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L-Tryptophan and Thiamine Hydrochloride as Vital Factors of the Indole Acetic Acid and Siderophores Produced by *Rhizobium leguminosarum* bv. *viceae* and Their reflection on Faba Bean growth Yield and some Soil Properties under Saline Soil

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Abstract

Plant growth promoting rhizobacteria such as *R. leguminosarum* bv. *viceae* strains no. 481 & 441 were shown to produce indole-3-acetic acid (IAA) and siderophores in bacterial culture supplemented with L-tryptophan (L-trp) and thiamine hydrochloride (Thch) concentrations. Maximum IAA production was found in the medium amended with 1.5mg ml^{-1} (L-trp). IAA production was not observed with the (L-trp) in free medium while (Thch) produced a maximum amount of growth and IAA at 3 mg ml^{-1} for both the two rhizobial strains. Biosynthesis of siderophore was maximum (44.23% and 35.75%) at 1.5mg ml^{-1} of (L-trp) while the production of siderophore was (40.49% & 34.68 % of siderophore units) at 3 mg ml^{-1} of (Thch) in *R. leguminosarum* bv. *viceae* strain 481 & 441 respectively. Rhizobial strain 481 was more active in the production IAA and siderophore than the other rhizobial strain 441.

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A field experiment on a newly reclaimed saline soil at Sahl El-Hussinia, El-Sharkia Governorate, Egypt, used to evaluate the effect of IAA and siderophores synthesizing by rhizobial strains on plant growth parameters and soil characteristics. The obtained results revealed that applying of rhizobial strains grown on 1.5mg ml^{-1} L-Trp and 3 mg ml^{-1} (Thch) led to improve soil properties which reflected on the crop growth and yield components. Soil respiration (CO_2 evolution) and proline content were also studied.

Keywords: Indole Acetic Acid; Siderophores; *Rhizobium*; Tryptophan; Thiamine**;** Faba bean and Saline Soil.

1. Introduction

Rhizobacteria such as *Rhizobium leguminosarum* bv. *viceae* has the ability to secrete benefit plant growth promoting substances such as indole acetic acid (IAA) and siderophores [1]. Both IAA and siderophores are useful when used in accelerating intensive agriculture practice against the negative environmental impact of chemical fertilizers and pesticides. The use of rhizobacteria as biofertilizers is one of the most promising biotechnologies. Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L- tryptophan metabolism by several microorganisms including rhizobia [2]. Thiamine hydrochloride was the most effective vitamin for production of IAA [3]. Siderophores are metabolites produced by microorganisms. These compounds bind ferric iron, promote the rate of Fe^{3+} transport, and thus alleviate the problem of iron unavailability [4]. Culture medium is one of the most important environmental factors that affect the sidrophores production [5-6]. The major environmental factor that reduces plant productivity is salinity. Salts in soil have a negative impact on soil physical, chemical, and biological properties and can ultimately deteriorate soil quality in both ecological and agricultural aspects [7]. The beneficial effect of rhizobia in terms of biological nitrogen fixation has been the main focus of study in the past. Besides biological nitrogen fixation, some strains of rhizobia are also involved in PGPR activity [8]. The application of biofertilizers is an acceptable approach for higher yield with good quality and safe for human consumption [9]. Concerning the effect of using mineral nitrogen fertilizer levels or bio-fertilization on soil salinity and salt distribution, the degrees of soil salinity were slightly affected [10]. This study aims to evaluate the ability of two strains of *R. leguminosarum* bv. *viceae* to produce IAA and siderophores in the presence of different concentrations of L-tryptophan and Thiamine hydrochloride, and to assess their influence on faba bean plant growth and seed yield productivity and some soil fertility.

2. Materials and Methods

2.1. Rhizobial strains

Two rhizobial strains of *R. leguminosarum* bv. *viceae* 481 & 441 were used in the present study. Strains were kindly provided from the biofertilizers production unit, Agricultural Microbial. Dept., Soils, Water and Environ. Res. Inst., Agric. Res. Center (ARC), Giza, Egypt.

2.2. Utilization of different concentrations of L-tryptophan and Thiamine hydrochloride

Bacterial cultures were grown in 100 ml Erlenmeyer flasks containing 25 ml of yeast extract mannitol (YEM) medium [11] with 1% mannitol at pH 7.0 for 72h (optimum time for IAA production). Different concentrations of (L-trp) (0.5, 1.5, 2.5, 3.0 and 5.0 mg ml^{-1}) were added to the basal YEM medium. The effect of different concentrations of (Thch) (1, 2, 3.0, 4 and 5.0 mg ml^{-1}) on growth, IAA production and siderophore production was also estimated. The growth was measured turbidimetrically at 540 nm after 72 h of incubation at 30 ± 1°C.

2.3. Bio assay for IAA

IAA was determined *in vitro* by the method of [12]. The absorbance for indole production was measured at 535 nm. The quantity of indole was determined by comparison with a standard curve using an IAA standard graph.

2.4. Siderophores assay

Quantitative estimation of siderophores was done by the method of [13]. Optical density was taken at 630 nm and siderophores content in the aliquot was calculated by the method of [6].

2.5. Field Experiment

During the winter growing season of 2012- 2013 a field experiment was carried out on the farm El-Rowad village at Sahl El-Hussinia, El-Sharkia Governorate (Latitude 31° 8′ 12.461″ N and Longitude 31° 52′ 15.496″ E, Egypt to study the efficiency of indole acetic acid and siderophore producing bacteria on faba bean (Var. Sakh 3) productivity and soil fertility under saline soils conditions. Seeds were inoculated with the bacterial strains on the same day of sowing and the plants were inoculated after 23, 46 and 60 days of planting by liquid based inoculants according to [14].

2.6. Soil analysis

The main soil properties of the experimental field were determined as shown in Table (1). Available nitrogen was measured according to the methods described by [15]. Soil pH and Total soluble salt (EC) was measured and Available P was determined colorimetrically according to [16]. Available K was determined using the Flame-Photometer, [17].

2.7. Determination of plant growth parameters

Samples of ten plants were collected from each plot and those divided into seeds and straw, dried at oven 70^{o} C, weighed to obtain their dry matter per plant. Samples of plant were digested using the methods described by [18]. N, P and K contents in seeds and straw were determined according to [20], and the Crude protein content of straw and seeds were calculated by multiplying the percentage of nitrogen by 6.25[18].

Table (1). Some physical and chemical properties of the experimental soil before planting

Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)	Texture	O.M (%)	$CaCO_3$ (%)
1.25	45.75	16.23	36.77	Clay	0.45	12.17

pH	EC	Cations (meq/l)				Anions (meq/l)		
(1: 2.5)	(dS/m)	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	HCO_3^-	Cl^-	SO_4^{--}
8.10	12.45	11.39	24.30	88	0.81	10.73	78	35.77

Macronutrients (mg/kg)			Micronutrients (mg/kg)			
N	P	K	Fe	Mn	Zn	Cu
33	4.89	187	6.68	3.51	0.82	0.59

2.8. Proline determination

Extraction and determination of proline was performed according the method of [20]. Proline content was measured by spectrophotometer at 520 nm and calculated as μ mole proline / g of fresh weight material using a standard curve prepared from proline.

2.9. Co_2 evolution

The amount of CO_2 evolved with the soil microbial respiration was determined according to the method of [21].

2.10. Statistical analysis

The obtained results were statistically analyzed using the general linear models procedure of [22].When significant effects were found (P=0.05), means were separated using Duncan's multiple range test.

3. RESULTS AND DISCUSSION

L-tryptophan is generally considered as an IAA precursor, which enhances IAA biosynthesis in the bacterial culture [23, 24]. The effect of different concentrations of (L-trp) revealed that maximum growth and IAA production were observed at 1.5 mg ml^{-1} (L-trp) for both rhizobial strains (Fig. 1a and Table 2).

Tryptophan at levels above 1.5 mg ml^{-1} resulted in a decline of IAA production. IAA production was not observed in the L-tryptophan free medium; this indicates that *Rhizobium* isolates differ in their utilization of different concentrations of L-tryptophan for IAA production**.**

Table (2). Effect of different concentrations of L-tryptophan on the IAA and siderophores produced by rhizobial strains

L-tryptophan concentration (mg ml^{-1})	*Rhizobium leguminosarum* bv. *Viceae*					
	Strain no. 481			Strain no. 441		
	O.D 540	IAA (µg ml^{-1})	% of Siderophore Unit	O.D 540	IAA (µg ml^{-1})	% of Siderophore Unit
Control Without tryptophan	0.176	1.09^{f}	10.12^{f}	1.06	1.01^{f}	9.87^{e}
0.5	0.337	94.6^{e}	25.80^{d}	1.08	42.06^{e}	14.40^{d}
1.5	0.825	102.9^{a}	44.23^{a}	1.66	92.40^{a}	35.75^{a}
2.5	0.292	97.2^{b}	36.32^{b}	1.56	86.90^{b}	24.87^{c}
3	0.273	87.5^{c}	29.40^{c}	1.50	77.70^{c}	26.17^{b}
5	0.261	85.9^{d}	15.90^{e}	1.33	73.40^{d}	8.48^{f}

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test

Similarly, [25] indicated that L-trp at a concentration of 1.2 mg ml^{-1} was best for IAA production whereas higher concentration of tryptophan exerted the adverse effects on IAA production by *Acetobacter diazotrophicus* while *Rhizobium* sp. isolated from root nodules of *Roystonea regia* has been reported to produce a maximum amount of IAA at 3 mg ml^{-1} L-trp [26], and *Rhizobium* sp. isolated from root nodules of *Dalbergia lanceolaria* produced a maximum amount of IAA at 2.5 mg ml^{-1} L-trp [27]. Thiamine hydrochloride produced a maximum amount of growth and IAA at the level 3 µg ml^{-1} for both the two rhizobial strains (Table 3) as [3] reported that (Thch) (1 mg ml^{-1}) was effective vitamin for growth, having also had a higher productivity of IAA which indicates that the production of IAA was highly dependent on thiamine.

Table (3). Effect of different concentrations of Thiamine hydrochloride on the IAA and siderophores produced by rhizobial strains

Thiamine hydrochloride concentration (mg ml^{-1})	*Rhizobium leguminosarum* bv. *Viceae*					
	Strain no.481			Strain no. 441		
	O.D 540	IAA (µg ml^{-1})	% of Siderophore Unit	O.D 540	IAA (µg ml^{-1})	% of Siderophore Unit
Control Without tryptophan	0.196	97.02^{f}	11.31^{f}	0.196	81.1^{d}	9.40^{f}
1	0.837	99.70^{c}	27.80^{e}	0.837	83.2^{c}	15.19^{e}
2	0.625	99.80^{b}	36.69^{b}	0.625	83.5^{b}	18.17^{d}
3	0.592	103.20^{a}	40.49^{a}	0.592	85.1^{a}	34.68^{a}
4	0.293	97.80^{d}	35.36^{d}	0.293	81.1^{d}	33.60^{c}
5	0.265	97.60^{e}	35.48c	0.265	80.6^{e}	34.51^{b}

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test

Under aerobic (oxidizing) conditions at a neutral to alkaline pH, Fe is present primarily in the form of the Fe (III) mineral, Fe $(OH)_3$, which is essentially insoluble [28]. The excretion of siderophores by rhizosphere bacteria may stimulate plant growth by improving the Fe nutrition of the plant or by inhibiting plant pathogens or other harmful microorganisms [29]. As dedicated in Fig (1b) and Table (2), the concentration of (L-trp) (1.5 mg ml^{-1}) was found to be optimum for siderophores production which produced (44.23% and 35.75%) in *R. leguminosarum* bv. *viceae* strains 481 & 441 respectively. Siderophore production was declined at higher concentrations. Amongst the various concentrations of (Thch), a gradual increase in growth and siderophore production was observed with the increasing of (Thch), 3mg ml^{-1} was found to be the optimum concentration for siderophores yields (40.49% & 34.68 %) of siderophore units in *R. leguminosarum* bv. *viceae* strain 481 & 441 respectively (Table 3). Among the two rhizobial strains, maximum amount of IAA and siderophore produced by the *Rhizobium* sp. Strain 481.These results are in agreement with [30] who indicated that amino acids stimulated growth and siderophores production in *Rhizobium* strains. Amino acids were found to stimulate bacterial growth as well as siderophore production in two fluorescent pseudomonades species [6]. Production of chelating agent as siderophores could represent a trait important to the successful interaction between microbe and its plant host [28].

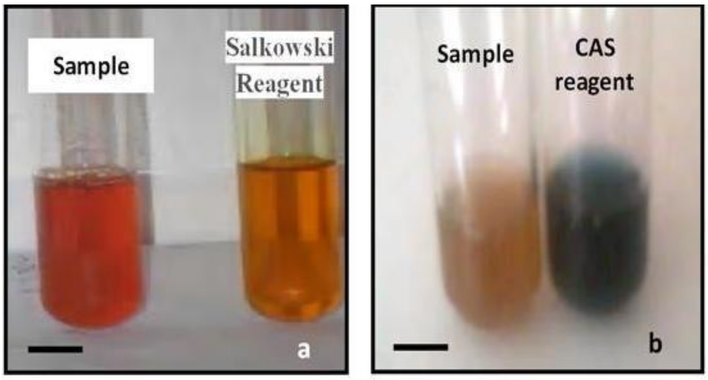

Figure (1). Quantitative estimation (a) indole acetic acid and (b) siderophores produced *by Rhizobium leguminosarum* bv. *viceae*

Results in Table (4) presented that plant height as well as shoot dry weight were significantly higher in case of tryptophan followed by thiamine hydrochloride comparing to full dose of nitrogen fertilizer. Application of rhizobia grown on mannitol was significantly lower than that of nitrogen fertilizer. These findings may be due to the synthesis of hormones like indole acetic acid by the inoculants under study that would have triggered the activity of specific enzymes that promoted early germination, such as a-amylase, which have brought an increase in availability of starch assimilation [31]. PGPR strains affect the growth by producing Indole-3 acetic acid (IAA) that led to plant root growth system development and subsequently uptake increase of nutrients by plant [32]. Indole-3-acetic acid is a phytohormone which is known to be involved in root initiation, cell division, and cell enlargement [33]. Most commonly IAA-producing bacteria are believed to increase root growth and root length, resulting in greater root surface area [34]. Siderophores producing microbes are involved in plant growth promotion of wheat where 10 % increase in the rate of germination, 20% increase in the root length was evident in the *P. fluorescent* inoculated wheat seeds over the control [6].

Proline is one the most diversely nitrogenous osmolytes accumulated under osmotic stress conditions in plants [35]. Induction of proline has been documented in legumes in response to salt stress [36]. The principal role of proline probably is not to reduce the osmotic potential, but to protect enzymes against dehydration and salt accumulation [37]. It is obvious from the present results that application of rhizobial strains grown on tryptophan and thiamine hydrochloride onto faba bean plants lowered the proline accumulation in these plants as compared to full dose nitrogen control (Table 4). These findings may be due to the synthesis indole acetic acid and siderophores by the experimental organisms. Proline content in NaCl-stressed plants was high. However, foliar application of IAA to salinity-stressed plants reduced the levels of proline [38].

CO_2 evolution is a sensitive indicator of abiotic controls, crop residue decomposition, and soil organic matter turnover [39]. It is clear from data presented in Figure (2) that the effects of inoculants of rhizobial strains grown on (L-trp) showed higher rate of CO_2 production (59 and 45μg CO_2 g^{-1} h^{-1}) untabulated, while inoculants of rhizobial that grown on (Thch) showed (40 and 35μg CO_2 g^{-1} h^{-1}) in strains 481and 441 respectively. These previous results are in accordance with the results found by [40] who reported that treatment of PGPR and humic with rice straw gave the highest values of all soil biological activity parameters (CO_2 evolution).

Table (4). Effect of adding tryptophan and thiamine hydrochloride inoculants on growth of faba bean after 70 days from planting

Treatments	Plant height (cm)		Dry weight of Shoot (g) plant^{-1}		Proline (μg mol g^{-1}FW)	
Strain no.	481	441	481	441	481	441
Nitrogen fertilizer (control)	36^{b}	35^{b}	6.3^{c}	7.9^{b}	31.84^{a}	32.32^{b}
Mannitol inoculants	34^{c}	32^{c}	5.9^{d}	5.2^{d}	31.60^{b}	86.02^{a}
Tryptophan inoculants	40^{a}	38^{a}	8.9^{a}	8.3^{a}	16.16^{d}	18.16^{d}
Thiamine hydrochloride inoculants	37^{b}	35^{b}	6.9^{b}	7.1^{c}	25.72^{c}	28.80^{c}

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test.

The application of mineral nitrogen fertilizer and inoculants of mannitol, tryptophan and thiamine hydrochloride have positive effect on macro elements (N.P.K.) concentration in faba bean plants after 70 days from planting. Data presented in Table (5) revealed that the N, P and K concentration was significantly higher in case of tryptophan and Thiamine hydrochloride inoculants respectively while mannitol gave lower results. These findings may be due to the synthesis of hormones like indole acetic acid and siderophores by the experimental organisms. the most important promotion mechanism by rhizobial strains is the production of indole phytohormons (IAA) which results the better root growth, which increase water and nutrient (N, P and K) uptake by the plant and which reflect on the plant growth [41].

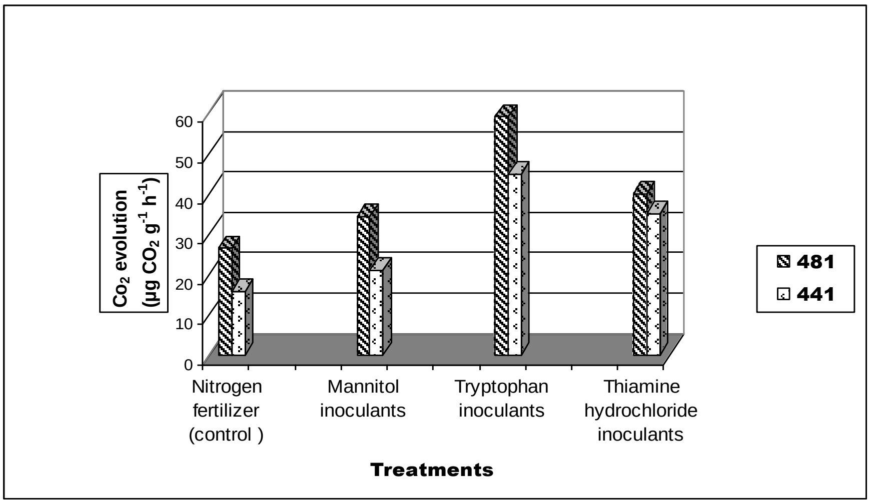

Figure (2). Co_2 evolution as affected by adding rhizobial strains grown on tryptophan and thiamine hydrochloride inoculants on faba bean growth

The obtained results are coincided with those of [42] that proved that auxins- produced by rhizobacteria can influenced plant growth, including root development which improve uptake of essential nutrients and increase plant growth. The concentrations of N, P and K of plant tissue nutrients were significantly increased by the bacterial plant growth rhizobacteria (PGPR) [43]. Inoculating broad bean plants with biofertilizer led to increased dry weight, grain yield, N, P and K content in grains and straw compared with uninoculated control treatment of broad bean plants [44]. Beans are great protein source; they are rich in minerals (especially iron and zinc) and vitamins [45].

Table (5). Concentration of nutrients contents in straw after 70 days after planting faba bean

Treatments	N (%)		P (%)		K (%)		Protein (%)	
Strain no.	481	441	481	441	481	441	481	441
Nitrogen fertilizer (control)	3.45^d	3.52^d	0.35^d	0.58^a	2.95^d	2.99^c	21.56^d	22.00^d
Mannitol inoculants	3.50^b	3.58^b	0.36^c	0.49^c	2.97^c	2.09^d	21.87^b	22.30^b
Tryptophan inoculants	3.56^a	3.63^a	0.42^b	0.48^d	3.06^b	3.13^b	22.25^a	22.68^a
Thiamine hydrochloride inoculants	3.47^c	3.55^c	0.56^a	0.53^b	3.28^a	3.30^a	21.68^c	22.18^c

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test

Data in Table (6) revealed that the results of total yield, seed yield, 100 seed weight of faba bean increased by application of rhizobial strain which grown on L-trp or Thch in comparison with the rhizobial strain which grown without amino acids and full nitrogen dose. PGPR can increase yield, growth and plant nutrients elements contents of raspberry [46]. Rhizobacteria selected for siderophores and IAA production may be useful in growth promotion of crop plants; inhibiting the colonization of roots by plant pathogens or other harmful bacteria and maximizing the yield dry weight [47, 13 ,48,53].

Table (6). Effect of adding tryptophan and thiamine hydrochloride inoculants on seeds and yields of faba bean

Treatments	Total yield kg /fed		Seed yield kg /fed		100 seed weight (g)	
Strain no.	481	441	481	441	481	441
Nitrogen fertilizer (control)	35^{c}	42^{b}	19.83^{c}	37.49^{b}	72.55^{c}	64.50^{c}
Mannitol inoculants	25^{d}	25^{d}	18.75^{d}	16.14^{d}	68.10^{d}	61.20^{d}
Tryptophan inoculants	50^{a}	43^{a}	29.54^{a}	38.53^{a}	94.75^{a}	73.41^{a}
Thiamine hydrochloride inoculants	45^{b}	40^{c}	27.22^{b}	20.65^{c}	74.20^{b}	69.90^{b}

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test

Chemical properties of the saline soil as affected by rhizobia grown on tryptophan and Thiamine hydrochloride were presented in Table (7) which revealed that rhizobia grown on tryptophan and Thiamine hydrochloride decreased both pH and EC as compared to the other treatments. This is expected due to the released of more organic acids from IAA over producing bacteria. These results are in agreement with those reported by [49] who proved that some PGPR microorganisms secrete different types of organic acids, e.g., carboxylic acid thus lowering the pH in the rhizosphere. Application of *Azospirillum brasilense* strain (salt tolerant PGPR strain) led to the high activity of dehydrogenase enzyme and the released carbon dioxide in the rhizosphere cause the formation of carbonic acids and the decrease of the soil pH of the root zone [14]. On the other hand, the results reflected the activity of microorganism to reduce salinity and simultaneously improving characterization of soil structure (increasing drainable porosity and aggregate stability) and consequently enhanced leaching process through irrigation fractions. Bio-fertilizers promote plant growth and had an effect to reduce the salinity stress [50]. Concerning, the pervious treatments increased the soil content of N, P, K elements due rhizobia grown on tryptophan and Thiamine hydrochloride over the control and rhizobia grown on mannitol. Nitrogen, phosphorus and potassium content increased in soil treated with bio-fertilizer (PGPR) as compared without bio-fertilizer [51].

The increase in P may have been due to the solubilization of insoluble P by inoculants come from tryptophan and Thiamine hydrochloride treatments. Some bacteria may solubilize inorganic P due to excretion of organic acids [34]. On the other hand, micronutrients available in soil (Fe, Mn and Zn were significant increase as affected by treatments bio-fertilizer. The increases in soil contents of the different nutrients are the final product of providing more favorable nutrients, i.e., reducing soil pH on one hand and decreasing soil salinity on the other. These results are in agreement with [10, 52].

Table (7). Chemical properties and macro-micronutrients content in soil at harvest stage

Treatments	pH (1:2.5)	EC (dSm^{-1})	Macronutrients ($mgkg^{-1}$)			Micronutrients ($mgkg^{-1}$)		
			N	P	K	Fe	Mn	Zn
Strain no.	***Rhizobium leguminosarum* bv. *viceae* 481**							
Nitrogen fertilizer (control)	**8.06**	**9.48**	53^{g}	5.23^{f}	197^{h}	6.82^{h}	3.93^{c}	0.91^{e}
Mannitol inoculants	**8.01**	**7.41**	60^{f}	5.31^{e}	200^{g}	6.87^{f}	3.97^{a}	0.98^{a}
Tryptophan inoculants	**7.97**	**7.29**	60^{f}	5.37^{b}	208^{f}	6.84^{g}	3.72^{h}	0.83^{h}
Thiamine hydrochloride inoculants	**8.04**	**6.40**	62^{e}	5.33^{d}	209^{e}	7.09^{c}	3.84^{e}	0.88^{f}
Strain no.	***Rhizobium leguminosarum* bv. *viceae* 441**							
Nitrogen fertilizer (control)	**8.04**	**5.16**	72^{d}	5.35^{c}	210^{d}	7.16^{b}	3.95^{b}	0.93^{d}
Mannitol inoculants	**7.95**	**5.06**	81^{b}	5.49^{a}	220^{b}	7.19^{a}	3.73^{g}	0.96^{b}
Tryptophan inoculants	**7.93**	**5.03**	84^{a}	5.49^{a}	223^{a}	7.08^{d}	3.77^{f}	0.84^{g}
Thiamine hydrochloride inoculants	**8.01**	**5.62**	77^{c}	5.37^{b}	214^{c}	6.93^{e}	3.92^{d}	0.94^{c}

Means in the same column followed by the same letters are not significantly different (P=0.05), according to Duncan's test

4. Conclusion

Applying bio-inoculants such as rhizobial strains which grown on 1.5mg ml^{-1} L-Trp and 3 mg ml^{-1} (Thch) in the cultural medium could be vital factor and advantageous that led to improve siderophores; auxin (IAA) production ; soil properties which reflected on the crop growth and yield components.

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