BIOREMEDIATION OF IMAZETHAPYR IN SOIL/WATER SYSTEM

DEVENDRA KUMAR

Thesis

Master of Science in Agriculture

(Soil Science and Agricultural Chemistry)

DEPARTMENT OF SOIL SCIENCE AND AGRICULTURAL CHEMISTRY

COLLEGE OF AGRICULTURE

RANI LAKSHMI BAI CENTRAL AGRICULTURAL UNIVERSITY
JHANSI-284003, UTTAR PRADESH,
INDIA

AUGUST, 2022

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Thesis

Submitted to the

Rani Lakshmi Bai Central Agricultural University, Jhansi (U.P.), INDIA, 284003

BY

DEVENDRA KUMAR

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science in Agriculture

(Soil Science and Agricultural Chemistry)

AUGUST, 2022

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Date:...12/11/2022

To,
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Subject: Submission of M.Sc. Soil science and Agricultural Chemistry thesis of Mr. Devendra Kumar.

Sir,

I am forwarding herewith thesis entitled "**Bioremediation of imazethapyr in soil/water system**" by **Mr. Devendra Kumar** in partial fulfilment for the award of degree of M. Sc. Soil science and Agricultural Chemistry from your university. The work has been carried out under the supervision of **Dr. Srinivasan R**, Principal Scientist, Division of Crop Production at ICAR-Indian Grassland and Fodder Research Institute, Jhansi.

Thanking you

(Amaresh Chandra)

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CERTIFICATE

Certified that **Mr. Devendra Kumar,** Id. No. **RLBCAU/AG/PG/0040/20** has satisfactorily pursued his course of research for a period of not less than IV semesters and that the thesis entitled **"Bioremediation of imazethapyr in soil/water system"** submitted by him to the Rani Lakshmi Bai Central Agricultural University, Jhansi – 284 003 (UP) in partial fulfillment of the requirements for the award of the degree of **Master of Science in Agriculture** in the subject of **Soil Science and Agricultural Chemistry** is the result of original research work conducted by him under my supervision and is sufficiently of a high standard to warrant its presentation to the examination.

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CERTIFICATE-II

This is to certify that the thesis entitled **"Bioremediation of imazethapyr in soil/water system"** submitted by **Mr. Devendra Kumar,** Id. No. **RLBCAU/AG/PG/0040/20** submitted to the Rani Lakshmi Bai Central Agricultural University, Jhansi – 284 003 (UP) in partial fulfillment of the requirements for the award of the degree of **Master of Science in Agriculture** in the subject of **Soil Science and Agricultural Chemistry** has been approved by the Student's Advisory Committee after the *viva voce* examination.

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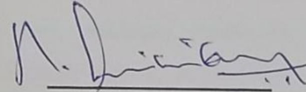

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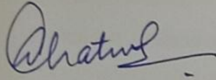

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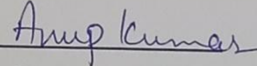

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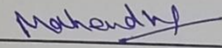

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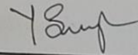

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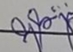

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Place: Jhansi **(Devendra Kumar)**

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CONTENTS

CHAPTER	TITLE	PAGES
1.	INTRODUCTION	1-5
2.	REVIEW OF LITERATURE	7-27
3.	MATERIALS AND METHODS	29-46
4.	RESULTS AND DISCUSSION	47-77
5.	SUMMARY AND CONCLUSION	79-82
	BIBLIOGRAPHY	83-99
	ANNEXURE	101-104
	ABSTRACT (ENGLISH)	105
	ABSTRACT (HINDI)	106

LIST OF TABLES

Table No.	Title	Page No.
	MATERIALS AND METHODS	
3.1	Metrological data of the crop growing season (2021-22)	**31**
3.2	Initial properties of experimental field soils	**34**
3.3	Genomic DNA isolation from bacterial isolates and 16S rDNA gene amplification	**45**
3.4	Genomic DNA isolation from fungal isolates and ITS gene amplification	**46**
	RESULTS AND DISCUSSION	
4.1	Impact of imazethapyr application on berseem plant growth (45 DAS)	**48**
4.2	Impact of imazethapyr application on berseem plant weight (45 DAS)	**49**
4.3	Impact of imazethapyr application on berseem nodule and chlorophyll	**50**
4.4	Impact imazethapyr spray on weed flora in berseem fields	**52**
4.5	Impact imazethapyr spray on soil physico-chemical properties	**53**
4.6	Impact imazethapyr spray on soil nutrient status	**54**
4.7	Soil texture and type of soil in study fields/sites	**54**
4.8	Testing the compatibility between selected isolates	**63**

4.9	Identification of selected isolates based on NCBI BLAST similarity	**67**
4.10	Biodegradation of imazethapyr in water *in vitro* using microorganisms	**68**
4.11	Biodegradation of imazethapyr in different soils *in vitro* by microorganisms	**70**
4.12	Biodegradation of imazethapyr by alginate bead bioformulation	**73**
4.13	Effect of contaminants concentration on imazethapyr bioremediation	**75**

LIST OF FIGURES

Figure No.	Title	Page No.
	MATERIALS AND METHODS	
3.1	Variation in weathers parameters during crop period (*Rabi* 2021-22)	**32**
3.2	HPLC system used for herbicide extraction	**41**
	RESULTS AND DISCUSSION	
4.1	Impact of imazethapyr application on berseem green fodder yield	**51**
4.2	Impact of imazethapyr application on soil bacterial population	**56**
4.3	Impact of imazethapyr application on soil fungal population	**56**
4.4	Impact of imazethapyr application on soil enzyme activity (FDA hydrolysis)	**57**
4.5	Impact of imazethapyr application on soil enzyme activity (dehydrogenase activity- DHA)	**58**
4.6	Impact of imazethapyr application on soil microbial biomass carbon	**59**
4.7	Screening bacterial isolates for imazethapyr tolerance	**61**
4.8	Screening bacterial isolates for imazethapyr tolerance	**62**
4.9	Biodegradation of imazethapyr in water *in vitro* using microorganisms	**69**
4.10a	Biodegradation of imazethapyr in different soils	**70**

4.10b	Biodegradation of imazethapyr in different soils	**71**
4.11	Microbial load in the alginate beads bioformulation	**73**
4.12	Biodegradation of imazethapyr by alginate bead bioformulation	**74**
4.13a	Effect of contaminants concentration on imazethapyr bioremediation	**76**
4.13b	Effect of contaminants concentration on imazethapyr bioremediation	**76**

LIST OF PLATES

PLATES No.	Title	Page No.
4.1	Berseem crop at different study sites	**48**
4.2	Soil samples collected from study sites for analysis	**55**
4.3	Isolation of microorganisms from imazethapyr treated soils	**60**
4.4	Colony morphology of pure cultures of selected imazethapyr degrading isolates	**60**
4.5	Slant cultures of total bacterial and fungal isolates	**61**
4.6	Screening bacterial isolates for imazethapyr tolerance	**62**
4.7	Screening fungal isolates for imazethapyr tolerance	**63**
4.8	Testing the compatibility between selected isolates	**64**
4.9	Microscopic observation of cell / sporangiophore of selected imazethapyr degraders	**65**
4.10	Identification of bacterial isolate (B15) using BIOLOG id system	**65**
4.11	PCR amplification of ITS and 16S rRNA genes for identification of selected isolates	**66**
4.12	Preparation of alginate beads based bioformulations	**72**

ABBREVIATIONS

1.	%	Percent
2.	*et al.*	Co-workers
3.	WHO	World Health Organization
4.	DTE	Down to Earth
5.	USEPA	United States of Environmental Protection Agengy
6.	FAO	Food and Agriculture Organization
7.	HCH	β-Hexachlorocyclohexane
8.	DDT	Dichlorodiphenyltrichloroethane
9.	MT	Metric Ton
10.	DPPQS	The Directorate of Plant Protection, Quarantine, and Storage
11.	GOI	Government Of India
12.	DNA	Deoxyribonucleic acid
13.	PPDB	Pesticide Properties Database
14.	h	hour
15.	$mg\ l^{-1}$	milligrams per Liter
16.	$q\ ha^{-1}$	quintal/hectare
17.	DGGE	Denaturing Gradient Gel Electrophoresis
18.	ITS	Internal transcribed spacer

19.	UNEP	United Nations Environment Programme
20.	OECD	Organisation for Economic Co-operation and Development
21.	SIB	Social Impact Bond
22.	*UNIDO*	United Nations Industrial Development Organization
23.	PCR	Polymerase chain reaction
24.	2, 4-D	2,4-Dichlorophenoxyacetic acid
25.	i.e.	that is
26.	$mg\ kg^{-1}$	milligrams per kilogram
27.	°C	degree Celsius
28.	HPLC	High-performance liquid chromatography
29.	≥	Greater than or equal to
30.	$mg\ l^{-1}$	Milligrams per Liter
31.	GC-MS	Gas chromatography–mass spectrometry
32.	$mmol^{-1}$	Millimolar per mole
33.	sp.	species
34.	IMZT	Imazethapyr
35.	$\mu g\ ml^{-1}$	Micrograms per Milliliters
36.	>	Greater than
37.	$g\ l^{-1}$	Gram per liter

38.	ml ha^{-1}	Milliliter per hectare
39.	kPa	Kilopascal
40.	UV	Ultraviolet
41.	mM	Milli meter
42.	N	Nitrogen
43.	C	Carbon
44.	w/w	weight for weight
45.	HA	Humic acid
46.	g ha^{-1}	Gram per hectare
47.	$T_{1/2}$	Half life
48.	nm	Nanometer
49.	µM	Micrometer
50.	cells g^{-1}	Cells per gram
51.	MSM	Mineral salt medium
52.	lbs/sq.in.	pounds/square inch
53.	SL	Soluble liquid
54.	ppm	Parts per million
55.	cm	Centimeter
56.	mm	millimeter
57.	km h^{-1}	kilometre per hour
58.	RH	Relative humidity

59.	Mg m^{-3}	Milligram per cubic meter
60.	kg ha^{-1}	Kilogram per hectare
61.	g	Gram
62.	OD	Optical density
63.	ABS	Absorbance
64.	TFC	Total fungal count
65.	TBC	Total bacterial count
66.	µg/gm/day	Microgram per gram per day
67.	v/v	volume per volume
68.	BOD	Biochemical oxygen demand
69.	QuEChERS	Quick Easy Cheap Effective Rugged Safe
70.	rpm	Round per minute
71.	SMW	Standard meteorological week
72.	CFU	Colony forming unit
73.	Fig.	Figure
74.	dS/m	Deci siemen per metre
75.	BD	Bulk density
76.	EC	Electrical Conductivity
77.	OC	Organic Carbon
78.	DHA	Dehydrogenase enzyme activity
79.	FDA	Fluorescein diacetate

80.	SMBC	Soil microbial biomass carbon
81.	t ha^{-1}	tonnes per hectare
82.	g a.i ha^{-1}	Gram active ingredient per hectare
83.	kg a.i. ha^{-1}	Kilogram active ingredient per hectare
84.	WAS	weeks after sowing
85.	μg g^{-1}	Micrograms per gram
86.	g/plant	Gram per plant

1. INTRODUCTION

Agriculture is always trying to maximize production and productivity of crops to fulfill the food demands of growing populations. Indian agriculture is changing significantly since after independence due to the use of high-yielding cultivars, agrochemicals, and improved irrigation systems which increases the agricultural production dramatically. Modern eras of agricultural techniques are consuming a greater number of pesticides and fertilizers to achieve the target production of food materials. Chemical forms of pesticides are now used to control various pest infestations and minimize pest losses. Among the different formulations of pesticides dusting powder form is having maximum share about 85% followed by water-soluble dispersible powder 12% and emulsification concentrates 2%. However, farmers often use these pesticides because they are easy to apply, widely available and quickly effective. Due to improper pesticide selection, hurried crop harvesting, and excessive pesticide application, many of these pesticides used, have negative effects on the environment and human health (Sharma, 1994). Pesticides are responsible for 2,20,000 fatalities and 30,000 poisoning cases annually as per the World Health Organization reports and it is majorly observed in the developing countries like India (Rosenstock *et al*., 1991; Pimental, 1992; Kishi *et al*., 1995; World Resources Institue, 1998; Down to Earth, 2001). The Long-term and even in low-dose exposure of pesticides having the negative consequences on human health like including immune suppression, hormone disruption, lowered intellect, anomalies in reproduction, and cancer etc. (Gupta, 2004). Pesticides constitute any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest. They can also serve as plant regulators, defoliants, or desiccants (USEPA, 2014). Effective pest management is one of the ways to boost crop productivity because pest infestations account for more than 45% of food production losses each year. A farmer survey says that pesticide is constantly applied for more than five years and observed that more than 60% surveyed farmers do not wait for two weeks between pesticide application to harvesting the crops.

Herbicides are one of the components of pesticides which are used to kill the unwanted plants present in the crop field for enhancing the crop production. Whereas, herbicides increase the crop production in one side but on another side it causes soil and water contamination which poses serious human health risks (Kanissery and Sims, 2011).

Lasserre *et al*. (2009) reported that the atrazine (2-chloro, 4-ethylamino, 6-isopropylamino, 1, 3, 5-triazine) is an endocrine disruptor that affects the immunological system, central nervous system, and endocrine system and it is also been obliged make male frogs reverse their sex (Hayes *et al*., 2003). Continuous use of herbicides causes its residue compounds to be detected in the soil and associated systems, which function as a pollutant in soil and other systems and ultimately degrade soil quality, especially soil microorganisms are influenced and soil physico-chemical characteristics are also altered. Heavy and improper application of pesticides may infiltrate in the food chain and contaminating the soil, air, water and other associated systems. The highest global market coverage of pesticides is majorly occupied by 42.48% herbicides followed by 25.57% insecticides, 24.19% fungicides, and 7.76% other types of pesticides. Globally, more than five lakh tonnes of unwanted and obsolete pesticides are harming today in the environment and public health as reported by the Food and Agriculture Organization (FAO) inventory and over the past one decade an increase in public awareness about pesticide residue and their side effect. India is currently struggling with the after effects of widely used chemical fertilizers and pesticides like HCH, DDT, endosulfan and phorate etc and herbicides use is expected to increase by 15-20% per annum in future (Duhan *et al*., 2019).

Imazethapyr [5-ethyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl) pyridine-3-carboxylic acid] is an imidazole compound used as a selective herbicide for soybean and other pulse crops. Locally, the imazethapyr is available in the market namely as Contour, Hammer, Overtop, Passport, Pivot, Pursuit, Pursuit Plus, and Resolve. Total consumption of imazethapyr was 61.07 MT in 2016-17 which is increased up to 74.00 MT in 2020-21(DPPQS, Govt. of India). It is applied as pre-plant incorporation, pre-emergence, and post-emergence to control the broad-leaved weeds and grasses including barnyard grass, crabgrass, cocklebur, pigweeds and foxtail millets. The compound controls weed by reducing the levels of three branched chain aliphatic amino acids, isoleucine, leucine and valine through the inhibition of aceto-hydroxy acid synthase. This inhibition causes a disruption in protein synthesis which leads to interference in DNA synthesis, cell growth and eventually to weed death (Breccia *et al*., 2013; Zhou *et al*., 2010). Among the applied pesticides less than 1% of pesticides reach the target weeds or other pests and remaining quantity is lost to the surrounding environments *via* spray drift, off-target deposition, run-off, and photodegradation which can have undesirable effects on some species,

communities and ecosystems which ultimately impact negatively on human health (Hernandez *et al*., 2013). This herbicide was reported to induce nuclear abnormalities such as blebbed and notched nuclei, when tadpoles were exposed for 96 h. The exposure level 0.39-1.17 mg l^{-1} increased the genetic damage index chances in tadpoles lasting for both 48 and 96 h, respectively (Perez-Iglesias *et al.*, 2015).

Berseem (*Trifolium alexandrinum L.*) known as king of the fodder crops, is one of the most important winter season leguminous fodder crop in North, North-West and central parts of India. India has the greatest area under berseem cultivation after Egypt and Pakistan (Muhammad *et al*., 2014). It is grown on over 2 million hectares of land in India (Pandey and Roy, 2011). Due to its excellent and quick re-growing ability and long durational nutritious green fodder availability (November to April), the crop is grown under irrigated condition. The berseem crop, grown under irrigation, gives 800–850 q ha^{-1} of highly palatable, succulent, and nutrient-rich green fodder yield in 5–6 cuts. Berseem fodder consists of 20% crude protein, 62% total minerals that are digestible, and 65% digestibility. It is well known that feeding dairy animals with green forage stimulates and improves milk production.

Because of its slow growth in the initial stages, yield reduction in the crop on account of weeds is well documented. Weeds particularly *Cichorium intybus* found associated with berseem and give more computational stress by robbing the crop of essential nutrients, light, moisture and space (Thakur *et al*., 1990). Weed competition substantially reduces the green forage yield and consequently, it causes reduction up to 30-40% besides deteriorating quality of green forage, if not controlled during critical period of crop-weed competition.

Pesticides can convert into simple compound by using a different method, including chemical deterioration, photolysis, volatilization, leaching, runoff, and microbial degradation. The improper handling and disposal of pesticides and their wastes may possible source of contamination in the surrounding environment. Imazethapyr is persistent with having half-life of 7 to 513 days (O'Sullivan *et al.*, 1998; Punia *et al*., 2011; Grichar *et al*., 2012) and has diferential sensitivity to successive sensitive crops such as wheat, spinach, mustard, canola, barley, pea and sugarbeet (Alister and Kogan, 2005). The soil pH, organic matter, soil moisture, soil type and clay texture in soil affect the adsorption and

persistent of imazethapyr in soil system. However, soil with less pH, high organic matter and high clay content increases the persistence of herbicide through decreasing their bioavailability. Various techniques used for the removal of herbicide from soil *i.e.* soil flushing, washing, extraction, bioremediation and phytoremediation. Herbicides may also interfere with biochemical processes of plants and affect the plants via seed germination, seedling development and growth of the plants. The factors like solubility in water, organic carbon coefficient, octanol- water partition coefficient, vapour pressure and biological transformations control the fate of applied herbicides in soil.

Bioremediation is a technique where pollutants can be degraded from the soil by using certain microorganism(s) which is cheap and effective way to remediate pesticide from soil and water system. Aerobic condition of soil promotes biodegradation process as compared to anaerobic conditions. Soil microorganism(s) *viz.*, fungi, bacteria, algae and protozoa are responsible to degrade the pesticides in soil and water. Many factors affecting the pesticides degradation in soil are structure of pesticides, concentration of pesticides, soil types, soil moisture, soil temperature, soil pH, soil salinity, soil organic matter, dissipation of pesticides and soil biotic components etc.

Microorganisms play pivotal role in the degradation of different herbicides and the isolation and identification of degrading microorganism is important for the development of suitable inoculants for the bioremediation. Only a few numbers of species have been identified as imazethapyr-degrading microorganisms. The characteristics of microbial associations in the contaminated site can be studied with the aid of denaturing gradients gel electrophoresis, bacterial pyrosequencing of 16S rRNA gene and fungal ITS regions.

A little information is available in the literature about the imazethapyr degradation by microorganism. Therefore, present investigation was conducted to evaluate the persistence of imazethapyr in soil and water system of berseem crop and imazethapyr degrading microorganism, isolations and identification from the imazethapyr contaminated site is undertaken with the following objectives:

1. Assessment of persistence of imazethapyr in Bundelkhand soils under berseem
2. Isolation and characterization of imazethapyr degrading microorganisms from soil of berseem crop
3. Bioremediation of imazethapyr using selected microorganisms in soil/water *in vitro*
4. Formulation of microbial consortium for remediation of imazethapyr

2. REVIEW OF LITERATURE

Agriculture is an important sector wherein 60% of the Indian population depends for their source of income and most of them belong to rural background. During cultivation of crops, the crop can be damaged by insects, fungi, bacteria, nematodes, weeds, and/or other pests and reduce crop production which harm agricultural enterprise. Herbicides constitute about 16% of the total pesticides used in Indian agriculture. Application of herbicides removes the unwanted plants from the cultivated field and helps to increase crop production. However, indiscriminate use of herbicides results in variety of negative effects on the agriculture and associated environments. Herbicide residues remain in the soil and water system for longer periods which are ultimately consumed by the human beings through different food sources.

Bioremediation is an effective measure to reduce the contaminant from the contaminated sites by using certain microorganisms. The bioremediation is the process of converting environmental pollutants into less hazardous forms using living organisms, primarily microorganisms like bacteria. It uses plants, naturally existing bacteria, and fungi to break down or detoxify pollutants that are harmful to the environment or to human health. By using natural biological activity that can remove or render harmless a variety of pollutants, through low-tech, low-cost methods that are typically well-liked and accepted by the general population and it may be used on the spot remediation. The literature relevant to the present study on "Bioremediation of imazethapyr in soil/water system" has been reviewed and presented in this chapter. An attempt has been made to cite as much literature as possible on microbial degradation of imazethapyr. However, due to paucity of adequate experimental evidences, the similar work on the other pesticides has also been reviewed wherever felt necessary. The literature pertaining to the pesticides contaminations and their bioremediations are highlighted in this chapter within the following heads:

2.1. Isolation and characterization of pesticides degrading microorganisms from contaminated soil

Smith and Adkins (1996) isolated six non-fermentative gram-negative bacilli by enrichment with diclofopmethyl from soils in Manitoba and identified them as *Sphingomonas paucimobilis, Acinetobacter baumannii, Chryseomonas luteola, Pseudomonas aureofaciens, Pseudomonas cepacia*, and *Pseudomonas fluorescens* by

microscopic examination, physiological and biochemical tests. All isolates could flourish in a minimum medium containing diclofop-methyl as a source of carbon and energy and could use diclofop-methyl (1.5 µg ml^{-1}) in 31 h of incubation at 25°C.

Digvijaya *et al*. (2017) collected soil samples, sieved, dried and then inoculated into MSM media suspended with imazethapyr and incubated for seven days and thus isolated imazethapyr degrading bacteria from six horticultural farms of Jalandhar and Phagwara region of Punjab.

Hernández *et al.* (2008) isolated a bacterium designated as *Pseudomonas* sp. strain MHP41, capable of degrading simazine and atrazine, from agricultural soil in the Quillota valley, central Chile. Strain MHP41 was able to grow in minimal medium, using simazine as the sole nitrogen source.

Ding *et al*. (2008) isolated one strain of imazethapyr degradable bacteria from the mixture of imazethapyr production factory's sludge and the soil that was contaminated with imazethapyr for a long time. The strain could degrade imazethapyr more than 90% within 72 h when it grew in 500 mg l^{-1} imazethapyr condition. After characterizing by physiological and biochemical properties as well as 16S rRNA sequence analysis, the strain was related and shared characteristics of the genus *Alcaligenes* sp.

Topp *et al*. (2000) isolated nine gram-positive bacterial strains from maize production area contaminated with groundwater of atrazine in four farms in central Canada, which could utilize this pesticide as their only supply of nitrogen. They characterized those strains by using genomic fingerprinting and repetitive extragenic palindromic (replication)-PCR method with ERIC and BOXA1R primers and the strains were split into two groups of *Nocardioides* sp. using 16S ribosomal DNA sequence analysis.

A *Pseudomonas aeruginosa* was isolated by Fernandes *et al*. (2014) from a preservation soil area of the Brazilian Amazon Forest, without usage of any pesticide and it was evaluated for atrazine mineralization and demonstrated capacity to use atrazine as a nitrogen source, having achieved a reduction of 44 % of the initial concentration of atrazine after 24 h.

The chlorimuron-ethyl-degrading bacterium LW3 was isolated from contaminated soil Ma *et al*. (2009) and was identified by 16S rRNA gene sequencing as *Pseudomonas* sp. When

chlorimuron-ethyl was provided as the sole nitrogen source, the degradation efficiency in liquid medium was about 81.0% after 7 days of inoculation with strain LW3.

Lü *et al*. (2017) isolated the strain IM9603 degrading herbicide imazethapyr, and optimize the degradation condition. IM9603 was identified as *Brevibacterium* sp. based on morphological, physio-biochemical characteristics, and analysis of 16S rDNA.

Musarrat *et al*. (2000) reported that the two bacterial isolates (*NJ 10* and *NJ 15*) isolated through enrichment culture could break down the herbicide 2, 4-Dichlorophenoxyacetic acid (2, 4-D) in agricultural soil. The isolates demonstrated significant growth on mineral salt medium when 2, 4-D was the only source of carbon and energy. The isolates *NJ 10* and *NJ 15* were identified as *Pseudomonas* species and *Pseudomonas aeruginosa*, respectively based on their morphological, cultural, and biochemical traits.

Rousseaux *et al*. (2001) reported that 8 out of 12 soils breakdown radiolabelled atrazine in laboratory incubations more quickly and 25 bacterial strains were isolated from 10 of these soils for atrazine degradation by enrichment technique. The isolates were identified as Gram-positive (*Arthrobacter crystallopoietes*) as well as Gram-negative species (*Chelatobacter heintzii, Aminobacter aminovorans*, and *Stenotrophomonas maltophilia*) by sequencing and aligning the 16S rDNA genes.

Sorensen *et al*. (2003) found that phenylurea herbicide isoproturon which is also known as 3-(4-isopropylphenyl)-1, 1-dimethylurea (IPU) was metabolized by soil a bacterium (strain SRS2) that was previously isolated from IPU-treated agricultural soil. The strain was identified as *Sphingomonas* sp. in the proteobacteria-subdivision by the rRNA gene and cellular fatty acid analyses. When given a source of carbon, nitrogen, and energy to strain *SRS2* was able to mineralize IPU as well as diuron and chlorotoluron herbicides.

Sette *et al*. (2005) reported that six of the 53 actinomycetes isolated from soil treated with alachlor by utilizing selective conditions were able to grow and breakdown ≥ 50% alachlor (72 mg l^{-1}) in mineral salts medium *in vitro*, demonstrating their tolerance to high doses of the herbicide up to 720 mg l^{-1}. The bacteria that degrade alachlor were identified as the genus *Streptomyces* according to morphological and phylogenetic studies. While strains LS143 and LS153 were related to *Streptomyces bikiniensis*, strain LS151 was related to the type strains of *Streptomyces capoamus / Streptomyces galbus*. The remaining strains, LS166, LS177, and LS182, shared many morphological traits and they were located in a

same cluster based on 16S rDNA sequence analysis. However, the genetic fingerprint data rep-PCR revealed that they were distinct from one another.

Kuklinsky *et al*. (2005) studied endophytic bacterial communities from soybean (*Glycine max*) plants grown in soil treated with and without glyphosate as preplanting applications and found that some microbial species had the ability to utilize glyphosate as a source of energy and nutrients although this herbicide might be hazardous to other groups, had created a shift in the bacterial balance in the plant-endophyte relationship. The *Acinetobacter calcoaceticus, A. junii, Burkholderia* sp*., B. gladioli, Enterobacter sakazaki, Klebsiella pneumoniae, Pseudomonas oryzihabitans, P. straminea, Ralstonia pickettii*, and *Sphingomonas* sp. were found among the cultivable endophytic bacteria in the soybean leaves, stems, and roots. Denaturing Gradient Gel Electrophoresis (DGGE) analysis of soybean roots revealed some groups not seen by isolation that were exclusive for plants grown in soil treated with glyphosate prior to planting such as *Herbaspirillum* sp., and other groups in plants grown in soil devoid of glyphosate such as *Xanthomonas* sp. and *Stenotrophomonas maltophilia*.

Acetochlor-degrading bacterial strain LCa*2,* identified as *Pseudomonas oleovorans* was isolated from acetochlor-contaminated soil by Xu *et al*. (2006) had 35°C and 8.0, respectively as optimum temperature and pH for the best growth. After 7 days of incubation, the strain was able to decompose 98.03% of acetochlor given at a concentration of 7.6 mg l^{-1} and could tolerate 200 mg l^{-1} of acetochlor and degradation cycle lengthened as the acetochlor content increased.

Harada *et al*. (2006) identified three strains namely *Nocardioides* sp. MFC-A, *Rhodococcus rhodochrous* MFC-B, and *Stenotrophomonas* sp. MFC-C which were found to be microorganisms capable of co-metabolically breaking down mefenacet (2-benzothiazol-2-yloxy-N methylacetanilide). The mefenacet can be broken down by a number of species from the genera *Nocardioides, Rhodococcus*, and *Stenotrophomonas*, indicating that the ability was not exclusive to these isolates but rather widespread at least in these genera. They discovered the metabolites *viz*., N-methylaniline and 2-benzothiazoloxy acetic acid by using liquid chromatography-mass spectrometry were. The isolates were also capable of breaking down flutolanil, mepronil, metolachlor, and pretilachlor in addition to mefenacet.

Durand *et al*. (2006) isolated bacterium from cloud water and identified based on 16S rRNA gene alignment as *Bacillus* genus and studied the kinetics of mesotrione degradation by using high-performance liquid chromatography and in situ ^{1}H nuclear magnetic resonance spectroscopy at various doses at a concentration of 5 $mmol^{-1}$ where mesotrione underwent complete biotransformation and reported the first rapid mesotrione biotransformation by a pure bacterial strain.

Two bacterial strains, *Pseudomonas* sp. and *Stenotrophomonas* sp. (IB78 and IB93), were isolated and demonstrated to degrade diuron in pure resting cells in a first-order kinetic response during the first 24 h of incubation by Batisson *et al*. (2007).

Six gram-positive bacteria belong to the genera *Nocardioides* and *Arthrobacter* having atrazine degradation potential were identified by Vibber *et al*. (2007). It was observed that when given glucose as a different carbon source along with atrazine all six isolates were able to use it as their only nitrogen source. None of the isolates could utilize atrazine as the exclusive source of carbon and nitrogen under the culture conditions that were employed.

Vaishampayan *et al*. (2007) isolated *Arthrobacter* sp. strain uses only atrazine as a nitrogen source and it also breaks down additional triazines, including ametryn, cyanizine, propazine, and simazine. This research establishes the existence of an atrazine-degrading microbial population in Indian soils which may be effectively applied to the cleanup of contaminated soils.

Rani *et al*. (2008) isolated chlorpyrifos degrading bacteria in a selective enrichment medium and identified as *Providencia stuartii* based on biochemical traits and 16S rRNA sequence analysis. The *P. stuartii* strain MS09 was grown in Luria-Bertani broth with chlorpyrifos doses ranging from 50 to 700 mg l^{-1} and was found that 50 to 200 mg l^{-1} of chlorpyrifos was ideal quantity that maintained bacterial growth throughout a 24-hour period. At a low dose of chlorpyrifos (50 mg l^{-1}) compared to the control and a significant increase in bacterial growth was detected. While, at higher concentrations (300–700 mg l^{-1}) an increased lag phase was seen without suppressing the development of the pesticide–using bacterium.

Huo *et al.* (2011) isolated an actinomyces strain (S181) from the samples of soil where imazethapyr was applied for a long-term by use of bottle enriched culture. The strain had strong ability to degrade imazethapyr and could grow using mazethapyr as the sole nitrogen. The strain was related and shared characteristics to genus *Streptomyces omiyaensis* according to the physiological and biochemical properties as well as 16S rRNA sequence analysis.

A simazine and atrazine-degrading bacteria strain MHP41 was isolated by Hernandez *et al.* (2008) from agricultural soil in the Quillota valley, central Chile. The strain could grow in media with simazine as its only nitrogen source. It was motile gram-negative bacterium, identified as a *Pseudomonas* sp. based on comparative sequence analysis of the 16S rRNA gene sequences.

Batisson *et al*. (2009) revealed that the soil microflora were able to biotransform mesotrione, a novel triketone herbicide, when it was applied in soil according to the dissipation kinetics of the herbicide. They isolated mesotrione degrading bacteria in mineral salt solutions that included mesotrione as the only source of carbon and identified as *Bacillus* sp. and *Arthrobacter* sp. *Bacillus* sp. was more capable of biotransforming mesotrione.

Sun *et al*. (2009) identified three bacterial strains isolated from soils, that can break down the herbicide isoproturon, 3-(4-isopropylphenyl)-1, 1-dimethylurea as *Sphingobium* sp. based on the comparative examination of the 16S rRNA gene, phenotypic analysis, and biochemical characterization. These strains performed best at pH 7.0 and 30°C for the breakdown of isoproturon. They also demonstrated the ability to eliminate additional phenylurea herbicides such as chlorotoluron, diuron, and fluometuron residues.

Huang *et al*. (2009) isolated imazethapyr (IMZT)-degrading bacterium IM-4 from the IMZT-contaminated soil and identified it as *Pseudomonas* sp. based on its physiological traits, biochemical tests, and phylogenetic analysis of the 16S rRNA gene. This strain could use IMZT as the only source of carbon and energy. After 7 days of inoculation with strain IM-4 approximately 73.4 % of the 50 mg l^{-1} of IMZT was broken down. Additionally, this strain also demonstrated the ability to break down the imidazolinone herbicides imazapyr, imazapic, and imazamox. It has been observed that whether the soil

was sterilized or not, the inoculation strain IM-4 to IMZT-treated soil led to a faster degradation rate than in non-inoculated soil.

Murugesan *et al*. (2010) studied the capacity of five bacterial isolates (*Pseudomonas aeruginosa, Klebsiella* sp.*, Escherichia coli, Bacillus* sp., and *Corynebacterium*) to degrade cypermethrin. *P. aeruginosa, Klebsiella* sp. and *E. coli* were very actively in using cypermethrin (1%) and *Bacillus* sp. and *Corynebacterium* were only moderately active (0.1%).

A bacterial strain that can mineralize isoproturon (IPU) was isolated by Hussain *et al*. (2011) by using enrichment cultures from soil and was identified as *Sphingomonas* sp. strain *SH* after 16S rRNA sequence. The pH had a significant impact on strain *SH's* ability to degrade IPUs and pH 7.5 being the most conducive to degradation. In contrast to other structurally related phenylurea herbicides including diuron, linuron, monolinuron, and chlorotoluron as well as their aniline derivatives, SH was only able to mineralize IPU and its recognized metabolites including 4-isopropylaniline.

Romdhane *et al*. (2016) isolated a bacterial strain that can utilize the herbicide, sulcotrione (β-triketone) as source of carbon and energy from soil sample. The isolate was identified as *Bradyrhizobium* sp. SR1 after a phylogenetic analysis using the 16S rRNA gene sequence analysis.

Besides many reports on degradation by bacteria, fungal systems can also effectively degrade paraquat. *Lipomyces starkeyi* Lod and Rij completely removed paraquat (2 mg l^{-1}) from the medium within 3 days. However, when the paraquat concentration was increased two fold (54 mg l^{-1}), biomass and paraquat degradation notably decreased to less than 10% (Alexander, 1999). It was noticed that *L. starkeyi* could degrade paraquat under aerobic conditions.

Microbial degradation of ^{14}C-imazaquin and ^{14}C-imazethapyr was monitored by Flint and Witt, (1997) by measuring $^{14}CO_2$ evolution compared to nonsterile soil. It was found that $^{14}CO_2$ evolution was greatest from carboxyl-labeled imazaquin and imazethapyr compared to ring-labeled imazaquin and imazethapyr. A corn root bioassay indicated nearly complete loss of herbicidal activity in nonsterile soil after 5 months, but herbicide activity was reduced only 14% in sterile soil. $^{14}CO_2$ evolution more than doubled when soil temperature

increased from 15 to 30° C. Total CO_2 production responded similarly. Degradation of imazaquin and imazethapyr increased as soil moisture increased from 15% (-2.4 MPa) to 75% of field capacity (-0.03 MPa).

Sharma *et al*. (2012) reported that *Aspergillus niger* survived in minimal broth containing chlorimuron at 2 mg ml^{-1}. *Aspergillus niger* degraded the herbicide to harvest energy through two major routes of degradation, while fungi such as *Fusarium* and *Alternaria* are unable to survive in artificial media containing chlorimuron-ethyl at 25 mg l^{-1}.

Penicillium chrysogenum and *Aspergillus niger* were isolated and identified from rhizospere soil of rice field, as potent pyrazosulfuron-ethyl degrading fungi by Sondhia *et al.* (2013). The rate of dissipation of pyrazosulfuron-ethyl was found higher in soil of rice field and soil inoculated with *P. chrysogenum*. This showed important route of degradation of pyrazosulfuron-ethyl by microbes apart from chemical degradation.

A large number of fungal species were found to be efficient to degrade phenylurea herbicides such as *Rhizoctonia solani*, *Bjerkandera adusta* and *Aspergillus* sp. (Sondhia *et al*., 2013). Rønhede *et al*. (2005) demonstrated effective degradation of isoproturon by *Alternaria* species.

Eman *et al*. (2013) isolated forty-five fungal isolates from eleven cultivated soil farms (Riyadh and Karj area, KSA) after enrichment with Mineral Salt Media (MSM) supplemented with the herbicide glyphosate. Certain fungal strains were tolerated to the herbicide up to 10,000 ppm where the growth inhibition reached up 47.9% in certain isolate. 800 ppm of glyphosate almost was degraded and metabolized in liquid Czapek Dox broth medium containing 1% sucrose by certain fungi *viz*. *A. flavus* WDCZ2 (99.6%) and *P. spiculisporus* ASP5 (95.7%) followed by *P. verruculosum* WGP1 (90.8%).

Sondhia *et al.* (2016) reported for the first time the biodegradation of penoxsulam by *Aspergillus* sp. of fungi. Penoxsulam was degraded at a faster rate in the soil inoculated with *A. flavus* than *A. niger*. Degradation kinetics showed that degradation of penoxsulam in the soil was followed by formation of several degradation products/metabolites by both the fungi. These results showed that *Aspergillus flavus* and *Aspergillus niger* can enhance microbial degradation of penoxsulam in the soil.

Camacho-Morales *et al*. (2017) isolated 54 macromycetes from southeastern Mexico, and only three (*Trametes pavonia* ECS-67, *Trametes versicolor* ECS-79, and *H. dispersum* ECS-705) presented 54.2, 54.1, and 70.7% of paraquat (100 mg l^{-1}) degradation within 12 days.

Sondhia and Waseem (2019) reported that pyrazosulfuron-ethyl was efficiently degraded by *Alternaria alternata* in the soil. Degradation of pyrazosulfuron-ethyl by *A. alternata* was achieved by the cleavage of Sulfonylurea Bridge and hydrolysis. This efficient *A. alternata* can effectively be used for the enhanced degradation of pyrazosulfuron-ethyl in agricultural soil or mixed with other microbial consortia for rapid degradation with half life of 7.9 days.

2.2. Bioremediation of pesticides/imazethapyr and formulation of microbial consortium

Cantwell *et al*. (1989) investigated abiotic vs. biotic degradation of ^{14}C-imazaquin and ^{14}C-imazethapyr in two soils, a Cisne silt loam and a Drummer silty clay loam. Herbicide degradation in gamma-irradiated soil was compared to fresh soil. After 12 weeks of incubation, 95% of the radioactivity could be extracted as parent from sterilized soil. In unsterilized soil, imazaquin and imazethapyr degraded at a similar rate which was dependent upon soil type. All herbicides degraded slower in the Drummer soil and triallate degraded two to three times faster than the imidazolinones in either soil. ^{14}C-imazaquin degradation products included $^{14}CO_2$ and unextractable residues. The major product from ^{14}C-imazethapyr degradation was $^{14}CO_2$.

A bacterium for degrading herbicide imazethapyr was developed as a carrier based formulation for application in bioremediation of imazethapyr in soil. The bacterium for degrading imazethapyr is *Methylobacterium* sp. PST1991, which is preserved in China General Microbiological Culture Collection Center by a preservation number of CGMCC No.7773. The *Methylobacterium* sp. PST1991 CGMCC No.7773 reaches a degrading rate of 71.06% on 8 mg l^{-1} of imazethapyr in an organic salt culture medium within 7 days, showing that the strain can effectively degrade the imazethapyr; the bacterium has a wide application prospect in an aspect of repairing imazethapyr pollution of soil (Patent No. CN103333834B, China, 2013).

Zayed *et al*. (1983) reported that under aerobic circumstances with the fungi, trifluralin was decomposed very quickly in a 10 days incubation period up to 9, 7, and 3% of the total ^{3}H-products produced by *Aspergillus earneus, Fusarium oxysporum* and *Trichoderma viride*, respectively.

Basham and Lavy (1987) tracked microbial breakdown of imazaquin 2 for 7 months in a controlled laboratory environment by detecting $^{14}CO_2$ evolution. In a Crowley silt loam up to 10% of the imazaquin with a ^{14}C chain label developed as $^{14}CO_2$ in 7 months was observed. A soil called Sharkey silty clay which had more clay and organic matter than silt loam soils had less evolution of $^{14}CO_2$ than silt loam soils. A Crowley silt loam incubated for 8 months at 18°C and 35°C which lost 66 to 100% of the applied imazaquin, respectively and indicated that alterations in metabolism with addition to CO_2 evolution were also taking place. Whenever, soils were maintained at warm and wet conditions that support to microbial growth (35°C and -33 kPa) and imazaquin phytotoxicity was rapidly lost. While in it stored under cool and dry conditions (18°C and -100 kPa) imazaquin was more permanent.

Barua *et al*. (1990) identified numerous soil fungus including *Aspergillus flavus, Aspergillus terreus, Fusarium solani, Fusarium oxysporum, Penicillium citrinum,* and *P. simplicissimum* which efficiently broken down pendimethalin. It was found that all six fungal isolates could break down at least 60% of the pendimethalin in just 15 days. The fungal species of *P. citrinum* produced the maximum degradation among them (66%) followed by *F. solani* (64.5%). *F. solani* demonstrated extremely high efficiency in terms of pendimethalin usage as it generated the highest cell dry matter yield.

Assaf and Turco (1994) identified a mixed enrichment culture of microorganisms that mineralized atrazine rapidly. In the presence of simple carbon sources, microbial consortium's liquid cultures mineralized 96% of the administered atrazine (0.56 mM) in just 7 days. Atrazine was entirely converted in 25 days in soil that had been exposed to the microbial consortia and atrazine at a concentration of 0.14 mM (concentration is based on total soil mass). About 60% of the sprayed atrazine was identified as $^{14}CO_2$ after 30 days of incubation. A total of 86% of the administered atrazine was accounted for as $^{14}CO_2$ after 145 days and soil extractable hydroxyatrazine was decreased to up to zero. They found that use of the mixed microbial culture increased mineralization by a factor of more than 20 than uninoculated soil.

Gan *et al*. (1996) recorded persistence of atrazine was higher at high concentration in both field and laboratory trials in the sandy loam from field and laboratory experiments conducted in two soil type in Webster clay loam (fine loamy, mixed, mesic typic Haplaquoll) and Estherville sandy loam (sandy, mixed, mesic typic Hapludoll). Atrazine was dissipated through mineralization at all concentrations in clay loam soil and at concentrations of 5 to 500 mg kg^{-1} in sandy loam. They proposed that the N or C from the s-triazine ring could be utilized by soil microorganisms. Based on the rise in soil respiration in the clay loam soil atrazine at 500 and 5000 mg kg^{-1} may have accelerated atrazine breakdown by increasing soil microbial proliferation and activity.

Moorman *et al*. (2001) studied on the effects of compost, corn stalks, corn fermentation byproducts, peat, manure, and sawdust at rates of 0.5 and 5% (w/w) on the biodegradation of soil additives like atrazine, metolachlor, and trifluralin. Atrazine is a mixture of the chemicals 2, 6-dinitro-N, N-dipropyl-4-(trifluoromethyl) benzenamine, and atrazine, metolachlor, and trifluralin had initial concentrations of 175±42 mg atrazine, 182±25 mg metolachlor, and 165±23 mg trifluralin kg^{-1} of soil. Compared to non-supplemented soils the soils amended with 0.5% manure, 5% peat, and 5% corn stalks was degraded atrazine more quickly and addition of compost, manure, and cornstalks at rate of 5% showed significantly increased the bacterial populations and dehydrogenase activity.

Zanardini *et al*. (2002) evaluated two microbial consortia that could grow on the two sulfonylureas, chlorsulfuron and metsulfuronmethyl under controlled laboratory conditions on soil samples that had previously been treated with sulfonylurea herbicides. They reported that a *Pseudomonas fluorescens* strain (B2) was able to breakdown roughly 21% of metsulfuron-methyl and 32% of chlorsulfuron from these mixed cultures under co-metabolic conditions with a rich medium in two weeks.

Sorenson *et al*. (2001) investigated in recent years seen an increase in interest on the environmental fate of phenylurea herbicides as a result of growing concern over the effects of water contamination on the environment and public health. In agricultural soils improved biodegradation of isoproturon (IPU) may occur with further field treatments. However, there was still a possibility of significant in-field spatial variation in the IPU-metabolizing community's distribution. The microbial mechanisms behind this geographical heterogeneity have been clarified by thorough research using soils from the Deep Slade field in combination with culture-dependent and molecular techniques.

Sorenson *et al*. (2001) reported that isoproturon (IPU)-degrading microorganisms either proliferated slowly or not at all in areas with slow IPU biodegradation. This pattern was effectively related to isolate IPU-degrading *Sphingomonas* sp. strain investigations conducted in pure culture. These findings imply that areas with unfavorable growth circumstances occur even in agricultural fields where phenylurea herbicides have been widely applied and where phenylurea-metabolizing microorganisms have adapted.

Piutti *et al*. (2003) identified *Nocardioides* sp. SP12 an atrazine-degrading bacterium from bulk soil and maize rhizosphere treated with atrazine.

Pattanasupong *et al*. (2004) used microbial consortium obtained from paddy soil samples in Japan and this consortium was immobilized on the commercial support Fabios® (Unitika, Osaka) to break down the herbicide 2, 4-D and the fungicide carbendazim. In a glass column reactor with 20 µM carbendazim and 2 mM 2, 4-D as the only carbon sources, this consortium was acclimated. In batch culture, this adapted consortium totally metabolized up to 100 µM carbendazim and 3 mM 2, 4-D in 36 and 24 h, respectively, while a lag period was noticed after pre-cultivation in a rich medium. Contrary to using free cells, the consortium ability to degrade was improved by being immobilized on a polyester support. Results suggested that the immobilized consortium on loofa sponge is a promising material for bioremediation of polluted water with these pesticides in paddy fields.

Torra-Reventos *et al*. (2004) isolated and identified a filamentous fungus able to breakdown the herbicide thiobencarb as *Aspergillus niger* van Tieghem (CRN) at the Centraal bureau voor Schimmel cultures in the Netherlands. The factors pH, temperature, and agitation speed were studied to determine the ideal conditions for thiobencarb breakdown by the isolated strain. At pH 5.5 the thiobencarb degradation induction duration was shorter and lower pH levels, a larger rise in biomass was seen 0.83, 0.41, and 0.36 g l^{-1} at pH 3.5, 4.5, and 5.5, respectively, and 0.24 and 0.14 g l^{-1} at pH 6.5 and 7.5 at 48 h after cultivation began.

Singh *et al*. (2004) isolated one bacterial strain B-14 belong to Enterobacteriales order, which used chlorpyrifos as its sole source of carbon and phosphorus while mineralizing chlorpyrifos under various growing conditions. The isolate was discovered to have phosphotriesterase, mono and diphosphatase activities. The initial rate of chlorpyrifos

breakdown was slowed down by the addition of other carbon sources (glucose and succinate). However, carbon and phosphorus added and the isolate was destroyed the DETP-containing organophosphates parathion, diazinon, coumaphos, and isazofos but not fenamiphos, fonofos, ethoprop, and cadusafos was observed. A faster rate of breakdown was seen when strain B-14 (10^6 cells g^{-1}) was added to soil that had a low native population of bacteria that break down chlorpyrifos and was treated with 35 mg of chlorpyrifos kg^{-1}. These findings demonstrate the bacterium potential for application in the removal of tainted pesticide waste from the environment.

Ermakova *et al*. (2010) evaluated the bioremediation effectiveness of glyphosate-contaminated Soddy podzol soil based on the outcomes of laboratory and field studies. Aerobic breakdown of glyphosate was carried out using bacterial strains *Achromobacter* sp. Kg 16 (VKM B-2534D) and *Ochrobactrum anthropi* GPK 3 (VKM B-2554D). They showed that high survivability in soil containing ten times the amount of glyphosate that is advised for a single in situ weed treatment. When compared to the local soil microbial community, the strains provided a two- to three-fold higher rate of glyphosate breakdown. Glyphosate content of the treated soil reduced and overall toxicity and phytotoxicity returned to levels of uncontaminated soil within 1-2 weeks of the introduction of the strain. A greater than two-fold rise in the dehydrogenase activity of native soil microorganisms and their biomass (1.2-fold and 1.6-fold for saprotrophic bacteria and fungi, respectively) showed that the reduction in glyphosate content restored soil biological activity. The glyphosate-degrading microorganisms utilized in this investigation do not produce total toxicity or phytotoxicity and are not harmful to the mammals.

Seeger *et al*. (2010) reported that several bacteria can break down PCBs and s-triazines in both aerobic and anaerobic conditions. Anaerobic bacteria can transform higher chlorinated PCBs by reductive dehalogenation and aerobic bacteria can oxidize lower chlorinated biphenyls. Toxic substances like PCBs and s-triazines have the potential to disturb the endocrine system and lead to cancer.

Du *et al*. (2011) examined that the biodegradation of atrazine-contaminated soil by *Rhodobacter sphaeroides* strain W_{16} under simulation circumstances and also evaluated the influencing elements of the soil repair process. The findings indicated that introducing strain W_{16} can speed up atrazine breakdown in soil. The amount of W_{16} inoculation and atrazine degradation were positively associated. The strain W_{16} having capacity to degrade

atrazine was noticeably diminished when the inoculation amount was decreased to $1.2x10^5$ cfu g^{-1} soil. The strain W_{16} atrazine-degrading rate on day 15 was 96.86% and the ideal temperature for strain W_{16} to degrade atrazine in soil was 30°C. The rate of atrazine degradation in soil by W_{16} increased as soil moisture content was increased.

Belal *et al*. (2013) isolated one bacterial strain from soil previously treated with pendimethalin using enrichment technique which was identified using 16S rDNA sequence as *Pseudomonas putida* (E15). It was found that 7 and 30°C were the optimal pH and temperature for the growth of the pendimethalin dissipating strain, respectively. Pendimethalin which has a half-life of 5.46 days in mineral liquid medium was released using *P. putida.* After being treated with *P. putida* or compost, pendimethalin was not harmful and has not been found in the soil on cucumber plants. This study said that bioremediation using *P. putida* and compost was considered to be an efficient way to remove pendimethalin from soil.

Sagarkar *et al*. (2014) found atrazine-degrading consortia as an efficient pollution eliminator and improved bioremediation. In soil that had previously been exposed to atrazine about 90% atrazine degradation was seen in six days but soil without a history of atrazine use took 15 days to remove the same quantity of modified atrazine. Three different bacterial strains with various genetic atrazine degradation capacities made up the bacterial consortium. Atrazine and its intermediary like cyanuric acid levels were measured in order to track the bioremediation process. The atzA, atzB, atzD, trzN, and trzD genes from the atrazine breakdown pathway were measured in every mesocosm over 60 days. However, atzA, atzB, atzD, and 16S rDNA were amplified from *P. citronellolis* and the trzN gene was amplified from the standard culture of *Arthrobacter aurescens* TC1 (ATCC BAA-1386), while *Pseudomonas* sp. AK CAN1 was used to amplify the trzD gene. The results showed that communities altered quickly after inoculation but after one month there was no significant change in the profile of the microbial communities. They reported that atrazine bioremediation utilizing a microbial consortium might be successfully scaled up to pilot scale.

Yang *et al*. (2014) isolated a bacterial strain *Hansschlegelia* sp. CHL1 that can use chlorimuron-ethyl as its only source of carbon and energy to use in soil bioremediation. By keeping track of the copy counts of bacterial and fungal marker genes as well as N-cycling functional genes during the bioremediation procedure, native microbial populations and N-

cycling activity in the soil were also studied (nifH, amoA, nirS, and nirK). They found that soils inoculated with CHL1 could breakdown >95% of chlorimuron-ethyl in 45 days. A single immunization had lower remediation efficiency and a shorter survival period than two inoculations. The results concluded that CHL1 was useful for cleaning up chlorimuron-ethyl-contaminated soil.

Nousiainen *et al*. (2015) investigated those two boreal non-agricultural soils to evaluate the methods for bioremediation of atrazine pesticide that frequently contaminates groundwater in low quantities. Without bioremediation procedures atrazine was not mineralized in soil. Atrazine was mineralized to a maximum of 52% in molasses biostimulation treatment at 10°C despite no increase in the copy numbers of the degradation genes. The results of micro-autoradiographic analysis after radioactively labeled atrazine incubations showed that bioremediation techniques increased the relative proportion of active degraders from 0.3 to 1.9% of the overall bacterial population. These findings suggested that the atzA/trzN-atzB genes may not be the only ones that assist atrazine breakdown. About 76% of atrazine was mineralized at 30°C in a combined biostimulation treatment using citrate or molasses and augmentation with *Pseudomonas citronellolis* ADP or *Arthrobacter aurescens* strain TC1. The atrazine degradation gene numbers increased to 10^7 copies g^{-1} soil. Atrazine degradation genes atzA and atzB were found in clone libraries created from passive samplers in groundwater monitoring wells together with previously known phylogenetic groupings that included atrazine degraders. These findings demonstrate that bioremediation techniques can mineralize atrazine at moderate temperatures and low concentrations in the groundwater zone.

Kumar and Singh (2016) used an atrazine-degrading enrichment culture to study degradation of atrazine metabolites *viz.* hydroxyatrazine, deethylatrazine, and deisopropylatrazine in mineral salts medium and found that the enrichment culture was able to degrade only hydroxyatrazine and it was used as the sole source of carbon and nitrogen. Hydroxyatrazine degradation slowed down when sucrose and/or ammonium hydrogen phosphate were supplemented as the additional sources of carbon and nitrogen, respectively. The enrichment culture could degrade high concentrations of atrazine (up to 110 µg ml^{-1}) in mineral salts medium, and neutral pH was optimum for atrazine degradation. Further, except in an acidic soil, enrichment culture was able to degrade atrazine in three soil types having different physico-chemical properties. Raising the pH of

acidic soil to neutral or alkaline enabled the enrichment culture to degrade atrazine suggesting that acidic pH inhibited atrazine-degrading ability. They reported that the enrichment culture can be successfully utilized to achieve complete degradation of atrazine and its persistent metabolite hydroxyatrazine in the contaminated soil and water.

Andleeb *et al*. (2016) studied atrazine bioremediation at different soil pH (5, 7 and 9) and temperature (20, 30, and 40°C) along with sodium citrate, *Arthrobactor* sp. strain DNS10, sawdust, and animal manure. The results indicated that atrazine remediation was generally optimum at pH 7 and 30°C for all treatments with the exception of sodium citrate because soil treated with sawdust was not temperature dependent but that the remediation process was slower at pH 5. Atrazine remediation was only 34% in soil with no extra amendment but at optimal pH and temperature it was 75.17, 89, 74.17, and 76.83% in soil treated with sawdust, DNS10, sodium citrate, and animal dung, respectively.

Saha *et al*. (2016) conducted a field experiment to examine how two post-emergence herbicides (imazethapyr and quizalofop-p-ethyl) degraded and their effect on soil ecosystems when used at half the recommended rate (HRE), recommended rate (RE), and the double the recommended rate (DRE) during the kharif peanut harvest. Herbicides had no effect on the soil microbial activity at HRE but they had a considerable impact at RE and DRE, which affected activity of the microbes that metabolized fluorescein diacetate and biomass carbon. At DRE dehydrogenase activity was also decreased for a shorter time and alkaline phosphatase activity was alternated between promotion and inhibition. The herbicides increased soil NH_4^+ and NO_3^- nitrogen at the first (after 7 days) and final phases (after 30 days), respectively. Urease activity had been initially inhibited but after 30 days, it had restored to the control level. Imazethapyr residue dissipation followed first-order rate kinetics at HRE but best fit bi-exponential order rate kinetics at DRE and RE. Only up to a day after application, quizalofop-p-ethyl residues were discovered, indicating a fast conversion to active acid metabolites.

An atrazine degrading enrichment culture, a consortium of bacteria of genus *Bacillus* along with *Pseudomonas* and *Burkholderia*, was immobilized in sodium alginate and was used to study atrazine degradation in mineral salts medium (MSM), soil and wastewater effluent by Kumar *et al*. (2017). Sodium alginate immobilized consortium, when stored at room temperature (24 ± 5 °C), was effective in degrading atrazine in MSM up to 90 days of

storage and population counts decreased to 1/5th on 120 days. Comparison of atrazine degrading ability of the freely suspended enrichment culture and immobilized culture suggested that the immobilized culture took longer time for complete degradation of atrazine as a lag phase of 2 days was observed in the MSM inoculated with alginate immobilized culture. The free cells resulted in complete degradation of atrazine within 6 days, while immobilized cells took 10 days for 100% atrazine degradation. Further, immobilized cultures were able to degrade atrazine in soil and wastewater effluent. Alginate beads were stable and effective in degrading atrazine till 3rd transfer and disintegrated thereafter.

Villaverde *et al*. (2017) found that the diuron was contaminant in sediments, water, and soil and reported as mildly poisonous to aquatic invertebrates as well as animals and birds. The compound 3, 4-dichloroaniline was main biodegradation byproduct which was more hazardous than diuron and its having high persistent in the environment.

Villaverde *et al*. (2017) assessed the possible ability of a unique diuron-degrading microbial consortium (DMC) to mineralize diuron both in solution and soil with various characteristics. The consortium was evaluated in a soil solution with diuron as the only carbon source and was observed that more than 98.8% of the diuron initially introduced was mineralized in few days. Three diuron-degrading bacteria made up the consortium were *Arthrobacter sulfonivorans, Variovorax soli,* and *Advenella* sp. which were isolated from a highly contaminated industrial site. The potential ability of *Advenella* to clean up pesticide-contaminated soil was demonstrated for the first time. However, none of the three strains individually were able to mineralize (ring-^{14}C) diuron in a mineral salts medium (MSM) with a trace nutrient solution. While, combined in pairs they were able to mineralize 40% of the diuron in solution but the most significant result was obtained in the presence of the three-member consortium where complete diuron mineralization was achieved after only a few days.

Villaverde *et al*. (2018) evaluated five microbial consortia (C1-C5), which were isolated from various agricultural soils to degrade diuron. Consortium C3 was only able to mineralize 22.9% of the diuron in solution and consortium C2 was able to do so for 78.6% of it. In all cases, with the exception of consortium C4, DT50 (the time needed for the diuron concentration to fall to half of its initial value) was significantly shorter than in the

non-inoculated control falling from 700 days to 546, 351, and 171 days for consortia C5, C2, and C1, respectively.

Cao *et al*. (2018) revealed that the genus *Pennisetum* hasten the removal of atrazine from its rhizosphere. By using biolog ecoplates and high-throughput 16S rRNA gene sequencing identify *Pennisetum americanum (L.) K. Schum (P. americanum)* as the test plant and examined the interaction between *P. americanum* and atrazine-contaminated soil were also focusing on the adjustment of the soil biochemical properties as well as bacterial functional and community diversity in the rhizosphere. The findings showed that at 28-day incubation the rhizosphere soil of *P. americanum* had greater catalase activity, urease activity, water soluble organic carbon (WSOC) content, and an appropriate pH for microorganisms. For rhizosphere soil, the Shannon and McIntosh bacterial functional diversity indices were 3.17 & 0.04 and 6.43 & 0.86, respectively, while 2.95 & 0.06 and 3.98 & 0.27, respectively, for non-rhizosphere soil. As a result, bacteria in the *P. americanum* rhizosphere were utilized carbon substrates more effectively than bacteria outside of the rhizosphere. Rhizosphere soil had stronger bacterial community features even though atrazine was reduced the diversity of the soil bacterial community. For instance, the soil rhizosphere and non-rhizosphere had Shannon diversity indices of 5.821 and 5.670, respectively. However, other bacteria such as those from the genera *Paenibacillus, Rhizobium, Sphingobium*, and *Mycoplana*, which aid in the breakdown of organic contaminants or the cycling of soil nutrients were not discovered in the rhizosphere soil until 28 days after remediation.

Olu *et al*. (2019) evaluated indigenous *Pseudomonas aeruginosa, Bacillus subtilis*, and *Aspergillus niger* as bioaugmentation agents and chicken droppings as a biostimulation agent for bioremediation of atrazine-contaminated agricultural soil. They found that bioaugmentation with *Bacillus subtilis, Aspergillus niger*, *Pseudomonas aeruginosa* using a bacterial-fungal consortia, poultry droppings as a source of biostimulation, as well as biougmentation and biostimulation were combined with *Pseudomonas aeruginosa* to maximize atrazine biodegradation was observed with these three organisms (*Bacillus subtilis, Aspergillus niger,* and bird droppings) which was degrading approximately 97, 95, 84, 99, 100, and 100%, respectively. The effectiveness of the various bioremediation techniques for atrazine biodegradation or removal were in order of combination of bioaugmentation and biostimulation > bacterial-fungal consortium bioaugmentation >

poultry droppings biostimulation > *Pseudomonas aeruginosa* bioaugmentation > *Bacillus subtilis* bioaugmentation > *Aspergillus niger* bioaugmentation > Natural bioattenuation.

Topp (2001) identified that three atrazine-degrading bacteria like *Pseudomonas* sp. strain ADP, *Pseudaminobacter* sp., and *Nocardioides* sp. in lab microcosms for their capacity to break down and mineralize this pesticide in a loam soil. All the bacteria were reported hydrolytically dechlorinate atrazine and breakdown it with similar specific activity in pure culture. Atrazine can be used as the single source of carbon and nitrogen by *Pseudomonas, Nocardioides*, and *pseudomonas*, but solely as a nitrogen source by *Pseudomonas*. The chemical was mineralized by the bacteria *Pseudomonas* and *Pseudaminobacter*, and N-ethylammelide is the byproduct of the *Nocardioides*' metabolism of atrazine. At inoculum densities of 10^5 cells g^{-1} soil, only the *Pseudaminobacter* and *Nocardioides* accelerated atrazine dissipation. Atrazine was quickly and completely mineralized by the *Pseudaminobacter* but it took several days for the *Nocardioides*-inoculated soil to catch up.

2.3. Effect of pH, carbon source and soil texture on bioremediation

2.3.1. Effect of pH

One significant environmental component that affects the growth of bacteria is the hydrogen ion concentration *i.e*. pH. The ideal pH range for microbial growth varies depending on the characteristics of certain microbe and the pH level can have an impact on the biological activity of microbial metabolites (Dongfeng *et al*., 2011).

Abd El-Rahim *et al*. (2009) investigated the bioremoval of textile direct violet dye by the fungus *Aspergillus niger*. At pH levels ranging from 2 to 11, the impact of pH on dye bioremoval was examined. After 24 hours of incubation, the direct violet dye bioremoval was at its highest with 92.4%, 64.0%, 91.4%, and 62.3% at pH values of 2, 3, 8, and 9, respectively. Removal rates at pH 2, 3, 6, 7, 8, and 9 were 98.9, 97.3, 94.0, 95.0, 97.0 and 97.3 %, respectively, after 72 hours of incubation. For direct dye removal, the optimum pH ranges were 2, 3, 8, and 9.

Joshi *et al*. (1996) observed biodegradation rates of pyrene by *Acinetobacter* sp. at pH levels of 5.0, 7.0, and 9.0 and found that the remediation at pH 7.0 was seen to be nearly complete after a three-week period. At pH 5.0, the remediation of pyrene was much slower

compared to other contaminants, which were fully degraded in a five-week period as well as remediation at pH 9.0 was slower than that at pH 7.0.

Boivin *et al*. (2004) reported that the bentazone and 2, 4-D were poorly sorbent by a range of soils shortly after application. However, the sorption of these and other weak organic acids was dependent on the pH of the soil and this was mostly due to their acid-base equilibrium. The sorption isotherms was also examined that the impact of soil variables (organic matter content, clay content, pH, etc.) on the sorption of weakly acidic, weakly basic, and neutral pesticides by a variety of soils.

2.3.2. Effect of carbon source

Fava *et al*. (1995) studied growth kinetic parameters of *Alcaligenes* sp. and *Pseudomonas* sp. CPE2 strain on 2,5-dichlorobenzoic acid and 2-chlorobenzoic acid degradation through batch and continuous growth tests in the presence or absence of yeast extract as a carbon source (50 mg l^{-1}). In the absence of yeast extract, strain CPE2 growth was inhibitive, whereas in the presence of yeast extract, it was noninhibitory. Culture affinity for the chlorobenzoic acids was significantly increased when yeast extract was present.

2.3.3. Influence of soil texture

Torrents and Jayasundera (1997) elaborated that appropriate bioremediation strategy particularly in a field environment was affected by soil texture. It has a direct impact on the uniform distribution of pollutants, water, microorganisms, oxygen, and nutrients in contaminated soil. A variety of inorganic and organic chemicals with varied compositions and surface activities make up soil which was a heterogeneous mixture. These substances could bind to insecticides and decrease their bioavailability. Redox potential and moisture content which can affect the rate of biodegradation was only two examples of how texture parameters can affect a variety of soil environmental conditions. The amount of adsorption and rate of desorption affect the bioavailability of adsorbed pesticides.

Martins and Mermoid (1998) showed that an increase in the organic content led to an increase in the sorption of the nitro aromatic insecticide dinoseb in their experiments.

Kumar and Philip (2006) reported that the different types of soil (sandy soil, red soil, composted soil, and clayey) had distinct capacities for adsorbing endosulfan isomers and

varied degrading capacities for them. They recorded that higher rate of pesticide adsorption in clay soil, could be attributed to its greater organic content.

Arshad *et al*. (2008) revealed that endosulfan isomers of sandy loam, loam, sandy clay loam, and clay loam soil were biodegraded @ 89, 85, 79, and 75% in 16 days. The results showed that soil texture had 10% impact on biodegradation. Additionally, clay loams soil had a higher endosulfan adsorption rate which decreased the substance's bioavailability to bacteria.

3. MATERIALS AND METHODS

3.1. Cleaning of glasswares

All glasswares used were rinsed thoroughly in tap water after being soaked for a short time in chromic acid cleaning solution (10% potassium dichromate solution in 25% sulfuric acid). It was then given a second detergent wash, followed by a distilled water rinse and air drying.

3.2. Sterilization of glasswares and media

All the media used in this study were sterilized in an autoclave, at 121°C under 15 lbs/sq.in. pressure for 20 minutes. Glasswares were sterilized for one hour at 170°C in a hot air oven.

3.3. Chemicals

Analytical grade imazethapyr (> 98% purity) was purchased from M/S Sigma Aldrich chemicals Pvt Ltd, Bangalore, India. Pesticide grade imazethapyr 10% SL were purchased from- Agarwal Beej Bhandar, Agricultural product wholesaler in Jhansi, Uttar Pradesh. HPLC grade acetonitrile, methanol and solvents (analytical reagents) like dichloromethane, acetonitrile and acetone were purchased from the Himedia. Reagents like calcium chloride and HPLC grade water were purchased fromthe SD Fine Chemicals Ltd., Mumbai.

3.4. Preparation of standard solution

Imazethapyr (51.02 mg, 98% purity) was weighed, transferred into a 50 ml capacity volumetric flask and dissolved in ~5 ml acetonitrile (HPLC grade). The volume was made up to the mark with additional acetonitrile. This gave a stock solution of 1000 μg ml^{-1}.

Working standard solutions of lower concentrations were prepared from the stock solution by serial dilution. One millilitre of the primary stock solution (1000 μg ml^{-1}) was taken in a 10 ml volumetric flask and volume was made up with acetonitrile to give a standard solution of 100 μg ml^{-1} concentration. Similarly, serial dilution was done to get working standards of lower concentration.

3.5. Experimental locations

3.5.1. Sampling area

Soil samples were collected from three fields of different soil types previously exposed to the pesticide imazethapyr from the Bundelkhand region of Jhansi, Uttar Pradesh, India. The Jhansi district is located between 25.44' 84°N and 78.56' 85°E longitude.

3.5.2. Samples and processing

Using a sterile zip lock plastic cover, random soil samples were taken from the surface to a depth of 0 to 15 cm. With a pH range of 7.09 to 7.64, soil was divided into sandy loam and sandy clay loam. A total of 24 soil samples (3 samples one each from 3 sites before imazethapyr spray) were collected from the IGFRI and RLBCAU fields at different days of interval (2, 4, 8, 16, 32, 45 and 50 days) after imazethapyr spray (21 samples) on 15 DAS of berseem in the field. Twigs and stones were removed, and the samples were dried in a dark environment. Representative soil samples were stored at 4°C for subsequent processing after being sieved through a 2 mm sieve.

3.5.3. Climate and weather

The study area comes under semi-arid climate with harsh summer and winter seasons. The weekly meteorological data for the crop growing season 2021-22 is presented in Table 3.1. The high and low temperatures fall between 40-45°C and 7-10°C, in May and January, respectively. The daily maximum temperature rises from February to June before progressively falling when the rainy season starts in July. The average wind speed varies between 3.47 km h^{-1} in November and 5.11 km h^{-1} in April. The region's long-term average annual rainfall is 887.8 mm, with the majority of that falling between June and September (90-95%) and the remaining 10–20% falling during the winter months (Fig. 3.1). Mean relative humidity peaks at 80–87 % or even higher during the wet season. Beginning in the first two weeks of February, there is a rising tendency in both the daily maximum and minimum temperature that continues through the month of June. The monsoon season brings mild temperatures, which continue to dip until the middle of February before rising once more until the monsoon season officially begins. The meteorological data gathered at the ICAR-Indian Grassland and Fodder Research Institute in Jhansi during the trial period are reported in the table and graphically represented.

Table 3.1 Meteorological data of the crop growing season (2021-22)

Weekly meteorological data for the crop growing season 2021-22

Month	Standard Week	Rainfall (mm)	Temperature (^0C)		RH (%)		Wind velocity (km h^{-1})	Evaporation (mm)
			Max	Min	I*	II#		
November	45	0.0	30.8	9.9	81	46	3.1	4.6
	46	0.0	27.9	10.6	84	50	3.1	4.0
	47	0.0	28.1	12	85	49	3.2	3.6
	48	0.0	27.5	9.3	85	57	2.8	3.3
December	49	0.0	24.7	11.2	88	56	3.2	2.8
	50	0.0	23.4	7.8	89	60	3	2.5
	51	0.0	22.9	4.4	88	61	2.8	2.4
	52	12	22.3	8.4	90	65	3.2	2.3
January	1	18.0	20.9	7.7	91	71	3.1	2.0
	2	23.8	19.2	10.4	91	71	2.9	1.7
	3	0.0	18.3	5.8	91	72	2.8	1.6
	4	3.6	19.9	7.6	91	71	2.6	1.6
February	5	0.0	26.4	7.3	89	59	2.9	3.0
	6	0.0	24.0	7.5	88	47	3.6	3.2
	7	0.0	25.8	8.0	87	46	4.4	3.7
	8	0.0	28.0	11.3	84	46	3.7	4.1
March	9	0.0	28.5	11.3	84	45	4.8	4.6
	10	0.4	30.0	12.6	81	44	5.1	4.7
	11	0.0	35.2	16.0	80	38	4.4	5.9
	12	0.0	38.0	17.4	79	38	4.2	7.1
	13	0.0	38.8	16.8	76	34	4.3	7.9
April	14	0.0	40.4	17.4	60	30	4.1	8.6
	15	0.0	42.7	20.4	53	29	4.8	9.6
	16	0.0	40.5	20.5	52	29	4.4	9.4
	17	0.0	42.3	21.3	49	26	4.7	10.5

* Observation taken at 07:16 h; # Observation taken at 14:16 h

Source: Meteorological observatory, Crop Production Division, ICAR-Indian Grassland and Fodder Research Institute, Jhansi.

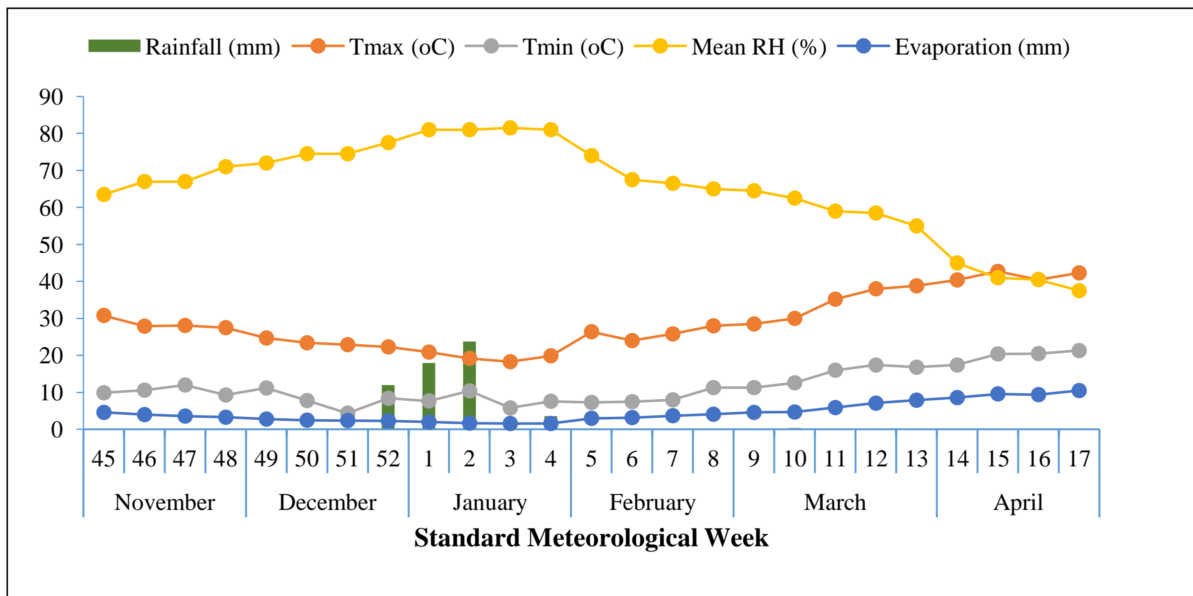


Figure 3.1 Variation in weathers parameters during crop period (*Rabi* 2021-22)

3.6. Application of the treatments

3.6.1. Land preparation

The field was leveled using a leveler after the area was disk-plowed, then it was cultivated using a cultivator, and finally it was leveled. To make manual seeding easier, the furrows were widened using a seed drill and sowing was done manually. Then the field was divided into two blocks, one control and another imazethapyr treatment block. Each treatment consisted of rectangular block (5 m x 6 m) of size 30 m^2. Before sowing, soil samples were taken randomly from different plots to determine the field's initial nutrient quality parameters.

3.6.2. Crop variety

The berseem cultivar Bundel Berseem-2 (BB-2) was used in this experiment.

3.6.3. Application of the fertilizers

At a rate of 80 kg N ha^{-1} through urea (NH_2CONH_2) and di-ammonium phosphate [$(NH_4)_2HPO_4$], 40 kg P ha^{-1} through diammonium phosphate was applied as the recommended dose of fertilizers. A base dose of N and P fertilizers was broadcasted prior to sowing. For each treatment of size 30 m^2, 158 g of urea and 260 g of DAP at the sowing was applied.

3.7. Cultural operations

3.7.1. Seed rate of berseem

On December 13, 2021, the fodder berseem was sown with a seed rate of 20-25 kg ha^{-1} at 3 different sites (S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block).

3.7.2. Irrigation

Utilizing the basin approach, irrigation was applied. The first irrigation was administered immediately following seeding, and successive irrigations were given every 10 to 15 days as well as after each harvest.

3.7.3. Cutting/harvesting

First cut for green fodder was done at 62 days after sowing. For regular and rapid regrowth of fodder, the height of the stubble was kept at 5–6 cm above the ground. Second cutting was done at about 30 days after first cut.

3.8. Analysis of physico-chemical parameters of soil samples

Table 3.2 Initial properties of experimental field soils

	Properties	**Value**			
1.	**Physical properties**	**Sand (%)**	**Silt (%)**	**Clay (%)**	**Textural class**
	S1*	61.12	22	16.88	Sandy loam
	S2	45.12	27	27.8	Sandy clay loam
	S3	57.12	23	19.88	Sandy loam
	Method followed: Sand/silt/clay: Hydrometer method (Buoyoucus, 1951), **Textural class:** Texture Triangle				

2.	**Chemical properties**	**pH**	**EC (dS/m)**	**BD (Mg m^{-3})**	**OC (%)**	**N (kg ha^{-1})**	**P (kg ha^{-1})**	**K (kg ha^{-1})**
	S1	7.21	0.013	1.45	0.51	241.5	7.9	255.4
	S2	7.29	0.019	1.35	0.63	205.4	8.65	266.3
	S3	7.47	0.021	1.48	0.61	216.9	7.8	244.1
	Methods followed: pH: Glass electrode pH meter (Jackson,1958); **EC:** Digital conductivity meter (Richards, 1954); **BD:** Pycnometer method (Black**,** 1965); **OC:** 1 N $K_2Cr_2O_7$ method (Walkley and Black, 1934); **N:** 0.32% $KMnO_4$ method (Subbiah and Asija method, 1956); **P:** 0.5 N $NaHCO_3$ method (Olsen *et al.*, 1954); **K:** 1 N CH_3COONH_4 method (Jackson, 1958)							

3	**Biological properties**	**TBC (CFU g^{-1} soil)**	**TFC (CFU g^{-1} soil)**	**DHA (µg TPF g^{-1} soil)**	**FDA hydrolysis (µg fluorescein g^{-1} soil)**	**SMBC (µg g^{-1})**
	S1	16.5×10^{7}	14×10^{4}	21.55	4.7416	901.6
	S2	27.5×10^{7}	14.5×10^{4}	10.29	4.8439	1059.8
	S3	22.5×10^{7}	17.5×10^{4}	13.95	6.6853	1002.3
	Methods followed: TBC: Waksman's method (1952); **TFC:** Beuchat method (1979); **DHA:** Cassida method (1964); **FDA:** Adam method (2001);**SMBC:** Jenkinson *et al*. (1976)					

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

3.8.1. Soil pH

The pH of the soil samples was determined using a digital pH meter. A glass rod was used to stir soil samples (10 g) with 100 ml of distilled water, and the pH of the suspension was noted.

3.8.2. Soil Electrical Conductivity

Using a digital EC meter, the EC of the soil sample was determined. A glass rod was used to stir soil sample (10 g) with 100 ml of distilled water, and the EC of the suspension was recorded.

3.8.3. Soil Organic Carbon

All samples were air-dried, gently crushed and passed through a 2 mm sieve. Soil Organic Carbon (SOC) was estimated following the classic dichromate oxidation method of Walkley & Black (1934). Organic C was oxidized by the heat of reaction during 30 min after adding potassium dichromate to the sample mixed with sulphuric acid. About 200 ml of distilled water and 10 ml of orthophosphoric acid was added slowly and 1ml of diphenylamine indicator was added and titrated with 0.5 N ferrous ammonium sulphate solution in 50 ml burette.

$$\text{Organic carbon \%} = \frac{10\ (B\text{-}S) \times 0.003 \times 100}{B \times \text{Weight of soil sample}}$$

Where, B- Blank reading; S- Sample reading

3.8.4. Available nitrogen

Alkaline potassium permanganate was used to evaluate the sample's available nitrogen (Subbiah and Asija, 1956). The sample's organic nitrogen was converted to ammonia by the oxidation of nascent oxygen produced by 0.32% $KMnO_4$ in the presence of 2.5% NaOH, which was then collected in a conical flask holding a boric acid cum mixed indicator solution. With 0.1 N H_2SO_2, the obtained distillate was titrated.

$$\text{Available Nitrogen (kg ha}^{-1}) = \frac{(S - B) \times 0.0028 \times 2.24 \times 10^{6}}{\text{Weight of soil sample}}$$

Where, B- Blank reading; S- Sample reading

3.8.5. Available phosphorus

Olsen's method was used to determine the amount of accessible phosphorus (Olsen *et al.*, 1954). Using the 0.5M $NaHCO_3$ reagent, the soil samples' usable phosphorus was recovered (1.5% Ammonium molybdate solution). By adding ammonium molybdate to the filtered extract and then reducing the molybdenum-phosphate complex with stannous chloride in an acid medium, phosphorus was colorimetrically determined in the sample. Blue phosphomolybdate complexes were produced throughout the formation process. At 660 nm, the intensity of the blue colour was determined using a spectrophotometer. After adding stannous chloride, the colour was measured after 10 minutes but before 12 minutes.

$$\text{Available P (kg ha}^{-1}) = \frac{Q \times V \times 2.24 \times 10^{6}}{A \times S \times 10^{6}}$$

Where, Q- Quantity of P in µg read on X – axis; V- Volume of extracting reagent used;
A- Volume of aliquot used for color development; S- Weight of soil sample

3.8.6. Available potassium

The amount of potassium that was readily available in the soil samples was determined by using the ammonium acetate method (Hanway and Heidel, 1952). After standardizing with potassium chloride (1.907 g KCl in 1 L distilled water), the amount of accessible potassium in the soil samples was extracted using neutral normal ammonium acetate as the extractant and measured using a flame photometer.

$$\text{Available K (kg ha}^{-1}) = \frac{\text{R x Ammonium acetate used x 2.24}}{\text{Weight of soil sample}}$$

Where, R- ppm of K in the extract

3.9. Isolation of bacteria, fungi and growth estimation in different concentration

3.9.1. Isolation of bacteria and fungi from soils exposed to imazethapyr

The serial dilution and standard plate count method was used to count the number of bacteria. Ten gram soil sample of imazethapyr applied field was taken and serially diluted up to a 10^{-7} dilution. Amounts of about 1 ml from each dilution were placed in a sterile petri dish and the sterile molten and cooled nutrient agar (NA) medium for bacteria and potato dextrose agar (PDA) for fungi was poured and rotated the plate clockwise and anticlockwise thrice for uniform mixing of inoculums with medium and was allowed to solidify. Then plates were incubated at 28±2°C for 24 to 48 h for bacteria and 3-7 days for fungi. After incubation, the plates were observed for the development of well isolated colonies. By repeatedly streaking across 300 ppm imazethapyr NA and PDA plates, different bacterial and fungal colonies that formed on the plates were seen, isolated, and purified. For further studies, the purified isolates were preserved in nutrient agar/PDA slants at 4°C and glycerol stock kept at -20°C.

3.9.2. Imazethapyr as carbon and energy source for growth of microbes

A modified growth medium containing ammonium nitrate (2 g l^{-1}), imazethapyr (1 g l^{-1}) and agar (18 g l^{-1}) was used to study the ability of degrading microbes to use imazethapyr as carbon and energy source for growth. The medium pH was adjusted to 7.0 for bacteria and 5.0 for fungi. Then media was autoclaved and poured in petri plates and bacterial and fungal isolates were inoculated and observed for bacterial growth in 1-2 days, and fungal growth in 2–3 days.

3.9.3. Screening bacterial isolates for imazethapyr tolerance

Bacterial isolates were screened for the tolerance towards imazethapyr based on their growth in presence of various concentrations (500, 1000, 1500 and 2000 µg ml^{-1}) of imazethapyr in nutrient broth (50 ml) by inoculating 1.0 ml of overnight grown bacterial isolates. The growth of bacterial isolates was measured by turbidimetric analysis after 24-48 h. The changes in the optical density (OD) in broth medium due to growth of bacteria were observed in spectrophotometer at 660 nm against uninoculated nutrient broth as blank.

3.9.4. Screening fungal isolates for imazethapyr tolerance

The fungal growth was measured as dry mycelial weight after the growth of isolates in presence of various concentrations (500, 1000, 1500 and 2000 µg ml^{-1}) of imazethapyr in potato dextrose broth (50 ml) media (aerated agitated cultures) for 6 days at 28–30°C with shaking (250 rpm) after inoculation of 1.0 ml fungus culture suspension. After the growth, the mycelia were filtered through pre-weighed filter papers. The mycelia with filter paper were dried at 105°C for 48 h. Filter papers with dry mycelium were reweighed, and actual fungal mycelia dry weight was calculated by subtracting the weight of filter paper.

3.10. Soil biological properties

3.10.1. Total bacteria count and total fungi count

Microbial population was enumerated from the rhizospheric soil of selected samples through culture dependent method following the serial dilution technique. About 10 g soil was suspended in 90 ml sterile water (10^{-1} dilution) and thoroughly shaken for 15 minutes in a horizontal shaker. From 10^{-1} dilution, 1.0 ml of the suspension was again diluted in 9.0 ml sterile water to get a dilution factor of 10^{-2}. Similarly, 10^{-3}, 10^{-4}, 10^{-6} and 10^{-8} diluted suspensions were prepared for fungal and bacterial population studies. 1.0 ml of 10^{-4} dilution was poured for total fungal count (TFC) in sterilized petri dishes and about 20-25 ml molten cooled up to 42-45 °C potato dextrose agar medium was poured and the plate was rotated clockwise and anticlockwise thrice and allowed to solidify. Similarly, 1.0 ml of 10^{-7} dilution was poured for total bacterial count (TBC) in sterilized petri dishes and about 20-25 ml molten cooled up to 42-45 °C nutrient agar medium was poured and the plate

was rotated clockwise and anticlockwise thrice and allowed to solidify. After solidification, the plates were incubated at 30+2 °C for 1-3 days for TBC, 1-7 days for TFC. The microbial colonies were counted and the population of fungi and bacteria were calculated in terms of colony forming units (CFU) per gram oven dry weight basis as described by Rolf and Bakken (1987).

3.10.2. Soil enzyme activities

3.10.2.1 Dehydrogenase enzyme activity

The method outlined by Cassida *et al*. (1964) was used to measure dehydrogenase (DHA) activity. Fresh soil samples (10 g) that had been homogenized were added to test tubes together with 2.5 ml of phosphate buffer, 0.2 g of $CaCO_3$, and 1.0 ml of substrate (3% TTC v/w, triphenyl-tetrazolium chloride). These test tubes were incubated for 24 hours at 28 ± 2°C. Similar steps were taken to prepare a blank sample, with the exception that 1 ml of a phosphate buffer solution with a TTC concentration of 3% was added. Following incubation, the samples were centrifuged for 10 minutes using at 3000 rpm. The liquid supernatant was discarded. Methanol was used to remove the triphenyl formazan (TPF) that had developed. Each of the tubes received 5 ml of methanol, which was added, and was forcefully shaken for a while. The procedure was carried out twice (10 ml of methanol was used for extraction). The tubes were centrifuged once more. A clean tube was used to contain the liquid supernatant once it had been produced, and the solution's absorbance was measured at 485 nm. Dehydrogenase activity was calculated and expressed in terms of µg TPF/g/day.

3.10.2.2. Fluorescein diacetate (FDA) hydrolysis

A 50 ml conical flask containing 2 g soil (fresh weight, sieved, 2 mm) was filled with 15 ml of 60 mM potassium phosphate buffer with a pH of 7.6. To begin the reaction, stock solution (0.2 ml 1000 µg FDA ml^{-1}) was added. A sufficient number of sample replicates and blanks without the FDA substrate were also produced. The contents of flasks were manually shaken. After that, the flasks were shaken for 20 minutes at 30°C in an orbital incubator shaker. The following procedure, containing methanol and chloroform, were completed in a fume cupboard. 15 ml of chloroform/methanol (2:1 v/v) were added right away to stop the reaction when the incubator was turned off. The flasks stoppers were

replaced and the contents were gently shaken by hand. The conical flasks contents were then transferred to 50 ml centrifuge tubes, where they were centrifuged at 2000 RPM for around three minutes. After filtering (Whatman, No. 2) the supernatant from each sample into 50 ml conical flasks, the filtrates were quantified at 490 nm using a spectrophotometer.

3.10.3. Soil microbial biomass carbon

Fumigation extraction method was used to estimate microbial biomass carbon in the soil. Moist samples were taken in duplicate (around 10 g of soil) in 50 ml glass beakers. One set was placed in a desiccator and fumigated with ethanol free chloroform for 24 hour (Jenkinson *et al*., 1976). One package was placed in a desiccator and fumigated with chloroform free of ethanol for 24 hours. Another set of samples had been stored as unfumigated samples in the refrigeration. Using 25 ml of 0.5 M K_2SO_4 solution (1:2.5; soil: extraction ratio), the fumigated and non fumigated soils were extracted for 30 minutes. The extract was filtered through Whatman Number 42 filter paper and digested in the presence of potassium persulphate ($K_2S_2O_8$) and 0.025 M H_2SO_4 in the digestive block for 2 h at 120°C. Evolved CO_2-C was trapped in vial containing 0.5 N NaOH kept over inside the digestion tube, and unused or untreated alkali titrated against 0.1N HCl.

The calculated amount for the MBC as,

MBC = (OCf – OCnf)/K_{EC}

OCf = Organic carbon extracted from fumigated soil

OCnf = Organic carbon extracted from non fumigated soil

K_{EC} = Efficiency of extraction (K_{EC} = 0.45)

3.11. Preparation of sodium alginate beads

Alginate beads were prepared according to the methods outlined in Bashan (1986). A mixture of 2.5 ml of 10% humic acid and 750 µl of 30% glycerol were added to 2% sodium alginate solution to obtain a final volume of 25 ml. Exactly 25 ml of cultures were centrifuged, the cell pellet was washed with saline (0.85% NaCl) and suspended in 25 ml of alginate – humic acid mixture and mixed thoroughly aseptically. This suspension was

extruded through a 26- gauze needle drop wise into a pre-cooled sterile 1.5% aqueous solution of $CaCl_2$ under mild agitation in laminar air flow chamber. The water-soluble sodium alginate along with microbial cells was converted into water insoluble calcium alginate beads. Thus instantaneously formed beads were allowed to harden for 3-6 hours at room temperature. Then, the beads were collected by sieving and were washed several times with sterile water and stored at 4°C in 0.85% saline for further studies.

3.12. Herbicide extraction

High performance liquid chromatography (HPLC) analysis

Fig. 3.2 HPLC system used for herbicide extraction

HPLC parameters

Imazethapyr was analyzed using HPLC (Young Ling 9100 HPLC System) equipped with Vacuum Degasser, Binary Pump, PDA detector and using Reverse phase Chromatopak 30 cm C-18 stainless steel column [250 mm × 4 mm (i.d.)], acetonitrile: 0.1% aqueous o-phosphoric acid (55:45) as a mobile phase at a flow rate of 1 mL min^{-1} at wave length of 250 nm.

3.12.1. Recovery of imazethapyr from soil samples

Recovery of imazethapyr was done with modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) as depicted by Marinho *et al.*, (2018) with slight modifications.

Fortification of soils with imazethapyr: About 50 g soil sample was taken in 250 ml conical flask and was fortified with addition of 100 μg ml^{-1} imazethapyr. Soil samples were obtained from fortified soil sample at 0, 3, 6 and 9 days intervals and were analyzed for imazethapyr by HPLC.

Extraction of imazethapyr from soil samples: About 5.0 g of soil was taken in 50 mL oak ridge tube. Soils were thoroughly mixed and 0.5-1.0 mL of distilled water to moisten the soil. Then 10 mL of HPLC grade acetonitrile (ACN), 4.0 g of anhydrous magnesium sulphate ($MgSO_4$), 1.0 g fine ground sodium chloride (NaCl), 1.0 g tri-sodium citrate dihydrate ($Na_3C_6H_5\ O_7.2H_2O$) and 0.5 g disodium hydrogen citrate ($Na_2C_6H_6O_7.1.5H_2O$) were added to each tube and the contents of tube were mixed by vortex mixer for 5 min. Further, samples were centrifuged at 5000 rpm for 5 min and 1.0 mL of supernatant from each tube was withdrawn for imazethapyr analysis.

3.12.2. Degradation of imazethapyr in MSM (Mineral salt medium) by sodium alginate beads based microbial formulation

To study the degradation of imazethapyr in MSM (Mineral salt medium) by sodium alginate beads, 20 ml mineral salt medium (broth) was taken in 100 mL conical flasks and fortified with 75 μg ml^{-1} imazethapyr and about 10 alginate beads based microbial formulation of each selected isolate and consortium were added separately in different flasks. Flasks were incubated at 28 ± 2°C in biological oxygen demand (B.O.D) incubator and about 2.0 ml sample from each treatment (flask) was taken 0, 3, 6, 9, 12 and 15 days interval. Beads untreated samples served as control. Each treatment was replicated thrice. The collected 2.0 ml samples were centrifuged at 10000 rpm for 5 minutes and after centrifugation, 1.0 ml of supernatant was transferred into HPLC vials and was analyzed by the HPLC.

3.12.3. Degradation of imazethapyr in MSM (Mineral salt medium) by pure culture

To study the degradation of imazethapyr in MSM (Mineral salt medium) by culture, 20 ml mineral salt medium was taken in 100 ml conical flasks and 25 μg ml^{-1} imazethapyr was added. The bacterial isolate was inoculated @ 1.0 ml overnight grown culture per flask and fungal mycelial balls @ 12/flask were inoculated. The inoculated flasks were kept in

B.O.D. incubator shaker and sampling @ 2.0 ml) was done in 0, 3, 6, 9, 12 and 15 days intervals. Culture untreated samples served as control. Each treatment was replicated thrice. The collected 2.0 ml samples were centrifuged at 10000 rpm for 5 minutes and after centrifugation, 1.0 ml of supernatant was transferred into HPLC vials and was analyzed by the HPLC.

3.12.4. Effect of solution pH on degradation of imazethapyr in MSM (Mineral salt medium) by sodium alginate beads based microbial formulation

Imazethapyr degradation is greatly affected by the different pH. The bacteria better grow in neutral pH and fungi in acidic pH and may have different effect on herbicide degradation at different pH. Therefore, degradation of imazethapyr in MSM (Mineral salt medium) at different pH levels (pH 4.0, 7.0 and 9.0) was studied. About 20 mL MSM was taken in 100 mL conical flasks and 25 µg ml^{-1} imazethapyr was added into each flask. Samples were incubated in B.O.D. incubator shaker and sampling (2.0 ml) was done in 0, 5, 10, and 15 days intervals. Beads untreated samples served as control. The collected 2.0 ml samples were centrifuged at 10000 rpm for 5 minutes and after centrifugation, 1.0 ml of supernatant was transferred into HPLC vials and was analyzed by the HPLC.

3.12.5. Influence of contaminants on degradation of imazethapyr in MSM (Mineral salt medium) by sodium alginate beads based microbial formulation

Imazethapyr degradation is greatly affected by the different contaminants. Degradation of imazethapyr in different contaminants (5, 15 and 25 µg ml^{-1}) was studied. 20 ml MSM broth was taken in 100 ml conical flasks and 5, 15 and 25 µg ml^{-1} imazethapyr was added in separate flasks (4 sets) for each isolate (B15 and F5) / consortium (F3, 13 and F10, 11) alginate beads. Flasks were kept in B.O.D. incubator shaker and sampling (2.0 ml) was carried out in 0, 3, 6 and 9 days intervals. Beads untreated samples served as control. The collected 2.0 ml samples were centrifuged at 10000 rpm for 5 minutes and after centrifugation, 1.0 ml of supernatant was transferred into HPLC vials and was analyzed by the HPLC.

3.13. Identification of imazethapyr degrading microbial isolates

3.13.1. Identification of imazethapyr degrading bacterial isolate by BIOLOG system

Using the Biolog GEN III system, tests were done to determine the biochemistry of the isolates. The MicroPlate test plate (Biolog) was inoculated with a single colony that was chosen and emulsified into inoculating fluid A (Biolog) (Wragg *et al.*, 2014). The inoculum was developed by using a turbidity meter to a specific transmittance as directed in the user manual. Using a multichannel pipette, 100 µl of the cell suspension from the bacterial isolate was inoculated into each well of the MicroPlate and incubated at 37°C for 20 hours. After 20 hours, Micro Plate was read in the semi-automated MicroStation reader, with the results analyzed by the identification systems software (GEN III database).

3.13.2. Identification of imazethapyr degrading microbial isolates by molecular method

3.13.2.1. Genomic DNA isolation from bacterial isolates and 16S rDNA gene amplification

The bacterial isolate was cultured in nutrient broth at 37 °C overnight and broth culture was centrifuged at 5000 rpm for 10 minutes to form pellets. The particle was cleaned, the supernatant was discarded, and genomic DNA was extracted using the lysis technique with SDS and NaOH from the pellet. The isolated genomic DNA's purity was tested by both qualitative (agarose gel assay) and quantitative analysis (spectrophotometric assay). Using two universal primers sets (F- 5'AGAGTTTGATCCTGGCTCAG-3' and R- 5'-AAGGAGGTGATCGACCCGCA-3'), the 16S rDNA was amplified. The reaction was conducted in a 50.0 µL reaction volume. PCR mixes included primers, Taq polymerase, four deoxynucleoside triphosphates (dNTPs), and isolated genomic DNA as a template. PCR (XP Thermal Cycler TC-XP-G, CHINA) assays were carried out in a Bio Era thermocycler programmed for the following PCR conditions: Initial denaturation at 94 °C for 1 minute; Denaturation at 94 °C for 1 minute; Annealing at 56 °C for 30 seconds; Extension at 72 °C for 4 minutes and Final extension at 72 °C for 10 minutes. Total number of cycles was 33.

Table 3.3 Genomic DNA isolation from bacterial isolates and 16S rDNA gene amplification

Steps	Temperature (°C)	Duration (minutes)	Number of cycles
Initial denaturation	94	1	1
Denaturation	94	1	30
Annealing	56	30 seconds	
Extension	72	4	
Final extension	72	10	1
Holding	4	30	1

3.13.2.2. Genomic DNA isolation from fungal isolates and ITS gene amplification

About 25 mL of potato dextrose broth was taken in 100 mL conical flasks and were sterilized. Selected fungal isolates mycelia were inoculated separately in each flask and were incubated for 72 hours at 28±2 °C. The mycelial mat was centrifuged for 5 minutes at 13,000 rpm in a microfuge to pellet it, and then it was washed with 500 µl of TE (Tris-EDTA) buffer and was washed the pellet once more. Following the decantation of the TE, 300 µl of extraction buffer was added. The same as this one (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS (Sodium Dodecyl Sulfate). The mycelium was crushed with a conical grinder that precisely fits the tube and was operated by a hand or electric potter for a few minutes at 200 rpm. After that, tubes were placed at -20°C for approximately five minutes while 150 µl of 3 M sodium acetate, pH 5.2, was added. The supernatant was then transferred to another tube after the tubes were centrifuged in a microfuge. The precipitated DNA was then pelleted by centrifugation in a microfuge after at least 5 minutes at room temperature and the addition of an equal amount of isopropanol. The pellet was washed with 70% ethanol and air dried for a few minutes and then resuspended in 50 µl of TE. The ITS sequences of the fungal isolates were amplified using ITS1 F: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 R: 5'-TCCTCCGCTTATTGATATGC-3') There were 33 cycles of cycling with the following parameters: Initial denaturation at 94°C for 5 minute, Denaturation at 94°C for 1 minute, Annealing at 56°C for 30 seconds, Extension 72°C for 45 seconds and a final auto

extension step of 72°C for 10 minutes. A 2% agarose gel was used to analyze PCR product, which was then stained with ethidium bromide to make it visible.

Table 3.4 Genomic DNA isolation from fungal isolates and ITS gene amplification

Steps	Temperature (°C)	Duration (Minutes)	Number of cycles
Initial denaturation	94	5	1
Denaturation	94	1	30
Annealing	56	30 seconds	
Extension	72	45 seconds	
Final extension	72	10	1
Holding	4	30	1

3.13.2.2 Sequencing of PCR amplified gene products

The amplified 16S rRNA gene and ITS1-ITS4 gene sequences were sequenced by Sanger sequencing procedure through outsourcing. The obtained sequences were analyzed using NCBI BLAST algorithm and the imazethapyr degrading selected bacterial and fungal isolates were identified using maximum sequence similarity with the existing sequence database.

4. Results and Discussion

The results obtained of the laboratory and field experiments of present study entitled on "Bioremediation of imazethapyr in soil/water system" conducted at ICAR-IGFRI, Jhansi are presented in this chapter. Major focus was given to isolate microorganisms including both bacteria and fungi capable of degrading imazethapyr and characterize them for possible bioremediation application under laboratory condition. The observations pertaining to crop growth parameters, fodder yield and soil physico-chemical and microbiological properties recorded in experimentation were statistically analysed and significance of results verified. The data have been given in Tables and also depicted graphically wherever necessary to provide better understanding of important results.

4.1. Growth and yield parameters

4.1.1. Plant height

The berseem plant growth parameters were studied at 45 days after sowing (DAS) by randomly selecting 6 plants from each soil type /field sites (Plate 4.1). All data regarding growth parameters were significant (Tables 4.1-4.3) except for chlorophyll (SPAD values). Plant height in control plots ranged from 18.33 to 34.03 cm and the maximum was recorded in RLBCAU - I_{14} Block field and the minimum was recorded in RLBCAU - H Block site (Table 4.1), while imazethapyr applied plots it ranged from 21.0-35.77 cm and followed similar trend (Table 4.1). Our results are corroborated by the findings of Dubey (2013), who found that application of imazethapyr and chlorimuron (@ 100+24 g ha^{-1}) in groundnut recorded maximum nodules $plant^{-1}$, plant height, pods $plant^{-1}$, pod yield, kernel yield, haulm yields followed by imazethapyr 300 g ha^{-1} and imazethapyr 200 g ha^{-1}.

4.1.2. Root length

Root length in control plots ranged from 6.47-8.63 cm while in imazethapyr applied plots it ranged from 7.73-9.57 cm (Table 4.1). Both S1 and S2 sites recorded significantly higher root length over S3 in control plots whereas in imazethapyr treated plots S1 was significantly superior to S3 and at par with S2, which was at par with S3. The differences among sites could be due to their inherent physico-chemical properties of the soils. However, a slight increase in shoot and root length in imazethapyr applied plots could be attributed to reduction in competition between crop plants and weeds due to herbicide

application. Our results are corroborated by the results of Ceballos *et al*. (2004), who reported that compared to the untreated control, plant height increased significantly for bentazon-treated red clover throughout the experimental period, resulting in plants that were 70 and 48% taller than the control by the end of experimentation for the 1X and 2X rates, respectively. Plants treated with 2, 4-DB, flumetsulam, haloxyfop-methyl and flumetsulam/ 2, 4-DB were all significantly taller than untreated plants by the end, at the recommended dosage, but were variably affected by the 2X rate.

Table 4.1 Impact of imazethapyr application on berseem plant growth (45 DAS)

Soil sites	Plant height (cm)				Root length (cm)			
	Control		Imazethapyr treated		Control		Imazethapyr treated	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
S1*	28.37	3.02	29.50	1.44	8.00	0.50	9.57	0.37
S2	34.03	2.47	35.77	1.42	8.63	0.15	8.73	0.12
S3	18.33	0.17	21.00	0.20	6.47	0.03	7.73	0.32
SE(m)	2.31		0.96		0.25		0.29	
C.D.	9.32		3.85		1.02		1.17	

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

Plate 4.1 Berseem crop at different study sites

4.1.3. Plant shoot and root fresh weight

The berseem shoot fresh weight recorded ranged from 1.97 (S3) to 5.89 g/plant (S2) in control and 2.33 (S3) to 7.88 g/plant (S2) in imazethapyr applied plots (Table 4.2). Similarly, root fresh weight ranged from 0.61 (S3) to 0.857 (S1) g/plant in control soils while it ranged from 0.613 (S3) to 0.857 (S1) g/plant in imazethapyr applied soils. Soils S1 and S2 recorded significantly higher shoot and root fresh weight over S3 both in control as well as in imazethapyr applied plots. It could be due to the soil physico-chemical properties which supports the plant growth. Our results are in line with results of Kumar *et*

al. (2016), who recorded significantly higher shoot and root dry weight in summer mungbean treated with imazethapyr over control. In general, both shoot and root fresh weight was recorded higher in imazethapyr applied plots over control plots (Table 4.2), which could be attributed to reduction in competition between crop plants and weeds due to herbicide application and more growth in length.

Table 4.2 Impact of imazethapyr application on berseem plant weight (45 DAS)

Soil sites	Shoot weight (g/plant)				Root weight (g/plant)			
	Control		Imazethapyr treated		Control		Imazethapyr treated	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
S1*	4.83	0.15	7.19	0.29	0.683	0.012	0.857	0.022
S2	5.89	0.14	7.88	0.04	0.747	0.022	0.793	0.015
S3	1.97	0.12	2.33	0.04	0.610	0.045	0.613	0.04
SE(m)	0.15		0.16		0.028		0.014	
C.D.	0.59		0.64		0.114		0.055	

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

4.1.5. Root nodule count

Number of root nodules per plant in berseem ranged from 16 to 22.7 in control plots and 14.3 to 19.0 in imazethapyr treated plots (Table 4.3). Tha data on nodule number was significant. Maximum root nodule count was recorded in S1 and least was in S3 in both control as well as treated plots. Overall control plots recorded slightly higher nodule number/plant than imazethapyr treatments. This could be due to the toxic effect of herbicide on microbial population until recovery period. Similar results *i.e.* various herbicide affects nodulation were also reported in many leguminous crops *viz*., *Lens culinaris* (Sandhu *et al*., 1991), *Pisum sativum, Vigna sinensis* (Agarwal *et al*., 1986). Gonzalez *et al*. (1996) observed in *Pisum sativum* that imazethapyr affected number of nodules per plant than nodule size. Tilgam and Shyam (2019) reported that maximum number of nodules (56.8/plant), dry weight of nodules (73.6 mg/plant) in black gram was registered with treatment (two hand weeding at 20 & 40 DAS), which was statistically identical to T8 [imazethyper + imazemox (pre-mix) 80 g ha^{-1} PoE] and T10 [imazethyper + pendimethalin (pre-mix) 1000 g ha^{-1} PE]. In black gram, Aggarwal *et al.* (2014) number of nodules, dry weight of nodules and leghaemoglobin content were the highest in

imazethapyr 100 g ha^{-1} sprayed at 15 DAS, being statistically at par with 2 hand-weedings (20 and 40 DAS).

4.1.6. Chlorophyll content (SPAD values)

The data on chlorophyll content as SPAD values were not significant (Table 4.3). Chlorophyll ranged from 35.28 (S1) to 36.96 (S2) in control plots and 34.54 (S1) to 36.62 (S3) in imazethapyr treated plots. There were no significant differences found among treatments found between soils or control and imazethapyr treatments. Imazethapyr (IM) is a chiral herbicide with reported enantioselective biological activities between its enantiomers. Qian *et al.* (2013) studied enantioselectivity between R- and S-IM in *Arabidopsis thaliana* on chlorophyll synthesis and photosynthesis and found that R-IM inhibited the transcription of chlM to a greater extent than S-IM, which reduced chlorophyll synthesis. R-IM also showed a stronger inhibitory effect than S-IM on the transcription of photosynthesis-related genes, affecting linear electron transport and CO_2 fixation. Though there are some reports on imazethapyr affect chlorophyll synthesis, it is in the early stage of growth only. Ramesh and Rathika (2015) also found that among the early post-emergence herbicides, Imazethapyr 10% SL @ 100 g a.i. ha^{-1} + Quizalofop-ethyl 5% EC @ 50 g a.i. ha^{-1} (Tank mix) on 15 DAS shown significant results in all the photosynthetic pigments in green gram.

Table 4.3 Impact of imazethapyr application on berseem nodule and chlorophyll

Soil sites	Nodule number				Chlorophyll content (SPAD values)			
	Control		Imazethapyr treated		Control		Imazethapyr treated	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
S1*	22.7	0.3	19.0	0.6	35.28	1.007	34.54	0.873
S2	17.0	1.5	16.3	0.9	36.96	0.432	36.46	0.638
S3	16.0	0.6	14.3	1.2	36.6	0.657	36.62	0.658
SE(m)	0.9		0.7		0.768		0.708	
C.D.	3.6		2.8		NS		NS	

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

4.1.7. Green fodder yield

Berseem green fodder yield (GFY) was recorded higher in imazethapyr treated plots invariably of soil types. GFY obtained from two cuts (late sown) ranged from 33.39 (S3) to 39.96 (S2) t ha^{-1} in imazethapyr treated plots and 28.03 (S3) to 33.16 (S2) t ha^{-1} in control plots. Weeds compete with berseem in early growth stage for nutrients, water and space

and dominate quickly. It affects overall yield and quality of fodder. Manual weeding or herbicide can be management options available. Our results are also supported by researchers' reports, Wasnik *et al.* (2017). Among herbicidal treatments, imazethapyr application @ 0.1 kg a.i. ha^{-1} recorded maximum green fodder (439.9 q ha^{-1}) and seed yield (3.4 q ha^{-1}), which were significantly superior compared to other treatments. Among different herbicide treatments maximum weed control efficiency (96.7%) was recorded with imazethapyr application (0.1 kg a.i. ha^{-1}) as post-emergence herbicide in berseem crop. Application of imazethapyr 100 g ha^{-1} and pendimethalin 750 g ha^{-1} at 14 DAS significantly improved green fodder yield of lucerne but failed to control *Cuscuta* infestation at reproductive stage resulting in poor seed yield (Mishra, 2012). Application of imazethapyr at 100 g ha^{-1} at 15 DAS resulted in 62.0% higher grain yield in black gram over the unweeded control (Aggarwal *et al.*, 2014).

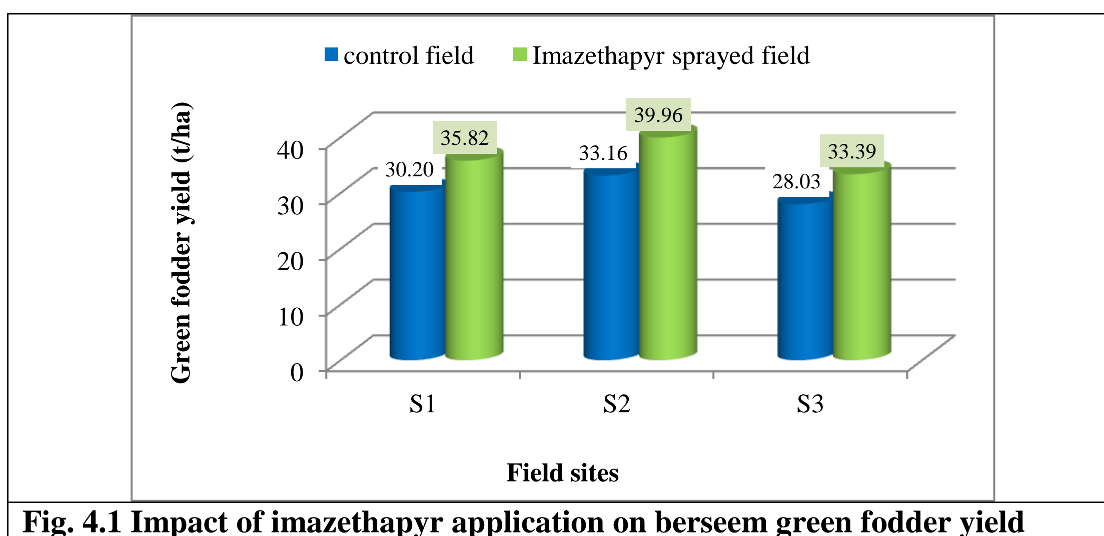


Fig. 4.1 Impact of imazethapyr application on berseem green fodder yield

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

4.2. Impact of imazethapyr spray on weed flora in berseem fields

Weed population was recorded for each soil and treatments. The maximum weed species (5, 3 and 2) and number (74, 62 and 48) were recorded in S2, S3 and S1 respectively in control plots. Whereas in imazethapyr applied plots, maximum weed species (3, 2 and 2) in (S3, S2 and S1), respectively and number (28, 22 and 18) in S3, S1 and S2, respectively. The maximum % weed reduction was recorded in S2 followed by S3 and S1. Similar results are reported by various workers in different crops. Kumar *et al.* (2016) reported that

weed control efficiency of imazethayr was more than 40% at a dose of 100 g ha^{-1} and the corresponding value of weed control index was more than 60% in summer mungbean. Priyanka *et al*. (2018) found that imazethapyr at 100 g ha^{-1} at 3 WAS (also imazethapyr at 75 g ha^{-1} at 3 WAS as second best treatment for yield (85 t ha^{-1}) and weed control efficiency (67.7-75.8) and butachlor 1500 g ha^{-1} as pre-emergence were significantly superior in controlling weed flora (weed control efficiency 69.7-77.3 and 68.7-75.8%) and recorded higher green fodder yield (86.0 and 82.1 t ha^{-1}) in berseem than other treatments. Kauthale *et al*. (2016) reported that treatment combination of oxyflourfen 0.1 kg ha^{-1} + imazethapyr 0.1 kg ha^{-1} immediately after harvest of first cut recorded lowest weed dry matter yield (0.05 t ha^{-1}) and highest weed control efficiency (80.97%). Goud (2016) found that Quizalofop-ethyl, fenoxoprop-ethyl and imazethapyr @ 0.05 kg ha^{-1} at 20-25 DAS did not provide satisfactory weed control in green gram fields.

Table 4.4 Impact imazethapyr spray on weed flora in berseem fields

Local name	Botanical name	Number of weeds/m^2		% weed control
		Control	Imazethapyr sprayed	
S1*				
Medick or burclover	*Medicago truncatula*	39	19	54.17
Canada thistle	*Cirsium arvense*	9	3	
S2				
Field spurry, Sand weed	*Spergula arvensis* var. *sativa*	1	0	75.68
Medick or burclover	*Medicago truncatula*	41	13	
Scarlet, Pimpernel	*Anagallis arvensis*	30	0	
Wheat	*Triticum aestivum*	1	0	
Mustard	*Brassica juncea*	1	0	
Canada thistle	*Cirsium arvense*	0	5	
S3				
Melde, Goose foot, Lamb's quarters	*Chenopodium album*	39	22	54.84
Field spurry,Sandweed	*Spergula arvensis* var. *sativa*	23	0	
Wheat	*Triticum aestivum*	1	0	
White top weed	*Parthenium hysterophorus*	0	4	
Bermuda grass	*Cynodon dactylon*	0	2	

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

4.3. Soil physico-chemical properties

The data pertaining to soil physico-chemical properties were not significant (Table 4.5). The pH of soils ranged from 7.21 (S1) to 7.47 (S3) in control plots and 7.09 (S1) to 7.64 (S3) in imazethapyr treated plots. EC ranged from 0.013 (S1) to 0.021 (S3) in control plots and 0.015 (S2) to 0.023 (S1) in imazethapyr treated plots. Bulk density (BD) ranged from 1.34 (S2) to 1.49 (S3) in control plots and 1.36 (S2) to 1.48 (S3) in imazethapyr treated plots. Soil organic carbon (SOC) ranged from 0.51 (S1) to 0.63 (S2) in control plots and 0.52 (S1) to 0.63 (S2) in imazethapyr treated plots. The observed properties did not vary much between control and treated plots. It could be due to difference in weed population and crop plant growth in 45 days may not be sufficient to make changes in physico-chemical properties of the soil.

Table 4.5 Impact imazethapyr spray on soil physico-chemical properties

Soil sample*	**pH**		**EC (dS/m)**		**BD (g/cm³)**		**SOC (%)**	
	Control	**Ima. sprayed**	**Control**	**Ima. sprayed**	**Control**	**Ima. sprayed**	**Control**	**Ima. sprayed**
S1*	7.21	7.09	0.013	0.023	1.46	1.44	0.51	0.52
S2	7.29	7.22	0.019	0.015	1.34	1.36	0.63	0.63
S3	7.47	7.64	0.021	0.016	1.49	1.48	0.61	0.61

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block; Ima: Imazethapyr

4.4. Soil nutrient status

The nutrient status between control plots and imazethapyr treated plots was also remained similar or there were very negligible changes (Table 4.6). The available N content ranged from 205.4 (S1) to 241.5 (S2) kg ha^{-1} in control plots as compared to imazethapyr treated plots [206.8 (S1) to 252.7 (S2) kg ha^{-1}]. The available P content ranged from 7.8 (S3) to 8.65 (S2) kg ha^{-1} in control plots as compared to imazethapyr treated plots [8.03 (S3) to 8.95 (S2) kg ha^{-1}]. The available K content ranged from 244.1 (S3) to 266.3 (S2) kg ha^{-1} in control plots as compared to imazethapyr treated plots [244.2 (S3) to 263.6 (S2) kg ha^{-1}]. This could be due to application of imazethapyr might have not influenced soil nutrients directly except impacting plants and thereby influencing indirectly. Most of the imazethapyr treated plots recorded a slight increase in N and P contents over control plots.

This could be due enhanced microbial activities at berseem plants rhizosphere which has led to enhanced N-fixation and P-solubilization. Lal *et al.* (2017) reported that spray of Imazethapyr (@ 75 g a.i. ha^{-1} with adjuvant) recorded higher uptake of nutrients (NPK) by green gram. Nutrients (NPK) uptake by weeds was higher in weedy check, but significantly lower in weed free, standard check and Imazethapyr treated plots.

Table 4.6 Impact imazethapyr spray on soil nutrient status

Soil sample*	Available N (kg ha^{-1})		Available P (kg ha^{-1})		Available K (kg ha^{-1})	
	Control	Imazethapyr sprayed	Control	Imazethapyr sprayed	Control	Imazethapyr sprayed
S1	205.4	206.8	7.9	8.12	255.4	256.2
S2	241.5	252.7	8.65	8.95	266.3	263.6
S3	236.9	238.6	7.8	8.03	244.1	244.2

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

4.5. Soil texture

The soil samples collected from all 3 sites are shown in Plate 4.2. The sand, silt and clay contents of the soil were 61.12%, 22% and 16.88% in S1 (IGFRI CR farm), 45.12%, 27% and 27.88% in S2 (RLBCAU – I_{14} Block) and 57.12, 23 and 19.88% in S3 (RLBCAU - H Block) soils (Table 4.7). Accordingly, they were classified as sandy loam (S1), sandy clay loam (S2) and sandy loam (S3).

Table 4.7 Soil texture and type of soil in study fields/sites

Soil sample*	Sand %	Silt %	Clay %	Type of soil
S1*	61.12	22	16.88	Sandy loam
S2	45.12	27	27.88	Sandy clay loam
S3	57.12	23	19.88	Sandy loam

*S1: IGFRI CR farm; S2: RLBCAU - I14 Block; S3: RLBCAU - H Block

Plate 4.2 Soil samples collected from study sites for analysis

4.6. Persistence of imazethapyr in Bundelkhand soils and microbiological properties

The persistence of imazethapyr in field soils could not be estimated directly effectively. This could be due to dilution and/or run off since December 2021 last week and January 2022 first two weeks received about 53.8 mm rainfall during crop period. However, the degradation in soil in vitro was done by spiking imazethapyr in the collected soil samples and results are presented at section 4.9 and table 4.10. The indirect effect of imazethapyr application on the soil microbial properties was studied and presented here. Assessing soil microbiological properties gives hints regarding the beneficial or harmful effects of the particular input/ treatment in environment or soil. Some of the microbial parameters like population counts (bacterial, fungal), enzyme activities (FDA hydrolysis and dehydrogenase) and microbial biomass (SMBC) contents are used to indicate the soil health especially biological health.

4.6.1. Impact of imazethapyr application on soil bacterial and fungal population

Lesser total bacterial count (TBC) was recorded from 2 to 50 days after imazethapyr spray on berseem at 15 DAS. There was a decline in TBC at 2 days up to 32 days from the original / control soil in all 3 sites (Fig. 4.2). The reduction in population was significant up to 16 days after spray. This could be due to the persistence of herbicide residues in soil which might be toxic to many organisms. These findings are in close conformity with the results of Babu *et al.* (2015), who reported that the residue of imazethapyr persists in soil up to 60 days at higher rates of application while it persists up to 30 days in plant with the

calculated half life of 2.8 to 7.4 days in soil and 5.1 to 5.9 days in plant. Our results are in line with Abbas *et al*. (2014), who reported that bacterial population ranged from 0.67 to $1.84x10^8$ and 0.87 to $2.37x10^8$ cfu g^{-1} soil in the soils A (contaminated soils *i.e*. exposed to bromoxynil herbicide for about last ten years) and B (soils without contamination), respectively.

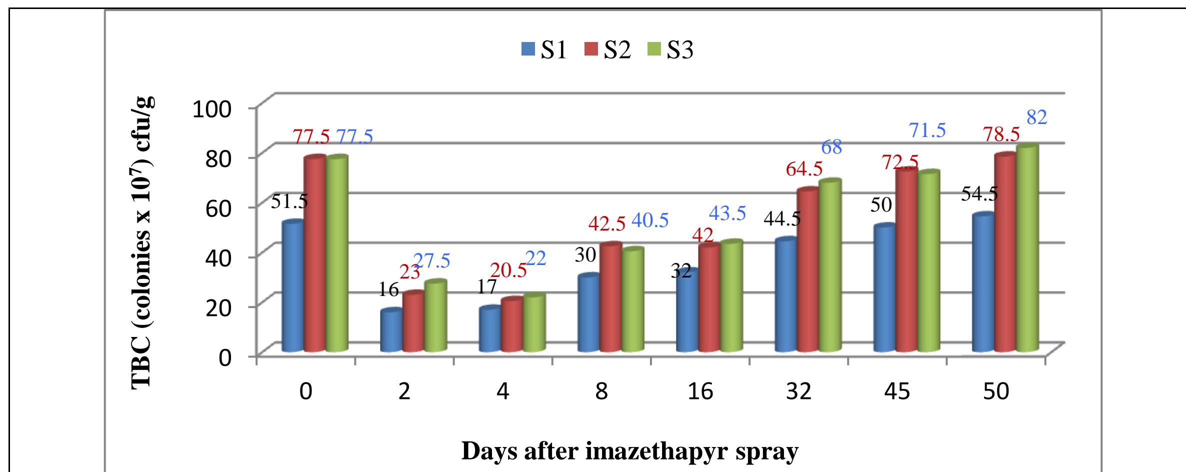


Fig. 4.2 Impact of imazethapyr application on soil bacterial population

Similar to total bacterial count, reduced total fungal count (TFC) was recorded from 2 to 16 days after imazethapyr spray on berseem at 15 DAS. There was a decline in TFC at 2 days up to 16 days from the original / control soil in all 3 sites (Fig. 4.3). Liu *et al*. (2019) reported that the diversity and evenness of the leaf microbiota were not affected by imazethapyr treatment, but the composition of community structure at the genus level was altered by IM spraying in *Arabidopsis thaliana* leaf surface.

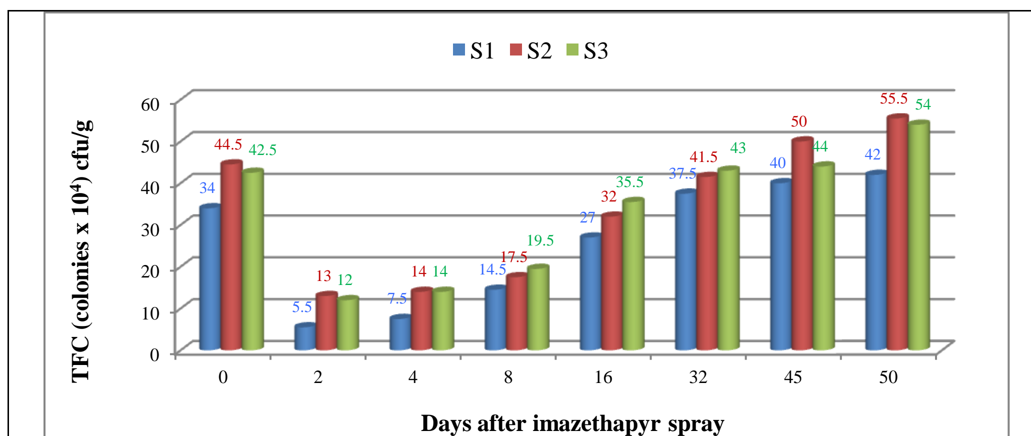


Fig. 4.3 Impact of imazethapyr application on soil fungal population

4.6.2. Impact of imazethapyr application on soil enzyme activity

Vekemans *et al*. (1989) and Perucci (1992) reported a close relationship between FDA-hydrolysis kinetics and soil microbial biomass. Dumontet *et al*. (1997) suggested that FDA-hydrolysis may be considered as a suitable tool for measuring the early detrimental effect of pesticides on soil microbial biomass, as it is a sensible and non-specific test able to depict the hydrolytic activity of soil microbes. In the present study, the FDA hydrolysis was affected from 2 to 16 days after herbicide spray and recovered from 32 days (Fig. 4.4). This could be due the hazardous effect of imazethapyr on total microbial biomass and activities till the residues are converted as C, energy and/or N source by microbes. Perucci *et al*. (2000) found that the herbicides (rimsulfuron and imazethapyr) applied at field rate were able to lower the FDA-hydrolytic activity measured at days 7 and 14 of incubation. Such an impairment of the microfloral metabolic activity had disappeared by the 30th day of incubation. Such behaviour could be related to the selection of resistant strains and/ or to the capability of microbes to use herbicides, or their degradation products, as a C source.

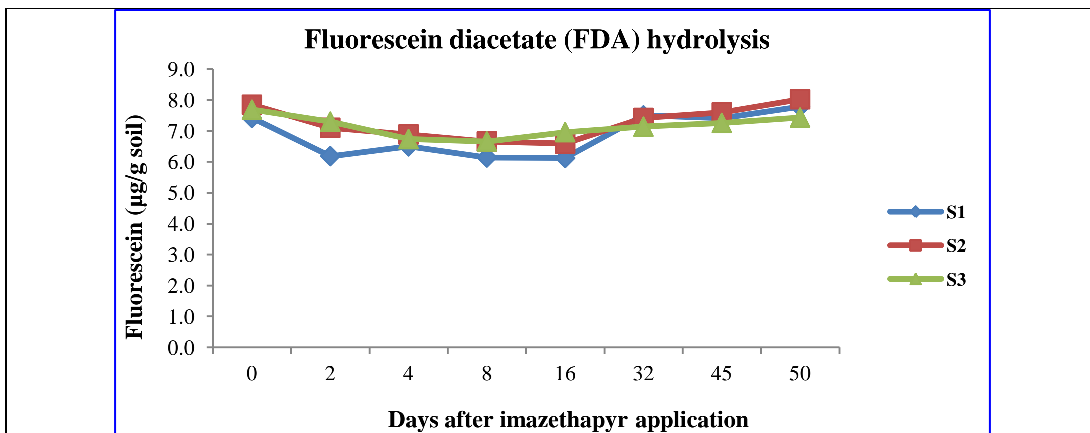


Fig. 4.4 Impact of imazethapyr application on soil enzyme activity (FDA hydrolysis)

However, the dehydrogenase (DHA) activity was not significantly affected by the imazethapyr application in all soils except S3 wherein significant reduction DHA was observed up to 16 days after spray (Fig. 4.5). Lal *et al*. (2017) reported that application of imazethapyr (75 g a.i. ha^{-1} with adjuvant) recorded lower dehydrogenase, urease and phosphatase activity of soil at seven and 15 days after spraying. Imazethapyr decreased the DHA from the 7th day onwards after the treatment and persisted only up to the 15th day in peanut (Saha *et al*., 2016). Imazethapyr suppression of DHA was observed, which may be due to the lethal action of Imazethapyr on soil microorganisms (Jyot *et al*., 2015). Whereas

Pertile *et al.* (2020) reported that soil respiration, respiratory quotient, and dehydrogenase activity increased significantly after the application of the herbicides (imazethapyr and flumioxazin) compared to the control.

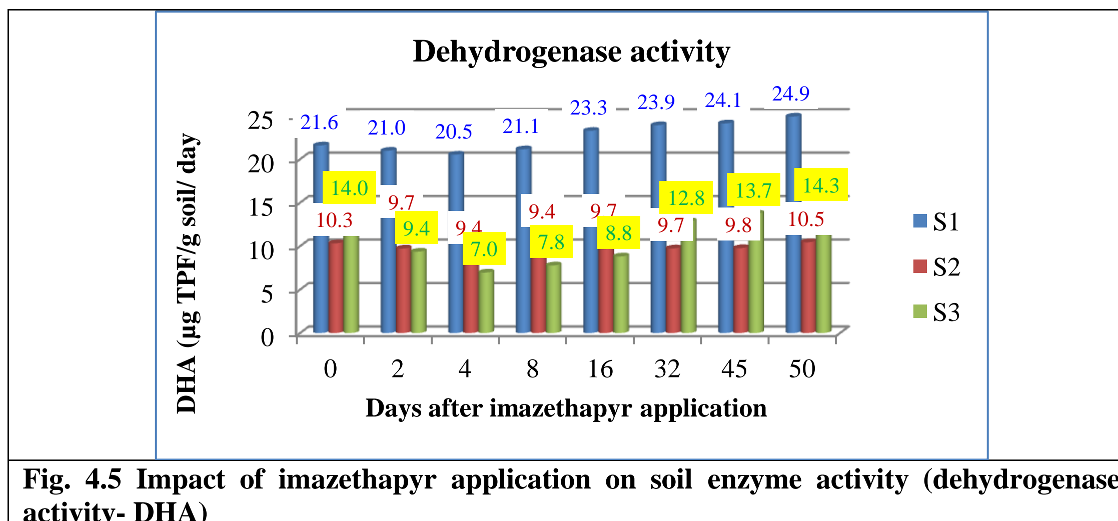


Fig. 4.5 Impact of imazethapyr application on soil enzyme activity (dehydrogenase activity- DHA)

4.6.3. Impact of imazethapyr application on soil microbial biomass carbon

Assessment of the microbial biomass carbon and nitrogen of the soil provide a mean of estimating the response of microbes to the changes in soil management operations. For sustainable agroecosystem, soil microbial biomass and biological productivity are most essential. Soil microbial biomass comprise only 2-6% of total organic matter of soil but being highly mobile constituent of the organic matter, it plays major role in nutrient cycling (Anderson and Domsch 1980). The SMBC content decreased up to 8 days and started increasing gradually from 16th day after spray in all 3 soil sites (Fig. 4.6). Pertile *et al.* (2020) evaluated the responses of soil microbial biomass and enzymes activity to the application of the herbicides imazethapyr and fumioxazin and reported that soil microbial biomass C (MBC) decreased, while microbial biomass N (MBN) was not afected after the application of the herbicides as compared to the control. The hydrolysis of fuorescein diacetate (FDA) was not signifcantly diferent between the control and the herbicide treatments. Abbas *et al.* (2014) reported that at all the sites MBC, MBN and MBP ranged from 131 to 457, 1.22 to 13.1 and 0.59 to 3.70 µg g^{-1} in the contaminated soils *i.e.* exposed to bromoxynil herbicide for about last ten years (Soil A), which was 187 to 573, 1.70 to 14.4 and 0.72 to 4.12 µg g^{-1} in the soils without contamination (soil B). It is evident from

this, that herbicide had left toxic effects on soil microbial parameters, thus confirmed that continuous use of this herbicide affected the quality of soil and sustainable crop production.

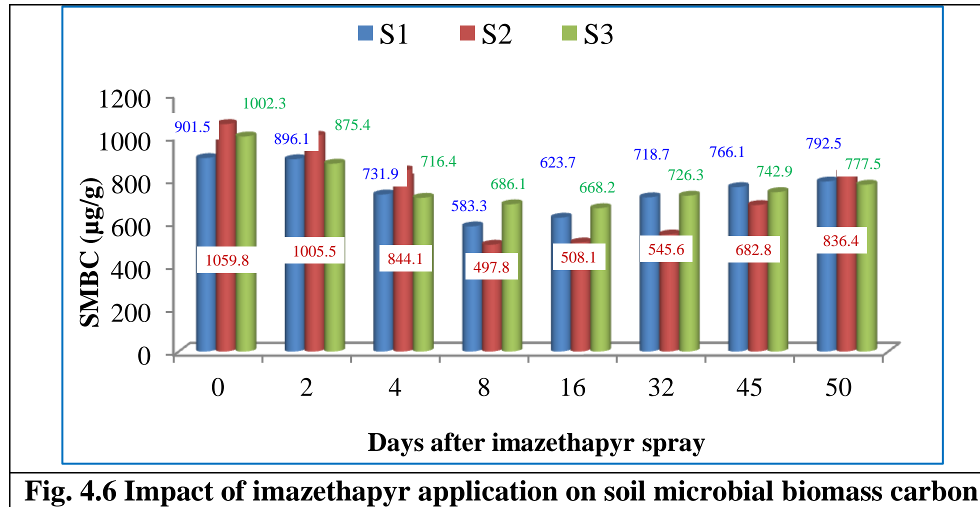


Fig. 4.6 Impact of imazethapyr application on soil microbial biomass carbon

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

4.7. Isolation of microorganisms from imazethapyr treated soils

Soil samples were collected from fields previously exposed to imazethapyr from Central research farm, ICAR-IGFRI, Jhansi for isolation of bacteria and fungi adapted to imazethapyr stress. More resembling colonies from all replicates, typically grown separately were picked up (Plate 4.3) and were purified by repeated streaking by four way streak plate method for bacteria and repeated transfer to fresh plates for fungi isolates (Plate 4.4). Many workers had isolated many microorganisms capable of growth and degradation of various pesticides including herbicides around the globe. Isolation of high numbers of microorganisms from an environment is commonly taken as an index that those organisms degrade the particular contaminant of the environment from which they were isolated (Ajao *et al*., 2014). Huo *et al*. (2011) isolated a strain of actinomyces from the samples of soil where imazethapyr had been applied for a long-term by use of bottle enriched culture and named S181. Digvijaya *et al*. (2017) collected soil samples from wheat ranch from different sites in Punjab region and isolated imazethapyr degrading microorganisms in pure culture by adopting streaking method.

Plate 4.3 Isolation of microorganisms from imazethapyr treated soils

About 16 bacterial isolates and 13 fungal isolates were obtained as pure cultures from more than 20 soil samples.

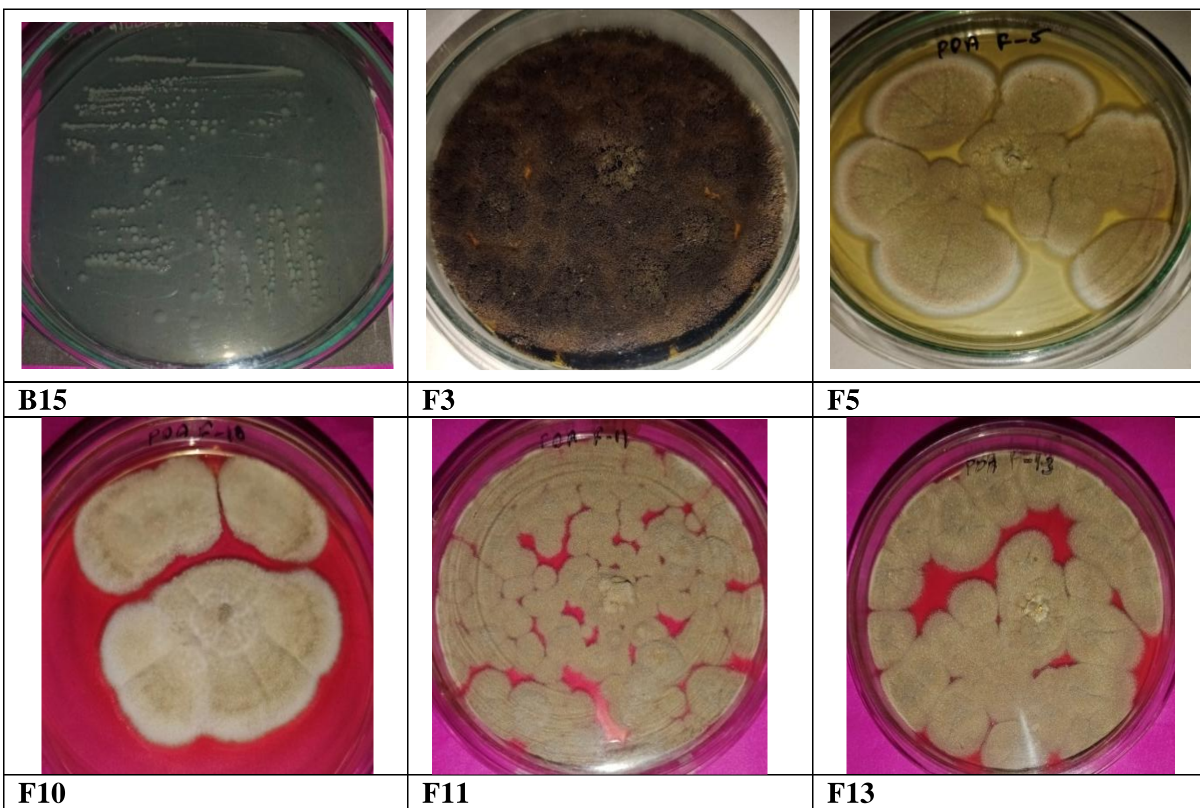
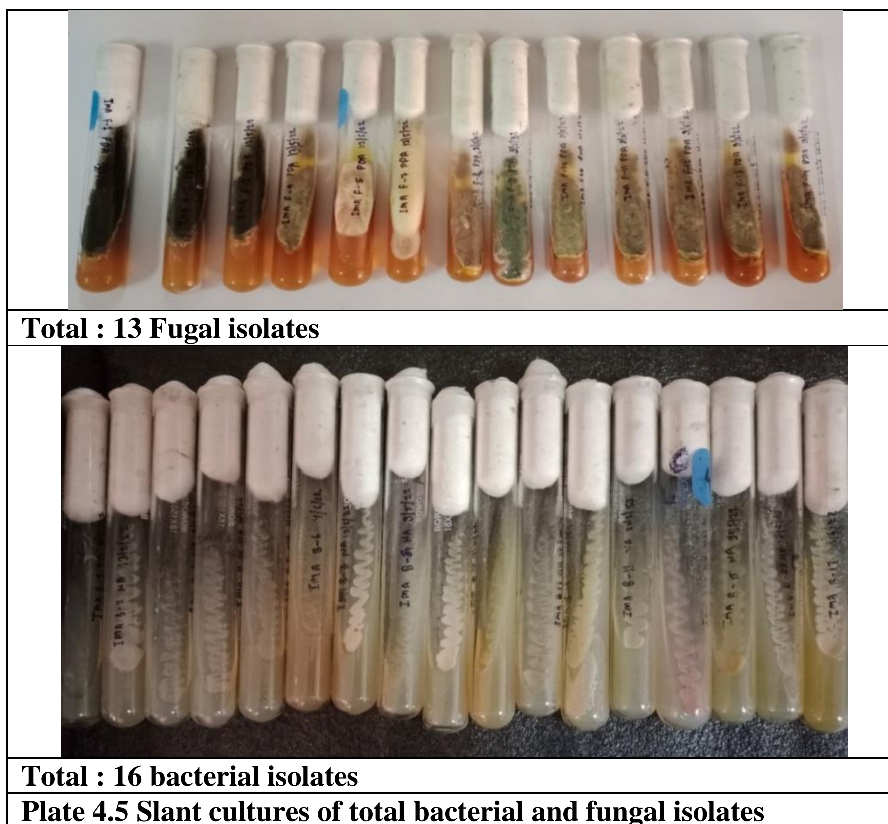


Plate 4.4 Colony morphology of pure cultures of selected imazethapyr degrading isolates

All bacterial and fungal pure cultures were properly labeled and preserved in slants (Plate 4.5) made of potato dextrose agar and nutrient agar, respectively at 4°C for further studies.



Total : 13 Fugal isolates

Total : 16 bacterial isolates

Plate 4.5 Slant cultures of total bacterial and fungal isolates

4.7.1. Screening microbial isolates for bioremediation

All the pure cultures of bacteria (16) and fungi (13) were further screened for tolerance and growth in presence of commercial grade imazethapyr at different concentrations (500-2000 µg ml^{-1}) in broth medium under laboratory condition. All tested bacterial isolates could able to survive at all tested concentrations (Fig. 4.7 and Plate 4.6).

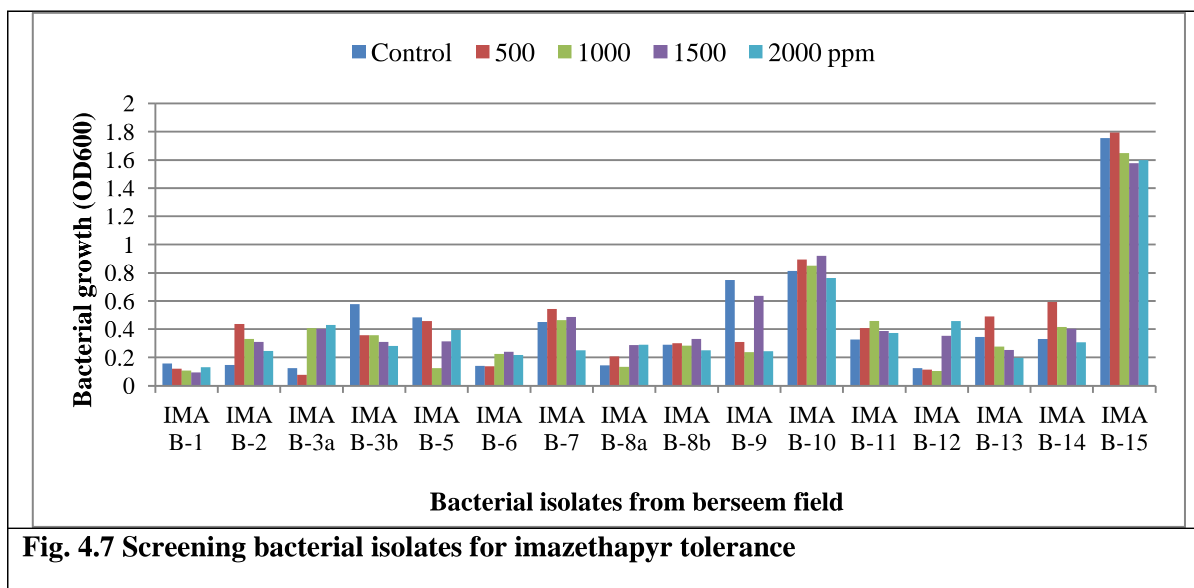


Fig. 4.7 Screening bacterial isolates for imazethapyr tolerance

However, only two bacterial isolates (IMA B-10 and B-15) could grow luxuriously (turbidity 0.8 and 1.6 at OD_{600}) in the higher concentration i.e. up to 2000 µg ml^{-1}. Maximum tolerance and growth was shown by bacterial isolate, B-15, which was selected for further studies. Huo *et al*. (2011) reported that the strain S181 had strong ability to degrade imazethapyr and could grow using imazethapyr as the sole nitrogen.

Plate 4.6 Screening bacterial isolates for imazethapyr tolerance

Similarly, all fungal isolates tested for their growth in presence of imazethapyr had shown growth based on their mycelia dry weight at all the concentrations (Fig. 4.8 and Plate 4.7).

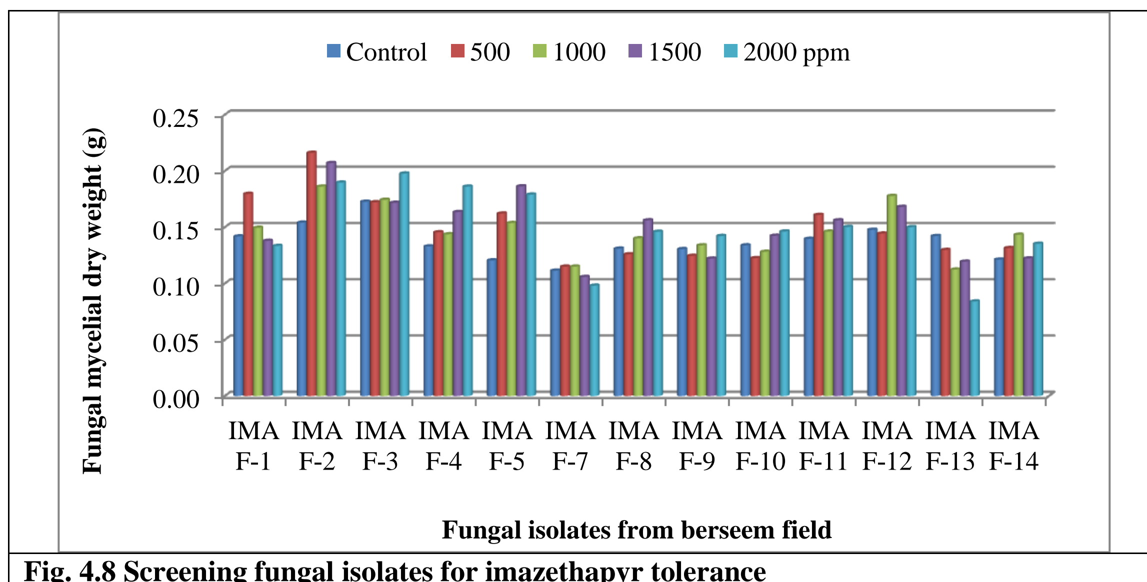


Fig. 4.8 Screening fungal isolates for imazethapyr tolerance

Fungal isolates mycelia dry weight recorded ranged from 0.1 to 0.2 g per 50 ml broth medium. All fungi recorded minimum about 0.1 g at 2000 µg ml^{-1} except IMA F-13.

Plate 4.7 Screening fungal isolates for imazethapyr tolerance

About 5 fungal isolates (IMA F-3, F-5, F-10, F-11 and F-13) were selected for further studies regarding bioremediation of imazethapyr and characterization.

4.7.2 Testing the compatibility between selected isolates

A total of 6 isolates (1-bacteria and 5-fungi) were selected based on their tolerance to imazethapyr and growth and degradation ability. All these selected isolates were tested for compatibility between them (Table 4.8 and Plate 4.8) so as to formulate consortium of isolates for rapid degradation of imazethapyr.

Table 4.8 Testing the compatibility between selected isolates

Isolates	F-3	F-5	F-10	F-11	F-13	B-15
F-3	++	--	--	--	++	++
F-5	--	++	--	--	--	--
F-10	--	--	++	++	++	++
F-11	--	--	++	++	++	++
F-13	++	--	++	++	++	++
B-15	--	--	++	++	++	++

The results of compatibility tests revealed that F3 was compatible with F13 and B15; F10 was compatible with F11, F13 and B15; F11 with F10, F13 and B15; F13 with F3, F10, F11 and B15; B15 with F10, F11 and F13. F5 was not compatible with any isolate. Compatibility between microbes is generally tested to use in formulation of more than microorganism so as to avoid any antagonistic activities. Sun *et al*. (2020) reported that

compatible combination of bacteria (*Alcaligenes faeca*lis) with biochar is an attractive and efficient approach for remediation of pesticide (tebuconazole)-contaminated soil and improvement of soil biological health. Darma *et al*. (2019) performed purity testing on the four diesel degrading bacterial isolates (*Bacillus subtilis, Staphylococcus aureus, Micrococcus roseus* and *Rhodococcus* species) and tested compatibility by cross-spreading them on nutrient agar prior to consortia formulation that can degrade diesel in polluted environment. Consortia formulation was made using bacteria resting cells in Phosphate Buffer Saline based on compatibility testing and mathematical permutations.

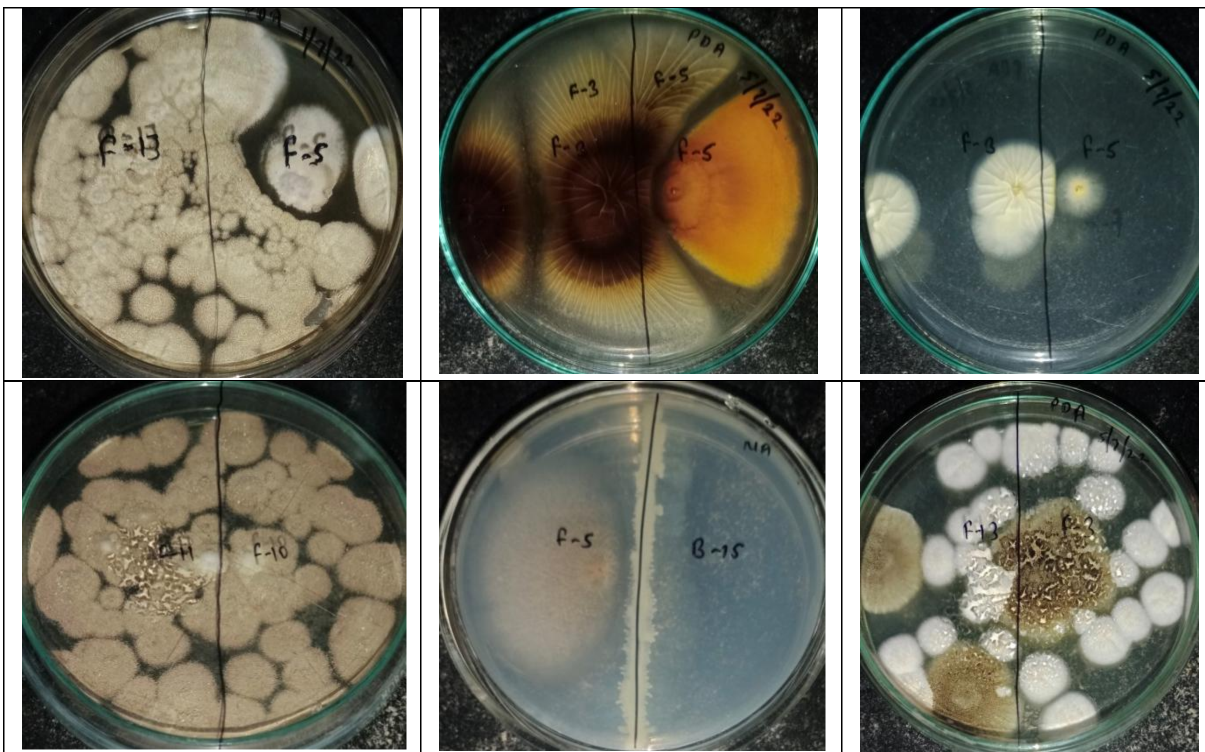


Plate 4.8 Testing the compatibility between selected isolates

4.7.3. Characterization of selected isolates

Morphological observation of selected isolates

The colony morphological features (Plate 4.4) and microscopic morphological features (Plate 4.9) of cell/sporangiophores of the selected microbial isolates were studied for identification of the isolates. The bacteria (B15) appeared as small, circular, raised colonies with translucent (from nearly colorless or creamy) colony. All fungal colonies appeared as white cottony, raised, circular colonies with uniform margin except F3, which appeared dark brown to black spores. Bacterial cell morphology was gram negative rod shaped cells.

All fungal sporangiophores seemed globose vesicle with phialides attached and releasing colorless spores.

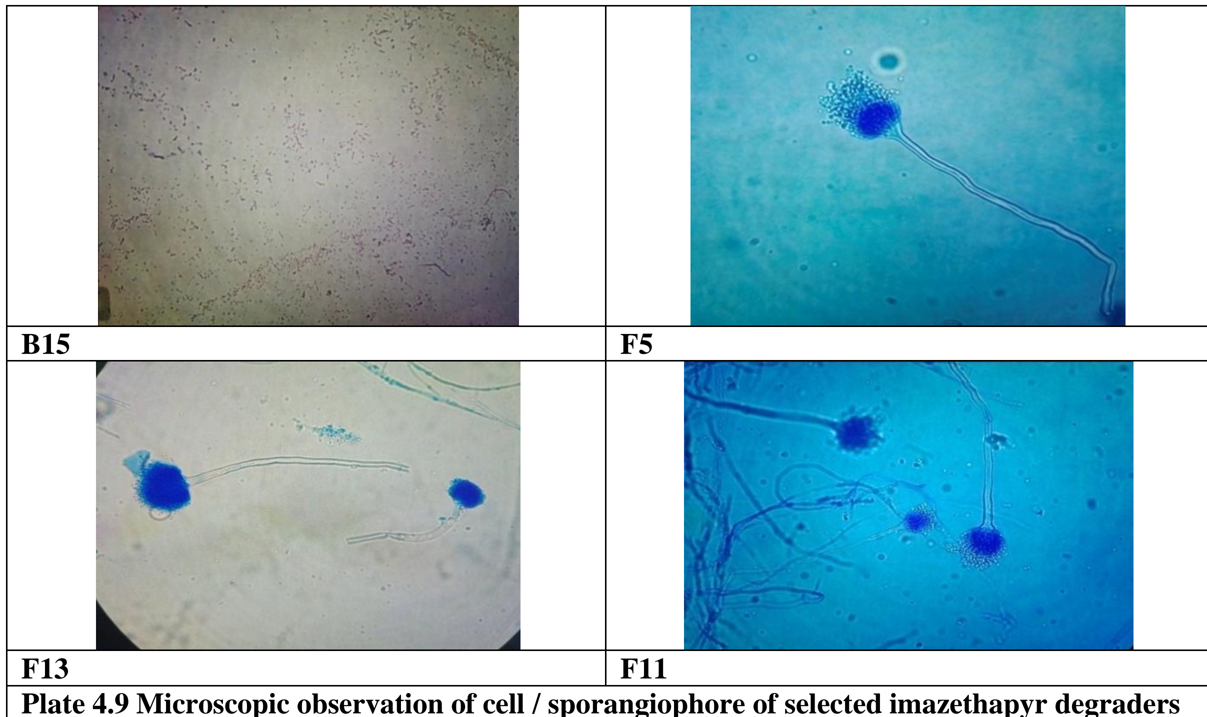


Plate 4.9 Microscopic observation of cell / sporangiophore of selected imazethapyr degraders

Identification of bacterial isolate using BIOLOG id system

The bacterial isolate (B-15) was grown in NA plate over night. The o/N grown colony was suspended in the buffer for bacterial suspension up to the recommended concentration and was inoculated in the 96 well bacterial id plate and was read after 24 hours and it was found that the bacteria was Gram negative, aerobic, rod probably belong to *Xanthomonas* or *Stenotrophomonas*. Digvijay *et al*. (2017) also carried out biochemical and molecular tests for identification of the isolated imazethapyr degrading microorganisms.

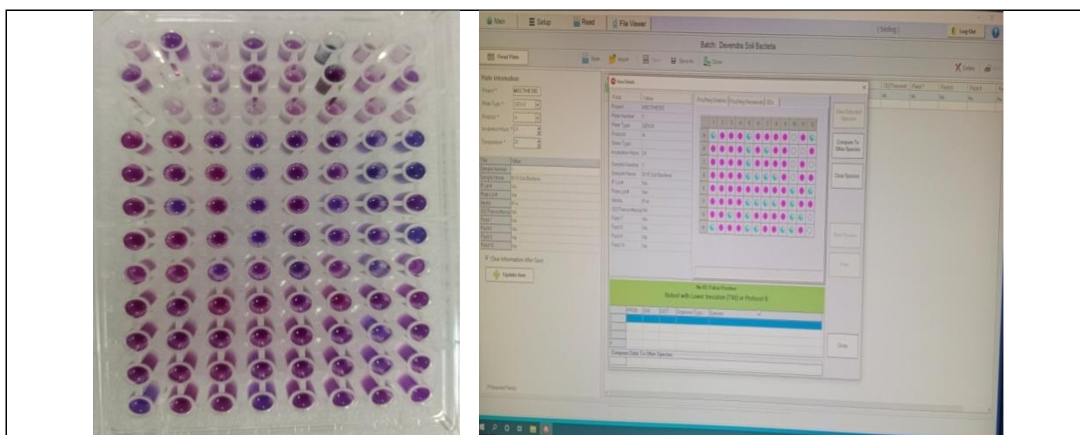


Plate 4.10 Identification of bacterial isolate (B15) using BIOLOG id system

Molecular characterization of selected isolates

For identification of the isolates, the genomic DNA was isolated from both bacterial (B15 and all selected fungal isolates (IMA F-3, F-5, F-10, F-11 and F-13). The internal transcribed spacer 1 (ITS1) and ITS2 regions and the 5.8S ribosomal DNA (rDNA) region of the fungi were amplified by using universal primers ITS1 and ITS4 and it was visualized in the agarose gel electrophoresis as about 600 bp (Plate 4.11, lanes 2-6). Similarly, the 16S rDNA gene sequences were amplified using universal 16S rRNA gene primers ans was visulaized as 1.5 kb band on agarose gel (Plate 4.11, lane 8).

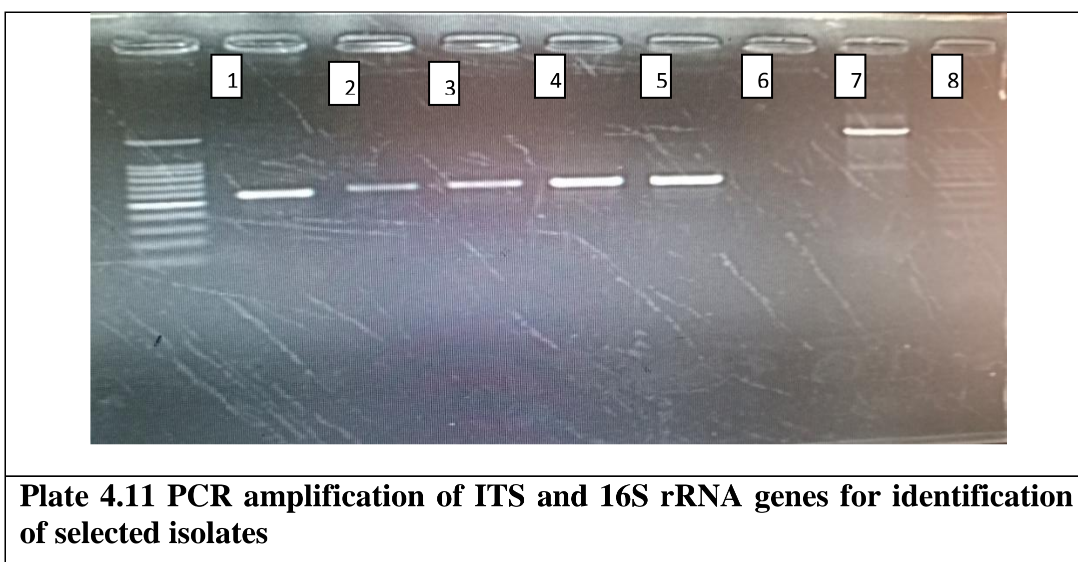


Plate 4.11 PCR amplification of ITS and 16S rRNA genes for identification of selected isolates

The amplified PCR products were sequenced using Sanger sequencing method by outsourcing. The isolates were identified based on 16S rDNA sequences and ITS1-ITS4 gene sequences homology search in NCBI BLAST similarity results and are given in Table 4.9. Following biochemical and especially molecular characterization methods for identification of various microbial isolates has become common approach recently. Huo *et al*. (2011) identified imazethapyr degrading bacteria belong to genus *Streptomyces omiyaensis* according to the physiological and biochemical properties as well as 16S rRNA sequence analysis.

Table 4.9 Identification of selected isolates based on NCBI BLAST similarity

Microbial isolate	Identified as
B15	*Stenotrophomonas maltophilia*
F3	*Aspergillus niger*
F5	*Aspergillus terreus*
F10	*Aspergillus terreus*
F11	*Aspergillus citrinoterreus*
F13	*Aspergillus terreus*

Sondhiya *et al*. (2016) identified *Aspergillus flavus* and *Aspergillus niger* and found that penoxsulam was degraded at a faster rate in the soil inoculated with *A. flavus* than *A. niger*. And reported that *Aspergillus flavus* and *Aspergillus niger* can enhance microbial degradation of penoxsulam in the soil. Sharma *et al*. (2012) reported that *Aspergillus niger* survived in the minimal broth having chlorimuron at the level of 200 mg per 100 mL media and it degraded the herbicide to harvest energy. El-Metwally *et al*. (2019) isolated a new fungal strain capable of imidacloprid degradation from agricultural wastewater drain. The fungal strain of YESM3 was identified as *Aspergillus terreus* based on ITS1-5.8S rDNA-ITS2 gene sequence by PCR amplification of a 500 bp sequence. Two autochthonous microbial strains, *Methylobacterium populi* VP2 and *Aspergillus sydowii* VP4, were isolated from a soil of a highly contaminated industrial site and used to degrade the aqueous extract of contaminants (aqueous extract of contaminants) obtained from the same polluted soil (Sannino *et al*., 2016). Ozdal *et al*. (2017) isolated *Stenotrophomonas maltophilia* OG2 from the intestine of cockroaches that was collected from a cow barn contaminated some pesticides belong to pyrethroid and organochlorine groups. *S. maltophilia* OG2 degraded α-endosulfan via a hydrolysis pathway and indicated that strain OG2 may have potential use in the biodegradation of pesticides contaminated environments. Zang *et al*. (2016) reported that *Stenotrophomonas maltophilia* D310-3 exhibited a high chlorimuron-ethyl-degrading capability.

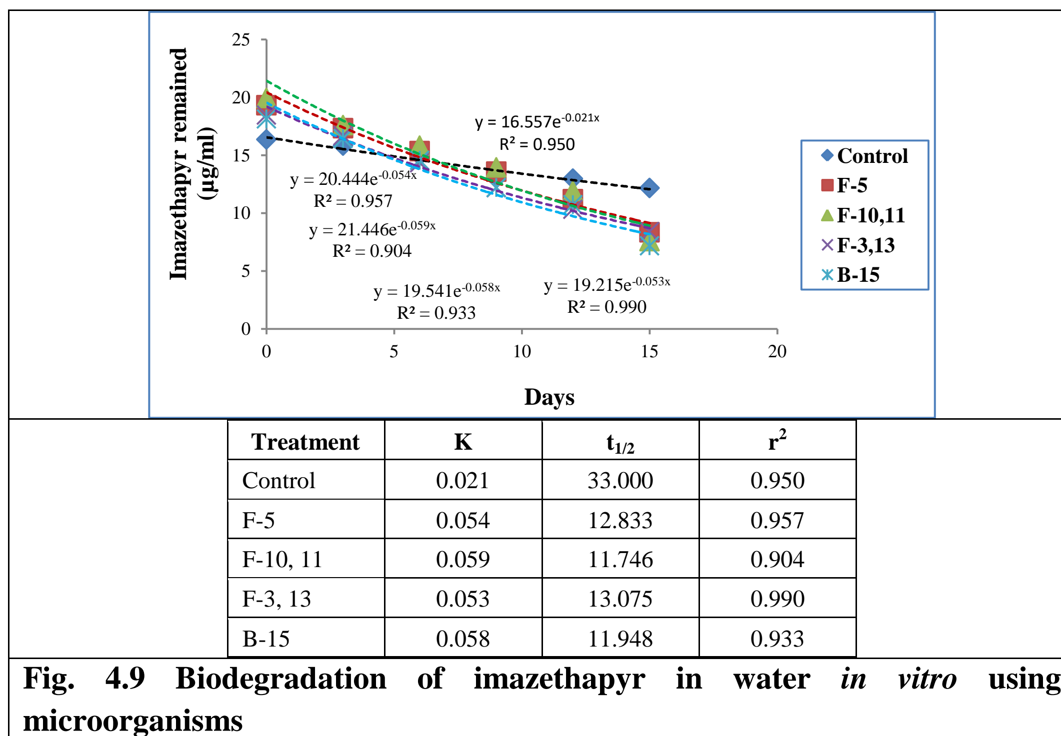
4.8. Biodegradation of imazethapyr in water *in vitro* using microorganisms

The ability of selected microorganisms to degrade imazethapyr incorporated @ 25 µg ml^{-1} in sterile MSM broth i.e. water based system was studied *in vitro* using actively growing inoculum sources up to 15 days. Percentage degradation of imazethapyr by selected cultures and consortia ranged from 66.4-71.32 wherein the bacterial isolate, B15 recorded the maximum degradation in 15 days (Table 4.10).

Table 4.10 Biodegradation of imazethapyr in water *in vitro* using microorganisms

Days	Per cent Imazethapyr degraded by microorganisms in water				
	Control	**F5**	**F10-F11**	**F3-F13**	**B15**
0	34.56	22.72	20.2	26.12	27.44
3	36.68	30.64	29.6	33.24	34.24
6	40.16	38.6	36.68	42.6	41.76
9	47.88	45.68	44.2	51.24	51.16
12	48.08	55.2	52.36	58.68	57.12
15	51.32	66.64	69.72	66.4	71.32

The consortium F10-F11 (11.75 d) recorded shortest half life ($T_{1/2}$) followed by B15 (11.95 d) which were significantly superior to control (33 d) (Fig. 4.9). The $T_{1/2}$ of other fungus F5 was 12.83 d and of consortium F3-F13 was 13.08 d. Kumar and Singh (2016) reported that enrichment culture was able to degrade only hydroxyatrazine, and it was used as the sole source of carbon and nitrogen. Hydroxyatrazine degradation slowed down when sucrose and/or ammonium hydrogen phosphate were supplemented as the additional sources of carbon and nitrogen, respectively. The enrichment culture could degrade high concentrations of atrazine (up to 110 µg ml^{-1}) in mineral salts medium, and neutral pH was optimum for atrazine degradation.



Treatment	K	$t_{1/2}$	r^2
Control	0.021	33.000	0.950
F-5	0.054	12.833	0.957
F-10, 11	0.059	11.746	0.904
F-3, 13	0.053	13.075	0.990
B-15	0.058	11.948	0.933

Fig. 4.9 Biodegradation of imazethapyr in water *in vitro* using microorganisms

4.9. Biodegradation of imazethapyr in different soils *in vitro* by microorganisms

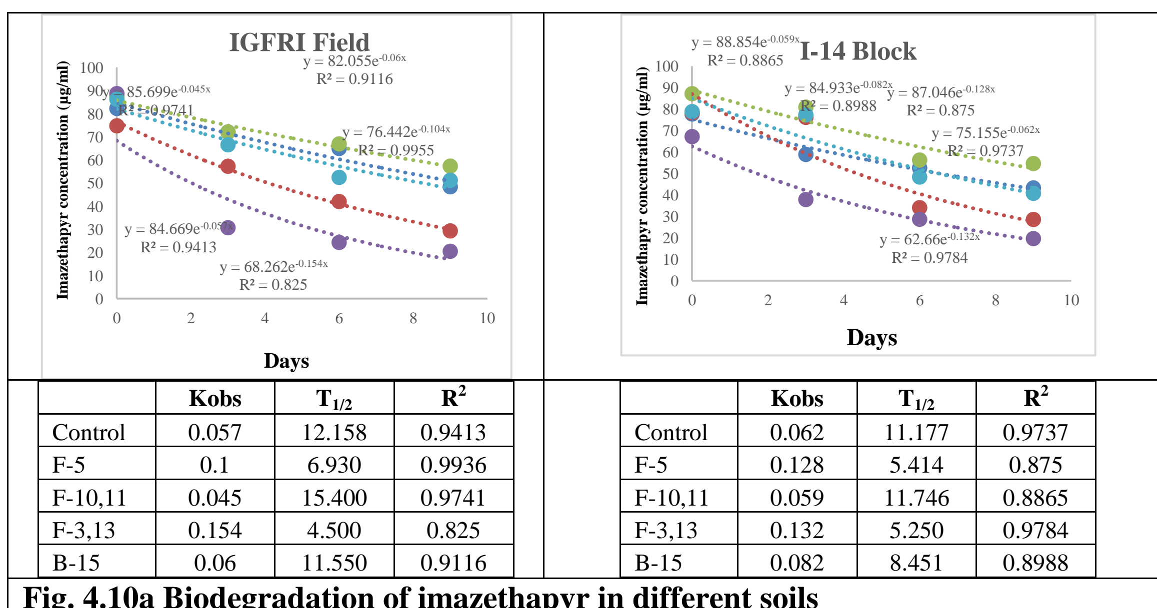
The selected organisms and consortia were also tested for degradation of imazethapyr applied @ 100 µg g^{-1} in 3 different Bundelkhand soils collected from different sites *viz.*, S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block, respectively. All the data presented in Table 4.10 was significant. The imazethapyr degraded in soil S1 ranged from 42.74 to 79.6% among the microbial treatments (Table 4.11). Fungal isolate F5 and F3-F13 consortium recorded 70.8 and 79.6% degradation in 9 days which were far better than F10-F11 (42.74%) and B15 (48.84%). This could be due to the variations in adaptability, survival and dominance of the introduced culture over native microbes present in the soil.

Table 4.11 Biodegradation of imazethapyr in different soils *in vitro* by microorganisms

	Imazethapyr degraded in soil S1 (%)				
Days	**Control**	**F5**	**F10-F11**	**F3-F13**	**B15**
0	17.8	25.3	12.6	11.38	13.81
3	27.99	42.8	28.0	69.37	33.5
6	35.1	58.05	33.13	75.73	47.61
9	41.66	70.75	42.74	79.57	48.84
	Imazethapyr degraded in soil S2 (%)				
0	22.33	21.85	12.89	32.88	21.24
3	31.19	23.91	19.16	62.21	22.75
6	37.5	66.01	43.81	71.41	51.72
9	46.82	71.5	45.43	80.41	59.32
	Imazethapyr degraded in soil S3 (%)				
0	22.5	23.06	17.88	23.15	16.4
3	32.37	37.0	35.5	37.26	33.62
6	39.59	78.95	42.82	52.49	45.93
9	44.47	90.84	52.56	67.52	53.24

*S1: IGFRI CR farm; S2: RLBCAU - I_{14} Block; S3: RLBCAU - H Block

The imazethapyr degraded in soil S2 ranged from 45.43 to 80.41% among the microbial treatments in 9 days (Table 4.10). Similar to soil S1, Fungal isolate F5 and F3-F13 consortium recorded 71.5 and 80.41% degradation in 9 days in soil S2, which were significantly higher than F10-F11 (45.43%) and B15 (59.32%).

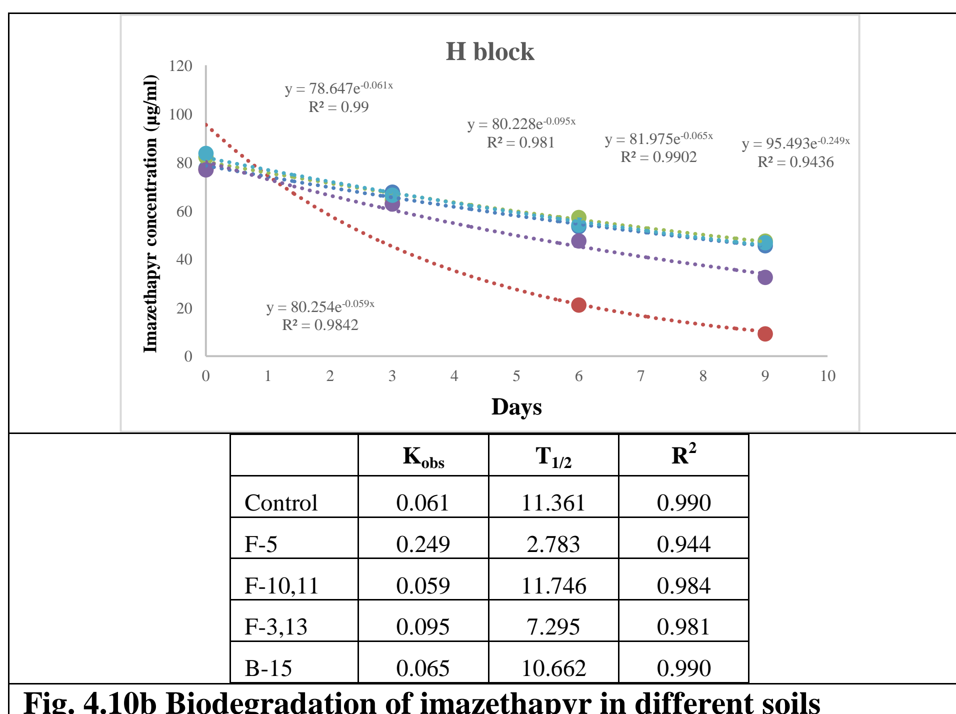


	Kobs	$T_{1/2}$	R^2
Control	0.057	12.158	0.9413
F-5	0.1	6.930	0.9936
F-10,11	0.045	15.400	0.9741
F-3,13	0.154	4.500	0.825
B-15	0.06	11.550	0.9116

	Kobs	$T_{1/2}$	R^2
Control	0.062	11.177	0.9737
F-5	0.128	5.414	0.875
F-10,11	0.059	11.746	0.8865
F-3,13	0.132	5.250	0.9784
B-15	0.082	8.451	0.8988

Fig. 4.10a Biodegradation of imazethapyr in different soils

The biodegradation of imazethapyr was followed the same trend in soil S3 as well. Fungal isolate F5 performed maximum degradation (90.84%) in 9 days of incubation, which was the highest among all microbial treatments in all 3 soils. However, the degradation by F3-F13 consortium was comparatively lesser in S3 as compared to S1 and S2 soils. This could be due significantly short half-life recorded by F5 at S3 soil compared S1 and S2 soils (Fig. 4.10a and b) and vice versa with F3-F13 consortium (Fig. 4.10a and b). Our results are supported by various reports regarding consortium and pure culture degradation of herbicides. A microbial consortium, mainly containing the genera *Bacillus, Phyllobacterium, Pseudomonas, Rhodococcus* and *Variovorax,* could use azimsulfuron as the sole nutrient source, degrading the herbicide better together than what was achieved using isolated pure cultures. This is likely due to complementary (synergistic) metabolism among bacterial consortia members for the degradation of the herbicide (Valle *et al*., 2006). Cantwell *et al*. (1989) reported that imidazolinone microbial degradation was regulated by the amount of herbicide in soil solution as determined by soil characteristics.



	K_{obs}	$T_{1/2}$	R^2
Control	0.061	11.361	0.990
F-5	0.249	2.783	0.944
F-10,11	0.059	11.746	0.984
F-3,13	0.095	7.295	0.981
B-15	0.065	10.662	0.990

Fig. 4.10b Biodegradation of imazethapyr in different soils

Effect of contaminants concentration on imazethapyr bioremediation

4.10. Preparation of alginate beads based bioformulation

Based on compatibility tests four alginate beads based microbial formulations were developed (Plate 4.12). Two formulations had single pure cultures separately (F5 and B15) and two formulations had dual culture consortium in alginate beads *viz*., F10 and F11 in single alginate bead and F3 and F13 in single alginate bead.

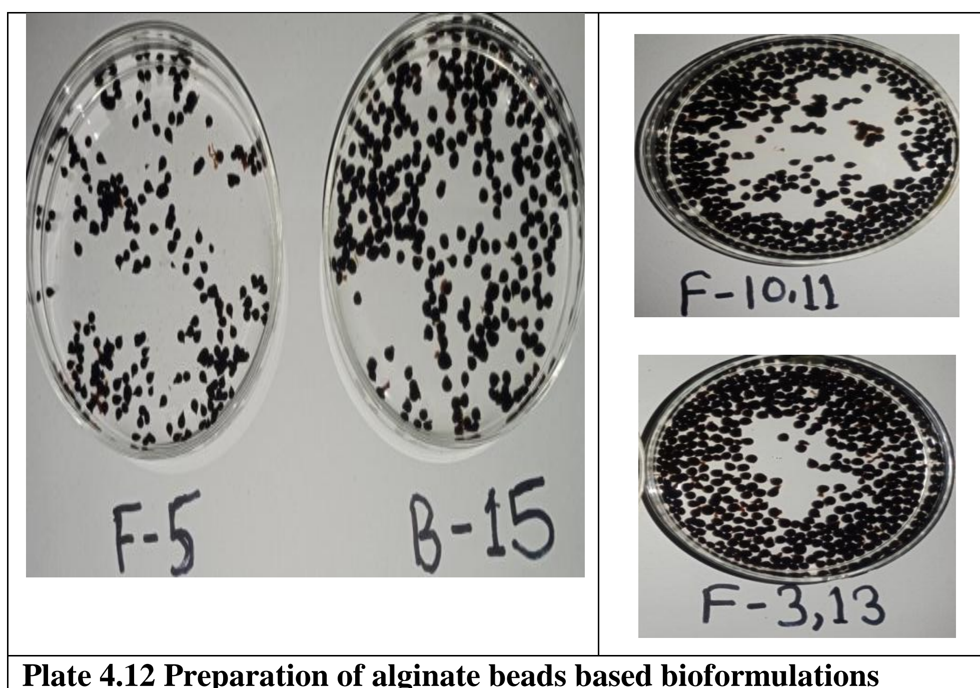


Plate 4.12 Preparation of alginate beads based bioformulations

The microbial load in the alginate beads was estimated by serial dilution and standard plate count method. B15 alginate bead formulation had maximum number of cells (>$1x10^7$ cfu/g) followed by F10-F11 and F3-F13 consortia and least was recorded in F5 alginate formulation (Fig. 4.11). Similar formulations using different carrier materials including alginate beads had been reported with many microbial pure culture as well as consortium for biodegradation applications. An atrazine degrading enrichment culture, a consortium of bacteria of genus *Bacillus* along with *Pseudomonas* and *Burkholderia*, was immobilized in sodium alginate and was used to study atrazine degradation in mineral salts medium (MSM), soil and wastewater effluent (kumar *et al*., 2017).

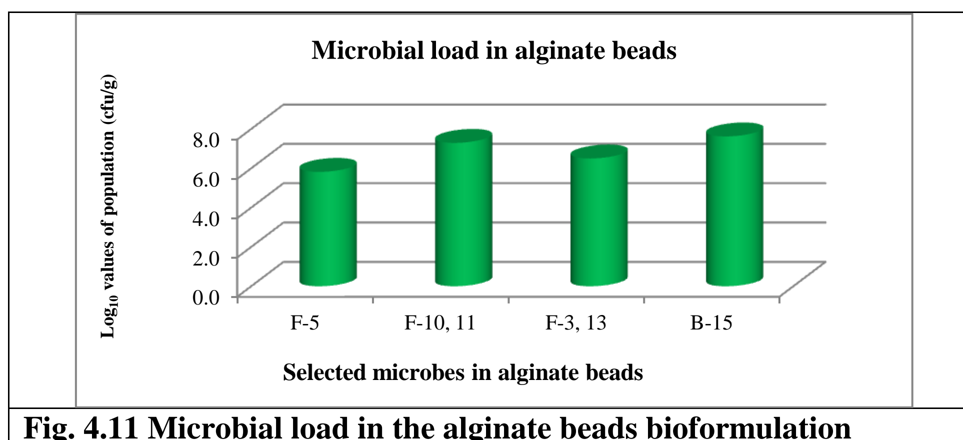


Fig. 4.11 Microbial load in the alginate beads bioformulation

4.11. Biodegradation of imazethapyr by alginate bead bioformulation

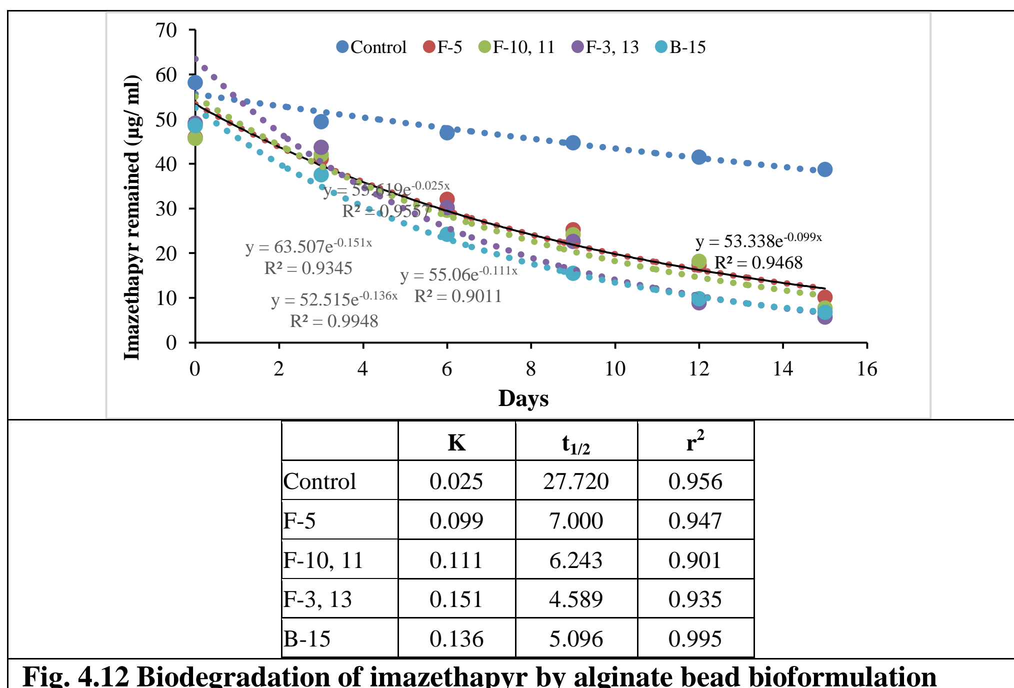
The alginate bead based bioformulations were evaluated for degradation of imazethapyr in vitro in MSM medium incorporated with imazethapyr @ 75 µg ml^{-1}. The results revealed that all bioformulations could degrade >85% imazethapyr in 15 days (Table 4.12). The consortium F3-F13 degraded the maximum (92.41%) followed by bacterial isolate B15 (90.96%), F10-F11 (89.72%) and F5 (86.52%).

Table 4.12 Biodegradation of imazethapyr by alginate bead bioformulation

	Per cent Imazethapyr degraded by alginate beads bioformulation				
Days	**Control**	**F5**	**F10-F11**	**F3-F13**	**B15**
0	22.05	38.8	39.10	34.57	35.29
3	34.73	45.17	43.97	41.77	49.96
6	36.88	57.26	60.54	59.77	67.72
9	39.78	66.36	67.85	69.84	79.34
12	44.13	76.93	75.77	88.14	86.93
15	46.26	86.52	89.72	92.41	90.96

Compared to previous results, F5 performed slightly lesser in this experiment, which could be due to lesser inoculums load of F5 in alginate beads compared to other formulations. Bacterial isolate B5 also shown better performance in liquid and sterile medium than soil, which might be due to poor survival and competing ability with soil microflora. The half-life ranged from 4.59 to 7.0 d among bioformulations (Fig. 4.12). Many bioremediation strategies are based on metabolic processes of isolated bacteria and sometimes fungi.

However, some factors may hinder the application of this microbiota to many environments. One issue is that the metabolic processes may depend on communication among microbial communities organized in biofilms and may depend on quorum sensing. *C. testosteroni, H. sulfonivorans* and *Variovorax* spp. cooperate in biofilm structures in soil for the synergistic degradation of the herbicide linuron. None of these species alone was able to degrade linuron (Flemming *et al*., 2016). Thus, the speed of this process may differ between isolated strains and those in communities. Degradation ability of the microbial consortium on fungicide (carbendazim) and herbicide (2, 4-dichlorophenoxyacetic acid: 2, 4-D) was increased by immobilization on loofa (*Luffa cylindrica*) sponge in comparison with that of free-living consortium Pattanasupong *et al.* (2004). Kumar *et al*. (2017) reported that the effect of number of beads on atrazine degradation in MSM suggested that degradation was affected by the number of beads inoculated and degradation increased with increase in the number of beads. On 6th day of incubation 18, 71 and 81% atrazine degradation was observed in the MSM inoculated with 10, 30 and 50 beads. It took around 10 days for ~90% atrazine degradation in the medium inoculated with 10 beads while in medium inoculated with 30 and 50 beads, atrazine concentration reached below detectable levels on 8th day.



	K	$t_{1/2}$	r^2
Control	0.025	27.720	0.956
F-5	0.099	7.000	0.947
F-10, 11	0.111	6.243	0.901
F-3, 13	0.151	4.589	0.935
B-15	0.136	5.096	0.995

Fig. 4.12 Biodegradation of imazethapyr by alginate bead bioformulation

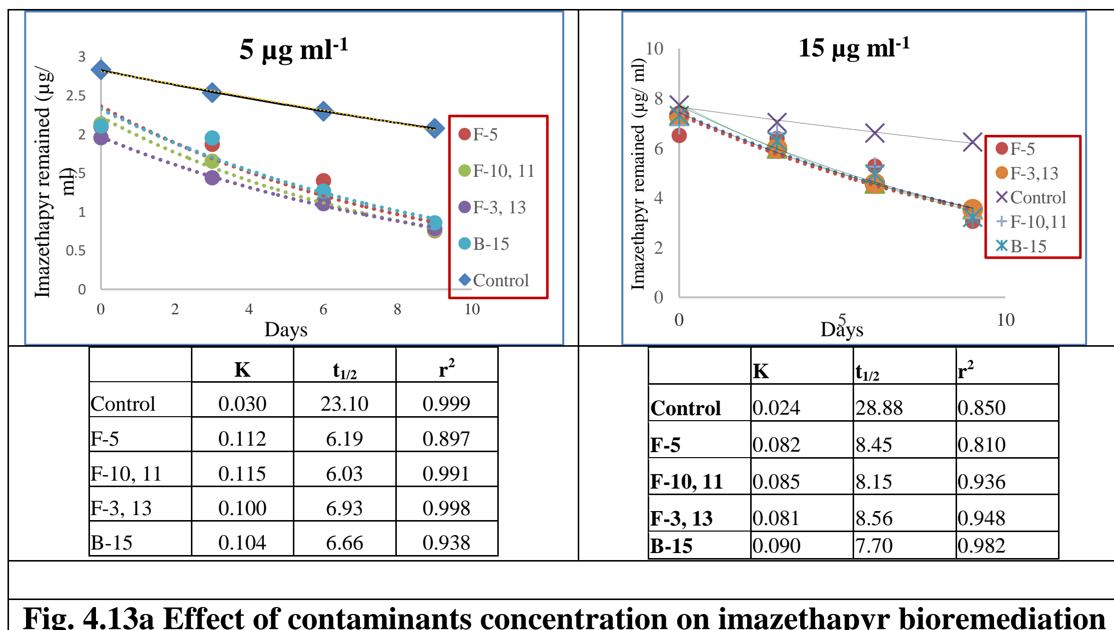
4.12. Effect of contaminants concentration on imazethapyr bioremediation

The effect of contaminants concentration on imazethapyr biodegradation was also studied in vitro in sterile liquid medium. Microorganisms use chemical contaminants as an energy source through their metabolic processes throughout the microbiological process. However, excessive amounts of inorganic nutrients in soil cause microbial inhibition (Ahirwar *et al.*, 2016). Khan *et al.* (1997) pointed out that the control and optimization of bioremediation processes are complex factors. These factors include the presence of a microbial population proficient in degrading pollutants, the availability of contaminants to the microbial population, and environmental factors like soil type, temperature, pH, and presence of oxygen or other electron acceptors, and nutrients. In the present study, all bioformulation treatments recorded similar level of degradation (82.82-84.86%) in 9 days with 5 µg ml^{-1}contaminant concentration, 76.4-79.6% in 9 days with 15 µg ml^{-1} contaminant concentrations, 67.68-72.9% in 9 days with 25 µg ml^{-1} contaminant concentration (Table 4.13).

Table 4.13 Effect of contaminants concentration on imazethapyr bioremediation

Days	Imazethapyr degraded at 5 µg ml^{-1} contamination (%)				
	Control	**F5**	**F10-F11**	**F3-F13**	**B15**
0	43.3	58.06	57.3	60.86	57.82
3	49.22	62.56	66.92	71.18	60.92
6	54.02	71.96	77.26	77.88	74.62
9	58.44	84.86	84.74	84.3	82.82
	Imazethapyr degraded at 15 µg ml^{-1} contamination (%)				
0	48.44	56.61	53.8	51.37	51.26
3	53.13	57.44	56.03	60.11	58.14
6	55.97	64.84	64.98	69.50	66.94
9	58.38	79.64	78.70	76.40	78.58
	Imazethapyr degraded at 25 µg ml^{-1}contamination (%)				
0	28.88	35.90	44.47	37.08	39.31
3	34.60	48.18	53.35	41.12	47.996
6	36.82	52.21	63.41	54.768	59.61
9	45.77	68.65	72.9	67.68	72.63

Similarly half-life was also recorded at par with all microbial formulations ranging from 6.03-6.93 d at 5 µg ml^{-1}, 7.70-8.56 d at 15 µg ml^{-1} and 7.88-9.36 d at 25 µg ml^{-1} concentration (Fig. 4.13a and b).

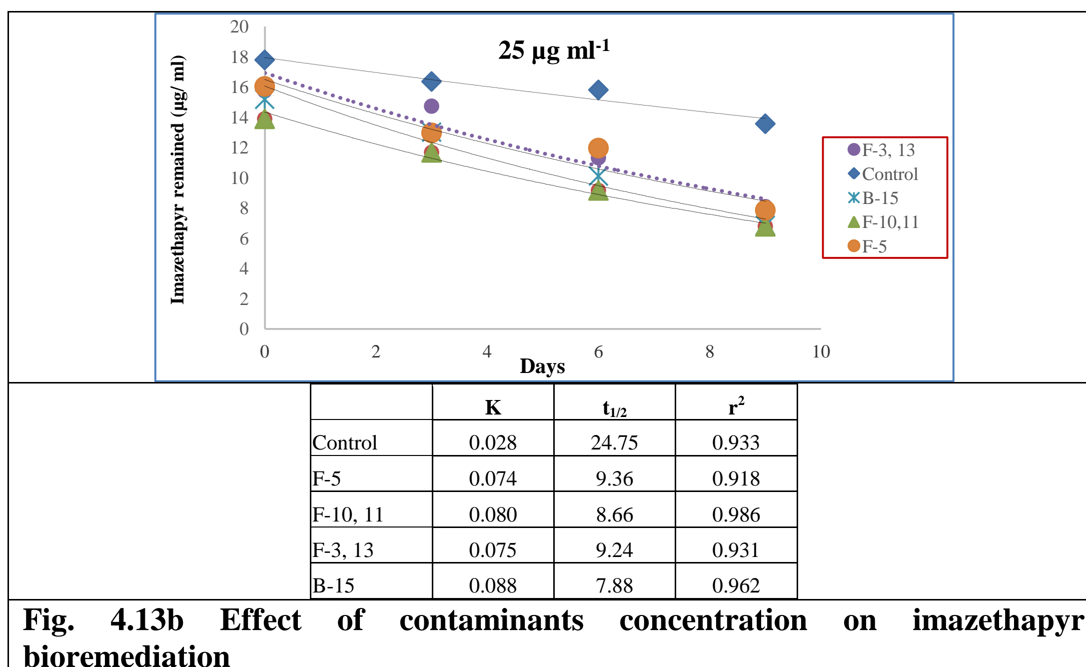


	K	$t_{1/2}$	r^2
Control	0.030	23.10	0.999
F-5	0.112	6.19	0.897
F-10, 11	0.115	6.03	0.991
F-3, 13	0.100	6.93	0.998
B-15	0.104	6.66	0.938

	K	$t_{1/2}$	r^2
Control	0.024	28.88	0.850
F-5	0.082	8.45	0.810
F-10, 11	0.085	8.15	0.936
F-3, 13	0.081	8.56	0.948
B-15	0.090	7.70	0.982

Fig. 4.13a Effect of contaminants concentration on imazethapyr bioremediation

There was a gradual increase in half-life values with increase in contaminant concentration in the medium. The lowest half-life was recorded by F10-F11 at 5 µg ml^{-1}, B15 at 15 µg ml^{-1} and 25 µg ml^{-1}, respectively.



	K	$t_{1/2}$	r^2
Control	0.028	24.75	0.933
F-5	0.074	9.36	0.918
F-10, 11	0.080	8.66	0.986
F-3, 13	0.075	9.24	0.931
B-15	0.088	7.88	0.962

Fig. 4.13b Effect of contaminants concentration on imazethapyr bioremediation

Since pollutants rarely exist singularly in contaminated environment, effective biodegradation requires the employment of a consortium (aggregate/group) of microorganisms, each capable of degrading one or more of the constituents of the contaminants or the use of a microorganism capable of degrading all the pollutants, with the latter being rare to find and the former desirable in biodegradation scenarios (Lee *et al*., 2019).

Findings of this study reveal that certain isolates have good potential to be developed as bioagents for biodegradation of imazethapyr. However, further characterization of studied potential isolates for degradation of other possible contaminants in environment and various other factors controlling biodegradation may be carried out for detailed understanding. Also the formulation has to be tested widely both in lab and environment before developed into a product for large scale applications.

5. Summary and Conclusions

The study comprised laboratory and field experiments on "Bioremediation of imazethapyr in soil/water system" was conducted at ICAR-IGFRI, Jhansi during 2021-22. The salient findings of the study are presented in this chapter.

Growth and yield parameters

Plant height in control plots ranged from 18.33 to 34.03 cm and imazethapyr applied plots it ranged from 21.0-35.77 cm. Root length in control plots ranged from 6.47-8.63 cm while in imazethapyr applied plots it ranged from 7.73-9.57 cm. The berseem shoot fresh weight recorded ranged from 1.97 (S3) to 5.89 g/plant (S2) in control and 2.33 (S3) to 7.88 g/plant (S2) in imazethapyr applied plots. Root fresh weight ranged from 0.61 (S3) to 0.857 (S1) g/plant in control soils while it ranged from 0.613 (S3) to 0.857 (S1) g/plant in imazethapyr applied soils.

Number of root nodules per plant in berseem ranged from 16 to 22.7 in control plots and 14.3 to 19.0 in imazethapyr treated plots. Chlorophyll (SPAD values) ranged from 35.28 (S1) to 36.96 (S2) in control plots and 34.54 (S1) to 36.62 (S3) in imazethapyr treated plots.

Berseem green fodder yield (GFY) obtained from two cuts (late sown) ranged from 33.39 (S3) to 39.96 (S2) t/ha in imazethapyr treated plots and 28.03 (S3) to 33.16 (S2) t/ha in control plots.

The maximum weed species (5, 3 and 2) and number (74, 62 and 48) were recorded in S2, S3 and S1 respectively in control plots. Whereas in imazethapyr applied plots, maximum weed species (3, 2 and 2) in (S3, S2 and S1), respectively and number (28, 22 and 18) in S3, S1 and S2, respectively.

Soil physico-chemical properties

The pH of soils ranged from 7.21 (S1) to 7.47 (S3) in control plots and 7.09 (S1) to 7.64 (S3) in imazethapyr treated plots. EC ranged from 0.013 (S1) to 0.021 (S3) in control plots and 0.015 (S2) to 0.023 (S1) in imazethapyr treated plots. Bulk density (BD) ranged from 1.34 (S2) to 1.49 (S3) in control plots and 1.36 (S2) to 1.48 (S3) in imazethapyr treated plots. Soil organic carbon (SOC) ranged from 0.51 (S1) to 0.63 (S2) in control plots and 0.52 (S1) to 0.63 (S2) in imazethapyr treated plots.

Soil nutrient status

The available N content ranged from 205.4 (S1) to 241.5 (S2) kg/ha in control plots as compared to imazethapyr treated plots [206.8 (S1) to 252.7 (S2) kg/ha]. The available P content ranged from 7.8 (S3) to 8.65 (S2) kg/ha in control plots as compared to imazethapyr treated plots [8.03 (S3) to 8.95 (S2) kg/ha]. The available K ranged from 244.1 (S3) to 266.3 (S2) kg/ha in control as compared to imazethapyr plots [244.2 (S3) to 263.6 (S2) kg/ha].

Soil texture

The sand, silt and clay contents of the soil were 61.12%, 22% and 16.88% in S1 (IGFRI CR farm), 45.12%, 27% and 27.88% in S2 (RLBCAU - I14 Block) and 57.12, 23 and 19.88% in S3 (RLBCAU - H Block) soils and were classified as sandy loam (S1), sandy clay loam (S2) and sandy loam (S3).

Microbiological properties of soil

There was a decline in TBC at 2 days up to 32 days from the original / control soil in all 3 sites. The reduction in population was significant up to 16 days after spray. Reduced total fungal count (TFC) was recorded from 2 to 16 days after imazethapyr spray on berseem at 15 DAS. FDA hydrolysis was affected from 2 to 16 days after herbicide spray and recovered from 32 days. Dehydrogenase (DHA) activity was not significantly affected by the imazethapyr application in all soils except S3 wherein significant reduction of DHA was observed up to 16 days after spray. The SMBC content decreased up to 8 days and started increasing gradually from 16th day after spray in all 3 soil sites.

Isolation and screening of microbial isolates for bioremediation

About 16 bacterial isolates and 13 fungal isolates were obtained as pure cultures from more than 20 soil samples. They were screened for growth in presence of commercial grade imazethapyr at different concentrations (500-2000 ppm) in broth medium. Two bacterial isolates (IMA B-10 and B-15) could grow well (0.8 and 1.6 at OD_{600}) in the higher concentration i.e. up to 2000 ppm. Maximum growth was shown by bacterial isolate, B-15, which was selected for further studies. All fungi recorded minimum about 0.1 g at 2000 ppm. About 5 fungal isolates (IMA F-3, F-5, F-10, F-11 and F-13) were selected for further studies.

Testing the compatibility between selected isolates

A total of 6 isolates (1-bacteria and 5-fungi) were selected and were tested for compatibility between them. Tests revealed that F3 was compatible with F13 and B15; F10 was compatible with F11, F13 and B15; F11 with F10, F13 and B15; F13 with F3, F10, F11 and B15; B15 with F10, F11 and F13. F5 was not compatible with any isolate.

Characterization of selected isolates

Colony and microscopic morphological features of the selected microbial isolates were studied. Bacterial isolate (B-15) was found as Gram negative, aerobic, rod probably belong to *Xanthomonas* or *Stenotrophomonas* by biochemical tests using BIOLOG bacterial id system. For identification of the isolates, the genomic DNA was isolated from both bacterial (B15 and all selected fungal isolates (IMA F-3, F-5, F-10, F-11 and F-13). The internal transcribed spacer sequences by universal primers ITS1 and ITS4 and 16S rDNA gene sequences using universal 16S rRNA gene primers were amplified and PCR products were sequenced.

Biodegradation of imazethapyr in water *in vitro* using microorganisms

Percentage degradation of imazethapyr by selected cultures and consortia ranged from 66.4-71.32 wherein the bacterial isolate, B15 recorded the maximum degradation in 15 days.

Biodegradation of imazethapyr in different soils *in vitro* by microorganisms

The imazethapyr degraded in soil S1 ranged from 42.74 to 79.6% among the microbial treatments. Fungal isolate F5 and F3-F13 consortium recorded 70.8 and 79.6% degradation in 9 days. The imazethapyr degraded in soil S2 ranged from 45.43 to 80.41% among the microbial treatments in 9 days. Fungal isolate F5 and F3-F13 consortium recorded 71.5 and 80.41% degradation in 9 days in soil S2. Fungal isolate F5 performed maximum degradation (90.84%) in 9 days of incubation in S3, which was the highest among all microbial treatments in all 3 soils.

Preparation of alginate beads based bioformulation

Based on compatibility tests four alginate beads based microbial formulations (two formulations had single pure cultures separately (F5 and B15) and two had dual culture consortium *viz*., F10 and F11 in single alginate bead and F3 and F13 in single alginate bead) were developed.

Biodegradation of imazethapyr by alginate bead bioformulation

All bioformulations could degrade >85% imazethapyr in 15 days. The consortium F3-F13 degraded the maximum (92.41%) followed by B15 (90.96%), F10-F11 (89.72%) and F5 (86.52%).

Effect of contaminants concentration on imazethapyr bioremediation

All bioformulation treatments recorded similar level of degradation (82.82-84.86%) in 9 days with 5 µg ml^{-1} contaminant concentration, 76.4-79.6% in 9 days with 15 µg ml^{-1} contaminant concentration, 67.68-72.9% in 9 days with 25 µg ml^{-1} contaminant concentration. Similarly half-life was also recorded at par with all microbial formulations ranging from 6.03-6.93 d at 5 µg ml^{-1}, 7.70-8.56 d at 15 µg ml^{-1} and 7.88-9.36 d at 25 µg ml^{-1} concentrations.

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ANNEXURE

1. Chemical composition of Nutrient Agar (NA) media

S.No.	Chemicals	For 1000 ml
1.	Peptone	5 g
2.	Beef extract	3 g
3.	Sodium chloride	5 g
4.	Agar	18 g
5.	Distilled water	1000 ml
6.	pH	7-7.2

2. Chemical composition of Potato dextrose agar (PDA) media

S.No.	Chemicals	For 1000 ml
1.	Potatoes, peeled and diced	200 g
2.	D-glucose	20 g
3.	Agar	18 g
4.	Distilled water	1000 ml
5.	pH	5.6

3. Chemical composition of Nutrient broth (NB)

S.No.	Chemicals	For 1000 ml
1.	Peptone	5 g
2.	Beef extract	3 g
3.	Sodium chloride	5 g
4.	Distilled water	1000 ml
5.	pH	7-7.2

4. Chemical composition of Potato dextrose broth (PDB)

S.No.	Chemicals	For 1000 ml
1.	Potatoes, peeled and diced	200 g
2.	D-glucose	20 g
3.	Distilled water	1000 ml
4.	pH	5.6

5. Chemical composition of ammonium nitrate for growth of bacterial and fungal culture

S.No.	Chemicals	For 1000 ml
1.	Ammonium nitrate	2 g
2.	Agar	18 g
3.	Distilled water	1000 ml

6. Chemical composition of Mineral Salt Medium (MSM) for degradation analysis of imazethapyr in HPLC

S.No.	Chemicals	For 1000 ml
1.	K_2HPO_4	1.8 g
2.	NH_4Cl	4.0 g
3.	$MgSO_4.7H_2O$	0.2 g
4.	NaCl	0.1 g
5.	$FeSO_4.7H_2O$	0.01 g
6.	pH	6.8

7. Chemical composition of reagents used for analysis of available nitrogen in soil

S.No.	Chemicals	Concentration
1.	$KMnO_4$	0.32 %
2.	NaOH	2.5 %
3.	Boric acid	2 %
4.	Potassium hydrogen phthalate	0.1 N
5.	NaOH	0.1 N
6.	H_2SO_4	N/50

8. Chemical composition of reagents used for analysis of available phosphorous in soil

S. No.	Chemicals	Concentration
1.	$NaHCO_3$	0.5 M
2.	Activated charcoal	1 g
3.	H_2SO_4	5 N
4.	Ascorbic acid solution	1.056 g ascorbic acid in 200 ml molybdate tartrate solution
5.	Molybdate tartrate solution	4.5 ml
6.	p-nitro phenol	0.5 g

9. Chemical composition of reagents used for analysis of available potassium in soil

S.No.	Chemicals	Concentration
1.	Ammonium acetate	1 N
2.	Standard potassium solutions	1000 ppm
3.	Working k solution	10,20,30,40 ppm

10. Chemical composition of reagents used for analysis of organic carbon in soil

S.No.	Chemicals	Concentration
1.	$K_2Cr_2O_7$	10 ml
2.	Ferrous ammonium sulfate	0.5 N
3.	H_3PO_4	10 ml
4.	H_2SO_4	20 ml
5.	Diphenyl indicator	2 ml

11. Chemical composition of reagents used for analysis of soil enzyme dehydrogenase activity

S.No.	Chemicals	Concentration
1.	2,3,5- Triphenyl tetrazolium chloride (TTC)	3 %
2.	Methanol	10 ml
3.	Glucose	1%

12. Chemical composition of reagents used for analysis of soil enzyme fluorescein diacetate hydrolysis

S.No.	Chemicals	Concentration
1.	K_2HPO_4	8.7 g
2.	KH_2PO_4	1.3 g
3.	Chloroform/methanol	2:1
4.	Fluorescein diacetate	1000 µg
5.	Fluorescein sodium salt	2000 µg

13. Chemical composition of reagents used for analysis of soil microbial biomass carbon

S.No.	Chemicals	Concentration
1.	Chloroform	20 ml
2.	0.5 M K_2SO_4	25 ml
3.	Orthophosphoric acid	5 ml

14. Chemical composition of reagents used for extraction of imazethapyr from soil sample

S.No.	Chemicals	Concentration
1.	Acetonitrile	10 mL
2.	Magnesium sulphate anhydrous	4.0 g
3.	Trisodium citrate	1 g
4.	Disodium hydrogen citrate	0.5 g
5.	Sodium chloride	1 g

15. Chemical composition of reagents made for Sodium alginate beads

S.No.	Chemicals	Concentration
1.	Humic acid	10 %
2.	Glycerol	30 %
3.	Sodium alginate	2 %
4.	Calcium chloride	1.5 %
5.	Sodium chloride	0.85 %

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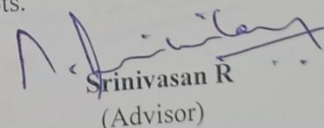
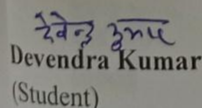
Semester and year of admission: IV, 2020-21 **Degree:** M.Sc. (Ag.)
Department: Soil Science and Agricultural Chemistry **Major subject:** Soil Science
Thesis Title: Bioremediation of imazethapyr in soil/water system
Advisor: Dr. Srinivasan R

ABSTRACT

Imazethapyr is an imidazole compound used as a selective herbicide for soybean and other leguminous crops. It is applied as pre-plant incorporation, pre-emergence, and post-emergence to control the broad-leaved weeds and grasses including barnyard grass, crabgrass, cocklebur, pigweeds and foxtail millets. Herbicides increase the crop production in one side but on another side it causes soil and water pollution which poses serious health risks to human being and livestock. The study comprised laboratory and field experiments on "**Bioremediation of imazethapyr in soil/water system**" was conducted at ICAR-IGFRI and RLBCAU, Jhansi during 2021-22. Six imazethapyr degrading microorganisms (F-3, F-5, F-10, F-11, F-13 and B-15) were isolated from different agricultural soils and tested for degradation. Results showed that consortium F10-F11 (11.75 d) recorded shortest half life ($T_{1/2}$) followed by B15 (11.95 d) for degradation of imazethapyr in water. Fungal isolate F5 performed maximum degradation (90.84%) in 9 days of incubation in sandy loam (S1 and S3) compared to sandy clay loam (S2) soil, which was the highest among all microbial treatments in all 3 soils. In sodium alginate formulation, the consortium F3-F13 (92.41%) recorded the maximum degradation of Imazethapyr followed by B15 (90.96%), F10-F11 (89.72%) and F5 (86.52%) in MSM broth. Bioformulation treatments recorded similar level of degradation 82.82-84.86% in 9 days with 5 $\mu g\ ml^{-1}$ contaminant concentrations, 76.4-79.6% in 9 days with 15 $\mu g\ ml^{-1}$ contaminant concentrations, 67.68-72.9% in 9 days with 25 $\mu g\ ml^{-1}$ contaminant concentration.

Imazethapyr also influenced plant height, root length, No. of root nodules and green fodder yield. Berseem plant height, root length, number of root nodules per plant in control plots ranged from 18.3 to 34.0 cm, 6.47-8.63 cm and 16-22.7, and in imazethapyr applied plots it ranged from 21.0-35.8 cm, 7.73-9.57 cm and 14.3-19.0, respectively. Berseem green fodder yield obtained from two cuts (late sown) ranged from 33.39 (S3) to 39.96 (S2) t ha^{-1} in imazethapyr treated plots and 28.03 (S3) to 33.16 (S2) t ha^{-1} in control plots.


Srinivasan R
(Advisor)


Devendra Kumar
(Student)

नाम: देवेंद्र कुमार

पहचान संख्या :RLBCAU/AG/PG/0040/20

सेमेस्टर और प्रवेश का वर्ष: चतुर्थ, 2020-21

डिग्री: एम.एस सी. (एजी.)

विभाग: मृदा विज्ञान और कृषि रसायन विज्ञान

प्रमुख विषय: मृदा विज्ञान

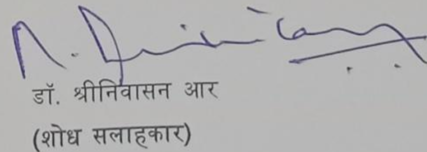
शोध का शीर्षक: मृदा/जल प्रणाली में इमाजेथापायर का जैव उपचार

सलाहकार: डॉ. श्रीनिवासन आर

सार

इमाजेथापायर एक इमिडाज़ोल यौगिक है जिसका उपयोग सोयाबीन और अन्य फलीदार फसलों के लिए एक चयनात्मक शाकनाशी के रूप में किया जाता है। यह बार्नयार्ड घास , क्रैब घास, कॉकलेबर, पिगवीड्स और फॉक्सटेल बाजरा सहित व्यापक-पत्ती वाले खरपतवारों और घासों को नियंत्रित करने के लिए पूर्व -पौधे निगमन (Pre plant incorporation), पूर्व-उद्भव (Pre emergence) और बाद के उद्भव (Post emergence) के रूप में उपयोग किया जाता है। शाकनाशियों से एक तरफ फसल उत्पादन बढ़ता है लेकिन दूसरी तरफ ये मृदा और जल प्रदूषण का कारण बनते हैं जो मानव और पशुधन के लिए गंभीर स्वास्थ्य जोखिम पैदा करते हैं । अध्ययन में 2021-22 के दौरान आईसीएआर-आईजीएफआरआई (ICAR-IGFRI) और आरएलबीसीएयू (RLBCAU), झांसी में **" मृदा/जल प्रणाली में इमाजेथापायर का जैव उपचार " (Bioremediation of imazethapyr in soil/water system)** पर प्रयोगशाला और क्षेत्र प्रयोग शामिल हैं । छः इमाज़ेथापियर क्षरण (Degrading) सूक्ष्मजीवों (एफ -3, एफ -5, एफ -10, एफ -11, एफ -13 और बी -15) को विभिन्न कृषि मृदाओं से अलग किया गया और क्षरण (Degrading) के लिए परीक्षण किया गया । परिणामों से पता चला कि संघ (consortium) एफ -10, एफ- 11 (11.75 दिन) ने पानी में इमाजेथापिर के क्षरण के लिए बी -15 (11.95 दिन) के बाद सबसे छोटा अर्द्ध आयुकाल ($T_{1/2}$) दर्ज किया। कवकीय (Fungal) पृथक एफ- 5 ने रेतीली मिट्टी की दोमट (एस 2) मृदा की तुलना में रेतीले दोमट (एस 1 और एस 3) में इनक्यूबेशन के 9 दिनों में अधिकतम गिरावट (90.84%) का प्रदर्शन किया , जो सभी 3 मृदा नमूने में सभी सूक्ष्मजीवों उपचारों में सबसे अधिक था। सोडियम एल्जिनेट सूत्रीकरण, संघ एफ -3, एफ- 15 (92.41 %) ने एमएसएम शोरबा में बी -15 (90.96 %), एफ-10,एफ -11 (89.72 %) और एफ - 5 (86.52 %) के बाद इमाजेथापिर की अधिकतम गिरावट दर्ज की गई।

जैव सूत्रीकरण उपचार ने 9 दिनों में 5 माइक्रोग्राम/ मिलीलीटर दूषित सांद्रता के साथ 9 दिनों में गिरावट के समान स्तर 82.82-84.86%, 15 माइक्रोग्राम/ मिलीलीटर दूषित सांद्रता के साथ 9 दिनों में 76.4-79.6%, 67.68-72.9%, 25 माइक्रोग्राम/ मिलीलीटर दूषित सांद्रता के साथ दर्ज किया। इमाजेथापायर ने पौधे की ऊंचाई , जड़ की लंबाई, जड़ गांठ की संख्या और हरे चारे की उपज को प्रभावित करता है। बरसीम पौधे की ऊंचाई , जड़ की लंबाई, नियंत्रण मृदाओ (control field) में प्रति पौधे जड़ गांठ की संख्या 18.3 से 34.0 सेमी, 6.47-8.63 सेमी और 16-22.7 तक थी और इमाजेथापायर लागू मृदाओ में यह क्रमशः 21.0-35.8 सेमी, 7.73-9.57 सेमी और 14.3-19.0 सेमी थी। दो कट (Two cutting) (देर से बोई गई) से प्राप्त बरसीम हरे चारे की उपज इमाजेथापायर उपचारित मृदाओ में 33.39 (एस 3) से 39.96 (एस 2) टन/हेक्टेयर और नियंत्रण मृदाओ में 28.03 (एस 3) से 33.16 (एस 2) टन/हेक्टेयर तक थी।



देवेंद्र कुमार
देवेंद्र कुमार
(शोधकर्ता)

डॉ. श्रीनिवासन आर
(शोध सलाहकार)

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Academic Qualification

DEGREE	BOARD/UNIVERSITY	INSTITUTION/SCHOOL	YEAR	%
M.Sc. (Ag.) SSAC	RLBCAU, Jhansi (U.P.)	College of Agriculture	2022	84.20
B. Sc. (Hons) Agriculture	SKNAU, Jobner (Jaipur)	College of Agriculture Lalsot, Dausa	2020	67.80
Sr. Secondary	Board of Secondary Education, Rajasthan	Govt. Sr. Sec. School, Bhusawar (Bharatpur)	2016	78.80
Secondary	Board of Secondary Education, Rajasthan	Govt. Sr. Sec. School, Bhusawar (Bharatpur)	2014	83.33

DECLARATION

I do hereby declare that all information given above is true to the best of my knowledge and belief.

Date: 30/11/2022

Your truly

देवेन्द्र कुमार

Devendra Kumar

Place: Jhansi

107