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RESEARCH ARTICLE
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Genetic transformation of Acinetobacter sp.strain AZS1 bacteria by bacterial plasmids of Bacillus sp. strain JA Leaves and Bacillus sp. strain JA seed using conjugation technique.

Jihan Khalil Khuder[1] Amjad Abdul-Hadi Mohammed[1]
[1]Department of Biology, College of Science, University of Mosul, Mosul, IRAQ.
*Corresponding Author: jihan.23scp100@student.uomosul.edu.iq.

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ABSTRACT

This study used Bacillus sp. strain JA Leaves, Bacillus sp. strain JA seed and Acinetobacter sp.strain AZS1. The strains belonging to the genus Bacillus were sensitive to antibiotic 10 µg ml^{-1} gentamicin and had the ability to grow on solid nitrogen-free medium (NF) and the opposite was true for Acinetobacter bacteria, which were used as genetic markers to conduct the conjugation process. Two conjugation processes were performed, the first was between Bacillus sp. strain JA Leaves and Acinetobacter sp.strain AZS1 and the second process was between Bacillus sp. strain JA seed and Acinetobacter sp.strain AZS1. The results showed that conjugation succeeded through the growth of transconjugant bacteria on solid nitrogen-free medium supplemented with 10 µg ml^{-1} gentamicin. The frequency of conjugation was 2.3 x 10-2 and 1.9 x 10-2, respectively. In addition to the success of conjugation process, Acinetobacter sp.strain AZS1 and the two types of bacteria resulting from conjugation were able to infect the roots of clover seedlings through inoculation and form root nodules on the main root in different proportions depending on the type of bacterial suspension and the time of inoculation..

***Keywords*:** Conjugation technique, Bacillus sp., Acinetobacter sp., clover seedling inoculate.

INTRODUCTION

Horizontal gene transfer (HGT) is a process that involves the transfer of genetic material between similar or different species and genera [1]. Evidence of the occurrence of HGT has been found between prokaryotic organisms such as bacteria and even between distantly related eukaryotic organisms such as fungi and animals or between plants and fungi [2, 3]. There are three main mechanisms of horizontal gene transfer described in prokaryotic organisms: transformation, transduction, and conjugation [4]. Conjugation is considered the main mechanism responsible for the transfer of genetic material in bacteria and the emergence of multidrug resistance in hospital environments, water and soil environments, and others, mediated by the so-called fertility factor (F) [5].

Conjugation requires direct contact between conjugated donor cell (F+) and the recipient cell (F-) this connection between cells results in the transfer of genetic material in one direction (from the donor cell to the recipient cell) [6]. This transfer is carried out by forming a bridge that allows communication between the cellular cytoplasm of both cells, called the sex pilus or conjugation bridge [7]. The two cells are connected by sex filaments (Pili) formed on the wall of the donor cell, forming what is called the fertilization tube (pilus) or conjugation bridge, which can control in the conjugation process and through the conjugation bridge one of the two strands of double-stranded plasmid DNA is transferred to the recipient cell [8].

The researchers also pointed out the possibility of the transfer of antibiotic resistance plasmids from the *R. leguminosarum* bacteria to the A. tumefaciens bacteria, which led to the emergence of new genetic characteristics in the bacteria resulting from conjugation, in addition, the use of conjugation plasmids for various types of bacteria belonging to the *Agrobacterium* genus within the *Rhizobiaceae* family in Both *A. tumefaciens* and *A. radiobacter* and *A. rhizogenes* and *A. vitis*, as well as plasmids encoding opine, which is the virulence factor of this bacterium [9]. The researchers were also able to successfully conjugation between standard and wild *A. tumefaciens* bacteria with *S. meliloti* bacteria, as the paired bacteria succeeded in infecting the root tips of Alfalfa seedlings and forming root nodules [10].

The present study aimed at determining the possibility of performing genetic transformation between bacterial species and studying the ability of the conjugated bacteria to form root nodules on clover plants after inoculating their seedlings with the bacterial suspensions specified in this study.

MATERIALS AND METHODS

Source of bacterial strains

The *Bacillus* sp. strain JA Leaves**,** *Bacillus* sp. strain JA seed and *Acinetobacter* sp.strain AZS1 were previously identified and submitted to NCBI under the accession numbers PP213275.1, PP215359.1, ONO76417, respectively. Bacterial strain was kindly provided from plant tissue culture laboratory in Department of Biology/ College of Science / University of Mosul/ Iraq. Bacterial species belonging to *Bacillus* genus are distinguished by their sensitivity to the antibiotic gentamicin at a concentration of 10 µg ml^{-1} and their ability to grow on nitrogen-free medium. In contrast, the opposite is true for the recipient bacteria, *Acinetobacter* sp. strain AZS1.

Bacterial Conjugation

A Conjugation experiment was conducted according to method described by [11] between *Bacillus* sp. strain JA Leaves and *Bacillus* sp. strain JA seed that can grow on nitrogen-free media and is sensitive to gentamicin at a concentration of 10 µg ml^{-1}, each individually as donor cells, and bacteria *Acinetobacter* sp.strain AZS1, which cannot grow on solid nitrogen-free medium (NF) medium and is resistant to gentamicin at a concentration of 10 µg ml^{-1} as recipient cells. The conjugation process was carried out by taking a separate loopful from *Acinetobacter* sp.strain AZS1and placed in two eppendorf tubes separately. Each tube contained 750 µL of normal saline (NS) and mixed well for one minute with a vortex device to disintegrate the cells. Then, two loopful from *Bacillus* sp. strain JA Leaves were taken and placed in the first tube and two loopful from *Bacillus* sp. strain JA seed were placed in the second tube, then the tubes were mixed well with the vortex device for a minute and incubated for 24 houres at 28 °C. After the process, 100 µL of each mixture was taken individually and spread with a sterile glass L-shaped on a plate containing nutrient agar medium containing gentamicin 10 µg ml-1, as well as on a plate containing solid nitrogen-free medium (NF) and supplemented with the antibiotic gentamicin 10 µg ml^{-1}. The plates were incubated at 28°C (recipient temperature) for 24 hours for NA medium and 72 hours for NF medium.

Preparation of cell suspensions of bacterial species

Suspension of *Acinetobacter* conjugated with *Bacillus* sp. strain was prepared by taking a loop full of each bacterial type and placing it in a 100 ml glass beaker containing 20 ml of nutrient broth medium individually. The samples were incubated at 28°C for 24 hours in a shaking incubator (New Brunswick Scientific Co., Inc. Edison, N.J. USA) with a 120 rpm/ min. rotational speed [12].

Surface sterilization of *Trifolium* spp. Seeds

Seeds of *Trifolium* spp. plant were surface sterilized by soaking in ethanol 96% for 2 min. Floated in 3% sodium hypochlorite (NaOCl) while stirring for 5 min. Seed were finally rinsed every minute for three times with sterilized distilled water [13] Sterilized seeds were placed on the surface of solidified Murashige and Skoog medium (MS) [14]. Samples were maintained in the dark at room temperature. After the complete seedlings were produced, they were transported to the same conditions but in 1500 lux with 16 hours light / 8 hours dark.

Inoculating the roots system of *Trifolium* spp. seedlings with bacterial suspensions used in this study

fifty of the growing clover seedlings were entirely raised with 6 days from their medium they inoculated individually by immersion the root in a flask containing 20 ml of bacterial cell suspensions for 10, 20 and 30 min separately. The seedlings were dried using sterile filter papers and transferred to the surface a nitrogen free medium (NF) in a 9.0 cm plastic petri dish at a rate of 2-3 seedlings / plate. The plates were coated with parafilm and kept vertically in the growth incubator at 24 ± 2 °C under 16 hours light / 8 hours of darkness conditions and intensity of light 1500 lux, with covering the root system with black tape.

RESULTS AND DISCUSSION

Plasmid inclusion of *Bacillus* sp. strain JA Leaves and *Bacillus* sp. strain JA seed within *Acinetobacter* sp.strain AZS1 by conjugation technique

In (Table.1) showed the success of the transfer of the nitrogen fixation genes from the donors to the recipient, *Acinetobacter* sp. strain AZS1, in terms of their growth on the NF solid medium containing the antibiotic 10 µg ml^{-1} gentamicin.

Table 1: Bacterial conjugation between Bacillus sp. Strain JA Leaves and Bacillus sp. Strain JA seed (donor cells) and Acinetobacter sp.strain AZS1 (recipient cells)

Antibiotic resistance test (µg ml^{-1})			
Donor bacteria (µg ml^{-1})	Received bacteria (µg ml^{-1})	Conjugated (µg ml^{-1})	Conjugation frequency X 10^{-2}
Bacillus sp. strain JA Leaves $Gen^{S}_{(10)}$/ NF^{+}	*Acinetobacter* sp.strain AZS1 $Gen^{R}_{(10)}$/ NF^{-}	*Acinetobacter* $Gen^{R}_{(10)}$ / NF^{+}	2.3
Bacillus sp. strain JA seed $Gen^{S}_{(10)}$ / NF^{+}		*Acinetobacter* $Gen^{R}_{(10)}$ / NF^{+}	1.9

Both *Bacillus* sp. JA Leaves and *Bacillus* sp. JA seed sensitivity to the antibiotic 10 µg ml^{-1} Gentamicin and the ability to grow on solid nitrogen-free medium (NF).

Acinetobacter sp.strain AZS1 bacteria is resistant to the antibiotic 10 µg ml^{-1} Gentamicin and is unable to grow on solid nitrogen-free medium (NF).

Production of roots nodules on *Trifolium* spp. Seedlings

The results indicated the ability of the three bacterial species (*Acinetobacter* conjugated with *Bacillus* sp. strain JA Leaves, *Acinetobacter* conjugated with *Bacillus* sp. strain JA seed and *Acinetobacter* sp.strain AZS1) to infect the roots of clover seedlings when treated with them, and the success of root nodules formation at different rates depending on the type of bacterial suspension and incubation times (Table.2).

Table 2: Production of root nodules on the root seedling of Trifolium spp. after inoculated by different types of bacterial suspension

Bacterial types	Medium	Inoculation time (min.)	Number of seedlings infected	Percentage of infected seedling (%)	Number of nodules	Rate of Number nodes/seedling
Acinetobacter conjugated with *Bacillus* sp. strain JA Leaves	NF	10	28/50	56	58	2.0
		20	32/50	64	67	2.0
		30	35/50	70	82	2.3
Acinetobacter conjugated with *Bacillus* sp. strain JA seed		10	26/50	52	49	1.8
		20	30/50	60	59	1.9
		30	33 /50	66	71	2.1
Acinetobacter sp.strain AZS1		10	20/50	40	33	1.6
		20	23/50	46	41	1.7
		30	27/50	54	54	2.0

Number of seedlings inoculated: 50 for every treatment.

In general, the period of treatment 30 min. was the most prominent for the root nodules formation, whereas the numbers of nodules in *Acinetobacter* conjugated with *Bacillus* sp. strain JA Leaves were higher than their numbers in seedlings treated with *Acinetobacter* conjugated with *Bacillus* sp. strain JA seed and *Acinetobacter* sp.strain AZS1 bacteria, were responded to the formation of nodules at a rate of 2.3, 2.1 and 2.0 nodules/plant after 12, 15, and 16 days, respectively, and their shapes were elongated on the main root.

The appearance of the root hairs changed as a result of inoculation with bacteria, which is represented by their transformation from a straight shape to a curved shape (A from Fig. 1, 2, 3) which continued to grow and formed a spherical shape represented by the nodules in (B from Fig. 1,2,3). After two weeks, it has an elongated oval shape, it developed into a complete root. Root nodules formed on clover seedlings was thus successful (C Fig. 1, 2, 3).

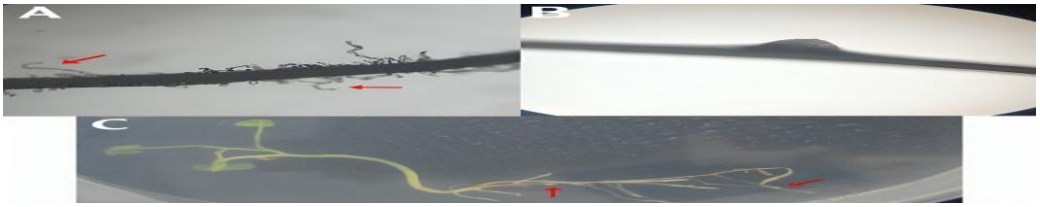

Fig. 1: Production of nodules on the clover seedling roots inoculated with Acinetobacter conjugated with Bacillus sp. strain JA Leaves

A: Root hairs malformed after 6 days of inoculation (arrows (and curling (like shepherd's hook (

B: Produced the primary root nodule after 7 days

C: Nodules on the main clover seedling root (arrows)

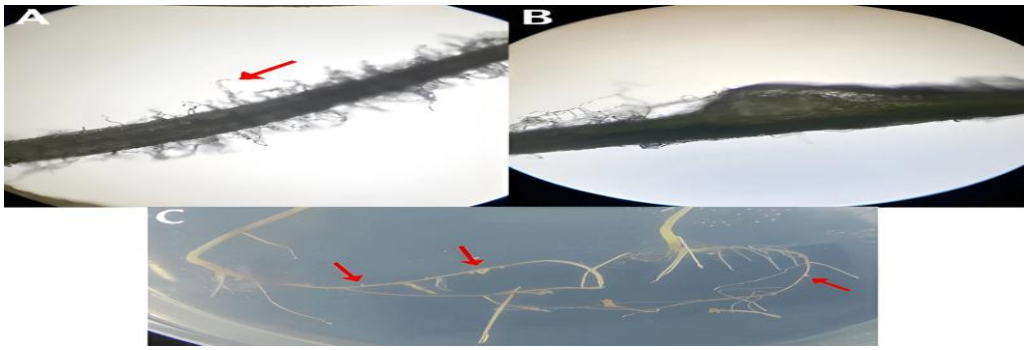

Fig. 2: Production of nodules on the clover seedling roots inoculated with Acinetobacter conjugated with Bacillus sp. strain JA seed

A: Root hairs malformed and curling after 7 days of inoculation (arrows) (like shepherd's hook)

B: Produced the primary root nodule after 11 days

C: Nodules on the main clover seedling root (arrows)

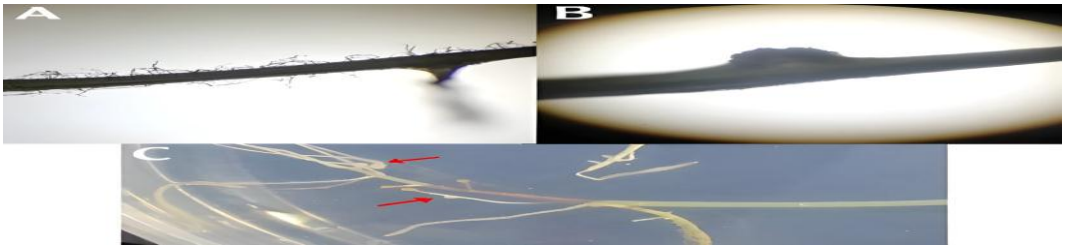

Fig. 3: Production of nodules on the clover seedling roots inoculated with *Acinetobacter* sp. strain AZS1

A: Root hairs malformed and curling after 9 days of inoculation

B: Produced the primary root nodule after 12 days

C: Nodules on the main clover seedling root

The formation of root nodules on clover seedlings inoculated with *Acinetobacter* conjugated with *Bacillus* sp. strain JA Leaves, *Acinetobacter* conjugated with *Bacillus* sp. strain JA seed and *Acinetobacter* sp.strain AZS1 represents the success of the symbiotic relationship between them [15].

The deformation of hairs and formation of root nodules of clover seedlings after treatment with the two conjugated bacteria confirm the transfer of *nif*H and pSym A plasmids from *Bacillus* sp. strain JA Leaves , *Bacillus* sp. strain JA seed and *Acinetobacter* sp.strain AZS1, to the two conjugated bacteria and these plasmids carried the genes responsible for nitrogen fixation [16] and nodules formation [17], and to transfer the plasmid pSym B encoding the Exo-polysaccharide compound [18] to the two conjugated bacteria, which are of great importance in the reproduction of bacteria within the roots of clover [19]. The infection thread forms inside the root hair and the infected cells divide to form root nodules [20].

A study indicated the possibility of transferring the symbiosis plasmid from the bacterium *Rhizobium Leguminosarum* bv. *trifolii* to *A.tumefaciens* bacteria and the success of the associated bacteria in forming nodules on the roots of clover plants [21].

This matches the results of this study, which are represented by the ability of two conjugated bacteria to form root nodules on clover seedling roots similar to the root nodules formed by *Acinetobacter* sp.strain AZS1 on clover plants. This indicates the success of transferring the symbiosis plasmid pSym from one of the bacterial groups within the conjugation process to them.This may also explain that the surfaces of the clover plant's root hairs have two types of receptors, the first of which is specialized in binding to the bacterium *R. leguminosarum bv. trifolii* and the second type enable the plant to associate with species other than *Rhizobium* [22].

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التحول الوراثي لبكتريا Acinetobacter sp.strain AZS1 بواسطة بلازميدات بكتريا Bacillus sp. strain JA Leaves و Bacillus sp. strain JA seed باستخدام تقنية الاقتران.

جيهان خليل خضر [1] امجد عبدالهادي محمد [1]

[1]قسم علوم الحياة، كلية العلوم، جامعة الموصل، الموصل، العراق.

الخلاصة

استخدمت هذه الدراسة كل من بكتريا *Bacillus sp. strain JA Leaves*، *Bacillus sp. strain JA seed* و *Acinetobacter sp.strain AZS1*، حيث كانت السلالات التابعة لجنس *Bacillus* حساسة للمضاد الحيوي 10 ميكروغرام/مل *Gentamicin* ولديها القدرة على النمو على الوسط الصلب الخالي من النيتروجين وكان العكس هو الصحيح بالنسبة لبكتريا *Acinetobacter*، والتي تم استخدامها كمعلمات وراثية لاجراء عملية الاقتران. تم إجراء عمليتين اقتران، الأولى كانت بين *Bacillus sp. strain JA Leaves* و *Acinetobacter sp.strain AZS1* والعملية الثانية كانت بين *Bacillus sp. strain JA seed* و *Acinetobacter sp.strain AZS1*. أظهرت النتائج نجاح عملية الاقتران من خلال نمو البكتريا المقترنة على الوسط الصلب الخالي من النيتروجين مضاف إليه المضاد الحيوي 10 ميكروغرام/مل *Gentamicin* وبتردد اقتران 2.3×10^{-2} و 1.9×10^{-2} على التوالي. كما نجحت بكتريا *Acinetobacter sp.strain AZS1* ونوعي البكتريتين المقترنتين بإصابة المجموع الجذري لبادرات نبات البرسيم بواسطة التلقيح وتكوين العقد الجذرية على الجذر الرئيسي وبنسب مختلفة اعتمادا على نوع اللقاح البكتيري وزمن التلقيح.

الكلمات المفتاحية : تقنية الاقتران، *Bacillus sp.*، *Acinetobacter sp*. ، تلقيح بادرات البرسيم.