Genetic Characterization of intergeneric fusant between *Agrobacterium* and *Bacillus* and its effects on sugar beet calli

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ABSTRACT

Protoplast fusion technique was applied between *Agrobacterium tumefaciens* and *Bacillus thuringiensis* DM55 for obtaining fusant bacteria. The characters of this fusant were studied comparing with parental strains via performing the Light microscope examination and six microbiological tests. Assessment of larvicidal effect on cotton leaf worm (*Spodoptera littoralis*) was carried out through bioassay for them. On the other hand, parental and fusant bacteria were characterized and compared using the SDS-PAGE technique, and the plasmidic patterns. The protein fingerprinting of the fusants bacteria exhibited recombination of both parent's profiles. However, *A. tumefaciens* have produced higher amount of Indole Acetic Acid (IAA) than that in the fusant while the *B. thuringiensis* Dm55 was neglected. High genetic similarity was detected between *B. thuringiensis* MD55 and the fusants, more than between *Agrobacterium tumefaciens* and the fusant bacteria. Also, peroxidase isozymes activity was detected in all of calli, which obtained via caulogenesis technique on adult sugar beet leaves of uninfected (control) after infection with bacteria.

INTRODUCTION

Agrobacterium tumefaciens and *Bacillus thuringiensis* (Bt) are the most important soil bacteria in genetic engineering field. However, *A. tumefaciens* germs had an exceptional ability to transfer and integrate their mobile segment of Ti plasmid (T-DNA) into plant chromosome. Therefore, any foreign DNA fragment placed between T-DNA borders can be transferred into plant cells. For this unique property, *A. tumefaciens*-mediated transformation technique was developed in many plants such as *Brassica juncea cv.* Pjk; Medicago truncatula cv. Jemalong; strawberry; *Pinus radiata* (Monterey pine); sunflower and Sugar beet (Jiang, 1984; Babu *et al.*, 2003; Chabaud *et al.*, 2003; Gabriel *et al.*,2003; Grant *et al.*, 2004).

Bacillus thuringiensis (a gram positive soil bacterium) was used as bio insecticidal via its ability to produce crystalline inclusions during sporulation. Most strains of this bacteria are active against larvae of certain members of Lepidoptera. Other strains show toxicity against Deptiran or Coleopteran species. The crystalline inclusions of Bt dissolve in larval midgut and release one or more insecticidal crystal proteins of 27 to 140 kD. The activation of this prototoxins generate pores in midgut membranes;

disturbance of osmotic balance; cell swell and lyses. Then, larvae stops feeding and dies. (Höfte and Whiteley, 1989).

Deacon (2002) reported that Bt is an important insecticidal bacterium against many important plant pests especially caterpillar of Lepidoptera (butter flies and moths); mosquito larvae and smuliid black flies. From province of Thuringia, Germany Bt was first discovered in 1911 as a pathogen of flour moths. In France, It was first used as a commercial insecticide in 1938. Then, it was used in USA in (1950). However, these early products were replaced by more effective commercials insecticides in 1960 when various highly pathogenic strains were discovered.

In bacteria, protoplast fusion technique was used as an effective tool of gene transfer. It can be induced by chemical fusogens like polyethylene glycol (PEG) after isolation from bacterial cells by digestion of cell wall with lysozyme in presence of osmotic stabilizers. Applying this technology for related species *Bacillus thuringiensis* var. *kurstaki* and Bt var. *aizawai* (Shweil *et al.*,1998), increased Egyptian cotton leaf worm (*Spedoptera littoralis*). Delta-endotoxin concentration of Bt fusion was 1.4 times more than wild type (Shamsi *et al.*, 2002). Also, protoplast fusion between *E.coli* strains (isolated from contaminated sites with organophosphate pesticides, OP) and Bt (MD55) and *A. tumefaciens*, showed a superlative increase in OP biodegradation. This increasing enhanced with time and showed a tremendous efficiency (100%) at high level of substrate concentrations (Mansee *et al.*, 2005). In addition the intergeneric protoplast fusion carried out between *A. tumefaciens* and Bt exhibited some properties of both parental strains. As shown by Puntambekar *et al.*, (1989) who repeated that one of the gram positive fusants with most of the *Bacillus* properties showed tumour induction capacity in pigeonpea (*Cajanas cajan*).

Sugar beet is considered as the second most important sugar crop in Egypt. Sugar beet var Saccharifera (altisima) belongs to the genus *Beta* family *Chenopodiaceae*. The species includes the fodder beet; and Swiss chard. The genus *Beta* contains 13 species; which are grouped into four sections: *vulgaris*; *Corrolinae*; *Patellares* and *Nanae*.

The present study aims at: (1) obtaining fusant bacteria from *A. tumefaciens* and *B. thuringiensis* DM55 via protoplast fusion technique; (2)characterizing ; (3)determining the plasmidic patterns and Indol Acetic Acid (IAA) production for the fusant bacteria and parental strains; (4)infection of adult sugar beet leaves and stems by parental and fusant bacterial cell suspension to detect peroxidase isozymes for infected callus and adult leaves; and (5)evaluation of the genetic relation ship between parental and fusant bacteria according to the result of microbiological tests.

MATERIAL AND METHODS

Bacterial strains:

- *Agrobacterium tumefaciens* strain was obtained from Phytopathology Department, Bacterial Disease laboratory, The faculty of Agriculture, Alexandria University.
- *Bacillus thuringiensis* strain (DM55) was obtained from EL-Helow *et al.* (2000).

Plant Material :

Sugar beet variety (Lados) was used in the present study. This cultivar has been obtained from Kuhn, Holland. Lados is polygem; triploid; moderate yield and sugar cultivar.

Protoplast isolation and regeneration :

Protoplast, were isolated according to the method of Puntambekar and Ranjeker (1989) with some modifications. *A. tumefaciens and B. thuringiensis* (MD55) strains were maintained for 16 hours (h) in 10 ml of YEB, LB broth respectively. The suspensions were centrifuged at 6000 rpm. Then, pellets were washed and resuspended in TES buffer after adding $MgCL_2$, protoplasts were visualized using light microscopic (100X). Protoplasts of *A. tumefaciens and B. thuringiensis* (MD55) strains were regenerated on YEB and LB agar plates, respectively, containing 0.6 M sucrose and 0.8 % agar.

Protoplast fusion:

From each strain, Identical numbers of protoplasts were gently mixed; fused in presence of 30% Polyethylene glycol (PEG) 6000(wt/vol) for 3 min and washed with TES buffer. The fused protoplasts were mixed in SM medium containing 0.6 M sucrose and 0.8 % agar. The mixture was poured in Petri dish and incubated at 30 C for 10 to 15 days. Grown colonies were transferred to SM medium containing 1.5 % agar.

Bacterial characterization:

In order to characterize *A. tumefaciens and B. thuringiensis* strains as well as their fusants, several bacterial tests were applied according to Abo-Eldahab *et al.*, (1965). Bacterial characterization included light Microscope examination and six microbial tests :i.e., Levan production; fats lysis; resistance to penicillin; starch hydrolysis; gelatine lysis; casein hydrolysis.

Genetic relationships based on microbial tests,

The genetic relationships among *A. tumefaciens; B. thuringiensis* and their fusants were estimated using Past program (2.1 Version).

Indole Acetic Acid (IAA) production:

Five concentrations of IAA (0.2; 0.4; 0.6; 0.8; and 1 mg/ l) were prepared and their standard curve was estimated using spectrophotometer at wave length 430 nm. In YEB medium, *A. tumefaciens* was cultured overnight with shaking at 120 rpm, the culture centrifuged at 4000 rpm for 5 min. However,1 ml of supernatant was taken into eppendorf tube, and 1 ml of gohar sapler was added and left for 1 hour at room temperature. For each sample, the concentration of IAA (dark red colour) was estimated using spectrophotometer .

Bioassay test of toxicity against *S. littoralis* :

The larvicidal effects of *A. tumefaciens, Bacillus thuringiensis* and their fusants on cotton leaf worm (Spodoptera *littoralis*) were estimated according to Puntambekar *et al.* (1989). Twenty 1st instars larvae of (Spodoptera *littoralis*) were transferred to Petri dishes containing 25 ml of insect medium supplemented with different dilutions (1, 0.5, and 0.25) of constant bacterial cell suspensions ($1X10^7$cells/ml). After 72 h, survival larvae were counted. For each treatment, three replicates were raised and LC50 was estimated.

Electrophoretic of the total protein :

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using discontinuous buffer system described by Laemmli (1970).

Plasmid Isolation and electrophoresis :

This method was described by (Kado and Liu, 1981). Agarose gel electrophoresis was performed as described by Mc Donnell *et al.* (1977) and Mickel *et al.,* (1977). Agarose at 1% was melted, briefly, in TBE-buffer, which used as an electrode buffer (40mM Tris acetate and 2mM EDTA pH 7.9) and poured into a horizontal apparatus. Electrophoresis was carried out at 75 volt for 2 h. The gel stained with ethidium bromide(0.5 μg/ml) and Photographed.

Tissue culture and transformation of sugar beet calli :

Sugar beet leaves were rinsed several times with tap water and finally with distilled water. According to Sharaf and ouf (1995) and using Laminar cabinet flow each leaf was cut into small discs (about 1 cm^2)

and sterilized in 0.05 % of HgCL2 for 10 - 20 min or Clorox (10%) for 20 min. Then, these explants were washed twice with sterile distilled water. Explants were cultured on B5 medium(Gamborg *et al*,1968), supplemented with phytohormones.

According to Draper *et al.*, (1988),10-12 leaf explants were placed on Gamborg medium. These plates were sealed with Parafilm and incubated under low light intensity (2000 Lux, 16 –h photoperiod) for 2 days at 24- 26 C.

Leaf explants were dipped into diluted suspension (1:50) of *A. tumefaciens, B. thuringiensis* and fusants strains, leaf explants were returned into original Gamborg medium plates; sealed with Parafilm and incubated at 24-26 °C. 2-4 days under low light- intensity (2000- 4000 Lux) plates were checked, regularly, for bacterial overgrowth. Then, explants containing visible bacterial colonies were transferred to Gamborg medium supplemented with penicillin and incubated under low light- intensity (2000-4000 Lux) at 24-26 °C.

Peroxidase isozymes :

In the present study, peroxidase isozyme patterns were detected in leaves of sugar beet infected plants and calli as well as non-infected plants. The activity of this isozymes estimated using Total Lab program (version 2.2).

RESULTS AND DISCUSSION

Protoplast fusion

Protoplast fusion technique was performed successfully between both *A. tumefaciens* and *B. thuringiensis* MD55 to produce the fusants bacteria. After using gram staining, high ratio of fusant cells (80-90%) was observed by light microscope. Hybrid cells were gram-positive stain then, after six months converted to be gram-negative bacterium. As shown in figure (1), there were differences in cells size between parental strains and their fusants. Fusant cells were, obviously, bigger than the parental cells and were individualized cells while parental cells were formed in chains. Similar results were observed by Puntambekar and Ranjekar (1989) through performing an intergenic protoplast fusion between *A. tumefaciens* and *B. thuringiensis*. Also, successful fusants were applied between different bacterial strains such as *Pseudomonas fluorescencs and B. thuringiensis* (Rajendran, 1994); *B. thuringiensis* israelensis (Shamsi *et al.*, 2002) and *E. coli; A. tumefaciens* and *B. thuringiensis* (Mansee *et al.*, 2005).

Figure (1): Cells of *A. tumefaciens* (A), *B. thuringiensis*., (B) Fusants after fusion (AB1) and after six months (AB2).

Bacterial characterization :

Table (1) shows the results of the different microbial tests for the parental bacteria and their hybrid. All of *B. thuringiensis MD55* and the fusants bacteria were positive in fat lyses, levan production and in the hydrolysis of starch, casein and gelatin, but *A. tumefaciens* was negative in these tests. Therefore, according to protoplast fusion, β-amylase gene probably transferred with *Bacillus thuringiensis (MD55)* genome into fusants and expressed in their cells. Similarly, fusants of the two genetically marked *Bacillus subtilis* strains (which produce noncomplementing heterodiploid bacteria) expressed only in the cells of parental markers (Grandjean *et al.*, 1996). On the other hand, *A. tumefaciens* and the fusants not were susceptible to penicillin, but *Bacillus thuringiensis MD55* was susceptible. These results indicated that pencilling resistance gene was existed only in *A. tumefaciens* and also through protoplast fusion this gene was expressed in fusant cells. Previous differences shown that hybrids retaining common genes from both parents (Chen et al., 1987, Puntamberekar and Ranjekar, 1989 and Mansee *et al.*, 2005).

Table (1): The results of the different microbial tests for *A. tumefaciens; B. thuringiensis* (DM55) and their fusants.

	Microbial test	Bacterial strain *Agrobacterium tumefaciens*	*Bacillus thuringiensis* (DM55)	fusant
1	Levan production	(+)	(-)	(+)
2	Starch hydrolysis	(-)	(+)	(+)
3	Fat lysis	(-)	(+)	(+)
4	Casein hydrolysis	(-)	(+)	(+)
5	penicillin Resistance	(+)	(-)	(+)
6	Gelatine hydrolysis	(-)	(+)	(+)

Genetic relationships based on microbial tests,

High genetic similarity was detected between *B. thuringiensis* MD55 and the fusants, more than in between *A. tumefaciens* and the fusant bacteria, as shown in Figure (2). These results were in agreement with those of Rajendran *et al.*, (1994) who found that hybrid produced by applying protoplast fusion between *P. fluorescens* and *B. thuringiensis subsp. kurstaki* HD was more closely related to *P. fluorescens* more than *B. thuringiensis* with 77% and 20 % of genetic relationships values, respectively.

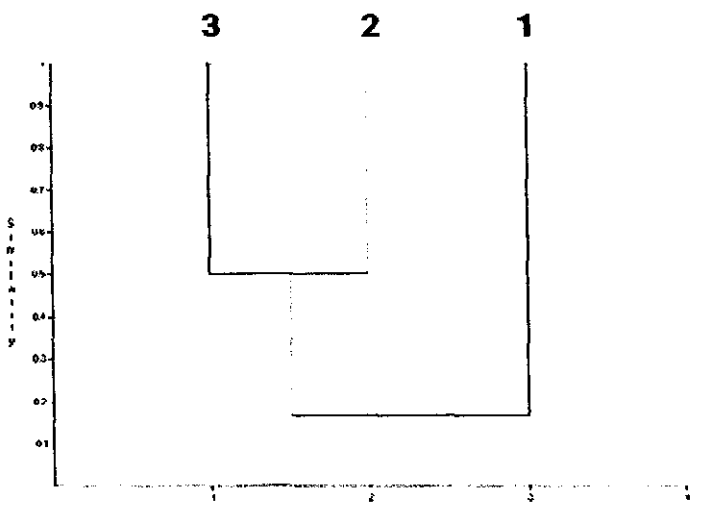

Figure (2): The genetic relationship among (1) *B. thuringiensis* (MD55); (2) *A. tumefaciens* and (3) their fusants. The dendogram based on the Microbial tests.

Indole Acetic Acid (IAA) production:

The production of IAA was compared between the parental strains and their fusants. However, *A. tumefaciens* had higher concentration of IAA (10.13 µg/l) than that in the fusant (6.22 µg/l), while the concentration of *B. thuringiensis* Dm55 was neglected (Table 2). These data proposed that genes responsible for IAA hormone production (tms1, tms2) were more active in *A. tumefaciens* strain than that of fusants. These results were in agreement with those of Yacout (1992) who calculated high concentration of IAA hormone (10 µg/l) for *A. tumefaciens* C58 strain.

Table (2): Concentrations (µg/L) of Indole Acetic Acid (IAA) hormone in *A. tumefaciens* and *B. thuringiensis* (MD55) strains and their fusants.

Strain	Concentration
A. tumefaciens	10.13
B.thuringiensis	------
fusants	6.22

Bioassay test of toxicity against *S. littoralis*

The larvicidal effects of *B. thuringiensis MD55* measured as 1st instar larvae, found survival percentages was higher than the fusants which was higher than the *Agrobacterium tumefaciens*. The LC50 for *Bacillus thuringiensis* MD55 and for the fusants bacteria was 0.2, and 0.41, respectively. These values indicated that the *Bacillus thuringiensis MD55* have 100% toxic to 1st instars larvae of the Egyptian cotton leaf worm (Spodoptera *littoralis*), when compared with the fusants (Table, 3).

Table (3): Average numbers of survivals first instar larvae and their percentages of Egyptian cotton leaf (Spodoptera littoralis) treated with different concentrations of *A. tumefaciens*; *B. thuringiensis* and their fusants.

Strain		*A. tumefaciens*		*B.thuringiensis*		fusants	
Concentration		larvae Number	%	larvae Number	%	larvae Number	%
	0.25	30	100	15 ± 0.3	50	22 ± 0.33	73
	0.50	29 ± 0.33	97	7 ± 0.88	23	14 ± 0.33	47
	1.00	29 ± 0.33	73	2 ± 0.88	7	6 ± 0.33	20
LC50 *		--		0.22		0.41	

* LC50= lethal dilution of cell suspension corresponding to (1 $X10^7$ cell/ ml) killing 50 % of treated Larvae.

Total protein electrophoresis :

In search for differences between and among total proteins for *A. tumefaciens, B. thuringiensis* MD55 and their fusants, detected by Sodium Dodysyl Sulphate Polyacrylamide gel electrophoresis (SDS- PAGE). The molecular weights (MW) of protein bands which extracted from *A. tumefaciens* ranged approximately from 23 to 60 KDa (Figures 3 and 4). While for *B. thuringiensis*, the protein band patterns varied from 13.5 to 160 KDa. On the other hand, the protein fingerprinting of the fusants bacteria exhibited recombination of both parent's profiles and found their protein bands ranged from approximately 13 to 160 KDa. Those results suggested the possibility of gene expression in fusant bacteria for insecticidal crystal proteins which ranged from 27 to 140 kDa according toHöfte and Whiteley, (1989).

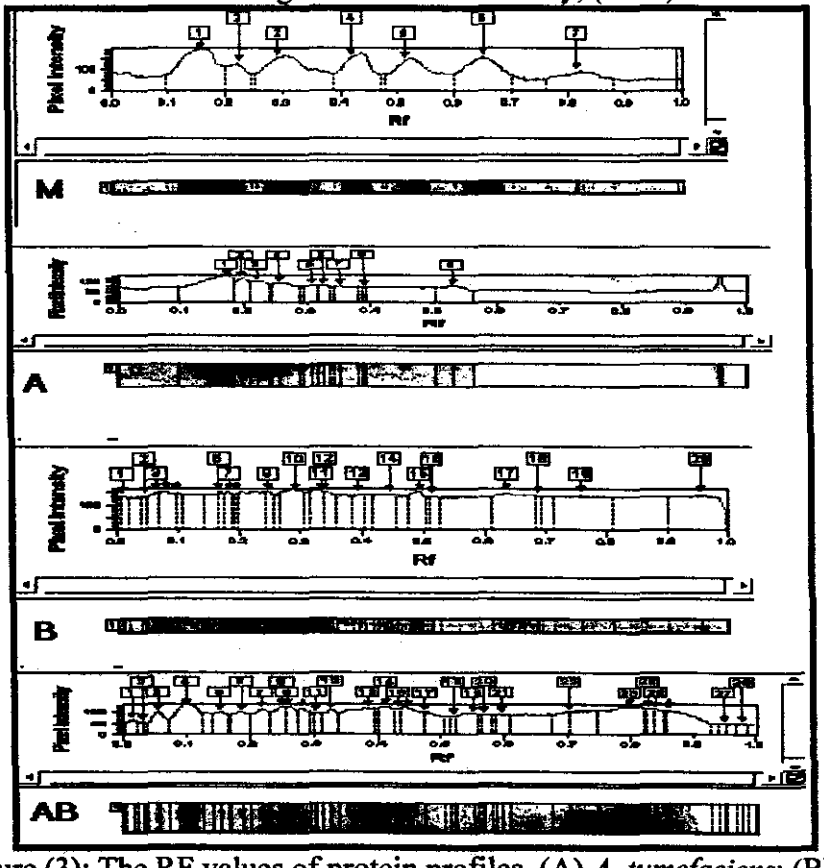

Figure (3): The RF values of protein profiles. (A) *A. tumefaciens*; (B) *B. thuringiensis*; (AB) their fusant and (M) Marker protein

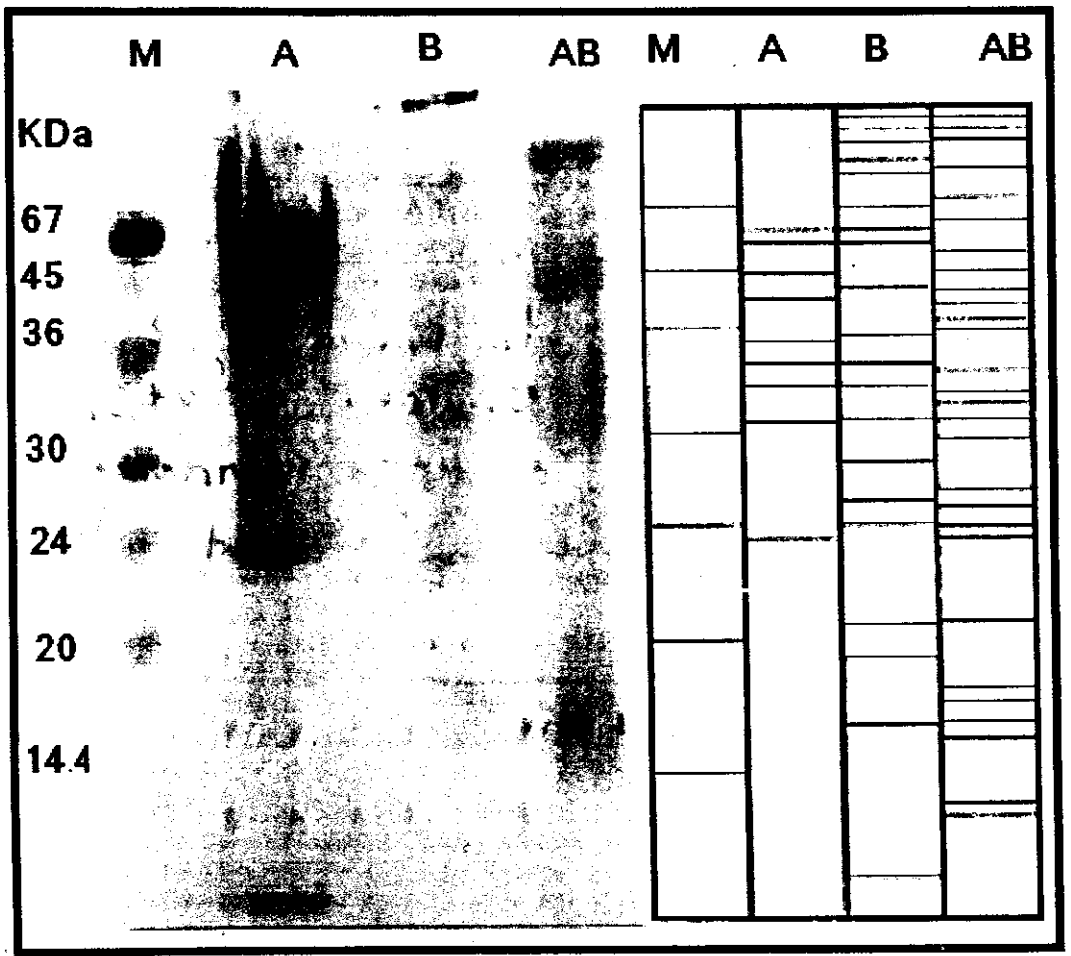

Figure (4): Photograph and descriptive diagram showing protein profile of *A. tumefaciens*(A), *(B) B. thuringiensis* MD55 (AB) their fusant and (M) Marker protein.

Plasmid Isolation and electrophoresis :

Plasmid isolation was performed for identifying the plasmidic patterns of *A. tumefaciens*, *B. thuringiensis* MD55 and their fusants. *A. tumefaciens* strain (A) revealed one band (Ti-plasmid) with MW 200 kbp. On the other hand, *B. thuringiensis* (B) has five bands, the molecular weights of these bands were 197, 150, 100, 50 and 18 kbp. After that, the fusants showed three plasmidic bands in their patterns which were in MW 150, 100 and 78 kbp. (Figure 5): Fusant bacteria contained two plasmids belonged to *B. thuringiensis* ,i.e. 150 and 100 kbp. The third plasmid of fusants was 78 kbp. which could be a result of recombination between

other plasmids in parental bacteria as reported by Boyd *et al.* (1996) in. study of plasmids from natural populations of *Escherichia coli*.

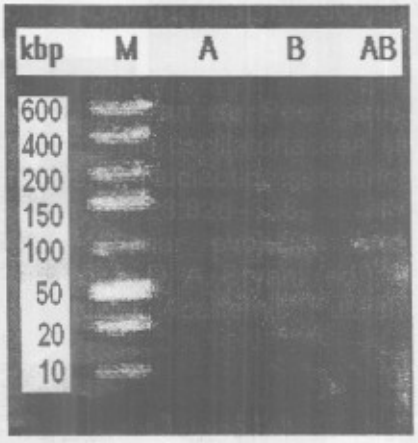

Figure (5): The agarose gel electrophoresis plasmidic patterns for (M) DNA marker (A) *A. tumefaciens*, (B) *B. thuringiensis* MD55 and (AB) the fusant.

Tissue culture and transformation of sugar beet calli :

The infection for adult sugar beet leaves and stems were performed via wounded leaves and stems by bacterial cells suspension of A. *tumefaciens, B. thuringiensis* MD55 and their fusants. Tumors formation were observed only in case of wounded leaves and stems with the suspension of *A. tumefaciens* (Figure 6A). Leaves of sugar beet (Lados Varity) used as explants for obtaining calli which were friable; white; ease to separate from explants and appeared as embryogenic calli (Figure 6B). These characters were mentioned before by Sharaf and Ouf (1995) when used similar medium with different varieties. On the contrast, different characters of sugar beet calli were described by Abdel-hamid (1997) who used B5 medium augmented with 0.1 mg/l BAP; 0.1 mg/l NAA and 0.05 mg/l 2-4 D as growth regulators.

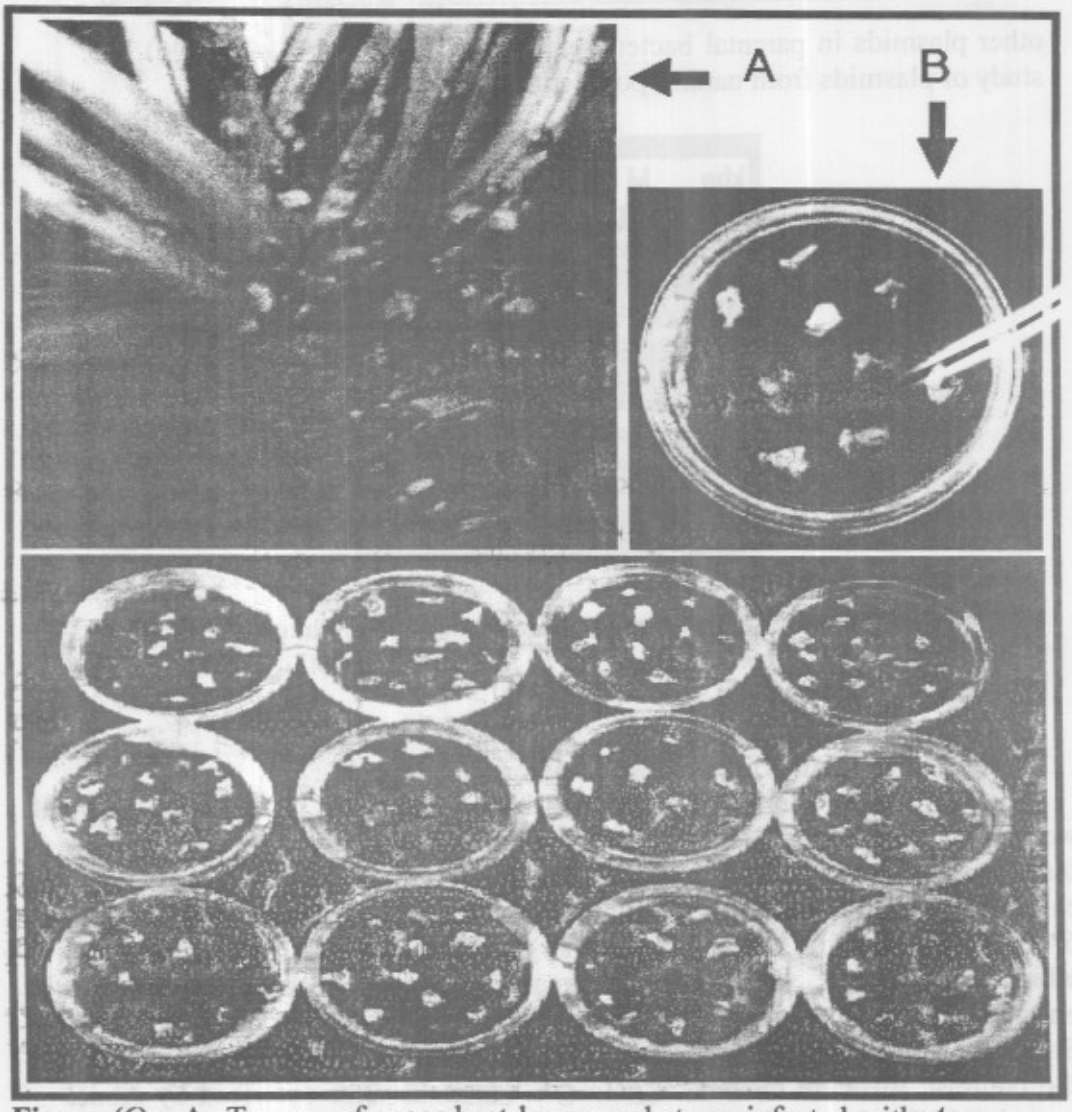

Figure (6) : A- Tumors of sugar beet leaves and stems infected with *A. tumefaciens*. **B-** Calli formed from leaves of sugar beet (Lados Varity).

Peroxidase isozymes :

Peroxidase isozymes activity was detected in all of calli and adult leaves, of un infected control and after infection with *A. tumefaciens, B. thuringiensis* and with their fusants. In general the peroxidase isozyming bands, showed higher activities in all calli than those of leaves, as shown in Figure (7). The cathodal bands controlled by three homozygous loci pox-1C, pox-2C and pox-3C, but the anodal bands may controlled by five loci pox-1A, pox-2A, pox-3A, pox-4A, pox-5A and pox-6A. The band numbers varied

among two to nine bands, the wounded leaves with suspension of *A. tumefaciens*, were lowest in activity and number of peroxidase isozymes when compared to the other treated leaves sample. Again, in case of calli, the non infected (control) and infected calli with suspension of *A. tumefaciens* has the highest numbers and activities of peroxidase isozymes when compared to the other calli of treated samples. The second, third and fourth cathodal bands present in all calli and not wounded leaves sample except infected calli with *A. tumefaciens, B. thuringiensis* and with their fusants. All genes that controlling the cathodaly and anodaly isozyming bands turned to be homozygous, except which controlled the fifth and sixth anodal bands was heterozygous in all calli wounded and non wounded samples and non wounded leaves (Table 4 and Figure 7).

These result were in agreement with those which mentioned by Bisbis *et al.*, (1999) found cells from primary normal (N) calli are always rich in activity of enzyme peroxidase than the cells of tissues or organs from the derive sugar beet cells. The total peroxidase activity of normal calli comparing with those of infected calli with *A. tumefaciens*; The infection with *A. tumefaciens* cause has the higher activity when comparing with the normal ones.

Table (4): Number of bands of peroxidase isozymes and their mobility (Rf) in non-infected sugar beet plants and calli that of infected with *A. tumefaciens*, *B. thuringiensis* and the fusant.

Type of samples	Type of treatment	Number of bands	Rf								
			Cathodal bands			Anodal bands					
			1	2	3	1	2	3	4	5	6
Leaves and stems	Control	7	0.5	1.0	1.6		2	2.8		5.0	5.5
	A. tumefaciens	2	0.6	-	-	-	-	-	-	5.0	-
	B. thuringiensis	4	0.6	1.3	1.5	-	-	-	-	5.2	-
	the fusant	4	0.7	1.2	1.7	-	-	-	-	5.1	-
Calli	Control	9	0.8	1.0	1.5	1.0	2.0	3.0	4.1	5.2	5.7
	A. tumefaciens	9	0.5	1.0	1.6	1.3	2.2	3.2	4.3	5.1	5.5
	B. thuringiensis	7	0.5	1.2	1.7	-	2.6	-	4.4	5.0	5.8
	the fusant	8	0.6	1.0	1.7	-	2.2	3.0	4.6	5.1	5.7

tobacco plants with *Agrobacoterium tumefaciens,* the activity level of ascorbic peroxidase (APX) was 3.8 folds than the wild type plants.

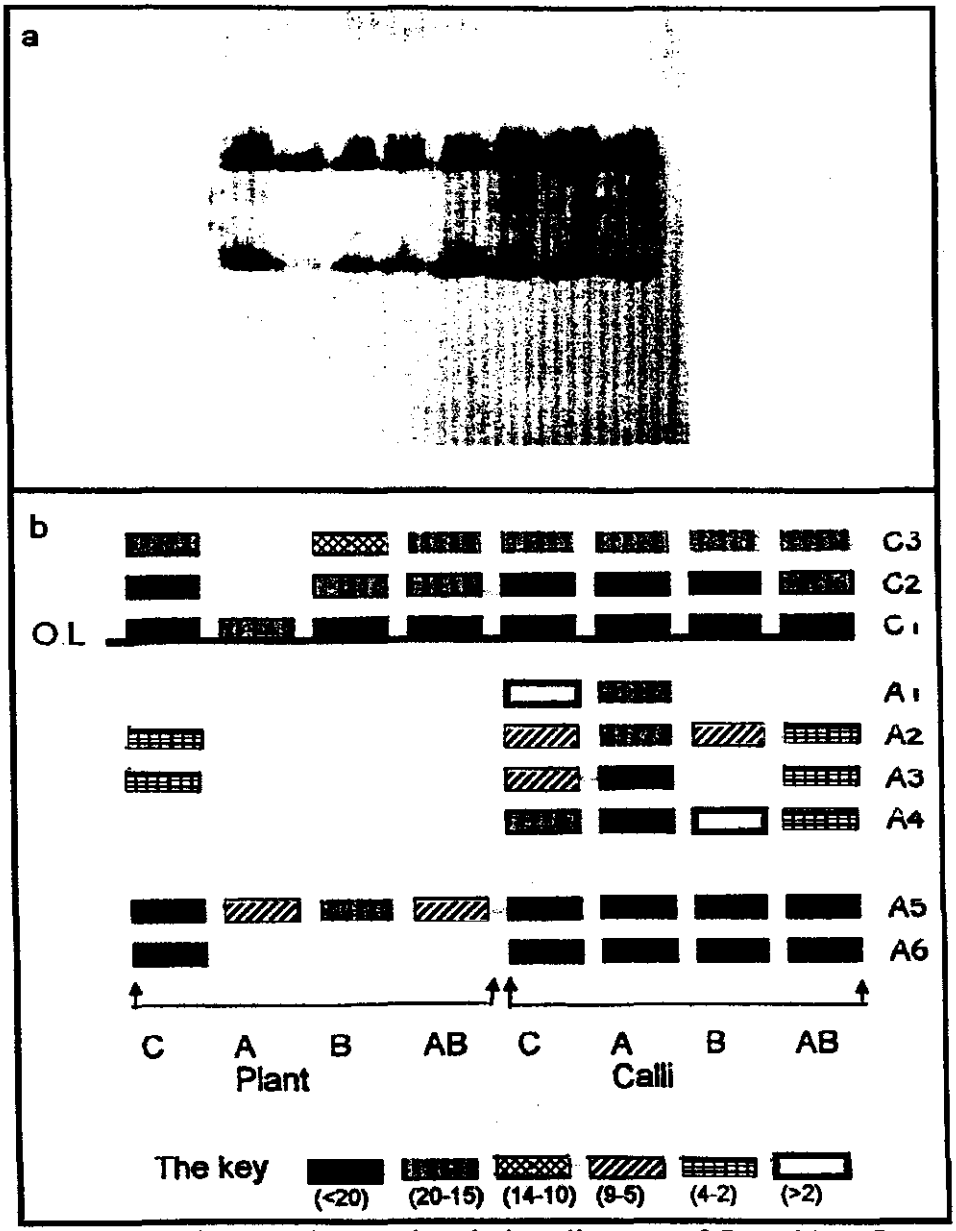

Figure (7): Photograph and descriptive diagram of Peroxidase Isoenzymes patterns for sugar beet leaves and calli.

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الملخص العربى

التوصيف الوراثى لهجين بين جنسى الاجروبكتيريم والباسليس وتأثيراته على كالس بنجر السكر

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تم الحصول على هجين بين أجناس بكتيرية intergeneric hybrid هي بكتيريا *Agrobacterium tumefaciens* النافعة وبكتيريا *Bacillus thuringiensis* و ذلك عن طريق إجراء الاندماج البروتوبلاستى لكل من البكتيريتين.ودراستها وراثيا بالمقارنة مع الاباء من خلال دراسة عدد من الصفات البيوكيميائية وهى انتاج الليفان وتفاعل صبغة جرام وتحليل النشا وتحليل الكازين وتحليل الجيلاتين وتحليل الدهن والمقاومة للمضاد الحيوى البنسلين وقياس تركيز هرمون اندول حمض الخليك(IAA بالاضافة الى دراسة التغيرات الناتجة عن عدوى نبات بنجر السكر والكالس الناتج من زراعة الأنسجة له بواسطتها .ثم تم عمل استخلاص للبلازميدات الخاصة بكل من البكتيريات الثلاثة و عمل تفريد لها على جيل الاجاروس لدراسة التغيرات الحادثة نتيجة عمل الاندماج البروتوبلاستى وكذلك مشابهات الانزيم بيروكسيديز. انتجت الاجروبكتريم كمية اعلى من اندول حمض الخليك كما اتضح التشابه بدرجة اعلى بين الباسليس والهجين بناء على الاختبارات المختلفة