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Isolation and Characterization of Fipronil Degrading *Acinetobacter calcoaceticus* and *Acinetobacter oleivorans* from Rhizospheric Zone of *Zea mays*

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Abstract An enrichment culture technique was used for the isolation of bacteria capable of utilizing fipronil as a sole source of carbon and energy. Based on morphological, biochemical characteristics and phylogenetic analysis of 16S rRNA sequence, the bacterial strains were identified as *Acinetobacter calcoaceticus* and *Acinetobacter oleivorans*. Biodegradation experiments were conducted in loamy sand soil samples fortified with fipronil (50 μg kg^{-1}) and inoculated with *Acinetobacter* sp. cells (45×10^7 CFU mL^{-1}) for 90 days. Soil samples were periodically analyzed by gas liquid chromatography equipped with electron capture detector. Biodegradation of fipronil fitted well with the pseudo first-order kinetics, with rate constant value between 0.041 and 0.051 $days^{-1}$. In pot experiments, fipronil and its metabolites fipronil sulfide, fipronil sulfone and fipronil amide were found below quantifiable limit in soil and root, shoot and leaves of *Zea mays*. These results demonstrated that *A. calcoaceticus* and *A. oleivorans* may serve as promising strains in the bioremediation of fipronil-contaminated soils.

Keywords Fipronil · Biodegradation · *Acinetobacter* · Kinetics · Metabolites

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Abbreviations

CFU mL^{-1}	Colony forming unit per mililitre
w/w	Weight/weight
w/v	Weight/volume
WHC	Water holding capacity
C_0	Maximum concentration
C	Concentration at time t
t	Treatment times in days
t_0	Treatment time of maximum concentration in days
k	Degradation rate constants ($days^{-1}$)

Fipronil {5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile} is a phenylpyrazole insecticide having broad spectrum activity against insects such as rice stem borer, leaf folder, cockroaches, mosquitoes, locust, ticks and fleas at both their larval and adult stages (Aajoud et al. 2003). It is one of the most persistent, lipophilic and toxic insecticides licensed for use since banned/restricted use of dieldrin, lindane and DDT (Chopra et al. 2011). Biochemical assay indicated that fipronil binds to the insect Gamma amino butyric acid (GABA) receptor with a higher affinity than the vertebrate site (Cole et al. 1993). The degradation of fipronil can involve oxidation, reduction, photolysis, hydrolysis and microbial degradation to yield sulfone, sulfide, desulfinyl and amide metabolites (Gunasekara et al. 2007). Studies on environmental fate of fipronil indicated that its half-life in soil varies from 3 days to 7 months (Tingle et al. 2003). Fipronil and its metabolites are reported to be present with variable contamination levels in environment providing multiple routes for chronic (and acute in some cases) exposure of non-targeted animals (Bonmatin et al. 2015). Thus, due to its persistent nature and potential

hazard to environment, it is necessary to investigate suitable treatment methods for cleanup of fipronil contaminated soil.

Biological treatment is considered to be a more feasible option in comparison to physicochemical methods as the latter involves high treatment costs and possibilities of causing secondary pollution problems (Uniyal et al. 2013; Paliwal et al. 2014; Thangadurai and Suresh 2014). Literature provides evidence on the microbial degradation of fipronil (Zhu et al. 2004; Masutti and Mermut 2007; Lin et al. 2008). However, to date, very few attempts were made to isolate and characterize specific bacteria that can use fipronil as a sole carbon and energy source. For instance, bacterial sp. viz. *Paracoccus* sp., *Bacillus firmus, Bacillus thuringiensis* and *Stenotrophomonas acidaminiphila* are reported to exhibit catabolic potential against fipronil by utilizing it as a sole source of carbon and energy (Kumar et al. 2012; Mandal et al. 2013, 2014; Uniyal et al. 2016). The present study was therefore, designed to isolate and characterize bacteria, capable of utilizing fipronil as a sole carbon and energy source and to investigate their fipronil degradation potential in soil. In addition, metabolites formed as a result of fipronil degradation were identified and fipronil degradation rate in soil inoculated with bacterial isolates based on the kinetics of degradation was studied. Furthermore, pot experiments were conducted to evaluate the fipronil degradation potential of bacterial isolates in the presence of maize (*Zea mays*). Maize was chosen as the model plant species because of its high biomass yields and ability to create particularly good environmental conditions for soil microorganisms and microfauna (Lynch and Whipps 1990; Benimeli et al. 2008). To the best of our knowledge, this is the first report on fipronil degradation by bacterial strains *Acinetobacter calcoaceticus* and *Acinetobacter oleivorans*.

Methods and Materials

Technical grade fipronil (Regent 0.3 % G, purity, 97.5 %), sulfone (purity 99.7 %), sulfide (purity 98.8 %), desulfinyl (purity 97.8 %) and amide (99.8 %) were procured from Bayer Crop Science Ltd, India. Solvents and other chemicals were of analytical grade. Dorn's broth media used for the isolation of bacteria contained the following (g L^{-1}): $Na_2HPO_4{\cdot}12H_2O$ 3.0 g, KH_2PO_4 1.0 g, $(NH_4)SO_4$ 1.0 g, $MgSO_4{\cdot}7H_2O$ 10.0 g, $CaCl_2{\cdot}2H_2O$ 2.0 g, $MnSo_4{\cdot}H_2O$ 3.0 g, $FeSO_4{\cdot}7H_2O$ 0.2 g, ammonium ferric citrate 0.01 g, yeast extract 0.1 g, distilled water, pH 7.0 and was sterilized at 121°C for 20 min (Kumar et al. 2012). Solid media plates were prepared by adding 2 % (w/v) of agar powder to the Dorn's broth medium.

The soil samples used for isolation of bacteria were collected from the rhizospheric zone of (0–20 cm) of *Z. mays* plantation, with previous history of fipronil application, situated at Crop Research Centre of the G.B.P.U.A.T. Pantnagar (29°3′0″N, 79°31′0″E). Soil samples were collected with the help of an alcohol sterilized (70 % ethanol) core sampler, mixed thoroughly to form a composite sample and kept in −20°C until bacterial isolation. Soil was passed through a 2 mm sieve and used for isolation of bacteria by the enrichment method (Wang et al. 2013) using Dorn's broth media supplemented with fipronil (50 μg L^{-1}). Serial dilutions of the enrichment cultures were spread out on Dorn's broth agar plate amended with 50 μg L^{-1} of fipronil. The isolates were purified and screened for fipronil degradation ability. Two isolates with maximum degradation potential were selected for further degradation study and encoded as S3 and S5. Bacterial isolates were initially identified on the basis of morphological, physiological and biochemical tests (Holt 1994) and stored in 10 % glycerol at −70°C.

Molecular identification of strains was done on the basis of 16S rRNA gene amplification (Barghouthi 2011). The PCR product was sent for sequencing to Chromus Biotech Ltd. Bangalore, India. The obtained sequenced data were edited using Chromas Lite 2.1.1. The individual 16S rRNA sequences were subjected to analysis according to homology search with sequences available in Genbank using blast function of National Center for Biotechnology Information (NCBI) available at www.ncbi.nlm.nih.gov/blast. Phylogenetic trees were generated using the neighbor-joining method according to the Kimura 2-parameter model (Saitou and Nei 1987). Bootstrap values were calculated from 1000 replicate runs, using the routines included in the MEGA 4.0 software. The strains sequences were submitted to NCBI GeneBank for accession number.

Pure culture of each isolate was inoculated in 2.5 mL of Dorn's broth media for 24 h at 30°C. The cultures of both strains were harvested by centrifugation at 6000 rpm for 5 min, washed twice with sterilized deionised water and resuspended in fresh Dorn's broth media ($OD_{600nm} = 1.5$).

Fipronil degradation study was conducted in loamy sand soil samples having pH 6.8, organic carbon 0.42 %, and total N content 0.06 %. Solution of fipronil in acetone was added to obtain a final concentration of 50 μg kg^{-1} soil and mixed thoroughly. After solvent evaporation soil samples were inoculated with 45×10^7 cells of both strains separately. Non inoculated soil samples maintained under same condition were kept as control. Soil samples were incubated at 30°C in dark and moisture content was adjusted to 40 % of the water holding capacity (w/w of dry weight of soil). All experiments were performed in triplicate. Soil samples were collected and extracted with acetonitrile-acetone at regular time interval as per method

described by Mohapatra et al. (2010). Analytical method was validated by performing fortification of fipronil and its metabolites in soil at the rate of 0.01, 0.1 and 1.0 μg kg^{-1}.

Zea mays-mediated fipronil remediation study was carried out in two different experiments: (1) Non-sterilized control (without microbial inoculation/0 μg g^{-1} fipronil) (2) Non-sterilized treatment (50 μg kg^{-1} fipronil + S3/S5). Soil samples were spiked with fipronil as per method described before in biodegradation study of fipronil in soil. Soil was inoculated with 45×10^7 cells of individual strain in non-sterilized treatment. Seeds of *Z. mays* were soaked in deionized water for 3 h and sown into treated soil filled in earthen pots. All the experiments were conducted in triplicate. Pots were regularly watered with deionised water. Soil moisture content of all pots was maintained at 40 % of the water holding capacity. Soil samples were collected at regular time intervals to evaluate the fipronil dissipation. Fipronil accumulation in plant samples was assessed at the time of harvest.

Fipronil degradation rate in soil was found to follow pseudo first-order kinetic reaction.

$$\ln {}^{C_0}/_{C} = -K(t - t_0) \quad (1)$$

where C_0 is the maximum concentration of fipronil (μg kg^{-1} in soil); C is the concentration of fipronil (μg kg^{-1}) in soil at the time of t; t is the treatment times in days; t_0 is the treatment time of maximum concentration in days; k is fipronil degradation rate constants ($days^{-1}$).

Fipronil concentration measurement and metabolite identification was performed by gas liquid chromatography with a Hewlett-Packard 5890 series II equipped with Electron Capture Detector U.H.P. grade. A capillary column, equity 5 was used. Split ratio was 10. Helium was used as the carrier gas and flow rate was maintained at 1 mL/min. Following temperature programming was used: the oven was initially held at 100°C for 0.5 min, and then for 3 min. Ion source and interface temperature were 230 and 260°C respectively. The retention time of pure standards of fipronil, fipronil desulfinyl, fipronil sulphide, fipronil sulfone, fipronil amide was found to be 6.36, 4.2, 6.14, 8.53 and 11.3 min respectively. Recovery of fipronil was in the range of 94 %–98 %.

Results and Discussion

Bacterial strains capable to utilize fipronil as a sole carbon and energy source were isolated from the maize rhizospheric soil. Both selected strains were initially characterized on the basis of morphological and physiological–biochemical traits. Bacterial strains S3 and S5 were found to be aerobic, gram-negative coccobacilli and are 0.5–1.5 mm in size. Strains produced circular, convex, off white colonies with entire margin on Dorn's broth agar plates. Strain S3 showed positive enzymatic reactions for H_2S production, oxidase, catalase with assimilation of citrate, lysine, ornithine, arginine, malonate, D-glucose, D-lactose and arabinose, while strain S5 showed positive tests for citrate, D-glucose, D-lactose, malonate and arabinose. Sugar assimilation efficiency of *Acinetobacter* sp. has been reported by several workers (Wang et al. 2009; Huang et al. 2013). The phylogenetic analysis revealed that strains S3 and S5 showed highest homology with *A. calcoaceticus* (99 %) and *A. oleivorans* (99 %) respectively (data not shown). The results were consistent with that of the morphological and biochemical characteristics. Therefore, strains S3 and S5 were identified as *A. calcoaceticus* and *A. oleivorans,* respectively. The nucleotide sequences of the bacterial strains S3 and S5 were submitted to GenBank nucleotide sequence databases under accession no KJ396944 and KJ396945, respectively.

Fipronil residues formed at different time interval in non-sterilized treatment and control are represented in Table 1. After 45 days, fipronil residues were reduced to minimum concentration of 10.3 and 7.12 μg kg^{-1} in soil inoculated with *A. calcoaceticus* and *A. oleivorans,* respectively, while for control, it was 25.60 μg kg^{-1}. A statistically significant ($p < 0.05$) difference in fipronil

Table 1 Residues of fipronil in non-sterilized soil inoculated with *A. calcoaceticus* and *A. oleivorans*

Days after treatment	Fipronil residues (μg kg^{-1})		
	Control	*A. calcoaceticus*	*A. oleivorans*
9	46.4 ± 2.19[a]	44.5 ± 2.44[a]	41.8 ± 2.26[a]
18	40.68 ± 2.05[a]	36.3 ± 2.26[a]	31.6 ± 2.32[a]
27	35.94 ± 1.89[a]	22.74 ± 1.72[b]	19.1 ± 1.64[b]
36	31.29 ± 1.64[a]	15.56 ± 1.91[b]	11.30 ± 2.02[c]
45	25.60 ± 1.12[a]	10.3 ± 1.45[c]	7.12 ± 1.32[c]
90	18.32 ± 1.12[a]	ND	ND

Values are mean ± SE of three replicates. For treatments marked with different letters differed significantly at $p < 0.05$ (ANOVA–DMRT)

ND Not detected

residue concentration was observed between control and treatment. No fipronil residues were observed in non-sterilized treatment after 90 days of time period, whereas for control, fipronil residues with concentration of 18.32 μg kg^{-1} were still present under same time period. In the present study, inoculation of *Acinetobacter* sp. seems to enhance fipronil degradation in non-sterilized soil. In a similar study, fipronil degradation by *Paracoccus* sp. was observed in soil (Kumar et al. 2012). The residues of fipronil were found to persist only up to 10 days in soils fortified with fipronil (20 μg kg^{-1}) and amended with *Paracoccus* sp., while in the soils fortified at the rate of 80 μg kg^{-1} fipronil, residues persisted up to 20, 30 and 30 days in loamy sand, sandy loam and clay loam, respectively. In another study, fipronil degradation in soil samples fortified with fipronil (0.50–1.50 mg kg^{-1}) and inoculated with *B. firmus* cell was studied (Mandal et al. 2014). Results indicated that fipronil residues were completely degraded after 35 days at lower doses of fipronil (0.50, 0.75 and 1.00 mg kg^{-1}). Uniyal et al. (2016) also studied biodegradation of fipronil in soil fortified with fipronil (50 mg kg^{-1}) and inoculated with *S. acidaminiphila.* Results showed complete degradation of fipronil residues after 90 days of time period.

Masutti and Mermut (2007) suggested that biodegradation seems to be dependent on the bioavailability of the fipronil. Bobe et al. (1997) has shown that fipronil can bind to soil particles and adsorption increased as the organic matter content in soil increased from 0.1 % to 6.5 %. Generally, soil-sorbed organic contaminants and pesticides have been considered unavailable for biodegradation without prior desorption (Smith et al. 1992). However, bacteria must either use sorbed molecules directly or facilitate desorption in some manner, for example, by producing surfactants (Park et al. 2003). Biosurfactants are the surface-active molecules which possess the ability to reduce the surface and interfacial tension between two immiscible fluid phases (Das et al. 2008). *Acinetobacter* sp. is reported to produce surfactants alasan and emulsan (Das et al. 2008), and thus might aid to fipronil degradation in non-sterilized soil by altering its bioavailability.

Fipronil metabolites were observed with a total concentration of 0.94 and 0.58 μg kg^{-1} in soil inoculated with *A. calcoaceticus* and *A. oleivorans,* respectively, after 45 days of time period (Table 2). All the metabolites were degraded below the quantifiable limit of 0.01 μg kg^{-1} in non-sterilized treatment, after 90 days of time period. Fipronil sulfide, sulfone and amide were formed as the metabolites of fipronil degradation. The detected metabolites indicated that reduction, oxidation and hydrolysis reactions were mainly responsible for fipronil degradation. Since desulfinyl metabolite was not formed, therefore results suggested that photolysis reaction had not contributed in the degradation process. Ying and Kookana (2002) found fipronil sulfide as a major metabolite while studying fipronil degradation in non-sterile soils with high moisture contents (>50 % WHC). Masutti and Mermut (2007) reported sulfone derivative (an oxidation product) as a predominant metabolite of fipronil degradation, but the sulfide (a reduction product) and amide (a hydrolysis product) derivatives were also formed under non-sterile conditions after 120 days of incubation in degradation experiments carried out under laboratory conditions. Similarly, Uniyal et al. (2016) also reported fipronil sulfone as a major metabolite followed by sulfide and amide, in the biodegradation study of fipronil.

The degradation kinetics of fipronil was studied and kinetic data were compared to reveal the impact of isolated strains on fipronil degradation in soil. Degradation process characterized the rate constant of 0.041–0.051 $days^{-1}$ for soil inoculated with *A. calcoaceticus* and *A. oleivorans* (Table 3). The regression coefficient (R^2) of the pseudo first-order kinetic reaction was 0.994 for *A. calcoaceticus* while 0.998 for *A. oleivorans*, which represented better

Table 2 Residues of fipronil metabolites (μg kg^{-1}) in non-sterilized soil inoculated with *A. calcoaceticus* and *A. oleivorans*

Days after treatment	Residues (μg kg^{-1}) during different time interval							
	A. calcoaceticus				*A. oleivorans*			
	Fipronil sulfide	Fipronil sulfone	Fipronil amide	Fipronil desulfinyl	Fipronil sulfide	Fipronil sulfone	Fipronil amide	Fipronil desulfinyl
9	2.62 ± 0.013^{a}	0.93 ± 0.014^{a}	0.4 ± 0.022^{a}	ND	2.28 ± 0.01^{a}	0.83 ± 0.019^{a}	0.35 ± 0.016^{a}	ND
18	2.12 ± 0.022^{a}	0.75 ± 0.026^{a}	0.31 ± 0.021^{a}	ND	1.66 ± 0.02^{a}	0.61 ± 0.014^{ab}	0.27 ± 0.012^{a}	ND
27	1.38 ± 0.019^{ab}	0.49 ± 0.013^{ab}	0.18 ± 0.015^{b}	ND	0.98 ± 0.017^{b}	0.34 ± 0.023^{b}	0.12 ± 0.017^{b}	ND
36	0.82 ± 0.012^{b}	0.3 ± 0.024^{b}	0.15 ± 0.012^{bc}	ND	0.57 ± 0.013^{b}	0.24 ± 0.018^{b}	0.089 ± 0.015^{c}	ND
45	0.59 ± 0.017^{bc}	0.24 ± 0.015^{b}	0.11 ± 0.014^{c}	ND	0.38 ± 0.02^{c}	0.13 ± 0.019^{b}	0.07 ± 0.009^{c}	ND
90	ND	ND	ND	ND	ND	ND	ND	ND

Values are mean ± SE of three replicates. For treatments marked with different letters differed significantly at $p < 0.05$ (ANOVA–DMRT)

ND Not detected

Table 3 Degradation kinetics of fipronil in soil inoculated with *A. calcoaceticus* and *A. oleivorans*

Strain inoculated	Parameter			
	Regression equation (year)	k ($days^{-1}$)	Half life (days)	Correlation coefficient
Control	0.016x − 0.085	0.016	43.31	0.989
A. calcoaceticus	0.051x − 0.400	0.051	13.59	0.994
A. oleivorans	0.041x − 0.337	0.041	16.90	0.998

fitness of experimental data. The values of regression coefficient (R^2) indicated that 99 % of the variability in the fipronil degradation rate for soil inoculated with both bacterial strains could be explained by the pseudo first-order kinetic reaction. The inoculated strains showed different impacts on fipronil degradation. *A. calcoaceticus* exhibited stronger acceleration on the degradation of fipronil than *A. oleivorans*, as it led to higher value of rate constant and is indicative of faster degradation. Tingle et al. (2003) reported that fipronil degrades slowly in soil and water, with a half-life ranging between 36 h and 7.3 months depending on substrate and conditions. Chopra et al. (2011) studied the kinetic of fipronil degradation in soil under field conditions and reported that dissipation of fipronil followed first order kinetics with half-life period of 23.35 days in single dose (56 g a.i.ha^{-1}) and 24.31 days in double dose (112 g a.i.ha^{-1}).

Fipronil accumulation by *Z. mays* was assessed at the time of harvesting (after 90 days) and fipronil was observed below quantifiable limit in all the treatments (data not shown). The dissipation of fipronil from sterilized and non-sterilized treatments was observed at different time interval (data not shown). Fipronil dissipation was significantly more ($p < 0.05$) in non sterilized treatment than in control. After 45 days, fipronil dissipation percentage in S3 and S5 inoculated soil was 86.6 % and 89.75 %. *Zea mays* was reported to be more tolerant to pesticide in comparison with other plant species like bitter lupine, sweet sorghum, radish, sweet lupine, oat, soya bean and wheat (Ibrahim et al. 2013). Besides production of root exudates, *Z. mays* has been reported to produce the phytosurfactant mucilage and thus might aid the fipronil dissipation by altering the bio availability of the pollutant, provide more substrates for co-metabolic degradation, and modify the soil environment to be more suitable for microbial transformation (Read et al. 2003). Furthermore, root growth and death promotes soil aeration, which can enhance oxidative degradation of recalcitrant organic compounds (Kuiper et al. 2004). Previous studies proved that plant and bacteria interactions are important in the enhanced degradation of many soil pollutants such as pesticides, chlorinated compounds, and polyaromatic hydrocarbons (PAHs; Johnson et al. 2004; Abhilash et al. 2011).

Present study demonstrated the ability of *A. calcoaceticus* and *A. oleivorans* to degrade and utilize fipronil as a sole source of carbon and energy. The inoculation of *Acinetobacter* sp. to non-sterilized soil amended with fipronil resulted in a higher degradation rate than that observed in noninoculated soils. Results of the degradation kinetics in non-sterilized soils were found to follow pseudo first order kinetics. Pot experiments further confirmed the ability of *A. calcoaceticus* and *A. oleivorans* to degrade fipronil. This work demonstrated bioremediation of fipronil-contaminated soil with *A. calcoaceticus* and *A. oleivorans*.

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