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RESEARCH PAPER

Productivity of Carboxymethyl Cellulase from *Rhizobium* Transconjugants

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ABSTRACT

Cellulase produced by rhizobia would soften the root hair cell wall enabling *Rhizobium* to penetrate and forming a trigger infection thread. In response the plant deposits a new layer of wall material at the infection site. Therefore, this study aimed to generate new recombinants of *Rhizobium* transconjugants that represent high cellulase activity to be used as bioinoculants in sustainable agriculture pathways. Cellulase activity was detected in 64 new recombinants isolated from eight conjugations compared with the mid-parent of nine parental strains. From each conjugation, eight genotypes were isolated to be evaluated for cellulase activity. The variability in cellulase activity has a very wide range which may be related to the variations in the bacterial genotype. Among all transconjugants evaluated in this study, cellulase activities over the mid-parent ranged between 0.023-0.087. The highest activity (0.087) over the mid-parent was shown by new recombinants resulting from $P_3 \times P_7$, followed by transconjugants released from $P_2 \times P_6$ (0.085) and transconjugants released from $P_4 \times P_6$ (0.084). Meanwhile, the lowest activity over the mid-parent was shown by transconjugants derived from $P_4 \times P_7$ (0.023). In the outlook, the superior genotypes in this guide may be functional to the elevated selection of novel cellulase activity, which allows transconjugants to better penetrate and form root nodules from the infection threads of their legume hosts.

HIGHLIGHTS

- *Rhizobium* can penetrate plant cells via producing cellulase and forming infection thread in this point.
- This study improved cellulase productivity from *Rhizobium* via inducing new recombinants to be select the superior genotypes.
- Generated transconjugants showed better expression in cellulase activity which allows to better penetrate and forming root nodules with their legume hosts from the infection threads.

Keywords: Cellulase activity, *Rhizobium* transconjugants, mid-parent, infection thread, bioinoculants, hybrid performance

Rhizobia are gram-negative bacteria developed morphologically nodules on legume plant roots. The symbiotic interaction resultedin the reduced atmospheric dinitrogen to ammonia which is then implied to be utilized by the host legume. The symbiosis between rhizobia and leguminous hosts is of agronomic and environmental importance and facilitates crop production in nitrogen-limited soils without needing fertilizer supply. Rhizobia may grow as free-living microorganisms as well as induced and colonized root nodules on the roots of leguminous plants thereby establishing a partnership that benefits both partners. This process begins when the plant flavonoids induced *Rhizobium nod* genes which are involved in the secretion of

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lipochito-oligosaccharide signal molecules, known as *Nod* factors. In response, plant root hairs exhibit a marked curling to facilitate bacterial infection and penetration. In the bacteriods, bacteria fix atmospheric dinitrogen into ammonia (Dazzo *et al.* 1984). A central stage in the formation of root-nodule symbiosis is the localized erosion of cellulosic plant cell walls from which the bacteria establish a nitrogen-fixing intracellular state within their legume hosts. Plant cell wall degrading enzymes participate in the infection event via two steps: in the first step primary infection of host root hairs induced infection thread formation and later step the bacterial cells were released from the infection points within host nodule cells. Plant cell wall degradation allows the localized penetration of the bacterial symbiont into the host cell without its overt lysis and destruction (Dazzo *et al.* 1984).

Several studies reflected that rhizobia produce enzymes capable of degrading plant cell wall polymers (Angle 1986). Carboxymethyl cellulase is probably involved in all of the infection processes (Chen *et al.* 2004). The enzyme from *Rhizobium leguminosarum bv. trifolli* degraded both carboxymethyl cellulose and polypectate. This facilitated direct bacterial penetration (Mateos *et al.* 1992). The cell wall degradation enzymes that are involved in the infection stage are associated with the bacteria. Cellulolytic enzymes contained at least two carboxymethyl cellulase (CMCase) isozymes (Mateos *et al.* 2001). Robledo *et al.* (2008) established that cellulases are produced by the wild-type strains of *Rhizobium leguminosarum* (biovars *viciae*, *trifolli* and *phaseoli*), *Mesorhizobium loti*, *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* (Jiménez-Zurdo *et al.* 1996). Further investigations on *Rhizobium leguminosarum* bv. *trifolli* reflected that the wild-type strain of clover nodulating rhizobia secreted at least two cellulase isozymes, C_1 and C_2, both enzymes are cell-bound (Mateos *et al.* 1992). Studies using the symbiotic plasmid indicated that gene (s) required for the production of cellulase C_1 are located in a symbiotic plasmid (pSym), whereas the locus of cellulase C_2 is not pSym-borne (Jiménez-Zurdo *et al.* 1996).

The infection of legume roots by *Rhizobium* shows a high degree of specificity in a complex process. Ljunggren and Fähraeus (1961) suggested that rhizobia induced the production of polygalacturonases by the plant. These enzymes would soften the cell walls in the root hair allowing *Rhizobium* to penetrate and infection thread formation. The involvement of polysaccharide degrading enzymes in the infection process was proposed by electron microscope results. *Rhizobium* appears to be a degraded plant cell wall (Callaham and Torrey 1981). Martinez-Molina *et al.* (1969) showed that rhizobia produces cellulases and hemicellulases. Meanwhile, Hubbell *et al.* (1978) found that rhizobia produces polygalacturonases. Cellulase is the most prominent group of hydrolytic enzymes that hydrolysis of -1, 4 linkages present in cellulose to release glucose. The cellulases are primarily produced in nature by bacteria, fungi, plants, nematodes, mollusks and even some protozoa (Watanabe and Tokuda 2001). The presence of cell wall-hydrolyzing enzymes in the *Rhizobium* legume system is widespread in nature (Bateman and Millar 1966). The enzymes are frequently inducible in the following sequence are as follows; pectinase, hemicellulase and cellulase (Cooper 1977). The first type of enzyme in the Rhizobium legume system prompted two types of enzymes (hemicellulase and cellulase) that could be expected to be present in rhizobia and active in the infection event (Hubbell *et al.* 1978).

The cellulase genes and their corresponding cellulolytic enzymes they encode are widespread and expressed in the bacteria establishing root nodule symbiosis (Robledo *et al.* 2008). Among them, the cel C gene is involved in bacterial cellulose biosynthesis (Matthysse *et al.* 1995). Cellulase was essential for symbiotic infection in the roots of legume host in the genus *Rhizobium*, thus playing a very significant role in the infection processes thatfacilitate the development of nitrogen-fixing endosymbiosis (Robledo *et al.* 2008). The infection process during the establishment of the rhizobia-legume symbiotic relationship was established through a complex molecular dialogue between the rhizobia and the legumehost, followed by a series of steps of reciprocal signal exchanges (Charpentier *et al.* 2016). Bacteria entry into their host root cells requires not only an appropriate recognition of rhizobia cells to maintain a compatibility relationship but also to preserve the balance between the host survival and bacterial viability, thus avoiding plant defense responses. The key step

in this stage is the local plant cell wall degradation required for *Rhizobium* penetration into root hairs leading to induce plant infection thread development and further release of *Rhizobium* into the cells of nodule primordia. More than 50 different strains forming diazotrophic plant symbiosis have been found to share cellulase activity (Robledo *et al.* 2008). All *Rhizobium* genomes sequenced to date harbor genes encoding glycosyl hydrolase enzymes. The genes encoding endoglucanases are widespread and commonly expressed in all species of rhizobia genera (Robledo *et al.* 2011). These hydrolytic enzymes played a primary role in the host infection through root-hairtips by rhizobia which produces two cell-bound cellulases designated $CelC_1$ and $CelC_2$ (Mateos *et al.* 1992). $CelC_2$ leads to localized hole formation, which matches the entry point of bacteria during the primary infection process (Mateos *et al.* 2001). $CelC_2$ belonging to glycosyl hydrolase family eight exhibits high substrate specificity for noncrystalline cellulose. In addition, *cel* C mutants can not invade their clover host through root hairs (Robledo *et al.* 2008). Besides this observation, $CelC_2$ over-producing strains induced a lower number of nodules in root tissues if compared with the wild-type infection suggesting that $CelC_2$ triggers plant defense responses (Robledo *et al.* 2011). Therefore, this study aimed to estimate cellulase produced by *Rhizobium* transconjugants *in vitro* which played a very significant role in the primaryinfection process required for the development of nitrogen-fixing symbiosis.

MATERIALS AND METHODS

Genetic materials

One genotype of each fenugreek (Giza 30) and lentil (Giza 9) was used in this study. These genotypes were kindly obtained from the Field Crops Research Institute, Agriculture Research Center, Egypt. These genotypes were used to isolate *Rhizobium* strains used in this study.

Bacterial strains and growth conditions

Wild-type isolates of *Rhizobium* sp. were isolated from their legume hosts and grown on yeast extract mannitol medium (YEMAM) at 28° C as previously described by Karanja and Wood (1988). This medium (YEMAM) was used as a full synthetic medium according to Allen (1959). Congo-red was added to these medium when needed for the purification of isolates according to Vincent (1970). Bacterial cultures were isolated from the root nodules of fenugreek and lentil grown on the farm of the Genetic Department, Faculty of Agriculture, Mansoura University through the winter season of the academic year 2022-2023. All *Rhizobium* colonies isolated from the root nodules of fenugreek were numbered; R_1, R_2, R_3, R_4 and R_5. Meanwhile, the *Rhizobium* colonies isolated from the root nodules of lentil were numbered; R_6, R_7, R_8, R_9 and R_{10}.

Carboxymethylcellulose medium (CMC) used for the cultivation of cellulolytic microorganisms

This medium consists (g/L) of; $(NH_4)_2$ SO_4 0.5, KH_2PO_4 10.0, K_2HPO_4 5.0, $MgSO_4$ 0.1, NaCl 0.2, Yeast extract 1.0, CMC 3.0 according to Liang *et al.* (2014).

Dinitrosalicylic acid (DNSA) reagent

This reagent was prepared by adding 1g of 3,5 – dinitrosalicylic acid, 1g sodium hydroxide, 200 mg crystalline phenol, 50 mg sodium sulphite and 40g potassium sodium tartrate in 100 ml distilled water (Miller 1959).

Citrate buffer (0.1 M, pH 6.0)

Citrate buffer was used to determine the cellulase activity of crude enzyme solution obtained from CMC medium. The components of this buffer are listed in Table 1.

Table 1: Required components of citrate buffer

Component	Weight	Concentration
Sodium citrate dihydrate (mW 294.10 g/mol)	24.269 g	0.0825 M
Citric acid (mW 192.12 g/mol)	3.358 g	0.0175 M

Prepare 800 ml of distilled water in a suitable container. 24.269 g of sodium citrate dihydrate was added to the solution. Add 3.358 g of citric acid to the solution. Adjust the solution to the desired pH using 0.1 N HCl (pH = 6.0). Adjust the volume to one litre using distilled water (Gomori 2004).

Isolation of rhizobia

Rhizobia were isolated from plant roots according

to Somasegaran and Hoben (1985). The authenticity of *Rhizobium* is confirmed by the nodulation test according to Somasegaran and Hoben (1985).

Plant infection test

The nodulation test was carried out during the growing season of the academic year 2022/2023. This experiment was done using pots containing sterilized sandy and clay soils with a ratio of 1:1, which were autoclaved at 121°C for two hours over three days. The sandy soil was washed with distilled water several times to diminish chloride ions. These pots were used for authentication of nodulation by bacterial isolates against their legume hosts.

Inoculation

Mid-log phase growing cultures in a nutrient broth of YEM were used for inoculating 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 two plants, per pot. The seeds planted in all experiments were inoculated with approximately 10 ml of rhizobia cells from the previous suspension before being covered with the soil. 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 plant roots reflected that inoculated isolates were rhizobia specified for this host plant.

Nodulation test

This trait was measured when the plants reached to 45 days plant-old. Three plants from each replicate were selected at random and the roots were washed by tap water. The number of nodules developed on plant roots reflected that inoculated cultures were rhizobia.

Genetic marking based on antibiotic susceptibility assays

Antibiotic susceptibility was measured by plate diffusion method according to Collins and Lyne (1985) using different antibiotics from the same concentration of 250 mg/ml as follows; Azithromycin (*Azm*), Ampicillin (*Amp*), Nitrofurantoin (*Nit*), Benzoyl Metronidazole (*Mtz*), Clindamycin (*Cli*) and Amoxicillin - Clavulanic acid (*Aug*). Conjugation was done between different bacterial strains harboring the opposite genetic markers according to Grinsted and Bennet (1990) as shown in Table 2.

Endoglucanase activity – determining carboxymethylcellulase activity

This method was prepared by Miller (1959) using 3,5 – Dinitrosalicylic acid (DNS) which modified by citrate buffer (0.1 M, pH 6.0), substrate concentration CMC (0.5 – 2.0 %), DNS reagent, biological material which was used in reaction mixture (0.2 – 1.0 cm^3) and incubation: temperature (30-50 °C), as well as, reaction time (10 – 30 min) (Carvalho *et al.* 2016). The following technique represents the modified method used to determine the activity of endoglucanase released by rhizobia. In this technique, three

Table 2: Conjugation between one *Rhizobium* isolate of fenugreek against one *Rhizobium* isolate of lentil both harboring the opposite genetic markers

Mating	Relevant genotype	Selective markers	Designation of transconjugant (Tr) colonies isolated
Rhizobium sp. (1) × *Rhizobium sp.* (6)	Cli^+, Aug^- × Cli^-, Aug^+	Cli^+, Aug^+	Tr_1, Tr_2, Tr_3, Tr_4, Tr_5, Tr_6, Tr_7, Tr_8
Rhizobium sp. (2) × *Rhizobium sp.* (6)	Mtz^+, Aug^- × Mtz^-, Aug^+	Mtz^+, Aug^+	Tr_9, Tr_{10}, Tr_{11}, Tr_{12}, Tr_{13}, Tr_{14}, Tr_{15}, Tr_{16}
Rhizobium sp. (3) × *Rhizobium sp.* (7)	Mtz^+, Aug^- × Mtz^-, Aug^+	Mtz^+, Aug^+	Tr_{17}, Tr_{18}, Tr_{19}, Tr_{20}, Tr_{21}, Tr_{22}, Tr_{23}, Tr_{24}
Rhizobium sp. (3) × *Rhizobium sp.* (9)	Mtz^+, AMP^- × Mtz^-, AMP^+	Mtz^+, AMP^+	Tr_{25}, Tr_{26}, Tr_{27}, Tr_{28}, Tr_{29}, Tr_{30}, Tr_{31}, Tr_{32}
Rhizobium sp. (3) × *Rhizobium sp.* (10)	Mtz^+, AMP^- × Mtz^-, AMP^+	Mtz^+, AMP^+	Tr_{33}, Tr_{34}, Tr_{35}, Tr_{36}, Tr_{37}, Tr_{38}, Tr_{39}, Tr_{40}
Rhizobium sp. (4) × *Rhizobium sp.* (6)	Mtz^+, Aug^- × Mtz^-, Aug^+	Mtz^+, Aug^+	Tr_{41}, Tr_{42}, Tr_{43}, Tr_{44}, Tr_{45}, Tr_{46}, Tr_{47}, Tr_{48}
Rhizobium sp. (4) × *Rhizobium sp.* (7)	Mtz^+, Aug^- × Mtz^-, Aug^+	Mtz^+, Aug^+	Tr_{49}, Tr_{50}, Tr_{51}, Tr_{52}, Tr_{53}, Tr_{54}, Tr_{55}, Tr_{56}
Rhizobium sp. (5) × *Rhizobium sp.* (7)	Mtz^+, Aug^- × Mtz^-, Aug^+	Mtz^+, Aug^+	Tr_{57}, Tr_{58}, Tr_{59}, Tr_{60}, Tr_{61}, Tr_{62}, Tr_{63}, Tr_{64}

samples are prepared with DNS reagent: control sample, actual sample and reagent sample, which is measured by spectrophotometry. In the control sample, 0.75 cm^3 of 1% carboxymethylcellulose prepared in citrate buffer (0.1 M, pH 6.0) is placed in a test tube and incubated at 50 °C for 30 min. First 1.5 cm^3 of DNS reagent and 0.75 cm^3 of supernatant are added after incubation. The reaction mixture is heated in water bath at 100 °C for 10 min and next cooled down. A similar methodology was applied with the actual sample, as well as, the supernatant was added to the CMC solution before the addition of DNS. All are incubated at 50 °C for 30 min and further steps are the same as in the control sample. 7.0 cm^3 of sterile H_2O was added for each the control and actual samples. The absorbance was estimated spectrophotometrically at 540 nm. Reducing sugars (glucose, oligosaccharides) formed caused the transformation of DNS to 3-amino, 5-nitrosalicylic acid, which caused the change of color from yellow to orange-red, depending on the quantity of generated reducing sugars (Marsden *et al.* 1982). A standard curve was prepared by the following concentrations of glucose; 200 μg/cm^3, 400 μg/cm^3, 600 μg/cm^3, 800 μg/cm^3, 1000 μg/cm^3, 1200 μg/cm^3, 1400 μg/cm^3, 1600 μg/cm^3, 1800 μg/cm^3 and 2000 μg/cm^3 (Fig. 1).

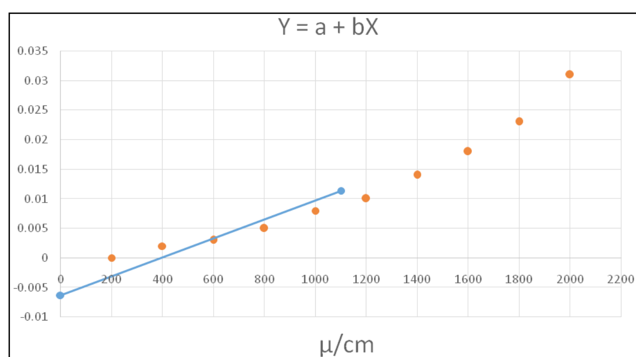

Fig. 1

The following formula was used to calculate cellulolytic activity according to Khoshnevisan *et al.* (2011) as follows;

$$\text{Activity} = \frac{W.1000}{V.t.M}$$

Where: W = the account of released glucose equivalents

M = the molecular weight of glucose

V = the volume measured sample

t = the reaction time

After accounting the amount of reducing sugars, cellulase activity was estimated and expressed in units (IU). One unit of enzymatic activity corresponds to the amount of enzyme required to liberate 1.0 μmol of glucose per one min under the test conditions (Manfredi *et al.* 2016).

Statistical analysis

Results are the mean values of three biological replicates. The data collected were subjected to analyses of variance to test the significance of differences between genotypic means using the F- test. In addition, the least significant difference (LSD) was used to test the significance of differences between two means at 0.05 and 0.01 levels of probability according to Steel and Torrie (1960).

RESULTS AND DISCUSSION

Cellulase activity in recombinants

Transconjugants of $P_1 \times P_6$

The results obtained in Table 3 showed significant differences in glucose concentration released from cellulose hydrolysis leading to significant differences in cellulase activity between rhizobial transconjugants. Cellulase activities produced by transconjugants ranged between 0.041 to 0.088 if compared with the mid-parent (0.055). The highest cellulase activity (0.088) was produced by transconjugant Tr_3 followed by Tr_2 (0.083), whereas the lowest (0.041) activity was produced by transconjugant Tr_6. Fifty percent of transconjugants as Tr_2, Tr_3, Tr_5 and Tr_8 appeared significant increase in cellulase activity in relation to the mid-parent. These results indicated that some *Rhizobium* transconjugants showed better expression in cellulase genes if compared with their parents. The higher activity of cellulase may be due to the high dosage of cellulase genes harboring transconjugants through the conjugation process either plasmid or chromosomal located. Extending the role of cellulase in the active transconjugants was reflected in the active penetration of transconjugants against plant cell walls. This agreed with McCoy (1932), who was the first to investigate the possible involvement

of hydrolytic enzymes in the infection of legumes by rhizobia. It was established that cellulases are produced by all types of strains of the official species of rhizobia (Robledo *et al.* 2008). The cellulase genes and their cellulolytic enzymes are commonly encoded and expressed among bacteria establishing the root nodule symbiosis (Robledo *et al.* 2011). Among the cellulase genes, the *cel* C gene encodes a cellulase ($CelC_2$) essential for symbiotic infection of legume host roots in the genus *Rhizobium* fulfilling a significant role in infection processes required for the development of nitrogen fixation endosymbiosis (Robledo *et al.* 2011). All species in the genus *Rhizobium* analyzed up to date carry a *cel* C gene in their chromosome (Robledo *et al.* 2011). The phylogeny of the *cel* C gene is completely congruent with those based on the chromosomal genes, but not with those based on plasmid-borne genes, in species of the genus *Rhizobium*. Furthermore, *cel* C represents a new phylogenetic maker useful for taxonomic studies in the genus *Rhizobium* (Robledo *et al.* 2011). For improving cellulase-producing activity, transconjugants may be more effective than their parents. Recombinant transconjugants gave various amounts of reducing sugar concentration through cellulose hydrolysis. Increasing the activity of transconjugants producing cellulase reflected that chromosomal genes may be transferred through the conjugation process leading to increasing the dosage of *cel* C genes in *Rhizobium* transconjugants because some of the cellulase genes were chromosomal located.

Therefore, transconjugants – plant symbioses play an important role in sustainable crop production through *Rhizobium*–legume symbioses which provide all N-requirements of many leguminous crops worldwide (Masson-Boivin *et al.* 2009). Thus, *Rhizobium* transconjugants are excellent plant growth-promoting rhizobacteria (PGPRs) when inoculated onto host crop legumes (Flores-Felix *et al.* 2013). Transconjugants from PGPRs can positively affect plant growth by both direct mechanisms, such as the acquisition of nutrients and biosynthesis of plant hormones, and by indirect mechanisms, such as the production of lytic enzymes e. g. cellulases which induced active penetration in plant cell walls during symbioses (Gaiero *et al.* 2013). This agreed with Hubbell (1981), who proposed that wall-degrading enzymes produce a localized degradation that completely traverses the root hair wall, creating a continuous portal of entry allowing direct penetration by the bacteria. The biochemical characteristics of $CelC_2$ cellulase restrict its symbiotically relevant activity during primary host infection (Mateos *et al.* 2011). The results provide compelling evidence that *Rhizobium* recombinants play a significant role in the primary infection required for

Table 3: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_1 \times P_6$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	**Hybrid performance**	**Glucose concentration mg/ml**	**Hybrid performance**	**Cellulase activity**
P_1		1.55	—	2.61	—	0.044
P_6		1.14	—	3.85	—	0.065
MP		1.35	1.00	3.23	1.00	0.055
Tr_1		1.14	0.85	3.35	1.04	0.057
Tr_2		2.48	1.85	4.91	1.52	0.083
Tr_3		1.37	1.01	5.22	1.61	0.088
Tr_4		2.17	1.61	2.75	0.85	0.046
Tr_5		2.13	1.58	3.85	1.19	0.065
Tr_6		2.01	1.49	2.42	0.75	0.041
Tr_7		2.05	1.52	3.37	1.05	0.057
Tr_8		2.48	1.84	3.93	1.22	0.066
F – Test		**	**	**	**	**
LSD	0.05	0.18	0.127	0.21	0.062	0.0034
	0.01	0.25	0.174	0.29	0.086	0.0047

***: Significance at 0.01 probability level. MP: Mid-parent.*

the development of symbiosis with their legume hosts (Robledo *et al.* 2008).

Transconjugants of $P_2 \times P_6$

The results tabularized in Table 4 showed significant differences in glucose concentration released from cellulose hydrolysis among the control and actual samples which led to significant differences in cellulase activities between rhizobial transconjugants. Cellulase activities produced by rhizobial transconjugants ranged between 0.051 to 0.134 if compared with the mid-parent (0.049). The highest cellulase activity (0.134) was produced by transconjugant Tr_{14} followed by Tr_{11} (0.127), whereas the lowest (0.051) activity was produced by transconjugant Tr_9. Most of transcojugants obtained from this conjugation revealed significant increase in cellulase activity in relation to the mid-parent. These results indicated that *Rhizobium* transconjugants showed better expression in cellulase-producing genes if compared with the mid-parent. This agreed with Robledo *et al.* (2018), who decided that rhizobial cultures release hydrolytic enzymes degrading plant cell wall polymers, which enable the primary infection of legume hosts. All rhizobial genomes to date harbor genes encoding glycosyl hydrolase enzymes. The genes encoding endoglucanases were commonly expressed in all species of *Rhizobium* (Robledo *et al.* 2011). *Rhizobium leguminosarum* bv. *trifolii* synthesize ß-(1–4)-endoglucanase, named cellulase $CelC_2$, that has been involved in the establishment of the symbiosis between this bacterium and its legume host (Robledo *et al.* 2008). Mateos *et al.* (2001) found that *Rhizobium* produced two cell-bound cellulases, designated $CelC_1$ and $CelC_2$, from which $CelC_2$ only can completely erode the root hair of the compatible legume host. $CelC_2$ has two described functions: (i) it modulates the length of cellulose microfibrils, affecting biofilm formation, and (ii) it catalyzes the localized hydrolysis of the root cellwall, thereby promoting primary and secondary infection into the host tissues (Menéndez *et al.* 2016). Moreover, Robledo *et al.* (2018) showed an impact of this cellulase in earlier stages of symbiotic signaling, interfering with calcium spiking and delaying nodulation.

Transconjugants of $P_3 \times P_7$

The results tabularized in Table 5 showed significant differences in glucose concentration released from cellulose hydrolysis which reflected significant differences in cellulase activity between rhizobial transconjugants. Cellulase activities produced by rhizobial transconjugants ranged between 0.075 to 0.149 if compared with the mid-parent (0.062). The

Table 4: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_2 \times P_6$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	**Hybrid performance**	**Glucose concentration mg/ml**	**Hybrid performance**	**Cellulase activity**
P_2		0.62	—	1.86	—	0.032
P_6		1.14	—	3.85	—	0.065
MP		0.88	1.00	2.86	1.00	0.049
Tr_9		1.80	2.04	3.06	1.07	0.051
Tr_{10}		3.17	3.60	5.49	1.92	0.092
Tr_{11}		4.43	5.03	7.51	2.63	0.127
Tr_{12}		4.32	4.91	6.42	2.25	0.108
Tr_{13}		3.60	4.09	6.04	2.12	0.102
Tr_{14}		3.68	4.19	7.97	2.79	0.134
Tr_{15}		4.12	4.68	7.20	2.53	0.121
Tr_{16}		3.79	4.31	6.33	2.22	0.107
F – Test		**	**	**	**	**
LSD	0.05	0.189	0.22	0.169	0.047	0.0028
	0.01	0.260	0.30	0.231	0.065	0.0038

***: Significance at 0.01 probability level. MP: Mid-parent.*

highest cellulase activity (0.149) was produced by transconjugant Tr_{20} followed by Tr_{18} (0.106), whereas the lowest (0.075) activity was produced by transconjugant Tr_{22}. Fifty percent from the transconjugants as Tr_{18}, Tr_{20}, Tr_{21} and Tr_{24} showed significant increase in cellulase activity if compared with the mid-parent. These results indicated that *Rhizobium* transconjugants showed better expression in cellulase encoding genes if compared with the mid-parent. Electron microscopic studies of the infection process confirm that the *bcs*Z gene also called *cel* C in *R. leguminosarum* bv. *trifolii* has been shown to encode a cellulase (Ridge and Rolfe 1985). Rhizobium *cel* C is also located in the celABC operon (Ausmees *et al.* 1999). Studies using the symbiotic plasmid (pSym) cured and nod-recombinant derivatives of *Rhizobium* indicated that genes required for cellulase C1 production are located in pSym but outside the *nod* region, whereas the locus of cellulase C2 is not pSym-borne (Jiménez-Zurdo *et al.* 1996).

Therefore, Mateos *et al.* (2001) summarized the symbiotic relevance of cellulase activity as follows: (i) its highly localized point of cell wall degradation at root hair tips of legume hosts, (ii) it was increased root hair infectibility, and (iii) it was less extensive when legume plants were grown at sufficient nitrate supply to inhibit primary infection (Dazzo *et al.* 1996).

Transconjugants of $P_3 \times P_9$

As shown from the results tabularized in Table 6 there were significant differences in glucose concentration released from cellulose hydrolysis leading to significant differences in cellulase activity between rhizobial transconjugants. Cellulase activities produced by transconjugants ranged between 0.092 to 0.123 if compared with the mid-parent (0.072). The highest cellulase activity (0.123) was produced by transconjugants Tr_{29} and Tr_{32} followed by Tr_{31} (0.118), whereas the lowest (0.092) activity was produced by transconjugant Tr_{26}. All transconjugants obtained from this conjugation showed significant increase in cellulase activity in relation to the mid-parent. The results indicated that *Rhizobium* transconjugants showed overexpression in cellulase genes if compared with their parents. This agreed with Dingle *et al.* (1953), who reported that rhizobia hydrolyzes the root cell wall of legumes at the highly localized site of infection. Meanwhile, root extracts stimulate the production of cellulases by *R. leguminosarum* bv. *trifolii* (Morales *et al.* 1984). Therefore, Robledo *et al.* (2008) reported that *Rhizobium cel* C null mutants were unable to canonically invade its legume host through root hairs, confirming the essential role of cellulase in primary infection required for the development of root nodule symbiosis.

Table 5: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_3 \times P_7$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	**Hybrid performance**	**Glucose concentration mg/ml**	**Hybrid performance**	**Cellulase activity**
P_3		1.70	—	2.77	—	0.046
P_7		2.79	—	4.62	—	0.078
MP		2.25	1.00	3.69	1.00	0.062
Tr_{17}		1.92	0.85	5.03	1.36	0.085
Tr_{18}		1.45	0.65	6.27	1.70	0.106
Tr_{19}		2.01	0.90	5.15	1.39	0.087
Tr_{20}		3.97	1.77	8.84	2.39	0.149
Tr_{21}		4.66	2.07	6.04	1.63	0.102
Tr_{22}		1.53	0.68	4.43	1.20	0.075
Tr_{23}		1.39	0.62	4.72	1.28	0.080
Tr_{24}		1.18	0.52	5.53	1.50	0.093
F – Test		**	**	**	**	**
LSD	0.05	0.132	0.058	0.166	0.044	0.0027
	0.01	0.180	0.081	0.228	0.061	0.0037

***: Significance at 0.01 probability level. MP: Mid-parent.*

In addition, Morales *et al.* (1984) showed that curing of pSym did not affect cellulase production in *R. leguminosarum* bv. *trifolii* indicated that the gene(s) coding for the endo-glucanase is not located in the sym plasmid, but these genes encoding CM-cellulase(s) are harboring on either the bacterial chromosome or cryptic plasmids. *Cel* C gene was located near putative cellulose synthase genes in a region of the chromosome (*cel* ABC) (Matthysse *et al.* 1995). Therefore, *Rhizobium* transconjugants fulfill a very significant role in the infection processes required for the efficient development of nitrogen fixation endosymbiosis (Robledo *et al.* 2008). In addition, rhizobial transconjugants showed better results for increased cellulase $CelC_2$ production allow to invade and occupy a higher proportion of formed nodules on their legume hosts than their parents. Cellulases are multi-enzyme complexes containing three main components that act synergistically to hydrolyze cellulose to glucose (Beguin and Aubert, 1994). New recombinants of rhizobia obtained in this study may carry a high dosage of cellulase genes especially that plasmid harboring or chromosomal located which must breach plant cell walls to colonize intact inner plant tissue (Robledo *et al.* 2011). This indicated that plasmids or either chromosomal segments harboring chromosomal genes encoding cellulase may transferred through conjugation leading to transconjugants overexpressing $CelC_2$ than their parents. This suggests that bacterial recombinant cellulases contribute to better breaking down plant cell walls to develop symbiotic phenotypes. Therefore, $CelC_2$ homologs have been identified in all species of the genus *Rhizobium* (Robledo *et al.* 2011). Thus, the model of *Rhizobium* transconjugants showed overexpressing $CelC_2$ than their parents could therefore explain the efficiency of developing symbiotic phenotypic. It is also plausible that $CelC_2$ directly impacts symbiosis signaling through the effect on NF-membrane-bound receptors as an extracellular domain. The cellulase encoding genes and the cellulolytic enzymes they encode are widespread and commonly expressed in rhizobia establishing root nodule symbiosis (Robledo *et al.* 2008). Cellulase is the most prominent group of hydrolytic enzymes that catalyze the hydrolysis of -1, 4 linkages in cellulose to release glucose which is the central metabolite pathway of different microorganisms to produce various bioproducts (Watanabe and Tokuda 2001). The recombinant transconjugants can be promising candidates for developing new cellulase systems with enhanced cellulase activity as a consequence exhibited symbiotic phenotype.

Table 6: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_3 \times P_9$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	**Hybrid performance**	**Glucose concentration mg/ml**	**Hybrid performance**	**Cellulase activity**
P_3		1.70	—	2.77	—	0.046
P_9		4.04	—	5.80	—	0.098
MP		2.87	1.00	4.29	1.00	0.072
Tr_{25}		1.55	0.54	6.98	1.63	0.117
Tr_{26}		0.76	0.27	5.49	1.28	0.092
Tr_{27}		3.87	1.35	6.00	1.40	0.101
Tr_{28}		2.24	0.78	6.44	1.50	0.108
Tr_{29}		0.98	0.34	7.31	1.70	0.123
Tr_{30}		0.83	0.29	5.78	1.35	0.097
Tr_{31}		1.65	0.58	7.06	1.65	0.118
Tr_{32}		0.89	0.31	7.29	1.70	0.123
F – Test		**	**	**	**	**
LSD	0.05	0.152	0.05	0.159	0.039	0.003
	0.01	0.208	0.07	0.218	0.053	0.004

***: Significance at 0.01 probability level. MP: Mid-parent.*

Transconjugants of $P_3 \times P_{10}$

The results tabularized in Table 7 showed significant differences in glucose concentration released from cellulose hydrolysis leading to significant differences in cellulase activity between rhizobial transconjugants. Cellulase activities produced by *Rhizobium* transconjugants ranged between 0.078 to 0.136 if compared with the mid-parent (0.059). The highest cellulase activity (0.136) was produced by transconjugant Tr_{38} followed by Tr_{36} (0.118), whereas the lowest (0.078) activity was produced by transconjugant Tr_{35}. All transconjugants obtained from this conjugation revealed significant increase in cellulase activity in compared with the mid-parent. The results obtained herein indicated that *Rhizobium* transconjugants showed better expression in cellulase encoding genes if compared with their parents.

Several studies in thiscriteria have detected cellulolytic (Saleh-Rastin *et al.* 1991), pectinolytic (Martinz-Molina and Olivares 1982) and hemicellulolytic (Lopez and Singer 1987) enzyme activities from pure cultures of rhizobia. In addition, Mateos *et al.* (1992) detected and measured cellulolytic and pectinolytic activities by different wild-type strains of *Rhizobium* isolated from different geographical regions. On the other hand, nitrogen fixation is energetically dependent on the available energy from plant residues that contain a high percentage of cellulose. For this, the production of cellulolytic enzymes was considered as an indirect mechanism to promote plant growth (Gupta *et al.* 2015). Furthermore, the establishment of rhizobia within legume root nodules needs disruption in plant cell walls (Robledo *et al.* 2008). Meanwhile, carboxymethyl cellulase (CMCase) is the enzyme used to establish the symbiosis process (Chen *et al.* 2004). The results obtained in this study agreed with Jiménez-Zurdo *et al.* (1996), who found that *Rhizobium* has two CM-cellulase isozymes-C1 and C2, C1 isozyme was produced by the pSym plasmid because cellulase C1 was not encoded by the pSym-cured derivative. The C2 isozyme was encoded by a gene not located on the pSym plasmid because C2 is produced by the pSym cured derivative. The cellulases C1 and C2 isozymes are not regulated by the *nod* D gene of *Rhizobium* because flavone induction of pSym *nod* genes is not required for this production. Furthermore, Finnie *et al.* (1998) isolated genes encoding carboxymethyl cellulase from *Rhizobium leguminosarum* bv. *viciae*, *ply* A and *ply* B. The infection stage of rhizobia in root nodule symbiosis involvement enzymes that degrade plant cell wall polymers has been an unresolved question. Endoglucanase is one of the important enzymes associated with plant cell wall degradation (An *et*

Table 7: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_3 \times P_{10}$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	Hybrid performance	Glucose concentration mg/ml	Hybrid performance	Cellulase activity
P_3		1.70	—	2.77	—	0.046
P_{10}		1.88	—	4.30	—	0.072
MP		1.79	1.00	3.54	1.00	0.059
Tr_{32}		4.30	2.40	5.44	1.54	0.091
Tr_{34}		1.57	0.88	6.40	1.80	0.108
Tr_{35}		2.07	1.16	4.62	1.31	0.078
Tr_{36}		3.04	1.70	7.02	1.98	0.118
Tr_{37}		4.30	2.40	5.11	1.44	0.086
Tr_{38}		4.18	2.33	8.11	2.29	0.136
Tr_{39}		3.81	2.13	6.40	1.81	0.108
Tr_{40}		3.02	1.69	5.17	1.46	0.087
F – Test		**	**	**	**	**
LSD	0.05	0.168	0.081	0.15	0.043	0.002
	0.01	0.230	0.111	0.21	0.060	0.003

***: Significance at 0.01 probability level. MP: Mid-parent.*

al. 2004). Studying these enzymes was essential to understanding the mechanism of host-*Rhizobium* interaction. The results suggested that plasmid transfer through the conjugation process led to an increase in the gene dosage of plasmids harboring gene *ply* A in transconjugants which induced higher activity of C1 isozyme. In addition, rhizobia may have a complex system of enzymatic hydrolysis that degraded cellulose in the mechanism of plant-*Rhizobium* interaction which needed multiple cell wall-degrading enzymes.

Transconjugants of $P_4 \times P_6$

The results presented in Table 8 showed significant differences in glucose concentration released from cellulose hydrolysis which reflected significant differences in cellulase activity between rhizobial transconjugants. Cellulase activities produced by rhizobial transconjugants ranged between 0.097 to 0.166 if compared with the mid-parent (0.082). The highest cellulase activity (0.166) was produced by transconjugant Tr_{46} followed by Tr_{48} (0.154), whereas the lowest (0.097) activity was produced by transconjugant Tr_{47}. All transconjugants obtained from this conjugation achieved significant increase in cellulase activity if compared with the mid-parent. The results obtained herein indicated that *Rhizobium* transconjugants showed better expression in cellulase-encoding genes if compared with the mid-parent.

This agreed with Htway *et al.* (2018), who found that the cellulolytic activity of transconjugants is two times higher than the wild-type cultures at their maximum growth rate by using cellulose substrate. In addition, the same authors used the agricultural cellulose waste as substrates, the reducing sugar produced by transposon mutagenized cultures was higher than the wild-type isolates. The results obtained in this study are also in harmony with Robledo *et al.* (2011), who decided that the observed increase of CM-cellulase activity in *Rhizobium* was due to the overproduction of the $CelC_2$ enzyme which increased the competitiveness of *Rhizobium* with their legume roots. The same authors demonstrated that most of the nodules formed after two months of inoculation were formed by strains containing pJZC2 plasmid, which overexpressed the $CelC_2$ cellulase. In this respect, Finnie *et al.* (1998) isolated two genes encoding CMCase from *Rhizobium* (*Ply* A and *Ply* B) which encode two extracellular glycanases (*Ply* A and *Ply* B) that are degraded exopolysaccharide (EPS) and carboxymethyl cellulose (CMC) as content of plant cell wall. In this criteria, Goormachtig *et al.* (2004) decided that rhizobia invade their plant hosts

Table 8: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_4 \times P_6$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	Hybrid performance	Glucose concentration mg/ml	Hybrid performance	Cellulase activity
P_4		0.70	—	5.84	—	0.098
P_6		1.14	—	3.85	—	0.065
MP		0.92	1.00	4.84	1.00	0.082
Tr_{41}		1.32	1.44	7.33	1.51	0.123
Tr_{42}		0.87	0.94	6.79	1.40	0.114
Tr_{43}		0.89	0.97	6.52	1.35	0.110
Tr_{44}		3.54	3.85	6.06	1.25	0.102
Tr_{45}		5.65	6.14	6.42	1.33	0.108
Tr_{46}		6.73	7.31	9.88	2.04	0.166
Tr_{47}		4.60	5.00	5.75	1.19	0.097
Tr_{48}		6.48	7.04	9.17	1.89	0.154
F – Test		**	**	**	**	**
LSD	0.05	0.159	0.179	0.170	0.03	0.003
	0.01	0.218	0.247	0.233	0.04	0.004

***: Significance at 0.01 probability level. MP: Mid-parent.*

through colonization of intercellular epidermal spaces or penetration into the root hairs via tubular structures called infection threads. The latter route is the best infection pathway characterized by a complex molecular interaction between both partners. In the early stage of infection, rhizobia secreted flavonoids from legume roots which induced the synthesis of nodulation factors (lipochito-oligosaccharide signal molecules) by the nodulation (*nod*) genes. In turn, morphogenetic responses occurred in the plant via reactivating their mitotic activity to divide, thereby generating nodule formation of the nodular meristem (Oldroyd and Downie 2008). The penetration of rhizobia into their legume hosts required the degradation of root hair cell walls via hydrolytic enzymes produced by rhizobia to invading bacteria in the primary penetration. In the secondary infection, rhizobia is released by endocytosis from the infection site into membrane-enclosed vesicles within the cytoplasm of some nodule cells. Released bacteria formed morphological bacteroids which are the mature nodules enclosed within the membrane vesicles of the host origin (Jones *et al.* 2007). Rhizobia grows at the tip of the infection thread which enables them to reach the growing nodule meristem. Rhizobia were released into nodule cells where they are differentiated and induce the genes required for nitrogen fixation (Gage *et al.* 1996). The results obtained in this study agreed with Robledo *et al.* (2011), who found that trans-overexpression of *celC*$_2$ instrain ANU843C$_2^+$ increased carboxymethyl cellulase activity by *R. leguminosarum* bv. *trifolii*to be disrupt plant cell wall for establishment the effective symbiosis. A high local concentration of cellulase may be required for localized degradation of plant cell walls to generate a point of infection (Robledo *et al.* 2011). Therefore, nitrogen-fixing bacteria can be used for the degradation of agricultural wastes by converting them into organic fertilizer via enzyme activities. The significant role of nitrogen-fixing bacteria on cellulose degradation may be useful in the biotechnological industry to convert cellulose wastes into nitrogen-rich compost (Htway *et al.* 2018).

Transconjugants of $P_4 \times P_7$

The results presented in Table 9 showed significant differences in glucose concentration released from cellulose hydrolysis leading to significant differences in cellulase activity between rhizobial transconjugants. Cellulase activities produced by rhizobial transconjugants ranged between 0.081 to 0.111 if compared with the mid-parent (0.088). The highest cellulase activity (0.111) was produced by transconjugants Tr_{53} followed by Tr_{51}, Tr_{52} and

Table 9: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_4 \times P_7$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	**Hybrid performance**	**Glucose concentration mg/ml**	**Hybrid performance**	**Cellulase activity**
P_4		0.70	—	5.84	—	0.098
P_7		2.79	—	4.62	—	0.078
MP		1.75	1.00	5.23	1.00	0.088
Tr_{49}		2.15	1.23	5.34	1.02	0.090
Tr_{50}		2.05	1.17	5.19	0.99	0.087
Tr_{51}		2.23	1.28	5.90	1.13	0.100
Tr_{52}		5.09	2.92	5.92	1.13	0.100
Tr_{53}		4.28	2.45	6.60	1.26	0.111
Tr_{54}		4.35	2.49	4.82	0.92	0.081
Tr_{55}		5.07	2.90	5.92	1.13	0.100
Tr_{56}		3.79	2.17	5.11	0.98	0.086
F – Test		**	**	**	**	**
LSD	0.05	0.168	0.097	0.154	0.028	0.0026
	0.01	0.230	0.134	0.211	0.039	0.0036

***: Significance at 0.01 probability level. MP: Mid-parent.*

Tr_{55} (0.100), whereas the lowest (0.081) activity was produced by transconjugant Tr_{54}. Fifty percent of transconjugants as Tr_{51}, Tr_{52}, Tr_{53} and Tr_{55} appeared significant increase in cellulase activity if compared with the mid-parent. These results indicated that some *Rhizobium* transconjugants showed better expression in cellulase encoding genes if compared with the mid-parent.

This agreed with Kumar *et al.* (2011), who found that *R. leguminosarum* and *E. meliloti* isolated from the root nodules of fenugreek showed better chitinase and β-1,3-glucanase activity. Similarly, Chaudhary *et al.* (2021) found that *R. pusense* isolated from chickpea and mung bean nodules exhibited protease, β-1,3-glucanase, as well as, cellulase activity. Furthermore, Sharma *et al.* (2024) reported that *Rhizobium* isolated from the root nodules of *Zornia gibbosa* exhibited positive results for inducing cellulose activity. Similarly, 50% of root nodule bacteria isolated from *Alysicarpus vaginalis* showed positive results for cellulase activity (Rathi *et al.* 2017). The production of hydrolytic enzymes as cellulases facilitates bacterial entry, colonization, and maintenance with legume hosts (Nascimento *et al.* 2018). A significant event in the establishment of the rhizobium-legume symbiosis is the localized disruption of a cellulosic plant cell wall by cellulase produced by *Rhizobium* species, by which the bacterial symbiont passes to develop a nitrogen-fixing intracellular endosymbiotic state with their legume hosts (Menéndez *et al.* 2019).

The results obtained herein are in harmony with Mir *et al.* (2024), who found that four isolates from eight rhizobial cultures like bacteria produced cellulase which can be used as bio-inoculants to increase the production of common bean in a sustainable way. In addition, Nascimento *et al.* (2018) decided that the production of hydrolytic enzymes as cellulases facilitates bacterial entry, colonization and maintenance. Therefore, Menéndez *et al.* (2019) reported that cellulase produced by *Rhizobium* species played a significant role in the establishment of symbiosis between *Rhizobium* and their legume hosts to be pointed disruption of cellulosic plant cell wall by cellulase, by which the bacterial symbiont passes to establish a nitrogen-fixing intracellular endosymbiotic event within their legume hosts. Additionally, Sharma *et al.* (2024) isolated 44 root nodule bacterial isolates from the root nodules of *Zornia gibbosa* and evaluated their biochemical activities as cellulase activity which showed significant diversity in cellulase activity. The type of inducing rhizobial recombinants is essential for developing superior genotypes of biofertilizers to enhance plant production in sustainable agriculture.

Transconjugants of $P_5 \times P_7$

As shown from the results presented in Table 10 there were significant differences in glucose

Table 10: Glucose released from cellulose hydrolysis by new recombinants of rhizobia resulted from the conjugation between $P_5 \times P_7$

Genotypes		Control sample		Actual sample		
		Glucose concentration mg/ml	Hybrid performance	Glucose concentration mg/ml	Hybrid performance	Cellulase activity
P_5		1.99	—	3.29	—	0.056
P_7		2.79	—	4.62	—	0.078
MP		2.39	1.00	3.95	1.00	0.067
Tr_{57}		1.18	0.49	3.10	0.79	0.052
Tr_{58}		2.38	1.00	3.50	0.89	0.059
Tr_{59}		0.72	0.30	2.50	0.63	0.042
Tr_{60}		1.22	0.51	2.94	0.74	0.049
Tr_{61}		2.05	0.86	3.52	0.89	0.059
Tr_{62}		0.74	0.31	2.73	0.69	0.046
Tr_{63}		1.72	0.72	5.80	1.47	0.098
Tr64		1.86	0.78	5.13	1.30	0.086
F – Test		**	**	**	**	**
LSD	0.05	0.173	0.071	0.135	0.031	0.0024
	0.01	0.238	0.098	0.184	0.043	0.0033

***: Significance at 0.01 probability level. MP: Mid-parent.*

concentration released from cellulose hydrolysis which reflected significant differences in cellulase activity between rhizobial transconjugants. Cellulase activities produced by *Rhizobium* transconjugants ranged between 0.042 to 0.098 if compared with the mid-parent (0.067). The highest cellulase activity (0.098) was produced by transconjugant Tr_{63} followed by Tr_{64} (0.086), whereas the lowest (0.042) activity was produced by transconjugant Tr_{59}. Some of transconjugants as Tr_{63} and Tr_{64} appeared significant increase in cellulase activity above the mid-parent. These results indicated that some recombinants showed better expression in cellulase-encoding genes if compared with the mid-parent. The differences in enzyme activity found among different genotypes of recombinants may reflect the differences in infection pathways (Chandler 1978). Production of cellulases and hemicellulases by *Rhizobium*, in addition to pectinase strongly supports that rhizobia infects plant legumes through hydrolyzing the root cell wall at a highly pointed site of infection. However, legume infection requires a complex biochemical interaction between the legume host and its homologous *Rhizobium* (Dingle *et al.* 1953). *Rhizobium*-legume specificity may be determined at both levels of cell attachment through specific lectins, as well as, penetration through specific hydrolytic enzymes (Dazzo *et al.* 1976).

Cellulose hydrolysis as a main component of plant cell wall takes place via the action of cellulases which consists of endoglucanases, exoglucanases and β glucosidases enzymes. Endoglucanases randomly hydrolyze the internal bonds in the cellobiose chain (Lynd *et al.* 2002). The results obtained by Morales *et al.* (1984) reflected that the gene (s) encoding endoglucanase by *Rhizobium trifolii* was not located on the symbiotic plasmid. The results obtained in this study agreed with Hooykaas *et al.* (1981), who found that the transfer of symbiotic plasmids from *R. trifolii* to a Ti plasmid cured derivative of *Agrobacterium tumefaciens* leading transconjugants be able to develop root nodules in clover plants. This reflected that all the genes needed for nodulation were plasmid-located.

In conclusion, rhizobium transconjugants generated in this study possessed excellent cellulase activity than their parents which further clarifies that they may increased the growth parameters of their legume hosts. This study clarified that 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. Most rhizobial recombinants exhibited positive results in cellulase activity revealing a remarkable degree of diversity in their activity. Further molecular analysis on the selected recombinants was beneficial for their marking to be used in agriculture-sustainable pathways.

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