Innovations in Pharmaceuticals and Pharmacotherapy

www.innpharmacotherapy.com

eISSN: 2321–323X

Research Article

Hexaconazole biodegradation by a soil isolate and its dehydrogenase study

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Abstract

A microbiological assay of hexaconazole (a triazole fungicide) was standardized using a fungus, *Fusarium oxisporum* . No spontaneous decay of the fungicide was observed in aqueous solution. At high concentration only, certain amount of degradation was observed at 15 µ g/ml level after 12 days. A soil isolate of *Penicillium frequentans* HDF –1 was able to degrade the hexaconazole in culture even at 2000 µg/ml level. The fungus was able to utilize fungicide as sole carbon source and to some extent total nitrogen source also. The culture filtrate containing fungicide (pretreated, 100 µ g/ml) was added during growth of *P. frequentance* with various ratios (1:9, 1:4, 3:7, 2:3 and 1:1) mixed with fresh culture medium and sterilized at 10 lb for 20 min. The final concentration remaining in the culture media were expected to be 100 µg/ml a sterile culture medium containing fresh fungicide solution (i.e. 1, 2, 3, 5 and 10 µg/ml) served as control. The sensitive *fungus Fusarium* was then inoculated in all experimental sets and incubated at 32 +2 0C for 16 days. It was observed that the *Fusarium* started to grow in medium containing culture filtrate up to 100µ g/ml of fungicide whereas in control, no visual growth was observed from 5 µg/ml onwards upto 8 days. Only faint growth was observed after 16 days at 10 ppm level. So, glucose dehydrogenase enzyme (GDH) was studied to test the differential effect on sensitive and resistant organisms. (E.coli HB 101) at 100µg/ml concentration, whereas in sensitive organisms the enzyme was inhibited (43.5% in *Fusarium* and 90.2%, in *B. subtilis* respectively) When compared to control at the identical concentration of fungicide (100 µ g/ml).

Keywords: Hexaconazole, *Fusarium oxisporum, Penicillium frequentans* HDF-1, Biodegradation, Residual Fungicide, Dehydrogenase.

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1. Introduction

Microbial degradation critically affects the fate and behavior of pesticides in soil. Whether a chemical is persistent, short lived, mobile, stationary, adsorbed, absorbed, activated, inactivated or eventually constitutes residue problem may depend upon its metabolism by soil micro-organisms. Microbial transformations have long been reported beneficial to mankind. More recently environmental implications and chemical applications of their transformation have been realized, especially in the degradation of toxic pollutants. In the present study, biodegradation of hexaconazole by a soil isolate was observed. In the Basel convention, it was adopted that the hazardous wastes should be minimized by environmentally sound management, and should be treated and disposed of as close as possible to their source of generation. UNEP strictly banned 12 chemicals, among which 7-8 chemicals are used as pesticides [1]. The toxic chemical can be used and disposed of in a cost-effective manner and with a high degree of safety for both human health and the environment. Under continuous use of any particular herbicide (or pesticide) there remains a potential risk of building up of residues in soil. Biodegradation is one of the most environment-friendly and cost-effective methods to detoxify xenobiotics. Bioassay techniques are widely used and suitable for detection of pesticide residues [2-4].

Hexaconazole is a protectant and irradicant systemic fungicide belonging to the class of triazole group. Chemically it is a 2-(2, 4 dichlorophenyl)-1-(1 H-1, 2, 4-triazole-1-yl) hexan-2-ol. Its melting point is 110-112 ^{0}C. It is a widely used pesticide and is very active against a variety of diseases like powdery mildew, rusts, leaf spot, brown spot etc. It is a systemic fungicide with a causative action and inhibits ergosterol biosynthesis. It is generally not phytotoxic when used in the recommended dose but some injury was noted in Mcintosh apple, mirror carp and *Daphnia* [5]. It was first introduced in 1987 in India. There are reports that it was degraded in the soil, with a half-life period ranging between 3-4 days at lower level, but the description of biodegradation of the fungicide by a particular microorganism was lacking. Not much is known about the microbial degradation and microbiological assay of this fungicide. The present investigation was undertaken to study the biodegradation of hexaconazole by a resistant soil isolate, a fungus *Penicillium frequentans* HDF-1. The present communication also deals with the standardization of a sensitive and rapid microbiological assay procedure, by using *Fusarium dampieri R-12* as a test organism from which the residual fungicide in the culture was estimated. Glucose dehydrogenase enzyme activity in intact cells of sensitive and resistant organisms was determined to compare the effect of hexaconazole on respiratory enzyme.

2. Materials and Methods

Microbial Cultures- *Fusarium oxisporum* ITCC no. 2367.2 K was collected from departmental stock culture. *Penicillium frequentans* HDF-1 was isolated from agricultural field soil of Burdwan locality. *Bacillus subtilis* and *Escherichia coli* HB 101 were taken from the stock culture maintained in our laboratory.

Fungicide- Pure hexaconazole was procured from Rallis (India) Limited, Bangalore.

Culture media- Czapek-Dox (CD) media containing [mg/l] glucose (0.5); $NaNO_3$ (2.0); K_2HPO_4 (1.0); $MgSO_4$ (0.5); KCl (0.5) [pH 7.2] and sterilized at 10 lbs for 20 min was used as growth medium. The test organism was grown in Czapek-Dox agar media in Petridishes.

Bioassay procedure:

A standard dose response curve for the microbiological assay of hexaconazole was prepared by carefully weighing out 10 mg of a pure hexaconazole supplied by Rallis India Ltd. and dissolving in 10 ml of methanol. From the stock solution various concentration grades (10, 20, 30, 40, 50, 60, 80 & 100 μg/ml) were carefully poured into the tubes. The tubes were then evaporated at 50^0 C in an oven. CD-agar (10 ml) medium was poured into the tubes and autoclaved at 10 lbs for 20 min. The molten agar was poured into sterile agar plates and dried at 40^{0}C in the oven. The final concentration remained as 1.0 to 10 μg/ml. The test organism, *Fusarium oxisporum* was grown in petri plates. After 48 h of incubation, mycelium was scratched and mixed with 10 ml sterile distilled water and spread on 8 cm sterile filter paper disc. The discs, soaked with mycelial suspension of *Fusarium* were placed on the agar plates having various concentrations in aseptically poured plates. Plates were incubated for 72 h at 30$\pm$2^0 C. The growth zones were measured and a dose response curve was prepared. For each concentration six replicates were taken. The readings were computed as the mean value lying between 5.0 and 31.5 mm. The quantity of hexaconazole in the test sample was determined from a standard curve prepared in the same way.

Biodegradation assay:

For the biodegradation experiment, the resistant fungi were isolated by enrichment culture technique. Six fungi were isolated by pre-treating the soil with the fungicide (100 μg/ml) for about three weeks and plating it on fungicide containing medium. Among the six isolates, one was found to grow at very high concentration of fungicide (2000 μg/ml) and this organism was identified as *Penicillium frequentance* (HDF-1) and was used in the biodegradation experiment. *P. frequentance* was grown in CD broth (pH 6.8, hexaconazole 0.1 %) in presence and absence of the carbon source. Samples were withdrawn after 4, 8, 12 and 16 days and analyzed for residual fungicide by the microbiological assay and mycelial dry weight (105^0 C, 18 h).

The fungus was able to utilize the fungicide as sole carbon source and to some extent also, the nitrogen source. The degradation of the fungicide was observed by the bioassay method. The test organism used in this experiment was the same sensitive fungus, *Fusarium oxisporum* as described above. The culture filtrate containing fungicide (100 μg/ml) was mixed with various ratios i.e.

1:9, 1:4, 3:7, 2:3, 1:1 with a fresh culture medium and sterilized. The final concentrations remaining in the culture media were expected to be 10 –100 μg/ml level. A sterile culture medium containing fresh fungicide solution (1-10 μg/ml level) served as blank. The fungus *Fusarium* was then inoculated in all experimental sets and incubated at 32 ± 2^0 C. Growth was measured after 72 hr. It was observed that the *Fusarium* was able to grow in medium containing culture filtrate up to 100 μg/ml of fungicide whereas in control (without culture filtrate) no visual growth was observed from 5 μg/ml onwards upto 8 days. Only faint growth was observed after 16 days at 10 ppm level. Percentage of residual hexaconazole was estimated by measuring the growth zone of *Fusarium* (Fig. 2).

Dehydrogenase enzyme assay:

Dehydrogenase activity in intact cells of the fungi was measured by the TTC method [2,3,5-Triphenyl tetrazolium chloride (TTC) reduction to Triphenyl formazan (TPF)] and used for comparing dehydrogenase activity of whole cells in sensitive and resistant fungi in culture tubes with some necessary modifications. Fungi were grown in Czapek-Dox agar plates for 4 days at $32+2^0$ C. Uniform diameter (9 mm) of the mycelial mats were cut by cork borer and were used for enzyme assay. The tubes contained mycelial mats equivalent to 80-100 μg protein/ml, phosphate buffer (0.033 M ; pH 7.0; 2.0 ml), glucose (0.05 M; 0.1 ml) and TTC (0.3 mM ; 0.5 ml). Pesticide was dissolved in ethanol and the desired amount was poured in culture tubes. The tubes were then dried at 50^0C and the solvent was evaporated. These tubes were used in enzyme assay. For bacteria, the tubes contained cell suspension equivalent to 100–120 μg protein/ml [6]. The protein contents of cell suspensions were determined using Coomasie brilliant blue reagent [7-8]. The contents of the tubes were thoroughly mixed and incubated at 37^0 C for 2 h. After incubation, glacial acetic acid (7.5 ml) was added followed by toluene (3.0 ml). The tubes were shaken vigorously and centrifuged at 4000 g for 5 min. The layer of toluene was separated and measured at 485 nm in Shimadzu UV-Spectrophotometer. Quantity of formazan was determined from the standard curve of formazan (Sigma).

3. Results and discussion

The influence of hexaconazole on growth of *Fusarium* was shown in Fig.1. From the dose response curve, it could be observed that the relationship was almost linear between the concentration 0 and 3.5 μg/ml (Fig. 1). The curve, therefore, was suitable for quantitative determination of hexaconazole up to a conc. of 3.0 μg/ml. The concentration required for 50% inhibition (IC_{50}) was found to be 2.2 μg/ml. The antifungal activity of the fungicide remained unaltered by autoclaving (10 lb, 20 min). Fungicidal activity decreased with highly alkaline pH (8.0 and above). No spontaneous decay of the fungicide was observed in aqueous solution within 10 days and only certain amount (about 15 %) of decay was observed after 12 days incubation at 15 μg/ml level (Table 1).

Residual fungicide was estimated by bioassay of the test organism from the culture media. It was observed that residual fungicide decreased with progression of time and remained at 10 % level after 16 days of incubation, when only hexaconazole served as carbon source. The organism was able to utilize fungicide as carbon and nitrogen source to some extent. But when glucose was added at 0.1 % level along with the fungicide, the rate of degradation was enhanced and only 4 % residual fungicide was recorded after 16 days of incubation. Some amount of fungicide was degraded spontaneously in control (18 %) and in aqueous suspension (16 %) after 16 days (Fig.2).

With the increasing duration of incubation, mycelial dry weight also increased up to 12 days and thereafter, the dry weight decreased possibly due to sporulation. In absence of glucose, where the fungicide is the only source of carbon, the growth was very slow up to the 8^{th} day and thereafter reached the log phase. In the experimental set, containing 0.1 % glucose, the growth was recorded after 4 days. The percentage of residual hexaconazole also declined with increase of time, but the degradation was rapid in presence of 0.1 % glucose (8 % residual fungicide) when compared to the data obtained in absence of glucose (10 % residual fungicide at 0.1% concentration and 28 % residual fungicide at 0.2% concentration). The dry wt. of the mycelium was found to increase with the concomitant increase of fungicide concentration to certain extent, but at very high concentration at 0.5 %, 56 % residual fungicide, even after 16 days

of incubation, it caused inhibition of growth (Fig.2). The mycelial dry weight was only 2 mg in comparison to control (without fungicide) where it was 44 mg after 12 days (Fig. 2).

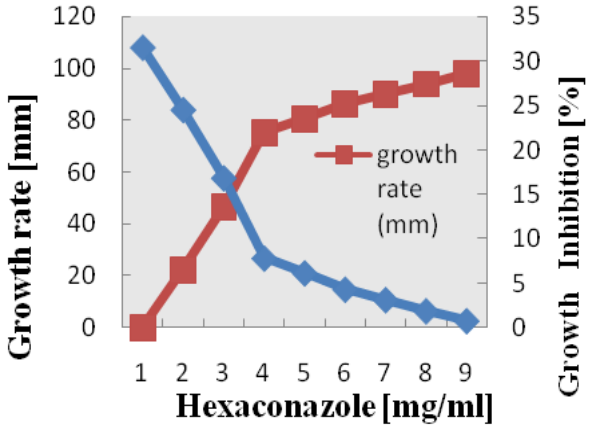

Fig: 1 Growth rates of *Fusarium* sp. on glucose in presence of hexaconazole.

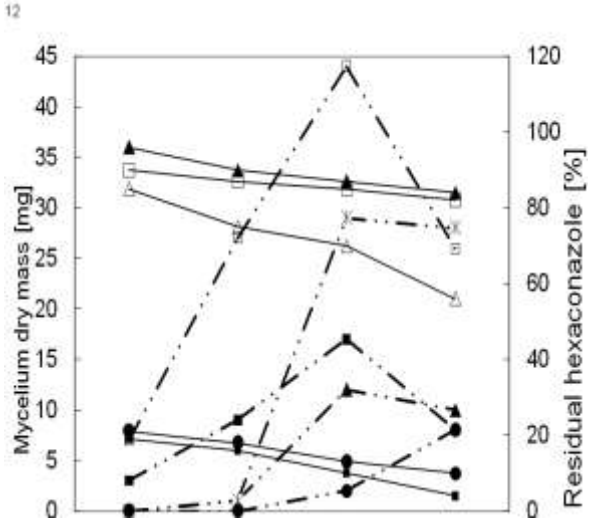

Fig: 2 Mycelia dry mass of *P. frequentance* and residual hexacconazole (%) as estimated from bioassay. Doted link represents Mycelia dry mass and solid lines represent residual hexacconazole

Dehydrogenase showed differential activities on sensitive and resistant organisms. In resistant organism (P. *frequentans* HDF-1), the activity was augmented (+2.85 %) at 100 µg/ml of concentration, whereas in sensitive organism (F. *oxisporum*), it was inhibited at about 43 % at the same concentration in comparison to control (without fungicide). Similar observation was recorded in case of bacterial study. The resistant organism (E. coli) was inhibited to about 18.5 % at 100 µg/ml whereas the inhibition went beyond 90 % in case of 100 µg/ml for the sensitive organism (B. *subtilis*) in comparison to control (without fungicide, Table 2).

Table 1. Effect of hexaconazole on glucose dehydrogenase enzyme of resistant and sensitive organisms.

Organisms	*MIC [µg/ml]	Enzyme inhibition [%]
Penicillium frequentans HDF-1	> 2000	+2.85
Fusarium sp	10	43.5
Bacillus subtilis	60	90.2
Escherichia coli HB 101	200	21.5

* MIC: minimum inhibitory concentration

The bioassay technique is a standard, fast and easy method and is being used since a long time [9-12]. Several workers have reported the estimation of residual toxicants by using bioassay technique. Bioassays, combined with pre-concentrated samples, proved to be useful screening and monitoring tools for initial assessment of water pollution by pesticides [13]. Bioassays of pesticides (Dimethoate, Alrazine, Simazine etc.) by *Tradescantia* stamen hair mutation assay and *Tradescantia* micronucleus assay was reported by Mohammed and MA [14].

Biodegradation of pesticides by microorganism is a standard tool to neutralize the adverse effects of pesticides. Degradation of pesticide by actinomycetes was reported by De and De [15]. Similarly, biodegradation of S-triazine (herbicide) by a stable mixed bacterial community was reported by Kontchou and Gschwind [16]. Cyanobacteria as agents for control of pollution by pesticides were reported by Kuritz [17]. No irreversible effects on non-target plants would be expected, if the use of herbicides could be under good agricultural practice [18]. On the other hand, there are few reports about the impacts of herbicides on the local flora [19-20] and also on the plant productivity [21]. Dose-response relationship, which demonstrates the sensitivity of different crop plants, has been determined for about 300 crop/herbicide combination and then integrated into a database [22-24]. The general effect of pesticides on earthworms is to suppress their selective feeding on the microbially colonized organic matter, denoting an indirect effect on microbial quality [25]. One of the present authors reported the biodegradation of tridemorph and an assay for residual fungicide by bioassay method [12]. Application of short term bioassay to detect dissolved extract in water

quality of agricultural land run-off was done by using *Daphnia* as test organism [26]. A carbendazim degrading fungi was isolated and half-life of carbendazim was estimated about 1.16 days by using *Alternaria alternata* as test organism [27].

Persistence of hexaconazole was studied by Singh & Dureja [28]. The residues and absorption-desorption of hexaconazole [29-30].

Conclusion

A microbiological assay of hexaconazole was standardized using a fungus *Fusarium oxisporum* in CD agar medium. The dose response relationship was linear upto a concentration of 3.5 μg/l (Fig.1). The antifugal activity was stable after sterilization. No spontaneous decay of the fungicide was observed in aqueous solution. The results presented in this paper showed that a soil isolate *Penicillium frequentans* HDF-1 was able to utilize hexaconazole as sole source of carbon and nitrogen. The degradation rate was enhanced in presence of 0.1% glucose (Fig.2). The dry mass of the mycelium was found to increase with the associated increment of fungicide to some extent, but at very high concentration (0.5 %, 56 % residual fungicide even after 16 days of incubation, Fig.2) it inhibited growth. Only 2 mg of mycelial dry mass was recorded after 12 days, whereas in control (without fungicide) set, it was 44 mg. Study of dehydrogenase revealed that there was some differential effect on sensitive and resistant organisms. Enzyme activity increased in case of resistant organism and declined in sensitive organisms (Table 1). From this observation, it might be concluded that partial inhibition/promotion of the enzyme might be responsible for resistance or susceptibility. Bioassay of this fungicide by *Penicillium frequentans* and partial mechanism of inhibition were not reported earlier. There are reports that it does not persist in soil, but its microbial degradation by a particular organism was not reported earlier.

Acknowledgement

We are grateful to Dr. Asok Mukherjee, Former Professor of Botany Department, Burdwan University for his valuable suggestions. Partial work of this paper was done in the Botany Department, Burdwan University. We would like to acknowledge the Botany Department, Burdwan University for allowing the work. The authors are also grateful to the Bidhannagar College for allowing us to use the infrastructure of the college where partial work has been done

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