Australian Journal of Basic and Applied Sciences, 3(2): 1386-1411, 2009
ISSN 1991-8178

Induction of *Rhizobium* Inoculants Harboring Salicylic Acid Gene

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Abstract: In this study eight antibiotics and six heavy metals were used for genetic marking of seven *Rhizobium* strains and six *Pseudomonas* strains to be used in conjugation. Six *pseudomonas* strains were selected on the basis of IAA and salicylic acid production to be used as a donor against seven *Rhizobium* strains as recipients. All the matins between *rhizobiunm* and *pseudomonas* strains were successed except for the four matings between; RL-3841 X *P.putida*21, RL-4406 X *P.putida*21, RL-4404 X *P.putida*21 and RL-4404 X PF-10 which did not appeared any recombinants on selective medium. Plasmids from seven *rhizobium* and six *Pseudomonas* strains were cured using elevated temperatures method. Some recombinants (Tr_{12}, Tr_{37}, Tr_{40}, Tr_{45}, Tr_{52}, Tr_{53}, Tr_{78} and Tr_{79}) appeared significant increase in IAA production usim the complete medium supplemented with tryptophan in relation to the mid-parents. All bacterial transconjugants harboring DNA from two sources produced high levels of salicylic, these have a beneficial effect in controlling plant parasites. Bacteriocin produced from all *Rhizobium* strains inhibit the growth of PF-50090 strain, except for that produced from two strains RL-3841 and RL-2070. In contrast bacteriocins produced from all *Rhizobium* strains did not inhibit on the growth of *P.putida*. Bacteriocins produced from *P. putida21* inhibit all rhizobium strains. All bacteriocines produced by bacterial strains caused growth inhibition against Agr1513 except for three *Rhizobium* strains; RL-2070, RL-2074 and RL-207. As well as, all bacteriocins produced by transconjugants had the same effect against Agr1513, except for the transconjugant Tr_{10}. All *Pseudomonas* strains were successfully to inhibit the growth of Agr1513 in Petri dishes. Whereas, only one *Rhizobium* strain RL-4404 had the same effect against Agr1513 in contrast, the other strains were failed to appear the same effect of antagonism. Six transconjugants (Tr_4, Tr_5, Tr_6, Tr_7, Tr_9 and Tr_{14}) were successfully to appeared antagonism against Agr1513. All *Rhizobium* strains and their transconjugants appeared significant increase in nodules number per plant above the uninoculated plants among both seasons. Successful nodulation was achieved by the transconjugants Tr_2, Tr_3, Tr_5, Tr_7, Tr_8 and Tr_{15} which produced a higher number of nodules developed per plant. Successful nodulation was achieved by transconjugants: Tr_2, Tr_3, Tr_5, Tr_7, Tr_8 and Tr_{15} which produced a higher number of nodules developed per plant. All bacterial strains play an important role to improve the concentration of chlorophyll a at 45 days plant-old during both seasons and total chlorophyll during the first season. *Rhizobium* transconjugants (T_1, Tr_3, Tr_5, Tr_7, Tr_8, Tr_9, Tr_{10} and Tr_{14}) appeared significant increase in shoot N content at 45 days plant-old above uninoculated plants among two season. All *rhizobium* strains and their transconjugants appeared significant increase in seeds content of total protein above uninoculated plants among both seasons except for Tr_{11} and Tr_1 at the first season.

Key words: *Agrobacterium*, antagonism, bacteriocines, conjugation, *Pseudomonas*, *Rhizobium,* recombinants, salicylic acid.

INTRODUCTION

Pea (*Pisum sativum* L.) is an important crop worldwide and has an excellent subject for genetic and physiological studies. Its ease of production, short generation cycle, and wealth of morphological variation have lent pea to numerous scientific investigations. (Zohary *et al.* 2000).

Rhizobium can establish an effective nitrogen-fixing symbiosis through exchanging signal molecules with its plant host. The presence of plasmids of different size in various *Rhizobium* species is fairly well established (Nuti *et al.* 1977), it generally contains 1 to 10 plasmids which vary in size from 30 kb to more than 800 kb (Mercado-Blanco and Toro 1996).

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Pea establishes a symbiotic association with *Rhizobium leguminosarum* bacteria (Tricot *et al*., 1997). The macrosymbiont genotype plays an important role in symbiotic nitrogen fixation. A combination of two symbiotic genes, *nod3* and *Nod5*, in the same genotype of pea (*Pisum sativum* L.) promotes supernodulation, active nitrogen fixation, and high productivity of the plants (Sidorova *et al*., 2008). The plant produces a chemical signal molecule (flavonoid) that is perceived by the bacterium and activates transcription of the nod genes. The nod genes produce lipo-chito-oligosaccharids (Nod factors), which provoke curling of the root hairs (Long, 1989). Upon activation by plant flavonoids, the *nod* genes are transcribed and their protein products participate in the biosynthesis of Nod factors (lipochito- oligosaccharides, LCOs) (Long 1996). These Nod factors induce at least the first steps of nodule formation. (Perotto *et al.* 1994). In addition to lipopolysaccharide (LPS) and exopolysaccharide (EPS), Nod factors might also play a role in controlling defense, as suggested by the fact that Nod factors can act as elicitors of phytoalexins biosynthesis (Savouré *et al.* 1997). Furthermore, Vasse *et al.* (1993) showed that, in a compatible interaction, the bacteria induce a hypersensitive response (HR), and propose that this is part of the plant mechanism controlling the number of successful infections.

At low iron availability, bacteria produce iron-chelating molecules, called siderophores, to acquire sufficient iron. Salicylic acid (SA) is another siderophore produced by *P. fluorescens* that could be involved in induced systematic resistance (ISR). SA is important in pathogen-induced systemic acquired resistance and can induce a systemic resistance to pathogens after root or soil treatment (Enyedi *et al.* 1992). Salicylic acid is another phenolic compound that has been implicated in plant defense against pathogens (Klessig and Malamy 1994).

SA is a key molecule in plant disease resistance. It is clear that SA is intimately involved in the induction of both the hypersensitive response (HR) and systemic acquired resistance (Feys and Parker, 2000). There is a correlation between an increase in SA levels and plant gene expression. Exogenous SA can induce simultaneous pathogenesis-related expression and resistance to pathogens, even in the absence of pathogenic organisms (Ward *et al.* 1991).

The acquisition of DNA by horizontal gene transfer is one of the evolutionary strategies that contribute to the formation of genetics variants in the environments (Curz and Davies 2000). Horizontal gene transfer apparently plays an active role in many biological processes including the emergence and spread of virulence (Dobrindt and Hacker, 2001), symbiosis (Sullivan and Ronson, 1998), the degradation of xenobiotic compounds (Tsuda *et al.* 1999) and resistance to antibiotics.

The present study aimed to induce recombinaants in *Rhizobium* strains harboring salicylic acid gene from *Pseudomonas* strains used as a donor against the recipients of *Rhizobium*, via conjugation as a horizontal gene transfer for better symbioses with *Pisum sativum*, as well as, enhanced the capability of plant defense against pathogens.

MATERIALS AND METHODS

Genetic Material:

Bacterial Strains and Growth Conditions:

Bacterial strains used in this study are listed in Table 1, which including their references, as well as, their origins.

Table1: Bacterial strains used in this study.

Strains	Source or Reference	Designation
Rhizobium leguminosarum (12612)	IAM culture collection, Univ. of Tokyo, Japan.	RL- 12612
Rhizobium leguminosarum (NRRL4406)	National center for Agriculture Utilization Research, , USA	RL-4406
Rhizobium leguminosarum (NRRL B-4404)	National center for Agriculture Utilization Research, , USA	RL-4404
Rhizobium leguminosarum (3841)	Kindly provided by Prof J P W Young , Department of Biology, University of Yourk ,UK.	RL-3841
Rhizobium leguminosarum (USDA2070)	Kindly provided by Dr. Peter van Berkum, Microbiologyist, National *Rhizobium* culture collection, USDA, Baltimore Avenue Beltsville	RL-2070
Rhizobium leguminosarum (USDA2074)	Kindly provided by Dr. Peter van Berkum, Microbiologyist, National Rhizobium culture collection, USDA, Baltimore Avenue Beltsville	RL-2074
Rhizobium leguminosarum (ARC 207)	Agric. Res. center, Dept. of Microbiology, Giza, Egypt.	RL -207
Pseudomonas putida (SWRI 18)	Kindly provided by Hadi Asadi Rehamani (PhD) Soil microbiology lab. Soil and Water Reasearch Inst., Tahran, Iran	*P.putida*18
Pseudomonas putida (NRRL B-21)	National center for Agriculture Utilization Research, , USA	*P.putida*21
Pseudomonas putida (NRRL B-8)	National Center for Agriculture Utilization Research, , USA	*P.putida*8

Table 1: Continued

Pseudomonas fluorescences (DSM50090)	Kindly provided by Hadi Asadi Rehamani (PHD), Soil microbiology lab. Soil and Water Reasearch Inst., Tahran, Iran	PF- 50090
Pseudomonas fluorescences (NRRL B-6bs)	National Center for Agriculture Utilization Research, , USA	PF -6bs
Pseudomonas fluorescences (NRRL B-23932)	National Center for Agriculture Utilization Research, , USA	PF-23932
Pseudomonas fluorescences (NRRL B-10)	National Center for Agriculture Utilization Research, , USA	PF-10
Pseudomonas fluorescences (NRRL B-1603)	National Center for Agriculture Utilization Research, , USA	PF-1603
Agrobacterium tumefaciens (NBIMCC1513)	National Bank for Industrial Nicroorganisms and Cell Cultures NBIMCC,Bulgaria	Agr1513

Media:

Rhizobial strains were grown at 28°C in yeast extract-mannitol medium (YEM) according to Vincent (1970).

Yeast extract-mannitol-congo red agar (YMCRA):

This medium was used to checked *rhizobium* strains according to Pattison and Skinner (1974).

TY Medium:

This medium was used in mating experiments, IAA determination and bacteriocin assay according to Beringer (1974).

Minimal Medium:

This medium was used for IAA assay according to Balassa (1963).

King's Medium:

This medium was used for the maintenance of *Pseudomonas* strains according to Merck (1994).
Nutrient broth-yeast extract medium: It was used to grow *Pseudomonas* strains in conjugation steps according to Merck (1994), as well as, in IAA determination (Ahmad *et al.* 2005).

The Basal Medium:

It was used for the production of bacteriocin from *Pseudomonas* strains according to Morse *et al.* (1976).

Standard Succinate medium (SSM):

It was used for evaluating salicylic acid production according to Mercado-Blanco *et al.* (2001).

M9 minimal medium: It was used as a minimal medium for IAA determination in *Pseudomonas* strains according to Leveau *et al.* (2005).

Agrobacterium was grown on the medium consists per liter of distillied water; glucose, 10g; Yeast extract, 10g; $(NH_4)_2SO_4$, 1 g; KH_2PO_4, 0.25g and Tap water1.0 ml. The pH of the medium was adjusted to 7.0 according to National Bank for Industrial Microorganisms and cell cultures NBIMCC, Bulgaria.

Plants:

Pea (*Pisum sativum* L.) seeds variety Master B were kindly supplied from Vegetable Research Institute, Agri. Res. Center, Dokki, Giza, Egypt.

Intrinsic Antibiotic Resistance Profiles:

Resistance to antibiotics was tested on YMA agar and nutrient agar medium supplemented with one of the antibiotics at the concentration listed in Table 2.

Heavy Metals Tolerance as a Genetic Markers:

Six heavy metals were used in this study with the concentration of 100µg/ml. They are as follow; Hg (HgCl), Cd ($CdSo_4$), Cu ($CuSO_4$), Zn ($ZnSO_4 7H2O$), Co ($CoSo_4$. H_2O) and Pb ($Pb(NO_3)_2$, they were filter sterilized into sterile test tubes (Sebat *et al.* 2001).

Nutrient solution:

This solution was used with the plants growing in the pots to improving the growth. It was consists (g/l); $Ca(NO_3)_2$.4H20, 1.18; KNO_3, 0.51; K_2HPO_4, 0.14; $MgSO_4.7H_20$, 0.49; H_3BO_3, 0.0029; MnCI2.4H_20, 0.0018; $ZnSO_4$, 0.00022; $CuSO_4.5H_20$, 0.00008; and H_2MoO_4, 0.00002; $FeC1_{3,}$ 0.02g. The pH was adjusted to 7.5 with 0.1M KOH and 1.0 g $CaCO_3$was added as buffering agent according to Allen (1959).

Table 2: Different antibiotic concentrations used in this study.

Antibiotics	abbreviations	Conc. (mg/ml)	Source of product
Chloramphenicol	*Cm*	30	Hoechst Orient SAE, Cairo, Egypt
Ampicillin	*Ap*	75	Hoechst Orient SAE, Cairo, Egypt
Tetracycline	*Tc*	100	Misrco. For pharm.Ind. S.A.a. Mataria. Cairo.A.R.E. C.C.R.32048
Streptomycin	*Str*	75	Hoechst Orient SAE, Cairo, Egypt
Neomycinsulphate	*Nm*	60	Pharco Pharmace Euticals under lience of Biochemiegmbh, Egypt
Erythromycin-ethlsuccinate	*Eryth*	50	Kahira Pharm& chem.. Ind.co underlicence of Abbott.Laboratories.U.S.A, Egypt.
Cephalexin	*Cp*	100	Pharco Pharmace Euticals under lience of Biochemiegmbh, Egypt
Rifampcillin	*Rif*	50	Pharco Pharmace Euticals under lience of Biochemiegmbh, Egypt

II. Methodology:

Tolerance to Antibiotics and Heavy Metals:

It was measured by plate diffusion method, according to Collins and Lyne (1985) with cultures grown to logarithmic growth phase in nutrient broth for each strain. The plates were incubated overnight at 28°C and the diameter of resulting zones of inhibition was measured according to Toda *et al.* (1989).

Plasmid Curing:

To determine the resistance to antibiotic is encoded by a plasmid or chromosomal genes elevated temperature treatment (35, 37, 40 and 45ºC) were applied. Culture were inoculated into TY broth medium at elevated temperatures for 48 h and then plated on TY medium containing 5% sucrose. Plates incubated at higher temperatures were transferred to 28°C for another 3 days. Single colonies from each temperature treatment were picked up and rechecked for the same antibiotic resistance pattern to ensure from the growth characteristics obtained before (Bastos *et al.* 1980).

Bacterial Mating:

Matings were performed between *Pseudomonas* strains as donors which may carring salicylic acid genes according to Maurhofer *et al.* (1998) and phenol degradative genes according to Kasai *et al*., (2001) with a standard recipient *Rhizobium* strains (Selvarathnan and Gealt 1993). Donors and recipients were mixed in a 1:2 ratio and incubated for the appropriate time. Samples of 3 ml from the serial dilutions of the mixture mating were plated on suitable selective medium according to Lederberg and Lederberg (1952). The plates were incubated for 3 days minimum until the transconjugants were appeared on the selective media. The appropriate time for appearing transconjugants was differed from one mating to another. Dilute samples from the mixture mating were taken from each conjugation tube every two days and plated on the appropriate media, to obtain the suitable time needed to construct transconjugants. It was differed from one conjugation to another, as shown herein. Matings were conducted according to Tun-Garrido *et al.* (2003).

In Vitro SA Production:

Rhizobium strains were grown in YEM medium and *Pseudomonas* strains were grown in SSM medium. After removal the cells by centrifugation, culture supernatants were acidified with 1 N HCl to pH 2, and SA was extracted into $CHCl_3$ upon vigorous shaking (culture supernatant: $CHCl_3$, 1:1). For low levels of SA production, 1 volume of $CHCl_3$ was used to extract SA from up to 3 volumes of spent medium. For quantitative measurements, 1 volume of H_2O and 1.25×10^{-3} volumes of 2 M $FeCl_3.6H_2O$ were added to the $CHCl_3$ phase. The absorbance of the purple iron-SA complex developed in the aqueous phase was measured at 527 nm. (Mercado-Blanco *et al.* 2001). SA concentration was calculated from the following formula of regression as follows:

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

Where; y = Optical density at 527 nm, x = Concentration of salicylic acid, b = Regression = 0.0107 and a = Absorbance at 530 nm when the concentration of salicylic acid equal zero = 0.0497.

IAA-detection with the Salkowski Colorimetric Technique:

Production of IAA in the supernatant was assayed using the PC method, as described by Pilet and Chollet (1970). This method was shown to be the most sensitive and most specific (Glickmann and Dessaux, 1995). Absorbance was measured at 530 nm. However, IAA concentrations were calculated from the following formula of regression as follows:

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

Where; y = Optical density at 530 nm, x = Concentration of IAA, b = Regression = 0.0385 and a = Absorbance at 530 nm when the concentration of IAA equal zero = 0.215.

Evaluating Bacteriocin:

Disk diffusion assay was used for the detection of bacteriocin activity. The diameter of inhibition zones was measured as the distance from the edge of the test bacteriocin to the end of the zone according to Delgado *et al.* (2001).

Testing the Antagonism

The antagonism of bacterial strains and their transconjugants against *Agrobacterium tumefaciens* was performed using well diffusion method according to Nedialkova and Naidenova (2004).

Plant Infection:

Pots Experiment:

Pots experiment were conducted during 2007 – 2008 and 2008 - 2009 seasons. Plants were grown in plastic pots containing the mixture of sterilizes sand and clay (1:1 w/w) with three replicates. Soil was washed with distilled water several times to diminishing chloride ions, as well as, autoclaved three times at 121°C for one hour among three days. Plants were inoculated with the parental strains and their transconjucants at the time of sowing using over night culture suspension growing at 28°C on rotary shaker (120 RPM) containing finally 10^8 FU/ml. Nodules developed on the plants were counted after 45-days-plant old according to Vincent, (1970). This experiment was conducted in Tag El- Aze Agriculture Research station, Agric. Res. Center, Egypt.

Nodulation and Vegetative Traits:

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

Average weight of nodule (AWON): This was estimated according to Pereira *et al.* (1989) using the following formula:

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

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

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

Leaf area/plant: It was determined using leaf fresh weight method according to Beadle (1993).

Photosynthetic Pigments:

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

Nitrogen determination: It was determined according to APHA, (1992). Color photometrically was measured as absorbance using a Spectrophotometer. Samples were reading at 425 nm for 1-cm light path. Calibration curve was prepared according to APHA (1992) using the linear regression equation as follows:

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

Where; y = optical density at 530 nm, x = concentration of nitrogen, b = regression = 0.14, a = absorbance at 425 nm when the concentration of N equal zero = 0.01 and crude protein in seed (%) = Seed N_2 percentage x 6.25.

Statistical Analysis:

Data were subjected to the analysis of variance according to Snedecor and Cochran (1955). Least significant difference (L.S.D.) was used to compare between means if the F-test was significant.

RESULTS AND DISCUSSION

Indole Acetic Acid and Salicylic Acid Production from Pseudomonas Strains:

Bacteria that inhabit the rhizosphere may influence plant growth by contributing to a host plant's endogenous pool of phytohormones, such as auxins. Production of the auxin indoleacetic acid (IAA) is widespread among plant-associated bacteria. Beneficial bacteria synthesize IAA predominantly by an alternate tryptophan-dependant pathway, through indolepyruvic acid (Patten and Glick. 1996). Beneficial effects of the rhizobacterium *Pseudomonas* strains can induce physiological changes throughout entire plants, making them more resistant to pathogens. This phenomenon, termed induced systemic resistance (ISR), has been demonstrated for various rhizobacteria in several plants (Alström. 1991). PGPR of the genus *Pseudomonas* can produce several siderophores such as pyoverdine (pseudobactin), pyochelin, and salicylic acid (SA), (De Meyer and Hofte, 1997).

Salicylic acid has been defined as a new potential plant hormone (Raskin, 1992a) and found to play an important role in disease resistance (Raskin, 1992b) and abiotic stress tolerances. SA is important in pathogen-induced SAR and can induce a systemic resistance to pathogens after root or soil treatment (Enyedi *et al.*, 1992). This study evaluated *Pseudomonas* strains, based on salicylic acid and IAA production to be used in matings as a donor against *Rhizobium* strains which were used as recipients. As shown from the results presented in Table 3, all *Pseudomonas* strains produced IAA and salicylic acid constitutively, except for two strains; PF-6bs and PF-23932 were produced low levels of salicylic acid. As shown herein, all *Pseudomonas* were produced high levels of IAA when grown in the media supplemented with tryptophane except for four strains (*P.Putida*21, PF-6bs, PF-23932 and PF-1603). The results obtained herein are in agreement with Patten and Glick (2002), who found that many plant-associated bacteria synthesize the phytohormone indoleacetic acid (IAA). Whereas, IAA produced by phytopathogenic bacteria, mainly by indoleacetamide pathway which has been implicated in the induction of plant tumors. Whether IAA synthesized by beneficial bacteria usually via the indolepyruvic acid pathway, is involved in plant growth promotion. IAA secreted by bacteria may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity. ACC deaminase, is involved in the stimulation of root elongation in seedlings (Li *et al.* 2000), it hydrolyzes plant ACC, the immediate precursor of the phytohormone ethylene, and thereby prevents the production of plant growth-inhibiting levels of ethylene (Penrose *et al.* 2001).

Table 3: Evaluate *Pseudomonas* strains in salicylic acid and IAA production to be used as a donor in matings with *Rhizobium* strains.

strains	IAA (µg/ml)	Salicylic acid (µg/ml)
P. putida 21	2.23	2.15
P. putida 8	24.36	6.88
P. putida 18	14.16	6.51
PF- 50090	25.47	7.17
PF -6bs	4.56	2.61
PF -23932	4.13	0.03
PF -10	15.92	10.96
PF -1603	3.44	6.88
F- Test	**	**
LSD 0.05	1.58	3.18
0.01	1.19	4.43

** = significant at 0.01 probability level.

Six *Pseudomonas* strains were selected out of eight to be used as a donors in matings with *Rhizobium* strains because of higher IAA and salicylic acid production.

Intrinsic Antibiotic Resistance Profiles:

Seven *Rhizobium* strains and six *Pseudomonas* strains used in this study were genetically marked using eight antibiotics. The results recorded in Table (4) showed that Tetracycline (*Tc*) was much more effective to inhibit the growth of all bacterial strains except for two strains (RL- 12612 and *P.putida*21) than the other antibiotics used herein (Fig 4). The resistance to tetracyclines was due to the presence of *tet* genes in the bacterial DNA. The characterized *tet* genes encode three mechanisms of resistance: efflux pump, ribosomal protection or enzymatic inactivation (Chopra and Roberts 2001). This was agreed with Brock (1964), who found that streptomycin inhibited plague formation when certain bacteriophages were plated on streptomycin-resistance host cells , as well as, Moskowitz (1963), found that streptomycin was much more effective than dihydrostreptomycin in precipitating nucleic acids, streptomycin has been one of the most frequently and markers for *rhizobium* (Borges *et al.*, 1990). However, all bacterial strains are resistance to *Rif* and *Eryth*. This agreed with Chin *et al* (2005), who found that all *Lactobacillus* strains exhibited varying degrees of resistance to chloramphenicol and erythromycin. However, Date and Hurse (1992) found that *Bradyrhizobium* strains are resistance to 30µg of rifampicin and 500µg of sterptomycin. Growth inhibition appeared in this study was not accompanied by cell death and could be readily reversed by removing the antibiotic (Fernandez and Anton 1987). Küçük and Kıvanç (2008) found that *Rhizobium* isolates appeared high level of resistance against erythromycin, kanamycin, penicilin and chloramphenicol.

The natural or intrinsic form of antibiotic resistance can be regarded as a genetically determined property of a cell, which matches a gap in the spectrum of action of an antibiotic agent. Intrinsic antibiotic (IAR) has been portrayed as a quick and simple method for strain identification within the Rhizobiaceae in comparison with standard serological methods (Chanway and Holl 1989). Antibiotic resistance, especially the resistance conferred by plasmids, is the least stable trait in the line of marker traits (Shirokikh and Shirokikh, 2007).

Results clearly show that RL- 12612 was resistance to all antibiotics except *Cm* and *Str*. Whereas; RL-4404 was resistance to all antibiotics except *AP*, *Tc* and *Cp.* This agreed with Khan and Malik (2001), who found high incidence of antibiotic resistance in *E. coli* and *Staphylococci* strains isolated from foodstuffs.

As noted in previous studies (Cresti *et al.* 2002), there was a correlation between the antibiotic resistance phenotype and the genotype for each isolate. As shown in this study there are variation between different bacterial strains in their resistance to different antibiotics, making strains are capable of intergenic or intragenic transfer. This indicated that rhizobial strains contained variations to the same antibiotic. The location (chromosomal or extrachromosomal) of drug resistance determinants was confirmed by plasmid curing strategies. Such broad host-range transferable plasmids play an important role in the spread of antibiotic resistance. However, conjugation (physical mating) strategies do not allow the pushable plasmids which need the sex pili provided by an independent F-plasmid (Rasool, 1992).

Table 4: Diameter (cm) of inhibition zones resulted from testing bacterial strains against different antibiotics.

Strains	Antibiotics							
	Cm	*Ap*	*Tc*	*Str*	*Nm*	*Eryth*	*Cp*	*Rif*
RL- 12612	1.25	0.00	0.00	1.50	0.00	0.00	0.00	0.00
RL-4404	0.00	1.43	2.58	0.00	0.00	0.00	1.77	0.00
RL-3841	0.00	0.00	2.65	0.00	0.00	0.00	2.17	0.00
RL-4406	1.22	0.80	2.27	1.18	1.88	0.00	0.00	0.00
RL-2070	2.58	2.50	2.83	1.10	0.00	0.00	3.50	0.00
RL-2074	0.00	1.95	4.87	1.78	0.00	0.00	3.50	0.00
RL -207	1.43	3.00	3.03	1.57	0.00	0.00	0.00	0.00
PF- 50090	0.00	0.00	1.27	1.18	0.00	0.00	0.00	0.00
*P.putida*18	0.00	0.00	1.43	0.00	0.00	0.00	0.00	0.00
*P.putida*8	0.00	0.00	1.00	1.93	1.03	0.00	0.00	0.00
*P.putida*21	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00
PF-1603	0.00	1.53	1.35	1.43	1.30	0.00	0.00	0.00
PF-10	1.60	0.00	2.13	0.00	2.00	0.00	0.00	0.00
F- test	NS	NS	*	NS	NS	NS	NS	NS
LSD 0.05			0.72					
0.01			0.92					

NS, * = Insignificant, significant at 0.05 probability level, respectively.

Heavy Metal Resistance:

Many species of bacteria have genes that control resistance to specific toxic heavy metals. This resistance often is determined by plasmids. The mechanisms of resistance occur in bacteria from soil, water, industrial waste and clinical sources Silver and Misra (1984). Bacterial plasmids encode resistance systems for toxic metal ions, including Ag, Cd, Co, Hg, Ni, Pb, Te, Zn and other toxic heavy metals (Silver and Phung 1996). Plasmid-determined resistance to toxic metal ions has been demonstrated for many bacterial species and is a useful selectable marker for these DNA molecules (Romero *et al.* 1998). In genetic studies, heavy metal resistance should be extremely valuable as positive selection markers.

As shown from the results presented in Table 5, all bacterial strains showed resistance to Zn, Pb (except *P.putida* 8) and Co (except *P.putida* 18). This agreed with Küçük and Kıvanç (2008), who found that 89.2% of *Rhizobium* sp. showed resistance to three metals; Zn, Cr and Ni. In addition, Zolgharnein *et al.* (2007) found that all of the strains belonging to the marine environment and enclosed industrial areas were resistant to all four metals including Cu, Pb, Cd, and Zn. However, Hungria and Vargas (2000) suggested that the high levels of Zn, Cu and Cd could be used as selective agents for some *Rhizobium* strains.

As shown in this study, Hg was much more effective to inhibit the growth of all bacterial strains except four strains; RL-2070, PF-50090, *P.putida*18 and PF-1603. This was harmony with Keasavan and Purrushothaman (1991), who found that *Bradyrhizobium* strains isolated from normal field were sensitive to Hg at concenteration below our moderate resistant concentration (50µg/ml). RL-2074 and RL-4404 were sensitive to Cu whereas; PF-50090 was more sensitive to Cd, cu and K, which revealed differences in their action on different bacterial strains. Coral *et al.* (2006) found that all Enterobacter strains showed more tolerance up to 25 mM copper and 14 mM cadmium.

Table 5: Diameter (cm) of inhibition zones resulted from testing bacterial strains against different heavy metals.

Strains	Heavy metals						
	Hg	Cd	Cu	K_2	Co	Zn	Pb
RL- 12612	1.87	0.00	0.00	0.00	0.00	0.00	0.00
RL-4404	2.37	0.00	2.43	0.00	0.00	0.00	0.00
RL-3841	1.97	0.00	0.00	0.00	0.00	0.00	0.00
RL-4406	1.80	0.00	0.00	0.00	0.00	0.00	0.00
RL-2070	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RL-2074	2.03	0.00	2.17	2.33	0.00	0.00	0.00
RL -207	3.17	0.00	0.00	2.83	0.00	0.00	0.00
PF- 50090	0.00	1.00	1.00	2.00	0.00	0.00	0.00
*P.putida*18	0.00	0.00	0.00	1.33	1.00	0.00	0.00
*P.putida*8	2.00	0.00	0.00	0.00	0.00	1.00	2.00
*P.putida*21	1.77	2.00	0.00	0.00	0.00	0.00	0.00
PF-1603	0.00	2.10	0.00	0.00	0.00	0.00	0.00
PF-10	1.00	0.00	0.00	0.00	0.00	0.00	0.00
F- test	NS	NS	NS	NS	NS	NS	NS

NS = Insignificant differences.

Horizontal Gene Transfer:

Horizontal gene transfer is the direct transfer of genetic material from one organism to another.

In order to construct recombinant bacterial strains *Pseudomonas* was used as a donor which have ability to phenol-degradative and salicylic acid production which can improve the efficiency of rhizobium strains in nodulation with *Pisum sativum* L. DNA transfer by bacterial mating technique was presented in Table (6).

Matings were performed using six *pseudomonas* strains selected on the basis of production of IAA and salicylic acid, which used as a donor against seven *Rhizobium* strains used as a recipients.

All matings between *rhizobiunm* and *Pseudomonas* were successed, except for the four matings between; RL-3841 X *P.putida*21, RL-4406 X *P.putida*21, RL-4404 X *P.putida21* and RL-4404 X PF-10, which failed to transfer genetic material from the donor to the receipient ones. This may due to the differences between the donor and the recipient bacterium in nucleotide sequences, gene expression, codon usages, posttranslational modifications and protein interactions (Nielsen and Townsend 2001). In addition, the mating strains may be from the same mating type. Different times were needed for different matings to appeared transconjugants on selective media. Five isolates were selected at random from each of the sixteen successed matings to be tested for their efficiency in IAA and salicylic acid production (Tables 9, 10, 11 and 12).

Table 6: Horizontal DNA transfer between the recipient *Rhizobium* against the donor of *Pseudomonas* strains.

Mating	Revelant geneotype of mating	Suitable time of mixed cultures mating needed for genetic transfer	Time needed to appeared trans- conjugants on selective media (day)	Recombinant genotype
RL- 12612 X PF-50090	Tc^{+} Cm^{-} Hg^{-} Cd^{+} X Tc^{-} Cm^{+} Hg^{+} Cd^{-}	3	4	Tc^{+} Cm^{+} Hg^{+} Cd^{+}
RL- 12612 X*P.putida*18	Tc^{+} Cm^{-} Str^{-} $\mathrm{Hg}^{-}\mathrm{Co}^{+}$ X Tc^{-} Cm^{+} Str^{+} Hg^{+} Co-	6	3	Tc^{+} Cm^{+} Str^{+} Hg^{+} Co^{+}
RL- 12612 X*P.putida*8	Tc^{+} Cm^{-} Nm^{-} *X* Tc^{-} Cm^{-} Nm^{+}	3	3	Tc^{+} Cm^{+} Nm^{+}
RL- 12612XPF-1603	Cm^{-} Nm^{+} Ap^{+} *X* Cm^{+} Nm^{-} Ap^{-}	3	3	Cm^{+} Nm^{+} Ap^{+}
RL- 12612 XPF-10	*Tc+ Str-* *X* *Tc+ Str-*	3	3	Tc^{+} Str^{+}
RL-3841X*P.putida*21	Tc^{-} St^{+} *X* Tc^{+} Str^{-}	†	†	†

Table 6: Continued.

RL-3841XPF- 50090	Str- Cp^+ X Str^+ Cp^-		3	3	Cp^+ Str^+
RL-4406 X *P.putida*21	Str- Ap^+ X Str^+ Ap^-	Str^+ Ap^+	†	†	†
RL-4404 X PF- 50090	Str+ Ap^- Cp^- X Str- Ap^+ Cp^+	Ap^+ Cp^+ Str+	6	3	Ap^+ Cp^+ Str+
RL-4404 X *P.putida*21	Tc^- Str+ Ap^- X Tc^+ Str- Ap^+	Ap^+ Tc^+ Str+	†	†	†
RL-4404 X *P.putida*8	Ap^+ Tc^- X Ap^- Tc^+	Tc^+ Ap^+	3	3	Tc^+ Ap^+
RL-4404 X PF-10	Ap^- Cm^+ X Ap^+ Cm^-	Ap^+ Cm^+	†	†	†
RL-2070 X *P.putida*8	$Ap^- Nm^+$ X $Ap^+ Nm^-$	Ap^+ Nm^+	3	3	Ap^+ Nm^+
RL-2070 X PF-1603	Nm^+ Cm^- X Nm^- Cm^+	Nm^+ Cm^+	3	3	Nm^+ Cm^+
RL-2074 X *P.putida*8	Nm^+ $Ap^- X$ Nm^- Ap^+	Ap^+ Nm^+	3	5	Ap^+ Nm^+
RL-2074 X PF-1603	Nm^+ Cm^- X Nm^- Cm^+	Nm^+ Cm^+	3	3	Nm^+ Cm^+
RL-2074 X PF-10	Nm^+ Ap^- X Nm^- Ap^+	Nm^+ Ap^+	3	4	Nm^+ Ap^+
RL -207 X PF-10	Nm^+ Ap^- X Nm^- Ap^+	Nm^+ Ap^+	3	3	Nm^+ Ap^+
RL -207 X PF-1603	Nm^+ Cm^- X Nm^- Cm^+	Cm^+ Nm^+	12	5	Cm^+ Nm^+
RL -207 X *P.putida*8	Nm^+ Ap^- X Nm^- Ap^+	Nm^+ Ap^+	6	3	Nm^+ Ap^+

† These matings were incubated for 3, 6 and 12 days at 28^{0}C before plating on selective media, without any resulted transconjugants.

Plasmid Curing

Attempts to cur plasmids from seven *rhizobium* strains and six *Pseudomonas* strains have been made using elevated temperatures method at 28ºC, 35 ºC, 37ºC, 40ºC and 45ºC. As shown From the results presented in Table 7, all strains were able to grow at 28ºC, 35 ºC and 37ºC, however, the RL-4404 and RL-2074 appeared little growth at 37ºC. Some *rhizobium* strains; RL-4404, RL-3841 and RL-2074 failed to grow at 40ºC and 45ºC, whereas, RL-2070 failed to grow at 45ºC. These results agreed with Pinto *et al.* (2000), who found that high temperatures can affect the survival, establishment and symbiotic properties of *Rhizobium* strains. In addition, Saha *et al*., (2000) found that *Rhizobium meliloti* was most tolerant to heat (35.5 to 42.5ºC) than *Rhizobium leguminosarum* and *R. trifolii*. However, Brockwell, (1963) observed that up to 40°C, there was no serious mortality, but beyond this temperature mortality rate was very high. Although, Iswaran *et al.* (1970) reported that counts of *R. japonicum* at temperature ranging from 28 to 35°C were appreciable, while at a temperature of 40°C the mortality rate was high. As shown in this study all *Pseudomonas* strains successfully able to grow at 40°C and 45°C, except for two strains; PF- 50090 and *P.putida*18, which failed to grow at 40°C and 45°C whereas, *P.putida*21 failed to grow at 45°C. The elimination of plasmid from a bacterial culture is the best method to substantiate the relationship between a genetic trait and carriage of the specific plasmid by the culture as the phenotypic characters which are associated with the plasmid into the cured strain the lost phenotype is re-appeared. For curing of plasmid, both physical and chemical agents have long been used to free or "cure" bacterial cells of plasmid DNA molecules (Stanisich, 1988).

The results presented in Table 8 revealed that treatment of RL- 12612 with different levels of temperature has been shown to result in the eliminate the *Ap* and *Rif* resistance genes at 40°C and 45°C, as well as, *Tc* resistance gene at 37°C, 40°C and 45°C, whereas *Nm* resistance was lost at 45°C, in contrast with the *Cp* resistance which was still stable at 45°C. These results are in agreement with Abdel-Salam *et al.* (2007), who found that all cured isolates had lost their antibiotic resistance to all five antibiotics tested. This indicated that genes of the resistance losted were located on plasmids. RL-4404 was eliminated all antibiotic resistance genes at 35 and 37°C, except for *Eryth* resistance, whereas 40 and 45°C have a lethal effect on these strain. *Ap*, *Str* and *Rif* resistance genes in RL-3841 were eliminated at 35 and 37°C, whereas, *Eryth* resistance genes were lost at 37°C, however *Cm* resistance genes was stable at 35 and 37 °C. However, RL-4406 was lost the *Rif* and *Cp* resistance genes at 35 and 37°C, although, the *Eryth* resistance genes were lost at 40 and 45°C. At 40°C *Eryth* and *Rif* resistance genes were lost in RL-2070. At 35 and 37°C RL-2074 strain was lost all antibiotic resistance genes. These results are in agreement with Sharma and Laxminarayana (1989), who found that high temperature "cured" the mutants from the single large plasmid present in the parent strain, all these cured mutants were nod$^-$, indicating that the genes for nodulation were present on the plasmid, which is readily cured at a high temperature (40°C). Hynes *et al.* (1989) reported that exposure of labelled cells to 44°C resulted in elimination the plasmid, while the symbiotic plasmid b was cured at 30°C. The results obtained herein appeared that at 37°C PF- 50090 strain was lost all resistance genes. This are in agreement with those reported by Chin *et al* (2005), who found that all of *Lactobacillus* strains exhibited varying degrees of resistance to chloramphenicol and erythromycin, plasmids curing suggested that the plasmids may be encoded genes (Baldani *et al.*1992).

Table 7: Effected of elevated temperature on plasmid curing.

Bacterial strains	Temperature (C °)				
	28	35	37	40	45
RL- 12612	++	++	++	++	++
RL-4404	++	++	+	-	-
RL-3841	++	++	++	-	-
RL-4406	++	++	++	++	++
RL-2070	++	++	++	+	-
RL-2074	++	++	+	-	-
RL -207	++	++	++	++	++
PF- 50090	++	++	++	-	-
*P.putida*18	++	++	++	-	-
*P.putida*8	++	++	++	++	++
*P.putida*21	++	++	++	++	-
PF-1603	++	++	++	++	++
PF-10	++	++	++	++	++

++, +, – = Normal growth, little growth and no growth, respectively.

Table 8: Effect of high temperaturs on plasmid curing .

Bacterial strains	Resistance antibiotic at 28°C	Diameter (cm) of inhibition zones			
		35	37	40	45
RL- 12612	*Ap*	0.0	0.0	2.3	2.4
	Tc	0.0	1.5	1.9	2.2
	Nm	0.0	0.0	0.0	1.2
	Eryth	0.0	1.8	2.0	3
	Cp	0.0	0.0	0.0	0.0
	Rif	0.0	1.3	1.5	1.9
RL-4404	*Rif*	1.3	2.1	LE	LE
	Nm	1.2	1.9		
	Cm	2.6	3.2		
	Str	3	3.5		
	Eryth	0.0	0.0		
RL-3841	*Cm*	0.0	0.0	LE	LE
	Ap	2.5	3.5		
	Str	3.2	4.0		
	Eryth	0.0	1.2		
	Rif	1.2	1.5		
RL-4406	*Eryth*	0.0	0.0	2.5	3.7
	Rif	2.5	2.9	3.5	3.9
	Cp	3.2	3.6	4.5	4.8
RL-2070	*Eryth*	0.0	0.0	1.5	LF
	Rif	0.0	0.0	1.3	
	Nm	0.0	0.0	0.0	
RL-2074	*Nm*	1.5	1.9	LE	LE
	Eryth	2.3	3.6		
	Rif	1.2	2.0		
	Cm	1.3	2.2		

Table 8: Continued.

RL -207	*Nm*	0.0	0.0	0.0	0.0
	Eryth	0.0	0.0	0.0	0.0
	Rif	0.0	0.0	0.0	3.5
	Cp	0.0	2.2	3.0	3.2
PF- 50090	*Nm*	1.9	2.5	LE	LE
	Eryth	3.00	3.5		
	Rif	2.5	3.0		
	Cp	1.5	3.0		
	Cm	0.0	1.2		
	Ap	0.0	1.0		
*P.putida*18	*Nm*	0.0	0.0	LE	LE
	Eryth	0.0	0.0		
	Rif	0.0	1.2		
	Cp	2.2	2.5		
	Cm	3.2	3.5		
	Ap	0.0	0.0		
	Str	1.1	2.0		
*P.putida*8	*Cm*	0.0	0.0	0.0	2.1
	Ap	0.0	0.0	0.0	1.3
	Cp	0.0	0.0	1.4	1.5
	Eryth	3.2	4.0	4.8	5.2
	Rif	2.3	3.2	3.4	3.7
*P.putida*21	*Cm*	0.0	0.0	2.3	LE
	Ap	0.0	0.0	3.2	
	Cp	0.0	1.5	4.2	
	Eryth	0.0	1.9	2.5	
	Rif	0.0	0.0	0.0	
	Tc	0.0	0.0	0.0	
PF-1603	*Cm*	0.0	0.0	0.0	1.2
	Cp	0.0	0.0	0.0	1.5
	Eryth	2.8	3.2	3.5	3.9
	Rif	2.2	2.5	2.6	3.2
PF-10	*Cp*	0.0	0.0	0.0	1.7
	Eryth	2.8	2.9	2.9	3.0
	Rif	2.3	2.9	3.0	3.1
	Ap	0.0	0.0	0.0	0.0
	Str	0.0	0.0	1.3	1.5

LF = lethal effect.

Regulation of Indole-3-acetic Acid and Salicylic Acid:

Production of the auxin indole-3-acetic acid (IAA) is widespread among plant-associated bacteria. Beneficial bacteria synthesize IAA predominantly by an alternate tryptophan-pathway, through indolepyruvic acid, its role in plant growth promotion. As shown as in Table 9 some bacterial transconjugants (Tr$_{12}$, Tr$_{37}$, Tr$_{40}$, Tr$_{45}$, Tr52, Tr$_{53}$, Tr$_{78}$ and Tr$_{79}$) harboring DNA from two sources appeared significant increase in IAA production and yield percentage in complete media supplemented with tryptophan in relation to the mid-parent. This agreed with Williams and Signer (1990), who found that the alfalfa symbiont *Rhizobium meliloti* produces indole-3-acetic acid in a regulated manner when supplied with exogenous tryptophan. However, no significant increase in IAA production was observed in minimal media. Interactions between IAA-producing bacteria and plants lead to diverse outcomes on the plant side, varying from pathogenesis to phytostimulation. Reviewing the role of bacterial IAA in different microorganism–plant interactions highlights the fact that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms. Moreover, several recent reports indicated that IAA can also be a signaling molecule in bacteria and therefore can have a direct effect on bacterial physiology. (Spaepen *et al.* 2008).

Both transconjugants Tr$_{52}$ and Tr$_{54}$ resulted from the mating between *P.putida*8 x RL-2074 appeared significant increase in salicylic acid production in relation to the mid- parents. Salicylic acid (SA) has been proposed as the systemic signal for the induction of systemic acquired resistance (SAR). Salicylate is a biosynthetic product and a precursor of secondary metabolites and siderophores (iron chelators) in several bacterial genera, *e.g. Pseudomonas*, *Burkholderia*, *Azospirillum*, *Vibrio*, *Yersinia*, and *Mycobacterium* (Gaille *et al.* 2002). In bacteria, SA synthesis proceeds from chorismate via isochorismate (Serino *et al.* 1995), and isochorismate synthase (ICS) is the rate-limiting enzyme for SA synthesis in *Pseudomonas aeruginosa* (Gaille *et al.* 2002). Indole acetic acid (IAA) is one of the most physiologically active auxins and a common product of L tryptophan metabolism by several microorganisms including rhizobia (Mandal *et al.* 2007).

Table 9: Evaluation of transconjugants resulted from the mating between *P.putida* 8 as a donor with different *Rhizobium* strains used as a recipients concerning IAA and salicylic acid production.

Strains	IAA (CM)		IAA (MM)		Salicylic acid	
	IAA (µg/ml)	Yield%	IAA (µg/ml)	Yield %	(µg/ml)	Yield %
*P.putida*8	6.33	100	4.43	100	6.87	100
RL- 12612	5.73	100	1.54	100	0.77	100
Mid parent	6.03	100	3.0	100	3.8	100
Tr_{11}	3.60	60	0.70	23	5.76	151
Tr_{12} †	11.50	191	2.50	84	4.64	121
Tr_{13}	5.23	87	2.03	68	0.00	0
Tr_{14}	1.70	28	1.17	39	11.02	289
Tr_{15}	2.00	33	2.07	69	6.41	168
*P.putida*8	6.33	100	4.43	100	6.87	100
RL-4404	19.33	100	2.40	100	2.83	100
Mid parent	12.83	100	3.0	100	5.0	100
Tr_{36}	6.07	50	3.40	104	15.20	318
T_{37}	16.00	123	6.60	191	10.47	232
Tr_{38}	7.40	63	4.23	121	13.21	409
Tr_{39}	3.97	30	3.53	105	10.15	204
Tr_{40} †	29.70	245	6.23	192	9.34	263
*P.putida*8	6.33	100	4.43	100	6.87	100
RL-2070	6.83	100	1.43	100	2.97	100
Mid parent	6.85	100	2.9	100	4.9	100
Tr_{41}	4.90	74	1.17	40	11.25	229
Tr_{42}	3.50	53	3.63	124	10.93	222
Tr_{43}	6.33	96	1.03	35	5.70	116
Tr_{44}	2.70	41	3.30	113	10.06	205
Tr_{45} †	16.70	254	14.27	486	12.33	251
*P.putida*8	6.33	100	4.43	100	6.87	100
RL-2074	9.43	100	0.93	100	5.33	100
Mid parent	6.45	100	2.2	100	6.1	100
Tr_{51}	6.57	83	7.47	337	16.93	278
Tr_{52} †	21.00	266	7.00	316	8.87	145
Tr_{53}	10.40	132	7.57	341	6.23	102
Tr_{54}	3.40	43	4.33	196	13.70	225
Tr_{55}	3.97	50	2.10	95	3.80	62
*P.putida*8	6.33	100	4.43	100	6.87	100
RL -207	5.33	100	2.83	100	1.60	100
Mid parent	5.83	100	2.6	100	4.2	100
Tr_{76}	7.00	120	1.30	36	0.00	0
Tr_{77}	3.43	59	1.53	42	10.30	243
Tr_{78} †	9.63	165	2.83	78	5.07	120
Tr_{79}	8.20	141	3.50	96	0.00	0
Tr_{80}	2.70	46	2.13	59	0.00	0
F-test	**	0	NS	NS	**	NS
LSD 0.05	2.59	22			7.71	
0.01	3.44	29			10.25	

NS,*,** = Insignificant differences , significant at 0.05 and 0.01 probability levels, respectively.

Transconjugants selected to be applied in pots experiments, based on salicylic acid and IAA production.

As shown from the results presented in the Table 10 transconjugant Tr$_4$ resulted from the cross between PF50090 x RL-12612 showed significant increase in IAA produced in complete medium above the mid-parents, whereas, Tr$_{35}$ resulted from the mating between PF50090 x RL-4404 reflected the same effect in IAA production. The results indicated that IAA production was markedly increased when Trp was included in the growth medium. This agreed with Kaneshiro *et al.* (1983) who reported that Rhizobia are able to convert the amino acid Trp to IAA, which is the primary naturally occurring auxin in plants. Therefore, it appears that although tryptophan are involved in the synthesis of IAA, because there is evidence that plant root exudates contains significant levels of Trp (Kittel *et al.* 1989). It is possible that most transconjugants could exhibit a symbiotically effective phenotype than their parental strains.

Table 10: Evaluation of transconjugants resulted from the mating between PF50090 as a donor with RL- 12612, RL-3841 and RL-4404 as a recipients concerning IAA and salicylic acid production.

Strains	IAA (CM)		IAA (MM)		Salicylic acid	
	IAA (μg/ml)	Yield %	IAA (μg/ml)	Yield %	Salicylic acid (μg/ml)	Yield %
PF50090	10.93	100	4.01	100	7.17	100
RL-12612	5.73	100	2.21	100	0.77	100
Mid-parent	8.33	100	3.11	100	4.0	100
Tr_1	2.83	34	2.84	91	8.03	203
Tr_2	2.47	30	3.79	122	7.54	190
Tr_3	4.33	52	2.88	93	9.16	231
Tr_4 †	11.93	143	2.10	68	7.51	189
Tr_5	4.90	59	3.48	112	4.14	104
PF50090	10.93	100	4.01	100	7.17	100
RL-3841	6.30	100	2.77	100	4.43	100
Mid-parent	8.62	100	3.39	100	5.8	100
Tr_{26} †	3.73	43	1.97	62	4.43	89
Tr_{27}	4.33	46	1.60	54	2.04	41
Tr_{28}	2.30	27	0.97	31	3.96	77
Tr_{29}	1.40	15	3.53	63	5.22	101
Tr_{30}	3.97	46	1.63	457	3.07	45
PF50090	10.93	100	4.01	100	7.17	100
RL-4404	19.33	100	2.40	100	2.83	100
Mid-parent	15.13	100	3.2	100	5.0	100
Tr_{31}	3.97	26	2.47	77	11.62	232
Tr_{32}	11.80	78	1.70	53	6.38	128
Tr_{33}	7.40	49	3.47	108	3.41	68
Tr_{34}	5.90	39	3.57	111	12.33	247
Tr_{35} †	29.70	196	6.87	214	15.23	305
F-test	**	NS	NS	NS	0	**
LSD 0.05	2.09				5.8	103
0.01	2.81				7.7	138

NS,*,** = Insignificant differences , significant at 0.05 and 0.01 probability levels, respectively.

† Transconjugants selected to be applied in pots experiments, based on salicylic acid and IAA production .

Three transconjugants Tr_3, T_{r31} and Tr_{35} resulted from the mating between; PF50090 with RL-12612 and RL-4404, appeared significant increase in salicylic acid yield in relation to the mid parents. This agreed with Berraho *et al.* (1997), who found that some strains of *Rhizobiumciceri*, specific to chickpea (*Cicer arietinum* L.) secreted salicylic acid and 2,3-dihydroxybenzoic acid as phenolate-type siderophores, as well as, under the conditions of iron limitation many rhizospheric bacteria produce siderophores, ferric iron-specific ligands. This may enhance plant growth by increasing the availability of iron near the roots screened for their ability to grow on iron-deficient medium and to produce siderophores.

The data presented in Table 11 appeared that Tr_9 revealed significant increase in IAA producted in both complete and minimal media in relation to the mid-parents. This agreed with Ghosh and Basu (2002), who found that *Rhizobium* sp. isolated from root nodules of *Dalbergia lanceolaria* also produced high amount of IAA at 2.5 mg/ml of L-tryptophan concentration. However, the same effect was appeared in salicylic acid production in relation to the mid parent. Whereas, Tr_8 appeared significant increase in IAA production in minimal medium. The importance of IAA and SA was demonstrated before by Zaghool *et al.* (2001), who found that the combination between IAA and salicylic acid led to increase in growth parameters of rosemary plants if compared with individual effect of phenols. Salicylic acid (SA) is a phenolic compound that affects a variety of biochemical and molecular events associated with induction of disease resistance. SA has been shown to play an important role in expression of both local resistance controlled by major genes and systemic induced resistance developed after an initial pathogen attack (Hammerschmidt and Smith-Becker 2000).

Data presented in Table 12 indicated that all bacterial transconjugants harboring DNA from two sources produced high levels of IAA in the complete medium supplemented with tryptophan. In contrast, the isolates produced very low levels of IAA in minimal medium than that produced in complete medium except two transconjugants (Tr_{56} and Tr_{57}) resulted from the mating between PF-1603 x RL-2074. This indicated that the genes responsible in this pathway in these isolates may be more amplified. Transconjugants Tr_{17} and Tr_{19} resulted from the mating between PF-1603 x RL-12612, as well as, transconjugant Tr_{71} resulted from mating between PF-1603 x RL-207 produced significant amounts of IAA in relation to the mid parents. This may be due to more amplification of *ipdc* gene in these isolates. The *ipdC* gene coding the key enzyme phenyl-3-pyruvate decarboxylase (PPDC) of IPyA pathway was identified from several strains. The *ipdc* gene encoding indolepyruvate decarboxylase, which catalyzes a key step in the indolepyruvic acid pathway for IAA synthesis. Indolepyruvic acid is the product of catalysis of tryptophan by tryptophan transaminase, the first step in the IAA biosynthetic pathway, and is the substrate for indolepyruvate decarboxylase (Spaepen *et al.* 2007).

Table 11: Evaluate transconjugants resulted from the mating between *P.putida*18 as a donor and RL- 12612 as a recipient in IAA and salicylic acid production.

Strains	IAA (CM)		IAA (MM)		Salicylic acid	
	IAA (μg/ml)	Yield %	IAA (μg/ml)	Yield %	Salicylic acid (μg/ml)	Yield %
*P.putida*18	5.8	100	3.0	100	6.5	100
RL-12612	5.7	100	2.2	100	0.8	100
Mid -parent	5.8	100	2.6	100	3.6	100
Tr$_{6}$	3.5	61	2.2	86.3	0.7	18.3
Tr$_{7}$	5.9	103	3.4	131	1.6	44.5
Tr$_{8}$	8.3	144	8.5	327	2.1	56.9
Tr$_{9}$ †	13.3	231	5.3	204	4.0	108.8
Tr$_{10}$	4.1	70	3.7	143	1.6	45.0
F-test	0	**	0	NS	0	NS
LSD 0.05	4.22	58.9	0.77		2.42	
0.01	5.92	82.7	1.09		3.4	

NS,*,** = Insignificant differences, significant at 0.05 and 0.01 probability levels, respectively.

† Transconjugants selected to be applied in pots experiment, based on salicylic acid and IAA production .

Table 12: Evaluate transconjugants resulted from the mating between PF-1603 as a donor with RL- 12612, RL-2070, RL-2074 and RL -207 as a recipients on the basis of IAA and salicylic acid production.

Strains	IAA (CM)		IAA (MM)		Salicylic acid	
	IAA (μg/ml)	Yield %	IAA (μg/ml)	Yield %	(μg/ml)	Yield %
PF-1603	15.90	100	8.23	100	6.87	100
RL- 12612	5.73	100	1.54	100	0.77	100
Mid-parent	10.82	100	4.89	100	3.82	100
Tr$_{16}$	5.77	53	4.73	97	6.82	179
Tr$_{17}$	13.87	128	5.63	115	4.83	126
Tr18	6.87	63	4.63	95	9.97	261
Tr$_{19}$†	19.03	176	6.93	142	8.72	228
Tr$_{20}$	5.37	50	0.53	11	3.61	95
PF-1603	15.90	100	8.23	100	6.87	100
RL-2070	6.83	100	1.43	100	2.97	100
Mid-parent	11.0	100	4.83	100	4.92	100
Tr$_{46}$	7.80	70	7.10	146	15.87	349
Tr$_{47}$†	7.77	71	3.00	86	12.83	253
Tr$_{48}$	7.23	64	2.17	42	17.77	471
Tr$_{49}$	2.60	25	2.43	47	21.90	415
Tr$_{50}$	12.37	112	3.80	103	8.40	210
PF-1603	15.90	100	8.23	100	6.87	100
RL-2074	9.43	100	0.93	100	5.33	100
Mid-parent	12.7	100	4.58	100	6.10	100
Tr$_{56}$	2.50	20	3.17	69	13.47	221
Tr$_{57}$	5.13	41	23.03	503	9.77	160
Tr$_{58}$	9.27	73	3.03	66	3.93	64
Tr$_{59}$†	7.60	60	6.43	140	14.40	236
Tr$_{60}$	11.90	94	2.13	47	4.50	74
PF-1603	15.90	100	8.23	100	6.87	100
RL -207	5.33	100	2.83	100	1.60	100
Mid-parent	10.62	100	5.53	100	4.23	100
Tr$_{71}$†	15.93	150	15.40	278	13.00	307
Tr$_{72}$	6.27	59	14.27	258	5.80	137
Tr$_{73}$	3.53	33	6.00	108	16.73	395
Tr$_{74}$	4.07	38	5.07	92	7.13	169
Tr$_{75}$	9.13	86	6.30	114	9.28	219
F-test	**	**	**	**	0	NS
LSD 0.05	1.63	8.30	1.98	38.14	5.7	
0.01	2.17	11.06	2.64	50.85	7.59	

NS,*,** = Insignificant differences , significant at 0.05 and 0.01 probability levels, respectively.

† Transconjugants selected to be applied in pots experiment, based on salicylic acid and IAA production.

All transconjugants resulted from the mating between PF-1603 x RL- 2070 appeared high significant increase in salicylic acid production, except for Tr$_{50}$. Whereas, transconjugants Tr$_{71}$ and Tr$_{73}$ revealed the same effect when grown under conditions of iron limitation. This agreed with Page (1993), who found that bacterial siderophores depends upon the siderophore affinity for iron, as well as, salicylic acid and citric acid produced by other soil bacteria were cited as low iron affinity ligands (Page 1993).

The results summarized in Table 13. show that four transconjugants; Tr_{23}, Tr_{64}, Tr_{66} and Tr_{69} appeared significant increase in IAA in complete medium. This result agreed with Jones *et al.* (1983), who found that IAA has been tentatively identified in culture supernatants of *Rhizobium* strains grown in the presence of tryptophan.

All transconjugants resulted from the mating between PF-10 x RL-2070, except for, Tr_{64} and Tr_{65}, produced significant values of IAA above the mid parents in minimal medium. The presence of IAA in the supernatant of transconjugant cultures, indicating the capability of these bacteria to produce IAA from different media. In particular, it has been postulated that IAA is the causal agent of root hair curling (Fahraeus and Liunggren 1968). This identification has been much speculation that indole-3-acetic acid (IAA) might play a role at various stages in the symbiotic relationship between *Rhizobium* and leguminous plants (Newcomb 1980).

As shown herein all bacterial transconjugants produced high levels of salicylic acid. Transconjugant Tr64 produced significant amounts of SA above the mid parents. Salicylate is also important in plants, where it induces flowering and is involved in resistance to systemic diseases through multiple signal transduction pathways (Klessig and Malamy, 1994). Salicylate appears to be synthesized by the members of only four genera: *Pseudomonas*, *Azospirillum*, *Yersinia*, and *Mycobacterium*. This study induced recombinans in *Rhizobium* strains harboring SA gene which was the main criteria of this investigation. This agreed with Leeman *et al.* (1996), who reported that *P. fluorescens* strain WCS374, produced SA in quantities that were iron dose–dependent, it can be conducted that all strains and their transconjugants tested, produced different values of IAA and salicylic acid in culture supernatants, which were of the same order of magnitude. The data presented herein reflected the increased transcriptional activity of *ipdC* genes during the growth of transconjugants harboring different parts of extra copy of DNA.

Bacteriocin Activity:

Bacteriocins are proteins or protein complexes with bacteriocidal activity directed a against species that are usually closely related to producer bacterium**.** Bacteriocins are often defined as narrow-spectrum antibiotics produced by bacteria and active against only closely related species or strains (Tagg *et al.* 1976).

The result presented in Table14 appeared that all *Rhizobium* strains were tested for bacteriocin production against *Pseudomonas* strains. Bacteriocin produced from most *Rhizobium* strains was inhibit the growth of PF-50090 strain. These results agreed with Gross and Vidaver (1978), who found that bacteriocin-like substances were commonly produced by slow-growing *Rhizobium japonicum* and cowpea rhizobia on L-arabinose medium. Bacteria including *Rhizobium* are known to produce antagonistic substances having narrow inhibitory spectrum called bacteriocin or bacteriocin like substances (Nirmala and Gaur 2000).

In contrast the bacteriocins produced from all *Rhizobium* strains did not inhibit the growth of *P.putida* 21. Oresnik *et al.* (1999) found that only 4 out of 33 *Rhizobium* strains did not produce small bacteriocin and only 6 of the 33 strains produced a medium bacteriocin. The bacteriocin of Rl-12612 inhibit the growth of three *Pseudomonas* strains (PF- 50090, *P.putida*18 and *P.putida*8), but it was not effect on the growth of other strains These results are in agreement with Gross and Vidaver (1978), who found that cowpea strains 8A9 and 32F1 inhibited *B. subtilis* St168, and 32F1, as well as, *Serratia sp*. strain Sp ST7; in these cases, all zones (8 to 10 mm) were turbid. Rodelas *et al.* (1998) investigated bacteriocin production of Nod^+ Fix^+ indigenous isolates of *R. leguminosarum* bv. V*iceae*. Many, if not all, species of *Rhizobium* produced bacteriocins, designated rhizobiocins (Roslycky, 1967). Oresnik *et al.* (1999) found that the bacteriocins appear to play a major role in determining competitiveness for nodulation when assayed against some strains.

As shown in Table 15, the Pf -50090 bacteriocin producer strain could inhibit the growth of two *rhizobium* strains; RL-4406 and RL-2074. Whereas, other *rhizobium* strains didn't affected, this indicated that they had immunity genes against this bacteriocin. This agreed with Jiang *et al.* (2000), who reported that the biocontrol strain *Pseudomonas fluorescens* F113 has been found to produce the *Rhizobium* small bacteriocin.

Although, *P.putida* 8 bacteriocin's inhibit the growth of two rhizobium strains; RL4404 and RL-207 whereas, other rhizobium strains didn't affected. Whereas, PF-10 bacteriocin appeared the same trend of growth inhibition against two rhizobium strains; RL-3841 and RL-2074.

In contrast, the bacteriocins produced from *P. putida*21 inhibit the growth of all rhizobium strains. This indicated that these strains did not have any immunity genes against this bacteriocin. This result agreed with Homma *et al.* (1989), who found that rhizobia was inhibited *in vitro* by *Pesudomonas* strains.

Rhizobium strain RL-2070 did not affected by any *Pseudomonas* bacteriocin producers except that, produced by *P.putida* 21 which inhibit the growth of this strains. This agreed with Padilla *et al.* (2002), who reported that bacteriocin PsVP-10 a 2.6 Kda peptide isolated and purified from *Pseudomonas sp.* possesses lethal activity against *Enterococcus faecalis, Salmonella typhimurium* and Shigella flexneri.

Table13: Evaluate transconjugants resulted from the mating between PF-10 as a donor with RL- 12612, RL-2074 and RL -207 as a recipients based on IAA and salicylic acid production.

Strains	IAA (CM)		IAA (MM)		Salicylic acid	
	IAA (µg/ml)	Yield %	IAA (µg/ml)	Yield %	(µg/ml)	Yield %
PF-10	3.43	100	3.00	100	11.0	100
RL- 12612	5.73	100	1.54	100	0.8	100
Mid-parent	4.58	100	2.27	100	5.87	100
Tr_{21}	1.97	43	0.97	38	2.4	40.1
Tr_{22}	4.87	106	1.90	78	3.4	57.8
Tr_{23}	13.17	287	2.93	128	10.1	171.8
Tr_{24}	1.90	41	0.57	21	3.4	57.1
Tr_{25}	6.93	151	1.40	79	1.8	30.5
PF-10	3.43	100	3.00	100	11.0	100
RL-2074	9.43	100	0.93	100	5.3	100
Mid-parent	6.43	100	1.97	100	8.15	100
Tr_{61}	3.27	53	4.50	229	8.4	101
T_{62}	3.37	56	4.13	210	8.9	112
Tr_{63}	6.40	95	3.93	200	5.2	63
Tr_{64}	12.20	190	2.30	117	11.2	124
Tr_{65}	6.67	103	2.87	146	18.0	160
PF-10	3.43	100	3.00	100	11.0	100
RL -207	5.33	100	2.83	100	1.6	100
Mid-parent	4.38	100	2.91	100	6.28	100
Tr_{66}	7.07	161	3.00	103	1.7	27
Tr_{67}	3.20	73	5.33	183	5.9	94
Tr_{68}	4.13	94	2.33	80	1.5	24
Tr_{69}	7.73	176	11.53	395	5.4	87
Tr_{70}	2.80	64	6.67	229	12.1	192
F-test	**	NS	**	**	*	**
LSD 0.05	1.86		1.6	62.35	6.62	92.26
0.01	2.50		2.15	83.55	8.87	123.62

NS,*,** = Insignificant differences , significant at 0.05 and 0.01 probability levels, respectively.

† Transconjugants selected to be applied in pots experiment, based on salicylic acid and IAA production.

Table 14: Diameter of inhibition zone resulted from *Rhizobium* bacteriocin against *Pseudomonas* strains.

Bacteriocin producers	*Pseudomonas* strains					
	PF- 50090	*P.putida*18	*P.putida*8	*P.putida*21	PF-1603	PF-10
RL- 12612	2.5	1.5	1.2	0.0	0.0	0.0
RL-4404	2.7	0.0	0.0	0.0	1.2	1.3
RL-3841	0.0	0.0	0.0	0.0	0.0	0.0
RL-4406	2.5	1.8	0.0	0.0	1.5	0.0
RL-2070	0.0	1.6	0.0	0.0	0.0	0.0
RL-2074	3	0.0	0.0	0.0	0.0	0.0
RL -207	2.5	0.0	0.0	0.0	3.4	0.0

Table 15: Diameter of inhibition zones resulted from *Pseudomonas* bacteriocin against *Rhizobium* strains.

Bacteriocin producers	*Rhizobium* strains						
	RL- 12612	RL-4404	RL-3841	RL-4406	RL-2070	RL-2074	RL -207
PF- 50090	0.0	0.0	0.0	1.5	0.0	2	0.0
*P.putida*18	1.5	0.0	3	2	0.0	0.0	0.0
*P.putida*8	0.0	1.3	0.0	0.0	0.0	0.0	1.5
*P.putida*21	2.3	1.2	3.5	1.5	3.7	2.3	4.5
PF-1603	0.0	1.5	0.0	0.0	0.0	0.0	2
PF-10	0.0	0.0	1.5	0.0	0.0	1.2	0.0

Evaluation of Bacterial Strains and Their Recombinants Against Agrobacterium Tumefaciens (Agr1513):

Members of the genus *Agrobacterium* are ubiquitous components of the soil microflora, the vast majority of which are saprophytic, surviving primarily on decaying organic matter. However, several species of agrobacteria cause neoplastic diseases in plants, including *Agrobacterium rhizogenes* (hairy root disease), *Agrobacterium rubi* (cane gall disease), *Agrobacterium tumefaciens* (crown gall disease) and *Agrobacterium vitis* (crown gall of grape). Infections of wound sites on dicotyledonous plants by the soil bacterium *Agrobacterium tumefaciens* result in the formation of crown gall tumors. An early step in tumor formation is the attachment of the bacteria to the plant cell surface. This attachment is required for bacterial virulence. All non attaching mutants are reduced in virulence.

In this study bacteriocin activity and antagonism were used to evaluate the efficiency of bacterial strains and their transconjugants against *Agrobacterium tumefaciens* (Agr1513),as shown in Table 16. The data appeared that all bacteriocines produced by parental strains and transconjugant isolates inhibit the growth of Agr1513 except for *Rhizobium* strains RL-2070, RL-2074 and RL-207, as well as, Tr10. These results are in agreement with Hirsch (1979), who found high frequency conjugal transfer of the ability to produce bacteriocins from three different *R. leguminosarum* field isolates to other strains of *R. leguminosarum*. Transfer of the determinat of medium sized bacteriocin production pRLIJI was correlated with the appearance of an extra plasmid with a molecular weight of about 130 x 10^6.

Rhizobium leguminosarum strains have been shown to produce bacteriocins which have been characterized as small, medium or large based on their assumed sizes and diffusion characteristics (Hirsch 1979). Oresnik *et al.* (1999) found that A 3-kb region containing the determinant for bacteriocin activity from *Rhizobium leguminosarum* 248 was isolated and characterized by Tn*5* insertional mutagenesis and DNA sequencing.

All *Pseudomonas* strains as shown herein were successfully inhibit the growth of Agr1513 growth in Petri dish as shown in Fig 14. Whereas, only one *Rhizobium* strain RL-4404 inhibit the growth of Agr1513, as well as, appeared antagonism against the same strains but the other strains unsuccessfully to do the same effect.

The results are harmony with Eastwell *et al.* (2006), who found that *Pseudomonas fluorescens*, *Bacillus subtilis*, as well as, *Bacillus* species significantly reduced gall size when applied 25 or 86 days before inoculation with *R. vitis*, and reduced the population of *R. vitis* relative to control plants. *P. fluorescens* '1100-6' also reduced the percentage of *R. vitis* inoculations that yielded galls during greenhouse studies for their effectiveness at preventing formation of galls induced by *Rhizobium vitis*, the gram negative bacterium that causes crown gall disease of grapevines.

However, six transconjugants (Tr_4, Tr_5, Tr_6, Tr_7, Tr_9 and Tr_{14}) were successfully in antagonism against Agr1513. These results agreed with Hafeez *et al.* (2005) who observed that *Rhizobium leguminosarum* bv. *viciae* produced a medium typed of bacteriocin that was found to be highly effective in growth inhibition against some strains of *R. leguminosarum* bv. *viciae* and *Agrobacterium* sp. during antagonism amongst mixtures of inoculant strains of *Rhizobiaceae* on the basis of bacteriocin production.

Table16: Diameter of inhibition zone due to bacteriocin produced by bacterial strains and their antagonism against *Agrobacterium tumefaciens* .

Bacterial strains and their recombinants	*Agrobacterium tumefaciens*	
	Diameter of inhibition zone(Cm) due to bacteriocin producers	Diameter of inhibition zone(Cm) due to antagonism
RL-12612	2.1	0.0
RL-3841	1.4	0.0
RL-4404	2.3	1.2
RL-2070	0.0	0.0
RL-2074	0.0	0.0
RL-207	0.0	0.0
P.putida8	3.2	2.6
P.putida18	3.1	3.5
PF-50090	1.2	1.4
PF-10	4.5	3.6
PF-1603	2.5	1.4
Tr_1	1.1	0.0
Tr_2	1.7	0.0
Tr_3	1.9	0.0
Tr_4	3.1	3.0
Tr_5	2.1	2.2
Tr_6	3.5	3.0
Tr_7	1.9	1.5
Tr_8	3.2	0.0
Tr_9	3.2	3.6
Tr_{10}	0.0	0.0
Tr_{11}	2.2	0.0
Tr_{12}	2.4	0.0
Tr_{13}	1.2	0.0
Tr_{14}	4.0	3.2
Tr_{15}	1.2	0.0
Tr_{16}	1.1	0.0

Testing Symbiotic Effictiveness of Rhizobial Recombinants in Pots Experiment:

Pea (*Pisum Sativum* L.), as most legumes, establishes in root nodules a symbiotic association with *Rhizobium leguminosarum* bacteria. The *Rhizobium*-legume symbiosis provides a good model system with which to study the molecular basis of bacterial competitiveness, because nodulation competitiveness is a readily quantifiable trait. The ability of a particular rhizobial strain to establish the development of a specialized plant organ, the root nodule, in which the bacteria fix atmospheric nitrogen in the presence of other strains is known as nodulation competitiveness (Mark and Handelsman, 2000).

As shown from the results presented in Table 17 all bacterial strains and their transconjugants resulted from all matings appeared significant increase in nodules number developed per plant except for Tr$_{11}$ which didn't successfully in nodulation with pea, however, parental strains significantly increase most of nodulation parameters developed on the root system of pea plants among both seasons above the uninoculated plants. This indicated the activity of nodulation (*nod*) genes in these strains to establish the symbiosis between pea plants and *Rhizobium* strains. The final product of nod genes are secreted lipo-chitin oligosaccharides, termed as Nod factors that represent the agent inducing initial nodule formation in the host root (Spaink, 2000). Higher activation of rhizobial nodulation (*nod*) genes giving significant increase in nodulation parameters. Whereas, Camerini *et al.* (2008) found that nodule growth rate is about 70% greater in *Rhizobium* RD20 than in the parental strain [267.35 § 0.03 mg/ (plant day) whereas, root nodule number per plant was 37% lower in *V. hirsuta* plants nodulated by the RD20 strain (7 ± 1) as compared with plants nodulated by the wild type strain (11 ±2).

Table 17: Means of nodulation parameters in pea plants grown in pots and inoculated with *Rhizobium*, *Pseudomonas* and their transconjugants .

Inoculum	Nodules/plant		Nodule DW†(mg/plant)		Average weigh of nodule (mg)	
	2007	2008	2007	2008	2007	2008
Uninoculated	0.00	0.00	0.00	0.00	0.00	0.00
RL-12612	9.33	7.00	10.00	13.33	0.93	2.00
RL-3841	8.00	8.33	9.33	10.00	0.88	1.21
RL-4404	7.33	8.00	9.42	9.75	0.78	1.24
RL-2070	10.33	11.33	18.33	20.00	0.58	1.77
RL-2074	7.00	5.33	10.87	7.00	0.73	1.34
RL-207	9.00	10.67	14.00	31.33	0.65	2.98
*P.putida*8	0.0	0.0	0.0	0.00	0.00	0.00
*P.putida*18	0.00	0.00	0.00	0.00	0.00	0.00
PF-50090	0.33	0.00	0.10	0.00	1.11	0.00
PF-10	0.33	0.00	0.00	0.00	0.00	0.00
PF-1603	0.00	0.00	0.00	0.00	0.00	0.00
Tr$_1$	10.00	11.67	15.83	23.50	0.68	2.01
Tr$_2$	12.33	14.00	12.33	35.00	1.00	2.50
Tr$_3$	12.00	10.33	16.67	20.00	0.74	1.91
Tr$_4$	8.67	8.00	16.67	11.00	0.58	1.48
Tr$_5$	18.00	18.00	43.33	41.67	0.42	2.31
Tr$_6$	8.67	9.33	16.67	18.33	0.52	1.98
Tr$_7$	11.33	10.00	28.33	16.67	0.40	1.82
Tr$_8$	13.33	11.00	28.67	15.00	0.49	1.58
Tr$_9$	7.00	8.33	17.00	9.33	0.41	1.17
Tr$_{10}$	8.67	8.67	11.67	15.00	0.77	1.74
Tr$_{11}$	0.00	0.00	0.00	0.00	0.00	0.00
Tr$_{12}$	10.67	12.33	19.33	32.00	0.57	2.64
Tr$_{13}$	10.00	8.33	33.00	11.67	0.30	1.67
Tr$_{14}$	10.00	13.00	29.33	32.00	0.34	2.50
Tr$_{15}$	14.00	15.67	40.00	43.00	0.35	2.89
Tr$_{16}$	8.67	8.67	11.87	11.20	0.74	1.30
F-test	**	**	NS	0	NS	NS
LSD 0.05	2.48	2.89		4.07		
0.01	3.30	3.84		5.41		

NS,*,** = Insignificant differences , significant at 0.05 and 0.01 probability levels, respectively.
†= Dry weight.

The rhizobacterium *Pseudomonas* used in mating experiments as a plasmid-donor is a strong candidate as a soil inoculant to enhance crop yields (Patten and Glick, 2002). Truchet *et al.* (1991) demonstrated that the Nod factors (NFs) are essential bacterial signaling for nodule induction. Martinez *et al.* (1987) found that genetic transfer between *Rhizobium* species has led to different results and the transconjugants produce fewer nodules and have lower acetylene reduction (25% as compared to the original *R. phaseoli* strain) and more amyloplasts per nodule.

The results summarized in Table 18 appeared the effect of bacterial strains and their transconjugants on leaf area per plant, plant height and number of branches. All bacterial strains and their transconjugants revealed significant increase in leaf area per plant above uninoculated plants at 45 days plant-old, except for, Tr_1 and Tr_2. These results are in agreement with Deshwal *et al.* (2003), who reported that rhizobia are known to increase nodulation and nodule dry weight in legumes along with increase in host plant growth and development. Besides protecting roots from the pathogens attack due to production of diverse microbial metabolites like siderophore, rhizobitoxin, plant growth enhancement through IAA production, uptake of phosphorus and other minerals. In addition, all bacterial strains appeared significant increase in plant height per plant above uninoculated plants at 100 days plant- old, except for RL-207, PF-50090, Tr_1, Tr_7 and Tr_{11} during the season of 2008. Dakora (2003) reported that rhizobia produce various metablites such as auxines, cytokinins, riboflavin and vitamins, their invasion of legume and non-legume plant roots should promote an increase in plant growth.

The results obtained herein are in agreement with the results obtained by Thakur and Panwar (l997), who found that vigna radiate cv ps l6 and Pusa l05 inoculated with *Rhizobium* had larger leaf area (l0%), higher chlorophyll content (11.40%) and photosynthesis activity (2l.65%), transpiration (l5.894) and stomatal conductance plants. In general, the parameters recorded herein were higher in *Rhizobium* inoculated plants.

Table 18: Mean of growth parameters parameters in plants grown in pots and inoculated with *Rhizobium* and *Pseudomonas* and their transconjugants.

Inoculum	L.A/P[†] (cm2)		Plant height (cm)		Number of branches/plant	
	2007	2008	2007	2008	2007	2008
Uninoculated	160.95	261.00	34.00	39.00	3	4
RL-12612	261.32	337.50	51.67	56.67	5	6
RL-3841	262.99	263.00	60.00	56.67	7	8
RL-4404	262.16	300.30	63.67	65.67	5	5
RL-2070	313.19	310.10	68.10	55.00	5	5
RL-2074	371.20	439.30	60.00	55.00	4	6
RL-207	437.86	384.00	55.00	55.00	4	5
*P.putida*8	422.24	360.00	59.33	59.33	6	6
*P.putida*18	286.70	377.60	45.00	45.00	7	6
PF-50090	276.66	361.20	43.33	52.00	5	5
PF-10	282.35	353.90	69.67	73.67	6	6
PF-1603	282.51	252.70	65.00	65.00	4	3
Tr_1	250.16	252.70	33.00	40.00	6	6
Tr_2	242.08	278.90	60.00	60.00	6	6
Tr_3	277.07	336.30	63.67	63.67	5	5
Tr_4	396.33	354.50	65.67	65.67	5	5
Tr_5	410.25	361.17	69.00	69.00	6	6
Tr_6	357.30	338.30	62.00	66.00	4	6
Tr_7	314.57	335.30	40.33	38.67	8	8
Tr_8	467.10	457.83	64.33	64.33	6	6
Tr_9	334.90	351.27	66.33	66.33	6	6
Tr_{10}	484.70	443.13	50.33	55.33	6	6
Tr_{11}	471.40	440.13	34.67	60.00	5	5
Tr_{12}	376.50	371.40	66.00	68.67	6	6
Tr_{13}	300.10	318.03	45.00	56.33	6	6
Tr_{14}	369.00	330.23	55.00	55.00	7	8
Tr_{15}	315.70	294.70	65.67	65.67	5	6
Tr_{16}	314.00	301.97	70.00	67.33	8	8
F-test	**	**	0	NS	**	**
LSD 0.05	52.37	62.20	6.80		1.8	1.3
0.01	69.65	82.72	9.01		2.4	1.7

NS,*,** = Insignificant differences , significant at 0.05 and 0.01 probability levels, respectively.

†= Leaf area per plant.

Response of Biochemical Traits to Inoculation:

Data presented in Table 18 showed that all bacterial strains play an important role to improve chlorophyll a at 45 days plant-old among two seasones and total chlorophyll during the seasones of 2008, whereas, all baterial strains didn't reported significant increase in Chl b, total chlorophyll and carotene in shoots at 45 days plant-old. These results agreed with Kosslak and Bohlool (1984), who found that the number of succful infections may be affected by photosynthetic capacity of the host plant.

Stancheva *et al.* (2006) demonstrated that dual inoculation of pea plants increased plant biomass, nodulation parameters, N_2 fixation activity at varying levels compared to plants submitted to single inoculation with *Rhizobium leguminosarum*. Dakora (2003) reported that the release phytohormones produced by rhizobia into cropping systems promoted plant growth through N_2 fixation by rhizobia. Vencatasamy (1984) reported that nitrogen fixation in beans was influenced by both the *Rhizobium* and host genotypes. Caers and Vendrig (1986) reported that application of cytokinins promote photosynthetic activity mainly by means of increase in leaf chlorophyll content. Hardy and Havelk (1975) and Bethlenfalvay and phillips (1978) reported the pivotal role of photosynthesis in nitrogen fixation.

Table 18: Mean of photosynthetic pigments of plants grown in pots affected by different bacterial strains and their transconjugants.

Inoculum		Chlorophyll content (mg/g)						Carotene	
		Chl†.a		Chl.b		Total Chl.			
		2007	2008	2007	2008	2007	2008	2007	2008
Uninoculated		0.078	0.108	0.376	1.248	0.454	1.356	1.195	1.356
RL-12612		0.507	0.153	2.668	1.537	3.175	1.690	1.941	1.690
RL-3841		0.470	0.284	2.353	1.402	2.823	1.687	1.949	1.687
RL-4404		0.297	0.211	2.079	1.593	2.376	1.805	1.061	1.805
RL-2070		0.392	0.465	1.904	1.849	2.296	2.314	1.705	2.314
RL-2074		0.419	0.185	2.557	1.491	2.976	1.675	1.542	1.675
RL-207		0.454	0.352	2.435	2.184	2.889	2.536	2.167	2.536
*P.putida*8		0.464	0.179	2.334	1.620	2.798	1.799	1.807	1.799
*P.putida*18		0.437	0.372	2.422	1.738	2.859	2.110	1.671	2.110
PF-50090		0.312	0.312	2.238	2.238	2.550	2.550	1.385	2.550
PF-10		0.428	0.428	2.330	2.330	2.758	2.758	1.640	2.758
PF-1603		0.456	0.456	2.492	2.492	2.948	2.948	1.783	2.948
Tr_1		0.421	0.390	1.772	1.979	2.193	2.369	1.553	2.369
Tr_2		0.405	0.206	2.391	1.291	2.796	1.497	1.768	1.497
Tr_3		0.430	0.202	1.974	1.547	2.404	1.749	1.607	1.749
Tr_4		0.339	0.245	2.446	2.523	2.786	2.769	1.865	2.769
Tr_5		0.362	0.418	2.429	2.204	2.791	2.621	1.696	2.621
Tr_6		0.504	0.322	2.404	2.188	2.908	2.510	1.622	2.510
Tr_7		0.515	0.262	2.629	1.559	3.144	1.821	2.332	1.821
Tr_8		0.545	0.231	2.401	1.675	2.946	1.907	1.999	1.907
Tr_9		0.515	0.315	2.629	2.136	3.144	2.452	2.332	2.452
Tr_{10}		0.488	0.282	2.600	2.708	3.088	2.990	2.086	2.990
Tr_{11}		0.488	0.203	2.600	1.206	3.088	1.408	2.086	1.408
Tr_{12}		0.427	0.351	2.263	1.839	2.690	2.191	1.851	2.191
Tr_{13}		0.498	0.394	2.493	1.895	2.991	2.289	2.471	2.289
Tr_{14}		0.493	0.456	2.640	2.294	3.133	2.750	1.620	2.750
Tr_{15}		0.474	0.420	2.319	1.810	2.793	2.229	2.204	2.229
Tr_{16}		0.407	0.347	2.450	1.606	2.857	1.953	2.266	1.953
F-test		0	**	NS	NS	**	NS	NS	NS
LSD	0.05	0.16	0.160			0.55			
	0.01	0.21	0.213			0.73			

NS,*,** = Insignificant differences , significant at 0.05 and 0.01 probability levels, respectively.
†= chlorophyll.

Data summarized in Table 19 revealed that *Rhizobium* transconjugants (T_1, Tr_3, Tr5, Tr_7,Tr_8, Tr_9, Tr_{10} and Tr_{14}) appeared significant increase in nitrogen content at 45 days plant-old above uninoculated plants among the second season. These results are in agreement with Matos and Schroder, (1989) who found that increases in total yield and N depended on the presence of root nodules and the assimilation of free nitrogen, this leading to increased shoot N content and improvement the soil. However, when evaluating the benefit of inoculation, increase in seed yield, as well as increase in grain protein content or total soil N should also be considered (Stevenson and Vankessel, 1997). Whereas, all *rhizobium* strains, except for RL-12612 and RL-207, appeared significant increase in nitrogen content among the second season above uninoculated plants at 45 days plant-old. The same effect was asheived by transconjugants Tr_{10}, Tr_{11} and Tr_{16}. All rhizobium strains and their transconjugants appeared significant increase in total protein of seeds above uninoculated plants among two seasons, except for Tr_{11} and Tr_1 at the first season. Solaiman and Rabbani (2005) found that the performance of *Rhizobium* inoculant alone was superior in relation to uninoculated control concerning protein content in green and mature seeds of pea.

Table 19: Mean of nitrogen and protein content of plants grown in pots affected by different bacterial strains and their transconjugants.

Inoculum	N% at 45 days plant old		N% at 100 days plant old		Protein in seeds	
	2007	2008	2007	2008	2007	2008
Uninoculated	1.10	0.93	0.20	0.20	15.10	16.77
RL-12612	1.52	1.51	0.30	0.23	24.90	24.90
RL-3841	2.06	1.95	0.50	0.34	26.60	26.60
RL-4404	1.77	1.80	0.50	0.26	23.20	23.20
RL-2070	2.07	2.00	0.40	0.30	23.10	23.10
RL-2074	2.16	1.50	0.30	0.25	20.10	20.10
RL-207	1.73	1.27	0.20	0.22	20.00	20.00
*P.putida*8	1.88	1.20	0.30	0.24	23.00	23.00
*P.putida*18	2.44	1.30	0.40	0.16	23.00	23.00
PF-50090	1.68	1.53	0.28	0.30	17.33	17.33
PF-10	1.89	1.30	0.28	0.22	18.00	18.00
PF-1603	1.56	1.43	0.60	0.21	16.33	17.67
Tr_1	2.77	1.37	0.20	0.33	15.33	18
Tr_2	1.59	1.40	0.30	0.40	18.67	19.00
Tr_3	2.44	1.33	0.20	0.26	23.67	24.53
Tr_4	1.54	1.37	0.30	0.28	22.00	23.37
Tr_5	2.30	1.30	0.40	0.21	25.50	25.07
Tr_6	1.80	1.53	0.50	0.26	24.60	21.10
Tr_7	2.40	1.40	0.40	0.33	22.27	21.03
Tr_8	3.16	1.47	0.40	0.25	22.00	20.70
Tr_9	2.60	1.40	0.20	0.35	24.33	22.70
Tr_{10}	3.01	1.99	0.60	0.36	20.00	23.03
Tr_{11}	1.22	1.02	0.20	0.34	16.33	18.00
Tr_{12}	1.26	2.00	0.30	0.36	25.00	22.03
Tr_{13}	1.68	1.90	0.20	0.24	23.50	23.70
Tr_{14}	2.72	1.50	0.30	0.29	23.87	22.77
Tr_{15}	1.40	1.56	0.73	0.31	26.00	23.00
Tr_{16}	2.00	2.07	0.37	0.30	24.67	22.00
F-test	**	**	NS	**	**	**
LSD 0.05	1.16	0.31		0.13	3.62	2.89
0.01	1.45	0.42		0.17	4.82	3.85

NS,*, ** = Insignificant differences, significant at 0.05 and 0.01 probability levels, respectively.

In conclusion, it is evident that it is possible to select rhizobial strains harboring recombinant genomes that were efficient in secrete IAA as a plant hormone, as well as, salicylic acid as a inhibitor agent against pathogenic microorganisms. Bacteriocins produced from these recombinant strains had a good performance to inhibit the growth of Agr1513 as an soil pathogen. In addition, rhizobial recombinants appeared symbiotic effectiveness leading to increase grain yield, as well as, improving biochemical traits such as grain protein contents and leaves chlorophyll concentration.

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