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GENETIC CONSTRUCTION OF BROAD HOST RANGE *Bradyrhizobium japonicum*

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ABSTRACT

Protoplast fusion and conjugation were used to expand the Rhizobium host range. *B.japonicum AS25* and *R.leguminosarum biovar vicia ICARDA441* were fused and six, genetically stable, fusants were isolated. No fusants were obtained when *B.japonicum BR20* and *Agrobacterium tumefaciens* CB were fused. Conjugation technique was done between *Agrobacterium tumefaciens* CB and twenty-five *B.japonicum* strains, which isolated from different Egyptian governorates. Fourteen conjugants were selected according to their variant antibiotic resistance (Genetic marker). The six fusants were able to nodulate both soybean and *Vicia faba*, while the fourteen conjugants were able to nodulate both clover and soybean. Plasmid profiles and phyllogenetic analysis of the parents; *B.japonicum AS25* and *R.leguminosarum biovar vicia ICARDA441,* and their six fusants were done. All fusants and conjugants were characterized and their relatedness were determined according to plasmid profiles.

Keywords: Genetic construction, host range, *Bradyrhizobium japonicum,* protoplast fusion, conjugation.

INTRODUCTION

Leguminous plants can obtain most of the nitrogen they need from the vast supply of gaseous nitrogen in the air symbiotically by interacting with special bacteria, *e.g.*. *Rhizobium* or *Bradyrhizobium* species that resides in nodules on the legume roots. The N_2- fixing partnership between these bacteria and legumes is very important to agricultural and sylvicultural practices. Legumes also leave some of the fixed nitrogen in the soil. They are by far is one of the largest sources of organic nitrogen in the global nitrogen cycle (Dreyfus *et al* ., 1988).

Bradyrhizobium japonicum has an economically important problem in Egypt. It has no persistence in the soil and / or lost its nodulation capability. Therefore, the traditional solution is to re-inoculate the soil with *Bradyrhizobium japonicum* every year. The most promising solution to come over this problem is the construction of new superior strains that can be associated in nitrogen fixing symbiotic relationship with more than one plant species. This broad-host range rhizobia may be more effective in competition and maintenance in soil.

The aim of this investigation is to construct new *Bradyrhizobium* strains with broad host range as they can nodulate *Vicia faba,* soybean and Egyptian clover. Intra-protoplast fusion and conjugation techniques were applied through this investigation.

MATERIALS AND METHODS

Materials

Strains

Twenty- five *Bradyrhizobium japonicum* Egyptian strains (Ibrahim *et al.*, 2001) and two parental strains ; *Rhizobium leguminosarum biovar vicia* ICARDA 441 (ICARDA 441) and *Agrobacterium tumefaciens CB;* rifampicin and neomycin resistant (Abo-aba.,1996) were used through this investigation. Characters of these strains are presented in table (1).

Table (1): Characterization of the studied bacterial strains

strains*	O.D.420 nm		Antibiotic resistance**					Heavy metal resistance
	At 30% PEG 6000	At 950 mM Na Cl	Cm	Sm	Ap	Tc	Rif	
ICARDA 441	0.121	0.088	+	-	+	-	-	-
B.japonicumDO4	0.154	0.071	+	-	+	-	+	Fe^+
B.japonicum SE8	0.237	0.023	-	+	+	+	-	Fe^+, Ni^+
B.japonicum SE9	0.319	0.016	+	+	+	+	-	Fe^+, Zn^+, Co^+
B.japonicumSE10	0.122	0.036	+	+	-	+	-	Fe^+, Zn^+, Co^+, Ni^+
B.japonicumQA11	0.199	0.762	-	+	-	+	-	Fe^+, Co^+, Ni^+
B.japonicum QA12	0.210	0.232	-	+	+	-	-	Fe^+, Co^+
B.japonicum QA13	0.203	0.231	-	+	-	+	-	Fe^+, Co^+,Ni^+
B.japonicum QA14	0.183	0.076	-	+	-	+	-	Fe^+, Co^+, Ni^+
B.japonicum QA15	0.203	0.116	-	+	-	+	-	Fe^+, Co^+
B.japonicum BR16	0.095	0.0632	+	-	+	-	-	Fe^+, Co^+
B.japonicum BR17	0.027	0.762	+	-	-	-	-	Fe^+,
B.japonicum BR18	0.060	0.659	+	-	+	-	-	Fe^+, Co^+, Ni^+
B.japonicum BR19	0.123	0.780	+	-	+	-	-	Fe^+, Zn^+, Ni^+
B.japonicum BR20	0.398	0.714	-	+	-	+	+	Fe^+, Zn^+, Co^+
B.japonicum BR21	0.087	0.697	+	-	+	-	-	Fe^+, Co^+, Ni^+
B.japonicum BR22	0.148	0.013	+	-	+	-	-	Fe^+, Ni^+
B.japonicum BR23	0.150	0.432	+	-	+	-	+	Fe^+, Ni^+
B.japonicum BR24	0.058	0.401	+	-	+	-	-	Fe^+, Ni^+
B.japonicum AS25	0.303	0.970	+	+	+	-	+	Fe^+, Co^+, Zn^+
B.japonicum QH26	0.171	0.088	+	-	+	-	-	Fe^+, Zn^+,Co^+, Ni^+
B.japonicum QH27	0.216	0.150	+	-	+	-	-	Fe^+, Zn^+,Co^+, Ni^+
B.japonicum QH28	0.217	0.464	+	-	+	-	-	Fe^+, Zn^+,Co^+, Ni^+
B.japonicum QH29	0.226	0.531	+	-	+	-	-	Fe^+, Zn^+, Ni^+
B.japonicum QH30	0.203	0.520	+	-	+	-	-	Fe^+, Zn^+,Co^+, Ni^+
B.japonicum AS31	0.298	0.988	+	+	+	-	-	Fe^+, Zn^+,Co^+, Ni^+

*** On YEMA medium**

**** Chloramphenicol (Cm), Streptomycin (Sm), Ampicillin (Ap), Tetracycline (TC), Rifampicin (Rif).**

plants

Soybean, *Vicia faba* and Egyptian clover plants were used in the nodulation experiments.

Media

1. Yeast extract-mannitol Agar medium (YEMA) was used as a complete medium of Rhizobia (Mohammad *et al*., 1991).
2- Minimal medium (MM) of Rhizobia was used as a selective medium during conjugation experiments (Plassa, 1963).
3- Jensen's medium was used as a nutrient medium for soybean in nodulation test experiments (Michiels *et al.,* 1993).
4- Nutrient solution was used for plant nutrition throughout the nodulation test (Norris and Date, 1976).

Buffers and reagents

1- TBE buffer was prepared according to Maniatis *et al.*(1982).
2- N-Lauroylsarkosine (0.3% in 1x TBE buffer).
3- Agarose-gel was prepared as 0.75% in TBE buffer.
4- Protoplast buffer was used according to EL-Gaali *et al*. (1995).

Methods

1-*Rhizobium* isolates were conjugated with AgCB according to Abo-Aba (1996).
2- Protoplast fusion**:** Protoplast fusion of rhizobia strains was carried out according to El-Gaali *et al*. (1995).
3- Protocol of Del Papa *et al*. (1999) for plasmid isolation was used.
4- Nodulation efficiency of the fusants was determined according to (Hynes *et al.,* 1988).
5- Plasmid DNA content and plasmid profile were carried out according to Hashem and kuykendall (1994).

RESULTS AND DISCUSSION

Attempts to induce Rhizobium strains with wider plant host range were done using protoplast fusion technique between *B. japonicum* AS25, *Sym* with G*lycin-max,R .leguminosarum bv.vicia* ICARDA441, *Sym* with *Vicia faba,* and conjugation techniques between *Ag. tumeficiencs CB* with twenty-five *Bradyrhizobium* strains.

Protoplast fusion between *B. japonicum* AS25 and *R. leguminosarum bv. vicia* ICARDA441

Intra-generic and intra-specific protoplast fusions are versatile general techniques to induce genetic recombination in a variety of prokaryotic microorganisms and hence strain improvement. It's a powerful tool for the location of markers and identification of linkage groups (El-Gaali *et al*., 1995).

The chosen two parents; *B.japonicum* AS25 and *R. leguminosarum bv. vicia* ICARDA441, have distinctive different characters which are beneficial in fusant selection; *e.g., B. japonicum* AS25 is tolerated salinity, drought and resisted iron and zinc in comparison with the other parent. On the other hand,

B.japonicum AS25 is resistant to streptomycin and rifampicin (400μg and 150μg /ml, respectively) which are lethal for *R. leguminosarum bv.vicia* while the two parents are sensitive to tetracycline, 10μg/ml, which causes lethality (Table 2).

The two parental protoplasts were mixed in the presence of 25% PEG 6000 (Foder and Alfoldi, 1976). The selective medium was YEMA supplemented with rifampicin and heavy metals; ferric Chloride and zinc sulfate, to eliminate *R. leguminosarum biovar vicia* and tetracycline to eliminate both parents (Table 2). The fusant is a new strain which may has all the characters of one or the two parents, or only has some of its parents characters (Spencer *et al*., 1988).

Six fusants were successfully isolated, their names and characterization are presented in table (2). To study the nature of the constructed fusants, the resistance of salt, drought and heavy metals were estimated. All obtained fusants were more salt tolerance, at 5.5% NaCl, than *R. leguminosarum biovar vicia* parent, which grown to OD 0.127. On the other hand, one fusant; *B.j*:: *R. l. v*- 25-441-2, which grown to OD 0.905, was nearly equal in salt tolerance to *B. japonicum* AS25 which grown to OD 0.975 (Table 2). These results are in agreement with those of Necasek *et al.* (1993) who found variations in drought and salt tolerance of the fusion products between *R. leguminosarum* and *R. meleloti.*

All fusants were more tolerance for drought than *R. leguminosarum biovar vicia* parent which grown to OD 0.209, except one fusant; *B. j* :: *R. l. v*- 25-441-1grown to OD 0.061, while three fusants; *B. j* :: *R. l. v*- 25-441-2, *B. j* :: *R. l. v*- 25-441-4 and *B. j* :: *R. l. v*- 25-441-6, showed more drought tolerance (grown to OD 0.434,0.472 and 0.352, respectively) than the other parent *B. j* AS25 and the rest three fusants; *B. j*::*R. l. v*25-441-1-,*B. j* :: *R. l. v*- 25-441-3 **,** *B. j* :: *R. l. v*-25-441-5, showed lower drought tolerance (grown to OD 0.061, 0.233 and 0.190, respectively) than the same parent.

Data in (Table 2) also revealed that all fusants acquired heavy metal resistance of the parent *B. j.* AS25.

Protoplast fusion between *B. j.* BR20 and *Agrobacterium tumefaciens CB* was attempted and no fusants were obtained. This negative result may be due to incompatibility due to internal environmental variations (Stackebrandt *et al*., 1988 and Kinkle *et al*., 1993).

Table 2

Conjugation

Conjugation is a major mode of gene transfer among prokaryotes, e.g, bacteria and as a tool of gene transfer in different environments (Van Elsas *et al.*, 1990).

Agrobacterium have been used as a recipient of *Rhizobium* plasmid because the genetic background of both strains share properties not found in all bacteria (Yelton *et al.*, 1983).

In order to obtain different conjugative strains, *Agrobacterium tumefaciens* CB*;* Nm^r,Rif^r ,Nod^+ and $Bact^+$ (Abo-aba 1996) was used in mating as a donor strain with the chosen twenty-five variants, *Bradyrhizobia* strains, which have multiple antibiotic resistant markers, as recipients.

Different obtained successful conjugated strains were selected onto minimal medium supplemented with rifampicin and neomycin to eliminate *Agrobacterium*, and selective antibiotics for each *Bradyrhizobium* parent.

Fourteen conjugants were successfully acquired the *Agrobacterium* plasmid out of twenty-five trials. Each of these selected conjugants proved to form nodules with clover and Soybean. These results are in harmony with those obtained by Mergeay *et al*. (1994). They showed that broad host range plasmids to be considered as powerful agents of heterospecific gene transfer. It is reasonable to consider that conjugational processes are generally relevant in gene release and dissemination of these elements i.e. plasmids and transposons.

Nodulation capability of the fusion products and conjugants.

The results in table (2) indicated that all the fusants are genetically stable and wider host range. The ability of the fusants and conjugants to nodulate the plants were tested. The six fusants were able to nodulate both soybean, and *vicia faba* (Fig.1) ,while the fourteen conjugants were able to nodulate both soybean and clover (Fig.2).these resutls indicating the accumulation and stability of *nod* genes for all plants in the fusants and conjugants. These results are in agreement with El-Gaali *et al*. (1995). They studied the intra protoplast fusion between *B. japonicum* and *R. fredii* and found that, after several repeated subcultures, the fusion products were capable of forming nodules against the host plants.

Plasmid profiles of the intra- generic protoplast fusion

The plasmid analysis of the intra-generic protoplast fusion between *B. j* AS25 with *R. l. bv. vicia* ICARDA441 and their fusants with the reference strain; *R. fredii* USDA201, which has three plasmids with molecular weights; 1000, 210 and 120 MDa were done (Fig 3).

Gel documentation system, image analysis Gel works 1 D advanced software, was used for more accurate analysis and comparison between the parents and their fusants obtained from intra-generic protoplast fusion pre-mentioned (Fouly and Wilkinson, 1999 and Ibrahim *et al*., 2001).

Fig (4) shows results of Gel-documentation for parents used in protoplast fusion depending on plasmids number and migration in Agarose-gel electrophoresis (Maniatis *et al*., 1982).

fig

fig

The analysis for the plasmid profiles of the parents; *R. l. bv. vicia* ICARDA441 and *B. j.* AS25 and their six obtained fusants, resulted in the tree dendrogram which shown in fig.(5). Two separate clusters were evident, one is consisting of F-1, F-3, F-4, F-6, F-2 and the other is consisting of F-5, *R. l. bv. vicia* ICARDA441 and *B. j.* AS25. F-5 is more related to the parent *B. j.* AS25 than to the other parent.

In conclusion, intra-generic genetically stable fusants retained nodulation efficiency were obtained *via* protoplast fusion studies and proved to be more efficient in tolerance for severe conditions; salinity and drought, and resistant to some heavy metals more spreaded in Egyptian soils. The obtained fusants were selected *via* genetic marker, antibiotic resistance, and characterized for their salt and drought tolerance, heavy metals resistance and compared on plasmid profile level. The genetic relatedness of fusants and their parents were detected by dendrogram analysis based on their plasmid content.

In addition, conjugation between *Agrobacterium CB* and the twenty-five variant strains of *Bradyrhizobia* were successfully occurred in fourteen trails and each of the selected fourteen conjugants proved to have a wider host plant range in *Sym* relationship.

It is recommended to use these new improved strains as biofertilizers, for their higher competence, in the native soils of their parents in addition to the expected success for their application in new reclaimed regions.

REFERENCES

Abo-Aba, S. (1996). Genetical studies on some *Rhizobium leguminosarum biov. trifolii* genes which influence nitrogen –fixation efficiency. M.Sc. thesis Fac. of Agric. Ain Shams Univ.

Del Papa , M. A. F; J. L. Balague ; S. C. Sowinski; C.Wegener; E. Segundo; M .Francisco ; K. Niehaus; P. Alfred; O. M.Aguilar; M. Gloria and I. Antonio (1999). Isolation and characterization of Alfalfa-nodulating Rhizobia presents in acidic soils of central Argentina and Uryguay.Appl. Eniviron. Microbiol.,.65: 1420 –1427.

Dreyfus, B.; J. Ký; L. Garcia and M. Gillis (1988). Characterization of *Azorhizobium caulinodans* gen. nov., a stem nodulating nitrogen - fixing bacterium isolated from *Sesbania rostrata* . Int. J. Syst. Bact., 38 : 89 - 98 .

El-Gaali Eisa; M.Kazuhiko and N. Mori (1995). Enhanced nitrogen fixation capability of Soybean Rhizobia by inter–and intra–specific cell fusion. Japanese J. of Crop Sci., 64: 273-280.

Foder , K. and L. Alfoldi (1976). Fusion of protoplasts of *Bacillus megaterium*. Proceedings National Academy of Science of the USA., 73 : 2147-2150.

Fouly, M. H. and T.W. Wilkinson (1999). Use of marker as a diagnostic tool in the identification of *Gaeumannomyces graminis* isolates that cause patch diseases of grasses. Arab. J. Biotech.,2:143-160.

Hashem, F.M and L. D. Kuykendall (1994). Plasmid DNA content of several agronomically important *Rhizobium* species that nodulate alfalfa, berseem clover, and *Leucaena*. In Symbiotic Nitrogen Fixation. Eds. P.H. Graham, M.J. Sadowsky and C P Vance.

Hynes, M. F.; K. Brucksch and U. Priefer (1988). Melanin production encoded by a cryptic plasmid in a *Rhizobium leguminosarum* strain. Arch. Microbiol., 150 : 326 - 332.

Ibrahim, S. A.; M.M.Abd El-Halim; M.S. Abd El-Salam; S.Kh.Abd -El-Aal and A.G. Attallah (2001). Differentiation for Egyptian *Bradyrhizobia* isolates and plasmid patterns. Arab Univ. J. Agric. Sci., 9 (in press).

Kinkle-B-k; M.J. Sadowsky; E.L. Schmidt and W.C. Koskinen (1993). Plasmids pJP4 and r68.45 can be transferred between populations of Bradyrhizobia in nonsterile soil. Appl. And Environ. Microbiol., 59 : 1762-1766.

Maniatis, T. E. ; F. Fritsch and J. Sambrock (1982). Molecular Cloning ,A Laboratory Manual , Cold Spring Harbor , New York.

Mergeay, M.; H. De Rore; E. Top; F. Springael; M. Hofter ; Van de Lelie; R. Dijkmans and W. Verstratet (1994). Plasmid biology and risk assessment: elements for modelling approach of plasmid-mediated gene release in soil environments. (c.f. Bazin and Lynch, 1994).

Michiels, J. ; P. De Wilde and J. Vanderleyden (1993). Sequence of the *Rhizobium leguminosarum* biovar *phaseoli syr* M gene. Nucleic Acids Res. , 21 (16) : 3893.

Mohammad, R. M.; M. Akhavan-K.; W. F. Campbell and M. D. Rumbaugh (1991). Identification of salt- and drought- tolerant *Rhizobium meliloti* L. strains. Plant and Soil, 134:271-276.

Necasek, J.; M. Nemcova; I.. Lisa ; J. Dusbabkova; V. Nasiec and D. Psarkova (1993). Drought tolerant of *Rhizobium leguminosarm* and *Rhizobium meliloti*. FOLIA Microbiologica, 38: 320-324.

Norris, D. O. and R. A. Date (1976). Legume Bacteriology, Commonwealth Bureau of pastures and Field Crop Bulletin No. 51 p. 134, Commonwealth Agricultural Bureau , England.

Plassa, G. (1963). Genetic transformation in *Rhizobium*. Bacterial Rev., 27 : 228 - 241.

Spencer,J. F.T; D. M. Spencer and R. Nicola (1988). Genetic manipulation of non- conventional yeast by conventional and non- conventional methods. J. Basic Microbiol., 28: 321-333.

Stackebrandt, E.; E. R. G. E. Murray and G.H. Truber (1988). Proteobacteria, classes nov. a name for the phylogenetic taxonomy including the purple bacteria and their relatives. International J. of Systematic bacteriol., 38 : 321 –325.

Van Elsas, J. D; M. Nikkel and S. L. Van Overbeek (1990). Transfer of plasmid RP4 between *Pseudomonas* after introduction into soil; influence of spatial and temporal aspects of inoculation. FEMS Microbiol ,. Eco ., 73 :1-12.

Yelton, M. M.; S. S. Yang; S. Edie and S.T. Lim (1983). Characterization of an effective salt tolerant fast- growing strain of *Rhizobium japonicum*. J. Gen. Microbiol., 129: 1537-1548.

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البناء الوراثى لبكتريا البرادى ريزوبيم ذات مدىعوائلى واسع .

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استخدم كل من الدمج البروتوبلاستى والتزاوج لتوسيع المدى العوائلى لبكتيريا البرادىريزوبيم. تم الدمج البروتوبلاستى بين سلالة البرادى ريزوبيم المصرية أسيوط25 وبين السلالة المستوردة ريزوبيم لجيومينوزارم أى سي إيه أر دى إيه 441 ونتج عن ذلك ستة مندمجات ثابتة وراثيا تم عزلها فى حين لم ينتج أى مندمجات بين السلالة البرادى ريزوبيم البرادعة 20 المصرية وبين السلالة المستوردة أجروبكتيريم تيوميفاشنس سي بي . كماتم عمل تزاوج بين السلالة اجروبكتيريم تيوميفاشنس سي بي و 25 سلالة مصرية من البرادىريزوبيوم سبق عزلها من عدة محافظات .كما تم انتخاب عدد 14 متزاوجة على أساس مقاومتهم للمضادات الحيوية . أظهرت المندمجات الستة مقدرة على تكوين العقد الجذرية مع كلا من الفول البلدى وفول الصويا بينما أظهرت الأربعة عشر متزاوجة مقدرة على تكوين العقد الجذرية مع كل من نبات البرسيم وفول الصويا . تم توصيف جميع المندمجات والمتزاوجات التى أمكن الحصول عليها وكذلك حددت القرابة بينها بمقارنة نماذجها البلازميدية بطريقة تحليل التفريع الشجرى. ويوصى باستخدام هذه السلالات الجديدة فى التسميد الحيوى لما لها من مقدرة تنافسية عالية فى أماكنها الأصلية التى تم العزل منها. اضافة لما هو متوقع من نجاح إستخدامها فى المناطق حديثة الاستزراع.

Table (2): Characteristics of *B.japonicum.*AS25 and *R.leguminosarum biovar vicia* ICARDA 441 and their fusants.

Strains	O.D.$_{420\ nm}$ At 30% PEG6000	Growth at 5.5% NaCl		Antibiotic resistance			Fe,Zin Resistance	Nodulation efficiency	
		YEMA*	At O.D. $_{420\ nm}$	Rif	Sm	Tc		*Glycin max*	*Vicia faba*
B.japonicum *AS 25*	0.306	+	0.975	+	+	-	+	+	-
R.leguminosarum biovar vicia ICARDA 441	0.209	-	0.127	-	-	-	-	-	+
*B.J::R.l.v.*25-441-1**	0.061	+	0.251	+	+	-	+	+	+
*B.J::R.l.v.*25-441-2	0.434	+	0.905	+	+	-	+	+	+
*B.J::R.l.v.*25-441-3	0.233	+	0.213	+	+	-	+	+	+
*B.J::R.l.v.*25-441-4	0.472	+	0.542	+	+	-	+	+	+
*B.J::R.l.v.*25-441-5	0.190	+	0.627	+	+	-	+	+	+
*B.J::R.l.v.*25-441-6	0.352	+	0.793	+	+	-	+	+	+

*** Supplemented with 2.05% NaCl**

**** Fusants between *B. japonicum AS 25* and *R. leguminosarum bv. vicia ICARDA 4***