GAMMA RADIATION INDUCED FUNGICIDE-SENSITIVE MUTANT OF PEANUT *RHIZOBIUM* STRAINS

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ABSTRACT

Two fast growing *Rhizobium* strains of peanut (*Arachis hypogea*) A.V. and 169 were subjected to increasing doses of γ-radiation. Dose response curve and sublethal dose were determined. Effect of the fungicide (Tilt) on the survival of the most radioresistant bacterial isolates was alsoexamined.

Results showed that the dose response curve of both strains was of the exponential type and the sublethal dose for 169 strain was 50 k rad while that for A.V. strain was 75 k rad.

Interaction of the most radioresistant bacterial isolates and the fungicide (Tilt) with different doses of 2.5, 5, 7.5, 10, 20, 25 and 30 ppm showed that γ-rays induced a fungicide sensitive isolates more than the wild type.

INTRODUCTION

Some authors (Jordan, 1952a; Dygdala, 1962, 1963; Russell and Jones, 1973; El-Zawahry, 1976; Srivastava *et al.*, 1980 and Barend and Henri, 1981) have studied the effect of radiation on growth and survival of the bacteria responsible for soil fertility through symbiotic association, suches thesymbiotic nitrogen fixing bacteria belonging to the genus *Rhizobium*.

The use of pesticides has become an integral and economically essential part of agriculture. Because of concern about possible side effects the attention of soil microbiologists has been focused on the effects of pesticides on non target soil microorganisms such as *Rhizobium*.

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Pareek and Shidu (1978) reported that the phenoxy herbicide 2,4-D reduced the growth of R. meliloti, R. trifolii and Rhizobium sp., oxygen suptake was stimulated though endogenous respiration was not affected.

Janos et al., (1979) studied the effect of the urea herbicide chlorobromuron and its hydrolysate product, chlorobromaniline, on R. meliloti and found that chlorobromuron inhibited growth at a concentration of 10 mg/L. However chlorobromaniline stimulated the growth of R. meliloti.

Kao and Wang (1981) studied the interaction between herbicides and Rhizobium in pure culture. They found that the fast-growing type rhizobia were more sensitive than the slow-growing ones in presence of the same herbicide dosage, however linuron with the dosage of 50 ppm was decomposed by Rhizobium-leguminosarum and Rhizobium japonicum (Cowpea strain) individually, and 2,4-D of 50 ppm was also decomposed by Rhizobium lupini. Mutants of R. meliloti, R. phaseoli and cowpea rhizobia that were resistant to the fungicides thiram, phygon and spergon have been isolated by Odeyemi and Alexander (1977a,b). These fungicide resistant mutants were able to destroy the pesticides and were highly effective on their host plants. So, these fungicide resistant mutants considered as better inoculants than their parental strains when applied to legume seeds that had been treated with these fungicides.

The present investigation aimed at the response of two strains of Peanut to increasing doses of γ-radiation as well as the effect of the fungicide (Tilt) on the survival of the most raioresistant bacterial isolates.

MATERIAL AND METHODS

Bacterial strains: Effective strains were obtained from the Microbiology Research Center, Ministry of Agriculture, Cairo, two strains were used A.V. and 169. Cultures were grown and maintained on yeast extract mannitol medium (YMA) (Allen, 1957) of the following composition: Mannitol, 10g; Nacl, 0.1g: $MgSo_4 \cdot 7\ H_2O$, 0.2g; $K_2H\ PO_4$, 0.5g: yeast extract, 0.5g; $CaCl_2$, 0.1 g, distilled water 1000 ml. The pH of the medium was adjusted to 7.2 using NaOH. Fifteen grams of Difco agar were added when desired.

Irradiation techniques:

Source of Gamma radiation: The used source of radiation was cobalt 60 (commercial Gamma cell Atomic Energy of Canada Limited, Located in the Physics Department of the Egyptian Atomic Energy Establishment), giving a dose of 15 rads per second at time of experiment.

Irradiation of the wild type Rhizobium strains A.V. and 169:

The adopted method was that recommended by Dygdala (1963), Schwinghamer (1969) and Russell and Jones (1973). The wild strains of Rhizobium A.V and 169 were allowed to grow individually untill the late log phase on yeast extract mannitol liquid medium. At the end of the growth period, the cells were hervested by centrifugation. The harvested cells were then washed with phosphate buffer at pH 7.0 for three times and finaly resuspended in the buffer to a concentration of 10^7 - 10^8 cells/ml. The bacterial suspension was divided under aseptic condition into 5 ml aliquots in sterile test tubes. Duplicate tubes of the above bacterial culture were exposed to increasing doses of γ-radiation (10, 20, 30, 40, 50, 75 and 100 Krad) at room temperature. Control culture tubes (of non irradiated culture) were kept at room temperature. Viable count for each of the irradiated and the control cultures were determined by the dilution plate (pour agar plate) method on yeast extract mannitol agar medium. Incubation of the agar plates continued for one week at 28°C before they were counted. Dose response curve was constructed by plotting log N/No VS: the irradiation dose where No and N were the intial and final viable count for each irradiation dose.

Isolation of the most radioresistant bacterial isolates:

Bacteria at the sublethal dose were subcultured three times on petridish containing yeast extract mannitol agar medium.

Effect of the fungicide (Tilt) on the survival of the most radioresistant bacterial isolates:

The effect of the fungicide "tilt" on the survival of the most radioresistant bacterial isolate of the wild type A.V and 169 strains was investigated. Bacteria were incoulated separately in yeast extract mannitol broth medium supplemented with tilt at concentrations of 2.5, 5, 7.5, 10, 15, 20, 25 and 30 ppm of active ingredient per ml medium. Cultures were prepared in 100 ml conical flask containing 50 ml of medium incoulated with 0.2ml of a culture of the bacteria (about 10^6 cells/ml) in yeast mannitol liquid medium and incubated at 28°C on a rotary shaker. Viability of the bacteria was monitored by plating on yeast extract-mannitol-agar.

Pesticide:

One comercially available pesticide was used in this investigation.

The fungicide (Tilt), a product of (GIBA-GEIGY) Switzerland. The active ingredient is 1-[2-(2,4 dichloropheyl)-4-propyl-1,3-dioxolan-2-yl methyl]-1H-1,2,4 triazole.

It is available as an emulsifiable concentrate with 25% active ingredient, and 0.25% recommended field application (Hussain, 1985).

RESULTS

Response of Rhizobium of Peanut A.Y. and 169 strains to increasing doses of γ-radiation.

This experment was conducted to measure the resistance of *Rhizobium* of Peanut strains to the following doses of γ-radiations 10, 20, 30, 40, 50, 75 and 100 K rad. The number of viable cells after irradiation was taken as criterion for resistance as compared with non irradiated control.

Fig. (1) and Table (1) show the effect of different doses of γ-radiation on the number of viable cells of the wild type of Rhizobium of Peanut 169 strain. These results suggested that this Rhizobium strain was highly sensitive to γ-radiation. Dose response curve is of the exponential type as the viable count decreased with the increase of γ-radiation. Vaible count begins with 1.3×10^8 and ends with 1.3×10^3 at non irradiated control and dose of 50 k rad respectively. No growth was recorded at 75 k rad. A dose of 50 k rad was estimated as the sublethal dose. It was found that a dose of 50 k rad reduced the number of survival cells by 5 log cycle.

Fig. (2) and Table (2) show the effects of different doses of gamma radiation on the number of viable cells of the wild type of Rhizobium of Peanut A.V. strain. The results suggested that this Rhizobium strain was less sensitive to γ-radiation. Dose response curve is of the exponential type because the viable count decreased with the increase of γ-radiation. Non irradiated control begins with 6.9×10^8 and reaches 3.1×10^2 at the dose of 75 krad. No growth was recorded at the dose of 100 k rad. A dose of 75 k rad was estimated as the sublethal dose and it reduced the viable count by 6 log cycle.

Effect of the fungicide (Tilt) on the survival of the most radio-resistant bacterial isolates of A.V. and 169 strains and their wild type.

Addition of the fungicide (Tilt) at different concentrations varying from 2.5 to 30 ppm to the yeast extract mannitol broth together with the most radioresistant bacterial isolate and its parent (Table 3) during the log phase results in an increase in viable count of the wild type of strain 169. Survival percentage increased gradually from 2.5 to 20 ppm then decreased at 25 and 30 ppm. Highest survivors recorded at 20 ppm. Survival percentage of the most radioresistant isolate increased at the lower doses of fungicide, while it decreased at the higher dosage. Highest survivors recorded at 7.5 ppm then it decreased gradually after that (Table 3).

Table (4) shows that survival of Rhizobium of Peanut A.V. in yeast extract mannitol medium supplemented with different doses of the fungicide (Tilt)-increased with the increase of the fungicide concentration from 2.5 to 7.5 ppm then decreased from 10 to 30 ppm. Highest survivors recored at 7.5 ppm. It was found that at this concentrations of the fungicide, the survivors increase 4-fold over that of the control. Survivors of the most radioresistant isolate increased gradually at the lower fungicide concentration from 2.5 to 7.5 then it decreased in presence of 7.5 to 30 ppm. Highest survivors recorded at 7.5 ppm.

It is clearly shown that the most radioresistant bacterial isolate either from 169 strain or from A.V. strain produced fungicide sensitive mutants while the wild type produced fungicide resistant mutants.

DISCUSSION

Many leguminous plants have been infected by certain fungi articulary Peanut (Arachis hypogea).

Aspergillus flavus, the common soil fungus may infest products uch as Groundnut and dried foods and produced a toxin called aflaoxin known to induce diseases to man and animals (Wogan 1965).

The purpose of this investigation was to manifest the effect of gamma radiation and the fungicide "Tilt" on the survival of the Peanut (Groundnut) Rhizobium strains for finding a fungi-cide resistance mutant used as an inoculant of Groundnut seeds as for the protection from Aspergillus flavus and other filamentous soil fungi.

Rhizobium strain 169 was foundmore sensitive to gamma radiation as the viable count was decreased with the increase of gamma radiation doses, thus it exihibited a simple exponential response curve with most radioresistant bacterial isolate at 50 k rad dose (Table 1). El-Zawahry (1976) found that radioresistant strains could be developed if the wild type of Rhizobium leguminosarum strain is subjected to different doses of gamma rays.

Rhizobium strain A.V. was found less sensitive to gamma radiation and it exihibited a simple exponential dose response curve with most radioresistant isolate at 75 k rad (Table 2). Barend and Henri (1981) studied the effect of gamma rays on the growth and survival of Peanut Rhizobium strain in peat culture and they found that the Rhizobium strain is not sensitive to gamma rays and it is not affected byincreasing the dose from 25 to 50 KGY.

As "Tilt" is a fungicide of many agriculture plants, it would be useful to have resistant strains to be used as inoculants. Tilt induced a fungicide resistant mutant with the wild type of Rhizobium strains and 20 ppm fungicidal concentration induced survival increase 3-fold over that of control (Table 3) in 169 strain while 7.5 ppm fungicidal concentration induced survival increase 4-fold in A.V. strain (Table 4).

The fact that pesticides inhibited and/or stimulated Rhizobium strains, many authors have suggested that it might be due to a lack of energy for cell synthesis because the pesticides acts as an uncoupling agent of oxidative phosphorylation (Pareek and Shidu 1978). While Odeyemi and Alexander (1977a,b) and Kao and Wang (1981) have suggested that the fungicide resistant mutants were able to destroy the pesticide.

The most radioresistant isolate did not induced a fungicide resistant mutants as all the fungicidal concentrations induced survival decrease in viable count of cells, survivals decreased with the increase of fungicidal concentrations (Tables

13 and 14). Since the most radioresistant isolate of 169 strain was achieved at 50 K rad and the most radioresistant isolate of A.V. strain was achieved at 75 K rad; survivors of 169 strain were more than those of strain A.V., they were 88% and 48% at the same level of fungicidal concentration (7.5 ppm) respectively (Tables 13 and 14).Golebiowska et al., (1967) reported that Rhizobium strains showed different sensitivities to the fungicide thiuram. They also reported that subculturing bacteria in media containing thiuram did not increase the fraction of thiuram resistant mutants in most Rhizobium strains. However the frequency of bacteria resistant to thiuram was increased among survivors of U.V. irradiation indicating that resistance could be induced by mutation. Ruiz-Sainz et al. (1984) studied the effect of the fungicide captafol on the survival and symbiotic properties of Rhizobium trifolii and they found that captafol resistant mutants were lost the smallest plasmid responsible for nodulatin and nitrogen fixation, hence it is not necessary that a fungicide resistant mutants form good nodulation and nitrogen fixation. Curley and Burton (1975) have made a simillar suggestion in a report in which they found that although captan was less toxic for Rhizobium japonicum than PCNB (pentachloronitrobenzene) the former was more inhibitory to nodulation when they were used as seed protectants for inoculated soybean.

If comparing the effect of certain chemicals and the effect of radiation on Rhizobium survival and activity, Khare et al. (1982)

found that mutagency by radiation was more pronounced than chemical mutagency in Cicer arietnum Rhizobium strains. The mutant produced differentfrom their parents in that the mutant colony size was bigger and gelatinase activity was higher, the number of nodules and its size were also higher.

Many studies have been done on the effect of pesticides on Rhizobium in which the measurment of the viability of a culture after a pesticide treatment has been the main method used to determine whether the assayed pesticides were harmful for Rhizobium or not (Ruiz-Sainz et al., 1984). Further work is needed to determine the effect of the fungicides on the survival and symbiotic properties of the Rhizobium strains.

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REFERENCES

1. Allen, O.N. (1957) Experiments in soil Bacteriology. 3rd ed. Minneapolis.

2. Barend, W.S. and Henri, J.V.R. (1981): Effect of steam sterilization and gamma irradiation of peat on quality of Rhizobium inoculants, Applied and Environmental Micobiology, 41(6), 1344-1347.

3. Curley, R.L. and Burton, J.C. (1975). Compatibility of R. japonicum with chemical seed protectants. Agronomy Journal 67, 807-808.

4. Dygdala, K. (1962). Morphological and cytological changes of Rhizobium as influenced by gamma rays and radiometric substances. Bull. Acad. Polon. Sci, Ser. Biol., 10, 381.

5. Dygdala, K. (1963). Morphological and cytological observations on gamma rays irradiated Rhizobium leguminosarum. Acta Micr. Pol., 12, 237.

6. El-Zawahry, Y.A. (1976). Study on the effect of gamma radiation on growth and activity of Rhizobium leguminosarum Ph.D. Thesis, Cairo University. Egypt.

7. Golebiowska, J., Kaszubiak, H. and Pajewska, M. (1967). Adaptation of Rhizobium to thiuram. Acta microbiologica polonica, 16, 153-158.

8. Hussain, A.A. (1985). Studies on the effect of some pesticides on the chemical composition of some yeasts. Ph.D. Thesis. Women's College, Ain Shams University. Egypt.

9. Janos, E., Cserhati, T. and Kecskes, M. (1979). Xenobiotics and soil micrbiota affected by xenobiotic interactions. Chlorobromuron and its breakdown product inhibiting the growth of different microorganisms. Acta microbiological academiae scientiarum, hungaricae, 26, 301-309.

10. Jordan, D.C. (1952a). Studies on the legume root nodule bacteria. II, The production and behavior of colonial mutants produced by X-rays irradiation. Cand. J. Botany, 30, 125-130.

11. Kao Te-chen and Chi-chu wang (1981): Studies on the effect of herbicides on growth of Rhizobia and development of root nodules III. The interaction between herbicides and Rhizobia in pure culture. Journal of the Agricultural association, 114, 45-66.

12. Khare, A.K., Shinoe, D.A. and Nayak, M.L. (1982). Comparative effeciency of chemical and radiation mutagens on 2 Cicer-arietnum rhizobial strains.

13. Odeyemi, O. and Alexander, M. (1977a). Resistance of Rhizobium strains to phygon, speryon and thiuram. Applied and Environmental Microbiology, 33, 784-490.

14. Odeyemi, O. and Alexander, M. (1977b). Use of fungicide-resistant rhizobia for legume inoculation. Soil Biology and Biochemistry, 9, 247-251.

15. Pareek. R.P. and Shidu, B.S. (1978). Uncoupling of phosphorylation in Rhizobium spp. by 2, 4-dichlorophenoxy acetic acid and Taficide-80. Indian Journal of Microbiology, 18, 97-100.

16. Ruiz-Sainz, J.E.; Beringer, J.E. and Gutierrez-Navarro, A.M. (1984). Effect of the fungicide captafol on the survival and symbiotic properties of Rhizobium trifolii. Journal of Applied Bacteriology, 57, 361-367.

17. Russell, J.A., and Jones, D.G. (1973). The effect of ultra violet light on the symbiotic effectiveness and some physiological characteristics of four strains of Rhizobium trifolii J. Appl. Bact., 36, 567.

18. Schwinghamer, E.A. (1969). Mutation to auxotrophy and prota-trophy as related to symbiotic effectiveness Rhizobium leguminosorum and R. trifolii. Cand. J. Microbiol. 15, 611.

19. Srivastava, R.C.; Mukerji, D.; Chandra, G. and Mathur, S.N. (1980). Radiation induced changes in the nitrate reductase activity in Rhizobium culture, leaves and nodules of Black-Gram (Vigna mungo (L) Hepper). Annual meeting of the American society of plant physiologists and the phytochemical society of North America.

20. Wogan, G.N. (ed.) (1965): Mycotoxins in foodstuffs. 291 pp. Cambridge, Mass.: M.I.T. Press.

Table (1) : Effect of different doses of Gamma radiation on the survival of <u>Rhizobium</u> of Peanut (Arachis hypogea) 169 strain .

Irradiation dose (Krad)	Number of surviving cells	Log number of surviving cells	Log surviving fraction (log $\frac{N}{No}$)
Control	1.3062×10^{8}	8.116	0
10	1.6443×10^{7}	7.215	- 0.900
20	1.5601×10^{6}	6.193	- 1.922
30	2.0057×10^{5}	5.302	- 2.813
40	1.5520×10^{4}	4.190	- 3.925
50	1.3172×10^{3}	3.119	- 4.996
75	No growth	-	-
100	No growth	-	-

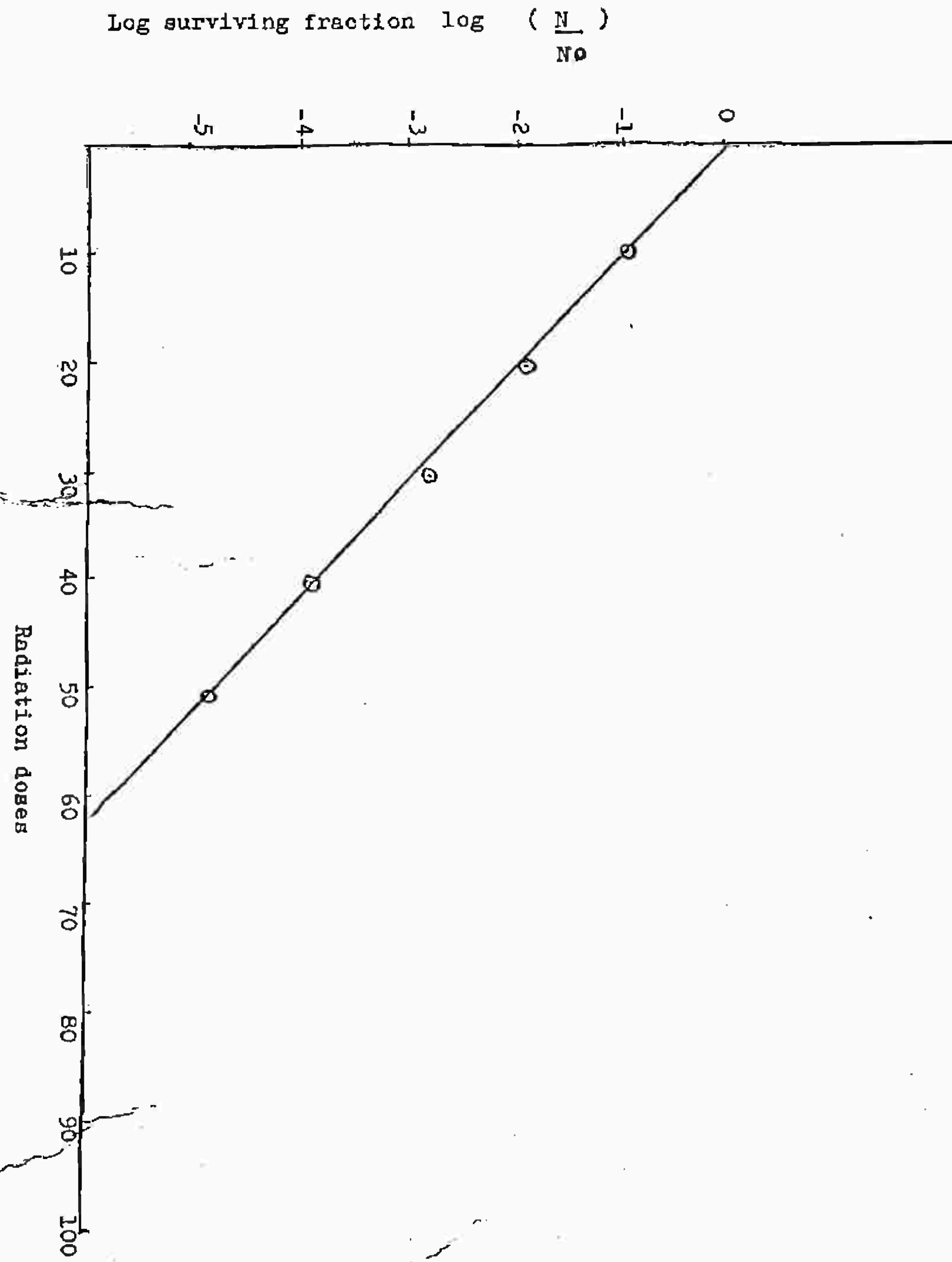

Fig (1) Dose response curve of peanut <u>Rhizobium</u> strain 169

Table (2) : Effect of different doses of Gamma radiation on the survival of *Rhizobium* of Peanut (Arachis hypogea) A.V strain

Irradiation dose (Krad)	Number of surviving cells	Log number of surviving cells	Log surviving fraction ($\log \frac{N}{N_0}$)
Control	6.9155×10^{8}	8.839	0
10	6.3480×10^{7}	7.802	- 1.037
20	4.4016×10^{6}	6.643	- 2.196
30	2.5259×10^{5}	5.402	- 3.437
40	2.689×10^{4}	4.422	- 4.410
50	3.1155×10^{3}	3.493	- 5.346
75	3.1020×10^{2}	2.491	- 6.348
100	No growth	-	-

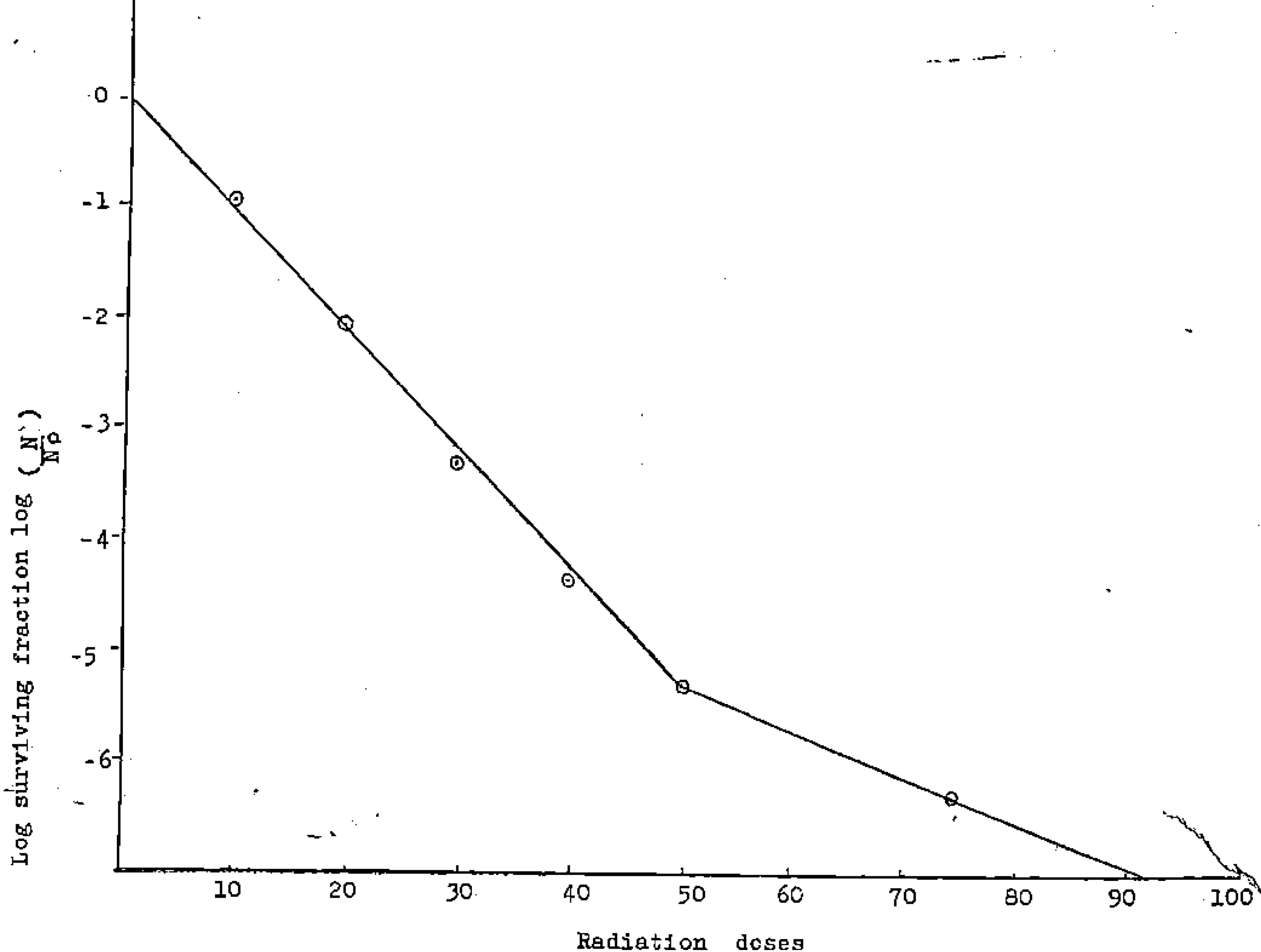

Fig (2) Dose response curve of peanut *Rhizobium* strain AV.

Table (3) : Survival of <u>Rhizobium</u> of peanut 169 strain in yeast extract mannitol medium supplemented with different concentrations of the fungicide tilt.

Conc. of fungicide (ppm)	Wild type		Most radioresistant isolate	
	Viable count (Cells/ ml) x 10^8	Survival (%)	Viable count (Cells/ ml) x 10^8	Survival (%)
Control	1.0635	100	0.8050	100
2.5	0.5140	48.33	0.4636	57.59
5	0.8281	77.86	0.6173	76.68
7.5	1.1708	110.08	0.7156	88.89
10	1.4277	134.24	0.6752	83.87
15	1.8764	176.43	0.5762	71.57
20	2.8433	267.35	0.4852	60.27
25	2.0688	194.52	0.3312	41.14
30	1.7188	161.61	0.2900	36.02

Table (4) : Survival of *Rhizobium* of peanut A.V strain in yeast extract mannitol medium supplemented with defferent concentrations of the fungicide tilt

Conc . of fungicide (ppm)	Wild type		Most radioresistant isol ate	
	Viable count (cells / ml) $x10^8$	Survival (%)	Viable count (cells/ml) $x\ 10^8$	Survival (%)
Control	1.2902	100	1.0300	100
2.5	0.9657	74.84	0.0570	5.53
5	3.1825	246.66	0.2489	24.16
7.5	5.6755	439.89	0.4991	48.45
10	4.4005	341.07	0.4911	47.67
15	1.9220	148.96	0.3251	31.56
20	1.4531	112.62	0.2483	24.10
25	1.4658	113.61	0.2478	24.05
30	1.2562	97.36	0.2310	22.42

انتاج سلاله حساسه لمبيد فطرى من سلالات ريزوبيم الفول السودانى بواسطة الاشعاع الجامى

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ملخص

استهدف هذا البحث تعريض سلالاتين من ريزوبيم الفول السودانى متميزه بالنمو السريع الى جرعات متزايده من اشعة جاما وقد تم تعيين منحنى الاستجابه وكذلك الجرعه تحت المميته ثم دراسة تاثير المبيد الفطرى (تلث) على نمو السلالات المقاومه لاكبر جرعه من اشعة جاما والتى يتوقف النمو بعدها تماما . هذا وقد اوضحت النتائج ما ياتى :

١ـ سلالات الفول السودانى ١٦٩ ، ٨٠٧ ذات حساسيه عاليه لاشعة جاما ومنحنيات تجاوب الجرعه من النوع (١) وكانت الجرعه تحت المميته ٥٠ كيلو راد بالنسبه للسلاله ١٦٩ ، و ٧٥ كيلو راد بالنسبه للسلاله ٨٠٧ .

٢ـ عندما تعرضت السلاله المقاومه لاكبر جرعه من اشعة جاما والتى يتوقف بعدها النمو تماما الى جرعات متزايده من المبيد الفطرى (تلت) وجد ان هذه السلاله كانت حساسه للمبيد الفطرى وان نسبة عدد الخلايا الحيه يقل بازدياد جرعاته بعد التركيز (٧,٥ جزء فى المليون) وقد اعطت هذه الجرعه من المبيد الفطرى اعلى نسبه من عدد الخلايا الحيه (٨٨,٨٦) بالنسبه للسلاله ١٦٩ بينما كانت (٤٥, ٤٨) بالنسبه للسلاله ٨٠٧ .

٣ـ كانت السلالات المتطوره من ١٦٩ ، ٨٠٧ اكثر مقاومه لجرعات متزايده من المبيد الفطرى وقد وجد ان نسبة عدد الخلايا الحيه يزداد بازدياد تركيز المبيد الفطرى وحتى تركيز (٢٠ جزء فى المليون) وقد اعطى هذا التركيز اكبر نسبه من عدد الخلايا الحيه بالنسبه للسلاله ١٦٩ بينما اعطى التركيز (٧,٥ جزء فى المليون) اكبر نسبه من عدد الخلايا الحيه بالنسبه للسلاله ٨٠٧ .