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EVALUATION OF IN VITRO MUTAGENIC ACTIVITY OF DIFENOCONAZOLE TECHNICAL, MANCOZEB TECHNICAL AND TRICYCLAZOLE TECHNICAL BY AMES *SALMONELLA* MICROSOME ASSAY

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ABSTRACT:
Fungicides are agrochemicals used in crop protection. Fungal infection to the crop significantly impact on crop yield and quality. For crop management fungicides are used to control disease during establishment and development of crop to increase the productivity. Mutagenic potential of three fungicides systemic with preventive and curative fungicide-Difenoconazole Technical, contact fungicide-Mancozeb Technical and systemic fungicide-Tricyclazole Technical was evaluated by using Ame's test. The mutagenicity was evaluated by study of its ability to induce reverse mutation on selected histidine loci in strains of Salmonella typhimurium viz. TA1535, TA97a, TA98, TA100 and TA102 with/without S9. A Preliminary Cytotoxicity Test was performed at 5000, 2500, 1250, 625, 312.5, 156.25, 78.125 and 39.0625 µg/plate in TA98 and TA100 strains. The fungicides were found to be non-cytotoxic in Preliminary Cytotoxicity Test at and up to 5000 µg/plate. Therefore, the doses selected for main study were half log difference (√10) interval, which were 5000, 1500, 500, 150, and 50 µg /plate in ±S9. The main study was performed as Trial I by plate incorporation method with 5% S9 and without S9. Trial II conducted by pre-incubation method using with 10% S9 and without S9. Results indicated that the revertant frequencies at all concentrations of fungicides in strains TA1535, TA97a, TA98, TA100, and TA102 in ±S9 were comparable to the revertant counts observed in the concurrent DMSO control. Difenoconazole Technical, Mancozeb Technical and Tricyclazole Technical are non-mutagenic at and up to 5000 µg/plate in all the strains of Salmonella typhimurium.
Key words: *Fungicides, Mutagenicity, Cytotoxicity, Salmonella typhimurium*
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INTRODUCTION:

Plant pathogenic fungi cause devastating damage to crop production worldwide. Fungicides are essential components of crop protection and have played a significant role in managing several devastating crop diseases and realizing optimum crop yields (11, 18). Their use has assumed importance in the control of more damaging plant pathogens against which host resistance is not easily available or is unstable, such as polycyclic oomycete pathogens. In some cases, the benefit gained through fungicide use is more critical to the extent that certain crops, such as potato, melons, and grapes, to name a few which cannot be cultivated in the absence of disease control that remains heavily dependent on the use of fungicides.

Most of the fungicides have low to moderate toxicity. However, several fungicides, such as alkyl dithiocarbamic acid (manganese, zinc, and ammonium salts), halogenated substituted monocyclic aromatics (dinocap), carbamic acid derivatives (maneb and zineb metabolites and ethylene ethiurammonosulfide) acid derivatives. More than 80% of all oncogenic risk from the use of pesticides derives from a few fungicides; only a small number of pesticide-related deaths from fungicides have been reported (1, 6, 14). Some fungicides are known to disrupt the endocrine system and may lead to reproductive and developmental abnormalities. Based on the pre-natal toxicity, several fungicides have been deregistered or banned in many countries but are still used in other, less regulated areas of the world.

Fungicides based on their translocation mode in plant, can either be contact (Mancozeb Technical) (6), translaminar (Tricyclazole Technical) (15) or systemic (Difenoconazole Technical) (14, 18). A systemic fungicide is the one which is taken up by a plant and is then translocated within the plant system, it can there by protect the plant from infections and restrict/control the further growth of existing fungal infection. Contact fungicides doesn't enter the plant, but controls the fungi when it comes in contact with

fungi during the application. Translaminar fungicides redistribute the fungicide from the upper, sprayed blue-leaf surface to the lower, unsprayed surface.

Looking at the severity of the fungicides, the group of different pathogenicity of fungicides are chosen for the study. **Difenoconazole Technical is a**triazole fungicide belonging to the demethylation inhibitor (DMI) group of fungicides (Group 3). Difenoconazole is used as a foliar fungicide and a seed treatment on field crops, fruits and vegetables. **Mancozeb Technical**is a mixture of Maneb (M163500) and Zineb, a manganese and zinc (1:1) complex mixture with the ethylene bis (dithiocarbamate) anionic ligand. Mancozeb is a foliate fungicide used to protect crops in agriculture. Mancozeb has a broader and more effective fungicidal activity than either of its component on their own. Mancozeb also significantly enhances the copper activity against several bacteriocins (4).**Tricyclazole Technical**considered standard for blast control and its application alone or in combination, was satisfactory because it provides systemic protection with a residual period of 30 days (12, 13, 19).

Ames test: The Microbial mutagenicity Ames test is a bacterial bioassay accomplished in vitro to evaluate the mutagenicity of various environmental carcinogens and toxins (2, 3). While Ames test is used to identify the revert mutations which are present in strains, it can also be used to detect the mutagenicity of environmental samples such as drugs, dyes, reagents, cosmetics, waste water, pesticides and other substances which are easily solubilized in a liquid suspension (15, 21, 24).The Microbial Ames test is a simple, rapid and robust bacterial assay consisting of different strains and applications of *Salmonella typhimurium/E. coli*, used for ascertaining the mutagenic potential (17, 18). In 1975, Ames and his followers standardized the traditional Ames assay protocol and reappraised in 1980's (Maron and Ames, 1983). Induction of new mutations replacing existing mutations allows restoring of gene function.The newly formed mutant cells are allowed to grow in the absence of histidine and form colonies, hence this test is also called as 'Reversion assay' (20,22, 25, 27).

MATERIALS AND METHODS:

The design of this study was based on the requirements of the following guideline: OECD Guideline 471, Bacterial Reverse Mutation Test adopted on July 21, 1997 and June 26, 2020 (24). The test employs histidine dependent strains of *Salmonella* each carrying different mutations in various genes in the histidine operon (2, 3, 4, 27). These mutations act as hotspots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (his+) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate increases, usually in a dose-related manner (5, 20, 22)

Bacterial Strains:

The tester strains used in the study were *Salmonella typhimurium* - histidine auxotrophs TA1535, TA97a, TA98, TA100 and TA102 (20)(Table 1).

Genetic markers of the test strain and the degree of its spontaneous reversion were checked each time before testing the samples. Average numbers of revertant formed spontaneously were close to those given by Maron and Ames (2, 3, 20. The strain sensitivity check was based on a positive response and performed by exposing the bacteria to diagnostic mutagens. The positive controls used in the study are listed in Table 2. Quantitative evaluation of a group of Fungicides was performed by using plate incorporation assay. It was designed to establish relationship between the number of induced revertant and the doses of test substance used. Fungicides understudy were tested using five tester strains viz.TA 1535, TA97a, TA98, TA100, TA102.

Procedure for *Salmonella* microsome assay described by Maron and Ames (1983) was adopted in this study (20). Preliminary Cytotoxicity Study (PCT) was performed as plate incorporation method with eight test concentrations viz. 5000, 2500, 1250, 625, 312.5, 156.25, 78.125 and 39.0625 µg/plate in presence (5% S9) and absence of metabolic activation system using tester strains of TA98 and TA100 in triplicate.

The Main study was performed as Trial I by plate incorporation method using five tester strains with (5% S9) and without metabolic activation and Trial II by pre-incubation method using all five tester strains with (10% S9). All the plates Trial I and Trial II studies were maintained in triplicates for each concentration (2, 3).

The condition of the bacterial background lawn was observed with microscope and revertant colonies for the tester strains of all test concentrations, vehicle and positive controls were counted using Colony

Counter. To ensure sterility of the vehicle and equipment, tests for evaluation of contamination were performed along with the assay. Genotypic characterization of the tester strains was performed before the test (Table 1).
Mutagenicity ratio (MR) was calculated as the ratio of the number of *Salmonella typhimurium* revertant grown in the presence of the tested sample to the number of spontaneously appeared revertant. The sample was considered mutagenic when MR ≥ 2 (5, 28).

RESULTS:
Preliminary cytotoxicity study:
A Preliminary Cytotoxicity Test was performed at test concentrations of 5000, 2500, 1250, 625, 312.5, 156.25, 78.125 and 39.0625 µg/plate in TA98 and TA100 tester strains (Figure 1). DMSO was used as vehicle control.The fungicides were found to be non-cytotoxic in Preliminary Cytotoxicity Test at and up to 5000 µg/plate. The doses for main study were selected with approximately half log difference (√10) interval keeping 5000 µg/plate as a highest dose (3, 5, 14, 28).
Histidine revertant counts in main study (Trial I):
Based on the results of Preliminary cytotoxicity study 5000 µg/plate was selected as the highest test item concentration for main study with four subsequent concentrations viz. 1500, 500, 150, and 50 µg /plate, in presence and absence of metabolic activation system, for all the five tester strains tested (Figure 2).
All the 5 strains with the group of fungicides namely Difenoconazole Technical, Mancozeb Technical, Tricyclazole Technical when tested in plate incorporation assay with (5% S9) and without metabolic activation at 5 different concentrations did not show any mutagenic effect on *Salmonella* strains. Mutation ratio was calculated with respect to vehicle control, did not show two or more-fold increase in the revertant (Figure 3).
In Trial I (Plate Incorporation method) the revertant colonies at all tested concentrations of fungicides were found to be comparable to those observed in the vehicle control plates in the tester strains TA1535, TA98, TA100, TA97a, and TA102 in presence and absence of metabolic activation system (Figure 4).
Histidine revertant counts in main study (Trial II):
With similar concentrations, like Trial I, 5000 µg/plate as the highest concentration with four subsequent concentrations viz. 1500, 500, 150, and 50 µg /plate, were selected in presence and absence of metabolic activation system, for all the five tester strains tested.
All the 5 strains with the group of fungicides namely Difenoconazole Technical, Mancozeb Technical, Tricyclazole Technical when tested in plate incorporation assay with (10% S9) and without metabolic activation at 5 different concentrations did not show any mutagenic effect on *Salmonella* strains. Mutation ratio was calculated with respect to vehicle control, did not show two or more-fold increase in the revertant (Figure 5, 6 and 7).
The revertant in the vehicle and positive controls were found to be within the range of the in-house historical control data for Trial I and Trial II. Significant increase in the revertant colonies observed in concurrent positive controls demonstrated sensitivity of the assay in presence and absence of metabolic activation system. Difenoconazole Technical, Mancozeb Technical, Tricyclazole Technical are non-mutagenic at and up to 5000 µg/plate in all the strains of *Salmonella typhimurium*.

DISCUSSION
Difenoconazole Technicalis a fungicide used for disease control in many fruits, vegetables, cereals and other field crops. Although potentially a mobile molecule, it is unlikely to leach due to its low aqueous solubility. It does however have potential for particle bound transport. It is slightly volatile, persistent in soil and in the aquatic environment. There are some concerns regarding its potential for bioaccumulation. Moderately toxic to humans, mammals, birds and most aquatic organisms (8, 9, 29), and showed cytotoxicity in zebrafish (23, 24, 29), human hepatocellular carcinoma HepG2 cells (16, 27). In present study Difenoconazole Technical did not show cytotoxicity in *Salmonella* neither mutagenic. Difenoconazole is unlikely to be genotoxic in vivo and unlikely to pose a carcinogenic risk to humans (9).
Mancozeb Technical is a broad-spectrum contact fungicide in crop management. In cytotoxicity studies like HepG2 cells, Mancozeb strongly reduces the cell proliferation. Also one of the parameter from mutagenicity battery like micronucleus test in HepG2 cell line showed significant increase in the micronucleus. But in Ames test all the parameters like cytotoxicity study, trial I and trial II it is non mutagenic and the background lawn of the salmonella was also uniform. Mancozeb is used against fungal

infections on many fruit, vegetable, nut, and field crops in Minnesota. It provides protection against a wide spectrum of fungal diseases, including potato blight, leaf spot, scab, and rust. It is also used as seed treatment for potatoes, corn, sorghum, tomatoes, and cereal grains (6, 7,19, 30, 32).
Tricyclazole Technical is found to be non-mutagenic at 10 to 5000 µg /plate in various studies conducted globally. Tricyclazole Technical, a new systemic fungicide used for the control of rice blast caused by Pyriculariaoryzae Cav., hardly inhibited the mycelial growth, conidial germination and appressorial formation of P. oryzae at concentrations less than 125ppm, but it protected the plants almost completely from the disease by foliage application at as low as 10 to 20ppm (1, 12, 13, 31).

CONCLUSION

The effects of a group of fungicides chosen for the study on the *Salmonella*/microsome assay are demonstrated by the results of exposure to fungicide, as in several concentrations were non cytotoxic and non-mutagenic.

CONFLICT OF INTEREST: NONE

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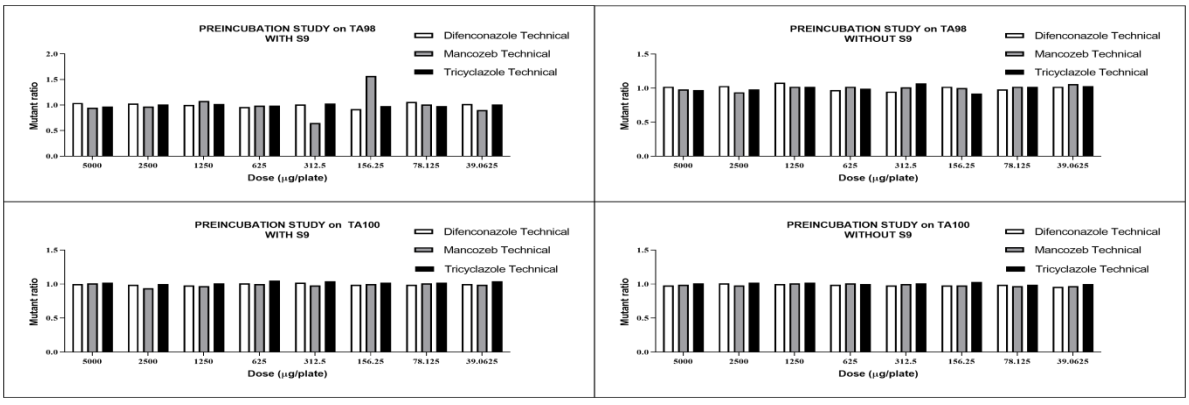
Table1: Characters of *the Salmonella typhimurium* strains

Tester Strains Genotypes				
Salmonella typhimurium				
Tester Strains	**his Mutation**	**Additional Mutations**		**Plasmid**
		Repair	**LPS**	
TA1535	*his*G46	*uvr*B	*rfa*	-
TA97a	*his*O1242	*uvr*B	*rfa*	pKM101
TA98	*his*D3052	*uvr*B	*rfa*	pKM101
TA100	*his*G46	*uvr*B	*rfa*	pKM101
TA102	*his*G428	-	*rfa*	pKM101 & pAQ1

Table 2: The combinations of positive controls were plated concurrently with the assay

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
Vehicle Control			
ALL	**Both**	DMSO	**10** µL
Positive Controls			
TA1535	+	2-Aminofluorene	10 µg
	-	Sodium Azide	1.5 µg
TA97a	+	2-Aminofluorene	10 µg
	-	4-Nitroquinolene-N- Oxide	0.5 µg
TA98	+	2-Aminofluorene	10 µg
	-	4-Nitroquinolene-N- Oxide	0.5 µg
TA100	+	2-Aminofluorene	10 µg
	-	Sodium Azide	1.5 µg
TA102	+	2-Aminofluorene	10 µg
	-	Methyl Methane Sulphonate	1.0 µg

Figure 1: Preliminary cytotoxicity study



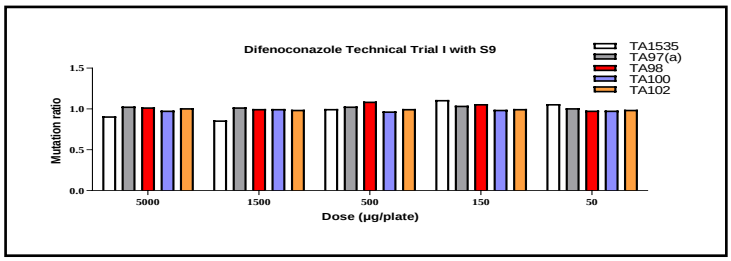


Figure 2: Histidine revertant counts in main study (Trial I) – Difenoconazole Technical

Figure 3: Histidine revertant counts in main study (Trial I) – Mancozeb Technical

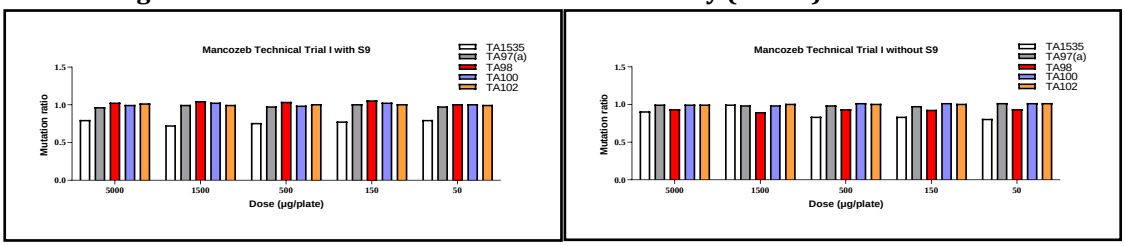


Figure 4: Histidine revertant counts in main study (Trial I) – Tricyclazole Technical

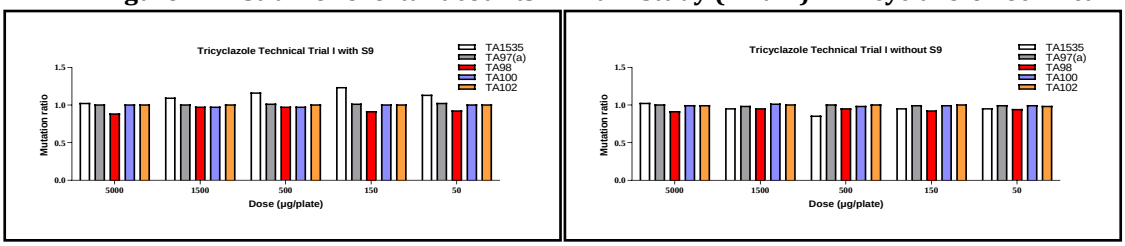


Figure 5: Histidine revertant counts in main study (Trial II) – Difenoconazole Technical

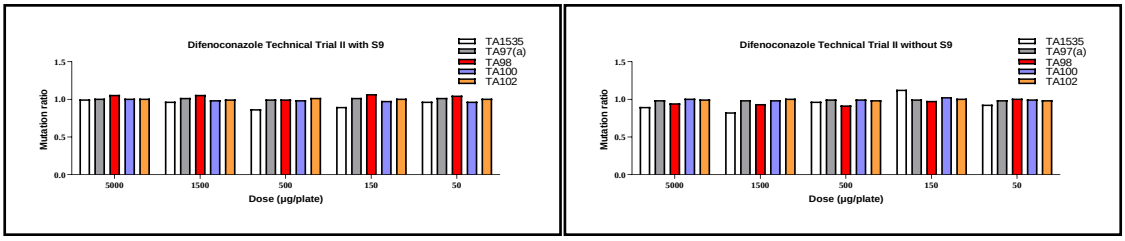


Figure 6: Histidine revertant counts in main study (Trial II) – Mancozeb Technical

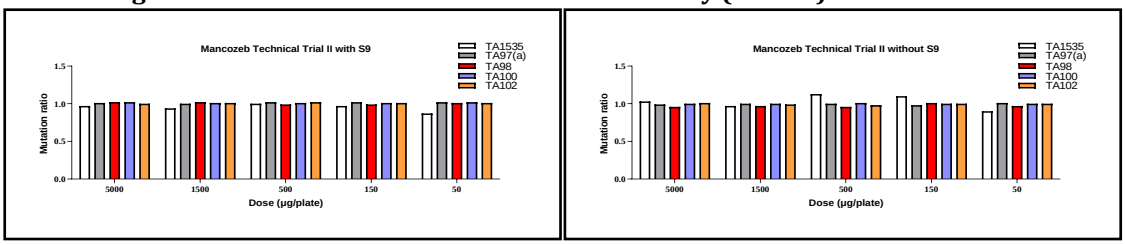
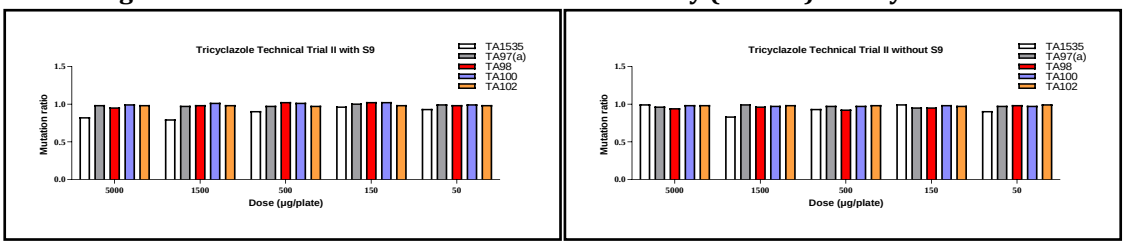
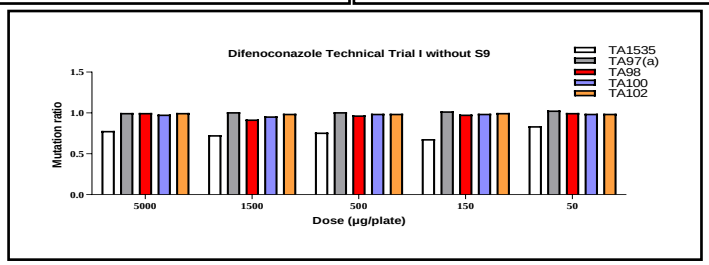


Figure 7: Histidine revertant counts in main study (Trial II) – Tricyclazole Technical





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