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AUTOBIOCHROMATOGRAPHY: A TECHNIQUE FOR DETECTION AND ESTIMATION OF THIRAM FROM TREATED SEEDS OF WHEAT

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Abstract

Wheat seeds treated with different fungicides are qualitatively and quantitatively estimated regularly by different bioassays such as thin layer chromatography, autobiochromatography and spectrophotometry. Autobiochromatography is a highly sensitive method for qualitative and quantitative estimation of small amount of fungitoxic material and useful for detecting a wide range of fungicide using a broad spectrum sensitive fungus. In present investigation double zone of inhibition was observed on chromatoplates in combination of *Curvularia lunata* and thiram system by autobiochromatography. Plates were spotted with thiram, developed in acetone: chloroform (60:40, v/v) solvent system and autobiochromatography was done with the PDA (5% agar) seeded with the spores of *C. lunata.* The results of autobiochromatography bioassay revealed that thiram exceptionally produced double zone of inhibition with *C. lunata* fungus at 100, 250, 500, 1000, 2000 µg/ml concentration. Hence, this information can be used as criterion for detection and identification of thiram extracted from treated seeds.

Introduction

One of the greatest accomplishments of plant disease management is seed treatment, which not only control the seed borne infection but also check the infection due to seed borne pathogens. It is a biological, chemical, mechanical or physical process designed to control externally or internally seedborne or soilborne microorganisms resulting in the emergence of a healthy seedling and consequently a healthy plant (Taylor & Harman, 1990). Effectiveness of seed treatment depends on suitable chemicals (fungicides), proper doses and its absorption by the seed. Fungicidal seed treatment forms a protective zone around seeds that reduces seed decay and seedling blight caused by seedborne as well as soilborne pathogens. The use of fungicides as seed treatment is the most widely followed disease control measure used in all crops globally (Agarwal & Sinclair, 1997; Vishunavat, 2009).

In India, breeder and foundation seeds are treated with broad spectrum (captan, thiram) as well as systemic fungicides (carbendazim, carboxin and tebuconazole) whereas, certified seeds are treated only with broad spectrum fungicides. For effective seed treatment it is essential to know about method of seed treatment, dose of fungicide, quantity of fungicide received by the seed, coverage of fungicide over the seed. Estimation of fungicides in treated seeds is being done in regular manner in countries like Denmark and USA (Jorgensen, 1966; Kulik & Crosier, 1964). However, in India reports on quantitative and qualitative estimation of fungicides in seeds treated with fungicides are lacking. Techniques like bioassay, thin layer chromatography (TLC) and spectrophotometry have been found useful for estimation of the individual fungicide to some extent (Chatrath and Gupta, 1992; Mathur & Kongsdal, 2003). The bioassay was conducted for the qualitative and quantitative estimation of captan, carbendazim, carboxin, congo red, deltamethrin, tebuconazole on treated seeds of wheat by using *Alternaria alternata, Aspergillus niger, Bipolaris sorokiniana, Colletotrichum falcatum, Curvularia lunata, Fusarium moniliforme, Phytophthora cactorum, Trichoderma viride, Trichoderma harzianum* and *Trichoderma virens* as test fungi (Gupta & Agarwal, 2003). Further, Autobiochromatography was attempted to identify and estimate the quantity of thiram present in treated wheat seed using *Curvularia lunata* as a test fungus. Autobiochromatography is also known as bioautography, biochromatography, thin layer chromatography bioassay, microbioassay chromatography. It is a sensitive technique to provide information about the nature of pesticides, antifungal properties of the metabolic products or the synthesis of the antifungal compounds by plants in response to fungicide application (Abdel-Motaal *et al*., 2010; Khan *et al*., 2012; Mishra & Verma, 2009; 2011).

This technique is potentially capable of detecting any other antifungal compound formed due to metabolism of the fungicide or produced by the host in response to a fungicide (Adinarayanan *et al*., 1988). Keeping in view the above considerations, this technique was performed for the detection and identification of thiram from treated seeds of wheat. The present investigation was undertaken to study the double zone formation exhibited in *C. lunata* - thiram system on potato dextrose agar medium and in autobiochromatography.

Materials and Methods

Autobiochromatography was carried out to devise simple, sensitive and reproducible technique for the estimation of thiram. This technique was performed only with *C. lunata* and thiram system because a typical double zone of inhibition was found only in this case on bioassay (Dhingra & Sinclair, 1986; Verma *et al*., 2004). TLC plates were prepared, prespotted with thiram and developed in acetone: chloroform (60: 40 v/v) solvent system. After development of plates, they were sprayed with the spore suspension of test fungi *C. lunata*. Forty gram of silica gel suspended in 100 ml chloroform and methanol (60 and 40% v/v) with the help of pestle and mortar for one minute. Few drops of methanol were added to slurry for uniform coating. The blended slurry was immediately poured into thin-layer spreader and applied on glass plates uniformly. The glass plates (20×20 cm) were coated by 0.25 mm thick layer of silica gel with the help of an applicator. The coated

plates were air dried for 5 min. at room temperature followed by oven drying at 50°C for 5 min. After complete drying the slides were removed from the oven, cooled and stored at the room temperature. Thiram with active ingredient of sigma was used in present study. Different concentrations of thiram i.e., 50, 100, 250, 500, 1000 and 2000 µg/ml were taken. Two hundred mg of thiram was dissolved in 100 ml acetone to obtain stock solution of 2000 µg/ml (active ingredient) concentration. Different concentrations of fungicides were obtained by diluting the stock solution in acetone.

Curvularia lunata was grown on potato dextrose agar (0.50 per cent agar) for 8 days at 25°C. Fungus was grown in 250 ml flask by taking 80 ml media. On 8th day fungus was scrapped by inoculation needle and 25 ml sterilized distilled water was added to the flask and shaken for 2-3 min. to release the conidia into the water and filtered through two layer of musclin cloth. Suspension was collected and mixed in PDA (at 40°C). The seeded medium was shaken to mix the conidia uniformly for use in biography. This spore suspension was sprayed on spotted TLC plates. Twenty microlitre of the each sample was spotted on TLC plates using micropipette at equal distances leaving 1.5 cm of the bottom edge of the TLC plates and the solvent (acetone) was evaporated off. The spot size was kept about 5 mm in diameter**.** Hair drier was used to check the unnecessary spread of spots. The spotted plates were developed in an air tight rectangular TLC chamber containing 200 ml of solvent system. Care was taken to see that the spots did not immerse in the solvent system at the bottom of the chamber. The plates were developed until the solvent front had moved about one inch below the top edge of TLC plates. Developed plates were air dried and kept in oven at 100°C temperature for 10 minutes to remove solvent completely. The spore suspension of *C. lunata* in PDA was sprayed on the TLC plates with the help of sprayer. Plates sprayed with seeded medium (*C. lunata* + PDA) were incubated at 28±2°C in a tray and covered from all the sides with three layers of sterilized and moist blotter paper. Observations were taken after every 24 hours of incubation for three days. The inhibition zones were demarcated with a needle on butter paper and spot diameter was measured. Diameters of the spots, as average of two directions at right angle to each other, were measured and then spot area was calculated.

Results and Discussion

Double zone of inhibition was first reported by Verma (1998) from *C. lunata*-thiram system. In present investigation double zone was observed on chromatoplates in the same combination of *C. lunata* and thiram. Plates were spotted with thiram, developed in acetone: chloroform (60:40, v/v) solvent system and autobiochromatography was done with the PDA (5% agar) seeded with the spores of *C. lunata*.

Active ingredient of thiram of different concentration (50, 100, 250, 500, 1000 and 2000 µg/ml) gave double zone of inhibition on autobiochromatography. Pattern of double zone formation was the same as revealed in bioassay (Fig. 2a). In autobiochromatography maximum zone of inhibition was obtained after 48 h of incubation as in case of bioassay. Table 1 revealed that in case of lowest concentration 50 µg/ml the zone pattern was not same. It got reduced from 15.91 mm^2 (24 h) to 6.29 and 3.15 mm^2 after 48 and 72 h of incubation. At higher dosages i.e. 100, 250, 500, 1000, 2000, the zone of inhibition increased after 48 h of incubation but reduced after 72 h. At 100 µg/ml area of inhibition zone was 24.86 mm^2 after 24 h which increased after 48 h (52.12 mm^2) and got reduced after 72 h (14.15 mm^2). On testing with other concentration of thiram i.e. 250, 500, 1000 and 2000 µg/ml the maximum zone of inhibition was 99.70, 172.10, 227.07 and 433.90 mm^2 developed after 48 h of incubation, respectively (Table 1, Fig. 1). Double zone of inhibition was also reported by various workers (Kaunt & Agarwal, 2002; Verma *et al*., 2004) from *C. lunata*-thiram system. In present investigation double zone was observed on chromatoplate at 48 h of incubation period at 100, 250, 500, 1000, 2000 µg/ml concentration, but at 50 µg/ml no double zone was observed. These double zones were scrapped and dissolved separately in acetone and autobiochromatography was done from the scrapped material. The extract of mid zone did not reveal double zone (Fig. 2b-B). It was inferred from the results that thiram was not present in the extract of mid zone whereas outer and inner zone extracts yielded thiram which gave double zone on autobiochromatography (Fig. 2b). Earlier autobiochromatography was performed for estimation of benomyl, carbendazim, organotin compounds, catechol and pyrocatechol with the respective test fungus *Penicillium* spp., *Neurospora crassa; C. lunata; Bipolaris carbonum*, (Adinarayanan *et al.*,1988; Balinova & Balinov, 1977; Chakraborty & Saha, 1994). However, none of these compounds exhibited double zone of inhibition like thiram. So it may revealed from the present investigation that double zone of inhibition as observed in *C. lunata*-thiram system could be used as a criterion for the detection and identification of thiram.

Table 1. Diameter and area of inhibition zone of *Curvularia lunata*-thiram system as revealed on autobiochromatography.

Concentration (µg/ml)	Period of incubation (hour)					
	24		48		72	
	Diameter (mm)	Area (mm^2)	Diameter (mm)	Area (mm^2)	Diameter (mm)	Area (mm^2)
50	4.50	15.91	2.83	6.29	2.00	3.15
100	5.63	24.86	8.15	52.12	4.25	14.15
250	8.00	50.28	11.27	99.70	6.20	30.20
500	12.67	126.03	14.80	172.10	10.50	86.62
1000	15.50	188.76	17.00	227.07	13.35	139.92
2000	19.85	309.43	23.50	433.90	17.65	244.62

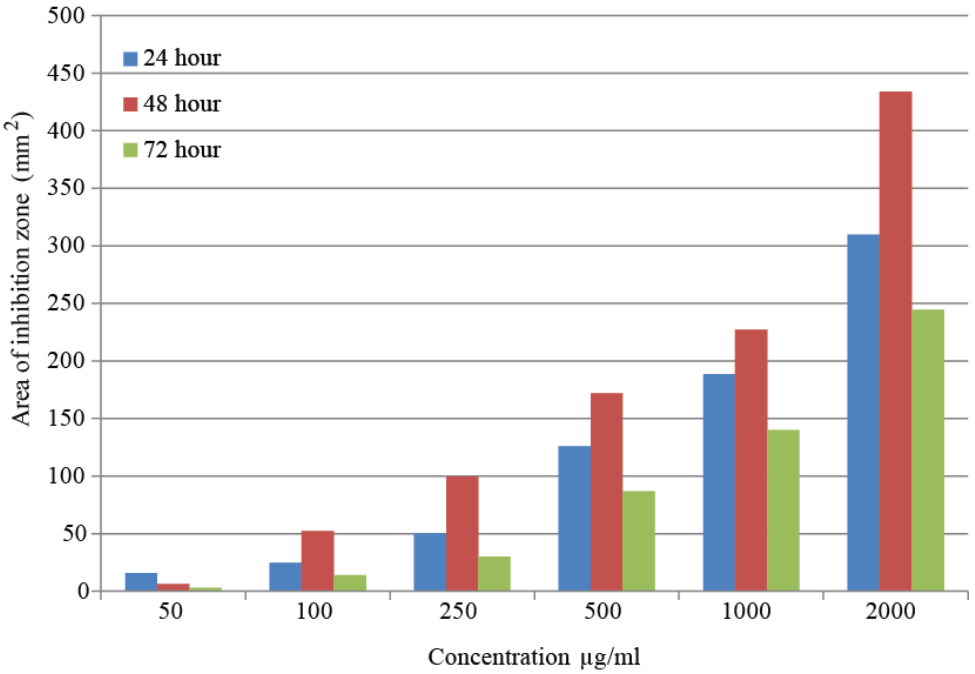

Fig. 1. Inhibition zone of *Curvularia lunata* - thiram system as revealed on autobiochromatography after 24, 48 and 72 hour of incubation

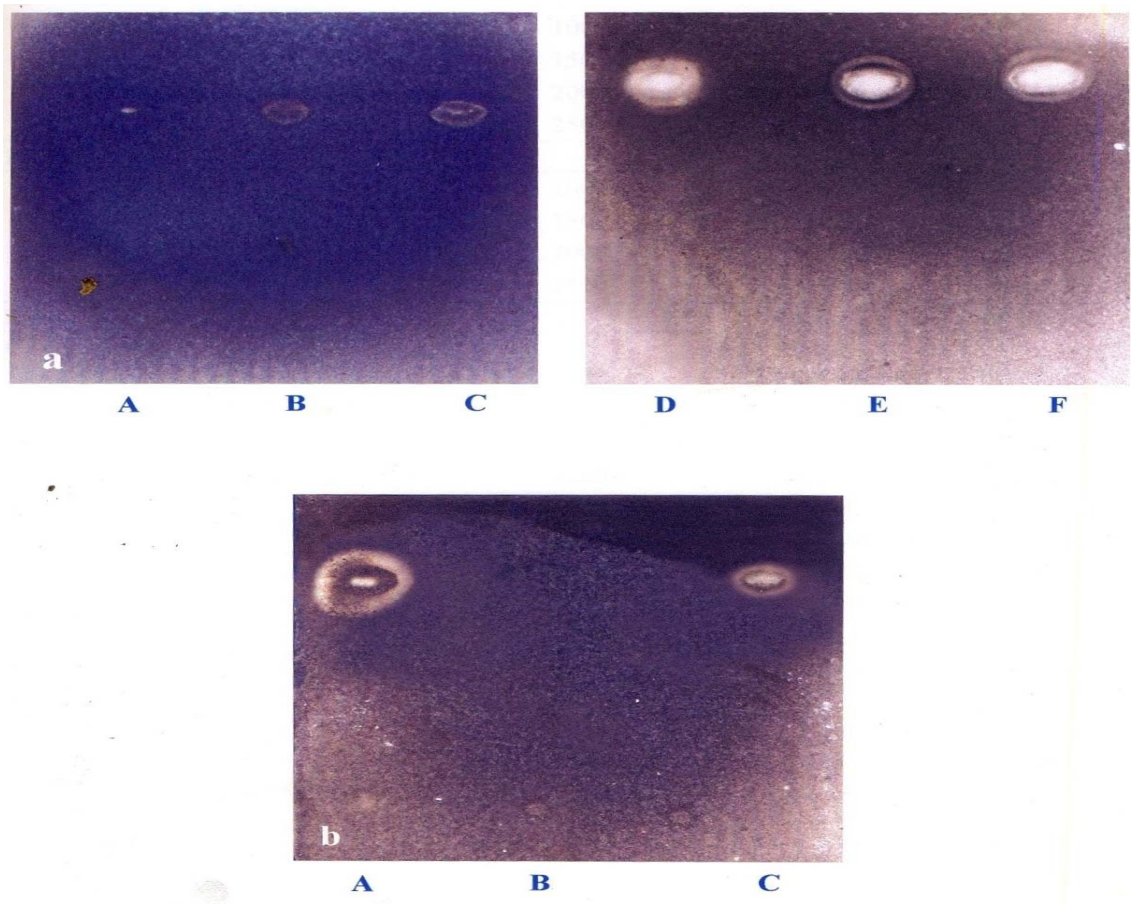

Fig. 2. a, b: Double zone of inhibition as exhibited on chromatoplate.

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