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Degradation of Monocrotophos in Sandy Loam Soil by *Aspergillus* sp.

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P A P E R I N F O

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The present study premeditated to explore and compare the potential of *Aspergillus* sp. to degrade monocrotophos in soil. Two different strains of *Aspergillus* sp. viz *Aspergillus niger* JQ660373 and *Aspergillus flavus*, were tested under anaerobic conditions for their monocrotophos (150 µg Kg^{-1}) degrading ability in sandy loam soil for a period of 30 days. The water-holding capacity was maintained at 60% and samples were incubated at 25 ± 4 °C. After regular time interval of 5 days, the samples were collected and estimated for residual monocrotophos concentration. Residual monocrotophos was extracted with ethyl acetate and estimated by spectrophotometric method at 254nm. The degradation of monocrotophos in soil was observed to be rapid and followed first order kinetics. A 99% of applied pesticide was degraded within 30 days of incubation. It was found that *Aspergillus niger* was more efficient for the degradation of monocrotophos than *Aspergillus flavus*. The half life of monocrotophos for *Aspergillus niger* and *Aspergillus flavus* was found to be 7.35 and 9.23 days, respectively. Degradation process was assessed by HPTLC and FTIR analysis. These revealed reduction in the peak of standard monocrotophos as a measure of hydrolytic cleavage of vinyl bond with the formation of inorganic phosphates and release of carbon dioxide and ammonia. It could be concluded from the study that these two fungal strains had the ability to degrade monocrotophos in soil.

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INTRODUCTION

Soil is a complex matrix containing different component viz. chemical, physical and biological. They undergo constant changes due to environmental factors and anthropogenic management [1-3]. Therefore, it is best known as the sink of pesticide residues.

Monocrotophos (MCP) is a broad-spectrum organophosphorus (OP) insecticide and acaricide, developed by Ciba-Geigy (now Novartis) and was first registered in 1965. MCP is widely used for agricultural and household purposes; it works systemically as multipurpose agrochemicals pesticides [4]. It is characterized by a P–O–C linkage and amide bond and is a perilous chemical especially for conditions of use in developing countries [5]. It has been barred due to its acute toxicity against beneficial and non-target insects such as honey bees [6, 7], fish [8] birds and mammals [9].

However, its usage for the control of major insect pests in agriculture has been continued in developing countries like India, primarily due to lack of alternative replacements [10]. Use of temporary suspension of pyrethroid insecticide resulted in the development of high resistance to pests [11]. This ultimately led to reliance on cheap insecticides such as MCP for control of pests in agriculture. It is surprising to know that MCP had a lion's share (one-third) of total sales of pesticides in 1996 alone in India.

Despite of its indiscriminative and wide usage, only limited number of studies were undertaken to find its impact on agroecosystem under tropical conditions. Although it has short-life on different agricultural crops [12-16] and in soil [17] but frequent exposure to pests developed moderate insecticide resistance in them [18]. But still nervous system of different pests as well as humans and soil ecology is adversely affected by this perilous chemical. In view of all these facts degradation

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of MCP from soil in the ambient environment is very necessary.

Microorganisms play a very major role in the degradation. Bioremediation has emerged as a slow but viable method due to the time lag that requires weeks to months and complexity of microbial mechanisms for degradation of organic pollutants [19]. Low levels of MCP residues in agroecosystem are rapidly degraded by chemical and biological means [20-22].

The use of bioremediation technology is typically less expensive than the traditional physical-chemical methods as this offers the potential to treat contaminated soil and groundwater on site without the need for excavation [23, 24]. Hence, bioremediation requires little energy input and preserves the soil structure [25].

Reduced impact on the natural ecosystems is perchance the most attractive feature of bioremediation, which should be more acceptable to the public [26]. MCP degradation is extensively studied in liquid culture medium by means of different bacteria [5, 27-30], algae [31], fungi [32-34] and extracellular fungal enzymes [35]. However, the biodegradation of MCP in soil remained a mystery for a long time.

Although, Gundi and Reddy [36] had reported, the degradation of MCP is naturally occurring in soil samples. Jia et al., [30] had also reported the degradation of MCP in fluovo aquic soil using *Paracoccus* sp. Owing to these studies, the present study is designed to analyse the potential of two different fungal strains viz *Aspergillus niger* and *Aspergillus flavus* for the degradation of MCP in sandy loam soil of Rajasthan under controlled conditions.

MATERIALS AND METHODS

Chemicals

MCP of analytical grade (99.5% purity) was procured from Sigma and its stock solution of 1 mg ml^{-1} was prepared in ethanol. All the other chemicals employed in the present study were of analytical grade and purchased from Himedia and Rankem, India.

Microbial culture

Two different fungi viz *Aspergillus niger* JQ660373 and *Aspergillus flavus* were used for the study of MCP degradation in soil. These were previously tested for their MCP degrading ability in liquid media by different workers [33, 34]. Spore suspension of each of these fungal strains was made up to 1×10^8 spores ml^{-1} in 0.85% saline.

Soil sampling

Soil samples were collected from eight different agricultural fields previously treated with OP pesticides. Soil samples (1 Kg each) were collected in different polyethylene bags from within 10 km circumference of Banasthali Campus. These different soil samples were dried at 40°C for 1 week. Dried sampled were meshed through 2mm mesh and mixed in equal proportion. This mixed sample named as SS_{mix}. The samples were stored at 4°C for further use.

Physico-chemical attributes of sampled soil

Physico-chemical properties of soil were analyzed using standard methods. Type of particles in soil, organic carbon percentage, exchangeable cations and pH were as following-

Particle size: Clay- 8.9%, silt- 5.3%, sand- 85.8%, Texture class-Loamy sand

Organic Carbon- 0.33%, Exchangeable Cations: Ca-7.5 m.e/100gm soil, Mg-2.00 m.e/100gm soil, Na-0.65 m.e/100gm soil, K-0.039 m.e/100gm soil, Soil reaction (pH) - 7.88.

Soil samples were sterilized at 200°C for 24 hours in a hot air oven to inhibit the growth of microorganism. Thereafter the samples were cooled down to room temperature for further use.

Experimental setup

Experiment was set in triplicates. A 50 g of soil sample was weighed by using physical balance. Each of this soil was put in 250 ml Erylmener flask. A 1 ml ($1 x10^8$ spores per ml) spore suspension of each isolate was inoculated in 50 g of sterilized soil containing 150 µg kg^{-1} MCP concentration and incubated at temperature 24±4 °C under static culture conditions for 30 days. The water holding capacity of soil was maintained at 60%. The flasks were incubated in dark to rule out the possibility of photo degradation. Effective antibiotics, streptomycin and penicillin (30 mg Kg^{-1}), were added in the sterile soil to avoid any bacterial contamination. Control sample containing sterile soil + MCP (150 µg Kg^{-1}) was prepared simultaneously. At regular time interval of 5 days each flask was removed and residual MCP was extracted and calculated.

Extraction of residual MCP

Residual MCP was extracted from soil with equal amount of ethyl acetate (1ml ethyl acetate for 1g soil). The solvent fraction was pooled. The solvent was allowed to evaporate and residues were dissolved in 2 ml ethyl acetate. Clean up of residual MCP was done by using florosil column and cyclo hexane: ethyl acetate (1:1 v/v) as solvent system. Again the purified fractions were collected and solvent evaporated to dryness and residue was re-dissolved in minimum amount of ethyl acetate. Purified samples of MCP were stored at -20ºC and used for further quantification by spectrophotometer. The results were confirmed by HPTLC and FTIR spectra. Residual MCP was quantified by spectrophotometer at wavelength of 254

nm. The concentration of the remaining MCP was then calculated using molar absorption coefficient. From the residual MCP concentration % of MCP degradation was calculated using the following formula:

$$\% \text{ of MCP Degradation} = \frac{\text{Concentration of MCP in control} - \text{Concentration of MCP in sample}}{\text{Concentration of MCP in control}} \times 100$$

Degradation kinetics

Degradation kinetics of MCP was studied as per the method used by Jain et al., [34].

Analytical tools

HPTLC (CAMAG Linomat 5, Switzerland) was used for analysis of residual MCP in samples after different incubation periods. The aqueous samples from MCP degradation flasks were extracted with ethyl acetate. The samples (20-25 ml) were inoculated on silica gel (60 F 254) TLC plates (E. Merck, India) using an applicator system. Twin trough glass chamber (20×20 cm) containing dichloromethane: methanol (9:1) as the mobile phase was used for development of the chromatogram. Spots were detected using a CAMAG TLC scanner-3 at the wavelength of 254 nm using a deuterium lamp.

Infrared spectra of the parent compound (MCP) and sample after fungal degradation were recorded at room temperature (25°C) in the frequency range of 4000–400 cm^{-1} with a Fourier transform infrared (**FTIR**) spectrophotometer (8400 Shimadzu, Japan, with Hyper IR-1.7 software for Windows) with a helium neon laser lamp as a source of infrared radiation. Aqueous samples (20 days of incubation) from MCP degradation flasks were extracted with ethyl acetate and solvent was evaporated using a rotary vacuum evaporator (Rotavapor R.214, Bu¨ chi, Switzerland). The contents were re-dissolved in acetone. A drop of this sample in acetone was placed in between two sodium chloride discs, after cleaning with ethyl acetate. The background spectrum for acetone was corrected from the sample spectrum.

Statistical analysis

The statistical analysis was done using Statistical Package for the Sciences System 17 (SPSS 17). The variables were subjected to Student t-test and One Way ANOVA.

RESULTS

Degradation of MCP by *Aspergillus niger* and *Aspergillus flavus*

MCP degraded rapidly in the soil samples inoculated with different fungal isolates. The rate of degradation was higher for *Aspergillus niger* than for *Aspergillus flavus*. It was observed that almost 99% of the applied MCP was degraded in soil after 30 days of incubation. It is clearly evident from the Figure 1 that initially the rate of MCP degradation was high i.e in exponential phase till 15 days. Between 15-20 days there was observed a lag/ stationary phase at which the degradation was lower and afterwards it again followed an exponential phase. It reaches to its maximum at 30th day. The percentage of degradation in the control sample was found negligible (1.34%) which exclude the possibility of auto degradation of MCP under controlled conditions. *Aspergillus niger* showed 99.41% whereas *Aspergillus flavus* degraded 96.6% of MCP after 30 days of incubation.

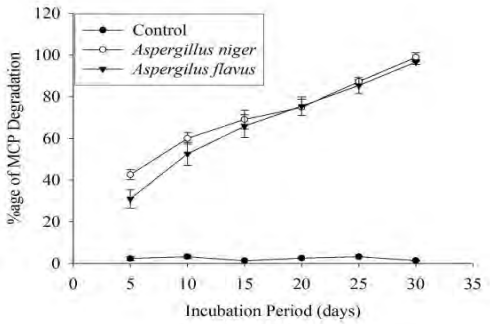

Figure 1. Percentage of MCP degradation in soil by Control, *Aspergillus niger* and *Aspergillus flavus* at different time periods. Error bars indicates standard deviation.

HPTLC

The results of the spectrophotometric determination of residual MCP concentration were further confirmed by HPTLC analysis. The results depicted in Figure 2 clearly indicated that increasing the incubation period decreases the peak of MCP. Standard peak of MCP was observed at 0.19-0.21 rf value. In case of *Aspergillus niger* no peak was observed at this rf value after 20 days of incubation. Though, in *Aspergillus flavus*, a short peak of MCP was seen after 20 days of incubation. This data clearly indicated the removal of MCP from the sample (*Aspergillus niger*) with increasing incubation period.

FTIR

Molecular insight of MCP degradation was studied by FTIR analysis. FTIR spectrum as shown in Figure 3 indicates hydrolytic cleavage of MCP with the formation of inorganic phosphates ($–PO_4$). Peak at 2973.33 cm^{-1} characteristic for vinyl bonds were completely reduced in the spectrum after degradation. Whereas a new peak at 1424.39 cm^{-1} was observed; characteristic of inorganic phosphates. One other peak at 1115.09 cm^{-1} was also observed which was characteristic for aliphatic amines. This clearly indicated the hydrolytic cleavage of MCP by the fungal strains.

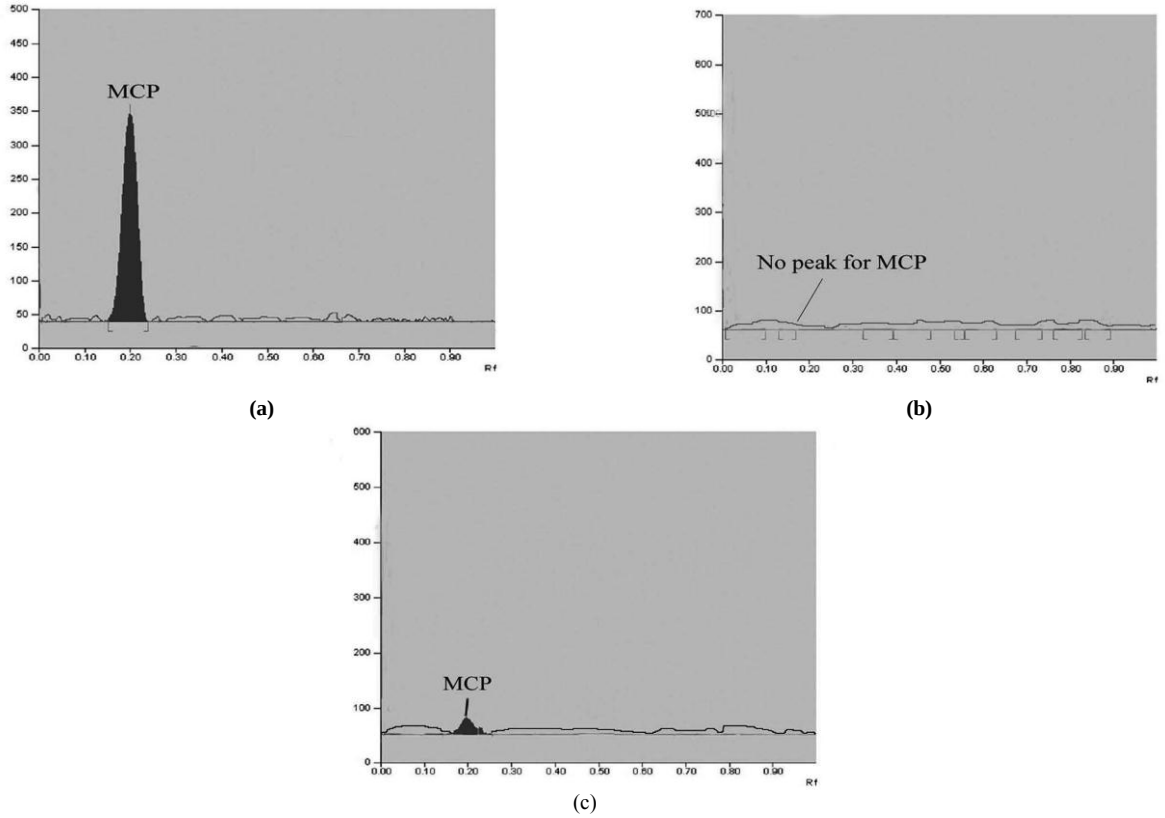

Figure 2. HPTLC chromatograms of residual MCP concentration in (a) Control (b) *Aspergillus niger* and (c) *Aspergillus flavus*, after 20 days of incubation.

Degradation kinetics

Degradation kinetics of MCP was studied by plotting logarithmic concentration of MCP as a function of time. A linear relationship was found between them as seen in Figure 4. Hence, it was concluded that the degradation of MCP in soil followed first order kinetics. Kinetic constant of degradation and half life were then calculated using straight order equation. Inoculation of spore suspension in soil leads to rapid degradation of MCP. The values of kinetic constants for control, *Aspergillus niger* and *Aspergillus flavus* were found to be 0.0069, 0.136, 0.108, day^{-1} with a calculated half life of 144.74, 7.35, 9.23 days, respectively. And hence it concludes that *Aspergillus niger* is more competent towards MCP degradation than that of *Aspergillus flavus.* The half life of MCP in control was observed to be too high which excludes the possibility of auto degradation of MCP in sterile soil.

DISCUSSION

The present study emphasizes the role of fungi in the degradation of MCP in soil. It explored and compared the potential of two fungi belonging to same genera viz *Aspergillus niger* and *Aspergillus flavus* for the degradation of MCP in sandy loam soil of Rajasthan. These fungi were previously tested for their MCP degradation ability in phosphorus free liquid growth medium. It was observed that almost 90% of the applied MCP concentration was degraded in soil within 15 days of treatment. This clearly demonstrates the efficiency of these fungal strains to degrade MCP.

In our knowledge this is the first study vis-à-vis the potential of two different fungal isolates to degrade MCP in sterile soil.

However, Jia *et al.*[30] had reported that the addition of *Paracoccus* sp. M-1 (10^6 $CFUg^{-1}$) to fluvo-aquic soil and a high-sand soil containing MCP (50 mg kg^{-1}) resulted in a higher degradation rate than that obtained from non-inoculated soil.

Gundi and Reddy [36] had also reported 96-98% degradation of MCP in black versitol and red alfinsol soil at 10 and 100 µg gm^{-1} concentration of pesticide. The amount of MCP dissipated from control soil sample in the present study was not considerable. This completely excludes the chance of auto degradation. The possibility of photo degradation was also ruled out as the samples were incubated in dark under restricted conditions

Long persistence of MCP in fumigated and sterile soils/aqueous systems than in corresponding non-sterile and non-fumigated systems was also reported by various researchers [37-41].

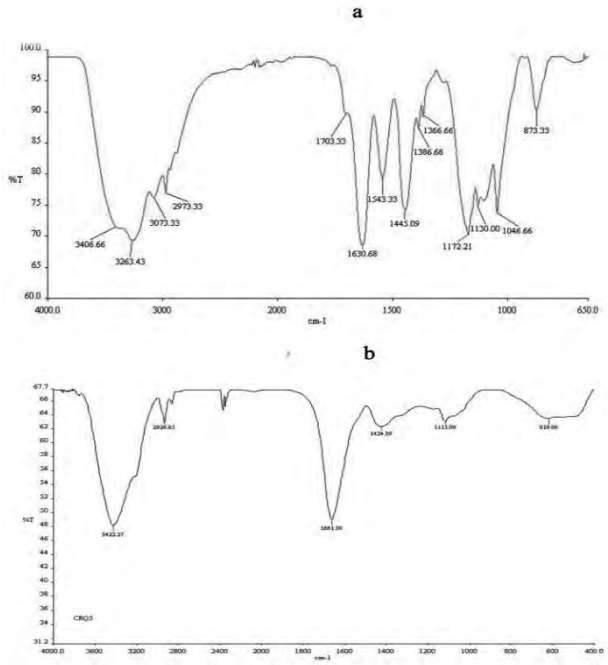

Figure 3. FTIR spectrum of MCP (a) before and (b) after degradation (20 days).

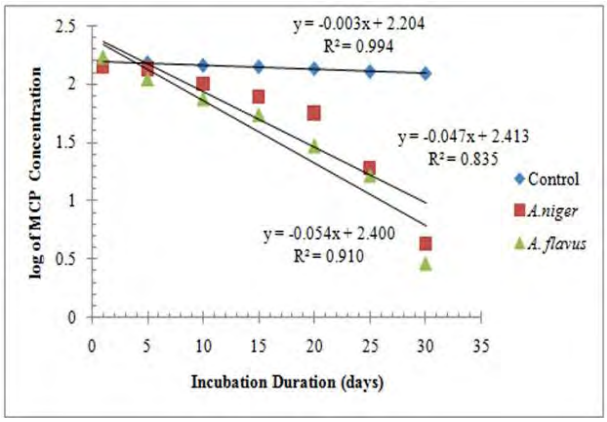

Figure 4. Degradation kinetics of MCP by Control, *Aspergillus niger* and *Aspergillus flavus* in soil at different incubation periods.

The results of spectrophotometric calculation were further confirmed by HPTLC and molecular insight was examined by FTIR. HPTLC chromatograms clearly indicated reduction in the standard peak of MCP at rf 0.19-0.21. No such peak was observed for *Aspergillus niger* and *Aspergillus flavus* after 20 days of incubation which clearly indicates complete detoxification of MCP by these isolates. The FTIR analysis revealed hydrolytic cleavage of vinyl bond with the formation of new peaks characteristic of $–PO_4$. The results are concurrent with the study of Bhalerao and Puranik and Jain *et al*., [32-34]which also correlated reduction in the standard peak of MCP with its degradation by hydrolytic cleavage of vinyl bond using *Aspergillus oryzae*.

Degradation of MCP followed first order kinetics. The kinetic constant for control, *Aspergillus niger* and *Aspergillus flavus* were 0.0069, 0.136 and 0.108, day^{-1} and hence the calculated half life of MCP was found to be 144.74, 7.35 and 9.23 days, respectively.

Concurrent to our studies less than 6 and 21% of the applied MCP concentrations were recovered from the Hanford soil after 16 days of aerobic and anaerobic incubation with the rate constants of 0.17 and 0.09 day^{-1}, and half-lives of 4 and 8 days, respectively [42]. Other researchers had also reported that the persistence of MCP in the paddy agroecosystem decreased in the following order of paddy soil > rice leaves > water with maximum half life of 10 days in paddy soil [16, 43]. Likewise, a granular formulation (5%) of MCP, applied at a rate of 1.5 g a.i. ha^{-1} to an Indian clay soil, also dissipated rapidly with a half-life of 10 days [44]. In horticultural and vegetable fruits the same insecticide was reported short-lived with 3-4 days of half-life by other researchers [14, 15].

Generally, the short persistence of MCP in terrestrial and aqueous environments was owed to its rapid degradation by physico-chemical and biological factors [22]. In the same way, faster degradation of MCP in moist and flooded soils than in dry soils indicated participation of microbes.

Therefore, the study concludes that both fungal isolates were efficient MCP degrader in sandy loam soil of Rajasthan. They degraded MCP within 20 days for *Aspergillus niger* and *Aspergillus flavus* with the half-life of 7.35 and 9.23 days, respectively. HPTLC and FTIR spectrum revealed detoxification of MCP as a result of hydrolytic cleavage of vinyl bond. The order of MCP degradation in soil was found to be *Aspergillus niger* > *Aspergillus flavus*.

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چکیده

این تحقیق می خواهد توانایی *Aspergillus sp* برای کاهش(تجزیه) مونوکروتوفوس در خاک را مشخص و مقایسه کند.دو گونه مختلف از *Aspergillus sp* به عبارت دیگر *Aspergillus niger* JQ660373 و *Aspergillus flavus* تحت شرایط بی هوازی برای تعیین قابلیت کاهش مونوکروتوفوس آن ها در خاک رس و شن به مدت ۳۰ روز مورد آزمایش قرار گرفتند.ظرفیت نگه داری رطوبت در ۶۰٪ ثابت نگه داشته شد و نمونه ها در ۴±۲۵ درجه سانتی گراد اینکوبه شدند.بعد از زمان معمول فاصله ۵ روزه نمونه ها جمع آوری شده و غلظت مونوکروتوفوس باقیمانده اندازه گیری شد. مونوکروتوفوس باقیمانده با اتیل استات استخراج شده و با اسپکتوفتومتر در 254nm اندازه گیری شد. مشاهده شد که تجزیه مونوکروتوفوس در خاک سریع بوده و از سینیتیک درجه اول پیروی می کند.۹۹٪ ضد افت ها در طول ۳۰ روز از بین رفتند.مشخص شد که *Aspergillus niger* برای کاهش مونوکروتوفوس نسبت به *Aspergillus flavus* کارایی بیشتری دارد.زمان نیمه عمر مونوکروتوفوس برای *Aspergillus niger* و *Aspergillus flavus* به ترتیب ۷٫۳۵ و ۹٫۲۳ روز است.فرایند تجزیه مونوکروتوفوس با HPLC و FTIR تخمین زده شد.این آنالیزها نشان می دهند که کاهش پیک های استاندارد مونوکروتوفوس به عنوان مقیاسی از تقسیم هیدولیتیکی پیوندهای وینیل با تولید فسفات معدنی و آزاد شدن کربن دی اکسید و بخار آمونیاک است.میتوان از این تحقیق نتیجه گرفت که این دو گونه قارچی توانایی تجزیه مونوکروتوفوس در خاک را دارند.