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The Production of Indole Acetic Acid from *Bacillus thuringiensis* and Application in Tissue Culture Technique for Two Cultivars of *Solanum tuberosum*

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Abstract
Bacillus thuringiensis is the most widely used bacterial pesticide, besides its activity against insects; the coming research designed for dual production of delta toxin and phytohormone production the indole acetic acid. A local isolate of *B. thuringiensis* kurstaki KS3 was earlier isolated and characterized for its activity against insects. The medium minimal salt (MS) was used for the production of IAA. IAA activity that evaluated in tissue culture technique for two varieties cultivars of *Solanum tuberosum* L and the results indicated that phytohormone IAA extract gave a positive response for the varieties cultivars Rivera and Bureen, that tissue cultures.Statistical analysis revealed no significant differences between the two varieties cultivars about different concentrations of extracted IAA and laboratory-prepared IAA and the negative control in response to plant high, branch number and length, and leaves number. Whereas, LSD showed that Rivira was superior in root number and length.

Keywords: Potato, *in vitro*, Phytohormone, Crystal protein
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1. Introduction

The emergence of a highly increased population and climate change worldwide needs the overproduction of crops by different and new techniques.In Iraq, potatoes are one of the most popular daily foods; many serious pests and fungi infested potatoes in fields and at stores. The tissue culture technique is one of the candidates to produce disease-free plants with somaclonal variation through in vitro propagation of callus culture with desired traits. to improve the recent cultivars.Also, tissue culture was used extensively to boost plant breeding. The tissue culture technique needs many factors to simulate traditional farming. Phytohormone and auxin growth regulators (Cytokinies and Auxins) are famous vital components in the composition of growth medium [1].

Many researchers clarified the ability of soil-borne bacteria to produce plant hormones. Bacillus bacteria are considered one of the most important species present in the soil environment that secrete and produce IAA [2]. One of the most important biocontrol agents belonging to soil microorganisms that are used in the field is *Bacillus thuringiensis*. Besides its bioactivity towards many insects, the bacterium biosynthesizes IAA. Anaturally widespread auxin that produces in larger quantities than all related compounds [3]. In their natural habitat rhizospheric bacteria use root secretions to produce IAA as part of the secondary metabolism process [3]. Microorganisms specifically produce indole acetic acid through a tryptophan-dependent pathway, where the process of metabolism of the amino acid tryptophan occurs by microorganisms to produce the hormone fromRhizosphere bacteria, while the concentration of tryptophan in the laboratory environment varies depending on several factors, including the time of inoculation [4–6].

In plant tissue culture technique requires the availability of medium, with its contents of salts, amino acids, vitamins, sugar and growth regulators (auxins, cytokines and gibberellins), whose presence is very necessary for plant growth. Auxins and Cytokines are the most widely used growth regulators in many studies [7,8] and they are usually added to the medium with specific concentrations either separately or in different combinations based on the gentotype, micropropagation stage, and the explants.

This research is designed to investigate the capability of local isolates belonging to *Bacillus thuringiensis* to produce indole acetic acid and studying the optimum requirement for production.

Also, studying the effectiveness of the extracted IAA and comparing it with the IAA widely used in micropropagation of potato crop.

2. Material and Methods

2.1 Screening for the production of indole acetic acid

The spores oflocal isolates belonging to B. thuringiensis kurstaki wereactivated and their phenotypic characteristics was determined, and some other biochemical characteristics were confirmed by growing it and cultivating it on a solid nutrient agar culture.

MS broth Medium containing 0.3% tryptophan was prepared, sterilized, and then inoculated with corresponding bacterial isolate. Each bacterial inocula was incubated at 30 °C for 24 h using shaker incubator at 120 rpm. For screening of IAA biosynthesis, bacterial growths were centrifuged for 10 min at 10000 rpm, and two ml of each supernatantwere mixed and incubated at dark for 30 min with one ml of Salkowski's reagent (prepared by mixing one milliliter of 0.5M $FeCl_3$ with 49 ml of 35% perchloric acid and storage at dark bottle [9]. The positive results of plant hormone the indole acetic acid was indicated by the appearance of a pink color. For estimation of IAA, the absorbance was recorded after 30 min at 530 nm using spectrophotometer Analytikjenaspecord 205 for three replicates. The concentration of IAA was quantified using a standard curve [10].

2.2 The effect of laboratory parameters on the production of indole acetic acid

Different concentrations of tryptophan were added to the basic MS medium 0, 0.1, 0.2,0.3%. The concentration of IAA was quantified after 24,48 and 72 h to suggest the effect of tryptophan concentration according to time interval.

To study the effect of temperatures on the production of IAA, the bacterial isolate *B. thuringiensis* KS3 and Bt ESP were incubated at 25, 30, 35, and 40 °C for 24 h with agitation. The best temperature was accounted for inthe study.

To qualify the aeration on the production of IAA from*B. thuringiensis*KS3 and BtESP, cultures wereincubated at an ambient temperature either in steady state fermentor without aerationor at shaking fermentor at 120 rpm/min.

2.3 Plant tissue culture experiment

Two varieties of Potato tubers the Riviera and Burren were purchased from Anhar al- Ward company. Tubers were cleaned using tab water for 30 min to remove external soil and dirt; tubers were stored in the lab at 25±2 °C until green puds (spourt) were recognized and became 1-2 cm, then removed and cultivated in the MS medium.

Potato tubers of Reverra and Bureen were brooked the dormancy and sprout. The sprouts were cleaned and sterilized by dipping in 2% sodium hypochlorite for 10 min [11]. Shoot tips at 0.1- 0.3 mm with leaf primordial were existed and placed on MS medium [12]. Incubated in growth room chamber at 25°C±2 under photoperiod of 16 h light and 8 h dark. Nodal cuttings (with length 1-2 cm in with one pair of shorten leaves planted in 20ml of soild MS salt supplemented with 0.4, 100, 2 and 2 mg L^{-1} of Thiamine HCL, Inositol, Glycin, respectively.With different concentrations of Indole Acetic Acid,(IAA) extracted 0.5, 1,1.5 and 2 mg L^{-1} comparing with 1,0 mg L^{-1} IAA normally used in micropropagation medium of potato. All cultures placed in growth room chamber at the previsouly condition.

The IAA extracted from the local isolate of Bt3 and imported BT were added separately to the MS medium at volume equivalent to concentrations 0.5, 1,1.5 and 2 mg L^{-1} concentration. three pods were cultured per tube containing 15 ml sterilized medium. The progress of cultivars was under observation for 6 weeks at ambient temperature 25 ±2 C, 1000 luks lightning for 16 h/ day. Treatments were subjected to randomized complete block design RCBD for five replicas under level 0.05[13].

Five different treatments were designed to predict the validity of local product of IAA on the performance of two varities of ***Solanum tuberosum* tissue culture**. The treatments were included 0 concentration of IAA, 1 mg/l of commercialized IAA that used in the tissue culture experiments, and four concentrations of laboratory-prepared IAA. The plant parameters were: plant high, number of branches root length, and branches.

Data for the experiments was subjected to ANOVA (Analysis of Variance) testing and analyzed as for the factorial completely randomized design in five replications. Analyzed using GenStat program and means were separated using Duncan's test at a probability level of 5%. Data of plant height , shoot, leaf and root number, shoot and root length were taken after 30 days.

3. Results and Discussion

3.1 Lap experiments

The qualitative investigation showed that*Bacillus thuringiensis* isolates produced Indol Acetic acid in the presence of 0.3% tryptophan forming a pink color after being treated with salkowski·s reagent (figure 1)and the intensity of color depended on the IAA concentration as recorded by Chagas et al [14], that production of IAA might vary among isolates of the same species when tested under same circumstances.

Our results indicated that the biosynthesis of IAA varied among isolates, we recognized that all isolatesbiosynthesized IAA through a tryptophan-dependent pathway. MS medium without tryptophan gave non- detectable concentration of IAA. This is

assured by using MS medium otherwise LB medium to overcome the interference of residual tryptophan in the components (tryptone + yeast extract) ofthe LB medium as mentioned earlier by Bulgarelli et al. [15].

Also, results showed that optimal production time differed among isolates, BtKS3 produced the highest concentration after 48h compared with Bt ESP which produced after 2 h. While was no difference in production time between 2h and 48h of the BtDS and Bt3G. Exceeding incubation time resulted in depletion of IAA concentration (table 1).

1 2 3 4 5

Fig. (1) Detection of indole acetic acid produced by*Bacillus thuringiensis*isolates grown on the MS medium augmented with 3% tryptophan

The production rates varied with the concentration of tryptophan and with time, as it was observed that production increases with the increase in the concentration of tryptophan indicating that isolates using tryptophan as a basicsubstrate for tryptophan-dependent pathway. It decreases with increasing incubation time till 72 h for the bacterial culture and all isolates. This may be due to the depletion of most of the tryptophan used in the culture, which the bacteria use as their pathway in producing indole acetic acid. Also, the production of IAA into the medium might cause fed-back inhibition and suppress the production of IAA at detectable concentration, especially after incubation for 24 h. This phenomenon was explained by Duca et al., [16] and Figueredo et al., [17]. On the other hand, prolonged incubation of culture might oxidate IAA by the activity of enzyme IAA oxidase excreted by bacteria.

We chose only two isolates the isolate Bt KS3 and BtEspto study the other parameter,.The production of IAA under study would differ according to the incubation temperature and between isolates. We recognized that the optimum temperature was 25 °C for the local isolate and IAA concentration decreased with higher temperature. The other isolate BtESP produced a higher concentration at 40°C followed by 25°C data presented in table (2). The variation in the production of indole acetic acid at different temperatures may be due to the behavior and pathways of the bacteria at temperature and the interactions that occur with the components of the medium, in addition to the physiological characteristics of each bacterial isolate.

A significant increase in IAA concentration resulted from the aeration of bacterial culture compared to the static cultures (table 3). The extent of the effect of the ventilation factor on the productivity of indole acetic acid becomes clear, as the productivity of indole acetic acid increased to almost double due to the effect of ventilation on the bacteria as they are aerobic,and therefore it is preferable to produce indole from bacteria under aerobic conditions.

Table (2) Investigation of the production of indole acetic acid from *Bacillus thuringiensis* isolates at different temperatures

Bacterial isolates	Temperature (°C)				Average
	25	30	35	40	
BtKS3	a0.9647	Cd0.8070	Cd0.8170	cd0.8140	B0.8506
BtEsp.	B0.9010	C1.8410	D0.7440	A0.9413	A0.8568

* *The means with similar letters for the main factors and their interactions within one trait do not differ from each other significantly, according to Duncan's multinomial test at the 5% probability level.*

Table (3) The effect of aeration of submerged fermentor on the production of indole acetic acid from Bacillus thuringiensis

Bacterial isolates	Concentration of Indole Acetic Acid (mgL^{-1})		Average
	Steady-state ferment	Aeration ferment	
BtKS3	c0.4710	a0.9340	a0.7025
BtEsp.	0.4460d	0.9170b	0.6815b
Average	b0.4585	a0.9255	

* *The means with similar letters for the main factors and their interactions within one trait do not differ from each other significantly, according to Duncan's multinomial test at the 5% probability level.*

3.2 in vitro testing

Data presented in table 4 indicated that Rivira var of potato more responding to commercial and extracted IAA than Bureen var and gave a significant performance. Plant high reached to11 cm at 1.5 mg/L of extracted IAA for Rivira var while no significant differences were accounted for Burrin var.Plant branches and their length recorded their highest number with commercial IAA followed by 1 mg/L of extracted IAA.

A recent study presented data on the *in vitro* application of bacterial-extracted IAA. At the same time, many researchers demonstrated the interaction between the bacteria in the rhizospheric region and plant rootsand exudates.Gomes et al. [18] found increased growth of lettuce in the greenhouse after treatment with B. thuringiensis. Later, Azizoglu, [19] proposed that B.thuringiensisplays an important role in plant development while, Figueredo et al., [17] have been investigating the production of IAA by Bt RZ2MS9 and its role in the beneficial effects ofapplying the PGPR Bt RZ2MS9 on a range of plant crops includingsoybean, tomato, and maize.

To the best of our knowledge, types of research related to the production of IAA from *B.thuringiensis*and its bioactivity as a biofertilizer worldwide [13,18]. In Iraq, this is the first trial to produce the auxin IAA from local *B.*

*thuringiensis*kurstaki KS3 at a laboratory-synthesizedmedium. We replaced commercial IAA in the growth medium for potato propagation with four different concentrations of IAA extracted from *B. thuringiensis* KS3 (Fig. 2). Our investigation revealed that root numbers, branches shoot and high height were respondents to local extract of IAA. The explanation is that the bacterial product of IAA may affect processes such ascell division, elongation, tropism, apical dominance, senescence, and stress response [20]. AL-Hussaini et al [8] found the importance of IAA in improving many parameters upon propagation of potato varieties using tissue culture.

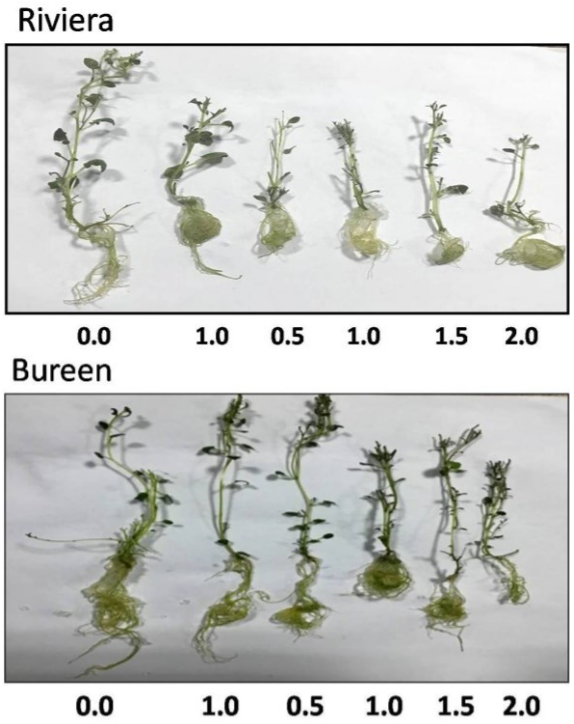

Fig. (2) The effect of IAA extract on the performance of two cultivars of potato under tissue culture propagation

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Table (1) Investigation of the production of indole acetic acid from some local Bacillus thuringiensis isolates using submerged fermentation

Time h	24				48			72				
	Tryptophan concentration g											
Isolate name	%0	%0.1	%0.2	%0.3	0%	%0.1	%0.2	%0.3	%0	%0.1	%0.2	%0.3
BtKS3	0	0.65	.85	0.97	0	0.90	0.93	0.95	0	0.815	0.91	0.92
Btesp	0	0.64	0.82	0.91	0	0.490	0.645	0.665	0	0.5	0.599	0.65
Bt 3G	0	0.30	0.75	0.86	0	0.473	0.786	0.894	0	0.26	0.64	0.76
Bt DS	0	0.49	0.69	0.87	0	0.690	0.777	0.90	0	0.60	0.779	0.812

Table (4) The comparison effect of the Indole acetic acid extract produced by the local isolate *Bacillus thuringiensis*KS3 and commercially IAA on the morphological characteristics of *in vitro* propagated cultivars (Riviera and Burren) after 30 days

Cultivars	0.0	IAA	Bacterial extract concentrations				Average
		1.0	0.5	1.0	1.5	2.0	
	Plant high (cm)						
Rivira	4.00 b	6.90 b	3.90 ab	4.70 b	11.00 a	6.20 ab	6.12 a
Burren	7.10 ab	7.60 ab	6.20 b	6.30 ab	7.40 ab	7.80 ab	7.07 a
Average	5.55 b	7.25 ab	5.05 b	5.50 b	9.20 a	7.00 ab	
	Shoot number plant^{-1}						
Rivira	3.60 bc	7.00 a	2.80 c	6.80 ab	4.00 abc	4.20 abc	4.73 a
Bureen	6.00 bc	5.40 abc	4.00 abc	3.00 bc	4.40 abc	4.20 abc	4.50 a
Average	4.80 ab	6.20 a	3.40 b	4.90 ab	4.20 ab	4.20 ab	
	Shoot length (cm)						
Rivira	3.60 bc	7.00 a	2.80 c	6.80 ab	4.00 abc	4.20 abc	4.73 a
Bureen	6.00 bc	5.40 abc	4.00 abc	3.00 bc	4.40 abc	4.20 abc	4.50 a
Average	4.80 ab	6.20 a	3.40 b	4.90 ab	4.20 ab	4.20 ab	
	Leaves number plant^{-1}						
Rivera	10.80 a	17.80 a	9.20 a	16.80 a	17.80 a	14.60 a	14.50 a
Burren	15.60 a	15.00 a	11.80 a	10.40 a	10.20 a	13.00 a	12.67 a
Average	13.20 a	16.40 a	10.50 a	13.60 a	14.00 a	13.80 a	
	Roots number plant^{-1}						
Rivira	14.60 a	8.80 bc	6.40 bc	8.00 bc	9.40bc	8.60 bc	9.30 a
Bureen	11.20 ab	8.00 bc	5.40 c	6.00 bc	4.80 c	7.60 bc	7.17 b
Average	12.90 a	8.40 b	5.90 b	7.00 b	7.10 b	8.10 b	
	Root length (cm)						
Rivira	10.20 c	11.00 bc	13.60 a	13.80 a	14.20 a	13.20 ab	12.67 a
Bureen	3.20 d	5.20 d	3.20 d	3.20 d	4.60 d	2.80 d	3.70 b
Average	6.70 b	8.10 ab	8.40 ab	8.50 ab	9.40 a	8.00 ab	

Means followed by the same letters are not significantly different (P<0.05) accordimg to Duncan's test.

