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Research Article

EXPRESSION OF PHOSPHATE SOLUBILIZING GENES IN RHIZOBIUM TRANSCONJUGANTS

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Abstract

Rhizobium as a Gram-negative bacteria play a significant role in phosphate solubilization. This study was designed to identify phosphate-solubilizing ability of *Rhizobium* transconjugants. About 10 of *Rhizobium* wild types were isolated from fenugreek and lentil hosts. The strains are genetically marking based on their resistance or sensitivity to different antibiotics. Conjugation was done between both strains nodulating the opposite hosts that harboring the opposite genetic markers. Eight conjugations were done, from each, eight recombinant transconjugants were evaluated for phosphorus solubilizing abilityon medium supported with tri-calcium phosphate as insoluble source of phosphate. Most transconjugants derived from the conjugations between P_2 x P_6 and P_5 x P_7 revealed high capacity in P solubilization after six days of incubation time and then auto consumption at nine days. All transconjugants derived from the conjugations between P_3 x P_7, P_3 x P_9, P_4 x P_6 and P_4 x P_7 appeared gradually increase in P solubilization till reached the maximum at nine days. High heritability estimates coupled with high genetic advance as a percent of mean were obtained in P solubilization at all time intervals, indicating the main impact of genetic effects on the gene expression of P solubilization. All *Rhizobium* transconjugants having a broad host range among fenugreek and lentil with better nodulation ability on both hosts. All transconjugants improved plant growth parameters in relation to both controls and their mid-parents. The results position *Rhizobium* recombinants as an excellent biofertilizer in sustainable agriculture to establish an effective nitrogen-fixing root nodule symbiosis with a broad host range.

Keywords: *Rhizobium* transconjugants, Phosphate solubilization, Genetic marking, conjugation, Biofertilization, Recombinant ratio, Tri-calcium phosphate.

INTRODUCTION

There are two components of phosphorous (P) in the soil inorganic and organic phosphates. A large values are present in insoluble forms which were not available for plant assimilation (Rodríguez *et al*., 2006). Inorganic phosphates were mostly present in insoluble mineral complexes. Some of them were appeared after chemical fertilizers applied to the soil. These are precipitated soon in unavailable forms and cannot be absorbed by plants. The organic matter of immobilized phosphate is an important reservoir that accounts for 20-80% of soil phosphates (Richardson 1994). Most lands are deficient in phosphorus solubility over the world. Phosphorus is an essential macronutrient to the development of plants and for their growth. P is the determining factor for crop production in sub topical and tropical locations (Schneider *et al.,* 2010). Phosphorous fertilizers are expensive due to their imported or industrialization from imported raw materials. Farmers are used chemical fertilizers to overcome P limitation. P present in many soils in the range of 1 µM, meanwhile, approximately 30 µM was required for optimum crop production (Sashidhar and Podile 2010). Phosphorous availability estimated only 0.1% of total phosphorous pool in the lands (Illmer and Schinner 1995). The organic forms of phosphorous was resulted from plant tissues, microorganisms and decaying animal cells (Behera *et al.,* 2013). The largest reserves of phosphorus are rock deposits. Organic acids produced by microorganisms as a metabolic by products can be dissolved the rocks. This process was beneficial to plants and microbes both (Chang and Li 1998). The used of phosphate from rock sourcesin association with phosphate solubilizing bacteria has became animportant alternative pathway to high expensive manufacture chemical fertilizers (FAI 2002). Phosphate fertilizers added to agriculture soil are converted in alkaline soils into Ca or Mg phosphates and Fe, Al and Mn phosphates in acid soils which are insoluble and unavailable to the plants (Arcand and Schneider 2006). Bacteria belonging to genera *Rhizobium* release phosphorous from insoluble organic forms via enzymatic pathway (Rossolini *et al.,* 1998). Moreover, mineral P solubilization was associated with low-molecular weight organic acids produced by *Rhizobium* mainly gluconic and 2-ketogluconic acids (Rodríguez *et al.,* 2006). These organic acids chelate the cations of Ca, Mg, Fe, Al bound with insoluble phosphate forms and then transform its into the soluble forms. Gram negative bacteria as *Rhizobium* has produced in the periplasm lower molecular weight organic acids via the direct oxidation of non-phosphorylated glucose pathway (DOPG). This pathway used gluconate dehydrogenase (GADH) and glucose dehydrogenase (GDH) which oriented outside cytoplasmic membrane to be oxidize glucose inside the periplasmic space (Anthony 2004). The factor redox pirroquinolin quinone (pqq) was required with these enzymes, whose biosynthesis operon consisted of at least 5-7 genes (Kim *et al.,* 2003). The pqq was important for bacteria that expressed on the phenotype of phosphate solubilization (Shen *et al.,* 2012). Plants cannot grow and development in the absence of a regular phosphorous supply because it is a main ingredient of important compounds in the cell as phospholipids, ATP and nucleic acids. It was involved in the different metabolic pathways as enzyme activity, energy transfer reactions, photosynthesis and cell division (Khan *et al.,* 2014). Gram negative bacteria as *Rhizobium*has released different organic acids into the land, leading them are more important keys than bacteria expressed gram-positive in solubilizing mineral phosphates (Kumar *et al.,* 2018). Metal ions chelation that linked with a phosphorous complex forms by inorganic and organic acids released by bacteria can solubilize phosphates of insoluble forms,

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enabling them to release the absorbed P (Pradhan and Sukla 2005). The solubility of organic phosphate complex into utilizable forms named mineralization mediated by bacterial enzymes that plays a main role in P cycling in the soil (Sharma *et al.,* 2013). The enzymes mediated mineralization of organic P including phospholipase, phosphatases, phosphoesterase, phytases and phosphonatases (Kalayu 2019). There are three gene families referred as pho A, pho D and pho X that express for phosphatases alkaline, common known in prokaryotes. Alkaline phosphatase produced free soluble inorganic P from various containing phosphateorganic compounds to be providing bacteria solubilize inorganic P as nutrient (Neal *et al.*2017). The genes expressed on pqq production are included in the phenotype of phosphate dissolving expression through glucose oxidation pathway that produced gluconic and 2-ketogluconic acids. Glucose oxidation pathway was dependent on glucose dehydrogenase (Rodríguez *et al*., 2000). The express of *gcd* gene was glucose dehydrogenase that responsible for inorganic P complex solubilization (Liang *et al.,* 2020). The *gab Y* gene is responsible for encoding gluconic acid and releasing mineral phosphate solubilization. Gluconic acid was produced through the direct oxidation of glucose which plays a principal role in P solubilization in the soil (Rodríguez*et al.* 2006). The introduced of genes or their over expression related to organic and inorganic P solubility is a promising technique for enhancing *Rhizobium* efficiency to be use as a biofertilizer inoculants (Sharma *et al.,* 2013). The expression of phosphate solubilizing genesin *Rhizobium* is a promising tool for achieving the mechanism of P dissolving which leading it as an attractive option in the field of biotechnology. Different plant growth promoting rhizobacteria (PGPR), as well as, rhizosphere bacteria are capable to increasing phosphorous availability in the soil either by solubilization of inorganic phosphate via organic acids production or by mineralization of organic phosphate (Rodríguezand Fraga 1999). *Rhizobium* is one of the most powerful P solubilizers. They are the first bacterial group attributed with their ability to solubilize insoluble phosphates (Johri *et al.,* 2003). Rhizobia involved in symbiotic nitrogen fixation require phosphorus as energy for their survival and growth (Sarker *et al.,* 2014).

Plants acquire phosphate in a solution form as phosphate anions (Schubert *et al.,* 2020). Different bacterial species as *Rhizobium* can solubilize insoluble P compounds as di-calcium phosphate, tri-calcium phosphate and rock phosphate within the soil (Li *et al.,* 2020). The role of *Rhizobium* in solubilizing inorganic phosphates making them available to plants is well documented as biofertilizers since the 1950s (Corpas *et al.,* 2013). It was played fundamental roles in biogeochemical phosphorus cycling in agricultural ecosystems. The application of phosphate-solubilizing *Rhizobium* transconjugants in soils and chemical fertilizers will release soluble phosphorus promote plant growth and productivity. These microbes can transform insoluble P to soluble forms of H_2PO^{-4} and HPO_4^{-2} through exchange reactions, acidification, polymeric substances formation and chelating (Lara *et al.,* 2013). In general, phosphorus should be the main focus in sustainable agriculture. These is a growing interest in the application of genetically improved microbial genotypes to reduced the cost of chemical fertilizers. One of the most essential macronutrients is phosphorus among all biota to maintain basic metabolic activities and ecosystem functions (Mogollón *et al*., 2021). The *gcd* gene exhibited quinoprotein glucose dehydrogenase was the most reliable biomarker for detecting the phenotype of phosphate-solubilizing bacteria (Wu *et al.,* 2022). The same authors found significant association between genome size and the copy number of *gcd*, as well as, with the metabolism of polysaccharides (fructose, starchand mannose metabolism). Increasing organic carbon availability in the soil exhibited the microbial solubilization of phosphates (Huang *et al.,* 2021). So, genome size increase with *gcd* gene copy number may play an adaptive trait toward phosphate solubilization that improve the ability of bacteria in P solubilization (Wu *et al.,* 2022).Therefore, this study was conducted to test phosphorus solubilization ability of *Rhizobium* transconjugants generated from the conjugation between fenugreek and lentil rhizobia.

MATERIALS AND METHODS

Genetic materials

Fenugreek (Giza 30) and lentil (Giza 9) seeds were obtained from Field Crops Research Institute, Agriculture Research Center, Egypt, to be used in this study as hosts for *Rhizobium* strains and their transconjugants.

Bacterial strains

Wild type isolates of *Rhizobium sp.* were isolated from the root nodules of fenugreek and lentil according to Karanja and Wood (1988), through the winter season of academic year 2022-2023 (Table 1).

Table 1. Bacterial strains used in this study and their sources

Strains	Source	Designation
Rhizobium sp.	Root nodules of fenugreek	R_1, R_2, R_3, R_4, R_5
Rhizobium sp.	Root nodules of lentil	R_6, R_7, R_8, R_9, R_{10}

The strains were affirmed as *Rhizobium* by growing on YEMA with congo red, phosphate solubilization capacity and nodulating capacity on homologous hosts by plant contamination tests. The authenticity of these isolates as pure cultures of *Rhizobium* is confirmed later by the nodulation test (authentication) under bacteriological controlled conditions according to Somasegaran and Hoben (1985).

Growth conditions

Yeast extract mannitol agar medium (YEMAM): It was used as a complete synthetic medium according to Allen (1959). However, YEMAM with Congo – red was used for purification of isolates according to Vincent (1970).

Rhizobium isolation

Rhizobium wild types were isolated from the root nodules of their hosts according to Somasegaran and Hoben (1985). Conjugation event was done between different genotypes of *Rhizobium* strains harboring the opposite genetic markers according to Grinsted and Bennet (1990). Transconjugants used in this study were described before by Mervat *et al.,* (2024).

Tri-calcium phosphate medium used for testing phosphate solubilizing *Rhizobium*

This medium was consists of: KCl 0.4g, $(NH_4)_2SO_4$ 0.5g, $MgSO_4.7H_2O$ 0.5g, $MgCl_2$ 0.01 g, $FeCl_3$ 0.01 g, $CaCl_2$ 0.1 g, Peptone 1.0g, yeast extract 1.0g, glucose 5.0g. Adjust the volume to 750 ml using distilled water. Adjust solution to desired pH using 0.1 N NaOH (pH = 7.4). Tri-calcium phosphate was added to each flask with the rate of 0.25 g/90 ml (Bunt and Rovira 1955) mediumas modified by (Abd El-Hafez 1966).

Reagents

Sodium dodecyl sulfate (SDS)

This solution is prepared by addition of 20 g sodium dodecyl sulfate and adjust the volume to 100 ml using distilled water.

Color forming reagents

All reagents were prepared with distilled water. The reagents of color reactions are prepared based on the study of Dick and Tabatabai (1977). For convenience and completeness, the reagents and procedures were as follows; Reagent A: Trichloroacetic acid (0.5 M) and ascorbic acid (0.1 M) were prepared daily. Dissolve 3.268 g of trichloroacetic acid (MW 163.4) and 0.704 g of ascorbic acid (MW 176.1) in about 10 ml water and adjust the volume to 40 ml. Reagent B (ammonium molybdate, 0.01 M)was prepared by dissolve 2.472 g of ammonium molybdate (MW 1235.9) in about 100 ml of water and adjust the volume to 200 ml. Reagent C (toxic): Sodium citrate (0.1 M), sodium arsenite (0.2 M), and acetic acid (5%). Dissolve 5.882 g of sodium citrate (MW 294.12) and 5.196 g of sodium arsenite (MW 129.9) in about 100 ml, add 10 ml of glacial acetic acid, and adjust the volume to 200 ml.

Standard phosphate solution

This solution was used for standard curve, prepared by dissolve 0.2195 g anhydrous KH_2PO_4 in oneliter distilled water to make stock solution with the concentration of 50 ppm. A standard curve with KH_2PO_4 as the model is prepared used the following concentrations of KH_2PO_4; 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 ppm (Sarkar and Haldar 2005).

Genetic marking

Antibiotic susceptibility test was used based on Collins and Lyne (1985) using differed antibiotics as, ampicillin (*Amp*), benzoyl metronidazole (*Mtz*), azithromycin (*Azm*), clindamycin (*Cli*), nitrofurantoin (*Nit*) and amoxicillin-clavulanic acid (*Aug*).

Carboxymethylcellulase activity

The activity of carboxymethylcellulase in *Rhizobium* strains and their transconjugants was assessed according to Miller (1959) as described before by Mervat *et al.,* (2024).

Screening hydrolysis of tricalcium phosphate

To study the phosphate dissolving ability of bacterial isolates and their transconjugants, these isolates were inoculated in phosphate liquid medium containing tri-calcium phosphate. The glucose required for this medium was sterilized separately by filtration and added to the medium after sterilization. The pH of this medium was adjusted to 7.4 for ensuring a minimal concentration of the soluble phosphorus. Aliquots of 90 ml medium were placed in 250 ml Erlenmeyer flasks. Tricalcium phosphate was added to each flask with the rate of 0.25 g. The flasks were autoclaved and sterilized glucose was added separately after the media were autoclaved. Flasks were inoculated with each bacterial isolate and incubated at 30^oC. Alteration in the value of soluble phosphorus was assessment after three, six and nine days of inoculation time. The quantity of soluble phosphorus was measuredas a biomarker for phosphate dissolving ability of bacterial genotypes tested. In addition, bacterial isolates given higher amounts of soluble phosphorus were considered as more efficient ones (Abdel-Nasser *et al.,* 1982).

Orthophosphate determination procedure

The methodology reported by Dick and Tabatabai (1977) was used as follows; 0.32 ml of supernatant, 0.40 ml of reagent A, 0.08 ml of reagent B and 0.20 ml of reagent C were added sequentially. After the addition of each reagent the solutions were stirred well with a cuvette stirring rod. The absorbance of the molybdenum blue was registered over time via read at 470 nm after 30 min. Alternatively, 0.10 ml of 20% SDS was added prior to reagent A and incubated for 30 min.

Soil sterilization method

The most commonly used method for soil sterilization involves moist heat. Moist heat is much more effective than dry heat for sterilization. Pressurized steam at 121°C as applied by an autoclave sterilization is the most commonly technique. Pre-incubate the moist soil for two to three days at laboratory condition to express the growth of microorganisms before autoclaving. Ideally, the moisture potential of the soil was adjusted to approximately 60% moisture-holding capacity. The moist soil was placed in stainless steel container and covered with aluminum foil to prevent its form microbial contamination. Steam sterilization of high quantities of soil must be avoided because of unequal heat distributed in the soil. High efficient sterilization was applied through spreading the soil at a depth of $\leq$ 2.5 cm in a stainless container for subsequent steam sterilization (Wolf and Skipper 1994).

The soil should be autoclaved at 121°C for one hour. After the first autoclaving, sterile distilled water was added to restore the desired moisture level of the soil. The moist soil was incubated for additional two days and then autoclaved a second time for one hour to eliminate any growth of microorganisms that were not destroyed in the first autoclaving. Sterility can be verified by confirming the absence of microbial growth (Wolf *et al.,* 1989).

Pots experiment

Pots experiment was carried out at the growing season of the academic year 2023/2024. This experiment was done using a plastic-pots containing sterilized sandy and clay soils with the ratio of 1:1, which autoclaved at 121°C for two hours among three days. Sandy soil was washed with distilled water several times to diminishing chloride ions.

Inoculation

Mid-log phase growing cultures in nutrient broth of YEM were used for inoculated the plants (Kucey 1989). Seeds were surface-sterilized with 10% ethanol and washed three times with sterilized distilled water (Dobert and Blevins 1993). Four-surface-sterilized seeds were planted in each plastic pot. The plants were thinned after complete emergence to one plant per pot. The nature suggested agricultural practices concerning fenugreek and lentil production were applied at the proper time. The seeds planted in all experiments were inoculated with approximately 10 ml of *Rhizobium* cells containing 10^7 cells/ ml before covered with the soil.

Preparation of samples

Wild type isolates and their transconjugants were used to determine their efficiency in nodulation. Six weeks after inoculation, the plants per each replicate were carefully uprooted and the roots were washed by tap water to remove the adhering clay particles. The nodules developed on every plant were counted and the mean number per plant was calculated. Shoots, roots and nodules were separated from every treatment and then dried at 70°C until reached to the constant weight (Pineda and Nolt 1990).

Plastid pigments

Chlorophyll and carotenoids in leaves were determined after six weeks of planting (DAP) using a spectrophotometric method according to Markinney (1941).

Nodule number

This trait was estimated when the plants reached to 45 days plant-old. From each replicate, three plants were selected at random and the roots were washed by tap water. Then the number of nodules was counted for each plant and then dried in oven at 70 °C till reached the constant weight and then weighted immediately.

Plant dry weight

This trait was recorded when the plants reached to 45 days plant-old. The plants were sun dried for five days and then put in oven with forced dry air circulation at 70°C for 24 hours until the weight was constant, and then weighted immediately.

Genetic parameters

The genotypic and phenotypic variances, as well as, genetic coefficient of variation and phenotypic coefficient of variation were measured according to Singh and Chaundry (1985).

Heritability in broad sense

Heritability in broad sense for quantitative traits was computed according to Allard (1999).

Genetic Advance Expected (GA)

The genetic advance expected under selection assuming selection intensity of the superior 5% of the genotypes was estimated, in addition the genetic advance as a percentage of mean was computed according to Allard (1999).

Statistical analysis

Results are the mean values of three biological replicates. The data were subjected to analyses of variance and the least significant difference (LSD) value was computed to test the differences between means at 0.05 and 0.01 levels of probability according to Steel and Torrie (1960).

RESULTS AND DISCUSSION

Genetic analysis of phosphate solubilization

Recombinants derived from P$_1$ x P$_6$

Phosphorus (P) was exist in the soil in both organic and inorganic forms as one of the most common metallic element. The plants cannot grow without this element which is important after nitrogen for plant growth and yield (Sharma *et al.,* 2013). P is a component of important compounds in the cell as nucleic acids, phospholipids and ATP. The better performance of P solubilization by transconjugants was appeared after nine days (Table 2). Phosphate solubilization by *Rhizobium* transconjugants was ranged between 28.83 to 65.95 ppm in relation to the mid-parent (48.52 ppm). Significant concentrations of solubilizing P after nine days were obtained by Tr$_1$, Tr$_2$, Tr$_3$, Tr$_6$, Tr$_7$, and Tr$_8$ above the mid-parent. This indicated that these recombinants were synthesize and secrete higher amounts of organic acids which is the principal tool of dissolving inorganic phosphate. The inorganic forms of P contribute more than 50% in the earth (Buch *et al.,* 2008).

Table 2. Tri-calcium phosphate solubilized in ppm by *Rhizobium* wild types and their transconjugants resulted from the conjugation between P$_1$ x P$_6$

Genotypes	Incubation time (day)		
	3	6	9
P-$_1$	16.26	37.63	52.34
P-$_6$	60.17	112.06	44.71
Mid-parents	38.22	74.85	48.52
Tr-$_1$	3.95	41.34	64.09
Tr-$_2$	12.34	43.54	59.14
Tr-$_3$	10.90	47.12	50.21
Tr-$_4$	1.96	37.63	30.14
Tr-$_5$	7.80	39.62	28.83
Tr-$_6$	2.17	33.64	55.50
Tr-$_7$	5.53	30.41	56.12
Tr-$_8$	17.84	41.55	65.95
Transconjugants mean	7.81	39.36	51.25
F – Test	**	**	**
LSD 0.05	0.78	0.72	0.82
0.01	1.07	0.99	1.12

**: Significance at 0.01 probability level.

Among the different organic acids produced, the principal factors in P solubilization are keto-gluconic and gluconic acids. The released of these organic acids caused microbial cells acidification and its surroundings medium (Lin *et al.,* 2006). The results are in harmony with Rodríguez *et al.* (2006), who found that low molecular weight organic acids separated from the growing culture of *Rhizobium leguminosarum*d is solved about the same value of phosphate that was dissolved by the culture. In addition, Buch *et al.,* (2010) reported that the genes directly or indirectly expressing organic acids are detrimental for mineral P solubilization. Halder *et al.,* (1990) supported the importance role of low molecular weight organic acids released in mineral phosphate solubilization. Moreover, Illmer and Schinner (1995) stated that proton translocation ATPase plays a major role in P mineralization through protons extruded to the outer membrane, in addition to an exchange of protons for cation uptake. Besides, Lin *et al.,* (2006) observed that organic acid protons are indicated in P solubilization. Bacterial cells released organic acids in their growing culture can be estimated by chromatography apparatus of high performance (Kumar and Rai 2015). Kpomblekou-A and Tabatabai (1994) decided that organic acids containing hydroxyl and carboxyl groups compete with Al, Ca and Fe cations that form complexes of chelate with metal ions to be transform the insoluble P into soluble forms. Besides, solubilization of inorganic phosphate occurs as a consequence of carbon dioxide evolution, nitrate formation (nitrogen assimilation) and oxidation of sulphur. This leading to produced sulphuric, nitric and carbonic acids. Pei-Xiang *et al.* (2012) reported that pH drop and soluble P released are not correlated, as a consequence acidification was not to be considered the sole tool of inorganic P solubilization. In addition, siderophores and exopolysaccharides secreted by phosphate solubilizing bacteria (PSB) bring out phosphate into dissolved form due to the interactions of charge related (Sharma *et al.,* 2013). Therefore, reducing molecules produced by transconjugants, organic acids and chelating are responsible for inorganic P solubilization. These organic acids produced by microbial recombinants are played a key roles in inorganic P solubilization that can be metabolized as an alternative energy source by PSB leading to increased the yield of biomass (Kumar and Rai 2015).

Recombinants derived from P$_2$ x P$_6$

Regarding to the results presents in Table 3, only one transconjugant (Tr$_{15}$) achieved significant increase in phosphate solubilization after nine days in relation to the mid-parent. This transconjugant appeared gradually increase in P solubilization among the incubation periods with similar to the parental strain P_2 genotype. All other transconjugants showed the high P solubilization after six days of incubation time. So, the optimum time of P solubilization by these recombinants was obtained at six days of incubation. After this time, the parental strain P_6 genotype and most of transconjugants were auto consumed high values of solubilizer P. Phosphate solubilization after six days of incubation time was ranged between 21.41 to 60.11 ppm if compared with the mid-parent (70.72). Meanwhile, this solubilization after nine days was ranged between 12.82 to 58.94 ppm if compared with the mid-parent (43.44 ppm). The results indicated that Tr_{15} inherited the gradually increase in P solubilization from their parental strain P_2 genotype. Meanwhile, all recombinants, except Tr_{15}, inherited the optimum time of P solubilization and the consumption of solubilized P after the optimum time from their parental strain P_6 genotype. Organic phosphate in the agriculture soils was resulted from the animal, plant tissues and microbial residues.

Table 3. Tri-calcium phosphate solubilized in ppm by *Rhizobium* strains and their transconjugants resulted from the conjugation between P_2 x P_6.

Genotypes		Incubation time (day)		
		3	6	9
P-$_{2}$		17.90	29.38	42.17
P-$_{6}$		60.17	112.06	44.71
Mid-parents		39.04	70.72	43.44
Tr-$_{9}$		16.19	48.42	39.07
Tr-$_{10}$		15.77	52.27	34.74
Tr-$_{11}$		1.00	60.11	28.77
Tr-$_{12}$		13.37	64.85	18.59
Tr-$_{13}$		3.88	38.39	16.05
Tr-$_{14}$		12.13	55.91	12.82
Tr-$_{15}$		9.25	21.41	58.94
Tr-$_{16}$		13.30	37.77	17.49
Transconjugants mean		10.61	47.39	28.31
F – Test		**	**	**
LSD	0.05	0.78	0.92	0.83
	0.01	1.07	1.26	1.14

**: Significance at 0.01 probability level.

This leading to mineralization that occurred by different enzymes. Besides, the basic mechanism contribute inorganic phosphate is the solubilization by organic acid released by bacterial cells (Kumar 2016). The enzymes involved in mineralization of organic P are phosphatases, phytases and phosphonatases (Behera *et al.,* 2013). Various number of genes are expressing alkaline and acid phosphatases with high substrate specificity (Nilgiriwala *et al.,* 2008). Behera *et al.,* (2013) stated that recent approach is the integration of screened genes into bacterial chromosome that encoding acid and alkaline phosphatases to obtain promising phosphate solubilizing bacterial genotypes avoiding the risk of horizontal gene transfer. Over than 50% of organic P in the soil was derived from phytate which is the main source of inositol phosphate. It is generated by microorganisms, pollen grains and plant seeds. Phytases secreted utilizable P from inositol phosphate as a phytate component. The use of phytase secreted PSB improved plant development and growth (Rodríguez and Fraga 1999). Behera *et al.,* (2013) found that genetically engineered *Arabidopsis* plants with phytase gene from *Aspergillus niger* acquired P from phytate. Phosphate solubilizing bacteria enhance crop production, as well as, plant growth through direct and indirect mechanism. The indirect mechanism of stimulating plant growth include prevention or decline the negative impact of pathogenic microorganisms through the secretion of siderophores or antibiotics (Sharma *et al.,* 2013). The direct mechanism of growth promotion involves P solubilization, availability of nutrients, phytohormone production and N-fixation. Therefore, the biofertilizer ability of transconjugant Tr_{15} shall enhance growth and crop productivity of the host legume. Thus, these recombinant genotype was recommended to be use as biofertilizer. So, P available in the land accounts 0.1% only of phosphorus total pool containing agriculture soil (Illmer and Schinner 1995). Most lands are deficient in available P to the plants in the time that soils containing a huge values of total P (Richardson 2001). Organic forms of phosphate contribute 30-50% of its total in the soil (Behera *et al.,* 2013). Most forms of high molecular weight organic P compounds could not be assimilated. They are degraded by microbial cells before utilization (Rodríguez and Fraga 1999). The higher quantities of inorganic phosphate reserves are rock deposits (FAI 2002). Organic acids as a metabolic byproducts released by microorganisms can dissolved rocks (Chang and Li 1998). Therefore, rock phosphate used with PSB become as an alternative method to high expensive manufacture chemical fertilizers (Sahu and Jana 2000).

Recombinants derived from P_3 x P_7

Regarding to the results tabulated in Table 4, all recombinant transconjugants achieved gradually increase in P solubilization among the incubation periods. The higher concentrations of solubilizing P by transconjugants were obtained after nine days of incubation. All transconjugants derived from the cross between P_3 x P_7 achieved highly significant increase in P solubilization over the mid-parent. *Rhizobium* transconjugants derived from this cross achieved P solubilization after nine days was ranged from 52.27 to 88.56 ppm in relation to the mid-parent (37.18 ppm). Therefore, phosphate solubilizationby *Rhizobium* transconjugants plays a key roles in increasing phosphorus availability to be uptake by the plants (Rodríguez *et al.* 2006). The results indicated that *Rhizobium* transconjugants obtained from this cross are more effective in P solubilization in relation to the parental strains. This because of enzymatic processes released by recombinant bacterial cells, as well as, the production of low molecular weight organic acids mainly gluconic and 2-ketogluconic acids (Rodríguez *et al*., 2006). This agrees with Stevenson (2005), who reported that organic acids chelate Al, Mg, Fe, Ca cations that bound with phosphate insoluble forms to be transform them into soluble forms of P with pH medium decrease.

Table 4. Tri-calcium phosphate solubilized in ppm by *Rhizobium* strains and their transconjugants resulted from the conjugation between P_3 x P_7

Genotypes		Incubation time (day)		
		3	6	9
P_{-3}		10.69	30.62	51.10
P_{-7}		54.40	76.81	23.27
Mid-parents		32.55	53.71	37.18
Tr_{-17}		18.04	27.73	52.27
Tr_{-18}		22.37	22.92	74.88
Tr_{-19}		22.44	25.54	76.19
Tr_{-20}		22.37	22.65	88.56
Tr_{-21}		18.04	24.43	72.41
Tr_{-22}		20.79	23.27	56.88
Tr_{-23}		10.62	25.67	79.55
Tr_{-24}		13.99	16.05	82.58
Transconjugants mean		18.58	23.53	72.91
F – Test		**	**	**
LSD	0.05	0.82	0.71	0.80
	0.01	1.12	0.98	1.09

**: Significance at 0.01 probability level.

This mechanism was found in Gram negative bacteria as *Rhizobium* which produced low molecular weight of organic acids in the periplasm via glucose direct oxidation pathway (DOPG) (Anthony 2004).The DOPG involves two enzymes, gluconate dehydrogenase (GADH) and glucose dehydrogenase (GDH). These enzymes are oriented toward outside the cytoplasmic membrane. Then they are oxidize glucose in the periplasmic space to be produce organic acids that transform phosphorous insoluble forms into soluble forms (Anthony 2004). These results agreed with Collavino *et al.,* (2010), who classified the isolates of phosphate solubilization into two groups, the first group included more than 75% of bacteria that produced the greatest values of soluble P secreted through the first three days of growth, the second group contained bacteria that reached the greatest phosphorous solubilization at seven days of growth. Therefore, transconjugants obtained from the mating between P_3 x $P_7$are belonging to the second group because they are reached to the greatest phosphorus solubilization ability at nine days of growth. These results are in line with Anzuay *et al.,* (2013), who suggested that the major organic acid indicated in mineral P solubilization is gluconic acid which was released by almost all bacteria. Whereas, the released of other organic acids are also indicated. The production of GDH and GADH enzymes that oxidize glucose into organic acids in the periplasm require the cofactor redox pirroquinolin quinone (pqq), whose expression indicates operon consisting of at least 5-7 genes (Kim *et al.,* 2003). This cofactor was essential for P solubilizing phenotype. Gram positive bacteria represented the decline levels of gluconic acid in their growing culture supernatants, as well as, the absence of pqq genes in their genomes. In contract, to Gram positive bacteria, Gram negative P solubilizing bacteria harboring both pqq C and pqq E genes in their genomes (Anzuay *et al.,* 2013).

Recombinants derived from P_3 x P_9

The results tabularized in Table 5 appeared that P solubilization was increased gradually among the incubation time by all *Rhizobium* transconjugants in similar with both parental strains. P solubilization reached the maximum values after nine days. At this time it was ranged between 50.82 to 133.23 ppm if compared with the mid-parent (55.33 ppm). The highest P solubilization (133.23 ppm) was produced by Tr_{29}.These results are in line with Johri *et al.,* (2003), who stated that rhizobia are often considered as one of most powerful P solubilizers due their attributed to solubilize insoluble phosphates. Some variations were observed in P solubilization among the different recombinants of transconjugants regarding to the differences in their genetic makeup that induced differences in their enzymatic activities. This agrees with Halder *et al.,* (1990), who found variations in enzymatic activities among *Rhizobium* strains. In addition, Jinturkar (2014) found that *Rhizobium* strains produced urease, amylase and protease are considered as better for nodulation ability and nitrogen fixation efficiency with their hosts. The same author found that P solubilization was glucose concentration response reached maximum at 2.0% glucose concentration, as well as, above this concentration P solubilization was decline.

Table 5. Tri-calcium phosphate solubilized in ppm by *Rhizobium* strains and their transconjugants resulted from the conjugation between P_3 x P_9

Genotypes		Incubation time (day)		
		3	6	9
P_{-3}		10.69	30.62	51.10
P_{-9}		40.24	53.44	59.56
Mid-parents		25.47	42.03	55.33
Tr_{-25}		5.60	6.22	105.88
Tr_{-26}		17.08	18.11	52.20
Tr_{-27}		2.51	14.88	124.02
Tr_{-28}		17.29	19.76	50.82
Tr_{-29}		4.02	10.55	133.23
Tr_{-30}		7.59	10.69	115.71
Tr_{-31}		5.95	8.56	120.04
Tr_{-32}		4.09	12.41	124.50
Transconjugants mean		8.02	12.65	103.30
F – Test		**	**	**
LSD	0.05	0.88	0.76	0.80
	0.01	1.20	1.04	1.09

**: Significance at 0.01 probability level.

The results appeared that the better P solubilization was obtained by Tr_{29} followed by Tr_{32}, Tr_{27}, Tr_{31}, Tr_{30}, and Tr_{32} which produced significant values of P solubilization. Any decrease in P concentration after the maximum values obtained herein at nine days may be due to soluble phosphate auto consumption by the growing bacterial population as reported by Jinturkar (2014). Therefore, better P solubilizing transconjugants have been considered to play a key role in plant nutrition through increasing P uptake by the plants. This because P was considered as a next factor after nitrogen, it is the second key macronutrient important for the promoting of plant growth and productivity (Rodríguez *et al.* 2006). Furthermore, Rossolini *et al.,* (1998) reported that bacterial strains transform insoluble organic phosphate compounds into dissolved forms released by enzymatic processes. Meanwhile, mineral P solubilization was related to the secretion of organic acids with low molecular weight, mainly gluconic and 2-ketogluconic acids (Rodríguez *et al.* 2006). The results indicated that most of *Rhizobium* transconjugants produced high values of organic and inorganic acids that solubilize the insoluble forms of phosphate via chelating calcium, Fe and Al ions complexed with phosphate, which enabling the secreted of adsorbed phosphate through ligand exchange processes (Pradhan and Sukla 2005). The ions connected with phosphate chelating leading to convert insoluble P into soluble forms (Walpola and Yoon 2012). The results obtained herein are in line with Kalayu (2019), who decided that carboxylic acids plays an important role in Al-P and Fe-P solubilization, whereas, 2-ketogluconic acid is a strong chelator of Ca-P. In addition, Jha *et al.,* (2014) stated that inorganic acids like hydrochloric acid can dissolve P with a less effective rate than organic acids. Therefore, the inorganic and organic acids transform tricalcium P into di and monobasic P that enhanced availability of P to the plant (Walpola and Yoon 2012).

Recombinants derived from P_3 x P_{10}

As shown from the results tabularized in Table 6 that all *Rhizobium* transconjugants, except Tr_{33}, showed gradually increase in P solubilization among incubation times. Transconjugant Tr_{33} is the only one induced the maximum P solubilization at six days and then auto consumed the solubilized P at nine days. Tr_{33} was similar in auto P consumption with the parental strain P_{10}. This agrees with Jinturkar (2014), who found that maximum P solubilization was obtained at 2.0% glucose and above this concentration TCP solubilization was decreased due to the auto consumption of soluble P by bacterial population, as the same found before in *Azospirillum brasilense*. *Rhizobium* transconjugants showed P solubilization was ranged between 34.19 to 86.33 ppm. The highest recombinant solubilizer genotypes are Tr_{40} followed by Tr_{37} and Tr_{35}. Among eight transconjugants derived from this conjugation, four of them showed significant P solubilization in relation to the mid-parent.

Table 6. Tri-calcium phosphate solubilized in ppm by *Rhizobium* strains and their transconjugants resulted from the conjugation between P_3 x P_{10}

Genotypes		Incubation time (day)		
		3	6	9
P-$_{3}$		10.69	30.62	51.10
P-$_{10}$		40.65	63.34	45.05
Mid-parents		25.67	46.98	48.08
Tr-$_{33}$		1.75	53.64	47.39
Tr-$_{34}$		15.16	15.91	34.19
Tr-$_{35}$		0.31	1.00	56.26
Tr-$_{36}$		7.39	12.68	47.73
Tr-$_{37}$		0.79	2.23	58.11
Tr-$_{38}$		0.24	1.62	47.80
Tr-$_{39}$		0.93	9.45	48.83
Tr-$_{40}$		1.34	10.55	86.36
Transconjugants mean		3.49	13.39	53.34
F – Test		**	**	**
LSD	0.05	0.71	0.96	0.95
	0.01	0.97	1.31	1.30

**: Significance at 0.01 probability level.

This indicated that these transconjugants released higher values of organic acids into the surrounding media induced cells acidification with their media, as a consequence releasing phosphate ions from the phosphate mineral through H^+ substitution with the cation connected to phosphate (Sharma *et al.,* 2013). However, mineralization is another tool used for solubilization of organic phosphate into utilizable form mediated by different enzymes produced by *Rhizobium* cells. These was carried out in the soil containing residues of microorganisms, animal and plant wastes. These residues containing higher quantities of organic P as protein, sugar phosphates, phospholipids and nucleic acids. One of the enzymes mediated mineralization is phosphatase which is produced extracellularly only by microorganisms (Sharma *et al.,* 2013). However, tri-calcium phosphate used in this study was inorganic source of phosphate. Therefore, its solubilization has often referred to the secretion of organic acids as ketogluconic acids, malic acids, glutamic acid, lactic acid, citric acidand succinic acid released by *Rhizobium* cells to dissolve insoluble P through anion exchange of phosphate by acid anion. Organic acids are by products of microbial metabolism pathway mostly resulted from oxidation pathway or from the fermentation of organic carbon sources on the outer face of cytoplasmic membrane. The results indicated that the value of organic acids produced and probably their types are differed among different genotypes of *Rhizobium* transconjugants (Kalayu 2019). Thus, P solubilization amount was depending on the nature of organic acids released by bacterial cells. Di- and tri-carboxylic acids are more efficient if compared with monobasic and aromatic acids. In addition, aliphatic acids are more efficient in P solubilization if compared with citric and phenolic fumaric acids (Kalayu 2019).

Recombinants derived from P_4 x P_6

The results tabularized in Table 7 achieved that the maximum P solubilization was appeared by both parental strains at six days of incubation time and then auto consumption at nine days. In contrast, all transconjugants showed gradually increase in P solubilization among the incubation periods. Phosphate solubilization by transconjugants was ranged between 66.29 to 104.5 ppm if compared with the mid-parent (52.10 ppm). All *Rhizobium* recombinants derived from this conjugation induced significant increase in P solubilization over the mid-parent. The cofactor necessary for P solubilization is pyrroloquinoline quinone (pqq). Their genes are involved in P solubilizing bacteria as *Rhizobium* used in this study (Anzuay *et al.,* 2013). The direct oxidation pathway of glucose was dependent on glucose dehydrogenase, which leads to release gluconic and 2-ketogluconic acids that have a wide range of specificity in P solubilization. The results are in harmony with Liang *et al.,* (2020), who found that the *gcd* gene was encodes quinoprotein glucose dehydrogenase mediates the solubilization of inorganic phosphate.

Table 7. Tri-calcium phosphate solubilized in ppm by parental strains of *Rhizobium* and their transconjugants resulted from the conjugation between P_4 x P_6

Genotypes		Incubation time (day)		
		3	6	9
P-$_4$		26.63	70.55	59.49
P-$_6$		60.17	112.06	44.71
Mid-parents		43.40	91.31	52.10
Tr-$_{41}$		25.26	57.56	77.36
Tr-$_{42}$		22.92	38.87	76.46
Tr-$_{43}$		30.96	56.32	71.99
Tr-$_{44}$		16.05	42.85	93.92
Tr-$_{45}$		27.66	28.70	104.50
Tr-$_{46}$		29.38	41.76	94.54
Tr-$_{47}$		34.88	63.68	66.29
Tr-$_{48}$		24.92	29.11	76.39
Transconjugants mean		26.50	44.86	82.68
F – Test		**	**	**
LSD	0.05	0.84	0.89	0.80
	0.01	1.15	1.21	1.10

**: Significance at 0.01 probability level.

The *gcd* is the most biomarker express on determining phosphate solubilizing bacteria (PSB). In addition, Rodríguez *et al.* (2006) decided that the gene of *gabY* is included in mineral phosphate solubilization and gluconic acid release. Moreover, gluconic acid plays a key role in P solubilization and it is the main organic acid released through the direct oxidation of glucose in the bacterial cells. The overexpression of organic acid genes in *Rhizobium* transconjugants leading these cells is a promising strategy in P solubilization when utilized as biofertilizers. This makes *Rhizobium* transconjugants from the better genotypes are an attractive option for biotechnology applications in sustainable agriculture. Therefore, *Rhizobium* can enhance plant growth and productivity through different mechanisms. One of these is the solubilization of insoluble P to be available for the plant nutrition (Glick 1995). Free inorganic P was released from organic P by enzymatic processes (Rossolini *et al.,* 1998). The production of organic acids as gluconic acid by the bacterial cells is the principal mechanism in this criteria (Rodríguez and Fraga 1999). Glucose oxidation into gluconic acid needs the metabolic function of pqq synthase that synthesize the cofactor pqq which is necessary in the assembly of glucose dehydrogenase (GDH) holenzyme, to be acts in the oxidation of glucose into gluconic acid (Goldstein 1995). The results are in harmony with Ostwall and Bhide (1972), who observed variations in P concentrations along the cultivation time. The results are also in agreement with Rodríguez *et al.* (2000), who found higher P values were detected in rhizobacterial transconjugants along the cultivation time with some intervals. The same authors found correlation between P solubilization drop and pH increase, as well as, vice versa. Inorganic phosphate solubilization, cellular phosphorus turnover and organic phosphate mineralization leading microbial populations central to soil P cycling (Oliverio *et al.,* 2020). The results are in line with Bashan *et al.,* (2000), who reported that the introduction or over-expression of genes involved in P solubilization in natural rhizospheric bacteria is very significant approach for improving their capacity to be used as inoculants. Furthermore, Illmer and Shinner (1995) stated that acid production as an important way in P solubilization was not the only mechanism of P solubilization by bacterial cells. Thus, microbial genetic techniques are an advantageous approach for releasing improved phosphate solubilizing bacteria (Igual *et al.,* 2001).

Recombinants derived from P_4 x P_7

The results presented in Table 8 achieved that the maximum phosphate solubilization by both parental strains was shown at 6 days of incubation after this time solubilized P was auto consumption. All transconjugants derived from this cross appeared gradually increase in P solubilization among the incubation time till reached the maximum values at nine days. Solubilizing P at nine days was ranged between 66.15 to 125.40 ppm if compared with the mid-parent (41.38). All *Rhizobium* transconjugants derived from this cross induced highly significant increase in P solubilization over the mid-parent. Therefore, biofertilizer with these transconjugants are gaining importance because they are non-toxic, eco-friendly, and non-hazardous products (Gichimu*et al.* 2020). However, plants acquire P in a solution form as phosphate anions. Thus, phosphate solubilizing bacteria (PSB) were used as biofertilizers since 1950s (Sharon *et al.,* 2016). These strains secrete different types of organic acids as carboxylic acid that lowering the rhizospheric pH and then dissociate the bound forms of phosphate as $Ca_3(PO_4)^2$ in calcareous soil (Sridhar *et al.,* 2005). This making inorganic P available to the plants (Corpas *et al.,* 2013). The results obtained herein agreed with Sarker *et al.,* (2014), who stated that *Rhizobium* require phosphorous as energy for survival and growth. Therefore, *Rhizobium* cells plays a fundamental roles in phosphorus cycling in agricultural ecosystems. So, Lara *et al.,* (2013) decided that PSB transform the insoluble P into soluble forms of $(HPO_4)^2$ and H_2PO_4 by acidification, exchange reactions, polymeric substances formation and chelating.

Table 8. Tri-calcium phosphate solubilized in ppm by *Rhizobium* strains and their transconjugants resulted from the conjugation between P_4 x P_7

Genotypes	Incubation time (day)		
	3	6	9
P-$_4$	26.63	70.55	59.49
P-$_7$	54.40	76.81	23.27
Mid-parents	40.52	73.68	41.38
Tr-$_{49}$	10.41	33.58	99.76
Tr-$_{50}$	26.98	51.44	125.40
Tr-$_{51}$	8.83	14.13	66.15
Tr-$_{52}$	7.73	60.17	99.69
Tr-$_{53}$	4.64	20.65	70.34
Tr-$_{54}$	11.31	13.30	82.24
Tr-$_{55}$	2.31	9.59	74.40
Tr-$_{56}$	14.47	18.46	91.31
Transconjugants mean	10.84	27.67	88.66
F – Test	**	**	**
** 0.05	0.80	0.97	0.94
0.01	1.10	1.32	1.29

**: Significance at 0.01 probability level.

For instance, the application of high phosphate solubilizing transconjugants around the plant roots has been release soluble P promote plant growth (Shamseldin and Werner 2005). So, this study provided a significant evidence for the selection of efficient phosphate solubilizing *Rhizobium* transconjugants biologically active in P solubilization. As a consequence, the application with better genotypes will be highly recommended to improve plant growth and their productivity. The discovered of these genotypes can be beneficial to the farmers to be used as biofertilizer inoculum in sustainable agriculture. The results obtained herein agreed with Djordjevic *et al.,* (1983), who reported that *Rhizobium* plasmids are self-transmissible between *Rhizobium* and nonrhizobial species. Plasmids transfer under field conditions have never been shown (Kinkle andSchmidt1991). In addition, Hardman and Gowland (1985) considered plasmids are usually efficient tool for gene transfer that leading to increasing biochemical versatility of the population. In fact, *Rhizobium* plasmids were carrying additional genetic information important at any stage of the life cycle in bacteria. It was surprising that P was required for different metabolic pathways as energy transfer reactions, cell division, enzyme activity regulation by phosphorylation of tyrosine, serine, histidine, threonine, aspartate, synthesis of biomolecules and photosynthesis (Raghothama 1999). Rodríguez and Fraga (1999) reported that plants absorb phosphate from the soil as P anions ($H_2PO_4^-$ or HPO_4^-). Precipitation of these anions is soil pH dependent. Phosphate anions were precipitated in acidic soil with hydroxides of iron and aluminum and free oxides. Calcium is the major factor indicated in P fixation in alkaline soils (Igual *et al.,* 2001). Most agriculture lands worldwide are decline in the availability of phosphorous. Farmers are used chemical fertilizers to overcome P deficiency in the soil. In developing countries P fertilizers are expensive because they are imported or generated from the raw materials imported (Sundara *et al.,* 2002). However, a huge amount of total P containing soils are locked with aluminum, calcium and iron through associations charge related which varies between 400-1200 mg/kg. These required an efficient *Rhizobium* transconjugants to be solubilize P by their acids released as a metabolic byproducts to be beneficial for bacteria and plants both (Chang and Li 1998). Rock phosphate used with association of P solubilizing bacteria has become as an alternative source to high expensive manufactured chemical fertilizers (Sahu and Jana 2000). This will serve as a cheap origin for phosphate fertilizer produced *in vitro* that leading to improve soluble forms of P availability in the agriculture lands. P solubilizing ability was varied from genotype to genotype of transconjugants. This leading to biodiversity in their physicochemical properties upon the soil. Therefore, the better transconjugant genotypes were necessary to be used as biofertilizer in sustainable agriculture. Inorganic forms of insoluble P contribute more than 50% in the soil. These needing to solubilization via secretion of organic acids (Buch *et al.,* 2008). Acid ionization takes place and proton produced was involved for phosphorous secreted from mineral phosphates through proton substitution of Fe, Al and Ca to be release phosphate anions (Kumar and Rai 2015). Therefore, the efficient P solubilizer transconjugants obtained in this study were over expressed in low molecular weight of organic acids which is takes place in the principal mechanism of inorganic P solubilization.

Recombinants derived from P_5 x P_7

The results tabularized in Table 9 achieved that the maximum P solubilization was obtained by both parental strains at six days of incubation time and then auto consumption at nine days. Most of transconjugants as Tr_{57}, Tr_{59}, Tr_{62}, Tr_{63}, and Tr_{64}, appeared the same trend as their parental strains. In contrast, both transconjugants numbered Tr_{60} and Tr_{61} showed gradually increase in P solubilization till reached the maximum value at nine days. Most of transconjugants derived from this conjugation between P_5 x P_7 produced highly significant increase in P solubilization over the mid-parent. P solubilization produced by *Rhizobium* transconjugants at nine days of incubation was ranged between 9.11 to 63.41 ppm in relation to the mid-parent (23.23 ppm). Due to the high values of P solubilization obtained by transconjugants, the use of these recombinant genotypes has shown to be a promising biofertilizer in sustainable agriculture. The efficient transconjugant genotypes can stimulate P solubilization in relation to the parental strains with exhibiting different activities. The results obtained herein agreed with Hamane *et al.,* (2020), who found that rhizobia isolated from *Hedysarum pallidum* formed clear halo zones around *Rhizobium* colonies on agar medium containing insoluble phosphate. The same authors demonstrated that these halos are due to organic acids released or to the activity of phosphatase enzymes secreted by *Rhizobium*strains(Khan *et al.,* 2009). The results are also in line with Hamane *et al.,* (2020), who found that P dissolving ability was differed among the eight isolates of PSB. In another investigation, Sridevi and Mallaiah (2007) found that the greatest P dissolving ability was obtained by *Rhizobium*strains isolated from *Cassia absus* (620 mg/L), followed by *Rhizobium* sp. No. 19 (391 mg/L) and *Rhizobium* sp. No. 26 (126 mg/ L), all of them were isolated from *Sesbania sesban.*

Table 9. Tri-calcium phosphate solubilized in ppm by *Rhizobium* strains and their transconjugants resulted from the conjugation between P$_5$ x P$_7$

Genotypes		Incubation time (day)		
		3	6	9
P-$_5$		14.81	24.85	23.20
P-$_7$		54.40	76.81	23.27
Mid-parents		34.61	50.83	23.23
Tr-$_{57}$		9.73	50.21	45.33
Tr-$_{58}$		59.21	35.77	34.75
Tr-$_{59}$		8.01	49.38	38.94
Tr-$_{60}$		5.88	16.33	50.48
Tr-$_{61}$		2.44	47.73	63.41
Tr-$_{62}$		14.33	56.26	10.97
Tr-$_{63}$		13.78	83.06	20.79
Tr-$_{64}$		9.45	43.27	9.11
Transconjugants mean		15.35	47.75	34.22
F – Test		**	**	**
LSD	0.05	0.85	0.97	0.82
	0.01	1.17	1.33	1.12

**: Significance at 0.01 probability level.

The same authors found that the pH of the growing media was acidic nature indicating pH drop from the start initial pH of 6.8 to 7.0 to 5.6-3.8 in the culture filtrate of seven incubation days. Hamane *et al.,* (2020) found significant association between the concentrations of solubilized P in growing media and the final pH of the same media. This indicated that if the final pH decline then dissolving P concentration was increased (Hamane *et al.,* 2020). These results are very well supported by Collavino *et al.,* (2010), who found that broth medium acidification was coincided with phosphate solubilization. Therefore, Liu *et al.,* (2012) supported these findings through P released from insoluble form of phosphate was negatively correlated with the solution pH. The results obtained in this study are also in harmony with Pérez*et al.* (2007), who stated that the acidification of growing culture supernatants is the major mechanism in phosphate dissolving. Therefore, *Rhizobium* solubilize insoluble P by secreting organic acids which showed overexpressed by their transconjugants in relation to their parents.

Genetic parameters of phosphate solubilization

According to Sivasubramanian and Madhavamenon (1973), who categorized genotypic coefficients of variation (GCV) and phenotypic coefficients of variation (PCV) as low (0-10%), moderate (10-20%) and high (> 20%). Based on this classification high PCV and GCV values were recorded for phosphate solubilization by *Rhizobium* transconjugants resulted from all conjugations at all time intervales (Table 10). The limited magnitude of variation between genotypic and phenotypic coefficient of variations indicating the limited influence of environmental conditions on the expression of phosphate solubilization genes. Though, selection depending on phenotypic expression of P solubilization would exhibited about considerable genetic important. This result is in conformity with the findings of Abebe *et al.,* (2017), who found higher PCV and GCV estimates for plant height, culm length, number of unfilled grains per panicle, grain yield and biomass yield. However, Johnson *et al.,* (1955) classified heritability above 60% as high heritability.

Table 10. Genetic variability parameters of tri-calcium phosphate solubilization by *Rhizobium* transconjugants

Genetic parameter	1 x 6			2 x 6		
	3 Days	6 Days	9 Days	3 Days	6 Days	9 Days
σ^2g	295.47	554.11	163.83	266.66	633.40	229.55
σ^2p	295.67	554.29	164.05	266.86	633.69	229.79
σ^2e	0.21	0.18	0.23	0.21	0.29	0.23
GCV%	123.74	50.67	25.24	100.21	48.35	48.35
PCV%	123.79	50.68	25.26	100.24	48.36	48.38
ECV%	0.043	0.008	0.017	0.039	0.011	0.025
h^2%	99.93	99.97	99.86	99.92	99.95	99.90
GA	35.40	48.48	26.35	33.63	51.83	31.20
GAM%	254.82	104.37	51.97	206.35	99.57	99.55
Genetic parameter	3 x 7			3 x 9		
	3 Days	6 Days	9 Days	3 Days	6 Days	9 Days
σ^2g	155.59	289.74	389.98	129.22	199.21	1255.34
σ^2p	155.81	289.92	390.20	129.48	199.40	1255.55
σ^2e	0.23	0.17	0.22	0.26	0.20	0.22
GCV%	58.35	57.57	30.03	98.78	76.19	37.81
PCV%	58.40	57.58	30.04	98.88	76.23	37.81
ECV%	0.043	0.017	0.008	0.100	0.037	0.003
h^2%	99.85	99.94	99.94	99.80	99.90	99.98
GA	25.68	35.05	40.67	23.39	29.06	72.98
GAM%	120.12	118.55	61.84	203.28	156.88	77.88

Table 10. Continued

Genetic parameter	3 x 10			4 x 6		
	3 Days	6 Days	9 Days	3 Days	6 Days	9 Days
σ^2g	158.79	489.69	185.29	138.26	613.22	315.27
σ^2p	158.96	490.00	185.60	138.50	613.48	315.49
σ^2e	0.17	0.31	0.31	0.24	0.27	0.22
GCV%	158.98	110.07	26.04	39.35	45.73	23.19
PCV%	159.07	110.10	26.06	39.38	45.74	23.20
ECV%	0.086	0.035	0.021	0.034	0.010	0.008
h^2%	99.89	99.94	99.84	99.83	99.96	99.93
GA	25.94	45.57	28.02	24.20	51.00	36.56
GAM%	327.33	226.67	53.59	80.99	94.19	47.76
Genetic parameter	4 x 7			5 x 7		
	3 Days	6 Days	9 Days	3 Days	6 Days	9 Days
σ^2g	243.75	657.10	768.95	408.92	427.01	311.83
σ^2p	243.96	657.41	769.25	409.17	427.34	312.06
σ^2e	0.22	0.32	0.30	0.25	0.32	0.23
GCV%	93.09	69.53	35.01	105.30	42.72	55.14
PCV%	93.13	69.55	35.02	105.33	42.74	55.16
ECV%	0.041	0.017	0.007	0.032	0.016	0.020
h^2%	99.91	99.95	99.96	99.94	99.92	99.93
GA	32.15	52.79	57.11	41.64	42.55	36.36
GAM%	191.68	143.19	72.11	216.85	87.98	113.55

σ^2g : genotypic variance, σ^2p : phenotypic variance, σ^2e : environmental variance, GCV (%) : genotypic coefficient of variation in percent, PCV (%) : phenotypic coefficient of variation in percent, ECV (%) : environmental coefficient of variation in percent, h^2 (%) : heritability in broad sense in percent, GA : genetic advance, GAM (%) : genetic advance as percent of mean at 5% selection intensity.

Based on this classification heritability estimates obtained in P solubilization by all transconjugants at all time intervales was considered as high heritability because all values obtained from all crosses exceeded 60%. This indicated that phosphorous solubilization activity by *Rhizobium* cells was less influenced by the environmental factors in their expression. Therefore, the direct selection of superior genotype in P solubilization will be effective based on phenotypic performance of this trait. This agreed with Fentie *et al.,* (2014), who found high broad sense heritability for 1000 grain weight. In addition, Rai *et al.,* (2014) also observed high heritability in broad sense for fertile tillers number per plant and harvest index in rice. According to Johnson *et al.,* (1955), who considered the genetic advance as percent of mean as high (> 20%). Based on this argument, the genetic advance as a percent of mean for P solubilization was high for all transconjugants released from all crosses at all time intervales. The results of P solubilization indicated high heritability values were coupled with high genetic advance. This reflected that this trait was governed by additive gene action and the direct selection will be effective. Thus, genetic variation is the occurrence of differences among the transconjugant isolates due to the differences in their genetic makeup and the environment in which they are raised (Falconer and Mackay 1996). The genetic variability present in released transconjugant genotypes is essential to P solubilization improvement which must be exploited by microbial geneticists for P solubilization improvement to meet the required P of crop producers.

Genetic parameters of cellulase activity

Genetic variation within bacterial populations has been exploited in improving bacterial productivity of cellulase through conjugation and selection. Information about the genetic parameters as heritability and genetic advance as shown in Table 11 is a foundation of choosing appropriate transconjugant genotypes for cellulase improvement strategy. The results indicated the estimates of genetic parameters of cellulase activity among 64 *Rhizobium* transconjugants resulted from eight crosses in addition to their parents. The genetic parameters including genotypic and phenotypic coefficients of variation (GCV and PCV) were greater for cellulase activity. These estimates showed the existence of variability among the genotypes with a significant contribution of genotypic variance to an observed phenotype. High values of phenotypic coefficient of variation coupled with high genotypic coefficient of variation indicates the existence of sufficient variation within the transconjugants obtained in this study. These results indicated that the phenotypic variation between transconjugants in cellulase activity was attributed to genetic not to environmental effects.

Table 11. Genetic parameters of cellulase activity produced by rhizobial recombinants resulted from different conjugations

Conjugation	σ^2g	σ^2p	σ^2e	h^2%	GCV%	PCV%	GA	GAM%
1 x 6	0.86	0.8750	0.0150	98.29	25.58	25.80	1.90	52.40
2 x 6	4.08	4.0897	0.0097	99.76	36.24	36.29	4.15	74.47
3 x 7	2.45	2.4617	0.0094	99.62	29.33	29.38	3.22	60.30
3 x 9	1.81	1.8186	0.0086	99.53	22.09	22.14	2.77	45.48
3 x 10	2.29	2.2980	0.0080	99.65	27.34	27.39	3.12	56.37
4 x 6	2.98	2.9898	0.0098	99.67	44.08	44.23	3.55	52.51
4 x 7	0.37	0.3771	0.0081	97.85	10.99	11.11	1.23	22.26
5 x 7	1.20	1.2062	0.0062	99.49	29.50	29.58	2.25	60.60
Mean	2.01	2.0145	0.0094	99.23	28.14	28.24	2.77	53.05

σ^2g : genotypic variance, σ^2p : phenotypic variance, σ^2e : environmental variance, h^2 (%) : heritability in broad sense in percent, GCV (%) : genotypic coefficient of variation in percent, PCV (%) : phenotypic coefficient of variation in percent, GA: genetic advance, GAM (%) : genetic advance as percent of mean at 5% selection intensity.

Thus, genotypic coefficient of variation and phenotypic coefficient of variation were very close to each other indicated that an observed phenotype in regard to cellulase activity was majorly attributed to genetic effects. These results agreed with Gedifew *et al.,* (2023), who reported that traits showed high coefficients of variation reflected that the genotypes under investigation had sufficient variability in these traits. The genotypic and phenotypic variances cannot be used directly for comparing the magnitude of variability. Thus, the coefficients of variation at genotypic and phenotypic levels has been used to compare the observed variability among the genotypes. High values of GCV coupled with high PCV indicated that there were a greater diversity for cellulase activity. Hence, the direct selection would be effective for the improvement of cellulase activity. The genotypic coefficient of variation alone does not give the heritable variation present in the population. Therefore, the important selection parameters are heritability and genetic advance were worked well in understanding the mode of inheritance of investigated trait. So, heritability express on the magnitude of association between the observed phenotype and genotype. Besides, the genetic gain indicates the anticipated response as a result of selection (Shukla *et al.,* 2004). All the heritability values obtained for cellulase activity exceeded 98%. Hence, high heritability values were coupled with high genetic advance as a percentage of mean. This indicated that cellulase activity was controlled by additive gene action, as well as, this trait revealed better scope for its improvement through selection. This are in harmony with Larik *et al.,* (2000), who stated that the best performance of selection are those traits with high genetic advance associated with high heritability. High heritability values obtained in cellulase activity validated the higher importance of genetic factors on the phenotype of cellulase activity. Therefore, genetic variability was essential for continued genetic improvement in any trait. Thus, biometrical techniques serves as a useful tool in evaluating the genetic variability and the extent of heritability in the segregating transconjugant colonies. The magnitude and direction on association between heritability and genetic advance as a percent of mean (GAM) will be useful to programme the selection technique. The results are in harmony with Kalaiyarasi *et al.,* (2019), who decided that traits observed high heritability coupled with high genetic advance indicated that the additive genes were governed these traits and the improvement could be brought by selection. Based on Yeshiwas and Negash (2017) who categorized heritability (h^2) as follows; < 30% is low, 30-60% moderately and > 60% considered as high. Thus, the heritability values obtained in this study for cellulase activity were considered as high heritability. Therefore, selection for cellulase activity will be more effective because there are a major portion of genetic variation exhibiting additive gene effects. Based on Saxena and Bisen (2017), who categorized genetic advance as an another important genetic parameter for selection as low (< 10%), medium (10-20%) and high (> 20%). Through, the genetic advance obtained in this study was considered as high that shows the expected amount of genetic gain in the progeny through selection. So, high GAM indicates the effectiveness of selection and rapid genetic improvement of cellulase activity in successive generations (Kiruthika *et al.,* 2018).

Nodulation and growth related traits in fenugreek

Table 12 describe nodulation and growth parameters in fenugreek inoculated with *Rhizobium* strains and their transconjugants. The number of nodules formed by parental strains was ranged between 10-19 nodule per plant. The number of nodules developed by transconjugants was ranged between 16.60 - 31.0 nodule per plant, if compared with that formed on the positive control grown in non-sterilized soil which formed 10.53 nodule per plant. This indicated that most of the parental strains and all transconjugants achieved significant increase in nodule numbers developed per plant in relation to the positive and negative controls. The results reflected that inoculation had a significant stimulatory effect on nodule formation especially with *Rhizobium* transconjugants compared with uninoculated plants in both controls. Most of *Rhizobium* transconjugants and most of the parental strains induced significant increase in plant dry weight. Meanwhile, all *Rhizobium* transconjugants and some of the parental strains showed significant increase in nodule dry weight and root dry weight if compared with both controls. This indicated the beneficial effect of transconjugants for agricultural application was to improve plant growth and productivity. However, transconjugants applied in this study harboring plasmids from fenugreek *Rhizobium* and lentil *Rhizobium*.

Table 12. Nodulation and growth parameters in fenugreek affected by inoculation with Rhizobium strains and their transconjugants

Treatments		Number of nodules per plant	Nodules FW per plant	Nodules DW per plant	Plant FW	Plant DW	Root FW	Root DW
Sterilized soil†		0.00	0.0000	0.0000	0.85	0.155	0.041	0.0141
Non- Sterilized soil††		10.53	0.0183	0.0034	1.25	0.193	0.065	0.0213
P_1		17.00	0.0247	0.0059	1.31	0.203	0.170	0.0291
P_2		10.00	0.0157	0.0026	0.97	0.188	0.058	0.0205
P_3		13.00	0.0213	0.0046	1.40	0.204	0.154	0.0246
P_4		14.07	0.0230	0.0054	1.42	0.211	0.167	0.0285
P_5		12.27	0.0193	0.0045	1.26	0.196	0.149	0.0233
Tr_3		11.27	0.0187	0.0044	1.31	0.197	0.111	0.0225
Tr_{14}		20.40	0.0367	0.0070	1.53	0.225	0.272	0.0353
Tr_{20}		28.33	0.0530	0.0116	1.79	0.271	0.288	0.0394
Tr_{29}		27.67	0.0470	0.0102	1.68	0.235	0.279	0.0362
Tr_{35}		16.60	0.0250	0.0064	1.47	0.216	0.172	0.0331
Tr_{46}		23.53	0.0267	0.0070	1.52	0.224	0.181	0.0343
Tr_{53}		26.60	0.0447	0.0085	1.59	0.229	0.206	0.0351
Tr_{63}		31.00	0.0677	0.0166	1.69	0.248	0.333	0.0403
F-test		**	**	**	**	**	**	**
LSD	0.05	0.84	0.0009	0.0006	0.016	0.0098	0.008	0.0008
	0.01	1.13	0.0013	0.0009	0.022	0.0132	0.011	0.0011

** : Significance at 0.01 level of probability. FW, DW: Fresh weight and dry weight, respectively.
†Sterilized soil: Negative control. ††Non-sterilized soil: Positive control.

Therefore, transconjugants showed a broad host range via nodulation of fenugreek and lentil as well. Thus, transconjugants showed specific fenugreek and lentil interactions. This reflected that transconjugants demonstrated nodulation competitiveness with both hosts which is dependent on different functions harboring on their two plasmid types that acquired from both origins (Triplett 1990). So, transconjugants are harboring two types of nodulation genes of both *Rhizobium* strains introduced in conjugation event. This agrees with Hahn and Hennecke (1988), who reported that *nod* genes deleted in *R. leguminosarum* bv. *trifolii* and *Bradyrhizobium japonicum* resulted in delayed nodulation and decreased competitiveness. In addition, Lagares *et al.,* (1992) stated that alteration in lipopolysaccharide structure delayed nodulation and decline the competitiveness of *Rhizobium*. Onishchuk *et al.,* (1994) found that nodulation competitiveness was connected with numerous cultural and symbiotic performance of *Rhizobium*as phage and antibiotic resistance, bacteriocin secretion, polysaccharides production, nodulation efficiency and host specificity. The interaction between *Rhizobium* transconjugants and their leguminous hosts that are symbiotically associated plays a significant role in crop production. Legume nitrogen fixation enhanced by coinoculation with *Rhizobium* transconjugants is a new tool to improve nitrogen availability in sustainable agriculture production systems. *Rhizobium* strains and their recombinants promoted plant growth and their production directly via the secretion of plant growth regulators and enhanced the uptake of plant nutrients, or indirectly by metabolites production as siderophores, antibiotics and the decline of phytopathogens growth (Glick 1995). The cooperative interaction between *Rhizobium* transconjugants and their hosts is of relevance in nitrogen fixation and nodulation improvement with both fenugreek and lentilif compared with the parental wild type strains. Therefore, *Rhizobium* transconjugants showed better enhancement nodulation in fenugreek and improved plant growth. This may be due to overexpressing organic acids solubilizing phosphate in addition to their activity in nodulation and nitrogen fixation. Since phosphorus is an insoluble compounds and unavailable to plant, high efficient P-solubilizing transconjugants appeared are important in plant nutrition. Thus, N_2-fixing and P-solubilizing transconjugants play an important role by increasing N and P uptake by the plants. Then, these transconjugants shall playing a key role in the nodulation with their host crop legumes, because they are able to exert a beneficial action on plant growth by P-solubilization and nitrogen fixation (Zaidi and Mohammad 2006). As shown from the results tabularized in Table 13, most of the parental strains and their transconjugants showed significantly stimulated plant height and root length if compared with both controls. Furthermore, no significant increase was obtained in chlorophyll and carotenoid pigments above the positive control. In this respect, Henriksson and Pearson (1981) reported that there were a close correlation between leaf chlorophyll content and leaf N content in the lichen *Peltigera canina*. In addition, Rajendran *et al.,* (2012) found that increased chlorophyll concentrations in plants inoculated with nodule associated bacteria (NAB) reflected the efficiency of nitrogen fixation occurring in the root nodules. This indicated that there were a plant growth enhanced rhizobacteria in the positive control which is a way to improve nitrogen availability that leading to increase chlorophyll and carotenoid pigments in plants grown in natural soil as a positive control. Meanwhile, the plants grown in sterilized soil and inoculated with *Rhizobium* strains and their transconjugants showed decease in their chlorophyll and carotenoid contents in relation to the positive control, due to the absence of other rhizospheric microorganisms which contribute in stimulating plant growth.

Table 13. Plant growth and chlorophyll pigment in fenugreek affected by inoculation with Rhizobium strains and their transconjugants

Treatments		Plant height (cm)	Root length (cm)	Chl a	Chl b	Total Chl	Carotenoids
Sterilized soil†		42.7	8.6	0.361	0.109	0.470	0.113
Non- Sterilized soil††		46.3	12.9	1.012	0.482	1.494	0.500
P_1		51.5	14.9	0.951	0.401	1.352	0.443
P_2		43.7	12.3	0.858	0.359	1.217	0.494
P_3		49.1	14.5	0.511	0.241	0.752	0.344
P_4		51.0	14.6	0.692	0.352	1.044	0.395
P_5		48.6	12.9	0.786	0.388	1.174	0.438
Tr_3		45.2	12.6	0.659	0.359	1.018	0.379
Tr_{14}		55.7	15.0	0.560	0.371	0.931	0.366
Tr_{20}		59.7	18.8	0.731	0.369	1.100	0.433
Tr_{29}		58.7	16.7	0.560	0.237	0.797	0.297
Tr_{35}		51.1	14.9	0.425	0.251	0.676	0.256
Tr_{46}		53.8	14.9	0.591	0.382	0.973	0.375
Tr_{53}		56.2	17.6	0.555	0.346	0.901	0.373
Tr_{63}		58.9	18.5	0.355	0.244	0.599	0.193
F-test		**	**	**	**	**	**
LSD	0.05	0.63	0.82	0.012	0.016	0.016	0.0034
	0.01	0.85	1.11	0.016	0.022	0.022	0.0046

** : Significance at 0.01 level of probability.

†Sterilized soil: Negative control. ††Non-sterilized soil: Positive control.

This enhancement was occurred in the positive control attributed to nitrogen fixation by *Rhizobium* and their associated of plant growth promoting rhizobacteria (PGPR) which is a way to increase nitrogen availability in the soil. Many works have shown simultaneous infection with *Rhizobium*and rhizospheric bacteria that enhanced nodulation and growth in a wide legume varieties(Bolton *et al.,* 1990). Such nodule assisting bacteria may be rhizobacteria reside free living or endophytic. The endophytic bacteria may reside intercellularly or intracellularly within the host tissues (Sturz *et al.,* 2000). Based on their influence on the host crop, endophytic bacteria can be divided into three categories: plant growth inhibiting, plant growth promoting and plant growth neutral. A major class of bacterial endophytes have plant growth promoting effect (Hallmann *et al.,* 1997). The parental strains and their transconjugants achieved significant increase in chlorophyll and carotenoid pigments in relation to the negative control grown in the sterilized soil. In the sterilized soil inoculated with *Rhizobium*no rhizospheric bacteria were associated with *Rhizobium* in contrast with the positive control where rhizospheric bacteria are present. These results are in line with the findings of Rajendran *et al.,* (2012), who decided that chlorophyll levels increased in the leaves of plants inoculated with NAB reflected the efficiency of nitrogen fixation occurring in the root nodules. The same trend was also obtained by Henriksson and Pearson (1981), who stated that there are a close association between leaf N content and leaf chlorophyll content. The results indicated that parental strains and their transconjugants revealed enhancement of plant growth, nodulation and chlorophyll concentration in the leaf of their host plant due to their effective in nitrogen fixation. Besides, if these genotypes were used to inoculate the plants grown in non-sterilized soil, it shall appear better results in relation to the positive control, due to the enhancement of root-associated bacteria, as well as, to their competitiveness with rhizospheric baceria. This because plant growth-enhanced rhizobacteria promoting nitrogen fixation, stimulating plant growth and phosphorous dissolving (Zaidi and Mohammad 2006). As indicated from the results tabularized in Table 14 that all the parental strains and their transconjugants showed highly significant increase in nodule formation and nodules dry weight per plant of lentil in relation to negative (sterilized soil) and positive (non-sterilized soil) controls. This indicated that genetic exchange and rearrangement among heterologous *Rhizobium* spp. leading to broadening the host range (Vlassak andVanderleyden 1997).

Table 14. Nodulation and growth parameters in lentil affected by inoculation with Rhizobium strains and their transconjugants

Treatments		Number of nodules per plant	Nodules FW per plant	Nodules DW per plant	Plant FW	Plant DW	Root FW	Root DW
Sterilized soil†		0.0	0.000	0.0000	0.83	0.131	0.039	0.013
Non- Sterilized soil††		12.7	0.016	0.0042	0.90	0.151	0.117	0.031
P_6		22.9	0.035	0.0073	0.88	0.137	0.108	0.023
P_7		30.2	0.051	0.0093	1.74	0.208	0.670	0.073
P_9		28.8	0.049	0.0091	1.57	0.177	0.425	0.063
P_{10}		25.3	0.040	0.0091	1.49	0.181	0.735	0.092
Tr_3		32.8	0.074	0.0141	1.79	0.213	0.868	0.101
Tr_{14}		40.1	0.106	0.0245	2.35	0.231	1.184	0.128
Tr_{20}		30.9	0.063	0.0103	1.72	0.202	0.663	0.056
Tr_{29}		44.7	0.105	0.0233	2.51	0.240	1.267	0.123
Tr_{35}		35.7	0.084	0.0145	2.20	0.225	0.996	0.104
Tr_{46}		68.9	0.114	0.0255	2.87	0.244	1.818	0.181
Tr_{53}		39.5	0.094	0.0151	1.96	0.215	0.909	0.103
Tr_{63}		48.7	0.098	0.0183	2.49	0.233	1.195	0.121
F-test		**	**	**	**	**	**	**
LSD	0.05	0.83	0.00085	0.0008	0.0078	0.006	0.008	0.0007
	0.01	1.12	0.00115	0.0011	0.0105	0.008	0.010	0.0009

** : Significance at 0.01 level of probability. FW, DW: Fresh and dry weight, respectively.
†Sterilized soil: Negative control. ††Non-sterilized soil: Positive control.

Such type of rhizobial transconjugants having broad host range in nodulating fenugreek and lentil with better efficiency could be beneficial in agriculture practices if compared with most of the commercially available *Rhizobium* strains that have a very fewer host range. Thus, selection of the best inoculant genotypes for a particular legume host is difficult for obtaining effective nodulation (Martinez-Romero and Rosenblueth 1990). Broadening of host range may be brought by manipulating both plant hosts and associated rhizobia to be creating an artificial rhizosphere (Ikbal *et al.,* 2017). Electroporation is a novel tool for generating recombinants in rhizobial strains through conjugation (Chitchanok *et al.,* 2011). These genotypes showing before better cellulase production (Mervat *et al.,* 2024). Therefore, transconjugant genotypes found to have a better performance on symbiotic interaction (Aggarwal *et al.,* 2000). The number of nodules formed on lentil was ranged between 22.9 to 68.9 if compared with the positive control which formed 12.7 nodule/ plant. Whereas, the negative control was free of nodule formation. Most of rhizobial transconjugants showed better nodulation ability on lentil as compared with the mid-parents.

This are in line with Ikbal *et al.,* (2017), who investigated that *Rhizobium* transconjugants showed better nodulation ability on chickpea, lentil and pea if compared with the respective parents, whereas all un-inoculated plants were free from nodules formation. The same authors found that plants inoculated with the transconjugant genotypes expressed high than 95%elongated pink nodules. The greatest nodule number of 68.9 was obtained on roods of lentil when it was infected with transconjugant Tr_{46}. This high number of nodule formation was expected to contribute more nitrogen fixation required to promoted plant growth under nitrogen deficient soils. Plant dry weight was ranged between 0.137 (P_6) to 0.244 g (Tr_{46}) per plant, whereas the positive control recoded 0.151 g and negative control reached to 0.131 g. All *Rhizobium* transconjugants were significantly increased plant dry weight if compared with both controls and their mid-parents. Root dry weight was ranged between 0.023 (P_6) to 0.181 (Tr_{46}) if compared with the positive control (0.031). All *Rhizobium* transconjugants were significantly increased root dry weight as compared with their mid-parents and both controls. *Rhizobium* transconjugants obtained in this study are harboring genetic materials from both parental strains. This agrees with Cresti *et al.,* (2002), who stated that there was an association between the genotype and antibiotic resistance performance. The di-parental transconjugants obtained in this study extend their better symbiosis with both hosts fenugreek and lentil. These results agrees with Pankhurst *et al.,* (1986), who found that plasmid transfer may be resulted to increase nodule formation and nitrogen fixation by *Rhizobium* genotypes. Moreover, Gonzalo *et al.,* (2011) stated that plasmid transferred from varied genomic backgrounds can extend nodulation host range. The results are in line with Ikbal *et al.,* (2017), who observed that all *Rhizobium* transconjugants except one achieved a greater increase in the shoot fresh weight of chickpea, pea and lentil that developed per plant over their mid-parents. The results are in harmony with Martinez *et al.,* (1987), who observed that genetic transfer between *Rhizobium* strains has led to greatly nodule fresh, nodule dry weight and nodulation process with increase the development and growth of host legume. Similarly, Truchet *et al.,* (1991) demonstrated that *Rhizobium* transconjugants showed significant increase in nodule numbers developed per plant and shoot dry weight above the mid-parents. In addition, Musiyiwa *et al.,* (2005) found better effectiveness and nodulation efficiency of genetically modified *Rhizobium* strains. The results obtained herein greatly expand with Saı¨d*et al.* (1998), who achieved that transfer of plasmid into *Rhizobium fredii* increased the host range of recipients to the non-hosts. The present investigation evidences towards variability and dynamics of transconjugants capability to nodulate both host plants. These transconjugants are highly enhanced nodulation and nitrogen fixing ability with both hosts. In contrast, Beynon *et al.,* (1980) found that transferred plasmid pJB5JI from *Rhizobium leguminosarum* nodulating peas into *Rhizobium phaseoli* produced transconjugants were poor in nodulating *Phaseolus vulgaris* indicates the presence of plasmid interferes with the nodulation of this host. The results are also agreed with Brewin *et al.,* (1980), who stated that the host-range determination in certain strains of *Rhizobium* are borne on specific plasmids. In addition, Harun-Or Rashid *et al.,* (2014) stated that *Rhizobium leguminosarum* is the original symbiont of lentils. The dispersal of *Rhizobium* cells with the seeds of legumes is a well-accepted hypothesis (Alvarez-Martinez *et al.,* 2009). Furthermore, Harun-Or Rashid *et al.,* (2014)reported that *Rhizobium leguminosarum* presence with the seeds of lentil at the center source and countries where lentil was introduced reflected that not only the same *R. leguminosarum* species were associated with lentil but also the same chromosomal genotype was present in three different countries including Turkey, Syria and Germany. This assumed that lentil seeds may have played a key role in the dispersal of *Rhizobium leguminosarum* symbiovar *viciae* to various countries and locations. The genetic diversity in *Rhizobium* species not only based on genetic factors located on bacterial chromosome but also on plasmid borne nodulation genes (Silva *et al.,* 2005). The *nodC* and *nodD* genes determine the host range and the relationship between rhizobia with their host (Iglesias *et al.,* 2007).

Regarding to the results recorded in Table 15, all *Rhizobium* transconjugants and most of the parental strains showed significantly increase plant height and root length in relation to both controls.Plant height of lentil at 45 day-plant old was ranged between 41.07 to (P_6) to 61.13 cm (Tr_{46}). This reflected that the higher plant length was shown under the effect of transconjugant Tr_{46}. The plant height in the control was equal 40.60 cm (negative control) and 42.87 cm (positive control). The root length was ranged between 12.27 (P_6) to 26.13 cm (Tr_{46}) in relation to negative (9.47 cm) and positive (13.40 cm) controls. All *Rhizobium* transconjugants induced highly significant increase in root length above the positive and negative controls. Most of *Rhizobium* transconjugants showed significantly increase chlorophyll a, b, total and carotenoids in relation to both controls and their mid-parents. This indicated that rhizobia transconjugants are agriculturally importants via stimulating plant growth, development and chlorophyll pigments because they are capable to forming nitrogen-fixing nodules on the root legumes of both lentil and fenugreek as well. The genes required for establishment this symbiosis (*nif*, *fix* and *nod* genes) are usually located on symbiotic plasmids.

Table 15. Plant growth and chlorophyll pigment in lentil affected by inoculation with Rhizobium strains and their transconjugants

Treatments		Plant height (cm)	Root length (cm)	Chl a	Chl b	Total Chl	Carotenoids
Sterilized soil†		40.60	9.47	0.338	0.166	0.503	0.172
Non- Sterilized soil††		42.87	13.40	0.384	0.205	0.589	0.203
P_6		41.07	12.27	0.369	0.171	0.539	0.188
P_7		50.07	18.27	0.401	0.182	0.583	0.201
P_9		49.20	16.47	0.372	0.193	0.565	0.199
P_{10}		43.07	16.07	0.337	0.180	0.517	0.183
Tr_3		53.73	18.60	0.449	0.221	0.670	0.231
Tr_{14}		58.93	24.47	0.444	0.203	0.647	0.211
Tr_{20}		52.87	18.47	0.509	0.461	0.970	0.380
Tr_{29}		59.87	24.80	0.459	0.347	0.807	0.289
Tr_{35}		56.33	21.47	0.447	0.233	0.680	0.215
Tr_{46}		61.13	26.13	0.513	0.233	0.746	0.249
Tr_{53}		54.13	20.47	0.372	0.186	0.557	0.189
Tr_{63}		58.20	21.73	0.206	0.181	0.387	0.116
F-test		**	**	**	**	**	**
LSD	0.05	0.77	0.56	0.008	0.014	0.013	0.0033
	0.01	1.03	0.75	0.011	0.019	0.018	0.0045

** : Significance at 0.01 level of probability.

†Sterilized soil: Negative control. ††Non-sterilized soil: Positive control.

Meanwhile, other large plasmids in *Rhizobium* are carrying the genes of fitness competitiveness as bacteriocin, lipopolysaccharide, exopolysaccharide productions and utilization of carbon sources (Baldani *et al.,* 1992). The results are in harmony with Ding *et al.,* (2013), who found that two smallest plasmids in *R. leguminosarum* bv. *viciae* were known to be transmissible from *Rhizobium* to *Agrobacterium tumefaciens*. Hynes and McGregor (1990) reported that the genes are necessary for forming nitrogen-fixing nodules have been located on two cryptic plasmids distinct from symbiotic plasmid in *Rhizobium leguminosarum* bv. *viciae.* Nodulation assays in this study showed that the large number of nodules and effective nodules were formed on lentil by the segregants of respective *Rhizobium* transconjugants. These recombinants exhibited high concentrations of chlorophyll pigments indicating their efficiency in nitrogen fixation because chlorophyll is biomarker for nitrogen fixation. The results are also in harmony with Selbitschka and Lotz (1991), who found that segregants isolated from effective root nodules of faba bean were stably expressed Nod^+ Fix^+ with the original host *Vicia faba*, as well as, with *L. sativus*, *V. angustifolia* and *L. culinaris*. In addition, Wang *et al.,* (1986) reported that changes in the symbiotic properties of *R. L.* bv. *trifolii* and bv. *phaseoli* are correlated with plasmid rearrangements. The results obtained in this study suggested that the different host-range genes are functionally compatible in the segregants of transconjugants. Therefore, the genetic exchange and rearrangement involved heterologous rhizobia leading to generated transconjugants broadening of host range which could be beneficial in sustainable agricultural practices as most of the commercially available genotypes that have a very decrease in their host range.

Concluding remarks

Rhizobium transconjugants generated in this study clarifies that they may increased the growth parameters of their legume hosts. Inoculation with *Rhizobium* recombinants may be an effective bioinoculant for their hosts to increase plant production in a sustainable way. In the future, these recombinants could be tested in the field to test their stability under various environmental circumstances to develop into promising green biofertilizer inoculants. *Rhizobium* recombinants may cause a promoting effect on plant growth parameters leading to enhanced plant yield. *Rhizobium* transconjugants obtained in this study showed better expression in phosphorous solubilization, as well as, expressed better ability in nodulation with both hosts of fenugreek and lentil. This work have suggested that host-range genes in certain *Rhizobium* strains are borne on specific plasmids. Increased chlorophyll concentrations in plants inoculated with transconjugants above both controls indicates the efficiencyof nitrogen fixation expressed in the nodules. Because there are a close association between chlorophyll concentration and nitrogen fixation. So, chlorophyll content is a biomarker for nitrogen fixation in nodules. Physical isolation and characterization of transconjugant genotypes may help to clarify their role in symbiosis. Fenugreek and lentil inoculated with better genotypes of transconjugants will improve consequently the quantity of the produced seeds especially in low soil nitrogen content. So, better nodulation and nitrogen fixation ability of *Rhizobium* genotypes are urgently requisite for improving the growth and development of legume crops in sustainable agriculture. Most of *Rhizobium* transconjugants showed maximum phosphate solubilization in relation to their mid-parents. This provided significant evidence for the importance of recombinant genotypes in phosphate solubilization. Before recommending the use of such genotypes further tests must be designed under field conditions (competitiveness, persistence, etc.) to examine their behavior. P availability in the soil which is limited to the plants for crop production. *Rhizobium*cells can convert P into utilizable form via inorganic P solubilization by organic acids, released or organic P mineralization mainly by phytases and acid phosphatases. So, the gene expression and regulation of P solubilizing ability in transconjugants is an important task in microbial genetics of soil biofertilizers. All P solubilizing *Rhizobium* harboring *pqq* genes in their genomes which is a potential molecular marker in gram negative bacteria as *Rhizobium*that solubilize tricalcium phosphate. These genes were absence in the genome of Gram positive bacteria leading them did not have P solubilizing mechanism.Conjugative transfer of symbiotic plasmids between fenugreek and lentil *Rhizobium* species generated transconjugants rendered them are able to exhibit better nodulation ability on both hosts. The analysis of infection process induced by transconjugants would also be important. Transconjugants are necessary for the development of better symbiosis ability in a broadhost. Therefore, conjugative transfer is one of the mechanisms allowing *Rhizobium* diversification and evolution.

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