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Research Paper

Biodegradation of 2,4-D and Trifluralin Herbicides by the Bacteria *Pseudomonas spp.* Using Factorial Design of Experiments

Hale Koksoy[1, *], Guven Uraz[2]

[1]*Department of Medical Biology, Medical Faculty, Karamanoglu Mehmetbey University, Turkiye;*
[2]*Department of Biology, Faculty of Science, Gazi University, Turkiye;*

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Abstract: Herbicides are commonly used to control unwanted weeds in fields, gardens, airports, parks, and railways. In addition to the benefits of herbicides that are applied to the ground with the help of agricultural tools, they also may be observed to have some damaging effects on the ecosystem. Herbicides may cause death and birth defects by getting mixed into drinking water. Studies show that numerous Pseudomonas spp. species isolated from various environments degrade hydrocarbon compounds. Degradation processes increase when environmental conditions become extreme. My purpose is to treat Pseudomonas ssp. isolated from environmental and clinical specimens. To clean herbicides by bacteria and contribute to cleaning nature economically. This study aims to establish the biodegradation of bacteria in the most effective medium in a statistical 23 multi-factorial testing apparatus created from four environmental and four clinical isolates selected from Pseudomonas aeruginosa, *Burkholderia cepacia*, Pseudomonas fluorescens, and Pseudomonas putida species. *Burkholderia cepacia* species was observed to degrade 2,4-D at a rate of 99.7% in the presence of activated carbon in the medium, and Pseudomonas aeruginosa species was found to degrade trifluralin at a rate of 99.3% in the presence of activated carbon in the medium. The presence of activated carbon and succinic acid in the medium increased the efficiency of bacteria in herbicide biodegradation. Consequently, it is believed that the use of Pseudomonades for eliminating toxic residues left by 2,4-D and Trifluralin herbicides may provide some benefits environmentally, clinically, and economically.

Keywords: *2,4-dichlorophenoxyacetic acid(2,4-D), Trifluralin, Pseudomonas aeruginosa, Burkholderia cepacia, Pseudomonas fluorescens, Pseudomonas putida.*

Introduction

2,4-dichlorophenoxyacetic acid (2,4-D) and trifluralin herbicides are commonly used in weed control in our country and around the world. These herbicides, which are found in soil, water, and factory wastes, cause unwanted mutations in various organisms in the environment. Some microorganisms in soil degrade 2,4-D, especially trifluralin under aerobic conditions. Some herbicides remain in the soil in varying amounts based on the variety of the soil. The residue is mixed in streams through rain and irrigation water and accumulates in lakes and seas. Anaerobic microorganisms degrade 2,4-D and trifluralin herbicides that permeate into the deep soil. In addition, microorganisms that degrade such herbicides are found in sediments in sea and lake bottoms (Kerner, 1971; Berry *et al.,* 1987).

The demand of human beings for food increases due to the upsurge in the world population. Agricultural production is not at the desired level. The main reason for low yield in agricultural production is due to weeds. In addition, damage by weed diseases and pests also causes low yield (Loser *et al*., 1999). In addition to their benefits, various chemical substances (e.g., pesticides and insecticides) used in the control of weeds and pests that lead to the loss of yield in agricultural produce cause environmental pollution and threaten the health of living organisms due to their toxic effects (Loser *et al*., 1999; Leahy *et al*., 1990).

For a chemical substance to create an effect, it must first be taken into the body in a certain way and then absorbed by the body. When given to a living organism a certain way and at a sufficient dose, every chemical substance can create detrimental effects. The severity of the effect is due to the amount of substance reaching the effect zone and the physical structure of an organism. When establishing the toxicity risk of a chemical substance, knowing just the type of effect is not sufficient. The main factors

*Corresponding: E-Mail: hkoksoy@kmu.edu.tr; Tel.05422926776

affecting toxicity are the dose of the toxic substance, the manner of administration, contact time, and frequency (Gomes *et al*., 2009).

The zone where Pseudomonas species bacteria are colonized, and the condition of the host are the most significant factors affecting their pathogenicity. The primary criterion for the disease to occur is the settlement of the pathogen in a suitable area. Colonization occurs when the skin and mucosa structure deteriorates or the immune system is suppressed, causing systemic disease through local invasion. Cellular damage plays a deterministic role in the colonization of Pseudomonas species bacteria in epithelial cells. Damage occurring in epithelial cells because of infection through a virus or endotracheal intubation causes similar results. This phenomenon is called "opportunistic adherence". Pseudomonas bacteria are a significant trigger in the pathogenesis of infections. Pseudomonas bacteria rarely cause any diseases in healthy persons. However, they can establish infection in every system and organ in persons with a deficient immune response and defense system, especially in a hospital environment; in other words, whenever they find an opportunity (Koneman *et al*., 1997).

Studies conducted on the genes of microorganisms that degrade pesticides demonstrated that these genes are carried on plasmids, transposons, and chromosomes. Catabolic genes are modified. Means of metabolic degradation were achieved through the purification of enzymes found in microorganisms (Colpella *et al*., 1990). Today, some Pseudomonas bacteria species commonly found in clinical environments (hospitals, burn units, and any environment with infection and wound risks) and environmental areas (soil, water, food, *etc*.) are popular for their ability to degrade pesticides biotechnologically. Another dimension of this research is the production of biotechnological metabolites by Pseudomonades through degradation to maintain life (Balows, 2003).

Photosynthetic microorganisms are commonly used for the purification of nitrogen, phosphorus, heavy metals, and other toxic products from industrial wastewater. The use of photosynthetic microorganisms that can develop without the need for any carbon source is economical for industrial research (Kerner, 1971).

Pseudomonas spp. bacteria that will constitute the basis of this study were isolated from various clinical and environmental areas. The fact that these bacteria are economical and easy to work with is a significant motivation in investigating their biodegradation abilities. Advanced statistical methods and HPLC measurements were used to establish the resistance of bacteria against toxic environments and to reveal the herbicide biodegradation capabilities of the most resistant strains. The biological capability of a combination of a commonly found herbicide and *Pseudomonas* spp. under different environmental conditions shall be discussed in light of experimental findings. In the next section, some important biodegradation studies shall also be reviewed in short. In the third section, information on materials and methods shall be summarized. Experimental results and findings shall be discussed in the fourth section.

A Brief Look at Biodegradation Studies

It can be seen in the literature that many varieties of pesticides and chemicals are broken down by different microorganisms. In a study conducted with Chlorobenzene, the Pseudomonas spp. RHO1 bacteria used the biodegradation end products of 2-chlorophenol and 3-chlorocatechol as carbon and energy sources (Fritz *et al*., 1992).In many studies conducted with pure and mixed cultures, it was reported that 2,4-D could be used as a carbon and energy source by species belonging to the Artrobacter, Pseudomonas, Xanthobacter, and Alcaligenes genera (Fisher *et al*., 1978). In a ten-day study conducted under light, the cyanobacteria *Microcystis aeruginosa* was able to survive at a 2,4-D dose of 1000-1500 ppm (Hoffmann *et al.,* 1996). The LC50 of two phytoplankton against the 2,4-D herbicide was determined. According to this evaluation, the LC50 of *Phaeodactylum tricornutum* against 2,4-D was determined as 362±9 ppm, and the LC50 of *Dunaliella tertiolecta* against 2,4-D was determined as 185±11ppm (Okay et al. 1996).In a 44 days study conducted with *B. cepacia* on sterile and non-sterile soil, it was reported that while 5 ppm of 2,4-D was toxic, bacterial growth continued at a 2,4-D dose of 500 ppm (Jacobsen *et al*., 1992).

By isolating the dioxygenase gene that breaks down naphthalene from the *Klebsiellaoxytoca*, *Herbaspirillum seopedicae*, and Bacillus megaterium bacteria, it was investigated whether the same gene would be effective for breaking down trifluralin. This article is the first to investigate the metabolic breakdown pathways for the pesticide trifluralin. However, because of sequence analysis with PCR and the use of other techniques (Clear zone), it was determined that the genes that breakdown naphthalene and trifluralin are not the same, and that these genes do not entirely breakdown trifluralin (Bellinaso *et*

al., 2003). It is seen in the literature that Pseudomonas bacteria breakdowns trifluralin at doses of approximately 500 ppm. In a study on the biodegradation of trifluralin, oxadiazon, and norflurazon in soil sediments, it was determined that pesticides fully absorbed into the sediments were biodegraded at a faster rate than pesticides that did not mix with the sediments. biodegradation of 2,4-D and trifluralin herbicide was studied with P. fluorescens and P. putida isolated from the Mississippi river. Although these bacteria did not break down 2,4-D, trifluralin was broken down to a considerable extent (Zablotowicz *et al.*, 2001). The trifluralin herbicide, once used against the plant disease *Rhizoctonia solani* in cotton, was broken down by the *B. cepacia* bacteria (Heydari et al. 1998). A study was conducted in which succinic acid was added to the media while evaluating the effect of *B. cepacia* on the biodegradation of the 2,4-D herbicide. According to the HPLC results, the addition of 0.2% succinic acid accelerated the bacterial breakdown of 2,4-D by 95% (Daugherty et al. 1994). In a study conducted with five pesticides and the *R. spharoides* and *R. pallustris* bacteria, 0.2% maltose was added as an inducer to the media, and it was observed that the rate of the breakdown reaction accelerated as a result (Chalam *et al*., 1997). In wastewater with high carbon content (COD=584.11), it was reported P. putida broke down more than 90% of 2,4-D and paraquat herbicides within 24 hours (Kopytko et al. 2002).

In a study that evaluated the effects of active carbon (NP5) on the biodegradation of phenoxy acid, it was observed that the 2,4-D, MCPA, and MCPP herbicides were effectively broken down (Wattanaphon *et al.,* 2008). It was reported that the rate of breakdown for soil samples contaminated with benzene increased with the aid of the biosurfactant obtained from the BSP3 strain of *Burkholderia cepacia* and by adding glucose to the media (Ignatowicz, 2009). Also, a study was published in 2008 that demonstrated the enzymatic breakdown of the 2,4-D herbicide by the *B. cepacia* bacteria (Smith et al. 2008). In a study that investigated the changes in the morphology, structure, and capability of removing the target contamination of the aerobic sludge granules cultured with mixed substrates of glucose and 2,4-dichloro phenoxy acetic acid (2,4-D) in a long-time running sequence batch reactor (SBR), when the carbon source transformed into the sole carbon source of 2,4-D (Ma et al. 2010).

In a study by Elizangela *et al*. (2021), Pseudomonas strains were isolated from environments with 2,4-D and its derivatives and the original Pseudomonas CMA-7.3. strain and all antioxidant capacity activities were studied. In the studies, it was observed that 36% of the bacteria cleaned the herbicide in the tanks with a biofilm layer. It is recommended that it can be used in ports or warehouses.

Studies investigated the potential genotoxic and retinal developmental effects of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on zebrafish (*Danio rerio*) during their early life stages. To assess genotoxicity, we measured DNA damage using the comet assay and analysed the mRNA expression of genes involved in apoptosis and DNA repair. Additionally, we evaluated retinal developmental toxicity through a histological approach. The results of our study revealed that exposure to 2,4-D caused alterations in the DNA integrity of zebrafish larvae. Furthermore, the transcriptomic data showed a significant increase in the expression of p-53 and casp-3 genes, which are associated with apoptosis, and a noteworthy reduction in the expression of the lig-4 gene, involved in DNA repair, in the larvae exposed to the highest tested concentration of 2,4-D (0.8 mg/L). These findings suggest that 2,4-D exposure may have detrimental effects on the genotoxicity and retinal development of zebrafish during their early life stages (Gaaied *et al*., 2022).

Microbial elimination of these herbicides is economically and environmentally feasible. Mycoremediation (bioremediation by fungi) is recognized as an effective approach to cleaning up harmful chemicals and converting them to non-toxic metabolites through various enzymatic pathways. Various fungi including *Phanerochaete chrysosporium, Phlebia aurea, A. niger, Phoma glomerata, Chrysosporium pannorum,* and *Trichoderma sp.* have shown that they have the potential to convert or break down harmful pesticides into harmless or less harmful compounds (Magnoli et al. 2020).

According to EPA regulations, the use of 2,4-D herbicides at currently recommended concentrations (< 2 ppm whole lake treatment) may pose a risk to freshwater fish (Dehnert *et al*., 2021).

Soil samples were taken from different fields in studies with Trifluralin herbicide, which is frequently used in cotton fields in China. Trifluralin residues in these soils were not sufficient to kill soil worms but were risky for barley, wheat, and alfalfa (Yang *et al*., 2021).

Recent studies have found that trifluralin may potentially affect mitochondrial function (Oliveira *et al.*, 2020), exhibit genotoxicity (Hakala et al., 2010), and act as a persistent biotoxin in mammalian cells (Bisceglia et al., 2018). Additionally, it has been associated with higher cancer incidence rates in agricultural workers exposed to the chemical (Weichenthal et al., 2012).

In the study of Kumar *et al*., (2016) significant progress has been made in understanding the biodegradation mechanisms of 2,4-dichlorophenoxyacetic acid (2,4-D). The 2,4-D biodegradation pathway has been elucidated in several microorganisms, including strains of *Cupriavidus necator* JMP134 (previously known as *Wautersia eutropha*, *Ralstonia eutropha*, and *Alcaligenes eutrophus*) and Pseudomonas. An alternative approach involves introducing suitable plasmid-derived catabolic genes into established and competitive natural bacterial populations. Therefore, further characterization of new local bacterial populations is needed for possible application in the bioremediation of 2,4-D. That's why it's an important article (Kumar *et al*., 2016).

Trifluralin is a widely used herbicide with significant environmental persistence and ecotoxicity, especially for aquatic organisms. It is insoluble in water and highly volatile, leading to rapid loss from soils when applied on the surface. The herbicide strongly binds to soil organic matter and shows minimal leaching into water. Trifluralin's structure contains a tertiary amino group, two nitro-groups, and a trifluoromethyl-group. Despite its xenobiotic nature, it can undergo biodegradation through dealkylation or nitro-group reduction by specific bacteria and fungi (Coleman *et al*., 2020).

Materials and Methods

Pseudomonas samples used in the research were divided into two groups clinical and environmental. Water and soil samples were taken from 10cm below the surface in sterile containers and polyethylene bags. The locations of the samples were recorded, and the samples were taken immediately to the laboratory environment. After the samples were taken, they were maintained at 4ºC for 1-2 days until analyses were carried out (Brock, 1979). Clinical samples were supplied by the Microbiology Laboratory of the Medical Faculty of Gazi University in sterile Eppendorf tubes containing 0.5ml glycerol maintained at -80ºC and were activated (Rajmohan *et al*. 2002).

Pseudomonas spp. bacteria were isolated from the environmental water and soil-based samples obtained from the surroundings of Ankara Province (Govan, 1989; Pier *et al*., 2005). In addition, 11 *Pseudomonas* spp. isolates (clinical samples) were supplied by the Microbiology Laboratory of the Medical Faculty of Gazi University (GUMF). The total number of isolated samples was 121 which is shown in Table 1.

Bacteria belonging to the genus Pseudomonas; Pseudomonas P (King A) giving pyocyanin pigment and Pseudomonas F (King B) giving Pyoverdin and Fluoressin pigment were incubated for 24 hours at 37° C in a selective medium (Kristiansen, 1983). Studies were conducted on environmental (4 isolates) and clinical (4 isolates) samples of *Pseudomonas aureginosa*, *Burkholderia cepacia*, *Pseudomonas fluorescens*, and *Pseudomonas putida* bacteria belonging to Pseudomonas species. For biochemical tests, the definition of isolate identifications was conducted by Analytical Profile Index (API20NE; Biomérieux, Marcy I' Etoilé, France) and "Microscan Auto-analyser".

For three days after isolation, 2,4-D herbicides in 25, 50, and 100ppm concentrations and trifluralin herbicides in 100, 250, and 500ppm concentrations were pipetted onto Elisa plates along with bacteria (0.5 MacFarland) in live colony count experiments. Live colony count was carried out by cultivating samples taken every 24 hours for three days on plate count agar (PCA) media (Bauer 1982; Claus 1989). Results were compared with control samples that did not contain herbicides.

Statistical Probit Analysis and Lethal Concentration (LC) values were determined, and a new experiment set was set up by selecting two herbicide-resistant strains (Levesque, 2007; Finney, 2009). In the statistical 23 testing apparatus designed to find out in which media bacteria best degrade herbicides (Hicks *et al*., 1999; Yates, 1937), the results were established by HPLC measurements (Bresolle *et al*., 1996) and were analysed and interpreted by Statistical Yates Algorithm (Yates, 1937).

The 23-design used in the study aims to establish statistically the differences between the biodegradation capabilities of herbicide-resistant bacteria under various media conditions. Independent variables of the design were established as 2,4-D or trifluralin herbicide concentration (low and high doses), succinic acid usage rate (0.2% and 0.4%), and the presence of activated carbon in media (present or absent) (Daugherty *et al*., 1994; Kopytko *et al*., 2002). In the multi-factor experiment, there were three factors (independent variables) and two levels of each factor. The dependent variables of the experiment design (responses) are 2,4-D and trifluralin biodegradation percentages. The 23 experiment was designed to answer the following questions: How does succinic acid affect biodegradation? Does activated carbon have the feature of absorbing (physicochemical) substances in the environment it exists? What kind of effects does an increase or decrease in herbicides in the environment pose?

Table 1. Areas from where Pseudomonades were isolated and isolation count.

Areas from where pseudomonades were isolated Environmental samples	**Isolation count**
1) WATER SAMPLES	
Lake Mogan	12
Ankara River	24
Lake Eymir	16
Kecioren Waterfalls 1 and 2	8
Gazi Medical Faculty Bayrakkale Pool	4
Total	64
2) SOIL SAMPLES	
Kecioren and Cubuk Municipalities Vegetable Gardens	18
AUAF Pulse Plantation (Barley, Wheat, Chickpeas) Areas	13
House Plants Pot Soil and Agricultural Mold	8
Agricultural Soil from the Surroundings of Lake Eymir	7
Total	46
CLINICAL (HOSPITAL SAMPLES)	
Cerebrospinal Fluid (CSF)	1
Wound culture	4
Blood Culture	2
Urine Culture	1
Sputum Culture	2
Abscess Culture	1
Total	11
Grand Total	121

Table 2. 2^3 experimental design set for 2,4-D

Experiment Numbers	Herbicide Consantration (X)	Succinic Acid (Y)	Activated Charcoal (Z)	Trial Combination	Bacteria
1	14 ppm	%0,2	NO	(1)	YES
2	27 ppm	%0,2	NO	X	YES
3	14 ppm	%0,4	NO	Y	YES
4	27 ppm	%0,4	NO	XY	YES
5	14 ppm	%0,2	YES	Z	YES
6	27 ppm	%0,2	YES	XZ	YES
7	14 ppm	%0,4	YES	YZ	YES
8	27 ppm	%0,4	YES	XYZ	YES
9	27 ppm	%0	NO	9	YES
10	27 ppm	%0	YES	10	YES
11	14 ppm	%0	NO	11	YES
12	14 ppm	%0	YES	12	YES

LC90 results were taken into consideration for the highest concentration of herbicides in the testing set. The division of high concentration by two obtained low herbicide concentration used in the experiment. Low and high values of succinic acid were established to be 0.2% and 0.4%, respectively. In the test design, 0.225gr activated carbon (1-disc 15gr/l) was added to the 15ml medium by calculation. A contrary result would indicate the absence of activated carbon (Kopytko *et al*., 2002). Tests 9,10, 11, and 12 were for control purposes (Tables 2 and 3).

HPLC measurements were conducted by Thermo Finnigan Surveyor brand HPLC device at the Instrumental Analysis Unit of Ankara University. For 2,4-D, analyses were conducted with RP18column, UV50 detector, 230nm wavelength, Methanol-0.1% Phosphoric Acid (60/40) at mobile phase, and flow rate of 1ml/minute (Yadav *et al*., 1993). For trifluralin, analyses were conducted with a C18 column, UV50 detector, 275nm wavelength, 80% acetonitrile, and 20% distilled water at mobile phase, and a flow rate of 1ml/minute (Bellinaso *et al*., 2003). During the biodegradation phase of

bacteria, a minimal salt medium was used to conveniently observe the products of degradations by not seeing a molecule that might arise from the media (Nam *et al*., 2003).

In the minimal salt medium of the experiment, the shaker continued to operate with 15ml tubes (3 parallels) at 100rpm and 37ºC incubator temperature for three days. A sample was taken every 24 hours and herbicide was extracted by the necessary method (Yadav *et al*., 1993; Bellinaso *et al.,* 2003; Nam *et al*., 2003). The biodegradation level of the bacteria was measured with the HPLC device. The results were calculated with a [y=ax+b] linear regression model based on the calibration values taken before the test (Bresolle *et al*., 1996). These were then transformed into percentile results by comparing them with the controls according to Abbott's formula (Abbott, 1925). HPLC tests were conducted for three days, however, the results for the third day in which the herbicides were best degraded by bacteria were taken into consideration. Yates' method was applied to the results of the two repeated results of the third day and an F statistic was calculated as a statistical significance control (Yates, 1937).

Table 3. 2^3 experimental design set for Trifluralin

Experiment Numbers	Herbicide Concentration (X)	Succinic Acid (Y)	Activated Charcoal (Z)	Trial Combination	Bacteria
1	180 ppm	%0,2	NO	(1)	YES
2	360 ppm	%0,2	NO	X	YES
3	180 ppm	%0,4	NO	Y	YES
4	360 ppm	%0,4	NO	XY	YES
5	180 ppm	%0,2	YES	Z	YES
6	360 ppm	%0,2	YES	XZ	YES
7	180 ppm	%0,4	YES	YZ	YES
8	360 ppm	%0,4	YES	XYZ	YES
9	360 ppm	%0	NO	9	YES
10	360 ppm	%0	YES	10	YES
11	180 ppm	%0	NO	11	YES
12	180 ppm	%0	YES	12	YES

Results

The live colony count results were compared with the controls, and the resistance of bacteria to herbicides was estimated (Figures 1, 2and 3). When conducting probit analysis, percent inhibition results from the second day, in which bacterial growth levels were higher, were used (Table 4). The lower the percent inhibition results were the higher the herbicide's resistance to bacteria. These results were inversely proportional to live colony count results. For instance, for trifluralin herbicide, P41 (*P. fluorescens*) bacteria in 100ppm concentration (66,73) were observed to be more resistant compared to P11 (*B. cepacia*) bacteria (71,35). During the evaluation stage, percent inhibition results were used, and LC levels on which the bacteria were the most resistant to the herbicide were established by SPSS statistical software.

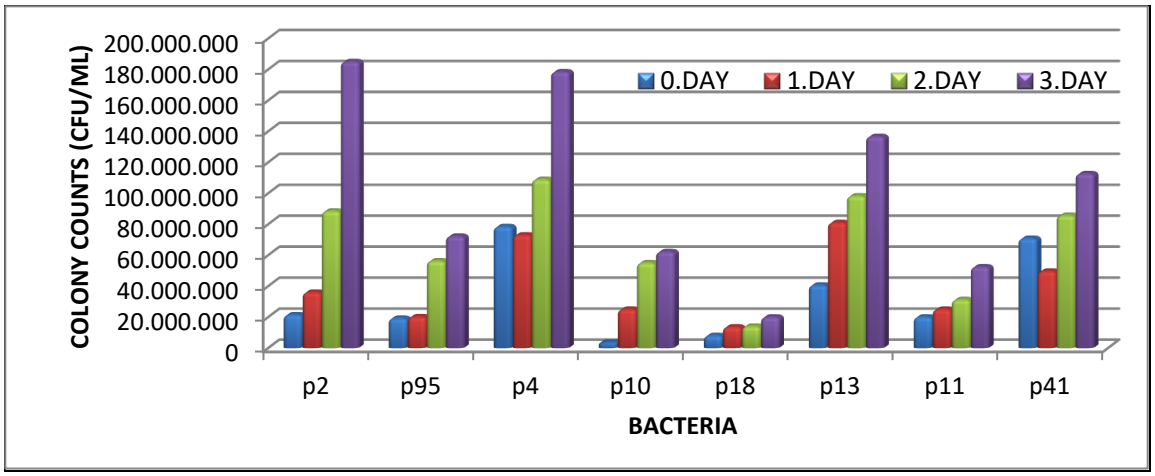

Figure 1. Control table for Pseudomonas spp. strains

LC 10, 50, 90, and 95 results were taken as the basis for probit analysis data. Figures 4 and 5 demonstrate the second-day probit analysis results of 2,4-D and trifluralin. Based on the results of probit analysis conducted with 2,4-D herbicide, the P11 (LC 95; 46,19ppm) bacteria strain was established to be the most resistant strain. In the probit analysis conducted with trifluralin herbicide, the P4 (LC 95; 609,41ppm) strain was found to be the most resistant.

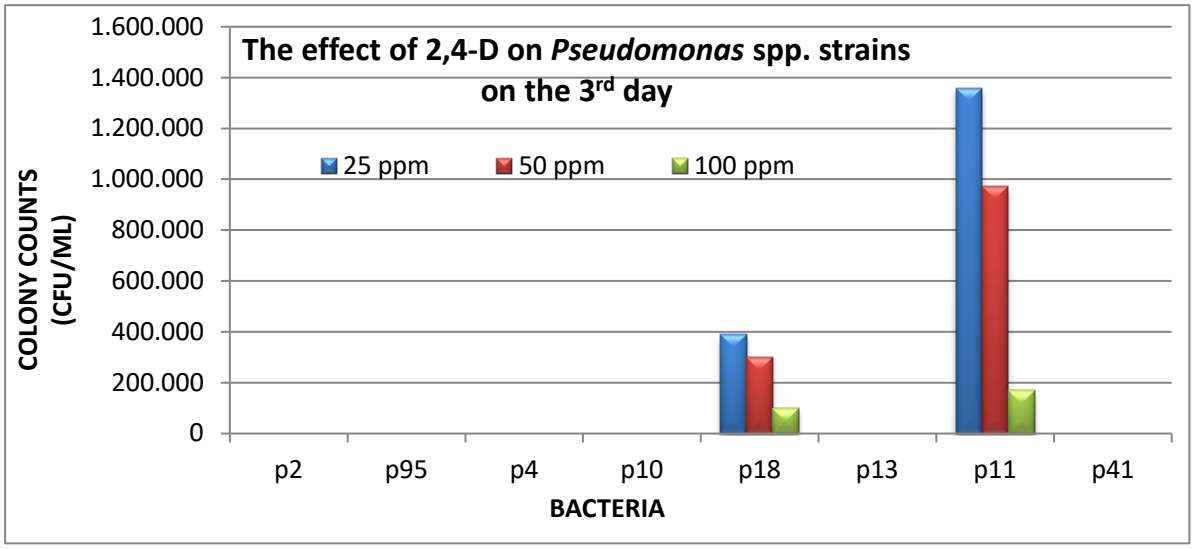

Figure 2. The effect of 2,4-D on Pseudomonas spp. strains on the 3rd day

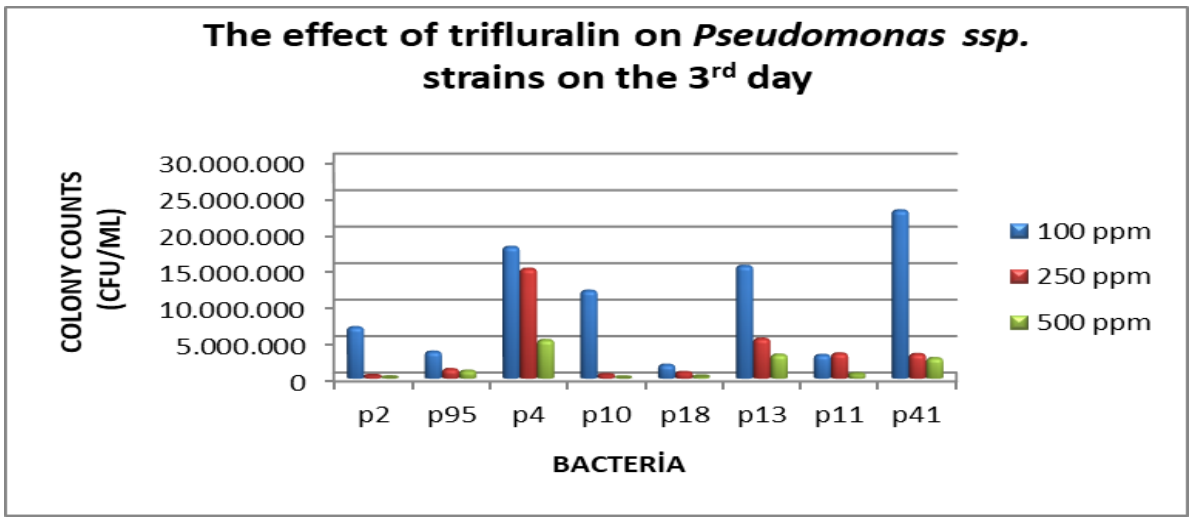

Figure 3. The effect of trifluralin on Pseudomonas spp. strains on the 3rd day

Table 4. 2nd day % inhibition results of 2,4-D and trifluralin herbicides

Second day	2,4-D			Trifluralin		
Bacteria	25 PPM	50 PPM	100 PPM	100 PPM	250 PPM	500 PPM
p2	100	100	100	90,82	99,15	99,88
p95	100	100	100	90,72	96,12	94,64
p4	100	100	100	68,71	77,98	96,86
p10	100	100	100	77,84	98,52	99,99
p18	94,24	95,20	99,07	77,21	94,21	97,12
p13	100	100	100	75,56	94,08	94,62
p11	89,66	94,16	99,13	71,35	91,23	94,39
p41	100	100	100	66,73	95,49	96,74

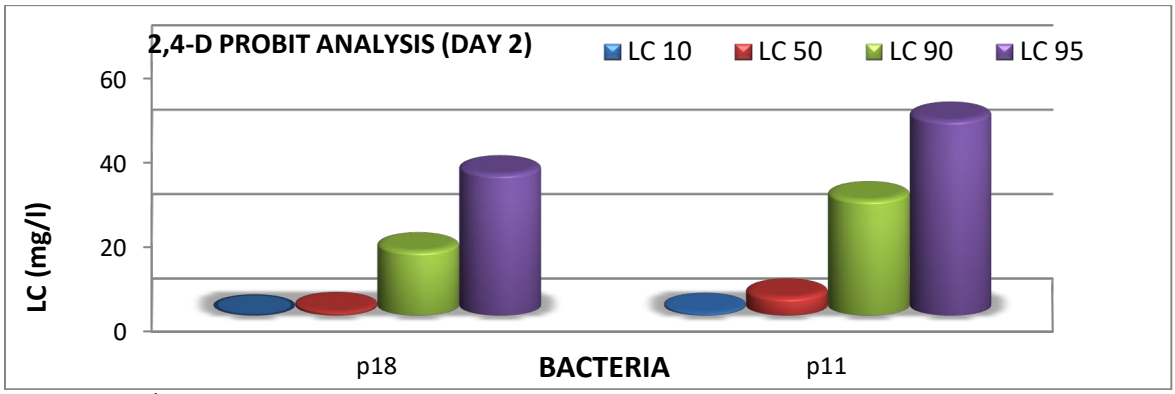

Figure 4. 2nd day probit analysis of 2,4-D

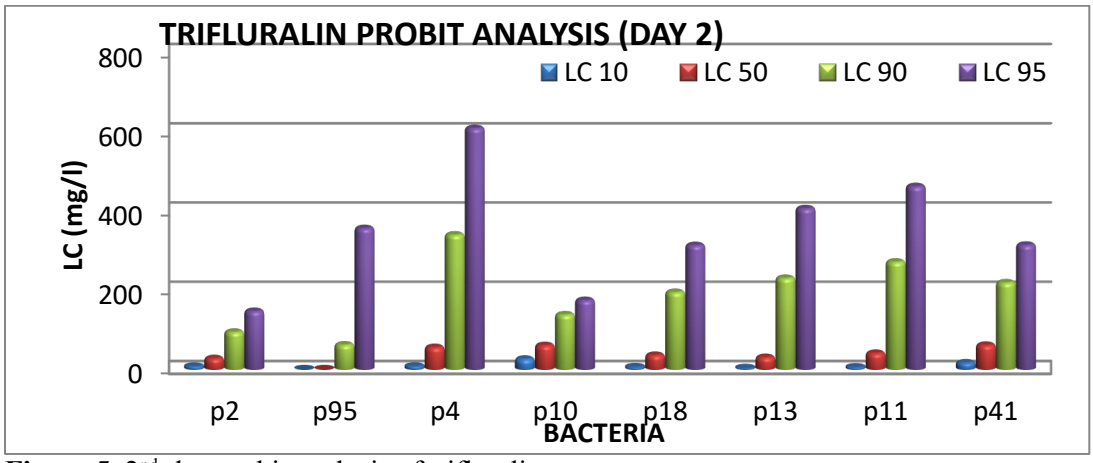

Figure 5. 2nd-day probit analysis of trifluralin

Clinical sample P11, which was resistant to the 2,4-D herbicide, was *Burkholderia cepacia*; the environmental sample P4 was *Pseudomonas aureginosa*. The HPLC method was adopted to measure the resistance of bacteria to herbicides. Table 5 shows the 2,4-D biodegradation percentages of Burkholderia *cepacia*. As 2,4-D herbicide is toxic, *B. cepacia* bacteria demonstrated significant degradation at a 14ppm low dose. As activated carbon showed a physicochemical absorption effect in the study, biodegradation occurred at over 95% in testing sets with activated carbon (5th - 8th experiments). Succinic acid being at 0.2% in the medium increased the biodegradation compared to its absence. It was observed in some sets that biodegradation was more effective at higher levels of succinic acid (0.4%). In testing sets in which activated carbon was absent (1st, 2nd, 3rd, and 4th experiments) the highest rate of 2,4-D degradation of P4 bacteria on the third day was only 33.3%.

Table 5. Biodegradation of 2,4-D herbicide by *B. cepacia*

	2,4-D Biodegradation (%) Experiment numbers											
Day	1	2	3	4	5	6	7	8	9	10	11	12
1	14,5	10,3	21,7	25,7	98,1	94,3	94,2	98,3	16,0	80,9	17,4	95,3
2	15,7	10,5	30,8	26,9	98,8	94,7	95,1	98,5	16,4	81,5	24,4	96,2
3	**16,4a**	**14,2**	**33,3**	**28,4**	**99,7**	**99,3**	**97,5**	**98,3**	**28,8**	**82,3**	**22,9**	**97,0**

a= Inhibition percent rate

Yates algorithm was applied to the estimated third-day biodegradation percentages (two-repetition) and results are shown in Table 6. According to the results in Table 6, herbicide concentration (X), succinic acid (Y), and activated carbon (Z) were established to be single significant factors in the 2,4-D biodegradation of *B. cepacia* (0.95F1.8= 5.32). The combination of herbicides and succinic acid (XY) was not found to be significant in terms of biodegradation, but bacteria demonstrated biodegradation at higher levels when herbicides and activated carbon (XZ) and succinic acid and activated carbon (YZ)

were in combination. The combination of herbicides, succinic acid, and activated carbon (XYZ) did not have a significant effect on the biodegradation of B.cepacia.

Table 6. Yates algorithm of 2,4-D herbicide

Exp.	response	[1]	[2]	[3]	SSTb	Trial Combination	Fc
1	32,86	61,3	184,86	974,92	59404,31	Identify Average	
2	28,44	123,56	790,06	-13,68	11,6964	X	6,817127
3	66,74	398,22	-14,34	55,88	195,1609	Y	113,7475
4	56,82	391,84	0,66	-2,96	0,5476	XY	0,319163
5	199,58	-4,42	62,26	605,2	22891,69	Z	13342,19
6	198,64	-9,92	-6,38	15	14,0625	XZ	8,196184
7	195,12	-0,94	-5,5	-68,64	294,4656	YZ	171,6263
8	196,72	1,6	2,54	8,04	4,0401	XYZ	2,354731

***a**= Total biodegradation percentage (3rd-day results of a study in 2 parallels)*
***b**= Squares total; **c**= F statistics; The significance level is 5% and the standard deviation is 1.31.*

In the trifluralin herbicide biodegradation of Pseudomonas aeruginosa, the medium in which the bacteria showed the most degradation was determined by creating different media through experimental design (Table 7). Since, in the study, activated carbon demonstrated a physicochemical absorption effect, biodegradation occurred at over 95% in testing sets that especially contained activated carbon (experiments 5, 6, 7, and 8). The succinic acid present in the medium at 0.2% increased biodegradation compared to its absence. In some sets, it was observed that biodegradation was more effective at higher levels of succinic acid (0.4%). The highest P11 degradation rate of trifluralin on the third day was at only 58.4% in test sets in which activated carbon was not present (experiments 1, 2, 3, and 4).

Table 7. Biodegradation of trifluralin by *P. aeruginosa*

	Trifluralin Biodegradation (%) Experiment Numbers											
DAY	1	2	3	4	5	6	7	8	9	10	11	12
1	32,4	26,0	27,0	34,9	98,7	96,3	97,9	97,4	27,2	98,2	6,4	93,7
2	34,1	36,0	40,9	41,3	99,0	96,8	98,4	97,9	30,5	98,7	14,9	93,9
3	**44,9a**	**47,9**	**58,4**	**54,2**	**99,3**	**98,9**	**99,0**	**98,7**	**40,1**	**99,3**	**24,4**	**93,8**

a= Inhibition percent rate

The Yates algorithm evaluated the significance of the established experiment design sets, single, double, and triple. According to the results given in Table 8, herbicide concentration (X) was not a significant factor by itself in the trifluralin biodegradation of P. aeruginosa (0.99F1.8= 11.3). If only succinic acid (Y) or only activated carbon (Z) is present in the medium, bacteria degrade trifluralin at higher levels. The combination of herbicide and succinic acid (XY) or the combination of herbicide and activated carbon (XZ) is not significant in terms of biodegradation. However, bacteria demonstrate higher levels of biodegradation when succinic acid and activated carbon (YZ) are in combination. The triple interaction of herbicide, succinic acid, and activated carbon (XYZ) is not significant in the biodegradation of *P. aureginosa*.

Table 8. Yates algorithm of Trifluralin herbicide

Exp.	Response	[1]	[2]	[3]	SSTb	Trial Combination	Fc
1	89,7	185,52	410,76	1202,54	90381,4	Identify Average	
2	95,82	225,24	791,78	-3,78	0,893025	X	0,120792
3	116,92	396,36	-2,48	38,78	93,99303	Y	12,71366
4	108,32	395,42	-1,3	-14,42	12,99603	XY	1,757865
5	198,58	6,12	39,72	381,02	9073,515	Z	1227,299
6	197,78	-8,6	-0,94	1,18	0,087025	XZ	0,011771
7	197,96	-0,8	-14,72	-40,66	103,3272	YZ	13,97622
8	197,46	-0,5	0,3	15,02	14,10003	XYZ	1,907194

a= Total biodegradation percentage (3rd-day results of a study in 2 parallels); **b**= Squares total; **c**= F statistics; The significance level is 1 % and the standard deviation is 2.72.

Discussion

Biological interventions for the degradation of pesticides would greatly contribute to the food chain, and thus, the natural cycle of nature. Biological intervention methods are more cost-effective and less harmful to the environment compared to other intervention methods. This and other similar studies conducted in a laboratory setting are a model for other large-scale studies. The application of bacteria in solution via pump to herbicide-intensive areas as the bacteria reproduce in a way that does not threaten other living organisms, is considered effective.

This study investigated the effectiveness of various Pseudomonas species in eliminating toxic residues left in the environment by 2,4-D and trifluralin herbicides used for agricultural pest control in our country. Only two of four environmental and four clinical isolates, succinic acid and activated carbon used in the study were established to have high degradability activity. It was found that B.cepacia (P11; clinical isolate, Blood Culture) degraded 2,4-D in the presence of succinic acid and activated carbon best in 72 hours and that the degradation reached 99.7%. Again, P. *aeruginosa* (P4; environmental isolate, Kecioren Vegetable Garden) was established to degrade trifluralin in the presence of succinic acid and activated carbon best in 72 hours, and the degradation reached 99.3%. Based on these results, both tested bacteria species were observed to be quite effective in the destruction of herbicides with toxic effects. It is recommended to conduct more comprehensive studies for their commercial uses. Contributions to the use of advanced experimental designs and analyses in biodegradation issues have been emphasized in this paper.

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