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TRANSFORMATION OF SUGARCANE CALLI WITH *B. thuringiensis* AND *A.tumefaciens* PROTOPLAST FUSIONTS

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ABSTRACT

This investigation was designed to induce a foreign genomic fragments of *Bacillus thuringiensis* (which is considered as important soil bacteria due to its ability to produce a lethal protoxin) to calli of three sugarcane varieties namely, C0 413, PH 8013 and GT 54-9. Thus, protoplast fusion technique was performed using *Agrobacterium tumefaciens* and *Bacillus thuringiensis* to produce a fusionts bacterium. Then, calli of different varieties under study were infected with parental and fusionts bacteria. To detect the transformants for sugarcane calli, SDS-PAGE technique was used. Consequently, successful expression of target gene was detected through produce a final protoxin (target band with 116 KDa) which characterize *Bacillus thuringiensis* as a bio insecticidal in all three varieties which were infected with delta endotoxin fusionts. Finally, polymorphism of DNA fragments, produced using arbitrary primers was study via RAPD technique and genetic similarity was recorded. In order to detect the differences between natural and transformant varieties, the percentages of polymorphism were recoded and found 18, 21, 5 and 45 % for first, second, third and fourth primers respectively.

INTRODUCTION

It is known that, sugarcane is the most important sugar crops in Egypt. Therefore, enthusiastic efforts have been directed toward the improvement of sugar crops either by using conventional breeding methods (Gaber *et al*., 1990 and Abu El Fath *et al.*, 1994) or via tissue culture techniques and their applications (Sharaf and Ouf. 1995 a & b; Sharaf and Ouf, 1998 and 1999; Sharaf et *al*., 2000 and Ouf et al., 2003). The improvement of any crop not only based on production and technological characters. But also to produce new cultivars which could be resist the different pests especially pests belong to (family Lepidoptera) especially porrers pests (Mark and Jones 1999) via applying genetic engineering techniques through introduce DNA fragment which included protoxin gene from *Bacillus thuringiensis* to commercial sugarcane varieties. Then, this gene was express in the sugarcane leaves and the insecticidal crystal proteins were released in the pest midgut membranes after eating which causing sudden die.

Agrobacterium tumefaciens and *Bacillus thuringiensis* (*Bt*) are the most important soil bacteria in the field of genetic engineering. *Agrobacterium tumefaciens* cells had an exceptional ability to transfer and integrate their mobile segment of Ti plasmid (T-DNA) into plant chromosome. Therefore, foreign DNA fragment placed between T-DNA borders can be transferred into plant cells. For this unique property, *Agrabacterium tumefaciens*-mediated transformation technique was developed in many plants such as *Saccahrum officinarum*L , *Brassica juncea cv. Pjk*; *Medicago truncatula cv. Jemalong*; strawberry; *Pinus radiata*; sunflower and sugar beet

(Jiang, 1984., Babu *et al*., 2003; Chabaud *et al.,* 2003; Gabriel *et al.,*2003 and Grant *et al*., 2004).

Bacillus thuringiensis (a gram positive soil bacterium) was used as bio insecticidel via its ability to produce crystalline inclusions during sporulation which 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).

Protoplast fusion technique was used as an effective tool for bacterial genome 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 the presence of osmotic stabilizers. Interestingly, it could be applied to improv the useful traits of bacteria as a type of microorganisms genetic engineering methods. For example, *E. coli* strains (isolated from contaminated sites with organophosphate pesticides, OP) and *Bacillus thuringiensis* (MD55) and *Agrobacterium tumefaciens*, showed a superlative increase in OP biodegradation. This increase enhanced with time and showed a tremendous efficiency (100%) at high level of substrate concentrations (Mansee and Yacout., 2005). In addition the intergeneric protoplast fusion carried out between *Agrobacterium tumefaciens* and *Bacillus thuringiensis* exhibited some properties of both parental strains. As shown by Puntambekar *et al*. (1995).

This work aimed to detecting the response of sugarcane calli to be transformed with *Agrobacterium*- transformation technique to produce new sugarcane cultivar which could produce final product protoxin (target band with 116 KDa) which characterize *Bacillus thuringiensis* as a bio insecticidal via infection calli of three sugarcane varieties with *Agrobacterium tumefaciens* and *Bacillus thuringiensis* MD 55 and their fusionts which produce trough protoplast fusion technique.

MATERIALS AND METHODS

In this investigation, protoplast fusion technique was employed to produce fusionts bacteria between *Agrobacterium tumefaciens* and *Bacillus thuringiensis* MD55. Then, the infection was carried out for three sugarcane varieties CO413, PH 8013 and GT54-9. In final, the protein profiles of three sugarcane varieties which infected with *Agrobacterium tumefaciens*; *Bacillus thuringiensis* and their fusionts were detected by using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS- PAGE) according to Laemmli (1970).

Plant Material:-

Calli of three sugarcane varieties namely, CO413, PH 8013 and GT54-9 (produced according to Sharaf and Ouf, 1995a) and were used in this study.

Bacterial strains:-

Agrobacterium tumefaciens strain kindly provided from Phytopathology Department, Bacterial disease laboratory, Faculty of Agriculture, Alexandria

University and *Bacillus thuringiensis* strain (DM55) was isolated by EL-Helow *et al*. (2000).

Bacterial fusion technique

Protoplasts, were performed after the treatment with lysozymes for hydrolysis the bacterial cell wall and isolated. Then, identical numbers of protoplasts were gently mixed; fused in presence of 30% polyethylene glycol (PEG) The fused protoplasts were mixed in SM medium for use according to Ranjekar and Puntambekar (1989). Furthermore, bacterial microscope examination was performed via spread of drop of cell suspension on clean slide, fixed and dried. Bacterial cells were stained by crystal violet for examination.

Callus infection:

Three sugarcane varieties namely, CO413, PH 8013 and GT54-9 were cultured on callus induction medium according to Sharaf and Ouf, 1995 (a) for obtaining calli which infected with *Agrobacterium tumefaciens, Bacillus thuringiensis* and the fusionts. Infection process was performed according to Draper *et al.,* 1999.

Protein electrophoresis patterns calli:-

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using discontinuous buffer system described by Laemmli (1970). Nevertheless, the genetic relationships among three sugarcane varieties which infected with *Agrobacterium tumefaciens; Bacillus thuringiensis* (MD55) and their fusionts were estimated using past program (2.1 Version).

Random amplified Polymorphic DNA (RAPD) technique

Four arbiters primers were used to detect the differences among varieties under study, features of this promers were listed in Table (1).

RESULTS AND DISSCUSION

High ratio of fusionts cells (80-90%) was observed by light microscope. As shown in figure (1) there were differences in cells size between parental strains and their fusionts. Fusionts cells were obviously bigger than the parental cells and it found in single cells while parental cells were found in chains. Similar results were observed by Puntambekar and Ranjekar (1995) through performing an intergenic protoplast fusion between *Agrabacterium tumefaciens* and *Bacillus thuringiensis*.

Depending on protein patterns results (Fig2,3), untreated calli of variety CO413 reflected seven bands with 75, 80,90 , 65, 52, 50 and 25 KDa. While, six bands were observed in case of infection with *Agrabacterium tumefaciens* 80, 72, 66, 51, 48, 30 and 23 KDa. Interestingly, target band with 116 KDa which present the final product (protoxin) which characterize *Bacillus thuringiensis* as a bio insecticidel was detected in the total protein which extracted from the calli of variety PH 8013 which infected with *Bacillus thuringiensis* beside three bands with 66, 55 and 48 KDa. In final, the same target band with 116 KDa was found as a result of callus infection with fusionts bacteria, additionally, eleven bands with 118, 100, 92, 80, 72, 66, 55,

50, 46, 30 and 27 KDa. Untreated variety PH 8013 showed 93, 80, 72,64, 50,46,30 and 27 KDa. In case of infection with *Agrabacterium tumefaciens* six bands were detected with 93, 77, 64, 50,46 and 27 KDa. As shown in the previous variety infection with *Bacillus thuringiensis* showed the target band with 116, 93, 77, 64, 50 and 19 KDa. Indicating the previous results, infection with fusionts bacteria showed the same interested band with 116 KDa (with 93, 77, 65, 50, 30 and 20 KDa) which indicate expression of *Bacillus thuringiensis* genomic which integrated in the sugarcane genome through *Agrabacterium tumefaciens* which was a genomic vector. The final infected variety GT54-9 showed six bands in case of infection calli with *Agrabacterium tumefaciens* (77, 69, 67, 52, 48 and 22 KDa) comparing with five bands (100, 69, 66, 46 and 20 KDa) in untreated calli. Moreover, six bands were detected with 100, 77, 68, 67, 50 and 48 KDa as the main of product infection with *Bacillus thuringiensis*. Interestingly, in case of infection calli with fusionts bacterium, protein band with 116 KDa was detected in protein profile of infected calli with fusionts (beside six bands with 77, 70, 69, 67, 49 and 47 KDa) which indicate the success of fusion and transformation for sugarcane calli.

Trough the exceptional ability of bacterial fusionts which induced via applying protoplast fusion technique between *Agrobacterium tumefaciens* and *Bacillus thuringiensis* three sugarcane varieties namely, C0 413, PH 8013 and GT 54-9 were transformed to express the lethal protoxin for larvae of the stem poorer. This result is considered as a promising base to produce regenerated plants could be resistant to stem borer and must be followed by bioassay.

Figure 1. bacteria, protoplasts and fusionts cells.
Where:

(A) *Agrobacterium tumefaciens* cells
(A1) protoplast of *Agrobacterium tumefaciens*
(B) *Bacillus thuringiensis* cells
(B1) protoplast of *Bacillus thuringiensis*
(F) Fusionts cells.

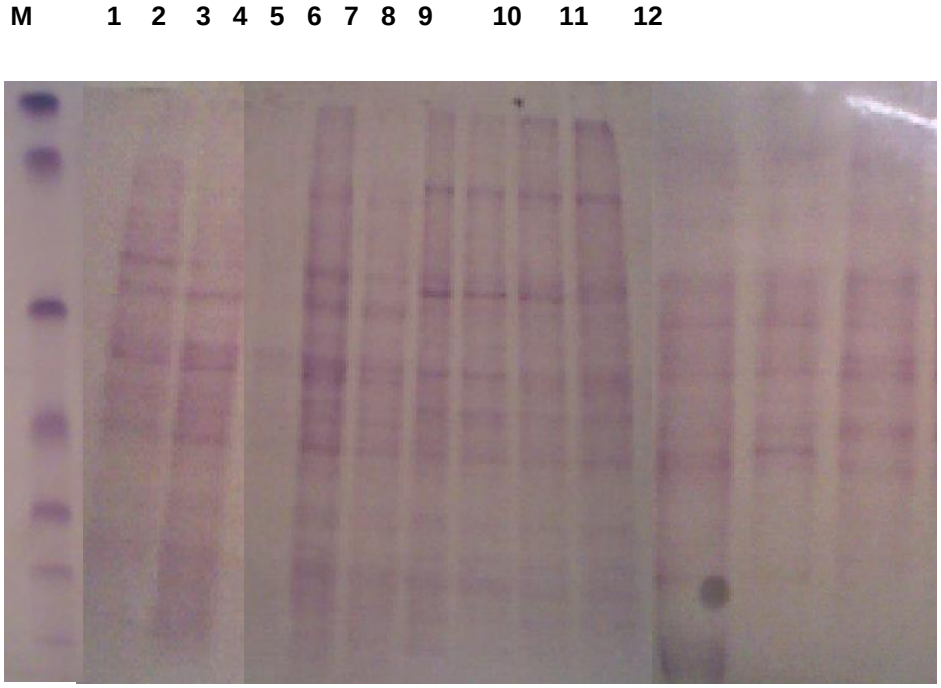

Figure 2a. Protein patterns of three calli of sugarcane varieties under different bacterial infection.

Where:
1- CO413 (untreated calli).
2- CO413 infected with *Agrobacterium tumefaciens*
3- CO413 infected with *Bacillus thuringiensis*
4- CO413 infected with fusionts bacteria
5- PH 8013(untreated calli).
6- PH 8013 infected with *Agrobacterium tumefaciens*
7- PH 8013 infected with *Bacillus thuringiensis*
8- PH 8013 infected with fusionts bacteria
9- GT54-9 (untreated calli).
10- GT54-9 infected with *Agrobacterium tumefaciens*
11- GT54-9 infected with *Bacillus thuringiensis*
12- GT54-9 infected with fusionts bacteria

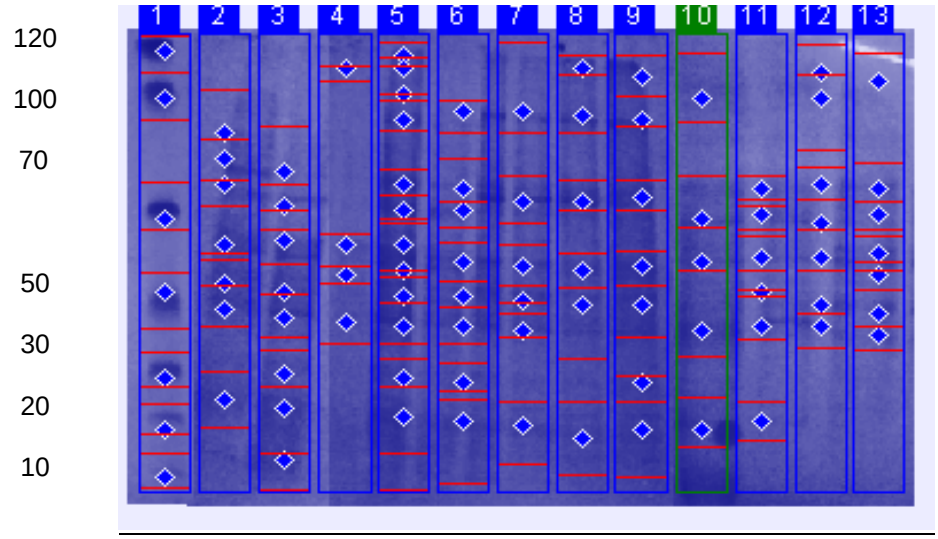

Figure 2b. Computerized analysis of protein patterns identification of three calli of sugarcane varieties under different bacterial infection.

Where:

1- CO413 (untreated calli).
2- CO413 infected with *Agrobacterium tumefaciens*
3- CO413 infected with *Bacillus thuringiensis*
4- CO413 infected with fusionts bacteria
5- PH 8013(untreated calli).
6- PH 8013 infected with *Agrobacterium tumefaciens*
7- PH 8013 infected with *Bacillus thuringiensis*
8- PH 8013 infected with fusionts bacteria
9- GT54-9 (untreated calli).
10- GT54-9 infected with *Agrobacterium tumefaciens*
11- GT54-9 infected with *Bacillus thuringiensis*
12- GT54-9 infected with fusionts bacteria

RAPD technique result:

As shown in Table (1) and Fig (4), the obtaining results could be summarized as the follow:

1- Primer 1:

Using primer 1 with the genomic DNA from three sugarcane varieties CO413, PH 8013 and GT54-9 which were infected with *Agrobacterium tumefaciens* and *Bacillus thuringiensis* MD55 and its fusionts , 38 bands with various size ranging from 1000 to 100 were observed. The number of Polymorphic bands were 7 bands with 18 % of Polymorphism % (Table 1 and Figure 1).

2- Primer 2:

Twenty nine bands were recorded for three three sugarcane varieties CO413, PH 8013 and GT54-9 after infection with *Agrobacterium tumefaciens* and *Bacillus thuringiensis* MD55 and its fusionts, interestingly, 21% of total bands were recorded as polymorphic bands.

3- Primer 3:

However the large number of amplified bands (95 bands), only 5 % showed as a polymorphic bands after the infection with *Agrobacterium tumefaciens* and *Bacillus thuringiensis* MD55 and its fusionts for different three sugarcane varieties CO413, PH 8013 and GT54-9.

4- primer 4 :

Significant superiority was noticed for application of fourth primer with 45 % of polymorphism (with total of 20 bands).

Table (1): Illustrate number of bands and % of polymorphism for samples.

primer	Number of bands	% of polymorphism
1 AACTGGTCAG	38	18
2 CGCGTTGTAA	29	21
3 TACCGTATGC	95	5
4 TTGTGCAAGT	20	45

Genetic similarity among three infected sugarcane varieties according to RAPD technique:

Based to RAPD technique for three sugarcane varieties CO413, PH 8013 and GT54-9 which infected with *Agrobacterium tumefaciens* and *Bacillus thuringiensis* MD55 and its fusionts, all samples were divided into two main clusters (at 30 % of genetic similarity). The first contains varieties CO413 infected with *Agrobacterium tumefaciens* , CO413 infected with *Bacillus thuringiensis*, CO413 infected with fusionts bacteria, PH 8013(untreated calli), PH 8013 infected with *Agrobacterium tumefaciens* and PH 8013 infected with Bacillus thuringiensis. The second divided into two sub cluster at 35 % of genetic similarity, the first sub cluster included PH 8013 infected with fusionts bacteria, GT54-9 (untreated calli) and GT54-9 infected with *Agrobacterium tumefaciens*. Furthermore, the second sub cluster composed of two groups (40 % of genetic similarity), GT54-9 infected with fusionts bacteria presented the first group. In final, CO413 (untreated calli) and PH 8013 infected with *Bacillus thuringiensis* were the second group.

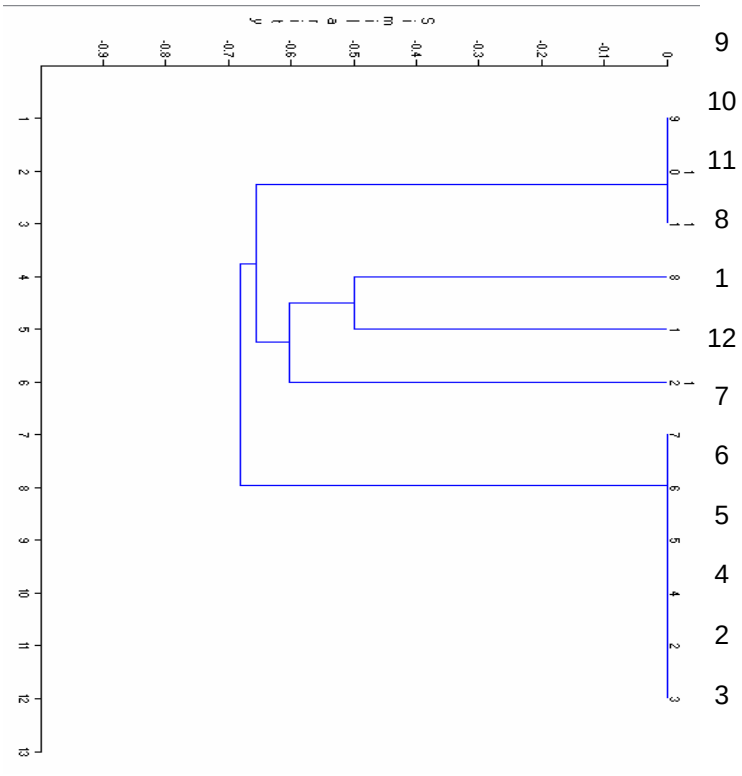

Figure 4. Genetic similarity among three sugarcane varieties CO413, PH 8013 and GT54-9 after *Agrobacterium tumefaciens* and *Bacillus thuringiensis* MD55 and its fusionts

Where:

1- CO413 (untreated calli).
2- CO413 infected with *Agrobacterium tumefaciens*
3- CO413 infected with *Bacillus thuringiensis*
4- CO413 infected with fusionts bacteria
5- PH 8013(untreated calli).
6- PH 8013 infected with *Agrobacterium tumefaciens*
7- PH 8013 infected with *Bacillus thuringiensis*
8- PH 8013 infected with fusionts bacteria
9- GT54-9 (untreated calli).
10- GT54-9 infected with *Agrobacterium tumefaciens*
11- GT54-9 infected with *Bacillus thuringiensis*
12- GT54-9 infected with fusionts bacteria

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التحول الوراثى لكالس قصب السكر باستخدام ناتج الاندماج البروتوبلاستى ما بين *Bacillus thuringiensis* و *Agrobacterium tumefaciens*

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يعتبر تكنيك واحد من اهم طرق التحول الوراثى التى تتميز بعدد من الخواص التى لا تتوافر فى باقى تقنيات الهندسة الوراثية المتعددة و من اهم هدة المميزات رخص تكلفتها و كفائها العالية. و تهدف هدة الدراسة الى ادخال الجين الخاص ببكتريا *Bacillus thuringiensis* و التى تعتبر من اهم بكتريا التربة المقاومة لعدد من الافات الزراعية مثل الثاقبات و الحشرة القشرية بما تنتجة من بروتينات سامة. و لادخال هدا الجين الهام تم عمل اندماج بروتوبلاستى بنجاح بين *Agrobacterium tumefaciens* المستخدمة كناقل فى الهندسة الوراثية و بكتريا *Bacillus thuringiensis* و قد بلغت نسبة نجاح هدا الاندماج حوالى ٩٠ %. ثم تم عمل العدوى لتحقيق التحول الوراثى المرغوب فى اكالس الخاص بالصنف C0 413, PH 8013و GT 54-9. بكل من البكتريا الابوية و الهجينة و يلى هدا فصل البروتين الكلى من عينات الكالس تحت الدراسة للتحقق من تاثير العدوى الخاصة بالبكتريا المرغوبة و لد نتج من هدة ادراسة التاد من حدوث التعبير الجينى للبروتين السام دو الوزن الجزيئى ١١٦ كيلو دالتون داخل الكالس الخاص بثلاثة اصناف عند العدوى بالبكتريا الهجينة بالاضافة لوجود عدد من الحزم البروتينية الاخرى دات الاوزان المختلفة. بالاضافة لهدا تم اسخدام تكنيك RAPD لدراسة اخلاف اطوال قطع من DNA الخاص بكل من العينا السابقة بعد مضاعفتها باربعة بادئات عشوائية و تم تقدير درجة القرابة الوراثية بين النباتات تحت الدراسة. و قد بلغت نسبة تعدد لاشكال المظهرية للقطع المتضاعفة للعينات تحت الدراسة ١٨ و ٢١ و ٥ و ٤٥ % للبادئات الاربعة على التوالى و يستدل من هدا ان البادىء رقم ٤ هو الاكثر كفاءة فى اظهار الاختلافات الوراثية الناتجة عن عدة اسباب منها عمليات زراعة الانسجة بالاضافة الى التحول الوراثى نتيجة العدوى.