TRANSFERRING *NOD*D2, *NOD*D3 GENES FROM *RHIZOBIUM LEGUMINOSARUM*, *NIF*H2 AND *NIF*H3 GENES FROM *AZOTOBACTER CHROOCOCCUM* TO *BACILLUS MEGATERIUM*

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Abstract. The present study aimed to transfer *nod* and *nif* genes from *Rhizobium* and *Azotobacter* to *Bacillus* by: the transformation technique which used to transfer *nod*D2 and *nod*D3 from *Rhizobium leguminosarum* to *Bacillus megaterium*; and the conjugation mechanism which performed the transfer of *nif*H2 and *nif*H3 from *Azotobacter chroococcum* to transformant *Bacillus megaterium*. The results showed that: 12 colonies were obtained after transformation on Sperber's agar plates which contained tetracycline and ampicillin. While after conjugation; 166 colonies were obtained on Sperber's agar plates which contained ampicillin and erthymycinem, indicating that the transformation and conjugation were successful. To confirm this, a molecular study was performed based on the followings: the extraction of the plasmid DNA from *Rhizobium leguminosarium* and transformant *Bacillus megaterium* to detect *nod*D genes; and the extraction of the genomic DNA from *Azotobacter chroococcum* and transformant-conjugant *Bacillus megaterium* to detect *nif*H genes by PCR and gel electrophoresis. The PCR products on gel electrophoresis showed that *Rhizobium leguminosarium* and transformant *Bacillus megaterium* contained *nod*D2 and *nod*D3; and *Azotobacter chroococcum* and transformant-conjugant *Bacillus megaterium* contained *nif*H2 and *nif*H3. Furthermore, the new *Bacillus megaterium* which obtained these genes successfully, can be used as biofertilizer for nitrogen fixation and phosphorus solubilization at the same time.
Keywords: *nod gene, nif gene, DNA transferring, PCR, Gel electrophoresis*

Introduction

Rhizobium and *Azotobacter* interact with a wide range of other soil microorganisms in the rhizosphere of plants. These interactions are sometimes stimulatory where they increase the growth response of the host in the presence of other microorganisms (Kennedy and Islam, 2001; Nosheen et al., 2011). *Azotobacter* and *Rhizobium* are known to be good non-symbiotic and symbiotic nitrogen fixers, respectively (Siddiqui et al., 2014). This biological nitrogen fixation process in *Azotobacter*, and between *Rhizobium* strains and their legume partners can happen under low levels of available nitrogen with the help of many different genes such as *nod*, *nif*, *fix*, production of polysaccharides, competition, infection process, and host specificity (Shamseldin, 2013). *Rhizobium nod* genes, and their product, *Nod* Factor (NF), have been recognized as essential for the development of nitrogen-fixing nodules on legume roots (Lerouge et al., 1990). *Nod*D is present in all *rhizobia* (Shamseldin, 2013). *Nod*D is the core signaling protein, reacting to

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plant flavonoids then binding to nod boxes, binding sites upstream of nod genes, typically *nod*A and/or *nod*B, trigger the expression of a *nod* gene cascade and thus the construction of the *Nod* Factor (Jones et al., 2007). The *nif* genes are able to fix nitrogen in both the free-living and symbiotic states, and the *nif* genes can transcript in both free-living and symbiotic diazotrophs (Dixon and Kahn, 2004). The expression and regulation of *nif* genes, while sharing common features in all or most of the nitrogen-fixing organisms in nature, have distinct characteristics and qualities that differ from one diazotroph to another (Spaink et al., 1998). The structural gene *nif*H, as an important *nif* gene, is involved in the formation of the Fe-protein complex (Cocking, 2003), and *nif*H is the gene that encodes the iron protein subunit of nitrogenase, which is highly conserved among all nitrogen-fixing groups and serves as an ideal molecular marker for these microorganisms (Deslippe and Egger, 2006). *Bacillus megaterium* has the ability to solubilize phosphorus, which is good for the plant (Velineni and Brahmaprakash, 2011). *Bacillus megaterium* produces organic compounds such as lactic acid, gluconic acid, citric acid, succinic acid, propionic acid and enzymes that help solubilize the fixed phosphorus into exchangeable form (Agrilife, 2008).

In the bacterial population, DNA can be transferred from one organism to another by the horizontal transfer mechanism. The DNA thus transferred by the lateral/horizontal method can be stably incorporated in the recipient, and changes the genetic composition of the recipient permanently. Three broad mechanisms mediate efficient movement of DNA between cells: transformation, conjugation, and transduction (Acharya, 2013). Bacterial plasmid transformation is a process by which genetic material, often a plasmid, is inserted into a bacterial cell. Though transformation does occur naturally, scientists have developed many techniques to ensure DNA uptake by bacterial cells (Yoo, 2010). Genetic transformation occurs when a cell takes up (takes inside) and expresses a new piece of genetic material—DNA, and it literally means change caused by genes and involves the insertion of one or more gene(s) into an organism in order to change the organism's traits, this new genetic information often provides the organism with a new trait which is identifiable after transformation (www.explorer.bio-rad.com). Transformation takes place to a limited extent in many bacteria. But laboratory techniques have been developed that increase the rate of DNA uptake. (www.kullabs.com). Bacterial transformation is a process which involves genetic alteration of bacteria by the incorporation and stable expression of foreign genetic material from the environment or surrounding medium. Since DNA is a very hydrophobic molecule, it will not normally pass through a bacterial cell membrane. In order to uptake foreign DNA, the bacterial cells must first be made competent. Competence is the ability of a cell to take up extracellular DNA from its environment (www.himedialabs.com. a). In bacteria, the haploid genome is a single circular chromosome. This differs from eukaryotic genomes like those of plants and animals, where the genetic material is diploid and arranged into linear chromosomes. Bacteria can also possess additional nonessential pieces of circular DNA called plasmids (www.towson.edu/cse). Plasmids are small circles of DNA that contain an origin of replication (ori) and a small number of genes, some of which may confer a survival advantage on a host. Some plasmids can transfer between different species; even between different kingdoms (Slonczewski, 2006). Plasmid transformation into bacterial competent cells is a key technique in molecular cloning (Tu et al., 2005).

Many bacteria from different species frequently exchange their genetic materials by a process called conjugation, which occurs by cell-to-cell contact (Fernandez-Lopez et al.,

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2005). Conjugation is a recombination process where two live bacteria come together, and the donor cell transfers genetic material to the recipient cell. Conjugating bacteria are of two mating types. Certain "male" types (designated as F+) donate their DNA, and other "female types" (designated as F-) receive the DNA, F- cells become F + when they acquire a small amount of DNA. Hence the F factor is called as the Fertility factor. In contemporary microbiology, the donor's F factors are known to be plasmids which are extra-chromosomal elements. The factors (plasmids) contain about 20-30 genes, most of which are associated with conjugation. These genes encode enzymes that replicate DNA during conjugation and structural proteins needed to synthesize special pili at the cell surface which known as F pili or sex pili, these hair-like fibers contact the recipient bacteria, and then retract so that the surfaces of donor and recipient are very close or touching one another. At the area of contact, a channel or conjugation bridge is formed. Once contact via sex pili has been made, the F factor (plasmid) begins replicating by the rolling circle mechanism. A single strand of the factor then passes over or through the channel to the recipient. When it arrives, enzymes synthesize a complementary strand, and a double helix is formed. The double helix bends to a loop and reforms an F factor (plasmid), thereby completing the conversion of the recipient from F- cell to F + cell. Meanwhile, back in the donor cell a new strand of DNA forms, to complement the leftover strand of the F plasmid. The transfer of F factors involves no activity of the bacterial chromosome; therefore the recipient does not acquire new genes other than those on the F factor (www.himedialabs.com).

Transformation process holds key step in molecular biology (Das et al., 2017), and it has been a handy tool in several areas of bacterial research because the genotype of a strain can be deliberately changed in a very specific way by transforming with an appropriate DNA fragment. For example, transformation is used widely in genetic engineering (Pimda and Bunnag, 2010). The goals of gene transfer experiments with other organisms are to study gene regulation and to obtain stable inheritance and expression of new characteristics (Moses, 1987).

Conjugation gene transfer is considered to be an important mechanism for the establishment of new genetic traits in diverse environments (Hausner and Wuertz, 1999). Conjugation is used in nature to share beneficial genetic material between bacteria, such as antibiotic resistance. However, manually inserting genes into the F-plasmid would allow for scientists to have bacteria transfer almost any gene to other cells (Griffiths et al., 2000).

Materials and methods

The country and location of the study

The present study was performed in Sulaimani city/Iraq. All bacteria were isolated from the organic farm of Bakrajow, and the molecular studies were done at the laboratories. The farm and the laboratories are located inside the College of Agricultural Sciences, University of Sulaimani.

Isolation of bacteria

Rhizobium leguminosarium spp. were isolated from healthy, unbroken, firm and pink nodules of Broad bean from the organic farm of Bakrajow using YMA medium in accordance with Agrawal et al., 2012, while *Azotobacter chroococcum spp.* were

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isolated from Bakrajow soil on modified Ashby's medium according to Marwa et al., 2010, and *Bacillus megaterium* was isolated from same soil using Sperber's medium based on Shiva et al., 2010.

Purification of isolated bacteria

A loop of actively growing of each bacterial species (48 h old surface film) was streaked on their selective media, the growth was observed depending on the type of bacteria, *R. leguminosarium* and *B. megaterium* after 24-48 h and *A. chroococcum* after 3-7 days at 28 °C of incubation. The well separated and apparently un-contaminated colonies appearing on the plates were streaked on agar medium, plating and picking were repeated at least 4-5 times.

Maintenance and storage of bacterial culture

The cultures were maintained for a short time at slant medium, and for a long time in 20% glycerol and stored at -70 °C (Ausabel et al., 2003) without losing their activity.

Identification of bacteria by molecular protocol

Depending on the molecular protocol, *B. megaterium* was identified by the extraction of genome DNA using PrestoTM Mini gDNA Bacteria Kit Protocol to detect and check the presence of *B. megaterium* in the region by two random primers, *R. leguminosarium* was identified by the extraction of plasmid DNA using Genetbio, PrmePrep Plasmid DNA isolation kit to detect the *nod*D2 and *nod*D3 genes which we wanted to transfer to the *B. megaterium* by the transformation process, and *A. chroococcum* was identified by extraction of genomic DNA using PrestoTM Mini gDNA Bacteria Kit Protocol to detect the *nif*H2 and *nif*H3 genes which we wanted to transfer to the transformant *B. megaterium* by the conjugation process using specific primers for each gene in order to obtain a new model of the *B. megaterium* which would contain both of the *nod* and *nif* genes and then could be used as bio-fertilizers to provide available nitrogen and phosphorus to plants.

PCR amplification conditions

The conditions of PCR amplification: for *nod*D2 and *nod*D3 were performed according to the modified method of Del Cerro et al., 2015a at cycling conditions consisted of a single cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 2 min, 60 °C for 30 s and 72 °C for 30 s, and a final extension cycle at 72 °C for 6 min, while PCR reactions for *nif*H2 and *nif*H3 were done in accordance with Setubal et al., 2009), and for random primers 1 and 2 of *B. megaterium* was carried out in accordance with Patil et al., 2013). The forward, reverse and random primers are shown in *Table 1*.

Gel electrophoresis (Helmut et al., 2004)

Gel electrophoresis was used to identify the extraction of each of genome DNA, chromosomal DNA, and plasmid DNA, and it was also used to check the DNA amplification of each product by dissolving 0.5, 1, 1.5 and 2% (w/v) of agarose gel depending on the size of amplified DNA in 100 ml (IX) TBE buffer by heating it in a micro-wave oven for 3 min, then cooling the solvent to 45 °C at room temperature. The gel was stained with 5 µl of 5 $mg.ml^{-1}$ ethidium bromide, and the gel was soaked in a

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gel tank containing TBE. The gel tank was covered by a lid, and electrophoresis was run at (80 to 100 V) for 2-3 h depending on the product. DNA fragments were visualized at 312 nm with a UV- transilluminator image.

Table 1. *The forward, reverse and random primers used*

Primer	Sequence (5'-3')	Nucleotide	Reference
*nod*D2- F-	(GTA GGC CAT AAT GTC CAG A)	19	Del Cerro et al., 2015a
*nod*D2- R-	(GCG GCT TTA TAC TCA CCA)	18	
*nod*D3- F-	(GAG CTA CCT CGA CTG CTA)	18	Del Cerro et al., 2015b
*nod*D3- R-	(CTA CCG CCA TGA TCA CCA)	18	
*nif*H2- F-	(CGCCGGCGCAGTGTTTGCGG)	20	Setubal et al., 2009
*nif*H2 -R-	(CACTCGTTGCAGCTGTCGGC)	20	
*nif*H3- F-	(CGATGACTGAAGACTGAACGAG**)**	22	Setubal et al., 2009
*nif*H3 -R-	(AAGGTGCGGTCAGGAGAGAA)	20	
Random primer1	(GGT GCG GGA A)	10	Patil et al., 2013
Random primer2	(GTA GTC ATA T)	10	

Antibiotics resistance test

Table 2 shows the 8 common antibiotics including Chloramphenicol, Gentamycin, Tetracycline, Ampicillin, Cefotaxim, Erthymycine, Streptomycin and Rifampicin which were used for testing the sensitivity of: *R. leguminosarium* and *B. megaterium* for transformation technique, and transformant *B. megaterium* and *A. chroococcum* for conjugation process.

Table 2. *The antibiotics used in accordance with Shoukry et al., 2013; American Type Culture Collection, 2013; Giacopello et al., 2016*

Antibiotic	Recommended stock concentration	Recommended working concentration
Chloramphenicol	10 mg/ml in ethanol	25 µg/mL
Gentamycin	10 mg/ml	10 µg/mL
Tetracycline	10 mg/ml in (50% D.W + 50% ethanol)	10 µg/mL
Ampicillin	10 mg/ml in water	20 µg/ml
Cefotaxime	10 mg/ml in water	30 µg/ml
Erthymycine	10 mg/ml in water	15 µg/ml
Streptomycin	10 mg/ml in water	10 µg/ml
Rifampicin	10 mg/ml in methanol	5 µg/ml

Preparation of competent cells from B. megaterium

A modified method of Sambrook et al., 1989 was used for the preparation of competent cells of *B. megaterium* for transformation. An overnight culture of *B. megaterium* was prepared by suspending a colony from a fresh Sperber's plate in 100 ml of Sperber's broth medium. The culture was incubated in a shaking incubator at 100 rpm for 24 h at 28 °C, then 10 ml of this culture was suspended in 90 ml of

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fresh Sperber's broth and grown at 28 °C for 90 min in a shaking incubator until an OD_{600} nm of approximately 0.3-0.6 was attained, then 10 ml aliquots was centrifuged at 4000 Xg for 10 min at 4 °C to pellet the cells. After that, the supernatant was discarded and cells were re-suspended in 10 ml of 0.1 M $CaCl_2$ over 1 h on ice, and re-centrifuged twice, following the third washing with 5 ml 0.1 M $CaCl_2$. Finally the cells were re-centrifuged and gently re-suspended in 2 ml of ice-cold 0.1 M $CaCl_2$ containing 20% glycerol. The cells could be used immediately for transformation or stored at -20 °C.

Bacterial transformation (Ausabel et al., 2003)

A total of 200 µl of competent cells (*B. megaterium*) were mixed with 2 µl of the extracted plasmid from *R. leguminosarium* in a tube, and the tube was left on ice for 30 min. After that the tube was incubated for 30 s at 42 °C in a water bath and then placed on ice for 5 min, then 800 µl of Sperber's broth was added and the tube was incubated at 28 °C for 1 h. Then the cells were centrifuged at 6000 Xg for 1 min. Next, the pellet was re-suspended in 200 µl of Sperber's broth. A total of 100 µl of the cells were plated out on Sperber's agar medium containing two antibiotics which were used as genetic markers. After post-incubation at 28 °C for 28 h the colonies were screened, then the molecular study was performed using PCR and gel electrophoresis techniques for detection of transferred *nod*D2 and *nod*D3 genes in transformant *B. megaterium* that transferred by transformation process.

Bacterial conjugation (Olsen et al., 1992)

A 10 ml of modified Ashby's broth medium was inoculated with a single colony of *A. chroococcum* (donor cell), and 10 ml of Sperber's broth medium was inoculated with a single colony of transformant *B. megaterium* (recipient cell), then they were incubated at 28 °C for 48 h. with shaking at 100 rpm. After incubation, 0.8 ml of the *A. chroococcum* (donor cells) were mixed with 0.2 ml of transformant *B. megaterium* (recipient cells) and 1 ml of Sperber's broth, the mixture was incubated at 28 °C for 3 h under aerobic condition. Then 0.1 ml of the conjugated mixture was spread on Sperber's agar medium plates containing two antibiotics that were used as genetic markers, and control plates were prepared by spreading 0.1 ml for each donor and recipient suspensions separately on agar plates containing the same markers, then all plates were incubated at 28 °C till the colonies appeared. To verify the transfer of nitrogen fixation genes (*nif* genes) from *A. chroococcum* to transformant *B. megaterium*, the transformant-conjugant colonies were screened, then the molecular study was performed using PCR and gel electrophoresis techniques for the detection of transferred *nif*H2 and *nif*H3 genes, that were transferred by conjugation process, in transformant-conjugant *B. megaterium*.

Results

DNA isolation from bacteria

The DNA was isolated from bacterial cultures, and the molecular method based on the extraction of the genomic DNA from *A. chroococcum* and *B. megaterium* and plasmid DNA from *R. leguminosarium*, which were detected by agarose gel electrophoresis, the results are shown in *Figure 1* lane 2, 4, 6 respectively.

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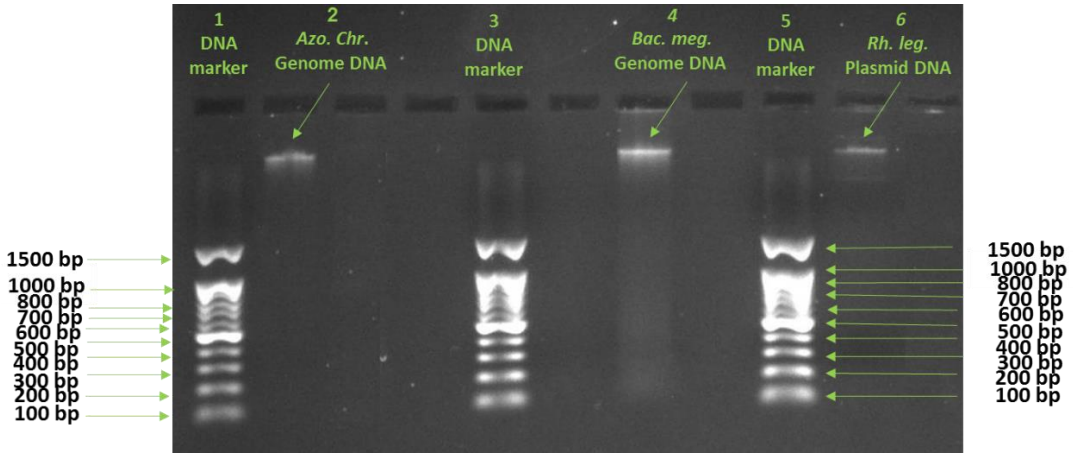

Figure 1. *Agarose gel electrophoresis shows DNA marker lanes 1, 3, and 5, extracted chromosomal DNA from A. chroococcum, genome DNA from B. megaterium and plasmid DNA from R. leguminosarium lanes 2, 4 and 6, respectively*

Molecular based identification of Bacillus megaterium

The PCR amplification was performed to check the presence of *B. megaterium* in the region using two random primers. The PCR products showed high amplification rate and reproducible banding pattern which confirmed the existence of *B. megaterium* (*Fig. 2*, lanes 2 and 3).

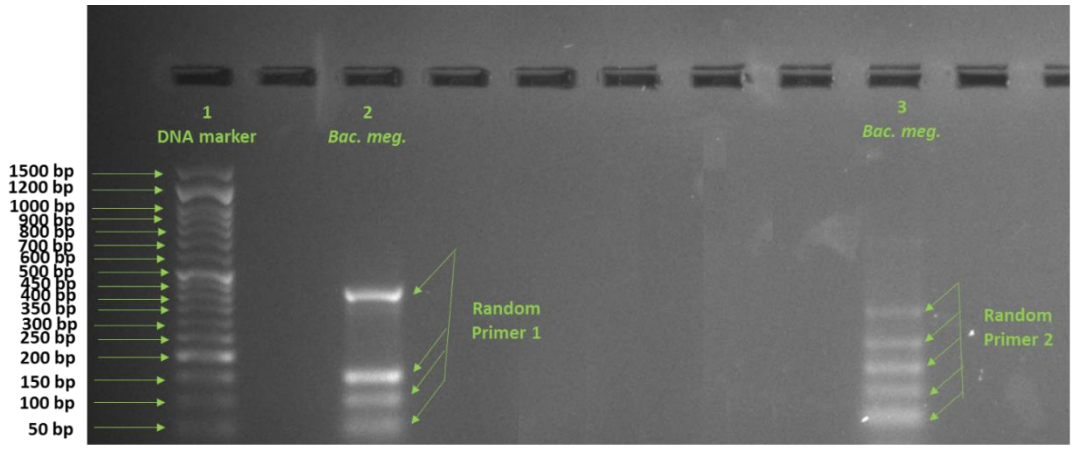

Figure 2. *Agarose gel electrophoresis shows the PCR amplified products of the B. megaterium generated using 10-mer random primers: Lane 1: DNA marker, lanes 2 and 3: random primers 1 and 2 respectively (+ve PCR products)*

Antibiotics resistance of R. leguminosarium, B. megaterium, transformant B. megaterium and A. chroococcum

The resistance screening of *R. leguminosarium, B. megaterium*, Transformant *B. megaterium,* and *A. chroococcum* was evaluated for eight antibiotics that are widely used. Depending on the results in a *Table 3*, tetracycline and ampicillin were used as genetic markers for transformation experiment, while ampicillin and erthymycine were used as genetic markers for the conjugation experiment.

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Table 3. *Antibiotics Resistance of R. leguminosarium, B. megaterium, Transformant B. megaterium and A. chroococcum*

Antibiotic	*R. leguminosarium*	*B. megaterium*	Transformant *B. megaterium*	*A. chroococcum*
Chloramphenicol	R	S	R	R
Gentamycin	R	S	R	S
Tetracycline	S	R	R	R
Ampicillin	R	S	S	R
Cefotaxim	R	S	R	S
Erthymycine	R	R	R	S
Streptomycin	R	S	R	S
Rifampicin	R	S	R	R

S = Sensitive R = Resistance

Transformation of plasmid genes (nodD2 and nodD3) from R. leguminosarium to B. megaterium by transformation process

Transformation experiment was carried out to transfer the genes responsible for the nodulation (*nod*D genes) which were *nod*D2 and *nod*D3 from *R. leguminosarium* to *B. megaterium.* After the transformation, 12 colonies were gained on Sperber's medium supplied with tetracycline and ampicillin as genetic markers and the colonies were resistant to both antibiotics. These results confirmed that the resulting colonies were transformant colonies and the transformation technique was successful between *R. leguminosarium* and *B. megaterium*. To confirm if *nod*D2 and *nod*D3 genes transferred from *R. leguminosarium* to *B. megaterium* by transformation technique, the molecular study was performed using specific primers to detect the *nod*D2 and *nod*D3 genes in transformant cells and compared to the *nod*D2 and *nod*D3 genes in *R. leguminosarium* by PCR technique and gel electrophoresis.

PCR amplification of nodD2 and nodD3

After extraction and purification of plasmid DNA from *R. leguminosarium*, *B. megaterium* and transformant *B. megaterium* using the Genetbio, PrmePrep Plasmid DNA isolation kit according to the manufacturer's protocol, PCR and gel electrophoresis were carried out in order to amplify and check the existenc of *nod*D2 and *nod*D3 in the plasmid DNA of *R. leguminosarium, B. megaterium* and transformant *B. megaterium* using specific oligonucleotide primers that flank the DNA sequence to be amplified.

Figure 3 shown positive PCR products on gel electrophoresis in *R. leguminosarium* and transformant *B. megaterium* which contained *nod*D2-F- and *nod*D2-R- primers with 100 bp in lanes 2 and 4, respectively, while in *Figure 4*, the results show the positive amplification on gel electrophoresis of *nod*D3 in *R. leguminosarium* and transformant *B. megaterium* with 150 bp in lanes 2 and 4, respectively using *nod*D3-F- and *nod*D3-R- primers, but the negative control and *B. megaterium* were negative PCR products and they did not produce bands, which can be seen in lanes 3 and 5, respectively in *Figures 3* and *4*.

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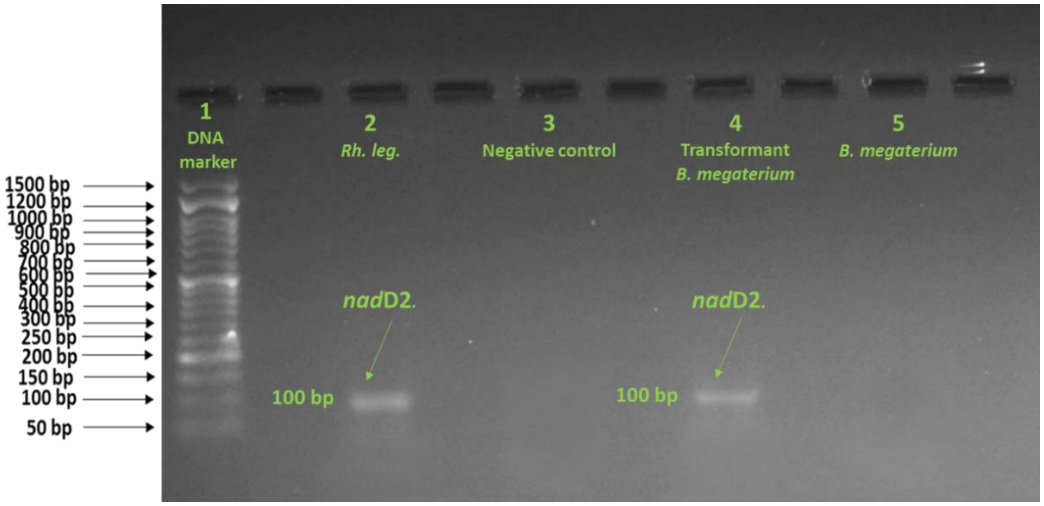

Figure 3. *Agarose gel electrophoresis shows the PCR amplified products of the nodD2 gene (100 bp). Lane 1: DNA marker, lane 2: R. leguminosarium (+ve PCR product), lane 3: negative control (-ve PCR product), lane 4: transformant B. megaterium (+ve PCR product) and lane5: B. megaterium (-ve PCR product)*

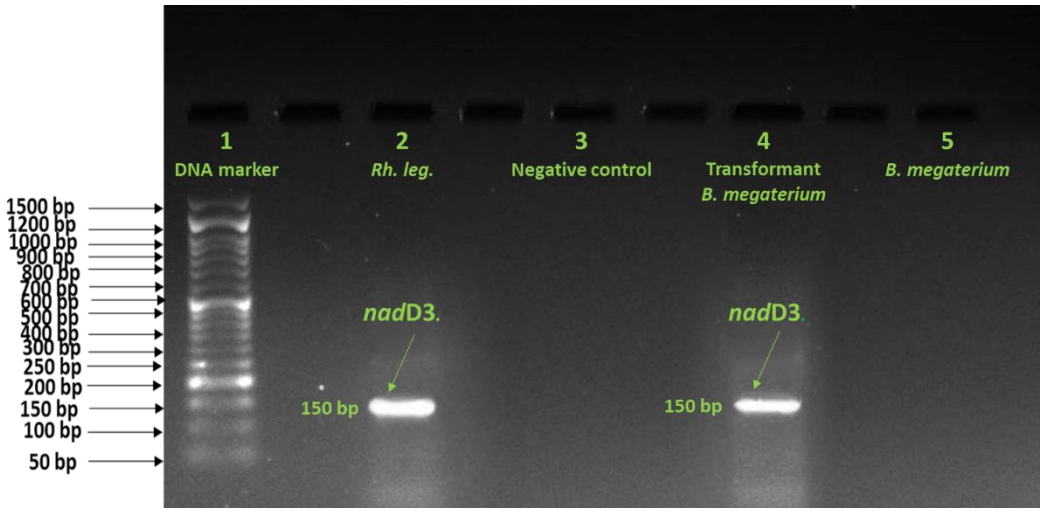

Figure 4. *Agarose gel electrophoresis shows the PCR amplified products of the nodD3 gene (150 bp). Lane 1: DNA marker, lane 2: R. leguminosarium (+ve PCR product), lane 3: negative control (-ve PCR product), lane 4: transformant B. megaterium (+ve PCR product) and lane5: B. megaterium (-ve PCR product)*

Transferring of chromosomal genes (nifH2 and nifH3) from A. chroococcum to transformant B. megaterium by conjugation process

Conjugation experiment was done between *A. chroococcum* as donor cells and transformant *B. megaterium* as recipient cells to investigate the transference ability of nitrogen fixation genes (*nif*H genes) which were *nif*H2 and *nif*H3 from isolated *A. chroococcum* to transformant *B. megaterium*. *A. chroococcum* was successful in conjugation technique with transformant *B. megaterium*, and (166) transformant-conjugant colonies were obtained on Sperber's agar plates containing ampicillin and

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erthymycine. In order to confirm the mobilization of nitrogen fixation genes (*nif*H2 and *nif*H3) from donor to recipient, a molecular study using PCR technique and gel electrophoresis was done for detecting these genes in each of *A. chroococcum, B. megaterium,* and transformant-*conjugant B. megaterium.*

PCR amplification of nifH2 and nifH3

After extraction and purification of genomic DNA from *A. chroococcum, B. megaterium* and transformant-conjugant *B. megaterium* using the PrestoTM Mini gDNA Bacteria Kit according to the manufacturer's protocol, PCR was performed for amplification of *nif*H2 and *nif*H3 via the use of specified oligonucleotide primers that flanked the DNA sequence to be amplified, and gel electrophoresis was carried out in order to check the presence of *nif*H2 and *nif*H3 in the chromosomal DNA of *A. chroococcum, B. megaterium,* and transformant-conjugant *B. megaterium*.

Results in *Figure 5* indicated that *A. chroococcum* and transformant-conjugant *B. megaterium* samples which contained *nif*H2-F and *nif*H2-R primers exhibit positive PCR products of *nif*H2 on the gel in lanes 2 and 4 respectively with (250 bp). Also the results in *Figure 6* indicated that the gel electrophoresis shows positive amplification of *nif*H3 (130 bp) in *A. chroococcum* and transformant-conjugant *B. megaterium* samples using *nif*H3 -F- and *nif*H3 -R- primers and they produced clear bands in lanes 2 and 4, respectively, while *B. megaterium* and negative control were shown negative PCR products in lanes 3 and 5, respectively and they did not produce bands in *Figures 5* and *6*.

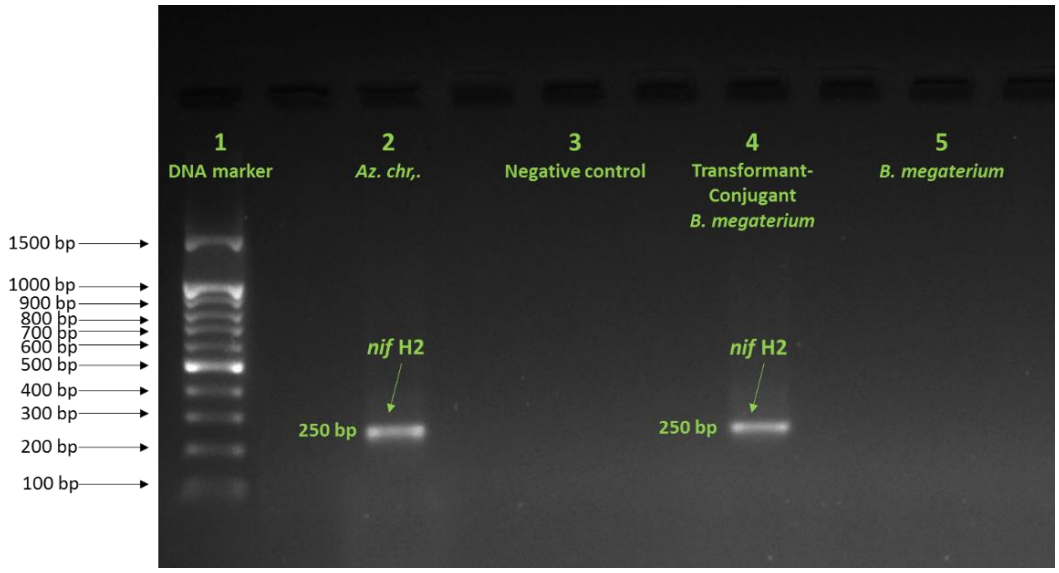

Figure 5. *Agarose gel electrophoresis shows the PCR amplified products of the nifH2 gene (250 bp). Lane 1: DNA marker, lane 2: A. chroococcum (+ve PCR product), lane 3: negative control (-ve PCR product), lane 4: transformant-conjugant B. megaterium (+ve PCR product) and lane5: B. megaterium (-ve PCR product)*

Discussion

This research work was carried out to find out the transference ability of *nod*D2 and *nod*D3 genes from *R. leguminosarium* by transformation technique, and *nif*H2 and

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*nif*H3 genes from *A. chroococcum* by conjugation process, to the *B. megaterium* in order to obtain a new model of *B. megaterium* which can then be used as a biofertilizer that provides available nitrogen and phosphorus for plants. *Nod*D genes act as a family of regulatory genes (Kidaj and Wielbo, 2010). The *nod* genes are controlled by *nod*D genes, *nod*D proteins act as transcriptional activators of inducible nod genes (Kumari and Sinha, 2011). *Nif*H acts as dinitrogenase reductase, obligate electron donor to dinitrogenase during dinitrogenase turn over and are required for FeMo-Co biosynthesis and apodinitrogenase maturation (Shamseldin, 2013). The region of chromosome which contain *nifK, nifD, nifM, nifA, nifN, nifB, nifQ, nifZ, nifP, nifF, nifW, nifB, nifL* and *nifY* genes are located between the fragment of chromosome which contains *nif*H1, *nif*H2, *nif*H3 and the fragment containing *nif*V, *nif*S and *nif*U (Hamilton et al., 2011). So the importance of the *nod*D genes which are located on the plasmid, and *nif*H genes which are located between fragments of chromosomal DNA, and their role in nitrogen fixation process gave importance to our choice of these genes for this study.

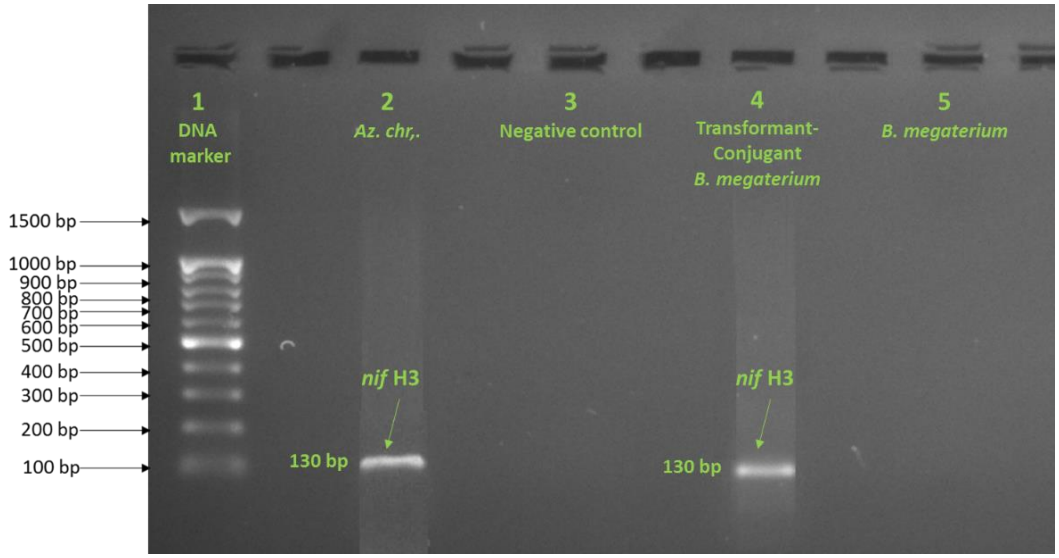

Figure 6. *Agarose gel electrophoresis shows the PCR amplified products of the nifH3 gene (130 bp). Lane 1: DNA marker, lane 2: A. chroococcum (+ve PCR product), lane 3: negative control (-ve PCR product), lane 4: transformant-conjugant B. megaterium (+ve PCR product) and lane5: B. megaterium (-ve PCR product)*

Firstly, *R. leguminosarium, A. chroococcum* and *B. megaterium* were isolated and identified by classical and molecular approach, classical approach included cultural, morphological and biochemical tests (the results did not show), while molecular characterizations of isolated bacteria based on the detection of genomic DNA of *B. megaterium* and *A. chroococcum* and the plasmid DNA of *R. leguminosarium*, have been successfully applied (*Fig. 1* lanes 2, 4, 6 respectively), and PCR technique and gel electrophoresis were carried out to detect and check the presence of *B. megaterium* by using two random primers 1 and 2. The banding patterns were intense, clear and reproducible which confirmed the existence of *B. megaterium* in the region (*Fig. 2*) lanes 2 and 3. Previous studies by Shiva et al. (2010) and Patil et al. (2013) indicated the presence of *B. megaterium* in a different area by these primers. Also the molecular study to identify *R. leguminosarium* was depended on the detection of the *nod*D2

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*nod*D3 on the plasmid using specific complementary primers by PCR technique and gel electrophoresis, because nodulation genes (nod genes) are located on the plasmid which is also called mega plasmid. Kumari and Sinha, 2011; Giraud et al., 2007 indicated that the common nodulation genes (*nod* ABCD) are found in all bacteria that nodulate legumes, and Black et al. (2012) reported that all fourteen species of *Rhizobium* contained *nodD*, as well Rossen et al. (1985) showed and confirmed the presence of *nod*D in *R. leguminosarium*, and for the detection of the *nif*H2 and *nif*H3 in the *A. chroococcum* using specific complementary primers to amplify them, PCR and gel electrophoresis were carried out after extracting the genomic DNA of the *A. chroococcum*. Previous studies by Dashti (2011), Khider (2012), Abid (2013) and Mohamed (2017) proved the existence of different *nif* H genes in *A. chroococcum* in some regions of Kurdistan. Iraq.

The transformation technique was based on the extraction of the plasmid from *R. leguminosarium* and preparation of the competent cells from *B. megaterium* in order to transfer *nod*D2 and *nod*D3 genes from *R. leguminosarium* to *B. megaterium*, The process succeeded when 12 colonies were gotten on Sperber's agar plates containing tetracycline and ampicillin which were used as genetic markers, these transformant colonies may be due to transferring the plasmid DNA from *R. leguminosarium* to *B. megaterium*, to confirm that, PCR technique and gel electrophoresis were carried out to amplify and detect the presence of the *nod*D2 and *nod*D3 genes in each of *R. leguminosarium*, *B. megaterium* and transformant *B. megaterium* using specific complementary primers after extracting the plasmid DNA from them. The results presented in *Figures 3* and *4* showed positive PCR products of *nod*D2 with 100 bp and *nod*D3 with 150 bp for *R. leguminosarium* in lane 2 and transformant *B. megaterium* in lane 4, and negative PCR products for negative control in lane 3 and *B. megaterium* in lane 5 and they did not produce any bands, these results indicated that the plasmid transferred to transformant bacteria by transformation process. Mohamed (2017) also transferred *nod*C from *R. leguminosarium* to *B. megaterium* by the transformation technique and proved the success of the process between these two bacteria.

Based on these results, the conclusion is that the plasmid with all the *nod* genes (*nod*A, *nod*B, *nod*C *nod*D, *nod*E, nodF, *nod*G, *nod*I, *nod*M, *nod*P, and other *nod* genes) were transferred to the *B. megaterium* and all of them expressed and encoded for nitrogen fixation process in transformant cells, because all of them were located on the plasmid DNA. Plasmid transformation into bacterial competent cells is a key in molecular cloning technique (Tu et al., 2005). Plasmids are replicating circular pieces of DNA, they are smaller than the bacterial genome which encoded their transfer by replication into another bacterial strain or species. They can carry and transfer multiple resistance genes, which may be located on a section of DNA capable of transfer from one plasmid to another or to the genome-transposon or jumping gene (Snyder and Champness, 1997). Chemical transformation includes the usage of calcium chloride, this mode of transformation is easy to perform and requires a minimum number of equipment (www.himedialabs.com a). Because bacteria hosts are not readily convertible to transgenic cells, they need to be treated chemically with calcium chloride and physically with heat shock (Jones & Bartlett Learning, LLC., 2016). Cohen and his colleges in 1973 successfully transformed R-factor and recombinant plasmids into *E. coli* cells using a calcium chloride method, since that time this method has been widely used due to its convenience (Tu et al., 2005).

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The conjugation technique was based on the transfer of *nif*H2 and *nif*H3 genes from *A. chroococcum* which acted as donor cells to *B. megaterium* which acted as recipient cells. The process succeeded when 166 colonies were obtained on Sperber's agar plates containing ampicillin and erthymycine that were used as genetic markers. The growth of the transformant-conjugant colonies may be due to the formation of the conjugation bridge between the donor and recipient bacteria and transmission of ori T genes across this bridge (Snyder and Champness, 1997). To prove the completion of the conjugation process, PCR technique and gel electrophoresis were carried out to amplify and detect the presence of the *nif*H2 and *nif*H3 genes in each of *A. chroococcum, B. megaterium* and transformant-conjugant *B. megaterium* using specific complementary primers. The results in *Figures 5* and *6* showed positive PCR products of *nif*H2 with (250 bp) and *nif*H3 with (130 bp) for *A. chroococcum* in lane 2 and transformant-conjugant *B. megaterium* in lane 4, and negative PCR products for negative control in lane 3 and *B. megaterium* in lane 5 and they did not produce any bands. The conjugation technique of *nif* genes have been proved and reported previously by Davis et al. (2000) when they transferred many *nif* genes from *A. chroococcum* to *Klebsiella pneumonia* and they observed the expression of these genes in transconjugant bacteria, while Khider (2011) and Dashti (2011) transferred some chromosomal *nif* genes by conjugation from *A. chroococcum* to *Lactobacillus planetarium*, also Mohamed (2017) proved this process by transferring some chromosomal *nif* genes from *A. chroococcum* to *B. megaterium.* Many bacteria from different species frequently exchange their genetic materials by a conjugation process which occurs by cell-to-cell contact. This process has been extensively studied because of its significance in genetic manipulation studies (Fernandez-Lopez et al., 2005) as well as in horizontal gene transfer and the resulting spread of multiple antibiotic resistance and virulence.

Conclusion

As a result, the two techniques: transformation to transfer *nod*D2 and *nod*D3, and conjugation to transfer *nif*H2 and *nif*H3 to *Bacillus megaterium* cells were succeeded perfectly. The aim of the presented manuscript is to obtain a new bacteria containing beneficial genes besides its advantageous genes that can be used as an important biofertilizer to plants. Our recommendation is using a new *Bacillus megaterium* bacteria that received *nod* and *nif* genes as a biofertilizer for providing nitrogen by two methods and solubilized phosphorus for plant nutrition, and improving the quality and quantity of plants.

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