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## Biodegradation of Paclobutrazol — A Plant Growth Regulator Used in Irrigated Mango Orchard Soil

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/60818>

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### Abstract

Paclobutrazol (PBZ), [2RS,3RS]-1-[4-chlorophenyl]-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pentan-3-ol, consists of a triazole ring and a benzene ring-chloro linked to a carbon chain open. It is a plant growth regulator widely used in many crops in order to produce fruit throughout the year by inhibiting gibberellin synthesis, a hormone responsible for the vegetative plant growth. Actually, studies are showing that paclobutrazol remains active in the soil for a long time, affecting the growth and development of subsequent crops by reducing plant vigor. Biodegradation is an effective and cheap process that can to degrade or transform contaminants to less toxic or nontoxic. In this work, the biodegradation of paclobutrazol was studied using in submersed culture and saturated and unsaturated soils. In these conditions, experiments with biostimulation and bioaugmentation were performed. In the experiments carried out in submersed culture, with biostimulation by addition of glycerol, the PBZ biodegradation was higher than that with PBZ as sole carbon source. The biodegradation of PBZ in unsaturated soils was more efficient when soil samples with a history of application of PBZ were used. The highest number of applications of PBZ favored biodegradation. The biodiversity of the microbiota in the soil favored the biodegra-

dation of PBZ aromatic rings. PBZ was not seen to be phytotoxic and the biodegraded products increased the germination index.

**Keywords:** paclobutrazol, semi arid region of Brazil, models, *Pseudomonas*

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## 1. Introduction

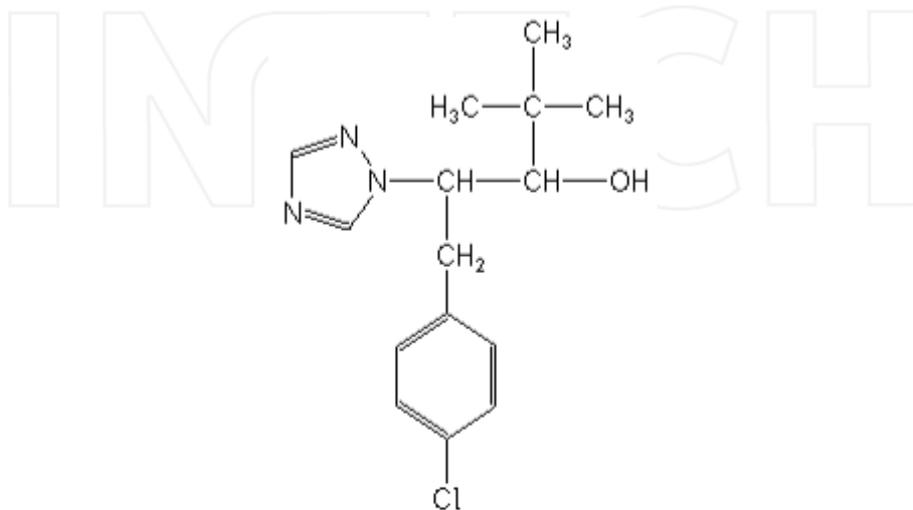
Brazil has a great potential for fruit production, as it has area, climate, and enough water for production throughout the year. The production of mango can be developed under different climatic conditions, but it is commercially viable only within a well-defined range of temperature, rainfall, altitude, insulation, relative humidity, and winds. The fruit is native to tropical climates, but it can be grown in subtropical regions of the planet. Mango (*Mangifera indica* L.) plantations in the country occupy about 74,000 ha, generating a production of over 1.1 million tons. Mango is produced in all regions of Brazil; however, the southeast and northeast regions account for 94% of the total [1]. The area cultivated with mango in the northeast region has increased by 20,000 ha in 10 years. In 2012, that amount alone was responsible for producing more than 85% of the total exported by Brazil [2]. To foster exportation, however, it is necessary to guarantee production whenever the market is receptive and to ensure that the quality of the fruit corresponds to international food safety requirements [3].

The production of mango in Brazil can be divided into two different phases: the first one characterized by extensive cultivation of local varieties with little or no use of technology; the second one characterized by a high level of technology, such as irrigation, floral induction, and improved varieties [4]. Mangos from the Brazilian semiarid region stand out in the national scenario due to high yields and fruit quality, and also to the possibility of year-round production taking advantage of the climatic conditions as well as management techniques (irrigation, pruning, and the use of growth regulators) for plant growth and blossom control [5].

The growth of the mango tree, as well as other tropical fruit trees, is not continuous but comes in vegetative flushes of the terminal and axillary shoots of the branches, before the period of dormancy. For the vegetative or floral growth to happen, two different processes occur in the plant: the growth of the buds and the initiation of the sprouting. The bud starts to grow, which includes the end of the dormancy and a quick development of the shoot. Along with the shoot initiation, the induction happens, and it will define the vegetative type, floral or mixed [6].

Flowering in mango is a process that may occur during an extensive period (up to several months) and can have its beginning altered, naturally or artificially, due to climatic conditions, yield of the former harvest, or use of specific crop management techniques, including plant growth regulators [7]. Most of the plant growth regulators inhibit the gibberellin synthesis and can therefore be used for plant growth and flowering management [8]. Among the plant growth regulators used in fruit production, paclobutrazol (PBZ) has shown efficiency in mango flowering management [9]. PBZ (Figure 1) must be applied directly to the soil due to its low solubility, long residual activity, and lack of efficient foliar

uptake [8]. The recommended doses range between 1.0 and 1.5 g, measured by tree crown diameter, and dependent on the cultivar, climate, soil type, and plant nutrition. Paclobutrazol is absorbed by the roots, conducted by the xylem to the leaves and buds, without mobility by phloem [10]. It is persistent in the plant and soil, highly stable in the soil, and its slow degradation lowers plant metabolism [8]. PBZ applied as a soil drench reduces internode lengths and causes earlier and enhanced flowering in mango trees. These results have been confirmed in different locations in the tropics [11].



**Figure 1.** Chemical structure of paclobutrazol.

Paclobutrazol doses applied, each year, are not always adequate because they do not take into account the residue from previous applications. Paclobutrazol increases the compaction of inflorescence in the 'Tommy Atkins' mango proportionate to the applied dose [12]. High dosage, which tends to reduce the panicle length of the treated plants (33% as compared to control), results in the formation of very compact inflorescences, creating appropriate conditions for the incidence of diseases and pests as well as making phytosanitary control difficult [9]. In addition to the phytosanitary problems, excessive doses of PBZ can inhibit vegetative and floral growth longer than desirable, requiring more nitrate sprays to stimulate flowering. The high cost of crop production, for all the reasons that have been mentioned, is only one of the problems, as there is also the question of the accumulation of a chemical in the soil and plant without knowing the consequences over the years, both for the production system and the environment.

Soil application rather than foliar application of paclobutrazol has been found to be more responsive in suppressing the vegetative growth and enhancing the reproductive growth in mango trees [12, 13]. Studies have shown that paclobutrazol needs to be applied annually to increase mango fruit yields [5]. However, the paclobutrazol treatments to the tree basins (soil

under the canopy drip area within a radius of 1.5 m of the tree trunk) may result in its uptake into the trees and thereby result in the persistence of its residues in the mango fruit and also in the soil at the tree basin [13]. Such persistence of paclobutrazol residues in mango fruit may lead to adverse effects on human health. The persistence of paclobutrazol residues in soil may influence the soil microbial activity too. Soil microbial count of a mango orchard soil where paclobutrazol was frequently applied has been shown to be reduced by up to 58% [14].

Soils are becoming polluted by pesticides because of the wide and, often indiscriminate, use of these xenobiotic molecules in agricultural practice. In the soil, pesticides may be involved in several stages, such as retention, transformation, and transport, the intensity of which will affect the potential activity of agrochemicals [15]. Bioremediation is an effective and cheap process that can degrade or transform contaminants to become less toxic or nontoxic [16–18]. Two processes have been found to increase the activity of microorganisms during bioremediation: biostimulation and bioaugmentation [19]. Biostimulation involves the addition of nutrients and/or a terminal electron acceptor to increase the weak activity of indigenous microbial populations by accelerating the decontamination rate since the addition of one or more rate-limiting nutrients to the system improves the degradation potential of the inhabiting microbial population [20, 21]. Bioaugmentation involves the addition of external microbial strains (indigenous or exogenous) that have the ability to degrade the target toxic molecules [22].

In this work, the biodegradation of paclobutrazol was studied using in submersed culture and saturated and unsaturated soils. In these conditions, experiments with biostimulation and bioaugmentation were performed.

## 2. Methodology

Soil samples were collected from irrigated mango orchards (*M. indica* L. cv. Tommy Atkins) at the Bebedouro and Mandacaru Experimental Stations of the Brazilian Agricultural Corporation (EMBRAPA Semiárido), in the Municipalities of Petrolina (9°09' S, 40°22' W), Pernambuco state, and Juazeiro (9°24' S, 40°24' W), Bahia state, both located in the São Francisco river valley (northeast of Brazil). The two representative soils were a Yellow Ultisol (Bebedouro) and a Vertisol (Mandacaru). These regions had been consecutively treated with PBZ, with an average dose of 3.57 g of active ingredient per plant. The soil samples were collected 30 days after the last application. An average of 1.5 kg of soil at depths of 15 and 30 cm was collected from four points around eight plants. These samples were stored in a refrigerator for isolation until the beginning of the experiments. Soil samples without a history of PBZ application were also taken from the same farms.

The bacteria were isolated in a mineral medium [23], containing 0.25 g/L paclobutrazol (Cultar 25 SC, containing 25% of the active compound), which was used as the sole carbon source. 10 g of each soil sample (MS—Mandacaru without historical application of PBZ; MC—Mandacaru with historical; BS—Bebedouro without historical; BC—Bebedouro with historical) were added to 100 mL medium in 500 mL flasks. These flasks were incubated at 30°C in a rotatory

shaker (200 rpm). Evidence for bacterium utilization of paclobutrazol was sought by streaking turbid enrichment broths onto a mineral agar medium (15 g/L), containing paclobutrazol or glucose as the sole carbon source, and then incubating these plates under the same enrichment conditions. Pure cultures of paclobutrazol utilizing bacteria were obtained by streaking distinct colonies present on the mineral agar medium plates onto Tryptone Soy Agar (TSA, Oxoid). The isolates were identified by Gram staining test. The Gram-negative isolates were streaked on three selective media for *Pseudomonas*: agar D4 [24]; agar Cetrimide (Merck) and King [25]. Biodegradation experiments were accomplished in a mineral broth with PBZ (1 g/L) and glycerol (5 g/L) or glucose (10 g/L).

Biodegradation experiments in saturated soils (Yellow Ultisol and Vertisol) were conducted in batch using paclobutrazol and paclobutrazol with added glycerol. The experiments were performed under sterile (by Gamma radiation) conditions using the mixed culture of *Pseudomonas* spp. Two concentrations of PBZ (10 and 25 mg/L) according to solubility in water (<26 mg/L) were used. The experiments were carried out in 60 mL flasks, where 5 g or soil was added to a 25-mL volume of liquid. The microorganisms were added with about  $10^7$  cells/mL. Control experiments were carried out only for the PBZ concentration of 25 mg/L without the addition of microorganisms. These experiments were placed in a rotary shaker (200 rpm) at 30°C for 35 days. Microbial concentration initial was  $1.10^7$  CFU/mL. This quantity corresponds to the inoculum of microorganisms added to the experiment.

Biodegradation experiments were conducted with the collected soil samples with and without history. Glycerol was added as the additional carbon source. To each 10 g of soil with (P-G: PBZ and glycerol; P-NG: only PBZ) and without (NP-G: PBZ and glycerol; NP-NG: only PBZ) PBZ application history, 30 µg/g of PBZ was added from a solution prepared with a commercial product (Cultar 25 SC). The experiments were carried out in 125 mL flasks at room temperature, without stirring, for 63 days and in triplicate. Samples were withdrawn at 0, 7, 14, 21, 35, 48, and 66 days for the quantification of native microbial and residual PBZ. In experiments with the addition of glycerol (P-G, NP-G), the concentration of this compound in the soil was 150 µg/g. Microorganisms were not added to the soil.

A  $2^4$  factorial design to study the biodegradation of paclobutrazol was applied. A two-level factorial design with 16 runs was employed to evaluate the individual and combined effects of the four factors: glycerol, mineral medium, inoculum, and soil (Table 1). The levels of the factorial design were glycerol ( $X_1$ ), with (+) and without addition (-); mineral medium [28] ( $X_2$ ), with (+) and without addition (-); inoculum ( $X_3$ ), with (+) and without addition (-); and region of soil collection ( $X_4$ ), A (+) and B (-).

Infrared spectra of the samples before (E1: 0 days) and after (E2: 70 days) the biodegradation process using only PBZ or PBZ and glycerol in unsaturated soils were measured with FTIR spectrophotometer (Vertex 70, Bruker). The analysis was done in IR region of 400 and 4000  $\text{cm}^{-1}$ .

The determination of the phytotoxicity was carried out with samples of biodegradation experiments (0 and 70 days), using only PBZ (4 µg/g) or glycerol (2.4 mg/g) as additional carbon source. Thirty seeds of *Allium cepa* (cv. Vale Ouro IPA-11) were germinated in individual Petri dishes containing 20 mL for each treatment at room temperature for 72 h. Distilled water was

Run	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	X <sub>4</sub>
1	-	-	-	-
2	+	-	-	-
3	-	+	-	-
4	+	+	-	-
5	-	-	+	-
6	+	-	+	-
7	-	+	+	-
8	+	+	+	-
9	-	-	-	+
10	+	-	-	+
11	-	+	-	+
12	+	+	-	+
13	-	-	+	+
14	+	-	+	+
15	-	+	+	+
16	+	+	+	+

**Table 1.** Conditions of factorial design 2<sup>4</sup> experiments for the biodegradation of PBZ.

used as negative control, totalizing five treatments. After 24 h of treatment, seed germination (%) and root length were measured per treatment, in order to determine seed germination index (GI), as described in Equation (1):

$$GI (\%) = \frac{S_t R_t}{S_c R_c} * 100 \quad (1)$$

where  $S_t$  is the seed germination of treatment (%),  $S_c$  is the seed germination of negative control (%),  $R_t$  is the root length of treatment (cm), and  $R_c$  is the root length of negative control (cm).

### 3. Results and discussion

#### 3.1. Isolation of bacteria from soil resistant to paclobutrazol

A total of 37 strains were isolated from the soil samples, in which 89% were Gram-negative bacteria. Eleven of these were identified as *Pseudomonas* (Figure 2). The growth curves of the 11 isolates of *Pseudomonas* showed exponential phase between 4 and 6 h. The maximum specific

rates growth ( $\mu_{max}$ ) were higher than  $0.30 \text{ h}^{-1}$  (Table 2). These bacteria were selected by their capacity to degrade diverse composites, as for example hydrocarbons [26], 2,4-dichlorophenol [27], naphthalene [28], and organophosphates [29]. *Pseudomonas* also participates in metabolic routes of compound degradation similar to paclobutrazol, as chlorobenzene [30] and atrazine [31, 32]. Jackson et al. [33], in research with paclobutrazol biodegradation, isolated nine *Pseudomonas* spp. with biodegradation capacity.

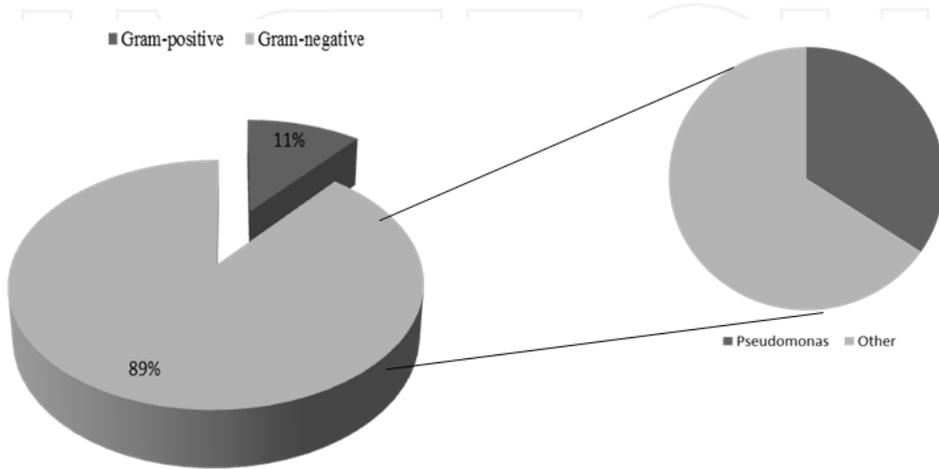


Figure 2. Bacteria isolated by enrichment of soil with paclobutrazol.

<i>Pseudomonas</i> spp.	$\mu_{max} \text{ (h}^{-1}\text{)}$
BC8	0.36
MS9	0.45
BS19	0.49
BC20	0.78
BC21	0.96
MS23	0.65
MS26	0.59
MC27	0.68
BS31	0.71
BS32	0.68
BS33	0.68

Table 2. Growth maximum specific rates

Two strains of *Pseudomonas* were identified as *Pseudomonas aeruginosa*; however, these were not used in this work due their pathogenicity [34, 35]. Experiments with mixed cultures of the soil samples MS, MC, BS, and BC were carried out using only PBZ as the carbon source to evaluate PBZ biodegradation. TSA broth was inoculated with the cultures to activate them. After 24 h, at 30°C, bacteria was inoculated into 40 mL of the nutrient broth. After a period of approximately 4 at 6 h of incubation, at 30°C and 200 rpm, mixed cultures were prepared and inoculated into 400 mL of mineral broth, as described by Ridgway et al. [23] and PBZ, 1 g/L. Temperature and agitation conditions of this stage were similar to those for the inoculum. Later, biodegradation experiments were accomplished in mineral broth with PBZ, 1 g/L and glycerol, 5 g/L.

Biodegradation was for MS and BC mixed cultures that had reached the maximum in 20 days of culture, with 47% (MS) and 43% (BC) of PBZ biodegradation. No relation was observed between the PBZ biodegradation and the soil to have a history of application, probably due the isolation to have been for enrichment. Since the results of the experiments with mixed cultures MS and BC were similar, the culture MS was selected to continue with biodegradation experiments.

The experiments carried out with glycerol as an additional carbon source grew and had PBZ biodegradation higher than those with PBZ as sole carbon source. The maximum biodegradation reached about 75% in 10 days of culture. Jackson et al. [33], using *Pseudomonas*, obtained a biodegradation of 79% in 39 days. On the other hand, Silva et al. [14] observed a 56% biodegradation in 90 days, with mixed cultures of *Bacillus*, in an isolated soil sample with a history of application. Table 3 presents PBZ biodegradation in relation to the time, found in experiments using submersed culture. Lee et al. [28] observed that pyruvate can be used as an additional carbon source to stimulate growth and aromatic hydrocarbons biodegradation for *Pseudomonas putida* PG7.

Microorganism	Biodegradation (%)	Time (days)	Additional carbon source	Reference
<i>Pseudomonas</i>	79	39	-	Jackson et al. [33]
<i>Bacillus</i>	56	90	-	Silva et al. [14]
<i>Pseudomonas</i>	47	20	-	Present work
<i>Pseudomonas</i>	75	10	Glycerol	Present work
<i>Pseudomonas</i>	0	-	Glucose	Present work

**Table 3.** Comparison of the PBZ biodegradation in submersed culture.

When glucose was used as an additional carbon source, the PBZ concentration remained almost constant throughout the observation period (Table 3). PBZ biodegradation did not occur probably due to glucose catabolic repression. The presence of a catabolic repressor, or the presence of a carbon source that represses the expression of certain genes and operons responsible for the utilization of alternative carbon sources, can result in a low concentration inducing specific cometabolic routes [32].

### 3.2. Paclobutrazol biodegradation in saturated soils

In the experiments with PBZ as the sole carbon source in saturated soils, there was no difference in growth between the two PBZ concentrations used (10 and 25 mg/L). However, in experiments containing glycerol, a higher growth was observed when the concentration of PBZ 25 mg/L was used as a glycerol concentration of 50 mg/L. This can be attributed to an increased amount of glycerol (125 mg/L) compared to experiments with the PBZ concentration of 10 mg/L. The biodegradation of PBZ without an additional carbon source and 10 mg/L of PBZ was approximately 43% after 14 days (Figure 3). However, with glycerol as an additional carbon source, the biodegradation reached 70% in 28 days (Figure 4). The glycerol concentration decreased rapidly and was completely consumed during 4 days, independently of the amount utilized (Figure 5).

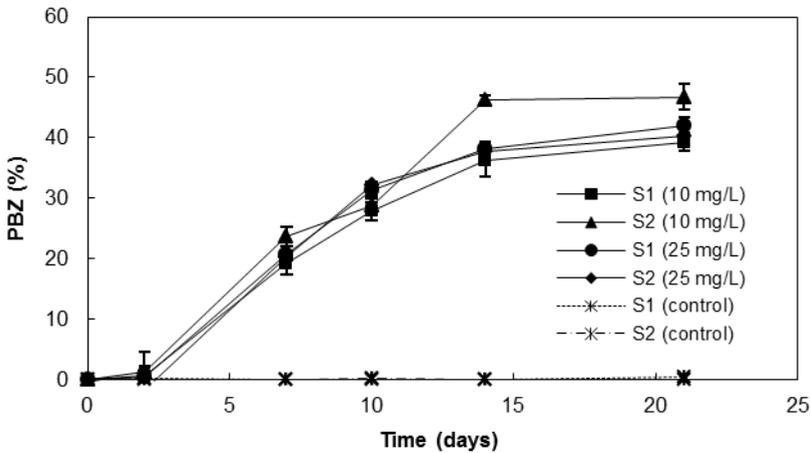


Figure 3. PBZ biodegradation. S1: yellow ultisol; S2: vertisol.

For the two soils and the two PBZ concentrations used (10 and 25 mg/L), there was a lag phase (the period in which there is virtually no biodegradation) of approximately 2 days, when PBZ was used as the sole carbon source. In all the experiments, biodegradation increased after a certain period of time, approximately 28 to 14 days, with only PBZ and PBZ with glycerol, respectively. The addition of a carbon source to the nutrient into the soil is believed to enhance in situ bioremediation by stimulating the growth of microorganisms that are indigenous to the subsurface and are capable of degrading contaminants [32].

### 3.3. Paclobutrazol biodegradation in unsaturated soils with and without a history of application

Figure 6 shows the PBZ biodegradation kinetics in unsaturated soil without and with a history of application, with and without the addition of glycerol. Experiments in P-G and P-NG soil showed a sustained reduction after the 14th day and only around 1% of the PBZ remaining on

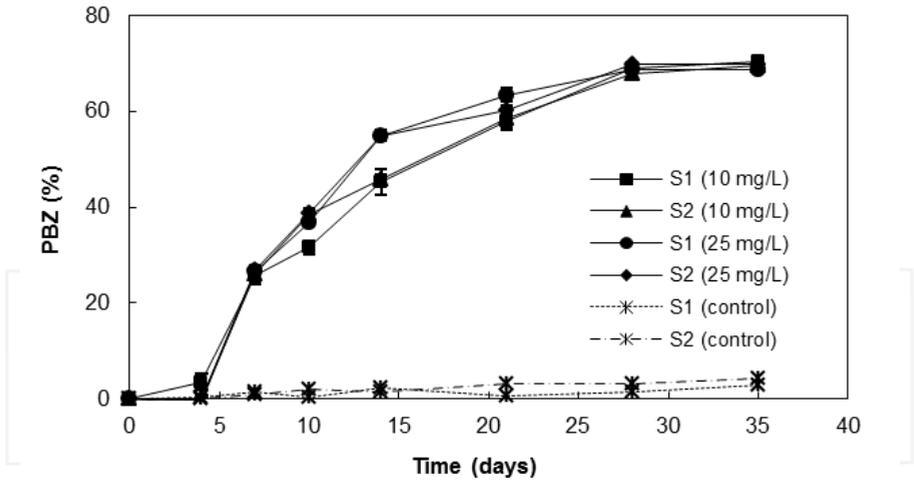


Figure 4. PBZ biodegradation with glycerol as additional carbon source. S1: yellow ultisol; S2: vertisol.

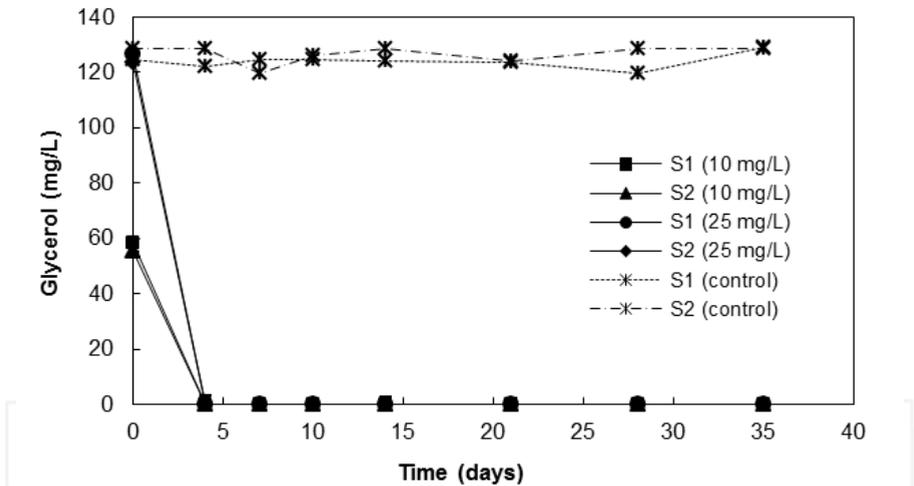


Figure 5. Glycerol consumption. S1: yellow ultisol; S2: vertisol.

the 63th day. This ability of the native microbiota to degrade paclobutrazol was probably due to the historical application. After repeated applications of some pesticides, the native microorganisms in the soil can degrade these compounds as they become suited for agrochemical use as a source of carbon for energy production and growth. Although there are some other factors affecting the persistence of agrochemicals in the soil, such as temperature, pH of the soil, chemical hydrolysis, and water content of the soil, microorganisms seem to play an important role in the degradation of these compounds [34].

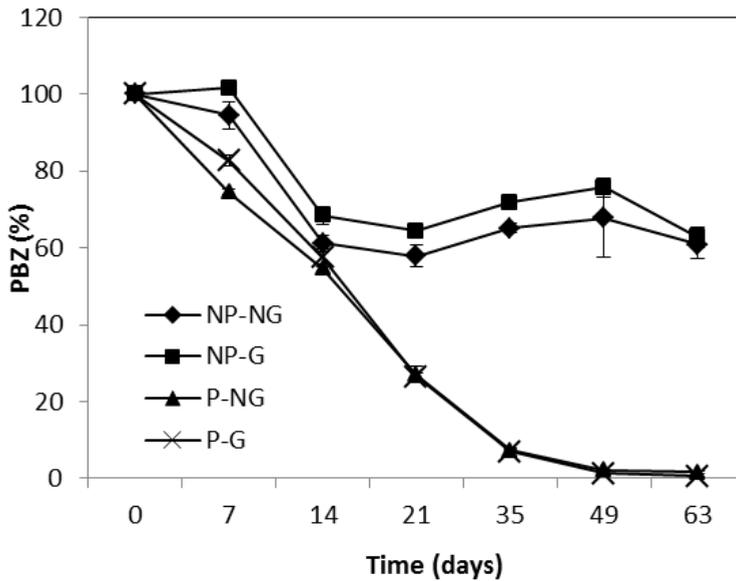


Figure 6. PBZ residual over 63 days.

The PBZ residue in soil without application history of PBZ, containing glycerol (NP-G) or not (NP-NG), was approximately 64% at 14 days. The lower biodegradation rate in NP-G and NP-NG was due to the microbial not being adapted to PBZ since this soil had no history of application.

The biodegradation in NP-G or NP-NG soils was clearly lower than that in P-G or P-NG soils. Biodegradation was not significantly different up to 49 days for experiments in NP-G and NP-NG soils. Similarly, in soil P-G and P-NG soils, biodegradation was not significantly different up to 14 days. Maximum biodegradation occurred in soil with a PBZ application history within 63 days, regardless of the presence of glycerol. This was probably due to the low concentration of glycerol added. Growth was similar; regardless of the addition of glycerol, comparing NP-G with NP-NG soils and P-G- with P-NG soils. However, with respect to the soil with and without history, there was higher growth to soil with history (P-G and P-NG).

The PBZ biodegradation kinetics modeling in the experiments without history (NP), but with (G) and without (NG) glycerol (Figure 7) was similar and presented the highest fit following a double first-order equation. PBZ was consumed at a rate  $k_1$  of 0.0894 and 0.1028 (Table 4), respectively, in experiments with and without glycerol [35].

The kinetic modeling for the experiments with history (P) and with and without glycerol (G, NG) differed greatly from experiments without history (NP) (Figure 8). Both followed a first-order kinetics, where the PBZ was degraded at a constant  $k$  rate of 0.0573 and 0.0538 (Table 4), respectively.

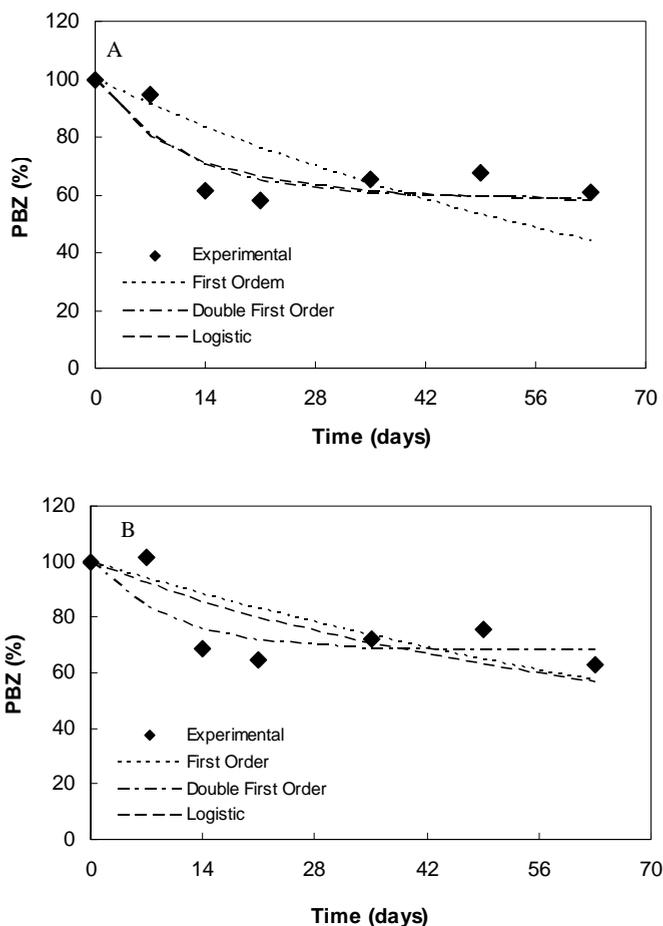


Figure 7. Modeling kinetics of soil without history with and without glycerol. (A) Without glycerol; (B) with glycerol.

Soils	$C_0$ (%)	First order		Double first order			Logistics			
		$K$	$R$	$f$	$k_1$	$k_2$	$R$	$K$	$k$	$R$
NP-NG	100	0.013	0.62	0.42	0.089	$7.12 \times 10^{-11}$	0.89	$5.76 \times 10^1$	$5.84 \times 10^{-2}$	0.88
NP-G	100	0.009	0.51	0.32	0.103	$2.69 \times 10^{-11}$	0.81	$5.00 \times 10^{-4}$	$6.09 \times 10^{-8}$	0.60
P-NG	100	0.057	0.99	1.60	0.057	$5.72 \times 10^{-2}$	0.99	$9.00 \times 10^{-4}$	$9.44 \times 10^{-7}$	0.95
P-G	100	0.054	0.98	1.00	0.054	$5.35 \times 10^{-2}$	0.98	$3.77 \times 10^{-5}$	$3.72 \times 10^{-8}$	0.93

Table 4. Constants and kinetic parameters of the biodegradation of paclitaxel; NP-NG: soil without history and without glycerol; NP-G: soil without history and with glycerol; P-NG: soil with history and without glycerol; P-G: soil with history and with glycerol.

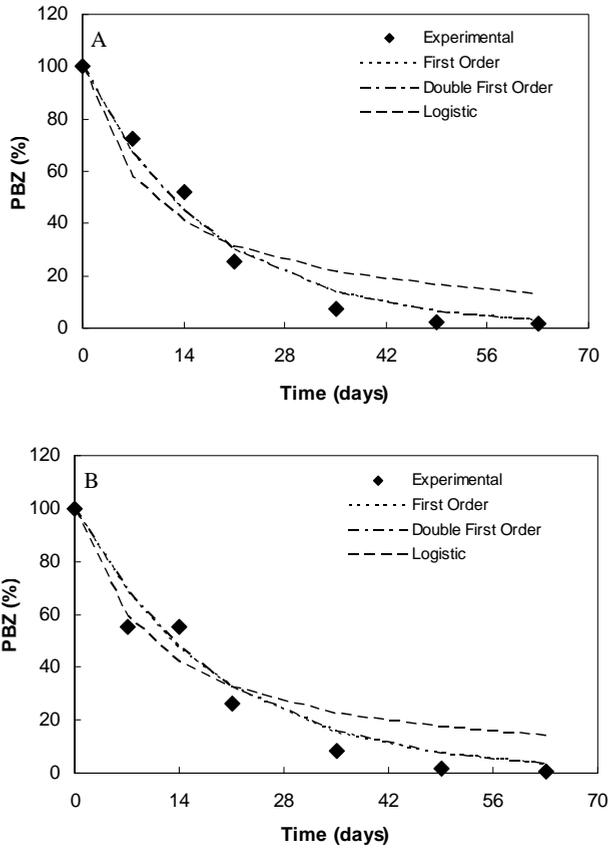


Figure 8. Modeling kinetics of soil with history with and without glycerol. (A) Without glycerol; (B) with glycerol.

Vaz et al. [36] obtained excellent fits using double first-order kinetic and logistic models in sterile soil and with addition of *Pseudomonas* spp., isolated from soil with no history. Mathematical models can help to identify high levels of toxic substances in soil or fruits of plants treated with pesticides and indicate that such substances are able to be systematically monitored.

Paclobutrazol has been shown to be efficient in treating mango trees in semiarid conditions [9]. Because it needs to be applied directly into the soil, it is inconvenient since it remains and affects future planting. Further, it is difficult to determine the dosage for each future use when only empirical methods are used, as there may remain residue from the previous cycle of application [8].

No quantification is done, nor is it always taken into consideration when deciding the dose. Thus, the amount of paclobutrazol applied to the soil is not always appropriate, and risks of

using doses above the recommended are great. The inflorescences on trees treated with high doses are very compact [5], creating suitable conditions for the incidence of diseases and pests, whose control is also hampered by the format of the panicles. Besides the phytosanitary problems, excessive doses of PBZ can inhibit vegetative and floral sprouting longer than desirable, requiring nitrate sprays to stimulate flowering. Thus, in addition to increasing the cost of crop production, for all the reasons that have been mentioned, there is accumulation of chemicals in the ground making the long-term consequences for the production system unknown.

### 3.4. Paclobutrazol biodegradation in unsaturated soils with a history of application—Effect of bioaugmentation and biostimulation

PBZ biodegradation under the conditions of  $2^4$  factorial design is shown in Figure 9, where it is possible to observe that biodegradation occurred under all conditions. Less biodegradation was obtained in runs 1 and 9. In these runs, glycerol, the mineral medium, or inoculum had not been added (control experiments). The biodegradation was possible probably due to action of the native microbiota in the A and B soils since these soils had an application history [35].

Biodegradation was 79% and 60%, when only glycerol was added, in runs 2 and 10, respectively. However, biodegradation was about 85% when glycerol and the mineral medium were added. The combination of bioaugmentation and biostimulation might be another promising way to speed up the biodegradation of recalcitrant compounds. On the other hand, biodegradation reached 94% (runs 3 and 11) with the addition of the mineral medium only. The lack of energy sources or electron acceptors or a lack of stimulation of the metabolic pathways responsible for degradation can inhibit or delay the bioremediation [17, 35].

In runs 5 and 13, biodegradation reached only 38% and 29%, respectively. In these runs, only the bacterial consortium was added. In runs with biostimulation and bioaugmentation simultaneous (runs 6, 7, 8, 14, 15 and 16), a high level of biodegradation was achieved. Values varied between 81% and 96%. Vaz et al. [37] studied the biodegradation of PBZ in two soils under saturation conditions. A maximum value of 70% biodegradation within 28 days was found in experiments where glycerol and the three strains of *Pseudomonas* spp. were used. In the present study, higher values were found probably due to using soils with history of application.

Glycerol ( $X_1$ ), mineral medium ( $X_2$ ), and inoculum ( $X_3$ ) were significant factors in the biodegradation (Figure 10). The addition of glycerol, mineral medium, and inoculum increased the biodegradation, regardless of soil used. However, higher biodegradation values (94% to 96% biodegradation for the runs 11, 14, 15, and 16) were found in Soil B. In these runs, biostimulation was applied by the addition of glycerol and/or the mineral medium. Among the main factors, the most significant was the mineral medium followed by glycerol and inoculum. Both soils (A and B) used in research were different with respect to the percentages of sand, silt, and clay. The factorial design, however, did not differentiate significantly among these soil types.

In relation to the factors of interaction, only those factors involving the mineral medium ( $X_2$ ) with glycerol ( $X_1$ ) or the inoculum ( $X_3$ ):  $X_1X_2$  and  $X_2X_3$ , respectively, were significant. These

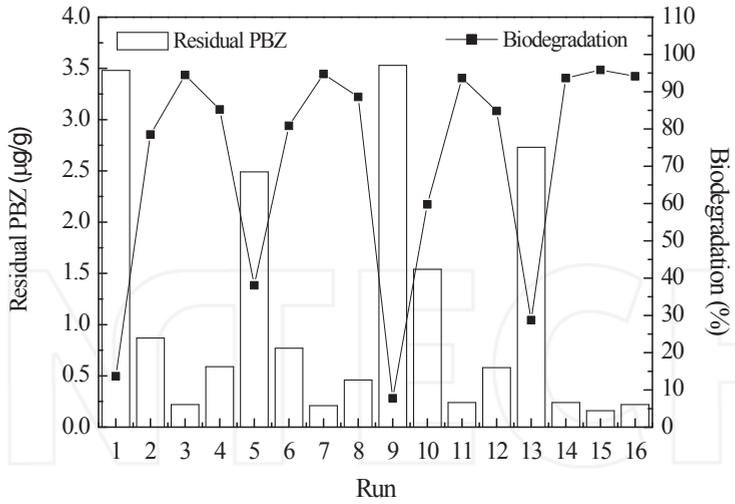


Figure 9. Biodegradation and PBZ residual for each run of factorial design.

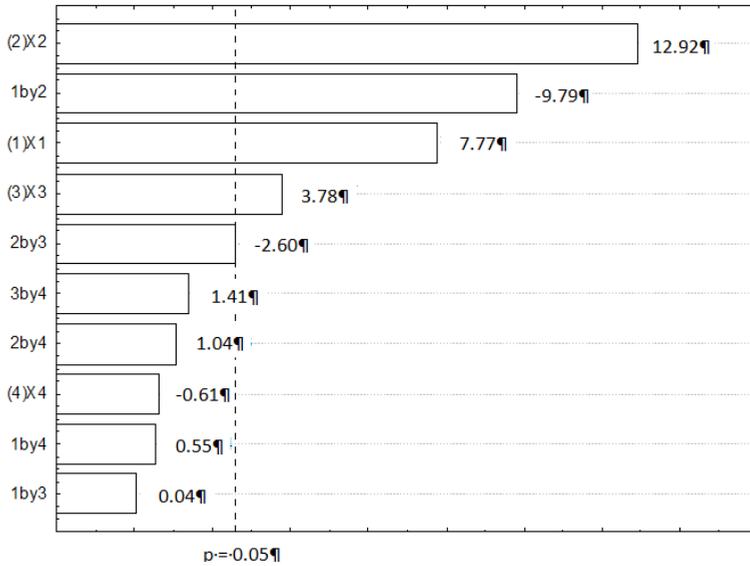


Figure 10. Analysis of significance of independent factors presented as standardized Pareto charts of biodegradation.

effects were negative, indicating that the addition of the mineral medium with glycerol or the inoculum did not favor the biodegradation of paclobotrazol. The addition of glycerol and mineral medium was more significant (-31.31) than the addition of the mineral medium and inoculum (-8.31).

By applying multiple regression analysis on the experimental data, a polynomial model in coded unit explains the role of each factor and its second-order interactions (Equation 2). The negative and positive signs of regression coefficients indicate the antagonistic effect and synergistic effect of each variable, respectively:

$$Y = 70.78 + 12.42X_1 + 20.65X_2 + 6.04X_3 - 15.66X_1X_2 - 4.16X_2X_3 \quad (2)$$

where  $Y$  is biodegradation (%) and  $X_i$  is level factor in coded unit (+1 or -1),  $i = 1-4$  for four factors. Equation (2) demonstrates that glycerol ( $X_1$ ), the mineral medium ( $X_2$ ), and the inoculum ( $X_3$ ) were responsible for the biodegradation observed. There are only two significant interaction effects ( $X_1X_2$  and  $X_2X_3$ ). This indicates the additional synergistic effect of these factors. The analysis of variance (ANOVA) was applied to the experimental data and simulated by the empirical model data. The  $F$  test was calculated as the ratio between the mean square of regression and the residual mean square. The high value of  $F$  (137.63) test and the low  $p$  value (0.012405) indicated the significance of the regression.

Figure 11 shows the Log UFCg<sup>-1</sup> observed in all runs of the factorial design 24 at zero and 40 days. The Log UFCg<sup>-1</sup> decreased in runs 1 and 9 due to depletion of nutrients in the soils since only sterilized water was added (control runs). In runs 5, 6, 7, 8, 13, 14, 15, and 16, there was also a decrease in the Log UFCg<sup>-1</sup> since in these runs bioaugmentation was performed and probably the nutrients were not sufficient. On the other hand, in the runs with the addition of glycerol and/or the mineral medium (2, 3, 4, 10, 11, and 12), there was an increase in Log UFCg<sup>-1</sup>, independent of the soil used.

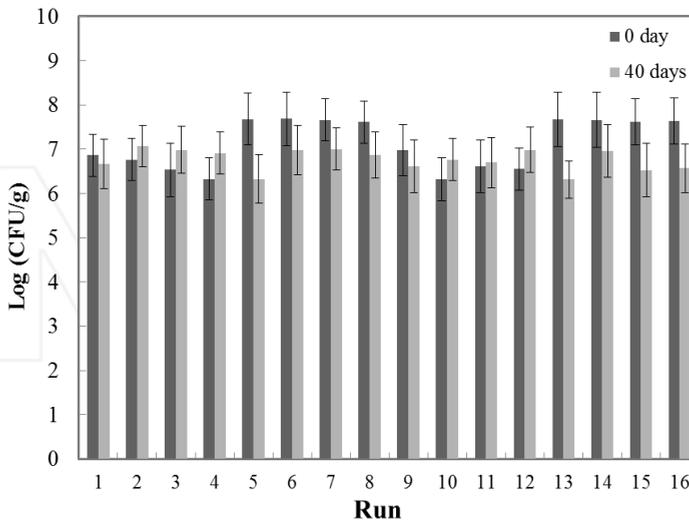


Figure 11. Colony forming unity in each run of factorial design.

### 3.5. Characterization of samples of biodegradation using FTIR spectroscopy

Figures 12 and 13 show an infrared analysis of PBZ dissolved in distilled water and only methanol, respectively. The solvent used in the preparation of samples was methanol. Comparing the spectra of PBZ and methanol, there is a band at  $1650\text{ cm}^{-1}$  only for spectra of PBZ.

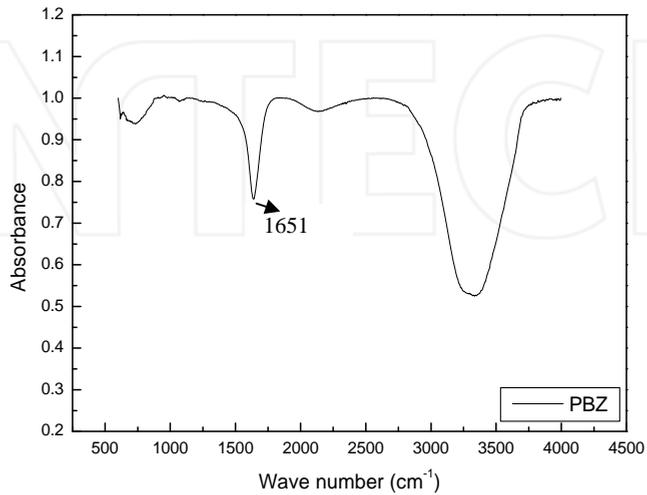


Figure 12. Fourier transformation infrared (FTIR) analysis of PBZ dissolved in distilled water.

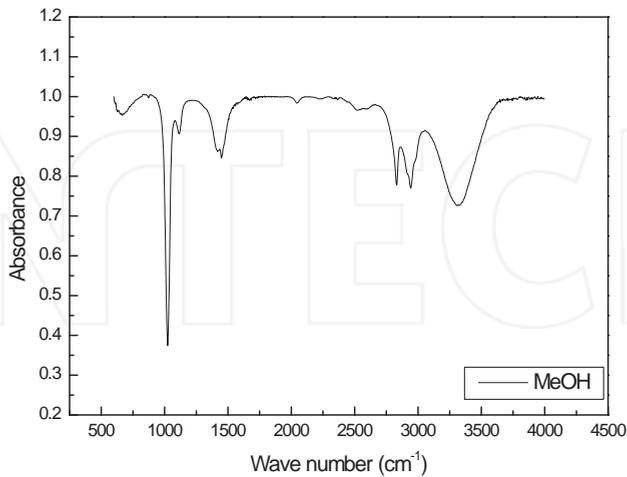
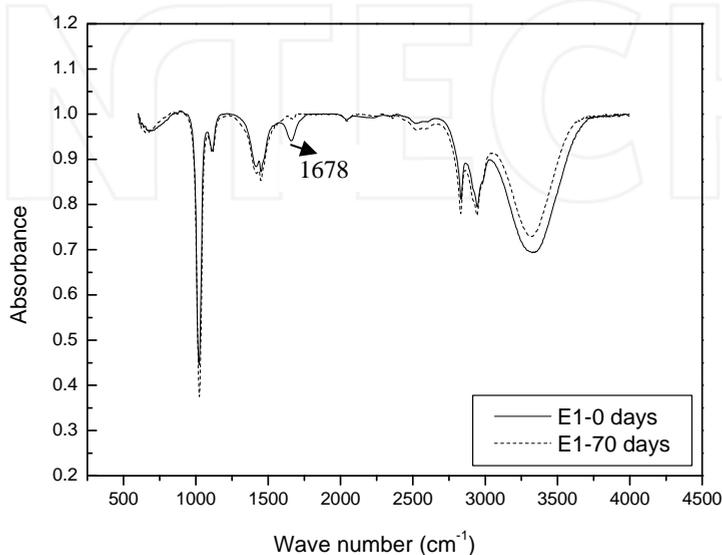


Figure 13. Fourier transformation infrared (FTIR) analysis of methanol.

Samples of soil E1 (only PBZ) and E2 (PBZ and glycerol) 0th day and 70th day of incubation are shown in Figures 14 and 15. A comparison of FTIR spectra of samples after biodegradation (70th day) and before biodegradation (zero day) revealed the lack of the band at  $1650\text{ cm}^{-1}$  corresponding to C=C and C=N stretching in the benzene and 1,2,4-triazole rings, respectively, which are observed in the structure of paclobutrazol (Figure 1). Therefore, examination of this particular band confirmed the reduction of paclobutrazol concentration in samples soils after 70 days of biodegradation.

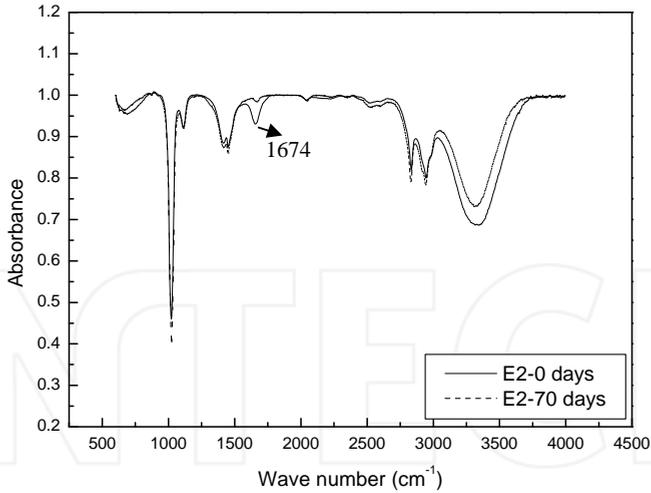


**Figure 14.** Fourier transformation infrared (FTIR) analysis of metabolites extracted with methanol before biodegradation of PBZ in experiments without (A) glycerol as carbon source additional.

Jackson et al. [33] observed 79% biodegradation after 39 days of incubation, possibly due mineralization of the [ $^{14}\text{C}$ ]-label to  $\text{CO}_2$ . Since the [ $^{14}\text{C}$ ]-label was located in the chlorobenzene ring of paclobutrazol, the observed loss of labeled carbon indicated some degree of degradation of this functional group by the *Pseudomonas* isolate. These authors concluded that PBZ is at least partly degradable by bacteria (*Pseudomonas* and *Alcaligenes*) in pure culture, with the chlorobenzene ring being catabolized but the 1,2,4-triazole ring was found to be resistant to attack.

### 3.6. Phytotoxicity studies

Phytotoxicity was evaluated based on the germination index, shown in Table 5. According Paradelo et al. [38], phytotoxicity between 50% and 80% is considered moderate, while above 80% is absent. These results indicated that the concentration of PBZ ( $4\text{ }\mu\text{g/g}$ ) used is not toxic since index of germination was 83.1%. On the other hand, when was used in PBZ and glycerol, this index decreased at approximately 3%. The metabolites produced during biodegradation



**Figure 15.** Fourier transformation infrared (FTIR) analysis of metabolites extracted with methanol after the biodegradation of PBZ in experiments with glycerol as carbon source additional.

did not show phytotoxicity and increased index of germination, independently of the addition of glycerol. However, higher increase was observed without addition of glycerol.

Sample	Germination index (%)
Soil before biodegradation experiments (with PBZ 4 µg/g)	83.1
Soil before biodegradation experiments (with PBZ 4 µg/g and glycerol 2.4 mg/g)	80.7
Extracted metabolites after biodegradation experiments in the soil with PBZ	96.6
Extracted metabolites after biodegradation experiments in the soil with PBZ and glycerol	85.2

**Table 5.** Phytotoxicity studies of PBZ and metabolites produced after biodegradation (70 days) in experiments with unsaturated soil with and without addition of glycerol.

#### 4. Conclusions

The *Pseudomonas* isolated presents a great potential of paclobutrazol biodegradation. The bacterial growth and the paclobutrazol biodegradation were higher in the experiments using paclobutrazol and glycerol as carbon sources. These results indicate that glycerol can be considered a carbon source that stimulates the growth and it does not inhibit the paclobutrazol degradation by *Pseudomonas* spp.

The biodegradation of PBZ in unsaturated soils was more efficient when soil samples with a history of application of PBZ were used. We concluded that this soil bacterium is better adapted

for the degradation of the compound. Mathematical models can help to identify high levels of toxic substances in soil treated with pesticides and indicate that such substances should be systematically monitored.

Soils microorganisms were able to degrade PBZ (control experiments: 1 and 2 of the factorial design), but only with a low increase in biodegradation (<25%). Simultaneous bioaugmentation and biostimulation is not the best strategy. The highest number of applications of PBZ favored biodegradation.

FTIR spectra indicate the biodegradation of PBZ aromatic rings. This probably happens because of the biodiversity of the microorganisms in the soil. This is different from research undertaken with a culture immersed in a mineral medium and a mixed *Pseudomonas* culture, where only benzene chlorate has been degraded. Concentrations of 4 µg/g of PBZ and 2.4 mg/g of glycerol were not phytotoxic, and the biodegradation products increased the germination index.

## Acknowledgements

The authors acknowledge the financial support from Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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