

## Annual Research & Review in Biology

17(1): 1-15, 2017; Article no.ARRB.36099  
ISSN: 2347-565X, NLM ID: 101632869

# Influence of Bio-fertilizers on Growth, Yield and Anthocyanin Content of *Hibiscus sabdariffa* L. Plant under Taif Region Conditions

A. A. Kahil<sup>1,2</sup>, F. A. S. Hassan<sup>1,3</sup> and E. F. Ali<sup>1,4\*</sup>

<sup>1</sup>Department of Biology, Faculty of Science, Taif University, Saudi Arabia.

<sup>2</sup>Legums Research Department, Field Crops Research Institute, ARC, Giza, Egypt.

<sup>3</sup>Department of Horticulture, Faculty of Agricultural, Tanta University, Egypt.

<sup>4</sup>Department of Ornamental Plants (Floriculture), Faculty of Agricultural, Assuit University, Egypt.

### Authors' contributions

This work was carried out in collaboration between all authors. Author AAK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors FASH and EFA managed the analyses of the study, managed the literature searches and sharing in wrote the protocol as well as sharing in wrote the first draft of the manuscript. All authors read and approved the final manuscript.

### Article Information

DOI: 10.9734/ARRB/2017/36099

#### Editor(s):

(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.

#### Reviewers:

(1) Tonye Sampson, University of Port Harcourt, Nigeria.

(2) M. Yuvaraj, Tamil Nadu Agricultural University, India.

Complete Peer review History: <http://www.sciencedomain.org/review-history/20937>

Original Research Article

Received 12<sup>th</sup> August 2017  
Accepted 27<sup>th</sup> August 2017  
Published 12<sup>th</sup> September 2017

## ABSTRACT

The production of chemical-free medicinal and aromatic plants has been the focus of interest of many researchers and producers in order to ensure the high quality and safety, not only for human, but also for the environment. Therefore, the aim of this study was to investigate the effect of some bio-fertilization treatments on the growth, yield and sepal quality attributes of *Hibiscus sabdariffa* L. plant under Taif region conditions.

The bacterial strains were isolated from the rhizosphere of plant roots grown in the study area and multiplied on media before treatment application. The bio-fertilizers applied in this experiment were *Azotobacter chroococcum* + *Azospirillum brasilense* as nitrogen fixing bacteria (NFB), *Bacillus megatherium* var. phosphaticum + *Bacillus polymyxa* as phosphate solubilizing bacteria (PSB) and the mixture between them as a combination treatment. Control plants were not treated with any bio-

\*Corresponding author: Dr. Esmat Farouk Ali Ahmed, E-mail: [esmatfarouk@yahoo.com](mailto:esmatfarouk@yahoo.com);

fertilizers. The results of this study showed that vegetative growth parameters, fruit number per plant and sepal yield were significantly increased due to NFB and/or PSB treatments applied relative to the control. The sepal quality attributes i.e. anthocyanin content and total soluble solids of inoculated plants were significantly enhanced in roselle juice compared with un-inoculated control however the pH value was reduced due to bio-fertilizer treatments. Moreover, the total chlorophyll, carbohydrates contents and macro elements of roselle leaves was significantly improved due to bio-fertilizer treatments relative to the control. Applying NFB and/or PSB treatment significantly reduced the nitrate and nitrite contents in roselle leaves relative to un-inoculated control. CO<sub>2</sub> evolution in the soil of roselle plants treated with NFB or PSB was significantly higher than untreated plants at any time point during the growth. The combined treatment of NFB + PSB was superior to solely application concerning all investigated parameters.

**Keywords:** Bio-fertilizers; growth; anthocyanin; chlorophyll; roselle; nitrate; nitrite.

## 1. INTRODUCTION

The cultivation of several medicinal and aromatic plants had noticeably increased in recent decades. Because the need of increasing the medicinal plant production all over the world, its production became an ultimate goal to meet the great increase of population to avoid chemical therapy side effects on human health through utilization of the medical herbs. However, the use of the most suitable and recommended agricultural practices in growing such crops could provide the producers with higher income, in comparison with many other traditional crops [1].

Roselle (*Hibiscus sabdariffa* L.) is a subtropical plant belonging to Family Malvaceae. The fruit calyxes of this plant are used for preparing refreshing beverage and jellies of brilliant red color with pleasant acidic taste. In addition, the drink has a laxative effect and the calyx extraction is of a great therapeutic action for curing heart and nerve diseases, high blood pressure and calcified arteries. Fertilization is a major factor affecting the production of this plant. One of the main problems of excessive use of N-fertilization is nitrate accumulation in the plant and it can directly inhibit oxygen transport by blood, a medical condition known as methemoglobinemia, because of the reduction of converting nitrate to nitrite [2]. Crop stimulation of different medicinal and aromatic plants by this plant growth promoting rhizobacteria has been demonstrated in different field trials. The vegetative growth parameters as well as sepal yield of roselle plant were increased as a result of using bio-fertilizers.

Although the importance of chemical fertilizers, many constraints have been raised due to their adverse impacts on the public health, environment and national income [2]. To confront

this problem, it was necessary to develop alternative methods of supplying nutrients to the growing plant. Many scientists consider the utilization of bio-fertilizers today as promising alternative techniques particularly for developing countries. Consequently, the production of chemical-free medicinal and aromatic plants has been the focus of interest of many researchers and producers in order to ensure the high quality and safety, not only for human, but also for the environment which we live in. Therefore, it has become essential to use untraditional fertilizers. It is well known that a considerable number of bacterial species, mostly those associated with the plant rhizosphere, are able to exert a beneficial effect upon plant growth. Therefore, their use as bio-fertilizers or control agents for agricultural improvement has been a focus of numerous researchers for a number of years [3]. Bio-fertilization is a very important method of providing the plants with their nutritional requirements without having undesirable impact on the environment. It also provides the means for stabilizing soil fertility (especially in newly reclaimed soils).

The utilization of bio-fertilizers is considered today by many scientists as a promising alternative particularly for developing countries. Bio-fertilizers are, generally, based on altering the rhizospher flora, by seed or soil inoculation with certain organisms, capable of inducing beneficial effect on a compatible host. Bio-fertilizers mainly comprise nitrogen fixer, phosphate solvers, silicate bacteria and others. These organisms may affect their host plant by one or more mechanisms such as nitrogen fixation, production of growth promoting substance or organic acids, enhancing nutrients uptake or protection against pathogens [3]. Recent awareness has been offered to reduce pollution practices in sustainable agriculture. One

of the ways to lessen soil pollution is the use of bio-stimulants compounds [4]. Bio-fertilizers compounds have beneficial return to increase population of soil microorganisms, especially in the surface layer of root rhizosphere, that create substances which stimulate plant growth [5].

The effective utilization of bio-fertilizers for crops not only provides economic benefits to the producers, but also improves, maintains the soil fertility and sustainability in natural soil ecosystem. The beneficial effects of plant growth promoting rhizobacteria on growth are not only through nitrogen fixed in the rhizosphere, but also related to the ability of these bacteria to synthesize antibiotics and growth-promoting substances including phytohormones and sometimes the ability to solubilize phosphates; the use of phosphate-solubilizing bacteria becomes necessary to minimize the dose of chemical P fertilizer that cause environmental pollution [1,6,7]. Bio-fertilizers being essential components of organic farming play vital role in maintaining long term soil fertility and sustainability by fixing atmospheric dinitrogen (N<sub>2</sub>), mobilizing fixed macro and micro nutrients or convert insoluble P in the soil into forms available to plants, there by increases their efficiency and availability [8]. Bio-fertilizers have fabulous tendency for decreasing the requirement of synthetic fertilizers without compromising on crop yield. Bio-fertilizers contain plant growth promoting rhizobacteria (PGPR) viz; *Azotobacter*, *Azospirillum* and phosphorus solubilizing bacteria (PSB) viz; *Pseudomonas sp.* and *Bacillus sp.* having the ability of atmospheric nitrogen fixing and solubilizing the soil phosphorus, respectively. Consequently, they fulfill the nitrogen and phosphorus requirement of cereals and also improve the soil fertility. So the utilization of nitrogen fixing and phosphate solubilizing bacteria as bio-fertilization has gigantic potential for using the atmospheric nitrogen and making use of fixed phosphorus present in the soil in crop production without causing any harmful effects on aerial and soil environment. Bio-fertilizers are more economical due to their low market prices comparing synthetic fertilizers, helpful in improving soil structure and the restoration of environment for leveraging agriculture [9].

During the past two decades, there have been extensive research efforts to increase and exploit biological N<sub>2</sub>-fixers by medicinal and aromatic plants. Recently, the world began to come back

to nature, in particular, for utilizing medical herbs instead of chemical materials therapy to avoid their side effects on human health. The beneficial effects of plant growth promoting rhizobacteria on plant growth are not only through nitrogen fixed in the rhizosphere, but also related to the ability of these bacteria to synthesize antibiotics and growth-promoting substances including phytohormones and sometimes the ability to solubilize phosphates; the use of phosphate-solubilizing bacteria becomes necessary to minimize the use of large doses of chemical P fertilizers that cause environmental pollution [10]. In this respect, Koreish et al. [11] found that phosphate-solubilizing bacteria inoculation is recommended to decrease the mineral P fertilizer dose. Many investigators pronounced the positive effect of bio-phosphatic fertilization such as Ibrahim [12] on fennel, Khater [13] on caraway plants and Ali and Hassan [7] on black cumin.

One of the recent achievements is the use of bio-fertilizer which retards nitrification for sufficiently longer time and increases the soil fertility [14]. Bio-fertilizer can provide an economically viable support to small and marginal farmers for realizing the ultimate goal of increasing productivity. Bio-fertilizer is low cost, effective and renewable source of plant nutrients to supplement chemical fertilizers. Their mode of action differs and can be applied alone or in combination. By systematic research, efficient strains are identified to suit to given soil and climatic conditions [15].

The amount of nitrogen available to plant in soil is usually small, for this reason, one of the main factors for increasing yield of agricultural crops is the availability of nitrogen supplies to plants, which can be achieved by using biologically fixed nitrogen fertilizers. In this concern, Abdou and Mahmoud [16], El-Ghawwas et al. [17] on fennel, Kandeel et al. [18] on dill and Kandeel et al. [19] on fennel plants found that the growth and yield of the previous plans were promoted as a result of applying organic or bio-fertilizers. Application of bio-fertilizers also increased the amount of N, P and K in plant tissues compared to non-inoculated plants. It can be concluded that bio-fertilizers that are considered as an important part of environment friendly and sustainable agricultural practice, reduce the application of chemical fertilizers by 50% without any reduction in growth and yield of fennel plants [20]. The growth parameters, yield components and oil content of rosemary were increased as a result of applying bio-fertilizer treatments [21].

During the past two decades, there have been extensive research efforts to increase and exploit biological N<sub>2</sub>-fixers by medicinal and aromatic plants. Recently, the world began to come back to nature, in particular, for utilizing medical herbs instead of chemical materials therapy to avoid their side effects on human health. The effective utilization of biological fertilizers for crops not only provides economic benefits to the farmers, but also improves and maintains the soil fertility and sustainability in natural soil ecosystem [22].

A positive response was shown on the growth and yield of roselle plants especially when seeds were inoculated with *Rhizobium* and *Azotobacter* [23]. Inoculation of roselle seeds with nitrobin (a bio-source of nitrogen) significantly increased the number of fruits as well as sepals yield compared to the control [24]. The anthocyanin content of roselle sepals was increased when bio-fertilizers were applied [23,24,25]. The application of bio-fertilizers enhanced the vegetative growth parameters, sepals and seeds yield of roselle plants, as well as their quality [6]. Recent awareness has been offered to reduce pollution practices in sustainable agriculture. One of the ways to lessen soil pollution is the use of bio-stimulants compounds [4]. Bio-fertilizers compounds have beneficial return to increase population of soil microorganisms, especially in the surface layer of root rhizosphere, that create substances which stimulate plant growth [5].

Application of biological fertilizers significantly increased seeds performance, biological yield per plant, plant height, number of spikes per plant, number of seeds per spike, number of seeds per plant, and the weight of 1000 seeds of *Anethum graveolens*. Among the treated plants, mixed treatment of nitroxin and *Pseudomonas fluorescens* caused maximum increase in most of the parameters under study [26]. They concluded that application of biological fertilizers plays a remarkable role in improving growth characteristics and yield compounds of and they can be viewed as a suitable replacement for chemical fertilizers. Akhiani et al. [27] reported that plant height, umbel numbers, plant dry weight, weight of 1000-seeds and seed yield of coriander were increased by using bio-fertilizer treatments. Some micro-organisms have positive effects on plant growth promotion, including the plant growth promoting rhizobacteria (PGPR) such as *Azospirillum*, *Azotobacter*, *Pseudomonas fluorescens* and several gram positive *Bacillus spp.* [28]. *Azotobacter* and *Azospirillum* are free-living N<sub>2</sub> fixing-bacteria in

the rhizospheric zone that have the ability to synthesize and secrete some biologically active substances that enhance root growth. It is well known that a considerable number of bacterial and fungal species possess a functional relationship and constitute a holistic system with plants. They are able to exert beneficial effects on plant growth also enhance plant resistance to adverse environmental stresses, such as water and nutrient deficiency and heavy metal contamination [29].

Plant height, branch number, and fresh and dry weights of leaves and shoots as well as seed and oil yield of coriander were significantly increased with application of *Azotobacter chroococcum* + phosphate solubilizing bacteria compared to single inoculation with N<sub>2</sub>-fixing or phosphate dissolving bacteria [1,30,31]. Fruit inoculation with *Azospirillum* and/or *Azotobacter* promoted vegetative growth and increased fruit yield of some Apiaceae family crops [32,33,34,35,36,37]. The essential oil content and its main components of caraway fruits were increased by using bio-fertilizers. In addition, carbohydrate percentage as well as nitrogen, phosphorus and potassium content in leaves were also promoted [13,38]. The same trend was observed by Badawi [39], Abou-Alyand Gomaa, [30], Kumar et al. [35] and Ali and Hassan [7]. Increasing the protein content as a result of applying bio-fertilizers has been previously reported [40,41,42].

The decrease of nitrate concentration is an important factor in medicinal and aromatic plant production. This is because consumption the leaves with high nitrate represent a health hazard to infants and adults. Applying organic and bio-fertilizers as alternative sources of chemical fertilizers reduced nitrate and nitrite contents of some plants [43,44].

Bio-fertilizers are known to play a pivotal role in many production oriented agricultural systems, but little is known about the interactive effects of both of them on growth and secondary metabolites accumulation in medicinal plants. To the best of our knowledge, few studies have been conducted so far to study the effect of bio-fertilizers on medicinal plants. Moreover, improving not only the quantity but also the quality of roselle yield still the main goal of several investigators. So, the objective of this study was to investigate the effect of bio-fertilizers treatments on the growth, yield and oil content as well as its composition of black cumin

plant to reveal the suitable treatment which maximizing seed and oil yields and obtaining the highest quality product.

One of the recent achievements is the use of bio-fertilizer which retards nitrification for sufficiently longer time and increases the soil fertility [14]. Bio-fertilizer can provide an economically viable support to small and marginal farmers for realizing the ultimate goal of increasing productivity. Bio-fertilizer are low cost, effective and renewable source of plant nutrients to supplement chemical fertilizers. Their mode of action differs and can be applied alone or in combination. By systematic research, efficient strains are identified to suit to given soil and climatic conditions [15].

Application of biological fertilizers significantly increased seeds performance, biological yield per plant, plant height, number of spikes per plant, number of seeds per spike, number of seeds per plant, and the weight of 1000 seeds of *Anethum graveolens*. Among the treated plants, mixed treatment of nitroxin and *Pseudomonas fluorescens* caused maximum increase in most of the parameters under study [26]. They concluded that application of biological fertilizers plays a remarkable role in improving growth characteristics and yield compounds of and they can be viewed as a suitable replacement for chemical fertilizers. Akhiani et al. [27] reported that plant height, umbel numbers, plant dry weight, weight of 1000-seeds and seed yield of coriander were increased by using bio-fertilizer treatments. Some micro-organisms have positive effects on plant growth promotion, including the plant growth promoting rhizobacteria (PGPR) such as *Azospirillum*, *Azotobacter*, *Pseudomonas fluorescens* and several gram positive *Bacillus spp.* [28]. *Azotobacter* and *Azospirillum* are free-living N<sub>2</sub> fixing-bacteria in the rhizospheric zone that have the ability to synthesize and secrete some biologically active substances that enhance root growth. They also increase germination and vigour in young plants, leading to improved crop stands [28]. It is well known that a considerable number of bacterial and fungal species possess a functional relationship and constitute a holistic system with plants. They are able to exert beneficial effects on plant growth [45] and also enhance plant resistance to adverse environmental stresses, such as water and nutrient deficiency and heavy metal contamination [29].

Seed inoculation with *Azotobacter* and *Azospirillum* with half dose of chemical fertilizer

positively influenced the growth characters and increased the fruit yield of *Coriandrum sativum*, *Foeniculum vulgare* and *Carum carvi* plants [46]. Inoculation of seeds with *Azotobacter* and *Azospirillum* in the presence of chemical fertilizers resulted in improving both growth and yield of anise plants; [47], *Foeniculum vulgare* plants [48], *Nigella sativa*, [49] and *Hibiscus sabdariffa* [25]. The environmental conditions of Taif city are suitable for cultivation of roselle plant and the anthocyanin content may be increased as well. This reason may give this plant an advantage to be an important economic plant in this region. If the cultivation succeeded this plant will need another national project for exporting to increase the national income.

For the previous reasons of the importance of bio-fertilizers, it is very important to study the effect of these bio-fertilizers on *Hibiscus sabdariffa* which consider one of the most important medicinal and aromatic plants. By applying bio-fertilizers we can decrease the production cost, protect the environment from pollution and obtain high quality product by reducing the quantity of chemical fertilizers.

## 2. MATERIALS AND METHODS

### 2.1 Plant Materials

A field study was carried out at a private farm during two successive seasons (2016 and 2017) in Taif region, Saudi Arabia to investigate the effects of different bio-fertilizer treatments on the growth, yield and sepal quality of roselle (*Hibiscus sabdariffa*, L) plants. Roselle seeds were sown directly in holes on the top of the ridge 70 cm apart in plots (2 x 2 m<sup>2</sup> including three rows and each row contained six hills at 25 cm apart. Three seeds were placed in each hill and one month later thinning was done leaving one seedling/hill. All other cultural practices were occurred when required. The physical properties of the soil used in this study were (sand, 75.27%, silt 8.84% and clay 15.89%). The chemical analysis of experimental soil was as follows; pH 8.02, EC, 1.98 dSm<sup>-1</sup>, OM, 0.14%, total CaCO<sub>3</sub>, 0.79%, total N<sup>+</sup>, PO<sub>4</sub><sup>-3</sup>, K<sup>+</sup> were 0.19, 0.048 and 0.057%, respectively, Na<sup>+</sup>, 3.07 meqL<sup>-1</sup>, Cl<sup>-</sup>, 0.53 meqL<sup>-1</sup>.

### 2.2 Treatments

The bio-fertilizers applied in this experiment were *Azotobacter chroococcum* + *Azospirillum brasilense* as nitrogen fixing bacteria (NFB), *Bacillus megatherium* var. phosphaticum +

*Bacillus polymyxa* as phosphate solubilizing bacteria (PSB) and the mixture between them as a combination treatment. Control plants were not treated with any bio-fertilizers.

The media of growing bacteria was prepared according to Hellal et al. [37]. Briefly, 5 gL<sup>-1</sup> peptone and 3 gL<sup>-1</sup> beef extract were added to distilled water in a flask and sterilized in autoclave at 121°C for 20 minutes. Then, the flask was left at room temperature for 2 h. The bacterial strains were isolated from the rhizosphere of plant roots grown in the study area. After that, the strains were individually inoculated in sterile room conditions. After inoculation, the flasks were incubated at 28°C for 7-10 days to obtain the highest growth (10<sup>9</sup> mL<sup>-1</sup>). To prepare NFB treatment, *Azotobacter chroococcum* and *Azospirillum brasilense* was mixed by 1:1 (v/v). The same procedure was followed to prepare PSB treatment. The combined treatment (NFB + PSB) was obtained by mixing them at 1:1 (v/v).

Roselle seeds were soaked in the inoculum suspension for 15 minutes before planting according to El-Zeiny et al. [50]. After 8, 12, and 16 weeks from planting, the soil will be inoculated with bacteria at side root zones of plants and irrigated immediately according to Gori and Favilli [51]. The treatments were arranged in a complete randomized design (CRD) with four replicates.

### 2.3 Data Recorded

Ten plants were randomly chosen from each experimental unit at harvest for determining plant height (cm), branch number/plant, herb fresh and dry weights (g/plant), fruit number/plant, sepal and seed yield/plant (g).

### 2.4 Total Anthocyanin Determination

Total anthocyanin content in the sepals was determined according to Fuleki and Francis [52] and developed by Du and Francis [53].

### 2.5 pH Values and Total Soluble Solids Determination

pH values and total soluble solids of sepals were determined as described by Diab [54].

### 2.6 Chlorophyll Content

Fresh leaf samples were taken for chlorophyll determination according to Sadasivam and

Manickam [55]. Extraction in acetone was repeated until all pigments extracted. The absorbance of extracts was determined by a spectrophotometer. The chlorophyll content was calculated as mg g<sup>-1</sup> FW.

### 2.7 Total Carbohydrates

Total carbohydrate percentages were determined in leaf samples dried in an electric oven at 70 °C for 24 hours according to A.O.A.C. [56]. Then, the fine powder will be used to determine total carbohydrate percentages according to Herbert et al. [57].

### 2.8 Leaf Mineral Content

Nitrogen, phosphorus and potassium were determined in leaf samples, digested using sulphuric and perchloric acids method [58,59,60].

### 2.9 Nitrate and Nitrite

Nitrate (NO<sub>3</sub>) and nitrite (NO<sub>2</sub>) concentrations in leaves were colorimetrically determined at 420 nm and 720 nm according to Jackson [60] and Follet and Ratcliff [61], respectively. The obtained values were assayed on fresh weight basis.

### 2.10 CO<sub>2</sub> Evolution

CO<sub>2</sub> evolution as an indicator for microbial activity was measured in samples obtained from the soil of all treatments at May 1<sup>st</sup>, June 1<sup>st</sup>, July 1<sup>st</sup>, August 1<sup>st</sup> and September 1<sup>st</sup> during the growth according to the method described by Promer and Schmid [62] and modified by Alef and Nannipieri [63].

### 2.11 Statistical Analysis

The analysis of variance (ANOVA) was performed and data were analyzed using SPSS 13.3 program. Means were compared by Duncan multiple range test at *P*=0.05 level.

## 3. RESULTS AND DISCUSSION

### 3.1 Vegetative Growth Parameters

It is evident from data in Table (1) that roselle plants inoculated with different bio-fertilizers recorded significantly higher vegetative growth characters compared with un-inoculated plants. Although the solely treatment of NFB or PSB

significantly increased plant height, branch number as well as fresh and dry weights of herb compared to the control, the highest values in this respect were obtained by applying the combination treatment between them.

These results may be ascribed to the roles of non-symbiotic N<sub>2</sub>-Fixing bacteria (NFB) and phosphate solubilizing bacteria (PSB) in exerting a positive effect on plant growth through the synthesis of phytohormones, N<sub>2</sub> fixation, reduction of membrane potential of the root, synthesis of some enzymes (such as ACC deaminase) that modulate the level of plant hormones as well as the solubilization of inorganic phosphate and mineralization of organic phosphate, which make phosphorus available to the plants [2]. Otherwise, the growth improvement of roselle plant due to bio-fertilizers could be explained through the synthesis of growth promoting compounds like cytokinins, gibberellins and indole acetic acid [48] and consequently the herb fresh and dry weights as a result of NFB or PSB were increased [21,39]. Similarly, Awad [5] reported that bio-fertilizers have beneficial return to increase population of soil microorganisms, especially in the surface

layer of root rhizosphere, that create substances which stimulate plant growth.

### 3.2 Yield Components

The fruit number per plant was significantly improved due to bio-fertilizer treatments applied relative to the control. The highest value in this concern (89.74/plant) was recorded by the interaction between NFB and PSB treatment. The fresh and dry sepal yield followed the same pattern of the fruit number. Application of any fertilizer treatment significantly increased the sepal yield compared with un-inoculated plants. The seed yield also was significantly higher as a result of bio-fertilizer application. The combination treatment of NFB and PSB resulted in the highest both sepal and seed yields (Table 2).

Increasing the fruit number of roselle plant due to bio-fertilizer inoculation may be attributed to the increment in branch number as our data indicated. Such increase in fruit number has been previously reported [33,64]. The promotion effect of bio-fertilizer application on vegetative growth of roselle is reflected in improving the sepal and seed yields through the increment in

**Table 1. Influence of different bacterial strains inoculation on vegetative growth characters of roselle plants**

Bio-fertilizer treatments	Plant height (cm)	Branch number/plant	Fresh weight (g/plant)	Dry weight (g/plant)
Un-inoculated	76.57d	3.36c	1254.36d	298.18d
NFB	93.12b	5.48b	1680.58b	436.75b
PSB	89.45c	5.62b	1542.69c	393.68c
NFB + PSB	98.58a	6.87a	1987.85a	508.15a

Un-inoculated; control plants, NFB; mixture of *Azotobacter chroococcum* and *Azospirillum brasilense* by 1:1 (v/v), PSB; mixture of *Bacillus megatherium* var. *phosphaticum* + *Bacillus polymyxa* 1:1 (v/v), NFB + PSB; mixture of NFB and PSB by 1:1 (v/v). Means followed by different letters were significantly different according to Duncan multiple range test at 0.05 level (n=8).

**Table 2. Influence of different bacterial strains inoculation on yield components of roselle plants**

Bio-fertilizer treatments	Fruit number/plant	Fresh sepal yield (g/plant)	Dry sepal yield (g/plant)	Seed yield (g/plant)
Un-inoculated	49.15d	66.34c	6.29c	52.15c
NFB	84.41b	82.57b	9.45b	70.67b
PSB	80.26c	81.96b	9.36b	71.69b
NFB + PSB	89.74a	95.59a	10.13a	83.94a

Un-inoculated; control plants, NFB; mixture of *Azotobacter chroococcum* and *Azospirillum brasilense* by 1:1 (v/v), PSB; mixture of *Bacillus megatherium* var. *phosphaticum* + *Bacillus polymyxa* by 1:1 (v/v), NFB + PSB; mixture of NFB and PSB by 1:1 (v/v). Means followed by different letters were significantly different according to Duncan multiple range test at 0.05 level (n=8).

fruit number or sepal growth improvement. It has been reported that NFB and PSB have the ability to synthesize and secrete some biologically active substances that enhance the growth [28]. Increasing the sepal and seed yields due to biofertilizer application has been observed [25,27,65,66].

### 3.3 Sepal Quality Parameters

To measure the sepal quality, total anthocyanin, pH values and total soluble solids were investigated. The statistical analysis of results clearly show that NFB or PSB treatments significantly enhanced the accumulation of anthocyanin pigments as well as increased the total soluble solids in roselle juice relative to the control however there is no significant difference between them in this regard. The combined treatment of NFB and PSB recorded the highest values of total anthocyanin ( $21.34 \text{ mgg}^{-1} \text{ DW}$ ) and total soluble solids (41.18 %). On the other hand, pH value was significantly reduced as a result of applying any bio-fertilizer treatments and the lowest pH value (2.98) was obtained when NFB was combined with PSB treatment (Table 3).

Increasing the anthocyanin content as well as total soluble solids of roselle sepals has been reported when bio-fertilizers were applied [23,24,67]. Reducing pH value as a result of bio-fertilizer treatments may be due to increasing the acidic taste of the fruit calyx because of the presence of several organic acids in the cell sap [65].

### 3.4 Chlorophyll and Carbohydrates Contents

The contents of chlorophyll and carbohydrates followed the same trend as a result of inoculation with NFB or PSB bio-fertilizers. The chlorophyll and carbohydrates contents of roselle leaves was significantly increased due to bio-fertilizer treatments relative to the control (Fig. 1 A and B). However, there were no significant differences between NFB or PSB treatments in this respect. Otherwise, when both treatments were combined the highest chlorophyll content was observed.

Bio-fertilizers have reduced chlorophyll loss and stimulated chlorophyll synthesis through encourages pyridoxal enzymes formation, that play an important role in  $\alpha$ -amino levulinic acid synthetase as a primary compound in chlorophyll synthesis [68]. These results support the other published data [7,44,69]. Increasing the growth

characters is correlated with improving the photosynthetic pigments as our data shown and hence the total carbohydrates were improved. The importance of bio-fertilizers in increasing the percentages of total carbohydrate in roselle plant tissues may be due to the role of these bio-fertilizers on the enzymatic systems responsible for the biosynthesis of these compounds [25]. A positive relationship between leaf carbohydrate and leaf pigments was recorded in this study. The results suggest that the synthesis of photosynthetic pigments in leaves is an induced factor for carbohydrate synthesis. These results are in accordance with the previous results [1,7,67].

### 3.5 Leaf Mineral Content

The contents of N, P and K in roselle leaves were significantly affected by bio-fertilizer application. The obtained results indicate that the contents of those macro elements were significantly increased in plants inoculated with NFB, PSB or the combined treatment of NFB and PSB compared with un-inoculated plants (Table 4). While, NFB treatment resulted in higher N content compared to PSB treatment, the latter treatment recorded higher P content than the first one. Generally, the highest values of macro elements measured in this study were observed with the combination between NFB and PSB treatments.

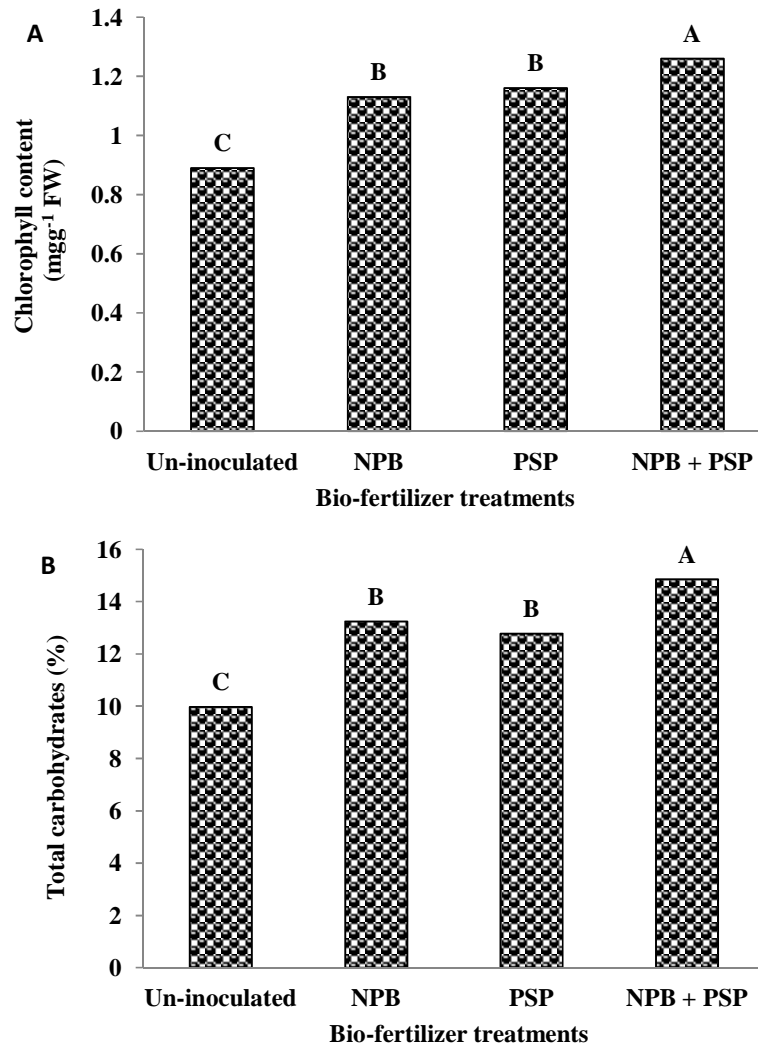
It is well known that increasing the microorganisms in the soil have a positive effect in converting the unavailable forms of nutrient elements to available forms, consequently both absorption and translocation could be increased. The microorganisms also produce growth promoting substances resulting in more efficient absorption of nutrients, which main components of photosynthetic pigments and consequently the chlorophyll content as well as N, P and K percentages were increased [47]. In addition, the non-symbiotic  $\text{N}_2$ -fixing bacteria produced adequate amounts of IAA and cytokinins with increasing the surface area per unit root length and enhanced the root hair branching with an eventual increase on the uptake of nutrients from the soil [3]. Phosphate solubilizing bacteria release organic and inorganic acids which reduce soil pH leading to change of phosphorus and other nutrients to available forms ready for uptake by plants [70]. Therefore, the percentages of N, P and K elements in the leaves were increased and this increment led to promote the growth and yield of roselle plants. Similar results have been reported [33,48,66,67].



**Table 3. Influence of different bacterial strains inoculation on sepal quality (anthocyanin content, pH value and total soluble solids) of roselle sepal juice**

Bio-fertilizer treatments	Anthocyanin content (mgg <sup>-1</sup> DW)	pH value	Total soluble solids (%)
Un-inoculated	16.89c	4.12a	29.13c
NFB	19.58b	3.57b	38.46b
PSB	19.21b	3.65b	37.72b
NFB + PSB	21.34a	2.98c	41.18a

Un-inoculated; control plants, NFB; mixture of *Azotobacter chroococcum* and *Azospirillum brasilense* by 1:1 (v/v), PSB; mixture of *Bacillus megatherium* var. *phosphaticum* + *Bacillus polymyxa* by 1:1 (v/v), NFB + PSB; mixture of NFB and PSB by 1:1 (v/v). Means followed by different letters were significantly different according to Duncan multiple range test at 0.05 level (n=8).



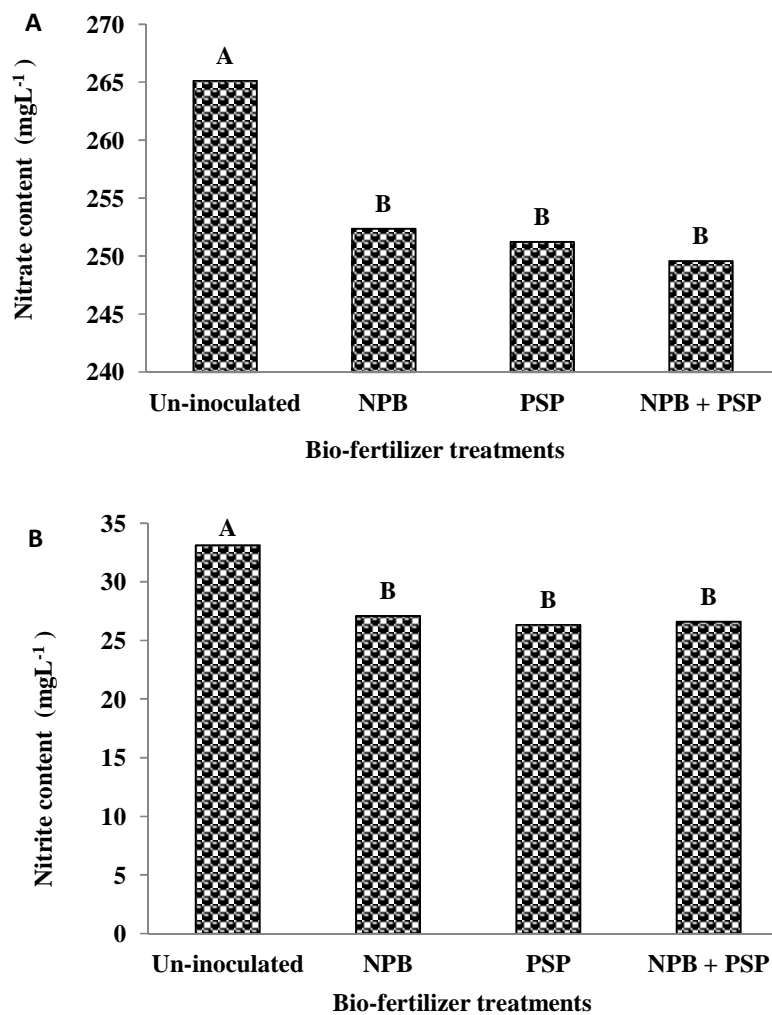
**Fig. 1. Influence of different bacterial strains inoculation on chlorophyll content (A) and total carbohydrate (B) of roselle leaves. Un-inoculated; control plants, NFB; mixture of *Azotobacter chroococcum* and *Azospirillum brasilense* by 1:1 (v/v), PSB; mixture of *Bacillus megatherium* var. *phosphaticum* + *Bacillus polymyxa* by 1:1 (v/v), NFB + PSB; mixture of NFB and PSB by 1:1 (v/v). Means followed by different letters were significantly different according to Duncan multiple range test at 0.05 level (n=8).**

### 3.6 Nitrate and Nitrite

Data presented in Fig. (2 A and B) show that applying any bio-fertilizer treatment significantly decreased the nitrate and nitrite contents in roselle leaves compared with untreated control. The analysis of results indicates that there is no difference between NFB, PSB or the combined application in this respect. The highest nitrate and nitrite contents (265.13 and 33.15 mgL<sup>-1</sup>) were recorded in un-inoculated plants.

Nitrate or even nitrite accumulation is dependent not only of agriculture system and respective

practices but also of soil properties, fertilizer usage, cultivation and weather conditions [71]. The decrease of nitrate and nitrite concentrations is an important factor in the production of medicinal and aromatic plants because of the health hazard of higher nitrate to human. The most important aspect for human health is related to nitrites contamination [72]. It is well documented that reducing nitrates to nitrites resulted in nitrite reaction with amines or amides to form carcinogenic compounds [73]. It has been reported that microbial fertilizer can effectively control nitrate content of Sheng [74]



**Fig. 2. Influence of different bacterial strains inoculation on nitrate (A) and nitrite contents (B) of roselle leaves. Un-inoculated; control plants, NFB; mixture of *Azotobacterchroococcum* and *Azospirillumbrasilense* by 1:1 (v/v), PSB; mixture of *Bacillus megatherium*var. phosphaticum + *Bacillus polymyxaby* 1:1 (v/v), NFB + PSB; mixture of NFB and PSB by 1:1 (v/v). Means followed by different letters were significantly different according to Duncan multiple range test at 0.05 level (n=8).**

that very important environment protection and produce healthy medicinal and aromatic plants. In this regard Hanafy Ahmed et al. [75] recorded a significant reduction in nitrate accumulation by the plants treated with bio-fertilizers. Consistent with our results, applying bio-fertilizers as alternative sources of chemical fertilizers reduced nitrate and nitrite contents of some plants [43,44,72].

### 3.7 CO<sub>2</sub> Evaluation

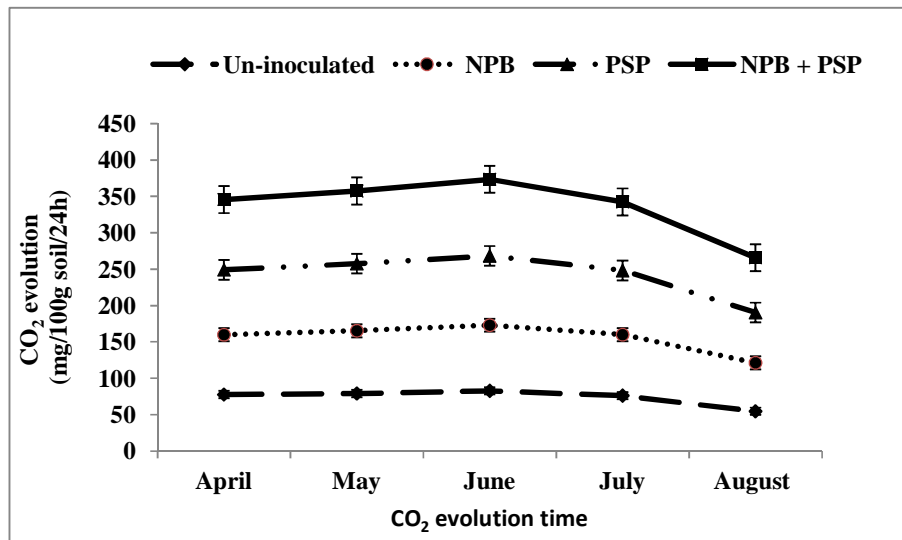
CO<sub>2</sub> evolution as an indicator of microbial activity is investigated under bio-fertilizer treatments in this study. The obtained results clearly show that, the CO<sub>2</sub> evolution in the soil of roselle plants inoculated with NFB or PSB was significantly higher compared with the control at any time

point during the growth. The highest CO<sub>2</sub> evolution was observed when both treatments were combined (Fig. 3). The CO<sub>2</sub> evolution in the soil of any treatment was gradually increased with the progressive of growth period from April to June and decreased thereafter during July and August which recorded the lowest values in this regard. These results are in accordance with those of Mazrou et al. [65] on roselle plants who found that application of bio-fertilizers significantly improved CO<sub>2</sub> evolution in the soil relative to untreated control. Otherwise, bio-fertilizers compounds have beneficial return to increase population of soil microorganisms, especially in the surface layer of root rhizosphere [5]. Moreover, El-Toukhy and Abdel-Azeem [76] on *Hordeum vulgare* observed the same previously trend.

**Table 4. Influence of different bacterial strains inoculation on some macro leaf mineral content of roselle plants**

Bio-fertilizer treatments	Macronutrients (mg g <sup>-1</sup> DW)		
	N	P	K
Un-inoculated	13.72d	2.06c	13.21d
NFB	19.88b	2.29b	17.62b
PSB	16.58c	3.15a	15.37c
NFB + PSB	20.01a	3.13a	18.85a

Un-inoculated; control plants, NFB; mixture of *Azotobacter chroococcum* and *Azospirillum brasilense* by 1:1 (v/v), PSB; mixture of *Bacillus megatherium* var. *phosphaticum* + *Bacillus polymyxa* by 1:1 (v/v), NFB + PSB; mixture of NFB and PSB by 1:1 (v/v). Means followed by different letters were significantly different according to Duncan multiple range test at 0.05 level (n=8).



**Fig. 3. Influence of different bacterial strains inoculation on CO<sub>2</sub> evolution in the experimental soil of roselle plants. Un-inoculated; control plants, NFB; mixture of *Azotobacter chroococcum* and *Azospirillum brasilense* by 1:1 (v/v), PSB; mixture of *Bacillus megatherium* var. *phosphaticum* + *Bacillus polymyxa* by 1:1 (v/v), NFB + PSB; mixture of NFB and PSB by 1:1 (v/v). Means followed by different letters were significantly different according to Duncan multiple range test at 0.05 level (n=8).**

#### 4. CONCLUSION

Conclusively, data obtained in this study showed that bio-fertilizer treatments improved the growth, yield and sepals quality of roselle plant. This treatment also enhanced the nutrient statuses in leaves as well as CO<sub>2</sub> evolution in soil. In addition, chlorophyll and carbohydrate contents were also promoted due to bio-fertilizer application. Otherwise, the content of nitrate and nitrite were significantly reduced as a result of bio-fertilizer inoculation which is the most important factor in the production of medicinal and aromatic plants because of the health hazard of higher nitrate to human.

#### ACKNOWLEDGEMENT

This study has been supported by Taif University, KSA project No. 1-437-5265 and it is appreciated. The authors are grateful to the Agency of Taif University for graduate studies and scientific research.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

#### REFERENCES

- Lyons DJ, Rayment GE, Nobbs PE, McCallum L. Nitrate and nitrite in fresh vegetables from Queensland. *J. Sci. Food Agric.* 1994;64:279-281.
- Rodriguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotech. Advan.* 1999;17:319-339.
- Hassan FAS, Ali EF, Mahfouz SA. Comparison between different fertilization sources, irrigation frequency and their combinations on the growth and yield of coriander plant. *Australian Journal of Basic and Applied Sciences.* 2012;6(3): 600-615.
- Fawzy ZF, El-Shal ZS, Yunsheng L, Zhu O, Sawan OM. Response of garlic (*Allium sativum* L.) plants to foliar spraying of some bio-stimulants under sandy soil condition. *J. Appl. Sci. Res.* 2012;8:770-776.
- Awad EM. Effect of compost and some bio-fertilizers on growth, yield and quality of potato crop (*Solanum tuberosum* L.). *J. Agric. Sci. Mansoura Univ.* 2002;27:5525-5537.
- Gendy ASH, Said-Al Ahl HAH, Mahmoud AA. Growth, productivity and chemical constituents of roselle (*Hibiscus sabdariffa* L.) plants as influenced by cattle manure and biofertilizers treatments. *Australian Journal of Basic and Applied Sciences.* 2012;6(5):1-12.
- Ali EF, Hassan F. Bio-production of *Nigella sativa* L. seeds and oil in Taifarea. *Int. J. Curr. Microbiol. App. Sci.* 2014;3(1):315-328.
- Mahdi SS, Hassan GI, Samoon SA, Rather HA, Dar SA, Zehra B. Bio-fertilizations in organic agriculture. *Journal of Phytology.* 2010;2(10):42-54.
- Yasin M, Ahmad K, Mussarat W, Tanveer A. Bio-fertilizers, substitution of synthetic fertilizers in cereals for leveraging agriculture. *Crop Environ.* 2012;3:62-66.
- Fallik E, Okon Y. Growth response of maize roots to *Azospirillum* inoculation: Effect of soil organic matter content, number of rhizosphere bacteria and timing of inoculation. *Soil Biol. Biochem.* 1988;20:45-49.
- Koreish EA, El-Fayoumy ME, Ramadan HM. Contribution of Vesicular Arbuscular Mycorrhiza and Phosphorien in acquisition of Phosphorus and micronutrient by maize (*Zea mays* L.) in a calcareous soil. *Egypt J. Soil Sci.* 1998;38(1-4):101-121.
- Ibrahim SM. Effect of seeding rate and bio-fertilization on fennel (*Foeniculum vulgare*, Mill) plants. M.Sc. Thesis., Fac. of Agric., Cairo Univ; 2001.
- Khater RM. Effect of some fertilizers treatment on the growth and volatile oil yield on *Carum carvi* (caraway) plant. M. Sc. Thesis. Agric. Zagazig Univ; 2001.
- Jat ML, Pal SS, Singh R. Crop residue treatment with mineral salts and its recycling under different tillage management practices and their impact on crop yields and soil health in a rice-wheat cropping system in Indo-Gangetic plains. In: 17<sup>th</sup> world Congress of Soil Science, August 14-21 2002, Bangkok, Thailand; 2002.
- Boraste A, Vamsi KK, Jhadav A, Khairnar Y, Gupta N, Trivedi S, Patil P, Gupta G, Gupta M, Mujapara AK, Joshi B. Biofertilizers: A novel tool for agriculture. *International Journal of Microbiology Research.* 2009;1(2):23-31.
- Abdou MA, Mahmoud AH. Growth and oil production of *Foeniculum vulgare* Mill. 2. Effect of number of irrigation and organic

- fertilizers. J. Agric. Sci. Mansoura, Univ. 2003;28(5):3868-3888.
17. El-Ghawwas EO, Eid MA, Mahmoud SM. Effect of different levels of organic manures and plant spacing on different (*Foeniculum vulgare*, Mill) Plants. Egypt J. Agric. Sci. 2002;5:198-219.
  18. Kandeel YM, Menesy FA, Khalafalla MM, Gad WM. Effect of some commercial biofertilizers on growth, seed, volatile oil yield and chemical composition of *Anethum graveolens* L. J. Agric. Res. Tanta Univ. 2004a;30(3):705-720.
  19. Kandeel YM, Menesy FA, Khalafalla MM, Gad WM. Effect of some commercial biofertilizers on growth, seed, volatile oil yield and chemical composition of *Anethum graveolens* L. J. Agric. Res. Tanta Univ. 2004b;30(3):721-737.
  20. Dadkhah A. Effect of chemicals and bio-fertilizers on yield, growth parameters and essential oil contents of funnel (*Foeniculum vulgare* Miller.). Journal of Medicinal Plants and By-products. 2012;2: 101-105.
  21. Abdullah AT, Hanafy MS, EL-Ghawwas EO, Ali ZH. Effect of compost and some biofertilizers on growth, yield, essential oil productivity and chemical composition of *Rosmarinus officinalis*, L. Plants. J Hort. Sci. Ornament Plants. 2012;4(2):201-214.
  22. Rosen, Carl J, Deborah L Allan. Exploring the benefits of organic nutrient sources for crop production and soil quality. Hort Technology. 2007;17:422-430.
  23. Harridy IM, Mervat, Amara. Effect of pre-sowing inoculation of seeds by nitrogen fixed bacteria on growth, fruit production, sepals yield and the chemical composition of roselle plants. Egypt. J. Appl. Sci. 1998;13(6):217-231.
  24. Shaalan MN, Abd-Ellatif TA, Soliman SG, El-Ghawwas EO. Effect of some chemical and biofertilizer treatments on roselle plants (*Hibiscus sabdariffa* L.). Egypt. J. Agric. Res. 2001a;79(2):587-606.
  25. Hassan F. Response of *Hibiscus sabdariffa* L. plant to some biofertilization treatments. Annals Agric. Sci. Ain Shams Univ. Cairo. 2009;54(2):437-446.
  26. Sokhangoy SH, Ansari K, Asli ED. Effect of bio-fertilizers on performance of dill (*Anethum graveolens*). Iranian Journal of Plant Physiology. 2012;2(4):547-552.
  27. Akhani A, Darzi MT, Hadi MHS. Effects of bio-fertilizer and plant density on yield components and seed yield of Coriander (*Coriandrum sativum*). International Journal of Agriculture and Crop Sciences. 2012;4-16:1205-1211.
  28. Chen J. The combined use of chemical and organic fertilizers and/or biofertilizer for crop growth and soil fertility. International Workshop on Sustained Management of the Soil-Rhizosphere System for Efficient Crop Production and Fertilizer Use. 16-20 October, Thailand; 2006.
  29. Wu SC, Caob ZH, Lib ZG, Cheung KC, Wong MH. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: A greenhouse trial. Geoderma. 2005;125:155-166.
  30. Abou-Aly HE, Gomaa AO. Influence of combined inoculation with diazotrophs and phosphate solubilizers on growth, yield and volatile oil content of coriander plants (*Coriandrum sativum* L.). Bull. Fac. Agric., Cairo Univ. 2002;53:99-113.
  31. Aly MK, Mohamed MA, Attia FA, El-Sayed AA, Abd El-Gawad MH. Effect of some organic and bio-fertilization treatments on coriander plants. The 1<sup>st</sup> Inter. Conf. on Desert Cultivation, Minia, Egypt, March 27-29; 2007.
  32. Yadav BD, Khurana SC. Effect of plant growth substances and Azotobacter on yield and yield attributing characters in fennel. Haryana Agricultural University Journal of Research. 2000;30(3):71-77.
  33. Kandeel YR, Nofal ES, Menesi FA, Reda KA, Taher MN, Zaki ZT. Effect of some cultural practices on growth and chemical composition of *Foeniculum vulgare*, Mill. Proc. Proc. 5<sup>th</sup> Arabian Hort. Conf., Ismailia, Egypt. 2001;24-28:61-72.
  34. Subramanian S, Vijayakumar M.. Effect of various levels of nitrogen and *Azospirillum* on growth and yield of coriander (*Coriandrum sativum* L.). South Indian Hort. 2001;49(3):191-194.
  35. Kumar SC, Choudhary GR, Chaudhari AC. Effects of nitrogen and biofertilizers on the yield and quality of coriander (*Coriandrum sativum* L.). Annals Agric. Res. 2002;23(4):634-637.
  36. Abd El-Kader HH, Ghaly NG. Effects of cutting the herb and the use of nitroben and phosphorein associated with mineral fertilizers on growth, fruit and oil yields, and chemical composition of the essential oil in coriander plants (*Coriandrum sativum*, L.). J. Agric. Sci. Mansoura Univ. 2003;28(3):2161-2171.

37. Hellal FA, Mahfouz SA, Hassan FAS. Partial substitution of mineral nitrogen fertilizer by bio-fertilizer on (*Anethum graveolens* L.) plant. Agri. Biol. J. North America. 2011;2(4):652-660.
38. Abdou MA, EL-Sayed AA,.Effect of planting date and biofertilization treatments on growth and yield characters of caraway crop (*Carum carvi* L.). Proc. 2<sup>nd</sup> Inter. Conf. Hort. Sci., Kafr El-Sheikh. Tanta Univ., Egypt. 2002;423-433.
39. Badawi AM. Effect of some agriculture treatments on growth and active ingredients in roselle plants in North Sinai. Ph.D. Thesis, Fac. Agric., Zagazig Univ; 2000.
40. Khalil FA, El-Aref KA. Bio-fertilizers as partner with mineral N-fertilizer for fertilizing wheat crop cultivar Sids. J. Agric. Sci. Mansoura Univ. 2001;26(12):8287-8295.
41. Shaalan MN, El-Ghawwas EO, Dessouky MM, Soliman SG. Effect of sources and levels of phosphorus fertilization on polish chamomile (*Matricaria chamomilla* L.). J. Agric. Sci. Mansoura Univ. 2001b;26(4): 2215-2233.
42. Susheela N, Dwivedi GK, Singh RV. Integrated nutrient management through biofertilizes, fertilizers organic manure and lime for vegetable pea in an acid inceptisol of cool temperate region of Uttaranchal. Legume Research. Agricultural Research Communication Center, Karnal, India. 2007;30(1):37-40.
43. Singh SR. Effect of organic farming system on yield and quality of brinjal (*Solanum melongena* L.) under mid-hill conditions of Himachal Pradesh. Haryana Journal of Horticulture Sciences. Horticultural Society of Haryana, Hisar, India. 2004;33(3):265-266.
44. Hassan FAS, Ali EF. A comparative study between traditional mineral nutrition and other alternative sources on anise plant. Europe. J. Sci. Res. 2013;106(2):201-212.
45. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 2003;255:571–586
46. Amin IS. Effect of bio and chemical fertilization on growth and production on *Coriandrium sativum*, L., *Foeniculum vulgare* L. and *Carum carvi* L. plants. Annals Agric. Sci. Moshtohor. 1997;35(4): 2327-2334.
47. Gomaa AO, Abou-Aly HE. Efficiency of biofertilization in the presence of inorganic and organic fertilizers on growth, yield and chemical constituents of anise plant (*Pimpinella anisum* L.). Proc. Fifth. Arab. Hort. Conf. Ismailia, Egypt. 2001;73-80.
48. Mahfouz SA, Sharaf-Eldin MA. Effect of mineral vs. biofertilizer on growth, yield and essential oil content of fennel (*Foeniculum vulgare* Mill.). International Agrophysics. 2007;21:361-366.
49. Shaalan MN. Influence of biofertilizers and chicken manure on growth, yield and seeds quality of *Nigella sativa* L. plants. Egypt. J. Agric. Res. 2005;83(2):811-828.
50. El-Zeiny OA, El-Behary UA, Zaky MH. Influence of biofertilizer on growth, yield and quality of tomato grown under plastic House. J. Agric. Sci. Mansoura Univ. 2001;26(3):1749-1763.
51. Gori A, Favilli F. First results on individual and dual inoculation with *Azospirillum* VI and related microorganisms, genetics-physiology-ecology, I. Fendrik, M. Del Gallo, J. Vanderleyden and M. de Zamaroczy (eds.), Germany. 1995;37:245-249.
52. Fuleki MA, Francis FJ. Quantitative method of anthocyanin. J. Food Sci. 1968;23:65-72.
53. Du CT, Francis FJ. Anthocyanin of roselle. J. Food Sci. 1973;38:8110-812.
54. Diab MA. The chemical composition of *Hibiscus sabdariffa* L. M. Sc. Thesis, Fac. Of Agric. Cairo Univ; 1968.
55. Sadasivam S, Manickam A. Biochemical methods for agriculture sciences. Wiley Eastern limited. 1992;181-185.
56. AOAC. Official methods of analysis of the association of official agricultural chemists (A.O.A.C.) 12<sup>th</sup> Ed. Washington. D.C.; 1980.
57. Herbert D, Phipps PJ, Strange RE. Determination of total carbohydrates. Methods in Microbiology. 1971;5(8):290-344.
58. Piper CS. Soil and plant analysis. 2<sup>nd</sup> Ed., Asia Pub. House, Bombay, India; 1967.
59. Black CA, Evans DD, Ensminger LE. Methods of soil analysis. Agron. J. Amer. Soc. Agron. Inc. Publ., Madison, Wisconsin, U.S.A.; 1965.
60. Jackson ML. Soil chemical analysis. Fall Indian Private. Ltd. New Delhi; 1978.
61. Follet RH, Ratcliff PW. Determination of nitrite and nitrate in meat products. J. Sci. Agric. 1963;14:138-144.

62. Promer D, Schmid EL. Experimental soil microbiology. Burgen Publishing Company, Minnesota, USA; 1964.
63. Alef K, Nannipieri P. Methods in applied soil microbiology and biochemistry. Academic Press. Harcourt Brace and Company Publishers, London. 1995;214-217.
64. El-Nagdy GA, Dalia Nassar MA, El-Kady AE, Galan El-Yamane SA. Response of flax plant (*Linum usitatissimum* L.) to treatments with mineral and bio-fertilizers from nitrogen and phosphorus. J. of American Science. 2010;6(10):207-217.
65. Mazrou M, Afifi M, Hassan F, Amer E. Effect of biofertilizers application on the growth and yield of *Hibiscus sabdariffa* L. plant. Minufiya J. Agric. Res. 2013;3(2): 775–784.
66. Getachew Z, Abera G, Beyene S. *Rhizobium* inoculation and sulphur fertilizer improved yield, nutrients uptake and protein quality of soybean (*Glycine max* L.) varieties on Nitisols of Assosa area, Western Ethiopia. African Journal of Plant Science. 2017;11(5):123-132.
67. Naser HM, El-Hosieny H, Elsheery NI, Kalaji HM. Effect of biofertilizers and putrescine amine on the physiological features and productivity of date palm (*Phoenix dactylifera* L.) grown on reclaimed-salinized soil. Trees 2016;30: 1149–1161.
68. Ramadan BS, Hassan HR, Abdo FA. Effect of minerals and biofertilizers on photosynthetic pigments, root quality, yield components and anatomical structure of sugar beet (*Beta vulgaris* L.) plants grown under reclaimed soil. J. Agric. Sci. Mansoura Univ. 2003;28(7):5139-5160.
69. Hassan EA, Ali EF, Ali AF. The enhancement of plant growth, yield and some chemical constituents of dill (*Anethum graveolens* L.) plants by filter mud cake and potassium treatments. Australian Journal of Basic and Applied Sciences. 2010;4(5):948-956.
70. Singh S, Kapoor KK. Inoculation with phosphate-solubilizing microorganisms and a vesicular *Arbuscular mycorrhizal* fungus improve dry matter yield and nutrient uptake by wheat grown in a sandy soil. Biol. Fertil. Soils. 1999;28:139–44.
71. Tamme T, Reinik M, Roasto M, Juhkam K, Tenno T, Kiis A. Nitrates and nitrites in vegetables and vegetable-based products and their intakes by the Estonian population. Food Addit. Contam. 2006;23: 355–361.
72. Aires A, Carvalho R, Rosa EAS, Saavedra MJ. Effects of agriculture production systems on nitrate and nitrite accumulation on baby-leaf salads. Food Science & Nutrition. 2013;1(1):3–7.
73. Savino F, Maccario S, Guidi C, Castagno E, Farinasso D, Cresi F, et al. Methemoglobinemia caused by the ingestion of courgette soup given in order to resolve constipation in two formula-fed infants. Ann. Nutr. Metab. 2006;50:368–371.
74. Sheng LI. Fertilization in the third millennium fertilizer, food security and environmental protection 12 International World Fertilizer Congress of CIEG. Beijing, China August 3-9 Proc. 2001;11:910-917.
75. Hanafy Ahmed AH, Mishriky JF, Khalil MK. Reducing nitrate accumulation in lettuce (*Lactuca sativa* L.) plants by using different biofertilizers. ICEHM, Cairo University, Egypt. 2000;509-517.
76. El-Toukhy SA, Abdel-Azeem HH. Response of Barley (*Hordeum vulgare*) to biofertilization technology. Annals of Agricultural Sci. Cairo. 2000;2:539-559.

© 2017 Kahil et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:  
The peer review history for this paper can be accessed here:  
<http://sciedomains.org/review-history/20937>