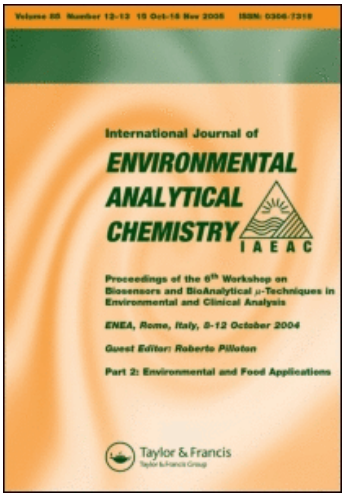

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Studies on the response of soil microflora to the application of the fungicide fenhexamid

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The aim of this work was to evaluate the impact of the fungicide fenhexamid (FEX) on the genetic structure of soil bacterial communities using the Ribosomal Intergenic Spacer Analysis molecular technique. Using real-time PCR, we also tried to quantify the pcaH sequences which encode the dioxygenases involved in the degradation process of a variety of aromatic compounds. Soil taken from a vineyard in the Etna Park (Sicily, Italy) was treated with FEX in the ratio 2 $\mu g\,g^{-1}$ soil every 7 days, the process being repeated four times. The analyses were carried out before treatment and 7 days after each further application of FEX. At the same time, the degradation rate was evaluated. The use of FEX determined a variation in the bacterial component of the soil which could be seen in an increase of some microbial strains and the inhibition of others. The pcaH sequence was already present in the genes of the soil microrganisms studied, but the use of FEX increased the number of the gene copies. These results suggest that the microbial population of the soil adapted to the presence of FEX with an increase in degradation potential. The measurements of the extent to which FEX was degraded confirm this hypothesis, showing that the molecule disappeared more quickly with successive applications.

Keywords: Fenhexamid; Soil microflora; RISA; Real-time PCR

1. Introduction

Telluric microflora play a key role in the degradation of pesticides in the soil and of many other pollutants from industrially produced organic substances. These substances can, in their turn, modify the biology of the soil microbial community inhibiting its growth or favouring the appearance and development of populations which are able to use them as a source of carbon [1]. Soil microorganisms also play a key role in the quality of agricultural soils which is usually defined as the sustained capacity of the soil to produce healthy and nourishing crops, resist erosion, and reduce the impact of

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environmental stresses on plants [2]. The interaction between soil microbial biodiversity and soil ecological functioning is poorly documented. For this reason, it would seem important to carry out studies which aim to evaluate links between the presence of specific microorganisms and the processes they catalyse [3]. A description of the structure, composition, density, and diversity of soil microbial communities is important to understand better the soil functioning [4].

The development of molecular techniques, based on the direct extraction of DNA from the soil and its amplification using the polymerase chain reaction (PCR), have made it possible to study soil microorganisms without the need for cultivation; this is of great importance considering that it is possible to cultivate only 1% of the bacteria in the soil microbial community [5]. In the past, the need to isolate microorganisms in order to carry out a biochemical and molecular characterization meant that only very partial information was acquired, regarding the actual consistency of the microbial population. Instead, direct extraction of DNA from the soil makes it possible to obtain much more detailed information on the microrganisms involved in the various steps of the degradation process by means of the identification and sequencing of some of the genes involved in each transformation [6, 7]. Sandmann and Loos [8] and Boundy-Mills *et al*. [9] succeeded, by means of this technique, in determining the degradation process of atrazine and 2.4-dichlorophenossiacetic acid. Different authors have demonstrated that DNA-fingerprint techniques are useful to study the effect of pesticides on soil microbial community [10–12]. El Fantroussi *et al*. [10] have shown that an effect of urea herbicides on the soil microbial community could be observed by using cluster analysis of DGGE profiles. They found that certain species were either eliminated or stimulated by the application of the herbicides, especially linuron. Crecchio *et al*. [12] have performed ARDRA and DGGE to assess the response of soil bacterial community to the application of propanil and prometryne herbicides and found that molecular approaches can represent differences among microbial populations as a consequence of anthropogenic activities as use of pesticides.

We had previously performed experiments on the degradation of fenhexamid (FEX), (*N*-(2.3-dichlor-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide), in water and in soil. Our investigations allowed us to highlight the fact that microrganisms have a great influence on the degradation of FEX in the soil. In effect, in the presence of microrganisms, the DT_{50} in soil proved to be about one day, while in sterile conditions, FEX did not degrade during the whole 152 days of the test. FEX proved to be reasonably stable in both sterile buffer solutions and in water samples collected in the Simeto river (Sicily, Italy), even if a good quantity of microrganisms was found in the latter. The isolation of a single microbial strain from the soil, the *Bacillus megaterium*, able to degrade the molecule, led to the hypothesis that there is a high degree of specialization in the microbial population involved in the degradation process [13]. FEX is a protective fungicide belonging to the hydroxyanilide group, used in the elimination of *Botrytis cinerea*, *Monilia fructigena*, and *M. laxa* in the cultivation of grapes, soft fruit, tree fruit, and vegetables.

With respect to these results, the aim of our work was to continue investigations into the environmental behaviour of FEX, evaluating its interaction with the microbial populations of the soil. We also investigated the genetic potential of the microrganisms of the soil studied in producing a dioxygenase which was able to cleave the aromatic ring of the molecule.

2. Experimental

2.1 *Soil sampling*

The soil was collected from a vineyard situated in the Etna Park (Sicily, Italy) at 670 m above sea level. This vineyard had never been treated with FEX. The soil was passed through a 2-mm sieve and stored at 4°C to maintain its biological activity. The soil was pre-incubated in its field moisture state for 1 week at 25°C before the experiments.

2.2 *Incubation*

The soil (150 g) was placed in polyethylene bottles, humidified to 50% of the Water Holding Capacity (WHC) and treated with FEX in the ratio of 2 $\mu g\,g^{-1}$ (according to good agricultural practice) every 7 days for 4 weeks. During this period, the soil was kept in the dark, in a thermostat at 30°C, in aerobiosis. The aerobic conditions were guaranteed by the partial closure of the screw top of the bottles. A part of the untreated soil was kept under the same experimental conditions. FEX concentration was determined after 15 min and every 7 days from the addition of the fungicide. The molecular analyses on the microbial population were performed on soil samples before treatment and 7 days after each addition of FEX. For each treatment, three replications have been done.

2.3 *Fenhexamid analyses*

In order to analyse the fungicide, diatom flour was added to the soil sample (Extrelut, Merck, Pavia, Italy) in a 1.0 : 1.6 ratio (w/w). The mixture was carefully homogenized, then extracted with 100 mL of a cyclohexane–ethyl acetate solution (95 : 5, v/v). The suspension was mechanically shaken for 30 min and left to sediment before decanting the supernatant, which was then transferred to a round-bottom flask before being passed through anhydrous sodium sulfate. The extraction was repeated twice with 75 mL of extraction mixture and shaking for 15 min. The three extracts were combined and dried in a rotary evaporator. The dry residue was solubilized in 10 mL acetonitrile. The concentration of the fungicide was determined by means of liquid chromatography (LC) using a Shimadzu instrument, model LC 10 AD VP, with a UV/VIS Shimadzu SPD-10 AVP detector and a Supelcosil-LC ABZ column (15 cm, 4.6 mm, 5 μm) (Milan). FEX was eluted with a mobile phase consisting of water acidified to pH 3 with orthophospheric acid (20%) and acetonitrile (80%), and detected at 290 nm.

2.4 *DNA extraction*

DNA was extracted directly from 250 mg of soil [14]. Briefly, soil samples were homogenized in 1 mL of extraction buffer [100 mM Tris, pH 8; 100 mM EDTA; 100 mM NaC1; 1% (w/v) polyvinylpyrrolidone; 2% (w/v) sodium dodecyl sulfate] for 30 s at 1600 rpm in a mini-bead cell disrupter (Mikro-Dismembrator S; B. Braun Biotech International). Soil and cell debris were removed by centrifugation (5 min at 14 000 g). Proteins were eliminated after sodium acetate precipitation. Nucleic acids were precipitated with cold isopropanol (v/v), then washed with 70% ethanol.

Soil DNA extracts were purified with a polyvinylpyrrolidone spin column and a Sepharose 4B spin column. The quality and integrity of the soil DNA were checked by electrophoresis on 1% agarose gel. DNA was quantified using standard concentrations of calf thymus DNA.

2.5 *Ribosomal intergenic spacer analysis (RISA)*

The genetic structure of the soil bacterial communities, whether treated or not with FEX, were studied using the RISA technique. This technique uses PCR to amplify the intergenic spacer between the ribosomal sub-units 16S and 23S, which are then separated on acrylamide gel. This region is useful for differentiating between the bacterial strains and relatively near species thanks to the heterogeneity of length and sequence of the intergenic spacer. Various authors have already demonstrated that such a technique is very useful for comparing microbial diversity in deforested soil [15], in mercury contaminated soil [16], and in plant rhizospheres [15].

The 16S–23S intergenic spacer of the bacterial rDNA was amplified in a final volume of 50 μL from 5 ng of soil DNA using 1 μM of 38_r (5′-CCG GGT TTC CCC ATT CGG-3′) and 72_f (5′-TGC GGC TGG ATC TCC TT-3′) universal primers [17], 5 mM $MgCl_2$ and 0.625 U of Taq polymerase (Applugene, Qbiogene, Illkirch, France) under the following conditions: 4 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, plus an additional 15 min cycle at 72°C in a PTC 200 gradient cycler (MJ Research, Waltham, MA). PCR aliquots were separated by electrophoresis on a native 6% acrylamide gel, run at 8 mA for 17 h. Gels were stained with SYBR Green II (Molecular Probes, Leiden, The Netherlands) and analysed with the IMAGE QUANT program (Molecular Dynamics). Ribosomal intergenic spacer analysis profiles were analysed with the ONE-D-SCAN 2.03 program (Scanalytics, Fairfax, VA) allowing the elaboration of matrices (presence–absence, size and relative intensity of each band). Principal-component analysis on the co-variance matrix was performed using ADE-4 software [18].

2.6 *Quantification of the protocatechuate 3,4-dioxygenase β-subunit (pcaH) gene sequence using quantitative PCR*

A quantitative PCR was used in order to verify whether the microbial community in the soil examined contained the pcaH genetic sequence for encoding the production of a dioxygenase which was able to catalyse the cleaving of the aromatic ring. This enzyme is involved in the catabolism of a variety of both natural and synthetic aromatic compounds [19] and can be found in diverse bacterial and fungal Philum [20].

Quantitative PCR analyses were carried out in an Abi Prism 7900 HT Sequence Detection System (Applied Biosystems) using the Smart Kit for Sybr Green I according to the manufacturer's recommendations. Briefly, quantitative PCR was realized in the presence of 5 mM $MgCl_2$, 0.625 μg of T4 Gene 32 product (Qbiogene, UK), 10 ng of soil DNA and 2 μM of $pcaH_f$ (5′-GAG RTS TGG CAR GCS AAY-3′) and $pcaH_r$ (5′-CCG YSS AGC ACG ATG TC-3′) primers yielding a PCR product of 395 bp. The amplification conditions were as follows: 15 min at 95°C; 6 cycles of 15 s at 95°C, 30 s at 60°C, 30 s at 72°C, and 15 s at 80°C followed by 40 cycles of 15 s at 95°C, 30 s at 57°C, 30 s at 72°C, and 15 s at 80°C, and finally a cycle of 15 s at 95°C, 15 s at 57°C, and

15 s at 95°C. Calibration of the quantitative PCR was conducted on serial dilution (from 10^2 to 10^8 copies) of pcaH PCR product generated from EP30 clones.

3. Results and discussion

The amount of FEX degraded in the 7 days following the first treatment was relatively low (30%); a significative increase of FEX degradation was observed in the 7 days following the second treatment (96%). The percentage of degradation was not significatively influenced by successive treatments (figure 1). This result makes it possible to hypothesize a single induction after the first application of FEX in the ability of the microbial population to metabolize the molecule.

The resulting RISA profiles appeared relatively complex, with 20–25 bands; in the treated samples, the number of bands dropped to 15–18 after the final treatment. The profiles of the samples treated with FEX taken 7 days after the first application were different from those of the untreated control. In particular, there was an absence of some bands (figure 2, striped arrow) and appearance of others (figure 2, checked arrow), together with an increase in the intensity of the third band group (figure 2, black arrow). Observing the electrophoretic profiles of the samples taken 7 days after the second treatment (T 14) and 7 days after the third treatment (T 21), the differences between the electrophoretic patterns of the control and treated samples were becoming more pronounced. In particular, an increase in the intensity of some bands was seen (figure 2, white arrow) leading us to suppose that FEX exhibits a selective pressure on some microbial populations. However, some differences among the replicates were observed. In order to obtain a better interpretation of the results, a statistical elaboration was carried out by means of the principal-components analysis (PCA) of the different electrophoretic patterns. For each electrophoretic profile, we considered the number, size, and intensity of the bands. If we observe the data relating to the soil samples taken 7 days after treatment (figure 3), the first principal component (PC1) explains 80.12% of the variance, while 12.18% is explained by the second principal component (PC2). The factorial map showed that the ellipses of control and

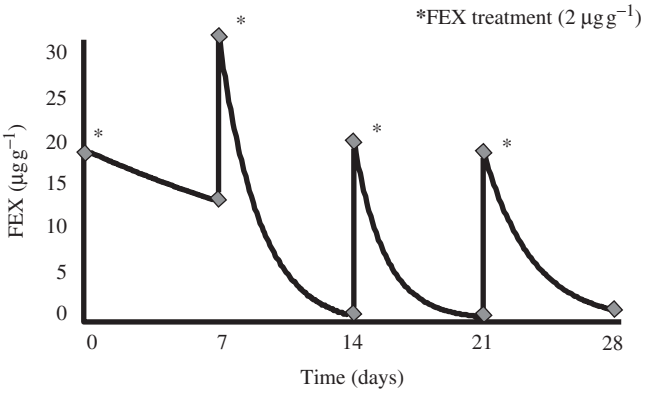

Figure 1. FEX degradation rate after successive treatments.

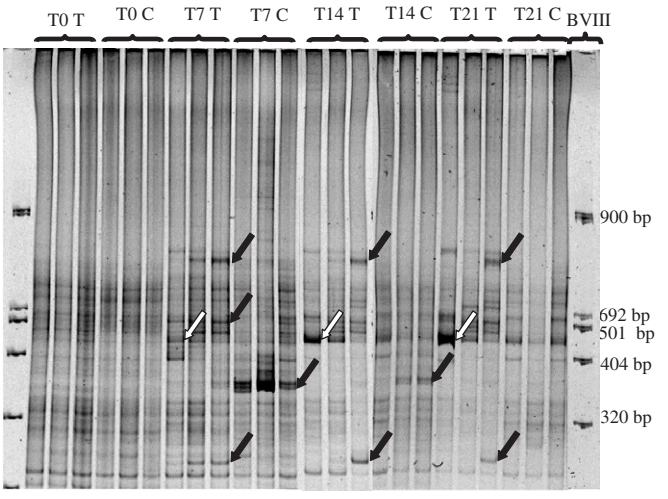

Figure 2. RISA electrophoretic profiles of 16S–23S rDNA amplicons obtained with universal primers of DNA extracted from FEX-treated and untreated soil samples; T: FEX-treated sample; C: control sample.

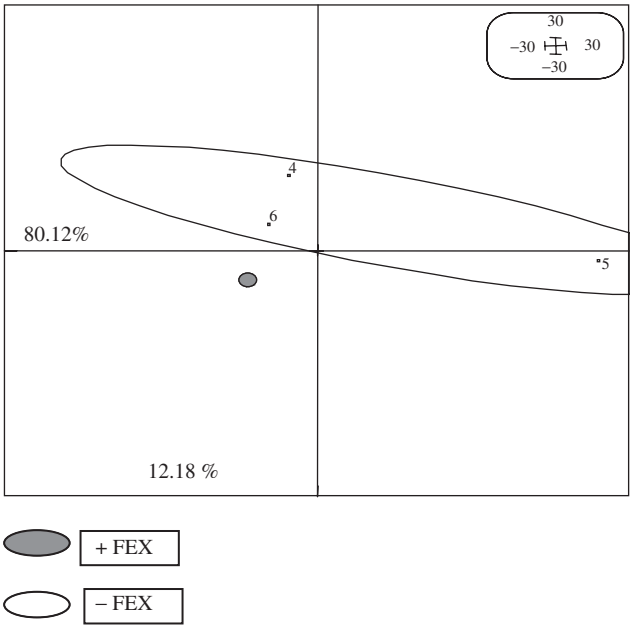

Figure 3. Principal-components analysis of the genetic structure of the bacterial communities of the soil 7 days after the addition of FEX.

FEX treated samples, representing the variance between RISA fingerprints replicates within a treatment, did not overlap, allowing their differentiation. This result showed that differences between control and FEX treated samples were significant, even if a variability among the replicates was observed. Similar results were obtained in samples taken after further treatments (data not reported).

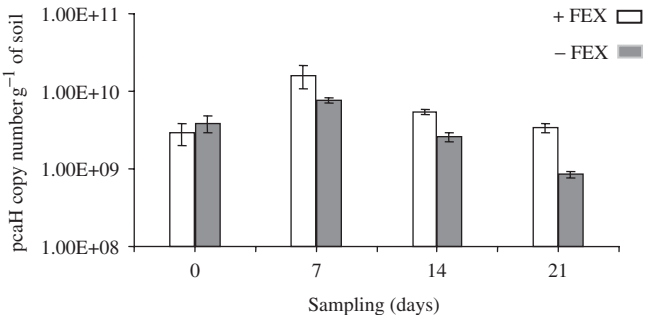

Figure 4. Quantification of the pcaH sequences after successive FEX treatments.

The RISA results suggest that the addition of FEX to the soil modifies the overall structure of the bacterial community. It can be hypothesized that the action of the fungicide has a toxic effect on some populations while stimulating specific degraders. The appearance of new bands could be due to the growth of new phylotypes able to degrade FEX, as previously observed in studies on the effect of 2,4-D and isoproturon on the soil microflora [1, 21]. In fact, when the decay of the active ingredient was examined, a progressive increase in the degradation rate was observed. At the same time, the disappearance of other bands could reflect the response of some microbial population to the toxic effects of FEX.

The results show how the RISA molecular approach, together with the PCA, can be a valid tool to evaluate the effect of FEX addition on the soil microflora. Quantitative PCR was used to quantify the bacterial community containing the pcaH sequences, coding for the production of a dioxygenase involved in the cleaving of the aromatic ring. The results, shown in figure 4, are expressed in numbers of copies of the pcaH gene/g^{-1} of soil. PcaH sequences were found in both the treated soil samples and the controls. However, a significant greater number of gene copies (one-way ANOVA analysis) was found in the treated soil samples for the whole of the test period, even if the greatest difference was found 7 days after the first treatment (1.61×10^{10} and 2×10^{9} genes g^{-1} of soil in the treated and untreated soils, respectively). This result can be correlated to the increase in the FEX degradation rate after the first treatment. The data obtained indicate that the pcaH sequence is present in the genes of the microbial soil components but that it increases when FEX is added.

On the basis of the indications given by Gonod *et al*. [1], the increase in the pcaH sequence could reflect an increase in the populations able to degrade FEX or an increase in the number of plasmid copies which transport the gene.

A positive correlation between the increase in the degradation rate and the number of genetic sequences encoded for specific enzymes has also been found for other pesticides [22, 23]. These results show that the soil microbial communities involved in the mineralization of xenobiotics are highly opportunistic, remaining at a low level in the absence of selective pressure but increasing rapidly when the xenobiotic is applied.

Quantitative PCR proved to be an appropriate strategy for the monitoring of the soil bacterial populations containing the pcaH sequence and for evaluating the quantitative modifications arising from the interaction of the microflora and an organic xenobiotic.

The use of molecular methods made it possible to understand more clearly the interaction that takes place between FEX and the soil microflora, confirming the presence of specific microorganisms able to degrade the fungicide, according to the observation of Abbate *et al.* [13] and Anderson *et al.* [24], who found a high degradation rate of FEX in soil. Nevertheless, this work has also demonstrated the sensibility of some microbial strains to FEX. Hypothesizing that these microbial populations play a key role in the nutrient cycles, further studies will be necessary in order to identify them and to evaluate probable changes in soil fertility due to FEX application.

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