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GENETIC IMPROVEMENT OF *B. japonicum* TO TOLERATE ABIOTIC STRESSES VIA PROTOPLAST FUSION

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ABSTRACT

Two distinguished *Bradyrhizobium japonicum* Egyptian strains, i.e. SE8 and DO4, each was fused with the fast growing *Rhizobium fredii* USDA201 strain. Nine fusants were obtained in the first attempt (USDA 201 and SE8) and four fusants were obtained in the second attempt (USDA201 and DO4). All fusants were characterized on the bases of nodulation efficiency, salt and drought tolerance, heavy metals resistance and antibiotic resistance. Electrophoretic plasmid profiles of these fusants and their parents were studied and the relatedness between them was determined according to their phylogeneric tree. Some fusants retained their nodulation efficiency and showed higher efficiencies in salt, drought and /or heavy metals resistance than their parents.

Keywords: *Bradyrhizobium japonicum , Rhizobium fredii,* Genetic improvement , Intergeneric, Protoplast fusion , Phylogenetic analysis .

INTRODUCTION

Soybean (*Glycin max*) is one of the most important leguminous crops in the world. Its importance is mainly due to the high free cholesterol oil (about 20%) and protein (about 40%) of seeds contents in addition to carbohydrates. Soybean seed-protein is characterized with high content of essential amino acids compared to many other crops. Soybean is considered one of the relatively new crops introduced into Egyptian Agriculture (Abo El-Kheir, 1990)

Rhizobium or *Bradyrhizobium* provide the leguminous plants, *e.g*., soybean, by gaseous nitrogen in the air via symbiotic interaction between the nitrogen fixation bacteria and the host plants. This symbiosis has the potentiality to free the soybean from dependence on nitrogenous fertilizers, as well as to increase soil fertility.

Soybean plants also leave some of the fixed nitrogen in the soil. Therefore, enhancing the biological nitrogen fixation (BNF) by soybean / *Bradyrhizobium* symbiosis is required through investigating the complex factors involved in this relation (Martinez-Romero 1994).

For improvement of *Bradyrhizobium japonicum* biofertilization efficiency, gene-transfer technique such as protoplast fusion which is an alternative option to improve its response to the environment. Protoplast fusion is the most effective technique since it facilitates the genetic material exchange, more than other mating methods, because the genetic exchange between the intact genomes of two couplet parents in every fused protoplast are possible (Hopwood, 1981).

Most of the genes required for effective symbiotic (*pSym*) are located on large plasmids in the fast growing *Rhizobium* species, whereas, the slow-growing *Bradyrhizobium* species carry these genes on the chromosome (Gussin *et al.,* 1986).

In 1985, Mark *et al*., found that *Rhizobium fredii*, fast-growing rhizobium; harbors nif-genes on 150 to 200 MDa plasmids.

This work aimed to improve the biofertilization system via genetic construction, using protoplast fusion technique, of new *Brodyrhizobium* strains of higher competitive efficiency and more tolerant to severe conditions.

MATERIALS AND METHODS

MATERIALS

Strains

Three Rhizobia strains were used through this investigation, *i.e., Rhizobium fredii USDA201* (Agriculture Research Center-ARC)*, Bradyrhizobium japonicum SE8 and Bradyrhizobium japonicum* DO4 (Ibrahim *et al.,* 2001).

Characters of these parental Rhizobia strains are presented in Table (1).

Table (1): Characterization of parental Rhizobia strains used in protoplast fusion.

Strains	O.D.$_{420\ nm}$ at 30% PEG6000	Max. NaCl conc. For Growth*	Antibiotic resistance	Heavy metal resistance	No. of nodules
R. fredii USDA201	0.049	2.05%	Sm^r,Rif^r,Ap^r,Tc^s,Cm^s	-	17
B. japonicum SE8	0.237	4.6%	Sm^r,Rif^s,Ap^r,Tc^r,Cm^s	Fe^{++},Ni^{+}	5
B. japonicum D0 4	0.154	2.05%	Sm^s,Rif^r,Ap^r,Tc^s,Cm^r	Fe^{++}	11

*** On YEMA medium**

Media

1. Yeast extract-mannitol Agar medium (YEMA) was used as a complete medium of Rhizobia (Mohammad *et al*., 1991).
2- Jensen's medium was used as a nutrient medium for soybean in nodulation test experiments (Michiels *et al.,* 1993).

Buffers and reagents

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

METHODS

1. Protoplast fusion**:** Protoplast fusion of rhizobia strains was carried out according to El-Gaali *et al*. (1995).
2. Protocol of Del Papa *et al*. (1999) for plasmid isolation was used.
3. Nodulation efficiency of the fusants were determined according to (Hynes *et al.,* 1988).

RESULTS AND DISCUSSION

Protoplast fusion

Protoplast fusion technique was hybrids of two or more species, which could not be obtained by classical mating . Polyethyelene glycol (PEG) was an effective and powerful fusogenic agent. Intergeneric or interspecific fusion provide a method for the introduction and transfer of desirable genes, *e.g*., the lactose permease and β- galactosidase genes (Borum and Jinke, 1990).

Strains *B.japonicum* SE8 and *B.japonicum DO4,* which isolated and characterized as distinctive strains by Ibrahim *et al.(*2001) were chosed in addition to strain *R.fredii USDA201* for genetic improvement attempt via protoplast fusion. The primary step was the estimation of parents strains capability in protoplast induction by enzymatic removal of the cell wall (El-Gaali *et al*., 1995). The results are presented in Table (2).

Table (2): Regeneration efficiency of induced protoplast.

Strains	No. / ml			
	Intact cells	Regenerated and intact cells	Protoplasts	Generated protoplast induction %
R.fredii USDA 201	250	900	650	72.22
B.japonicum DO 4	350	650	300	46.15
B.japonicum SE 8	340	730	390	53.42

Protoplast induction and regeneration

Table (1) presents Rhizobia strains used and there identifying characters. Strain *R.fredii USDA201* was chosen as a common parent with strains *B.japonicum* SE8 and DO4 essentially because it is a fast-growing bacterium which carry their genes required for effective symbiotic (*pSym*) on large plasmids, whereas the other two parents are belongs to slow-growing bacteria carrying these genes on the bacterial chromosome (Gussin *et al.*, 1986). This chosen design may increase the probability of increasing the gene dosage in fusants.

The highest protoplast induction reached 72.22% for the parent *R. fredii USDA201,* while it was 53.42% for *B. j*aponicum SE8 and 46.15% for *B. j*aponicum DO4. This result presented in Table (2) is considered as a positive factor in addition to the previously mentioned reasons for choosing strain USDA201 as a parent in common to increase the probability of fusants induction.

1. Protoplast fusion between *R. fredii* USDA 201 and *B. japonicum* SE8

The chosen two parents; *R. fredii* USDA 201 and *B. japonicum* SE8, have distinctive different characters illustrated in Table (1).

In order to select fusants between parents the protoplast suspension was mixed in presence of PEG 25% and 100 mM $CaCl_2$. Sample; 100 µl, was

added to YMEA medium supplemented with Tc, Rif, Fe and Ni. Cells that resisted both antibiotics were selected as fused cells.

All obtained genetically stable nine fusants were more salt tolerant on 0.46 % NaCl than the parent *R. fredii* USDA 201 which could grow to 0.007 at OD 420 nm. Three fusants out of them ; *R.f*:: *B.j*. 201-8-2, *R.f*:: *B.j*. 201-8-7 and *R.f*:: *B.j* 201-8-9, showed more tolerance reached 0.034, 0.052 and 0.038 at OD 420 nm, respectively than the other parent , *B.japonicum* SE8, which reached 0.031 at OD 420 nm (Table 3).

Table (3): Characteristics of *R. fredii.* USDA 201 and *B. japonicum* SE8 and its fusants.

Strains	O.D.$_{420\ nm}$ At 30% PEG6000	Growth at 4.6% NaCl		Antibiotic resistance				Fe,Ni resistance	Nodulation efficiency
		YEMA*	At OD $_{420\ nm}$	Sm	Rif	Ap	Tc		
R. fredii.USDA201	0.054	-	0.007	+	+	+	-	-	+
B. japonicum. SE8	0.233	+	0.031	+	-	+	+	+	+
*R.f::B.j.*201-8-1**	0.046	+	0.025	+	+	+	+	+	+
*R.f::B.j.*201-8-2	0.057	+	0.034	+	+	+	+	+	+
*R.f::B.j.*201-8-3	0.172	+	0.012	+	+	+	+	+	+
*R.f::B.j.*201-8-5	0.128	+	0.016	+	+	+	+	+	+
*R.f::B.j.*201-8-6	0.010	+	0.013	+	+	+	+	+	+
*R.f::B.j.*201-8-7	0.082	+	0.052	+	+	+	+	+	+
*R.f::B.j.*201-8-8	0.068	+	0.029	+	+	+	+	+	+
*R.f::B.j.*201-8-9	0.235	+	0.038	+	+	+	+	+	+
*R.f::B.j.*201-8-10	0.182	+	0.014	+	+	+	+	+	+

* **Supplemented with 4.6% NaCl.**

**** Fusants of the *R. fredii USDA201* and *B. japonicum* SE 8.**

All fusants were more tolerance for drought than *R. fredii* USDA 201 parent, which could grow to 0.054 at OD 420nm, except two fusants, *i.e., R.f*.:: *B.j* 201-8-1 and *R.f*:: *B.j* 201-8-6 which reached 0.046 and 0.010 at OD 420nm, respectively. On the other hand, only one fusant, *R.f*.:: *B.j*. 201-8-9 could grow to 0.235 at OD 420 nm which was nearly equal in drought tolerance to the parent *B. japonicum* SE8 (Table 3).

Data presented in Table (3) showed that all obtained genetically stable fusants acquired resistance for Fe and Ni from the parent *B. japonicum* SE8. These results are in harmony with that obtained by Mahmoud (2000). Who obtained successful intergeneric hybrids in bacteria by protoplast fusion.

2. Protoplast fusion between *R. fredii U*SDA 201 and *B. japonicum* DO4.

In another attempt to induce inter-generic fusants, two parents; *R. fredii* USDA 201and *B. japonicum* DO4, were used and their characters were shown in table (1). Cells that resisted both Cm and Sm antibiotics were selected as fusants.

Results presented in Table (4) proved the success of fusants formation between the two parents. Two of the four genetically stable fusants

obtained were more salt tolerance on 2.05 % NaCl than the parent *R. fredii* USDA 201,*i.e., R.f*:: *B.j* 201-4-1 and *R. f*.: *B.j.*201-4-4,while the other two fusants were more sensitive. Only one fusant, *R. f*:: *B. j*. 201-4-1, showed high salt tolerance in comparison with the two parents. Also, two fusants; *R. f*.::*B. j*. 201-4-1 and *R. f*::*B. j*. 201-4-2, were more tolerant for drought than the parent *R. fredii* USDA 201, they could grow to 0.293 and 0.113,respectivelly at OD 420 nm .

Table (4): Characteristics of *R.fredii* USDA 201 and *B.japonicum* DO4 and its fusants.

Strains	O.D.$_{420\ nm}$ At 30% PEG6000	Growth at 2.05% NaCl		Antibiotic resistance				Fe resistance	Nodulation efficiency
		YEMA*	At OD $_{420\ nm}$	Sm,	Rif	Ap	Cm		
R. frediiUSDA201	0.054	+	0.028	+	+	+	-	-	+
B. japonicum DO4	0.149	+	0.085	-	+	+	+	+	+
*R.f::B.j.*201-4-1**	0.293	+	0.094	+	+	+	+	+	+
*R.f::B.j.*201-4-2	0.113	+	0.027	+	+	+	+	+	+
*R.f::B.j.*201-4-3	0.012	+	0.023	+	+	+	+	+	+
*R.f::B.j.*201-4-4	0.014	+	0.031	+	+	+	+	+	+

*** Supplemented with 2.05% NaCl**
**** Fusants of the *R.fredii USDA201* with *B.japonicum* DO 4.**

Also, data in table (4) showed that the fusant, *R. f*:: *B. j*. 201-4-1, is considered as the more balanced tolerant fusant on both parameters, salinity and drought, as it reached the higher rate for drought and salt tolerance than the parent *B. japonicum* DO4.These results are in agreement with those of Nair *et al.*(1993) who that some rhizobia strains had been extremely tolerated high levels of salt and drought through screening 45 fusants. All genetically stable fusants acquired iron resistance of the parent *B. japonicum* DO4.

Nodulation efficiency of obtained fusants

The inter-generic fusion products were tested for their nodulation capability. These fusants showed nodulation with soybean which confirmed successful transfer of symbiotic characters between nitrogen fixing rhizobia (Table 4). A slight decrease in nodulation efficiency of fusants with soybean than both parents was detected in correlation with salinity increase which is in agreement with the results obtained by Esechie (1999).

Plasmid profiles of the inter-generic protoplast fusants

Plasmid analysis of the first inter-generic protoplast fusion between *R. fredii USDA* 201 and *B. japonicum* SE8 was done via agarose-gel electrophoresis for the two parents strains and their fusants with reference strain *R. fredii* USDA 201. (Fig 1).

Fig (1) shows that all plasmids have high molecular weights in comparison with the plasmids of the reference strain, *R.fredii* USDA 201, which have three plasmids with molecular weight of 1000,210 and 120 MDa.

Fig (2) shows the plasmid analysis of the two parents; *R.fredii* USDA 201 and *B. j*aponicum DO4 and their fusants.

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 via plasmid profiles analysis. This method is recommended to determine the relationship within and between of species (Fouly and Wilkinson, 1999).

Phylogenetic analysis for the plasmid profiles of the parents *R. fredii* USDA 201, *B.japonicum* SE8 and their fusants from one to four resulted in the tree dendrogram which shown in Fig (3) and of the parent *R.fredii USDA201* , *B.japonicum DO4* and their fusants (Fig.4).

The dendrogram tree which shown in Fig (4) evidenced that the fusants; F-3, F-4, F-2 and F-1, are cluster arranged in a descending manner according to their relatedness to the parent *B.japonicum* SE8 and parent *R.f* USDA201, while for the other cluster tree dendrogram; fusants F-7, F-5, F-9, F-10 and F-8, are arranged in a descending manner according to parent *R. fredii* USDA201.

Similarity analysis for the plasmid profiles of the parent *R. fredii* USDA 201 with *B. j*aponicum DO4 and their fusants resulted in the tree dendrogram which shown in Fig (4). Two separate clusters were evident, the first consisting of F-1 and F-5 which were more related to the parent *B.japonicum* DO4, while the second consisting of F-2 and F-4 which more related to the other parent, *R. fredii* USDA201.

All fusants were produced between Egyptian indigenous strains with an imported strain. It is proved that some of the obtained genetically stable fusants are more efficient than one or both parents. To establish successful symbiotic relatioship, *Rhizobium* adapted to the specific environment has to be used. It is advisable to use the obtained fusants in the original site of the Egyptian parent to increase their competition capability. Furthermore, all obtained fusants could establish efficient symbiotic relationship in new reclaimed soils with soybean , due to the absence of indigenous Rhizobia and for their abiotic stresses resistance.

Fig1,2

Fig3,4

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التحسين الوراثى لبكتيريا البرادى ريزوبيم جابونيكم لتحمل الضغوط الغير حيويه بواسطة الدمج البروتوبلاستى

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تم الدمج البروتوبلاستى للسلالة ريزوبيم فريدياى يو إس دى إيه 201 سريعة النمو مع سلالتين متميزتين مصريتين هما سنديون 8 ، دمياط 4 . تم الحصول على تسعة مندمجات من المحاولة الأولى (سنديون 8 مع ريزوبيم فريدياى يو إس دى إيه 201) كما أمكن الحصول على أربعة مندمجات من المحاولة الثانية (دمياط 4 مع ريزوبيم فريدياى يو إس دى إيه 201) . تم توصيف جميع هذه المندمجات و عددها ثلاثة عشر على اساس مقدرتها عل تكوين العقد الجذرية و تحملها للملوحة و الجفاف و مقاومتها لتأثير المعادن الثقيلة و المضادات الحيوية. تم دراسة نماذج البلازميدات بطريقة التفريد الكهربى لكل المندمجات الناتجه و أبائها ثم حددت القرابة بينها تبعاً لطريقة التحليل الشجيرى. أظهرت بعض من هذه المندمجات كفاءه أعلى فى تحمل الملوحة و الجفاف و كذلك مقاومة تأثير المعادن الثقيلة مقارنة بالأباء مع إحتفاظها بمقدرتها على تكوين العقد الجذرية .