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Effects Of The Transconjugant Pseudomonas Fluorescens Carrying 1-Aminocyclopropane-1-Carboxylate Deaminase Gene On Drought Tolerance Of Wheat

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ABSTRACT

To increase drought tolerance of efficient plant growth promoting Pseudomonas fluorescens isolated from Iraq- Erbil Kurdistan soils, ACC-deaminase genes were transferred from Pseudomonas putida to P. fluorescens. ACC deaminase containing plant growth promoting rhizobacteria are capable to decrease ethylene inhibition of plant growth following a wide range of abiotic and biotic stresses. For P. fluorescens and P. putida isolations, different Soil samples were collected in Erbil Governorate. According to microscopical, morphological, biochemical and molecular examinations 75 P. fluorescens isolates and 65 P. putida isolates were identified from 110 Soil samples. Most P. fluorescens isolates illustrated efficiency in phosphate solubilization, hydrogen cyanide, siderophores and indol acidic acid production. According to results the most efficient P. fluorescens (Mpf16) was selected for conjugation process. Potential transferring of ACC - deaminase genes were considered in this research. ACC-deaminase genes were moved completely from P. putida into P. fluorescens by conjugation technique after confirming via molecular techniques using selected primers. The effective of transferred ACC-deaminase genes from transconjugant P. fluorescens was confirmed in pot experiments under un-irrigation condition by evaluating growth rate and chemical analysis of wheat crops. The highest growth rate of root length(cm), plant height(cm), spike number/crops, root dry weight (gm/crops), total seed weight (gm/crop) and shoot dry weight (gm/crop) were 25.90, 54.10, 32.62, 18.15, 14.55 and 28.15 respectively, and they were recorded by inoculated transconjugant P. fluorescens treatments and significantly differed from control treatment. The new mutated P. fluorescens (transconjugant P. fluorescens) can be proposed as an efficient bio-fertilizer agent for increasing plant growth and drought tolerance of crops.

Keywords: ACC deaminase gene, conjugation, Pseudomonas putida, Pseudomonas fluorescens, Transconjugant Pseudomonas fluorescens. Drought tolerance.

INTRODUCTION

Drought tolerance is the capability to which a plant maintains its biomass production during drought conditions. Some plants are naturally adapted to dry conditions, surviving with protection

mechanisms such as desiccation tolerance, detoxification, or repair of xylem embolism (Tardieu et al., 2018). Other plants, specifically crops like corn, wheat, and rice, have become increasingly tolerant to drought with new varieties created via genetic engineering (Hu and Xiong, 2014). Among the main mechanisms which can be used by plants to resist drought condition is the presence of the enzyme 1-aminociclopropane-1-carboxylase (ACC) deaminase, which converts ACC into ammonia and α-ketobuty rate. The ACC is a precursor of ethylene in all higher plants, and both ACC and ethylene typically increase in plants when they are under stress. Therefore, the ACC deaminase activity is one of the key traits to decrease ethylene levels under a wide range of stress conditions. It is widely reported that certain ACC deaminase-containing plant growth-promoting bacteria have ability to decrease a part of ethylene inhibition of plant growth following a wide range of abiotic and biotic stresses (Glick, 2012). Therefore, crops which treated with ACC deaminase-containing plant growth-promoting bacteria usually have taller roots and shoots and are more resistant to growth inhibition by a variety of ethylene-inducing stresses. Reduction of ethylene ranges induce the crops to be more resistant to a wide variety of environmental stresses especially drought (Glick et al., 1998).

Pseudomonas fluorescens can be found in soil and water frequently. Additionally, it is one of the plant growth-promoting bacteria that have been employed widely as a biological control for phytopathogen as well as enhancing resistance and yields of crops. Genetically, this bacterium has ability to uptake exogenous genes through different transgenic techniques (Heinaru et al., 2009). Transgenic of this bacterium with 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene can be proposed to add extra characteristic to increase drought tolerance of treated some crops with transconjugant P. fluorescens.

Conjugation process has been utilized frequently to transfer desired genes among bacteria species such as; transferring chromosomal genes between Azotobacter chroococcum to Xanthomonas citri as well as between Bacillus subtilis species (Lotareva and Prosorov, 2006; Khider and Khidher, 2011). Additionally, for implementing conjugation process successfully, it is highly recommended that to be conducted among similar genus of bacteria (Griffiths et al., 2005). Therefore, Pseudomons putida UW4 which expresses ACC deaminas enzyme can be selected for conjugation process as a donor of ACC deaminase gene to P. fluorescens as a recipient. P. putida UW4 has been shown to promote plant growth of different environment stresses, including flooding and drought, as well as the presence of heavy metals and organic contaminants, salt and phytophatogens due to have ACC deaminase genes (Gontia-Mishra et al., 2014).

The essential objective of this research was to produce a kind of bio-fertilizer that proposed to have all above mentioned characteristics for progressing and reducing agricultural drought soils and increase crop yields generally. There was attempting in this research to transfer ACC deaminase gene from P. putida UW4 strain into P. fluorescens by conjugation process in order to add another high expressing ACC deaminase enzyme properties to P. fluorescens and generate transconjugant P. flourences.

MATERIALS AND METHODS

Isolations and Identifications of Bacterial strains

For rhizospheric P. fluorescens and P. putida isolation from collected Soil samples, soil suspensions were prepared and serial dilution processes were made up to 10^{-9} grades. about 100 µL of each dilution was spread on to pre-prepared King's B agar medium (Protease peptone 20.0g., Magnesium sulphate.

heptahydrate 1.50g, Dipotassium hydrogen phosphate 1.50g., Agar 20.0g. per liter and final pH 7.2±0.2) (Patel et al., 2013) and Pikovskaya (PVK) agar medium (Yeast extract 0.50g., Dextrose 10.0g., Calcium phosphate 5.0g., Ammonium sulphate 0.50g., Potassium chloride 0.20g., Magnesium sulphate 0.10g., Manganese sulphate 0.0001g. and ferrous sulphate 0.0001g. all in Liter) to isolate P. fluorescens and P. putida, respectively . After 72h of incubation at 28±2˚C, several colonies were randomly selected on the basis of colony morphology, and further purified by streaking on to King's B and PVK 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, sugars utilization, catalase and oxidase test, gelatinase formation (Thompson et al., 2019; Nordstedt et al., 2020). Additionally, each of P. fluorescens and P. putida were identified via API 20e according to API 20e codebook and the results were interpreted through API 20e online codebook application system. Moreover, both bacteria were identified to species level by using Vitek 2 technique according to Biomerieux-diagnostics protocol.

Molecular identifications techniques

The genomes of the selected bacteria were isolated based on the Pure Link Genomic DNA protocols. The amount and purity of the isolated genomes were assured through Nano Drop mechanism. The isolated genomes were amplified by the 16S rRNA universal primers in polymerase chain reaction (PCR). The sequences of the primers were F- 5' AGAGTTTGATCMTGGCTCAG 3', and R- 5' CTGCTGCSYCCCGTAG 3' (Waldeisen et al., 2011). The amplified genes were visualized on a 1.5% '(w/v)' agarose gel after staining with ethidium bromide and treated with UV light. The visualized sizes of PCR products were purified for sequencing process by utilizing EXOSAP-IT (Ambion, CA) prior to bi-directional sequencing and using primers 27F and 1392R (Srinivasan et al., 2015). Sanger sequences were produced at the Oligomer Biotechnology (https://oligomer.com.tr/). The produced sequences of both extracted genomes were analyzed via ChromasPro software.

For the ACC deaminase gene in P. putida strain UW4, the primers and sequences were selected and designed by [National Center for Biotechnology Information (NCBI), https://www.ncbi.nlm.nih.gov/tools/primer-blast/] (Figure 1). Additionally, for the ACC deaminase gene in P. flourences, the primers and sequences were chosen via (NCBI) as well, in order to be confirmed that the selected P. flourences bacteria do not contain the gene (Figure 2). Amplifications of the genes in both bacteria were performed by PCR technique. 20uL reactions for PCR were prepared and the protocols were installed based on the primer melting temperatures (T_m). Annealing temperatures for binding primers with ACC deaminase gene were 73.5˚C and 46˚C in P. putida genomics and P. fluorescens genomics respectively. 1.5% '(w/v)' agarose gel was run to confirm the amplified gene sizes. The amplified genes were utilized as positive controls during confirmations of conjugation techniques.

AY823987.1 Pseudomonas putida strain UW4 1-aminocyclopropane-1-carboxylate deaminase gene, complete cds 1017nt.

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1 ATGAACCTGAATCGTTTTGAACGTTATCCGTTGACCTTCGGTCCATCCCCCATCACTCCCTTGAAACGCC
71 TCAGCGAGCACCTGGGCGGCAAGGTGGAACTGTATGCCAAGCGTGAAGACTGCAATAGCGGCCTGGCCTT
141 CGGCGGGAACAAAACGCGCAAGCTCGAATATCTGATTCCCGAGGCCATCGAGCAAGGCTGCGACACCTTG
211 GTGTCCATCGGCGGTATCCAGTCGAACCAGACCCGCCAGGTCGCTGCGGTCGCCGCCCACTTGGGTATGA
281 AGTGTGTGCTTGTGCAGGAAAACTGGGTGAACTACTCCGACGCTGTATATGACCGCGTCGGCAACATCGA
351 GATGTCGCGGATCATGGGAGCGGATGTGCGGCTTGATGCTGCAGGTTTCGACATTGGAATTCGGCCGAGC
421 TGGGAAAAGGCCATGAGCGATGTCGTGGAGCGCGGCGGCAAACCGTTTCCAATTCCGGCGGGCTGTTCCG
491 AGCATCCCTATGGAGGGCTCGGGTTTGTCGGCTTCGCTGAGGAAGTGCGGCAGCAGGAAAAGGAGTTGGG
561 CTTCAAGTTTGACTACATCGTGGTCTGCTCGGTGACCGGCAGTACCCAGGCCGGCATGGTCGTCGGTTTC
631 GCGGCTGACGGTCGCTCGAAAAACGTGATCGGGGTCGATGCTTCGGCGAAACCGGAGCAAACCAAGGCGC
701 AGATCCTGCGTATCGCTCGACATACCGCTGAACTGGTGGAGCTGGGGCGCGAAATCACTGAAGAGGATGT
771 GGTGCTCGATACGCGTTTCGCCTATCCGGAATATGGCTTGCCCAACGAAGGGACGCTGGAAGCGATTCGC
841 CTGTGCGGCAGTCTTGAGGGGGTGTTGACCGATCCGGTCTACGAGGGCAAATCCATGCACGGCATGATTG
911 AAATGGTACGCCGCGGGGAATTCCCTGACGGCTCCAAAGTTCTTTATGCCCACCTGGGCGGCGCACCTGC
1017   GTTGAACGCCTACAGCTTCTTGTTTCGCAACGGCTGA
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Primers:

ACC-F (5′- CGCGCGCATATGATGAACCTGAATCGTTTTGAACGTTATCCG -3′)
ACC-R (5′- GCGATACTCGAGTCAGCCGTTGCGAAACAAGAAGCT -3′)

Analysis of predicted ACC deaminase gene product:

Sequences : 1017, Gene: ACC deaminase gene, No. of amino acids: 338 a.a

AAV73804.1 1-aminocyclopropane-1-carboxylate deaminase [*Pseudomonas putida*] 338 a.a

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MNLNRFERYPLTFGPSPITPLKRLSEHLGGKVELYAKREDCNSGLAFGGNKTRKLEYLIPEAIEQGCDTL
VSIGGIQSNQTRQVAAVAAHLGMKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPS
WEKAMSDVVERGGKPFPIPAGCSEHPYGGLGFVGFAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVGF
AADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVELGREITEEDVVLDTRFAYPEYGLPNEGTLEAIR
LCGSLEGVLTDPVYEGKSMHGMIEMVRRGEFPDGSKVLYAHLGGAPALNAYSFLFRNG
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Figure 1: Sequences of ACC deaminase gene in P. putida strain UW4 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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FJ465155.1 Pseudomonas fluorescens 1-aminocyclopropane-1-carboxylate
deaminase gene, complete cds 1017nt.

1 ATGAATCTGAATCGTTTTGAACGTTATCCATTGACCTTCGGTCCTTCGCCCATCACGCCCTTGAAGCGCC
71 TGAGTAAACATCTGGGGGGCAAGGTCGAGCTGTATGCCAAACGTGAAGACTGCAACAGTGGCCTGGCCTT
141 TGGTGGGAACAAGACACGCAAGCTCGAATACCTCATTCCCGAAGCGCTCGAGCAAGGCTGCGATACGCT
211 GTCTCCATCGGTGGCATTCAGTCGAACCAGACCCGCCAGGTTGCCGCAGTCGCTGCCCACTTGGGCATG
281 AGTGCGTGTTGGTGCAGGAAAACTGGGTGAACTATTCCGACGCGGTGTATGACCGGGTAGGCAACATCG
351 GATGTCGCGAATCATGGGCGCTGACGTGCGGCTTGATGCAGCTGGCTTCGATATTGGTATTCGGCCAAG
421 TGGGAAAAGGCCATGAGCGATGTCGTGGAGCAAGGTGGCAAACCGTTTCCGATTCCGGCGGGTTGTTCC
491 AACACCCCTACGGCGGCCTCGGTTTCGTCGGCTTTGCCGAAGAAGTGCGGGAGCAGGAAAAGGAACTGG
561 CTTCAAATTTGACTACATCGTGGTCTGCTCGGTGACCGGCAGCACGCAGGCGGGCATGGTGGTCGGTTT
631 GCGGCTGACGGTCGTTCAAGAAATGTGATTGGCATTGATGCTTCGGCCAAGCCGGAACAGACCAAGGCA
701 AGATCCTGCGCATTGCCCGGCACACGGCTGAGCTGGTGGAACTGGGGCGCGAGATTACGGAGGAGGATG
771 AGTGCTCGATACGCGTTTCGCCTATCCGGAATATGGCTTGCCCAACGAAGGCACATTGGAAGCCATCCG
841 CTGTGCGGCAGCCTTGAAGGGGTGCTGACGGATCCGGTGTACGAAGGTAAATCGATGCACGGCATGATT
911 AAATGGTGCGTCGGGGTGAATTCCCCGAAGGCTCGAAAGTGCTTTACGCACACTTGGGCGGGGCGCCGG
1017 GCTGAACGCCTACAGCTTCCTGTTCCGCAACGGCTGA
```

Primers:

ACCP-F 5′ - ATGAATCTGAATCGTTTTGAACG -3′

ACCP-R 5′ - TCAGCCGTTGCGGAAC -3′

Analysis of predicted ACC deaminase gene product:
Sequences : 1017, Gene: ACC deaminase gene, No. of amino acids: 338 a.a

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ACJ69586.1 1-aminocyclopropane-1-carboxylate deaminase [Pseudomonas
fluorescens] 338a.a

MNLNRFERYPLTFGPSPITPLKRLSKHLGGKVELYAKREDCNSGLAFGGNKTRKLEYLIPEALEQGCDTL
VSIGGIQSNQTRQVAAVAAHLGMKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRPS
WEKAMSDVVEQGGKPFPIPAGCSEHPYGGLGFVGFAEEVREQEKELGFKFDYIVVCSVTGSTQAGMVVGF
AADGRSRNVIGIDASAKPEQTKAQILRIARHTAELVELGREITEEDVVLDTRFAYPEYGLPNEGTLEAIR
LCGSLEGVLTDPVYEGKSMHGMIEMVRRGEFPEGSKVLYAHLGGAPALNAYSFLFRNG
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Figure 2: Sequences of ACC deaminase gene in P. fluorescens 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.

Antibiotic genetic marker tests

To know antibiotic genetic markers between P. fluorescens and P. putida, some types of antibiotics were examined manually such as; Cefixime (5), Levofloxacin (5), Moxifloxacin (5), Ampicillin (10), Amoxicillin - Clavulanic Acid (20), Amikacin (30), Ciprofloxacin (5), Tigecycline (15), Gentamicin (10) and Nitroxoline (30) all in (µg/mL). The isolated P. fluorescens and P. putida were cultured on King's B and PVK medium agar respectively. The cultured media were treated with the above antibiotics under conditions of air, at 28±2˚C and 48 hours. Moreover, the differentiations of sensitivities and resistances between both

bacteria were confirmed automatically by using VITEK-2 compact techniques which testing different types of antibiotics (https://www.biomerieux.com/).

Conjugation process

To transfer ACC gene from P. putida as a donor bacteria to P. fluorescens as a recipient bacteria, the process was conducted according to the proposed method (Olsen et al., 1992; Holmes and Jobling, 1996). Single colony of P. putida was used for inoculation of 10 mL of PVK medium, and incubated at 28±2˚C for 48 hours with shaking at 100rpm. For the P. fluorescens, same steps were performed using King's B medium. Then, 700µL of P. putida culture medium mixed with 300µL of the P. fluorescens culture as recipient cells. The mixture culture was added by 1.5 ml of fresh enrichment broth medium and incubated at 28±2˚C for 4 hours under aerobic condition. After incubation, 0.1 mL of conjugated mixture was streaked on the fresh King's B agar plates containing the selected antibiotic genetic markers. Further, control plates for both donor and recipient bacteria were prepared separately and treated with the same antibiotic genetic markers. All culture plates were incubated for two days at 28±2˚C. Isolation and purification steps were conducted on the conjugation mixture culture after incubation. Then, the transconjugant colony numbers were chosen and the conjugation frequency was measured (Simon, 1984).

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

$$\textit{Frequency of conjugation} = ___\%$$

Molecular identifications of the conjugation process

Based on the previous mentioned protocol, the genome of the transconjugant bacteria was isolated. The amount and purity of the isolated genome was confirmed via Nano Drop mechanism. Transferred ACC deaminase gene was amplified and visualized via running PCR and 1.5% '(w/v)' agarose gel based on the previous mentioned process. The functionality of the transferred ACC deaminase gene in transconjugant bacteria was known by capsule formation under staining in microscope.

Co-inoculations of wheat plants with transconjugant P. flourences

In order to know the effect of transconjugant P. flourences on wheat crop in field trial, pot experiment were conducted in Technical institute of Khabat – Erbil during January to June- 2021. Bio-fertilizers from the transconjugant P. flourences were prepared according to the technique which was mentioned in (Khider and Khidher, 2011). The isolated transconjugant P. fluorescens were inoculated with 250 ml of the King's B broth medium and shaking incubation was done at 28°C 125 rpm, and then Sub culturing were performed. In order to get inoculum with >108 cells/ml of the bacterial populations, spectrophotometry counting method at wave length 600nm was used (Kumar and Chandra, 2008). For bio-fertilizer production, specific carrier was prepared (%20 $CaCO_3$, %20 compost, %20 clay, %20 Charcoal, %19 sand , and %1 gum), and then bacterial inoculums were mixed with carrier (1ml/100gm). All mentioned steps were conducted for pure P. fluorescens and P. putida as well. Surface sterilized wheat seeds were inoculated with prepared biofertilizers (100gm seed/ 1gm biofertilizer) before sowing. In this experiment

there were five treatments with four replications included (transconjugant P. fluorescens, p. putida, P. flourescens, non- inoculated and non-irrigated control treatment, non-inoculated and irrigated control treatment. Treatments were arranged in three randomized flower pots. The plants were harvested at the end of May. The plants and soil were subjected to different tests included roots and shoots length, root and shoot dry weight, number of spikes and seeds, nitrogen, phosphorus and potassium uptake, total proteins in seeds, and soil nitrogen, phosphorous content.

RESULTS AND DISCUSSIONS

Soil samples collection were performed to isolate P. and P.putida from different locations in Erbil governorate. Among 110 Soil samples, only 75 isolates were selected on King's B medium based on their similarities in morphological and biochemical tests. All isolates negative to gram nature, rod shaped, greenish yellow colony, motile, aerobic, non-spore former. Isolates were biochemically characterized for their ability to produce catalase, gelatinase, arginine dihydrolase, oxidase, starch and urea hydrolysis, and had ability to utilize trehalose, levan production from sucrose, nitrate reduction, and growth at 4^{o}C and 41^{o}C. All these strains have been reported to use galactose, dextrose, citrate and mannose but showed varying degree of utilization towards other carbon sources such as xylose, lactose, fructose, melibiose, glycerol, Larabinose, ribose, D-arabinose, xylitol, malonate, sorbose, sorbitol, trehalose, mannitol, and glucosamine. These strains did not utilize maltose, sucrose, inositol, cellobiose, and rhamnose. According to the results, all isolates revealed extensive phenotypic characterization with P. fluorescens (Soesanto et al., 2011; Patel et al., 2013). On the PVK medium, 65 isolates were recognized from Soil samples which were taken from drought locations that most of the colonies were similar morphologically and biochemically to each other. They were characterized by creamy and smooth colony, negative reaction to Gram staining, short rods, had one flagella and did not create endospores, reduced nitrates, motile, did not liquefy gelatin, positive to oxidase and catalase test, produced HCN, could not hydrolysis starch, they hydrolyzed urea, unable to utilize trehalose, could not grow at 41ºC but grew at 4 ºC. Results of morphological, physiological and biochemical characterization indicated that all isolates belonged to the bacterium P. putida according to methods described in Bergey's Manual of Determinative Bacteriology.

Bacterial isolates were identified by API20E technique, and the result indicated that the bacterial isolates from both culture media were belonged to the genus Pseudomonas spp. Vitek2 compact technique was utilized for identifications of both bacteria to species level. The results demonstrated that both bacteria were P. fluorescens and P. putida. Vitek2 compact has ability to identify the majorities of bacteria and antibiotic susceptibility automatically (https://www.biomerieux-diagnostics.com/).

Molecular process was performed to identify the species and strains of the bacteria. One of the 16s rRNA universal primer was utilized in PCR, and the sizes of the PCR products were confirmed by 1.5% '(w/v)' agarose gel. The size of the PCR products were about 620bp which were closely comparable to the size of 16S rRNA gene in Pseudomonas spp. (Figure 3, lane 3 and 5), (Waldeisen et al., 2011; Patel et al., 2013).

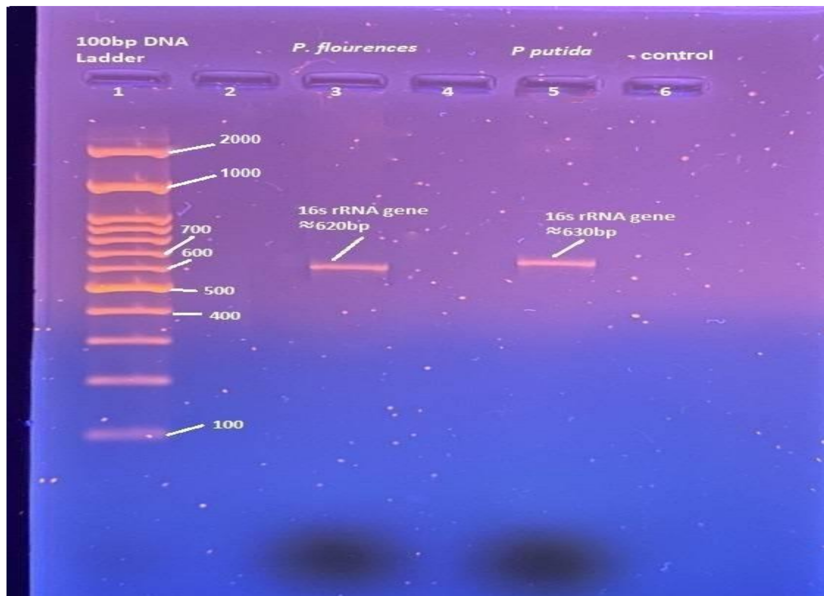

Figure 3: Gel for PCR products of 16s rRNA gene. Using 1.5% '(w/v)' agarose gel with Ethidium bromide. Lane1, 100bp DNA Ladder. Lanes 2 and 4 are blanks. Lane3, PCR products from P. fluorescens genomics. Lane5, PCR products from P. putida genomics. Lane6, negative control.

Sanger sequence was conducted on the amplified genes for identifications of isolated bacterial species and strains. (https://oligomer.com.tr/). P. putida strain UW4 was confirmed to have ACC deaminase gene. The gene size with sequences of the primers was around 1017bp (Figure 4, lane 3). Choosing this strain of the bacteria was due to have capability to survive in drought soil and express high expression amount of ACC deaminase enzyme compared with other types of strains (Cheng et al., 2008). Depending on the results in (Figure 4, lane 2), it was showed that our P. fluorescens isolates were lack of the ACC deaminase gene. Lack of ACC deaminase gene in P. fluorescens had to be confirmed in order to success the conjugation process, as there are many strains of P. fluorescens that contain this gene (Ahmad et al., 2018). For transferring of chromosomal ACC gene from P. putida to P. fluorescens conjugation process was performed.

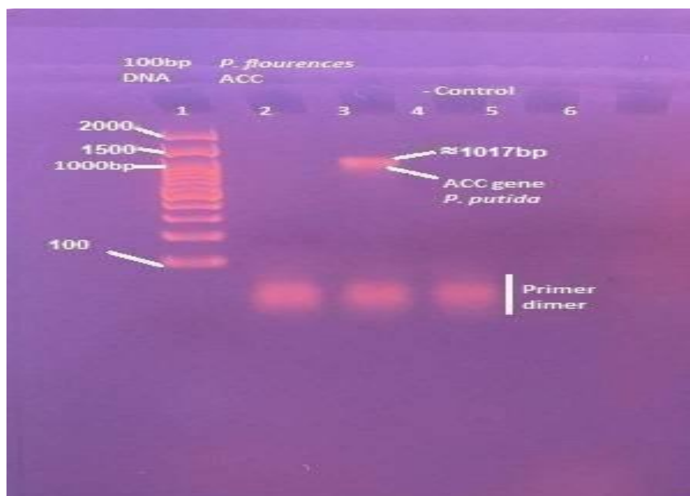

Figure 4: Gel for PCR products of ACC deaminase genes in both bacteria. Using 1.5% '(w/v)' agarose gel with Ethidium bromide. Lane1, 100bp DNA Ladder. Lanes 2, amplified ACC deaminase from P. fluorescens. Lane 3, amplified ACC deaminase from P. putida. Lane 4, negative control. Lane 5 and 6 are blanks.

For conjugation technique between both bacteria, antibiotic genetic markers were selected by antibiotic resistance test. Two distinguish antibiotic reactions were recorded towards ciprofloxacin (5 µg /mL) and Piperacillin/Tazobactem antibiotics among all antibiotic testing manually. P. fluorescens was resistance to this antibiotic, in contrast P. putida was sensitive. Meanwhile, P. fluorescens was sensitive to Piperacillin/Tazobactem antibiotic, but P. putida was resistance to this antibiotic during running automatically in Vitek2 compact technique (https://www.biomerieux.com/). This result showed that the Piperacillin/Tazobactem and ciprofloxacin can be used as genetic antibiotic markers in conjugation technique. Because, there is an condition that the recipient bacteria should be sensitive in one or two various antibiotics in conjugation technique (Peter et al., 2017).

Conjugation process was conducted between both selected bacteria in BHI enrichment liquid media with using selected genetic antibiotic markers. This media can be used for antimicrobial sensitivity purposes, as many bacterial species can be cultured on this media (Atlas, 2004). There were some bacterial colonies which had considerable phenotypic similarities between P. fluorescens and P. putida in terms of morphological and biochemical. Additionally, there was no colony on the control plates due to the susceptibility to the antibiotic. Molecular techniques on the transconjugant bacteria were conducted to know that ACC deaminase gene was moved from P. putida to P. fluorescens successfully. The amplified gene size of ACC deaminase in transconjugant bacteria was around 1017bp which was similar to the positive control gene size (Figure 5, lane 2 and 3), and there were no any amplified gene sizes in isolated P. fluorescens and negative control except primer dimers (Figure 5, lanes 4 and 5). This result indicated that the ACC deaminase gene has been moved completely from P. putida to P. fluorescens by conjugation process, as there are evidences that conjugation gene transfer can be happened among species and strains of Pseudomonas spp. (Heinaru et al., 2009). Furthermore, transferring of chromosomal DNA between some bacterial cells have been mentioned by conjugation process (Lotareva and Prosorov, 2006; Khider and Khidher, 2011). Conjugation process is occurred among bacteria due to the generation of the conjugation bridge between the donor and the recipient bacteria and transmission of oriT genes across this bridge (Cabezón et al., 2015).

Figure 5: Gel for PCR products of ACC deaminase genes in transconjugant P. fluorescens. Using 1.5% '(w/v)' agarose gel with Ethidium bromide. Lane1, 100bp DNA Ladder. Lanes 2, amplified ACC deaminase from P. putida used as a positive control. Lane 3, amplified ACC deaminase from transconjugant P. fluorescens. Lane 4, amplified ACC deaminase from P. fluorescens. Lane 5, negative control. Lane 6, blank.

Growth effects of transconjugant P. fluorescens on wheat crops (Triticum aestivum spp.) in the pot experiment field were evaluated according to the mentioned arrangement techniques. The data were analyzed and the results demonstrated that the average length of wheat roots in transconjugant P. fluorescens was 25.90cm which was the longest roots compared with other treatments (Table 1 and Figure 6). However, the lowest average length of wheat roots was 17cm in control without irrigation (Table 1 and Figure 6). The average root length of P. fluorescens treatment was 20.15cm which was smaller than P. putida treatment (Table 1). Moreover, the average root length of control with irrigation was 19.13cm which was longer than control with un-irrigation and smaller than other treatments. This result indicated that transconjugant P. fluorescens had significant effects on the wheat roots in the pot experiment filed due to uptake ACC deaminas gene. The longest roots could be due to lowering ethylene levels in roots, as has been mentioned that ACC deaminase activity could reduce approximately 60% stress stimulated ethylene levels and its associated growth inhibition in French beans (Gupta and Pandey, 2019). (Glick et al., 1998) demonstrated that ACC deaminase enzyme has ability to bind the surface of plants (usually seeds and roots), and induce the phyto-hormone indole-3-acetic acid (IAA) of plants. The IAA hormone can stimulate plant root extensions and proliferations of root cells (Penrose et al., 2001; Tahir et al., 2022). Therefore, this result illustrated that wheat plant treated with transconjugant P. fluorescens under pot experiments can reach the soil moister in the depth nearly 26cm without irrigation by its root elongations.

Figure 6: Comparison between the length of wheat roots of Transconjugant P. fluorescens treatment and control without irrigation treatment.

As can be noticed from the table (1), the plant heights of inoculated treatments with P. fluorescens and P. putida were 49.55cm and 50.85cm respectively. Additionally, the plant heights of controls with irrigation and without irrigation treatments were 49.20cm and 38.55cm respectively as well (Table 1). In contrast, the plant height of transconjugant P. fluorescens treatment was 54.10cm which was bigger than all other treatments significantly especially with control (without irrigation) treatment. This result revealed that transconjugant P. fluorescens had effect positively on the plant height under pot experiment due to expression of ACC deaminse enzyme. (Glick, 2012) demonstrated that ACC deaminase-containing plant growth-promoting bacteria can reduce ethylene which is responsible for decreasing of plant growth under a variety of abiotic and biotic stresses. Therefore, crops which treated with ACC deaminase-containing plant growth-promoting bacteria usually have taller roots and shoots and are more resistant to growth inhibition by a variety of ethylene-inducing stresses.

According to the table (1), transconjugant P. fluorescens treatment had positive effects on spike number, total seed weight and shoot dry weight of the wheat plants compared with other treatments. However, there were insignificant differences in tiller numbers of all wheat plant treatments (Table 1). This result illustrated that transconjugant P. fluorescens had positive impacts on increasing growing ratio of all organs of wheat crops due to the ACC deaminase activity under drought stress, as this enzyme can decrease plant stresses (biotic and abiotic), and eventually reduce Reactive Oxygen Species (ROS) of crops which is responsible for damaging plant cell organelles (Fischer and Maurer, 1978; Hussain et al., 2022).

Table 1: Morphological effects of single and co-inoculation of (P. fluorescens, P. putida, Transconjugant P. fluorescens, Controls with irrigation, and Controls without irrigation) on the growth of wheat (Triticum aestivum spp.) plants. Only five wheat crops were selected to be evaluated

Treatments	Root length	Plant height	Tiller number	Spike number	Root dry	Total seed	Shoot dry weight

	(cm)	(cm)		/5crops	weight	weight	(gm /5 crops)
T1 (P. flourences)	**20.15^{ab}**	**49.55^{ab}**	**18.25^{ab}**	**17.25^{ab}**	**13.90^{c}**	**9.40^{bc}**	**26.30^{a}**
T2 (P. putida)	**22.250^{ab}**	**50.85^{ab}**	**19.50^{a}**	**18.25^{ab}**	**16.65^{ab}**	**9.30^{bc}**	**26.57^{a}**
T3 (Transconjugant P. flourences)	**25.90^{ab}**	**54.10^{a}**	**18.25^{ab}**	**32.62^{b}**	**18.15^{ab}**	**14.55^{b}**	**28.15^{a}**
T4 (Controls with irrigation)	**19.13^{ab}**	**49.20^{ab}**	**18.20^{ab}**	**17.30^{ab}**	**14.10^{ab}**	**9.70^{bc}**	**25.90^{a}**
T5(Controls without irrigation)	**17.00^{b}**	**38.55^{c}**	**15.50^{b}**	**14.50^{c}**	**13.42^{c}**	**7.57^{c}**	**17.15^{b}**

Chemical effects were evaluated based on percentages of NPK and protein contents. The amount of seed protein contents in transconjugant P. flourences treatment was 7.820% which was the highest amount compared to other treatments (table 2). Meanwhile, the lowest quantity of seed protein contents was 6.675% in control treatment without irrigation (Table 2). This result illustrated that transconjugant P. flourences had ability to increase the amount of seed protein contents without irrigation, as the quantity of protein in wheat seed is responsible for the quality of the wheat seed and there are strong relationship between protein content and seed quality in wheat crops (Ayers et al., 1976; Asseng et al., 2019).

Quantity of nitrogen was measured in wheat shoots. As can be observed from the table (2), the highest amount of nitrogen was 3.32% in transconjugant P. flourences treatment, while the lowest quantity of nitrogen in wheat shoots was 2.79% in controls without irrigation treatment (Table 2). However, there were no significant differences between all treatments except transconjugant P. flourences treatment. Therefore, this result demonstrated that transconjugant P. flourences was capable to increase the amount of nitrogen in wheat shoots, as nitrogen is the most essential nutrient, and it has vital role in promoting tellering, increasing photosynthesis process and protein production substances in grain (Sanchez-Bragado et al., 2017).

The percentages of phosphorus were evaluated in wheat shoots. The highest amount of phosphorus was 0.20% in transconjugant P. flourences treatment (Table 2). Meanwhile, the lowest quantity of phosphorus was 0.17% in control without irrigation treatment (Table 2). This result showed that transconjugant P. flourences has ability to increase the of amount phosphorus in wheat shoots without irrigation. Phosphorus is an important nutrient in plant generally, and this nutrient has crucial roles in wheat growing in seedling stages to maturity and seed formations (Liu et al., 2020). Further, phosphorus is involved in transfer genetic characteristics to offspring, sugar and starch transformation, photosynthesis process, energy transfer to all plant organs and has a vital role in wheat cell division (Shabnam et al., 2018; Umar, 2021).

Potassium quantity was measured in the wheat shoots. There was a significant difference between the quantities of potassium in transconjugant P. flourences treatment to all other treatments. As can be observed in the table (2), the larger amount of potassium was 3.10% in transconjugant P. flourences,

whereas, the lowest quantity was 2.35% in P. putida treatment. The potassium quantity in P. fluorescens treatment was 2.82% which was bigger than the amount of potassium in controls with irrigation 2.65% and controls without irrigation 2.37% treatments (Table 2). This result indicated that transconjugant P. fluorescens had significant effect in increasing the quantity of potassium in the wheat shoots. Usually, wheat crop requires sufficient amount of potassium for optimal growth and enhancement, and it has crucial role in increasing resistance of wheat against particular pathogens and pests (El-Lethy et al., 2013; Burhan and Al-Hassan, 2019). In addition, potassium enhances photosynthesis process, produces grains rich in starch, maintains normal balance between proteins and carbohydrate, and decreases water lose due to drought stress in barely (Hosseini et al., 2016).

Table 2: Chemical effects of single and co-inoculation of (P. fluorescens, P. putida, Transconjugant P. fluorescens, Controls with irrigation, and Controls without irrigation) on the N,P, K and protein contents of wheat (Triticum aestivum spp.) plants. Only five wheat crops were selected to be evaluated

Treatments	Seed protein%	Nitrogen%	Phosphorus%	Potassium%
T1 (P. flourences)	7.3500^{cd}	2.8125^{b}	0.1850^{bc}	2.8250^{bc}
T2 (P. putida)	7.5600^{bc}	2.8775^{b}	0.1850^{bc}	2.3500^{d}
T3 (Transconjugant P. flourences)	7.8200^{bc}	3.3200^{a}	0.2000^{bc}	3.1000^{ab}
T4 (Controls with irrigation)	7.4000^{cd}	2.8000^{b}	0.1775^{bc}	2.6500^{cd}
T5 (Controls without irrigation)	6.6750^{d}	2.7925^{b}	0.1700^{c}	2.3750^{d}

CONCLUSION

According to the results of this research article, the ACC deaminase gene was transferred successfully via conjugation process from P. putida to P. fluorescens, and mutated P. fluorescens (transconjugant P. fluorescens) was generated after confirming via molecular identification process. The functionality of transferred ACC deaminse gene was confirmed by inoculating with wheat crops (Triticum aestivum spp.) in pot experiments. Growth rate and chemical effects of transconjugant P. fluorescens with ACC deaminase gene were evaluated. The transconjugant P. fluorescens had significant effects positively on wheat plant in terms of growth rate (root length, plant height, tiller number, spike number, root dry weight, total seed weight and shoot dry weight) and chemical (N,P, K and protein contents). The mutated transconjugant P. fluorescens now has ability to induce plant growth, drought tolerance, and it can be utilized as bio-fertilizer agents especially for drought and lack of nutrient soils.

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