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Interaction Between Gamma-irradiated Macrosymbiont and Recombinants of Microsymbiont to Improve Nodulation and Nitrogen Fixation in *Pisum sativum*

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Abstract: In this study, gamma irradiation was used to create genetic variations in pea variety victoria with better characteristics in nodulation and nitrogen fixation process via gamma irradiated the macrosymbiont and conjugation between microsymbiont as a mechanism of DNA transfer, to release a variety of recombinant rhizobia into the environment for such agriculture purposes to be improving nodulation and nitrogen fixation, in victoia variety. Fourteen antibiotics were used for screening six bacterial strains to determine the genetic differences between strains to be used in mating experiments as a selectable markers for isolating resu transconjugants. All bacterial strains were sensitive to streptomycin, tetracycline and cefadroxilmonhydrate and all bacterial strains were resistant to nystain. Some of di-parental transconjugants resulted from the mating between RL-ARC-207 X *P. putida* appeared significant increase in the number of nodules developed per plant in pots experiments. Although, most transconjugants, showed significant increase in shoot dry weight. The interaction between doses of gamma-rays and biofertilizer strains showed significant effect on the number of nodules developed per plant among M1and M2 populations. All bacterial strains appeared significant increase in total chlorophyll. Rhizobial strain RL-ARC-207 can improve shoot nitrogen content at 50 and 150 days plant-old through two generations. Inoculation with *Rhizobium* strains (RL-ARC-1601, RL-12612, RL-ARC-207 and RL-ARC-1604) can improve seeds protein content among M1 and M2 generations, whereas, RL-ARC-207 can improve seeds protein content in M1 population. Significant increase in pollen sterility was achieved in M2 population treated with 1 and 2 K- rad, whereas, the same trend was also shown in M1 treated with 3K-rad of gamma-rays. Doses of gamma-rays appeared significant effect in chlorophyll formation, shoot N content (50 days plant-old) and pollen sterility percentage. Biofertilization appeared significant effect on chlorophyll formation, shoot nitrogen content and seeds protein content. The interaction between doses of gamma rays and biofertilization appeared significant effect on pollen sterility among gamma-irradiated generations (M1&M2).

Key words: biofertilization, di-parental transconjugants, DNA transfer , gamma rays, improving nodulation.

INTRODUCTION

Field pea (*Pisum sativum* L.) belongs to the family leguminosae, its haploid chromosome number n = 7 have high levels of the essential amino acids, lysine and tryptophan, which are usually low in cereal grains. Consequently, therefore field pea can supplemented the low amount of protein present in food and feed processed from cereal. A cool growing season is necessary for optimum field pea production (a mean temperature of 55° to 65° F). On average, it requires 60 days from planting until bloom, and 100 days to mature the dry seed (Oelke *et al*.1991).

Mutation is any heritable alteration in genetic material, includes such diverse phenomena as change in the number of chromosomes, changes in the structure of chromosomes and changes within the genes themselves. Although change in the number and structure of the chromosomes are of considerable importance to the evolutionary geneticist and to the plant breeder. Point mutation always occur within a gene and it should be the smallest possible change in the structure of the genetic material that is detectable as a mutation. Point mutation involving a change in a single nucleotide pair (the smallest possible change) and a larger change involving a small number of adjacent nucleotides. Every gene has its own characteristic rate of mutation, some genes mutating more frequently than others.

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Gamma rays are high energy electromagnetic waves with very considerable penetrating powers. Ionizing radiation induces both gene mutations and chromosomal breaks and that the frequency of each of these aberrations is directly proportional to the dose. The important conclusion indicated that their effects of radiation are accumulative (Smith-keary,1975).

Better N_2-fixing symbiosis may be brought by manipulating both rhizobia and plant hosts and by eventually creating an artificial rhizosphere. Specificity of the rhizobium-legume symbiosis is also governed by specific genes on the bacterial chromosome that code for proteins involved in recognition and uptake of specific signal molecules present in root exudates (Rosenblueth *et al*. 1998). Gamma rays was used in this study as a tool for induction of genotypic variation in the germplasm of pea seeds which may effect on components of the signaling pathway. The genotypic specificity of the nodulation process depends on gene interaction between rhizobia and the host. Genotypic variation in flavonoids, betonies and other nod-gene inducers indicates a potential for genotype selection (Phillips *et al*., 1995). Symbiotic interaction between *Rhizobium* and leguminous plants is very specific. The formation of nitrogen-fixing root nodule involves a complex series of genes expression in both the rhizobial symbiotic and the plant host. The necessary genes of rhizobia for nodulation, *nod* genes, and for nitrogen fixation, the fix and *nif* genes.

Pisum sativum L. variety victoria used in this study showed little success in nodulation in Egyptian soils. Their nodulin genes may be silent, therefore symbiosis-related properties are cryptic. This study try to induce genetic variations in victoria variety to be select for better symbiosis in Egyptian soils through alteration the expression of nodulin genes using gamma irradiated-seeds to improve the symbiosis through the effects on nodulins referred to the first steps mechanism of nodule formation, as well as, inducing recombinations in bacterial strains through conjugation to be select the efficient one in symbiosis with victoria variety to improve nodulation process.

MATERIALS AND METHODS

Genetic Material:

Bacterial Strains and Growth Conditions:

The genotypic and phenotypic properties of different bacterial strains are shown in Table 1, together with references of their origin.

Table1: Bacterial strains used in this study.

Strains	Phenotype	Relevant genotypic markers	Source or reference	Designation
Rhizobium leguminosarum (12612)	Wild-type	*Ap*$^+$ *Eryth*$^+$ *Str*$^-$	IAM culture collection, Univ. of Tokyo, Japan.	RL- 12612
Rhizobium leguminosarum (ARC 1601)	Wild-type	*Ap*$^+$ *Eryth*$^+$ *Str*$^-$	Agric. Res. center, Dept. of Microbiology, Giza, Egypt.	RL-ARC-1601
Rhizobium leguminosarum (ARC 1604)	Wild-type	*Ceph*$^+$ *Eryth*$^+$ *Str*$^-$	Agric. Res. center, Dept. of Microbiology, Giza, Egypt.	RL-ARC-1604
Rhizobium leguminosarum(ARC 207)	Wild-type	*Epanu*$^+$ *Eryth*$^+$*Cm*$^-$	Agric. Res. center, Dept. of Microbiology, Giza, Egypt.	RL-ARC-207
Azorhizobium caulinodans (13624 T)	Ap^r	*Ap*$^+$ *Eryth*$^+$ *Cm*$^-$	IAM culture collection, Univ. of Tokyo, Japan.	Azo-24
Pseudomonas putida (B13)	Wild-type	*Str*+ *Ap*$^-$ *Eryth*$^-$ *Ceph*$^-$ Utilization Research,, USA	National center for Agriculture	*P.putida*

Ap^r =Ampicillin resistance.

Antibiotics Used:

Fourteen antibiotics [Nystain (Ny), Chloramphenicol (Cm), Cefradine (Ceph),Streptomycin (Str),Tetracycline(Tc), Amoxyicillin (Am), Neomycinsulphate (Nm), Ampicillin (Ap), Epanutin (Ep), Erythromycin-ethlsuccinate (Eryth), Amoxycillin and flucloxacillin (Am-Fluc), Erythromycin (Erth), Cefadroxilmonhydrate (Cefa), Ampicillin and Cloxacillin (Ap-clox)] were used in this study with the concentration of 100µg/ml for genetically marking the different bacterial strains used in this work Table 4.

Table 2: Di-parental mating between *Rhizobium* strains with *Pseudomonas putida.*

Mating	*Revelant geneotype of mating*	*Selective markers*	*Suitable time by days of mixed cultures incubated in each* mating *that needed for genetic transfer.*	*Time needed to appeared* transconjugants on selective media (day)	Designation
Rhizobium leguminosarum 12612 x Pseudomonas putida	Ap^+ $Eryth^+$ Str^- X Str+ Ap- $Eryth^-$	Ap+ Eryth+ Str	10	3	DPM-Tr1 DPM-Tr2 DPM-Tr3 DPM-Tr4 DPM-Tr5 DPM-Tr6 DPM-Tr7 DPM-Tr8 DPM-Tr9 DPM-Tr10
Rhizobium leguminosarum 1601 x Pseudomonas putida	Ap^+ $Eryth^+$ Str^- X *Str+ Ap- Eryth-*	*Ap+ Eryth+ Str*	4	3	DPM-Tr11 DPM-Tr12 DPM-Tr13 DPM-Tr14 DPM-Tr15 DPM-Tr16 DPM-Tr17 DPM-Tr18 DPM-Tr19 DPM-Tr20
Rhizobium leguminosarum 1604 x Pseudomonas putida	$Ceph^+$ $Eryth^+$ Str^- X *Ceph- Eryth- Str+*	*Ceph+ Eryth+ Str*	15	5	DPM-Tr21 DPM-Tr22 DPM-Tr23 DPM-Tr24 DPM-Tr25 DPM-Tr26 DPM-Tr27 DPM-Tr28 DPM-Tr29 DPM-Tr30 DPM-Tr31
Rhizobium leguminosarum 207 x Pseudomonas putida	$Epanu^+$ $Eryth^+$ Cm^- X *Epanu- Eryth- Cm+*	*Epanu+ Eryth + Cm*	4	6	DPM-Tr32 DPM-Tr33 DPM-Tr34 DPM-Tr35 DPM-Tr36 DPM-Tr37 DPM-Tr38 DPM-Tr39 DPM-Tr40 DPM-Tr41
Azorhizobium caulinodans x Pseudomonas putida	Ap^+ $Eryth^+$ Cm^- X *Ap- Eryth- Cm+*	*Ap+ Eryth+ Cm*	4	3	DPM-Tr42 DPM-Tr43 DPM-Tr44 DPM-Tr45 DPM-Tr46 DPM-Tr47 DPM-Tr48

Media:

Rhizobial strains were grown at 28°C in yeast extract-mannitol medium (YEM) (Vincent 1970). *Azorhizobium* strain was grown in tryptone-glucose-yeast extract (TGYE) medium (Ladaha *et al* 1989).

Plant Material:

Pea (*Pisum sativum* L.) seeds variety Victoria were kindly provided from Vegetable Research Institute, Agri. Res. Center, Giza, Egypt, through Mr. Essam El-Din Salam, Agric. Engineering in Water soil and Environmental Res. Institute, Agric Res. Center. This variety was selected to be used in this study because of lower nodulation in Egyptian soils. This study aimed to improve nodulation and the symbiosis in this variety through genetic modifications-induced by gamma irradiation in pea plantlets, which may effect on the root exudates affecting on development of nodules on root system, to be select the efficient genotype developed nodules. To application this aim, seeds were soaked in running water for ten minutes before treated with three

doses (1, 2 and 3 K-rad) of gamma-rays from a cobalt-60 source at the radioisotope center, Maddenet Nasser, Cairo, Egypt. It is conceivable that mutations induced is accompanied with changes in plant flavonid metabolism. It may lead to the aberrant regulation of *nod* genes of the rhizobial partner (Hungeria and Phillips 1993) and associated effects on symbiosis development.

Methodology:

Bacterial Mating:

Intergeneric mating was performed using *Pseudomonas putida* as donors for the possible transfer of phenol-degradative plasmids to a standard recipient *Rhizobium* strains (Clewel, 1993). This plasmid play an important role in phenol degradation compounds secreted by the plant roots to stimulate nodulation through the degradation of inhibiting substances which may present in root exudates. Conjugation between bacterial strains was done di-parentally as shown in Table 3. Conjugation experiments were performed on YEM plates for *Rhizobium* conjugated with *Pseudomonas putida*, as well as, TGYE plates was used for *Azorhizobium* conjugated with *Pseudomonas putida*. Representative media were supplemented with appropriate antibiotics for each cross and the transconjugants appeared on selective medium were picked up for testing in their production of IAA. Conjugation experiments used in this study were carried out according to Tun-Garrido *et al*. (2003). Hybridization technique was repeated several times through seven months until reached to appropriate time required for genetic transfer and appearing transconjugants on selective media as listed in Table 2.

Table 3: Analysis of variance of split-plot experiment.

Source of variation	Degrees of freedom (D.F)	MS	EMS
Main plots			
Replication	(r-1)	MS6	$\sigma^2e + r\,\sigma^2d + d\,\sigma^2r$
Doses of gamma rays	(d-1)	MS5	$\sigma^2e + r\,\sigma^2d$
Main plots error	(r-1) (d-1)	MS4	σ^2e
Sub plots			
Biofertilization	(b-1)	MS3	$\sigma^2e + r\,\sigma^2db + rd\,\sigma^2b$
Doses of gamma rays x Biofertilization	(d-1) (b-1)	MS2	$\sigma^2e + r\,\sigma^2db$
Sub main plots error	d (r-1) (b-1)	MS1	σ^2e

Where: r, d and b are the number of replications, doses of gamma rays and biofertilization, respectively.

Table 4: Genetic markers in six bacterial strains leading to 14 antibiotics.

Antibiotics	Bacterial strains					
	Rl-ARC-1601	Rl-12612	Rl-ARC-1604	Rl-ARC-207	Azo-24	*p.putida*
Streptomycin	-	-	-	-	-	0
Tetracycline	-	-	-	-	-	-
Cefadroxilmonhydrate	-	-	-	-	-	-
Erthomycin	+	+	+	+	-	-
Epanutin	+	+	+	+	-	-
Ampicillin-Cloxacillin	+	+	+	-	+	-
Amoxyicillin	+	+	+	+	-	-
Chloramphenicol	+	+	+	-	-	+
Neomycinsulphate	+	+	+	-	-	-
Amoxycillin-flucloxacillin	+	-	+	-	-	-
Cefardine	-	-	+	+	-	-
Ampicillin	+	+	+	+	+	-
Nystain	+	+	+	+	+	+
Erythromycinethlsuccinate	+	+	+	+	+	-

+, – = Means resistant and sensitive to antibiotics, respectively.

IAA-detection with the Salkowski Colorimetric Technique:

Rhizobium and *Pseudomonas putida* strains were grown overnight in YEM medium and Kings-B broth, respectively at 28°C. Production of IAA in the supernatant was assayed by using the PC method, as described by Pilet and Chollet (1970). IAA concentrations was calculated from the standard curve using the following formula of regression as follows:

$$x = \frac{y - a}{b}$$

Where:

- y = Optical density at 530 nm
- x = Concentration of IAA
- b = Regression =0.29
- a = Absorbance at 530 nm when the concentration of IAA equal zero = -0.01

Plant Infection:

Pea plants were grown in plastic pots in mixture of sterilizes sand and clay (1:1 w/w) with three replicates. Soil was washed with distilled water several times to diminishing chloride ions, as well as autoclaved three times at 121°C for one hour at three days. Plants were inoculating with parental strains and their transconjucants as listed in Table 3, 3 ml from cell suspension of over night culture growing at 28°C on rotary shaker (120 rpm) containing finally 10^8 FU/ml was used to inoculated plants at the time of sowing. Nodules developed on the plants were counted after 45-days-plant old. The accuracy of this method depends on the ability of a single *Rhizobium* cell to form nodule on the host. Plant infection technique is commonly applied in this study to determine the efficiency of different transconjugants (resulted from di-parental mating) of inoculants in symbiosis (Vincent, 1970). Pots experiment was carried out using a plastic pots according to Vincent (1970). This was conducted at the experimental form of Agric. Res. Horticulture Research Institute (El-Barammon station).

Field Evaluation:

This was conducted through the two winter seasons 2003/2004 and 2004/2005. Once after the seeds were gamma-irradiated with 1, 2 and 3 k rad from a ^{60}Co source, in addition to un-treated seeds as controls, seeds were surface sterilized with 10% ethyl alcohol (Dobert and Blevins 1993) for 10 minutes and then washed extensively three times with tap water to remove the residual alcohol before planting and planted in split plot design (SPD) consisted of three replications. Soil was covered with 1 cm of sand before planting the seeds. Plants were thinned after 3-days of germination for two plants per hill. The plants were watered to field capacity with water as needed until harvest, and fertilized with phosphorus 80kg/feddan, without any addition of nitrogen fertilizer. The plants of M1 and M2 generations were inoculated after germination with rhizobia and azorhizobia suspentions (10^8 CFU/ml) to four times with the rate of one ml/plant, five days separated each of them. The M1 observations were: nodule numbers /plant, nodule dry weight, shoot, root fresh and dry weight, plant height, number of pods and seeds weight per plant, pollen abortion, chlorophyll concentrations and number of branches per plant.

Field Evaluation of M2 Population:

Seeds from each M1 individual plants at each dose in addition to the control were selected on the basis of higher production of seed yielding per plant were bulked and saved for cultivation to obtain M2 plants. Seeds of each individual plant were field planted in an independent row in a split plot design consists of three replications. The same observations in M1 were recorded in M2 population. Protein estimations were recorded in M1 and M2 seeds (%NX6.25) according to Association of Official Analytical Chemists (1970).

Definition and Traits Studied:

Nodulation and Vegetative Traits:

Nodulation Test:

After forty days of inoculation, three plants from each replicate were removed and washed carefully with tap water. Nodule number appeared were counted, dried and weighted according to Novak *et al*. (2004).

Average Weight of Nodule (AWON):

This was estimated according to Pereira *et al*. (1989) using the following formula:

Average weight of nodule (AWON)= Nodule dry weight/Nodule number.

Nodulation index (NI):

An index of nodulation, which removed the effect of plant size was estimated according to Herridge and Betts (1988) as follows:

Nodulation index = Nodule mass/Shoot mass X100.

Table 5: Production of indole compounds by transconjugants of *Rhizobium* harboring DNA from two sources.

Mating	Parental strains and resulted transconjugants	Amounts of IAA (µg/ml)		
		Tryptophan	Ethanol	Lactic acid
Azorhizobium	P1	9.76	5.01	2.43
caulinodans	P2	4.73	14.57	2.45
&	M.P	7.25	9.79	3.44
pseudomonas	DPM-Tr1	29.19	8.25	0.43
putida	DPM-Tr2	27.58	12.48	0.43
	DPM-Tr3	10.56	16.87	0.71
	DPM-Tr4	28.42	18.83	0.50
	DPM-Tr5	10.64	3.93	2.48
	DPM-Tr6	26.09	3.61	0.47
	DPM-Tr7	22.06	6.87	0.45
	F-test	**	**	**
	LSD 5%	3.68	2.88	0.27
	1%	5.17	3.96	0.37
Rhizobium	P1	2.97	0.43	0.43
leguminosarum 12612	P2	4.73	14.57	2.45
&	M.P	3.85	7.5	1.44
pseudomonas	DPM-Tr8	48.11	0.48	0.43
putida	DPM-Tr9	43.90	0.59	0.43
	DPM-Tr10	43.74	0.67	0.53
	DPM-Tr11	43.39	0.71	0.43
	DPM-Tr12	44.57	0.63	0.43
	DPM-Tr13	56.60	0.45	0.45
	DPM-Tr14	42.82	0.70	0.48
	DPM-Tr15	49.01	0.72	0.57
	DPM-Tr16	94.30	0.72	0.43
	DPM-Tr17	57.12	0.64	0.59
	F-test	**	**	**
	LSD 5%	20.57	1.24	0.43
	1%	27.08	1.64	0.59
Rhizobium	P1	4.36	0.43	0.43
leguminosarum 207	P2	4.73	14.57	2.45
&	M.P	4.55	7.5	1.44
pseudomonas	DPM-Tr18	44.66	0.51	0.43
putida	DPM-Tr19	49.32	0.56	0.55
	DPM-Tr20	46.49	0.67	0.47
	DPM-Tr21	30.22	0.56	0.43
	DPM-Tr22	18.86	0.49	0.45
	DPM-Tr23	41.91	0.43	0.45
	DPM-Tr24	35.49	0.52	0.62
	DPM-Tr25	70.68	0.87	0.69
	DPM-Tr26	3.67	0.94	0.84
	DPM-Tr27	40.94	0.43	0.43
	F-test	**	**	**
	LSD 5%	2.86	0.91	0.10
	1%	3.89	1.24	0.14
Rhizobium	P1	17.73	0.43	1.29
leguminosarum 1604	P2	4.73	14.57	7.35
&	M.P	11.23	7.5	4.32
pseudomonas	DPM-Tr28	81.13	0.43	1.29
putida	DPM-Tr29	83.31	0.43	1.36
	DPM-Tr30	65.85	0.43	1.40
	DPM-Tr31	69.95	0.58	1.36
	DPM-Tr32	71.68	0.43	1.48
	DPM-Tr33	39.53	0.47	1.29
	DPM-Tr34	73.77	0.31	1.29
	DPM-Tr35	73.27	0.90	1.50
	DPM-Tr36	53.40	0.43	1.76
	DPM-Tr37	60.23	0.93	1.73
	DPM-Tr38	71.60	0.81	2.05
	F-test	**	**	**
	LSD 5%	11.85	0.89	0.10
	1%	16.10	1.21	0.14

Table 5: Continued

Rhizobium	P1	2.07	0.43	0.43
leguminosarum 1601	P2	4.73	14.57	2.45
&	M.P	3.4	7.5	1.44
pseudomonas	DPM-Tr39	4.97	0.60	0.47
putida	DPM-Tr40	5.20	1.07	0.52
	DPM-Tr41	3.23	0.62	0.46
	DPM-Tr42	4.28	0.56	0.52
	DPM-Tr43	5.04	0.64	0.57
	DPM-Tr44	8.20	0.43	0.43
	DPM-Tr45	6.13	0.43	0.43
	DPM-Tr46	4.60	0.48	0.46
	DPM-Tr47	6.14	0.43	0.43
	DPM-Tr48	4.13	0.43	0.48
	F-test	NS	**	**
	LSD 5%		0.93	0.11
	1%		1.27	0.15

NS, **= Not significant, P<0.01.

Shoot and Root Dry Weight per Plant:

Different parts of plants (shoots and roots) at 50-days-plant-old were oven dried at 70°C until reached to a constant mass and then turned immediately to weight.

Crop growth rate (CGR) [g/day]:

The following formulae was used to estimate CGR according to Sharma (2001)

$$\text{Crop growth rate (CGR g/day)} = \frac{W_2 - W_1}{(t_2 - t_1)}$$

Where W_2 and W_1 means total dry matter at successive stages, however, t2 and t1 means time interval.

Harvest Index:

It was measured according to Sharma *et a*l. (2001) using the following formulae:

Harvest index = Seed yield / biological yield X 100.

Shoot/root Ratio:

This trait was calculated according to Pererira *et al*. (1989) as follows:

shoot/root ratio = Shoot dry weight / Root dry weight.

Plant Height:

This trait was measured when the plants became to blooming at harvest time by centimeters from the first leaf to the apex.

Leaf Area/plant:

It was determined using leaf fresh weight method according to Beadle (1993). Leaf area was estimated using the following formula:

$$\text{Leaf area in } cm^2 = \frac{\text{Leaves fresh weight}}{\text{Disks fresh weight}} \times \text{Leaf area of disks in } cm^2$$

Chemical Traits:

Photosynthetic Pigments:

Chlorophyll (chl. a, b and total) was extracted in 80% methanol. The pigments were determined spectrophotometrically after stored the extracted solution for twenty four hours in a refrigerator and calculated according to the Lichtenthaler and Wellburn (1983) formulae.

Nitrogen Assays:

Nessler Reagent Used for Total Nitrogen Determination:

Dissolve 100g HgI_2 and 70 g KI in a small quantity of water and add this mixture slowly, with stirring, to a cool solution of 160 g NaOH dissolved in 500 ml water. Dilute to 1 L. Store in rubber-stoppered borosilicate glassware and out of sunlight to maintain reagent stability for up to a year under normal laboratory conditions. Check reagent to make sure that it yields the characteristic color with 0.1 mg NH_3-N/L within 10 min after addition and does not produce a precipitate with small amounts of ammonia within 2 hrs (APHA, 1992).

Sulphuric and Perchloric Acid Digestion:

Weight 0.1g of the plant sample into a 100 ml conical flask, add 5 ml conc. H_2SO_4. Boil and evaporate on a hot plate. Cool and add 1 ml of perchloric acid. Continue heating and adding drops of perchloric acid as necessary until digestion is complete as shown by a light colored, clear solution. Do not sample dry during digestion. Wash down flask walls with distilled water and then filter if necessary. Transfer filtrate to a 50 ml volumetric flask, cool, dilute to mark and mix thoroughly.

Procedure:

Mixed well 0.1 ml of sample diluted to 10 ml with di-distilled water, then add 1 ml Nessler reagent and mixed thoroughly. Keep such conditions as temperature and reaction time and also, the same conditions were used in the blank, samples and standards let reaction proceed for at least 10 min after adding Nessler reagent. If NH_3-N is very low use 30 min contact time for sample, blank and standards. Measure color photometrically as absorbance using a Spectrophotometer. Samples were reading at 425 nm for 1-cm light path. Calibration curve was prepared at the same temperature and the same reaction time used before for samples.

Determining the Concentration of Nitrogen:

Determination of nitrogen in the shoot samples was carried out according to (APHA, 1992) using a standard curve of colorimetric technique at OD 425 using different concentrations of NH_3Cl, as a source of nitrogen. Nitrogen concentrations was calculated from the linear regression equation as follows:

$$x = \frac{y - a}{b}$$

Where:

- y = Optical density at 530 nm.
- x = Concentration of nitrogen.
- b = Regression =0.14
- a = Absorbance at 425 nm when the concentration of N equal zero = 0.01

Crude protein in seed (%) = Seed N_2 percentage x 6.25

Pollen Fertility:

Mature anthers are collected from 60-days plant-old treated with different doses of gamma rays each inoculated with one of rhizobial strain (Cramer, 1991) and fixed in 70% alcohol. Each sample was tested for pollen fertility status by using the aceto-carmine staining method. To determine pollen fertility, darkly stained pollen grains were recorded as fertile and viable, and unstained or very lightly stained ones were considered as sterile or non-viable. Pollen sterility was calculated by dividing the number of non-viable pollen grains by the total number of grains counted in the field. Pollen viability was expressed as percentage of pollen fertility in each treatment. Pollen unviability leading to male sterility which is characterized by nonfunctional pollen grains, while fermale gametes function normally. Male sterility induced by gamma irradiation may be conditioned due to cytoplasmic or genetic factors or due to interaction of both.

Percentage pollen sterility is compared as follows:

$$\frac{\text{Number of (unstained whitered + unstained spherical + partially stained round)}}{\text{Total number of pollen grains (including fertile)}}$$

Experimental Design and Statistical Analysis:

Field experiment was designed in split-plot design involves assigning the treatments of one factor gamma irradiated plants in the main plots arranged in a completely random, where biofertilization was assigned to subplots within each main plot. This design usually sacrifices precision in estimating the average effects of the doses of gamma irradiation assigned to main plots. It often improves the precision for comparing the average effects of bioferitilizer with different bacterial strains assigned to subplots and, when interactions exist, for comparing the effects of subplot treatment. This arise from the fact that experimental error for main plots is usually large than the experimental error used to compare subplot treatments. Often, the error term for subplot treatments is smaller than would be obtained if all treatment combinations were arranged in a randomized complete block design. The source of variation and degrees of freedom of the analysis of variance of split-plot design has shown in Table 3.

RESULTS AND DISCUSSIONS

Screening Bacterial Strains for Resistance And/or Sensitivity Profiles of Antibiotics:

Six bacterial strains used in this study were genetically marked using 14 antibiotics. The results (Table 4) showed that streptomycin, tetracycline and cefadroxilmonhydrate were much more effective to inhibit the growth of all bacterial strains (fig 1) than the other antibiotics used. This is in agreement with Moskowitz (1963), who found that streptomycin was much more effective than dihydrostreptomycin in precipitating nucleic acids. All other antibiotics, except streptomycin, tetracycline and cefadroxilmonhydrate revealed differences in their action on different bacterial strains. As noted in previous studies (Cresti *et al.* 2002), there was a correlation between the antibiotic resistance phenotype and the genotype for each isolate.In this study it is demonstrated that there are variation between different bacterial strains in the resistance genes making strains are capable of intergenic or intragenic transfer. Filter-mating experiment showed that 5 were able to transfer genes encoding resistance to a recipient. Also, this study has shown that rhizobial strains contain a variety of resistance genes to the same antibiotic. The location (chromosomal or extrachromosomal) of drug resistance determinants was confirmed by plasmid curing strategies. Such broad host-range transferable plasmids play an important role in the spread of antibiotic resistance (Rasool, 1992).

Regulation of Indole-3-acetic Acid Production in Different Rhizobium Transconjugants:

Bacteria that inhabit the rhizosphere such as *Rhizobium*, *Azospirillium*, *Azotobacter*, *Pseudomonas*…….. etc may influence plant growth by contributing to a host plants endogenous pool of phytohormones, such as auxins. Production of the auxin indole-3-acetic acid (IAA) is widespread among plant-associated bacteria. In phytopathogenic bacteria, such as *Agrobacterium tumefaciens*, IAA is produced from tryptophan via the intermediate indoleacetamide and has been implicated in the induction of plant tumors. Beneficial bacteria synthesize IAA predominantly by an alternate tryptophan-pathway, through indolepyruvic acid, its role in plant growth promotion (fig 2).

As shown from the results presented in Table 5, all bacterial transconjugants harboring DNA from two sources produced high levels of IAA in the media supplemented with tryptophan. However, these isolates produced very low levels of IAA than that produced from tryptophan when grown in the media supplemented with ethanol or lactic acid, except for three transconjugants (DPM-Tr2, DPM-Tr3 and DPM-Tr4) resulted from the mating between *Azorhizobium caulinodans* X *Pseudomonas putida*, produced high levels of IAA from ethanol. However, all transconjugants resulted from other matings was not capable in producing significant amounts of IAA. Indolepyruvic acid is the product of catalysis tryptophan by tryptophan transaminase, the first step in the IAA biosynthetic pathway, and is the substrate for indolepyruvate decarboxylase. The genes responsible in this pathway may be higher amplified in all transconjugants resulted from the first four matings because of significant amounts of IAA produced from this pathway by most of them. Transconjugants resulted from the mating between *Rhizobium leguminosarum* (1601) X *Pseudomonas putida* produced lower amounts of IAA from tryptophan than that produced by the other transconjugant resulted the first four matings. This may be due to the lower amplification of *ipdc* gene in these isolates, that encodes indolepyruvate decarboxylase, a key enzyme in the indolepyruvic acid pathway. The suppression of *ipdc* gene in the transconjugants resulted from the fifth mating between *Rhizobium leguminosarum* (1601) X *Pseudomonas putida*, this could be due to transcription termination sequence just downstream of the translation stop codon. It is reasonable to conclude that the enzymes involved in the indolepyruvic acid pathway are not expressed from an operon because multiple copies of the gene encoding the first enzyme in the pathway, an aromatic aminotransferase. The higher amounts of IAA produced by different transconjugants from tryptophan are due to the enzyme indolepyruvate decarboxylase encodes by the *ipdc* gene prefers amino acid tryptophan

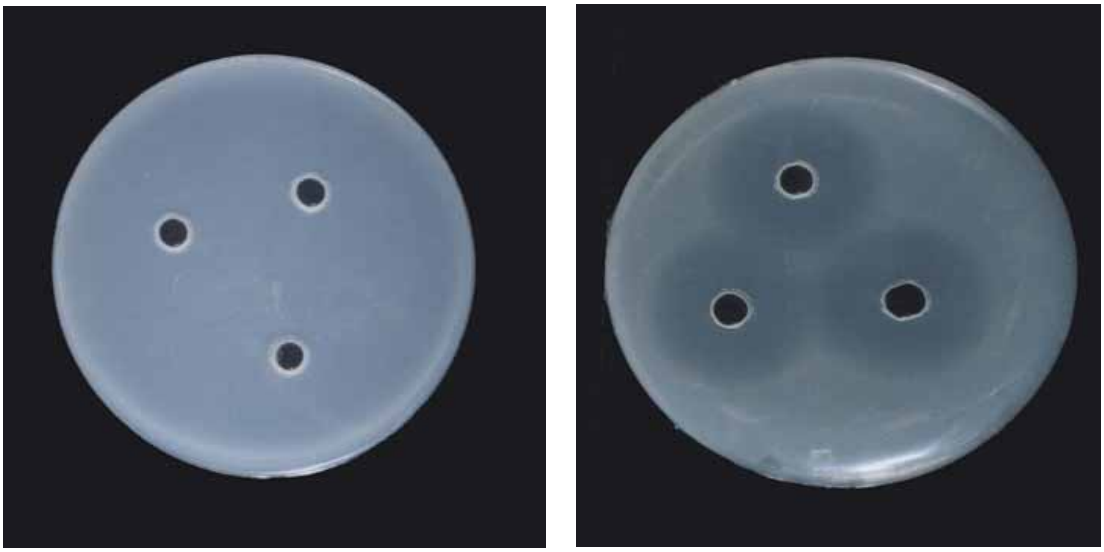

Fig. 1: Photograph representing the inhibition zones (below) resulted from tetracycline-treated Rhizobium leguminosarum (ARC-1601) if compared to nystain resistance (above).

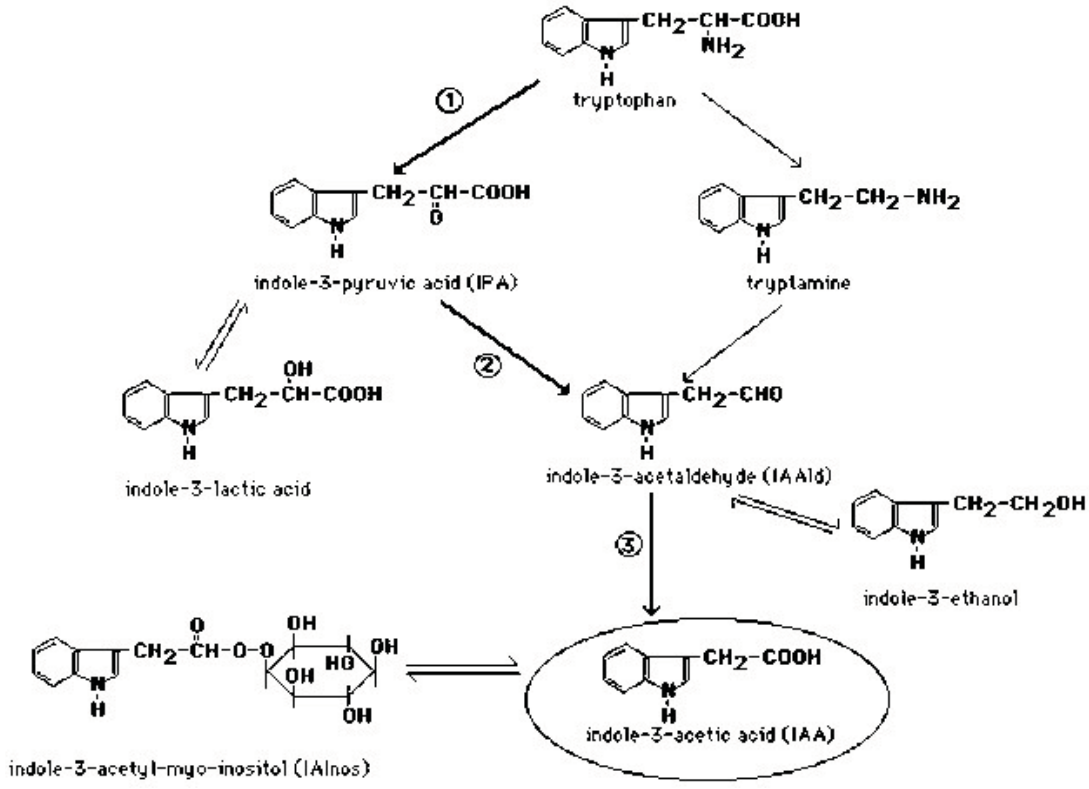

Fig. 2: The biosynthetic pathway for auxine (IAA) (Brandl and Lindow 1996)

other than ethanol and lactic acid. Thus, this aromatic aminotransferase is not solely an IAA biosynthesis enzyme. The results obtained herein are in agreement with Patten and Glick (2002), who found that many plant-associated bacteria synthesize the phytohormone indoleacetic acid (IAA). Whereas, IAA produced by

phytopathogenic bacteria, mainly by indoleacetamide pathway which has been implicated in the induction of plant tumors. Whether IAA synthesized by beneficial bacteria usually via the indolepyruvic acid pathway, is involved in plant growth promotion. IAA secreted by bacteria may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. ACC deaminase, is involved in the stimulation of root elongation in seedlings (Li *et al*. 2000. Production of IAA, a plant hormone in bacterial cells, may have evolved in bacteria, because it is important in the bacterium –plant relationship.

In this study the use of efficient-producing IAA transconjugants such as that resulted from the matings number 2, 3 and 4 may stimulates the development of the host plant root system. Higher levels of IAA produced from tryptophan were obtained from the crosses between; *Rhizobium leguminosarum* (12612) x *Pseudomonas putida*, *Rhizobium leguminosarum* (207) x *Pseudomonas putida* and *Rhizobium leguminosarum* (1601) x *Pseudomonas putida*. The results indicated that IAA production was markedly increased when Trp was included in the growth medium. Therefore, it appears that although tryptophan are involved in the synthesis of IAA, all transconjugants resulted from the first four cross (except one isolate, DPM-Tr26, resulted from the third cross) produced significant amounts of IAA above their mid-parents. Because there is evidence that plant root exudates contain significant levels of Trp (Kittell *et al*. 1989), it is possible that most transconjugants could exhibit a symbiotically effective phenotype in the presence of the plant, indicated that there was a competitive advantage associated with these strains. It was therefore concluded that most of transconjugants may symbiotically better than their parents.

Recent biochemical evidence suggested that some strains of *B. japonicum* can synthesize IAA by more than one pathway, including a pathway utilizing an indoleacetamide intermediate (Sekine *et al*. 1988) similar to the pathway utilized by *Pseudomonas savastanoi* and the T-DNA loci in *Agrobacterium tumefaciens* after transfer to plant cells. Clearly, the isolation of efficient transconjugants would be of interest.

The colorimetric assay used in this study revealed significant amounts of indole compounds in 40 out of 48 culture supernatants of transconjugants above their mid-parents. The higher amounts produced by transconjugants resulted from the mating between *Rhizobium leguminosarum* 1604 x *Pseudomonas putida* was ranged between 39 µg/ml and reached to 83.31 µg/ml in the presence of tryptophan. Significant amounts above the mid parents produced from ethanol were released by the transconjugants resulted from mating between *Azorhizobium caulinodans* X *Pseudomonas putida*, which ranged between 12-48 up to 18.83 µg/ml. Significant amounts of IAA were produced from the medium supplemented with tryptophan if compared with medium supplemented with other sources. This is in agreement with other results showing that this amino acid is a precursor for IAA in most studied IAA-producing bacteria (Omer *et al*. 2004).

The presence of IAA in the supernatant of transconjugant cultures, indicating the capability of these bacteria to produce IAA from different precursors. In particular, it has been postulated that IAA is the causal agent of root hair curling (Fahraeus and Liunggren 1968). This identification has been much speculation that indole-3-acetic acid (IAA) might play a role at various stages in the symbiotic relationship between *Rhizobium* and leguminous plants (Newcomb 1980).

Symbiotic Characterization of Rhizobial Strains and Their New Recombinant Isolates in Pots Experiment:

As shown from the results presented in the Table 6, all transconjugants resulted from di-parantal mating between Azo-24 and *P. putida* failed to nodulate pea (*Pisum sativum* L.). Truchet *et al*. (1991) demonstrated that the Nod factors (NFs) are essential bacterial signaling for nodule induction**.** Data (Table 7) illustrated that transconjugants; DPM-Tr10 and DPM-Tr11 resulted from di-parental mating between RL-12612 and *P.putida* showed significant increase above the mid-parents in nodulation parameters (number of nodules per plant and dry weight of nodules per plant), growth parameters (leaf area per plant (cm2) and dry weight of shoot and root) and chlorophyll content (chlorophyll a and total chlorophyll). These are in agreement with Martinez *et al*., (1987), who found that genetic transfer between *Rhizobium* species has led to different results The results are in accordance with Kimani (1988), who suggested that effective nodulation depends on the *Rhizobium* strain and the crop genotype. Many of transconjugents except DPM-Tr17 appeared significant increase in total chlorophyll above their mid-parents. The results are agreed with Sharma *et al*., (1997), who reported that nitrogen increased the synthesis of photosynthates.

Data presented in Table 8, clearly demonstrate that some transconjugants (DPM-Tr18, DPM-Tr19, DPM-Tr20 and DPM-Tr24) caused significant increase in the number of nodules developed per plant above the mid-parents. Whereas, All Transconjugants (except DPM-Tr19) appeared significant increase in shoot dry weight per plant (g) above their mid-parents. Roest *et al* (1995) suggesting that indole-3-acetic acid (IAA) might play a role at various stages in the symbiotic relationship between *Rhizobium* bacteria and leguminous plants (Newcomb, 1980). Although, DPM-Tr18 and DPM-Tr20 showed significant increase in chlorophyll b and total

Table 6: Mean of different growth parameters of plants (45-day-old) grown in pot experiment infected by *Azorhizobium* transconjugants resulted from di-parental mating between *Azorhizobium caulinodans* x *Pseudomonas putida*.

Inoculum	Nodulation parameters		Growth parameters			Chlorophyll content (mg/g)		
	Nodules /plant	Nodule DW† (g/plant)	L.A/P†† (cm2)	Shoot DW (g/plant)	Root DW (g/plant)	Chl. a	Chl.b	Total Chl†††.
Un.	0.0	0.0	261.0	1.02	0.55	0.17	0.58	0.75
Azo-24	0.0	0.0	266.1	1.33	0.51	0.17	1.00	1.17
P. putida	0.0	0.0	263.0	0.91	0.65	0.20	0.67	0.87
Mid-parent	0.0	0.0	264.6	1.12	0.58	0.19	0.84	1.02
DPM-Tr1	0.0	0.0	291.7	1.38	0.67	0.20	1.18	1.38
DPM-Tr2	0.0	0.0	250.2	1.31	0.63	0.96	0.80	1.08
DPM-Tr3	0.0	0.0	202.5	1.30	0.47	0.09	0.92	1.07
DPM-Tr4	0.0	0.0	212.5	1.34	0.63	0.19	0.66	0.85
DPM-Tr5	0.0	0.0	247.1	1.48	0.57	0.30	0.62	0.85
DPM-Tr6	0.0	0.0	242.4	1.33	0.50	0.29	0.79	1.08
DPM-Tr7	0.0	0.0	338.9	1.25	0.43	0.28	0.95	1.22
F-test	NS	NS	NS	NS	NS	NS	NS	NS

Ns = Not significant,†= Dry weight††= Leaf area per plant†††= Chlorophyll

Table 7: Mean of different nodulation, growth and chlorophyll content of plants (45-days-old) grown in pots experiment inoculated with transconjugants resulted from the mating between *Rhizobium leguminosarum* bv. *viciea* (12612) x *Pseudomonas putida*.

Inoculum	Nodulation parameters		Growth parameters			Chlorophyll content (mg/g)		
	Nodules /plant	Nodule DW† (g/plant)	L.A/P†† (cm2)	Shoot DW (g/plant)	Root DW (g/plant)	Chl.a	Chl.b	Total Chl†††.
Un.	0.0	0.000	260.95	1.02	0.55	0.17	0.58	0.75
RL-12612	3.0	0.014	261.32	1.19	0.62	0.24	0.81	1.05
P. putida	0.0	0.000	262.99	0.91	0.65	0.20	0.67	0.87
Mid-parent	1.5	0.007	262.16	1.05	0.64	0.22	0.74	0.96
DPM-Tr8	3.3	0.006	313.19	1.36	0.66	0.17	0.95	1.13
DPM-Tr9	2.3	0.004	371.20	1.32	0.57	0.23	0.87	1.10
DPM-Tr10	5.3	0.028	437.86	1.36	0.73	0.22	1.23	1.45
DPM-Tr11	7.0	0.019	422.24	1.56	0.66	0.17	0.94	1.11
DPM-Tr12	1.7	0.002	410.25	1.25	0.54	0.21	0.91	1.12
DPM-Tr13	0.7	0.001	276.66	1.03	0.48	0.17	0.90	1.08
DPM-Tr14	2.3	0.003	282.35	1.21	0.59	0.22	1.05	1.26
DPM-Tr15	2.0	0.003	282.51	1.19	0.46	0.27	0.87	1.14
DPM-Tr16	0.7	0.001	250.16	1.30	0.66	0.21	0.98	1.18
DPM-Tr17	2.7	0.003	242.08	1.25	0.56	0.20	0.79	1.00
F-test	**	*	*	**	*	NS	**	**
L.S.D 0.05	1.68	0.01	182.42	0.20	0.15		0.16	0.17
0.01	2.28	0.02	134.21	0.27	0.20		0.22	0.23

NS, *, ** = Not significant, p<0.05, p<0.01†= Dry weight††= Leaf area per plant†††= Chlorophyll

Table 8: Effect of inoculation with transconjugants resulted from the mating between, *Rhizobium leguminosarum* bv. *viciea* (207) x *Pseudomonas putida* on different nodulation, growth and chlorophyll parameters.

Inoculum	Nodulation parameters		Growth parameters			Chlorophyll content (mg/g)		
	Nodules /plant	Nodule DW† (g/plant)	L.A/P†† (cm2)	Shoot DW (g/plant)	Root DW (g/plant)	Chl.a	Chl.b	Total Chl†††.
Uni.	0.00	0.000	261.0	1.02	0.55	0.17	0.58	0.75
RL-12612	4.33	0.005	310.4	1.50	0.67	0.19	0.89	1.08
P. putida	0.00	0.000	263.0	0.91	0.65	0.20	0.67	0.87
Mid-parent	2.17	0.0025	286.7	1.21	0.66	0.195	0.78	0.98
DPM-Tr18	10.67	0.047	357.3	1.92	0.83	0.18	1.38	1.56
DPM-Tr19	5.00	0.008	447.9	1.44	0.48	0.21	0.94	1.15
DPM-Tr20	5.33	0.004	467.1	1.71	0.67	0.23	1.15	1.38
DPM-Tr21	8.67	0.023	334.9	1.82	0.81	0.18	1.02	1.21
DPM-Tr22	3.33	0.003	484.7	1.56	0.70	0.24	0.99	1.23
DPM-Tr23	1.67	0.002	271.4	1.51	0.64	0.20	0.77	0.96
DPM-Tr24	6.67	0.007	376.5	1.68	0.87	0.23	0.87	1.10
DPM-Tr25	2.33	0.003	300.1	1.69	0.76	0.18	0.95	1.13
DPM-Tr26	1.67	0.002	369.0	1.51	0.59	0.19	0.93	1.11
DPM-Tr27	3.00	0.003	315.7	1.66	0.75	0.19	1.02	1.21
F-test	**	**	**	**	**	NS	**	**
L.S.D0.05	2.49	0.010	91.3	0.28	0.14		0.32	0.28
0.01	3.38	0.014	124.0	0.38	0.19		0.43	0.38

NS, **= Not significant, p<0.01†= Dry weight††= Leaf area per plant
†††= Chlorophyll

chlorophyll, above their mid-parents. The results obtained herein are in agreement with the results obtained by Thakur and Panwar (1997), who reported that vigna radiate cv ps l6 and Pusa l05 inoculated with *Rhizobium* had larger leaf area (l0%), higher chlorophyll content (11.40%) and photosynthesis activity (21.65%), transpiration (l5.894) and stomatal conductance plants. These parameters were higher in *Rhizobium* inoculated plants.

Table 9: Mean of different growth parameters of plants (45-day-plant old) grown in pots, biofertilized with transconjugants resulted from the mating between *Rhizobium leguminosarum* bv. *viciea* (1604) x *Pseudomonas putida*.

Inoculum	Nodulation parameters		Growth parameters			Chlorophyll content (mg/g)		
	Nodules /plant	Nodule DW† (g/plant)	L.A/P†† (cm2)	Shoot DW (g/plant)	Root DW (g/plant)	Chl.a	Chl.b	Total Chl†††.
Uni.	0.0	0.000	261.0	1.0	0.55	0.174	0.578	0.752
RL-ARC-1604	3.0	0.006	337.5	1.5	0.67	0.221	0.848	1.070
P. putida	0.0	0.000	263.0	0.9	0.65	0.201	0.669	0.870
Mid-parent	1.5	0.003	300.3	1.2	0.66	0.211	0.758	0.97
DPM-Tr28	3.0	0.003	310.1	2.0	0.64	0.159	1.115	1.275
DPM-Tr29	3.7	0.007	439.3	1.8	0.71	0.188	0.973	1.161
DPM-Tr30	6.0	0.014	384.0	2.0	0.65	0.214	1.066	1.281
DPM-Tr31	4.3	0.006	360.0	1.7	0.55	0.227	0.868	1.095
DPM-Tr32	4.0	0.007	377.6	1.8	0.66	0.189	0.833	1.021
DPM-Tr33	7.7	0.019	361.2	2.3	0.76	0.210	0.853	1.063
DPM-Tr34	6.7	0.008	353.9	2.0	0.66	0.245	1.269	1.514
DPM-Tr35	2.0	0.002	252.7	2.0	0.57	0.271	0.823	1.095
DPM-Tr36	2.0	0.002	252.7	1.6	0.58	0.201	0.787	0.988
DPM-Tr37	1.0	0.001	278.9	1.6	0.59	0.208	0.800	1.008
DPM-Tr38	0.7	0.001	336.3	1.6	0.48	0.175	0.938	1.114
F-test	**	*	NS	**	**	*	**	**
L.S.D0.05	2.12	0.008		0.308	0.148	0.058	0.245	0.332
0.01	2.86	0.011		0.416	0.199	0.078	0.331	0.246

NS, *, ** = Not significant, p<0.05, p<0.01† = Dry weight†† = Leaf area per plant
††† = Chlorophyll

Table 10: Means of different nodulation, growth and Chlorophyll parameters of plants (45-day-old) grown in pots, inoculated with transconjugants resulted from the mating between *Rhizobium leguminosarum* bv. *viciea* (1601) x *Pseudomonas putida*.

Inoculum	Nodulation parameters		Growth parameters			Chlorophyll content (mg/g)		
	Nodules /plant	Nodule DW† (g/plant)	L.A/P†† (cm2)	Shoot DW (g/plant)	Root DW (g/plant)	Chl.a	Chl.b	Total Chl†††.
Uni.	0.0	0.000	261.0	1.02	0.55	0.17	0.58	0.75
RL-ARC-1601	2.3	0.002	287.5	1.25	0.66	0.16	0.75	0.91
P. putida	0.0	0.000	263.0	0.91	0.65	0.20	0.67	0.87
Mid-parent	1.2	0.001	275.3	1.08	0.66	0.18	0.71	0.89
DPM-Tr39	2.0	0.003	296.5	1.37	0.62	0.18	0.94	1.12
DPM-Tr40	5.7	0.041	292.6	1.52	0.69	0.21	0.85	1.06
DPM-Tr41	1.0	0.001	193.5	1.07	0.54	0.19	0.75	0.94
DPM-Tr42	1.7	0.008	256.6	1.44	0.80	0.24	0.95	1.19
DPM-Tr43	1.7	0.002	256.6	1.24	0.55	0.19	0.82	1.01
DPM-Tr44	2.3	0.019	287.0	1.44	0.52	0.30	0.98	1.28
DPM-Tr45	4.7	0.020	270.2	1.70	0.81	0.22	0.91	1.14
DPM-Tr46	4.3	0.003	253.5	1.40	0.51	0.34	0.83	1.17
DPM-Tr47	0.7	0.003	273.3	1.12	0.53	0.18	1.07	1.25
DPM-Tr48	1.0	0.001	193.3	0.97	0.53	0.18	0.80	0.98
F-test	**	**	NS	**	NS	**	**	**
L.S.D0.05	2.16	0.02		0.31		0.07	0.17	0.18
0.01	2.93	0.03		0.42		0.10	0.23	0.24

NS, ** = Not significant, p<0.01† = Dry weight†† = Leaf area per plant
††† = Chlorophyll

The results summarized in Table 9 demonstrated that di-parental transconjugants DPM-Tr29, DPM-Tr30, DPM-Tr31, DPM-Tr32, DPM-Tr33 and DPM-Tr34 appeared significant increase in the number of nodules developed above their mid-parents. Two di-parental transconjugants (DPM-Tr30 and DPM-Tr33) appeared significant increase in nodule dry weight per plant above their mid-parents. However, most transconjugants induced significant increase in shoot dry weight above their mid-parents.

Di-parental transconjugants DPM-Tr28, DPM-Tr30 and DPM-Tr34 appeared significant increase in the concentration of chlorophyll b and total chlorophyll. However, transconjugant DPM-Tr35 appeared significant increase in chlorophyll a above the mid-parents. This results are in harmony with Beynon *et al*. (1980), who transferred the conjugative *Rhizobium leguminosuarm* plasmid *pJBJI* which carried genes that determine the ability to nodulate peas, from strain T3 into *R. phaseoli* strains 1233, and they found that the majority (about 97%) of transconjugants produced pigment and nodulated both peas and phaseolus beans poorly.

The results presented in Table 10 showed significant increase in the number of nodules developed above the mid parents. In addition, DPM-Tr40 induced significant increase in the weight of nodules developed per plant above that developed by the mid-parents. These results are in agreement with Deshwal *et al* (2003), who reported that rhizobia are known to increase nodulation and nodule dry weight in legumes along with increase the growth of host plant growth and its development. Besides protecting roots from the pathogens attack due to production of diverse microbial metabolites like siderophore, rhizobitoxin, plant growth enhancement through IAA production, uptake of phosphorus and other minerals.

Taken together, some transconjugants (DPM-Tr39, DPM-Tr42, DPM-Tr44, DPM-Tr45, DPM-Tr46 and DPM-Tr47) resulted in significant increase in total chlorophyll concentration. This results agreed with Philips and Torrey (1970) and Dakora (2003), who reported that rhizobia produce various metabolites such as auxines, cytokinins, riboflavin and vitamins, their invasion of legume and non-legume plant roots should promote an increase in plant growth.

Nodulation Parameters of Pea Mutants:

Data presented in Table 11 did not show any significant increase in the nodulation parameters (number of nodules per plant, nodule dry weight, average weight of dry nodule and nodulation index) meaning that gamma rays did not affect on the development of nodulation. This dis-agreed with Sagan *et al*. (1995), who found significantly greater number of nodules developed on pea mutants treated with gamma rays than the control.

Table 11: Means of nodulation parameters affected various gamma rays doses at 50 days-old.

Nodulation parameters	Doses (K-rad)									
	0		1		2		3		F- test	
	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Nodules/plant	0.90	0.90	1.19	0.86	0.90	1.38	0.95	1.14	NS	NS
Nodule DW†(mg/plant)	8.38	9.95	9.8	5.29	6.34	10.95	10	4.67	NS	NS
Average weight of nodule(mg)	4.68	6.17	3.90	3.38	3.78	4.73	7.12	1.62	NS	NS
Nodulation index	0.69	0.66	0.95	0.42	0.68	0.53	0.94	0.45	NS	NS

NS= Not significant. †= Dry weight

Table 12: Means of nodulation parameters affected by all doses of gamma rays treated pea at 50 days.

Biofertilizers	Nodules/plant		Nodule DW†(mg/plant)		Average weight of nodule (mg)		Nodulation index	
Plant-old.	M1	M2	M1	M2	M1	M2	M1	M2
Uninoculated	0.33	0.17	0.43	0.17	0.43	0.08	0.06	0.02
RL-ARC-1601	1.67	1.33	14.75	7.33	6.51	5.47	1.41	0.65
RL-12612	0.92	1.75	10.33	14.92	7.64	6.60	1.08	1.25
RL-ARC-1604	0.83	1.67	8.83	12.00	6.63	6.22	0.66	0.90
RL-ARC-207	2.42	2.17	24.84	18.58	11.72	8.64	2.36	1.11
Azo-24	0.25	0.17	0.50	0.50	0.50	0.33	0.05	0.06
P.putida	0.50	0.25	0.67	0.50	0.67	0.50	0.09	0.05
F-test	**	**	**	**	**	**	**	**
L.S.D 0.05	0.76	0.52	7.97	5.68	5.46	2.52	0.75	0.44
0.01	1.01	0.69	10.63	7.58	7.28	3.37	1.00	0.58

** = p<0.01†= Dry weight

The results presented in Table 12 indicated that pea plants inoculated with different *Rhizobium* strains significantly increase most of nodulation parameters developed among M1 and M2 generations above the uninoculated plants. This indicated the activity of nodulation (*nod*) genes to establish the symbiosis between legume plants and nodule bacteria (rhizobia). Higher activation of rhizobial nodulation (*nod*) genes giving significant increase in nodulation parameters. This may be due to molecular changes in flavonoid compounds present in root exudates activated rhizobial *nod* genes. This because flavonoids interact specifically with the

Table 13: Means of nodulation parameters in M1 and M2 populations treated with different doses of gamma rays under the effect of biofertilization.

Doses	0				1				2				3			
	Nodules /plant		Nodule DW (mg/plant)		Nodule s/plant		Nodule DW (mg/plant)		Nodules /plant		Nodule DW† (mg/plant)		Nodules /plant		Nodule DW (mg/plant)	
Bio.	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Uninoculated	0.3	0	0.3	0	0.3	0.7	0.7	0.7	0.3	0	0.3	0	0.3	0	0.3	0
RL-ARC-1601	1	1	12.3	5.3	0.7	1	4.3	8.7	2.3	1.3	18.3	12.3	2.7	2	24	3
RL-12612	0.7	2.3	12.3	26.7	1	1	10.7	4.3	1.3	2.3	10	19.7	0.7	1.3	8.3	9
RL-ARC-1604	0.7	0.7	10	7.3	1	1	6.7	8.7	0.7	2.7	0.7	19.3	1	2.3	18	12.7
RL-ARC-207	3.7	1.7	23.7	28	4.3	2	44.3	14.3	0.7	3.3	14	24.7	1	1.7	17.3	7.3
Azo-24	0	0.3	0	1	0.3	0	1	0	0.3	0	0.3	0.7	0.3	0.3	0.7	0.3
P.putida	0	0.3	0	1.3	0.7	0.3	0.7	0.3	0.7	0	0.7	0	0.7	0.3	1.3	0.3
F-Test	*	**	NS	NS	*	**	NS	NS	*	**	NS	NS	*	**	NS	NS
LSD 1%	1.52	1.04			1.52	1.04			1.52	1.04			1.52	1.04		
5%	2.03	1.39			2.03	1.39			2.03	1.39			2.03	1.39		
Uninoculated	0.3	0	0	0	0.7	0.3	0.1	0.1	0.3	0	0.1	0	0.3	0	0	0
RL-ARC-1601	10	5.3	0.9	0.5	2.2	6.7	0.2	0.1	6.6	8.5	2.1	0.8	7.3	1.4	2.5	0.3
RL-12612	12.3	11.4	1.3	2.1	5.7	2.7	1.1	0.5	4.2	9.1	1.1	1.6	8.3	3.2	0.8	0.9
RL-ARC-1604	5	7.3	0.4	0.5	6.7	6.3	0.7	0.5	0.3	7.7	0.1	1.3	14.5	3.5	1.4	1.2
RL-ARC-207	5.1	16.8	2.2	1.3	10.4	7.3	4.3	0.9	14	7.8	1.3	1.8	17.3	2.6	1.6	0.5
Azo-24	0	1	0	0.1	1	0	0.1	0	0.3	0	0	0.1	0.3	0.3	0.1	0
P.putida	0	1.3	0	0.1	0.7	0.3	0.1	0	0.7	0	0.1	0	0.7	0.3	0.2	0
F-Test	NS	*	**	NS	NS	*	**	NS	NS	*	**	NS	NS	*	**	NS
LSD 1%		5.05	1.51			5.05	1.51			5.05	1.51			5.05	1.51	
5%		6.73	2.01			6.73	2.01			6.73	2.01			6.73	2.01	

NS, *, ** = Not significant, p<0.05, p<0.01†= Dry weight

Table 14: Mean squares of nodulation parameters from split plot analysis.

S.V.	D.F	No. of nodules/ plant		Nodule DW†(mg/plant)		Average weight of nodule (mg)		Nodulation index	
		M1	M2	M1	M2	M1	M2	M1	M2
Main plot									
Rep.	2	2.87	3.46	247.20	87.46	52.41	7.47	2.78	0.70
Doses	3	0.39	1.22	59.46	214.76	50.56	79.05	0.47	0.71
Error	6	3.49	2.07	577.36	53.85	236.09	24.29	4.91	0.31
Sub plot									
Biofertilizars	6	7.48**	8.79**	999.8**	699.2**	233.6**	152.7**	9.06**	3.42**
Doses x Bio	18	2.27*	1.03**	145.73	83.62	34.53	20.53*	1.58**	0.44
Error	48	0.86	0.40	94.45	48.06	44.39	9.48	0.84	0.28

*, ** = p<0.05, p<0.01†= Dry weight

protein product of the *nod* D gene and the active form of Nod D is believed to activate transcription through promoters of *nod* operons. This response is even clearer in rhizobia. The *nod* D activated by the Nod-D-flavonoid complex, such as *Rhizobium leguminosarum* bv. *viciae*, a garden pea (*Pisum sativum* L.) microsymbiont (Burn *et al*. 1987). The effect of molecular changes in flavonoid compounds due to gamma irradiation on the symbiosis affecting on nodulation parameters corresponded to the behavior from their interaction with *nod* genes of *Rhizobium* strains. The results obtained are in agreement with Burn *et al*. (1987), who reported that overproduction of *nod* gene products can be deterimental to the effective nodule formation as well. This might be the case of M2 generation in which higher flavonoid inducer excess is accompanied with conditional nodulation and an aberrant nodule phenotype.

Data presented in Table 13 showed that the increase in the activity of nodulation genes was appeared in M2 generation than in M1. This may be due to switched on genes that lead to increase of nodulation in M2 than in M1. The results obtained in this study are in agreement with Sagan et al. (1995), who found that symbiosis mutants could be easily selected after gamma-rays mutagenesis.

The results obtained in Table 14 are in accordance with Kimani (1988), who suggested that effective nodulation depends on the *Rhizobium* strain and crop genotype among other factors. This indicated that the formation of nitrogen-fixing root nodules involves a succession of signal exchanges between the host plant and symbiotic. Discrepancies in this molecular communication result in impaired symbiosis. Whereas, interaction between doses of gamma rays x biofertilizeres induced significant effect on both the average weight of nodule (M2) and nodulation index (M1).

Potential Use of Bacterial Strains as Promoters of Growth Parameters of Pea Mutants:

The results presented in Table 15 appeared that doses of gamma rays were significantly affect on leaf area/plant and plant height among M1 and M2 generations. However, crop growth rate (M1) was significantly affected by doses of gamma rays. This agreed with Ahloowailia *et al.* (1998), who reported that radiation and other chemical mutagens have been used to induce variability in crop plants. Other authors have clearly shown that the growth inhibition after gamma irradiation with high or low doses is correlated to more or less severe decrease in production of growth hormone, indolacetic acid (AIA) (Chandorkar and Clark, 1986). But the mechanisms involved in plant response are still unclear (Kuzin *et al*., 1991).

Biofertilizer strains revealed significant effect on all traits of M1 and M2 populations except for number of branches/plant. However, the interaction between doses of gamma rays and biofertilizer strains achieved significant effect on crop growth rate (M1 and M2), number of branches per plant (M1) and leaf area/plant (M2).

Table 15: Mean squares of some growth parameters from split plot design.

S.V	D.F	L.A/P† (cm2)		Plant height (cm)		CGR ††(g/day)		Number of branches/plant	
		M1	M2	M1	M2	M1	M2	M1	M2
Main plot									
Rep.	2	562.0	480.96	52.1	232.7	0.0006	0.0002	0.57	0.01
Doses	3	8121.7*	6288.35*	871.8*	1365.6*	0.0018*	0.0003	0.77	0.55
Error	6	1387.6	792.19	165.3	165.5	0.0003	0.0004	0.29	0.65
Sub plot									
Biofertilizers	6	6905.3**	4321.19**	703.6**	322.9**	0.0017**	0.0008**	0.96	0.31
Doses x Bio.	18	2508.9	1726.80*	18.2	31.9	0.0010*	0.0005**	1.20*	0.50
Error	48	1837.6	933.50	52.5	40.6	0.0005	0.0002	0.66	0.59

*, ** = $p<0.05$, $p<0.01$†= Leaf area††= Crop growth rate

Yield Parameters Influenced by Different Bacterial Strains under the Effect of Gamma Irradiation:

Gamma-irradiation stress induced significant changes in the plant physiological and biochemical processes. Gamma-irradiation affect on the synthetic plant growth regulators (auksins, giberlines, cytokinins, etc) in plants (Stoeva *et al*., 2001).

The data presented in Table 16 show the effect of different doses of gamma rays on the yield components through two generations under the effect of biofertilization. The result revealed significant increase in the number of pods per plant in M1 population at all treatment doses of gamma rays above unirradiated plants. Although, the number of pods per plant in M2 population was significantly increase at the dose of 3 K-rad.

These result agreed with Nalampang *et al*., (1982), who found that gamma-rays irradiated seeds of mung been appeared successive increase in dosage, the emergence, plant height and number of nodule were correspondingly decreased in M_1 generation, however, number of branches increased with 0 to 60 K-rad and remained steady between 60 to 90 k-rad, the maximum pod number per plant was obtained at 60 k-rad.

Whereas, there are no significant increase in seeds dry weight and harvest index through two generations. These results differed with Al-Safida and Simon (1996), who observed that gamma-irradiation induced variation on two generations in carrots. These variations concerning germination, plant size and seed production, differed by the nature of the irradiated material.

The data summarized in Table 17 revealed that inoculation of pea with some rhizobial strains; RL-ARC-1601, RL-12612, RL-ARC-1604 and RL-ARC-207 exhibited significant increase in the number of pods per plant (among M1 and M2 population) and seeds dry weight in M2 generation above uninoculated plants. Whereas, rhizobial strains; RL-ARC-1604 and RL-ARC-207 appeared significant increase in harvest index of M1 population above uninoculated plants. Seeds dry weight per plant was significantly increase above that in uninoculated ones among M2 generation in response to all biofertilizer strains, with the exception of Azo-24 and *P. putida*. However, both RL-12612 and RL-ARC-207 strains produced the same trend for seeds dry weight/plant in M1 population. These results agreed with Mozumide*r et. al.* (2003), who reported that inoculated summer mung bean with *Bradrhizobium* produced significantly higher number of pods (40.8) per plant if compared to uninoculated plants (35.5). Popescu (1998) found that grain yield of *Phaseous vulgaris* L. were significantly ($p < 0.05$) affected by inoculation with *Rhizobium* strains. Conversely, there were significant effects of biofertilizers strains on the yield traits.

Data presented in Table 18 did not show any significant increase on the performance of yield components in response to the interaction between doses of gamma rays and biofertilizer strains through two generations. This means that the genetic variation induced by gamma rays did not significantly affect on the interaction with biofertilizer strains. These results agreed with Jensen (1987), who found that inoculation of pea plants with

Table 16: Mean performance of yield parameters affected by different doses of gamma ray under the effect of biofertilizer strains.

Parameters	Doses (K-rad)													
	0		1		2		3		F-test		L.S.D			
	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1		M2	
											0.05	0.01	0.05	0.01
Number of pods/plant	29.18	29.67	33.74	30.33	34.65	34.33	35.95	37.48	*	*	4.42	6.69	4.79	7.25
Seeds DW† (g/plant)	9.65	10.26	9.80	11.69	11.71	11.43	9.70	10.76	NS	NS				
Harvest index (g)	100.64	102.95	93.75	125.66	111.85	121.24	90.16	109.36	NS	NS				

* = p<0.05.†= Dry weight.

Table 17: Mean performance of yield parameters affected by different biofertilizers strains under the effect of different doses of gamma rays

Biofertilizers	Number of pods/plant		Seeds DW†(g/plant)		Harvest index (g)	
	M1	M2	M1	M2	M1	M2
Uninoculated	28.48	28.17	7.95	8.41	85.97	84.01
RL-ARC-1601	34.61	35.17	10.2	13.04	85.28	118.31
RL-12612	36.28	37.75	11.79	11.31	114.9	110.82
RL-ARC-1604	36.37	35.33	12.23	11.99	133.57	136.89
RL-ARC-207	36.33	37	10.83	12.72	91.26	134.33
Azo-24	31.79	29	9.78	9.91	99.78	111.2
P.putida	29.79	28.25	8.75	9.87	82.94	108.04
F-test	**	**	**	**	**	**
L.S.D 0.05	4.35	4.74	2.27	2.34	27.8	27.75
0.01	5.8	6.32	3.02	3.12	37.08	37.01

Table 18: Mean performance of yield parameters affected by interaction between doses of gamma rays and biofertilizers strains.

Doses	0						1					
	Number of pods/plant		Seeds DW† (g/plant)		Harvest index (g)		Number of pods/plant		Seeds DW (g/plant)		Harvest index (g)	
Bio	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Uninoculated	25.7	24.3	7.2	7.4	94.1	80.7	28.3	23.3	7	8.5	67.4	82.6
RL-ARC-1601	29.7	31.3	8.3	13.2	76.8	119.7	33.1	30.3	10.5	13.9	74.2	103.2
RL-12612	29.3	33	10.1	9.7	88.9	88	35.2	35	13.3	11.8	145.8	107.9
RL-ARC-1604	32.7	30.3	13.5	10.3	152.3	132.8	40.7	33	12.3	14.8	171.2	153.8
RL-ARC-207	30.1	34	10.2	11.1	91.7	106.3	37.9	35	9	13.3	67	180.5
Azo-24	31	28.7	8.9	10.4	99.3	105.8	33.4	30.7	9.6	9.2	89.2	125.4
P.putida	25.8	26	9.4	9.7	101.3	87.3	27.7	25	7	10.4	41.5	126.2
F-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Uninoculated	29.4	31	9.9	8.6	105.5	86	30.6	34	7.8	9.3	76.9	86.7
RL-ARC-1601	36	36.7	12.1	11	98.6	117.8	39.7	42.3	10	14.1	91.6	132.5
RL-12612	40	38.7	13.1	13.1	127.6	144.4	40.7	44.3	10.6	10.7	97.3	103
RL-ARC-1604	31.5	35.3	11.9	11.5	105.9	119.3	40.6	42.7	11.2	11.4	104.8	141.6
RL-ARC-207	41.5	39.7	13.9	14.5	120.7	143.8	35.9	39.3	10.3	11.9	85.7	106.7
Azo-24	34	30	10.9	10.2	104.9	111.8	28.7	26.7	9.8	9.8	105.8	101.8
P.putida	30.1	29	10.3	11.2	119.9	125.4	35.6	33	8.3	8.1	69.1	93.2
F-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS= Not significant †= Dry weight

three *Rhizobium* strains did not significantly influence on the yield of seeds (mean yield: 5.81 t DM/ha) but the yield and N-concentration in seeds were slightly increased. In contrast Vaishy *et al*., (1983) reported that inoculation by *Bradyrhizobium* increased seed yield of (*Vigna radiate* L.) from 4.3 to 162%.

Biochemical Traits of Pea Plants:

Data presented in Table 19 demonstrated that the doses of gamma rays appeared significant effect on chlorophyll content (mg/ml), nitrogen concentration in shoot dry weight at 50 days plant-old among M1 and M2 populations, seeds protein content (mg/g) (M1) and pollen sterility among two generations**.** The results are in agreement with Malavia and Shukla (1984), who reported that pollen fertility was ranged between 66-87% in different lentil plants. Awasthi and Dubey (1985) reported that viable pollens were fully turgid and

Table 19: Mean squares from the analysis of variance of biochemical traits in M1 and M2 populations of pea plants resulted from irradiated seeds under the effect of biofertilization.

S.V.	D.F	Chlorophyll content (mg/g)						Shoot N content							
		Chl.a		Chl.b		Total Chl.		I		II		Seeds protein content (mg/g)		Pollen Sterility %	
		M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Main plot															
Rep.	2	0.003	0.004	0.005	0.04	0.011	0.06	0.04	0.27	0.08	0.002	29.7	0.2	9.8	79.8
Doses	3	0.014*	0.084*	0.827**	1.11**	0.958**	1.39**	7.52**	7.44**	0.02	0.043	19.8*	16.3	328.2**	845.1**
Error	6	0.002	0.006	0.01	0.07	0.007	0.08	0.23	0.1	0.2	0.037	2.6	4.1	11.4	38.4
Sub plot															
Biofertilizers	6	0.007*	0.02	0.388*	0.51**	0.478**	0.48**	1.33$_{**}$	1.48**	0.44**	0.190**	33.8**	38.1**	57.2*	39.4
Doses x Bio.	18	0.003	0.015	0.050*	0.1	0.049*	0.09**	0.13	0.27*	0.01	0.042	3.5	4.7*	30.9*	44.3*
Error	48	0.003	0.011	0.023	0.06	0.025	0.04	0.32	0.15	0.08	0.05	7.8	2.6	17	20.2

*, ** = p<0.05, p<0.01 I, II = 50 and 150 days plant-old, respectively

were stained uniformly by acetocarmine or iodine, while sterile pollens, which do not have any cytoplasm content fail to pick up the stain, analysis of variance showed that pollen fertility is highly significant due to lines. However, biofertilizers produced significant effect on all biochemical traits, except chlorophyll a (M2) and pollen sterility (M2). In addition, the interaction between doses and biofertilizers appeared significant increase in chlorophyll b (M1), total chlorophyll (M1 and M2), nitrogen concentration in shoot dry weight at 50 days plant-old (M2), seeds protein content (mg/g) (M2) and pollen sterility (M1 and M2). This are in harmony with Zaka *et al*. (2004), who demonstrated that the effect of low doses of gamma irradiation at an early stage of plant development may be explained by point mutations occurred in cells of the apical meristem. In fact, low doses of x rays have been shown to cause such mutations in *Arabidopsis thaliana* (Kovalchuk *et al*., 2000). These mutations are then transmitted via mitotic divisions during plant development, and the mutated genes are expressed during flowering, leading to diverse meiotic abnormalities and consequently to a decreased fertilization.

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