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The role of bacteria *Bacillus subtilis* in improving rooting response of Mung bean (*Vigna ratiata*) cuttings

Khalid Ali Hussein,[a] Najeh Hashem Kadhum,[a] Yass Kudhir Yasser[a]

[a]Department of Biology, College of Science, University of Karbala, Karbala, Iraq.
Correspondence to Khalid Ali Hussein (email: kalbio1970@yahoo.com).
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Objectives This study includes the investigative ability of *Bacillus subtilis* isolated from soil to produce Indole acetic acid (IAA) and effect on rooting response of mung bean cuttings.
Methods The some of the optimal conditions for IAA production have been indicated. The effect of indole acetic acid production in *Bacillus subtilis* filtrate on the rooting response of mung bean cuttings. The effect of IAA production in *Bacillus subtilis* filtrate In carbohydrate, protein, IAA contents and activity of IAAO in cutting hypocotyls.
Results Results revealed that, maximum IAA production was obtained by inoculating with an inoculum size of 125 µl/10 ml production culture and incubation at 40°C for 72 hrs. IAA produced by bacteria was significantly enhanced the average of root number/cutting to its levels in control treatment and caused an increase in IAA level and a decrease IAA oxidase activity in hypocotyls, which were significantly different from the control. Whereas, carbohydrate and protein contents in hypocotyls were increased significantly to its levels in control. The highest level values were found in synthetic auxin treatment.
Conclusion The bacterial auxin can be used to improve rooting response of mung bean cuttings and this method very easy and not costly compared with synthetic hormones.
Keywords Mung bean, Indole acetic acid, rooting response and *Bacillus subtilis*

Introduction

Bacteria that colonize plant roots in many cases are useful for the growth and development and plant productivity,[1] and the synthesis of plant hormone growth regulators such as auxin, gibberellins and cytokines) by microorganisms, believed as one of the main forms of interaction between the plant and the microorganisms. The ability of production has been found in different types of bacteria (pathogens and commensally as well as free is common with the plant, Khalid (2004)[2] revealed that many bacteria rhizophere are able to synthesis IAA including *Streptomyces*, *Bacillussp*, *Azotobatersp.* In addition to bacteria, fungi and algae have shown to stimulate plant growth by the synthesis of IAA. The bacteria synthesize and release auxins as secondary metabolites.[3] Indole-3-acetic acid (IAA) is the main member of the auxin family that controls many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity.[4] The auxin, IAA has been identified as a rooting hormone. Exogenous application of IAA induces rooting in several stem cuttings, formation of adventitious roots can be induced by the treatment with auxins.[5] IAA produced by the bacteria works in conjunction with the internal auxin plant to stimulate root proliferation and division of cells and nutrient uptake from the culture media.[6]

The main precursor for the synthesis of IAA in plants and bacteria is tryptophan. High production of IAA results in adding tryptophan to the growth medium.[7] Dobbelare et al., (2003)[8] reported the bacteria plant growth promoting rhizobacteria (PGPR) led to an increase in the number of root hairs and lateral roots and decrease of the lengths of the roots. These changes attributed to the effect of auxin produced by the bacteria root colonies of strains of bacteria, *B. subtilis,* which enhanced rooting in plants. Roots are the most the sensitivity of to changes IAA levels, by elongation primary roots and the formation adventitious roots and lateral roots.[9] The hormone produced by the bacteria can be used as a relationship between bacteria and plants.[10] Overall, industrial rooting hormones are widely used in vegetative propagation of stem cuttings which show variation in rooting ratios. So the goal of this study is to use *B. subtilis* bacteria in the production of IAA in growth medium as well as with the study some of the conditions that affect the production of bacteria that isolated from soil and to find out the role of the bacteria IAA in rooting response of mung bean cuttings and improvement the ability of the Mung bean cuttings rooting by using relatively easy and inexpensive way.

Materials and Methods

Bacillus subtilis, bacteria was used that was obtained from the Department of biology Sciences / College of Education / University of Karbala isolated from soil and dried to form a dry powder and loaded with a substance calcium carbonate and working bacterial suspension in normal saline solution 0.85% by dissolving 50 mg of bacteria in 4.5 ml of physiological salt solution. Bacteria active in nutrient broth by taking 1 ml of suspension Bacteria was added to 9 ml of nutrient broth and incubated for 24 hours for the parapets incubated[10] to identify the optimal conditions for indole acetic acid production by bacteria *Bacillus subtilis*

A number of factors were studied affecting indole acetic acid production of by *Bacillus subtilis* in nutrient broth media. After sterilization, it was supported by amino acid Tryptophan in the concentration of 1 mg/ml. Some of the optimal culture conditions of production were studied.[10]

Effect of Incubation Period on IAA Production

100 µL of 24 h bacterial broth was inoculated in 9.9 mL of nutrient broth medium in 25 mL flask containing 0.1 mg mL^{-1} L-tryptophan. The flask was incubated at 35°C temperature for (24, 48, 96, 120 and 196 hr), to determine the optimum period

for the production. The concentration of indole acetic acid was estimated according to Bent et al., (2001)[11] in production media (Nutrient broth support by tryptophan), the bacteria was removed from the culture by use of the centrifuge (10000 r/min), then the amount of auxin estimated in the bacterial filtrate using Salkowski reagent ($FeCl_3$1 ml, $HClO_3$ 35 ml) absorbance was noted at 530 nm. The amount of IAA produced was calculated based on standard graph prepared from IAA solution (0–100 μg ml^{-1}).

Effect of Incubation Temperature on IAA Production

The bacteria Bacillus subtilis was incubated for 48 hours at various temperatures (30, 35, 40, 45 and 50°C) to determine the optimum temperature for the production of auxin.

Effect of Inoculum Size on IAA Production

Production medium of indole acetic acid incubate by inoculum size (25, 50, 75, 100, 125 and 150) μl/ 10 ml production culture and incubation at 40°C for 72 hrs to statement effect of the inoculum size IAA production from bacteria.

The Effect of Indole Acetic Acid Production in Bacillus Subtilis Filtrate on the Rooting Response of Mung Bean Cuttings

After determining the optimal conditions for IAA production, temperature, period incubation and inoculum size, its effect on rooting mung bean cuttings were being tested. Seeds of Mung bean (*Vigna ratiata* L. Wilezek) were germinated in sterile sawdust supplied with water after soaking overnight. In addition to seedling growth were carried out in growth cabinet at 25 ± 1°C, under continuous illumination supplied by warm white fluorescent tubes (1500–1800 Lux) and relative humidity of 60–70% (Binder GMBH, Germany). Stem cuttings were prepared according to[12] from 10-day-old light grown seedlings (Fig. 1). The cutting had apical bud, pair of fully expanded primary leaves, epicotyls and 3 cm of hypocotyl under cotyledonary nodes, after removal of root system (Fig. 2). Boric acid was prepared at concentrations 5 μg/ml (as rooting medium) Auxin solution (IAA) was prepared at concentration 10^{-4} M. Basal part of the hypocotyl of fresh cuttings were treated for 24 h with tested solutions (distilled water, bacterial filtrate and synthetic auxin) thereafter, cuttings were transferred to boric acid (5 μg/ml) for 6 days due to its necessity in the formation of root primordia and its subsequent growth and development to visible roots.[13] Twelve cuttings per treatment for rooting test were placed 4 per glass vial containing 15 ml (3 cm depth) of the appropriate solution under the same conditions of growing seedlings, then calculate the number root/cuttings.

Fig. 1 **Mung bean seedling in culture media (left) and Mung bean seedling (right).**

Fig. 2 **Mung bean cuttings were prepared from 10-day-old light grown seedlings after removal of root system.**

The Effect of IAA production in Bacillus Subtilis Filtrate in Carbohydrate, Protein, IAA Contents and Activity of IAAO

The carbohydrate contents of hypocotyle according to Dubois et al. (1956)[14] and proteins according to Bishop et al., (1985)[15] and according to the method Unyayar et al. (1996)[16] IAA was estimated, the activity of IAAO estimated according to Sequeria and Nineo, 1966)[17], IAAO activity is represented by the amount of IAA degraded (μg) starting from 1 mg.

All experiments were designed completely randomized, Data were subjected to analysis of variance (ANOVA) and the means were compared using L.S.D test ($P \leq 0.05$) by SAS software.

Results and Discussion

The IAA production by bacteria in a growth medium was watched for five days, and then the model was to draw every 24 hours to measurement IAA produced by bacteria. Figure 3 shows that the IAA production begins after 24 hours of incubation. IAA production reached to (55.34) mg/ml and then rose gradually to a maximum in the third day, when they reached to (106.38) mg/ml, then began to decline, reaching below the fifth day with a concentration of (71.62) mg/ml. The highest value of auxin production was obtained at 72-hours (the third day) which is not significantly different compared to 48-hours. Mutluru and Konada, (2007)[18] observed that the combination of bacteria with plant roots produces IAA to the liquid medium, and the amount of auxin product depends on the growth conditions, phase growth of the bacteria and the abundance of material basis. Bharucha et al., (2013)[19] showed

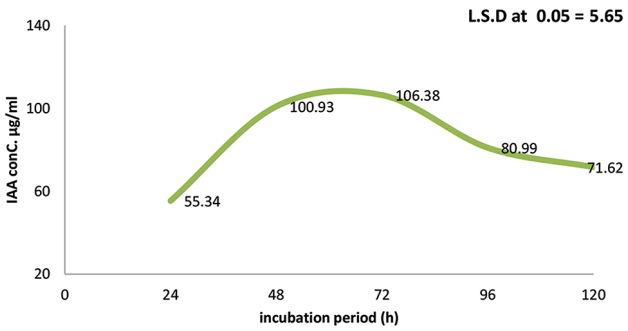

Fig. 3 **The effect of incubation periods on IAA production from *Bacillus subtilis*.**

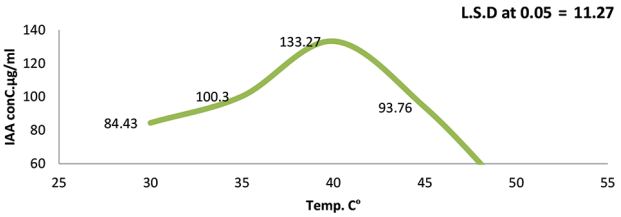

Fig. 4 **The effect of temperature on IAA production from *Bacillus subtilis*.**

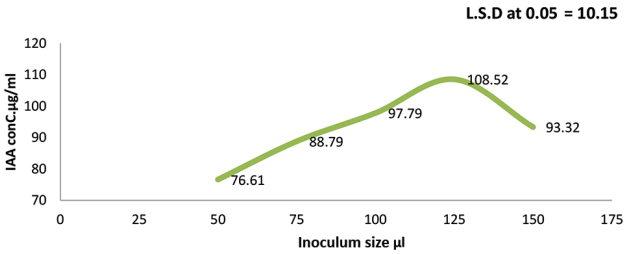

Fig. 5 **The effect of inoculum size on IAA production from *Bacillus subtilis*.**

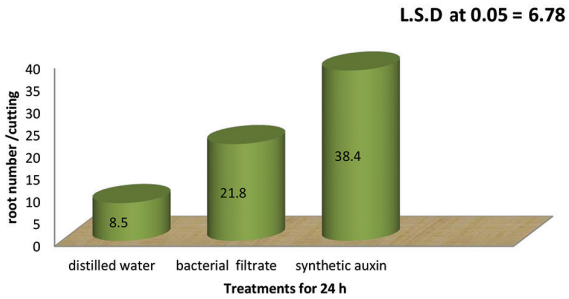

Fig. 6 **Effect of IAA produced by bacteria in rooting response of mung bean cuttings.**

that the higher productivity of IAA at 96 hours by bacteria *Pseudomonas putida*. Swain et al., (2007)[20] indicated that the IAA production by the *Bacillus subtilis* increased linearly from 2 to 8 days, and then fell with a decreased bacteria in a culture which was supported with amino acid Tryptophan. Decline in IAA production may be arising from the release of analysis enzymes for auxin such as peroxidase and IAA oxidase.[3]

2. Temperature

Figure 4 indicates that the highest value of IAA production was obtained at a temperature of 40°C, and gave (133.27) mg/mL, and then IAA production decrease significantly on level of 0.05. So this degree was adopted in all experiments. Varied thermal grades used in the production of indole acetic acid of microbiology, Shahab et al. (2009)[21] found that the 30°C is the optimal for auxin production from *Bacilllus thuringiensis*. While the use of 36 ± 2 temperature in the auxin production from *Azotobacter*, *Rhizobium*, *Bacillus* and Pseudomonas.[22] On the other hand, while Sudha et al., (2012)[23] showed that 37°C is the optimum temperature for IAA production by *Bacillus* ssp. Any change in the optimum temperature affected on the biosynthesis enzymes of IAA and thus reflected on the auxin production.[24]

3. Inoculum size

Results in Fig. 5 show that the IAA production increases the inoculum size until it reached its maximum by using 125 µl, where the auxin production reached (108.52 µg/ml) thereafter IAA declined, so the inoculum size, period of incubation for 2 days and at a temperature of 40°C were adapted in practical experience on mung bean cuttings. Volumes of the inoculum size used varied in IAA production from microorganisms, Swain et al., (2007)[20] had used the inoculum size 2% in IAA production from *B. subtilis*. Huu Da et al., (2015)[25] indicated that the percentage of inoculum size 1.65% gave the highest IAA productivity from *B. subtilis* TIB6 isolated from root rhizosphere of the pepper plant.

4. The Effect of IAA Produced by Bacteria in Rooting Response of Mung Bean Cuttings

Figure 6 shows a significant increase in rooting response of mung bean cuttings terms number of adventitious roots per cutting (8.5, 21.8, 38.4) root/cutting each of the control, synthetic auxin, respectively. Data show that the number of roots increased three-fold compared with the roots of cuttings treatment with distilled water and an increase percentage 156.47% when using bacterial filtrate attributed the cause to increase rooting percentage in bacterial filtrate to the bacteria ability to produce auxin, which is impact effective in rooting response. Many of microorganisms can interact with the plant and produced hormones similar to those produced by plant such as regulated growth auxins and gibberellins and cytokines,[26] among these hormones and most important in initiation and development roots is auxin.[27] Results of the study showed that *Bacillus subtilis* bacteria produces growth regulator (IAA), that impact on rooting response and thus success of rooting. This is agreeing with Bae (2007)[28] which found that *Bacillus subtilis* stimulate the emergence and differentiating adventitious roots in Cucumber cuttings. Tavkelova et al., (2005)[29] showed that kidney bean cuttings treated with liquid growth media of *Bacillus subtilis* enhanced adventitious root formation from 4.7 to 4.13 times more than control samples. Srinivasan et al., (1996)[30] showed that the treated *Phaseolus vulgaris* plant with *Bacillus* (non pathogenic) enhanced adventitious root formation. Also Erturk et al., (2010)[31] found that *Bacillus* RC23 and *Bacillus subtilis* and *Bacillus* RC03 and *Bacillus* simplex RC19 stimulates adventitious root formation in kiwifruit cuttings.

5. Effect of Bacterial and Synthetic IAA in Hypocotyl Content of IAA

Figure 7 shows the effect of each bacterial and synthetic filtrate auxin in Hypocotyl cutting content of IAA, the IAA content in

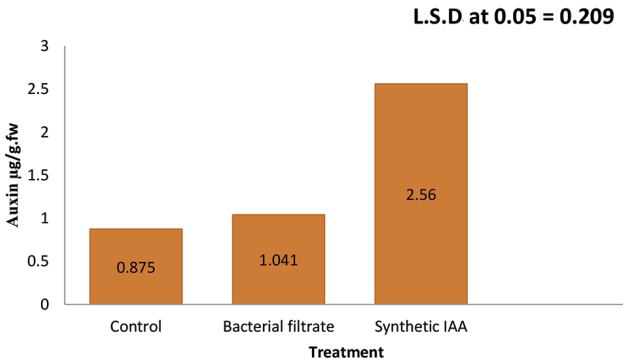

Fig. 7 **Effect of bacterial and synthetic IAA in Hypocotyl content of IAA.**

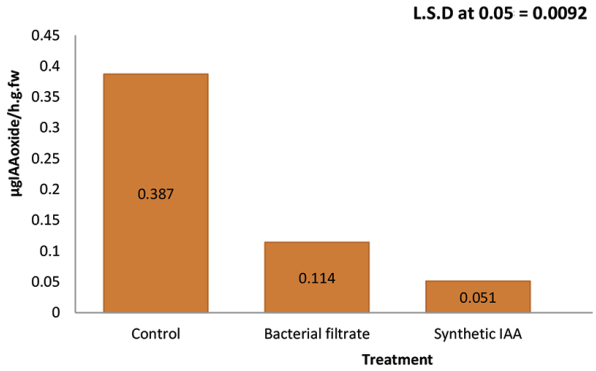

Fig. 8 **Effect of bacterial and synthetic IAA in IAA Oxidase activity in Hypocotyl.**

Hypocotyl as the primary content papers in control treatment (distilled water for 24 hours) is 0.875 µg/g fresh weight, this content rise to 1.041 µg/g fresh weight of Hypocotyl cutting in treated with bacterial filtrate and increase percentage 19%. However in the synthetic auxin treatment, IAA content has increased significantly for both treatment control and bacterial filtrate and increased by 145.91% compared to the treatment of bacterial filtrate as 100%, increase percentage 334.36% compared to the control treatment as 100%. Internal auxin produced in the vegetative and transport basipetally to Hypocotyl may be complementary to the exodogenous IAA levels, which improved the rooting percentage,[32] the increase in IAA content may be due to the decline in IAAO activity Fig. 8, and this was confirmed by[33] auxin also known as the key signal initiation and development adventitious roots formation,[34] lower IAAO activity in the cuttings might be among the critical factors that improve rooting and this is reflected in rooting response.

6. Effect of Bacterial and Synthetic IAA in IAA Oxidase Activity in Hypocotyl

The activity of IAAO in Hypocotyl cutting is 0.337 µg IAA oxidase/1 h. g. fw in cuttings that treated with distilled water for 24 hours. This activity lowered to 0.114 µg IAA oxides/1 h. g. fw in cuttings treatment of bacterial filtrate, reduce percentage 66.17%, lowers activity is 0.051 µg IAA oxides/1 h.g. fw. It was recorded in the treatment in synthetic IAA Fig. 8. This reflects increase IAA in Hypocotyl (Fig. 5), which revealed the highest number of adventitious roots (Fig. 6). Yan et al., (2014)[33] found reduced IAAO activity in *Hemarthria compressa* plant when treated with auxin.

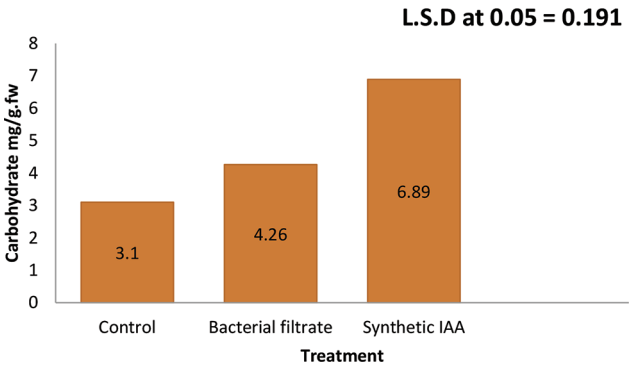

Fig. 9 **Effect of bacterial and synthetic IAA in Hypocotyl content of carbohydrate.**

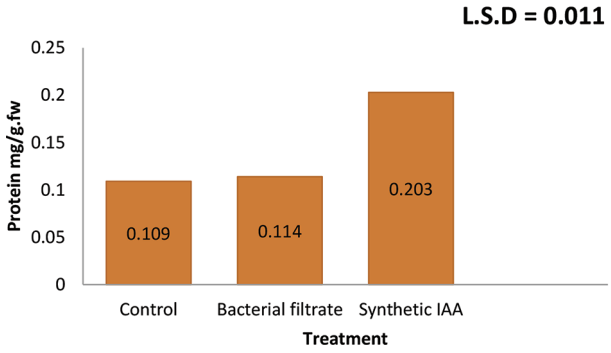

Fig. 10 **Effect of bacterial and synthetic IAA in Hypocotyl content of protein.**

7. Effect of Bacterial and Synthetic IAA in Hypocotyl Content of Carbohydrate

The effect of bacterial filtrate in Hypocotyl content of carbohydrate illustrated in Fig. 9. The results showed that the bacterial filtrate raise carbohydrate content from 3.10 mg/g f.w in control sample to 4.26 when exposed to bacterial filtrate for 24 hours, which increased significantly whereas carbohydrate content in Hypocotyl cuttings treated by synthetic IAA Hypocotyl raise to 6.89 mg/g. f.w. Adventitious root formation seems that depends on a sufficient supply of carbohydrates to adventitious roots formation zone where the roots processed energy and carbon necessary to initiation and development adventitious roots formation.[35] The relationship between auxin and carbohydrate metabolism by creating spin-off roots have studied the outward processing of auxin and control carbohydrate levels and the transfer of carbon and stimulate the enzymes used to metabolize sugar in the rooting zone and the increase observed in the base of the stem in carbohydrates mind Alqrnql levels after outward processing of auxin and suggested that auxin stimulates the new sink of carbohydrates, which directly contributes to the formation of the root primordial,[36] Druge et al., (2009)[37] found that the accumulation of auxin in the rooting zone contributes to the establishment of a new sink of carbohydrates in the rooting zone by stimulating the activity of enzymes Invertase cell wall and Vascular.[38]

8. Effect of Bacterial and Synthetic IAA in Hypocotyl Content of Protein

Figure 10 shows the effect of the bacterial filtrate synthetic auxin in protein content of Hypocotyl mung bean cuttings increased

from 0.109 in the control treatment to 0.114 when treated with bacterial auxin. An increase percentage 4.58%. in case supplying cuttings with a solution of synthetic IAA in concentration 5×10^{-5} for 24 hours, protein content in Hypocotyl was increased by 82.23% compared to treatment with distilled water. Bacterial filtrate is succeeded in increasing the protein content, but did not reach the level of a protein Hypocotyl cuttings-treatment of synthetic auxin. These ratios were significant at the 5% the *Bacillus subtilis*. The ability to produce IAA quantities improved when Tryptophan percent increases in growth media,[25] the bacterial filtrate containing IAA form the bacteria isolated from the soil efficiently improving rooting mung bean response by increasing rooting requirements such as auxin carbohydrates, protein and reduced activity of IAAO analysis for auxin in basal part cutting. Thus we can recommend their use in raising rooting rates in stem cuttings as an alternative to synthetic auxin in the plant propagation which is resulting environmental problems and may increase the cost of propagation. ■

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