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Response of *Azadirachta indica* (Neem) seedlings to biofertilizer compared to mineral nutrition

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Abstract

Growth of ***Azadirachta indica*** **(Neem)** transplants was stimulated by inoculation with Plant Growth Promoting Rhizobacteria **(PGPR).** Results showed that significant increases in the vegetative growth, biomass and chemical constituents per seedling were observed in the seedlings inoculated with mixture of ***Azospirillum brasilense, Azotobacter chrococcum and Bacillus polymixa*** **(PGPR),** and received half dose from mineral nutrients (NPK). This treatment gave higher parameters**,** the percentages of increases as a result of using PGPR with half dose from mineral nutrition as compared to full dose, were 25, 22, 44, 21and 40% for plant height, stem diameter, root length, fresh and dry weight per seedling over the seedling which received full doses of mineral nutrition. It can be also concluded that the previous treatment (PGPR and half mineral fertilizer) exceeded the growth parameters by 75, 43, 47, 86 and 101% respectively over control. Also, the above treatment gave the highest values of NPK up take, total carbohydrate and chlorophyll content. These results suggest that the growth promoting substances provided by PGPR may enhance the growth of *Azadirachta indica* seedlings and consequently produce healthy seedling in short time**.**

Key words**:** *Azadirachta indica*, PGPR*, Azospirillum brasilense, Azotobacter chrococcum and Bacillus polymixa*.

Introduction

Azadirachta indica commonly known as **Neem** belongs to the family of **Meliaceae** and has been used in medicinal treatments for more than 4000 years ago **(Pankaj *et al*, 2o11). Neem** tree with deep roots are medium - sized tree (up to a height of 30 meters and 2.5 meters in circumference), leaves broad evergreen vehicle, bark gray color, neem tree has a wide range of beneficial uses medically and chemically, neem tree has hard wood forces used in the wood industries, as well as fuel, in addition to therefore, tree grow well in most environmental conditions and any soil., calcareous soil with PH, up 8.5 **(Debashri and Tamal, 2012).** Fertilization is one of the major factors that affect on the growth and productivity of plants. Over the past few years, the use of chemical fertilizers or pest control in agriculture has been increasingly criticized, that the chemicals may adversely affect the formation of chemicals for agricultural products that are consumed by humans or animals, in addition to having the effect of unfavorable environment. Many studies have confirmed the use of biofertilizers as an alternative to conventional NPK fertilization in a number of plants. **(El-Maadawy and Moursy, 2007).**

Gutierrez Manero *et al.*, (1996) reported that influence of native rhizobacteria on the growth of **European alder *(Alnus glutinos*a Gaertn**). **PGPR** (Plant Growth promoting Rhizobacteria) has become a new class of biofertilizers and physiological stimulators in recent years**. PGPR** have been a renewed interest for inoculation of agricultural crops, **PGPR** hold great promise as potential agricultural and forestry inoculants and could reduce the use of fertilizers and pesticides **(Zahir *et al*., 2004).** Inoculation with Plant Growth Promoting Rhizobacteria **(PGPR**) comparatively enhance the growth of ***Taxodium distichum*** transplants and shortening the time of seedling growth **(Hammad *et al*., 2011)**. Many strains have been catalogued as **(PGPR**)due to their effect on plant growth pathogens **(Bashan and de Bashan, 2002 and Al-Kahal *et al*., 2003)** or to their ability to induce plant growth promoting effect **(Bashan, 1999 and Mekhamar, 2001)**. Most of these strains belong to ***Bacillus, Pesudmnas, Azotobacter, and Azospirillum*** **(Reddy and Rahe 1989).** Phytohormones such as indol -3- acetic acid (**IAA)** or cytokinins are among the plant growth promoting compounds often produced by bacteria **(Hubble *et al*., 1979 and Muller *et al*., 1989).** However, other compounds, known as auxin-like **IAA-1** are often responsible for the promoting effects **(Oberhansli *et al*., 1990 and Selvadurai *et al*., 1991).**

The present study aimed to investigate response of ***Azadirachta indicia*** (**neem)** seedlings to bio-fertilizer compared to mineral nutrition under calcareous soil as a new reclaimed soil condition.

Materials and Methods

This study was carried out at the Experimental Nursery of Horticultural Research Station at El-Kanater El-Khayria during two successive seasons of 2012/ 2013 and 2013/2014, to study the response of neem seedlings to biofertilizer and mineral nutrition grown in new reclaimed soil transplanted from 10th of Ramadan Desert in El-Sharkia Governrate.

Physical and chemical properties of soil

Physical and chemical analysis of used soil was done according to the Pipette method as described by **(Piper, 1947)** as shown in Tables (A) and (B).

Table A. The mechanical analysis of the calcareous sand used for growing *Azadirachta indica* seedlings

Soil type	Coarse sand %	Fine sand %	Silt %	Clay %	Texture class
Calcareous sand	39.40	57.33	2.17	1.10	Calcareous sand

Table B. The chemical characteristics of the calcareous sand used for growing *Azadirachta indica* seedlings.

Soil type	pH	E.C. (ds/m)	$CaCO_3$ %	Cations (meq/l)				Anions (meq/l)		
				Ca^{++}	Mg^{++}	Na^{+}	K^{+}	HCO_3	Cl^{-}	SO_4^{--}
Calcareous sand	8.51	2.44	15.17	1.2	0.7	3.2	0.3	1.1	2.34	1.96

Micro-organisms

Organisms culture strain (***Azospirillum brasilense, Azotobacter chrococcum and Bacillus polymixa***) were supplied by the unit of bio fertilizers, Faculty of Agriculture Ain Shams, University, Cairo Egypt. Biofertilizer strains were grown on modified Ashby's agar medium (**Abdel-Malek and Ishac, 1968**) and semi-solid malate medium **(Dobereiner, 1978**), respectively after that incubated at 30°C for 7 days until the bacteria numbers reached about $1x10^7$ cfu ml^{-1} over wise.

Chemical and bio-fertilization treatments

Seeds of ***Azadirachta indica*** were divided into two groups, the first one did not receive any microorganism but another one was inoculated with the culture of microorganisms PGPR (*Azotobacter chroococcum, Azospirillum brasilense and Bacillus polyixa*),on the first of June 2012 and 2013,seeds of both two groups were planted in plastic cups 5 cm diameter filled with sterilized media, at the nursery of Horticultural Research Station at El–Kanater El-Khayria.On the first of August 2012 seedlings were transplanted in plastic cups 10 cm diameter .The treated group were inoculated again by the same bacterial culture. On the first of October 2012 and 2013, homogenous seedlings were transplanted into plastic bags 25 cm diameter and 35 cm depth filled with 6 kg of soil, and the inoculation repeated another one by the same bacterial culture. The seedlings were placed in a shaded area and after two weeks from transplanting, seedlings were removed outdoors to a sunny area and irrigated (twice weekly in winter and daily in summer). Every seedling received nutrition NPK 0.5 gm. from Kristalon 19: 19: 19 as starter for bacterial activity. The seedlings received different mineral and biofertilization treatments, as follows

1- Control: Seedlings did not received any nutrition

2- Mineral fertilization: Seedlings receive Nitrogen, Phosphorus and Potassium, to all seedlings. Phosphorus applications was added monthly as calcium phosphate (15 % P_2O_5), at rate of o.5 gm./ seedling, while Potassium fertilizer was added as Potassium sulphate (48% K_2O) at rate 0.25 gm./seedling. Nitrogen fertilization was added as ammonium sulphate (20.5% N) at rate of 1 gm/ seedling.

3- Bio–fertilization: Seedlings were inoculated once with the culture of microorganisms PGPR (*Azotobacter chroococcum, Azospirillum brasilense and Bacillus polyixa*), at previous stages.

4- Bio – fertilization with complete dose of mineral fertilization.

5- Bio – fertilization with half dose of mineral fertilization.

Data concerned:

In both seasons 2012/2013 and 2013/2014, the vegetative growth parameters were recorded including:

- Plant height from soil surface (cm).
- Stem diameter above 5cm from soil surface (cm).
- Root length, (cm).
- Biomass fresh and dry weights (gm/plant).

Chemical composition

The chemical constituents in oven dried shoot samples were determined according to **A.O.A.C., 1980).**

Microbial analysis

Densities of *Azospirillum* and *Azotobacter* microbial were determined on modified Ashby's medium (**Abdel-Malak and Ishac, 1968**) and semi-solid malate **(Dobereiner, 1978),** respectively using the most probable number technique.

Nitrogenase activity in the rhizosphere of the tested plants were determined according to **(Hardy *et al.*, 1973).**

Carbon dioxide evaluated by soil micro-organisms was assayed using the method described by (**Page *et al*, 1982).**

Layout of the experiment

The lay out of the experiment was complete randomized block design, with 5 treatments; and three replicates each replicate included 25 seedlings.

The obtained data were subjected to analysis of variance ANOVA according to **(Snedecor and Cochran, 1980).** The means were compared by Duncan's multiple range test described by (**Duncan,**

1955) at 5% properly between means of various treatments

Results and Discussion

Vegetative growth.

Data presented in Tables (1 & 2) indicated that biofertilization by inoculation with (PGPR) as well as half dose of mineral nutrition enhanced vegetative growth much more than the other treatments. This fact was pronounced in both seasons in which significantly the highest values of plant height, stem diameter, root length, biomass fresh and dry weight were 76.85 cm; 1.13 cm, 27.13 cm, 41.71 g and 12.63 g in the first season. Whereas, the values were 71.18 cm, 1.66 cm, 31.00 cm, 44.53 g and 10.80 g in the second season, respectively. It means that the previous treatment resulted in increases as percentages of 75, 43, 47, 86 and 101 % in plant height, stem diameter, root length, biomass fresh and dry weight over control, respectively. These results indicate that the PGPR exhibited positive effects on the growth parameters of the inoculated host plant as previously reported by (Zahir et al., 2004) and (Mekhamar et al., 2007).

Table 1. Effect of chemical and bio-fertilization treatments on plant height, stem diameter and root length (cm) of *Azadirachta indica* seedlings during 2012/2013 and 2013/2014 seasons.

Treatments	First season			Second season		
	Plant height (cm)	Stem diameter (cm)	Root length (cm)	Plant height (cm)	Stem diameter (cm)	Root length (cm)
Control	43.80E	0.79D	18.40C	50.01D	0.97D	19.53D
Mineral	61.40C	0.93C	18.88C	63.18B	1.19C	21.01D
Bio-fertilizer	49.40D	0.81D	20.07C	56.40C	1.00D	23.44C
Mine. + Bio	68.11B	1.05B	22.18B	69.61A	1.31B	26.12B
1/2Mine. + Bio	76.85A	1.13A	27.13A	71.18A	1.66A	31.00A

Means followed by the same letter (s) within the same column are not significantly different

Table 2. Effect of chemical and bio-fertilization treatments on biomass fresh and dry weight (g) of *Azadirachta indica* seedlings during 2012/2013 and 2013/2014 seasons.

Treatments	First season		Second season	
	Biomass fresh weight (g)	Biomass dry weight (g)	Biomass fresh weight (g)	Biomass dry weight (g)
Control	22.42D	6.26D	23.35D	6.58D
Mineral	34.35B	9.00B	37.43B	9.50B
Bio-fertilizer	29.26C	8.36C	32.73C	7.93C
Mine. + Bio	35.35B	9.10B	39.00B	10.00B
1/2Mine. + Bio	41.71A	12.63A	44.53A	10.80A

Means followed by the same letter (s) within the same column are not significantly different

Many results are inconclusive, but encouraging enough to improve selection procedures and the production of quality inoculate for practical application. As PGPR mediated processes involved in nutrient cycling include those related to non-symbiotic nitrogen-fixation, and those responsible for increasing the availability of phosphate and other nutrients in the soil. Many symbiotic diazotrophic bacteria have been described and tested as bio fertilizers **(Kennedy *et al*., 2004)**. The selection of effective PGPR diazotrophs is critical for further development of this technology. ***Azospirillum* species** are also considered to be **PGPR (Lucy *et al*., 2004**) and **(Zahir *et al*., 2004).** A significant activity of these bacteria is the production of auxin-type phytohormones that affect root morphology and, thereby, improve nutrient uptake from the soil. This may be more important than their N2-fixing activity. ***Azospirillum species*** are being used as seed inoculants under field conditions **(Dobbelaere *et al*., 2001, Lucy *et al*., 2004, Zahir *et al*., 2004).** Despite many studies reporting that, the benefits of ***Azospirillum*** inoculation, some studies present inconsistent results. However, it can be assumed that, upon establishing appropriate management practices, the use of these inoculants will have a beneficial effect on plant fertilizer. It has recently been postulated that an additional mechanism for plant growth promotion by PGPR could be their altering of microbial rhizosphere communities **(Ramos *et al*., 2003).** Agreeing with such as indirect mechanism, it would be interesting to evaluate the actual impact of this activity in rhizosphere biology. Rhizobacteria that exert beneficial effects on plant growth and development are referred to as plant growth promoting rhizobacteria **PGPR**. In recent years, the use of **PGPR** to promote plant growth has increased in various parts of the world.

Chemical analysis

Data presented in Table (3) indicate that significant increases in N% were observed in the seedlings inoculated with PGPR with half doses from NPK which showed higher N content being 44% and

45% than those recorded for transplants grown in the control media which un-inoculated with PGPR, in two experiment of seasons, respectively. It indicates that ***Azadirachta indica*** seedlings responded positively to inoculation with PGPR, the N content varied from 1.33% in the transplants grown in an un-inoculated in control media and did not receive mineral fertilizer to 1.56% in the transplants grown in mineral fertilized media, with full doses from NPK, and without inoculation to 1.46% in the transplants grown in the inoculated media only, to 1. 91% in the transplants grown in the inoculated with PGPR and half doses from mineral nutrition, in the first season, the second season gave the same trends approximately. Data in **Table (3)** showed that no significant differences between N% content in the transplants grown in mineral nutrition medium and those grown in bio-fertilizer media in both seasons.

Table 3. Effect of chemical and bio-fertilization treatments on N, P and K (%) of *Azadirachta indica* seedlings during 2012/2013 and 2013/2014 seasons.

Treatments	First season			Second season		
	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)
Control	1.33B	0.156C	1.00C	1.41C	0.173C	0.98B
Mineral	1.56B	0.186B	1.30BC	1.66BC	0.194B	1.51A
Bio-fertilizer	1.46B	0.177B	1.09C	1.55C	0.188BC	1.13B
Mine. + Bio	1.86A	0.281A	1.58AB	1.93AB	0.291A	1.63A
1/2Mine. + Bio	1.91A	0.283A	1.64A	2.05A	0.296A	1.71A

Means followed by the same letter (s) within the same column are not significantly different

Additionally, it is well demonstrated that in both seasons chemical composition of dried transplants which were grown in different fertilizers types which reported in **Table (3)** that the P content was considerably increased referee to addition of ***Bacillus polymixa*** to the bio-fertilizes media. The P content varied from 0.156% in the transplants grown in an un-inoculated control media to 0.177% in transplants grown in media inoculated PGPR strains. This means that the addition of PGPR strains to the media increased NPK contents in the transplants as shown in **Table (3)** compared to control. Solubilization of mineral nutrients such as phosphorus and potassium by PGPR made them more readily available for plant uptake, and this should be considered as a mechanisms for enhanced plant growth **(Glick, 1996**). It worthily observed from data of **Table(3)** that, applying full or half dose of mineral fertilizers as well as bio fertilization association on increment in N,P,K content in plants means that scarce minerals applying specially half dose resulted in more activities for microorganisms around roots rhizosphere zone consequently increased absorbed nutrition minerals. Several authers suggested that PGPR can stimulate plant growth by increasing solubilization via releasing siderophores or organic acids and facilitate the uptake of mineral nutrition such as **(Chabot *et al*., 1996, Biswas *et al*., 2000 a and b, Hammad *et al*., 2011).**

These results are in agreement with the finding of several authors **(Abo El-Soud *et al*., 2007)** and **(Mekhamar *et al*., 2007)** who explained that improving effects arising from microbial inoculation are due to producing growth promoter substances such as auxins, gibberellins and cytokinins.

Table (4) indicates that the inoculation with (PGPR) treatments with half dose of mineral nutrition significantly exceeded leaf total chlorophyll concentration to maximum values in the two seasons. This result is beneficial in photosynthesis which ameliorate growth characters better than of control. Similar results, were obtained by **(Sorial and Abd El-Fattah 1998**) and (**El Khyat and Zaghloul 1999)**.

The results in Table (4) also indicate that, the inoculation with **PGPR** treatments with either half or full mineral nutrition gave significantly the highest values of total Carbohydrate of *Azadirachta indica* seedlings which reached the maximum values in both seasons.

Table 4. Effect of chemical and bio-fertilization treatments on total chlorophyll (mg/g. f.w) and total carbohydrate % of *Azadirachta indica* seedlings during 2012/2013 and 2013/2014 seasons

Treatments	First season		Second season	
	Total chlorophyll (mg/g. f.w.)	Total carbohydrate (%)	Total chlorophyll (mg/g. f.w.)	Total carbohydrate (%)
Control	0.836D	4.31C	0.893E	4.82C
Mineral	0.935C	6.25AB	1.044C	6.81A
Bio-fertilizer	0.860D	5.53B	0.968D	5.91B
Mine. + Bio	1.100B	7.03A	1.151B	7.50A
1/2Mine. + Bio	1.192A	7.14A	1.222A	7.53A

Means followed by the same letter (s) within the same column are not significantly different

The increasing in total Carbohydrate may be attributed to the positive effects of bio-fertilization on photosynthesis and consequently the plant metabolism. The same results were observed by (**Haggag and Azzazy 1996**) and **(Sorial and Abd El-Fattah 1998).**

As a general conclusion the results of this research indicate that, inoculation of ***Azadirachta indica*** seedlings by PGPR had a beneficial effect on the growth, chemical composition and strength of the seedlings.

Microbial Analysis.

Effect of (PGPR) inoculation and mineral fertilization on CO_2 evolution and Nitrogenase activity

The results presented in **Table** (5) show that, the rate of CO_2- evaluation in rhizosphere of *Azadirachta indica* seedling as an indication for microbial activity was remarkably increased by inoculation with (PGPR) and receiving half dose of mineral nutrition compared to any other treatments. This result is in harmony with **(Neweigy *et al*., 1997**) and **(Ashoub and Abd El-Ghany 1994**), they found that bio-fertilization treatments recorded the highest values of CO_2-evaluation.

Table 5. Effect of chemical and bio-fertilization treatments on CO2 evolution d µg/g dry soil/hr and Nitrogenase activity of *Azadirachta indica* seedlings during 2012/2013 and 2013/2014 seasons**.**

Treatments	First season		Second season	
	CO_2 evoluted g/g dry soil/hr	Nitrogenase activity	CO_2 evoluted g/g dry soil/hr	Nitrogenase activity
Control	46.90E	11.77E	47.40E	11.80E
Mineral	62.55D	21.14D	73.16D	20.44D
Bio-fertilizer	110.12C	55.17C	115.80C	56.93C
Mine. + Bio	122.40B	59.10B	129.80B	61.44B
1/2Mine. + Bio	131.50A	68.80A	139.40A	71.10A

Means followed by the same letter (s) within the same column are not significantly different

Results in **Table** (5) also show that the N_2-ase activity in rhizosphere of *Azadirachta indica* seedlings increased on all bio-fertilization treatments as compared to uninoculated treatments. Inoculation with (PGPR) with half dose of mineral fertilizers gave the highest records of N_2-ase activity. These results matched well with those of (**Pederson *et al*., 1978**) and **(El-Sawy *et al*., 1998),** they found that, heavy doses of mineral fertilizers inhibited N2-ase activity of N_2-fixers. Also, these results agree with **(Ishac *et al*., 1986) and (Saleh *et al*., 1998**), they found that low doses of N-fertilizer exhibited a positive effect on biological N_2-fixation.

As a general conclusion the results of this research indicated that, inoculation of ***Azadirachta indica*** seedlings by PGPR had beneficial effects on the growth, chemical composition and strength of the seedlings.

It can be recommended from the previous results that, the combination of bio-fertilizer and half dose of mineral fertilizer can be used as substitutes for full dose of environment and reduce the high cost of chemical fertilizers.

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استجابة شتلات اشجار النيم للتسميد الحيوى مقارنة بالتسميد المعدنى

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اجرى هذا البحث على شتلات اشجار النيم خلال موسمين زراعيين متتاليين2013/2012 & 2013 / 2014 بهدف دراسة تأثير خليط من بعض سلالات البكتيريا المشجعة للنمو وهى :

Azotobacter chroococcum, Azospirillum brasilense and Bacillus polymixa

اوضحت النتائج المتحصل عليها ما يلى:

1- حدوث زيادة معنوية فى قيم نمو الشتلات وقيم التحليل الكيميائى عند استخدام اللقاحات البكتيرية مقارنة بالكنترول.
2- ادى استخدام اللقاحات البكتيرية مع نصف جرعات السماد المعدني الى الحصول على أعلى قيم النمو والمكونات الكيميائية داخل الانسجة النباتية وكانت الزيادة 25 و22 و44 و21 و40% بالنسبة لارتفاع النبات ومحيط الساق وطول الجذر والوزن الطازج والجاف على الترتيب مقارنة بتلك التي أضيف إليها جرعات كاملة من السماد المعدني، وكانت نسبة الزيادة في النمو للشتلات التي عوملت باللقاحات البكتيرية مع نصف جرعة التسميد المعدني هي 75 ، 43 ، 47 ،86 ، 101% على التوالي مقارنة مع الكنترول .
3- وبناء على ما تم التوصل اليه من نتائج فانه يوصى بتلقيح بذور وشتلات اشجار النيم باللقاحات البكتيرية المشجعة للنمو للحصول على شتلات قوية بالمشتل فى فترة زمنية قصيرة بالاضافة الى توفير الاسمدة المعدنية للمحاصيل الاخرى وهذا سوف يكون له مردود اقتصادى كبير على عملية الانتاج.