since 1998 when isolates resistant to the phenylamide fungicide metalaxyl-M (mefenoxam) were detected. Isolates of *P. erythroseptica* were collected from infected tubers in 2001 and 2002 from six Idaho counties and tested for resistance to metalaxyl-M on amended agar. Metalaxyl-M resistant (MR) and metalaxyl-M-sensitive (MS) isolates were identified in six counties; 160 isolates were highly resistant, seven moderately resistant and 57 sensitive to metalaxyl-M with mean EC_{50} values of 182, 23 and 0·5 mg L^{-1} ai metalaxyl-M, respectively. Mycelial growth rates and oospore production in agar were assessed for 20 MS and 20 MR isolates at 10, 15, 20, 25 and 30°C. Growth rates of MR isolates were between 2·5 and 3·1 times greater ($P < 0·05$) than those of MS isolates at 10, 15, 20 and 25°C, and oospore production was between 6·8 and 20·5 times greater ($P < 0·0001$) for MR than for MS isolates at the same temperatures. Colony growth in V8 broth at 18°C was greater for MR than MS isolates ($P < 0·0032$). However, zoospore production at 18°C was greater for MS than for MR isolates ($P < 0·0109$), and zoospore production mM^{-1} of colony circumference was also greater for MS than for MR isolates, 14 191 and 9959, respectively ($P = 0·0109$). Sexual reproduction of MR isolates in nature may be greater than MS isolates, but MS isolates may be more asexually fit based on the fitness parameters studied.

Keywords: fungicide resistance, mefenoxam, metalaxyl-M, oospore and zoospore production, potato pink rot

Introduction

Pink rot, caused by several *Phytophthora* species, but most commonly by *P. erythroseptica*, was first identified in Ireland in 1913 (Pethybridge, 1913) and has since been found worldwide in potato growing regions (Cairns & Muskett, 1933). In the United States, pink rot was first identified in Oklahoma and Kentucky in 1923 (Dreschler, 1929) but now is found in most potato production areas (Taylor *et al*., 2002). Infected tuber tissue is characterized by a rubbery texture and a pink discoloration that appears 15 to 20 minutes after the tissue is exposed to air (Goss, 1949). Pink rot is most common in areas of potato fields where excessive water collects but has also been found on high dry ridges (Blodgett, 1945).

The phenylamide fungicide metalaxyl was labelled for use in the United States on potatoes in 1982 to control late blight caused by *P. infestans* and in 1987, pink rot was added to the label of diseases managed by this fungicide (P. McCain, Syngenta Crop Protection, Greensboro, N.C. U.S.A., personal communication). Metalaxyl, a racemic fungicide that contains both R- and S-enantiomers, was replaced with metalaxyl-M (commonly called mefenoxam) in 1997. Metalaxyl-M (Ridomil Gold EC, Syngenta Crop Protection) contains only the pure R-enantiomer as the active ingredient (Nuninger *et al*., 1996) which is more effective than the S-enantiomer in controlling oomycete plant pathogens (Hubele *et al*. 1983).

Metalaxyl resistance was first reported in isolates of *Pseudoperonospora cubensis* from cucumber plants grown in plastic houses in Israel (Reuveni *et al*., 1980) and under field conditions in isolates of *P. infestans* in 1981 in Ireland (Dowley & O'Sullivan, 1981) and the Netherlands (Davidse *et al*., 1981). Development of resistance to metalaxyl in nature has been confirmed in at least six other species of *Phytophthora* and has also been induced in an additional five species or subspecies of *Phytophthora* under laboratory conditions.

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Metalaxyl-resistant isolates of *P. erythroseptica* were first identified in Maine in 1993 (Lambert & Salas, 1994) and in Idaho in 1998 (Salas *et al*., 2000). Currently, resistant isolates have been found in Minnesota (Salas *et al*., 2000), New Brunswick, Canada (Salas *et al*., 2000) and New York (Goodwin & McGrath, 1995).

The purpose of this research was to identify how widespread metalaxyl-M-resistant (MR) isolates of *P. erythroseptica* are in southeastern Idaho, quantify the resistance of isolates to metalaxyl-M, and determine if differences in growth rate and asexual and sexual reproduction exist between metalaxyl-M-sensitive (MS) and MR isolates under controlled laboratory conditions.

Materials and methods

Isolation of *Phytophthora erythroseptica* from potato tubers

Tubers with pink rot symptoms were collected from commercial potato fields and storage facilities from six counties in Idaho, USA during 2001 and 2002. Tubers were cut in half from the stolon to the bud end and allowed to sit for 20 to 30 minutes to allow for symptom expression (Goss, 1949). Approximately 3 mm^3 of tuber tissue was excised with a sterile scalpel from the interface of discolored and healthy looking tissue and dipped in 70% ethanol for five seconds, rinsed in sterile distilled water for five seconds, blotted dry with paper towels and placed on 1·5% water agar. Mycelial growth characteristic of *P. erythroseptica* was then sub-cultured to V8 agar after incubation at room temperature (23 to 25°C) for 2 to 4 days. V8 agar was produced following the methods described for clarified V8 juice agar (Erwin & Ribeiro, 1996) with the exception that the dilution rate was 1:10 and there was no further clarification of agar using filter paper. Zoospores of each isolate (see later) were transferred to 1·5% water agar in Petri dishes and maintained at room temperature (20°C) for 24 hours. Single germinated zoospores were then selected and transferred to V8 agar to establish single zoospore cultures. Isolates were identified as *P. erythroseptica* using the methods of Newhook *et al*. (1978). No additional fungal species were isolated from the infected tubers using this technique.

Evaluation of isolates for metalaxyl-M sensitivity

Petri dishes (100 × 15 mm) were filled with 14 mL of autoclaved V8 agar using a Unispense Microprocessor Controlled Dispenser (Wheaton Science Products). V8 agar was amended with 0, 0·01, 0·1, 1·0, 10, 100 or 250 mg L^{-1} of metalaxyl-M (Ridomil Gold EC, 47·6% a.i., Syngenta Crop Science). Stock solutions of Ridomil Gold suspended in water at various concentrations were used to create the concentration gradient. Agar was stirred for two minutes prior to dispensing to ensure uniform distribution of metalaxyl-M after which V8 agar plugs measuring 7 mm in diameter were then taken from the leading edge of a four-day-old colony of *P. erythroseptica* using a cork borer and placed mycelia-side down in the centre of each Petri dish. Agar plate stacks containing the various isolates, at the seven metalaxyl-M concentrations, were arranged in a randomized design on trays and these were placed in plastic bags to reduce water loss. Colonies were allowed to grow for 4 days in the dark at 23 to 25°C, and two perpendicular measurements of colony diameter were made. Three replicate plates were used for each isolate and metalaxyl-M concentration. The test was performed twice and EC_{50} values were calculated by averaging subsample and replicate values from both tests.

EC_{50} calculations

EC_{50} values were defined as concentrations of active ingredient (ai) metalaxyl-M in agar at which growth was reduced by 50% as compared to growth on unamended agar. EC_{50} values were calculated as a function of concentration using nonlinear regression. Inhibition plots of EC_{50} values supported an exponential function:

$$y_{ij} = ae^{(-b\times \mathrm{conc}^{c})} + e_{ij} \quad \text{(Eqn. 1)}$$

where 'y_{ij}' is the percentage of growth at the *j*th replication of the *i*th concentration, '*a*' is the expected growth at a concentration of zero, '*b*' is a rate parameter, '*c*' is a shape parameter of the function and 'e_{ij}' is an error term under the usual assumptions of regression, $e_{ij} \sim \mathrm{NID}(0, \mathrm{sigma}^2)$ (Ratowsky, 1990). This model generally describes a process that initially decreases rapidly, but gradually approaches zero as concentration increases. Models for each isolate were estimated and assessed separately. Following model fits, EC_{50} values were estimated by equation 2.

$$\left(\frac{(\log(50) - \log(a))}{b}\right)^{\frac{1}{c}} \quad \text{(Eqn. 2)}$$

All computations were carried out using SAS (SAS Institute Inc. 2004. SAS OnlineDoc 9·1·2).

Selection of isolates for fitness tests

Twenty metalaxyl-M-sensitive (MS) and 20 metalaxyl-M-resistant (MR) isolates were selected from among the 237 isolates collected in 2001 and 2002 to assess mycelial growth rates and oospore production in V8 agar. Ten MS and 10 MR isolates were selected from each year for a total of 40 isolates (Table 1). The greatest potential diversity between and among MR and MS isolates was purposefully selected by choosing isolates collected from the greatest number of counties and diverse fields within those counties (Table 1). The 40 selected isolates were assessed for mycelial growth rates and oospore production. A selected subset of five MR and five MS isolates were selected from among the 40 isolates and further assessed for zoospore production, zoospore germination rate and oospore viability (see selected isolates with an asterisk in Table 1). These ten isolates were also selected so as to represent the greatest number of diverse counties and fields within those counties from which isolates of *P. erythroseptica* were collected.

Table 1 Isolate identification, year collected, field and county of collection, EC_{50} value, standard error of EC_{50} value, and sensitivity to metalaxyl-M of isolates of *Phytophthora erythroseptica* collected in southeastern Idaho

Isolate[a]	Year	Field[b]	County	EC_{50} (mg L^{-1})[c]	Standard error	Sensitivity[d]
01–1B*	2001	1	Fremont	144·67	2·585	R
01–9	2001	2	Fremont	0·46	0·050	S
01–15	2001	3	Minidoka	0·58	0·073	S
01–16	2001	4	Minidoka	0·03	0·137	S
01–17	2001	4	Minidoka	1·181	0·6957	S
01–21*	2001	5	Power	1·61	0·298	S
01–22A	2001	5	Power	0·89	0·443	S
01–22F	2001	5	Power	195·06	3·913	R
01–22G*	2001	5	Power	140·76	2·456	R
01–31A	2001	5	Power	0·26	0·023	S
01–34B	2001	6	Fremont	191·75	3·416	R
01–37	2001	7	Fremont	158·95	2·809	R
01–38D	2001	8	Fremont	201·99	2·798	R
01–39A*	2001	9	Fremont	0·42	0·050	S
01–39E*	2001	10	Bonneville	154·17	3·435	R
01–41C	2001	11	Fremont	183·31	6·021	R
01–50a	2001	44	Fremont	178·00	5·500	R
01–50b	2001	44	Fremont	157·00	3·200	R
01–54	2001	45	Bonneville	0·28	0·020	S
01–56	2001	45	Bonneville	0·39	0·080	S
02–01	2002	14	Minidoka	0·33	0·059	S
02–10	2002	14	Minidoka	0·61	0·157	S
02–11*	2002	14	Minidoka	0·33	0·040	S
02–25*	2002	15	Fremont	0·41	0·051	S
02–28*	2002	16	Fremont	183·19	3·429	R
02–33	2002	17	Fremont	185·44	2·986	R
02–41	2002	18	Fremont	216·46	2·604	R
02–49	2002	19	Power	0·35	0·018	S
02–51	2002	19	Power	0·12	0·137	S
02–58	2002	20	Fremont	160·39	2·629	R
02–63	2002	21	Fremont	203·82	3·308	R
02–67	2002	22	Fremont	209·54	1·874	R
02–79	2002	23	Fremont	0·43	0·066	S
02–105*	2002	24	Madison	179·85	2·734	R
02–106	2002	24	Madison	178·11	2·397	R
02–130*	2002	25	Madison	0·44	0·022	S
02–131	2002	25	Madison	0·41	0·021	S
02–133	2002	25	Madison	0·54	0·076	S
02–152	2002	26	Fremont	199·50	1·956	R
02–163	2002	27	Fremont	187·96	2·465	R

[a]Isolate identification. Those isolates with an asterisks were assessed for all fitness parameters studied, and those without an asterisk were used only to assess growth rate and oospore production.

[b]Fields with different numbers represent unique fields.

[c]EC_{50} is the effective concentration active ingredient metalaxyl-M (mg L^{-1}) at which mycelial growth is inhibited by 50% when compared to growth on unamended agar.

[d]Sensitivity identifies whether the isolate is resistant (R), intermediately resistant (IMR), or sensitive (S) to metalaxyl-M. Isolates were considered resistant, intermediately resistant and sensitive to metalaxyl-M if the EC_{50} values were 83 mg L^{-1} or greater, between 2 and 83 mg L^{-1}, and 2 or less mg L^{-1}, respectively.

Mycelial growth rate and oospore production

Mycelial growth rate and oospore production in V8 agar were determined at 10, 15, 20, 25 and 30°C for each of the 40 isolates. Agar plugs measuring 7 mm in diameter were taken from the leading edge of a colony of each *P. erythroseptica* isolate using a cork borer. Plugs were placed mycelial-side down in the centre of each Petri dish. Temperatures within incubators were monitored using a data logger (Watch Dog Data logger, Spectrum Technologies) and were all within 1·0°C of the set temperature during the course of the experiments. Five sets of Petri dishes were randomly arranged on plastic trays, with each tray containing all 40 isolates. Each tray was then placed in a clear plastic bag in an incubator operating at one of the five growth temperatures. The mean radial growth

rate of colonies was assessed after four days of growth as previously described. The radial growth rate in mm per day was determined by dividing the mean colony diameter (minus the diameter of the original plug) by four. Petri dishes were then returned to their respective temperatures for an additional 11 days. Oospores were observed to be uniformly distributed throughout the agar so three 1·6 mm^3 agar plugs, immediately adjacent to the 7 mm plug used to initiate the culture, were removed and placed in 3·7 mL vials containing 1 mL of 70% alcohol to preserve the oospores. The volume of the plugs was standardized by using a pippette tip with a 1 mm diameter and pouring the agar to a uniform depth using an agar dispenser. The plugs were flattened on microscope slides using a cover slip. The total number of oospores contained in each plug was counted using a light microscope at 400 X with the aid of a KR-406 10 mm square grid reticle divided into one hundred 1 mm square sections (Microscope Depot). The experiment was repeated five times.

Asexual reproduction, colony growth in V8 broth and germination of zoospores

Zoospore production was determined for five MS and five MR isolates of *P. erythroseptica* by growing the isolates on V8 agar in 100 × 15 mm Petri dishes filled with 23 mL of agar. Four 7 mm agar plugs were removed from the leading edge of the expanding colony of each isolate after four days of growth and placed in separate 100 × 15 mm Petri dishes and 10 mL of V8 broth were added to each dish. One replicate of each isolate was randomly placed on four separate trays, and trays were placed in the dark at 18°C for four days. Cultures were then rinsed twice with 10 mL of sterile distilled water, and refilled with 10 mL of sterile soil extract (see later) and placed at 18°C for 2 days, 30 cm below cool white fluorescent lights (Sylvania deluxe L 40 W FT40DL/841/RS, Sylvania). One of the four trays containing the replicates of each isolate was removed from the incubator every 30 min. and placed in a refrigerator at 4·4°C for 1·5 h. The isolates were then placed at room temperature for 45 min. The soil extract was then poured from the Petri dishes of each isolate into separate 15 mL conical tubes and weighed. Two 10 μL samples of the soil extract were removed from the surface (between 0 and 1 mm deep) in each conical tube and a haemacytometer was used to estimate the concentration of zoospores. A 1 mL sample of the soil extract containing zoospores was then spread onto 1·5% water agar in a 100 × 15 mm Petri dish using a glass spreader which was sterilized between each sample by dipping in 96% alcohol and flaming. Petri dishes with zoospores were placed inside a plastic bag and placed at 18°C in the dark for 24 h. Germination of 100 zoospores was observed under a light microscope at 400 x for each replication. The diameter of each fungal colony of each isolate was also measured in millimetres in two locations perpendicular to each other and a mean diameter was calculated.

Soil extract was prepared by placing 200 grams of a silt loam soil in 1 L of water, mixing the solution for 20 minutes on a stir plate and allowing the soil to settle for 24 hours. The solution was then screened through four layers of cheesecloth with care not to disturb the sediment at the bottom of the bottle and filtered through Whatman #1 filter paper (Whatman Inc.). Distilled water was added to bring the total volume back to 1 L and the extract was autoclaved for 20 minutes. The pH of the soil extract was 7·9.

Oospore viability

Tetrazolium stain method

Oospore viability was assessed using a tetrazolium bromide staining technique (Jiang & Erwin, 1990). V8 agar plugs measuring 5 mm in diameter were removed from the edges of expanding colonies of five MS and five MR isolates as previously described. Plugs were placed into 70 mL test tubes containing 10 mL of V8 broth. There were four replicates for each isolate. Test tubes were placed at 20°C in a randomized complete block design with replicates being the block. Cultures were incubated for forty days and the mycelial mats within each test tube were then washed twice with autoclaved distilled water and the water was decanted. Ten millilitres of a 0·4% solution of Lysing Enzymes from *Trichoderma harzianum* (Sigma-Aldrich) were placed in each test tube and incubated at 18°C for two days in the dark to dissolve the mycelia and leave only oospores. Test tubes were lightly vortexed and the contents transferred to 18 mL plastic centrifuge tubes. Test tubes were centrifuged at 2000 ***g*** for 5 min and the supernatant was removed. Ten millilitres of sterile distilled water were added to each test tube, the test tube was lightly vortexed and centrifuged again and the supernatant was decanted. This process was repeated to remove the lysing enzymes from the surface of the oospores. Two millilitres of 0·1% MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) solution in a 0·1 M phosphate buffer (pH 5·8) were then added to each test tube and incubated in the dark at 35°C for two days. The solution was vortexed lightly and a 10 μL droplet of the solution was placed onto a microscope slide. Viability of 100 oospores was observed under a light microscope at 400 X for each replication. Viable oospores stained red in colour. Two MS and two MR isolates with four replicates of each were autoclaved for 15 min to serve as non-viable controls.

Plasmolysis method

Oospore viability was also assessed using plasmolysis (Jiang & Erwin, 1990). V8 agar plugs from the edges of expanding colonies of five MS and five MR isolates were placed into 60 × 15 mm plastic Petri dishes with 5 mL of V8 broth. The five MS isolates selected were from Power, Minidoka, Madison and Fremont counties. There were four replicates for each isolate. Cultures were grown in the dark at 23 to 25°C for four days, rinsed twice with 5 mL of distilled water, and incubated in 10 mL of soil extract for an additional fifteen days. Soil extract was removed and 5 mL of 4 M NaCl were then added to the

Petri dishes. The percentage of spores undergoing plasmolysis was observed under the light microscope at 400 x after incubation for 1 h. One hundred oospores were observed for each replicate. After incubation for 1 h in the 4 M NaCl solution, the Petri plates were rinsed once with distilled water and refilled with distilled water until the fungal colonies were completely submerged and oospores were once again observed under the light microscope.

Statistical analysis

Differences between oospore production and growth rates for MS and MR isolates were compared based on the calculated standard error of the mean of treatments using SAS. Colony growth, zoospore production and zoospore germination of MS and MR isolates was assessed using ANOVA assuming a randomized complete block design with the PROC GLM feature in SAS, with replications being the blocks.

Results

Evaluation of isolates for metalaxyl-M sensitivity

Of the 224 isolates of *Phytophthora erythroseptica* tested for metalaxyl-M resistance, 160 isolates were metalaxyl-M resistant (MR), seven isolates demonstrated intermediate metalaxyl-M resistance (IMR) and 57 were metalaxyl-M sensitive (MS). Isolates that were resistant, intermediately resistant and sensitive had EC_{50} values that ranged from 0 to 2, 2·1 to 83 and 83·1 to 230 mg L^{-1} ai metalaxyl-M, respectively. Isolates classified as either MR, IMR or MS had mean (mean ± standard error) EC_{50} values of 182·9 ± 20·9, 22·5 ± 29·0 and 0·5 ± 0·32 mg L^{-1} ai metalaxyl-M, respectively. MR isolates were found in five of six Idaho counties from which potatoes were sampled: Bonneville, Fremont, Jefferson, Madison and Power counties (Table 1). Only sensitive isolates were found in samples from Minidoka County. Resistant (MR) isolates were found in potatoes from 35 of 45 potato fields evaluated.

Mycelial growth and oospore production

Growth rates of MR isolates were between 2·5 and 3·1 times greater ($P < 0·05$) than MS isolates on V8 agar at 10, 15, 20 and 25°C (Fig. 1). Mycelial growth rate was greatest at 25°C for both MR and MS isolates (Fig. 1). However, there was no significant difference between growth rates at 20 and 25°C for the resistant isolates and 15, 20 and 25°C for the sensitive isolates (Fig. 1). Colony growth in V8 broth at 18°C was also greater for MR than for MS isolates ($P < 0·003$, Table 2). Oospore production was 6·8 to 20·5 times greater ($P < 0·0001$) for MR than for MS isolates at 10, 15, 20 and 25°C (Fig. 2). Maximum oospore production occurred at 20°C for MS and 15°C for MR isolates (Fig. 2). However, there were no significant differences in oospore production among individual isolates within either the MR or the MS isolate classification at four of the five temperatures evaluated. Oospores were not produced by either MR or MS isolates at 30°C.

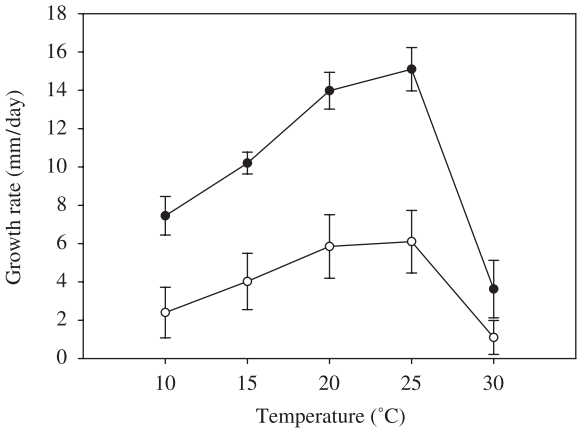

Figure 1 Mean mycelial growth rate of twenty mefenoxam-resistant (●) and twenty mefenoxam-sensitive (○) isolates of *Phytophthora erythroseptica* incubated at five temperatures on V8 agar for four days. Error bars represent the standard error of the mean.

Table 2 Colony growth, zoospore production, and zoospore germination of metalaxyl-M-resistant and -sensitive isolates of *Phytophthora erythroseptica*

Fitness parameter/Type[a]	Value	LSD[b]	*P*-Value
Colony growth[c]		1·2	0·003
Sensitive	51·4*[d]		
Resistant	53·2		
Zoospore production[e]		3238·2	0·011
Sensitive	14191*		
Resistant	9959		
Zoospore germination[f]		0·0135	0·96
Sensitive	95·3		
Resistant	95·3		

[a]Type = Sensitivity to metalaxyl-M. Five sensitive and five resistant isolates were tested.
[b]LSD = least significant difference between resistant and sensitive isolates for a given fitness parameter.
[c]Diameter of colony (mm) after four days of growth in V8 broth followed by two days of growth in soil extract.
[d]Asterisk denotes a significant difference ($P < 0·05$) between resistant and sensitive isolates.
[e]Number of zoospores produced per 1 mm of colony circumference.
[f]Percentage of zoospores that germinated out of 100 zoospores per replication of each isolate after a 24 hour incubation period at 18°C on water agar.

Asexual reproduction and germination of zoospores

Zoospore production at 18°C was significantly greater for MS than for MR isolates ($P < 0·011$, Table 2) but zoospore germination was not significantly different ($P < 0·96$). Significant differences among isolates within the same sensitivity class were not observed for either variable.

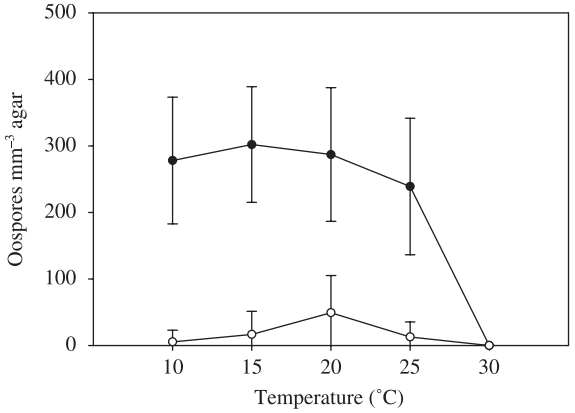

Figure 2 Mean oospore production of twenty mefenoxam-resistant (●) and twenty mefenoxam-sensitive (○) isolates of *Phytophthora erythroseptica* contained in a 1·6 mm^3 agar plug taken from V8 agar plates incubated at five temperatures for 15 days. Error bars represent the standard error of the mean.

Oospore viability

Oospore viability assessed using the tetrazolium stain method was inconclusive since both heat-killed and non-heat-killed oospores of MR and MS isolates stained red indicating false positives for viability. The plasmolysis technique for oospore viability was also inconclusive since MS and MR isolates were not observed to swell or shrink when external salt concentrations were increased or decreased.

Discussion

Metalaxyl-M, the active ingredient in Ridomil Gold EC, is commonly used in potato production in southeastern Idaho. Metalaxyl-M is most commonly applied as a foliar application at tuber initiation and a second application is applied two weeks later. The purpose of the applications is to reduce the risk of tuber rot due to *Pythium* sp. and *P. erythroseptica*. Despite the development of MR isolates of *P. erythroseptica*, many growers still apply metalaxyl-M to potatoes because they feel they are receiving additional potato health benefits. Also, the wheat, barley and seed of other crops in rotation with potatoes are commonly treated with metalaxyl or metalaxyl-M to prevent pre-emergence damping off due to *Pythium* sp. These treatments allow for the exposure of *P. erythroseptica* isolates to metalaxyl-M on a yearly basis.

Potato growers in southeastern Idaho were concerned about an increase in potato pink rot in their fields which initiated the current research which has found that MR isolates of *P. erythroseptica* are widespread in southeastern Idaho and were present in 78% of the fields sampled from six counties during 2001 to 2002. As of 2006, resistant isolates have now been found in seven of nine Idaho counties. Genetic diversity between four MR and MS isolates of *P. erythroseptica* from the collection in Idaho and other regions in North America were assessed using RAPDs (Peters *et al*., 2005), but major differences or correlations between MR and MS isolates were not found.

EC_{50} values of MR isolates in Idaho were consistent with previously published values of resistant isolates of *P. erythroseptica* from New Brunswick (Canada), Maine and Minnesota (Taylor *et al*., 2002) indicating that MR isolates across the United States and southern Canada may be similar in the level of metalaxyl-M insensitivity. *Phytophthora* species are generally sensitive to 0·01 to 1 mg L^{-1} of metalaxyl-M (Erwin & Ribeiro, 1996). EC_{50} values of natural MR isolates (not laboratory-induced) of species in the order Peronosporales are commonly over 100 times more resistant than those of MS isolates (White *et al*., 1988; Ferrin & Kabashima, 1991; Klein, 1994; O'Brien & Weinert, 1995; Pankhurst *et al*., 1995; Sujkowski *et al*., 1995; Timmer *et al*., 1998), and EC_{50} values of MR isolates of *P. erythroseptica* from Idaho in the present study were on average 380 times more resistant than MS isolates.

Isolates with intermediate resistance to metalaxyl-M consisted of only three percent of the isolates screened. Intermediate resistance is believed to be associated with the heterozygous state of the gene conferring resistance to metalaxyl-M (Goodwin & McGrath, 1995). Since isolates with intermediate resistance were rare, this study focused on isolates that were either sensitive or highly resistant to metalaxyl-M.

Asexual and sexual fitness parameters were assessed for both MR and MS isolates of *P. erythroseptica* in the present study. Growth rates of MR isolates of *P. erythroseptica* were significantly greater than those of MS isolates in V8 broth at 18°C, and at five temperatures when grown on an agar medium. Growth rates of MR isolates of *P. infestans* from British Columbia, Canada, were also reported to be significantly greater than those of MS isolates (Chycoski & Punja, 1996). Growth rates of MR and MS isolates of additional *Phytophthora* species need to be assessed to determine if a trend towards increased growth rates of MR isolates is common. Isolates with faster growth rates may be considered to be more fit based on the ability to compete for space and colonization of plant tissue. However, increased growth rates of MR *P. infestans* isolates on artificial medium in British Columbia did not correlate with increased leaf colonization rates (Chycoski & Punja, 1996). Therefore, the faster growth rates of MR isolates of *P. erythroseptica* may or may not correspond to increased aggressiveness in tuber rotting capabilities in the field and in storage. Furthermore, the optimal temperature for growth, although not statistically different from other evaluated temperatures, was the same for MS and MR isolates and was consistent with the optimal growth temperature published for *P. erythroseptica* isolates from Northern Ireland (Cairns & Muskett, 1939) and Peru (Vargas & Nielsen, 1972).

Oospore production of MR isolates of *P. erythroseptica* was 6·8 to 20·5 times greater than that of MS isolates in artificial medium depending on the incubation temperature, indicating greater potential sexual fitness for MR than for MS isolates of *P. erythroseptica*. To the authors'

knowledge this is the first report of fungicide resistance developed by any Peronosporales pathogen that increases the sexual reproductive capabilities of that pathogen above that of sensitive phenotypes. A previous study observing sexual fitness of MR and MS isolates of *P. infestans* observed that the lowest oospore production was obtained when crossing two MR isolates, while the highest oospore production was obtained when crossing two MS isolates (Hanson & Shattock, 1998). However, there may be a different response in oospore production between MR isolates that are homothallic (*P. erythroseptica*) verses those that are heterothallic (*P. infestans*). If the high oospore production observed for MR *P. erythroseptica* isolates *in vitro* is similar to the oospore production of MR isolates in nature in root and tuber tissue, and if the oospore viability of MS and MR isolates are the same, then there would be an increased risk in generating higher *P. erythroseptica* soil inoculum levels at a faster rate in soil containing MR isolates than in soil with only MS isolates. Higher sexual propagation by MR isolates, in addition to metalaxyl-M insensitivity, could potentially be related to the increased incidence of pink rot reported by growers in southeastern Idaho in their potato fields. In addition to potentially higher sexual propagation by MR isolates in nature, potato growers in Idaho have also moved from planting potatoes every three or four years to either planting potatoes consecutively or every other year due to economic reasons. An increased production of oospores by MR isolates followed by a shorter crop rotation would significantly increase soil inoculum levels in addition to what has previously been stated. Also, the majority of potato growers in southeastern Idaho are on a two-year rotation of either wheat or barley followed by potatoes. Wheat and barley are hosts of *P. erythroseptica* (Whelan & Loughnane, 1969) which would allow the pathogen to survive and reproduce from year to year between potato crops. In the spring of 2002, MR isolates of *P. erythroseptica* were isolated by this laboratory from the roots of wheat and barley plants collected in the early spring from six commercial wheat fields and one barley field in southeastern Idaho, thereby indicating possible carry over of *P. erythroseptica* from year to year in hosts commonly found in potato cropping systems.

Viability tests in this study were conducted using a viability stain or plasmolysis; however, these tests were inconclusive. Heat-killed oospores stained red when using the tetrazolium bromide viability stain, resulting in false positives for viability, as described by others (Williams *et al*., 1980; Sutherland & Cohen, 1983; Pittis & Shattock, 1994). In addition, oospores staining red were observed to burst releasing the red-stained cytoplasmic contents, resulting in oospores that were originally stained red, but changed in appearance to an oospore appearing non-stained and would therefore not be counted as a viable spore. Oospore viability determined by plasmolysis resulted in the same observations of membrane detachment from oospore walls for both positive and negative controls of MS and MR isolates. Therefore, these two techniques were not reliable in determining viability of *P. erythroseptica* oospores.

Sporangial production was not quantified between MR and MS phenotypes. However, zoospore production was significantly greater for MS than for MR isolates of *P. erythroseptica* under the laboratory conditions tested, indicating greater asexual fitness for MS isolates. However, asexual fitness based on zoospore germination rates was similar. Zoospore production previously assessed for MS and MR isolates of *P. infestans* indicated that MR isolates released zoospores more rapidly than MS isolates, but after four hours there were no significant differences in the number of zoospores released (Bashan *et al*., 1989). The findings here for *P. erythroseptica* are contrary to those determined for *P. infestans* since MS isolates released more zoospores. Zoospore release of *P. erythroseptica* cultures was not observed beyond 45 minutes since previous research indicated the duration of actively swimming zoospores at 22°C ceased after one hour (Vujicic & Colhoun, 1966).

Inheritance studies on phenylamide resistance in *P. infestans* (Fabritius *et al*., 1997; Judelson & Roberts, 1999) suggests different loci controlling resistance with interacting epistatic minor genes (see Shattock, 2002). In the pink rot pathogen recent analysis of two generations of selfed *P. erythroseptica* isolates also suggested that metalaxyl-M sensitivity might be controlled by more than one major gene, along with additive effects of minor genes (Samen *et al*., 2005). Such studies are useful in fully understanding the factors affecting increasing incidence of disease and changing frequency of phenotypes in pathogen populations, as observed here in pink rot in Idaho.

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