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Enhanced Nodulation and Nitrogen Fixation in Common Bean (*Phaseolus vulgaris* L.) Via Conjugation and Azide Resistant Mutants in Rhizobial Strains

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Abstract: Conjugal transfer of DNA was carried out in this study by inducing diparental and triparental transconjugants of *Rhizobium* to improve the symbiotic phenotype of the microsymbiot. Here, this study describe also the isolation and symbiotic characterization of Az^r mutants of *Rhizobium phaseoli* with enhanced symbiotic nitrogen fixation capability. Tri-parental transconjugants were involved DNA from *Pseudomonas putida* to supported the role of antibiotics in disease suppression of the isolates colonize root system to protect it against pathogens and also to degrade phenolic compounds present in root exudates, which may affect on the suppression nodulation process, thereby improving nodulation, plant growth and yield. All new recombinants induced including transconjugants and azide resistant mutants were evaluated in pots experiments, thus leading to select the efficient strains to be evaluated under field conditions. All new recombinants derived from the mating between; RLbp7 x *Pseudomonas putida* x *R. L.* bv. *viciae* significantly stimulated the formation of chlorophyll a and total chlorophyll. Three recombinants out of five exhibited high nodulation, in addition, two out of five produced significant increase in root dry matter production above the mid-parents. All tri-parental transconjugants resulted from the cross between; RLbp9 x *Pseudomonas putida* x *R. L.* bv. *viciae*, produced significant amounts of IAA above the mid-parents when grown in the presence of exogenous trypton and ethanol. Three out of five tri-parental transconjugants resulted from the cross between; RLbp9 x *Pseudomonas putida* x *R. L.* bv. *viciae* were efficient in symbiosis because of higher number of nodules developed on the root system of the host plant, which ranged between 22-46. The same recombinants increased root dry matter yield above uninoculated plants. Four out of five tri-parental transconjugants resulted from the cross between; RLbp10 x *Pseudomonas putida* x *R. L.* bv. *viciae*, synthesize significant amounts of IAA over their mid-parents in the presence of exogenous tryptophan and trypton. However, three out of five produced significant amounts of IAA from ethanol and all five recombinants produced significant amounts of IAA from lactic acid above their mid-parents. In addition, three recombinants (RLbp10 x *Pseudomonas putida* x *R. L.* bv. *viciae*), developed significant number of nodules above the mid-parents on the root system, which ranged between 16-25. Plants inoculated with some of azide resistant mutants (Az^r_4, Az^r_5 and Az^r_6), di-parental transconjugants ($DPM\text{-}Tr_3$ and $DPM\text{-}Tr_7$) and tri-parental transconjugants ($TPM\text{-}Tr_8$) appeared significant increase in grain weight per plant over uninoculated plants.

Key words: *Rhizobium leguminosarum* bv. *Phaseoli*, nitrogen fixation, conjugation, *Phaseolus vulgaris* L

INTRODUCTION

Rhizobium sp. are gram-negative soil bacteria studied primarily for their ability to establish nitrogen-fixing symbioses with leguminous plants. Intensive genetic analysis during the past decade has led to the identification of genes essential for the nodulation (*nod* genes) and nitrogen fixation (*nif* and *fix* genes) processes. In all the fast-growing *Rhizobium* species, these genes are carried on large plasmids, the so-called *sym* plasmids or *pSym*[1]. One interesting characteristic of the *Rhizobium* genome is the presence of a large number of reiterated DNA sequences. For *Rhizobium phaseoli*, the symbiont of the common bean (*Phaseolus vulgaris* L.) Romero *et al.*[2] estimated the presence of about 700 reiterated elements, belonging to 200 different families[3]. It has been shown that recombination between pairs of repeated elements may lead to different kinds of genomic rearrangement, including additions, amplifications, deletions and inversions[4]. These rearrangements, which are frequently deletions, may affect the symbiotic properties of the strain, either for nodulation or for nitrogen fixation[5]. *Phaseolus vulgaris* is considered to be a poor fixer of atmospheric N compared with other crop

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legumes[6] and generally responds poorly to inoculation in the field with *Rhizobium leguminasarum* bv. *phaseoli* strains[7]. The inability of inoculant strains to form a significantly large proportion of the nodules in the presence of an indigenous *Rhizobium* population is a frequently cited explanation for the lack of plant response to inoculation. Inoculant strains compete at different levels in the rhizosphere and in nodule formation. First, there is competition for survival and multiplication in the soil and rhizosphere. This depends on the capacity of each strain to adapt to environmental stress factors such as high soil temperature, drought, low pH, Al and Mn toxicity and micronutrient deficiency.

The main problem in *Phaseolus vulgaris* L. have shown in numerous field assays that the inoculation with *Rhizobium* has not been successful in increasing nitrogen fixation of bean and the failure may be attributed to the presence of native rhizobia with high competitive ability, which form the majority of the nodules[8].

The present study aimed to induce new genetic recombinants of *Rhizobium leguminosarum* bv. *phaseoli* for better symbioses with *Phaseolus vulgaris*. New recombinants induced including azide resistance mutants, transconjugants resulted from one direction of mating (diparental mating) and from two direction mating (triparental mating). All of these new recombinants were used as genetic tools for evaluating in better symbioses and nodulation with enhanced symbiotic nitrogen fixation capability with *phaseolus vulgaris*.

MATERIALS AND METHODS

Genetic materials

Macrosymbiotic plant: Commercial variety of common bean (*Phaseolus vulgaris* L. Giza 6) kindly provided from Horticulture Research Institute, Agriculture Research Center, Giza, Egypt, was used in three pots (2001 and 2002) experiments and substituted with Nebraska variety in the fourth pots (2003) experiment and the field experiment (2003).

Bacterial strains (Microsymbiotic) and growth conditions: Eleven strains of *Rhizobium leguminosarum* bv. *phaseoli*, one strain of *Rhizobium leguminosarum* bv. *viciae* and *Pseudomonas putida* strain listed in Table 1, are used in this study. These strains were kindly provided as follows; all USDA strains were obtained from Dr. Van Berkum through Mr. Douglas K. Jones USDA/ARS Beltsville Rhizobium Germplasm Collection, United States, Department of Agriculture, Agricultural Research Service, USDA. However, ARC strains were obtained from Prof. Samir Mohamed Abd El-Wahab, Soil, Water and Environmental Research Institute, Agriculture Research Center, Giza, Egypt. and the NRRL B-13 strain was kindly provided from Prof. Dr. Nakamura, United States, Department of Agriculture, Agricultural Research Service, USDA.

Table 1: Bacterial strains used in this study

Bacterial strains	Designation
Rhizobium leguminosarum bv. *phaseoli* USDA 2669	RLbp1
Rhizobium leguminosarum bv. *phaseoli* USDA 2671	RLbp2
Rhizobium leguminosarum bv. *phaseoli* USDA 3070	RLbp3
Rhizobium leguminosarum bv. *phaseoli* USDA 3074	RLbp4
Rhizobium leguminosarum bv. *phaseoli* USDA 3471	RLbp5
Rhizobium leguminosarum bv. *phaseoli* ARC 301	RLbp6
Rhizobium leguminosarum bv. *phaseoli* ARC 305	RLbp7
Rhizobium leguminosarum bv. *phaseoli* ARC 310	RLbp8
Rhizobium leguminosarum bv. *phaseoli* ARC 3612	RLbp9
Rhizobium leguminosarum bv. *phaseoli* ARC 3644	RLbp10
Rhizobium leguminosarum bv. *phaseoli* ARC 1899	RLbp11
Rhizobium leguminosarum bv. viceae ARC 207	RLbv
Pseudomonas putida NRRL B-13	Psdp

RLbpI to RLbpII were used in the first plant infection experiment

Table 2: Different antibiotic concentrations used for genetic marking in rhizobial strains

Antibiotic	Concentration (μg mL^{-1})	Designation
Streptomycin	100	Strep
Ibiamox	50	Ibim
Neomycin	50	Neomy
Amoxycillin	50	Amoxy
Ibidroxil	50	Ibidro
Velosef	50	Velo
Epicocillin	50	Epico
Ampicillin	80	Ampi
Erythromycin	25	Eryo
Tetracycline	25	Tetra
Chloramphenicol	80	Chloraph
Totacef	100	Tota
Cefazone	100	Cefa
Erythrin	70	Eryth
Mycostatin	70	Mycos

Media: Complete Medium (CM) was used as a full synthetic medium for cell growth[9] and the maintenance of cultures according to Vincent[10]. TY medium:was used for the induction of azide resistant mutant according to Sharma *et al.*[11]. Kings B medium was used for the maintenance of *Pseudomonas putida* culture and nutrient broth medium was used to grow *Pseudomonas putida* in the conjugation steps according to Meyak *et al.*[12]. Although nutrient solution was used to irrigate the pots experiment according to Allen[9].

Mutagenic agent (sodium azide): Sodium azide product of Sigma Chemical Company, USA, was used in this study as a mutagenic agent. It is a highly efficient mutagen in bacteria. The mechanism of mutagenic action of azide remains obscure. It was therefore suggested that either azide is metabolized *in vivo* to the ultimate reactive mutagen or it exerts its mutagenic effect only on replicating DNA. The latter possibility seemed that DNA

Table 3: Horizontal DNA transfer between *Rhizobium leguminosarum* bv. *phaseoli* strains and *Pseudomonas putida* that having the opposite genetic markers

No.	Matings	Appearance of transconjugants	Transconjugants genotype	Designation
1	RLbp2 (Strep^{S} Neomy^{R} Chloraph^{S} Eryth^{S}) X Psdp (Strep^{R} Neomy^{S} Chloraph^{R} Eryth^{R})	-		
2	RLbp5 (Neomy^{R} Chloraph^{S} Mycos^{S}) X Psdp (Neomy^{S} Chloraph^{R} Mycos^{R})	+	Neomy^{R} Chloraph^{R} Mycos^{R}	DPM-Tr$_1$ DPM-Tr$_2$ DPM-Tr$_3$ DPM-Tr$_4$ # DPM-Tr$_5$
3	RLbp7 (Amoxy^{R} Chloraph^{S}) X Psdp (Amoxy^{S} Chloraph^{R})	+	Amoxy^{R} Chloraph^{R}	DPM-Tr$_1$ DPM-Tr$_2$ # DPM-Tr$_3$ DPM-Tr$_4$ DPM-Tr$_5$
4	RLbp8 (Tetra^{R} Tota^{R} Strep^{S} Eryth^{S}) X Psdp (Tetra^{S} Tota^{S} Strep^{R} Eryth^{R})	-		
5	RLbp9 (Ibim^{R} Chloraph^{S}) X Psdp (Ibim^{S} Chloraph^{R})	+	Ibim^{R} Chloraph^{R}	DPM-Tr$_1$ DPM-Tr$_2$ DPM-Tr$_3$ DPM-Tr$_4$ # DPM-Tr$_5$
6	RLbp10 (Strep^{S} Eryo^{R}) X Psdp (Strep^{R} Eryo^{S})	+	Strep^{R} Eryo^{R}	DPM-Tr$_1$ DPM-Tr$_2$ DPM-Tr$_3$ # DPM-Tr$_4$ DPM-Tr$_5$
7	RLbp11 (Strep^{S} Epico^{R} Tetra^{R} Chloraph^{S}) X Psdp (Strep^{R} Epico^{S} Tetra^{S} Chloraph^{R})	+	Strep^{R} Epico^{R} Tetra^{R} Chloraph^{R}	DPM-Tr$_1$ DPM-Tr$_2$ DPM-Tr$_3$ DPM-Tr$_4$ DPM-Tr$_5$ #
8	RLbp2 (Ibim^{S} Neomy^{R} Velo^{S} Epico^{S}) X Rlbv (Ibm^{R} Neomy^{S} Velo^{R} Epico^{R})	+	Ibim^{R} Neomy^{R} Velo^{R} Epico^{R}	DPM-Tr$_1$ DPM-Tr$_2$ DPM-Tr$_3$ DPM-Tr$_4$ # DPM-Tr$_5$
9	RLbp8 (Velo^{S} Epico^{S} Tetra^{R} Chloraph^{R}) X Rlbv (Velo^{R} Epico^{R} Tetra^{S} Chloraph^{S})	-		

- , + = Mating failed to success and successed, respectively, # = Di-transconjugant isolates selected for conjugated with the third parent (tri-parental mating)

replication in gram-negative bacteria is inhibited both *in vivo* and *in vitro* by a moderate concentration of azide. It was considered that azide in a concentration sub-optimal for DNA synthesis inhibition may increase the error frequency during chromosome replication[13].

Induction and isolation of azide resistant mutants (Az^{r}): Three strains (RLbp7, RLbp9 and RLbp11) were overnight growing in TY medium using rotary shaker at 120 revolutions per min. (RPM) and 28°C giving finally 10^{8} cfu mL^{-1}. One milliliter culture suspension was plating on TY agar medium supplemented with different concentrations of sodium azide (40, 60, 80 and 100 μg mL^{-1}) and then incubated for two weeks at 28°C. Three single colonies from that appeared in every concentration were picked up and sub-cultured on TY slant agar medium. These resistant colonies were purified on the same medium containing the same concentrations of sodium azide.

Genetic marking in rhizobial strains based on antibiotic susceptibility assays: Antibiotic susceptibility was measured by plate diffusion method, according to Collins and Lyne[14]. Different antibiotics from the product of Hoechst Orient SAE, Cairo, Egypt were used with the different concentrations as shown in Table 2.

Conjugation procedure: Mix 0.2 mL of the donor strain culture with 1.8 mL of the recipient strain culture in a large test-tube (18 x 150 mm). This ensures adequate aeration without shaking, which might disrupt mating pairs. Incubate at 28°C for the required time which differed from one conjugation to another. Stop the mating by placing the tubes in ice and plate dilutions on selective plates according to Grinsted and Bennet[15]. The conjugation procedure has been done in two steps as follows:

First step: Di-parental mating (Table 3), which carried out between *Rhizobium leguminosarum* bv. *phaseoli*

Table 4: Tri-parental matings conducted in this study

No	Matings	Source or reference of di-transconjugants	Appearance of transcon-jugants	Genotype	Designation
1	DPM-Tr$_4$ (Zinc^S Cd chl^R Zn sul^S)	*Rhizobium leguminosarum* bv.	+	Zinc^R	TPM-Tr$_1$ #
	X	*Phaseoli* RLbp5 x *Pseudomonas putida*		Cd sul^R	TPM-Tr$_2$
	Rhizobium leguminosarum bv. viciae			Zin sul^R	TPM-Tr$_3$
	(Zinc^R Cd chl^S Zn sul^R)				TPM-Tr$_4$
					TPM-Tr$_5$
2	DPM-Tr$_2$ (Zinc^S Cd chl^R)	*Rhizobium leguminosarum* bv.	+	Zinc^R	TPM-Tr$_1$
	X	*Phaseoli* RLbp7 x *Pseudomonas putida*		Cd chl^R	TPM-Tr$_2$
	Rhizobium leguminosarum bv.				TPM-Tr$_3$ #
	viciae (Zinc^R Cd chl^S)				TPM-Tr$_4$
					TPM-Tr$_5$
3	DPM-Tr$_4$ (Zinc^S Cd chl^R Zn sul^S)	*Rhizobium leguminosarum* bv.	+	Zinc^R	TPM-Tr$_1$
	X	*Phaseoli* RLbp9 x *Pseudomonas putida*		Cd sul^R	TPM-Tr$_2$
	Rhizobium leguminosarum bv. viciae			Zin sul^R	TPM-Tr$_3$
	(Zinc^R Cd chl^S Zn sul^R)				TPM-Tr$_4$ #
					TPM-Tr$_5$
4	DPM-Tr$_3$ (Zinc^S Cd chl^R)	*Rhizobium leguminosarum* bv.	+	Zinc^R	TPM-Tr$_1$
	X	*Phaseoli* RLbp10 x *Pseudomonas putida*		Cd chl^R	TPM-Tr$_2$ #
	Rhizobium leguminosarum bv. *viciae*				TPM-Tr$_3$
	(Zinc^R Cd chl^S)				TPM-Tr$_4$
					TPM-Tr$_5$
5	DPM-Tr$_5$ (Cd chl^R Zn sul^S)	*Rhizobium leguminosarum* bv.	+	Cd chl^R	TPM-Tr$_1$
	X	*Phaseoli* RLbp11 x *Pseudomonas putida*		Zn sul^R	TPM-Tr$_2$
	Rhizobium leguminosarum bv. *viciae*				TPM-Tr$_3$
	(Cd chl^S Znsul^R)				TPM-Tr$_4$ #
					TPM-Tr$_5$
6	DPM-Tr$_4$ (Zinc^S Cp sul^R Cd sul^R Zn sul^S)	*Rhizobium leguminosarum* bv.	+	Zinc^R	TPM-Tr$_1$
	X	*Phaseoli* RLbp2 x *R. leguminosarum* bv. *Viciae*		Cp sul^R	TPM-Tr$_2$
	Pseudomonas putida			Cd sul^R	TPM-Tr$_3$ #
	(Zinc^R Cp sul^S Cd chl^S Zn sul^R)			Zin sul^R	TPM-Tr$_4$
					TPM-Tr$_5$

+ = Mating successed, # = Tri-transconjugant isolates selected to be use in the field experiment

(Rlbp2), *Rhizobium leguminosarum* bv. *phaseoli* (RLbp5), *Rhizobium leguminosarum* bv. *phaseoli* (RLbp7), *Rhizobium leguminosarum* bv. *phaseoli* (RLbp8), *Rhizobium leguminosarum* bv. *phaseoli* (RLbp9), *Rhizobium leguminosarum* bv. *phaseoli* (RLbp10) and *Rhizobium leguminosarum* bv. *phaseoli* (RLbp11) with *Pseudomonas putida* (Psdp). While, the other two strains; *Rhizobium leguminosarum* bv. *phaseoli* (RLbp2) and *Rhizobium leguminosarum* bv. *phaseoli* (RLbp8), which failed to success with *Pseudomonas putida* has been conjugated with *Rhizobium leguminosarum* bv. *viciae* strain. Though, just one conjugation was successed, this is the conjugation carried between *Rhizobium leguminosarum* bv. *phaseoli* (RLbp2) with *Rhizobium leguminosarum* bv. *viciae*. Five isolates from each of the six successed matings were selected at random to test for their efficiency in IAA production. Di-parental transconjugants giving a good results in the evaluation experiment were selected to be conjugated with the third parent (*Rhizobium leguminosarum* bv. *viciae* or *Pseudomonas putida*). The two parents used in tri-parental mating were genetically marking used different heavy metals; zinc (Zn), zinc sulphate (Zn sul), cadmium sulphate (Cd sul), cadmium chloride (Cd chl), cupper sulphate (Cu sul) and lead acetate (Ld acet). This tolerance was measured by a plate diffusion method which described before according to Collins and Lyne[14]. Each heavy metal was used with a concentration of 100 μg mL^{-1}.

Second step: Tri-parental mating: Tri-parental mating was performed between di-parental mating transconjugants with the third parent as shown in Table 4. All the above matings were successed. From each of six matings, five isolates were picked up at random to be used for application in the third pot experiment to evaluate their efficiency in nodulation with *Phaseolus vulgaris* L.

Indole 3-acetic acid (IAA) detection with Salowski colorimetric technique: Bacterial strains, their transconjugants and mutants used in this study were grown overnight in nutrient broth medium at 28°C for all *Rhizobium* strains and their new recombinants and at 30°C for the *Pseudomonas putida*. Production of IAA in the culture supernatant was assayed by using the PC method, as described by Pilet and Chollet[16].

IAA produced by bacteria strains and their transconjugants was calculated from the results standard curve of indole-3-acetic acid used the concentration ranged between 2 up to 20 μg mL^{-1}. IAA concentrations was calculated from the formula of regression as follows; Y = 0.029x-0.01.

Where, x, concentration of IAA; y, optical density at 530 nm; a, absorbance at 530 nm when the concentration of IAA equal zero and b, regression.

Plant infection: This technique involves growing the plants in a sterile sandy soily in plastic pots with three replicates. The plants were inoculating one time at sowing with the overnight culture suspension growing at 28°C on rotary shaker (120 rpm), containing finally 10^8 cfu mL^{-1}. The nodules developed on the plants were counted after 25-day-plant old. The accuracy of this method depends on the ability of a single rhizobia cell to form a nodule on the host. This plant infection technique is commonly applied in this study to determine the efficiency of different recombinants of inoculant in symbiosis[10]. Strains, their transconjugants and mutants were evaluated through plant infection technique four times as follows:

First, was performed to evaluate the efficiency of eleven strains of *Rhizobium leguminosarum* bv. *phaseoli* to form nodules on *Phaseolus vulgaris* variety Giza 6, when inoculated one time during the sowing. Second, was done to asses the response of *Phaseolus vulgaris* to form nodules, which inoculated with parental strains, resulted from three parental strains presented in Table 3. Third, was conducted to test the efficiency of the parents and their transconjugants (di- and tri-parental matings) in nodulation with the host plant to select the efficient recombinants to be evaluated under field conditions including the competition with other strains in the soil. Fourth, was performed to test the efficiency of the parents, their transconjugants resulted from di- and tri-parental matings and azide resistant mutants with Nebraska variety. All plastic pots experiments were carried out[10]. These pots were containing sterilized sandy soil, autoclaved three times at 121°C for 1 h at three days, at the Experimental Agriculture Research Unit of Plant Pathology Department, Faculty of Agriculture, Mansoura University, sandy soil was washed with distilled water several times to diminishing chloride ions.

Field experiment: The field experiment was carried out in grown season of 2003. This experiment was done at El-Saadia Village, Shirbeen Center, Dakahlia Governorate, in natural clay soil (non-sterilized), the experimental design was a Randomized Complete Block with three replicates. Each replicate containing 27 plots, each plot was four meter long and 40 cm between rows. Seeds were sown at one side in one seeded hill, 5 cm apart.

Inoculation: Cultures in a mid-log phase grown in nutrient broth of CM were used for inoculated plants according to Kucey[17]. Cultivated plants in the plastic pots were irrigated using free nitrogen nutrient solution of Bond's modified Cron's stock salts mixture[9]. Plants were irrigated when water as needed until harvest.

Preparation of samples: Since the parental strains, their transconjugants and azide resistant mutants were used to evaluate their efficiency in nodulation and nitrogen fixation, at 35 days plant-old for the field experiment and 25 days plant old for the pot experiments, two plants per each replicate were carefully uprooted and the roots were washed with tap water to remove the adhering clay particles. The nodules on every plant were counted, the mean number per plant was recorded. Shoots and roots dry weight were recorded at the same plant age. The plant parts were oven dried at 70°C for 72 h according to Pineda and Nolt[18]. Chlorophyll a, b and total were determined after 25 days of planting for the pot experiments. Spectrophotometric method, using Optical Density (OD) measurements according to Markinney[19].

Nitrogen determination: Nessler method was used to determination the concentration of the nitrogen in the sample[20].

Standard curve for determining the concentration of nitrogen: Determination of nitrogen in the shoot samples was carried out from the standard curve. of colorimetric technique at OD 425 using different concentrations of NH_3Cl ranged between 0.2 up to 2.2 ppm. Where; $Y = 0.01+0.14X$

Where, y, Optical density at 530 nm; x, Concentration of nitrogen; b, Regression and a, Absorbance at 425 nm when the concentration of N equal zero. However, Crude protein in seed (%) = N_2 % x 6.25.

Plant yielding: At harvest time, five individual guarded plants were taken at random for each experimental plot, on which the mean of the following traits was recorded protein in seed (%) and seed yield per plant (gram).

Statistical evaluation: Data were subjected to statistical analysis by the technique of analysis of variance. The treatment means were compared at 0.05 and 0.01 probability levels using the Least Significant Difference method as mentioned by Gomez and Gomez[21].

RESULTS AND DISCUSSION

Evaluation of rhizobia strains: As shown form the result presented in Table 5 four Rhizobium strains (Rlbp2, Rlbp5, Rlbp8 and Rlbp10) failed to nodulate whitebean Giza-6 variety. However, three strains; RLbp7, RLbp9 and

Table 5: Mean number of nodules developmented on Giza-6 variety using sandy soil in pots experiment

Treatments	Number of nodules
Control	0.00
RLbp1	4.00
RLbp2	0.00
RLb3	3.00
RLbp4	2.00
RLbp5	0.00
RLbp6	4.67
RLbp7	7.00
RLbp8	0.00
RLbp9	15.00
RLbp10	0.00
RLbp11	8.67
LSD 5%	3.76
1%	5.13

RLbp7, RLpb9 and RLbp11 were selected for inducing azide resistant mutants, however RLpb2, RLbp5, RLbp7, RLbp8, RLbp9, RLbp10 and RLbp11 were used in di-parental mating experiment

Rlbp11 were efficient than other stains, because of significant number of nodules developed on the root system of whitebean, for this these strains were selected for inducing azids-resistant mutant. This are done to isolate azide-resistant mutants from the efficient strains. The other strains which failed in nodulation in addition to that achieved significance in nodulation were used in conjugation experiments between di-parental strains.

Evaluation of tri-parental transconjugants in IAA production and plant growth in pots experiment: The results presented in Table 6 indicated the colorimetric assay used revealed that tri-parental transconjugants (TPM-Tr_3 and TPM-Tr_4) induced significant amounts of indole compounds from lactic acid in relation to their mid-parents. Although, non of the other transconjugants achieved any significant amounts from all precursors. The data obtained demonstrated the presence of IAA from lactic acid in the supernatant of some tri-parental transconjugant cultures. The finding that only two out of five isolates tested produced significant amounts of IAA in comparison with their mid-parents, under the conditions chosen further demonstrate a clear variability among the isolates derived from the same cross in their capability to produce IAA from the same precursor.

It proved to be useful for screening tri-parental transconjugants affected in auxin production and related indolic compounds such as indolepyruvic acid (IPyA), indolelatic acid (ILA), indoleacetic acid (IAA) and indoleethanol (or tryptophol). The presence of significant amounts of IAA in the supernatant of some strains may have some relevance to its ability to colonize plant surfaces successfully. Brandle and Lindow[22] reported that IAA pathway in *E. herbicola* 299R is induced under conditions of low-nitrogen availability in culture, a situation which is probably common on leaves. It seems likely that the release of IAA benefits these bacteria by enabling them to modify their microhabitat, such as by increasing the rate of nutrient leakage from plant cells in their vicinity.

The results obtained herein are in agreement with Patten and Glick[23] who found that the phytohormone indole-3-acetic acid (IAA) accumulates in the culture medium of the plant growth-promoting bacterium *Pseudomonas putida* GR12-2 only when grown in the presence of exogenous tryptophan. This suggested that expression of indolepyruvate decarboxylase, a key enzyme in IAA biosynthesis pathway in this bacterium, may be regulated by tryptophan.

Total chlorophyll concentrations recorded a significant increase in response to inoculation with tri-parental transconjugants (TPM-Tr_1, TPM-Tr_3 and TPM-Tr_4). The increase in total chlorophyll was shown also in response to inoculation with the parental strain *Pseudomonas putida*. This increase in the yields of total chlorophyll concentrations above the uninoculated plants could be due to the increase in chlorophyll a and or chlorophyll b.

Dry matter (shoot) productivity recorded significant increase with the application of tri-parental transconjugants (TPM-Tr_1, TPM-Tr_3, TPM-Tr_4 and TPM-Tr_5). Although, root dry weight was significantly increased above the mid-parents with the application of triparental transconjugants (TPM-Tr_2 and TPM-Tr_5). This increase in developmental characters will result of co-ordinate interplay of yield. Because vigorously growing nitrogen fixation plants inoculated with tri-parental transconjugants were able to absorb a large quantity of mineral nutrients through their well developed shoot and root system as seen in this study in response to inoculation with some of new recombinants. Nitrogen increased the synthesis of photosynthates and the storage organ-seeds in this case were well developed[11]. In contrast to the failure of di-parental transconjugants in nodulation, the application of tri-parental transconjugants had directly beneficial effect on nodulation leading to well developed of nodule number on root system.

Increasing the beneficial effect of inoculation with triparental transconjugants on the developmental characters might have contributed to higher biological yield. The results obtained herein indicated that rhizobia harboring recombinant genomes from through tri-parental mating, with potentially improved symbiotic capabilities. These studies suggest that a particular symbiotic phenotype, in this case the capacity to nodulate *Phaseolus vulgaris* effectively, is present in tri-parental transconjugants constructed in this study, in contrast to other di-parental transconjugants and the parental strains,

Table 6: Effect of recombinant transconjugants resulted from tri-parental mating between (*Rhizobium leguminosarum* bv. *phaseoli* (RLbp2)x*Rhizobium leguminosarum* bv. *viciae*)x*Pseudomonas putida* on indole-acetic acid production and growth parameters of plants (25-day-plant-old) grown in pots experiment

	IAA (μg mL^{-1})				Chlorophyll (mg g^{-1})					
Inoculum	Tryptophan	Trypton	Lactic acid	Ethanol	Chl a	Chl b	Total Chl	Nodules No.	Shoot DW (g plant^{-1})	Root DW (g plant^{-1})
Control					0.025	1.011	1.036	0.000	0.773	0.277
Psdp	21.48	10.25	1.60	8.530	0.277	2.817	2.863	0.000	0.473	0.290
DPM-Tr4	32.38	6.74	1.04	0.583	0.028	1.230	1.258	1.670	0.663	0.357
Mid-parent	26.93	8.50	1.32	4.560	0.153	2.024	1.960	0.833	0.568	0.323
TPM-Tr1	16.58	2.89	1.51	0.000	0.155	1.340	1.495	31.670	0.92	0.297
TPM-Tr2	18.11	5.21	1.30	0.000	0.250	0.904	1.154	61.330	0.537	0.430
TPM-Tr3	13.22	4.57	3.34	0.510	0.478	1.913	2.391	22.000	0.753	0.370
TPM-Tr4	13.97	5.21	2.73	0.593	0.234	1.588	1.823	20.330	1.000	0.310
TPM-Tr5	20.49	4.98	1.00	0.690	0.068	1.297	1.365	11.000	0.870	0.410
F-test	**	**	**	**	**	**	**	**	**	**
LSD 5%	1.96	0.71	0.44	0.329	0.026	0.177	0.359	6.810	0.065	0.062
1%	2.74	0.99	0.62	0.461	0.035	0.245	0.498	9.450	0.090	0.087

TPM-Tr$_3$ was selected to be used in the field experiment naming TPM-Tr$_7$, ** = p< 0.01

Table 7: Effect of recombinants resulted from tri-parental mating between *Rhizobium leguminosarum* bv. *phaseoli* (RLbp5)x*Pseudomonas putida* x *Rhizobium leguminosarum* bv. *viciae*, on indole-acetic acid production and growth parameters of plants (25-day-plant-old) grown in pots experiment

	IAA (μg mL^{-1})				Chlorophyll (mg g^{-1})					
Inoculum	Tryptophan	Trypton	Lactic acid	Ethanol	Chl a	Chl b	Total Chl	Nodules No.	Shoot DW (g plant^{-1})	Root DW (g plant^{-1})
Control					0.025	1.011	1.144	0.00	0.773	0.277
RLbv	10.05	5.48	1.21	0.000	0.059	1.605	1.665	0.00	0.857	0.467
DPM-Tr1	25.08	3.41	1.56	0.653	0.130	1.909	2.039	8.00	0.853	0.357
Mid-parent	17.56	4.44	1.38	0.326	0.094	1.757	1.852	4.00	0.855	0.412
TPM-Tr1	12.12	4.31	2.53	0.657	0.290	1.223	1.513	47.33	0.950	0.560
TPM-Tr2	14.58	0.68	1.45	0.000	0.506	1.548	2.054	0.00	0.820	0.147
TPM-Tr3	12.86	1.21	2.15	0.000	0.447	1.627	2.08	0.00	0.247	0.683
TPM-Tr4	8.24	1.16	1.40	0.000	0.322	1.286	1.608	1.00	1.207	0.227
TPM-Tr5	8.48	1.58	1.30	0.590	0.307	1.568	1.875	0.00	0.797	0.300
F-test	**	**	**	**	**	**	**	**	**	**
LSD 5%	3.87	0.36	0.37	0.037	0.050	0.171	0.220	3.12	0.174	0.089
1%	5.42	0.51	0.52	0.051	0.070	0.237	0.305	4.33	0.242	0.123

TPM-Tr$_1$ was selected to be used in the field experiment naming TPM-Tr$_8$, ** = p< 0.01

which failed in nodule formation. Reduced nitrogen fixation of the nodules formed by such altered bacteria may be due to loss of some *nif* regions results[24]. The results proposed that genomic rearrangements are the molecular basis for the variability and instability of *Rhizobium* strains. Research efforts carried in this study focused on understanding the mechanisms of such rearrangements, di- or tri-parental transconjugants trying to stabilize relevant symbiotic relationship.

It seems from the results obtained that tri-parental transconjugants of *Rhizobium* genome can generate and tolerate repeated DNA and that some repeated sequences get fixed during evolution in relation to specific physiological demands in particular strains. The results indicated that the exchange of genetic information among tri-parental strains could increase the adaptability of the *Rhizobium* genome, in regard to survival under certain soil conditions or interaction with particular plant host.

As shown from the results summarized in Table 7 that there was a significant increase in the productivity of indolic compounds by a tri-parental transconjugants TPM-Tr$_1$ from lactic acid and ethanol, TPM-Tr$_3$ from lactic acid and TPM-Tr$_5$ from ethanol. This indicated that the phytohormone indole-3-acetic acid (IAA) accumulates with significant amounts in the culture medium containing lactic acid (TPM-Tr$_1$ and TPM-Tr$_3$) and ethanol (TPM-Tr$_1$ and TPM-Tr$_5$). Plant growth-promoting IAA production from new recombinants of *Rhizobium*, suggesting that expression of indolepyruvate decarboxylase, a key enzyme in the IAA biosynthesis pathway, may be regulated by the presence of exogenous tryptophan, trypton, lactic acid and ethanol. The occurrence of IAA production from tryptophan, trypton, lactic acid and ethanol supplemented cultures is common[25].

Increased amounts of IAA productivity from lactic acid and ethanol by the previous tri-parental transconjugants over the mid-parents indicated increased transcriptional activity of *ipdC* during the growth. On the basis of these observations, it was expected that these recombinations will be useful in inoculation for the production of increased levels of IAA from different precursors.

Inoculation treatment with all tri-parental transconjugants exhibited significant amounts increased

Table 8: Effect of recombinant transconjugants resulted from tri-parental mating between, *Rhizobium leguminosarum* bv. *phaseoli* (RLbp7)x*Pseudomonas putida* x *Rhizobium leguminosarum* bv. *viciae*, on indole-acetic acid production and growth parameters of plants (25-day-plant-old) grown in pots experiment

	IAA ($\mu g\ mL^{-1}$)				Chlorophyll ($mg\ g^{-1}$)					
Inoculum	Tryptophan	Trypton	Lactic acid	Ethanol	Chl a	Chl b	Total Chl	Nodules No.	Shoot DW ($g\ plant^{-1}$)	Root DW ($g\ plant^{-1}$)
Control					0.025	1.011	1.036	0.00	0.773	0.277
RLbv	10.050	5.48	1.21	0.000	0.059	1.605	1.665	0.00	0.857	0.467
DPM-Tr2	1.160	0.74	0.71	0.530	0.228	1.596	1.824	0.00	0.483	0.173
Mid-parent	5.605	3.11	0.96	0.265	0.143	1.301	1.744	0.00	0.670	0.320
TPM-Tr1	13.700	1.42	0.62	0.550	0.472	1.179	1.651	8.00	0.673	0.283
TPM-Tr2	16.610	1.77	2.35	0.667	0.398	1.603	2.000	0.00	0.757	0.183
TPM-Tr3	13.870	1.49	0.96	0.560	0.557	1.644	2.202	9.00	1.043	0.803
TPM-Tr4	15.980	2.67	2.55	0.540	0.276	1.413	1.689	1.67	0.793	0.440
TPM-Tr5	15.920	0.80	1.77	0.530	0.231	1.245	1.476	12.33	0.480	0.230
F-test	**	**	**	**	**	**	**	**	**	**
LSD 5%	3.050	0.25	0.18	0.045	0.037	0.201	0.213	2.07	0.136	0.071
1%	4.270	0.35	0.26	0.064	0.051	0.278	0.295	2.88	0.189	0.099

TPM-Tr_3 was selected to be used in the field experiment naming TPM-T_9, ** = $p < 0.01$

in chlorophyll a concentration above the mid-parents, leading to significant increase the concentration of total chlorophyll above the uninoculated plants. The increase of total chlorophyll may resulted a significant increase in photosynthetic efficiency attributed to inoculation with ea new recombinants of *Rhizobium*. This provided highest photosynthetic efficiency, which may be attributed to adequate availability of nutrients during the growth, flowering and maturity stages. New recombinant isolate, which were superior to other strains (TPM-Tr_1) enhanced the higher number of nodule formation, may enhanced the nutrient uptake, adequate nutrition synthesis and synthesized more carbohydrates as, well as, proteins and finally protoplasm as it adapted well in the soil. For this tri-parental transconjugant (TPM-Tr_1) was selected to be used in the field evaluation.

The significantly highest dry matter production (shoot) over the mid-parents and uninoculated plants was observed from inoculation with TPM-Tr_4. Plants inoculated with TPM-Tr_1 and TPM-Tr_3 exhibited significant increase in dry matter production (root) over the mid-parents and uninoculated plants. Nodules appeared on the root system of the plants inoculated with DPM-Tr_1 and TPM-Tr_1 confirming that these genotypes were suitable for nodulation. This supports the hypothesis of a constant lag time required from *Rhizobium* infection to the appearance of a macroscopic nodule.

The results obtained are in agreement with Tricot *et al.*[26], who found a relationship between the presence/absence of nodules on a root segment and root elongation rate during the period between infection and appearance of nodules on the considered root segment. Kasperbauer and Hunt[27] showed for soybean and southern pea that greater photoassimilate allocation to roots was associated with formation of more nodules. Moreover, Merck[28] showed for pea and other legumes that increasing CO_2 atmospheric concentrations from 330 to 1000 $\mu l\ L^{-1}$ was associated with the formation of more nodules. Calvert *et al.*[29] observed that many infections formed on soybean roots, but relatively few developed into nodules. Kosslak and Bahlool[30] have shown that the number of successful infections may be affected by photosynthetic capacity of the host plant. In this regard, it has been shown that recombination between long, reiterated sequences in direct orientation leads to either amplifications or deletions both in plasmids[31] or chromosomal[4]. This study demonstrated that triplications and higher-order amplifications are formed after the intermediate generation of a duplication by either of the models presented here (di- and tri-parental mating).

The results summarized in Table 8 revealed that all tri-parental transconjugants accumulates significant amounts of the phytohormone indole-3-acetic acid in the culture medium when grown in the presence of exogenous tryptophan and ethanol in relation to the mid-parents. However, no significant amounts above the mid-parents were produced from trypton. Although, tri-parental transconjugants; TPM-Tr_2, TPM-Tr_4 and TPM-Tr_5 appeared significant amounts of IAA above the mid-parents in the presence of exogenous lactic acid. In addition, the amounts produced from tryptophan was greater than that produced from the other precursors because the enzyme indolepyruvate decarboxylase produced by the *ibdC* gene,a key enzyme in the indole pyruvic acid pathway, prefers tryptophan other than another precursors

It is reasonable to conclude that the enzyme involved in the indoleacetic acid pathway are good expressed from an operon in tri-parental transconjugants above their parents in the presence of tryptophan, lactic acid and ethanol. These new recombinants will be useful for inoculation to promote plant growth. This are in accordance with Patten and Glick[23] who found that high

Table 9: Effect of recombinants resulted from tri-parental mating between, *Rhizobium leguminosarum* bv. *phaseoli* (RLbp9)x*Pseudomonas putida* x *Rhizobium leguminosarum* bv. *viciae*, on indole-acetic acid production and growth parameters of plants (25-day-plant-old) grown in pots experiment

	IAA (μg mL^{-1})				Chlorophyll (mg g^{-1})					
Inoculum	Tryptophan	Trypton	Lactic acid	Ethanol	Chl a	Chl b	Total Chl	Nodules No.	Shoot DW (g plant^{-1})	Root DW (g plant^{-1})
Control					0.025	1.011	1.036	0.00	0.773	0.277
RLbv	10.05	5.48	1.21	0.000	0.059	1.605	1.665	0.00	0.857	0.467
DPM-Tr4	53.10	3.22	0.72	0.550	0.268	1.620	1.888	2.67	1.000	0.360
Mid-parent	31.58	4.35	0.97	0.275	0.163	1.612	1.775	1.33	0.928	0.413
TPM-Tr1	42.88	8.85	2.58	0.560	0.144	1.368	1.512	22.33	0.850	0.450
TPM-Tr2	31.71	10.51	1.41	0.700	0.031	1.185	1.216	41.67	0.930	0.457
TPM-Tr3	17.08	9.10	1.09	0.720	0.155	1.551	1.706	0.00	0.850	0.283
TPM-Tr4	53.10	11.22	1.00	0.500	0.038	1.251	1.289	46.33	0.737	0.443
TPM-Tr5	13.36	7.33	1.57	0.583	0.162	1.439	1.601	21.67	0.867	0.340
F-test	**	**	**	**	**	**	**	**	**	**
LSD 5%	3.10	1.22	0.34	0.034	0.028	0.114	0.125	4.66	0.122	0.123
1%	4.35	1.71	0.48	0.047	0.039	0.158	0.173	6.46	0.169	0.171

TPM-Tr$_4$ was selected to be used in the field experiment naming TPM-Tr$_{10}$, ** = $p < 0.01$

levels of bacterial IAA, whether from IAA-overproducing mutants or strains that naturally secrete high levels or from high-density inocula, stimulate the formation of lateral and adventitious roots. Mathis *et al.*[32] found that *P. putida* GR12-2 cells that produce wild-type levels of IAA stimulated the formation of many short adventitious roots on mung bean cuttings and an IAA-overproducing mutant stimulated the formation of even more adventitious roots than the wild-type strain.

All new recombinant isolates produced significant concentrations in chlorophyll a and most of them revealed (except TPM-Tr$_1$) significant increase in chlorophyll b, this leading of all new recombinations to appeared significant increase in total chlorophyll formation. This is an important indirect characteristic in response to rhizobial inoculation for the selection and evaluation of rhizobial new recombinations to ensure that not only are the rhizobia matched with the legume host, but also to the soil conditions. This is especially important in strain selection is considered.

Selection of rhizobia exhibiting high nodulation such as; TPM-Tr$_1$, TPM-Tr$_3$ and TPM-Tr$_5$, has an enormous impact on the economics of commercial grain production. This indicated that these strains were suitable for field evaluation, for this, TPM-Tr$_3$ was selected for field evaluation because it was exhibited significant increase in most parameters studied. There was considerable variation in nodulation between new recombinants.

Significant increase in dry matter production (root DW) above the mid-parents was shown in response to inoculation with TPM-Tr$_3$ and TPM-Tr$_4$. This may be due to significant amounts of IAA produced by these strains stimulating root elongation. This are in harmony with Jacobson *et al.*[33], who reported that 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, ACC deaminase, produced by many plant growth-promoting bacteria, is involved in the stimulation of root elongation in seedlings[34]. The benefits of constructed new recombinant transconjugants developed in this study include improved nitrogen fixation, high protein, high-value cash crops, disease breaks and reduced growth of weed species.

As shown in Table 9, the phytohormone indole-3-acetic acid (IAA) accumulates significant amounts above the mid-parents in the culture medium of all tri-parental transconjugants when grown in the presence of exogenous trypton and ethanol. However, higher amounts of IAA were produced by all tri-parental transconjugants in the presence of exogenous tryptophan above that produced from trypton, lactic acid and ethanol. Significant amounts of IAA above the mid-parents were produced by tri-parental transconjugants in the presence of exogenous tryptophan (TPM-Tr$_1$ and TPM-Tr$_4$) and lactic acid (TPM-Tr$_1$, TPM-Tr$_2$ and TPM-Tr$_5$). Higher levels of IAA produced from tryptophan and trypton suggesting that expression of indolepyruvate decarboxylase may be regulated by tryptophan and trypton, confirming that *ipdc* is induced by tryptophan and trypoton and prolonged a higher level of transcription at the different stages of cell cycle.

All bacterial cells inoculated plants showed significant increase in the concentrations of chlorophyll a (except TPM-Tr$_2$), chlorophyll b and total chlorophyll, in relation to uninoculated plants. Although, many of tri-parental transconjugants (TPM-Tr$_1$, TPM-Tr$_2$ and TPM-Tr$_4$) showed higher number of nodules developed on the root system of *Phaseolus vulgaris*. Nodulation of *Phaseolus vulgaris* ranged from non-infective, with a score of 0, to a highly effective with a nodule score of 46. Nodulation scores and shoot dry matter (showed significant increase above the uninoculated plants by DPM-Tr$_4$ and TPM-Tr$_2$) response to inoculation showed considerable variation between new recombinant isolates. Large host strain interactions were evident larger scores

Table 10: Effect of recombinants resulted from tri-parental mating between, *Rhizobium leguminosarum* bv. *phaseoli* (RLbp10)x*Pseudomonas putida* x Rhizobium leguminosarum bv. *viciae*, on indole-acetic acid production and growth parameters of plants (25-day-plant-old) grown in pots experiment

	IAA (μg mL^{-1})				Chlorophyll (mg g^{-1})					
Inoculum	Tryptophan	Trypton	Lactic acid	Ethanol	Chl a	Chl b	Total Chl	Nodules No.	Shoot DW (g plant^{-1})	Root DW (g plant^{-1})
Control					0.025	1.011	1.036	0.00	0.773	0.277
RLbv	10.05	5.48	1.21	0.000	0.059	1.605	1.665	0.00	0.857	0.467
DPM-Tr1	36.76	10.43	3.66	0.500	0.092	1.544	1.636	10.00	1.313	0.240
Mid-parent	23.40	7.96	2.44	0.250	0.075	1.574	1.650	5.00	1.085	0.353
TPM-Tr1	37.72	18.37	2.27	0.600	0.048	1.473	1.521	25.67	1.100	0.670
TPM-Tr2	44.95	17.22	1.25	0.000	0.316	2.790	3.106	23.33	0.943	0.450
TPM-Tr3	44.47	24.41	1.30	0.653	0.139	1.531	1.669	0.33	0.957	0.340
TPM-Tr4	38.64	19.35	1.48	0.000	0.151	1.776	1.928	6.67	0.897	0.367
TPM-Tr5	12.08	1.58	0.65	0.633	0.046	1.486	1.531	16.33	0.623	0.363
F-test	**	**	**	**	**	**	**	**	**	**
LSD 5%	2.06	1.27	0.33	0.024	0.007	0.266	0.266	2.82	0.185	0.063
1%	2.89	1.78	0.47	0.034	0.010	0.369	0.369	3.91	0.257	0.088

TPM-Tr$_2$ was selected to be used in the field experiment naming TPM-Tr$_1$11, ** = $p < 0.01$

of nodulation. These strains were effectively nodulated and increased dry matter yields of roots (TPM-Tr$_1$, TPM-Tr$_2$ and TPM-Tr$_4$) relative to the uninoculated control.

The results suggested that bacterial IAA plays a major role in the development of the host plant root system. Production of IAA is important in the bacterium-plant relationship. Certainly, while many adventitious roots are desirable, longer roots with more surface area through which the plants can absorb nutrients and water from the soil would be advantageous. The advantageous for shoot surface associated bacteria is a rich supply of carbohydrates through the photosynthesis, as much of the metabolic products of the carbon fixed by the plant surface, which is lost from roots and moves into the rhizosphere as exudates, lysates and mucilage[35].

Many new recombinations of tri-parental transconjugants (TPM-Tr$_1$, TPM-Tr$_2$, TPM-Tr$_3$ and TPM-Tr$_4$) as shown in Table 10, synthesize significant amounts of IAA above their parents in the presence of exogenous tryptophan and trypton. While, the amounts produced from tryptophan were higher than that produced from trypton. However, some new recombinants (TPM-Tr$_1$, TPM-Tr$_3$ and TPM-Tr$_5$) produced significant amounts of IAA from ethanol in relation to the mid-parents, while all tri-parental recombinations can not produced significant amounts from lactic acid above their mid-parents.

The presence of significant amounts of IAA produced from tryptophan, trypton and ethanol may have some relevance to its ability to colonize plant surface successfully, as shown from the significant number of nodules developed on the root system by TPM-Tr$_1$, TPM-Tr$_2$ and TPM-Tr$_5$.

The results indicated that tri-parental transconjugants may contain amplification of the *ipdC* leading to prolonged a higher level of transcription at the later stages of the cell cycle. The ability to synthesize high quantities of the plant growth regulator indole-3-acetic acid (IAA) may be due to significant amount produced from a gene encoding an aromatic aminotransferase responsible for the conversion of tryptophan to indole-3-pyruvic acid. However, the pathway of indol-3-pyruvic acid appears to be the main pathway for IAA synthesis. The synthesis of IAA via indole-3-pyruvic acid (IPyA) and indole-3-acetaldehyde (IAAld) has been proposed to occur in several non-pathogenic plant-associated bacteria[36] and in the tumorigenic *A. tumefaciens*[37].

Lower levels of IAA produced from lactic acid and ethanol in relation to the higher values produced from tryptophan and trypton, suggested the lower presence of catalytic domains for the recognition of these precursors, affecting on the gene encodes an indolepyruvate decarboxylase, a key enzyme in the IAA biosynthesis pathway[23], provided strong evidence for the importance of the IPyA pathway in IAA synthesis. Tri-parental transconjugants producing high amounts of IAA may have evolved a common mechanism to do so via transcriptional or post-transcriptional regulation of *ipdC* gene, whereas low IAA producing strains (as shown from the lower amounts produced from lactic acid and ethanol) may possess an *ipdC* gene that either is downregulated *in vitro* or encodes an *ipdC* of lower efficiency, because of lower presence of catalytic domains for the recognition of these precursors.

The common presence of amplified *ipdC* suggests that amplification of this gene confers sufficient selective advantage on the cells in their natural habitat for the maintenance of indolepyruvate decarboxylase activity in these strains. Consequently, to produce significant amounts of IAA, which has a significant role related to the growth and/or survival of bacterial strains on the plants.

All rhizobial strains including parents and tri-parental transconjugants affect to increase the concentration of

Table 11: Effect of recombinant transconjugants resulted from tri-parental mating between, *Rhizobium leguminosarum* bv. *phaseoli* (RLbp11)x*Pseudomonas putida* x *Rhizobium leguminosarum* bv. *viciae*, on indole-acetic acid production and growth parameters of plants (25-day-plant-old) grown in pots experiment

	IAA (μg mL^{-1})				Chlorophyll (mg g^{-1})					
Inoculum	Tryptophan	Trypton	Lactic acid	Ethanol	Chl a	Chl b	Total Chl	Nodules No.	Shoot DW (g plant^{-1})	Root DW (g plant^{-1})
Control					0.025	1.011	1.036	0.00	0.773	0.277
RLbv	10.05	5.48	1.21	0.000	0.059	1.605	1.665	0.00	0.857	0.467
DPM-Tr5	52.32	3.27	1.84	0.500	0.150	2.739	2.889	5.00	0.783	0.287
Mid-parent	31.19	4.38	1.53	0.250	0.104	2.172	2.277	2.50	0.820	0.377
TPM-Tr1	11.57	3.36	2.44	0.703	0.257	1.514	1.771	85.33	1.260	0.433
TPM-Tr2	20.91	1.5	1.34	0.517	0.175	1.333	1.508	46.00	1.007	0.163
TPM-Tr3	20.57	5.7	3.11	0.000	0.336	1.812	2.148	22.33	0.670	0.293
TPM-Tr4	15.21	7.85	1.22	0.640	0.193	1.035	1.228	87.33	1.420	0.553
TPM-Tr5	10.96	9.72	2.34	0	0.277	1.324	1.601	43.00	0.630	0.310
F-test	**	**	**	**	**	**	**	**	**	**
LSD 5%	2.59	0.68	0.22	0.030	0.025	0.268	0.265	6.02	0.347	0.079
1%	3.62	0.96	0.31	0.042	0.034	0.372	0.368	8.35	0.481	0.109

TPM-Tr$_4$ was selected to be used in the field experiment naming TPM-Tr$_{12}$, ** = $p < 0.01$

chlorophyll a, b and total above the uninoculated plants. Rhizobial strains produced significant dry matter (root) yield above uninoculated plants included all strains present in Table 10, except for DPM-Tr$_5$.

The results indicated that nodulation and dry matter (shoot or root) responses varied between strains. New recombinant isolates that produced the highest concentration of chlorophyll a (TPM-Tr$_2$, TPM-Tr$_3$ and TPM-Tr$_4$), chlorophyll b (TPM-Tr$_2$), total chlorophyll (TPM-Tr$_2$ and TPM-Tr$_4$), nodules (TPM-Tr$_1$, TPM-Tr$_2$ and TPM-Tr$_5$) and root dry weight (TPM-Tr$_1$ and TPM-Tr$_2$) above the mid-parent indicated highlighting the importance of matching new recombinant isolates to the host legume and the need for ongoing evaluation of rhizobia new recombinations. However, these pots results must be verified in the field, because environmental conditions are controlled in pots experiments.

Strong correlations between pots and field results were not always evident[38], the 1998 field study of these authors showed that the performances of some highly-effective strains in the glasshouse could not be duplicated in the field. However, there was considerable variation in most of symbiotic parameters, the tri-parental transconjugants (TPM-Tr$_2$) performing better symbiosis than the mid-parents was selected to be used in field evaluation. The effective nodulation, often an indicator of the grain yield responses to inoculation in the field as good nodulation generally produced high grain yields[38].

In spite of prevailing negative results in the survey of legume nodulation, it is still reasonable to assume that a full deficiency in *nod* gene inducers in the host will markedly inhibit nodulation as shown by TPM-Tr$_3$ and the parental strain (RLbv), which can not formed nodules on the root system of *Phaseolus vulgaris*[39]. The *nod* gene inducing activity observed in the host plant upon inoculation with rhizobia is related to increased nodulation gene-inducing activity. A corresponding increase in flavonoid production has been shown to be elicited by simple Nod factor, the product of *nod* genes[40]. The results obtained in this study are in agreement with Novak *et al.*[41] who found that the reductions in *nod* gene-inducing activity found are associated rather with the pleiotropic action of a mutation on the root growth instead of the nodulation block itself. It should be noted that the observed decrease in the inducer level appears not to be sufficient to cause a significant block of nodulation. On the other hand, over-production of *nod* gene products can be detrimental to the effective nodule formation as well[42], whereas flavonoid metabolism was affected in a less extent.

From the results presented in Table 11, it is well established that many tri-parental transconjugants; TPM-Tr$_3$, TPM-Tr$_4$ and TPM-Tr$_5$ (in the presence of exogenous tryptophan); TPM-Tr$_1$, TPM-Tr$_3$ and TPM-Tr$_5$ (in the presence of exogenous lactic acid); TPM-Tr$_1$, TPM-Tr$_2$ and TPM-Tr$_4$ (in the presence of exogenous ethanol) are able to synthesize significant amounts of the phytohormone indole-3-acetic acid (IAA). All of these new recombinations can produce and excrete significant amounts of IAA in their cultures more than the parental strains. However, the amounts of IAA produced from tryptophan were higher than that produced from the other precursors.

The results obtained herein are in harmony with Omer *et al.*[3] who found that IAA producing strains accumulated the phytohormone in amounts ranging from 6 to 13.3 mg L^{-1} in the presence of L-tryptophan and when L-tryptophan was not supplemented to the culturing medium, the production of IAA was in the range 1.1-2.4 mg L^{-1}. This agreement with other results amino acid is a precursor for IAA in most studied IAA-producing bacteria.

The importance of significant amounts produced from IAA is involved in the stimulation of root growth.

Table 12: New recombinant isolates of azide resistant mutants, di- and tri-parental transconjugants used in the field experiment

Original name of the isolates in pots experiment	Source or reference	Naming in the field experiment after selection
Az^r_9	RLbp7	Az^r_1
Az^r_{12}	RLbp7	Az^r_2
Az^r_2	RLbp9	Az^r_3
Az^r_{11}	RLbp9	Az^r_4
Az^r_{25}	RLbp11	Az^r_5
Az^r_9	RLbp11	Az^r6
DPM-Tr_4	RLbp2 x RLbv	DPM-Tr_1
DPM-Tr_4	RLbp5 x *Pseudomonas putida*	DPM-Tr_2
DPM-Tr_1	RLbp7 x *Pseudomonas putida*	DPM-Tr_3
DPM-Tr_3	RLbp9 x *Pseudomonas putida*	DPM-Tr_4
DPM-Tr_3	RLbp10 x *Pseudomonas putida*	DPM-Tr_5
DPM-Tr_5	RLbp11 x *Pseudomonas putida*	DPM-Tr_6
TPM-Tr_3	RLbp2 x RLbv x *Pseudomonas putida*	TPM-Tr_7
TPM-Tr_1	RLbp5 x *P. putida* x RLbv	TPM-Tr_8
TPM-Tr_3	RLbp7 x *P. putida* x RLbv	TPM-Tr_9
TPM-Tr_4	RLbp9 x *P. putida* x RLbv	TPM-Tr_{10}
TPM-Tr_2	RLbp10 x *P. putida* x RLbv	TPM-Tr_{11}
TPM-Tr_4	RLbp11 x *P. putida* x RLbv	TPM-Tr_{12}

Efficient producing strains were active in the *ipdC* gene encoding indolepyruvate decarboxylase, which catalyzes a key step in the indolepyruvic acid pathway for IAA synthesis. The lower amounts of IAA were produced from trypton, lactic acid and ethanol in comparison to that produced from tryotophan may be due to the enzymes involved in the IAA pathway which may not good expressed from an operon.

This provides more support for the hypothesis[44] that plant growth-promoting bacteria, produce IAA via the indolepyruvic acid pathway, in contrast to plant pathogens, which seem to preferentially synthesize IAA via the indoleacetamide pathway[45] .

All rhizobial strains (parental) and their new recombinants affect to significant increase the concentration of chlorophyll a, chlorophyll b (except for TPM-Tr_4) and total chlorophyll (except for TPM-Tr_4), in relation to uninoculated plants. However, all tri-parental transconjugants affect to significant increase the concentration of chlorophyll a and the mean number of nodules per plant, in relation to the mid-parents.

It can be concluded that all new recombinants presented in Table 11 revealed higher activity of *nod* gene in response to an exogenous *nod* gene inducer. Novak *et al.*[41] demonstrated that the symbiotic blocks in the studied mutant lines of pea are caused neither by the changed inducer level in exudate nor by the constitutive activity of the defense mechanism. The losses of naringenin present in the root exudates may be due to the degradation by the bacteria and by the plant[46] and from the absorption by the root. The concentration (1 μg mL^{-1}) of naringenin was shown to affect positively nodulation in the wild-type plants[47].

The results indicated that higher number of nodules ranged between 22-87 per plant appeared on the root system of the plants, thus confirming that these genotypes were efficient for nodulation. Nodulation scores produced by all tri-parental transconjugants affect to significantly increase shoot dry matter in response to inoculation with TPM-Tr_1 and TPM-Tr_4 and root dry matter in response to inoculation with TPM-Tr_4, relative to the mid-parents.

Large host-strain interactions were evident in nodule scores and dry matter production. The recombinant strain that produced the highest number of nodule per plant and the highest dry matter yield (shoot and root) was tri-parental transconjugant TPM-Tr_4. The best productive strain in terms of nitrogen fixation and symbiotic parameters (IAA production, chlorophyll a, nodulation, dry matter production including shoot and root) was TPM-Tr_4. This highlighting the importance of this rhizobial strain for matching the host legume and the need for ongoing evaluation these strain under the field conditions. Though, this recombinant (TPM-Tr_4) was selected to be evaluated in the field.

Azide-resistant mutants derived from three strains of *R. leguminosarum* bv. *phaseoli*, di- and tri-parental mating isolates were evaluated for symbiotic characterization with *Phaseolus vulgaris* as described before in Tables from number 7 to number 11. Thus, the recombinants (2 Az^r mutants from each strain and one isolate from each mating) showed improvement in most traits related to the efficiency of legume-*Rhizobium* symbiosis, is likely to result in increased availability of nitrogen for production of high quality food for human consumption, were selected to be evaluated for competitiveness in the field. Selected recombinants used in field experiments taken were another names differed from that in the pots experiment as shown in Table 12.

The new recombinant rhizobial isolates presented in Table 12 are selected on the basis of the results obtained from previous results in pots experiment using the variety

Table 13: Effect of new recombinants on different economic traits of common bean variety Nebraska grown in pots at 25-day-plant-old

Strain	Chl a (mg g^{-1})	Chl b (mg g^{-1})	Total (mg g^{-1})	Leaf Area (cm^2)	Nodule No./plant	Root DW (g $plant^{-1}$)	Shoot DW (g $plant^{-1}$)
Uninocu.	0.23	2.16	2.39	1078	0	0.14	0.41
Psdp	0.24	2.54	2.77	1324	0	0.40	0.66
RLbv	0.24	2.20	2.44	1508	0	0.18	0.40
RLbp2	0.30	2.37	2.67	847	26	0.30	0.88
RLbp5	0.19	2.28	2.47	1357	22	0.45	0.67
RLbp7	0.27	1.69	1.96	1437	13	0.29	0.99
RLbp9	0.24	2.53	2.77	1258	57	0.49	1.31
RLbp10	0.23	1.97	2.19	1366	19	0.42	1.16
RLbp11	0.24	2.46	2.69	1489	4	0.32	0.95
Az^r_1	0.37	2.86	3.23	1521	35	0.43	0.96
Az^r_2	0.53	2.25	2.78	1024	42	0.41	1.37
Az^r_3	0.29	1.87	1.91	718	5	0.23	0.55
Az^r_4	0.41	3.53	3.94	1029	50	0.25	0.91
Az^r_5	0.58	2.54	3.12	709	6	0.24	0.65
Az^r_6	0.40	2.59	2.99	1078	0	0.22	0.46
DPM-Tr_1	0.13	1.54	1.67	1146	9	0.52	0.86
DPM-Tr_2	0.36	2.54	2.90	1790	13	0.54	1.26
DPM-Tr_3	0.35	2.39	2.74	1023	24	0.34	0.57
DPM-Tr_4	0.26	2.06	2.32	1170	53	0.37	1.08
DPM-Tr_5	0.30	2.33	2.63	1355	24	0.26	0.96
DPM-Tr_6	0.26	2.56	2.82	1481	45	0.26	1.06
TPM-Tr_7	0.38	2.95	3.33	1160	20	0.46	1.13
TPM-Tr_8	0.39	2.80	3.19	1112	20	0.36	1.21
TPM-Tr_9	0.35	2.83	3.18	1514	45	0.44	1.17
TPM-Tr_{10}	0.31	2.50	2.81	1557	11	0.67	1.41
TPM-Tr_{11}	0.08	1.76	1.84	1197	0	0.46	1.13
TPM-Tr_{12}	0.24	2.44	2.68	981	51	0.33	1.12
F-test	**	**	**	*	**	**	**
LSD 5%	0.114	0.38	0.39	400	4	0.12	0.22
1%	0.152	0.51	0.52	533	5	0.16	0.30

* and ** = $p< 0.05$ and $p< 0.01$, respectively

Giza 6 were evaluated again in pots experiment using variety Nebraska of common bean.

The data presented in Table 13 revealed the effect of different inoculant genotypes on nodulation, chlorophyll concentrations and growth rate of common bean (Nebraska). The obtained data revealed that all new recombinant isolates affect to significantly increase two parameters (except Az^r_3) or more from seven measured in this experiment. However, three isolates (Az^r_1, DPM-Tr_2 and TPM-Tr_9) affect to significantly increase all the seven traits measured in this experiment and two isolates (TPM-Tr_7 and TPM-Tr_8) were affect to significantly increase six of seven traits. This indicated that the variety Nebraska of common bean was observed to be a good responsive variety to inoculation.

This could probably be due to the ability of this variety to adapt with rhizobial inoculant. The number of nodules developed on the root system of common bean Nebraska variety were ranged between; 3-57 (parental strains), 0-50 (Azide resistant mutants), 8-52 (di-parental transconjugants) and 0-51 (tri-parental transconjugants). These differences in nodule formation may be due to a strangely conserved regulatory sequence found upstream of all inducible *nod* genes[48]. However, negative regulation of *nod* gene expression has been reported for some genetic backgrounds is mediated by a putative repressor recognizing the RNA polymerase binding site[49]. The mechanism of interaction between Nod D, the *nod* box, the flavonoids, the repressor (if such is the case) and RNA polymerase to promote expression is not yet well established[50].

The common nodulation genes *nod* A, *nod* B and *nod* C have been found in different *Rhizobium* species. Recent studies on the function of the Nod proteins suggest that the Nod A and Nod B proteins are involved in generating small, heat-stable compounds that stimulate the mitosis of various legume and non-legume protoplasts[51]. The Nod A protein is found in the cytoplasm and cell envelope[52] and the Nod B protein is also located in the cytosol[51]. Nod C is a cell surface protein with a eukaryotic receptor-like structure, which may serve as a transducer of an intracellular bacterial signal to root cells[53]. The product of the *nod* D gene, acting as a positive regulator in combination with plant flavonoid compounds, induces the expression of common *nod* ABC genes and other *nod* genes.

Many flavonoid compounds produced by the seeds or roots exudates have been described as having a particular capacities to induce or repress transcription of the common *nod* ABC genes and other *nod* genes[54]. This specificity depends on the presence of the different *nod* D products described for each species. The R.

Table 14: Effect of inoculation with new recombinant rhizobial inoculant on the biochemical traits of *Phaseolus vulgaris* variety Nebraska grown under the field conditions

Inoculum	Shoot nitrogen (mg g^{-1})	Shoot nitrogen (%)	Seed protein (mg g^{-1})	Seed protein (%)	Grains dry weight (g $plant^{-1}$)	100-grain weight (g)
Uninocu.	12.12	1.21	230.5	23.05	22.50	40.07
Psdp	17.21	1.72	245.7	24.57	21.83	41.40
RLbv	22.42	2.24	253.0	25.30	31.17	43.71
RLbp2	21.84	2.18	258.8	25.88	17.63	41.80
RLbp5	20.80	2.08	273.2	27.32	24.83	38.63
RLbp7	15.94	1.59	306.5	30.65	20.17	43.73
RLbp9	21.26	2.13	247.9	24.79	17.30	37.80
RLbp10	13.04	1.30	236.3	23.63	22.80	37.87
RLbp11	22.65	2.27	276.8	27.68	21.87	39.60
Az^r_1	12.58	1.26	307.9	30.79	22.77	39.13
Az^r_2	17.33	1.73	304.3	30.43	20.40	38.13
Az^r_3	16.86	1.69	279.7	27.97	16.74	37.47
Az^r_4	21.49	2.15	270.3	27.03	29.20	45.00
Az^r_5	20.57	2.06	234.2	23.42	30.41	37.53
Az^r_6	12.58	1.26	297.8	29.78	31.83	41.73
DPM-Tr_1	20.57	2.06	266.7	26.67	17.23	43.87
DPM-Tr_2	14.55	1.45	268.9	26.89	16.80	43.87
DPM-Tr_3	12.81	1.28	279.7	27.97	26.40	40.00
DPM-Tr_4	16.75	1.67	310.8	31.08	12.57	42.93
DPM-Tr_5	19.53	1.95	336.2	33.62	32.67	47.67
DPM-Tr_6	12.70	1.27	313.0	31.30	23.07	43.67
TPM-Tr_7	12.81	1.28	348.5	34.85	20.17	45.63
TPM-Tr_8	16.17	1.62	276.8	27.68	33.47	43.47
TPM-Tr_9	18.02	1.80	336.2	33.62	17.33	40.15
TPM-Tr_{10}	16.28	1.63	314.5	31.45	18.23	45.93
TPM-Tr_{11}	22.65	2.27	347.7	34.77	19.87	38.87
TPM-Tr_{12}	17.21	1.72	375.9	37.59	18.67	41.07
F-test	**	**	**	**	**	**
LSD 5%	3.50	0.35	18.6	1.86	2.40	2.86
1%	4.65	0.47	24.8	2.48	3.20	3.81

** = $p < 0.01$

leguminosarum bv. *phaseoli* group has been classified into two types. Type I strains are defined by the reiteration of the *nif* HDK genes and a narrow host range of nodulation. In contrast, type II strains have one copy of the *nif* HDK genes and have a broad range of nodulation[55].

Hence, failure nodulation in some strains RLbv, Az^r_6 and TPM-Tr_5 may be due to repress transcription of the common *nod* ABC genes. In contrast to these strains, other strains appeared good nodulation ability, may be due to induce transcription of the common *nod* ABC genes. This specificity depends on the presence of the different *nod* D protein binds to the nod box, a strongly conserved regulatory sequence found upstream of all inducible *nod* genes.

Symbiotic effectiveness of new recombinations induced in *Rhizobium leguminosarum* under the field conditions: As shown from the results presented in Table 14 that there was a significant increase in chlorophyll a, b and total, shoot nitrogen and seed protein percent, above the uninoculated plants in response to inoculation with RLbv and DPM-Tr_1. All rhizobial strains, azide resistant mutants, di- and tri-parental transconjugants (except for Psdp, RLbp9 and RLbp10) were affect to markedly increase seed protein percent above that from uninoculated plants. This indicated effectiveness of N_2 fixation which all may had multiple copies of the structural nitrogenase genes. In addition, all parental strains (except for RLbp10), Az^r_1, Az^r_6, DPM-Tr_2, DPM-Tr_3, DPM-Tr_6 and TPM-Tr_7 revealed significant increase in shoot nitrogen percent above the uninoculated plants.

The results obtained herein provided novel information on the symbiotic effectiveness of these strains, which found from symbiotic characteristics to be effective in N_2 fixation with their host. The mean percent of seed protein in inoculated plants showed markedly increase ranged between 25.30-37.59, these values were significantly above that observed (23.05) in uninoculated plants. Interestingly, the parental strains (RLbv, RLbp2, Rlbp5, Rlbp9 and Rlbp11), azide resistant mutants (Az^r_2, Az^r_3, Az^r_4 and Az^r_5), di-parental transconjugants (DPM-Tr_1 and DPM-Tr_5) and tri-parental transconjugant (TPM-Tr_9 and TPM-Tr11) were shown to be highly efficient in N_2 fixing and induces an efficient translocation of fixed N to both the shoot and grains[56]. However, it is clear from the results that there were some strains does not induces an efficient translocation of fixed N to the grains, these including the parental strain (RLbp9 and RLbp10) and azide mutant (Az^r_5).

This emphasizes that strains recommended for commercial inocula must be able to perform and

translocate fixed N to all plant parts and grains very well under the soil conditions in which they are to be used. This need to acclimatize strains to be more competitive than the original ones, this may indicate a method for improving the competitivity of strains that have particularly desirable properties. However, some of di-parental transconjugant (DPM-Tr_2) isolates is capable to increase nitrogen fixation and induces well translocation of fixed nitrogen to the grains. Interestingly, most strains (RLbv, RLbp10, RLbp11, DPM-Tr_1 and TPM-Tr_{12}) are capable to significant increase grain protein percent above that in uninoculated plants. These results suggest that the rhizobia genotype plays a major role in determining the outcome of *P. vulgaris*, however, the host genotype plays a similar role in this respect.

Grain protein percent was significantly affected by inoculation with 17 new recombinants out of 18 isolates and by five out from eight parental strains used for evaluation. Grain yields and their contents of protein reflected nitrogen fixation efficiency. Higher shoot and grains N concentration indicates an efficient uptake of nitrogen by rhizobia-inoculated plants[57]. The concentration of shoot nitrogen is directly linked to protein content in grains among plants subjected to different inoculant strains. Nitrogen-fixing bacteria in close association and nodulated legume plants such as *P. vulgaris* are beneficial to plant growth and yield. These effects are attributed primarily to improved root development and enhanced water and mineral uptake.

Application of some new recombinants had marked influence on the number of pods per plant, 100-grain weight and grains yield per plant (Table 14). Application of some new recombinants including azide resistant mutants (Az^r_4, Az^r_5 and Az^r_6), di-parental transconjugants (DPM-Tr_3 and DPM-Tr_5) and tri-parental transconjugant (TPM-Tr_8) significantly increased grains dry weight per plant over uninoculated plants. However, similar increase in grains dry weight per plant over uninoculated plants was shown in response to only one parental strain out of eight parental strains evaluated. This indicated that plants inoculated with new recombinants improved the grains weight per plant. Thus, three new recombinants (Az^r_4, DPM-Tr_5 and TPM-Tr_8) out from six (Az^r_4, Az^r_5, Az^r_6, DPM-Tr_3, DPM-Tr_5 and TPM-Tr_8) given significant increase in the grains yield per plant

Further strains efficient in nitrogen fixation and increased accumulation of dry matter might have helped for better translocation of photosynthates and activation of enzymes like dehydrogenases, aldolases, RNA and DNA polymerases required for the synthesis of nucleic acids and proteins. For this, the presence of effective *Rhizobium* population in the root zone is considered essential. Therefore, artificial seed and plant inoculation of effective strains of *Rhizobium* near the root zone. Inoculation with efficient new recombinant isolates might have increased the nitrogen concentration, which ultimately led to improved growth and photosynthetic surface and finally increased the crop growth leading to increased the output of the plants such as, grain protein content and grains yield per plant.

The results obtained herein are in agreement with Popescu[58] who found that all inoculated plants, independent of fertilization applications, enhanced grain yields as compared to the non-inoculated control. The same author found two bacterial strains, FL_{100} and $CIAT_{45}$, were the most efficient in increasing the yield of *Phaseolus vulgaris* L. The significant yield increases induced by the two strains were noted at 0 and 30 kg N ha^{-1} for $CIAT_{45}$ and at 30 kg N ha^{-1} for FL_{100}, greater enhancing nitrogen fertilization rates being non-economic.

However, common bean (*Phaseolus vulgaris* L.), is one of the major sources of protein in Egypt, usually achieves grain yields that are less than the genotypic potential of the various cultivars. High temperatures in the summer season in Egypt influence symbiotic nitrogen fixation and assimilation and reduced yield. These negative effects need to be investigated further, bearing in mind that common bean only derives approximately 30% of its N from symbiotic N_2 fixation[59]. For this, this study investigated the symbiotic contribution to common bean production in the field using genetically modified rhizobial strains in the presence of large native rhizobial populations.

The results obtained revealed that common bean productivity is markedly affected by rhizobial inoculant. In addition, grain yields were significantly influenced by six out from 18 new recombinant isolates and by one out of eight parental strains. The significant amount of grain yields per plant was ranged between 26.4-33.47 gram/plant. Popescu[58] also found that grain yields of common bean were significantly affected by inoculation with strains, by experimental years and by the interaction between bacterial strains and N-fertilization treatments, but not by the fertilization applications, independently of the N-fertilization treatments, seed inoculation with strain FL400 significantly increased yield.

In conclusion, from the results presented in this study, it is evident that isolation of azide resistant mutants (Az^r) of *Rhizobium* in the present work support the hypothesis that in nitrogen fixing organisms, there can be an additional class of *azi* mutations, which affects the nitrogen. Similar Az^r mutants with improved symbiotic effectiveness have also been reported before in *R. leguminosarium* bv. *viciae*[61] and in *R. phaseoli* [60]

Identification of hyper-nitrogen fixing Azr mutants in three different *Rhizobium* species indicates that the resistance to sodium azide can be used as a technique to enrich populations of mutations with enhanced symbiotic effectiveness[61].

The fact that nitrogen fixation is a greater in a strains containing two functional operons as compared to a mutant with only one functional operon suggests that genetic manipulation of *Rhizobium* is needed to amplify nitrogen fixation genes, as shown in this study concerning the isolates resulted from di- and tri-parental matings. The exchange of genetic information among strains could increase the adaptability of the *Rhizobium* genome, in regard to survival under certain soil conditions or interaction with particular plant hosts. However, it is not known at what frequency transfer of genetic information occurs in nature. Understanding the degree and limits of genetic exchange in nature will have very important implications in view of the interest in introducing strains harboring recombinant genomes in the soil. Though, tandem amplifications in prokaryotes are commonly viewed as a rapid and reversible adaptation of a cell population to certain environmental factors that require over-expression of some genes[62]. The often observed phenomenon of rhizobia losing some of their ability to nodulate or fix dinitrogen as shown in the parental strains of *R. phaseoli* used in this study, is more likely due to genomic instability[5] than actual plasmid loss. Deletions or rearrangements within chromosomal DNA may also result in reduced symbiotic abilities[63].

In the current study, use of transconjugants resulted from di- and tri-parental mating as inoculant proved to be of significant importance in nodulation, which shown better in triparental transconjugants than the parental strains and diparental transconjugants, this leading to improving biological nitrogen fixation and the yield of common bean probably because the use of inoculant harboring recombinant genomes including tandem amplifications producing overexpression of some genes has enhanced the adaptation and competitiveness of introduced inoculant over the indigenous rhizobia. The competitive ability of rhizobial strains is reported to be the key factor in inoculation success of rhizobial strains[64].

All tri-parental transconjugants which constructed and evaluated in this study harboring recombinant genomes from *Pseudomonas putida* to be able to bioremediation of phenolic compounds, which may present in root exudates, thus illustrating the natural phenomenon of adaptation to phenolic compounds present in their nearby environment, which stimulate symbiosis, nodulation and nitrogen fixation by the constructed tri-parental transconjugants. The present investigation focus on the stimulaneous nodulation of these inoculant as compared to the parental strains. For this, the symbiotic performance of *Phaseolus vulgaris* was correlated with the percentage of nodules occupied by the effective strains. This study indicates the possibility of enhanced N_2 fixation and, consequently, the yield of *Phaseolus vulgaris* under field conditions. The present study suggested that it is possible to select rhizobial strains harboring recombinant genomes with good field effectiveness and stability and that their contribution to grain yield could lead to enhanced results in responsive cultivars.

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