Extension of Rhizobial / Plant Host Range and Symbiosis Improvement *via* Plasmid Transfer

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Abstract: Leguminous plants gather and use gaseous nitrogen by working symbiotically with special bacteria (rhizobia) in nodules on their roots where each species of Rhizobia nodulate certain plants. In a previous study the *Tn_5 - mob - sac B* system was used to label and transfer plasmids of indigenous *Rhizobium leguminosarum biov. trifolii* to *Agrobacterium tumefaciens*. Among *Agrobacterium tumefaciens* transconjugants, *Agrobacterium tumefaciens* CB and CE strains were characterized to harbor nodulation/bacteriocin plasmids. In the present study, plasmid transfer was attempted by conjugation between *Agrobacterium tumefaciens* CB and CE strains and seven indigenous *Rhizobium leguminosarum* bv. *trifolii* and six *Rhizobium meliloti* strains in order to amplify nodulation gene and/or to increase their plant host-range specificity. Results obtained indicated that all strains tested could receipt CB or CE plasmids from *Agrobacterium tumefaciens*. The transconjugants obtained were characterized by testing them for their abilities to nodulate Egyptian clover plants. All *Rhizobium leguminosarum* bv. *trifolii* (*R.l.* bv.*trifolii*) transconjugants produced more nodules in Egyptian clover roots than their original strains, while all *R. meliloti* transconjugants had acquired the ability to nodulate Egyptian clover as new host plant. Symbiotic efficiency of transconjugants were studied in both Egyptian and sweet clover plants, results showed that most of *R. l.* bv. *trifolii* transconjugants improved the plant growth parameters and that *R. meliloti* transconjugants had much better symbiotic efficiencies with Egyptian clover. The obtained modified Rhizobial strains were genetically stable, and their field application will more probably improve plant growth and yield.
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1. Introduction

Legumes are very important plants both ecologically and agriculturally because they are able to interact symbiotically with soil microorganisms, rhizobia, to form root nodules where biological nitrogen fixation (BNF) takes place **(Spaink, 2000; Perret *et al*., 2000).** During this process, an exchange of molecular signals occurs between the two partners, leading to the formation of the root nodule, where biological nitrogen fixation takes place **(Gage, 2004).**

The amounts of nitrogen fixed by legume / *Rhizobium* association are varying depending on the legume bacterial strain and the soil. In Egypt a deficiency in clover / *Rhizobium leguminosarum* bv. *trifolii* symbiotic system was reported **(El-Haddad *et al*., 1988)**. Enhancing the biological nitrogen fixation in this symbiotic system requires bacterial improvement.

Plasmids of different indigenous *Rhizobium leguminosarum biov. trifolii* were labeled using the *Tn_5 - mob - sac B* system which produce neomycin resistance, sucrose sensitivity and mobilization ability **(Ried & Collmer, 1987).** These plasmids were transferred to *Agrobacterium tumefaciens* UBAPF2 (plasmid free) using the triparental mating with *E. coli* S17-1 (pRP 4-4) as helper plasmid as described by **Hynes *et al*., (1989).** Two *A. tumefaciens* transconjugants, i.e., AGCB and AGCE, were selected they were able to nodulate clover roots and contain the helper plasmid pRP 4-4 along with either CB or CE plasmid of *R. leg. trifolii* **(Ibrahim *et al.*, 1998).**

This study aimed to genetically improve indigenous *Rhizobium leguminosarum* biovar *trifolii* and *Rhizobium meliloti* strains for their nodulation and symbiotically performances through nodulation – plasmid transfer.

2. Materials and Methods

Bacterial strains:

The used bacterial strains and their characterization are listed in Table (1).

Plant material:

Egyptian clover, *Trifolium alexandrinum* local variety (Meskawy) has been used in the inoculation experiments.

Media and growth conditions:

Trypton Yeast Extract (TY) medium **(Beringer, 1974)** wase used for routine cultivation of *Rhizobia* and *Agrobacterium* strains.

Minimal medium (MM) of rhizobia **(Plassa, 1963)**, **was** used as a selective medium during conjugation experiments.

Growth temperature of both *Rhizobium a*nd *Agrobacterium* was 30^0C**.**

The following concentrations of antibiotics were used (μg/ml) when required: ampiciline (AP) 100, chloramphenicol (CM) 50, neomycin (NM) 100, rifampcin (RF) 150, streptomycin (SM) 400 and tetracycline (TC) 10.

Conjugation:

Agrobacteium AGCB and AGCE strains, containing the *R. leguminosarum* bv. *trifolii* nodulation/ bacteriocin plasmid **(Ibrahim *et al*., 1998)** were used as plasmid donors. Different indigenous *Rhizobia* strains were used as recipients.

Rhizobia and *Agrobacterium* strains were grown overnight on TY solid medium at 30^0C. A loopfull from each *Agrobacterium* donor and *Rhizobium* recipient strains were mixed together, spreaded over the TY plates and incubated overnight. The bacterial mixture was then collected and resuspended in 1 ml of saline. In each conjugation, 0.1 ml of the bacterial mixture was spreaded over minimal medium plates containing selectable markers, the grown rhizobial colonies were reisolated and nominated.

Plasmid isolation

Plasmid DNA isolation was performed using the Miniscreen method of **Rodriguez and Tait (1983).**

Nodulation test

The seeds of Egyptian clover were sterilized by soaked them in concentrated H_2SO_4 for10 min. A large volume of water was used for the first rinse, poured off immediately and followed quickly by more water (Rothamsted Collection of Rhizobium (RCR), England). Seeds were soaked in sterile Petri dish with distilled water for two hours to imbibe the water. The enclosed tube method was used for plant inoculation. The nutrient medium of **Jensen (1942)** was used as slants in test tubes (150 mm X 18 mm). Clover seeds were pre-germinated to give 5-mm radicals, inoculated by soaking in the tested bacterial strain culture and then planted through a small hole in the tube cap. The radical grows on the top of the agar slope and the cotyledons grow outside. About 20 ml of nutrient solution of **Norris and Date (1976)** were added through the watering hole in the aluminum foil cap and stopped up as necessary.

Table 1 *Bacterial strains, their source and Nomination used in this study*

Bacterial strain	Reference	Resistance
Rhizobium leguminosarum biovar *trifolii*		
Rt nit	**Abdel-Salam *et al.*, 2010**	Cm, Sm, Ap
Rt1	**Abdel-Salam *et al.*, 2010**	Cm, Sm, Nm
Rt 10	**Abdel-Salam *et al.*, 2010**	Cm, Nm
Rt 14	**Abdel-Salam *et al.*, 2010**	Cm, Sm, Ap
Rt H1	**Abdel-Salam *et al.*, 2010**	Cm, Zn
Rt M1	**Abdel-Salam *et al.*, 2010**	Cm, Sm, Ap
Rt M2	**Abdel-Salam *et al.*, 2010**	Cm, Sm, Tc, Ap
Rhizobium meliloti		
Rm A-2	**Abdel-Salam *et al.*, 2010**	Cm, Ap
Rm 1	**Abdel-Salam *et al.*, 2010**	Cm, Ap
Rm 2	**Abdel-Salam *et al.*, 2010**	Co, Zn, Ap
Rm 3	**Abdel-Salam *et al.*, 2010**	Cm, Ap, Tc
Rm 4	**Abdel-Salam *et al.*, 2010**	Cm, Sm, Ap
Rm 5	**Abdel-Salam *et al.*, 2010**	Cm, Ap
Agrobacterium tumefaciens UBABF 2		
AGCB	**Ibrahim *et al*., 1998**	Rif, Ap, Sm, Nm
AGCE	**Ibrahim *et al*., 1998**	Rif, Ap, Sm, Nm

Cm: Chloramfinicol, Sm: Streptomycin, Nm: Neomycin, Tc: Tetracycline, Amp: Ampicilin, Rif: Rifampicin. Co: Cobalt, Zn: Zinc.

3. Results and Discussion

Clover, *Trifolium alexandrium* (L), can obtain most of the nitrogen it needs from the vast supply of air gaseous nitrogen. It gathers and uses this nitrogen by symbiotically working with *Rhizobium leguminosarum* bv. *trifolii* in its root nodules. A deficiency in this symbiotic system was reported **(El-Haddad *et al*., 1988)** where positive yield response of clover was only obtained when high densities of *Rhizobium* were used. Different ecological and biological factors are dramatically affecting the legume / *Rhizobia* symbiosis. Therefore, enhancing the biological nitrogen fixation by clover / *Rhizobium*

symbiosis requires through investigation of the complex factors involved in this symbiosis.

The effect of additional nodulation genes in improving the symbiotic efficiency of different indigenous *Rhizobium leguminosarum* bv *trifolii* and *Rhizobium meliloti* strains was studied.

Rhizobial conjugation

In a previous study **(Ibrahim *et al*., 1998)**, *Agrobacterium tumefacience* CB and CE strains harboring nodulation/bacteriocin plasmid of *Rhizobium leguminosarum* biovar *trifolii* were constructed. The two *Agrobacterium* strains also contain the helper plasmid Rp4-4 and hence both CB and CE plasmids could be transferred to other gram-negative bacterial strain via conjugation.

Attempts to transfer the CB and CE plasmids to *Rhizobium leguminosarum* biovar *trifolii* and *Rhizobium meliloti* were done in order to produce new transconjugants with amplified nodulation genes and or to increase the plant host-range which *R. meliloti* could nodulate.

Rhizobium leguminosarum biovar *trifolii* or *Rhizobium meliloti* strains were inoculated with both *Agrobacterium* strains and transconjugants were selected using the appropriate antibiotics, which counter selects the parental strains.

All seven *Rhizobium leguminosarum* biovar *trifolii* used could receipt CB/ or CE plasmid except Rt nit strain (Table 2).

Among the six *Rhizobium meliloti* strains used, four receipted CB or CE plasmid. Only two strains, i.e., Rm1 and Rm5, could not stably harboring CB or CE plasmids which may reflects incompatibility between their indigenous plasmids and the two studied ones.

Results indicated that *Agrobacterium tumefacience* AGCB donor strain was more efficient in plasmid transfer than *Agrobacterium tumefacience* AGCE, where ten transconjugants could be obtained from both *Rhizobia* strains, comparing with only six transconjugants obtained with the latter donor strain.

Transconjugants characterization

Nodulation efficiency

The sixteen transconjugants obtained were tested for their ability to nodulate Egyptian clover plants (Table 3 and Figure 1).

Results indicated that all *R. leg.* bv. *trifolii* – transconjugants produced more nodules, in Egyptian clover roots, than their original parental strains except in Rt 10 (CB) transconjugant which produce only 12 nodules/ plant comparing with 14 nodules per plant produced by the parental strain Rt 10. The best transconjugant was Rt M2 (CE) which produced 24 nodules per plant. i.e., about 1.6 times that produced by its parental strain Rt m2.

The improvement of nodulation efficiencies of Rt transconjugants is more probably due to the nodulation gene amplification via CB or CE plasmid transfer.

Rhizobium meliloti could not nodulate Egyptian clover. Since CB and CE plasmids contain *Rhizobium leguminosarum* biovar *trifolii* nodulation genes **(Ibrahim *et al*., 1998)**, *Rhizobium meliloti* transconjugants were tested for their ability to nodulate Egyptian clover (Table 3 and Fig. 1).

Nodulation experiments showed that all obtained *Rhizobium meliloti* transconjugants had acquired the ability to nodulate Egyptian clover by different efficiencies, ranging from 15 to 39 nodules/ plant. The best *Rhizobium meliloti* transconjugant was Rm A-2 (CB) which produced 39 big nodule/ plant.

In a previous work, CB and CE plasmids gained *Agrobacterium* the ability to nodulate Egyptian clover. *Agrobacterium tumefacience* (CB) produced 13-nodules/ plant while *Agrobacterium tumefacience* (CE) could produce 10 nodules per plant **(Abdel-Salam *et al.*, 2002).** In the present study both plasmids gained *Rhizobium meliloti* more nodulation efficiencies than that of *Agrobacterium*. This result could be due to the effect of the genetic background of both bacterial strains where *Rhizobium meliloti* had different addition nodulation genes. It could be due to the replication relaxation of CB and CE plasmids in their related new host, i.e., *Rhizobium meliloti,* than that in *Agrobacterium*.

Symbiotic efficiency

The symbiotic effects of Rhizobia transconjugants on clover were studied by measurement of different plant parameters. These parameters included plant height, weight, root size and root branches. Results are present in Tables 4.

Table 4 illustrated different symbiotic efficiencies among *Rhizobium leguminosarum* biovar *trifolii* transconjugants. Most transconjugants improve the plant growth parameters. Only Rt 10 (CB) had less symbiotic efficiency with Egyptian clover than its parental strain Rt 10. This latter finding may be due to changes in CB plasmid structure occurred during conjugation, e.g., some of its nodulation genes were turned off.

All *Rhizobium meliloti* transconjugants had much better symbiotic efficiencies with Egyptian clover than their parental strains which could not nodulate Egyptian clover. Plant height was increased from 4 to 25 cm. The best *Rhizobium meliloti* transconjugant was Rm A-2 (CB) which increased plant height more than 25 cm and increased plant weight more than 14 times.

Plasmid visualization

Plasmid DNA was isolated from some transconjugants including *A. tumefacience* harboring CE and CB plasmids (Fig 2). Figure 2 presented the plasmid patterns of the transconjugants Rt 10 (CB) and Rm A-2 (CB) compared with *A. tumefacience* CB and CE strains.

The two plasmids showed in *A. tumefacience* AGCE and AGCB are representing CB or CE plasmids side by side with the helper plasmid RP4-4. While the parental strain Rt 10 did not showed any plasmid, the other Rhizobia transconjugants were containing the CB *nod* plasmid. The results illustrated the co-transfer of RP4-4 plasmid in each of the Rhizobial strains. The latter finding gained each *Rhizobium* transconjugant the ability to transfer CB plasmid to other gram-negative bacterial strains.

In conclusion, the results confirmed the transfer and stably maintenance of *nod* plasmids in *R. leg.* bv. *trifolii* and *Rhizobium meliloti*. Amplification of *nod* genes resulted in increasing the nodulation efficiencies of *R. leguminosarum* bv. *trifolii* transconjugants and in their symbiotic performance with clover plant. The *nod* plasmids also gained *Rhizobium meliloti* the ability to nodulate clover.

Table 2. Transconjugants obtained, and their parental strains antibiotic resistance patterns

Strain	Antibiotic resistance					Transconjugant with	
	Rif&Nm	Amp	Cm	Tc	Sm	CB	CE
AGCB	+	+	-	-	+	00	00
AGCE	+	+	-	-	+	00	00
Rm A-2	-	+	+	-	-	√	√
Rm 1	-	+	+	-	-	00	00
Rm 2	-	+	-	-	-	√	00
Rm 3	-	+	+	+	-	√	√
Rm 4	-	+	+	-	+	√	00
Rm 5	-	+	+	-	+	00	00
Rt 1	-	-	+	-	+	√	00
Rt 10	-	-	+	-	-	√	√
Rt Nit	-	+	+	-	+	00	00
Rt M1	-	+	+	-	+	√	√
Rt M2	-	+	+	+	+	√	√
Rt H1	-	-	+	-	-	√	00
Rt 14	-	+	+	-	+	√	√

√= Transconjugants obtained. + = Antibiotic resistant. - = Antibiotic sensitive.

Table 3. Nodulation efficiency of parental and transconjugants Rhizobia with Egyptian clover

Rhizobium leguminosarum biovar trifolii			
Bacterial strain	Mean no. of nodules/ plant	Bacterial strain	Mean no. of nodules/ plant
Rt 1	15	Rt H1	14
Rt1 (CB)	20	Rt H1(CB)	19
Rt 10	14	Rt M1	17
Rt 10 (CB)	12	Rt M1 (CB)	21
Rt 10 (CE)	16	Rt M1 (CE)	18
Rt 14	21	Rt M2	15
Rt 14 (CB)	26	Rt M2 (CB)	20
Rt 14 (CB)	25	Rt M2 (CE)	24
Rhizobium meliloti			
Rm 2	00	Rm 4	00
Rm 2(CB)	22	Rm 4(CB)	28
Rm3	00	Rm A-2	00
Rm 3 (CB)	15	RmA-2 (CB)	39
Rm 3 (CE)	17	RmA-2 (CE)	29

Table 4. Symbiotic efficiency of *Rhizobium leguminosarum biovar trifolii* transconjugants with Egyptian clover

Rhizobium leguminosarum biovar trifolii									
Bacterial strain	Plant height (Cm)	Plant weight (g)	Root Size*	Root branches*	Bacterial strain	Plant height (Cm)	Plant weight (g)	Root Size*	Root branches*
Rt1	16	0.353	+	+	RH1	26	0.79	++	+++
Rt1(CB)	18	0.371	+	+	RtH1(CB)	31	0.96	+++	+++
Rt10	21	0.85	++	++	Rt M1	26	0.83	++	+++
Rt10(CB)	17	0.45	+	++	RtM1(CB)	28	1.09	++	+++
Rt10(CE)	26	0.911	+++	+++	RtM1(CE)	22	0.98	+++	+++
Rt14	27	0.96	+++	+++	RtM2	21	0.92	++	++
Rt14(CB)	29	1.13	+++	+++	RtM2(CB)	26	1.072	+++	+++
Rt14(CE)	34	1.24	+++	+++	RtM2(CE)	33	1.23	+++	+++
Rhizobium meliloti									
Rm2	16	0.242	+	+	Rm 4	16	0.133	+	+
Rm2(CB)	26	0.452	++	++	Rm4(CB)	20	0.945	++	++
Rm3	16	0.135	+	+	RmA-2	17	0.123	+	+
Rm3(CB)	20	0.354	++	++	RmA-2 (CB)	44	1.79	+++	+++
Rm3(CE)	24	0.76	++	++	RmA-2 (CE)	44	1.65	+++	++

* Number of + represent root size or root branches

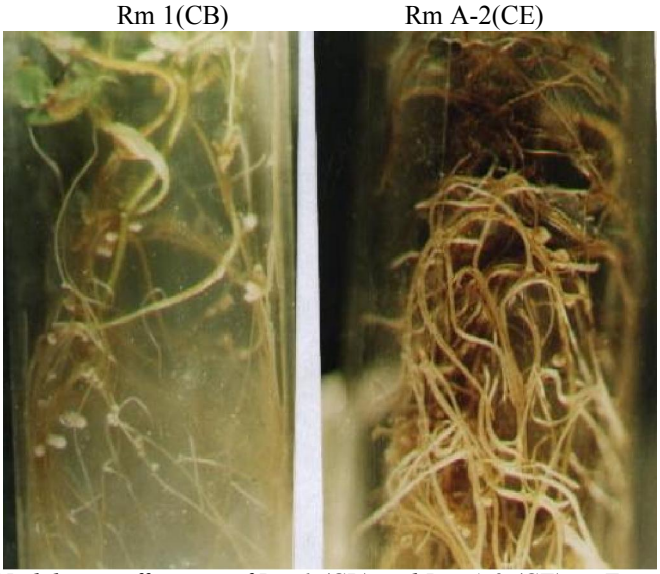

Figure 1: *Nodulation efficiency of R m1 (CB) and Rm A-2 (CE) on Egyptian clover.*

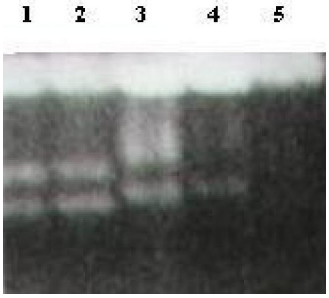

Figure 2: *Plasmid content of Rt 10 (CB) and Rm A-2 (CB) transconjugants.*
Lane 1, Rt 10 (CB); lane 2, Rm A-2 (CB); lane 3, AGCB; lane 4, AGCE; lane 5, Rt 10.

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