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DIAZOTROPHY AND GROWTH OF BEANS (PHASEOLUS VULGARIS) GENOTYPES INOCULATED WITH RHIZOBIA AND LACTIC ACID BACTERIA

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ABSTRACT: ***In a laboratory trial, the influence of inoculation with Rhizobium etli , Lactococcus lactis, and Rh. etli plus L. lactis, as well as, a control (without bacterial inoculation) on root nodulation and plant growth of two genotypes of Phaseolus vulgaris was investigated. Results, obtained at 12-day plant age, revealed that inoculation with Rhizobium etli induced root nodulation, while Lactococcus lactis alone did not produce any nodules . Moreover, the co-inoculation with Rh. etli mixed with L. lactis exhibited salient superiority in enhancing the nodulation process and nitrogenase activity. Increases in number of nodules reached 18.4 and 45.3%, and in acetylene reduction activity (ARA) were 360 and 64.8% above the inoculation with Rh. etli alone for the two genotypes tested BAT477 and DOR364, respectively.***

The results gained denoted that the bacterial inoculation increased shoot and root fresh and dry weights of the 12-day old plants, compared to the control. It was observed that the genotype BAT477 gave higher increases than those of DOR364 for such measures. Increases in shoot fresh weight, referring to, the control were (14.8, 6.1& 5.3 %) and (28.8, 7.6& 2.1 %) and those of shoot dry weights were (25.4, 24.5 & 21.5%) and (16.1, 14.8& 11.8 %) for the co-inoculation with both bacterial agents, for the bean genotypes BAT477 and DOR364, respectively. On contrast, DOR364 scored higher increases than BAT477 for root fresh and dry weights. The increases were more pronounced for the fresh weights, average increments, as compared with control, were (115, 86.4 &5.9 %) and (215.6, 155& 39.6 %) for the co-inoculation, Lactococcus lactis, Rhizobium etli, respectively .

Key words: ***Rhizobium etli, Lactococcus lactis, Co-inoculation, Nodulation, Acetylene Reduction Activity , Phaseolus Genotypes .***

INTRODUCTION

The beneficial effects of inoculating legumes with *Rhizobium* and *Bradyrhizobium* are well known and widely used in agriculture for crop improvement, due to their ability to fix molecular nitrogen, (Roughley *et al*., 1983). Co-inoculation of legumes with *Rhizobium* and plant growth promoting rhizobacteria *(PGPR)* has had an increasing attention in recent years. Plant growth –promoting bacteria are free-living in rhizosphere, rhizoplane , and phylosphere, that under some conditions are beneficial for plants. PGPR affect plant growth in two different direct and indirect ways, for the metabolism of the plants by providing substances that are usually in short supply. These bacteria are capable of fixing nitrogen, solubilizing phosphorus and iron, and producing plant hormones, such as auxins, gibberellins, cytokinins and ethylene. Additionally, they improve plant tolerance to stresses such as drought, high salinity, metal toxicity, and pesticide load. A second way is biocontrol-PGPR, that indirectly improve plant growth by preventing the deleterious effects of phytopathogenic microorganisms (bacteria, fungi, and viruses) (Bashan, 2005). So, the benefits observed following inoculation with associative bacteria are mainly attributed to improved root development and enhanced water and mineral uptake, (Okon and Vanderlyden, 1997). Mixed inoculation of *Azospirillum* with *Rhizobium* led to increase nodule stimulation and function , total number and weight of nodules, epidermal cell differentiation in root hairs , straw and grain yield and root surface area (Bashan, 2005). The lactic acid bacteria (LAB) family is composed of a heterogeneous group of Gram-positive, nonsporing , catalase and cytochrome negative , anaerobic or aerotoleont bacteria (Axelsson,1998). Antimicrobial compounds produced by lactic acid bacteria (lactic and acetic acids, hydrogen peroxide and bacteriocins) play an important role in preventing the growth of food spoilage and food –borne pathogenic bacteria. Bacteriocins are of special interest due to their potential use as natural food preservatives (Klaenhammer, 1988). Bacteriocin is one of the importants factor that affect proliferation of bacteria in rhizosphere as a cyst formation and siderophore and phytohormone production. Bacteriocins are proteic molecules synthesized for various lineages of Gram-positive and Gram-negative bacteria when exposed to stressful conditions. Bcteriocins have been characterized as molecules of high antimicrobial property even at low concentrations, provoking the microbial survival inhibition by antibiosis (Souza *et al*., 2005). Two bacteriocin-producing strains of *Lactococcus lactis* isolated from vegetables were active from pH 2to 9 and inhibited species of *listeria*, *Lactobacillus*, *lactococcus*, *Pediococcus*, *Leuconostocc*, *Carnobacterium* ,*Bacillus* and *staphylococcus*(Uhlman et al., 1992). The

potential application of antimicrobial –producing lactic acid bacteria as biopreservatives of ready to use vegetables is suggested (Vescovo et al., 1996).

In this study, we evaluated the effect of inoculation with *Rhizobium etli* individually or mixed with *Lactococcus lactis* on diazotrophy represented by nodulation status (number and dry weight of plant root nodules)and nitrogenase activity, as well as shoot and root fresh and dry weights, of two faseolus genotypes under aseptic laboratory conditions.

MATERIALS AND METHODS

Bacterial strains

***Rhizobium etli* "CNPAF512 (Embrapa,Barazil)" isolated from root nodules of *Phaseolus vulgaris* plants and *Lactococcus lactis* isolated from coal rhizosphere cabbage , Oost –Vlaonder, Belgium, (a PGPR that produces phytohormons and promotes lateral root and root hair formation) were employed in this study.**

Media

Trypton yeast extract (TY) medium, for *Rhizobium etli* (Beriger,1974) was introduced into one liter of distilled water (5g of trypton and 3g of yeast extract). After autoclaving the medium at 121°C, $CaCl_2$ was added to each of a final concentration of 7mM. MRS medium was used for *Lactococcus lactis.*

Plant hydroponic growth medium

The culture medium (based on Snoeck, 2001) contained the following : 0.70mM $MgCl_2$,1.06mM $CaCl_2$,0.48µM $MgSO_4$, 20.32 µM FeHEDTA, 14.08 µM $MnSO_4$,0.198 µM $CuSO_4$, 0.602 µM $ZnSO_4$, 10.003 µM H_3BO_3 and 0.02744 µM $(NH_4)_6Mo_7O_{24}$. This medium was used for growing plants in a growth chamber.

Preparation of cultures

***Rhizobium etli* (CNPAF512) stored at -80 ° C was grown for two days at 30 °C on agar plates with Ty medium. The plated cells were suspended in 5 ml of Ty medium and cultured overnight at 30 ° C on a shaker. The optical density (OD)of the overnight culture was measured at 595nm using a spectrophotometer and OD was adjusted to 0.4, 1ml of the cell suspension having OD of 0.4 was centrifuged at 6000 rpm for 5 minutes, then the supernatant was discarded and the cells were washed two times with 1ml 10M MgSO4. The supernatant was discarded and the cells were resuspended in 1ml 10M $MgSO_4$. The washed cells suspension was diluted to 10-12 in 10mM $MgSO_4$, and 200µl of the bacterial suspension,**

corresponding to approximately 10^3 to 10^4 cells was used to inoculate each plant growing in plate as single inoculm and 100µl of the bacterial suspension from *Rh.etli* and L. *lactis* as mixed inoculm.

Plant materials and growth conditions

***Phaseolus vulgaris* is a largely consumed grain legume. BAT477 and DOR364, used in this study, are two Meso-American phaseolus beans genotypes. Plant seeds were sterilized for 1min in 100%ethanol, then in 15% NaOCl for 12 min and finally rinsed 10 times in a sterile distilled water . Seeds were germinated on 10% water agar. Two days old seedlings were transferred to each of plates, (50 ml medium), and bottles (250 ml) for N_2-ase-activity assay. The plates were sealed with parafilm to avoid contamination and by sterile forceps and knife, then small holes were made, whereas the bottles were wrapped with aluminum foil to maintain darkness in the rooting environment. At 12-day plant age fresh and dry weights of the growing plants were determined and 21-dayplant age for N_2-ase-activity assay.**

RESULTS AND DISCUSSION

Diazotrophy

A) Number and dry weight of root nodules of phaseolus beans

Numbers and dry weights of nodules developed on *Phaseolus* roots are illustrated in Figs.(1,2 and 3). It was apparent that nodulation status was affected by *Rhizobium etli* inoculation. Co-inoculation with *Rh. etli* and *L.lactis* led to increases in number and dry weight of nodules, while the control treatment, inoculated with *L. lactis* alone did not produce any nodules. The highest values of nodulation aspects were attained for the genotype DOR 364, compared to BAT477 .Increases in the number of nodules reached up to 18.4 and 45.3 % over those inoculated with *Rhizobium* alone. For instance, the values of nodular tissues accumulated due to sole inoculation with *Rhizobium* and co-inoculation with *Rh. etli* plus *L. lactis* were 365& 379 and 380 & 389 mg/plant for BAT477 and DOR364, plant genotypes, respectively. Also, the numbers of nodules were 43.4&46.5 and 51.4 & 67.6, respectively. These results demonstrated that inoculation of beans with *Rhizobium* enhanced nodulation process. However, co inoculation of *Phaseolus vulgaris* with *R.etli* together with *L. lactis* might be an efficient and effective approach for mignifying nodulation. Indeed, *L. lactis* may increase the nodulation status by widening the root surface area leading to create more infection sites on the root system(Srinivasan *et al.,*1997). Root exudates also provided the chance for a better nodulation

process. On the other hand, *L.lactis* produced bioprotecting substances that could enhance plant healthy resulting in improved nodulation.

Similar finding, confirming that the co-inoculation increased nodulation of legume roots, was observed by Hassanein and Mekhemer(2003), El-Howeity (2004) and Abdel-Wahab et al.(2006) who reported that co-inoculation of some legumes with rhizobacteria exerted beneficial effects on nodulation of pea, faba bean, and peanut.

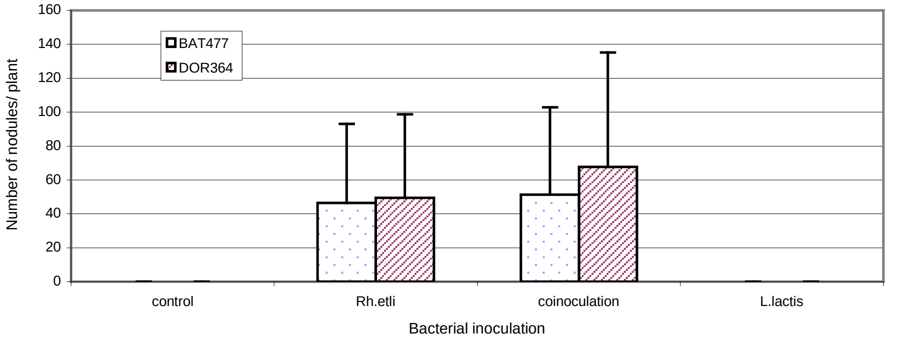

Fig.(1)Effect of inoculation with *Rh.etli* and *L.lactis* on nodules number of bean genotypes.

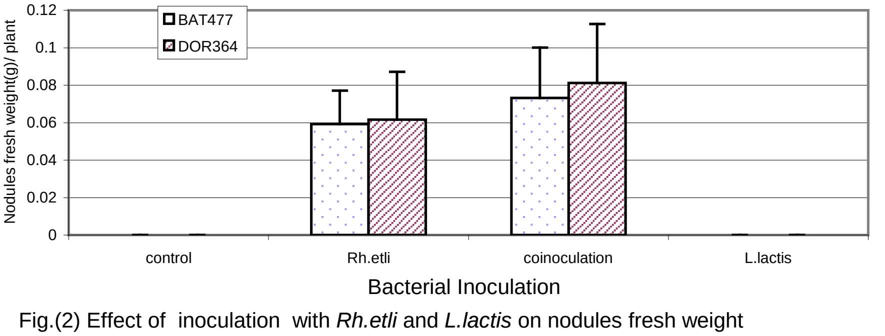

Fig.(2) Effect of inoculation with *Rh.etli* and *L.lactis* on nodules fresh weight of bean genotypes.

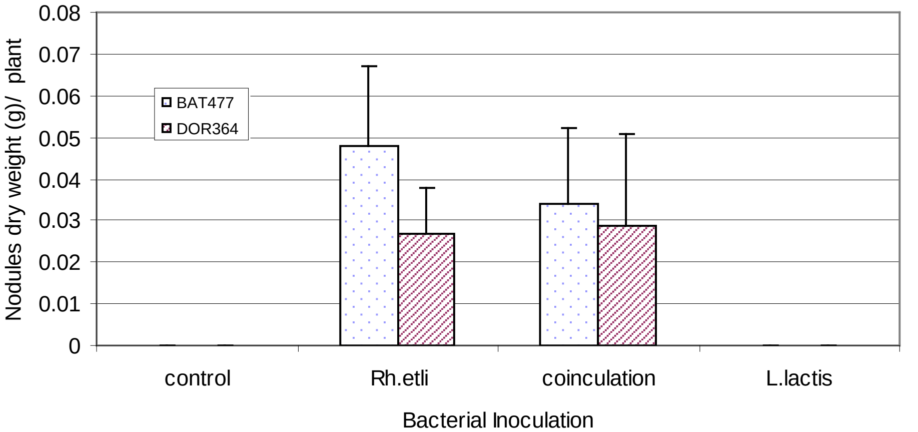

Fig.(3) Effect of inoculation with *Rh.etli* and *L.lactis* on nodules dry weight of bean genotyps.

B) Acetylene Reduction Assay (ARA)

N_2-Fixation capacity was evaluated by ARA and presented in Fig. (4). Plants of both genotypes inoculated with *Rh. etli* alone or *Rh. etli* plus *L. lactis* showed an acetylene reduction, while the plants un-inoculated or inoculated with *L. lactis* alone were negative to ARA. Co-inoculation treatments gained higher values of ARA for the two genotypes, where gave 0.4058& 0. 4236 and 1.868 & 0.6983 µl/h with DOR364 and BAT477 inoculated with Rhizobium only and *Rh. etli* + *L.lactis*, respectively. .Increases in ARA reached up to 360 and 64.8% over *Rhizobium* alone for the two genotypes BAT477 and DOR364, respectively. These increases in ARA might be due to increases in number of nodules and dry weight of roots . Lactic acid bacteria could also stimulate the growth and activity of rhizobia. Sindhu et al. (1999) observed that a culture supernatant of *Pseudomonas* isolates contained a fluorescent compound which influenced the root flavonoid content of green gram (*Vigna radiate*) and concluded that the production of flavonoid –like compounds by plant roots augmented nodule formation by *Bradyrhizobium .* Chebotar et al. (2001) found that co-inoculation of *P.fluorescens* 2137 and *B.japonicum* A1017 increased the colonization of *B.japonicum* A1017 on soybean roots, nodule number, and acetylene reduction activity (ARA) at 10 and 20 days after inoculation. Thus, we suggest that *Lactococcus lactis* produced compounds which stimulated the growth of *Rhizobium etli*.

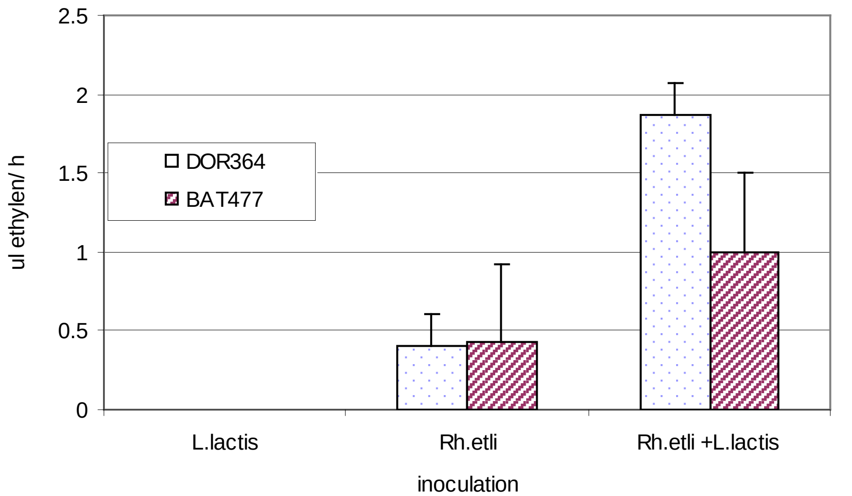

Fig.(4) Effect of inoculation with *Rh.etli* and *L.lactis* on nitrogenase activity of bean genotypes.

Shoot and root fresh and dry weights

Results illustrated in Figs.(5 thru 8) show the effect of inoculation with *Rh. etli* ,*L. lactis* and co-inoculation with both agents on the growth of the two genotypes of *Phaseolus vulgaris* BAT477 and DOR 364, under aseptic conditions. The results revealed that inoculation caused increases in shoot and root fresh and dry weights of both plant genotypes, but the effect was clearer with BAT 477 than with DOR 364 for shoot fresh and dry weights. However, the genotype DOR 364 showed higher values in root fresh and dry weights, compared to BAT477. The increases occurred in the fresh and dry weight of shoot due to aole rhizobial inoculation were 5.3 and 21.5 for c.v DOR 364, respectively and they were 2.1 and 11.8 % for c.v BAT 477 over control. The corresponding values attained as a result of inoculation with *L.lactis* were 6.1&24.5 % and 7.6 & 14.8 %, respectively.

Co-inoculation with *Rh. etli* and *L. lactis* gave higher increases in the shoot fresh and dry weights, as compared with every single inoculation and control. Increases in the shoot fresh weight were(up to 14.8 &28.8%) and for the shoot dry weight (up to24.4& 16.1) for BAT 477 and DOR 364,bean genotypes, respectively. On the other hand, increases in the root fresh and dry weights were clearer for DOR364, compared to the BAT 477 cultivars.

These increases reached (up to5.6& 39.6%) over the control for the root fresh weight and (up to 6.2& 2.4%) for the root dry weight under inoculation with *Rh. etli. Lactococcus lacti*s gave increases in the root fresh weight (up to 85.8 & 155.9%) and its dry weight (up to 14.4& 11.7%). Co inoculation with *Rh. etli* plus *L. lactis* led to higher increases in the root fresh and dry weights, reaching (up to114.7& 215.6%) over the control and for the root dry weight (up to23.7& 43.4%) for BAT 477 and DOR 364, respectively. The promotive effect of L.lactis on the plant vigour might be elucidated by its ability to produce efficient bioactive substances, which acting tostimulate and regulate the plant growth. These growth promoting substances comprised several bacterial metabolites such as hydrogen peroxide ,bacteriocins, siderophores, phytohormones and plant hormones regulators (Lowe and Arendt, 2004). Also, Ray (1996) noticed that lactic acid bacteria produce nisin and pediocin as examples of bacteriocins owner antimicrobial spectrum which is able to exert inhibitory action on the growth of bacteria such as *Lactobacillus Plantarum*, *Pediococcus acidilactici, Leuconostoc mesenteroides*, *Listeria monocytogenes* and *Mirococcus luteus*. Souza et al.(2005) noted that bacteriocins are proteic molecules synthesized by various lineages of Gram-positive and Gram-negative bacteria when exposed to stressful conditions. Bacteriocins have been characterized as molecules of high antimicrobial property even at low concentrations, provoking the microbial survival inhibition by antibiosis. Antimicrobial compounds produced by lactic acid bacteria (lactic and acetic acids, hydrogen peroxide and bacteriocins) play an important role in preventing the growth of food spoilage and food borne pathogenic bacteria . Bacteriocins are of special interest due to their potential use as natural food preservatives, (Klaenhammer, 1988). Such increases in the shoot and root fresh and dry weights might be due to amine groups produced by LAB. These results are in agreement with those obtained by El-Tarabily (2004) who found that growth promotion of beans *(Phasealus vulgaris*) occurred by polyamine produced by isolate of *Streptomyces griseoluteus* . Polyamines are ubiquitous low molecular weight, aliphatic nitrogenous polycations that contain two or more amine groups. The suppressive effect of bacteriocin produced by lactic acid bacteria against various pathogenic bacteria has been reported by many investigators (Uhlman *et al*., 1992; Franz *et al*., 1997; Yildirim and Johnson 1998; Lowe and Arend 2004, Souza *et al*., 2005 and Sajur *et al*., 2007).

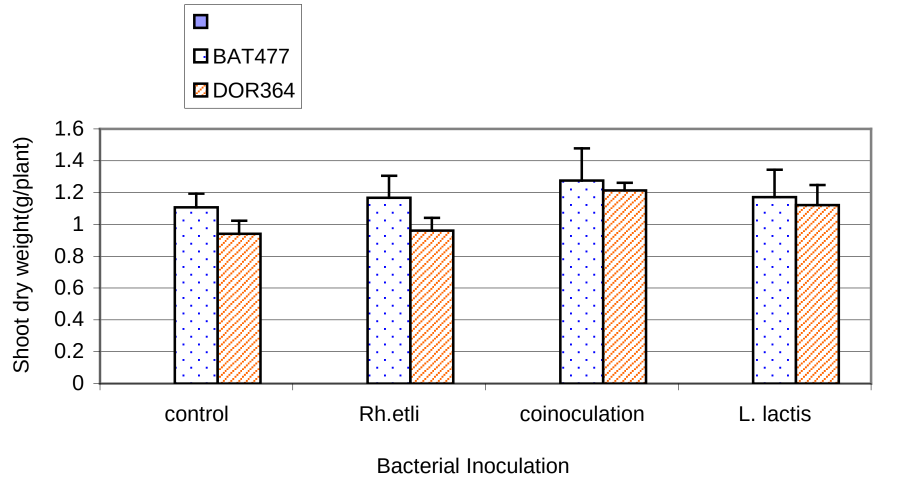

Fig.(5).Effect of inoculation with *Rh.etli* and *L.lactis* on shoot fresh weight of bean genotypes

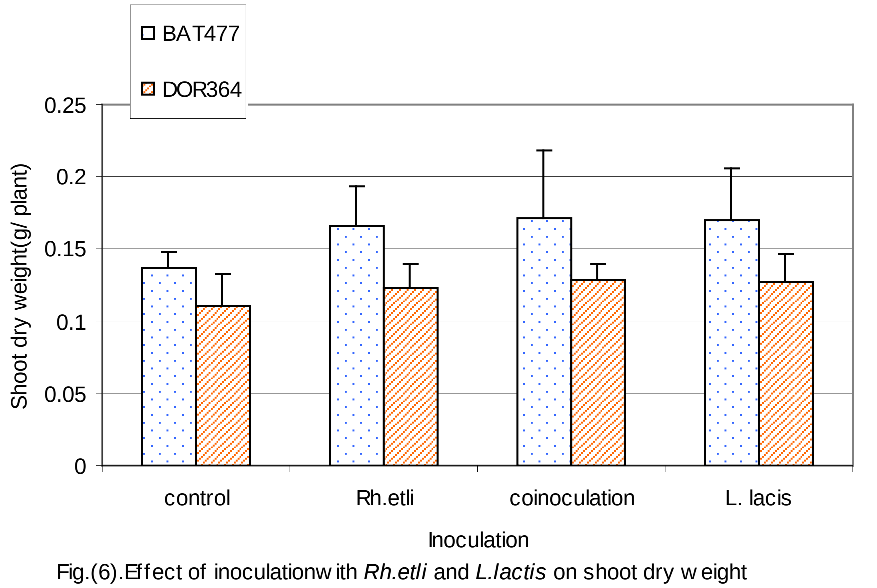

Fig.(6).Effect of inoculationwith *Rh.etli* and *L.lactis* on shoot dry weight of bean genotypes.

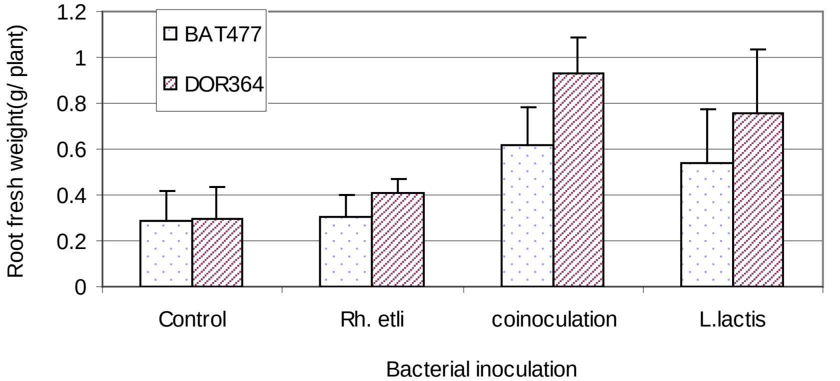

Fig.(7). Ecffect of inoculation with *Rh.etli* and *L.lactis* on root fresh weight of bean genotypes.

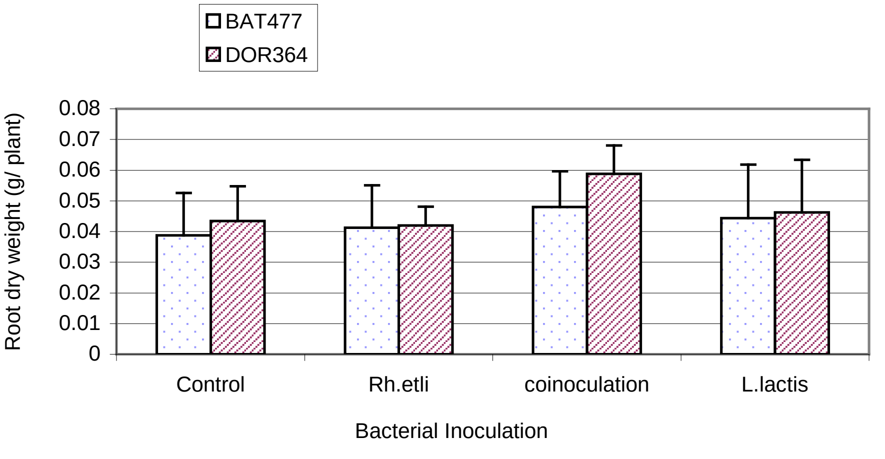

Fig.(8). Effect of inoculation with *Rh. etli* and *L.lactis* on root dry weight of bean genotypes.

Effect of bacterial inoculation on water absorption by the plants

Results presented in Table(1) demonstrated the effect of inoculation with *Rh.etli, L.lactis* and Co-inoculation with the two bacterial strains on water absorption by the two *Phaseolus vulgaris* genotypes BAT477 and DOR 364 at 12-day age.

Table (1) Effect of the experimental treatments on absorption of water by the whole plants of phaseolus bean genotypes tested at 12-day age.

Treatments	Whole plant weights(g / plant)							
	BAT 477				DOR364			
	Fresh	Dry	Difference *	Moisture ** %	Fresh	Dry	Difference *	Moisture ** %
control	1.31	0.189	1.121	593.12	1.23	0.206	1. 077	703.92
Rh.etli	1.47	0.206	1.264	613. 59	1.37	0.165	1.205	730.30
L.lactis	1.71	0.213	1.497	702.81	1.76	0.172	1.588	923. 25
Coinoculation	1.89	0.219	1.671	763. 01	2.14	0.186	1.954	1050.53
Sum	6.38	0.837	5..553	2672.53	6.50	0.729	5.824	3408.00

Each value is a mean of six replicates.
*** Difference between fresh and dry weights of the plants represents the amounts of water absorbed at the assigned age of vegetative growth.**
*** *Moisture content of the plants is based on the dry weight.**

Results showed that, inoculation with *Rh.etli* increased moisture content in the plants by 613.59 % compared with control (593.12 %), followed by *L .lactis* and Co-inoculation which gave 702.81 and 763.01 %, respectively for BAT477. The same trend was observed for DOR364, but with higher valus, i.e. 730.30, 923.25 and 1050.53 % , respectively. These results coincided with those obtained by Vessey (2003), Dobbelaere et al.(2003), Bashan(2005)and Karlidag et al.,(2007) who suggested that plant growth promoting rhizobacteria stimulate plant growth by facilitating the uptake of minerals and microelements, by the plant. The present study revealed the superiority of the Phaseolus cultivar DOR364 above the counterpart BAT477 in regard to water absorption . On the other hand, the bacterial inoculation with both examined genera together greatly favored water absorption and consequently uptake of nutrients for a better plant growth.

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تثبيت النيتروجين الجوى فى أصناف نباتات الفاصوليا الملقحة بالريزوبيا وبكتيريا حمض اللاكتيك

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الملخص العربي

فى تجربة معملية تم دراسة أثر تلقيح نباتات الفاصوليا النامية على بيئة اصطناعية بالريزوبيا (ريزوبيم اتلى) وبكتيريا حمض اللاكتيك (لاكتوكوكس لاكتس) على عملية تكوين العقد الجذرية (عدد العقد ووزنها)، وكذلك نشاط إنزيم النيتروجينيز بالجذور والنمو الخضرى للنباتات النامية .

وقد أظهرت النتائج أن تلقيح نباتات الفاصوليا بالريزوبيا أعطى عقدا بكتيرية على الجذور بينما لم تظهر أى عقد على جذور النباتات النباتات الملقحة فقط ببكتيريا اللاكتوكوكس لاكتس وكذا الكنترول. وأعطى التلقيح المشترك بين الريزوبيا وبكتيريا اللاكتوكوكس لاكتس تفوقا واضحا فى عدد العقد ووزنها مقارنة بالريزوبيا منفردة، حيث ووصلت الزيادة الى ، ١٨.٤ ، ٤٥.٣ %، وكذا وصلت الزيادة فى النشاط الانزيمى الى٣٦.٥ و ٦٤.٨ % مقارنة بالريزوبيا منفردة لكلا الصنفين المستخدمين فى هذة الدراسة وهما BAT477& DOR364 على التوالي

وأدى التلقيح البكتيرى إلى زيادة الوزنين الرطب و الجاف للنباتات مقارنة بالكنترول، وكان الصنف BAT477 أعلى استجابة عن الصنف DOR364 فى القياسات الخضرية، حيث كانت متوسطات الزيادة عن الكنترول فى المجموع الخضرى هى (١٤.٨ ، ٦.١ و ٥.٣%)، (٢٨.٨ ، ٧.٦ ، ٢.١ %)، والزيادة فى الوزن الجاف هى (٤.٢٥ ، ٢٤.٥ ، ٢١.٥%) ، (١٦.١ ، ١٤.٨ ، ١١.٨ %) للمعاملات الآتية: التلقيح المشترك للريزوبيا مع بكتيريا اللاكتوكوكس لاكتس يليها اللاكتوكوكس لاكتس منفردة ثم الريزوبيا منفردة لكلا الصنفين على التوالى و على العكس تفوق الصنف DOR364 على الصنف BAT477 فى مجموع الاوزان الرطبة والجافة للجذور ، حيث سجلت متوسطات زيادة عن الكنترول بمقادير (٠. ١١٥

و ٨٦.٤ ، ٩.٥%) ، (١٥.٦ ، ١٥٥.٠ ، ٣٩.٦%) عند استخدام التلقيح البكتيرى المشترك ثم بكتيريا اللاكتوكوكس لاكتس ثم الريزوبيا فقط على الترتيب .