GENOTOXICITY OF SOME SYNTHETIC AND BIO-FUNGICIDES ON *VICIA FABA* AND THE YEAST TESTER STRAIN (D_7)

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Abstract

The present work was carried out to evaluate the cytotoxicity and genotoxicity of two synthetic fungicides (Dithane M-45 and Rizolex T 50%) and two bio-organic fungicides (Clean root derived from *Bacillus subtilis*, and Blight stop derived from *Trichoderma harzianum*) which are commonly used in Egyptian Agriculture. The treatments were applied on two biological systems, one included a higher plant (*Vicia faba*) while the other included a microbial strain, (*S. cerevisiae* D_7). Cytological testes on *Vicia faba* plants showed that there were no significant differences between treated and untreated for parent and F1 in chiasma frequency/cell for all used fungicides. The mean frequency of chiasmata/cell was higher at diakinesis than that of metaphase I in all tested plants. This means that there were no effected by any treatment. Concerning the genotoxic effect on the tester *S. cerevisia* D_7 strain, the percentages of survivals decreased as the concentration and/or the treatment periods increased for all tested treatments. The used concentrations of Blight stop (*Trichoderma* filtrate) could not induce any convertants or revertants. All of Clean root, Dithane M-45 and Rizolex T 50% doses induced converted colonies in *S. cerevisiae* D_7 stain. None of the tested fungicides doses induced *S. cerevisiae* D_7 reverting colonies except the Dithane treatment. From the cytological and genetical points of view, results suggested that the use of bio-fungicides as an alternative of the synthetic fungicides may be more safe.

INTRODUCTION

Pesticides have some hazardous effects in addition to their benefits. Their undesirable residue in water, food and environment may cause health problems. Higher plants provided useful genetic system for screening and monitoring environmental pollutants. Mutagenic activity of chemicals has been analyzed with different plant systems such as *Allium cepa*, *Vicia faba, Arabidopsis thaliana* and *Hordeum vulgare*. Pesticides may be effective to change plant genetic system as a result of their mutagenicity (Badr, 1988). The majority of mutations induced by these pesticides may be recessive and have little significance to the individual carrying them but when brought together at the time of conception they may produce harmful effects to the developing embryo (Grant 1970). Many workers studied the meiotic behavior, chromosome pairing, chromosome aberrations and chiasma frequency in meiocytes of *Vicia faba*. [Baker *et al.*, (1976), Birch *et al.*, (1985), Raina and Ress,

(1983), Raina and Narayan, (1984), Chapman (1984), Raina, (1990), Hanelt and Mettin, (1991) and Maxted *et al.*, (1991)]. The cytological and genetical effect of Dithane fungicides on onion (*Allium cepa*) were analyzed by Mann (1977). Barakat and Hassan, (1997) reported that Pendimethalin herbicide induced proportion of abnormal pollen mother cells (PMCs) in all meiotic stage of *Vicia faba* flowering buds and reduced the mitotic index in *Allium cepa* root-tips.

The yeast *Saccaromyces cerevisia* is an eukaryotic microorganism that lends itself to the study of allelic recombination, occurring between related sequences on homologous chromosomes in a diploid cell. The diploid tester strain D_7 of *S. cerevisiae* was constructed (Zimmereman *et al.,* 1975) for the efficient detection of mitotic gene conversion of the *trp* locus, mitotic crossing over and gene conversion at *ade*-2 and point mutation causing reversion of the *ilv*-92 allele. The genetical effects of many different chemical compounds on yeast (*S. cerevisia*) were extensively studied. Bertini *et al.,* (2000) tested the genotoxic effects of Meneb (fungicide) on *S. cerevisiae* strain D_7 in logarithmic phase and showed that treatment of yeast cells with 0.5mM reduced survivals to 50%, and two of the four concentrations employed increased the mitotic gene conversion and all concentrations caused a significant increase in revertants. Della Croce *et al.,* (1996) examined the genotoxic effects of two commercial pesticides (Atrazine and Zineb) on *S. cerevisiae* D_7 strain. It was found that the two compounds increased both gene conversion and point mutation frequencies in the logarithmic phase cells, while in the stationary phase no genotoxic effect was observed. Schmid and Wolf, (1995) evaluated the genotoxicity of three carbamate insecticides (Benfuracarb, Carbosulfan and Furathiocarb) on *S. cerevisiae* strain D_7. They found that none of the three insecticides had an influence on the frequencies of gene conversion and reverse mutation when tested with and without metabolic activation.

The bio-safety of the recent used bio-fungicides hasn't been clarified yet. The aim of present work is determining cytogenetic and mutagenic effects of two synthetic fungicides (Dithane M-45 & Rizolex T-50%) in comparison with two bio- fungicides (Blight Stop & Clean Root). This target was realized by using two biological systems (faba bean plants and D_7 yeast tester strain).

MATERIALS AND METHODS

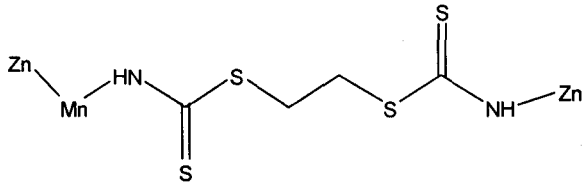
I. Fungicides

Two different groups of agro-chemicals (synthetic and bio-fungicides) were used as follow:-

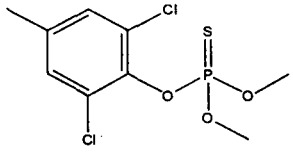
I. a. Synthetic fungicides (Fig. 1) which included:

1-Dithane M-45, which is an ethylene(bis)dithiocarbamate fungicide. [Manganese Ethylenebis (dithiocarbamate) polymeric complex with zinc salt]. It was applied with three concentrations, (1, 2.5 and 4 gram /Litter D.W).

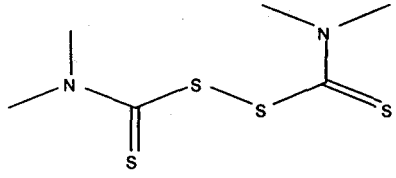
2- Rizolex T 50% WP (mixture with thiram), which is an organophosphorus fungicide. [Tolclofos methyl: O-2,6-dichloro-p-tolyl O,O-dimethyl phosphorothioate and Thiram : tetra methyl thiuram disulfide]. Three concentrations, (1, 3 and 5gram /Litter D.W) were used.



Dithane M-45



O-2,6-dichloro-p-tolyl O,O-dimethyl phosphorothioate



tetra methyl thiuram disulfide

Fig. 1. Chemical formula of Dithane M-45, Rizolex T 50% and Thiram synthetic fungicides.

I. b. Bio-fungicides

Two bio-fungicides (Blight stop & Clean root) and their active ingredients (*Trichoderma* filtrate & *Bacillus* filtrate, respectively) were prepared in the Central Lab of Organic Agriculture, Agriculture Research Center (ARC) according to the method of Brain and Hemming (1945) and Dowson (1957). The obtained culture filtrates from *Trichoderma harzianum* and *Bacillus subtilis* were used with three concentrations, 0.5, 1.0 and 1.5% in D.W. (v/v).

II. Plant system: Pure line seeds of *Vicia faba* (v.Masr1) were obtained from the Crop Research Institute, Agriculture Research Center (ARC), Giza, Egypt to whom our thanks are due.

II a- Field Experiment (Season 2006-2007)

The concentrations and treatments of synthetic and bio-fungicides in field experiment were carried out according to the recommendations of Egyptian Ministry of agriculture and Central lab. of Organic Agriculture (2000)*. The treatments were distributed in plots in Randomized Complete Block Design (RCBD).

Flowering buds from ten plants from each fungicide treatment and their control plants (i.e. parent plants) were gathered separately, when they were 45 days old after planting, for each treatment and the control. Fixation was done in a fresh fixative solution of ethanol / glacial acetic acid 3: 1 (v / v), for 24 hours, then storing in 70% ethanol at 4^0C in refrigerator.

Cytological preparations of PMCs were made using the aceto-carmine smear methods (Belling, 1926). Data were obtained from at least 100 cells/plant. The rest of plants were allowed to produce seeds. The data were statistically analyzed (in RCBD) using MSTAT program (Version 4).

II. b. Field Experiment (Season 2007-2008)

F1 seeds were cultivated and the flowering buds were taken for making F1 meiotic analysis. The F1and F2 plants were not treated by any pesticides.

III. Microorganism system

III. a. *Saccharomyces cerevisiae* tester strain (D_7)

An isolate of *S. cerevisiae* D_7 strain (Zimmermann *et al.,* 1975) was to whom our thanks are due obtained from Genetics department, Faculty of agriculture, Assiut University. This strain is a diploid and triple requiring mutant (Fig. 2), heteroallelic for both tryptophane (trp5-12/trp5-27) and adenine (ade2-40/ade2-119) and homoallelic for isoleucine (ilv1-92/ilv1-92). The genotype of D_7 strain is as shown in fig (2):

Fig. 2. illustration of three hetroallelic loci in D7 strain that used to measure the genetic effect of the tested fungicides.

III. b. Media

The complete growth medium **(CGM)** contained 2% d-glucose, 1% yeast extract, 2% peptone, 2% agar was used for routine culture growth and maintenance

Planting of Faba bean. pamphlet of ARC: 627 (2000)

of cultures (Zimmermann *et al.,* 1975). The synthetic complete medium **(SCM)** [2% d-glucose, 2% agar,0.17% yeast nitrogen base without amino acids and 0.5% $(NH_4)_2SO_4$] was supplemented with 5mg adenine/L medium, 60 mg isoleucine/L for detection of *trp*$^+$ gene convertants, or 5 mg adenine/L medium and 10 mg tryptophane/L for the detection of *ilv*$^+$ revertants.

III. c. Treatment of yeast cells

Strain *S. cerevisia* D_7 was inoculated as single colonies into 50 ml flasks each containing 25 ml of liquid **CGM** medium and incubated overnight at 30 C°. Ten ml of cell culture were centrifuged at 3500 rpm for 5-7 min, washed twice and resuspended in equal volumes of sterile distilled water. One ml of cell suspension was mixed with 8 ml sterile distilled water and one ml of the tested concentration of the fungicide. One ml of cell suspension was mixed with 9ml sterile distilled water to be counted as a control. The mixtures were incubated for 6, 12 and 24 hours in a shaker water bath at 30°. At the end of the treatment period, one tenth ml samples with suitable dilution were plated onto **CGM** plates to score survivors after 3 days incubation at 30 C°. One tenth ml samples with suitable dilutions were spread on **SCM** tryptophane free and isoleucine free plates with three replicates. After 4-6 days, the plates were checked for the spontaneous convertants and revertants. The converted and reverted colonies were counted in each plate and their mean number of colonies, accompanied with L.S.D was calculated for each treatment (Zimmermann, 1971 and Mousa *et al.*, 1989) relatively to their survivals.

IIII. d. Statistical analysis

The data were statistically analyzed according to Gomes and Gomes, (1984) (in RCBD) using MSTAT program (Version 4).

RESULTS

I. Effect of fungicides on chiasma frequency of *Vicia faba* plant

The mutagenic potential of the fungicides was evaluated using cytological data of chiasma frequency. The mean frequency of chiasmata per cell at both diakinesis and metaphase I after treatments with the synthetic and the bio-fungicides in parent and their F1 plants are shown in Table 1 and Figure 3. There were no significant differences between treated and control plants in chiasma frequency/cell for all used fungicides. The mean frequency of chiasmata/cell was higher at diakinesis than that of metaphase I in all tested plants. This relation wasn't affected by any fungicide treatment.

Table 1. Chiasma frequency in treated parent with four fungicides and their F1 plants.

Treatment	Parent		F1	
	Diakinesis	Metaphase I	Diakinesis	Metaphase I
Control	20.84	17.74	19.28	15.70
Dithane	20.60	17.87	18.75	14.90
Rizolex	21.27	18.07	18.43	15.20
Blight Stop	20.81	17.83	18.47	15.50
Clean Root	19.97	17.73	18.67	15.60
$L.S.D_{0.05}$ =	2.60	2.17	0.86	0.86

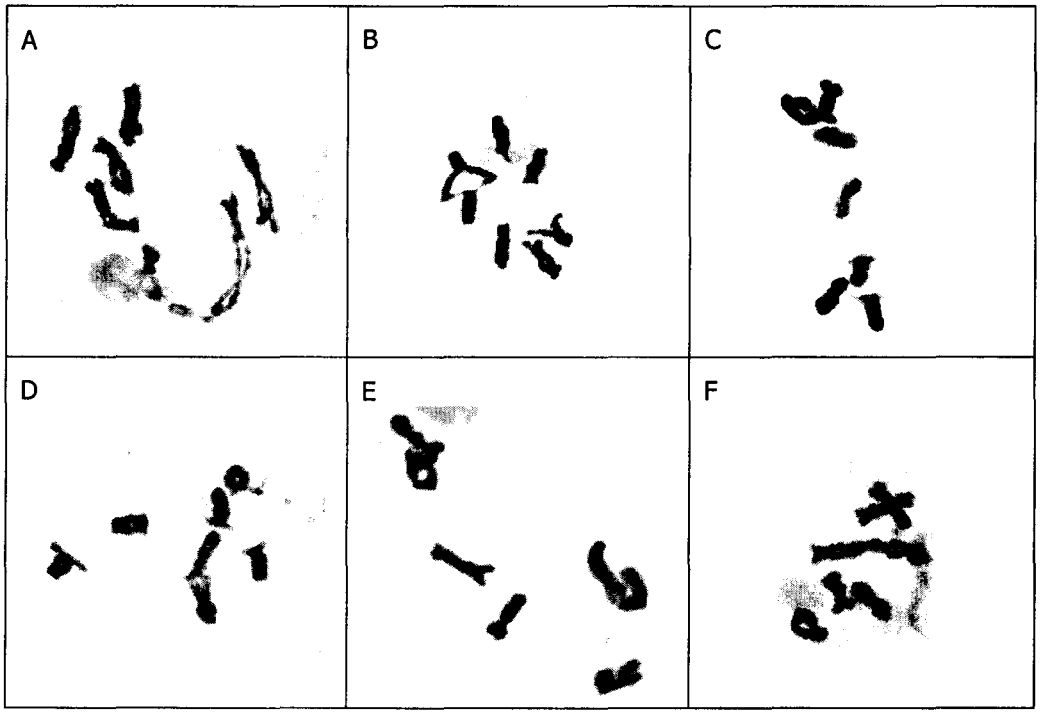


Fig. 3. Chiasma formation at both diakinesis (A) and metaphase I (B, C, D, E and F) of *Vicia faba* plants.

II. Genotoxicity of fungicides on *S. cerevisia* tester strain D_7

The four fungicides, Blight stop, Clean root, Rizolex T 50% and Dithane M- 45 were tested for their genotoxicity on the tester *S. cerevisiae* D_7. The obtained results are shown in Tables 2, 3, 4 and 5 respectively. Data in Table (2) show that the percentages of survivals decreased as the concentration and/or the treatment periods increased.

Table 2. Mean numbers and percentages of *S. cerevisiae* D_7 Survived, three concentrations with three periods of Blight stop (*Trichoderma* filtrate) treatments.

Treat.	Time	Conc. v/v	Survivals		Convertants	Revertants
			Mean values × 10^4	%		
BLIGHT STOP	6 (hr)	Zero	128	100	0	0
		0. 5%	117	91.41	0	0
		1.0%	103	80.47	0	0
		1.5%	101	78.91	0	0
	12 (hr)	0. 5%	112	87.50	0	0
		1.0%	111	86.72	0	0
		1.5%	112	87.50	0	0
	24 (hr)	0. 5%	75	58.59	0	0
		1.0%	78	60.94	0	0
		1.5%	75	58.59	0	0

The percentages of survivals relative to the control ranged from 91.41% in the treatment of 0. 5 for 6 hours to 58.59% in the treatment of 1.5% for 24 hours. The *Trichoderma* filtrate (Blight Stop) with the used concentrations could not result in inducing any convertant or revertants.

It can be concluded that *Trichoderma* filtrate has no detectable mutagenic effect by using different concentrations of fungicide Blight stop.

Data in Table (3) show that a relatively high percentages (95.15- 70.07%) of survivals could be obtained after treatment with different concentrations (0.5, 1.0 and 1.5) of *B. subtilus* filtrate (Clean Root) for 6 or 12 hours.

Table 3. Mean numbers and percentages of survivals and *trp* convertants resulted after treatments with different concentrations and periods of Clean root (*Bacillus subtilus* filtrate).

Treat.	Time	Conc. v/v	Survivals		Convertants × 10^4 survivors		Revertants
			Mean values × 10^4	%	Mean values	%	
CLEAN ROOT	6 (hr)	Zero	1527	100	0	0.00	0
		0. 5	1240	81.20	2.00	0.19	0
		1.0%	1150	75.31	2.00	0.20	0
		1.5%	1290	84.48	14.00	1.09*	0
	12 (hr)	0. 5%	1453	95.15	6.00	0.39	0
		1.0%	1070	70.07	10.00	0.90	0
		1.5%	1247	81.66	9.00	0.72	0
	24 (hr)	0. 5%	324	21..21	5.00	1.43*	0
		1.0%	364	23.83	3.00	0.84	0
		1.5%	297	19.45	10.00	3.32**	0
					$LSD_{0.05}$ =	1.079	
					$LSD_{0.01}$ =	1.479	

On the contrary the treatment with the same concentrations (0.5, 1.0 and 1.5) but for 24 hours decreased survivals ratios to 21.21, 23.83 and 19.45% respectively. On the other hand data in Table (3) revealed that *trp* convertants ranged from 0.19 to 3.32% relative to their survivals. It can be observed that percentages of *trp* convertants increased as concentrations or period of treatment were increased (Table3). These results indicate that *Bacillus subtilus* filtrate (Clean Root) treatment was effective as a mutagen particularly in a period more than 12 hours. The statistical analysis as shown in Table (3) ensures this conclusion.

Data in Table (4) show that survivals ratios still relatively high (48.8-72.8%) after treatments with 0.01 or 0.03 of Rizolex for all periods (6, 12 and 24hours).

Table 4. Mean numbers and percentages of S. *cerevisiae* cells Survivals and *trp* convertant after treatment with different concentrations and different periods of Rizolex T.50%.

Treat.	Time	Conc. g/L	Survivals		Convertants × 10^4 survivors		Revertants
			Mean values × 10^4	%	Mean values	%	
RIZOLEX T 50%	6 (hr)	Zero	4960	100	7.00	0.15	0
		0.01	3607	72.7	16.00	0.43*	0
		0.03	2953	59.5	18.00	0.62**	0
		0.05	686	13.8	0.30	0.05	0
	12 (hr)	0.01	3610	72.8	23.00	0.64**	0
		0.03	2563	51.7	16.00	0.64**	0
		0.05	680	13.7	0.20	0.3	0
	24 (hr)	0.01	3247	65.5	16.00	0.5**	0
		0.03	2420	48.8	10.00	0.41*	0
		0.05	369	7.4	0.10	0.03	0
					$LSD_{0.05}$ =	0.24	
					$LSD_{0.01}$ =	0.33	

The treatment with the Rizolex concentration 0.05 for any period decreased severely the survivals ratios to become 13.8, 13.7 and 7.4% of the control for the periods 6, 12 and 24 hours respectively. Obtained results (Table4) revealed that *S. cerevisiae* cells converted spontaneously in *trp* locus with ratio 0.15% of control survivals.This ratio of *trp* convertants has increased after treatment with Rizolex 0.01 and 0.03 to be 0.43% and 0.64% of survivals for any period. The treatment with concentration 0.05 resulted in 0.05, 0.3 and 0.03% *trp* convertants for 6, 12 and 24hr respectively. These results declare that Rizolex is effective as a mutagen in most cases with the used concentrations and treatment periods. Statistical analysis ensures the significant differences between the control and the treated *S. cerevisiae* with Rizolex.

Data in Table (5) show that ratios of survivals decreased as the concentration of Dithane was increased for each period of treatment.

Table 5. Mean numbers and percentages of survivals and *trp* convertants and *ilv* revertants after treatment with different concentrations and different periods of Dithane M 45.

Treat.	Time	Conc. g/L	Survivals		Convertants $\times 10^4$ survivors		Revertants $\times 10^4$ survivors	
			Mean values $\times 10^4$	%	Mean values	%	Mean values	%
DITHANE M-45	6 (hr)	Zero	7333	100	11.33	0.15	7.00	0.10
		0.010	5733	78.18	14.00	0.24	20.00	0.35
		0.025	4633	63.18	27.00	0.58*	37.00	0.80**
		0.040	3800	51.82	36.00	0.94**	31.00	0.82**
	12 (hr)	0.010	4500	61.37	21.00	0.47	25.00	0.56**
		0.025	3933	53.63	27.00	0.70**	31.00	0.78**
		0.040	2167	29.55	11.00	0.49*	15.00	0.71**
	24 (hr)	0.010	3000	40.91	22.00	0.73**	19.00	0.64**
		0.025	2267	30.92	14.00	0.63**	7.00	0.32
		0.040	800	10.91	37.00	4.6**	4.00	0.50*
					$LSD_{0.05}$ =	0.33		0.33
					$LSD_{0.01}$ =	0.45		0.45

The highest ratios (78.18- 51.82%) of survivals resulted from the treatment for 6 hours by different concentrations of Dithan fungicide. The lowest ratios 10.91-40.91% were obtained by treatment for 24 hours. Ratio of spontaneous *trp* convertants was 0.15% of control survivals in the cultures of *S. cerevisiae* D_7 used in this experiment. Spontaneous revertants of isoleucine also were obtained with the ratio 0.1 %. It can be observed that after treatments by Dithane M-45 both *trp* convertants and *ilv* revertants ratios increased, though variably (Table5). *trp* convertants were obtained with 0.24-4.6% after treatment with different concentrations and periods and *ilv* revertants were obtained with 0.32-0.82% after treatment. These results confirm that Dithane has a mutagenic effect with the concentrations and periods used in present work. The statistical analysis supported these results.

DISCUSSION

There is a general agreement that the agrochemicals, particularly synthetic pesticides are very dangerous and may cause tumors, cancers and teratogenic abnormalities (Grant, 1970 and 1971). However, in the last few decades, some alternative bio- products were used as safe pesticides in agriculture (Abd-El-Moity and Shatla, 1981). In the present work the genotoxic effects of bio-fungicides (e.g. Blight Stop and Clean Root) were examined in comparison with two partner synthetic fungicides (Dithane M-45 and Rizolex T-50%). Two biological systems (chiasma frequency in *Vicia faba* PMCs and mutagenicity of identified loci in D_7 *S. cerevisiae*) were applied to measure the cyto- and genotoxic effects of the given fungicides.

The chiasma frequency per cell was not affected by the treatments with both synthetic and bio-fungicides in parent and F1 plants while the frequency of uni- and multi-valents which was studied elsewhere (Ata *et al*, 2008) appeared to be significantly affected by Dithane treatment either in parent or in F1 plants. It was concluded that these features might have occurred due to the disturbance effects of the synthetic fungicides on genetic control of chromosome pairing during meiosis.

Concerning the obtained results with *S. cerevisiae* one of the tested bio-fungicides Blight stop (*Trichoderma* filtrate) proved not to be effective since no revertant or convertant were resulted. On the contrary the remaining tested three fungicides (Clean root, Dithane M-45 and Rizolex T 50%) proved to have mutagenic effect on *S. cerevisia* cells particularly the synthetic fungicide Dithane M-45 which induced both convertants and revertants with significantly higher frequencies than the control. Minet *et al.* (1980) concluded that there is heterogeneity in the tendency to undergo recombination among cells of *S. pombe* and it was proposed that a fraction of the population is in a "parameiotic state" in which recombination frequencies are more typical of meiosis than of mitosis.

Several studies (Hurst and Fogel, 1964 and Montelone *et al.* 1981) showed that elevated frequencies of coincident recombination apply to linked genes as well as unlinked genes. Heterogeneity in recombination competence has also been considered as an explanation for coincident mitotic recombination in yeast (Wilkie and Lewis 1963, Montelone *et al.* 1981 and Fabre and Roman, 1977). Probability of both haploid yeasts, arising under the experimental conditions, and cell cycle stage may contribute to variation in susceptibility to induction of genetic alternation (conversion and reversion). Freeman and Hoffman (2007) reported that modest influences of cell cycle stage have not been excluded while excluded effect of haploid or aneuploid yeast and reported that permeability to the mutagen affect convertants and revertants.

Studies of Freeman and Hoffman (2007) found that the indicator genes *trp*-5, *ilv*-I and *ade*-2 in yeast strain D_7 are on different chromosomes and the spontaneous frequencies of the recombinational events are at least 100 times higher than typical mutation frequencies. Moreover they reported that mutagen treatment can substantially increase the recombinant and revertant frequencies. This was observed in present findings specially with the chemical fungicides (Dithane M-45 and Rizolex T 50%).

It could be concluded, from the cytogentical and mutagenicity points of view, that the use of bio-fungicides as an alternative agricultural material instead of the synthetic pesticides may be more safety.

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السمية الوراثية لبعض المبيدات الفطرية المخلقة والحيوية على نبات الفول البلدي وعلى سلالة الخميرة المختبرة (D_7)

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أجريت هذه الدراسة لتقييم السمية الخلوية والوراثية لاثنين من المبيدات الفطرية المخلقة هما الديثان م- ٤٥ والريزولكس تى ٥٠٪ واثنين من المبيدات الفطرية الحيوية هما الكلين روت (مشتق من بكتريا الباسلس) والبلايت ستوب (مشتق من فطر التريكوديرما) التى تستخدم على نطاق واسع فى الزراعة المصرية. تم استخدام ثلاث جرعات من كل مبيد بالإضافة إلى معاملة للمقارنة (كنترول) لم يستخدم فيها أي من تلك المبيدات. تم استخدام نظامين بيولوجيين مختلفين كنماذج اختبار سريعة هما نبات الفول البلدي *V. faba* و سلالة D_7 *S. cerevisiae* (خميرة الخبز).

أظهرت نتائج الاختبارات الخلوية على نباتات الفول البلدي أن هناك اختلافات غير معنوية بين النباتات المعاملة بكل المبيدات الفطرية المستخدمة والكنترول فى تكرارات الكيازما لكل خليه وان متوسط تكرارات الكيازما / خليه كانت أعلى فى الطور التشتتى عنه فى الاستوائي الأول. بينما أظهرت الاختبارات الوراثية على Tester strain *S. cerevisiae* D_7 أن جميع المعاملات بالجرعات و أوقات التعريض المختلفة لجميع المبيدات تحت الدراسة أدت الى تثبيط نسبه الحياة لخلايا الخميرة كلما زادت الجرعات ومدة التعريض و أن المعاملة بالمبيد الفطري الحيوي البلايت ستوب (مشتق من فطر التريكوديرما) لم تنتج اى مستعمرات خميرة مرتدة أو ذات عبور أحادى غير متبادل بينما الجرعات المستخدمة من المبيدات الكلين روت و الديثان م-٤٥ و الريزولكس تى ٥٠ ٪ أنتجت مستعمرات ذات عبور أحادى غير متبادل من *S. cerevisiae* D_7 و لم تنتج مستعمرات مرتدة من *S. cerevisiae* D_7 فيما عدا المعاملة بالمبيد الفطري المخلق الديثان م-٤٥ الذى انتج مستعمرات ذات عبور احادى غير متبادل بالاضافه الى مستعمرات مرتده.

ويستخلص من هذه الدراسة أنه من وجهتى النظر الخلوية و الوراثية (التأثير الطفري) أن البديل الحيوي للمبيدات الكيماوية ربما يكون أكثر أمنا فى الاستخدامات الزراعية.