JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

EVALUATION OF SALT-TOLERANT *RHIZOBIUM* AS BIOINOCULANTS FOR ENHANCING PLANT GROWTH IN SALINE SOILS

Mrunal Posam[1], Rajendra Choure[2], Rupendra Jadhav[3]

[1,2,3]Department of Microbiology, Institute of Science, 15, Madam Cama Road, Mumbai 400032.
[1]Research Student, Department of Microbiology, Institute of Science, Fort, Mumbai, India.
[2]Assistant Professor, Department of Microbiology, Institute of Science, Fort, Mumbai, India.
[3]Professor, Department of Microbiology, Institute of Science, Fort, Mumbai, India.

Abstract: Salinity is one of the main factors responsible for soil deterioration and poor agricultural productivity. Damaged soil not only affects plants but also the microorganisms associated with it. Plant growth-promoting rhizobacteria (PGPR) are bacteria present in the rhizosphere of soil that are beneficial to the plant associated with it. Enhancement of plant growth by PGPR can be direct, providing the plant with essential nutrients, or indirect, protecting it against the harmful effects of plant pathogens. Ethylene levels can be regulated by ACC Deaminase by dissociation of stress-induced ACC (secreted as root exudates) into Ammonia and α- ketobutyrate. A wide spectrum of beneficial bacteria produces phytohormones such as indole-3-acetic acid (IAA), which are involved in plant growth promotion. *Rhizobium* is known to enhance plant growth by both direct and indirect mechanisms. The present study focused on studying different *Rhizobium* isolates for their salt tolerance and plant growth-promoting properties. Root nodules from various plants were collected from different locations and used to isolate *Rhizobium*. 17 isolates were obtained, and each isolate was grown in Yeast Mannitol Broth with different salt concentrations to determine its salt tolerance. These isolates were further screened for IAA production, ACC Deaminase production, and Phosphate solubilization.

Keywords: *Rhizobium*, Plant growth-promoting rhizobacteria, Salt stress, IAA, ACC

1. Introduction

Salinity is a major abiotic constraint limiting agricultural productivity worldwide, affecting over 20% of irrigated lands and impairing plant growth through osmotic stress, ion toxicity, and nutrient imbalance (Singlenton et al., 1982). Leguminous crops are particularly sensitive to salt stress because their productivity depends not only on plant physiology but also on the efficiency of symbiotic nitrogen fixation (Luca et al., 1998; Nascimento et al., 2016). In this context, *Rhizobium* spp.—soil bacteria capable of establishing symbiotic associations with legumes—play a pivotal role in sustainable agriculture by converting atmospheric nitrogen into bioavailable forms (Evidence That the *Rhizobium* Regulatory Protein RirA Binds to Cis-Acting Iron-Responsive Operators (IROs) at Promoters of Some Fe-Regulated Genes (Yeoman et al., 2015). However, elevated salt concentrations adversely affect *Rhizobium* survival, nodulation efficiency, and nitrogenase activity, ultimately reducing host plant performance.

Salt stress influences multiple stages of the legume–*Rhizobium* symbiosis, including rhizobial colonization, infection thread formation, and nodule development (Bhise et al., 2017). High salinity can disrupt signal exchange between host roots and rhizobia, reduce root hair curling, and inhibit bacterial motility and exopolysaccharide production—key factors required for successful symbiosis (Badenoch-jones et al., 1982).

Additionally, ionic imbalance, particularly due to excess Na^+ and Cl^- ions, can impair membrane integrity and enzyme function in both partners. Despite these challenges, certain salt-tolerant *Rhizobium* strains have evolved adaptive mechanisms such as osmolyte accumulation, ion homeostasis, and stress-responsive gene regulation, enabling them to survive and function under saline conditions (Nadeem et al., 2006; Tavakkoli et al., 2010).

The exploitation of salt-tolerant *Rhizobium* strains represents a promising strategy to enhance legume productivity in saline soils. Such strains can improve nodulation, maintain nitrogen fixation efficiency, and promote plant growth through additional mechanisms, including phytohormone production and stress alleviation (Chakraborty et al., 2011; Chookietwattana & Maneewan, 2012). Therefore, isolating and characterizing salt-tolerant *Rhizobium* from diverse environments is critical for developing bioinoculants tailored to stress-prone agroecosystems. The present study focuses on evaluating the salt tolerance and functional potential of *Rhizobium* isolates, aiming to contribute to sustainable crop production under salinity stress.

2.Materials and Methods

2.1 Isolation of *Rhizobium* from fenugreek

Rhizobium was isolated according to the methodology described by Anjum et al. (2011). The fresh and plump root nodules were collected from different plants retrieved from different locations in Maharashtra, India. Roots of all these host plants were washed under tap water, and healthy nodules were selected. Nodules were detached from the roots, rinsed in tap water, and surface-sterilized using 70% ethanol for 30 seconds followed by 0.1% $HgCl_2$ for 2 mins. The nodules were then washed thrice with sterile distilled water, crushed in sterile saline, and streaked on sterile Congo Red Yeast Extract Mannitol Agar (yeast extract 1.0 g, mannitol 10 g, K2HPO4 0.5 g, MgSO4 0.2 g, NaCl 0.1 g, Congo red 0.025 g, Agar 20 g, D/W 1 L, pH 6.8) plates to obtain isolated colonies. After 2 days of incubation, isolated colonies were picked up and used for further studies. Colony characteristics of isolates were studied, and Gram staining was carried out to ensure culture purity of rhizobia.

2.2 Determination of salt tolerance

Sterile Yeast Extract Mannitol (YEM) broth (yeast extract 1.0 g, mannitol 10 g, K2HPO4 0.5 g, MgSO4 0.2 g, NaCl 0.1 g, D/W 1 L, pH 6.8) was used to check the effect of various salt concentrations on selected isolates, where 100 ml of YMB flasks were prepared for each isolate. In this method, the flasks with varying salt concentrations (N, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 M) were inoculated with the same number of cells of a 24-hour fresh culture of *Rhizobium*. The medium was supplemented with salt to achieve the specified concentrations prior to sterilization by autoclaving. Following inoculation, cultures were incubated in a rotary shaker (120 rpm) at 28 °C±2 for 48 hrs. Growth was subsequently monitored by measuring absorbance at 580 nm via UV spectrophotometry.

2.3 Screening for Indole Acetic Acid (IAA) production

Different IAA concentrations were prepared as an aqueous solution of IAA ranging from 10 to 100 micrograms/ml. To each 1 ml of the standard, 2 ml of Salkowski reagent (i.e., 2 % 0.5M FeCl3 in 35% perchloric acid) was added, and readings were taken after 25 minutes at 535nm. The standard graph was prepared by plotting the concentration of IAA in micrograms/ml Vs. Optical density at 535nm(Gordon & Weber, n.d.) (Gordon and Weber, 1950). The bacterial culture was inoculated in 100ml of YMB containing 0.1% tryptophan and salt (0.1-0.6M) and incubated on the shaker at room temperature. Fully grown bacterial cultures were centrifuged at 5000 rpm for 10 min. The supernatant (2ml) was mixed with 2 drops of ortho-phosphoric acid and 4ml of Salkowski reagent (98 ml of 35% perchloric acid and 2ml of 0.5 M FeCl3). The development of pink colour indicates IAA production. Optical density measured at 535nm was used to plot the standard graph of IAA.

2.4 Screening for ACC deaminase production

Sterile M9 media was used for the screening of ACC deaminase producing isolates. Two sets of M9 media were used, the first one acted as the control with Ammonium chloride as the nitrogen source, and the second without Ammonium chloride. The plates made from the second set of media were surface spread with filter-sterilized ACC solution to get a final concentration of 3.0 mM. (Penrose & Glick, 2003)In order to study all the isolates conveniently, the spot inoculation method was used. Colonies were transferred with the help of a toothpick to the control and the test plates. The plates were incubated at RT for 24-72 hours, and results were noted periodically

3. Results

3.1 Isolation of *Rhizobium* from fenugreek

Rhizobium was isolated from the root nodules of leguminous plants like Crotalaria, Horsegram, Vigna radiata, Sesbania, Fenugreek, Faba/Fava bean, Arachis, Phaseolus, Cicer, etc. Colonies obtained on Congo Red Yeast extract Mannitol Agar after incubation at 27°C for 2 days. Colony morphology showed round, medium-sized, colorless watery colonies (Figure 1.a). Isolates were observed to be slow growing, with the colonies becoming

visible within 48 hours of plating. The colony characteristics of the isolates obtained on CRYEMA were studied (Table 2), and each isolate was numbered according to the host plant, as shown in Table 1. A total of 24 *Rhizobium* isolates were used for further studies. General microscopic observation of the isolates showed them to be Gram negative rods (Figure 1.b).

Table 3.1. List of the isolates obtained with their respective leguminous host plant

ISOLATE NUMBER	HOST PLANT
1	*Macrotyloma uniflorum*
2	*Sesbania sesban*
3	*Trigonella foenum-graecum*
4	*Trigonella foenum-graecum*
5	*Trigonella foenum-graecum*
6	*Vicia Faba*
7	*Vigna radiata*
8	*Vicia Faba*
9	*Vigna radiata*
10	*Vicia Faba*
11	*Sesbania sesban*
12	*Phaseolus vulgaris*
13	*Crotalaria juncea*
14	*Trigonella foenum-graecum*
15	*Trigonella foenum-graecum*
16	*Trigonella foenum-graecum*
17	*Trigonella foenum-graecum*
18	*Arachis hypogaea*
19	*Cicer arietinum*
20	*Crotalaria juncea*
21	*Phaseolus vulgaris*
22	*Cicer arietinum*
23	*Arachis hypogaea*
24	*Arachis hypogaea*

Purified isolates were then maintained on CRYEMA and used for further study. These were examined for colony characteristics. All isolates obtained had the following colony characteristics.

Table 3.2. Colony morphology of isolates from different leguminous hosts

Colony Characteristics	Observed Characteristics
Size	Medium (1-2mm)
Shape	Circular
Color	Colorless watery
Margin	Entire
Elevation	Elevated
Consistency	Smooth
Opacity	Translucent
Grams nature	Gram negative short rods

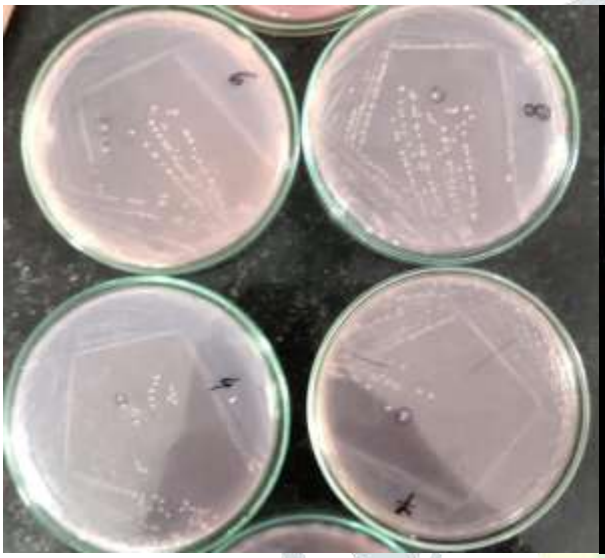

Fig. 3.1.a. Rhizobial Colonies on CRYEMA

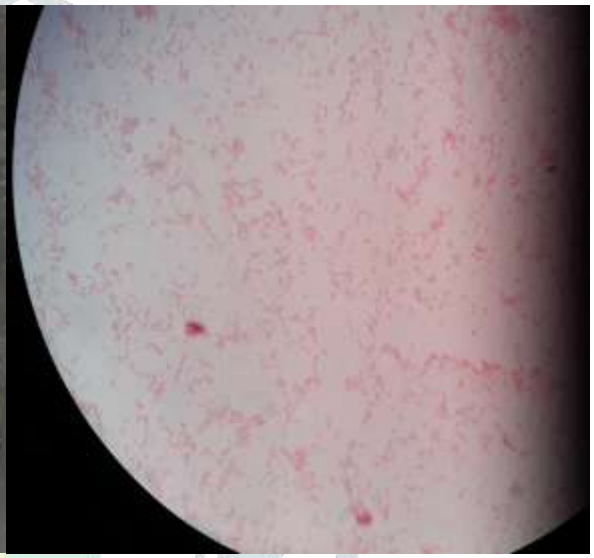

Fig. 3.1.b. Gram staining of *Rhizobium*

All the isolates were grown in YMB with varying salt concentrations (N, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 M) and incubated at RT under shaking conditions for 48 hrs. Absorbance was measured using UV spectrometer at 580 nm at an interval of 24 and 48 hrs to observe the growth. Different rhizobia show different levels of salt tolerance (Marinkovich et al., 2013).

Table 3.3. Salt tolerance of each *Rhizobium* isolate

ISOLATE NUMBER	HOST PLANT	SALT TOLERANCE (M)
1	*Macrotyloma uniflorum*	0.4
2	*Sesbania sesban*	1.2
3	*Trigonella foenum-graecum*	0.2
4	*Trigonella foenum-graecum*	1.0
5	*Trigonella foenum-graecum*	0.4
6	*Vicia Faba*	0.8
7	*Vigna radiata*	0.6
8	*Vicia Faba*	1.0

9	*Vigna radiata*	0.4
10	*Vicia Faba*	0.6
11	*Sesbania sesban*	0.6
12	*Phaseolus vulgaris*	0.2
13	*Crotalaria juncea*	1.0
14	*Trigonella foenum-graecum*	1.0
15	*Trigonella foenum-graecum*	1.2
16	*Trigonella foenum-graecum*	0.6
17	*Trigonella foenum-graecum*	1.0
18	*Arachis hypogaea*	0.2
19	*Cicer arietinum*	0.8
20	*Crotalaria juncea*	0.6
21	*Phaseolus vulgaris*	0.6
22	*Cicer arietinum*	0.4
23	*Arachis hypogaea*	0.2
24	*Arachis hypogaea*	0.4

The isolates exhibited a wide range of salinity tolerance, spanning from 0.2 M to 1.2 M. This diversity suggests significant physiological variation among the strains, even when isolated from the same host species. The most salt-tolerant strains were Isolate 2 (*Sesbania sesban*) and Isolate 15 (*Trigonella foenum-graecum*), both of which flourished at 1.2 M. Other robust performers reaching 1.0 M were found in *Crotalaria juncea* (Isolate 13), *Vicia faba* (Isolate 8), and multiple *Trigonella* strains (Isolates 4, 14, and 17). These isolates are primary candidates for biofertilizer development in saline soils.

3.2 Screening for Indole Acetic Acid (IAA) production

Since drought and salt stress responses in plants are often mediated by phytohormones, it is important to study IAA-producing, root colonizing plant growth-promoting bacteria in saline conditions, which could facilitate plant growth in such harsh environments (Egamberdieva, 2009). IAA production was studied in the obtained isolates and the concentration of IAA produced was estimated using the standard IAA estimation protocol. Varying concentrations of IAA were observed in isolates from different legume hosts.

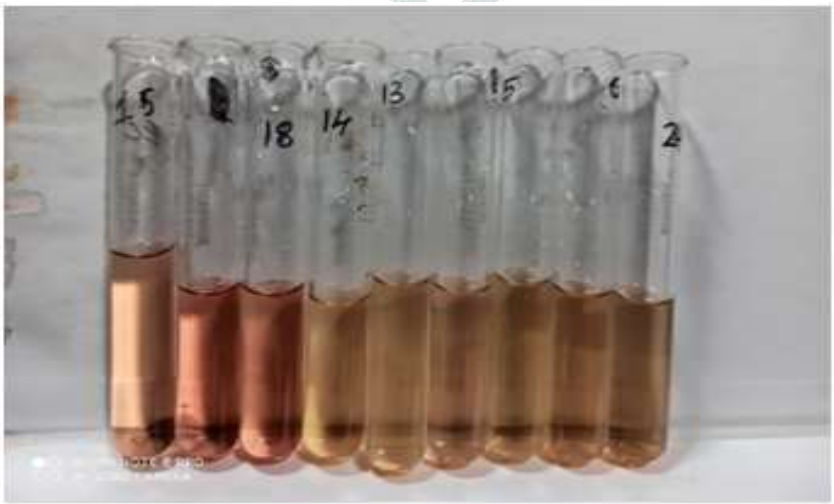

Fig. 3.2. IAA production by *Rhizobium*

Table 3.4. *Rhizobium* isolates showing IAA production

ISOLATE NUMBER	HOST PLANT	IAA Production (µg/ml)
1	*Macrotyloma uniflorum*	62
2	*Sesbania sesban*	12
3	*Trigonella foenum-graecum*	6
4	*Trigonella foenum-graecum*	16
5	*Trigonella foenum-graecum*	14
6	*Vicia Faba*	21
7	*Vigna radiata*	18
8	*Vicia Faba*	7
9	*Vigna radiata*	21
10	*Vicia Faba*	16
11	*Sesbania sesban*	9
12	*Phaseolus vulgaris*	24
13	*Crotalaria juncea*	12
14	*Trigonella foenum-graecum*	6
15	*Trigonella foenum-graecum*	39
16	*Trigonella foenum-graecum*	23
17	*Trigonella foenum-graecum*	19
18	*Arachis hypogaea*	49
19	*Cicer arietinum*	8
20	*Crotalaria juncea*	16
21	*Phaseolus vulgaris*	25
22	*Cicer arietinum*	11
23	*Arachis hypogaea*	32
24	*Arachis hypogaea*	27

3.3 Screening for ACC deaminase production

Twenty-four rhizobial isolates were spot inoculated on Sterile M9 plate containing ACC as a sole source of nitrogen (test) and on another sterile M9 plate without ACC but containing NH4Cl as a nitrogen source (Control). Both the plates were incubated at 28°C ± 2°C. The plates were scored as + or - (plus or minus) for presence or absence of growth. Out of this, isolates no. 2, 6, 9, 23, 24 were showing growth on ACC +ve plate after a period of 24 h, which suggests that they might be fast utilizers of ACC. Whereas isolates no. 3, 10, 11, 18, 20 showed no growth in any of the plates, which indicates these isolates were unable to grow under sterile M9 media. This should be further investigated to better understand the results observed.

Fig 3.3. M9 plates with and without ACC (left and right, respectively) showing grown colonies after 48 hours of incubation.

Size of the colony observed increased with an increase in incubation time, indicating that the isolates utilized more ACC and grew as incubation proceeded. Hence, it can be said that the isolates can utilize ACC as a carbon source, but they need to use more time and energy than Ammonium chloride in M9 medium.

Table 3.5. *Rhizobium* isolates showing ACC production

	After 24 hours		After 48 hours		After 72 hours	
Isolate No.	ACC +ve	ACC -ve	ACC +ve	ACC -ve	ACC +ve	ACC -ve
1	-	+	-	++	-	+++
2	+	+	+	++	++	+++
3	-	-	-	-	-	-
4	-	+	-	++	++	+++
5	-	+	++	++	+++	+++
6	+	+	++	++	+++	+++
7	-	+	++	++	+++	+++
8	-	+	++	++	+++	+++
9	+	+	++	++	+++	+++
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	+	++	++	+++	+++
13	-	+	++	++	+++	+++
14	-	-	-	++	+++	+++
15	-	-	-	-	+++	+++
16	-	-	-	-	+++	+++
17	-	-	++	+	+++	+++
18	-	-	-	-	-	-
19	-	+	++	++	+++	+++
20	-	-	-	-	-	-
21	-	-	-	-	-	++
22	-	-	-	++	-	++
23	+	+	++	++	+++	+++
24	+	+	++	++	+++	+++

Key: + = minute growth; ++ = growth; +++ = heavy growth; - = no growth

4. Discussion

The isolation and characterization of 24 *Rhizobium* strains from diverse leguminous hosts—including *Trigonella foenum-graecum*, *Sesbania sesban*, and *Vicia faba*—demonstrate significant functional diversity, particularly regarding salt tolerance and plant growth-promoting (PGP) traits. The isolates exhibited classic *Rhizobium* morphology on CRYEMA: medium-sized, circular, elevated, and colorless watery colonies. Their inability to absorb Congo Red and their Gram-negative rod status confirm their identity as members of the Rhizobiaceae family. The relatively fast growth (visible within 48 hours) suggests these may belong to the fast-growing genera such as *Sinorhizobium* or *Rhizobium* sensu stricto, which are common in temperate and subtropical legumes. Further analysis can be done to characterize and identify these isolates. A critical finding is the high degree of salt tolerance observed in several isolates. While typical soil bacteria struggle beyond 0.2 M NaCl, isolates from *Sesbania sesban* (Isolate 2) and *Trigonella foenum-graecum* (Isolate 15) flourished at 1.2 M. This extreme tolerance is likely an evolutionary adaptation to saline environments. Interestingly, *Trigonella* isolates showed the widest range of tolerance (0.2 M to 1.2 M), suggesting that the host plant can associate with a broad spectrum of strains depending on the soil's osmotic pressure (Marinkovich et al., 2013).

Phytohormone production is vital for mitigating abiotic stress. By producing Indole Acetic Acid (IAA), these bacteria stimulate root proliferation, increasing the surface area available for nutrient and water uptake under salt stress (Egamberdieva, 2009). The IAA concentrations ranged from a minimum of 6 µg/ml (Isolates 3 and 14 from *Trigonella foenum-graecum*) to a maximum of 62 µg/ml (Isolate 1 from *Macrotyloma uniflorum*). Isolate 1 stood out as the most potent producer, followed by Isolate 18 (49 µg/ml) and Isolate 15 (39 µg/ml). High IAA production is particularly critical under salt stress, as exogenous auxin can help maintain root development and counteract the inhibitory effects of salinity. The variation observed across isolates from the same host (e.g., *Trigonella foenum-graecum*, ranging from 6 to 39 µg/ml) suggests that IAA production is strain-specific rather than solely dependent on the host plant species. These high-performing isolates (1, 18, and 15) serve as promising candidates for developing bio-fertilizers aimed at improving crop resilience in saline environments.

A standout result was the ACC deaminase activity in isolates 2, 6, 9, 23, and 24. These "fast utilizers" can degrade 1-aminocyclopropane-1-carboxylate (the ethylene precursor). By lowering stress-induced ethylene levels in the host plant, these strains prevent premature senescence and root inhibition, which are common symptoms of salinity and drought.

5. Conclusion

This study successfully identified highly resilient *Rhizobium* isolates with potent bio-remediating capabilities. Isolates such as No. 2 (*Sesbania sesban*) and No. 15 (*Trigonella foenum-graecum*) are particularly noteworthy for combining extreme salt tolerance (1.2 M) with plant growth promoting traits like ACC deaminase activity. These strains represent promising candidates for the development of multi-functional biofertilizers aimed at reclaiming saline agricultural lands and improving the yield of legumes in arid and semi-arid regions.

References

Anjum, S. A., Xie, X. Y., Wang, L. C., Saleem, M. F., Man, C., & Lei, W. (2011). *Morphological, physiological, and biochemical responses of plants to drought stress. African Journal of Agricultural Research*, *6*(9), 2026–2032.

Badenoch-Jones, J., Summons, R. E., Djordjevic, M. A., Shine, J., Letham, D. S., & Rolfe, B. G. (1982). *Mass Spectrometric Quantification of Indole-3-Acetic Acid in Rhizobium Culture Supernatants : Relation to Root Hair Curling and Nodule Initiation Mass Spectrometric Quantification of Indole-3-Acetic Acid in Rhizobium Culture*

Bhise, K. K., Bhagwat, P. K., & Dandge, P. B. (2017). Synergistic effect of Chryseobacterium gleum sp. SUK with ACC deaminase activity in alleviation of salt stress and plant growth promotion in Triticum aestivum L. *3 Biotech*, *7*(2), 1–13. https://doi.org/10.1007/s13205-017-0739-0

Chakraborty, U., Roy, S., Chakraborty, A. P., Dey, P., & Chakraborty, B. (2011). *Plant Growth Promotion and Amelioration of Salinity Stress in Crop Plants by a Salt-Tolerant Bacterium*. *3*(11), 61–70.

Chookietwattana, K., & Maneewan, K. (2012). *Selection of efficient salt-tolerant bacteria containing ACC deaminase for promotion of tomato growth under salinity stress bacterium Effect of the selected salt-tolerant bacterium Screening bacterium*. *31*(1), 30–36.

Egamberdieva, D. (2009). Alleviation of salt stress by plant growth-promoting bacteria in crop plants. *Journal of Plant Growth Regulation*, 28(4), 318-324.

Gordon, S.A. and Weber, R.P. (1951) Colorimetric Estimation of Indoleacetic Acid. Plant Physiology, 26, 192-195

Luca, N. G. De, Wexler, M., Pereira, M. J., Yeoman, K. H., & Johnston, A. W. B. (1998). *Is the fur gene of Rhizobium leguminosarum essential ? 168*, 289–295.

Marinkovich, S., et al. (2013**).** Salt tolerance of *Rhizobium* strains isolated from different ecological zones. *Applied and Environmental Microbiology*.

Nadeem, S. M., Zahir, Z. A., Naveed, M., Arshad, M., & Shahzad, S. M. (2006). *Variation in growth and ion uptake of maize due to inoculation with plant growth promoting rhizobacteria under salt stress*. *25*(2), 78–84.

Nascimento, F. X., Brígido, C., Glick, B. R., & Rossi, M. J. (2016). The Role of Rhizobial ACC Deaminase in the Nodulation Process of Leguminous Plants. *International Journal of Agronomy*, *2016*. https://doi.org/10.1155/2016/1369472

Penrose, D. M., & Glick, B. R. (2003). *Technical focus Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria*. 10–15.

Singleton, P. W., Swaify, S. A. E. L., & Bohlool, B. B. (1982). *Effect of Salinity Rhizobium Growth and Survivalt*. *44*(4), 884–890.

Tavakkoli, E., Rengasamy, P., & Mcdonald, G. K. (2010). *High concentrations of Na + and Cl – ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress*. *61*(15), 4449–4459. https://doi.org/10.1093/jxb/erq251

Yeoman, K. H., Wisniewski-dye, F., Timony, C., Stevens, J. B., Nicola, G., Downie, J. A., & Johnston, A. W. B. (2015). *Analysis of the Rhizobium leguminosarum siderophore-uptake gene fhuA : differential expression in free-living bacteria and nitrogen-fixing bacteroids and distribution of an fhuA pseudogene in different strains*. (2000), 829–837.