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Application of zeolite as a rhizobial carrier under saline conditions

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Salinity is a major abiotic stress on most plants and microorganisms. The objective of this work is studying the adverse impact of salinity on the capability of nodule formation, plant growth, survrcomplex (Z-FOMa, Z-FOMb). the adhesion of *Rhizobium* on the zeolite has been studied under scanning and transmission electron microscopy. The survivals of both strains were followed on zeolites kept at 25°C for 90 days and results indicated that the carrier exhibited a high capacity to maintain an adequate survival rate for the rhizobial strains. Serial dilution concentrations from seawater (100, 90, 80, 70, 60, 50, 40, 30, 20 and 10%) during 30 days was conducted to evaluate the survival of immobilized *Rhizobium* on zeolites under saline condition and continuous increase up to the last date under most tested concentrates was observed. A pot experiment was conducted to evaluate the response of bean plant grown in saline soil to inoculation with either 2 strains at four rates (R0, R1, R2, R3). After 45 days of sowing, it was observed that a weakness in plant growth and failed of nodulation forming, these referred to the used soil was infected by nematode and led us to study the effect of Zeolite- *Rhizobium* complex on mortality of nematode.

Keywords: Zeolites - *Rhizobium* - Soil - Salinity stress – Bean plant

INTRODUCTION

Soil salinity seriously constraints agricultural production in arid and semi-arid tropics world-wide, as nearly 40% of the world's land surface are now impaired with unsustainability due to soil salinity-related problems. Abou-Baker et al., (2011) confirmed that soil management is an important practice to sustain agricultural production. Certainly, soil salinity adversely impacts the formation of nodules Rh legumes due to reducing root hair growth and limiting nutrient supply via photosynthesis (Ogutcu et al., 2010).

Adverse effects of salinity on N_2-fixing plants could include reductions in root nodulation, the N_2 fixation capacity of the Rhizobia and the amount of N fertilizer required.

A few of rhizobia are tolerant to salt stress and promote plant growth, but the mechanisms underlying these effects are poorly described (Dong et al., 2017). One of the most valuable methods to mitigate the environmental problem of salinity is the isolation of novel salt-tolerant rhizobia (Qu et al., 2016).

There are various types of mordent's frequently used as carrier for *Rhizobium* inoculants (Uma Maheswari et al., 2015 and Swelim et al., 2010). Numerous synthetic and natural mordents, i.e., alginate, ceramics and zeolitized tuff had been previously investigated. Natural zeolites, on the other hand, are alkali crystalline, characterized with high roughness, high porosity, vast ability to reversibly hydrate and dehydrate, high porosity and large surface area that increases their water-holding capacity and

nutrient adsorption as well as buffer soil pH might be useful as microbial mordents (Hrenovic et al., 2007). Furthermore, the dimensions of bacteria are comparable to the sizes of zeolites crystallites, as well as to the corresponding inter-crystalline pores, besides natural zeolites provide a large surface area that microbes are strongly adsorbed and adhered with another by extracellular excretions (Hrenovic et al., 2005 and Smedt et al., 2015). In a mixture with compost, zeolites have been shown to promote plant growth and to increase, at the sametime, the accumulation of nutrients in their aerial parts. Worthy to mention that after a few years of zeolites application crop yields increased might be due to its fertilization values (Ramesh et al., 2010).

In Egypt, root-knot nematode is becoming a real threat to almost all vegetable crops, especially in newly reclaimed areas and they have been considered a limiting factor in crop production (Ibrahim, 2011). Little, if any, work had been done to examine the action of bacteria in controlling these nematodes. Oostendrop and Sikora (1990) stated that certain types of rhizobacteria are able to reduce the infection by various plant parasitic nematodes. On the other hand Reitz et al. (2000) reported that root dipping in LPS solution of *Rhizobium etli* G12 decreased nematode infection at concentrations as low as 1 and 0.1 mg ml^{-1}. In a split-root experiment, the bacterium-free LPS of *Rhizobium etli* G12 were in most part responsible for systemic induced resistance of potato to *G. pallida* infestation. The LPS of *Rhizobium etli* G12 might be the decisive resistance inducer, since it is well known that for many gram-negative bacteria LPS is released into the soil ecosystem during bacterial growth (Schröder, 2000).

Sustainable farming under saline conditions using natural products like clay minerals (zeolites) and *Rhizobium* as a biofertilizer is a new approach. So that, production of zeolites-rhizobium bio-agent as a new product for improving bean growth under biotic (salinity) is a main purpose of this study.

MATERIALS AND METHODS

Formulation of Zeolites-*Rhizobium* complex

Rhizobium sp. Sources

Two isolates of *Rhizobium* were used as inoculants in the formulation of zeolites-rhizobium complex. The first isolate, was got from the Agricultural Research Center (FOMa) isolated from *Vicia faba* plant grown in El-Giza governorate and the second isolate was a local isolate from National Research Center (FOMb) isolated from *Vicia faba* plant grown in Sinai governorate.

Molecular identification of isolates

The isolates were identified using molecular biology tools. Genomic DNA was extracted by enzymatic lyses using lysozyme (20 mg/ml) and Proteinase K (1 mg/ml). Total genomic DNA was purified using isopropanol buffer as described by Darwesh et al. (2014). Polymerase chain reaction (PCR) amplification of the 16S rRNA genes was conducted using extracted DNA in the presence of the forward primer (5′ d AGAGTTTGATCCTGGCTCAG 3′) and the reverse primer (5′ d TACGGTTACCTTGTTACGACTT 3′). The final 50 µl reaction mixture contained 1× PCR buffer (NEB, England), 1 nmol of dNTPs, 1 pmol of 2 mM $MgSO_4$, 0.25 pmol of forward and reverse primers, 1 unit Taq DNA polymerase (NEB, England) and 5 µl template DNA (Kheiralla et al., 2016).

The PCR amplification included initial denaturation of DNA at 95°C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 45 sec. And then the mixture was kept for 10 min at 72 °C for complete extension. The PCR product was purified by QIAquick Gel Extraction Kit (QIAGEN, USA) and run on agarose gel to evaluate the purification method for 16s rRNA fragments for sequencing. Identification was achieved by comparing the contiguous 16s rRNA obtained sequences with the 16S rRNA sequence data from the reference and type strains available in public databases in Gene Bank using the BLAST program (National Centre for Biotechnology Information). The sequences were aligned using Jukes Cantor Model. The phylogenetic reconstruction was done using the neighbour-joining (NJ) algorithm; with bootstrap values. The obtained sequences were submitted to Gene Bank (Barakat et al., 2016).

Growth media

a- Yeast Mannitol Broth (YMB) medium composed of 0.5g K_2HPO_4, 2g $MgSO_4$, 0.1 NaCl, 0.4g Yeast extract, 2g Mannitol, 1000 ml distillate water.

b- Yeast Mannitol Agar (YMA) medium media supplemented with 15 g agar per liter

Cultural media

Rhizobium strains FOMa and FOMb were grown on (YMB) in an incubator shaker at 28 °C for 48 hrs, and washed with sterile water before their intensity were adjusted with sterile water to 10^9 cells ml. Initial rhizobial count reached 10^9 cells/ml by the serial dilution plate count method.

SEM and TEM before and after inoculation

Clay mineral of zeolites was obtained from Alex Company. Micrographs of zeolites surfaces before and after adhesion with *Rhizobium* (1:1 V:W) were obtained using Quanta FEG 250 a scanning electron microscope (SEM) and JEOL JEM-2100 a high resolution transmission electron microscope (TEM).

***Rhizobium* viability in Zeolites-*Rhizobium* complex**

Ten grams portion zeolites were used in estimating the viability of *Rhizobium* cells in zeolites-rhizobium bio-agent initially and after 7, 14, 21, 30, 60 and 90 days incubation on YMA medium at 28 ± 2°C for 72 hrs (Mala, 2003) using serial dilution plate method.

Monitoring the growing cultural characteristics of *Rhizobium* under saline conditions

Fifty grams of sandy soil were irrigated with serial dilution of seawater (100, 90, 80, 70, 60, 50, 40, 30, 20 and 10%) to evaluate the survival of *Rhizobium* which has been immobilized in zeolites under saline condition, after incubation for 7, 14, 21 and 30 days at room temperatures. The counts of *Rhizobium* bacteria were determined after the inoculum of sterilized zeolites using serial dilution plating method.

Greenhouse experiment

A pot experiment was carried out at the greenhouse of National Research Center to evaluate the consequences of zeolites- *rhizobium* complex under salt stress (soil salinity) on faba bean growth as well as on the survival of nematode.

A salty soil with an EC=7.18 dSm^{-1} (1:5 soil: water extract) from El-adlia region, Belbeis, Sharkia Governorate was collected. The soil sample was air-dried, crushed, sieved to pass through 2mm sieve and preserved for analyses. Some physical and chemical characteristics of the investigated soil (Klute, 1986 and Page et al., 1982) are given in Table (1). PVC pots of 20cm for both diameter and height were filled with 3kg soil/pot. Basal doses of N, P and K fertilizers, corresponding to recommended dose of ministry of agriculture (20kg N, 30 kg P_2O_5 and 24kg K_2O per fed. were added as ammonium nitrate, super-phosphate and potassium sulphate, respectively) and mixed thoroughly with the soils, before sowing.

Ten grams of sterilized solution of micro zeolites were mixed with a growth medium at a density of about 10^9 cells ml per gram of carrier. Faba bean seeds (*Vicia faba* L. cv. Giza 716) were inoculated with two *Rhizobium* strains (FOMa and FOMb) at rates of Rh0: (without *Rhizobium*), Rh1(1:1), Rh2(2:1), Rh3(3:1) (Rh:Zeolites) in presence and absence of zeoiltes as a carrier for *Rhizobium*. Seeds were coated with inoculum strains, directly before seeding using 40% Arabic gum as sticker. Four treated seeds were planted and thinned to two plants/pot, after germination. Un-inoculated control was prepared with sterile water. Soil moisture content was kept near field capacity using tap water for irrigation. Plants were harvested after 45 days of sowing and the fresh and dry weights of shoot and root, and total dry weight of bean plant were registered.

Table (1): Some physical and chemical properties of the investigated soil ecosystem

pH (1:2.5)	EC dSm^{-1}(1:5)	Cations and anions meq./L							
		Na^+	K^+	Ca^{++}	Mg^{++}	CO_3^{--}	HCO_3^-	Cl^-	SO_4^{--}
7.49	7.18	50.61	0.37	14.5	5.5	-	1.65	65.0	4.33
OC%	OM%	$CaCO_3$%	Particle size distribution			Texture	Available (ppm)		
			Sand	Silt	Clay		Fe	Zn	Mn
0.3	0.52	4.6	94.7	1.8	3.5	Sandy	3.78	1.09	1.51

Bioassay screening of nematodes

Rhizobium strains isolated from Faba bean plant grown in Sinai (FOMb) was chosen to evaluate their effect on nematode survival. The direct effect of zeolites- *rhizobium* complex (Z-FOMb) as well as of sole zeolites control without *Rhizobium* (ZC) against the root-knot nematode, *Meloidogyne incognita,* second stage larvae was estimated in a laboratory bioassay study. Five g of each of both tested materials (Z-FOMb and ZC) were dissolved in 50 ml distilled water to prepare a suspension with a concentration of 10%, which considered being a standard solution (S) of 100% concentration. Dilutions of S/2and S/10were freshly prepared by adding distilled water. Ten ml portion of each solution was added to one ml of a nematode suspension containing 200 *M. incognita* second stage larvae in 50 ml plastic vial. Control was done by adding 10 ml of distilled water plus 1ml of the nematode suspension. Each treatment was replicated for three times. Counts of the viable and dead nematodes in each treatment were recorded under a light microscope after 24, 48 and 72 hrs at 25±1°C and the nematode mortality percentages were calculated for each treatment. After 72 hrs, the nematodes in each concentration level were transferred to other plastic vial containing distilled water and were investigated after 24 hrs to be sure that those nematodes did not restore their viability. Only the nematode did not restore viability and was considered dead. Mortality percentage was calculated according to the Abbott's formula (Abbott, 1925).

- Each value represents mean of three replicates.

$$\% \text{ mortality} = \frac{n-n}{100-n} \times 100$$

Where m and n stand for the % mortality in treated sample and control, respectively.

- %net mortality= % mortality (72 hrs) - % recovery.

where m and n stand for the percentages of mortality in treated and control soils, respectively, the percentages of net mortality were also calculated.

Statistical analysis

All pots arranged in completely randomized design. Analysis of variance (ANOVA) and least significant difference (LSD) at 0.05 probability level was calculated (Gomez and Gomez, 1984).

RESULTS

Molecular identification of isolates

Two isolates of *Rhizobium* as potent strains, was analyzed for its 16S rRNA gene. The genomic DNA of tested strains was isolated and purified using isopropyl method. The 16S rRNA gene was amplified by PCR technology using forward and reverse primers and the PCR product was purified. The size of PCR product was approximately 1000 bp. After sequencing for 16S rRNA gene, the obtained sequences were compared with sequences available in Gen Bank using BLAST program (NCBI web page) and the similarity percentage of this isolate accounted for 93% with strain *Rhizobium leguminosarum* biovar *viciae*. The sequences of these strains were submitted to Gen Bank and recorded under accession number MG791929- MG791930 for *Rhizobium leguminosarum* biovar *viciae* strain FOMa and *Rhizobium leguminosarum* biovar *viciae* strain FOMb, respectively. The phylogenetic relationship (Fig 1a, Fig1b) showed that this strains very close to the type strains of *Rhizobium* genera deposited in culture collection centre of National Centre for Biotechnology Information.

SEM and TEM before and after inoculation

The scanning electron microscope (SEM) and transmission electron micrograph of zeolites before and after inoculation by FOMb strain are shown in Figures (2, 3 and 4). It's clear that batch of *Rhizobium* cells are attached to zeolites surfaces that have a slightly negative charged surface strengthening the adsorption of more microbes and endows zeolites with greater removal ability. The negatively charged zeolites became a superior supporting mordant for microbe attachment. At the same time, the hexagonal pores of zeolites were covered by *Rhizobium* cells; this is a double edges sword. The hydro-physical and chemical properties of zeolites may be decreased in contradict line of biological characteristics by adhesion process.

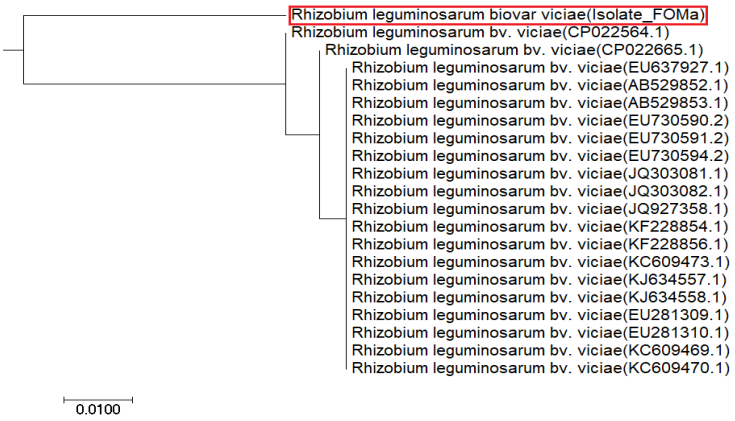


Figure 1a: Phylogenetic tree constructed from the 16s rRNA sequence of *Rhizobium leguminosarum* biovar *viciae* strain FOMa and their related strains in Gene Bank.

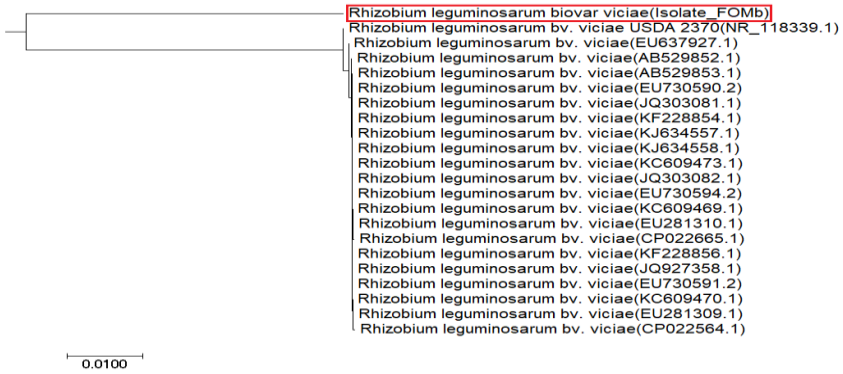


Figure 1b:Phylogenetic tree constructed from the 16s rRNA sequence of *Rhizobium leguminosarum biovar viciae* strain FOMb and their related strains in Gene Bank.

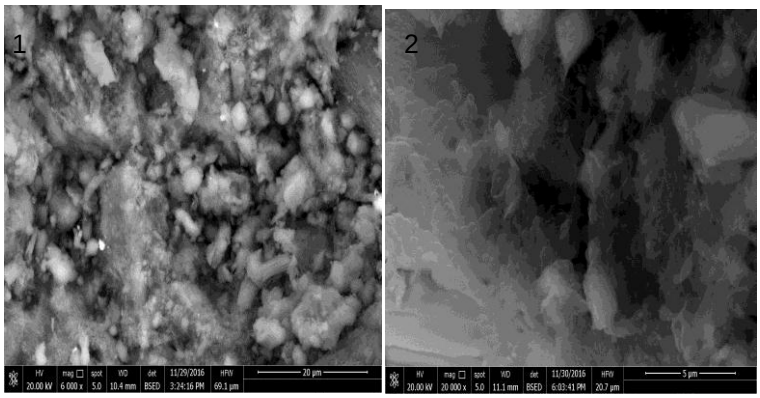


Figure 2: Scanning electron micrograph of micro zeolites-*rhizobium* bio-agent before (1) and after (2) cell adhesion.

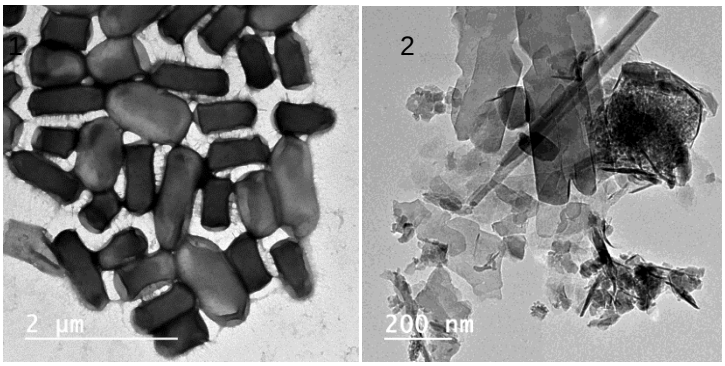


Figure 3: Transmission electron micrograph of *Rhizobium* (1) and micro zeolites (2) before adhesion

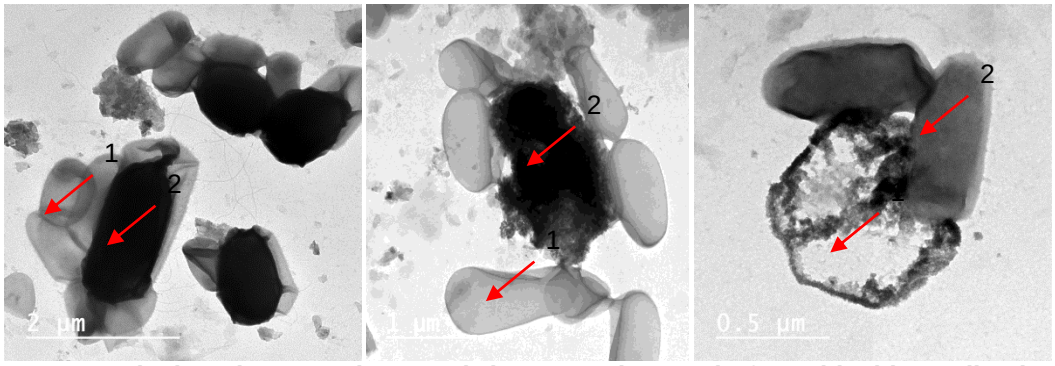


Figure 4: Transmission electron micrograph (at 2, 1 and 0.5 µm) after *Rhizobium* adhesion (1) on micro zeolites carrier (2)

Zeolites- *Rhizobium* complex monitoring

The survival of both strains of *Rhizobium* (FOMa-FOMb) was followed on zeolites mordent kept at 25 °C for 90 days (Table 2). Results indicated that the carrier exhibited a high capacity to maintain an adequate survival rate for the rhizobial strains as high population of *Rhizobium* was detected. Comparing with the initial 10^9 CFU/g, *Rhizobium* intensity increased from 10^9 CFU/g initially to 0.3 log CFU/g and despite the remaining populations at the end of storage for both isolates were almost unchanged but the number of FOMa (0.3 log $cfug^{-1}$) slightly diminished to less than the number population of FOMb (1.3 log CFU/g).

Monitoring of growing cultural intensity under saline soil conditions

Results given in Table (3) show the population intensity, measured by the log 10 CFU/g after 7, 14, 21 and 30 days of inoculation, for both trailed *Rhizobium* strains at the different tested concentrations prepared by diluting sea-water. The initial population intensity displayed a continuous increase up to the last date under most tested concentrates. Under the 100% sea water concentration, the population density reached 7.0 log CFU/g for both studied *Rhizobium* strains, while under the lowest concentration (10%), the population intensity reached 10.0 and 10.3 log CFU/g in FOMa and FOMb, respectively. Zeolites maintained higher population intensity for the two tested *Rhizobium* isolates probably because it had more available nutrients and maximum inherent moisture content as well as higher water holding capacity and retention characteristics besides having chemical and physical uniformity and being nontoxic to inoculants isolates.

Table (2) Survival of the two trailed species of *Rhizobium leguminosarium* bv. *viceae* in zeolites-rhizobium bio-agent during storage

Time	initial	7 d	14 d	21 d	30 d	60 d	90 d
	Plate count ($x10^9$cfu g^{-1})						
FOMa	1	9.0	8.5	7.8	6.6	0.3	0.3
FOMb	1	11.6	10	8.0	7.3	1.3	1.3

Table (3) Growth of both strains of Zeolites- *Rhizobium* **bio-agent under saline soil conditions**

Salinity %	FOMa				FOMb			
	Plate count ($x10^9$cfu g^{-1})							
	7d	**14d**	**21d**	**30d**	**7d**	**14d**	**21d**	**30d**
100	7.0	7.1	7.0	7.0	7.2	7.2	7.0	7.0
90	7.8	7.7	7.3	7.3	8.1	8.0	7.9	7.8
80	8.0	8.2	8.2	8.1	8.3	8.3	8.3	8.3
70	8.0	8.2	8.4	8.4	8.2	8.3	8.5	8.7
60	8.8	9.0	8.7	8.9	9.0	9.1	9.3	9.3
50	8.7	9.2	9.5	9.5	9.1	9.3	9.5	10.0
40	9.8	9.7	10.0	10.0	9.8	10.1	10.3	10.3
30	9.8	9.8	9.8	9.9	9.7	10.1	10.1	10.1
20	9.5	9.7	9.9	10.2	9.8	10.2	10.5	10.7
10	9.12	9.15	10.0	10.0	9.85	9.88	10.1	10.3

Greenhouse experiment

Fresh weight of bean shoots and roots

The zeolites-*rhizobium* complex doses as well as the interaction between them affected the fresh weight of bean plants without significant difference as illustrated in Fig. (5). Irrespective of *Rhizobium* strains and trailed doses of Zeolites- *Rhizobium* complex, addition of zeolites increased shoots and roots fresh weight by 34.3 and 13.0%, respectively compared to control. Both of *Rhizobium* strains led to an increase in fresh weight of shoot and root, the second strain (FOMb) was superior. The fresh weight of bean plant increased gradually with increasing the inoculation dose and follows the order: Rh3>Rh2>Rh1>Rh0. Under control conditions (without zeolites application), the fresh weight increased by the two isolates, whereas, zeolites-*rhizobium* complex at low doses (Rh1 and Rh2) led to a decrease shoot weights before increasing again with increasing Zeolites- *Rhizobium* complex rate (Rh3). Statistically, it is worthy to state that the effect of sole application of zeolites and *Rhizobium* was un-significant, while, the second interaction between zeolites and strains (Z x S; *p* values 0.008 and 0.031) as well as zeolites and doses (Z x D; *p* values 0.031 and 0.018) were significant for shoot and root, respectively.

Dry weight of bean shoots and roots

The dry weights of bean shoot were significantly affected by Z (*P*=0.02), Z x D (*P*=0.014) and S x D (*P* =0.004) treatments, while the individual effects of Rh strains (I) and its doses (D) were not significant (Table 4 and Fig. 6). The action of FOMb inoculant was greater than of FOMa on the dry weights of shoot at low doses (Rh1 and Rh2), while Rh3 of RhE exhibited the highest effect. In absence of zeolites, the dry weights of shoots increased by increasing Rh doses, but inoculation with Zeolites- *Rhizobium* complex at low doses slightly reduced shoot dry weights. The effect of zeolites (*P*= 0.000), the inoculations rates with *Rhizobium* (*P*= 0.015) and the second interactions Z x S and Z x D (*P*= 0.001 and 0.002) on root dry weights were highly significant. The second rate of *Rhizobium* exhibited a good effect on the dry weights of root especially under the application of FOMb that was the best treatment compared to FOMa.

The total dry weights were significantly affected by zeolites application as well as by the second interactions ZxS, ZxD and SxD. The effect of S, D and the third interactions (Zx S xD) on total dry weights of bean plants was not

significant.

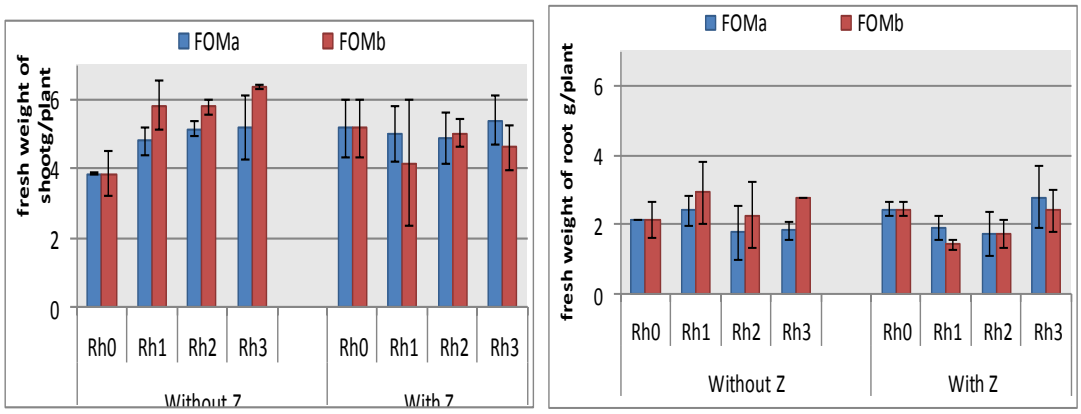
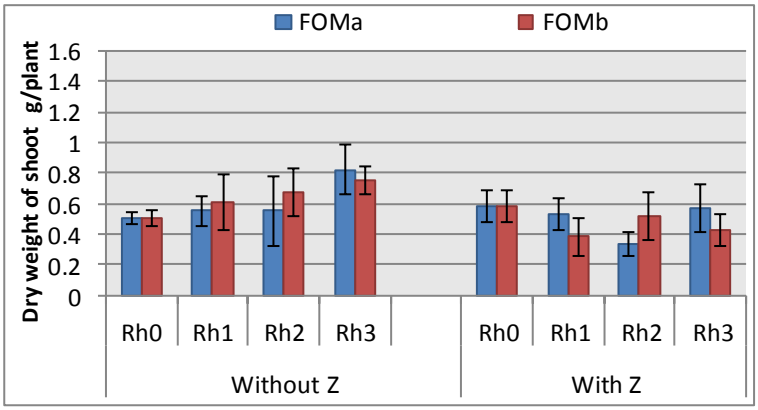


Figure. (5) Effect of zeolites-*rhizobium* bio-agent on fresh weight of bean shoots and roots (g/plant)

Table (4) *P* values of fresh and dry weight of shoot and root as well as total dry weight

Source	Df	P values				
		Shoot FW	Root FW	Shoot DW	Root DW	Total DW
Replicates	2	0.732	0.180	0.347	0.180	0.209
Zeolite	1	0.221	0.269	**0.020**	**0.000**	**0.000**
Strain	1	0.235	0.408	0.306	0.067	0.208
Dose	3	0.168	0.096	0.118	**0.015**	0.265
Z x S	1	**0.008**	**0.031**	0.792	**0.001**	**0.003**
Z x D	3	**0.031**	**0.018**	**0.014**	**0.002**	**0.000**
S x D	3	0.956	0.887	**0.004**	0.219	**0.024**
Z x S x D	3	0.568	0.462	0.073	0.058	0.361



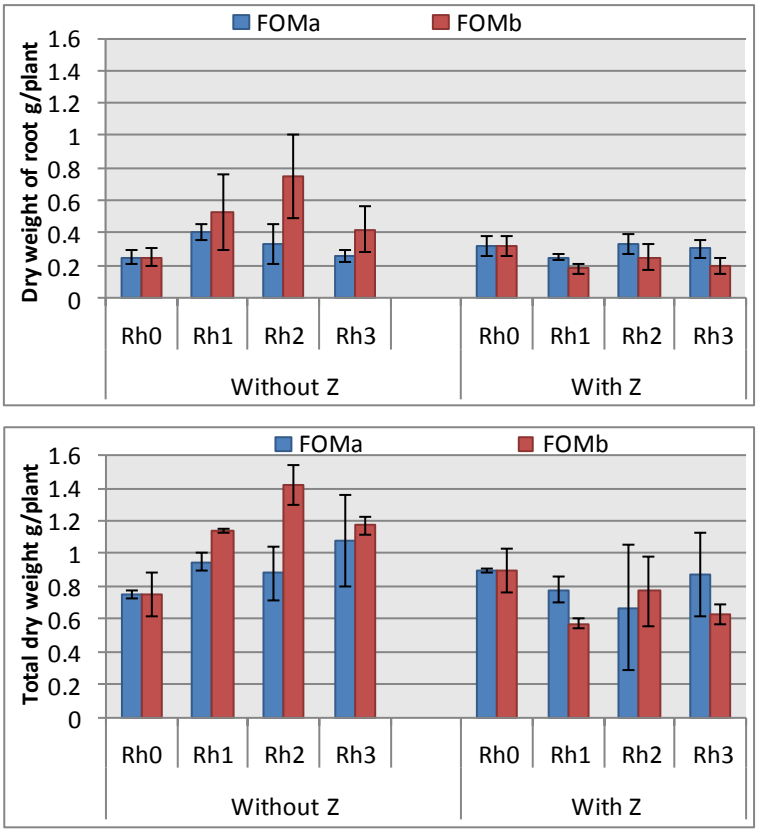


Figure. (6) Effect of Zeolites- *Rhizobium* bio-agent on bean shoot, root and total dry weight (g/plant)

Although, sole application of zeolites increased the total dry weights and the addition of *Rhizobium* without zeolites led to an increase in the total dry weights, their mixture (zeolites-*rhizobium* complex) decreased total dry weights under the lowest rate then turned to increase with increasing inoculation rate.

Infection intensity of bean root by nematode

Application of zeolites-*rhizobium* complex decreased the infection intensity of root by nematode (Table 5). Although all plants were infected, the application of zeolites-*rhizobium* complex led to a decrease the infection intensity. The infection decreased by increasing the inoculation rate. FOMb isolate was superior to FOMa in alleviating the adverse impacts of nematode in root.

The results confirmed the above mentioned findings and pointed to that the high infection rates with nematode is a critical reason of the reduction in plant growth and found insignificant differences between trailed treatments in addition to the impact of high salinity of used soil (EC=7.18 dSm^{-1}in 1:5 soil: water extraction).

Table (5) Effect of Zeolites-*Rhizobium* bio-agent on the infection intensity of bean root by nematode

Zeolite (Z)	*Inoculation Dose(D)*	*Rhizobium strains(S)*	
		FOMa	FOMb
Without	Rh0	++++	++++
	Rh1	+++	+++
	Rh2	+++	++
	Rh3	+++	+
With	Rh0	+++	+++
	Rh1	+++	++
	Rh2	++	++
	Rh3	+	+

Effect of Zeolites-*rhizobium* complex on resistance of nematodes

Different concentrations of zeolites-*rhizobium* complex (Z-FOMb) and zeolites control without *rhizobium* (ZC) were *in vitro* testedfor their direct effect on the mortality rate of *M. incognita* juveniles. Results presented Table (6), indicat that the percentages of net mortality of *M. incognita* juveniles were 97, 32 and 33% at the

concentrations of S, S/2 and S/10, respectively,

Table (6) Antagonistic effect of *Rhizobium leguminosarium* against the second stage larvae of root-knot nematode, *Meloidogyne incognita*

Treatments	Dilutions	Percentages mortality after (hrs)			Recovery (%)	Net Mortality (%)
		24	48	72		
Z-FOMb	S	31	46	97	-	97
	S/2	-	34	100	68	32
	S/10	-	28	100	67	33
ZC	S	100	77	81	45	36
	S/2	85	56	47	12	35
	S/10	-	2	4	-	4
Distilled water	-	5	4	6	-	-

while in ZC the percentages mortality were 36, 35 and 4% at the same concentrations; respectively compared with distilled water control. Data showed also that, the percentages mortality of Z-FOMb were positively joined with the exposure intervals, while the percentages mortality of ZC decreased with increasing the exposure intervals except at concentration of S/10.

DISCUSSION

Sustainable farming necessitates the application of certain conservative tools that keep soil fertility. The most important tools are flourishing the soil microbial population and maximizing their roles. The role of *Rhizobium* bacteria in legume crop production is unquestionable. In addition, the PGP microbes contain useful variation for tolerating abiotic stresses like extremes of temperature, pH, salinity and drought; potential toxic elements and pesticide pollution (Gopalakrishnan et al., 2015).

Nitrogen fixation is highly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full efficiency under unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing that impose limitations on the vigor of the host legume (Brockwell et al., 1995). Salinity affects the photosynthetic rate and nodule metabolism, it not only decreases the agricultural production of most crops, but also, affects soil physicochemical properties, and ecological balance of the ecosystem. Such impacts led to low agricultural productivity, low economic returns and soil sickness (Hu and Schmidhalter, 2002).

Image by SEM and TEM for the complex of *Rhizobium* and zeolite indicated that *Rhizobium* adhered to zeolite have a capability to aggregate and form larger particles. The rhizobial cells were partially covered by zeolite aggregate. Like aggregates could not be found in the SEM image of pure zeolite. It isreasonable that these aggregates are Zeolite-*Rhizobium* - complex, these results also found by Belaabed et al. (2016).

Incorporation of microorganisms in a proper mordent enables easy-handling, long-term storage and high effectiveness of *Rhizobium*. There are various types of mordents used as carrier. A good mordent must be: (I) good moisture absorption capacity, (II) non-toxic to inoculant bacterial strain, (III) easy to process and free of lump-forming materials, (IV) available in adequate amounts, (V) easy to sterilize by autoclaving or gamma-irradiation, (VI) good adhesion to seeds, and (VII) good pH buffering capacity, Needless to say, it should also be non-toxic to plant (Uma Maheswari et al., 2015). The material used as a mordent should enhance the proliferation of rhizobia and preserve their capacity to form nodules and to fix nitrogen (Swelim et al., 2010). A high-grade mordent should also have high water retention and stable pH, and be inexpensive, constitutive, nontoxic for the strain or ecosystem and easy to sterilize. It is responsible for osmotic imbalance, declining water availability for plants, causing ion-specific toxicities or imbalance, decreasing nutrients uptake, and thus, affecting plant physiology (Abou-Baker and El-Dardiry, 2015).

In highly saline ecosystems, leguminous plants growth requires the free-living rhizobia associated with a host tolerant to salt. Many *Rhizobium* strains can tolerance the upper limits of salinity than those of their host legumes (Singleton et al., 1982).

Salts might adversely influence symbiosis process by antagonizing the growth and survival

of rhizobia in the soil ecosystem, restricting root colonization, inhibiting infection and nodule development and/ or impairing active nodule functioning. These effects might be mediated through an effect of salt on the host, or through a specific effect on the micro-symbiont itself. The probability for the success of the partnership of selecting either a salt tolerant rhizobial strain or a salt tolerant legume plant had failed. This is because legume- *Rhizobium* symbiosis and nodule formation on the legumes is more sensitive to salt or osmotic stress than the rhizobia or the plant (Zahran, 1991).

The salt effect on the symbiotic interaction does not only inhibit the formation of the nodules, but also leads to the reduction of the host plant growth. Salinity effects on nodulation, includes formation of non-functional nodules with abnormal structure, degradation of peribacteroid membrane (Bolaños et al., 2003). Bacterial chemotaxis, colonization, root hair curling (Zahran, 1986) and deformation (Singleton et al., 1982), reduction in nodular respiration (Walsh, 1995) and impaired N-fixing activity (Bordeleau and Prevost, 1994) have also been observed as adverse effects of salt.

There are some factors affective on the symbioses relationship between *rhizobium* and their host plant such factors as nitrogen fertilization, sometimes needed to achieve an essential yield of legumes like soybean, when the symbiotic N_2fixation is unable to provide sufficient nitrogenHowever,fertilizer rates exceeding those exerting a beginning nitogen effect generally reduce nodulation and N_2 fixation (Afza, et al., 1987), and salinity is one of the important factors, however many parameters were affected nodule formation, nitrogen fixation, that is like occur in this study, although zeolite had the ability to retain the rhizobia and its vitality in these saline conditions, but the rhizobial strains failed to formation of nodules.

There are about 50 different types of natural zeolites with different mineralogical composition depending on their structure and silicon to aluminum ratio (Baker et al., 2009). Zeolites-*Rhizobium* mordent is fabricated from a combining complex, where *Rhizobium* attaches to the surface of zeolites. Zeolites application increased bean plant growth compared to without application. This might be ascribed to the characteristics of natural zeolites such as high cation exchange capacity, ability to hydrate and dehydrate reversibly and to exchange some of their constituent cations and ability to reserve water in rhizosphere zone. Under normal conditions (without zeolites application), most growth parameters increased with *Rhizobium* inoculation. This could be referred to the ability of *Rhizobium* to fix atmospheric nitrogen. While, zeolites-rhizobium complex especially with low doses of *Rhizobium* led to unexpected results, whereas, the growth values decreased and turned to increase again with increasing *Rhizobium* inoculation rate. These finding might be attributed to that both of zeolites and *Rhizobium* inhibited each other. *Rhizobium* entered into hexagonal gaps between clay mineral layers, but with increasing inoculation rate theses gaps were fulfilled and extra *Rhizobium* began to run again.

On the other hand, the successful symbiotic association between legumes and *Rhizobium* can be affecting by abiotic and biotic factors such as water stress, pH, soil's temperatures and infected of pest and diseases. Among important pests are nematodes which play an important role in limiting the production of legumes by reduce the nitrogen fixation which followed by failure to form nodules. The plant infections by nematodes causes negative effectson plant growth, nodule development, nitrogen contents of shoot and root,bacteroids, and nitrogenase activity and it can result in the production of factorsthat suppress nodulation (Bhat et al., 2009), and the discover of nematodes which infected soil perhaps may be is principal cause to reduce the growth of plant and failure to form the nodules. Previous studies demonstrated that specific rhizobacteria reduce infection by various parasitic nematodes (Neipp and Becker, 1999). Rhizobacterium *R. etli* G12 impaired infection by potato cyst nematode *G. pallida* indirectly by inducing systemic resistance (Hasky-Gu¨nther et al., 1998).

Several strategies have been developed in order to decrease the toxic effects caused by high salinity on plant growth, includingplant genetic engineering (Wang al., 2003), and recently theuse of plant growth-promoting bacteria (Dimkpa et al., 2009). The role of microorganisms in plant growth promotion, nutrient management and disease control is well known and well established.

Every organ of the higher plant (roots, stems, trunk, leaves, and inflorescences) is susceptible to be attacked by one or more plant-parasitic nematode (PPN) species. Since most PPN affect root functions, most symptoms associated with them are the result of inadequate water supply or mineral nutrition to the tops. For instancese, when plants are severely infected by meloidogyne, the normal root system is reduced to a limited number associated with severely galled roots with a

completely disorganized vascular system and rootlets are almost completely absent. The roots are seriously hampered in their main functions of uptake and transport of water and nutrients (Netsher and Sikora, 1990). Some plant-parasitic nematodes have specific symptoms, the typical ones are stunted growth, wilting, leaf discoloration and deformation. The increased metabolic activity in giant cells mobilizes photosynthetic products from shoots to roots (Hofmann and Grundler, 2007). Reduced yield is manifested in changes in quantity and/or quality.

Concerning the effect of the biotic agents on the mortality of *M. incognita* juveniles, Siddiqui and Shaukat (Siddiqui and Shaukat, 2003) stated that the effect of the biotic agents on the mortality of *M. incognita* juveniles were exposure of root-knot nematode, *M. javanica* to culture filtrate of *P. fluorescens* as biotic agent under *in vitro* conditions, significantly reduced egg hatch and caused substantial mortality of juveniles. They added that the antimicrobial metabolites 2,4 diacetyl phloroglucinol (2, 4 DAPG) and phuoluteorin contribute to the ability of *P. fluorescens* to induce mortality of juveniles under *in vitro* conditions. While the effect of the abiotic agents on the mortality of *M. incognita* juveniles, might be ascribed to changes in pH and/or the osmotic pressure (Loewenberg et al., 1960), this osmotic pressure at higher concentrations with prolonged exposure periods might be toxic to nematodes (Al-Sayed and Thomason, 1988). Second stage juveniles of root-knot nematode might not be tolerate these extremes. Siddiquiet al. (2001) noted that, the genus of *Rhizobium* could significantly reduceegg hatching and cause mortality for*M. javanica* invitro. Oligosaccharides of the core-region of *Rhizobium* are the main trigger of systemic resistance in potato roots towards *Globodera pallida* (Martina et al., 2001). Using *Rhizobium* plus*Pseudomonas putida* caused maximum reduction in *Meloidogyne javanica* galling and multiplication onlentil plants (Siddiqui et al., 2006).

CONCLUSION

Sustainable farming faces great challenges, at the time being, such as biotic stresses that led to increasing diseases intensity. Sustainable agricultural relies partially on the application of proper conservative inputs like bio-fertilizers and bio-pesticides. Although the negatively charged zeolites might be considered as a superior supporting input as a microbial mordent, it is governed by its rate of inoculation as a critical factor. Results confirmed that Zeolites- *Rhizobium* bio-agent proved its ability to reduce the impact of salinity and nematode on bean plant. Although, sole application of both zeolites-rhizobium complex increased total dry weight, their mixture (Zeolite- *Rhizobium*) led to decrease the total dry weight of faba beans especially under the lowest inoculation rate - as an unexpected result- and tend to increase the weights under increasing inoculation doses. Hence it is recommended to increase the inoculation rate to enhance of the characteristics of zeolites-rhizobium complex as both bio-fertilizer and bio-pesticide. Thus, the current study confirmed that *Rhizobium* could be immobilized on mineral carriers (Zeolites). In addition, zeolites-*rhizobium* complex (Z-FOMb) proved to be tolerant to salinity stress and could successfully be used as a bio-control agent against nematodes even in saline soil ecosystems. Our gained results recommend more studies to reach the proper inoculation rate of Zeolites- *Rhizobium* complex, as well as, evaluate its ability to control other pests rather than nematodes

CONFLICT OF INTEREST

The authors declared that the present study was performed in absence of any conflict of interest".

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AUTHOR CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors FHA and NHA designed the study, prepared the figures and tables and author NHA performed the statistical analysis. Authors FHA and NHA managed the analyses of the study, managed the literature searches, wrote the first draft wrote the protocol, author AMSL designed the experiment of nematode test. All authors wrote the read and approved the final version.

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