RESEARCH PAPER

Transferring of Exopolysaccharide (EPS) Gene Cluster from *Rhizobium leguminosarum* to *Pseudomonas fluorescens* by Horizontal Gene Transfer Techniques

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A B S T R A C T :
To increase drought resistance of efficient plant growth promoting *Psuedomonas fluorescens* isolated from Erbil governorate soils, exopolysaccharide (EPS) gene cluster was transferred from *Rhizobium leguminosarum* to *P. fluorescens*. EPS gene cluster is responsible for generating of capsular polysaccharides which are tightly attached to the cell surface and extracellular polysaccharides that are directly secreted to the environment. For *P. fluorescens* and *R. leguminosarum* isolations, different soil samples were collected in Erbil governorate. According to morphological, biochemical and molecular examinations 75 *P. fluorescens* isolates and 58 *R. leguminosarum* isolates were identified from 110 soil samples. Most *P. fluorescens* isolates showed efficiency in phosphate solubilization, hydrogen cyanide, siderophores and indol acidic acid production, the most efficient *P. fluorescens* (Mpf16) was selected for conjugation process. Potential transferring of EPS genes, included EPS1, EPS2, and EPS3, was investigated in this study. Gene cluster was transferred successfully from *R. leguminosarum* into *P. fluorescens* by horizontal conjugation gene transfer technique after confirmation via molecular techniques using different primers and enzymes. The expression of transferred EPS gene cluster from transconjugant *P. fluorescen*s was confirmed by testing capsule formations from viable cells. The new generated capsule former *P. fluorescens* can be used later on as an efficient bio-fertilizer agent for enhancing plant growth and crop production in drought cultivated soils.

1.INTRODUCTION :

EPSs are usually composed of polysaccharide and different types of proteins with some macro molecules. There are several types of microbes that express polysaccharides, and these types of polysaccharides can be classified by their biological function into intracellular storage polysaccharides,

capsular polysaccharides that usually connected to the surface of cells and extracellular bacterial polysaccharides which have crucial roles in formation of biofilms and pathogenicity (Cuthbertson et al., 2009). Therefore, polysaccharides can be noticed on the almost all cell surfaces, and they are collaborated in several processes such as improving immune avoidance systems and interactions between bacteria and hosts especially for pathogenic organisms (Morona et al., 2009).

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All Gram- negative soil α-proteobacteria which are belonging to the *Rhizobium* genera*,* have capability to infect legume plant roots and promote to generate new nodulation plant organs in which endo-symbiotic bacteria decrease atmospheric nitrogen to ammonia (Dudeja et al., 2012). According to the investigations so far, the strains of *Rhizobium leguminosarum* have ability to produce several types of polysaccharides such as cellulose fibrils, capsule polysaccharide (CPS), cyclic glycans, galactomannan, lipopolysaccharide, gel-forming polysaccharide (GPS), and acidic exopolysaccharide (EPS) due to have EPS genes in cluster form (Zevenhuizen and Scholten-Koerselman,1979).

The *R. leguminosarum* strains are capable to produce two types of EPS which are low molecular weight (LMW) and high molecular weight (HMW) as well as generating biofilms (Staehelin et al., 2006). Lardon et al., (2011) proposed that biofilms can enhance the rate of higher horizontal gene transfer between bacteria especially in conjugation gene transfer techniques. Therefore, this suggestion can be taken as a key to transfer EPS gene cluster from *R. leguminosarum* into *Pseudomonas fluorescens.*

Pseudomonas fluorescens is one of the gram negative, obligate aerobic and rod-shaped bacterium that can be found in soil and water (Anzai et al., 2000). It is also one of the plant growth-promoting bacteria that have been employed widely as a biological control for soil borne plant pathogens as well as enhancing resistance and yields of crops (Lucy et al., 2004). It was proposed that this bacterium can uptake genes by different types of gene transfer techniques in order to mutate its genomics and characteristics (Heinaru et al., 2009). There is no evidence that this bacterium has been mutated by EPS gene cluster in order to add extra performance characteristics by the gene.

The main purpose of the current research was to generate transconjugant *P. fluorescens* in order to form capsule and biofilm, resist drought condition, and induce plant drought resistance when it is used as biofertilizer through EPS production. There was an effort in this research to transfer EPS gene cluster from *R.*

leguminosarum as a donor cell into *P. fluorescens* as a recipient cell by horizontal conjugation gene transfer techniques.

2. MATERIALS AND METHODS:
2.1.Soil Sample Collections

Rhizospheric soil samples were collected from different locations according to climate conditions, cultivar type, and agriculture importance in different sites in Erbil governorate - Kurdistan region- Iraq from August to October. Soil samples were collected at a depth of 3-10 cm. 110 soil samples were collected from different sites randomly. Collected samples were performed in sterilized and aseptic bags and immediately transported to microbiological lab under condition of (4 ˚C) for further process.

2.2.Isolations and Identifications of Bacterial Strains

For rhizospheric *P. fluorescens* isolation from collected soil samples, soil suspensions were prepared and serial dilution processes were made up to 10^{-9} grades. On pre-prepared King's B medium agar (Proteose peptone 20.0, Dipotassium hydrogen phosphate 1.50, Magnesium sulphate. heptahydrate 1.50, Agar 20.0 and Final pH 7.2±0.2 all in Gms / Liter) about 0.1ml of each dilution was spread (Johnsen and Nielsen, 1999, and Jia Xie et al., 2009). After 48h of incubation at 28±2˚C temperature, several colonies were randomly selected on the basis of colony morphology, and further purified by streaking on King's B medium agar plates. To identify bacterial colonies, purified colonies were subject to microscopical, cultural and biochemical tests included gram staining, cell shape, flagellum observation, capsule formations, edges, colony surface, fluorescent under UV light, sugar utilization, catalase test, oxidase test and gelatinize formation (Jia Xie et al., 2009). Additionally, *P. fluorescens* was identified automatically by Vitek2 technique according to Biomerieux-diagnostics protocol. For rhizobial isolation, we selected pink nodules from pea plant root (*Pisum sativum* L) and rhizospheric soil from field-grown pea in Erbil governorates. After serial dilution of soil and nodule sample, we isolated and purified rhizobia following standard protocols using yeast extract mannitol

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agar medium (Yeast extract 1.000, Mannitol 10.000, Dipotassium phosphate 0.500, Magnesium sulphate 0.200, Sodium chloride 0.100, Calcium carbonate and 1.000 Agar 15.000 all in Gms / Liter), by streaking on agar plates and incubated at 28±2°C for 48 hours. Morphological and biochemical identification tests were conducted on the colonies.

2.3. Molecular Identifications of Bacteria and EPS gene Cluster

The genomics of both bacteria were extracted according to the protocols of PureLink Genomic DNA Kits based on gram negative bacterial samples. The quantity and quality of the extracted genomics were confirmed by NanoDrop technique. The extracted genomics of the bacteria were amplified using polymerase chain reaction (PCR) technique and using one of the 16S rRNA gene universal primers "(Forward:- 5' AGAGTTTGATCMTGGCTCAG 3', and Revers:- 5' CTGCTGCSYCCCGTAG 3')" (Waldeisen JR et al., 2011). PCR products were visualized on a 1% agarose gel stained with ethidium bromide under UV light to confirm the size of amplified genes. PCR products were purified using EXOSAP-IT (Ambion, CA) prior to bi-directional sequencing using primers 27F and 1392R (Srinivasan et al., 2015). Sanger sequences were generated at the Oligomer Biotechnology sequencing service (https://oligomer.com.tr/). The generated sequences were analyzed by ChromasPro application.

The primers and sequences of three EPS gene cluster from *R. leguminosarum* were selected and designed by https://blast.ncbi.nlm.nih.gov/Blast.cgi, (Figures 1, 2 and 3), in order to be confirmed that this bacteria strain contains EPS gene cluster. PCR technique was conducted for amplifications of the genes. The protocol for PCR in 20uL reactions was installed according to the primer milting temperatures (T_m). Annealing temperatures for binding primers in all genes (**EPS1-** Forward:- 5'-ATAATAGGATCCATTGCGGCAGATGGCC GCCGACCAC-3', and Revers:- 5'-CCGGACAAGCTTTTAGGCAAGCCATCGC AGGACGAGATAGA-3', **EPS2-** Forward:- 5'-CGCATAGGATCCATTGCCCGATACTTCGT

CGTCCGCATATG-3' and Revers:- 5' CGCGACAAGCTTCTAATGCCGATGCTCC GGACTTTCT-3' and **EPS3** Forward:- 5'-TATTAAGGATCCAATGAATCTATCGCAC CGCGCGCCTC-3' and Revers:- 5'-CGCTATAAGCTT TCACCGGCTTACCTGACTGATATTGGCC-3') in *R. leguminosarum* genomics were 51.5, 57 and 64.5 respectively. The sizes of amplified genes were confirmed by utilizing 1% of agarose gel, and staining with ethidium bromide to visualize under UV light. The PCR products of amplified genes were utilized as positive controls during confirmations of horizontal conjugation process.

Figure 1: Sequences of EPS1 in *R. leguminosarum* genomics with amino acids of the gene products. Sequences with yellow color are sites for forward primer. Sequences with red color are sites for revers primer.

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Exopolysaccharide biosynthesis protein-2 [*Rhizobium leguminosarum*] 891

TTGCCCGATACTTCGTCGTCCGCATATGCGAAACAGCCGGTGAGAAAACCGCGGATTCCCGTTGTCTTCT
GGTTGCTGCTGACGATTTCCCTCTCGCTGGTTATGGGAACGGTGCTGCTTTCCAACGACATGAAACACTT
AAAGACGGTCAGCCATTATTTTGGCTTCGATCTATTTCCCCCTGAGGTTAGGCCGCCGCCGCCCAAAGCT
CTTCCCCGTCCGGCGCCTCCAGCCACCTTCGCGCTTCCCTTGCATGTCATTGAACCGCCGGCCGCACAGA
CCGCGTCGACTTTTCTACGGACATGGCGGATATCCGGCGCCGCAATGTGTGCGGCGCTGCGCAATGCCG
CATCGAAACCACCGATTGGGCGGCGACGAGCTTCAACGCCGATACGTTCGAATGCTTTTTCGAGCAGAG
GGCAAGCGGGAAAAGGATCAGCTCCCGAACTCCATCTTCGTCATCGTTCGCGGCGACGCCGCCGGCACG
TCAACAATATGCGCGTGAAGATCGTCAATCCCGAGACCGATCAAAATGGCCAGCTCGATCCCGGCATTC
GCGAATTTTCCAAATCATGCTGCGGCAACCGCAATGGCTCGATTTTCACGAGACCCTGAACGCCATCAAC
AACCTGAGGGATATCAAGGAAGACGGCTCCATTCTAGTCAGTATCAGCTTCACCCGCGAGGTGCTCAATC
GGAATTACAATTTCACACTCTCGCTGGATGCGACTTCAGGACCGCAGAAAAGAACGAGAAATTACTTAT
CGGCAGAATATGGCTGCCATTGCCCGACCATGCGGTCGACATCAACAGCGCGCAACCGGAATCAGTCTC
GAGCCTTCCGATGCCGAGGCCCCGGCAGAAAGTCCGGAGCATCGGCATTAG

Primers:-

EPS2-F 5'- CGCATA GGATCCATTGCCCGATACTTCGTCGTCCGCATATG -3'

EPS2-R 5'- CGCGACAAGCTTCTAATGCCGATGCTCCGGACTTTCT -3'

Sequences: 891 , Gene; EPS2, No. of amino acids; 296 a.a

MPDTSSSAYAKQPVRKPRIPVVFWLLLTISLSLVMGTVLLSNDMKHLKTVSHYFGFDLFPPEVRPPPPKA
LPRPAPPATFALPLHVIEPPAAQTASTFLRTWRISGAAMCAALRNAGIETTDWAATSFNADTFECFFEQS
GKREKDQLPNSIFVIVRGDAAGTINNMRVKIVNPETDQNGQLDPGILRIFQIMLRQPQWLDFHETLNAIK
NLRDIKEDGFGASISFTREVLNPGNYNFTLSLDATSGPQKRTRNYLSGRIWLPLPDHAVDINSAQPESVS
EPSDAEAPAESPEHRH

Figure 2: Sequences of EPS2 in *R. leguminosarum* genomics with amino acids of the gene products. Sequences with yellow color are sites for forward primer. Sequences with red color are sites for revers primer.

Exopolysaccharide biosynthesis protein-3 [*Rhizobium leguminosarum*] 1311

ATGAATCTATCGCACCGCGCGCCTCGTGTGTCCTTTCGCGCGACGTCTGTCATCAGTGCACTCGTCCTTC
TTGCCGGAGCGGTGTCGCCAGCTCTCGCCGATAGCGCCCCCTTGGCTCCGCAAACAAGAATCCGCCTGAC
GATCGTCCAATGGATAGCTTCCAAGGGGCAGTTTGAACGATGGGATGGGATTGGAGGCGACTATACGAT
TCTGATGCGGGTGTAGTCTCCCTGCCCTTTCTGGGGTCGCTGTCGGTTGGAAATCTGGACAATGAGGAGC
TCACCAGCGAGATCGGCAGGCGCCTTCAGGCGAAGATGGGTCTGGCTCAGGCACCTGCAGTGACCATCG
CATTCTCGACTATCCCTCCATTTATGTCGTCGGAGACGTGGTGGCGCCGGGAGAGTACAAGTTTCGGTCC
GGCCTCAGCGTCCTGCAGTCCCTGGCGATGAGCGGCGGCCCGTTGCGGGCTGCGGCACTGCAGCAATCGC
AGACGATCAGACTTGCCGGGGATTTGCGGGAAATCGACCATTCGCTGCTGCGCAGCTCGGCAAAGCTCG
GCGTCTGCAAACGGAAATGGCCGGCGCGAAGGAAATCGTCTTTGATCCGCCGCCCACTGCCGATCGGCAA
TATGCCGAGAGTCTCTACCAGGAGGAGCGGGTCATTTTTCAGGCTCGTGCAAACGCGCTGGACAAGCAGT
CGGTGGCTCTCGCCGAATTACGCAATCTCCTGACGGCGCGGAAATCGATACACTGGAAGAAAAGCTGAAAGC
CTCGGATGACAATATCCGGTCGGTCGAAGAGCAACTGACGAGCGTGAAGACGCTGGTCCAGAAGGGCCT
ACGATCACGTCACGCCAGATGGATCTGGAACGATTGCTCACCACCTATCGCTCCGACCGGCTCGACCTCG
TGACGGCCATCATGCGGGGCCGTCAAGCGATCAACGAGACGACGCGAAATCTCGAGGGACTTTCCGATAG
TCGCCGGAGCGAGATCGCTTCCGAAGTGCAGGCGGAAAAGGCCAATCTCGATCAGCTCAAATTGAAGCG
GACACCACGCAACAACTGCTTCTCGAAGACCTGTCGAACGGCGCCAACGTGAATAGCCGCGTTGAAGAAC
TCCCCCTGACATTCCTCGTGAACCGGCGGGACAAGGGACAGGTCAATCAATTCCAGGCTTCCGAAACGAC
CGAGCTAGCACCGGGCGACGTGATCAGGGTCAGCCGGGGCCGCATCGCCGATGCGCCGTCTGAAGACGC
GCCGCGCTGCCTGTTCAGACAGAGGCCAATATCAGTCAGGTAAGCCGGTGA

Primers:-

EPS3-F 5'- TATTAAGGATCCAATGAATCTATCGCACCGCGCGCCTC -3'

EPS3-R 5'- CGCTATAAGCTT TCACCGGCTTACCTGACTGATATTGGCC -3'

Sequences: 1311 , Gene; EPS3, No. of amino acids; 436 a.a

MNLSHRAPRVSFRATSVISALVLLAGAVSPALADSAPLAPQTRIRLTIVQWIASKGQFERWDGIGGDYTI
SDAGVVSLPFLGSLSVGNLDNEELTSEIGRRLQAKMGLAQAPAVTIDILDYPSIYVVGDVVAPGEYKFRS
GLSVLQSLAMSGGPLRAAALQQSQTIRLAGDLREIDHSLLRSSAKLARLQTEMAGAKEIVFDPPPTADRQ
YAESLYQEERVIFQARANALDKQSVALAELRNLLTAEIDTLEEKLKGSDDNIRSVEEQLTSVKTLVQKGL
TITSRQMDLERLLTTYRSDRLDLVTAIMRGRQAINETTRNLEGLSDTRRSEIASEVQAEKANLDQLKLKR
DTTQQLLLEDLSNGANVNSRVEELPLTFLVNRRDKGQVNQFQASETTELAPGDVIRVSRGRIADAPSEDA
AALPVQTEANISQVSR

Figure 3: Sequences of EPS3 in *R. leguminosarum* genomics with amino acids of the gene products. Sequences with yellow color are sites for forward primer. Sequences with red color are sites for revers primer.

2.4. Preservations of Isolated Bacteria

The purified cultures of studied bacteria were preserved at −70 °C with 25% glycerol and at 4 °C on agar slants for further study (Delves et al., 1996).

2.5. Plant Growth Promoting Activities of *P. fluorescens* Isolates

2.5.1. Determination of Indole Acetic Acid (IAA)

The formation of IAA was assayed by using Salkowski method. Formation of red color indicated presence of IAA in the culture medium.

2.5.2. Phosphate Solubilizing Efficiency

The colony of isolates were streaked on the plates of PVK agar medium and incubated at $28\pm2°C$ for 5 days. After incubation period, the plates were then examined for formation of clear zone around the colony.

2.5.3. Detection of Hydrogen Cyanide (HCN)

HCN formation was assayed by the Lorck and Castric method. For the production of HCN, isolates were streaked on King's B agar plates.

2.5.4. Siderophore Production

Siderophore production was tested and evaluated using Chrome Azurole S (CAS) agar.

2.6. Antibiotic Resistance Tests

Several types of antibiotics were utilized manually for both bacteria in order to know the differentiations of sensitivities and resistances between both bacteria and using as genetic markers. All types of antibiotics were utilized according to the following concentrations (μg /mL), ciprofloxacin (5), levofloxacin (5), moxifloxacin (5), ampicillin (10), amoxicillin - clavulanic acid (20), cefixime (5), amikacin (30), gentamicin (10), tigecycline (15) and nitroxoline (30). The isolated *R. leguminosarum* was cultured on Yeast extract mannitol medium agar, and the isolated *P. fluorescens* was cultured on King's B medium agar as well as both cultured bacteria were treated with all mentioned antibiotics under aerobic, $28\pm2°C$ and 24 hours conditions.

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Moreover, the sensitivity and resistance of *P. fluorescens* towards antibiotics was confirmed automatically by using Vitek2 techniques (Biomerieux-antibiotic test protocol).

2.7. Horizontal Conjugation Technique

To transfer EPS cluster genes from *R. leguminosarum* as a donor bacteria to *P. fluorescens* as a recipient bacteria, the process was conducted according to the proposed methods (Olsen et al., 1992; Holmes and Jobling, 1996). Single colony of *R. leguminosarum* was inoculated with 10 mL of Yeast mannitol medium without agar, and incubated at 28±2°C for 48 hours with shaking 100rpm. For the *P. fluorescens,* same steps were performed using King's B medium without agar instead of using Yeast mannitol medium. Then, 0.7ml of the growth *R. leguminosarum* was used as donor cells mixed with 0.3ml of the growth *P. fluorescens* as recipient cells, and 1.5 ml of fresh enrichment liquid broth medium was added to the mixture. The mixture was incubated at 28±2°C for 4hours under aerobic condition. After incubation, 100 µL of conjugated mixture was spread on the fresh King's B agar plates containing the selected antibiotics which were chosen as genetic markers. Additionally, control cultural plates were provided from 100 µL of each donor cells and recipient cells suspended on media agar plates that contained the same genetic markers. Then the plates were incubated at 28±2°C for 48 hours. After incubation, purification steps were performed several times on the same properties plates, and then the numbers of transconjugant colonies were selected. According to the mentioned equation in Puhler and Timis (1984), the conjugation frequency was calculated.

$$\text{Equation:} \quad \frac{Number\ of\ transconjugants \times ml^{-1}}{Viable\ count\ of\ donors\ in\ the\ mix \times ml^{-1}}$$

$$\text{Frequency of conjugation} = \_\_\_\%$$

2.8. Molecular Confirmations of the Horizontal Conjugation Process

The genomics of transconjugant cells was extracted according to the protocol which has been mentioned previously. The quantity and quality of the extracted genomics was known by NanoDrop technique. PCR and agarose gel techniques were used for amplification and visualization of EPS genes according to the above protocols which were performed previously for confirmation of the transferred genes. The expression of the transferred genes was confirmed by staining of capsule formations from transconjugant cells under microscopic condition.

3. RESULTS AND DISCUSSIONS:

3.1 Isolation and Identification of *P. fluorescens* and *R. leguminosarum*

From collected soil samples, a total of 75 isolates were selected on King's B medium and they were recognized. All isolates produced fluorescent pigment on King's B medium while exposed with UV light, gram negative and rod shaped clearly. In each sample collection zone, one isolate was selected to further evaluation. These organisms were found to be quite similar in size, lengths and diameters. All isolates were non spore and capsule former, motile, aerobic, could not grow at 41°C but could grow very well at 4°C, and all isolates showed positive response to catalase, oxidase, starch, arginine, urease and nitrate reduction, and were able to utilize glucose, but they were differ from xylose, rhibose, rhamnose, gelatin and sucrose utilization. Also Vitek2 compact technique was used for identifications of *Pseudomonas* isolates to species level, as Vitek2 compact has ability to identify automatically the majorities of bacteria and antibiotic susceptibility (Figure 4), (https://www.biomerieux-diagnostics.com/). According to microscopical, cultural, and biochemical characteristics, they were classified as *P. fluorescens* (Patel et al., 2013 and Nepali et al., 2020). On Yeast mannitol agar, 58 isolates of *Rhizobium* were recognized from studied rhizospheric soil samples and pea plant nodules. The majority of the colonies had phenotypic similarities to each other and they had strong similarities in morphological and biochemical characteristics with *R. leguminosarum*. The isolates revealed circular colonies, creamy to white in color on YEMA. Furthermore, all the isolates were found to be gram negative, none spore former, capsule producer, motile, aerobic

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and rod shaped clearly. They indicated negative reaction to oxidase, while showed positive reaction to catalase test, urea hydrolysis and citrate utilization, and could utilize glucose, sucrose, lactose, fructose and maltose, but they were differ in mannitole, and rhibose (Gopalakrishnan et al., 2012 and Shahzad et al., 2019). In each sample collection zoen for this bacterium, one isolate was selected to further investigations.

Figure 4: Results of Vitek2 compact for identification of *Pseudomonas fluorescens*.

3.2. Plant Growth Promoting Traits of *P. fluorescens*

3.2.1. Indole-3-Acetic Acid Production

Most *Pseudomonas* isolates produced IAA in vitro in tryptophan supplemented medium (Table 1). Changing color of the broth culture from pink to red indicated to IAA production. Quantity of IAA produced by isolates was estimated and IAA concentrations range was varied from 59.4 to 8.7mg.ml-at 7 days of incubation. Statistical analysis showed that there was significant difference among isolates in IAA production. The highest amount of IAA was produced by isolate Psh11. It has been recorded that the amount of IAA compounds produced in vitro depends on the particular bacterial genus, species, strain, or the conditions of the culture media such as aeration and pH (Radwan et al., 2002). Also, this is opposing views on account of the miscellaneous

metaboilic pathways, position of the genes complicated, and the presence of enzymes to convert active free IAA into conjugated forms (Islam et al., 2009).

3.2.2. Siderophores Production

The *P. fluorescens* is known to generate many secondary metabolites, such as siderophores which have antagonistic properties against many phytopathogenic. Most studied isolates, were positive for siderophores production which was emphasized by production of yellow/orange-colored zone surrounding the bacterial colony (Table 1). Results indicated that there were differ among isolates in siderophores production ability, and usually siderophores which produced by *P. fluorescens* were antagonistic to pathogenic fungi (Suryakala et al., 2004).

3.2.3. Production of Hydrogen Cyanide (HCN)

All of the 22 tested *P. fluorescens* isolates showed high ability in hydrogen cyanide production which indicated by the discoloration of the filter paper from orange to brown after incubation period (Table 1). According to the result in table (1), few isolated *P. fluorescens* demonstrated weak HCN production, while the majority of isolates indicated moderate HCN production. Hydrogen cyanide is produced by many rhizobacteria and is postulated to play a vital role in biological control of plant pathogens (Shaikh et al., 2014). Although cyanide acts as a general metabolic inhibitor, host plants are not harmfully affected by rhizobacterial production of hydrogen cyanide and host specific rhizobacteria can act as biological control agents (Saharan and Nehra, 2011).

3.2.4. Phosphate Solubilizing Activities

Results of P solubilizing activity on solid PVK agar media revealed that all *P. fluorescens* isolates were able to solubilize P (Table 2). The highest P solubilizing activity (88.8%) was recorded by (Mpf16) treatment. This may be attributed to the efficiency of *Pseudomonas* isolate to produce higher quantity of acid which led to increase the available phosphorus. This result is in agreement with Ortiz-Castro et al.

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(2009) who observed the role of *Pseudomonas* species to solubilize fixed phosphorus to available phosphorus. Ability of isolates to solubilize inorganic phosphate due to the production of organic acids such as gluconic acid and ketogluconic acid, also possess some genes which encoded for several enzymes that have also been shown to be involved in making insoluble phosphorous compounds available for cell growth such as phosphatases enzymes (Shaikh et al., 2016).

Table I:- Plant growth promoting traits of *P. fluorescens*.

Isolate code	IAA Produc.	HCN Produc.	Siderphore produc.	IAA produc. mg.ml^{-1}	P. Solubilization (%)
Qpf1	+	+	+	13.55^{lm}	47.6^{lm}
Qpf2	+	±	+	32.60^{fg}	66.2^{i}
Cpf3	++	±	+	20.36^{ij}	76.9^{cd}
Cpf4	+	+	+	29.10^{g}	46.1^{m}
Cpf5	++	+	+	20.19^{hi}	70.1^{gh}
Wpf6	+	-	++	18.19^{jk}	52.9^{l}
Wpf7	+	+	+	12.10^{n}	39.10^{no}
Wpf8	++	+	+	15.99^{jk}	53.4^{k}
Kpf9	+	+	+	33.19^{ef}	64.8^{i}
Kpf10	+	++	+	41.09^{cd}	56.8^{j}
Hpf11	+	-	+	27.18^{g}	60.3^{j}
Hpf12	+	+	+	20.56^{h}	43.2^{n}
Spf13	+	-	++	33.80^{f}	78.1^{ef}
Gpf14	+	±	-	13.77^{m}	44.8^{n}
Gpf15	++	±	++	49.49^{b}	81.9^{b}
Mpf16	+++	+++	+++	49.90^{a}	88.8^{a}
Mpf17	+	++	++	20.05^{ij}	73.6f^{g}
Dpf18	+	-	+	33.99^{e}	46.4^{m}
Dpf19	+	±	+	14.10^{m}	59.9^{j}
Dpf20	+	+	+	25.99^{g}	77.8^{de}
Apff21	+	±	+	18.87^{ij}	47.8^{n}
Apf22	++	++	++	41.59^{c}	76.07^{c}

3.3. Molecular Identifications of Bacteria and EPS Gene Cluster

Molecular identification technique was conducted in order to confirm the species and strains of the bacteria. 16s rRNA universal primer was used, and amplified genes were visualized by agarose gel. The amplified genes were approximately 1300bp and 620bp which were similar to the size of 16S rRNA genes in *R. leguminosarum* and *P. fluorescens* bacteria respectively (Figure 5), (Shahzad et al., 2019; Waldeisen JR et al., 2011; Patel et al., 2013 and Wolde-Meskel et al., 2005). The amplified genes were sequenced by Sanger sequence, and the species and strains of the isolated bacteria were confirmed (https://oligomer.com.tr/).

R. leguminosarum was confirmed to have EPS gene cluster. The sizes of amplified EPS1, EPS2 and EPS3 with sequences of the primers were around 579bp, 891bp and 1311bp respectively (Figure 6). Selecting this strain of the bacteria was due to have ability to expression high amount of biofilms, capsule biosynthesis, solubilize large quantities of phosphorus and highly induce nitrogen fixation in soil (Naseem et al., 2018 and Jia Xie et al., 2009).
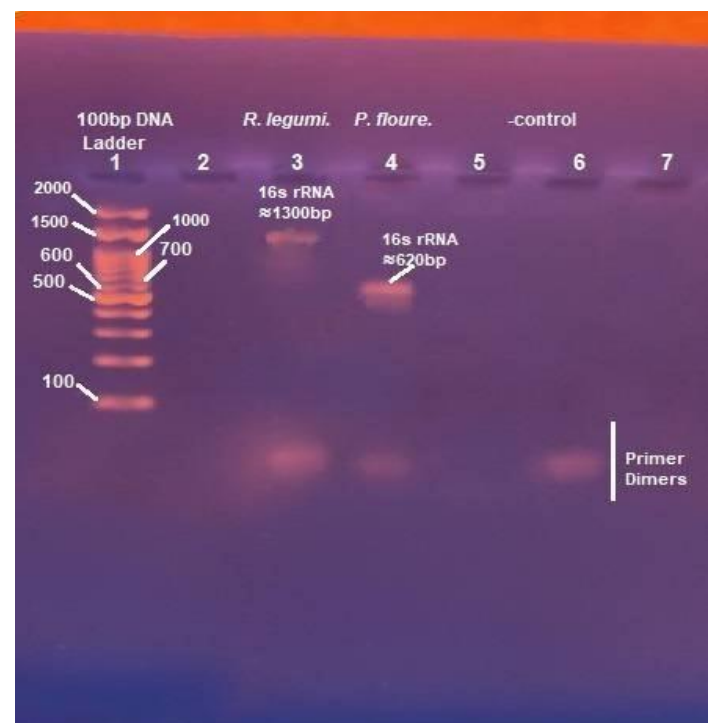

Figure 5: Gel for PCR products of 16s rRNA gene. Using 1% of agarose gel with Ethidium bromide. Lane1, 100bp DNA Ladder. Lanes 2, 5 and 7 are blanks. Lane3, PCR products from *R. leguminosarum* genomics. Lane 4, PCR products from *P. fluorescens* genomics. Lane 6, negative control.
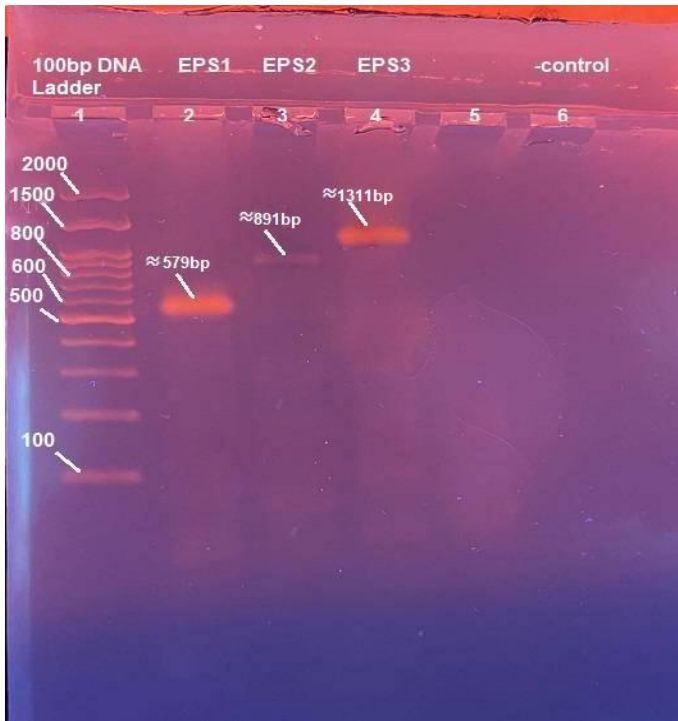
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Figure 6: Gel for PCR products of EPS gene cluster in *R. leguminosarum* genomics. Using 1% of agarose gel with Ethidium bromide. Lane 1, 100bp DNA Ladder. Lanes 2, amplified EPS1 gene. Lane 3, amplified EPS2 gene. Lane 4, amplified EPS3 gene. Lane 5, blank. Lane 6, negative control.

Antibiotic susceptibility testing was conducted for both bacteria to select genetic markers for horizontal conjugation gene transfer process. Among all antibiotic testing manually, they showed different reaction towards Piperacillin and Tazobactem (5 μg /mL) antibiotic. *P. fluorescens* was sensitive to this antibiotic in this quantity, whereas, *R. leguminosarum* was resistance to this antibiotic in the same quantity. Furthermore, *P. flourences* was sensitive to Piperacillin and Tazobactem antibiotic in Vitek2 compact system as well, (https://www.biomerieux.com/en/search/apachesolr_search/antibiotic). This result indicated that the Piperacillin and Tazobactem can be selected as genetic markers during horizontal conjugation process, as the recipient bacteria supposed to be sensitive in one or two different antibiotics in conjugation process (Peter et al., 2017).

Horizontal conjugation gene transfer technique was performed between *P. fluorescens* as a recipient and *R. leguminosarum* as a donor cells in BHI enrichment liquid media, as this media can be utilized for antimicrobial sensitivity, and several species of bacteria can grow in this media (Atlas,

2004). After treating with one of the genetic markers, there were some colonies which had extensive phenotypic characteristics with *P. fluorescens* and had some similar characteristics with *R. leguminosarum* in terms of morphological and biochemical. In order to confirm that the EPS gene cluster was transferred from *R. leguminosarum* into *P. fluorescens*, molecular techniques were performed on transconjugant cells. As can be noticed from (Figure 7), the size of amplified EPS1 gene in transconjugant cells was around 579bp which was similar to the size of the gene in positive control (Figure 7). While, there was no EPS2 amplified gene in transconjugant cells except light unknown gene was amplified (Figure 7). In the same time, there was amplified gene in positive control. The unknown amplified gene might be due to the using long sequences of the primers and integrated with different genomics, as has been mentioned that using long sequences of primers could bind to the unknown genes and slow hybridizing rate during running in PCR (Green et al., 2018 and Bustin et al., 2020). EPS3 gene was amplified in which its size was approximately 1311bp, and it was similar to the size of amplified gene in positive control (Figure 7). Further, there was no amplified gene size in negative control (Figure 7). This result demonstrated that the EPS1 and EPS3 genes which are the most important genes in EPS gene cluster have been moved successfully from *R. leguminosarum* into *P. fluorescens* by horizontal conjugation process and integrated with the genomics of transconjugant *P. fluorescens*, as EPS1 (succinoglycan) was identified as a pathway for the polysaccharide biosynthesis in modification, capsule biosynthesis, producing biofilms, exporting signaling and polymerization (Long, 2001 and Jones et al., 2007). Viable Transconjugant *P. fluorescens* cells were stained under microscopic conditions to ensure the expression capacity from capsule formation. There were some formed capsules due to the expression of transferred EPS gene cluster, as this gene cluster has ability to form capsules (Jones et al., 2007).

This result indicated that the transconjugant *P. fluorescens* has been generated successfully, as there are evidences that horizontal gene transfer can be occurred between species and strains of *Pseudomonas spp.* (Eeva et al., 2009 and Hall

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James et al., 2020). Moreover, chromosomal DNA transfers have been reported between bacteria cells by horizontal conjugation gene transfer process (Khider, 2011; Rhon-Calderon et al., 2016, and Lotareva and Prosorov, 2006). The successes of achieving transconjugant cells are probably due to the generation of the conjugation embankment between the donor and the recipient bacteria and transmission of oriT genes across this embankment (Snyder and Champness, 1997).
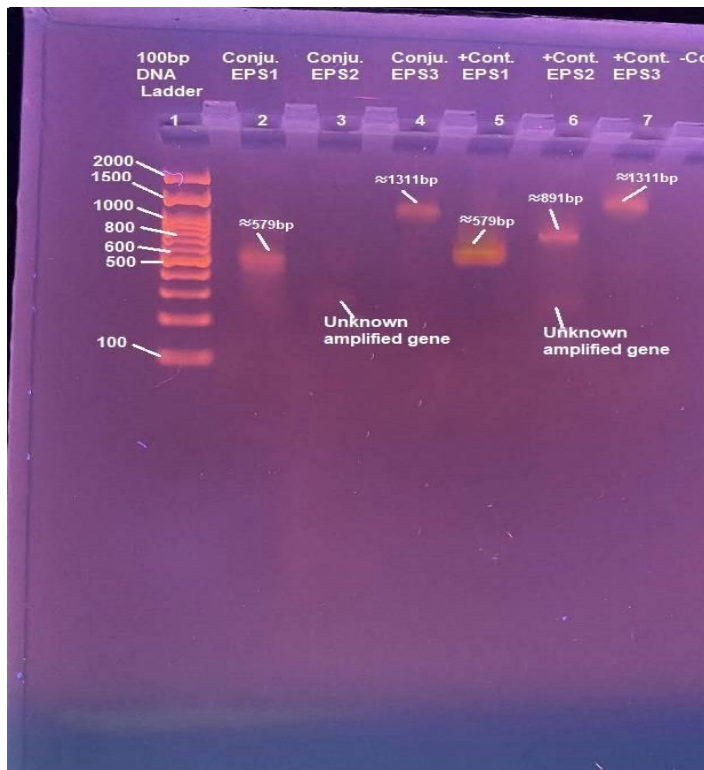

Figure 7: Gel for PCR products of EPS gene cluster in *R. leguminosarum* genomics. Using 1% of agarose gel with Ethidium bromide. Lane 1, 100bp DNA Ladder. Lanes 2, amplified EPS1 gene in transconjugant *P. fluorescens* genomics. Lane 3, amplified EPS2 gene in transconjugant *P. fluorescens* genomics. Lane 4, amplified EPS3 gene in transconjugant *P. fluorescens* genomics. Lane 5, amplified EPS1 gene product used as a positive control. Lane 6, amplified EPS2 gene product used as a positive control. Lane 7, amplified EPS3 gene product used as a positive control. Lane 8, negative control.

Generated transconjugant *P. fluorescens* bacteria from the EPS gene cluster could have indefinite positive characteristics for enhancing plant yields, as *P. fluorescens* itself has various distinctive properties. For instance, *P. fluorescens* can stimulate and enhance plant growth during association with plants by producing growth promoting plant hormones, increase plant disease resistances, damaging pathogenic soil born microorganisms, enhance nutrient availability in soil, potential relative water contents, increasing root tissues and improving plant biomass (Jimtha John et al., 2017; Wang et al., 2021 and Preston, 2004). Therefore, transferring EPS gene cluster into *P. fluorescens*, extra characteristics might be added to the *P. fluorescens* bacteria since EPS gene cluster has ability to express vital enzymes. For example, this gene cluster is capable to produce biofilm layer on the surfaces of the cells which have ability to protect cellular against environmental stress, nutrient aggregations for the plants and enhance surface attachment of the nutrients for the roots (Tanya et al., 2012). Additionally, symbiotic signaling for the molecules can be enhanced by extracellular polymers which can adjust the responses of the plants during infections, as chemically extracellular polysaccharides has special structural recognitions and physic-chemical possessions (Fraysse et al., 2003). Moreover, EPS gene cluster is responsible for forming capsules by capsule biosynthesis process (Song et al., 2018).

4. CONCLUSION:

According to the main results of this study, the ESP1, EPS2 and EPS3 among exopolysaccharide gene cluster were transferred successfully through horizontal conjugation gene transfer from *R. leguminosarum* into *P. fluorescens*, and transconjugant *P. fluorescens* was generated after confirming by molecular confirmation techniques (PCR), and expression of transferred genes were confirmed by staining of formed capsules. The generated transconjugant *P. fluorescens* now have vital roles in increasing plant growth promotion, generating biofilms, capsule formations and it is maybe secondhand later on as a biography-dressing to aid production of crops power particularly for drought and lack of source of nourishment soils.

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