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Effects of Bio- and Chemical Nitrogenous Fertilizers on Yield of Anise *Pimpinella anisum* and Biological Activities of Soil Irrigated with Agricultural Drainage Water

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TWO field experiments were conducted (during winter seasons of 2002 and 2003) to investigate whether chemical nitrogen fertilizer sources and/or N_2 fixing bacteria (*Azotobacter* and *Azospirillum*) using agricultural drainage water could affect anise plants quantity and quality. Besides, the effect of different nitrogen sources on biological activity in the rhizosphere of this medicinal plant.

The results proved significant difference among the treatments with regard to the nitrogen forms and activities of rhizosphere bacteria. Inoculation with N_2 fixing bacteria with half doses of chemical nitrogen fertilizers affected the quantity and quality of anise plants yield. The mean values of combination between chemical fertilizers and N_2 fixating bacteria were more or less equal and in sometimes higher than those obtained when using the recommended mineral N dose with any form of nitrogen. On the other hand, microbiological analysis indicated that the plants with high N content harbored higher populations of nitrogen-fixing bacteria and highest enzymatic activities of soil. Under different form of N fertilizer, the rhizosphere of anise plants, which differed in biological activities and their bacterial densities, harbored almost identical population densities of nitrogen-fixing bacteria. Interestingly, the total bacterial counts were greatly stimulated following the application of bio- and half dose of ammonium nitrate as nitrogen fertilizer.

Keywords: Anise plants (*Pimpinella anisum*), *Azotobacter* and *Azospirillum*, Dehydrogenase and urease activities, Rhizosphere.

Anise (*Pimpinella anisum*, L) is an umbelliferous aromatic plant contains essential oil used as a flavouring ingredient in licorice candy-expectorant and sedative and analgesic (Twaij *et al.*, 1987). Besides, the perfumery industries as an order of cod liver oil and other rancid order (El-Mesiry & Mazher, 2000). The productivity of anise is depending on both quantity and quality of the product. Cutting (1961) mentioned that irrigation; nitrogen and phosphorus fertilization might increase the yield and oil content of anise but reduced the oil quality. Salinity of irrigation water adversely affected mineral nutrients uptake and utilization, which intern affect negatively the physiological processes and reduce plant growth.

Among the mineral nutrients, nitrogen is one of the most widely limiting elements for crop production and its uptake could be more severely affected than other nutrients under stress conditions. To overcome acute N deficiency in anise soils, this element is usually supplied to the anise plants as the commercially available fertilizer urea. But unfortunately a substantial amount of the urea-N is lost through different mechanisms causing environmental pollution.

The use of microbial products has some advantages over conventional chemicals, since they considered safe than many chemicals now in use. They didn't accumulate in the food chain and target organisms seldom develop resistance as is the case when chemical agents are used. However, microorganisms have been applied to soil to increase nitrogen fixation because of their ability to transform atmospheric N_2 into ammonia that can be used by the plant, researchers were originally very optimistic about the potential of associative diazotrophic bacteria to promote the growth of many cereals and grasses. Application of biological N fixation (BNF) technology can decrease the use of urea-N, reducing the environmental problems to a considerable extent. Different BNF systems have different potentials to provide the required N, and it is necessary to design appropriate strategies in order to use BNF systems for efficient N supply to anise plants.

However, multiple inoculation experiments during recent decades failed to show a substantial contribution of Biological Nitrogen Fixation (BNF) to plant growth in most cases. It is now clear that associative diazotrophs exert their positive effects on plant growth directly or indirectly through a combination of different mechanisms. Apart from fixing N_2, diazotrophs can affect plant growth directly by the synthesis of phytohormones and vitamins, inhibition of plant ethylene synthesis, improved nutrient uptake, enhanced stress resistance, solubilization of inorganic phosphate and mineralization of organic phosphate. Indirectly, diazotrophs are able to decrease or prevent the deleterious effects of pathogenic microorganisms, mostly through the synthesis of antibiotics and/or fungicidal compounds, through competition for nutrients (for instance, by siderophore production) or by the induction of systemic resistance to pathogens (Sylvia *et al.*, 1998 and Attia *et al.*, 2004). In addition, they can affect the plant indirectly by interacting with other beneficial microorganisms.

The aim of this study was to determine whether nitrogen fertilizer sources and/or N_2 fixing bacteria (*Azotobacter* and *Azospirillum*) under saline conditions could affect anise plants quantity and quality. Besides, the effect of different nitrogen sources on biological activities in the rhizosphere of this medicinal plant.

Material and Methods

Biofertilizer

Identified strains of *Azotobacter chroococcum* and *Azospirillum lipoferum* were selected from microbial culture collection of the Agriculture Microbiology Dept. NRC. The selected strains were grown on their specific media to obtain of approximately 10^8 CFU ml^{-1} media. Microbial inoculant was prepared by mixing the two microbial strains in proportions of 1:1 (v:v) before applied to the soil. Mixed bacterial cultures inoculation was done by plating the seeds with a peat-based inoculant using gum Arabic.

Experimental methods

Two field experiments were carried out during winter seasons 2002 and 2003 at Tamiya village, El-Fayoum governorate. The soil was a clay loam with a pH of 8.12 (1:1, soil: water) and EC of 4.8 dS cm^{-1}. Soil nutrient determinations (Kalra & Maynard, 1991) included 40.4 mg N kg^{-1}; 20 mg P kg^{-1} and 35 mg K kg^{-1}. The experiments were laid in complete randomized block design with four replicates, each of five plots (1/400 fed). The plot area was 10.5 m^2 (3.5m in legth and 3.0m in width) with six ridges, 50 cm apart. Recommended dose of superphosphate (200 kg fed^{-1}) was added to the uninoculated and inoculated plots before sowing. Potassium sulphate (48% K_2SO_4) at rate of 100 kg fed^{-1} was applied during soil preparation. However, nitrogen fertilizer was added in three equal doses after 21, 35, and 60 days of sowing to all treatments. Seeds of anise were sown in hills 25 cm apart within the ridge (3 seeds $hill^{-1}$). The plants were thinned to one plant $hill^{-1}$ after 25 days of sowing. The irrigation was carried out whenever needed using the agriculture drainage water from drainage. Nitrogen treatments were as following:

1- Untreated (control treatment).
2- Fertilized with urea (46% N) as recommended dose.
3- Fertilized with ammonium nitrate (33.5% N) as recommended dose.
4- Biofertilization with the mixed culture of *Azotobacter* and *Azospirillum*.
5- Fertilized with half dose of urea plus biofertilizer.
6- Fertilized with half dose of ammonium nitrate plus biofertilizer.

Plant harvest and analysis

At flowering stage, rhizosphere samples were taken from each treatment for microbiological analyses. Dry weight of shoots and shoot nutrients content (N, P, K, Ca and Mg) were determined after drying at 70^{o} for 48 hr. At harvesting time, the seed yield and essential oil content were recorded. Fruits yield was determined in two complete rows from the middle of each plot.

Rhizosphere soil samples were analyzed microbiologically using the standard procedures described by Black *et al.* (1965) and Page *et al.* (1982). Plate counts of rhizosphere soils were done on selective media for total bacteria, fungi, actinomycetes, *Azotobacter* and *Azospirillum* (Campbell *et al.*, 1997) and the plates were counted after 7 days of incubation at appropriate temperature degrees. Dehydrogenase activity (as a measure of microbial activity) was determined by Garcia *et al.* (1998) method and expressed as µg INTF/g dry soil. Urease activity was measured according to the method of Nannipieri *et al.* (1980) and expressed as µmol of NH^{+}_{4}-N/g soil/h.

Shoot tissues were digested in H_2SO_4 and H_2O_2 for determination of P and N concentration (Horowitz, 1980). Nitrogen content of the digest was determined by Kjeldahl method and P-content was measured by the molybdate blue method. Total K, Ca and Mg were extracted with ammonium acetate and measured by atomic absorption spectrometry (Kalra & Maynard, 1991).

The essential oil content of anise seed was determined according to the method described in the British Pharmacopea (1963). The identification and composition of the essential oil components were determined using gas liquid chromatography.

Statistical analysis

Data were analyses using SPSS for Windows (Statistical Package for the Sciences System) by means of a one-way ANOVA and subsequently differences between treatments (multiple comparisons) were determined using Duncan's multiple range tests.

Results and Discussion

Enzymatic activities

Soil enzymatic activity was considered as a parameter to evaluate the biological activity of the microorganisms. Dehydrogenase activity is an indicator of biological redox-systems and can be taken as a measure for the intensity of microbial metabolism in the soil (Tabatabai, 1982). Dehydrogenase and urease activities varied widely among treatments (Fig. 1). In the 1st season, maximum activities of dehydrogenase and urease in the rhizosphere of anise plants were found in the soil inoculated with mixed culture of *Azotobacter* and *Azospirillum* plus urea, followed by combined bioferterization plus ammonium nitrate (Fig. 1). In the 2nd season, dehydrogenase and urease activities in these two treatments had increased relative to the 1st season (Fig. 1). Dehydrogenase and urease activities were apparently less in the un-fertilized soil (control) than the soil fertilized with mineral or biofertilizer (Fig. 1).

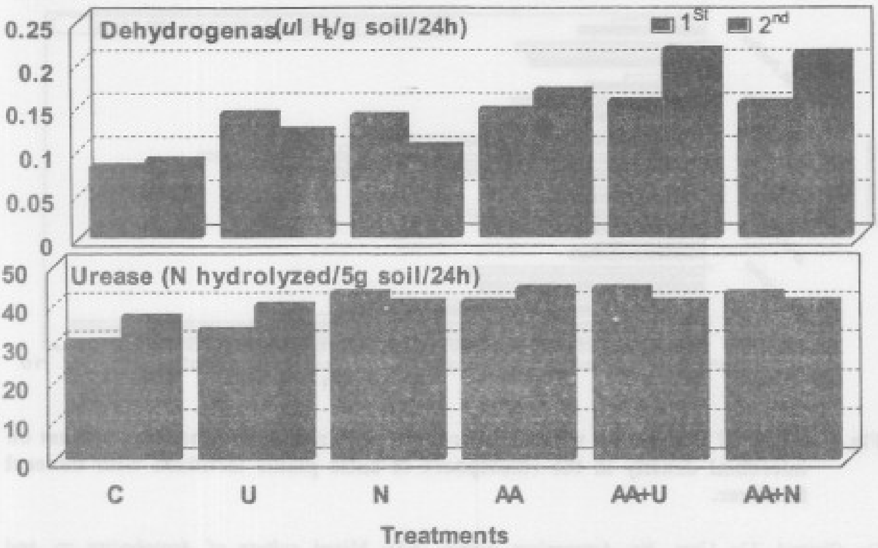

Fig. 1. Effect of inoculation with mixed culture of *Azotobacter* and *Azospirillum* on dehydrogenase and urease activities in the rhizosphere of anise plants in two seasons.

C= Control, U= Urea, N= Ammonium nitrate, AA= Mixed culture of *Azotobacter* sp. and *Azospirillum* sp.

The application of mineral nitrogen could lead to disequilibrium in the microbial population in the soil; but some studies have showed the beneficial effects of this kind of mineral fertilizer on enzymatic activities because the nutrient increment can stimulate the synthesis of enzymes (Chander & Brookes, 1991 and Pascual *et al.*, 1997). Our results up to some extent are in agreement with the above mentioned results. The results suggest that inoculation with mixed cutlure of dizotrophic bacteria improved enzymatic soil activities.

Microbiological analyses

Fertilization of soil with the two nitrogen sources or inoculated with nitrogen fixing bacteria affected densities of *Azotobacter* and *Azospirillum*. Populations of *Azotobacter* and *Azospirillum* were higher in the rhizosphere soil of anise plants inoculated with mixed bacterial cultures singly and were lower in soil fertilized with any source of nitrogen (Fig. 2) but had increased relatively to the unamended control. Highest population densities occurred at 2nd season in soil inoculated with biofertilizer and soil fertilized with urea in combination with biofertilizer (Fig. 2). Comparisons of population densities of *Azotobacter* and *Azospirillum* from 1st and 2nd seasons indicate that these bacteria were one to two orders of magnitude higher in 2nd season than in 1st season.

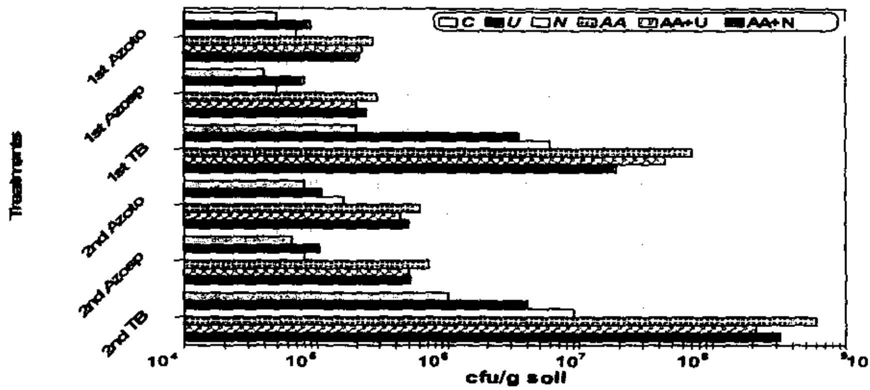

Fig. 2. Effect of inoculation with mixed cultures of *Azotobacter* and *Azospirillum* on microbial density in the rhizosphere of anise plants fertilized with mineral fertilizer.

C= Control, U= Urea, N= Ammonium nitrate, AA= Mixed culture of *Azotobacter* sp. and *Azospirillum* sp., TB= total bacteria

Tripathi *et al.* (1998) have studied in detail salinity stress response of *Azospirillum* sp. They have shown that in order to adapt to the fluctuations in soil salinity/osmolarity the bacteria of the genus *Azospirillum* accumulate compatible solutes such as glutamate, proline, glycine betain, trehalose, etc. Proline seems to play a major role in osmoadoptation. With increase in osmotic stress the dominant osmolyte in *A. brasilense* shifts from glutamate to proline. Accumulation of proline in *A. brasilense* occurs both uptake and synthesis. It is anticipated that understanding the mechanism of osmoadaptation in *Azospirillum* sp. may contribute to long-term goal of improving plant-microbe interactions for the exploration of salinity affected fields for crop productivity.

Densities of total bacteria were lowest in rhizosphere soil of the untreated control compared with all treatments. On the other hand, bacterial counts were higher in rhizosphere of anise plants inoculated with biofertilizer and/or fertilized with mineral nitrogen form plus biofertilizer than in soils fertilized with each form of mineral nitrogen alone in both years (Fig. 2). Highest bacterial counts were recorded in both years in soils fertilized with the two forms of mineral nitrogen plus biofertilizer. Marschner *et al.* (1986) found that addition of mineral nitrogen to the soil increased total bacteria. A decrease in bacterial population was recorded in soil treated with ammonium nitrate than soil treated with urea. Our results based on the method of "most probable number" (MPN) show distinct differences in the proportion of bacteria between the treatments (Fig. 2).

Addition of mineral nitrogen to the soil increased the proportion of total to diazotroph bacteria. Comprising population densities of *Azotobacter* and *Azospirillum* with densities of total bacteria in the rhizosphere of anise plants in two seasons, indicate that (Fig. 2) the total bacterial counts were one to two orders of magnitude higher than the two N_2 fixing bacteria in soil fertilized with the bio- plus chemical fertilizers. This shift between treatments in the proportion was the result of both, a decrease in the number of diazotroph bacteria and an increase in number of total bacteria. The decline of microbial inoculant population in soil results from several mechanisms including intrinsic physiological characteristics of the organisms, abiotic and biotic soil factors have been suggested to be many cause (van Elses *et al.*, 1992 and Evans *et al.*, 1993).

Population density of fungi was low and density not exceeding 12.5 x 10^3 CFU g^{-1} soil. Urea raised the fungal counts reached (24 x 10^3 CFU g^{-1} soil) followed by mixed cultural of N_2 fixing bacteria. No significant difference in fungal count between biofertilizer and biofertilizer plus ammonium nitrate treatments. Fungal population reached its minimum density in the treatment of biofertilizer plus urea at the first harvest and at in the second harvest in the non-biofertilized soil. Similarly, in 2003, population densities of fungi also were negatively affected by soil inoculation with biofertilizer, and were higher in soils fertilized with mineral nitrogen than biofertilizer (Fig. 3). Population densities of actinomycetes were not affected by treatments over the course of the experiment (Fig. 3).

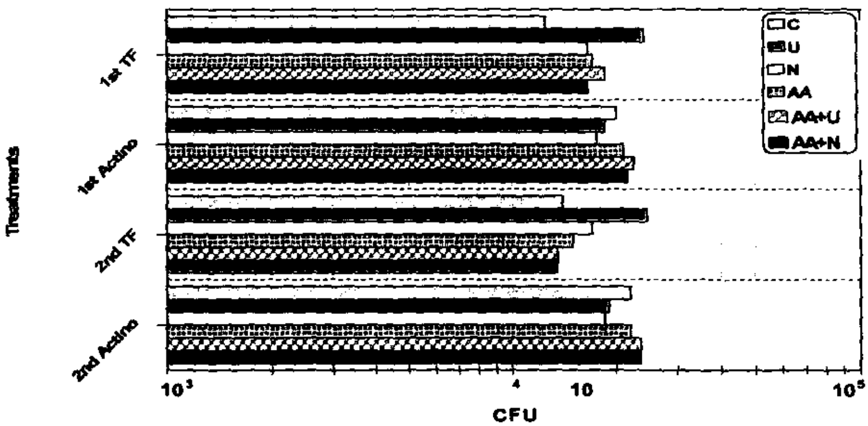

Fig. 3. Effect of inoculation with mixed cultures of *Azotobacter* and *Azospirillum* on total fungi and actinomycetes densities in the rhizosphere of anise plants fertilized with mineral fertilizer.

C= Control, U= Urea, N= Ammonium nitrate, AA= Mixed culture of *Azotobacter* sp. and *Azospirillum* sp.

Effect on shoot dry weight and yields of seeds and oil

Under the conditions of using of agriculture drainage water for irrigation, both the mineral and biofertilizer nitrogen sources showed pronounce favourable effect upon the shoot dry weight, seed yield and essential oil content of anise seeds (Table 1) compared to un-fertikized control. The data revealed that in both seasons, ammonium nitrate and urea plus mixed cultures of N_2 fixing bacteria treatments gave the highest values of shoot dry weight, fruit yield and essential oil compared with other treatments (Table 1). On the other hand, biofertilizer and ammonium nitrate significantly increased shoot dry weight, fruit yield and essential oil compared with urea. Similar results were reported by Hawkins & Lewis (1993) and Abou Zeid *et al.* (1999). When *A. lipoferum* and *Azotobacter chroococcum* inoculants were combined, their effect on seed yield was significantly higher than control (Table 1). It was noted that chemical fertilizers in combination with mixed cultures of N_2 fixing bacteria gave significantly higher yield than the every source alone. The interaction effect of increased nitrogen and inoculant on the plant growth parameters was not significant.

Okon & Labandera-Gonzalez (1994) found that a significant effect on wheat plant growth was obtained in 60-70% of the trials, with yield of seeds increases of up to 30%. They stated that, yield increases have been attributed to mechanisms such as nitrogen fixation, phytohormone production, and nitrate reduction. This response to biofertilizers inoculation depends on various different factors, including inoculation methods, chances for microbes survival and motility, competition with indigenous populations of rhizospheric microorganisms, and soil fertility (Okon, 1985 and Bashan *et al.*, 1995).

Some soil microorganisms, like *Azospirillum* sp. (Zimmer & Bothe , 1988), *Enterobacter* sp., *Azotobacter* sp., *Pseudomonas* sp. (Gamliel & Katan, 1992 and Arteca, 1996)) have shown to encourage plant growth by promoting the outbreak of secondary roots, acting as protectors against phytopathogenic microorganisms via plant hormones release and siderophores (Amstron *et al.*, 1993).

TABLE 1. Effects of different mineral nitrogen sources and biofertilizer on the vegetative growth, fruit yield of anise plant irrigated with agriculture drainage water.

Treatments	Shoot dry wt. (g/plant)	Umbels dry wt. (g/plant)	Seeds yield (kg fed^{-1})
Control	7.02a*	2.44a	141.54a
Urea	9.43a	2.66a	310.33a
Ammonium Nitrate	10.85ab	3.49ab	350.64a
Biofertilizer	10.84ab	4.52b	354.12b
Biofer.+Urea	14.26cd	6.78c	345.49b
Biofer+Amm.nitrate	16.64d	9.29d	590.67c

*Number not followed by the same letter are significantly different at P= 0.05.

Essential oil

Data presented in Table 2 show that all treatments had increased essential oil percentage and yield (L fed^{-1}) when compared with the control plants in both seasons. Chemical fertilization with urea or ammonium nitrate gave higher oil contents than the control. Inoculated plants with mixed cultural of N_2 fixing bacteria plus ammonium nitrate significantly increased the oil content compared to that fertilized with ammonium nitrate alone. In general, chemical fertilization increased the oil yields steadily, but bio- plus chemical fertilization was generally more effective than chemical fertilization (Table 2).

The main components of anise oil was anethole, followed by α-pinene whereas terpenyl acetate, was of lesser importance. The effect of chemical and biofertilization treatments on these three oil components was show in Table 2. Fertilization with ammonium nitrate gave higher anethole contents than biofertilization alone. Combination of ammonium nitrate and biofertilization gave the highest anethole contents in both seasons compared to all other treatments (Table 2). Biofertilization gave higher α-pinene contents than chemical fertilization.

TABLE 2. Effects of different mineral nitrogen sources and biofertilizer on the essential oil (percentage, yield and composition % in essential oil) of anise plants irrigated with agriculture drainage water.

Treatments	Essential oil		Anethole	α-pinene	Terpenyl acetate
	%	Yield (L fed^{-1})	%		
Control	3.1	4.39a*	25.53	6.67	0.41
Urea	4.8	14.90c	56.33	8.18	0.58
Ammonium Nitrate	3.5	12.27b	61.70	9.37	0.12
Biofertilizer	3.4	12.04b	58.77	11.31	0.27
Biofer.+Urea	4.3	14.86c	64.60	9.94	0.20
Biofer+Amm.nitrate	2.6	14.58c	68.52	10.91	0.33

*Number not followed by the same letter are significantly different at P= 0.05.

Nitrogen content and protein percentage

A perusal of data in Table 3 indicate that there was a significant difference in the effect of nitrogen source on nitrogen content and protein percentage. The data indicated that all treatments increased N content and protein percentage in anise plants compared with the control treatment. Nitrogen fertilization, in general, affects the amounts of nitrogen content as well as the quantity of protein produced by shoots. Whereas, plants inoculated with mixed culture of N_2 fixing bacteria and/or with urea or ammonium nitrate showed N content and protein percentage significantly higher than the rest of the treatments (Table 3). The concentration of N in the shoots of inoculated plants was lower than that of the chemical fertilized plants. The low shoot N contents for biofertilized plants led to suggest that inoculated plants had a faster growth rate which resulted in a lower percent N content, but a higher dry matter yield (Table 1).

Mozafar (1993) reported that when plant is presented with a lot of nitrogen, it increase protein production. Tyler *et al.* (1983) concluded that in soils where plants were fertilized 100% with the compound, the nitric form was found in a large proportion than any other concentration, due to the excessive application of the fertilizer, in amounts that the plant dose not really need. Thakur & Singh (1996) noticed that the use of biofertilizers had an important role in the utilization of nitrogen through higher biological N fixation and increasing nitrogen availability and uptake.

Nitrogen derived from BNF in combination with the chemical fertilizers increased P and K content compared to other treatments. The highest content of P was attained by using biofertilizer plus ammonium nitrate. The lowest value of P was attained from the plant fertilized with ammonium nitrate. Enhanced P contents following biofertilization suggests that theses rhizosphere bacteria increase the availability of nutrients through altering root surface characteristics involved in nutrient uptake. In those treatments in which the inoculation was carried out, bacterial populations at 120 day had increased from 10^3 to 10^5 CFU g^{-1} soil when compared to control treatment (Fig. 2). On the other hand, counts for all other treatments remained constant, indicating that the inoculated bacterial populations that were responsible for the increase could also be responsible for changes observed in the plants. Reduction in root permeability and consequent decrease in water and nutrient uptake under high salt condition (O'Leavy, 1974 and Frota & Tucker, 1978) has been associated with impaired N absorption. However, low levels in salts in the presence of N, P and K stimulated growth and increased yield of cotton plant.

TABLE 3. Effects of different mineral nitrogen sources and biofertilizer on the nutrients content of anise plant irrigated with agriculture drainage water.

Treatments	N	Protein	P	K	Ca	Mg
	%	%	(%)	%	%	%
Control	0.744a*	4.65a	0.804a	1.73a	0.85a	0.60a
Urea	1.732b	10.83b	0.834b	2.0b	0.93ab	0.98b
Ammonium nitrate	2.184e	11.80c	0.812ab	2.28b	0.86a	0.90b
Biofertilizer	1.888c	13.65e	1.134c	2.62b	1.47d	0.90b
Biofer.+Urea	1.956d	12.23d	1.288d	2.78bc	0.97b	0.90b
Biofer+ammonium nitrate	2.272e	14.20e	1.476f	3.35c	1.21c	1.08c

*Number not followed by the same letter are significantly different at P= 0.05.

The increase in both phosphorus and potassium due to the addition of biofertilizer may be partially explained according to the findings of Sundara Rao (1974) where they showed that, some microorganism tend to reduce the pH, since they produce organic acids, which may have a possible role in solubilizing phosphates and minerals.

Conclusions

The dual inoculum applied in combination with chemical fertilizers to anise plants enhanced the integral development of the plant, due to the bacteria's ability to make the nitrogen and phosphorus compounds available, and to the simultaneously production of growth-promoting substances (phytohormones). This suggests that biofertilization together with the addition of smaller quantities of the chemical fertilizers that are generally applied, is a real alternative that may increase crop production and, at the same time, of low cost compared to 100% chemical fertilization.

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تأثير التسميد الاحيائى والكيميائى النيتروجينى على محصول الينسون والنشاط البيولوجى للتربة المروية بمياه الصرف الزراعى

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أجريت تجربة حقلية خلال شتاء موسمين ٢٠٠٢-٢٠٠٣ لتقدير هل الأسمدة النيتروجينية منفردة أو مع البكتريا المثبتة للنيتروجين الجوى باستخدام مياه الصرف الزراعى يمكن أن تأثر على محصول الينسون بالإضافة إلى تقييم تأثير المعاملات النيتروجينية على النشاط البيولوجى فى ريزوسفير هذه النباتات الطبية.

وقد أظهرت النتائج وجود لختلافات معنوية بين المعاملات ولاحظ ذلك فى معاملات التسميد المعدنى على النشاط الميكروبى فى منطقة الريزوسفير، حيث وجد أن التثبيت النيتروجينى مع نصف الكمية الموصى بها من الأسمدة المعدنية تحسن من محصول الينسون. حيث كانت متوسطات القيم فى التسميد البيولوجى مع التسميد المعدنى متماثل وأحيانا أكبر من المتحصل عليه عند التسميد بالأسمدة المعدنية منفردة.

ومن ناحية أخرى أظهرت التحاليل الميكروبيولوجية للريزوسفير نباتات الينسون زيادة النيتروجين الممتص والذى يؤول إلى فاعلية الإمداد الكبير من الميكروبات المثبتة للنيتروجين الجوى والنشاط الانزيمى العالى فى التربة.