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Arch Environ Contam Toxicol (2013) 65:67–77
DOI 10.1007/s00244-013-9877-5

Degradation of Metalaxyl and Folpet by Filamentous Fungi Isolated From Portuguese (Alentejo) Vineyard Soils

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Received: 21 August 2012 / Accepted: 29 January 2013 / Published online: 22 February 2013
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Abstract Degradation of xenobiotics by microbial populations is a potential method to enhance the effectiveness of ex situ or in situ bioremediation. The purpose of this study was to evaluate the impact of repeated metalaxyl and folpet treatments on soil microbial communities and to select soil fungal strains able to degrade these fungicides. Results showed enhanced degradation of metalaxyl and folpet in vineyards soils submitted to repeated treatments with these fungicides. Indeed, the greatest degradation ability was observed in vineyard soil samples submitted to greater numbers of treatments. Respiration activities, as determined in the presence of selective antibiotics in soil suspensions amended with metalaxyl and folpet, showed that the fungal population was the microbiota community most active in the degradation process. Batch cultures performed with a progressive increase of fungicide concentrations allowed the selection of five tolerant fungal strains: *Penicillium* sp. 1 and *Penicillium* sp. 2, *mycelia sterila* 1 and 3, and *Rhizopus stolonifer*. Among these strains, *mycelium sterila* 3 and *R. stolonifer* presented only in vineyard soils treated with repeated application of these fungicides and showed tolerance >1,000 mg l^{-1} against commercial formulations of metalaxyl (10 %) plus folpet (40 %). Using specific methods for inducing sporulation, *mycelium sterila* 3 was identified as *Gongronella* sp. Because this fungus is rare, it was compared using csM13-polymerase chain reaction (PCR) with the two known species, *Gongronella butleri* and *G. lacrispora*. The high tolerance to metalaxyl and folpet shown by *Gongronella* sp. and *R. stolonifer* might be correlated with their degradation ability. Our results point out that selected strains have potential for the bioremediation of metalaxyl and folpet in polluted soil sites.

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Metalaxyl [methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacethyl)-D,L-alaninate] is a systemic acylanilide effective against Oomycetes (*Plasmopara viticola* Berlese et Toni) that cause downy mildew (Tomlin 1997). This fungicide is a stable compound with good photo-stability (Sukul and Spiteller 2001a), is soluble in water at 20 °C (7.1 g l^{-1}), with a vapour pressure of 0.29 mPa (20 °C) and an octanol–water partition coefficient (K_{ow}) of 1.75 (O'Neil et al. 2001). Metalaxyl was classified as a class III-E compound, with an oral LD_{50} in rats of 669 mg kg^{-1}, and it is considered nontoxic to nontarget arthropod and vertebrate species [United States Environmental Protection Agency (USEPA) United States Environmental Protection Agency 1994]. However, some studies related the genotoxic and mutagenic effects of metalaxyl (Hrelia et al. 1996). Because of its low adsorption and high mobility, metalaxyl lixiviated easily in soil and can contaminate groundwater, so its residues are a serious environmental threat (Andrades et al. 2001; Sukul 2006; Martin et al. 2012; Wightwick et al. 2012). Indeed, metalaxyl residues have been found in groundwater at concentrations ≤0.49 µg l^{-1}, which exceeds the 0.1 µg l^{-1} European Union limit (Hildebrandt et al. 2008).

Folpet [*N*-(trichloromethylthio)-phthalimide] is a contact fungicide with preventive action against downy mildew, one of the most important vine obligate parasites (Tomlin 1997; DGADR 2010). This fungicide is practically insoluble in water (0.8 mg l^{-1} at 25 °C), slightly soluble in organic solvents, and has low vapour pressure at 20 °C (O'Neil et al. 2001) and low stability in soil (USEPA United States Environmental Protection Agency 1999). The acute oral toxicity of folpet is 2.44 g kg^{-1} for mice and 10 g kg^{-1} for rats (USEPA United States Environmental Protection Agency 1999). Folpet was classified as a class II-B compound, and probable human carcinogenic, with carcinogenic and mutagenic activity in mice (USEPA United States Environmental Protection Agency 1999; Nazir et al. 2003). Commercial formulations containing metalaxyl and folpet as active products are widely used to protect vineyards against *P. viticola*.

The degradation of fungicides in soil is influenced by many factors, such as soil type, physicochemical properties, microbiota composition, and environmental conditions (Sukul and Spiteller 2001a; Arbeli and Fuentes 2007). Soil microorganisms contribute to soil quality and play an important role in soil ecosystem processes, such as nutrient cycling, organic matter decomposition, and bioremediation (Chirnside et al. 2007; Kadian et al. 2012). The persistence and the behaviour of metalaxyl residues in soil depends on the soil characteristics, namely mineral and organic content and microbial population (Sukul and Spiteller 2000; Monkiedje and Spiteller 2002; Demanou et al. 2004). Some studies have established that accelerated degradation of metalaxyl can take place in some soils or in liquid cultures and involves a wide range of microorganisms, but most of the them did not report the identification of specific microbial strains being responsible for metalaxyl degradation (Bailey and Coffey 1986; Sukul and Spiteller 2001b; Demanou et al. 2004). Zheng et al. (1989) report that a culture of *Syncephalastrum racemosum* could degrade metalaxyl, but when the concentration increases to 0.1 g l^{-1} the degradation decrease to less than 5 %. Firmino (2000) reported the dynamics of folpet in soil of Alentejo (Évora), in the South of Portugal, a region with intensive vine culture that requires frequent applications of this pesticide, combined with metalaxyl, to prevent downy mildew. Furthermore, Alentejo soils are usually poor in microbiota and organic content; thus, frequent application of pesticides in agricultural fields might be problematic to the soil ecology and consequently to the sustainability of ecosystems. In Portugal, despite the extensive application of metalaxyl and folpet, the impact of repeated treatments on soil microbiota composition and soil biotransformation of these fungicides have not been studied.

The aim of this work was to evaluate the impact of repeated application with metalaxyl and folpet on degradation ability by soil microorganisms and to select fungal soil strains capable of degrading these fungicides.

Materials and Methods

Chemicals

The technical-grade formulation of commercial fungicide Alister Combi (Makhteshim Agan, Portugal) containing folpet 40 % and metalaxyl 10 % (w/w) as active compounds in wet powder formulation was obtained directly from the Portuguese market. This commercial formulation, which has been repeatedly applied in vineyards according to Portuguese recommendation procedures (DGADR 2010), was used for the laboratory soil experiments and for fungal enrichment cultures.

The standards metalaxyl and folpet PESTANAL reagent grade (99 %) used for high-performance liquid chromatography (HPLC) analyses were purchased from Fluka (Germany). All of the others reagents and solvents used were of analytical grade.

Soil Samples

The study was performed using soils samples collected from three adjacent vineyards (1°34′N, 38°31′W) in Alentejo, Portugal. Two vineyards, submitted for 16 (site A) and 8 (site B) years to annual application of the commercial fungicide and a recent vineyard (site C) without history of fungicide application were sampled for this study. Soil samples taken from 0 to 20 cm below the surface, which had not received any fungicide treatment for at least 10 months, were freshly collected from the three vineyards, gently air-dried at room temperature, homogenized by sieving through an approximately 2-mm steel mesh, and stored at 4 °C before use (Rowell 1994). The predominant soils of both analysed sites are Mediterranean Luvisols (FAO/ISRIC/ISSS 2006). The physicochemical properties (texture, pH, conductivity, organic carbon, nitrogen, phosphorus, potassium and cation exchange capacity [CEC] and, calcium (Ca)-, magnesium (Mg)-, sodium (Na)-, and potassium (K)-exchangeable bases) of the three soil sites were determined as described elsewhere (Rowell 1994; McKenzie et al. 2008). Results are expressed as a mean of triplicate determinations.

Degradation of Metalaxyl and Folpet in Soil Samples

The degradation kinetics of metalaxyl and folpet were evaluated in soil samples from the three vineyards sites under nonsterilised and sterilised conditions. For experiments under sterilised conditions, which were performed

aseptically, soil samples were autoclaved at 121 °C during 15 min on three consecutive days. Concentrations of metalaxyl and folpet were determined in soil samples for monitoring the fungicide residues.

Laboratory experiments were performed with a commercial formulation of metalaxyl (10 % wet weight; w/w]) plus folpet (40 % [w/w]) applied in a concentration of 0.5 mg g^{-1} dry soil, corresponding to 50 μg g^{-1} of metalaxyl and 100 μg g^{-1} of folpet. This selected fungicide concentration corresponds to the maximal single permitted dose of commercial formulation by the Portuguese Regulatory Agency (DGADR 2010). The addition of fungicide to the soil samples was performed according Sukul and Spiteller (2001b). Calculated amounts of commercial fungicide in 1 ml of methanol, previously sterilized by filtration (pore size 0.2 μm), were distributed in portions of approximately 25 g of fresh soil in a porcelain dish to avoid potential effects of solvents on the soil biological activity. The treated subsamples of each soil were thoroughly mixed until complete evaporation of solvent (approximately 10 min). Each subsample was subsequently added to the total soil mass (100 g of dry soil) and transferred to individual Erlenmeyer flasks (250 ml). Erlenmeyer flasks were plugged with sterilised cotton pads and mixed using an orbital shaker (50 rev min^{-1}) for 1 h. The moisture content was adjusted to 60 % of maximum water-holding capacity. To negate the effect of light, sterilised and nonsterilised samples were incubated at 25 ± 2 °C in the dark. Remaining metalaxyl and folpet residues in treated soil samples were measured periodically during 70 days. Soil samples (20 g) were extracted with acetonitrile (50 ml) on an orbital shaker (Heidolph Unimax 2010) for 2 h in 250-ml Erlenmeyer flasks. Acetonitrile extracts were centrifuged at 10,000 g for 10 min (Hermle Z380 centrifuge), decanted, filtrated using 0.45-μm Whatman filter membrane, transferred to screw-top vials, and stored at −20 °C for further HPLC analyses.

Metalaxyl and folpet were quantified using an HPLC system equipped with a Rheodyne injector, a mobile phase with acetonitrile and ultra-pure water (1:1) with a flow rate of 1 ml min^{-1} (Merck Hitachi L-7100 pump), a reversed-phase column Merck Supersher (100 RP-18, 250 × 4.6 mm, 5 μm), and an Isco V_4 wavelength ultraviolet (UV) detector set at 210 nm. A Varian chromatography integrator was used to record and analyse the area of the peak. The retention times were 6.4 min for metalaxyl and 17.6 min for folpet. Quantification of metalaxyl and folpet residues were determined by external standard based on the analyses of metalaxyl and folpet standard solutions ranging from 0.5 to 6 mg l^{-1} and 2 to 24 mg l^{-1}, respectively. Solutions were prepared in triplicates and run four times on the high-pressure liquid chromatographer. Recovery percentages of metalaxyl and folpet on soil samples spiked with commercial fungicide were 98 ± 2 % ($p < 0.01$) and 96 ± 3 % ($p < 0.05$), respectively.

Isolation and Identification of Soil Microbial Populations

Aliquots of soil (10 g) sampled from each vineyard field were suspended with 90 ml of NaCl (0.85 % w/v). After shaking at 25 °C for 2 h at 180 rev min^{-1}, the supernatant was diluted 10- to 10^6-fold with the same saline solution, and five 0.2 ml aliquots of each suspension were spread onto 9-cm Petri dishes (Germida 1993). Bacteria isolates were enumerated in tryptic soy agar medium (Merck, Darmstadt, Germany) after 48 h of incubation at 30 °C and characterised according morphological features and tests, namely Gram stain, catalase, and oxidase (Krieg and Holt 1984). In addition, Biolog MicroLog3 4.01 C technique was used for bacteria species identification. Filamentous fungi isolates were enumerated in Cooke Rose Bengal agar medium (CRB) with chloramphenicol 0.01 % w/v (Merck, Darmstadt, Germany) after 5 days of incubation at 25 °C (Pereira et al. 2002). Results were expressed as colony forming units (CFU)/gram of soil (dry weight). In addition, the frequency of fungal isolates was determined as a cumulative number of isolates recorded on CRB plates in 0.2-ml aliquots of soil suspension diluted at 10^3 after plate incubation for 5 days at 25 °C. Frequency of isolates, designated as fungal species abundance, was expressed as the number of individual isolates of a species. Filamentous fungi were subcultured onto malt extract agar (MEA [malt extract 2 %, mycological peptone 1 %, glucose 2 %, and agar 2 %]) and identified according to standard methods (Domsch et al. 2007) based on macromorphological and micromorphological characteristics, such as colony diameter, texture, colour, dimensions and morphology of hyphae, and reproductive structures. Filamentous fungi were grouped based on their morphological characteristics; for genera *Aspergillus* and *Penicillium*, different strains were designed with numerical indices according to their morphological differences. Nonsporulating strains were classified as *mycelia sterila* numbered according to the similarity of the colonies' morphology when isolates were compared under identical incubation conditions.

Due to the interest in future studies, two isolates were deposited in the Culture Collection of Industrial Microorganisms (CCMI; Lisbon, Portugal) with the numbers CCMI 1104 and CCMI 1100 for *R. stolonifer* and *mycelium sterila* 3 (*Gongronella* sp.), respectively.

Soil Respiration Activities

Soil respiration activities were measured by rates of consumption of O_2 or production of CO_2. Soil suspensions (3 g of soil in 20 ml of sterilised water) were prepared with

samples collected from site A, which received the greatest number of repeated treatments. Three different concentrations of commercial fungicide (0.25, 0.5, or 1 mg ml^{-1}) were added to the soil suspensions to evaluate the effect of fungicide concentration in soil microbiota. A soil suspension without fungicide was used as control. In addition, soil suspensions containing a nontoxic commercial fungicide concentration (0.25 mg ml^{-1}) were amended with the antibiotic chloramphenicol (100 μg ml^{-1}) or cycloheximide (100 μg ml^{-1}), and soil respiration activities were measured to determine the active soil microbiota component in the degradation process (Roper and Ophel-Keller 1998; King and Dutka 2000). Quantifications of O_2 consumption and CO_2 production were performed using a gas chromatographer (HACH CARLE) with an exclusion molecular column (Altech), a helium C-50 mobile phase at 20 ml min^{-1} carrier flow, and a thermoconductivity detector. Microbial growth was evaluated by the quantification of O_2 consumption or CO_2 production in soil aqueous suspensions at 0, 12, 24, 48, 72 h or daily until cultures exceed the exponential growth phase. At the end of each assay, microbial growth was quantified as described in "Isolation and Identification of Soil Microbial Populations" section.

Enrichment Fungi Assays

The fungal isolates were submitted to progressively increasing concentrations of the commercial fungicide formulation in batch cultures (Pereira et al. 1996; Roper and Ophel-Keller 1998). The general procedure for fungal enrichment was performed as follows: under aseptic conditions, 25 ml of malt extract broth (MEB; Merck), supplemented with 0.1 g l^{-1} commercial fungicide, was inoculated with 1 ml of each fungal isolate (10^6 CFU). Experiments were performed in triplicate. Cultures were incubated at 25 °C in an orbital shaker at 180 rpm for 6 days. After incubation, flasks were subcultured by aseptically transferring 1 ml of culture in 25 ml of fresh MEB medium containing 0.2 g l^{-1} of the commercial fungicide. This serial procedure was repeated to concentrations of 0.4, 0.6, 0.8, 1.0, and 1.5 g l^{-1} of the commercial fungicide. For each fungicide concentration, a sample was inoculated onto MEA and CRB, and the plates were incubated for 5 days at 25 °C for identification of isolates as described in "Isolation and Identification of Soil Microbial Populations" section.

Identification and Molecular Characterisation of *mycelium sterila* 3

To promote sporulation and to identify the isolate *Gongronella* sp., previously designated as *mycelium sterila* 3, several assays were performed that included solid agar (1.5 %), sterile plant pieces (e.g., leaf fragments of vineyard and grains of corn, wheat, and rice), and aliquots of sterile soil. After inoculation of the fungus, a set of Petri dishes were incubated at 25 °C for 7 days, and another set were incubated at room temperature for 10 days. After these periods, samples of the fungal colonies were studied under an optical microscope to observe reproductive structures.

The molecular characterization of *Gongronella* sp. was performed by csM13-PCR fingerprinting. *G. butleri* (ATTC 8989) and *G. lacrispora* (ATCC 24412) were used as reference strains. For DNA extraction, fungal isolates were previously grown in MEA at 25 °C, for 7 days. *Mycelium* was scraped from plates with a sterile scalpel, collected into microtubes, treated with lyses buffer (50 mM Tris, 250 mM NaCl, 50 mM ethylene diamine tetraacetic acid [EDTA], 0.3 % sodium dodecyl sulfate (w/v) [pH 8.0]), and mixed with glass beads (425–600 μm) at 60 °C. After centrifugation, the supernatant was transferred to clean tubes with Tris-EDTA (10 mM Tris and 1 mM EDTA) and RNAse (Sigma) (60 μg ml^{-1}) and incubated for 30 min at 37 °C. Chloroform/isoamyl alcohol (24:1 ratio) was then added for extraction. The supernatant was transferred to a new tube, and DNA was precipitated with cold ethanol. The resulting pellet was air-dried and resuspended in Tris-EDTA. The genomic DNA was verified by 1 % agarose gel electrophoresis. csM13 PCR was performed in 25-μl reaction volumes comprising the following: sterilized ultrapure water, *Taq* polymerase buffer 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 0.2 mM of csM13 primer (5′-GAGGGTGGCGGTTCT-3′), 1U *Taq* DNA polymerase (Fermentas), and 250 ng of template DNA (Caldeira et al. 2009; Guimarães et al. 2011). The DNA was amplified in a thermal cycler according to the following program: 94 °C for 5 min; 50 cycles consisting of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; and a final extension step at 72 °C for 2 min (Progene; Techne, UK). PCR products were separated electrophoretically through a 1.2 % (w/v) agarose gel at 80 V. The DNA fragments were visualised on a UV transilluminator (Bio-Rad, Hercules, CA, USA) using Quantity One 1-D Analysis software (Bio-Rad, Hercules, CA, USA). Phylogenetic tree was generated by UPGMA method using the Dice coefficient of similarity.

Metalaxyl and Folpet Degradation by Selected Fungi Strains

Batch cultures of *R. stolonifer* and *Gongronella* sp. in MEB, supplemented with 1.5 g l^{-1} of the commercial fungicide, were incubated for 28 days at 25 °C in an orbital shaker at 180 rev min^{-1} and sampled periodically for the quantification of remaining metalaxyl or folpet by HPLC. Equivalent experiments performed without fungal

inoculum were used as abiotic control. Fungicides were extracted with dichloromethane, and the organics extracts were evaporated to dryness, suspended in 1 ml of acetonitrile, filtrated using 0.45-μm Whatman filter membrane, transferred into screw-top vials, and stored at −20 °C until further HPLC analyses. Recovery percentages of metalaxyl and folpet in MEB samples spiked with commercial fungicide were 97 ± 2 % ($p < 0.01$) for metalaxyl and 96 ± 3 % for folpet ($p < 0.05$).

Data Analysis

One-way analyses of variance were performed with degradations rates of metalaxyl and folpet for each soil site (expressed as μg day^{-1} g^{-1}, dry soil), with soil respiration activities, and with degradation rates in batch assays to determine statistically significant differences at the 99 % ($p < 0.01$) and 95 % ($p < 0.05$) confidence levels. Multiple comparisons of media were evaluated by Tukey test, and differences between values at $p < 0.01$ were considered statistically significant. IBM, SPSS software (SPSS, Chicago, IL), version 18.0, was used to perform these analyses.

Results and Discussion

Soil Characterisation

The physicochemical properties of the three soil samples (site A, B, or C) are listed in Table 1. The three soil sites presented clay-loam surface texture and showed similar physicochemical characteristics. The pH values were 6.5 to 7.3. The three soil sites showed poor organic carbon content at 12 g kg^{-1} for sites A and B and 15 g kg^{-1} for site C. Soil samples from site A showed the lowest (0.1 g kg^{-1}) and site C the highest (0.9 g kg^{-1}) concentrations of NH_3–N, but the concentrations of NO_3–N were similar among the three soil sites. Low concentrations of P_2O_5 were found in all soil sites, and concentrations of K_2O ranged between 60 and 144 mg kg^{-1} (Table 1). The CEC was similar for the three soil samples. Soil samples collected directly from the three analysed sites (sites A, B, C) had no detectable amounts of metalaxyl and folpet.

Degradation of Metalaxyl and Folpet in Soil Samples

Dissipation of metalaxyl and folpet (%) in soil samples are shown in Fig. 1. Results showed a linear correlation of metalaxyl and folpet degradation during the first 42 days of the assay; however, after that a lower decrease of concentrations of both analysed fungicides was observed. At 42 days of the assay, the degradation of metalaxyl was 26, 17, and 9 % for sites A, B, and C, respectively, whereas abiotic degradation was only 4 % (Fig. 1a). For the same incubation time, the degradation of folpet in soil was 56, 43, and 27 % for sites A, B and C, respectively, whereas abiotic degradation was 10 % (Fig. 1b). Table 2 lists the metalaxyl and folpet degradation rates at sites A, B, C as determined for the first 42 days of assay under biotic and abiotic conditions. Results show that metalaxyl and folpet degradation rates were significantly different for each soil site ($p < 0.01$). The highest degradation rates were observed for site A at values of 0.37 and 2.59 μg g^{-1} day^{-1} for metalaxyl and folpet, respectively. In contrast, independent of site, degradation rates in the abiotic control were 0.03 μg g^{-1} day^{-1} for metalaxyl and 0.42 μg g^{-1} day^{-1} for folpet (Table 2). The three analysed soil sites showed different ability to degrade the fungicides. Sites A and B showed greater degradation rates of metalaxyl and folpet than site C (the site without previous application of fungicide). Enhanced degradation ability was observed in soil samples from site A (with greater number repeated treatments) compared with site B ($p < 0.01$).

Results showed that degradation of metalaxyl or folpet was mainly due to resident soil microorganisms despite abiotic degradation. The low metalaxyl abiotic degradation observed could be correlated with its photostability (Sukul and Spiteller 2001a). Abiotic degradation observed for folpet could be due to the low stability of folpet in soil as has been reported in a few studies about the degradation of this fungicide in soils (USEPA 1999). Studies related that the biodegradation process of pesticides in soil depends on the relation between soil physicochemical parameters, namely, clay content and soil biomass, that influence the persistence and fate of pesticides in the environment (Andrades et al. 2001; Demanou et al. 2004; Sukul 2006). In addition, repeated application of some fungicides plays an important role in the degradation process compared with single applications (Bischoff et al. 2005; Hole and Powles 2007). Enhanced microbial degradation occurring after a previous application of the same pesticide could be due to an adaptation of one or more indigenous species of microorganism (Chung and Ou 1996; Arbeli and Fuentes 2007; Chirnside et al. 2007). In particular, metalaxyl in soil is dependent on soil type and environmental conditions (Bailey and Coffey 1986; Sukul and Spiteller 2001a). However, studies about metalaxyl degradation in clay loam soil, namely, in the Iberian Peninsula, are scarce. To our knowledge, there are few studies about the folpet degradation in soil. In addition, folpet in aqueous solution is converted to phthalimide as its major metabolite (Viviani-Nauer et al. 1997; Cabras et al. 2001).

Microbial Soil Populations

Bacterial isolates in soil sites A, B, and C were 1.9×10^6, 1.5×10^7 and 1.3×10^7 CFU g^{-1}, respectively. Fungal

Table 1 Physicochemical properties of soils samples and initial fungicide content in soil samples

Soil samples	Site A	Site B	Site C
Texture (%)			
Clay	32.75	31.63	36.96
Silt	21.83	27.24	23.43
Sand	45.43	41.1	39.61
Textural class	Clay loam	Clay loam	Clay loam
pH (H_2O)	7.0	7.3	6.5
Conductivity (mmhos cm^{-1})	0.06	0.04	0.03
Organic carbon (g kg^{-1})	12.0	12.0	15.0
NH_3–N (g kg^{-1})	0.1	0.6	0.9
NO_3–N (mg kg^{-1})	19.0	17.0	16.0
P_2O_5 (mg kg^{-1})	17.0	7.0	16.0
K_2O (mg kg^{-1})	144.0	118.0	60.0
CEC ($cmol_{(c)}$ kg^{-1})	19.7	16.1	16.7
Exchange Ca^{2+} ($cmol_{(c)}$ kg^{-1})	5.88	6.14	8.19
Exchange Mg^{2+} ($cmol_{(c)}$ kg^{-1})	3.44	3.23	1.25
Exchange Na^{+} ($cmol_{(c)}$ kg^{-1})	0.22	0.22	0.22
Exchange K^{+} ($cmol_{(c)}$ kg^{-1})	0.32	0.29	0.13
[Metalaxyl] (μg g^{-1})	Not detected	Not detected	Not detected
[Folpet] (μg g^{-1})	Not detected	Not detected	Not detected

isolates were 3.2×10^4, 2.4×10^4, and 8.2×10^4 CFU g^{-1} in soils sites A, B, and C, respectively. Results showed an increase of bacteria in soils from sites B and C compared with soil site A, which was submitted to a greater number of repeated treatments. In contrast, a decrease of fungi in soils from sites A and B, compared with soil from site C (without previous fungicide treatment), was observed.

The predominant bacterial strains isolated in soil samples from all analysed sites were from the genus *Bacillus*. However, one strain of *Actinomycetes* from site B and two bacterial strains, Gram-negative bacilli and Gram-positive cocci, were isolated simultaneously from soil samples of sites A and B. The Gram-negative and Gram-positive bacteria were identified as *Enterobacter cloacae* and *Staphylococcus arlettae*, respectively.

Table 3 lists the most abundant fungal isolates in soil from sites A through C. The fungal strains presented were the most abundant in each one of the three soils. Results showed that the frequency of fungal isolates changed with the number of repeat treatments. The most frequent fungal strains in site C were *Cladosporium* sp. 1, *Penicillium* sp. 3, and *Aspergillus* sp. 2; conversely, these taxa were isolated infrequently at sites A and B. Moreover, *R. stolonifer*

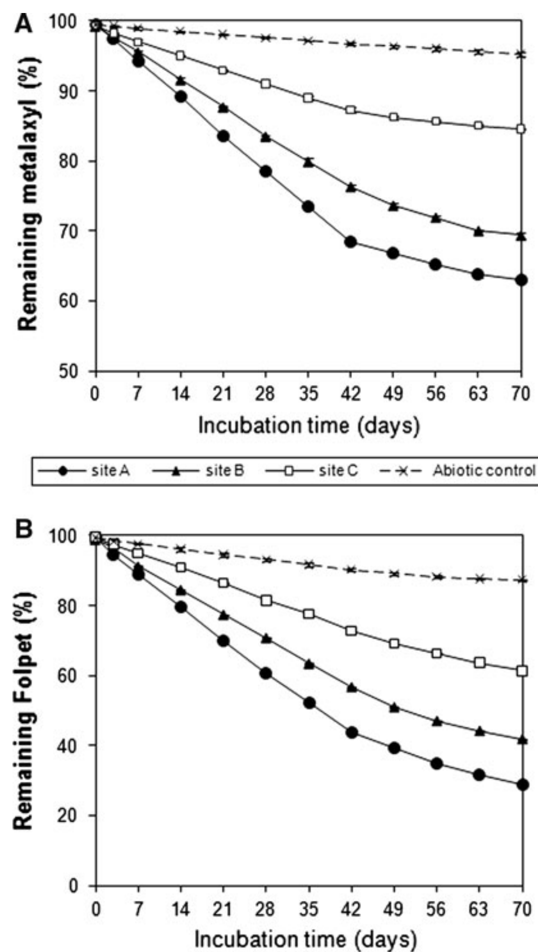

Fig. 1 Degradation kinetics of metalaxyl (**a**) and folpet (**b**) in nonsterilised soil samples after application of commercial fungicide at 0.5 mg g^{-1} (dry weight). Abiotic control is the mean of sterilised samples of the three soil sites (A, B, and C). Data are the mean of three replicates for each soil site ± SD

and *mycelium sterila* 3 were the strains most frequent in the oldest vineyard soil (site A), which were submitted to the greatest number of fungicide treatments, but they were not isolated in site C.

Effect of Commercial Fungicide on Soil Microorganisms

The effect of commercial fungicide concentration in soil suspensions containing metalaxyl plus folpet as the main carbon source on soil microbiota is shown in Fig. 2a, b. Respiratory activities were greater in presence of fungicide compared with the control, but O_2 consumption and CO_2 production rates decreased with the increase in fungicide concentration. The lowest fungicide concentration of commercial fungicide (0.25 g l^{-1}) tested, which has shown nontoxic effects for microorganisms, was selected for the study with antibiotics. Results obtained in the presence of specific antibiotics (chloramphenicol or cycloheximide)

Table 2 Metalaxyl and folpet degradation rates (0–42 days) in soil samples from soil sites A through C

Fungicide	Soil degradation rates (μg fungicide g^{-1} day^{-1})					
	Site A		Site B		Site C	
	Sample	Abiotic control	Sample	Abiotic control	Sample	Abiotic control
Metalaxyl	0.372 ± 0.003^{a}	0.032 ± 0.001^{d}	0.278 ± 0.002^{b}	0.033 ± 0.002^{d}	0.140 ± 0.003^{c}	0.032 ± 0.003^{d}
Folpet	2.588 ± 0.003^{a}	0.420 ± 0.002^{d}	1.983 ± 0.006^{b}	0.417 ± 0.001^{d}	1.272 ± 0.002^{c}	0.418 ± 0.002^{d}

Sample corresponds at soil samples with indigenous populations and abiotic control corresponds at sterilised soil samples (without microorganisms). Data are the mean of three replicates ± SD. Significant degradation rates are indicating by different superscript lower-case letters in each row

Table 3 Identification and abundance of fungi isolated from soil samples at sites A through C

Fungi strains	Isolates (CFU)		
	Site A	Site B	Site C
A. niger van Tieghem	10	9	16
Aspergillus sp. 1	4	2	0
Aspergillus sp. 2	0	2	18
Cladosporium sp.	0	0	82
Fusarium oxysporum Schlecht	5	4	2
Gliocladium catenulatum Gilman and Abbott	1	1	0
Penicillium sp. 1	4	2	0
Penicillium sp. 2	3	4	0
Penicillium sp. 3	2	2	38
R. stolonifer (Ehremb.) Lind	10	8	0
mycelium sterila 1	3	4	0
mycelium sterila 2	6	2	0
mycelium sterila 3	14	6	0
mycelium sterila 4	2	2	8
Total	64	48	164

showed decreased microbial population with decreased O_2 consumption and CO_2 production rates (Fig. 2c, d). O_2 consumption rates changed from 0.030 mmol day^{-1} in soil samples without antimicrobial agents (control) to 0.027 and 0.005 mmol day^{-1} in soil samples amended with chloramphenicol or cycloheximide, respectively (Fig. 2c). CO_2 production rates changed from 0.031 mmol day^{-1} in soil samples without antimicrobial agents (control) to 0.028 or 0.005 mmol day^{-1} in soil samples amended with chloramphenicol or cycloheximide, respectively (Fig. 2d). The bacteria population decreased from 6.0×10^6 to 1.0×10^6 CFU in the presence of chloramphenicol (Fig. 2e), and fungi decreased from 1.0×10^4 to 0.2×10^4 CFU in the presence of cycloheximide (Fig. 2f). The decrease of respiration activity was observed mainly in the presence of cycloheximide showing that fungal population was the microbiota community most active in the degradation of commercial fungicide containing metalaxyl plus folpet.

Selection of Fungi Able to Degrade Metalaxyl and Folpet Fungicides

The fungi strains isolated in presence of each fungicide concentration are listed in Table 4. Five fungal isolates—*Penicillium* sp. P_1 and P_2, *mycelium sterila* 1 and 3, and *R. stolonifer*—were able to grow at 0.4 g l^{-1} of commercial fungicide. Among these strains, *R. stolonifer* and *mycelium sterila* 3, isolated only in soils of vineyards treated with repeated application of this commercial fungicide, showed tolerance to >1.0 g l^{-1} of this formulation. Results showed that the most frequent fungal strains in site C soil samples were present at low frequency at sites A and B and that these strains did not tolerate greater fungicide concentrations (Table 4). Nevertheless, the two of the most tolerant strains, *R. stolonifer* and *mycelium sterila* 3, were isolated only in vineyard soils treated with repeated application of commercial fungicide (sites A and B), and their frequency was greater in samples from site A, which was submitted to a greater number of previous treatments.

Despite the number of some fungal isolates decreasing proportionally with the number of repeated treatments, the frequency of some fungal strains could be associated with a selection process simultaneous with fungicide application. The dissipation of metalaxyl and folpet in batch cultures of *R. stolonifer* and *mycelium sterila* 3 containing 1.0 g l^{-1} of commercial fungicide showed that, at day 20 of the assay, was significantly greater compared with the abiotic degradation ($p < 0.01$). *R. stolonifer* and *mycelium sterila* 3 (*Gongronella* sp.) showed greater tolerance and an ability to degrade metalaxyl and folpet. According to Zheng et al. (1989) *S. racemosum* showed an ability to degrade metalaxyl in aqueous solutions. It is important to point out that *S. racemosum* belongs to the *Zygomycetes* family as do the two selected isolates in the present study. These findings suggest that there may be a correlation between these fungi and their ability to degrade metalaxyl.

Identification of *Mycelium Sterila* 3

Once the isolate *mycelium sterila* 3 showed good performance regarding metalaxyl elimination, several procedures were

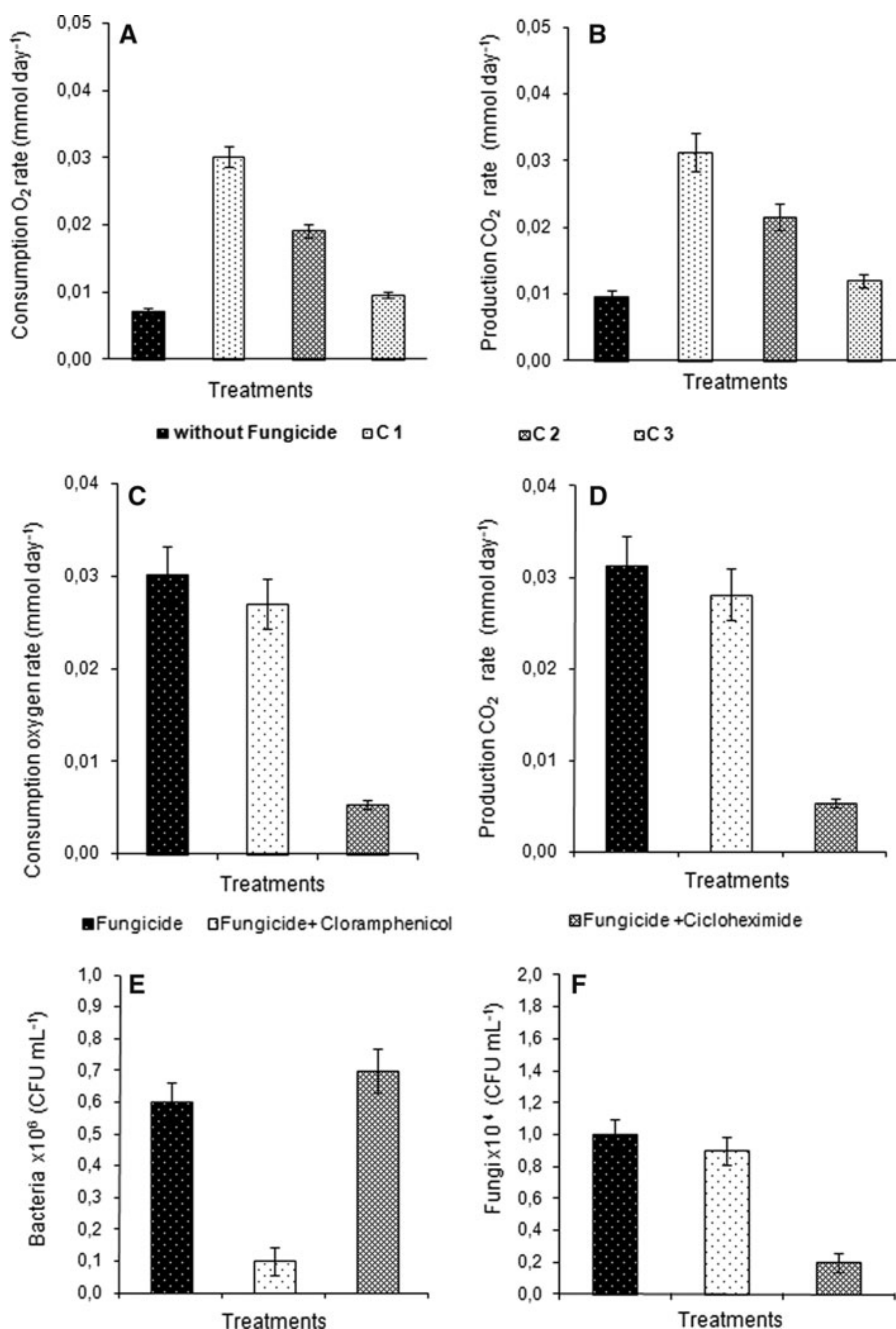

Fig. 2 Effect of the fungicide application on microbial soil respiration activity. Effect of concentration of commercial fungicide (C1 = 0.25 mg ml^{-1}; C2 = 0.5 mg ml^{-1}; C3 = 1 mg ml^{-1}) on O_2 consumption (**a**) and CO_2 production (**b**) rates in soil suspensions. Effect of selected antibiotics on microbial activity of soil suspensions containing 0.25 mg ml^{-1} of fungicide as main carbon. O_2 consumption (**c**) and CO_2 production (**d**) rates and total viable bacteria (**e**) and fungi (**f**) expressed (CFU ml^{-1}). Data are the mean of three replicates of each concentration ± SD

performed to identify this strain. Microscopic observation showed coenocytic mycelium. After induced sporulation onto medium with sterile soil, it was possible to observe sparse sporulated structures. This allowed identification of this isolate in the genus *Gongronella*. The csM13-PCR molecular approach showed differences in the fingerprinting profile of the isolate *Gongronella* sp. compared with those of *G. butleri* (ATCC 8989) and *G. lacrispora* (ATCC 24412) (Fig. 3).

The *Gongronella* genus has a worldwide distribution with moderated frequencies in subtropical regions and warm climates, which is the case of Alentejo region. These fungi have been isolated from arable soils, marshes, wasteland, anthills, garden soil, and palm plantations (Domsch et al. 2007). The csM13-PCR fingerprinting approach suggests that enriched *Gongronella* sp. isolate (CCMI 1100) is probably a new species of the genera *Gongronella*, for which only two species are currently described. Further studies should be developed to confirm if this isolate is a new species.

Metalaxyl and Folpet Degradation by Selected Fungi Strains

The strains *R. stolonifer* and *Gongronella* sp. were selected to study their ability to degrade metalaxyl and folpet. The

Table 4 Selective enrichment of soil fungi strains with commercial fungicide formulation containing metalaxyl (10 % [w/w]) and folpet (40 % [w/w])

Fungi strains	Commercial fungicide concentration (g l^{-1})						
	0.1	0.2	0.4	0.6	0.8	1.0	1.5
A. niger	+	+	+	–	–	–	–
Aspergillus sp. 1	+	+	+	–	–	–	–
Aspergillus sp. 2	–	–	–	–	–	–	–
Cladosporium sp.	+	–	–	–	–	–	–
F. oxysporum	+	+	–	–	–	–	–
Gliocladium catenulatum	+	+	–	–	–	–	–
Penicillium sp. 1	+	+	+	+	–	–	–
Penicillium sp. 2	+	+	+	+	–	–	–
Penicillium sp. 3	+	+	+	–	–	–	–
R. stolonifer	+	+	+	+	+	+	+
mycelium sterila 1	+	+	+	+	–	–	–
mycelium sterila 2	+	+	–	–	–	–	–
mycelium sterila 3	+	+	+	+	+	+	–
mycelium sterila 4	+	–	–	–	–	–	–

+ growth, – no growth

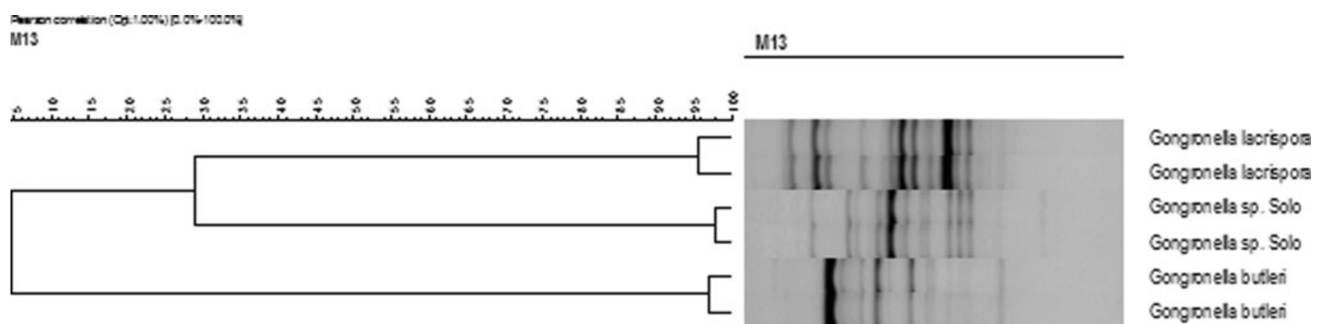

Fig. 3 Dendogram and csM13-PCR fingerprinting of *Gongronella* spp.

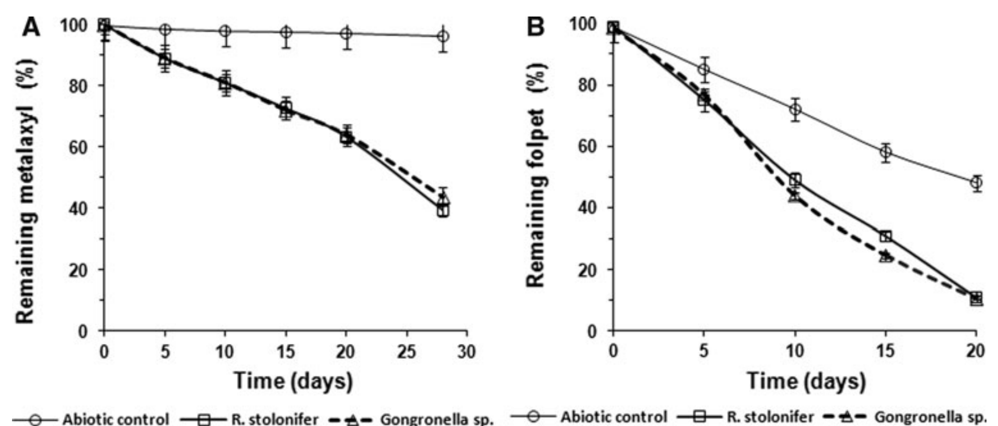

Fig. 4 Degradation kinetics of metalaxyl (**a**) and folpet (**b**) in cultures of *R. stolonifer* and *Gongronella* sp. containing 0.1 g l^{-1} of commercial fungicide formulation. Abiotic control was assayed in the same conditions without fungal inoculum. Data are the mean of three replicates ± SD

dissipation of metalaxyl and folpet in these cultures were evaluated for 20 days (Fig. 4). At the end of the assay, cultures with *R. stolonifer* and *Gongronella* sp. decreased the remaining metalaxyl from 98 % to approximately 70 % (Fig. 4a) and the remaining folpet from 78 to 10 % (Fig. 4b). Results showed that cultures of *R. stolonifer* and *Gongronella* sp. can significantly decrease metalaxyl and folpet concentrations compared with abiotic control

($p < 0.01$), but no significant differences on metalaxyl and folpet concentrations were observed in cultures of *R. stolonifer* compared with cultures of *Gongronella* sp. ($p > 0.05$) for the same incubation time.

Conclusion

The present study reports the impact of repeated metalaxyl and folpet treatments on soil microbial communities and the selection of soil fungal strains able to degrade these fungicides. In conclusion, our results showed that repeated application of the fungicides metalaxyl and folpet increases the ability of soil to degrade the fungicides metalaxyl and folpet and that this process in mainly a biotic degradation. Although the number of isolates decreased with the number of repeated treatments, the frequency of some fungal strains were associated with a selection process due to the repeated fungicide application. From the strains isolated from soil submitted to a greater number of treatments as well as selective enrichment with fungicide, *R. stolonifer* and *Gongronell*a sp. showed the ability to tolerate and to degrade either metalaxyl or folpet, even in high concentrations. Therefore, these selected fungal strains could have valuable applications for the remediation of metalaxyl and folpet in polluted sites.

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