Effect of Chemical Mutagens on Some Bacteria and Fungi Strains to Induce Para-Nodules in Wheat Plants

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ABSTRACT

The effect of Dimethoite and N-methyle-nitro-N-nitrosoguianidine (MNNG) upon *Trichoderma viride (T. viride*) and *Azotobacter chroococcum (A. chroococcum)* was studied. Four concentrations from dimethoite (0.1, 0.3, 0.5 and 0.7 ml/9 ml spore suspentions of *T. viride* were studied after 0, 10, 20, 30 and 60 minutes and four concentrations from (MNNG) 0.002, 0.003, 0.004 and 0.006 gm/10 ml spore suspentions from *T. viride* and *A. chroococcum* were studied after 0, 15, 30, 45 and 60 minutes. Production of para nodule in wheat plant by *T. viride* and *Azotobacter Azospirillum* was also studies in present work. The results showed that dimethoite has a positive effect on the survival perecent and the concentration at a level of 0.3 ml/9 ml had high effect on *T. viride* at all different time. On the other hand, the effect of MNNG at different concentrations and at different times increased lethality ratio in *T. viride* and induced auxotrophic mutant in *T. viride*.

Key words: Chemical mutagens, Fungi Strains, dimethoite, Para-nodules.

INTRODUCTION

Nitrogen fixation is one of the most important biological processes in the natural environment. It is the major pathway for the reduction of nitrogen molecules from air to give ammonia and subsequently glutamine and other nitrogen molecules. More than 14 years ago it was announced by Tchan and Kennedy (1989) that wheat plants from nodule-like tumors (para nodules) by inoculation with *Rhizobium* and treatment with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Since then, the study on this phenomenon opend new and exciting aspects concerning the extension of biological nitrogen fixation in graminaceous plants. It has been demonstrated that para-nodules are potentially inhabited by introduced different diazotrophic bacteria which create an increased level of nitrogen fixation (Tchan and Zeman, 1995; Abdel-Waha *et al*.,1995). Development mutants of *Trichoderma viride* by exposing the conidia of *T. viride-Tv6 (Tv.WD*) to physical and chemical mutagens such as uv and gamma rays and nitrosoguanidine were done. The mutants were screened for antagonistic potentiality by developing an antagonism index (Nakkeeran *et al.*, 2005). The mutant of *Trichoderma* showed enhanced production of fungal cell wall degrading enzymes: chitinases, B-1,3-glucanases and proteases (Zaldivar *et al*., 2001). Investigations on the possibility of cyanobacteria to inhabit and form para-nodules with cereals are sporadic and scanty (Ganter, 2000 a,b). The heterocyctous cyanobacterium Nostoc *sp.* is characterized by its ability to form light association with wheat roots and to improve significantly its N_2-fixing capacity and consequently plant growth (El-Shahed, 2005). The induction of mutation and the auxotrophic mutant was studied also by (Kanemaru and Migamoto, 1990) as a result of chitosan treatment. Chitosan (poly-β-1,4 glucosamine) could promote the plant to defend itself besides; it interferes with the growth of the pathogen. The present study aims to evaluate the effect of *Trichoderma, Azotobacter and Azospirillum* with rice straw for the productivity of wheat. Also induction of mutants in *Azotobacter* and *Trichoderma* and using these mutants to know their effects to induce para-nodules in wheat plants.

MATERIALS AND METHODS

A) Plant materials:

Pure grains of wheat variety"Gemaza 9" was donated from Wheat Research Center, Sakha, Kafer-El_Shaikh and used in this study.

B) Bacteria and Fungus strains:

Two wild types of bacteria and two wild types of fungi were used in this study. A local strain of *Azotobacter chroococcum* "K43"and *Azospirillum lipoferium* were donated by National Research Center, Dokky, Gizza, Egypt. Fungal Strain; *Trichoderma viride* and *T.harzianum* were kindly provided by Plant Pathology Department- Faculty of Agriculture, Al-Azher- University.

C) Chemical mutagens:

1- Dimethoite (D); the different concentrations used were 0.1, 0.3, 0.5 and 0.7 mL/ 9mL spore suspension.

2- N-methyl- N-nitro- N-noitrosoguanidine (MNNG) four concentrations were used to induce mutation. These concentrations were 0.002, 0.003, 0.004 and 0.006 gm/ 10mL spore suspension.

3- N-methyl-N-nitro-N-nitrosoguanidine (MNNG) were used to induce mutation of *Azotobacter Chrococcum*. These concentrations were 0.002, 0.003, 0.004 and 0.006 gm/100mL minimal media of *Azotobacter*.

D- Media:

1- Media for *Azospirillum lipoferum*: Semi-solid malate medium (Döbereiner 1978).

2- Media for *Azotobacter chroococcum*: 2-1- (Complete media CM) (Abdel-Malek and Ishac 1968).

2-2- Minimal media: Burk's minimal media (Harvey *et al.* 1967).

3- Media for *Trichoderma* PDA (Potato 200gm- Glucose 20gm- Agar 20gm).

Methods

1) Induction of mutation for *Azotobacter chroococcum*:

100mL of Burk's minimal media (Harvey *et al.,* 1967) inoculated with *Azotobacter chroococcum,* one mL cell suspension plated on (Complete media CM)**:** (Abdel-Malek and Ishac 1968) as control and 99 mL cell suspension were exposed to different concentrations of mutagen for different treatments. Samples of one mL cell suspension from each treatment were plated on complete media. Concentrations of mutagen were 0.002, 0.003, 0.004 and 0.006 as mentioned by Palsaha and Paul (2003). The plates were incubated at 28^0C. After incubation for three days at 28^0C, colonies were tested for mutation on MM& CM plates during five days. The colonies which showed growth on complete media but not on minimal media were considered as auxotrophic (mutants).

2- Induction of mutation for *Trichoderma viride*:

Conidia from *Trichoderma viride* were taken after grown for one week on PDA and spores were suspended in 10mL distilled water. One mL spore suspension was plated on PDA as control and 9mL spore suspension were exposed to different concentrations of mutagen for different treatments. Samples of one mL spore suspension for each treatment were plated on PDA as complete media. The plates were incubated at 28^0C. After incubation for two days at 28^0C, colonies were counted and survivals were calculated. About 300 colonies were tested on MM& CM plates for mutation after five days of incubation. The plates were incubated at 28^0C for two days and mutants were determined.

Induction of para-nodules in wheat plants:

Rice straw were cutted to short pieces, four replica of glass bottle "500mL" for each treatment, each bottle contained 5gm rice straw and 25mL of distilled water and were autoclaved at 121^0C for 40 minutes. Wheat grains were sterilized with colorox for 10 minutes, and washed with sterilized distilled water for three times. The grains were added for each bottle (seven grains/ bottle) and incubated with (4mL of 1.1×10^7/mL) from each microbe. Then bottles were incubated at 25^0C and the rate of germination was determined, the bottles were taken after 15 days and 30 days to determine para-nodules in wheat plant.

The treatments were:

1-Control without any inoclum.
2-A mutant of *Trichoderma viride (T.v 14).*
3-Trichoderma viride + (T.v 14).
4-Trichoderma harzinum+ (T.v 14).
5-Azospirillum lipoferum + (T.v 14).
6-Azotobacter chroococcum + (T.v 14).
7-T. viride +A. lipoferum + (T.v 14).
8-*T. viride +A. chroococcum + (T.v 14).*
9-*T. viride + A. lipoferum + A. chroococcum + (T.v 14).*
10-*T. harzinum + A. lipoferum + (T.v 14).*
11- *T. harzinum + A. chroococcum (T.v 14).*
12- *T. harzinum + A. lipoferum+ A. chroococcum + (T.v 14).*
13- *T. viride + T. harzinum+ A. lipoferum+ A. chroococcum+(T.v 14).*

RESULTS AND DISCUSSION

Table(1) and Figure (1) show the effect of dimethoite (as chemical mutagenic agent) at the different tested concentrations, i.e. 0.1, 0.3, 0.5, and 0.7 ml/ 9 ml spore suspensionon of the *Trichoderma viride.* gave the mean number of survivals and percentages obtained from treating *T. viride* with different concentrations of dimethoite at different time (0, 10, 20, 30 and 60 minute). From each treatment were tested approximately of 100 colonies for mutations. They all proved to be prototrophs, dimethoite concentration didn't have any effect to induce auxotrophic or morphological mutant.

From the obtained results it was observed that dimethoite concentrations have a positive effect on the survival percent, where the concentration of dimethoite 0.3 ml/ 9 ml spore suspension had a high effect on the survival of *T. viride* at all different time used and this effect was higher than 0.5 and 0.7 ml.

It was also observed that the increase of time for all concentrations reduced the survival of *Trichoderma* and increased lethality.

Table (2) and figure (2) show the effect of N-methyle – N-nitro –N-nitrosoguanidine "MNNG" concentrations (0.002, 0.003, 0.004 and 0.006 gm/ 10 ml spore suspension) at different times (0. 15. 30. 45 and 60 minute) on the survival percent and induction auxotrophic mutant in *Trichoderma viride.*

Table 1: Induction of mutations in *Trichoderma viride* by different concentrations of dimethoite at different time.

Time	Incubation period/ minute									
	0		10		20		30		60	
Concentration.	Survival/ml susp.		Survival/ml susp .		Survival/ml susp		Survival/ml susp		Survival/ml susp	
	M.C $\times 10^6$	%	M.C $\times 10^6$	%	M.C $\times 10^6$	%	M.C $\times 10^6$	%	M.C $\times 10^6$	%
0.1	177	100	140	79.1	140	79.1	97	54.8	52.3	29.5
0.3	190	100	22	11.6	10.33	5.4	3	1.6	0.7	0.4
0.5	183	100	113.3	61.9	35.7	19.5	10.7	5.8	0.17	.09
0.7	67	100	11.3	16.9	5	7.5	3.3	4.9	.00097	0.001

M.C = mean of colonies/ml spore suspension

Table 2: Induction of mutations in *Trichoderma viride* by different concentrations of N-methyle-N-nitro-N-nitrosoguanidine "MNNG" at different time.

Time	Incubation period/ minute														
	0			15			30			45			60		
MNNG Concentration	Survival/ml susp. (Control)		N.M.	Survival/ml susp.		N.M.	Survival/ml susp.		N.M.	Survival/ml susp.		N.M.	Survival /ml susp.		N.M.
	No.$\times 10^6$	%		No.$\times 10^6$	%		No.$\times 10^6$	%		No.$\times 10^6$	%		No.$\times 10^6$	%	
0.002	16	100	0	2.23	14	0	1.2	7	3	0.5	3	3	0.13	0.8	0
0.003	50	100	0	37	74	0	18.7	37	0	10.3	20.6	0	8	16	0
0.004	83	100	0	16	19.3	1	2.3	2.8	4	0.19	0.23	3	0.07	0.1	4
0.006	53	100	0	25	47.2	1	2.73	5.2	2	0.48	0.91	0	0.26	0.49	4

N. M. =number of mutants

Table 3: Induction of mutations in *Azotobacter chroococcum* by different concentrations of N- methyl-N-nitro- N-nitrosoguanidine (MNNG)

Time	0		15		30		45		60	
MNNG Concentration	Survival/ml susp.		Survival/ml susp.		Survival /ml susp		Survival/ml susp.		Survival /ml susp	
	No.$\times 10^4$	%	No.$\times 10^4$	%	No.$\times 10^4$	%	No.$\times 10^4$	%	No.$\times 10^4$	%
0.002	35.33	100	7.4	20.95	2.313	6.55	0.599	1.7	0.476	1.35
0.003	14.5	100	10	68.966	7.7	53.1	8.7	60	2.5	17.24
0.004	58	100	48	82.76	20.7	35.69	12	20.69	4	6.9
0.006	28.3	100	6.1	21.6	1.6	5.7	0.2	0.71	0.06	0.21

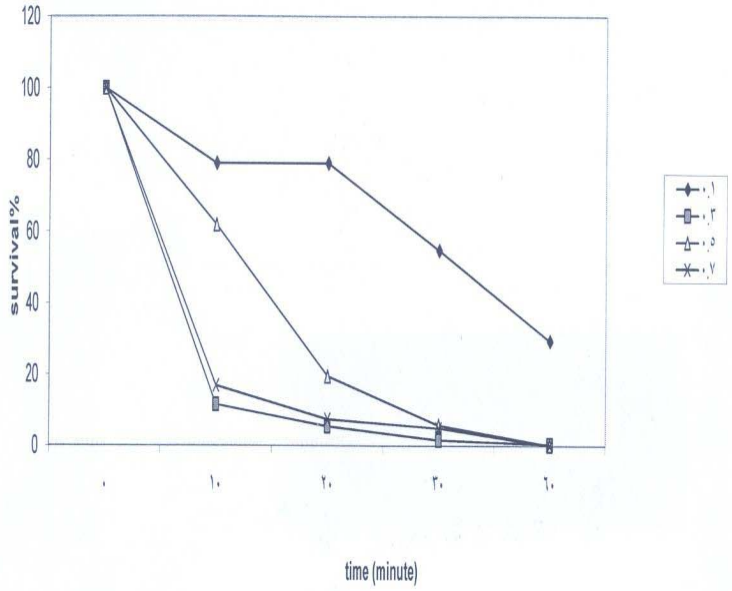

Figure1: Effect of dimethoite cocentration at different time on the survival of *Trichoderma viride*

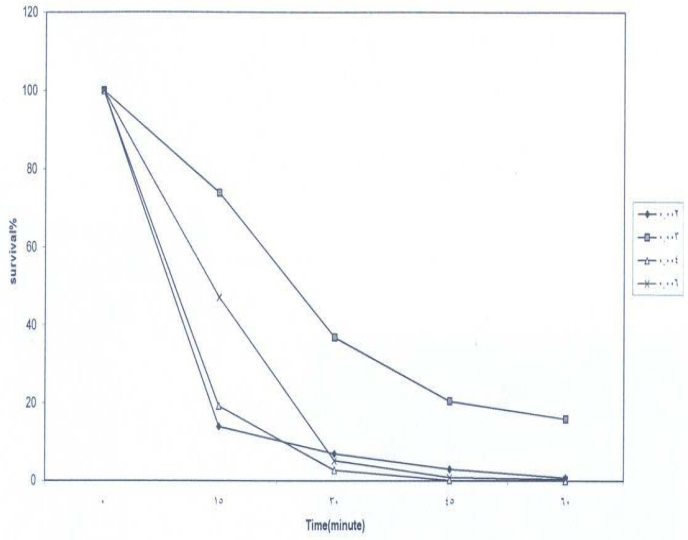

Figure 2: Effect of N-methyle-N-nitro-N-nitrosoguanidine (MNNG) concentration at different time on the survival of *Trichoderma viride*

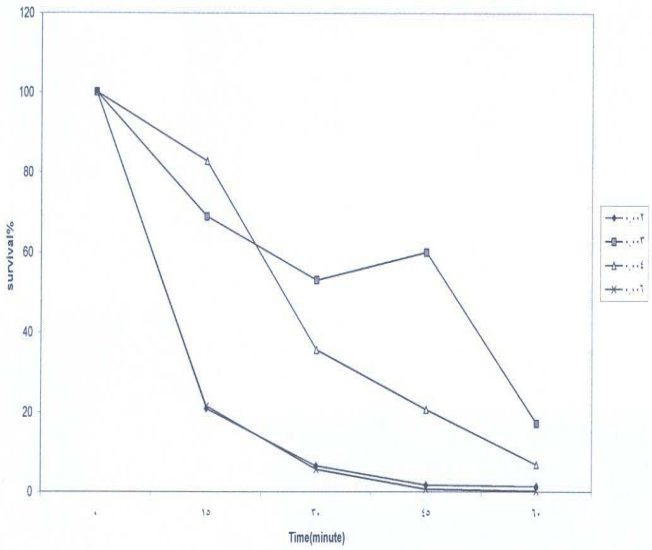

Figure 3: Induction of mutation in *Azotobacter chroococcum* by different concentrations of N- methyl-N- nitro- N- nitrosoguanidine

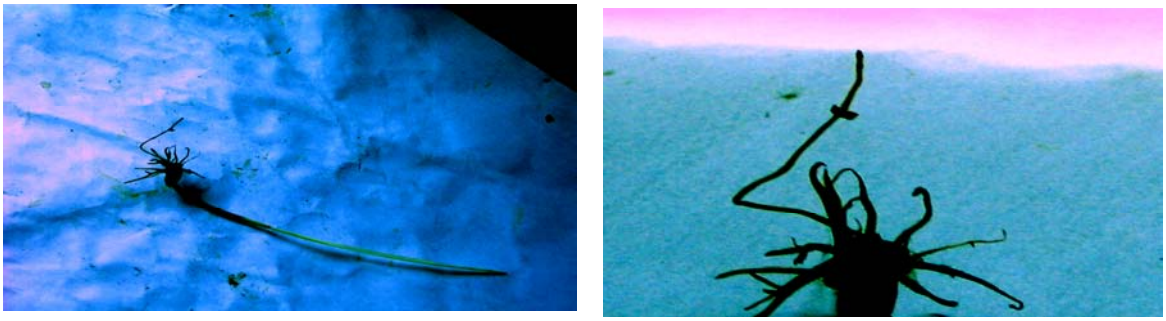

Figure 4: Wheat para-nodule induced by *T. hrazianum* +*Azotobacter chroococcum* +*T. v. 14* (*in vitro*)

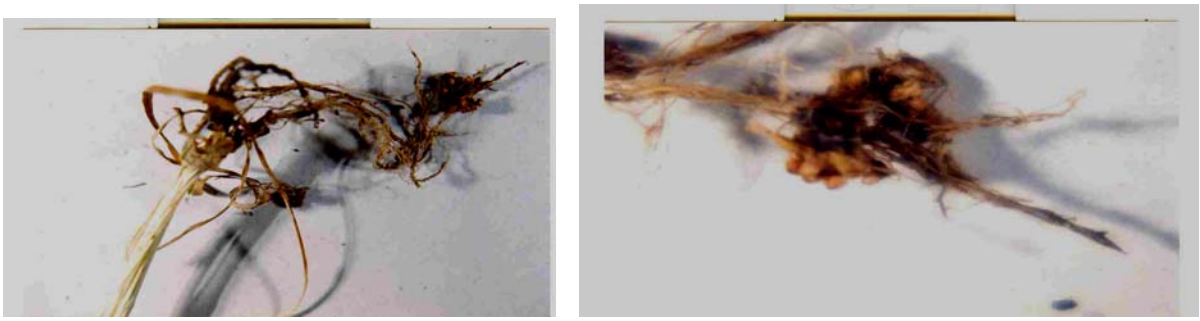

Figure 5: Wheat para-nodule induced by *T. hrazianum* +*Azospirillum lipoferum* +*Azotobacter chroococcum* (*in vivo*)

It was clear that all mutants which achieved with MNNG were morphologically by variant and only one was believed to be auxotrophic mutant because of the slowly growing on MM media, where it was grown after 1 to 2 weeks compared with that of wild type which was grown on MM in the next day from culturing.

Morphological and auxotrophic mutants produced after the treatment with MNNG were proven to be in accordance with (Talkhan *et al*., 2003) who investigated the possibility of inducing genetic variability in the fungus *Trichoderma reesei " T. longibrachiatum*"**,** when the NTG mutagen was applied, 48 colonies were isolated, 20 of them are morphological variants and only one is auxotrophic mutant. It was also observed from the present study that the increase of time for all concentrations reduced the survival of *Trichoderma* and increased lethality. Table (2) shows that the number of mutants which was obtained from treated with MNNG was 25 mutants and the concentration 0.004 gm had the highest number of mutants which gave 12 mutants. It was also observed that the concentration 0.003 gm didn›t give any mutants.

Table (3) and Figure (3) show that all concentrations increased lethality ratio with the increasing of time except of concentration 0 .003 gm.

The survival % at 15 minutes, the concentration 0.004 was higher than 0.003 and other concentrations but at 30 minutes the concentration 0.003 was higher than the other treatments and also, at 45 and 60 minutes, which indicated that *Azotobacter* was high tolerance for 0.003 gm than other concentrations.

It is clear that concentrations 0.006 and 0.002 were semi coincident at all different times which indicated that *Azotobacter* didn›t tolerance the low and high concentration of mutagensis MNNG.

Induction of para-nodules in wheat plant by combination between bacteria and fungi without chemical material

1- *In vitro* experiment

Figure (4) shows the para-nodule of wheat plant after using *Trichoderma viride* mutant *(T. v14)* with other microorganisms where the para-nodules showed in the treatments *(T. viride + Azospirillum lipoferum + T. v14* and *T. harzianum + Azotobacter chroococcum+ T.v 14*, these results were obtained in sterilized material (rice straw). Induction of para-nodules in wheat plant using microorganisms may be the execration of auxin by mutant of *Trichoderma viride* (IAA) was agreed with that reported by Nakkeeran (2005) who developed mutants of *Trichoderma viride* by exposing the conidia of *T. viride* and *T.v6* to physical and chemical mutagens such as UV rays, gamma rays and nitrosoguanidine. The mutants were screened for antagonistic potentiality by developing an antagonism index. Antagonism index is the product of competitive saprophytic ability, colonization behavior, percent inhibition of pathogen, propagule lysis, speed of over growth on pathogen and inhibition zone. Strains MG3, MG6, UV10 and

MNT7 recorded the highest antagonism index ranging from 192- 480 with increased activity of cellulose, chitinase, β-1,3 glucanase and IAA. Production IAA by *Trichoderma viride* induced para-nodules in wheat plant and capable of the other microorganisms such as *Azotobacter* or *Azospirillum* to penetrate this para-nodules and live in these para-nodules.

2- Pots experiment:

Para nodules showed in the treatment of *T. harzianum +Azospirillum lipoferum +Azotobacter chroococcum* after four months in pots experments as show in Figure (5). Cytological examination didn't show any nematode in these tumors but showed only bacteria, which was isolated and cultured on *Azotobacter* medium, *Azospirillum* medium and *Rhizobium* medium. Those bacteria grow on *Azotobacter* medium and *Rhizobium* medium.

The present results confirm that root-tumor induction offers a suitable method of establishing diazotrophs endophytically in the roots of gramineous crops (Christiansen-Weniger, 1996).

In conclusion, it is demonstrated that gramineous plants are potentially capable of developing an endophytical diazotrophic symbiosis through para-nodules formation. (Christiansen-Weniger, 1998).

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الملخص العربى

تأثير بعض المطفرات الكيماوية على بعض سلالات البكتيريا والفطريات الموثرة على تكوين شبه عقد جذرية فى نبات القمح

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تم دراسة تأثير استخدام بعض المطفرات الكيماوية Dimethoite و N- methyle-nitro-N-nitrosoguianidine (MNNG) على فطر التريكودرما وبكتيريا الأزوتوباكتر بتركيزات مختلفة من المادة المطفرة Dimethoite.أوضحت النتائج أن أستخدام هذه المادة بتركيز٠.٣مليجرام كان أعلى تأثير فى نسبة الخلايا الحية لكل ٩مل من المعلق الجرثومى من التريكودرما فى الأوقات المختلفة ولم يتحصل على طفرات منها. أما تأثير المادة الأخرى (MNNG) فقد أنتجت عدة طفرات بأستخدام تركيزات مختلفة وفى أوقات مختلفة كما زادت نسبة الخلايا الميتة من التريكودرما وبالتالى انخفضت عدد الطفرات فيها بزيادة التركيز والزمن. وقد تكونت عقد خارجية على جذور نبات القمح وأوضح ذلك أن النجيليات لديها القدرة على تكوين شبه عقد جذرية.