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Effect of Gamma Irradiation on Physiological and Biochemical Traits in Cowpea, *Vigna unguiculata* (L.) Walp Inoculated with New Recombinant Isolates of *Bradyrhizobium*

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Abstract: The symbiotic interaction between rhizobia and legume roots is characterized by a high degree of specificity. Two varieties of cowpea were gamma irradiated as a one method to create genetic variation resulting in new varieties with better characteristics in nodulation and nitrogen fixation processes. Conjugation is the second method used in this study, a cell contact-dependent DNA transfer mechanism, which has served as elegant tool in the development of genetic engineering technology. The possibility of horizontal gene transfer to other rhizobia, revealed that it is necessary, in view of possibility of deliberate release of a variety of recombinant rhizobia into the environment for such agricultural purposes as improving nitrogen fixation. New recombinants revealed higher amounts of indole compounds from tryptophan above the mid-parents in two out of six transconjugants resulted from the cross between P_1 x P_3. Significant number of nodules were developed on the root system of V_2-variety in M_4 generation treated with 20 krad in response to inoculation with the parental strains (P_2 and P_3) and also in response to inoculation with triparental transconjugants (Tr_4 and Tr_5), above that developed on the plants fertilized with recommended dose of N. The results revealed the success of rhizobial strains and their recombinants to colonize and infect roots of cowpea, because of significant dry weight of nodules per plant which can be obtained in V_1-variety treated with 20 krad in M_4 generation inoculated with the parental strain (P_3), above that on the plants fertilized with recommended dose of N. Total chlorophyll formation in V_1-variety inoculated with di-parental transconjugants (DPM-Tr_2 and DPM-Tr_3) at all doses of gamma irradiation was significantly increase above that in the plants fertilized with recommended dose and the mid-parents, with the exception at 30 krad if compared with the mid-parents. Significant increase was resulted in fresh weight of pods developed per plant above the mid-parents in M_3 generation of V_1-variety at doses zero and 10 krad, in response to inoculation with di-parental transconjugant, DPM-Tr_2. While, the same trend was also achieved above the full dose in M_3 generation at 10 krad in response to inoculation with DPM-Tr_2, DPM-Tr_3, TPM-Tr_4 and TPM-Tr_5. The highest nitrogen content was appeared in the shoots of V_1-variety at all doses of gamma irradiation in response to inoculation with diparental transconjugant (DPM-Tr_2). However, V_2-variety had the lowest nitrogen content in relation to the plants fertilized with recommended dose of nitrogen and to the mid-parents of rhizobial transconjugants. The genetic variability of grain-protein content appeared that V_2-variety treated with 10 krad had significant increase in protein content above that in the plants fertilized with recommended dose of N among M_3 and M_4 generations, in response to inoculation with parental strains and most of their transconjugants. The same trend was also shown in M_4 generation of V_1-variety treated with 20 and 30 krad above the plants fertilized with recommended dose of nitrogen, in response to inoculation with di-parental transconjugants. All biochemical traits studied were more affected by biofertilization than the doses of gamma rays and the interaction between biofertilization x doses. This indicated that the significance of treatments was mainly due to inoculation and particularly to gamma irradiation and the interaction between both of them.

Key words: *Bradyrhizobium*, conjugation, cowpea, gamma rays, nodulation triparental mating

INTRODUCTION

The legume root nodule is a specialized organ on the plant root and is formed as a result of specific signal exchanges between the rhizobial microsymbiont and the host plant. The signal exchange between rhizobia and legumes and the processes leading to a nitrogen-fixing symbiosis involve several steps: (i) growth of the rhizobia in the rhizosphere of the host, (ii) induction of rhizobial nodulation genes by plant exudate, (iii) production of the

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bacterial signal molecule, the nod factor, (iv) attachment of rhizobia to the roots of the host, (v) induction of cell division in the plant, (vi) penetration by rhizobia into the plant via the infection thread and (vii) engulfment of rhizobia into intracellular symbiosomes[1]. In the root nodule, the bacterial nitrogenase enzyme system can reduce atmospheric dinitrogen to ammonia, which is then transported to the plant

Cowpea, *Vigna unguiculata* (L.) Walp. is a tropical grain legume which plays an important nutritional role in developing countries of the tropics and subtropics, especially in sub-saharan Africa, Asia, Central and South America[2]. Because of its high protein content (20-25%), cowpea has been referred to as poor man's meat. Cowpea young leaves, pods and peas contain vitamins and minerals which have fuelled its usage for human consumption and animal feeding[3]. Despite its importance, the production of cowpea which is about 1000 kg ha^{-1} does not meet the need of consumers[4]. The low yield is the result of poor soil, particularly in nitrogen and the high cost of chemical fertilizers. However, cowpea establishes symbiotic association with *Bradyrhizobium* bacteria enabling it to fix atmospheric nitrogen. Nevertheless, breeders and agronomists have not given high priority to cowpea varieties with high nitrogen fixing potential in breeding program in order to improve production and maintain soil fertility[5].

The protein in cowpea seed is rich in the amino acids, lysine and tryptophan, compared to cereal grains; however, it is deficient in methionine and cystine when compared to animal proteins. Therefore, cowpea seed is valued as a nutritional supplement to cereals and an extender of animal proteins[6]. The main objective of this study was to explore and discuss the possibilities for enhancing N_2 fixation among the plant host and the microbial symbiont that illustrate best practices and experiences for enhancing biological nitrogen fixation. The genetic contribution of the cowpea plants to establishment of a successful N_2-fixing symbiosis in the legume-*Rhizobium* system has been clearly demonstrated through genetic variations induced in macro- and micro-symbiont using gamma irradiation and horizontal gene transfer, respectively

MATERIALS AND METHODS

Plant materials: M_3 grains used in this study were obtained from a plants treated with different doses of gamma irradiated cowpea including 0, 10, 20 and 30 krad[7].

Table 1: Bacterial strains used in this study

Bacterial strains	Source/reference	Designation
Bradyrhizobium sp. TAL 1577	WSAERI	P_1 (TAL 1577)
Bradyrhizobium sp. UK 2101	WSAERI	P_2 (UK 2101)
Bradyrhizobium sp. ARC 4010	WSAERI	P_3 (ARC 4010)
Pseudomonas putida NRRLB-13	USDA	PSP (NRRLB-13)

WSAERI: Water, Soil and Environmental Research Institute, Agric. Res. Center, Giza, Egypt, USDA: United States, Department of Agriculture, Agricultural Research Service, USDA, through Dr. Nakamura

Bacterial strains: Four bacterial strains used in this study are listed in Table 1.

Genetic markers based on antibiotic susceptibility assays: Antibiotic susceptibility was characterized using a plate diffusion method, according to Collins and Lyne[8]. with cultures grown to logarithmic growth phase in nutrient broth for each strain. Bacterial suspension (1.0 mL) was mixed carefully with 15 mL of nutrient agar medium in petri dishes. Wells (8 mm diameter) were punched in the agar using stainless steel borer with one mL diameter and were filled with 0.1 mL of the antibiotic concentration (40 μg mL^{-1}). The plates were incubated overnight at 28°C and the diameter of resulting zones of inhibition was measured using three replicates for each bacterial strain according to Toda *et al.*[9]. Different antibiotics from the product of Hoechst Orient SAE, Cairo, Egypt were used in this study.

Conjugation procedure: Nutrient broth cultures, in the late-exponential growth phase were used. Quantitative spot mating of conjugal transfer was carried out according to Lessel *et al.*[27] by inoculating 10 μL samples of the donor cultures onto the surface of selective medium, previously seeded with 100 μL of the recipient culture. A single colony of appeared transconjugants was picked up and transferred to slant nutrient agar medium. Conjugation procedure has been done in two steps for isolating di- and tri-parental transconjugants.

Di-parental mating: This was carried out between *Bradyrhizobium* strains shown in Table 2. Three isolates were obtained and picked up from, the mating No.1, six isolates from the mating No. 2 and four isolates from the mating No. 3.

Two isolates from each mating were selected to be conjugated with the third parent (*Pseudomonas putida*). Each parent used in tri-parental mating was genetically marketing on the basis of tolerance to different heavy metals, cadmium chloride (CdCl), cadmium sulphate (Cd sul), zinc sulphate (Zn Sul) and lead acetate (Ld acet) at 50 μg mL^{-1}.

Table 2: Horizontal DNA transfer between *Bradyrhizobium* strains

Mating	Transconjugant genotype	Designation
Bra-1 (Velo^{R} Epico^{S} Entro^{R} Tetra^{R})	Velo^{R} Epico^{R} Entro^{R} Tetra^{R}	DPM-Tr$_1$†
x		DPM-Tr$_2$
Bra-2 (Velo^{S} Epico^{R} Intro^{S} Tetra^{S})		DPM-Tr$_3$†
Bra-1 (Eryth^{R} Septa^{S} Rif^{R} Thio^{S} Osp^{S})	Eryth^{R} Septa^{R} Rif^{R} Theo^{R} Osp^{R}	DPM-Tr$_1$
x		DPM-Tr$_2$†
Bra-3 (Eryth^{S} Septa^{R} Rif^{S} Thio^{R} Osp^{R})		DPM-Tr$_3$
		DPM-Tr$_4$†
		DPM-Tr$_5$
		DPM-Tr$_6$
Bra-2 (Eryth^{R} Septa^{S} Epico^{R} Myco^{S})	Eryth^{R} Septa^{R} Epico^{R} Myco^{R}	DPM-Tr$_1$
x		DPM-Tr$_2$
Bra-3 (Eryth^{S}Septa^{R} Epico^{S} Myco^{R})		DPM-Tr$_3$†
		DPM-Tr$_4$

† : Di-parental transconjugants selected to be used in tri-parental matings.,which appeared high endole productivity

Table 3: Tri-parental mating experiments conducted in this study

Mating	Source or reference of di-transconjugants	Appearance of transcon-jugants	Genotype	Designation
DPM-Tr$_3$ (Cdsul^{S} Ldact^{R})	Bra-2 x Bra-3	Zero		
x				
Pse (Cdsul^{R} Ldact^{S})				
DPM-Tr$_4$ (Cdchl^{R} Cdsul^{S} Znsul^{R})	Bra-2 x Bra-3	+	Cd chl^{R} Cd sul^{R} Zin sul^{R}	TPM-Tr$_1$
x				TPM-Tr$_2$
Pse (Cdchl^{S} Cdsul^{R} Zn sul^{S})				
DPM-Tr$_2$ (Cdchl^{R} Cdsul^{S} Ldact^{R})	Bra-1 x Bra-3	Zero		
x				
Pse (Cdchl^{S} Cdsul^{R} Ldact^{S})				
DPM-Tr$_4$ (Cdsul^{S} Znsul^{R})	Bra-1 x Bra-3	Zero		
x				
Pse (Cdsul^{R} Znsul^{S})				
DPM-Tr$_1$ (Cdchl^{R} Cdsul^{S})	Bra-1 x Bra-2	Zero		
x				
Pse (Cdchl^{S} Cdsul^{R})				
DPM-Tr$_3$ (Ldact^{R} Cdsul^{S})	Bra-1 x Bra-2	Zero		
x				
Pse (Ldact^{S} Cdsul^{R})				

Tri-parental mating: Tri-parental mating was performed between selected diparental transconguants with the third parent. Six matings were performed, just one mating from all was successed (Table 3), while the other matings were failed to conjugata two isolates from this mating were picked up at random to be applied in the field experiment.

Indole 3-acetic acid (IAA) detection with Salowski colorimetric technique: Bacterial strains and their transconjugants used in this study were grown overnight in nutrient broth medium at 28°C for both *Rhizobium* strains and their transconjugants and at 30°C for the *Pseudomonas putida*. Production of IAA in culture supernatant was assayed by using the PC method, as described by Pitel and Chollet[11]. This method was shown to be the most sensitive and most specific Salkowski-based colorimetric technique[12]. For the reaction, 1 mL of reagent R1 consisting of 12 g $FeCl_3$ L^{-1} in 79 M H_2SO_4, was added to 1 mL of the sample supernatant, mixed well and left in the dark for 30 min at room temperature. Absorbance was measured at 530 nm. IAA concentrations was calculated from the standard curve using the regression equation formula.

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

Where:
x (Concentration of IAA) = The value needed to calculate
y= Optical density obtained at 530 nm.
a (Absorbance at 530 nm when the concentration of IAA equal zero.) = -0.01, b (Regression.) = 0.029, r = 0.99

Chemical traits

Chlorophyll contents (Chl. a, b and total): For chlorophyll analysis, leaf tissue collected at random was put in 10 mL of 80% methanol over night in the dark and then the extract was reading at 650 and 665 nm using a spectrophotometer (Spekol 11, Carl Zeiss). Chlorophyll content was calculated by using formula[29], as follows:

Chl. (a) = 16.5 E 665 – 8.3 E 650 mg L^{-1}
Chl. (b) = 33.8 E 650 – 12.5 E 665 mg L^{-1}
Total Chl. = 25.5 E 650 – 4.0 E 665 mg L^{-1}

Determination of nitrogen content in plant

Sulphuric and perchloric acid digestion: Weight 0.1 g of the plant sample into a 100 mL conical flask, add 5 mL pure reagent of H_2SO_4 (97-99%). 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 dist. H_2O 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 D.D.W. 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. The samples were reading at 425 nm for 1 cm light path. Calibration curve was prepared using different concentrations of NH_3CL ranged between 0.2 up to 2.2 ppm. at the same temperature and the same reaction time used before for samples according to APHA[13]. The following formula was used to calculate nitrogen concentration (mg g^{-1});

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

Where:
y = Optical density obtained at 530 nm
x (Concentration of nitrogen) = unknown value
b (Regression) = 0.14
a = Absorbance at 425 nm when the concentration of N equal zero. = 0.01
r = 0.99

RESULTS AND DISCUSSION

Indole compounds production: It is well established that many soil and plant-associated bacteria are able to synthesize the phytohormone indole-3-acetic acid (IAA). Nitrogen fixing bacteria produce and excrete IAA in their cultures (Table 4). The results indicated that the

Table 4: Production of indole compounds by different rhizobial strains and their recombinant isolates harboring DNA from two and or three sources

	Production of IAA (μg mL^{-1})			
Strains	Tryptophan	Trypton	Ethanol	Lactic acid
P_1	22.16	2.00	2.23	1.27
P_2	34.43	2.33	2.45	1.06
Mid-parent	28.29	2.16	2.34	1.16
DPM-Tr_1 (Tr_1)	23.45	1.87	1.80	0.95
DPM-Tr_2	17.54	1.88	1.49	1.71
DPM-Tr_3	18.33	0.98	1.98	1.02
P_1	22.16	2.00	2.23	1.27
P_3	20.84	1.48	2.71	0.80
Mid-parent	21.50	1.73	2.47	1.03
DPM-Tr_4	0.82	1.00	1.81	1.29
DPM-Tr_5 (Tr_2)	29.00	1.08	1.57	1.70
DPM-Tr_6	0.71	0.90	2.01	1.07
DPM-Tr_7	16.49	0.87	1.66	1.02
DPM-Tr_8	25.02	1.22	1.71	0.92
DPM-Tr_9	5.29	1.27	2.52	0.99
P_2	34.43	2.33	2.45	1.06
P_3	20.84	1.48	2.71	0.80
Mid-parent	27.63	1.90	2.58	0.93
DPM-Tr_{10}	16.49	1.79	2.07	2.21
DPM-Tr_{11}	20.28	1.61	1.57	2.30
DPM-Tr_{12} (Tr_3)	25.19	2.07	1.58	1.47
DPM-Tr_{13}	23.58	1.66	1.34	1.67
P_4	20.15	2.16	1.62	1.15
DPM-Tr_2	17.54	1.88	1.49	1.71
Mid-parent	18.84	2.02	1.55	1.43
TPM-Tr_1 (Tr_4)	24.87	2.30	1.57	1.37
TPM-Tr_2 (Tr_5)	22.23	2.80	1.48	1.72
F test	**	**	NS	NS
LSD 5%	3.87	0.56	---	---
1%	5.19	0.75	---	---

Selected isolates used for plants inoculation in the field experiment, were DPM-Tr_1, DPM-Tr_5,DPM-Tr_{12}, TPM-Tr_1 and TPM-Tr_2,It was namely Tr_1 Tr_2 Tr_3, Tr_4, Tr_5, respectively, in the field experiment, NS = Non-significant, ** =$p<0.01$

colorimetric assay used revealed high amount of indole compounds from tryptophan above the mid-parents in two out of six resulted from the cross between P_1 x P_3.

Diparental transconjugant DPM-Tr_5 accumulated the phytohormone above the mid-parents in the presence of tryptophan, was selected to be applied in the field experiment. From the cross between P_1 x P_2, only one transconjugant (Tr_2) accumulated the phytohormone above the mid-parents in the presence of lactic acid. All transconjugants resulted from the cross between P_2 x P_3 accumulated the phytohormone above their mid-parents in amounts ranging from 1.47 to 2.30 μg mL^{-1} in the presence of lactic acid and trypton supplemented the culture medium, the production of IAA was higher procedure above the mid-parents in one (DPM-Tr_{12}) out of four tested culture supernatants. The diparental transconjugant (DPM-Tr_{12}) produced higher amounts of indole compounds above the mid-parents in the presence of trypton and lactic acid was selected to be used for plants inoculation in the field experiment. One triparental transconjugant (TPM-Tr_2) resulted from the cross between P_4 x DPM-Tr_2 representative IAA production over their mid-parents in the presence of lactic acid. The two triparental transconjugants produced higher amounts of IAA from tryptophan and trypton over their mid-parents were selected to be used for inoculation in field experiment.

The obtained data demonstrated the presence of IAA in the supernatant of a new recombinant cultures, revealing the capability of some recombinant isolates to produce IAA over their mid-parents by some of them. The widely used Salkowsky reagent is not specific to IAA, but can also react with other indole derivative such as indole-3-acetamide and indole-3-pyruvic acid, which be long to the auxins. The occurrence of IAA production in tryptophan-supplemented cultures of rhizobial strains and many other non-pathogenic plant associated bacteria is common[14]. Insignificant IAA production above the mid-parents indicated decreased transcriptional activity of *ipdC* gene during the growth of transconjugants. This indicated the importance of increased transcriptional activity of *ipdC* gene and also the importance of the *ipdC* gene amplification in the production of IAA. The finding of this study is in agreement with Brandl and Lindow[14]. who reported that bacterial IAA synthesis can affect the normal physiology of plant cells. Recent biochemical evidence suggested that some strains of *B. japonicum* can synthesize IAA by more than one pathway[15].

The movement of the *ipdC* gene to other strains, particularly those of other genera, may be fairly restricted if the gene is generally located on the chromosome in *E. herbicola*. This may explain the lack of homology of *ipdC* in many bacterial species[16]. Patten and Glick [17] found that the phytohormone indole-3-acetic acid (IAA) accumulates in the culture medium of the plant growth-promoting bacterium *Pseudomonas putida* GR 12-2 only when grown in the presence of exogenous tryptophan, suggesting that expression of indole-pyruvate decarboxylase, a key enzyme in the IAA biosynthesis pathway in this bacterium, may be regulated by tryptophan. The beneficial bacteria usually synthesized IAA via the indole-pyruvic acid pathway, which is involved in plant growth promotion and enhances root development. The key enzyme in the indole-pyruvic acid pathway, indole-pyruvate decarboxylase, encodes by the *ipdC* gene (Fig. 1).

While, IAA produced by phytopathogenic bacteria, was mainly produced by the indoleacetamide pathway, which has been implicated in the induction of plant tumors. The results obtained in this study revealed the importance of improving IAA production by beneficial bacteria such as *Rhizobium* because IAA plays a major role in the development of the host plant root system. Patten and Glick[17] demonstrated directly that bacterial IAA plays a major role in promotion of root elongation when a bacterium is associated with its host plant. IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. Low levels of IAA stimulate root elongation, high levels of bacterial IAA stimulate the formation of lateral and adventitious roots[18]. High levels of exogenous or bacterial IAA and therefore high levels of ethylene have also been shown to inhibit elongation growth in roots[19].

The phytohormone produced from tryptophan by all isolates tested in this study was higher than that produced from other precursors. Although, the amounts of indole compounds produced from trypton, ethanol and lactic acid pathways were approximately similar, but it was less than that produced from tryptophan pathways.

Nodulation parameters: The results presented in Table 5 and Fig. 2 and 3 as a yield percentage revealed that DPM-Tr_1 appeared significant increase in the number of nodules developed on the root system of unirradiated cowpea (V_1) in M_3 and M_4 generations above the plants fertilized with recommended full dose and the mid-parents. Extending the ability of parental strains, di- and tri-parental transconjugants exhibit significant number of nodules on the root system of V_1-variety-non irradiated plants above the plants fertilized with the recommended dose. M_4 irradiated generation with 20 krad appeared significant number of nodules developed on the root system of V_1 variety above the plants fertilised with

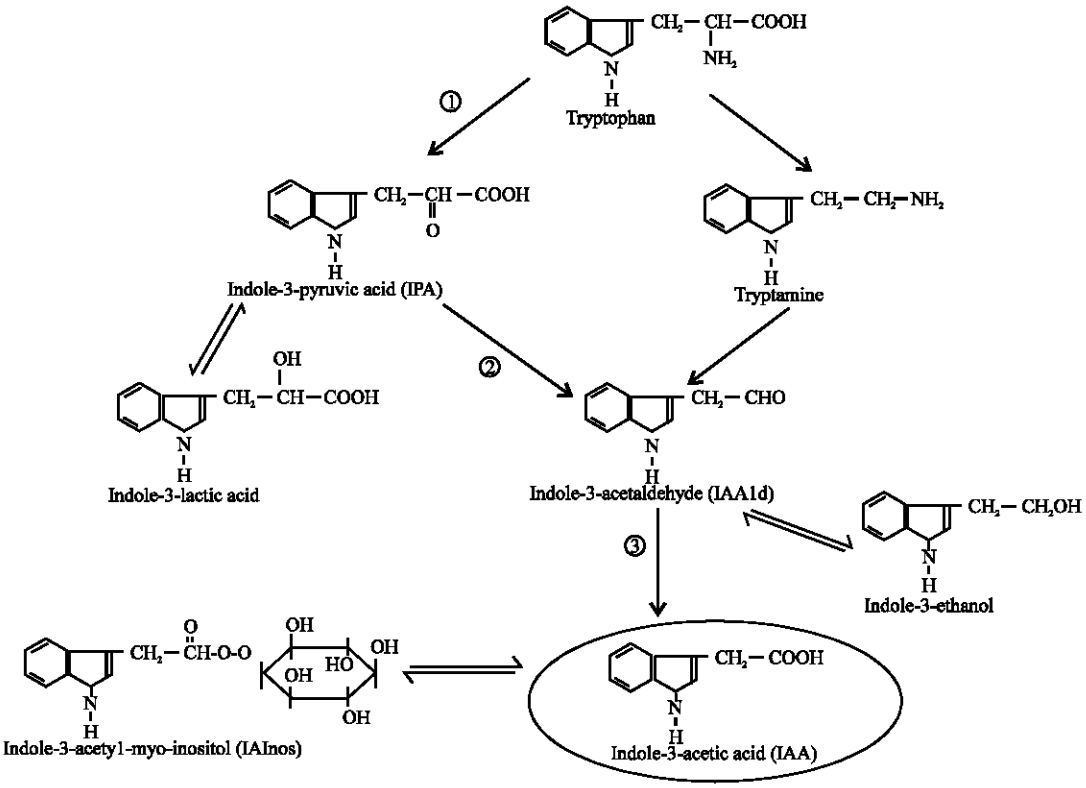

Fig. 1: The biosynthetic pathway for auxin (indole-3-acetic acid)

Table 5: Mean performance of nodule numbers developed on the root system of plants inoculated with bacterial strains and their transconjugants

	0		10		20		30	
Treatments	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4
V_1								
Uninoculated	1.00	9.67	3.67	11.00	0.00	10.00*	14.67	5.33*
Full dose	6.33	2.33	5.67	1.00	2.67	2.00	1.00	0.67
P_1	5.00	10.67	6.00	13.33	9.67	10.33*	8.67	6.00**
P_2	2.33	10.67	7.33	12.00	8.67	9.00*	0.00	3.00
P_3	6.33	13.33	17.67	13.00	32.67**	16.00**	3.67	8.67**
DPM-Tr_1	23.67**	24.00	0.00	10.67	7.67	10.00	0.33	6.67*
DPM-Tr_2	7.00	11.67	11.67	14.33	1.00	7.67	1.67	4.33
DPM-Tr_3	3.67	13.00	5.67	10.67	2.67	8.00*	5.00	7.67*
TPM-Tr_4	1.33	14.33	6.67	9.67	4.67	8.67*	1.00	5.00*
TPM-Tr_5	4.67	15.00	2.00	10.67	1.33	8.67*	2.00	6.67
F-test	**	**	NS	NS	**	*	NS	*
LSD 5%	7.27	6.62	---	---	13.84	5.95	---	4.19
1%	9.97	9.08	---	---	18.98	8.16	---	5.74
V_2								
Uninoculated	8.00	10.00	30.33	25.00**	21.00**	10.33*	4.67	9.33
Full dose	0.00	5.00	0.00	3.33	0.00	2.67	4.33	3.67
P_1	25.67**	27.33**	11.33	15.33	4.00	6.67	6.67	6.00
P_2	27.00**	29.33**	10.33	12.00	2.67	13.33**	21.67**	10.00
P_3	17.33*	20.00**	24.67	26.00**	7.00	11.00**	19.33**	15.33
DPM-Tr_1	9.00	16.67*	9.67	14.67	4.67	12.67*	0.33	7.33
DPM-Tr_2	1.33	13.33	6.67	10.00	1.00	6.33	0.00	6.33
DPM-Tr_3	17.33*	22.33**	2.67	7.67	0.00	4.33	1.00	5.67
TPM-Tr_4	12.67	17.00	15.00	15.33*	12.67**	17.67**	12.00	15.00
TPM-Tr_5	8.33	21.33**	4.33	11	12.33**	11.33*	4.00	9.00
F-test	**	**	NS	**	**	*	**	NS
LSD 5%	13.33	9.53	---	9.51	7.11	7.35	10.42	---
1%	18.28	13.06	---	13.05	9.75	10.08	14.29	---

NS = Non-significant, * =$p<0.05$, ** =$p<0.01$

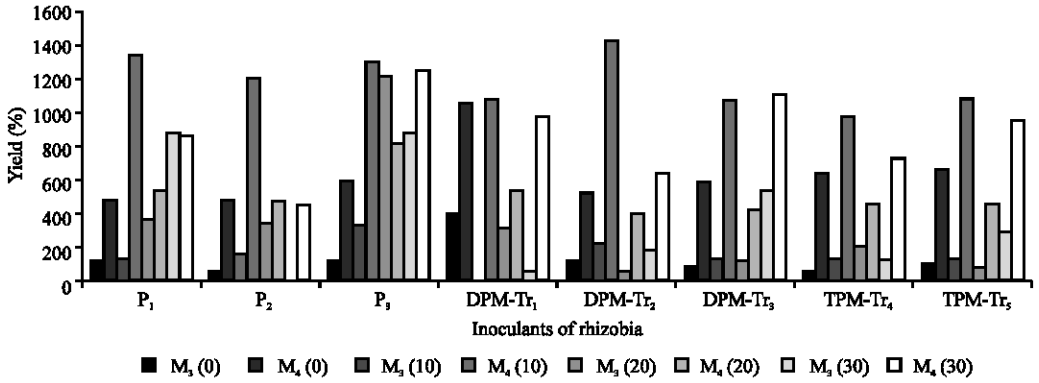

Fig. 2: Yield percentage in the number of nodules in relation to that developed on the root system of plants fertilized with recommended dose of N in V_1 irradiated with gamma rays and inoculated with different rhizobial recombinants

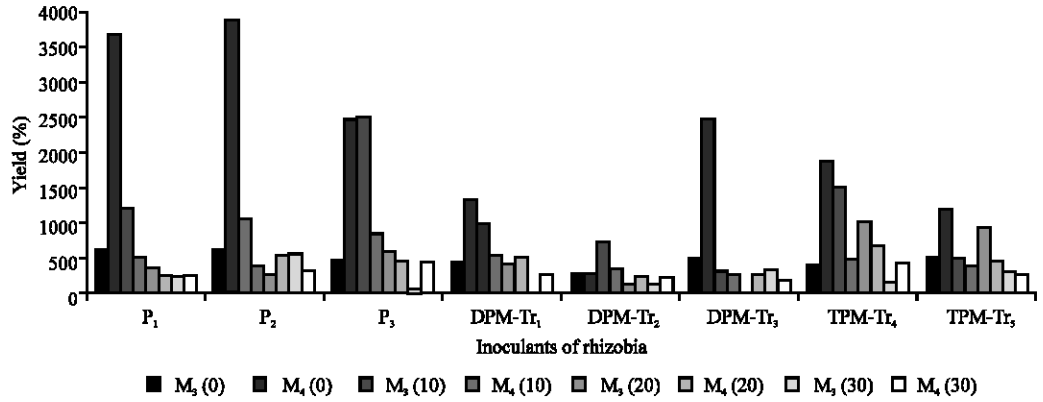

Fig. 3: Yield percentage in the number of nodules in relation to that developed on the root system of plants fertilised with recommended dose of N in V_2 irradiated with gamma rays and inoculated with different rhizobial recombinants

recommended dose of nitrogen in response to inoculation with parental strains, di- and tri-parental transconjugants (except for DPM-Tr_2). However, the following rhizobial strains; P_1, P_3, DPM-Tr_1, DPM-Tr_3, TPM-Tr_4 and TPM-Tr_5 developed significant number of nodules on the root system of V_1-variety treated with 30 krad.

Effective nodulation has also been observed in unirradiated plants of V_2-variety following inoculation with; P_1 (M_3, M_4), P_2 (M_3, M_3), P_3 (M_3, M_4), DPM-Tr_1 (M_4), DPM-Tr_3,(M_3,M_4) TPM-Tr_4(M_4) and TPM-Tr_5(M_4). significant number of nodules above that developed on the roots of the plants fertilized with recommended dose of N has also been observed in M_4 generation of V_2-variety irradiated with 10 krad in response to inoculation with parental strains (P_1 and P_3), di- and tri-parental transconjugants (DPM-Tr_1 and TPM-Tr_4).

In addition, plants resulted from 20 krad of V_2-variety developed significant number of nodules on the root system above that developed on the plants fertilized with the recommended dose of N in response to inoculation with parental strains (P_2 and P_3) in M_4 generation and also in response to inoculation with tri-parental transconjugants (TPM-Tr_4 and TPM-Tr_5) in M_3 and M_4 generations. Although, the plants resulted from 30 krad of V_2-variety revealed significant number of nodules above that fertilized with the recommended dose of N in response to inoculation with the parental strains P_2 and P_3.

The variations obtained herein in response to develop an effective symbiosis are in agreement with Mandal *et al.*[20] who reported that significant variation in cowpea rhizobium strains has been observed for nodulation in cowpea, but the local rhizobia invariably outpopulate the introduced strains. Therefore, in recent years, major efforts have concentrated on exploiting genetic variability in cowpea as a host for effective nodulation and nitrogen fixation[21]. Mandal *et al.*[20] also observed significant varietal differences in cowpea for

Table 6: Mean performance of nodule dry weight (g) developed on the root system of plants inoculated with bacterial strains and their transconjugants

	0		10		20		30	
Treatments	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4
V_1								
Uninoculated	0.006	0.016	0.036	0.012	0.000	0.021	0.026	0.005
Full dose	0.019	0.004	0.026	0.002	0.013	0.004	0.002	0.001
P_1	0.030	0.016	0.021	0.023	0.034	0.014	0.030	0.007
P_2	0.003	0.017	0.013	0.017	0.033	0.017	0.000	0.004
P_3	0.038	0.048	0.036	0.061	0.151	0.115*	0.009	0.01
DPM-Tr_1	0.072	0.051	0.000	0.014	0.024	0.009	0.001	0.007
DPM-Tr_2	0.023	0.013	0.008	0.044	0.004	0.014	0.023	0.008
DPM-Tr_3	0.012	0.017	0.022	0.050	0.002	0.010	0.023	0.01
TPM-Tr_4	0.003	0.063	0.011	0.037	0.011	0.009	0.003	0.008
TPM-Tr_5	0.008	0.122	0.002	0.010	0.013	0.009	0.018	0.007
F-test	NS	*	NS	NS	NS	**	NS	NS
LSD 5%	---	0.065	---	---	---	0.052	---	---
1%	---	0.089	---	---	---	0.071	---	---
V_2								
Uninoculated	0.012	0.012	0.090	0.019	0.041*	0.016	0.006	0.011
Full dose	0.000	0.012	0.000	0.011	0.000	0.007	0.08	0.007
P_1	0.048	0.079*	0.011	0.085**	0.008	0.062	0.009	0.008
P_2	0.027	0.091*	0.034	0.103**	0.003	0.049	0.175	0.053
P_3	0.083	0.100**	0.023	0.112	0.031	0.071	0.028	0.068
DPM-Tr_1	0.05	0.073*	0.042	0.088**	0.007	0.031	0.000	0.050
DPM-Tr_2	0.002	0.060	0.021	0.010	0.001	0.034	0.000	0.030
DPM-Tr_3	0.114	0.058	0.005	0.027	0.000	0.007	0.001	0.007
TPM-Tr_4	0.030	0.079*	0.071	0.039	0.060**	0.041	0.047	0.024
TPM-Tr_5	0.034	0.108**	0.01	0.013	0.042*	0.013	0.01	0.01
F-test	NS	*	NS	**	*	NS	NS	NS
LSD 5%	---	0.060	---	0.052	0.036	---	---	---
1%	---	0.082	---	0.072	0.050	---	---	---

NS=Non-significant, * =$p<0.05$, ** =$p<0.01$

nodule number and nodule weight, as well as, for nitrogenase activity indicating a good possibility of breeding improved cowpea varieties with enhanced N-fixation. For this gamma irradiation was used in this study for inducing varietal differences in cowpea, which may enhanced the development of symbioses and nitrogen fixation through developing significant number of nodules on the root system of the plants in response to inoculation with recombinant rhizobial isolates derived from mating.

The results presented in Table 6 achieved that the parental strains and their transconjugant; P_1, P_2, P_3 and DPM-Tr_1 appeared significant increase in the weight of nodules developed on the root system/plant above that developed on the plants fertilized with N recommended dose in M_4 generation of V_2-variety from unirradiated plants and that irradiated with 10 krad. Significant weight of nodules per plant were developed on the root system of V_1-variety treated with 20 krad of gamma irradiation in M_4 generation responded to inoculation with the parental strain (P_3). This indicated the success of rhizobial strains and their recombinants to colonize and infect roots of cowpea.

In addition, inoculation with two tri-parental transconjugants (TPM-Tr_4 and TPM-Tr_5) produced significant weight of dry nodules and dry weight of unirradiated V_2-variety (M_4) and that irradiated with 20 krad (M_3) above that developed on the root system of the plants fertilized with N full dose. Although, all transconjugants did not appeared significant nodules dry weight above their mid-parents.

These results are in agreement with Deshwal *et al.*[22] who reported that rhizobia are known to increase nodulation and nodule weight in legumes along with increase in host plant growth and development, besides protecting roots from the attack of pathogens due to production of diverse microbial metabolites like siderophore, rhizobitoxin, plant growth enhancement through IAA production, uptake of phosphorus and other minerals. The observation of the same authors clearly suggested that the siderophore and IAA-producing and phosphate-solubilizing *Bradyrhizobium* strains (AHR-2, AHR-5 and AHR-6) are good root colonizers and possess a strong antagonistic activity against *M. phaseolina*. *Bradyrhizobium* strains have been found effective antagonists *in vitro* and *in vivo*, besides enhancing seed germination, seedling biomass, nodulation nodule weight and weight per nodule.

The beneficial effect of *Rhizobium* and *Bradyrhizobium* in legumes in terms of biological nitrogen fixation has been a main focus in the recent past. Obviously, rhizobia are known to increase nodulation and

Table 7: Effect of bacterization with parental strains and their new recombinant isolates on root dry weight (g/plant)developed in response to inoculation

	0		10		20		30	
Treatments	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4
V_1								
Uninoculated	2.40	1.88	1.94	1.31	2.00	2.18	1.72	2.84
Full dose	2.17	2.39	2.13	4.19	1.91	3.06	1.96	1.38
P_1	2.15	3.62	2.04	5.01	1.77	3.00	1.91	2.71
P_2	2.21	2.37	2.43	1.89	2.56	1.84	2.31	1.47
P_3	2.12	2.50	1.96	2.01	2.27	2.23	2.53	2.85
DPM-Tr_1	2.54	1.93	2.34	2.13	1.91	2.23	1.96	2.27
DPM-Tr_2	2.31	2.42	2.40	1.94	1.59	2.39	1.62	2.29
DPM-Tr_3	2.20	1.81	2.65	1.65	2.13	1.04	1.96	1.62
TPM-Tr_4	2.04	1.52	2.52	1.12	1.92	1.53	1.99	1.99
TPM-Tr_5	2.35	1.78	2.47	1.85	1.97	1.23	2.03	1.51
F-test	NS	NS	NS	**	NS	**	NS	NS
LSD 5%	---	---	---	1.32	---	0.94	---	---
1%	---	---	---	1.81	---	1.29	---	---
V_2								
Uninoculated	2.06	2.29	2.07	1.79	2.03	3.16**	2.10	2.17
Full dose	2.36	2.10	1.67	1.45	2.50	1.85	2.08	1.48
P_1	3.41	1.49	2.00	2.07	1.97	2.29	2.09	1.25
P_2	2.44	1.59	2.22	2.08	2.34	2.01	2.50	4.36**
P_3	2.29	1.16	1.38	1.97	2.10	1.19	1.96	1.77
DPM-Tr_1	2.80	1.22	1.14	1.24	2.09	1.28	2.03	1.71
DPM-Tr_2	2.01	1.23	1.77	1.54	2.14	1.80	2.04	1.88
DPM-Tr_3	2.04	2.09	2.07	1.48	1.97	1.36	1.59	1.33
TPM-Tr_4	2.03	1.16	2.18	1.25	2.05	1.20	1.75	1.07
TPM-Tr_5	2.05	1.32	1.93	1.9	2.00	1.33	1.86	1.41
F-test	NS	NS	NS	NS	NS	**	NS	**
LSD 5%	---	---	---	---	---	0.63	---	0.97
1%	---	---	---	---	---	0.87	---	1.34

NS = Non-significant, ** =$p<0.01$

nodule weight in legumes along with increase in host plant growth and development[22], besides protecting roots from the attack of pathogens due to production of diverse microbial metabolites like siderophore, rhizobiotoxin, plant growth enhancement through IAA production, uptake of phosphorus and other minerals.

A few strains of rhizobia are reported to inhibit sclerotia germination of *Sclerotium rolfsii* and colony growth of *Phytophthora megasperma*, while *Rhizobium meliloti* and *Bradyrhizobium japonicum* bacterized seeds are known to have reduced *Macrophomina phaseolina* infection[22]. for this rhizobial inoculation used in this study have a beneficial effect on the production of cowpea.

Rubaihayo[23] obtained mutants for resistance to dehiscence, reduced height and prematurity in irradiated soybeans. These mutants showed high productivity in the M_2, M_3 and M_4 generators, one of them produced 40% more than the control. An increase in genetic variance of quantitative characters has been induced by irradiating cowpea[24] and wheat[25].

The results obtained in this study are in accordance with Kimani[26], who found significant inoculation effects during the short rains season of *Phaseolus vulgaris* L., however, inoculated plants had higher seed weights compared to the control. The results presented in this study showed that biochemical traits in cowpea can be improved through mutation breeding. The trials were carried out during two seasons to determine the performance of gamma irradiation in different segregated generations. It is plausible that irradiation of such loci would create new variability upon which selection was effective.

Root dry weight: The results presented in Table 7 appeared the same trend in root growth promotion, while only the parental strain (P_2) revealed significant increase in root dry weight of V_2-variety in M_4 generation treated with 30 krad. This indicated that root growth of M_4 generation treated with 30 krad appeared better response to inoculation with the parental strain (P_2) than that of plants fertilized with recommended dose of N.

Gunasekaran *et al.*[27] treated seeds of the cowpea variety CO4 with gamma rays and ethidium bromide and analyzed M_1 and M_2progenies for different agronomic traits. They observed a great deal of variation in M_2 population for different traits and further noticed that gamma rays were more effective in inducing mutation than ethidium bromide. However, non of the other rhizobial strains and their recombinants revealed significant promotion in root growth like the parental strain P_2 inoculated V_2 plants treated with 30 krad. This was in

Table 8: Mean performance of total chlorophyll formation in plants inoculated with the parental strains and their new recombinant isolates

	0		10		20		30	
Treatments	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4
V_1								
Uninoculated	4.430**	4.394**	3.767	3.741	5.029**	5.059**	3.472	4.900**
Full dose	2.673	2.666	3.798	3.779	3.312	3.304	2.813	2.825
P_1	3.439*	3.454	3.847	3.800	3.674	3.671	3.281	3.271**
P_2	3.139*	3.089	3.056	3.041	3.378	3.385	4.588*	4.574
P_3	4.043**	3.970**	3.911	3.869	2.836	2.835	3.861	3.833**
DPM-Tr_1	4.591**	4.526**	4.110	4.056	4.257	4.220	4.739*	4.727**
DPM-Tr_2	4.336**	4.391**	5.823**	5.385**	5.092**	5.176**	4.693*	4.701**
DPM-Tr_3	5.152**	5.106**	5.088**	4.988**	5.243**	5.184	4.813**	4.824**
TPM-Tr_4	5.056**	5.039**	5.054*	5.017**	4.885**	4.883**	5.072**	5.091**
TPM-Tr_5	5.016**	4.979**	4.031	4.046	5.131**	5.145**	5.396**	5.333**
F-test	**	**	**	**	**	**	*	**
LSD 5%	0.080	0.830	1.125	1.015	0.967	0.970	1.450	1.070
1%	1.100	1.130	1.543	1.392	1.326	1.330	1.989	1.467
V_2								
Uninoculated	5.520*	4.867	5.292	4.947	4.852	4.936	5.129	5.328
Full dose	4.753	4.360	4.306	4.323	4.435	4.546	4.743	5.127
P_1	4.387	4.689	3.913	4.166	4.958	4.948	4.585	4.703
P_2	3.985	4.296	5.133	4.993	3.593	3.874	4.269	4.84
P_3	4.142	4.408	4.520	4.465	3.585	3.731	5.542	5.383
DPM-Tr_1	4.426	4.808	4.213	3.846	5.093	4.883	4.499	5.19
DPM-Tr_2	5.058	5.086**	4.512	4.607	4.429	4.324	5.183	5.106
DPM-Tr_3	3.879	4.498	4.193	4.469	4.645	4.701	4.909	4.996
TPM-Tr_4	5.263	5.480**	5.037	4.859	4.742	4.157	5.323	5.255
TPM-Tr_5	5.225	5.318	4.764	4.856	5.382	4.997	5.352	5.366
F-test	**	**	NS	NS	**	NS	NS	NS
LSD 5%	0.770	0.450	---	---	0.848	---	---	---
1%	1.060	0.610	---	---	1.163	---	---	---

NS=Non-significant, * =p<0.05, ** =p<0.01

accordance with Graham and Scott[28] who observed a major genetic differences for nodulation and dry matter and N accumulation among 12 cowpea varieties. Therefore, Manda *et al.*[28] also observed significant varietal differences in cowpea for nodule number and nodule weight as well as for nitrogenase activity, indicating a good possibility of breeding improved cowpea varieties with enhanced N fixation.

Sanginga *et al.*[29] screened 94 cowpea lines and observed major varietal differences in cowpea for growth, nodulation and arbuscular mycorrhizal fungi root infection as well as for performance under low and high phosphorus. The improved cowpea variety IT86D-715, by the same authors, showed equally good growth under low as well as high phosphorus levels. It also showed better N-fixation than others. Based on its adaptability to grow in low P soils and overall positive N balance, they recommended cultivation of IT86D-715 cowpea variety in soils with low fertility

Total chlorophyll formation in plants: Table 8 results showed that inoculation with di-parental transconjugants DPM-Tr_2 and DPM-Tr ¡s almost always produced significant increase above the full dose and the mid-parents in total chlorophyll formation in V_1-variety at all doses of gamma irradiation with the except of at 30 krad in comparison with the mid-parent. However, DPM-Tr_1 revealed significant increase in chlorophyll formation of unirradiated plants above that at the full dose and the mid-parents.

The same trend above the mid-parent was also shown in M_4 generation of v1 plants treated with 30 krad and unirradiated V_2 plants in response to DPM-Tr_2 and also M_3 generation of V_2-variety treated with 20 krad. Great chlorophyll formation above that at the full dose was also obtained at all doses of gamma irradiation in response to inoculation with TPM-Tr_4 and TPM-Tr_5, with the except of plants treated with 10 krad and inoculated with TPM-Tr_5. This indicated that rhizobial inoculation of different new recombinants have a great influence on the formation of chlorophyll, which related to N_2 fixed.

However, inoculation will all new recombinants exhibited significant increases in photosynthetic pigments at all doses of gamma irradiation. This indicated the positive effect of rhizobial genetic recombinants in increasing physiological traits of cowpea. The importance of tri-parental transconjugants was due to its containing a genetic material from *Pseudomonas putida*, which played a dual role by reducing disease incidence and promoting plant growth, resulting in increased biomass and yield. It has been established that *Pserudomonas* enhance plant growth in several ways, viz producing plant

Table 9: Mean performance of fresh pods weight developed per plant in response to bacterization with the parental strains and their new recombinant isolates

	0		10		20		30	
Treatments	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4
V_1								
Uninoculated	203	199	178	210	175	175	160	141
Full dose	184	205	155	180	170	170	114	163
P_1	168	199	167	189	162	162	129	134
P_2	164	198	146	176	175	175	129	143
P_3	155	170	184	186	146	146	127	128
DPM-Tr_1	156	197	160	186	134	134	120	135
DPM-Tr_2	214	198	217	168	149	149	165	154
DPM-Tr_3	179	213	193	158	156	156	122	139
TPM-Tr_4	168	197	194	182	186	186	134	130
TPM-Tr_5	183	197	194	168	157	157	158	166
F-test	*	NS	*	NS	NS	NS	NS	NS
LSD 5%	35.14	---	35.22	---	---	---	---	---
1%	48.19	---	48.31	---	---	---	---	---
V_2								
Uninoculated	248	240	231	205	241	155	208	141
Full dose	238	236	163	209	167	189	185	151
P_1	260	242	226	213	213	170	184	156
P_2	276	239	235	248	231	226	180	177
P_3	197	222	166	201	172	185	166	171
DPM-Tr_1	238	237	239	254	202	210	126	163
DPM-Tr_2	211	257	171	261	191	211	161	162
DPM-Tr_3	244	263	227	259	197	183	198	162
TPM-Tr_4	264	253	200	237	186	229	165	157
TPM-Tr_5	250	238	236	179	176	200	174	174
F-test	NS	NS	NS	*	*	NS	NS	NS
LSD 5%	---	---	---	51.85	44.90	---	---	---
1%	---	---	---	71.12	61.58	---	---	---

NS, * and ** : Non-significant, p< 0.0.05 and p< 0.01, respectively

growth regulators, such as gibberellins, cytokinins and indole acetic acid, which can directly or indirectly modulate the plant growth and development[30].

Fresh pods weight/plant: Di-parental transconjugant, DPM-Tr_2 appeared significant increase in fresh weight of pods developed per plant above the mid-parents in M_3 generation of V_1-variety at zero and 10 krad of gamma irradiation. In addition, the same trend was also achieved above the full dose in M_3 generation resulted from 10 krad in response to inoculation with DPM-Tr_2, DPM-Tr_3, TPM-Tr_4 and TPM-Tr_5 (Table 9).

Only one recombinant (DPM-Tr_2) exhibiting significant increase in fresh pods above the mid-parents and the full dose in M_4 generation treated with 10 krad. However, other recombinant (DPM-Tr_3) corresponding the same results in M_4 generation of V_2-variety treated with 10 krad. Although, the two parental strains P_1 and P_2 achieved the same trend above the full dose in M_3 generation of V_2-variety treated with 20 krad. However, no significant number of pods were developed on the plants inoculated with the different rhizobial strains, but some of inoculants produced significant increase in fresh pods weight/plant.

It is important to note that because rhizobia produce phytohormones such as auxins, cytokinins, gibberllins and abscisic acid, it is likely that their release into cropping systems promotes plant growth including pod growth and possibly increases yield even through no N_2 fixation by rhizobia has been detected[31]. This is in addition to the role of microbial metabolites in making nutrients available to plants[32]. Mujeeb and Greig[33] demonstrated significant differences in the yield components at the higher dosage of gamma irradiation, which have been attributed to poor and delayed germination, inhibited plant growth and presumably to sterility from genetic imbalance (Common at higher dosage).

At some treatments of biofertilization growth characteristics (fresh pods weight) was reduced with increased radiation exposures, this have been reported by Ramulu[34]. More promise of being directly evaluated as a higher quality bacterization bean than the uninoculated control, because it yielded significant weight of fresh pods and seeds. It has been established that yield is the result of co-ordinated interplay of developmental traits. Vigorously growing of *Rhizobium*-inoculated plants were able to absorb a large quantity of mineral nutrients through their well developed root system. For this nitrogen fixed by rhizobia increased the synthesis of photosynthates as shown in this study and the storage organ-seeds in this case were well developed. The highest

Table 10: Mean performance of nitrogen content (mg/plant) in plant shoots inoculated with the parental strains and their new recombinant isolates

	Shoot nitrogen content (mg/plant)															
	V_1								V_2							
	0		10		20		30		0		10		20		30	
Treatments	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4	M_3	M_4
Uninoculated	20.27	18.82	20.54	20.30	25.45	24.12	17.42	18.25	52.51	55.8	56.29	55.37	34.38	35.47	44.20	43.3
Full dose	11.79	11.93	10.79	12.21	11.84	12.01	6.53	6.39	40.64	39.36	58.59	57.99	35.85	36.28	48.29	48.03
P_1	38.39	40.42	6.68	6.32	19.97	17.57	18.62	16.16	71.79	70.56	63.32	64.57	33.27	32.60	41.77	42.77
P_2	24.42	22.74	42.13	38.96	31.21	28.23	21.91	20.13	37.84	40.19	40.58	45.09	46.82	42.07	61.70	63.93
P_3	19.27	20.48	32.16	32.43	12.20	12.70	19.79	21.43	47.82	48.98	31.32	33.82	52.99	53.12	38.32	36.49
DPM-Tr_1	17.28	17.28	41.60	38.55	26.83	27.48	44.88	44.13	58.88	54.48	17.27	18.56	19.42	17.62	30.62	29.94
DPM-Tr_2	71.18	71.43	55.29	49.74	36.50	38.23	39.56	39.96	40.91	40.09	55.19	55.81	48.01	47.72	52.06	53.60
DPM-Tr_3	22.52	22.15	39.55	38.40	16.86	16.24	34.15	34.73	29.56	27.85	38.99	41.55	15.67	15.65	38.37	43.30
TPM-Tr_4	69.47	68.15	81.37	77.33	55.19	54.91	57.53	56.28	20.12	20.49	26.3	24.35	37.73	37.21	34.44	33.62
TPM-Tr_5	68.43	66.54	28.2	26.40	32.93	33.20	29.38	28.37	66.44	65.22	39.45	42.84	38.76	36.19	26.14	23.95
F-test	*	*	**	**	**	**	**	**	**	**	*	**	*	**	NS	**
LSD 5%	13.14	5.32	19.22	7.66	15.85	5.50	13.29	5.22	21.45	8.48	19.35	6.40	19.28	7.11	---	8.29
1%	18.03	7.30	26.35	10.50	21.73	7.55	18.23	7.16	29.41	11.63	26.54	8.78	26.44	9.74	---	11.36

NS=Not significant differences , * =p<0.05, ** =p<0.01

weight of fresh pods (260 g/plant) was recorded in M_4 generation of V_2-variety resulted from the treatment with 10 krad.

Nitrogen content in plant shoots: Table 10 revealed that the parental strain P_2 and some of transconjugants (DPM-Tr_2, TPM-Tr_4 and TPM-Tr_5) significantly improved nitrogen content of V_1-variety at all doses of gamma irradiation above the plants fertilized with recommended dose of nitrogen fertilizer. However, di-parental transconjugants (DPM-Tr_2) significantly improved shoot nitrogen content at all doses of gamma irradiation above the mid-parents, this strain appeared the highest nitrogen content in relation to the parental strains and other di-parental transconjugants. Whereas, DPM-Tr_1 and DPM-Tr_3 had significantly improved nitrogen content of V_1-variety at 30 krad of gamma irradiated plants above their mid-parents.

In addition, V_2-variety had the lowest nitrogen content in relation to the plants fertilized with recommended dose of nitrogen and to the mid-parents of rhizobial transconjugants. This suggests that effective nodulation and nitrogen fixation depends on the *Rhizobium* strain and the crop genotype among other factors. This are in agreement with Vencatasamy[35], who reported that nitrogen fixation in beans was influenced by both the *Rhizobium* and host genotypes.

Di-parental transconjugant (DPM-Tr_2) showed consistently significant concentration of shoot nitrogen in M_4 generation of V_2-variety treated with 10 and 30 krad compared to the mid-parents. These results indicated that inoculation with rhizobial transconjugants affect to significantly increased shoot nitrogen content in V_1 than in V_2 variety, above the plants fertilized with recommended dose of nitrogen and the mid-parents. From these results, it is interesting to note that V_1-variety appeared higher response to inoculation than V_2, supporting the earlier observation of higher nitrogen content in the shoots. Johnson *et al.*[36] pointed out that continued improvement in productivity at the expense of protein content and quality has dubious net value from a nutritional standpoint.

Protein percentage in seeds of plants: As shown from the results presented in Fig. 4 and 5, three di-parental transconjugants exhibited significant increase in grain protein content in M_4 generation of V_1-variety treated with 20 and 30 krad (except for DPM-Tr_2 at 30 krad), above the plants fertilized with recommended dose of N, although DPM-Tr_1 appeared the same trend above the mid-parents in M_3 generation treated with 30 krad. In V_1-variety, the protein content ranged from 20.54-33.25, 21.07-32.02, 22.69-33.18 and 20.79-29.65% at the doses 0, 10, 20 and 30 krad.

The genetic variability for protein revealed that V_2-variety treated with 10 krad had significant protein content above those in the plants fertilized with recommended dose of N among M_3 and M_4 generations, in response to inoculation with the parental strains and their transconjugants (except for DPM-Tr_2). However, similar trend was also observed in M_3 generation treated with 30 krad in response to inoculation with all rhizobial transconjugants, except for TPM-Tr_4. Although, the parental strain P_2 and rhizobial transconjugant TPM-Tr_4 appeared the same trend in M_4 generation treated with 20 krad. The protein content in V_2-variety ranged from 21.50-57.05, 21.05-34.19, 24.57-35.67 and 24.59-35.28% at the doses 0, 10, 20 and 30 krad, respectively.

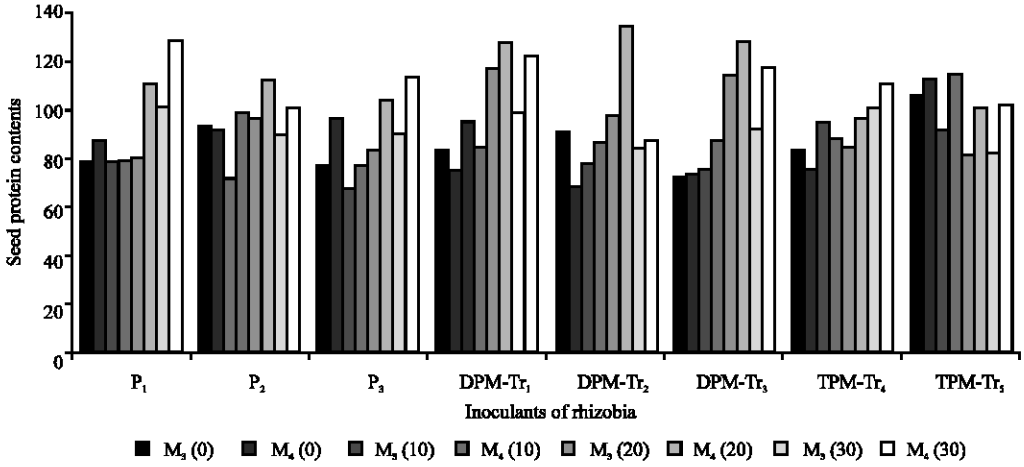

Fig. 4: Yield percentage of seed protein content in bioinoculated V_1 in relation to the plants fertilized with recommended dose of nitrogen

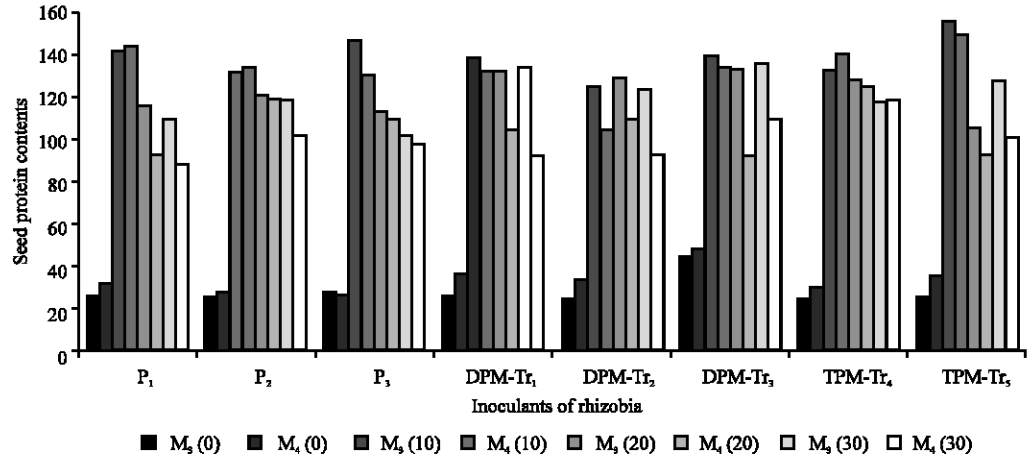

Fig. 5: Yield percentage of seed protein content in bioinoculated V_2 in relation to the plants fertilized with recommended dose of nitrogen

This are in agreement with Singh *et al.*[37] who reported that on the fresh weight basis (about 10% moisture), the protein content in cowpea ranged from 20 to 26% and the improved cowpea varieties IT89KD-245, IT89KD-288 and IT97K-499-35 had the highest protein content (26%), whereas the local varieties like Kanannado, Bauchi early and Bausse local had the lowest protein content (21 to 22%), although one of the local varieties, IAR 1696, had high protein content (24.78%).

Appropriate inoculation with efficient rhizobial strains have been made in this study to improve the content of protein. Breeding program can be used for improving this quality trait. Fashakin and Fasanya[38] analyzed 10 cowpea varieties and observed a range for protein content from 21.5 to 27.0% and for iron from 8 to 15 mg/100 g dry seeds.

These observations indicated that high shoot nitrogen content (V_1-variety) is negatively associated with improved nutritional quality traits such as protein, because of insufficient transport of nitrogen fixed from the shoots to the grains. Therefore, V_2-variety had lower nitrogen content in the shoots because of its sufficient transport to the grains inducing high protein content. Thus, the higher protein content in grains could be attributed to effective nitrogen transport from the shoots to the grains resulted in protein improvement. However, protein like most other traits considered in this study, is polygenic and considerably affected by environmental influence such as rhizobial inoculation and gamma irradiation. This indicated that there were significant inoculation and cultivar effects on protein content as shown in the differences between two varieties of cowpea

Table 11: Combined analysis of variance and mean squares for nodulation and growth traits in M_3 and M_4 populations of V_1 and V_2 varieties irradiated seeds

		V_1			V_2		
SOV	D.F	Nodules/plant	Nodule DW (g/plant)	Root DW (g/plant)	Nodules/plant	Nodule DW (g/plant)	Root DW (g/plant)
Generation	1	721.07**	0.001	0.10	592.2**	0.010*	8.18**
Repsx Gener.	4	38.98	0.0001	0.78	19.14	0.001	0.51
Treatments	39	142.50**	0.003**	0.94**	323.77**	0.005**	0.86**
Condition	9	206.39**	0.005**	1.95**	575.47**	0.008**	1.76**
Doses	3	263.31**	0.004**	1.06*	798.02**	0.0009**	0.49
Cond. x Doses	27	107.78**	0.003**	0.59	187.17**	0.003*	0.61**
Treat. x Ener.	39	49.84**	0.001	1.19**	36.37	0.003*	0.65**
Cond. x Ener.	9	67.65**	0.001	3.11**	53.10	0.003	0.70**
Doses. x Gener	3	71.69	0.002	1.04	47.02	0.003	1.29**
Cond. x Doses x Gener.	27	41.48	0.001	0.68	29.60	0.004	1.57**
Error	156	30.67	0.001	0.39	51.67	0.003	0.23

*, ** : $p< 0.0.05$ and $p< 0.01$, respectively

inoculated with the same rhizobial strains and their transconjugants

The combined analysis of variance for nodulation parameters and growth traits in M_3 and M_4 generations of V_1 (Table 11) revealed that the mean squares of generation were significant for number of nodules/plant indicated that there was a significant differences in variation between the M_3 and M_4 generations for both traits. However, the mean squares for treatments and the condition of biofertilization revealed significant effect among all the parameters of nodulation and growth traits. Therefore, the doses of gamma irradiation mean squares were significant for all parameters of nodulation and growth traits Although, the interaction mean squares (condition x doses) was significant for number of nodules/plant, nodule DW/plant. While, the interaction mean squares (treatment x generation) was significant for number of nodules/plant, root DW. In addition, the interaction mean squares (condition x generation) revealed significant effect for number of nodules/plant, root DW. The interaction mean squares of; doses x condition and condition x doses x generation, revealed insignificant effect among all the parameters of nodulation and growth traits.

The results indicated that the significance obtained in all traits of treatments were mainly due to condition (bioinculation) and doses of gamma rays, however sometimes due to condition x doses, treatment x generation and condition x generation. Therefore, the significant effect of condition and doses on most parameters of nodulation and growth traits in the treated population is an important indication of the efficiency of rhizobial strains and their recombinants in nodulating cowpea under the effect of competition with other rhizobial strains in the field, as well as, the efficiency of gamma irradiation treatment in inducing genetic variability. Mandal *et al.*[20] observed significant varietals differences in cowpea for nodule number and nodule weight as well as for nitrogenase activity indicating a good possibility of breeding improved cowpea varieties with enhanced N-fixation. Therefore, in recent years, major efforts have concentrated on exploiting genetic variability in cowpea as a host for effective nodulation and nitrogen fixation

Combined analysis of variances of M_3 and M_4 of V_2 variety irradiated generations demonstrated that the mean squares of generation were significant for all traits of nodulation parameters and growth rate. This indicated that there were significant differences in variation between the M_3 and M_4 irradiated generations. However, the treatments mean squares appeared significant effect for all studied traits. Significant effect of treatments on the number of nodules/plant was mainly due to the effects of biofertilization, doses of gamma rays and the interaction between both of them. In addition, the significance effect of treatments on nodule DW/plant was mainly due to the effect of condition, doses, the interaction between condition x doses and treatment x generation. The significant effect of treatment on root DW was mainly due to the significance effect of all sources of variation with the exception of doses effect of gamma irradiation. The interaction between condition x doses appeared significant effect on the number of nodules developed per plant, nodule DW/plant and root dry weight.

In addition, Interaction between treatment x generation was significantly affected on nodule DW/plant and root DW. Interaction between condition x generation reported significant mean squares on the root DW. Although, Interaction between doses x generations appeared significant effect on root DW, while interaction between condition x doses x generations achieved significant effect on root dry weight. The results obtained herein indicated that root DW was more effective with most sources of variations than the other parameters.

Significant effect of biofertilization on root DW as shown in this study was due to effective symbiosis leading to mature indeterminate nodules contain all of the stages of nodule development, including infection thread

Table 12: Combined analysis of variance and mean squares for yield and its components in M_3 and M_4 populations of V_1 and V_2 varieties irradiated seeds

		V_1		V_2	
SOV	D.F.	Weight of pods/plant (g)	100-seed dry weight (g)	Weight of pods/plant (g)	100-seed dry weight (g)
Generation	1	3825.45**	0.15	12.16	4.47**
Reps x Gener.	4	744.33	0.73	3824.18**	1.02
Treatments	39	2819.65**	1.14	6087.74**	2.06**
Condition	9	1569.32**	1.84**	3304.42**	2.01**
Doses	3	26981.5**	1.80*	60311.11**	10.62
Cond. x Doses	27	551.77	0.84	990.70	1.13**
Treat. x Gener.	39	888.25*	1.46**	1728.36*	1.15**
Cond. x Gener.	9	1225.4*	2.33**	3602.84**	2.05**
Doses x Gener.	3	1007.49	4.28**	2229.42	1.09
Cond. x Doses x Gener.	27	762.62	0.86	1047.86	0.86*
Error	156	550.11	0.61	1065.56	0.51

*, ** : p< 0.0.05 and p< 0.01, respectively

ramification, bacterial release and maturation into bacteroids, nitrogen fixation and senescence, all of this with IAA production stimulating root growth. Recently, Roest *et al.*[39] showed that the process of bacteroid differentiation differs, at the molecular level, between determinate- and indeterminate-nodulating hosts, suggesting that the signal exchange pathways may be unique to each type. There has been much speculation that indole-3-acetic acid (IAA) might play a role at various stage in the symbiotic relationship between *Rhizobium* and leguminous plants[40]. The results obtained herein are in agreement with Kimani[26] who reported that inoculation increased the grain yield of all the bean lines except GLP_2-Roko and also the M_5 and/or M_6 mutant populations also showed higher seed weight compared to the non-irradiated Canadian Wonder. The results obtained in this study are in harmony with Kimani[26] who stated that the mutant lines responded to *Rhizobium* inoculation with increases in grain yield, seed weight, pods per plant and seeds per pod. This suggested that nitrogen fixation was a limiting factor for growth and productivity of cowpea at the experimental site.

Combined analysis of variance for yield and its components in M_3 and M_4 generations of cowpea gamma irradiated V_1 and V_2 (Table 12) revealed that there was a significant mean squares for fresh weight of pods in V_1 and 100-grain weight in V_2 except for 100-grain weight in V_1 and fresh weight of pods in V_2 Treatments showed significant mean squares for all traits of the yield components except for 100 grain weight in V_1 Condition of biofertilization achieved significant mean squares for all yield components

In addition, the doses of gamma irradiation revealed significant effect for all parameters of yield components except for 100-grain weight in V_2. This indicated that breeding for quantitative traits is successful when there is genetic variability in the populations. The use of physical and chemical mutagens, or in combination of both, has been an important tool for the increase of variability in agronomic traits. The mean squares of interaction between condition x doses were insignificant except for 100-grain weight in V_2 However, the interaction mean squares for treatment x generation revealed insignificant effect for all parameters of yield components except for 100-grain weight in V_2. Although, the interaction mean squares (condition x generation) revealed significant effect on the weight of pods/plant, 100-grain weight, the interaction between doses x generation achieved insignificant mean squares for all parameters of yield components except for 100-grain weight in V_1.Therefore, the results indicated that the significance of all traits/components of yield (except for 100 grain weight) was mainly due to the doses of gamma rays and the condition of biofertilization, respectively. Thus, it could be concluded that the following traits, fresh weight of pods per plant was more effective with most sources of variations. However, 100-grain weight was less effective than fresh weight of pods per plant with most sources of variations. The significant effect of biofertilization (condition) and the interaction between condition x generation on most parameters of yield components may be due to the use of improved rhizobial strains carrying a genetic material from two and/or three parents, which may has a great potential to increase N_2 fixation. However, the competition between indigenous rhizobia and the selected strains can limit response in established production areas.

The results obtained herein are in agreement with Kessel and Hartley[41] who reported that maximizing N_2 fixation and managing fixed N may become more attainable by concentrating on the overall growing conditions of the host plant and the other crops in rotation rather than trying to increase the potential amount of N_2 fixation through improvement of the effectiveness of the rhizobia-host plant symbiosis. The results are also in harmony with Kimani[26] who found that the M_5 and M_6 lines of *Phaseolus vulgaris* L. showed increased grain yield (11%), 100-seed weight compared to non-irradiated

Table 13: Combined analysis and mean squares of biochemical traits in M_3 and M_4 populations of V_1 and V_2 varieties irradiated seeds

		V_1			V_2		
SOV	D.F.	Total Chlorophyll content	Protein content in seeds	Total N content Sh./P	Total Chlorophyll content	Protein content in seeds	Total N content Sh./P
Generation	1	0.005	31.55	29.94	0.145	457.03	0.39
Reps x Gener.	4	0.112	10.45	274.68**	0.346	871.13	3.42
Treatments	39	3.912**	55.76**	2059.37**	1.266**	1047.99**	1119.39**
Condition	9	12.617**	56.59**	6242.58**	1.899**	732.75	1682.52**
Doses	3	0.332	27.77*	1273.93**	3.156**	1551.84*	1196.37**
Cond. x Doses	27	1.408**	58.60**	752.24**	0.845	1097.09**	923.12**
Treat. x Gener.	39	0.088	14.35*	3.99	0.135	63.39	6.76
Cond. x Gener.	9	0.088	22.52*	4.85	0.142	45.73	4.65
Doses. x Gener.	3	0.122	14.99	7.57	0.2003	146.76	12.98
Cond. x Doses x Gener.	27	0.084	11.55	3.31	0.126	59.99	6.77
Error	156	0.383	9.03	50.37	0.228	473.35	84.70

* , ** : $p< 0.0.05$ and $p< 0.01$, respectively

controls. Inoculation increased grain and seed weight, but delayed maturity

The results obtained here are in agreement with Kimani[26], who evaluated advanced generation lines derived for radiation-treated bean cultivar Canadian Wonder for their performance and response to *Rhizobium* inoculation in replicated trials, results showed that the M_5 and M_6 lines appeared increased grain yield (11%), 100-seed weight and matured earlier compared to non-irradiated controls. The significance of biofertilization (condition) on the fresh weight of pods/plant, 100-seed weight and number of branches/plant mainly due to biological nitrogen (N_2) fixation, which is an important aspect of sustainable and environmentally friendly food production and long-term crop productivity

M_3 and M_4 generations of gamma irradiated v1 indicated that the mean squares of conditions (biofertilization) were significant for all biochemical traits studied herein. However, the doses mean squares were significant for all studied traits, except for total chlorophyll and N%, which are insignificant (Table 13).

These results appeared that significance of treatments was mainly due to biofertilization and particularly to gamma irradiation and the interaction between condition x doses. The interaction between condition x doses appeared significant mean squares for all biochemical parameters. The interaction between treatments x generations appeared significant effect on N% and seed protein content, while the interaction between condition x generation achieved significant effect on N% and seed protein content. It is appeared that the source of variants; condition, doses of gamma irradiation and the interaction between both of them achieved significant effect on most biochemical traits of cowpea V_1 variety. This also is in harmony with Antoun *et al.*[42] who found that applying *Bradyrhizobium japonicum* to radish significantly increased plant dry matter by 15%, but without nodulation. This is in addition to the role of microbial metabolites in making nutrients available to plants[32]. The inoculation of symbiotic N-fixing rhizobia enhances the nodulation capacity of plant roots of legumes and thereby promotes the plant yields and N availability in soil[43]. Furthermore, one can understand that the significant effect of biofertilization on biochemical traits of the plants results in a large part from the suppression of deleterious microorganisms and soil-borne pathogens by inoculated bacteria.

The beneficial effect of *Rhizobium* and *Bradyrhizobium* in legumes in terms of biological nitrogen fixation has been a main focus in the recent past. Obviously, rhizobia are known to increase nodulation and nodule weight in legumes along with increase in host plant growth and development[22], besides protecting roots from the attack of pathogens due to production of diverse microbial metabolites like siderophore, rhizobiotoxin, plant growth enhancement through IAA production, uptake of phosphorus and other minerals.

The results presented in Table 13 presented the combined analysis of variance of M_3 and M_4 generations of V_2 variety The doses mean squares appeared significant effect on all biochemical parameters, except for nitrogen percent in the seeds, which have insignificant mean squares. The interaction between condition x doses appeared significant effect on all biochemical parameters, except for total chlorophyll, which revealed insignificant mean squares. Although, the interaction between treatments x generation achieved significant mean squares for N% in the seeds.

The results appeared that biofertilization, doses of gamma rays and the interaction between biofertilization x doses of gamma rays was mainly affected on most biochemical traits studied herein. It can be concluded that the significance of treatments for all biochemical traits studied herein was mainly due to the effect of biofertilization, doses of gamma rays and their interactions.

The rhizobacterium *Pseudomonas putida* strain used in this study in mating experiments as a plasmid-donor (phenol degradative plasmid) is a strong candidate for development as a soil inoculant to enhance crop yields[17]. The ability of this strain to enhance plant growth and improve biochemical quality contribute to the ability of *P. putida* to enhance plant growth include the capacity to synthesize siderophores and thereby provide iron for the plant[44], the capacity to lower growth-inhibiting levels of ethylene in plant tissues by production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase[45] and the capacity to secrete IAA[19]. Nitrogen fixation by symbiotic rhizobia is of enormous economic importance, being responsible for the global fixation of about 50 million metric tons of nitrogen each year[46]. Van Elsas *et al.*[47] found that the increase in economic traits due to biofertilization may be due to increased metabolic activity due to root exudates and to colonization of the root surfaces, which may improve cell contact. Davison[11] reported that no evidence was found that gene transfer can take place in the nodules. Deshwal *et al.*[22] found that among ten strains of *Bradyrhizobium* (*Arachis*) sp. in peanut, only three produced siderphore and IAA and exhibited phosphate solubilization *in vitro*. The significant effect of cowpea biofertilization in this study was mainly due to the biochemical products of bacterial strains and their recombinant isolates

In conclusion, the transfer of genetic material between *Rhizobium* strains and between *Rhizobium* with *Pseudomonas putide* resulted in the conversion of about 20% of forming an effective symbiosis with the host plants. Improving nitrogen fixation of cowpea-*Rhizobium* interaction can improving quality, increasing yield, improving production through converting atmospheric nitrogen into a chemical form, thus reducing the need for applied nitrogen fertiliser.

The results presented in this study indicated that biochemical traits and growth of cowpea can be improved through efficient nitrogen fixation process and probably by mutation breeding, because the mutant lines responded to *Rhizobium* inoculation with increases in grain yield, seed weight, pods per plant and seeds per pod. This suggested that nitrogen fixation was a limiting factor for growth and productivity. It can be concluded that these plant/bacterial associations involving rhizobia can be genetically manipulated for increased plant growth and possibly grain yield.

Many of the observed differences between the horizontal transfer of informational and operational genes can be explained by complexity. The extensive amount of horizontal transfer between *Rhizobium* observed in this study makes it clear that horizontal transfer must be a major contributor to the evolution of genomes. Gamma-ray was efficient in increasing variability in M_3 and M_4 generations. Combined treatments (gamma irradiation+bioinoculation) effectively increased the variance of traits studied in this investigation, however it was not possible to discern exactly the superiority of the combined treatments over individual performance, because single treatments also were efficient.

Although, the use of improved rhizobial strains has great potential to increase N_2 fixation where crops have been newly introduced to a region, competition between indigenous rhizobia and the selected strains can limit response in established production areas. Biotechnology and gene manipulation techniques were able to provide potential means to improve the commercial inoculant strains. Biological N_2 fixation is an environment friendly food production and long-term crop productivity.

The use of improved host-rhizobia combination has great potential to increase N_2 fixation. Interaction between a range of traits and N_2 fixing symbiosis will require particular care in breeding and selection programs aimed at alleviating environmental and management practices that reduce Biological Nitrogen Fixation (BNF) using programmes for host plant selection and for *Rhizobium* selection. These methods available to enhance BNF was shown in this study. The transfer of genetic material between rhizobia via conjugation is necessary for the generation of genetic diversity and provides the raw material of natural selection the efficient strains in symbiosis and also evolution.

The bacterial IAA produced by transconjugants with a larger amounts above the mid-parents stimulates the development of the host plant root system and also stimulated the symbiotic relationship. The associations between two major symbiotic partners are considerable importance in agriculture, making the plant more tolerant to various stresses (low fertiliser levels, drought, pathogens, etc.) and mostly independent of N-fertilization.

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