Evaluation of Bacteria Isolated from Olive Mill Wastewater as Plant Growth Promoter on Basil (*Ocimum basilicum* L.) plant

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ABSTRACT

The aim of this study was evaluate *Enterobacter asburiae* as plant growth promoter, through a field experiment conducted at Baloza Research Station at Season 2017/2018 on the production of Basil (*Ocimum basilicum* L., Family: Lamiaceae). The results showed increasing in growth parameters and essential oil yield by *Ent. asburiae* treatment and *Ent. asburiae* + half dose of N_2 treatment than the control. *Ent. asburiae* produced phytohormones and amino acids which increased plant growth parameters. The enhanced essential oil has antibacterial activity which showed as a zone of inhibition around bacterial growth on agar plates. *Enterobacter asburiae* produced ammonia which decreased fungi counts. The more positive response of the oils extracted of treated plant showed essential the presence of gram negative strains (*Pseudomonas* sp.) and gram positive strains (*Listeria sp.* and *Bacillus cereus*) by using *Enterobacter asburiae* treatment.

Keywords: Basil, *Enterobacter asburiae*, growth promoting bacteria, biofertilizer, essential oil as antibacterial.

INTRODUCTION

Genus *Enterobacter* spp. as plant growth promoting bacteria is a Gram-negative, short rod and motile with flagella are having characteristics in nitrogen fixation, plant growth and enhancement. *Enterobacter* sp. is highly phosphate solubilizer, soil phosphorus solubilisation, producer of indole acetic acid (IAA), exopolysaccharides which increase soil porosity, ACC deaminase, HCN antibiotics, siderophore and chitinase, (Kumer *et al*., 2011).

Basil (*Ocimum basilicum* L., Family: Lamiaceae) is a spicy herb used in fresh and dried forms as seasoning. In Mediterranean countries, the herbal medicine is used also in cosmetic industries and perfumes, and basil essential oil used as antibacterial (Toaima and Hamed, 2016).

Microorganisms have an important role in recycling of nutrients. They reduce the use of chemical fertilizers. The relationship between plant growth promoting bacteria and medicinal plants is still to be explored. There are beneficial bacteria stimulate plants to growth, (Baljeet and Shuchita, 2015).

Baljeet and Shuchita (2015) described that *Enterobacteriaceae* as plant growth promoting bacteria**.** Laura Buckler (2018) reported that the chemical fertilizers are important for the cost production of commercial crops, in the last century. However, using chemical fertilizers caused damage and accumulation on long-term. One of the problems with chemical fertilizers is contamination and pollution the soil and the groundwater and other water sources. Now, NPK in small quantities is non-toxic, but a lot can damage the balance of nature in many ways. So it's important to decrease using chemical fertilizers and supplementation biofertilizers as a safe way to keep the environment and take high plant productions. The objective of this study was to evaluate the strain of *Enterobacter asburiae* as a plant growth promoter on Basil parameters under field conditions.

MATERIALS AND METHODS

1- Isolation of *Enterobacter asburiae*

Sample of olive mill wastewater (OMWW) was collected and *Enterobacter asburiae* was isolated for its evaluation as a growth promoting bacteria on basil plant. Ramsay's medium (Ramsay *et al*., 1983) was used for isolating phenolic compounds degrading bacteria.

The 16S rRNA gene sequences as for used to study bacterial phylogeny and taxonomy. The most common, *Enterobacter asburiae* was identified using methods described by Bergy's Manual of Determinative Bacteriology (1994). The bacterial isolate was also identified by partial 16S rRNA gene sequence analysis according to Berg *et al*. (2002). It was identified by partial (16S rRNA) gene sequence analysis as *Enterobacter asburiae* according to Karpouzas *et al*. (2000). El-Asli *et al*. (2005) suggested that the phylogenetic analysis of 16S ribosomal DNA showed that all the related sequences are members of the *Enterobacteriaceae* family.

2- Field experiment

Field experiment was conducted at Baloza Research Station - Desert Research Center, Egypt during summer 2017/2018. The main physical and chemical properties of the soil of field experiment are shown in Table (2). Basil (*Ocimum basilicum* L.) was the task crop. Field experiment involved 3 plots (3 treatments in 3

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replicates), each plot has $6m^2$ ($2x3m^2$). Inorganic fertilizers were added during soil field preparation. Six m^3 of organic compost were applied through preparation of soil. The applied inorganic fertilizers were ammonium nitrate (33.3% N) at 150Kg/feddan (added half dose), superphosphate (15.5% P_2O_5) at 200 Kg/fed. and potassium sulphate (48.5% K_2O) at 100Kg/fed. The plant yield was collected after 5 months from cultivation for first cut and after 45 days from the first cut for the second cut. The plants were soil treated with 100 ml of bacterial inoculum (10^8 CFU).

The grains of Basil (*Ocimum basilicum* L.) were provided by Agriculture Research Center, Ministry of Agriculture and Land Reclamation (MALR), Cairo - Egypt. Throughout this work, germination test was carried out to make sure of the viability of grains.

3- Plant growth parameters

The following plant growth parameters were measured in the field fresh weights and dry weights of total biomass were determined. Biological yield was determined after 150 and 195 days from planting and the result was expressed as per cent ratio of oil yield.

4- Chemical analysis

Total nitrogen content in shoots and grains was determined by modified Kjeldahl method (Chapman and Pratt, 1961), while phosphorus was measured according to Watanabe and Olsen (1965) and potassium was determined by flame photometer (Bremner and Mulvaney, 1982).

Essential oil chemical constituents: Volatile oils for the 1st and 2nd cuts were analyzed by Gas Chromatography –Mass Spectrometry instrument (GC-MS analysis) at National Research Center, Egypt. TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), (Toaima and Hamed, 2016).

Phytohormones: The auxin group phytohormones (indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA)**)** were determined by HPLC Ultimate 3000 Thermo dionex, Germany, at central laboratory of Desert Research Center.

Exopolysaccharides production was determined by sucrose medium **(**Amellal *et al*., 1998).

Total amino acid content: The acid hydrolyzed amino acids by amide bond breakage were determined according to Pellet and Young (1980).

5- Microbiological analysis

Counting of different microbial densities in samples from soil rhizosphere was conducted on different media according to the following methods: Ashby's medium (Abd- El – Malek and Ishac, 1968) was used for counting of nitrogen fixers by M.P.N technique and calculated using Cochren's Tables, (Cochran, 1950), MacConkey's medium (Windle, 1958) was used for *Enterobacteriacae* counting at 37 °C for 24h., in broth tubes counting and at 44°C for 24h., by plate count technique, and total microbial counts did by nutrient agar medium (Jacobs and Gerstein, 1960).

6-The antibacterial activity of essential oil in vitro: It was confirmed by determining basil essential oil on growing bacteria using plates incubated at 24h. for treatments and 48h for control according to Sharma and Sihag (2013).

Statistical analysis

The obtained data were subjected to statistical analysis by ANOVA using the method described by Sndecor (1966).

RESULTS AND DISCUSSION

1- Identification of *Enterobacter asburiae*

Table (1) showed the close data base match and similarity of *Enterobacter asburiae*.

Table 1. Identification of *Enterobacter asburiae*

Quality Sequence	16S rRNA gene sequencing		
	Identification	Closest database match	Similarity
CTTCTTTTGCAACCCACTCCCATGGTGTGAC GGGCGGTGTGTACAAGGCCCG	*Enterobacter asburiae*	NR_024640	99%

2-Field experiment

Table 2. The main Physical and chemical properties of the experimental soil at Baloza station

Particle size distribution (%)			Texture	EC (dS/m)	pH	Nutrients content			Water soluble ions (mg/L)					
Sand	Silt	Clay				P %	Na^+ %	K^+ %	Ca^{++} (mg/l)	Mg++ (mg/l)	CO_3^{--}	HCO_3^- (mg/l)	SO_4^-	Cl^-
92	6	5	Sand	1.35	8.30	0.43	4.78	0.54	3.65	4.40	nd	3.84	6.5	3.2

3- Plant growth parameters

Figure 1 showed significant increase in fresh weight of second cut as a result of *Ent. asburiae* treatment and by using *Ent. asburiae* + half dose of N_2 treatment. As shown in Fig 2 there is significant increased in dry weight of the first cut by using *Ent. asburiae* treatment to (80%), and at the second cut by using *Ent. asburiae* + half dose of N_2 treatment to (48.2%), Kumer *et al*. (2011) found significant increasing in plant dry weight by *Ent. asburiae* treatment compared with uninoculated (control) Khalifa *et al*. (2016) inoculated *Pisum sativum* with *E. cloacae* which significantly improved the growth parameters (dry weight) including grain legume compared to the non-treated plants.

4- Chemical analysis

Nitrogen: Fig 3 showed that treatment by *Enterobacter asburiae* increased N content in plant of the first and second cut (0.35 and 0.92%, respectively). The treatment of *Ent. asburiae* + half dose of N_2 gave high ratio of N_2 compared with control in the second cut (0.895%). Ogbo and Okonkwo (2012) reported that the higher concentration of nitrogen in maize seedlings treated with inoculums by *Enterobacter* sp. compared with those grown without it inoculation.

Phosphorus: Fig 4 showed that treatment by *Enterobacter asburiae* increase phosphorus content of the first cut (0.115%) and treatment by *Ent. asburiae* + half dose of N_2 increased phosphorus content. Deepa *et al*., (2010) found that *Enterobacter asburiae* (NII-0934) was producing IAA, P-solubilizers and HCN. They reported that stimulation of P-solubilizing activity were plant growth of cow pea (*Vigna unguiculata* (L.)".

Potassium: Fig 5 showed that the treatment by *Enterobacter asburiae* increased in potassium (2.9%) in the first cut. The second cut *Ent. asburiae* + half dose of N_2 treatment increased (1.7%).

Basil essential oil: Figure 6 showed increase in the yield of oil due to using *Ent. asburiae* treatment at first and second cuts results which were (1.04 and 1.49%, respectively). *Ent. asburiae* + half dose of N_2 treatment increased the oil yield in the first cut of plant to (1.03%). Swamy *et al*. (2016) reported that the essential oils have high potential in the field of biomedicine as they effectively destroy many of bacterial, fungal, and viral pathogens, due to several types of aldehydes, terpenes, phenolics and other antimicrobial compounds so that the essential oils are effective against a diverse range of pathogens**.**

Qualitative analysis of essential oil

Tables (3 and 4) showed wide variation in the percentage of the different essential oil compounds as a result of treatment by *Ent. asburiae*. Ana Cristina *et al*. (2012) found that the minimal concentration of the antimicrobial agent presenting complete growth inhibition. Pinene has activity of antimicrobial against *C. neoformans, C. albicans* and *R. oryzae*. The significant inhibition of pinene could be related to the potent antimicrobial action of pinene against this fungus. The antimicrobial activity was even more promising against biofilm formation, which makes pinene useful in formulating strategies to limit *C. albicans* biofilm formation.

Phytohormones

Figure 7 showed that *Ent. asburiae* produced the auxin group phytohormones: indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), Relative heights were 88.10% and 11.90%, respectively. Abraham and Silambarasan (2015) showed that *E. asburiae* JAS5 and *E. cloacae* JAS7 strains had the ability as plant growth promoting traits such as indole-3-acetic acid (IAA) production, organic acids production and solubilization of various inorganic phosphates. *E. asburiae* JAS5 solubilized of tricalcium phosphate, dicalcium phosphate and zinc phosphate, whereas *E. cloacae* JAS7 solubilized tricalcium phosphate, dicalcium phosphate and zinc phosphate".

The quantity of Exopolysaccharides (EPS) produced by *Enterobacter asburiae* was (4mg/ml) this agrees with Mu'minah *et al.* (2015) who indicated that the improved soil structure by aggregating soil through microorganisms such as bacteria producing exopolysaccharides (EPS) which are grouped into gram-negative bacteria. The EPS are necessary for microbial life, protection of environmental stresses; like drought and salinity, and important for chemical reactions, Microbial EPS can increase the soil granules and improve plants growth by availability of nutrients, keep soil humidity and therefore enhance soil fertility, (Ohana Costa, *et al*. 2018).

Total amino acids content

Figure 8 showed the concentration of amino acids in *Ent. asburiae* broth culture. Aspartic, glutamic acid and glycine were the greatest components (425.4, 354.78 and 401.44). Ogbo and Okonkwo (2012) found that the production of ammonia is frequently reported for PGPR, a process most probably resulting from the deamination (ammonification) of amino acids present in the peptone used for this assay. It has been suggested that ammonia may have a role in antagonism against competing flora, particularly the fungi.

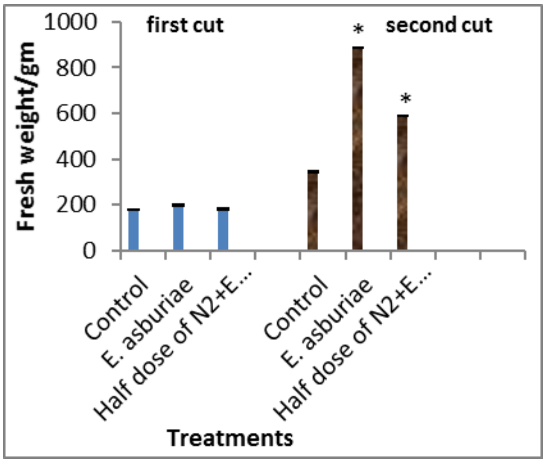

Fig. 1. Fresh weights (gm) in Basil plants

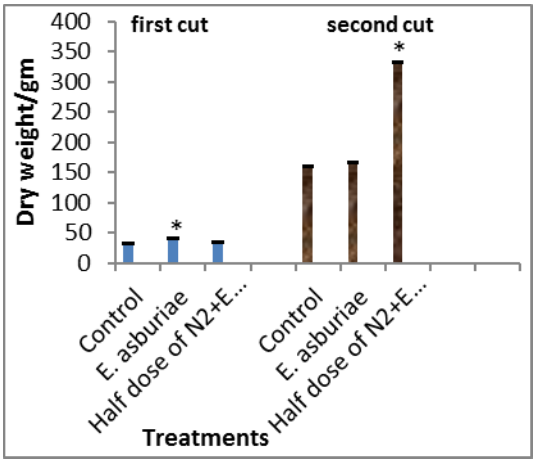

Fig. 2. Dry weights (gm) in Basil plants

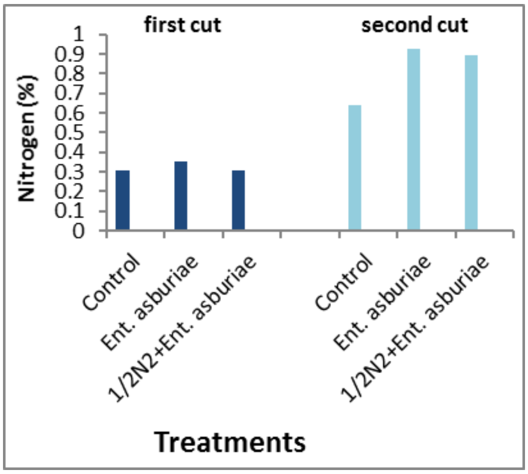

Fig. 3. Nitrogen contents in Basil plant

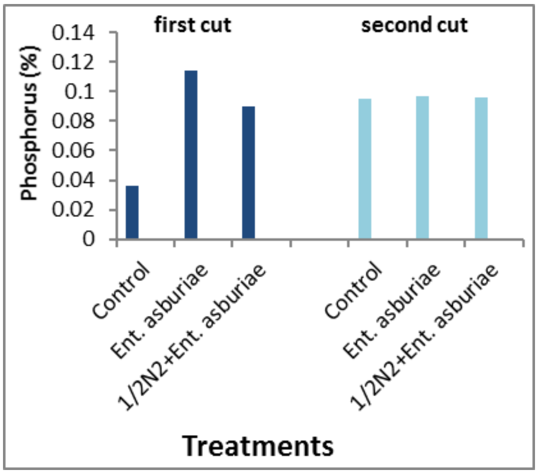

Fig. 4. Phosphorus contents in Basil plant

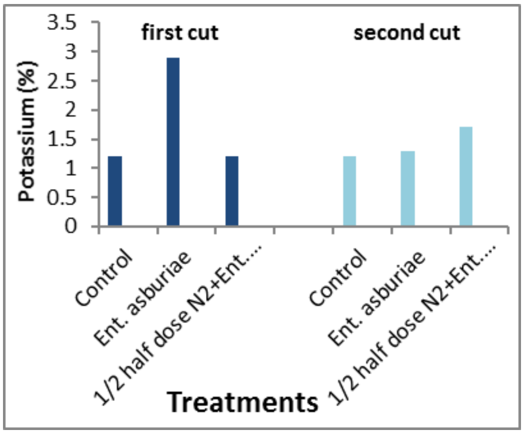

Fig. 5. Potassium contents in Basil plant

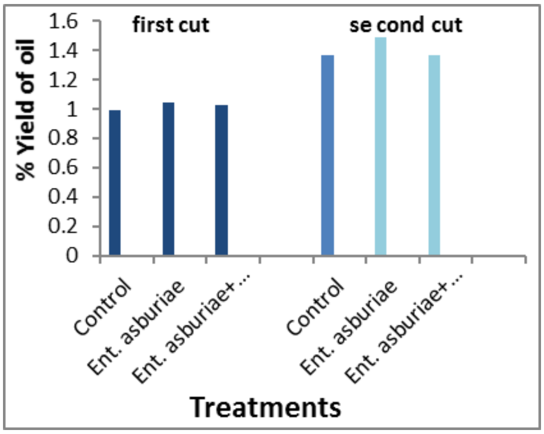

Fig. 6. Basil oil extracted essential yield

Table 3. The analysis of essential basil oil of the control plant

Control compounds name	Retention Time	AREA%
1,8-Cineole	7.18	14.39
Eucalyptol	7.18	14.39
LINALOOL L	9.64	41.40
1,6-Octadien-3-ol, 3,7-dimethyl-	9.64	41.40
Benzene, 1-methoxy-4-(2-propenyl) (CAS)	13.72	23.71
Estragole	13.72	23.71
2-Propenoic acid, 3-phenyl-, methyl ester	21.76	7.72
ç-Muurolene	31.50	2.16
1-Naphthalenol	31.50	2.16
1,2,3,4,4a,7,8,8a-octahydro-1,6 dimethyl-4-(1-methylethyl)	31.50	2.16
à-Cadinol	31.50	2.16

Table 4. The analysis of essential basil oil of the treated plant

Treatment compounds name	Retention Time	AREA%
1,8-Cineole	7.21	11.18
Eucalyptol	7.21	11.18
LINALOOL L	9.68	36.36
1,6-Octadien-3-ol, 3,7-dimethyl-	9.68	36.36
Benzene, 1-methoxy-4-(2-propenyl)-(CAS)	13.76	22.35
Estragole	13.76	22.35
Methyleugenol	22.37	7.72
Benzene	22.37	7.72
1,2-dimethoxy-4-(2-propenyl)- (CAS)	22.37	7.72
ç-Muurolene	31.53	2.42
1-Naphthalenol	31.53	2.42
1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)	31.53	2.42
à-Cadinol	31.53	2.42

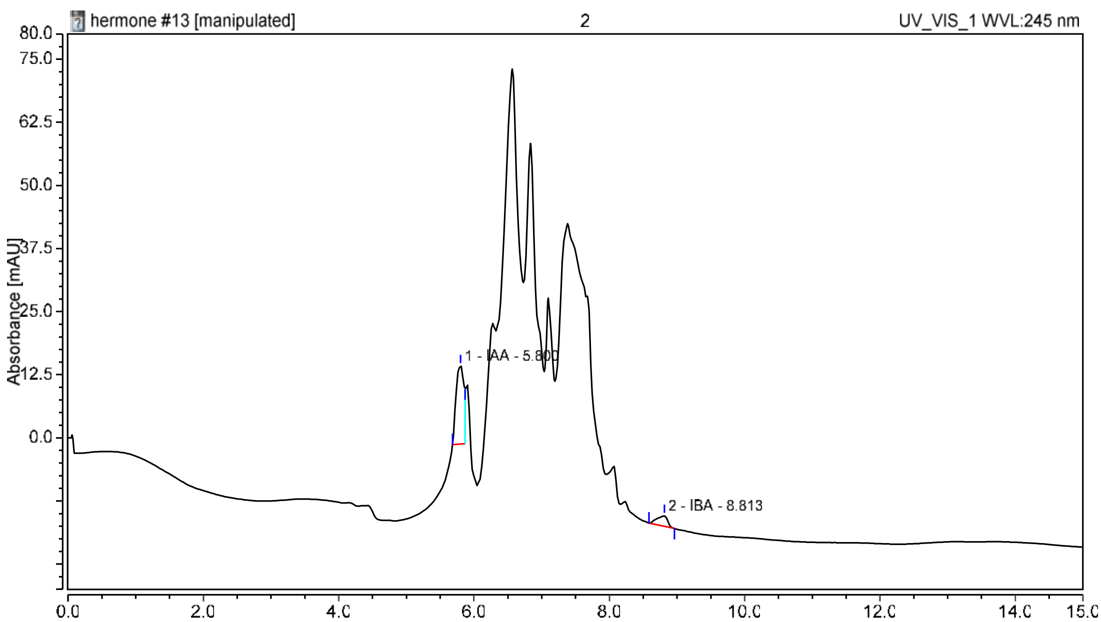

Fig 7. Phytohormones produced by *Ent. Asburiae*

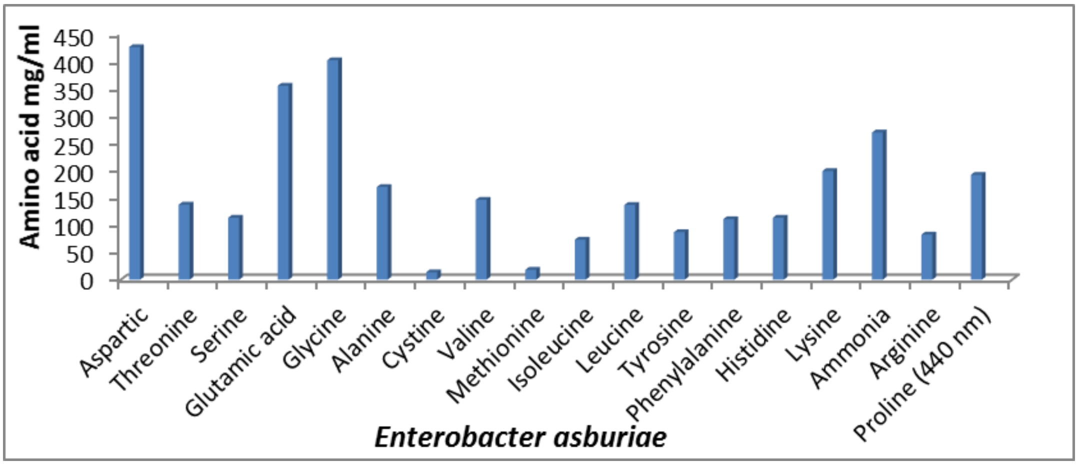

Fig 8. Amino acids determined in *Ent. asburiae* broth culture

These agree with Teixeira *et al*. (2018) who estimated that application amino acids glutamate, cysteine and glycine can act as signaling amino acids in soybean plants. Small doses are enough to increase the activity of the antioxidant enzymes, glutamate stands out for being the first amino acid in which the nitrogen absorbed by the plants a range of amino acids can be obtained through the activity of aminotransferases, In addition, amino acids such as glutamate, cysteine, and glycine may act directly or indirectly in the attenuation of plant oxidative stresses".

5- Microbiological analysis

Table 5 showed that at the first cut of rhizosphere Basil plant, there were increases in nitrogen fixers, total microbial counts and that at *Enterobacteriaceae* due to inoculation with half dose N_2+ *Ent. asburiae* treatment ($210*10^5$, $181*10^4$and $260*10^2$, respectively). By using *Ent. asburiae* treatment there was high Nitrogen fixers ($290*10^5$), total count ($132*10^4$) and *Enterobacteriaceae* ($61*10^2$) more than the control. At the second cut, there were increases in nitrogen fixers, total microbial counts and *Enterobacteriaceae* as the results of rhizosphere Basil plants by using inoculation with half dose N_2+ *Ent. asburiae* treatment ($30*10^6$, $158*10^4$ and $183*10^3$, respectively), Estrada *et al*. (2004) found that although the half dose of chemical fertilizer gave a marked increase in the microorganism counts however, nitrogen addition changed microbial group's composition by reducing the prorated abundance of gram-negative bacteria in forests.

6- Antibacterial activity of essential oil in vivo: The antibacterial activity of essential oil showed inhibition zone around bacterial growth on agar plates. The more positive response oil extracted (diameter of inhibition zone) increased by treated the plant with *Enterobacter asburiae*. The results showed the presence of gram negative strains (*Pseudomonas aeruginosa* and *E. coli*) and gram positive strains (*Listeria sp.* and *Bacillus cereus*). The response of control treatment has positive effect on *E. coli*.

Table 5. Rhizosphere microbial counts

Treatments		Nitrogen fixers (MPN/gm dry soil)	Total count (CFU/gm dry soil)	*Enterobacteriaceae* (CFU/gm dry soil)
First cut	Control	$43*10^5$	$117*10^4$	$32*10^2$
	Enterobacter asburiae	$290*10^5$	$132*10^4$	$61*10^2$
	Half dose of N_2+Ent.	$210*10^5$	$181*10^4$	$260*10^2$
Second cut	Control	$20*10^6$	$115*10^4$	$30*10^3$
	Enterobacter asburiae	$23*10^6$	$48*10^5$	$36*10^3$
	Half dose of N_2+Ent.	$30*10^6$	$158*10^4$	$183*10^3$

The results showed that basil oil have a higher strength to control bacterial growth, and the essential oil is strong versus all of the estimated strains of bacteria as *Escherichia coli* as reported by Monika Sienkiewicz *et al*. (2013). Ahmad and Khan (2010) estimated *Enterobacter* as anti fungal agent and plant growth promoting producer. Sakkas and Chrissanthy (2017) evaluated the activity of essential basil oil's as antibacterial, because its high content of estragole and linalool Thus, The action of antimicrobial depended on some specific bacteria *P. aeruginosa* and *Enterobacter* spp.

CONCLUSION

Evaluation of *Enterobacter asburiae* isolated from Olive Mill Wastewater as Promoting Growth Bacteria under Baloza Station Research conditions on Basil (*Ocimum basilicum* L.) plant, gave good positive results for producing phytohormones (IAA and IBA), exopolysaccharides, amino acids, phosphate release, increased nitrogen and potassium to enhance plant growth and production. It also produced ammonia which caused antagonism against fungi. The essential oil yield by using treatments of *Ent. asburiae* or half dose N_2+ *Ent. asburiae* changed the more effect on decrease the harmful microorganisms than control. This was clear in the zone of inhibition on Petri dishes. The essential oil has an antibacterial effect on Gram negative and Gram positive bacteria, but the effect on Gram negative bacteria (*Pseudomonas* sp.) was more than on positive samples (*Listeria* sp. and *Bacillus cereus*).

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الملخص العربي

تقييم البكتيريا المعزولة من مخلف ماء عصر الزيتون كبكتيريا معززه للنمو على نبات الريحان

غادة أمين زكي إبراهيم

تهدف هذه الدراسة الى تقييم السلالة البكتيرية (*Enterobacter asburiae*) والمعزولة من إحدى عينات مخلف ماء عصر الزيتون(المجمعة من معصرة الزيتون بمركز البحوث الزراعية) كبكتيريا معززه لنمو النبات. وقد تم اختبارها على نمو وإنتاجية نبات الريحان.

تمت التجربة الحقلية فى محطة بحوث بالوظه – مركز بحوث الصحراء فى موسم ٢٠١٧/ ٢٠١٨م. وكانت المعاملات كالآتى: معاملة كونترول تروى بالماء فقط، معاملة باستخدام (*Enterobacter asburiae*) ومعاملة باستخدام (*Enterobacter asburiae* + نصف جرعة التسميد الأزوتى الموصى به). وقد اظهرت النتائج أن بكتيريا الإنتيروباكتر اسبوري لها القدرة على انتاج هرمونات نباتية "أواكسينات" (اندول اسيتيك اسيد واندول بيوتيريك اسيد) والتى تسهم فى استطالة النبات وتحسين نموه وزيادة انتاجيته. كما تنتج البكتيريا احماض امينية، وسكريات خارجية عديدة لها دور فى تحسين مسامية التربة. وكذلك تيسر بكتيريا الإنتيروباكتر للفوسفات. كما كانت هناك زيادة فى النيتروجين والبوتاسيوم مما حسن نمو وانتاجية النبات وساهم فى زيادة محصول الزيت. كما وقد زاد ايضا النشاط الميكروبي بمنطقة الجذور. بالإضافة لإنتاج الإنتيروباكتر للأمونيا والتى بدورها تقاوم الفطريات.

أدت معاملة الريحان ببكتيريا الإنتيروباكتر إلى زيادة فعالية تأثير الزيت الطيار للريحان مما زاد من أثره المضاد على البكتيريا الممرضة السالبة لجرام (*Pseudomonas* sp.)، والموجبة لجرام (*Listeria* sp. and *Bacillus cereus*)، ويظهر ذلك فى حجم قطر هالة التثبيط حول الميكروب الممرض فى اطباق بتري.