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Research Article
Biodegradation of Monocrotophos Pesticide Using Nitrogen Fixing Bacteria and Their Effect on Plant Metabolism

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Abstract

Background and Objective: Modern agriculture employs a large number of monocrotophos as a result of rising food demand. This has resulted in great advancements in food production, but it has also resulted in a slew of environmental and health issues. The main objective of this study is the isolation of pesticide-degrading biofertilizer (nitrogen fixation) from root nodules and its effect on plant growth. **Materials and Methods:** Monocrotophos biodegradation by nitrogen-fixing bacteria was isolated from root nodules of *Arachis hypogaea* (groundnut) and *Vigna unguiculata* (black-eyed pea). The isolates were tested for monocrotophos biodegradation among 5 organisms. Morphologic observation and biochemical analysis of the organism were carried out. Biodegradation of monocrotophos by the bacteria was monitored using a UV-spectrophotometer and HPLC. Enzyme assays of nitrate reductase, glutamine synthetase and glutamate dehydrogenase were performed by extracting the enzyme from the leaves of the cultivated plants after 15 days. Statistical analysis was done by One-way Analysis (ANOVA) using GraphPad Prism 5.0 software. **Results:** Monocrotophos biodegradation in M9 medium was 41%, at the end of 48 hrs, 53% of monocrotophos degraded from the optimization of the media with pH 8 and temperature 35°C. There was no significant change in the biodegradation of monocrotophos with the addition of trace elements. Plants grown with the addition of monocrotophos showed a decline in enzyme activity than control. However, plants inoculated with both monocrotophos and the isolated nitrogen-fixing strain expressed similar enzyme activity to those enzyme activities manifested by the plants inoculated only with bacteria. **Conclusion:** This bacterial strain can degrade monocrotophos, enhance plant metabolism by nitrogen fixation and be a potential biofertilizer.

Key words: Glutamine synthetase, glutamate dehydrogenase, monocrotophos, nitrate reductase, nitrogen fixation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Agricultural crops were protected by using pesticides in larger quantities throughout the world. Extensive use of these pesticides causes many drastic effects on crops[1]. A pesticide involves economic loss or harm to people's physical well-being (like neurological, hormonal imbalances, immune system dysfunction, reproductive system defects and cancer). Pesticides are the primary destroyers of crops and contain chemicals that are used to eliminate undesired insects, herbicides to weeds, rodenticides to rodents and fungicides to control fungi and mildew in intensive agriculture and bio-farming[2]. Some are organism-specific, with a specialized mechanism of action for eradicating pests. Pesticide hazard is determined by the chemical's lethal dosage (LD), the period of contact and the mechanism of entrance or absorption into the body. There are several pesticides in use today, each with a unique mode of action and toxicity level[3]. Pesticides would however play a significant role in the destruction of food and fiber by contaminating soil, water, grass and other plant life. In hopes of killing insects and weeds and also harming a number of animals notably birds, fish, pollinating insects and non-target plants[4].

Pesticides with respect to their chemical composition, can be differentiated as organophosphates, organochlorines, carbamates and pyrethroids. Organophosphates such as azinophos-methyl, chlorpyrifos, diazinon, monocrotophos and disulfoton are widely used in agriculture, horticulture, pest management, plastic manufacturing, flame retardants and a variety of domestic uses[5]. Organophosphates rigidity and durability can lead to their build-up in soils when pesticides have been sprayed with supposedly moderate amounts. As a result, organophosphate pesticides are found in water and air all around the globe[6]. Monocrotophos is one such example of organophosphate applied to keep crops viable[7]. It's a non-specific acaricide and organophosphorus insecticide that's made from dimethyl-oxon. Monocrotophos brand focuses on its croton-amide structure and it is considered as cis-isomeric form accounts for roughly 75-80%, although trans-isomer accounts for less than 9%, monochloro-monomethyl acetoacetamide, trimethyl phosphate and others. Monocrotophos is a reddish-brown liquid or solid in its chemical state, even though it is colorless in its pure form[8]. It has a P-O-C linkage and an amide bond, as well as being very water soluble and hydrophilic by nature and is listed as extremely toxic by the World Health Organization (WHO). Because of its moderate cost and efficiency, it has been designed to regulate a range of insects on crops like cotton, sugarcane, peanuts and tobacco since its introduction in 1965[9].

As per statistics, Asia is first in monocrotophos consumption, accounting for 90% of all usage in nations such as South America, China, India and Southeast Asia[10]. The CIBRC has formally certified monocrotophos for 14 crops in our country, making it the most widely used pests in Asia[11]. Because of its efficiency in avoiding pests, cost-effective manufacture and absence of other substitutes, it is particularly popular in Andhra Pradesh and Punjab for the growing of diverse crops and vegetables. The EPA classifies it as a class I element, with typical fatal doses for birds and mammals. As a result, its absorption by the soil is reduced, resulting in its leaking into the groundwater and providing a significant risk of pollution[10]. It is critical to break down these monocrotophos to address problems produced by them on several levels. To solve these complications degradation of monocrotophos is essential and many physicochemical technologies are implied such as incineration, chemical treatment and volatilization to eliminate monocrotophos residue[5]. Many physicochemical technologies are implied such as incineration, chemical treatment and volatilization to eliminate monocrotophos residue but these processes are opposed as they are equally hazardous (produces alkalis and acids), expensive and inefficient. As a result, biodegradation is a potential effective therapy. This method depends on the microorganism's capability to transform complex chemical compounds into simple and non-toxic compounds. Biodegradation is adaptable, efficient and affordable[11]. Since Plant Growth Promoting Rhizobacteria (PGPR) is dominant in the nearby rhizosphere, where the percentage of monocrotophos break is amplified. The chemicals in the monocrotophos may be used as substrates (carbon and nitrogen sources) by some PGPR strains like *Azotobacter*, *Rhizobium*, *Pseudomonas* and *Bacillus*, etc., to generate enzymes for plant growth[12]. Such bacteria operate like a bio-fertilizer, boosting the accessibility of nutrients; escalating root biomass and its ability to gain the nourishment[11]. Among these bio-fertilizers, nitrogen-fixing bacteria, show remarkable tolerance, as they can survive under extreme monocrotophos concentration[13].

Nitrogen (N_2) is a necessary micronutrient for crop production, development and tolerance to biological and chemical stressors. Huge quantities of nitrogen are present in the atmosphere, yet plants cannot absorb it directly. Crops may acquire nitrogen in a variety of forms including nitrate, ammonium, urea, nitrite or perhaps a mixture of these[14]. Nitrogen influences on plant progress and infection hostility. As a result, it's critical to understand how N_2 metabolism works and how it's regulated. A variety of enzymes and compounds help in the absorption of N_2. Most of these bacteria produce stimulants that are helpful for their capacity, thus these enzymes are nitrogenase, nitrate reductase (NR), glutamine

synthase (GS) and glutamate dehydrogenase (GDH)[15]. They're often called diazotrophs because they produce nitrogenase, which is the key enzyme for organic nitrogen production, a set enzyme that catalyzes the renovation of N_2 from the atmosphere to useful components, which plants utilize. Nitrogen-fixing bacteria provide the most nitrogen available to legumes, as well as non-legume plants through their symbiotic relationship[13]. Biological nitrogen fixation (BNF) is a microbe-mediated N_2 conversion mechanism for roots to consume/hold. Symbiotic rhizobacteria cause structural and physiological changes in bacterial cells and plant roots, resulting in specialized structures known as nodules. Other N_2 fixing bacteria are free-living fixers with a wide range of species and a worldwide distribution in agriculture[16]. In natural and economic contexts wherein microbial nitrogen fixation isn't feasible, they represent a major biological supply of nitrogen fixation. This emphasized the importance of free-living N_2 fixing bacteria and potential bacterial bio-fertilizers of proven efficiency on both plant efficiency and organic soil fertility[2]. The properties of nitrogen-fixing bacteria that are valuable for the crops (e.g., nutrition utilisation productivity, defense in opposition phyto-pathogens, phyto-hormone bio-synthesis, etc.)[15]. To promote plant nourishment and bio-fertilizer-based products on a broad scale, PGPR is required. Several nitrogen-fixing bacteria can directly impact plant development by producing plant growth hormones such as indole acetic acid (IAA), gibberellins, naphthalene acetic acid (NAA) and cytokinins, in addition to BNF. In growth regulators research, moderate quantities of PGR are necessary[17]. The main aim of this experiment was to determine the nitrogen-fixing bacteria that have the potential to degrade monocrotophos and enhance the plants metabolism.

MATERIALS AND METHODS

Study area: The study was conducted from November, 2023 to May, 2024 for a period of seven months and the entire experiment was conducted in Azyme Biosciences Laboratory, Bengaluru, Karnataka, India.

Preparation and isolation of nitrogen fixing bacteria: The two root nodule samples were collected from two separate agricultural fields. *Arachis hypogaea* (groundnut) and *Vigna unguiculata* (black-eyed pea) from Kallambella, Sira taluk and another set of *Arachis hypogaea* (groundnut) from Kaggalipura, Kanakapura taluk, where in more pesticides were used for the production of yield. Six samples were collected for the isolation of microbial strains. The root samples were rinsed with 70% ethanol and twice washed with distilled water to remove surface soil and then the root nodules were separated.

Root nodules (1 gm each) sample was taken and homogenized with 1% sterile saline water. Then subjected to bacterial isolation by using the pour plate technique, where selective N_2-free mannitol agar media (yeast extract mannitol agar and Ashby's media) was poured into Petri plates containing 100 µL of the samples. These media were used for the isolation of nitrogen fixing bacterial strains. After solidification of the agar, the plates were incubated for 24 hrs at 37°C in an incubator (Thermo Fisher Scientific, Bengaluru, Karnataka, India). The appearances of colonies were observed and further subculture was done in fresh Luria Bertani (LB) agar plates to isolate and purify the prominent colonies[18,19].

Nitrogen fixation ability analysis from isolated organisms: The isolated colonies were later inoculated into semi-solid nitrogen-free bromothymol blue malate media (NfB) for screening to identify the nitrogen-fixing bacteria. Sterile media were poured and the cultures were streaked in zig-zag pattern followed by incubation for 48 hrs at 37°C. On NfB media, nitrogen fixing was defined by a blue discolouration of the media around the colonies. This property reveals that bacterial activity fixes N_2 and shifts the pH of the medium to alkaline. The organism showing discoloration is streaked on LB agar for pure culture[20].

Screening of monocrotophos degrading nitrogen fixing bacteria: Screening was done to examine the ability of nitrogen-fixing bacteria to degrade the monocrotophos. For this experiment, M9 broth was prepared and poured into a conical flask. After autoclaving the media, 100 µg of monocrotophos and a loop full of organisms were introduced. A negative control was also prepared without microbial inoculation. All cultures were incubated at 35°C in a rotatory shaker at 150 rpm. The 1.5 mL of the broth was centrifuged at 6500 rpm for 10 min, only supernatant was taken and the absorbance of degradation for every 24 hrs was measured at 330 nm using a UV-spectrophotometer (Thermo Fisher Scientific, Bengaluru, Karnataka, India) and degradation rate was calculated using the formula[21]. The organism showing the highest degradation rate was inoculated in LB broth for further testing:

$$\text{Degradation (\%)} = \frac{\text{Before degradation} - \text{After degradation}}{\text{Before degradation}} \times 100$$

Identification of organism by morphological and biochemical parameters: Maximum degradation of monocrotophos organism identified using morphological

characters such as gram stain, colour, shape and margin[22]. Biochemical tests such as indole, methyl red, voges-proskaueer, citrate utilization, hydrogen supplied, gelatin liquefaction, urease, triple sugar iron, nitrate reduction, glucose, sucrose, lactose, mannitol utilization test, cellulose, casein and starch hydrolysis, oxidase and catalase tests[23].

Optimization of physical parameters for monocrotophos degradation by the organism

Effect of different concentrations of pesticides: Analysis of maximum concentration of degradation by the microbes, 100 mL of M9 medium along with aliquots of monocrotophos such as 50, 100, 150, 200 and 250 µg and inoculated the organisms and incubated in incubator for 48 hrs. Degradation rate was calculated at each time interval by centrifuging the broth at 6000 rpm for 10 min and analyzing the supernatant at 330 nm in UV-spectrophotometer[24].

Estimation of incubation time: The time required by the organism to degrade monocrotophos can be studied by inoculating the organism along with 100 µg of the monocrotophos in the M9 medium. A control was also prepared lacking the organism. These broths were incubated for 24, 48 and 72 hrs at 30°C. The degradation rate was calculated at each time interval by centrifuging the broth at 6000 rpm for 10 min and analyzing the supernatant at 330 nm in UV-spectrophotometer[25].

Effect of pH: The pH at which the highest degradation rate of monocrotophos was optimized by incubating the M9 broth containing 100 µg of monocrotophos as well as the isolates. The pH was set ranging from 5-9 by adding either acetic acid or sodium hydroxide. Controls without isolates were also served for each pH. After optimized incubation of the isolates, centrifugation was done at 6000 rpm for 10 min, then supernatant with retained monocrotophos was validated using HPLC and biodegradation rate at exact pH was noted[25].

Effect of temperature: The optimization of temperature for degrading the monocrotophos was observed by inoculating the culture in M9 broth with optimized pH, containing 15 µL of monocrotophos. Similarly, media deprived of the cultures were also composed. Then broths were incubated at optimum times at different temperatures like 25, 30, 35, 40 and 45°C. To assess the biodegrading rate, broths were centrifuged for 10 min at 6000 rpm. The supernatant obtained was used for HPLC analysis and optimum temperature was recorded[25].

Effect of trace elements: The effect of trace elements on the degradation of monocrotophos was examined by adding 0.100 g of each metal such as magnesium chloride, zinc chloride, nickel chloride, ferric chloride, manganese sulphate and cupric sulphate individually in addition to the organism and 100 µg of monocrotophos in M9 media with optimum pH. In parallel, control without organisms was prepared. These broths were incubated at optimum temperature and time. The broth was then centrifuged to remove the pellet at 6000 rpm for 10 min and the supernatant was introduced into HPLC for analysis of monocrotophos degradation[25].

Effect of monocrotophos degrading nitrogen fixing bacteria on plant metabolism: The bacterial cultures were grown in an Erlenmeyer flask containing 250 mL of sterile M9 media with ideal pH. The broth was incubated under optimized temperature for the appropriate incubation time on a rotatory shaker (ThermoFisher Scientific, Bengaluru, Karnataka, India) providing proper aeration and agitation. Under optimum conditions, the bacteria grow and replicates in tremendous amounts that was further used for inoculating them in soil[26].

Effect of microbe on plants and analysis of nitrogen metabolism enzyme: To examine the degradation of monocrotophos by nitrogen fixing bacteria in soil 3 different plants were cultivated. About 1 gm each seed of *Vigna radiate*, *Vigna mungo* and *Cicer areietinum* were washed and soaked in water for a week, succeeding the germination, the seeds were sown in cock pits filled with manure in 4 sets. The first set was taken as a control that lacks pesticide as well as the organism. In the second set, 15 µL of pesticide was introduced. Only the organism was inoculated in the third set and in the last set, both the organism and 15 µL of pesticide were added. The plants were grown for 2 weeks with an adequate amount of water and sunlight. The organism produces certain nitrogen fixing enzymes that help in the elevation of plant metabolism. For analysis of enzyme, 1 g of leaf sample was homogenized with motor in 4 mL of cold extraction buffer which contains 50 mM Tris HCl (pH 7.8), 15% (v/v) glycerol, 0.1% Triton-X-100, 1 mM EDTA and 14 mM 2-mercaptoethanol. The homogenate was filtered through miracloth and further centrifuged at 6000 rpm for 10 min. The supernatant was utilized for the estimation of the enzyme activities[27].

Nitrate reductase (NR) assay: The NR enzyme assay media was in final volume of 4 mL, prepared by adding 50 mM potassium phosphate buffer (pH 7.5), 5 mM potassium

nitrate, 5 mM EDTA, 20 µL of NADH and 0.1 mL of enzyme extract. Excluding the NADH, the blank includes all the assay reagents. The reaction was executed at room temperature (25°C) and incubated for 20 min. The 0.6 mL of 1:1 (v/v) mixture of sulphanilamide (1% in 3 N Hydrochloric acid w/v) and NED (0.1% w/v) was added to halt the reaction. This was further incubated for 15 min at room temperature for the appearance of pink and was measured at 540 nm[28].

Glutamine synthetase (GS) assay: The synthetase assay buffer was formulated for 0.5 mL in 100 mM Tris-HCl (pH 7.8), by adding 50 mM glutamate, 50 mM magnesium chloride and 20 mM ATP. The 0.2 mL of enzyme extract was pipetted to initiate the reaction and incubated for 5 min at 30°C, to stop the reaction 0.7 mL of ferric chloride in 0.02 N hydrochloric acid was added. For control, all the components were mixed except ATP and OD was measured at 540 nm[29].

Glutamate dehydrogenase (GDH) assay: The reaction mixture was prepared in the total volume of 3 mL in 0.1 M tris-HCl (pH 7.5), 0.3 M α-ketoglutarate, 3 M ammonium chloride, 0.2 mL of enzyme extract and 0.2 mL NADH was mixed to commence the reaction. This was incubated at room temperature for 5 min, followed by measuring OD at 340 nm. All the mixture without ammonium chloride was served as blank[30].

Percentage of degradation by High Performance Liquid Chromatography (HPLC): After optimization, the monocrotophos degradation rate was analyzed on shimadzu reverse phase HPLC system with LC-10AD pump containing C18 column (symmetry, 4.6×250 mm) in an isocratic mode. The mobile phase was acetonitrile-water (70:30 v/v) managed at a flow rate of 1 mL/min. Controls and samples (1 mL) were dissolved in the mobile phase, 20 µL was injected and elution was measured at 330 nm. A graph was obtained, retention time and area of the sample were compared with control and biodegradation efficiency was calculated[31].

Statistical analysis: All experiment was done in triplicate and average data was taken. The three different plants were grown in four sets of three replicates. Statistical analysis was carried out by using GraphPad Prism 5.0 software and multiple comparison tests were performed through One-way Analysis of Variance (ANOVA) with <0.05 pearson correlation and regression near 0.99.

RESULTS

Preparation and isolation of nitrogen fixing bacteria: The root nodules were removed and measured for isolation of diazotrophs (Fig. 1a-b). After 24 hrs of incubation, a total number of 40 colonies were streaked on LB agar media for further growth and screening of nitrogen-fixing bacteria. Furthermore, these 40 isolates were streaked on NfB agar to qualitatively test their potentiality to fix nitrogen. Among them 11 cultures were found to be diazotrophs estimated by the color alteration of the media from green to blue, suggesting the production of ammonia. As ammonium was alkaline, it raised the pH hence change in the media colour was observed after inoculating the organisms (Fig. 2a-c).

Screening of monocrotophos degrading nitrogen fixing bacteria: The degradation of monocrotophos by the 11 isolates along with control was monitored using spectrophotometry at 330 nm for a period of 48 hrs. After 48 hrs many organisms showed rapid degradation of monocrotophos, furthermore with an increase in incubation time, slower degradation of monocrotophos was observed. The degradation rate was calculated to check the percentage of monocrotophos being reduced by the organisms. Monocrotophos degradation indicates that the organisms can tolerate the pesticide and use it as a nutrient source to support cell growth. Specimen 5 favored 20% degradation being the highest. The degradation of monocrotophos in M9 broth by different microorganisms was recorded (Fig. 3).

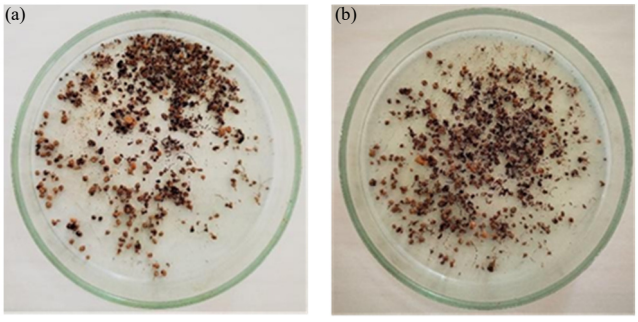

Fig. 1(a-b): Root nodules of (a) *Arachis hypogaea* and (b) *Vigna unguiculata*

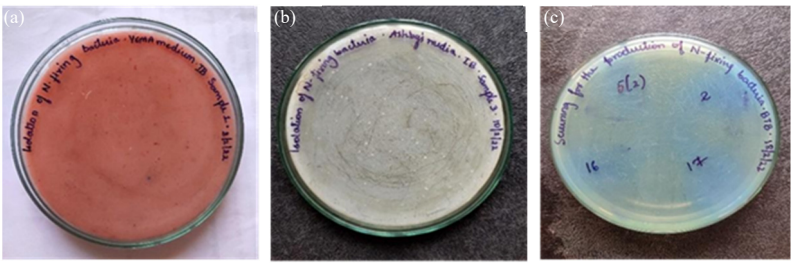

Fig. 2(a-c): Isolation of nitrogen fixing bacteria on (a) YEMA, (b)Ashby's media and (c) Screening of the nitrogen fixing bacteria on nitrogen free bromothymol blue media

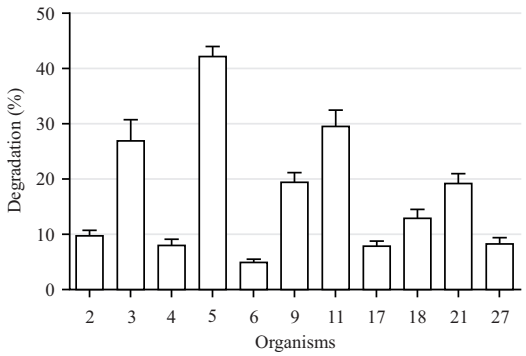

Fig. 3: Percentage of monocrotophos degradation absorbed at 330 nm

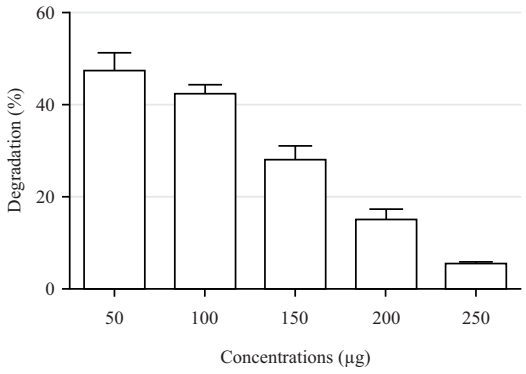

Fig. 4: Percent degradation efficiency of monocrotophos solutions at different concentrations

Identification of bacterial isolates: Morphological character of isolated organism colony appeared creamy white colour, small, opaque and flat with circular configuration, gram-positive cocci. Biochemical tests such as Indole (-ve), Methyl red (-ve), Voges-proskauer (-ve), Citrate utilization (+ve), Hydrogen supplied (-ve), Gelatin liquefaction (-ve), Urease (-ve), Triple sugar iron (+ve), Nitrate reduction (+ve), Glucose (-ve), Sucrose (-ve), Lactose(-ve), Mannitol utilization test(-ve), Cellulose (-ve), Casein (+ve), Starch hydrolysis (+ve), Oxidase (-ve) and Catalase (+ve) tests. The isolated organism with its biochemical character and gram-positive cocci was similar to *Micrococcus* spp.

Optimization of different concentrations of monocrotophos: Maximum degradation showed in the 50 µg 48.2% and 100 µg 42.5%, after that concentration increasing the percentage of degradation was also decreased, for the further optimization physical parameter 100 µg concentrations was used (Fig. 4).

Optimization of incubation time for degradation: Time required for the organism to degrade monocrotophos was determined by incubating the sample as well as control for 24, 48 and 72 hrs. When compared to control containing media and monocrotophos, the bacterial strains accelerated the degradation by 44.5% in 48 hrs. To check the maximum degradation rate 72 hrs incubation readings were also noted. During 72 hrs incubation, bacterial strain removed 45.66% of the initially added monocrotophos. No considerable rise in degradation rate was detected after 48 hrs. Hence, the optimum incubation time was 48 hrs. Monocrotophos degradation was estimated by UV-spectrophotometry. The elimination of monocrotophos by the bacteria at different incubation time was exhibited in the (Fig. 5).

Optimization of pH: The ideal pH on the removal of monocrotophos was studied by altering pH of the media from 5-9. Retention time along with the peak area from chromatogram was compared to sample and control of each pH. The organism did not degrade at pH 5. Highest degradation was observed at pH 8 with 55% and considered as optimum pH for growth and degradation of monocrotophos. Thus, monocrotophos degradation can be achieved by neutral to mild alkaline pH when compared to acidic pH (Fig. 6).

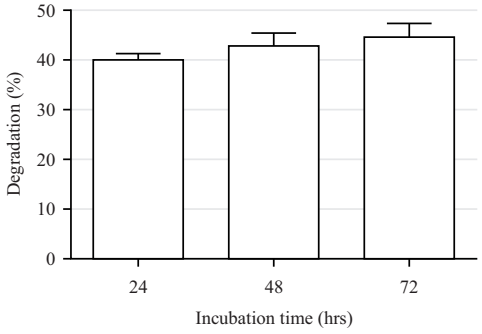

Fig. 5: Effect of incubation time on degradation of monocrotophos

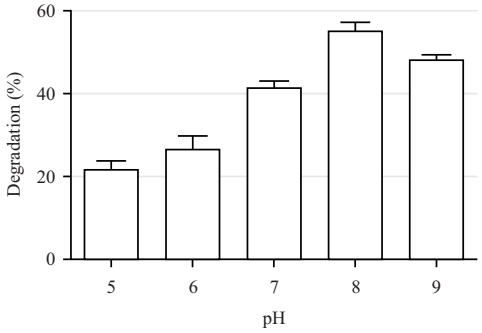

Fig. 6: Effect of pH on degradation of monocrotophos

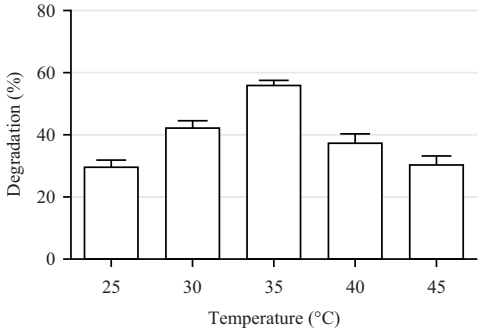

Fig. 7: Effect of temperature on degradation of monocrotophos

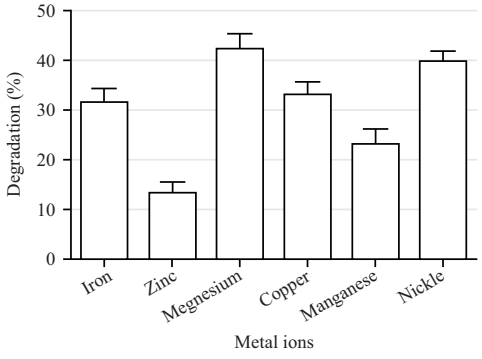

Fig. 8: Effect of trace elements on degradation of monocrotophos

Optimization of temperature: The optimization of temperature on the degradation of monocrotophos was investigated from 25-45°C at pH 8 after 24 hrs. During HPLC analysis the graph was obtained with retention time and area and a comparison was made between the samples and control. It was observed that as the temperature increased, degradation of monocrotophos also increased and maximum was at 35°C with 58% degradation rate. There was a drastic decline in the degradation rate (39%) at 40°C and 35% degradation at 45°C. Optimum temperature was required for the stability of pesticide and growth of organism to tolerate the malignant effect of pesticide. The ideal temperature taken was 35°C, at temperature of 35°C highest degradation of 58% was observed (Fig. 7).

Optimization of trace elements: The trace elements were added in small amounts in order to check their effect on depletion of monocrotophos. As HPLC analysis was done, it was observed that even in presence of certain trace elements like ferric chloride, magnesium chloride, cupric sulphate and manganese sulphate; the bacteria were able to degrade monocrotophos and no degradation was evident in presence of nickel chloride and zinc chloride. The presence or absence of trace elements was compared between them, it was noticed that degradation was highest in the absence of trace elements (58%). Some trace elements may have been toxic to the degeneration of monocrotophos by the bacteria (Fig. 8).

Effect of monocrotophos degrading nitrogen fixing bacteria on plant metabolism

Monocrotophos degeneration in soil and enhancement of plant metabolism: The ability of the isolated bacteria to degrade monocrotophos and magnify the plant metabolism was determined by growing 3 sets of 3 different plants: *Vigna radiate*, *Vigna mungo* and *Cicer areietinum*. The addition of monocrotophos affected the plant growth, while plants grown with monocrotophos and bacterial strain supplemented manure exhibited significant increase in plant growth may be due to the organism. The enzyme activity of all the 3 plants was analysed by comparing the samples with controls of the respective plants.

Nitrate reductase (NR) assay: The graphs exhibited the enzyme activity of the plants, the plants inoculated with bacteria showed increased NR activity and similar NR activity was seen in the plants inoculated with organism and pesticide but only pesticide-sprayed plants didn't show any increase in the NR enzyme activity (Fig. 9a-c).

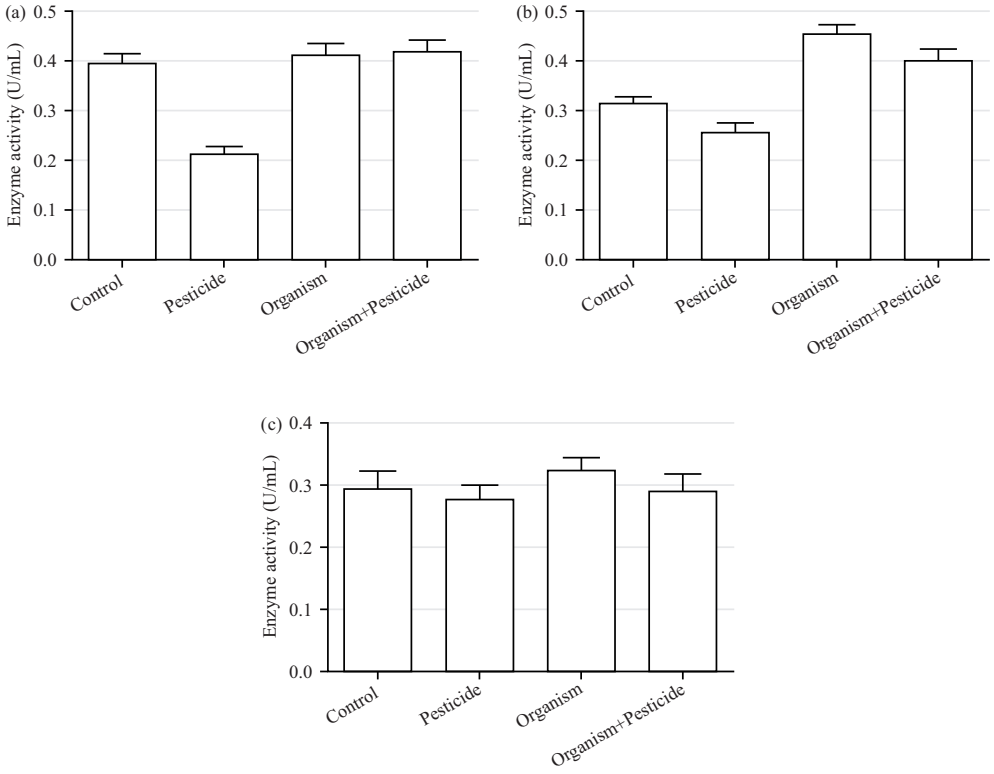

Fig. 9(a-c): Bar graph showing nitrate reductase activity in (a) Green gram, (b) Black gram and (c) Chick pea plants

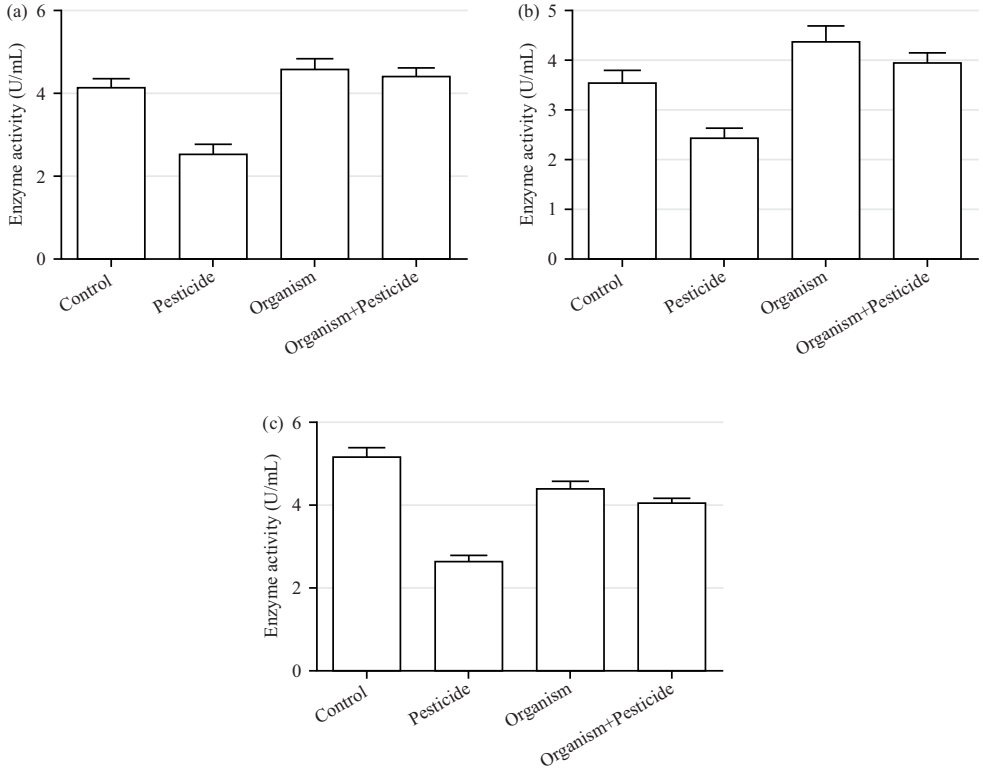

Fig. 10(a-c): Bar graph showing glutamine synthetase activity in (a) Green gram, (b) Black gram and (c) Chick pea plants

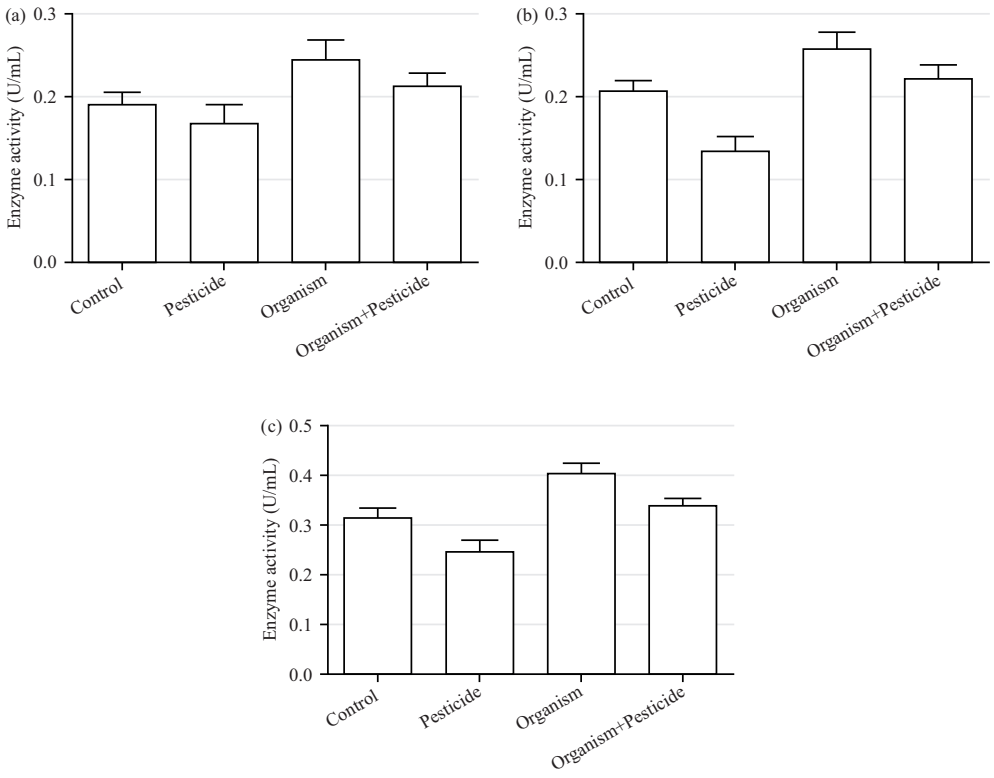

Fig. 11(a-c): Bar graph showing glutamate dehydrogenase activity in (a) Green gram, (b) Black gram and (c) Chick pea plants

Glutamine synthetase assay: The graph represented that, GS activity was not detected in plants with monocrotophos. The plants supplied with pesticides and organism increased the GS activity that was similar to the GS activity of organism containing plants (Fig. 10a-c).

Glutamate dehydrogenase assay: The data presented in the graphs of glutamine dehydrogenase of all plants showed that the pesticide affected the activity of glutamine dehydrogenase in the plants. The nitrogen-fixing bacteria inoculated plants enhanced the enzyme activity, hence nitrogen-fixing bacteria supplied to the pesticide-infected plants exhibited a significant increase in the enzymatic activity (Fig. 11a-c).

DISCUSSION

Organophosphorus pesticides are the major pest control approach, accounting for 34% of the world market. At present, monocrotophos is most frequently used due to its high effectiveness, although it is acutely toxic[32]. Due to their ubiquitous and persistent nature, extensive use of monocrotophos has triggered hazardous effects on the environment. It is very much essential to eliminate these chemical residues from the ecosystem. When compared to other degradation techniques, biodegradation is more suitable because microorganisms may absorb the xenobiotic compounds of the monocrotophos, for their development and these are eco-friendly[33,34]. Biodegradation of pesticides is higher near the rhizosphere as many bacteria are present that have a vital role in enhancing soil fertility and plant development[35]. To degrade monocrotophos and enhance plant development, a symbiotic bacterium was essential; hence nitrogen fixing bacteria was utilized in this study. The nitrogen fixing bacteria are associated with the formation of root nodules in leguminous plants and the pesticides applied roots were utilized in this investigation. As a result, the organisms from these roots would be able to tolerate pesticides and may have the ability to degrade them. Similarly, many researchers have collected the root nodules infected with pesticides from different agriculture fields for the isolation of nitrogen fixing bacteria which were able to tolerate the pesticide[36,37].

The isolation of nitrogen fixing bacteria was particularly done on YEMA and Ashby's media, mannitol was the main carbon source that helped in the growth of these bacteria.

Rhizobium, *Azotobacter* and *Klebsiella* were isolated from these bacteria in several research reports[38-43]. The NfB media was used for screening as sodium molybdate in the media helps in increasing nitrogen fixing activity of the organism. *Paenibacillus mucilaginosus* bacteria was confirmed as nitrogen fixing bacteria when isolated in NfB media[22]. The data were compatible with the report about *Azomonas agilis* and *Lysobacter enzymogenes* isolation by NfB media. In the initial screening using NfB media, 11 isolates confirmed their ability of nitrogen fixation and these isolates were studied for their monocrotophos degradation. In M9 media, 9 isolates exhibited degradation among them. Organism 5 shows the highest degradation rate of 48.2%. It was reported that monocrotophos biodegradation by *Pseudomonas synxantha* (67.8%), *Bacillus subtilis* (17%) and *Salmonella enterica* (6.67%), also described biodegradation and tolerance of monocrotophos by the bacteria[44]. The isolated bacteria were gram positive cocci and with biochemical analysis, it was found as *Micrococcus* sp.

Optimization was done for an efficacious breakdown of monocrotophos. In the present results, at 48 hrs the bacteria degraded 48.2% of monocrotophos. This incubation was also confirmed by Bhadbhade *et al.*[45] where many organisms showed the highest number of monocrotophos degradation. It was reported by Horne *et al.*[46] that the enzyme involved in degradation may be continuous due to rapid incubation time. *Starkeya novella* isolated from a paddy field was able to degrade 56% of monocrotophos in 24 hrs[47]. The optimization of bacteria to degrade monocrotophos was monitored by HPLC after 24 hrs of incubation. The highest degradation of 54% was achieved in alkaline pH 8, this was confirmed by earlier works as the activity of monocrotophos degrading enzyme was active at pH 8[48,49]. According to the pesticide property database at Hertfordshire University, all the pesticides are stable at pH 5-7[50], hence degradation at this pH may be difficult. Different works suggested that *Bacillus subtilis*, *Klebsiella* sp. and *Paracoccus* sp., showed highest degradation of monocrotophos at 35°C[51,52]. Similar to this in the present results, the isolated organism showed 58% degradation of monocrotophos at 35°C was the highest degradation. When this degradation rate was compared with degradation rate in the presence of trace elements, it was found that there was a decrease in the degradation of monocrotophos by the organism. A contradictory result was reported that in the presence of copper and zinc oxide, there was an increase in the degradation of monocrotophos[53].

As nitrogen is essential for plant metabolism, the nitrogen fixing bacteria converts the atmospheric nitrogen into nitrates, urea and ammonia for the uptake by plants, the enzymes like NR, GS and GDH are involved[54-56]. Therefore, the enzymes were extracted from the plant's leaves and enzyme assay was performed to identify the activity of nitrogen fixing enzymes in all the 3 plants used in the experiment. Similar enzyme extraction was done to obtain enzyme from wheat[57] and barley leaves[58]. Other works were done on the extraction of enzymes from nodules of *Phaseolus vulgaris*[59] and soybean[60]. This study revealed that with the application of monocrotophos the enzymatic activity was decreased and harmful to all the plants. In contrast to the control, the plants applied with bacteria had enhanced enzyme activity. As these plants are sensitive to insecticides[61,62], the survival rate was very low. With the inoculation of the isolated nitrogen-fixing bacteria, plants were able to uptake organic form of nitrogen from the soil that was converted by nitrogen-fixing bacteria even in the presence of monocrotophos. With respect to NR and GS activity, black gram evinces better activity, while GDH activity was elevated in chickpea plants. From this study, it is evident that *Micrococcus* sp., can degrade monocrotophos and helps in improvement of plant metabolism.

CONCLUSION

Ultimately, present results revealed that this is the first report on nitrogen fixing bacteria that could be employed successfully to remove monocrotophos from polluted soil. The isolated bacteria may belong to *Micrococcus* sp. In optimum pH 8, temperature (35°C) and at 48 hrs incubation, the bacteria had 88% biodegradation efficiency. The efficiency of this *Micrococcus* sp., found as a promising choice in developing monocrotophos tolerant organisms as a biofertiliser. This process is eco-friendly and cost-effective. Various biotechnological methods can be employed to advance this strain at field conditions. Genomic sequencing can be done to identify the gene responsible for biodegradation and an ample quantity of strains can be produced by recombinant DNA expertise.

SIGNIFICANCE STATEMENT

The main findings of this study are to detect the nitrogen-fixing microbes which has the ability to degrade the pesticides. A pesticide destroys the soil microbes, thereby decreasing soil fertility and soil fertility may play a pivotal role in plant growth. For this reason, an attempt has been made in this study to isolate the nitrogen fixing microbes which has the ability to degrade pesticides and increase soil fertility.

In addition to that, a microorganism helps in the uptake of nitrogen for plant growth. The present study recommends for the future that *Micrococcus* sp., is a promising choice in developing monocrotophos tolerant organisms as a biofertilizer.

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