Full Length Research Paper

Evaluation of various pesticides-degrading pure bacterial cultures isolated from pesticide-contaminated soils in Ecuador

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Due to the intensive use of pesticides within the greenhouse-rose production, remediation of polluted soils has become a hot topic for researchers in recent decades. Several bacterial strains having the ability to utilize various pesticides as a sole source of carbon and energy were isolated from pesticide-contaminated soils of Urcuquí, Ecuador. According to phenotypical, physiological, and biochemical characterizations, and 16S rRNA gene sequence analysis, the isolated pure bacterial cultures were identified as *Pseudomonas putida* sp. strain B1, *Acinetobacter* sp. strain B2 and *Arthrobacter* sp. strain B3. The bacterial isolates were used individually and mixed cultures were used in the laboratory and field experimentations for the degradation of various pesticides like Ridomil MZ 68 MG, Fitoraz WP 76, Cleaner, Decis 2.5 CE, Score 250 EC, Zero 5 EC, Bravo 720 SC, Meltatox, Mirage 45 CE and Teldor Combi at 50 mgL^{-1}. The bacterial growth was monitored on both liquid culture medium (in laboratory) and field experiments by spectrophotometer method, *Neubauer camera* and colony-forming units. In addition, the degradation of individual pesticides (50 mgL^{-1}) was determined by ultra-high performance liquid chromatography-tandem mass-spectrometry and UV-VIS spectrophotometer. The results showed that the highest growth rate of microbial consortium was observed during degradation of various pesticides than individual pure bacteria for both experiments. In addition, most pesticides were completely degraded by microbial consortium after 60 days.

Key words: Bioremediation, spectrophotometer, bioreactors, pesticides, consortium.

INTRODUCTION

In Ecuador, flower farming was started with a non-surpassing area of 30 hectares in 1970. This is how Ecuadorian valleys were devoted to the diversification of flowers which intern changing the old existing landscape for greenhouse production systems, focused on enhancing the production of flowers (Lopez, 2011). This enhancement turned out to be such a profitable business that led to the beginning of Ecuadorian flower exportation

in 1985. However, like any high-production crop, floriculture requires intensive farming which along with monocultural systems, may lead to pests and diseases outbreaks. This is why pesticides are required in flower cultivation to counter any diseases or pests (Seoanez et al., 2004). These pests can potentially cause damage during storage, transportation or commercialization of roses. Furthermore, pesticides are used during most phases of rose-cultivation to minimize any pest infestation, and there by protect crops from any potential yield losses or reduction of product quality (Hernandez and Hansen, 2010).

Despite the beneficial impacts that pesticides have, such as subduing pest infestation to stabilize and improve agricultural productivity, overusing them may lead to the contamination of agricultural land, water, crops, and adversely end up affecting non-target organisms (Gooty et al., 2015; Xiao et al., 2013). Toxic chemical use in soils represents a serious threat to the health of human beings, animals and aquatic systems around the globe. Subsequently, it makes both publicly and scientifically concerns become noticeable all over the world (Aparicio, 2015).

Bioremediation is an eco-friendly and cost-effective method, that involves use of microorganisms, such as bacteria and fungi, to degrade or mineralize the hazardous/toxic chemicals which might be harmful to the environment (Mulla et al., 2012). Hence, in this technique, the organisms break down the toxic substances in the presence of enzymes, turning them into absorbable energy and nutrients for their growth, nowadays, this biological remediating method has become widely used.

The isolation of microorganisms involved in pesticide/fungicide degradation has been broadly described in the literature (Megadi et al., 2010a, b; Yu et al., 2013; Tallur et al., 2015). In several cases, a prevailing microorganism is likely to have a key role in the degradation process (Wang et al., 2010). In other instances though, such as the well-known case of the Carbendazim fungicide, a variety of microorganisms has been known to be isolated as degraders (Zuniga, 2015). These microorganisms show high efficiency as pesticide-degraders. Keeping in view of this, the present study was focused on the isolation and characterization of bacterial cultures having the ability to degrade the most commonly-used pesticides in flower farming.

MATERIALS AND METHODS

Chemicals

Several pesticides, such as Ridomil MZ 68 MG (64% Mancozeb +

Table 1. Physicochemical properties of the soils.

Properties	Sample soil
Texture	Sandy-clay
pH (in H_2O)	7.2
Organic matter (%)	6.8
Ion exchange capacity (meq/100 g)	19.2
Electrical conductivity (mS/cm)	0.31
Nitrates (NH_3)	37.1
Phosphates (PO_4)	19.1
Chlorides (Cl^-)	8.7

Agroprojekt, consultancy and Laboratory services, 2012.

4% Metalaxil) by Sygenta, Fitoraz WP 76 (6% Cymoxanil + 70% Propineb) by Bayer Crop Science, Cleaner (180 g/L Cyhalofop Butyl) by Consul LMZ, Decis 2.5 EC (2.8% Deltamethrin) by Bayer Crop Science, Score 250 EC (23.5% Difenoconazole) by Sygenta, Zero 5 EC (50 g/L Lambda cyhalothrin) by Ecuaquimica, Bravo 720 SC (72% clorotanonil) by Sygenta, Meltatox (425 g/L Acetato de Dodemorph) by Basf, Mirage 45 CE (45% Prochloraz) by Proficol Andina, Teldor Combi (350 g/L Fenhexamid + 66.7 g/L Tebuconazole) by Bayer Crop Science were used in this study.

Soils

The pesticide-contaminated soil samples were collected from pencaflor floricultural farm, located in Urcuquí, province of Imbabura, Ecuador. Latitude: 0° 22' 31.0906 "; Length: - 78° 13' 54.4923 "; altitude: 2149.00 m; area: 17UTM to a depth of 20 cm, air dried at room temperature before use. Physico-chemical characteristics of the soil samples were analyzed by standard methods (Agroprojekt, 2012) and are shown in Table 1.

Observation of microorganisms in soil samples under the microscope

In order to observe the microorganisms present in the soil samples, microscopic observation was performed. Four small parts of the soil samples were taken and placed in test tubes containing 1 ml of sterilized distilled water and tightly covered by aluminum foil. The sample was homogenized by vortex at a rate of 600-revolution-per-minute for 60 s. An aliquot was further observed by microscope.

Experimental design and soil incubation studies

Isolation of microorganisms

In order to isolate the microorganisms capable of degrading various pesticides by enrichment technique, the following protocol was adopted, 20 mg of soil samples were placed into 10 Petri plates, one for each pesticide (50 mg L^{-1}). The soils were supplemented with the pesticides dilution. The Petri plates were incubated at 50°C for 24 h under sterilized condition. 2 mg soil sample were taken from Petri plates and placed in a clean and sterilized test tube with

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appropriate distilled water (sterilized), mixed and filtered. The few micro liters of filtrate was cultivated onto pesticide (50 mg L^{-1})-mineral-salts agar plates and incubated at 30°C for 3 days. The well grown different colored colonies were picked and transferred for further purification on pesticide-mineral salts agar plates. The bacterial strains (B1, B2 and B3), which are capable of utilizing pesticides as a sole carbon and energy source, were isolated from soils samples taken for the production of roses.

Microorganisms identification

The purified organisms were determined by their physiological as well as morphological characterizations and then biochemical tests. The morphology of the isolated colony was monitored for 3 days at 25°C after incubation, by using light microscopy on mineral salt medium plates with pesticides. Cells were stained according to the classical Gram-stain procedure. Oxidase activity was determined according to Garcia et al. (2010). Catalase activity was determined as described by Rodriguez and Gamboa (2009). Fermentation of glucose, King B and Indol tests were performed according to methods described by Faddin (2007).

Growth of microorganisms

Microbial counts in liquid culture

The experimental study of Mendoza et al. (2011) was adapted with little modification in the present study. In order to examine the bacterial growth, 150 ml of sterilized minimal salts medium (K$_2$HPO$_4$, 3 g; KH$_2$PO$_4$, 0.5 g; (NH$_4$)$_2$SO$_4$, 1.25 g; NaCl, 0.01 g; MgSO$_4$, 0.1 g; FeSO$_4$.7H$_2$O, 0.001 g for each liter) (pH 7.0) containing 79 µl of pesticides (50 mg L^{-1}) was transferred into sterilized flasks. Further, each flask were inoculated separately with approximately 27 µl of *Pseudomonas*, 2.7 µl of *Acinetobacter*, 28 µl of *Arthrobacter* and 2.8 µl of bacterial consortium (*Pseudomonas* + *Acinetobacter* + *Arthrobacter*, 1:1:1 ratio), in order to reach biomass level of CFU = 100000. All the flasks were incubated in a rotary shaker at 90 rpm and 25°C under dark condition and the samples were withdrawn at different intervals like 15, 30, 45 and 60 days, respectively, to measure growth rate of the organisms. The calculation of the pesticide was taken into account in the dosage of the pencaflor for the treatment of disinfection of roses.

Microbial counts in solid cultures

150 g of soil samples were weighed and autoclaved so that examination of material growth found in soils is achieved. The sample then was dried at 450°C for 24 h. The soil subsequently, was placed in a polypropylene flask. 10 ml of sterilized mineral-salts-medium (pH 7.0) was supplemented with 50 mg L^{-1} of pesticides as the sole source, according to the humidity loss. Each flask was inoculated with a suspension of 27 µl of *Pseudomonas putida* sp. strain B1, 2.7 µl of *Acinetobacter* sp., 28 µl of *Arthrobacter* sp. and 2.8 µl of bacterial consortium (*P. putida* + *Acinetobacter* sp. + *Arthrobacter* sp.), in order to reach biomass level of CFU= 100000. All the flasks were incubated into an automated greenhouse at 21.1°C having 54.4% of humidity. By utilizing the spectrophotometry method, the bacterial quantification process was performed. Three repetitions per treatment were employed.

Chromatography for determination of pesticides in cultures

The residual analysis of pesticides degradation were performed for

both liquid and soils samples. All the treatments were replicated three times, samples were collected on 0 day and subsequently at 1, 15, 30, 45, 60 days after inoculation. For Score, Teldor, Mirage, Cleaner, Bravo and Ridomil, the measurement was performed following an ultra-high performance liquid chromatography-tandem mass-spectrometry (LC–MS/MS). Chromatographic separation was achieved by Agilent C$_{18}$ column. The mobile phase contained the solvent A: H$_2$O/0.1% formic acid and solvent B acetonitrile/0.1% formic acid in a composition of 95% solvent A in 5% Solvent B at a flow rate of 0.20 mL/min. For Decis and Zero pesticides, the mobile phase was: water/methanol 90:10 with 5 mM ammonium formate at a flow rate of 400 µl/min. The retention time were: Score (8.35 min), Teldor (Fenhexamid 7.04 min + Tebuconazole 7.8 min), Mirage (8.29 min) and Ridomil (6.13 min), Cleaner (8.2 min), Bravo(2.3 min), decis (15 min), Zero (15 min). On the other hand, for the pesticides Fitoraz and Metatox, UV-VIS spectrophotometer was used, the wave length of maximum absorption used was 284-293 nm according to the pesticide.

RESULTS AND DISCUSSION

Identification of microorganisms

The bacterial cultures were isolated from soil samples by enrichment with pesticides (Ridomil, Cleaner, Decis, Meltatox, Mirage, Bravo, Zero, Fitoraz, Score and Teldor) as a sole source of carbon and energy. The three strains were characterized by their cultural, morphological and biochemical properties. The B1 strain is Gram negative, aerobic, shows catalase, oxidase and King B test positive, while glucose and Indol production turned out negative. The B2 strain is Gram negative, aerobic, catalase and King B test was positive, while the oxidase, glucose fermentation, Indol was negative. The B3 strain is Gram positive, aerobic, catalase, oxidase and King B test were positives, while glucose fermentation and Indol were found to be negatives. The isolates were further identified by phylogenetic analysis based on 16S rRNA gene sequences (Figure 1). The complete sequence of 16S rRNA gene of the isolated organisms (B1, B2 and B3) was determined. Analysis of sequences was done at NCBI, where relevant sequences were downloaded for further analysis in order to identify the isolates (Mulla et al., 2011). The isolate strain B1 was identified as *P. putida*, strain B2 as *Acinetobacter* sp. and strain B3 as *Arthrobacter* sp.

Growth of microorganisms

Microbial measurement in liquid culture

The growth of *Acinetobacter*, *Arthrobacter*, *Pseudomonas* and bacterial consortium showed similar growth rate in the presence of 10 different pesticides in the liquid culture media. By utilizing the SPSS Statistics software package, the data was evaluated with a 95% confidence interval using a marginal means analysis. The analysis showed that microbial consortium had reached the highest cell proliferation rate on the liquid media containing various
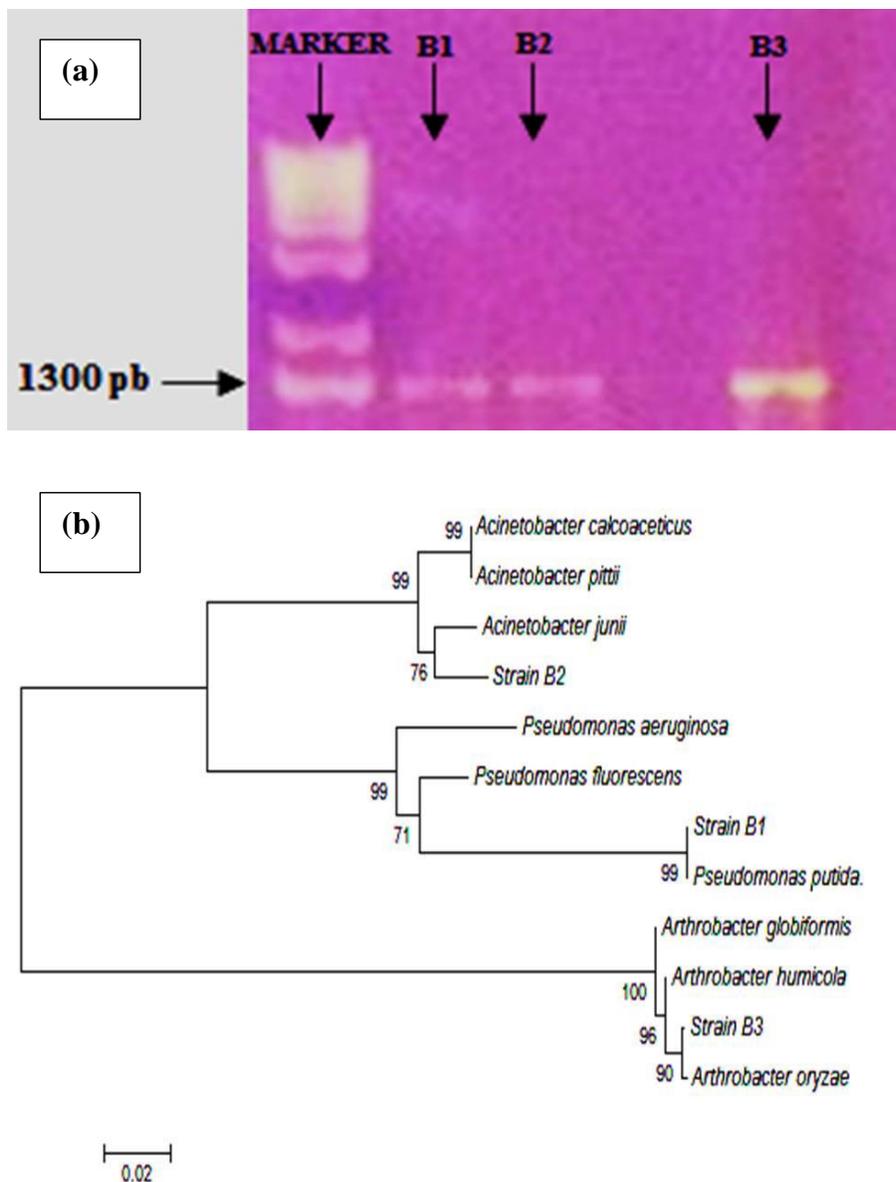
Figure 1. Agarose gel electrophoresis of 16S rDNA gene amplified from the isolate B1, B2 and B3 (a). Neighbour joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships among the B1, B2 and B3 strains, along with their closest relatives (b). The Bootstrap values (expressed as percentages of 1000 replications), which surpassed the 50%-range, are shown at branch point. The bar indicates a 2%-sequence divergence (b).

pesticides like Ridomil, Bravo, Cleaner, Mirage, Score and Teldorat (50 mg L^{-1}). Following *P. putida* strain B1, this showed the highest microbial growth on Decis and Zero pesticides (50 mg L^{-1}). *Acinetobacter* sp. strain B2 showed high growth rate on Mitaraz and Meltatox (50 mg L^{-1}), whereas *Artrobacter* sp. strain B3 showed the lowest bacterial growth on the studied pesticides. Number of colonies of consortium reached a maximum of 8.40 $\log_{10}$ CFU mL^{-1} on 30th day. After 30th day, all three strains and consortium decreased in growth rate (Figure 2).

Microbial measurement in solid culture

The solid phase showed a higher bacterial growth rate than the liquid phase. At day 15, strains B1, B2, B3 and consortium were inoculated in the autoclaved soils, the bacterial consortium of colonies reached highest rate of 9 $\log_{10}$ CFU g^{-1}. Further, all three strains and consortium decreased in growth rate. Results indicated that *P. putida* strain B1, *Acinetobacter* sp. strain B2, *Arthrobacter* sp. strain B3 and consortium exhibited similar population
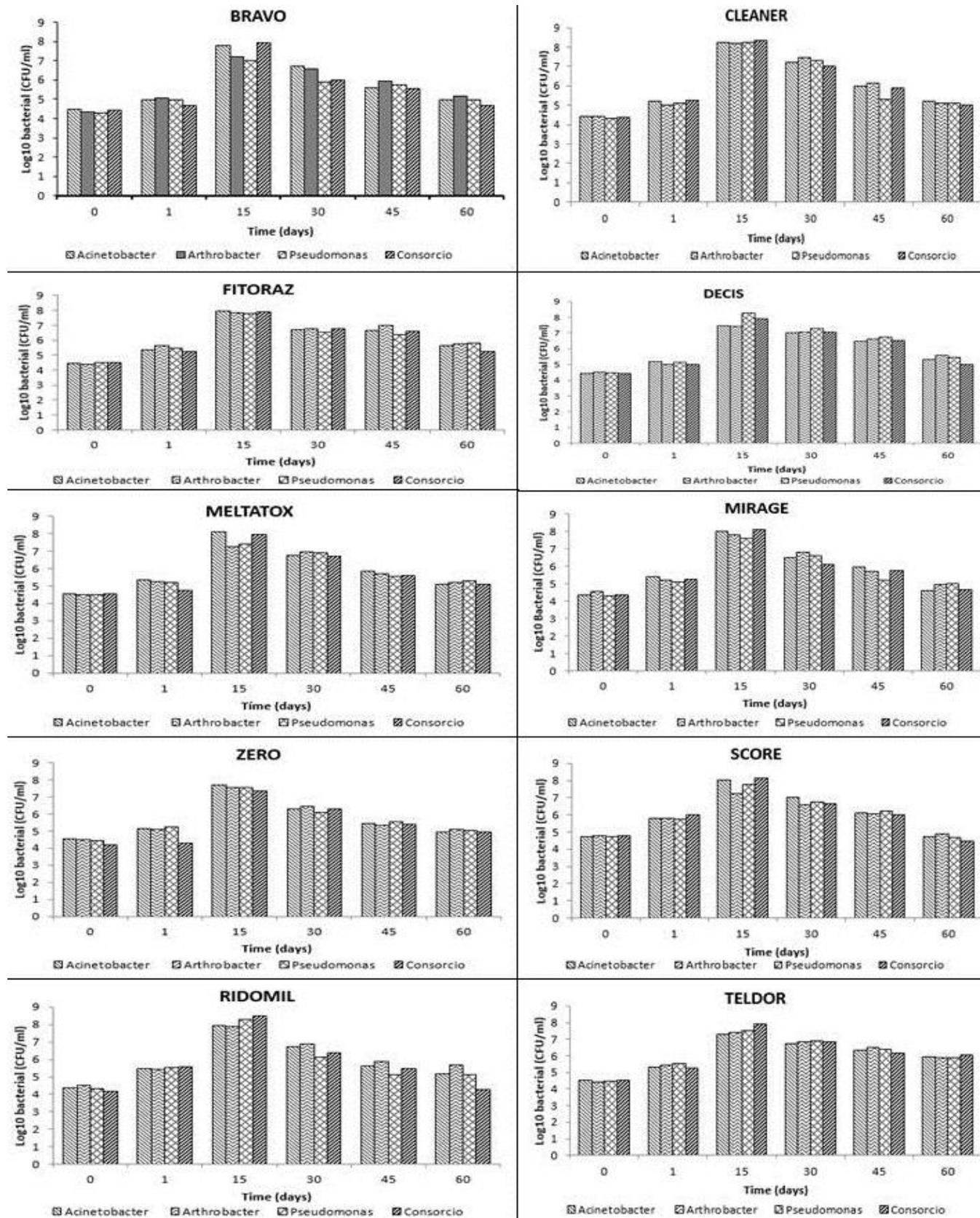
Figure 2. *Acinetobacter*, *Arthrobacter*, *Pseudomonas* and bacterial consortium growth of in liquid culture incubated at 25°C at 90 rpm in the presence of 10 different pesticides.

dynamics throughout the time of study (Figure 3). In solid culture, the bacterial consortium showed the highest growth rate on Ridomil, Bravo, Mirage, Cleaner, Score, Teldor, Fitoraz pesticides than individual pure cultures. The controls did not show a significant growth.

Degradation of pesticides

The growth of the bacterial cultures such as strains B1, B2, B3 and consortium on various pesticide-solid phase, confirmed a pesticide concentration reduction. The solid media showed higher cell proliferation rate in contrast to the liquid media.

The ability of *P. putida* sp. strain B1, *Acinetobacter* sp. strain B2, *Arthrobacter* sp. strain B3 and consortium to degrade different pesticides in solid media is shown in Figure 4. The organisms were able to use the 10 different pesticides as a sole carbon source. Additionally, the consortium managed to grow and had better degrading-pesticide ability than pure bacterial strains.

LC-MS/MS and spectrometer analysis indicated pesticides, such as Ridomil, Bravo, Mirage, Cleaner, Score and Fitoraz could no longer be detected after 30 days of incubation in the consortium culture filtrates. Teldor was degraded up to 93% by the bacterial consortium. Meltatox and Zero were degraded upto 98% rate by *Acinetobacter* sp., Decis was degraded up to 98% by *P. putida* sp, while the *Artrobacter* sp. isolate only degraded at 94% after a 30-day incubation period in all the treatments. In all the controls without the isolates, the hydrolysis percentages of pesticides were less than 15%. As compared to the controls without inoculation, degradation of all pesticides was enhanced obviously by the addition of isolates.

The *Pseudomonas* species has shown high metabolic adaptability to several toxic pollutants such as mancozeb (Pirahuata et al., 2006), metalaxyl (Massoud et al., 2008), methyl parathion, endosulfan, dimetoato, malation (Hussaini et al., 2013); parathion (Rosenberg and Alexander, 1979), monocrotophos (Bhadbhade et al., 2002a), lambda cyhalothrin (Manigandan et al., 2013), cymoxanil (Derbalah and Belal, 2008), cyhalop of butyl (Nie et al., 2011), chlorothalonil (Wang et al., 2011), and also consortium of *Pseudomonas* reduced Chlorothalonil (Rios and Penuela, 2015). However, there is no report on degradation of propineb, deltamethrin, dodemorph acetate, prochloraz, fenhexamid or tebuconazole by *Pseudomonas* sp. The present study indicates that *P. putida* strain B1 was able to show a higher degradation ratio in pesticides such as Meltatox, Fitoraz, Decis, Mirage and Teldor.

There are studies on the use of *Acinetobacter* to degrade pesticides such as atrazine (Pooja et al., 2004), methilparation (Liu et al., 2007), chloroanilines (Hongsawat and Vangnai, 2011), malation (Ahmed et al., 2015) and metalaxil (Zuno-Floriano et al., 2012). However, no studies mention the use of *Acinetobacter* to

degrade pesticides such as mancozeb, cyhalopof butyl, deltamethrin, difenoconazole, lambda cyhalothrin, clorotanolil, dodemorph acetate, prochloraz or mixed-up pesticides (Fitoraz, Teldor). In strain B2, the rate degradation of various pesticides reached between 90 and 98% (Figure 4). *Acinetobacter* degrade 98% of dodemorph acetate and lambda cyhalothrin than strain B1, strain B3 and consortium. However, *Acinetobacter* sp. strain B2 showed the lowest degrading rate only up to 90% of Teldor.

There are reports on the degradation of various chemicals like diazinon (Barik et al., 1979), fenitrothion (Ohshiro et al., 1996), parathion (Nelson et al., 1982), and monocrotophos (Bhadbhade et al., 2002b) by *Arthrobacter*. However, *Arthrobacter* has not been tested before on any of the currently studied pesticides. Despite showing low bacterial growth unlike other strains, *Arthrobacter* sp. strain B3 has been able to degrade up to 94% of all 10 pesticides.

The bacterial consortiums are able to complement each other's metabolic functions by recycling the whole pesticide. A microbial consortium contains degraders of target compound and can utilize the metabolic intermediates of the parent compound (Pattanasupong et al., 2003). Toxic intermediates may sometime continue to persist when using a pure bacterium. In this study, the microbial consortium was able to quickly adapt to the stress caused by the xenobiotic on solid media, showing a 100% degradation of ridomil, bravo, mirage, cleaner, score and fitoraz within 60 days of investigation. These results imply that the microbial consortium will therefore significantly enhance the soil conditions by reducing the pesticide concentration.

As shown in Figures 2 and 3, a higher bacterial growth was achieved by the cells in solid phase within 30 days, than free cells suspended on liquid media (both in pre-cultivated conditions). The exponential phase takes place during day 15 on liquid media, while on solid media, it can be perceived on day 13. This result matches what Salunkhe et al. (2014) mentioned: the *B. subtilis* strains experience an exponential phase 3 days after inoculation on liquid media. Nevertheless, this phase takes place on the sixth day in soil. This may be caused by masses-transference intensification and a high microorganism-pollutant interaction. The toxic-metabolites transference, which precedes degradation, is faster on liquid media. This ends up affecting bacterial growth by reducing the population. On liquid media though, the thicker contact surface leads to bacteria growth in a shorter period of time using the pesticide as a carbon source.

Conclusions

Microbial degradation is one of the most important techniques to dissipate pesticides found in soils. The present study concluded that *P. putida* sp. strain B1, *Acinetobacter* sp. strain B2, *Artrobacter* sp. stain B3 and
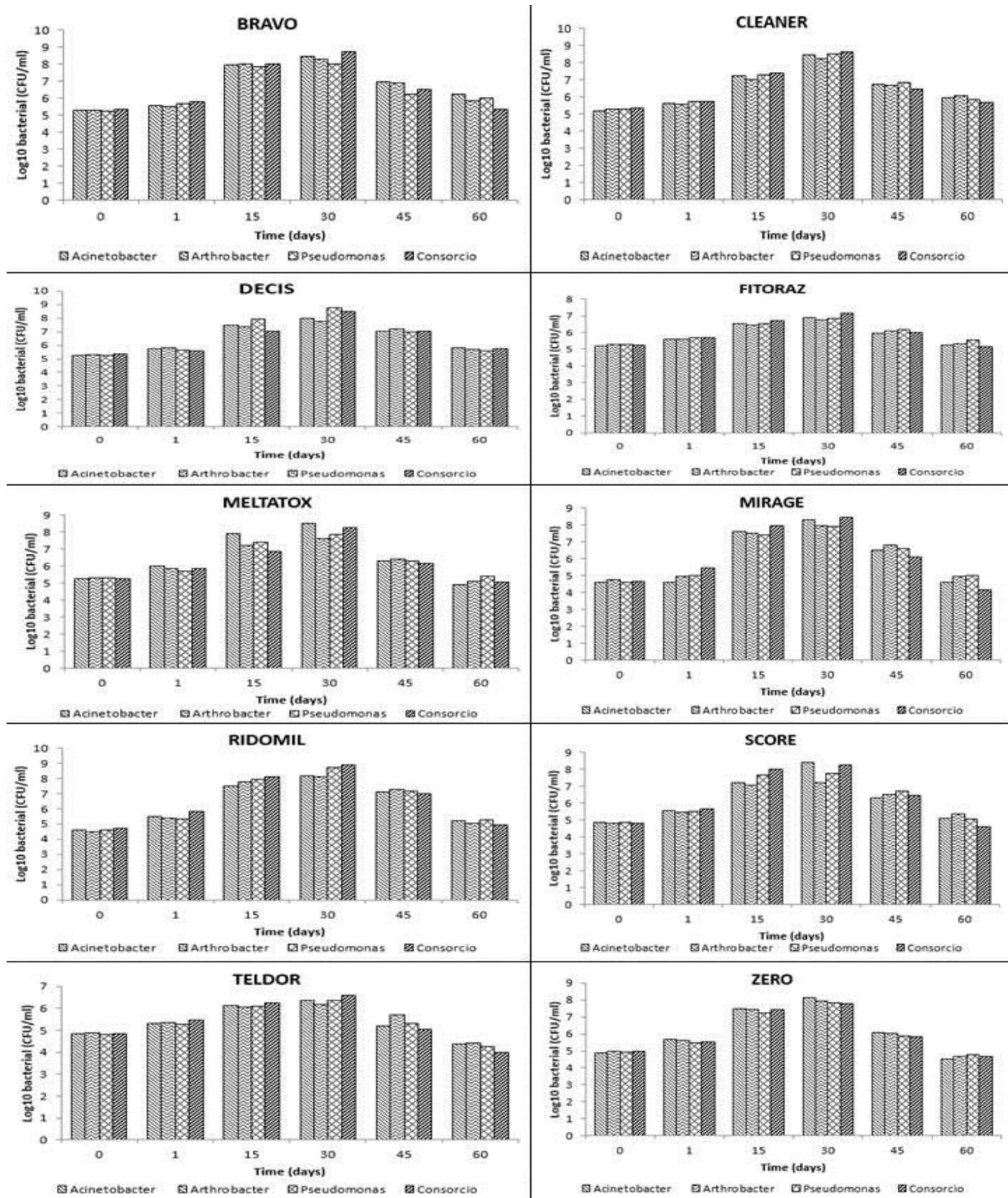
Figure 3. *Acinetobacter*, *Arthrobacter*, *Pseudomonas* and consortium growth in solid culture (soils) incubated at 21.1°C and 54.4% humidity in the presence of 10 different pesticides.
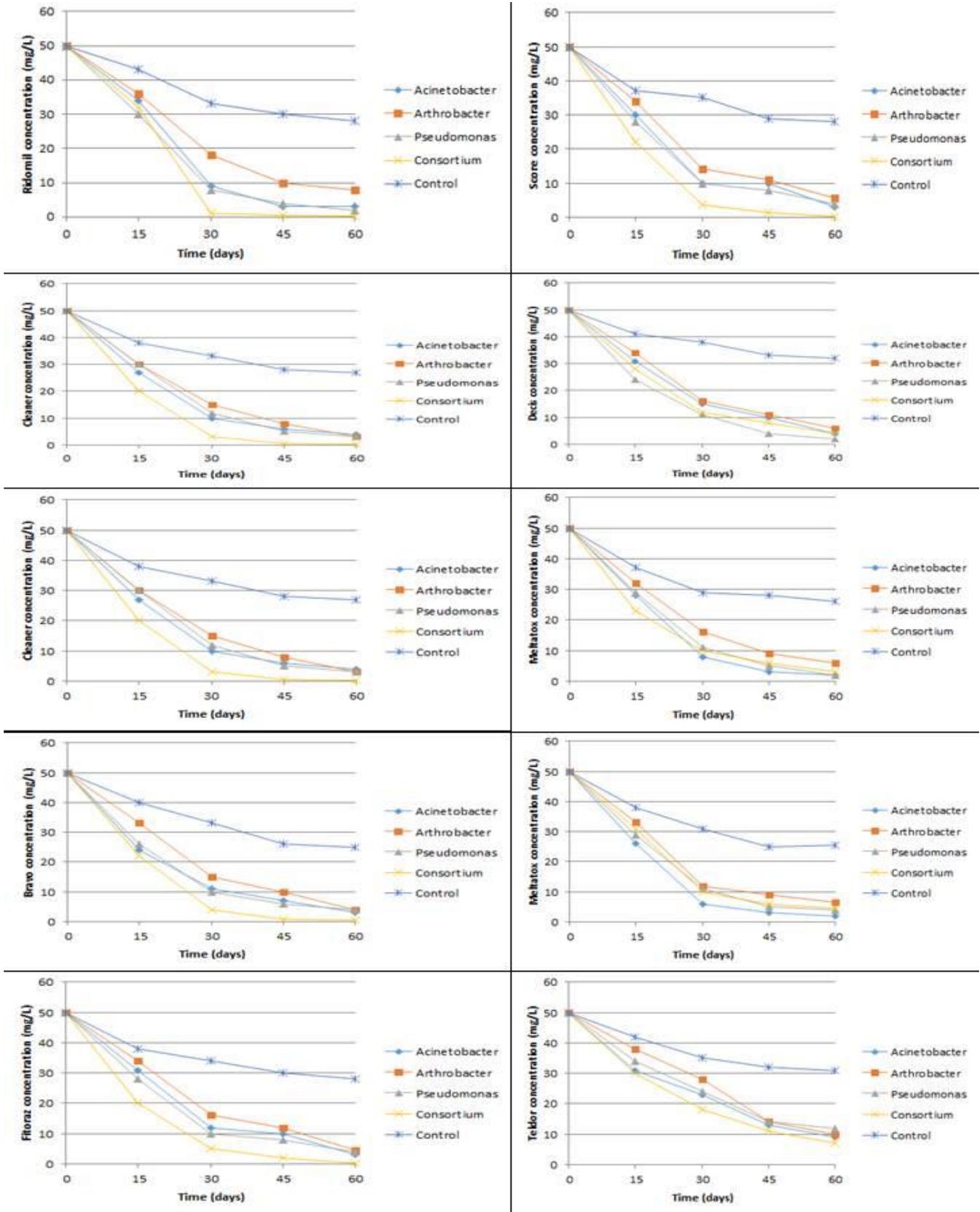
Figure 4. Various pesticides residual concentration observed in *Acinetobacter*, *Arthrobacter* and bacterial consortium in autoclaved soils (solid media). Each value is mean of three replicates.

the bacterial consortium have proven to have the capacity to utilize different pesticides as a carbon source and enhance the dissipation in soils and liquid medium. The bacterial consortium was more effective. All the strains can be applied in flower farming as a bioaugmentation process in order to reduce the pesticide residues in soils.

Conflict of Interests

The authors have not declared any conflict of interests.

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