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# Resistance to metalaxyl-M and cymoxanil in a dominant clonal lineage of *Phytophthora infestans* in Huánuco, Peru, an area of continuous potato production

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**Abstract** Fifty-eight isolates of *Phytophthora infestans* were evaluated *in vitro* and on detached leaves of potato for their sensitivities to metalaxyl-M and cymoxanil. The isolates belonged to the clonal lineage, EC-1, which is dominant on potato in Peru and Ecuador. All isolates were collected in Huánuco, Peru, an area of year-round potato production, where the potential for development of fungicide resistance is high. All isolates were resistant to metalaxyl-M, with *in vitro* $EC_{50}$ values ranging from 468.30—813.57 mg $l^{-1}$. In contrast, we found no evidence for resistance to cymoxanil for which *in vitro* $EC_{50}$ values ranged from 0.03—1.11 mg $l^{-1}$. Resistance to each fungicide was also evaluated for five isolates in a detached leaf assay in which the fungicide was sprayed on the leaf surface prior to inoculation. With metalaxyl-M, the range of $EC_{50}$ values was 158.85—828.29 mg $l^{-1}$, similar to that for the *in vitro* assay. For cymoxanil, $EC_{50}$ values ranged from 1.41 to 2.31 mg $l^{-1}$, which was higher than in the *in vitro* assay but still two orders of magnitude lower than the concentration applied by farmers in the field.

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## Introduction

The continuing changes in populations of *Phytophthora infestans* worldwide have made the management of late blight increasingly difficult. Due to the introduction and subsequent global migration of new genotypes of *P. infestans*, pathogen populations are now generally characterised by greater aggressiveness, earlier outbreaks of disease and increased resistance to fungicides (Hannukkala et al. 2007; Fry 2008).

The initial high efficacy of the phenylamide fungicide, metalaxyl, against oomycete pathogens led to its widespread use for many diseases, including potato late blight. However, resistance to metalaxyl was reported shortly after its introduction (Davidse et al. 1981) and has since been reported in most potato growing regions of the world (Fry et al. 2009).

The population of *P. infestans* in the Andes north of Bolivia is dominated by the EC-1 clonal lineage (Forbes et al. 1998; Perez et al. 2001), first described in Ecuador (Forbes et al. 1997). Many of the isolates belonging to this lineage have been characterised as metalaxyl-resistant in studies done in Peru (Perez et al. 2001; Garry et al. 2005a) and Ecuador (Forbes et al. 1997). In Peru, a decline in the efficacy of metalaxyl was also reported (V. Otazú and R. Egúsquiza, personal communication) and the use of the compound decreased, especially where the disease pressure was high.

During the last decade metalaxyl-M (mefenoxam) was introduced into the market in Peru. This compound contains a much higher concentration of the active enantiomer of the original metalaxyl, and is therefore more effective in the field. The introduction of metalaxyl-M apparently led to a resurgence in use of both metalaxyl and metalaxyl-M in some areas of Latin America, particularly where the disease pressure was moderate. Based on 1998—1999 surveys, the combination of metalaxyl with a contact fungicide was reported as one of the most frequently used potato (*Solanum tuberosum*) late blight fungicides in Morochata (Bolivia), Bolivar (Ecuador) and Cajamarca, Huaraz and Cuzco (Peru) (O. Ortiz, personal communication). Recent surveys carried out in Carchi (Ecuador) indicated that metalaxyl combined with mancozeb, was the third most commonly used among late blight fungicides (Kromann et al. 2008). Peruvian farmers from La Libertad and La Encañada, where disease pressure is moderate, indicated that metalaxyl-M-containing products were the most commonly used fungicides for control of potato late blight (L. Maldonado, personal communication).

The translaminar fungicide, cymoxanil, is also commonly used for potato late blight control in Peru and Ecuador (Kromann et al. 2008). In areas of high late blight pressure, cymoxanil is frequently the most commonly used of the translaminar and systemic fungicides (Kromann et al. 2008). Cymoxanil is sold throughout the Andes in a number of different mixtures, which always contain at least one contact fungicide, such as mancozeb.

Unlike for metalaxyl-M, there is little evidence for resistance to cymoxanil in populations of *P. infestans* (Sujkowski et al. 1995; Reis et al. 2005); however, resistance to cymoxanil in *Plasmopara viticola*, the cause of grape downy mildew, has been reported (Gullino et al. 1997; Klinkenberg et al. 1998). Even though no resistance to this compound has been demonstrated for *P. infestans,* there are several factors common to potato production in parts of Peru and much of the northern Andes that could lead to high selection pressure for resistance to fungicides in the pathogen population. First, plants generally must be protected from emergence on, and most cultivars require about 130 days from emergence to maturity. Therefore, the total number of sprays per season is high, ranging between four and 20 (depending on weather) for a susceptible cultivar (Kromann et al. 2008). Second, the pathogen population is comprised primarily of one clonal lineage (Forbes et al. 1997; Perez et al. 2001). Fungicide resistance, which has been selected in the population, would not be lost because of genetic recombination, migration from other populations, or seasonal bottlenecks. Third, disease is present at relatively high levels most of the year. Therefore, the pathogen population size is large, which is an important factor determining the probability of resistance occurring (Fry et al. 1992). Finally, farmers do not practice a management scheme designed to reduce selection pressure for resistance, as is done in other parts of the world. Most practices designed to reduce selection for resistance are routinely violated (e.g., systemic compounds are used repeatedly and as curatives). As examples of indiscriminant use of fungicides, in Ecuador the susceptible cv. Capiro is frequently sprayed exclusively with cymoxanil-based compounds, often more than 15 times in one season (J. Carrillo, personal communication), and in Huasahuasi (Peru), the cv. Canchan was sprayed 11 times in one season with the same class of fungicides (N. Bustamante, personal communication). Huánuco is one of the primary potato producing regions of Peru. Due to its location on the eastern slopes of the Andes, Huánuco has a mild climate appropriate for potato production all year round, which creates a situation like that described above where selection pressure for fungicide resistance in the pathogen population should be high. Compounds containing both metalaxyl-M and cymoxanil are frequently used in Huánuco. One survey indicated that in Chaglla, a community in Huánuco, both cymoxanil- and metalaxyl-M- based compounds are sprayed between three and seven times per crop for late blight control (L. Maldonado, personal communication).

Results of fungicide-resistance assessments on a sample of isolates of *P. infestans* collected in Huánuco are reported in this paper. Isolates were assessed for resistance to both cymoxanil and metalaxyl-M. The study was designed to test the hypothesis that resistance to these two systemic fungicides has been selected in the EC-1 clonal lineage in Huánuco. The results reported here should also establish current sensitivities for the EC-1 lineage for both fungicides in this geographic region, and provide a preliminary evaluation of the risk of the *P. infestans* population developing resistance to cymoxanil under conditions of continuous potato production in the Andes.

## Materials and methods

### Isolates

The collection strategy was designed to provide a general overview of the current sensitivity to metalaxyl-M and cymoxanil in the most important potato growing provinces in the department of Huánuco. Fifty-eight isolates of *P. infestans* were collected between February and March 2003 from the provinces of Pachitea ($n$=21 isolates), Ambo ($n$=15 isolates) and Huánuco ($n$=22 isolates). For each province, five to eight fields were sampled, with three to seven samples taken at random per field. The fields in each province were selected so as to be geographically representative and to represent both modern cultivars (recently released from plant breeding programmes, $n$ = 42) and native cultivars of uncertain origin ($n$ = 16). The fields were sampled just before flowering and an effort was made to register the number of fungicides that had been applied.

Leaflets with single lesions were taken from the diseased plants and maintained until processing in sealed Petri dishes containing a layer of 1.5% water agar (WA) in the base. Prior to isolation, plates were incubated for 5 days at 18°C with a photoperiod of 12 h to promote sporulation. Sporangia were washed from leaves in distilled water and collected via filtration using a 10 µm filter. After further rinsing to reduce bacterial contamination, sporangia were inoculated in a distilled water suspension on tuber slices of potato cv. Huayro. Slices were then incubated for 6 days in moist chambers at 18°C with a 12 h photoperiod. Mycelial fragments were then transferred aseptically from the tuber slices to V-8 agar plates (Perez et al. 2001). Isolates were maintained for short periods on V-8 agar (<6 months) and for longer periods in liquid nitrogen in 15% dimethyl sulphoxide (DMSO). Prior to evaluation, cryogenically-stored isolates were recovered and propagated on tuber slices and then transferred to V-8 media.

All isolates were analysed for mating type using a polymerase chain reaction (PCR) based assay with a marker linked to the mating-type locus (Judelson 1996) and RFLP fingerprinted using the probe RG57 (Goodwin et al. 1992).

In addition to the isolates collected in 2003 in Huánuco, two isolates with known sensitivity (one resistant and one sensitive) to metalaxyl were used as controls. There is cross-resistance between metalaxyl and metalaxyl-M (isolates resistant to one form are resistant to the other), although the level of resistance may vary (Parra and Ristaino 2001).

### Fungicides

Cymoxanil and metalaxyl-M were used in all assays as technical grade, with purities of 98.3 and 95.9%, respectively, as recommended by Georgopoulos (1982). Stock solutions of each fungicide were prepared (0.5 g of fungicide were suspended in 19.5 ml of DMSO) before use. Further concentrations are expressed in mg $l^{-1}$.

### *In vitro* assays

Dilutions in distilled water of the stock solution of metalaxyl-M 95.9% (a.i.) were added to V-8 agar to give final concentrations ranging from 0.01 to 1000 mg $l^{-1}$ (Fig. 1). DMSO (0.1%) was added to V-8 agar as a control. Fungicide solutions were added to molten V-8 agar and continuously agitated while pouring to ensure even distribution in Petri dishes. A plug of mycelium (1 mm diam) from zones of active growth of 10 day-old cultures was placed in the centre of each fungicide-amended plate and in the control. Plates were incubated in darkness at 18°C for 10 days. Four replications for each isolate by concentration combination were placed randomly in the incubator. Mean colony diameter (minus the diameter of the inoculation plug) was measured for each plate. Relative mycelial growth (RMG) was calculated for each isolate as follows:

$$RMG = 100Dx/Dy;$$

where $Dx$ is the mean diameter on the fungicide-amended plate and $Dy$ is the mean diameter of the control. Diameters were first corrected by subtracting the diameter of the inoculation plug (Sujkowski et al. 1995; Daayf and Platt 2002). The criteria of Therrien et al. (1993) were used to classify isolates as sensitive (radial growth < 40% of control with 5 and 100 mg $l^{-1}$), intermediately resistant (radial growth > 40% with 5 mg $l^{-1}$, but < 40% with 100 mg $l^{-1}$), and resistant (radial growth > 40% with both concentrations). $EC_{50}$ values were determined for each isolate by

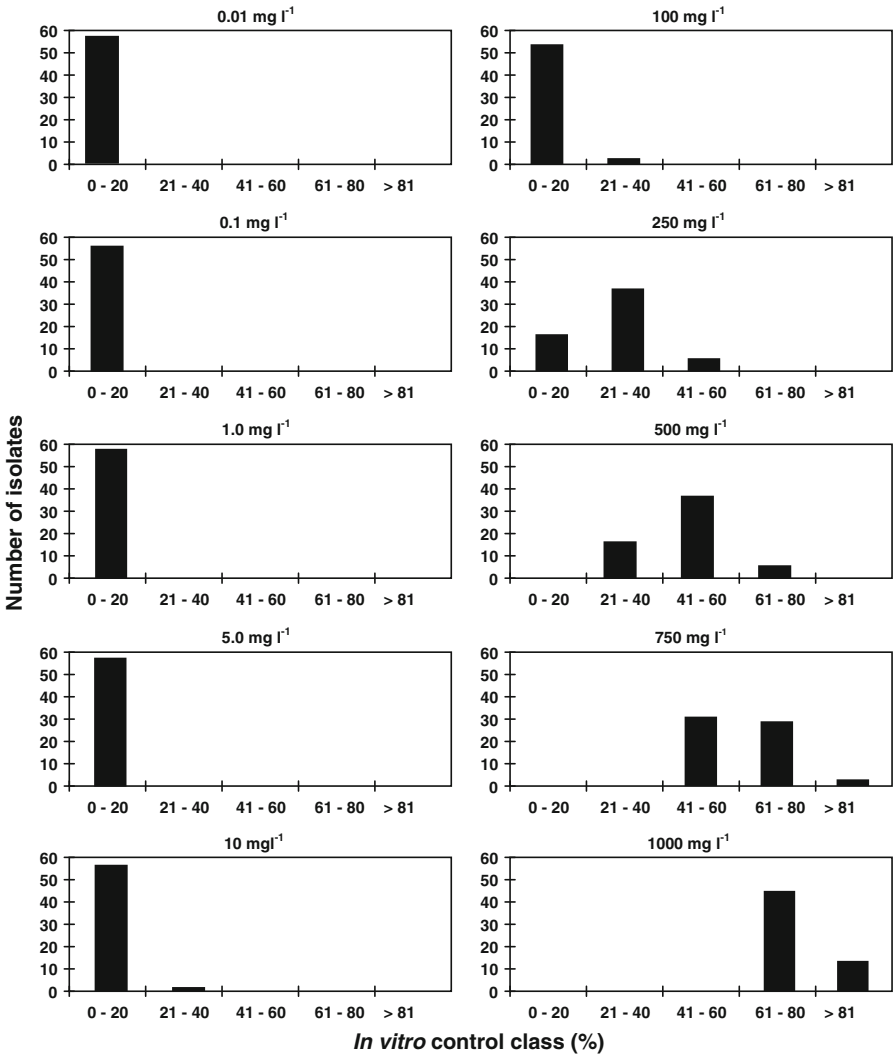


**Fig. 1** Frequency distributions of 58 *Phytophthora infestans* isolates from central Peru for *in vitro* sensitivity to different concentrations of metalaxyl–M. Sensitivity is expressed as the percentage mycelial growth of an isolate on fungicide-amended agar relative to growth on fungicide-free agar

calculating the inhibition: =1- (the mean colony diameter on amended media divided by the mean colony diameter on unamended media) in proportion and then subjecting the data to Probit analysis (Hsiang et al. 1997) using the statistical programme R (R Foundation for Statistical Computing, Vienna, Austria). Ten concentrations of metalaxyl-M were used to calculate $EC_{50}$ values (Fig. 1).

The method for measuring resistance *in vitro* to cymoxanil was the same as that for metalaxyl-M described above, except that six concentrations of cymoxanil 98.3% (a.i.) ranging from 0.01 to 4 mg $l^{-1}$ from stock solution were used (Fig. 2). Evaluations of relative mycelial growth and calculation of the $EC_{50}$ values were as described for metalaxyl-M.

Detached leaf assays

Detached leaf assays were done with 6 week-old plants of the late blight susceptible potato cv. Yungay. Five isolates were selected for each compound that represented the range of sensitivities in the *in vitro* assay. For metalaxyl-M, plants were sprayed with the same ten concentrations used in the *in vitro* study (Fig. 1), made from stock solution dissolved in 1 l of distilled water. The fungicide was allowed to dry for 12 h before plants were inoculated. A suspension of each selected *P. infestans* isolate (3,000 sporangia $ml^{-1}$) was sprayed until run-off on two replicates of each concentration x isolate combination. Ten leaflets from the middle part of each plant were immediately

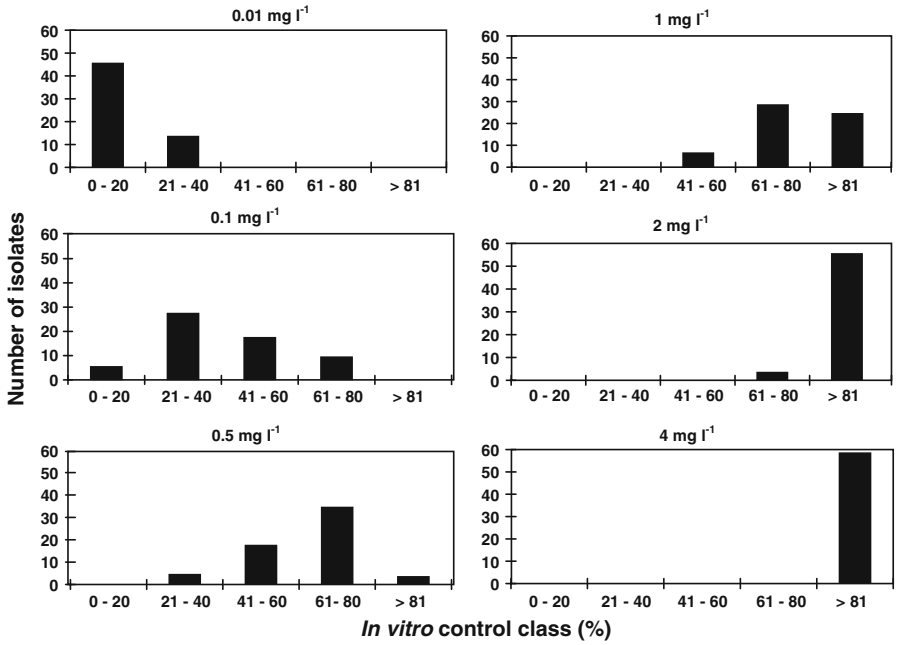


**Fig. 2** Frequency distributions of 58 *Phytophthora infestans* isolates from central Peru for *in vitro* sensitivity to different concentrations of cymoxanil. Sensitivity is expressed as the percentage mycelial growth of an isolate on fungicide-amended agar relative to growth on fungicide-free agar

removed and placed in inverted Petri dishes with 1.5% WA in the base, such that leaflets lay in the lids below the agar layer. The plates were incubated at 18°C for 6 days with a 12 h photoperiod (Georgopoulos 1982; Perez et al. 2001). The fungicide-free control plant was sprayed with distilled water. Infected foliar area of each leaflet was calculated seven days after inoculation using Sigma Scan Pro (Systat Software Inc. Richmond, California, USA) and expressed as percentage of each total leaflet area. The mean of measurements from ten leaflets from each concentration was subjected to Probit analysis (PROBIT Procedure, R) to obtain $EC_{50}$ values.

For cymoxanil, six concentrations ranging from 0.01 to 4 mg $l^{-1}$ (Fig. 2) from stock solution dissolved in 1 l of distilled water were sprayed onto two replicate plants for each concentration x isolate combination. The conditions for the assays, evaluation and analysis were the same as described for metalaxyl-M.

## Results

Using the criteria of Therrien et al. (1993) described above, all isolates were classified as resistant to metalaxyl-M. Metalaxyl-M had virtually no effect on *in vitro* growth up to 100 mg $l^{-1}$ (Fig. 1). From 250 to 1000 mg $l^{-1}$, increasing concentrations clearly caused greater restrictions of growth, with the highest concentration restricting growth by 60 % or more of the control. $EC_{50}$ values for metalaxyl-M ranged from 468.30 to 813.57 mg $l^{-1}$ with a mean of 643.82 mg $l^{-1}$ (Table 1).

The isolates were more sensitive to cymoxanil, as restriction in growth occurred even with 0.01 mg $l^{-1}$. Severe restriction of mycelial growth *in vitro* occurred in all isolates at concentrations > 1 mg $l^{-1}$, and growth was virtually stopped at 4 mg $l^{-1}$ (Fig. 2). *In vitro* $EC_{50}$ values ranged from 0.03 to 1.11 mg $l^{-1}$ (Table 1).

**Table 1** Sensitivity to metalaxyl-M and cymoxanil among isolates of *Phytophthora infestans* collected in Huánuco, Peru

| | $EC_{50}$ values (mg $l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Metalaxyl-M | | | Cymoxanil | | |
| Assays | Range | Mean | SD | Range | Mean | SD |
| *In vitro*[1] | 468.30–813.57 | 643.82 | 68.02 | 0.03–1.11 | 0.41 | 0.23 |
| Detached leaf[2] | 158.85–828.29 | 526.27 | 282.37 | 1.41–2.31 | 1.9 | 0.4 |

[1] Data from 58 isolates.
[2] Data from 5 selected isolates

Evaluation of sensitivity in detached leaves gave generally similar results based on the ranges of $EC_{50}$ values (Table 1). Sensitivity was in the same range for both types of assays for metalaxyl-M, and slightly higher for cymoxanil (1.41–2.31 mg $l^{-1}$).

The relative responses of isolates to the two compounds is best viewed graphically (Fig. 3). $EC_{50}$ values for metalaxyl-M were orders of magnitude higher than those for cymoxanil and there was no overlap in the frequency distributions (Fig. 3).

## Discussion

The results presented here demonstrate that a sample of isolates of *P. infestans* from Huánuco, Peru, was much more sensitive to cymoxanil than to metalaxyl-M. As with all fungicide evaluations, however, there are a number of factors that must be taken into consideration in the interpretation of results, for example: type of assays used, fungicide mode of action and interpretation of monitoring data, especially in the absence of baseline sensitivity data.

Cymoxanil is locally systemic, with penetrant and translaminar activity, but the mode of action is not yet known. Cymoxanil was introduced 30 years ago and has been used for control of late blight in many European countries for more than 15 years without reports of resistance (Power et al. 1995). Metalaxyl-M contains primarily the active enantiomer found in metalaxyl. Both metalaxyl and metalaxyl-M have the same specific mode of action, inhibiting fungal ribosomal RNA polymerases (Marucchini and Zadra 2002). Metalaxyl-M has about twice the activity of metalaxyl and therefore is used at half the rate. The level of resistance to metalaxyl-M found in Huánuco can be considered high, although comparing levels of resistance to metalaxyl-M to previous reports is difficult because many are based on the older compound metalaxyl and also because results are frequently presented only as resistance classes and not as $EC_{50}$ values.

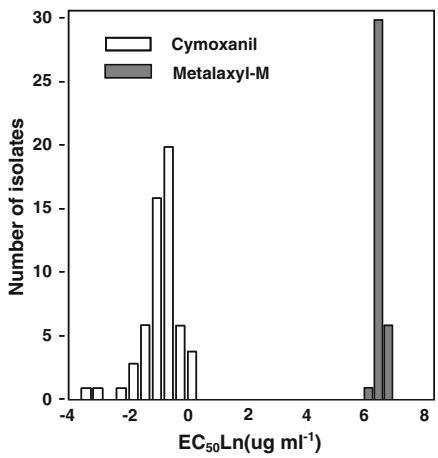


**Fig. 3** Graphical comparison of frequency distributions of $EC_{50}$ on a log normal scale for 58 *Phytophthora infestans* isolates from central Peru tested for *in vitro* sensitivity to different concentrations of metalaxyl-M (mefenoxam) and cymoxanil

Based on resistance classes, the Huánuco population would appear highly resistant to metalaxyl-M, compared to previous studies which have generally found some portion of the population to be sensitive or intermediate. A recent study in Brazil found relatively similar proportions of sensitive, intermediate and resistant isolates (Reis et al. 2005). Another recent study in Mexico found a range of sensitivity levels in both a background population and one sampled immediately after repeated fungicide applications (Grünwald et al. 2006). Previous studies in the EC-1 population in the Ecuadorean Andes have found a high proportion of resistant isolates but still within a range of sensitivity classes (Forbes et al. 1997; Adler et al. 2004) However, studies in the Peruvian Andes have found primarily resistant isolates (Perez et al. 2001; Garry 2005a). This could be an indication of greater use of metalaxyl-M in Peru, but sufficiently precise fungicide usage data are lacking to test this hypothesis.

The results of the *in vitro* and detached leaf assays were proportionally more similar with metalaxyl-M than with cymoxanil (Table 1). This may be due to a greater sensitivity of *P. infestans* to cymoxanil *in vitro* than *in planta*, which apparently is not the case for metalaxyl-M. However, since the levels of overall sensitivity were much greater for cymoxanil, comparison between the two compounds is difficult. Other published studies also demonstrated that *in vitro* and *in planta* assays give comparable results for the former metalaxyl; however we have not found studies using both types of assays for cymoxanil (Table 2). It is noteworthy, however, that the highest average $EC_{50}$ we found for cymoxanil in the literature was measured *in planta* on detached leaves (see Table 2, Gisi et al. 1997) and was higher than what we found on detached leaves.

**Table 2** Selected references reporting $EC_{50}$ values for sensitivity of *Phytophthora infestans* isolates to cymoxanil

| $EC_{50}$ Values mg $l^{-1}$ | | Method | Reference |
|---|---|---|---|
| Range | Mean | | |
| 0.06–1.48 | 0.42 | Petri dish | Hamlen and Power (1998) |
| | | | Hamlen and Power (1998) |
| 0.22 [b] | 0.46 [c] | Petri dish | Hamlen and Power (1998) |
| 0.22 [b] | 0.50 [c] | Petri dish | |
| < 10 [a] | 0.8 | Petri dish | Sujkowski et al. (1995) |
| | 3 | Detached leaf | Gisi et al. (1997) |
| | 1 | Petri dish | (Ziogas & Davidse 1987) |
| 0.04–0.52 | 0.17 | Petri dish | Power et al. (1995) |
| 0.1–1.0 | | Petri dish | (Reis et al. 2005) |
| 0.10–1.48 | 0.3 | Petri dish | Power et al. (1995) |

[a] Values not considered as a range. Values reported as sensitivity or more than this value.

[b] Values reported pre-commercialisation of the fungicide.

[c] Values reported after 10 or more years after commercialisation.

$EC_{50}$ values obtained in this study for cymoxanil were consistent with others reported in the literature (Tables 1 and 2). *In vitro* values were similar to those of Hamlen and Power (1998), and the detached leaf values were lower than those of Gisi et al. (1997). However, the ensemble of studies worldwide looking at resistance in cymoxanil presents a picture of consistency of $EC_{50}$ values and of a reduced risk of resistance developing with this fungicide.

One problem with fungicide resistance assays of any type is the degree to which biological significance can be attributed to the results (Taylor et al. 2006). In an effort to improve interpretation, the results have been compared with concentrations applied in the field under Andean conditions (Table 3). Results obtained would suggest that the population of *P. infestans* in Huánuco cannot be controlled by metalaxyl-M. Even the highest concentration applied in the field (metalaxyl, Table 3) is below the average $EC_{50}$ we found for the detached leaf assay. Ineffectiveness of metalaxyl in Peru may be somewhat masked by mixture with a contact fungicide and this may explain why it is still used. Indeed, metalaxyl-M and the former metalaxyl are still used in other locations where research has demonstrated insensitivity in the pathogen population (Mukalazi et al. 2001). Field experiments are needed to relate the results of laboratory and greenhouse studies to practical performance of metalaxyl-M. However, this can be difficult because of contamination with background inoculum of pathogen genotypes of unknown sensitivity. It would appear, however, that the probability of this occurring in Huánuco would be minimal, since virtually all isolates are resistant, and this could be a good location for field validation of our results.

In contrast to metalaxyl-M, our $EC_{50}$ values for cymoxanil for both detached leaves and whole plants were much lower than concentrations generally applied in the field. Gisi et al. (1997) developed a classification system for sensitivity to cymoxanil where: $EC_{50}$ values > 1000 mg $l^{-1}$ are resistant; >20 but <1,000 are intermediately resistant; and <20 mg

**Table 3** Fungicide formulations and doses of metalaxyl, metalaxyl-M and cymoxanil commercialised in Peru or Ecuador*

| Active ingredients [a] | Commercial dose (g/200 l) | Concentration (mg $l^{-1}$) [b] |
|---|---|---|
| Metalaxyl 35% | 150–250 | 262.5–437.5 |
| Mancozeb 64% + metalaxyl-M (mefenoxam) 4% | 500 | 100 |
| Copper hydroxide 46% + cymoxanil 6% | 500 | 150 |
| Mancozeb 64% + cymoxanil 8% | 500 | 200 |
| Propineb 70%+ cymoxanil 6% | 500–600 | 150–180 |

[a] Current active ingredients commercialised in Peru.

[b] 1 ppm = 1 mg $l^{-1}$.

* Data based on local fungicide usage guides.

$l^{-1}$ are sensitive. Using this system, all of our isolates would be considered sensitive based on *in vitro* and detached leaf tests. Overall, however, our results are consistent with reports of little or no resistance to cymoxanil in *P. infestans* in Europe (Hamlen and Power 1998). Our results are also consistent with others that demonstrated resistance to metalaxyl-M and cymoxanil are not linked (Samoucha and Cohen 1988); this information is useful for developing resistance management programmes. Recently, Grünwald et al. (2006) demonstrated directional selection for resistance to cymoxanil after repeated field applications of the compound, and indicated the potential for resistance within *P. infestans* in Mexico, the putative centre of origin of the pathogen.

The general predominance of the new (sensu Spielman et al. 1991) clonal lineage (EC-1 lineage) in Peru and elsewhere in the Andes is probably due to differences in pathogen fitness relative to the old lineage (US-1) (Kato et al. 1997; Andrade-Piedra et al. 2005). However, resistance to metalaxyl is another possible factor that could explain, at least in part, the rapid replacement of US-1 in central Peru (Huánuco, Pasco and Junín) by the EC-1 lineage (Perez et al. 2001), as it would appear that the US-1 lineage had much higher levels of sensitivity to this compound (Perez et al. 2001; Garry et al. 2005a, b).

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