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INFLUENCE OF DIFFERENT ALGAL SPECIES APPLICATION ON GROWTH OF SPINACH PLANT *(SPINACIA OLERACEA* L.*)* AND THEIR ROLE IN PHYTOREMEDIATION OF HEAVY METALS FROM POLLUTED SOIL

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Abstract

Pot experiment was conducted to evaluate the effect of algal species, concentration and application methods on the growth and quality of spinach *(Spinacia oleracea L.)* plant. This experiment was carried out at greenhouse of National Research Centre, Dokki, Egypt. Algal species used in this study were: *Chlorella vulgaris* and *Anabaena sphaerica*, with the following concentration: 1% and 2% and two different application methods: soil and foliar application. Soil samples were collected from El-Gabal El-Asfar-Egypt contaminated soil with sewage sludge.

The obtained results showed that:

- All the tested algal strain had promotive effect on growth of spinach plants as compared with control, indicating that nutrients and extra cellar substances excreted by algae had beneficial effect on plant growth.
- Application of *Chlorella vulgaris* at concentration 2% under soil application was more effective for spinach growth while application of *Anabaena cylindrical* was the best under foliar application.
- The addition of algae resulted in promotive effect in fresh and dry weight of spinach plant, nutrients concentration and uptake.
- Application of algae reduced the concentration of heavy metal in spinach plant.

Keywords: Bio-fertilizers- *Chlorella vulgaris, Anabaena sphaerica* algae, application method, Spinach, growth and quality.

Introduction

Spinach has been used by various cultures throughout history, notably Mediterranean, Middle-Eastern, and South East Asian cuisines. It can be incorporated quite easily into many diets because it is cheap and easy to prepare.

Spinach contains an antioxidant known as alpha-lipoic acid, which has been shown to lower glucose levels, increase insulin sensitivity, and prevent oxidative stress-induced changes in patients with diabetes (Machowsky, 2017)

Spinach and other green vegetables contain chlorophyll, which has been shown to be effective at blocking the carcinogenic effects of heterocyclic amines, which are generated when grilling foods at a high temperature. Due to its high potassium content, spinach is recommended for people with high blood pressure; it can help reduce the effects of sodium in the body. A low potassium intake may be just as big of a risk factor for developing high blood pressure as a high sodium intake.

Spinach is high in vitamin A, which is necessary for sebum production to keep hair moisturized. Vitamin A is also necessary for the growth of all bodily tissues, including skin and hair. Spinach and other leafy greens high in vitamin C are imperative for the building and maintenance of collagen, which provides structure to skin and hair. Iron deficiency is a common cause of hair loss, which may be prevented by an adequate intake of iron-rich foods, like spinach. Machowsky, 2017) Chemical fertilizers improve crop yields by providing essential plant nutrients which are easily available to plants; however, their abuse can be harmful for the environment and their use implies increased production costs which reduce the economic viability of agricultural products Adesemoye *et al.*, 2009). Microalgae may be use to decrease negative environmental impacts resulting from continued use of chemical fertilizers, Microalgae, help in replacing chemical fertilizers through benefits to plant growth and crop yield and may contribute to CO_2 sequestration because they add organic matter to the soil (Zaccaro *et al.*, 1999; Maqubela *et al.*, 2009).

Fresh water green microalgae extracts appeared to be promising natural fertilizers. They contain high macro and micronutrients concentrations in addition to the natural enzymes and hormones (Shaaban, 2001a). Kannan and Tamil (1990) observed that soil application of seaweed liquid fertilizer of *Enteromorpha clathrata* and *Hypnea musciformis* increased the growth characteristics of green gram, black gram and rice. Fayza and Zeinab (2008) found that addition of *chlorella vulgaris* to the soil significantly increased fresh and dry weight of seedling as well as pigments.

Moreover, vegetative growth of olive transplants was markedly enhanced as their root zoon was surrounded by pre-digested *Scenedesmus* bulk at the concentration equal to the recommended nitrogen dose. Nutrient concentrations and balance were also improved by the partial substitution of nitrogen by algal bulk (Abdel-Maguid *et al.*, 2004).

In addition, microalgae biomass is a rich source of metabolites in agriculture (Nirmal *et al.*, 2018) and also freshwater microalgae such as *Chlorella vulgaris* have been shown to provide high amounts of macro- and micronutrients and proteins (Elarroussia *et al.*, 2016) and growth promoting factors, such as cytokinins (Stirk *et al.*, 2002). Another work of Grzzesik *et al.* (2017) indicated that foliar application with *chlorlla sp.* caused an increase in growth and physiological Performance of willow plants.

Shaaban *et al.* (2010) showed that the best uptake, nutrient balance and dry matter accumulation was recorded with combined algal extract and micronutrient fertilizer treatment.

Blue green algal extract excretes a great number of substances that influence plant growth and development (Ordog, 1999). These microorganisms have been reported to benefit plants by producing growth promoting regulators (the nature of which is said to resemble gibberellin and auxin), vitamins, amino acids, polypeptides, antibacterial and antifungal substances that exert phytopathogen biocontrol and polymers, especially exopolysaccharides, that improve plant growth and productivity (Zaccaro *et al.,* 1999). Moreover, Zaccaro *et al.* (2001) reported that, foliar application of biochemical organic substances, which supply macro and micro nutrients, of increased demand. Fresh water algae contain high percentage of macro and micronutrients bounded in their major biochemical constituents and metabolites such as carbohydrates and proteins (Wake *et al*., 1992). In this respect Adam (1999) found that algal filtrate of the cyanobacterium *Nostoc muscorum* significantly increased germination of wheat seeds as well as their growth parameters and nitrogen compounds, compared to controls. On the other hand,

Faheed and Abd-El Fattah (2008) observed increases in germination, fresh and dry weights, and pigment content of *Lactuca sativa* seedlings treated with *Chlorella vulgaris*.

Organic pollutants and heavy metals are considered to be a serious environmental problem for human health. The contamination of soils and aquatic systems by toxic metals and organic pollutants has recently increased due to anthropogenic activity. These problems of pollution can be partially solved by the application of phytoremediation technologies using algae or aquatic plants to remove pollutants from the environment (Abd El-Aty *et al*., 2013; Abd El-Aty *et al*., 2015; Kaoutar and Mourad, 2008).

Some algal strain show a high capacity for accumulation of heavy metals as results of tolerance mechanisms and many algae synthesize phytochelatins and metallothioneins that can form complexes with heavy metals and translocate them into vacuoles (Suresh, 2004) Recently, the use of aquatic plants especially micro and macro algae has received much attention due to their ability to absorption of metals and taking up toxic elements from the environment or rendering them less harmful (Matagi *et al*., 2012).

Objectives of this work focused on the effect of different algal biomass (bio-fertilizer) as soil and foliar additive on growth, yield quality and some nutrient uptake of spinach plant and determine the ability of algae to reduce the accumulation of heavy metals in spinach plant.

Materials and Methods

Soil samples were collected from ElGabal ElAsfar, Egypt. Particle size distribution and soil texture along with soil moisture constants of the representative soil samples were determined according to Klute, (1986). Contents of organic matter and $CaCO_3$ as well as EC and pH were evaluated according to Black *et al.* (1982). Total N and available P, K, total Cu, Pb, Cd and available Cu, Pb and Cd were also determined according to Jackson (1973). Some physical and chemical of the used soil are shown in Table (1).

Table 1 : Some physical and chemical properties of El-Gabal El-Asfar soils.

% Sand	% Silt	% Clay	Soil texture	pH 1:2.5	E.C dS/m	% $CaCO_3$	% O.M	%			Total heavy metals (ppm)			Available heavy metals (ppm)		
								N	P	K	Pb	Cd	Cu	Pb	Cd	Cu
74.83	8.84	16.3	Sandy loam	7.1	0.27	0.4	4.9	0.39	0.05	0.12	70	0.6	47.6	3.42	0.17	14.7

Isolation, Purification and culture condition of algal species

The algal strains selected for the study was a fresh water micro-alga, which were isolated from phytoplankton community of the River Nile. Two microalgae *Anabaena sphaerica*, N_2-fixing *Cyanobacterial* species and *Chlorella vulgaris* which belongs to green algae *(Chloropyta)*. The algal species transferred and cultivated in a fresh algal nutrient medium BG11 (Carmichael, 1986). *Chlorella vulgaris* isolated in 100 % $NaNO_3$ (1.5 g/l), where $NaNO_3$ was excluded completely from *Anabaena sphaerica* media (Abd El-Aty *et al*., 2015).

The algal species were in the maximum growth when introduced to the standard media. The cultures were incubated at 24 ± 2 and 30 ± 2 ^oC for *Chlorella vulgaris* and *Anabaena sphaerica* under continuous white fluorescent illumination (33.3 E/m^2/s). Cultures were shaken once daily to prevent clumping of algal cells and adherence to the flasks.

Cultivation was carried out in sterilized conical flasks .The cultivation time differed from one strain to another depending on the optimum growth rate till reaching stationary phase which always ranged between (10-15) days.

Preparation of biomass for extract

At optimal growth, the algae were harvested by centrifugation at 3000 rpm for 15 min. Then, after removal of clear liquid, the pellet of algae was washed several times by distilled water till the effluents became almost transparent. The washed biomass was then dried in an oven at 40^{o}C until a constant weight was reached. The dried biomass was then ground into fractions. The algae were stored in an air tight container in a dry place.

Cell extracts were made by grinding the algae in distilled water with a pestle and blender. The blended material was filtered to remove debris.

Algal suspension containing 10 and 20 g dry algal material in 1000 mL of distilled water is referred to as 1 and 2% extract respectively.

Table 2 : Chemical composition of algae expressed on a dry matter basis (%).

Strain	Protein %	Carbohydrates %	Lipids	Surface area m^2/g	Total polysucrose
Chlorella vulgaris	51-58	12-17	14-22	29.18	0.36
Anabaena sphaerica	43-56	25-30	4-7	52.2	0.593

Pot experiment was conducted in the greenhouse of National Research Centre (NRC), Dokki, Giza, Egypt, to study the effect of algae (*Chlorolla vulgaris* and *Anabena sphaerica*), chemical composition of algal strain presented in table (2). Two algal concentrations were used (C1=1% and C2= 2%) and method of algal application (foliar and soil application) on plant growth and quality.

The treatments were arranged in a randomized complete block design factorial, with two factors and three replications. Factors of the experiment were as follows: (A): algal strain and (B) method of application. Used plastic pots, 5kg capacity of air dried soil ten seeds of spinach *(Spinach Olerecea)* were sown in each pot by pressing them into soil to depth of 0.5 cm and the following treatments were applied: 1- Control, 2- *Chlorolla vulgaris* 1% as foliar application, 3- *Chlorolla vulgaris* 2% as foliar application 4- *Anabena sphaerica* 1% as foliar application, 5- *Anabena sphaerica* 2% as foliar application, 6- *Chlorolla vulgaris* 1% as soil application, 7- *Chlorolla vulgaris* 2% as soil application 8- *Anabena sphaerica* 1% as soil application and 9- *Anabena sphaerica* 2% as soil application.

The pots were watered daily to 70% of the water holding capacity, and then thinned out to 5 seedlings per pot after 10 days then treated by algae and allowed to grow for a period of 75 days.

The basic amounts of mineral fertilizers were applied (20 kg fed^{-1} ammonium sulphate, 100 kg fed^{-1} super phosphate and 50 kg fed^{-1} potassium sulphate) for each pots.

At harvesting, plants were carefully removed, washed with tap water, to remove any attached particles, rinsed twice with distilled water, fresh and dry weight were record. Nitrogen ,phosphorus, potassium and trace elements (Cd, Pb, Cu) in plant were analyzed . All the analysis were determined using standard methods by Jackson (1973), Lindsay and Norvell (1978). Statistical analyses were conducted as described by Snedecor and Cochran (1982).

Results and Discussions

Fresh and dry weight: Effect of the algal strain and methods of application on the fresh and dry weight of the spinach plants shown in the table (3). Fresh and dry weight of spinach plants were increased by using different algae strain, concentrations and two methods of application. All addition of algae resulted in promotive effect in fresh and dry weight of spinach plants.

Table 3 : Effect of species, concentrations and methods of addition algae on fresh and dry weight of the spinach plants.

Treatments	Weight of spinach plants (g)							
	Chlorella vulgaris				*Anabaena sphaerica*			
	Fresh		Dry		Fresh		Dry	
	C1	C2	C1	C2	C1	C2	C1	C2
Control	28.00	28.00	10.00	10.00	28.00	28.00	10.00	10.00
Foliar application	32.20	36.27	11.16	11.75	37.80	36.20	13.58	13.10
Soil application	36.50	38.80	12.86	13.20	34.80	31.90	12.65	12.30

The results indicated the fresh weight of spinach plants increased by 21% and 25% in treatment of *Chorella vulgaris* and *Anabaena sphaerica* algal, while addition of two species of algae increased the dry weight of spinach plants by 24 and 29% as compared with control.

General observation in growing plants indicated that algal application enhanced plant growth .it seems that nutrients or bioactive compound in algae were effective in improving the growth of spinach plants as compared to control treatment. These results were in agreement with findings of Rajaa Kholssi *et al*. (2018) who stated that treatments with filtrate of *Chlorella soroiniana* improved the total biomass by 22% and 51% as compared to control, indicating that nutrients and extra-cellular substances excreted by algae were pertinent to the beneficial effects on plant growth. These results are in harmony with those obtained by Brahmbhatt and Kalasariya (2015) they reported that addition of all algal extracts can enhance plant growth of radical.

The best result of spinach growth founded at algal extract concentrate 2% with *Chlorella vulgaris* under soil application while the highest fresh and dry matter of spinach plants was found at *Anabaena sphaerica* under foliar application with extract concentrate 1%. These observations are consistent with previous reports obtained by Brahmbhatt and Kalasariya (2015)

Furthermore, biofertilizers enhanced the growth parameter–which leads to improve the crop productivity (Ghosh and Mohiuddin, 2000). These results are in agreement with those obtained by Mahmoud and Amara (2000) and Das *et al*. (2001). Also, Ali and Mostafa (2009) found that the effect of foliar spray or soil application methods of potassium-humate and *Spirulina platensis* (individually or combined) as bio-organic fertilizer on sesame yield and its attributes. They found that combined foliar application recorded the highest values of plant height, number of capsule/ plant, number of branches/ plant, seed weight/ plant and 1000 seed weight. While, combined soil application gave the highest values of seed and straw yield.

Data in Figure (1) show that the highest percentage increase in dry weight for spinach plants was recorded at foliar application with strain *Anabaena sphaerica* under concentration 1% which amounted to 35.8% while the lowest rate of increase was at foliar application with *Chlorella vulgaris* under concentration 1% (11.6%). The obtained results are good accordance with those which were recorded by Brahmbhatt and Kalasariya (2015) reported that each strain of algae is given a different effect on vegetable growth.

It was observed that soil additives of *Chlorella vulgaris* extract improve plant nutrients which in turn enhances all the physiological reactions that lead to a good growth, on the other hand the *Anabaena sphaerica* gave the best results when used as foliar as soil additives and there are slightly different effect between the two stains of algae on the growth of spinach . In this concert Nikolaos *et al*. (2016) showed the mineralization of soil organic carbon increased by 16.2-35.9% at all rates of algae addition compared with control.

Macronutrients concentration and uptake: Data in Table (4) show that all treatments led to increased spinach content of the macronutrients (N, P and K) compared with untreated treatments (control). Concentrations of macronutrients in spinach plants was affected by different concentration of algal extract where N, P and K content increases with the increase in concentration of *Chlorella vulgaris* extract, but the content of N, P and K in spinach plants did not affect by increasing the concentration of algae extract of *Anabaena sphaerica*.

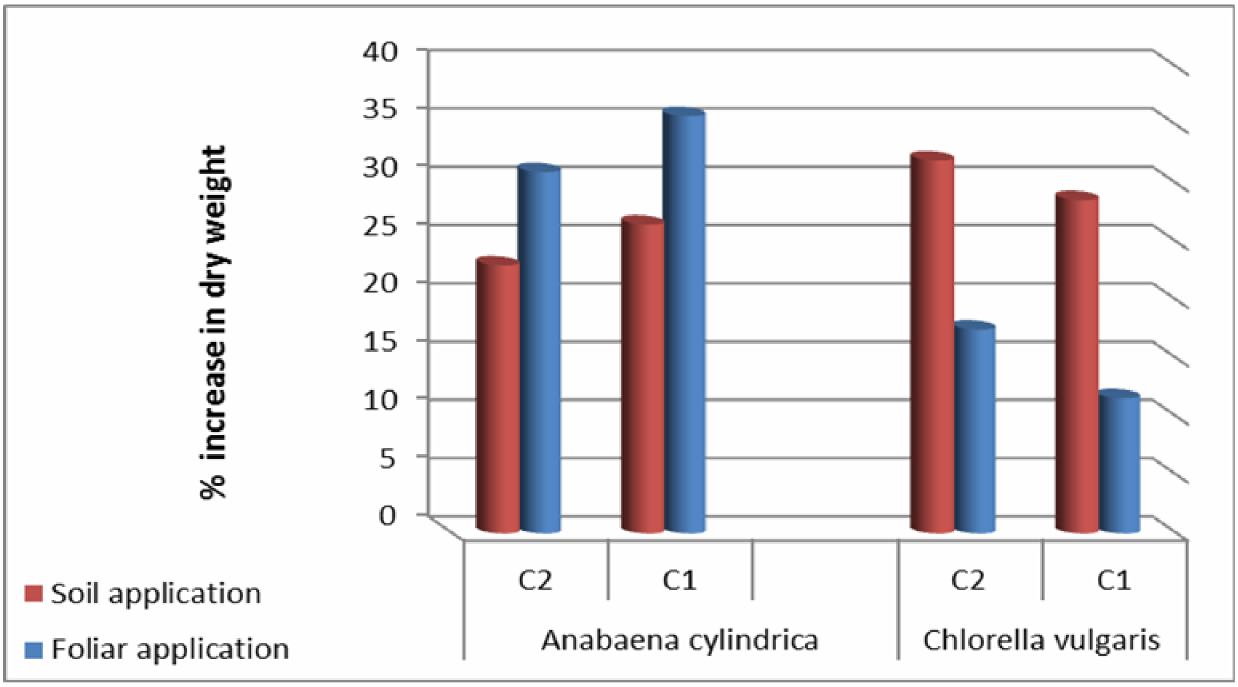

Fig. 1 : Effect of algal species, concentration and method of application on the rate of-increase in dry weight percentage of spinach plants.

Table 4 : Effect of algal species, concentration and method of addition on macronutrients concentration (%) in spinach plants.

Treatment	Macronutrients concentration (%)											
	Chlorella vulgaris						***Anabaena sphaerica***					
	N		**P**		**K**		**N**		**P**		**K**	
	C1	**C2**	**C1**	**C2**	**C1**	**C2**	**C1**	**C2**	**C1**	**C2**	**C1**	**C2**
control	2.00	2.00	0.10	0.10	1.9	1.9	2.00	2.00	0.100	0.100	1.9	1.9
Foliar applied	2.23	2.46	0.12	0.138	2.5	3.3	2.80	2.66	0.172	0.166	3.3	3.1
Soil applied	2.50	2.51	0.13	0.142	3.0	3.4	2.46	2.38	0.156	0.152	3.2	3.0

These results were in good harmony with those obtained by Ordog (1999) who documented that the suspension of cyanobacterial and microalgal extract contain a special set of biologically active compounds including plant growth regulators, which can be used for treatment to decrease senescence, transpiration as well as to increase leaf, chlorophyll, protein content and root development, also, Ghallab and Salem (2011) found that the two biofertilizers increased nutrients content in wheat plant.

Data in Figure (2-4) shows that N, P and K uptake in spinach plants under all treatments were increased compared to control. The highest increase of N uptake was recorded when *Chlorella vulgaris* added with C2 concentration by soil application. These results are in agreement with those reported by (Nirmal *et al.*, 2018 and Kholssi *et al.*, 2017) who reported that micro algal biomass is a rich source of metabolites in agriculture and also produces extracellular polymeric substances (EPS). In addition freshwater microalgae such as *Chlorella vulgaris* have been shown to provide high amounts of macro- and micronutrients, as constituents or metabolites, such as carbohydrates and proteins (Elarroussia *et al.* 2016) and growth promoting factors, such as cytokinins (Stirk *et al.*, 2002). Similar trend was found by Adam (1999) who showed that the improvement of the nitrogen contents was observed in response to application of cyanobacteria as biofertilizers on seed and related processes of wheat, sorghum, maize and lentil.

Although the highest increase of P and K uptake were observed when *Anabaena sphaerica* added at extract concentrated C1 by foliar application. This corresponds to (Bloemberg *et al.*, 2000) who reported that microbiological fertilizers are important to environment friendly stainable agricultural practices. Also, (Goel *et al.*, 1999) found that the bio-fertilizers include mainly the nitrogen fixing, phosphate solubilizing and plant growth promoting microorganisms.

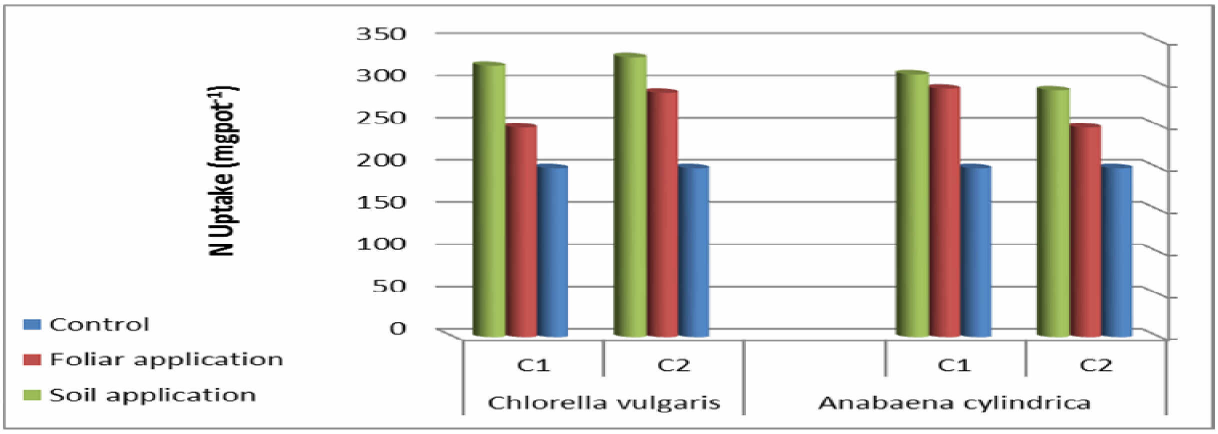

Fig. 2 : Effect of algae species, concentration and method of application on N uptake in spinach plants.

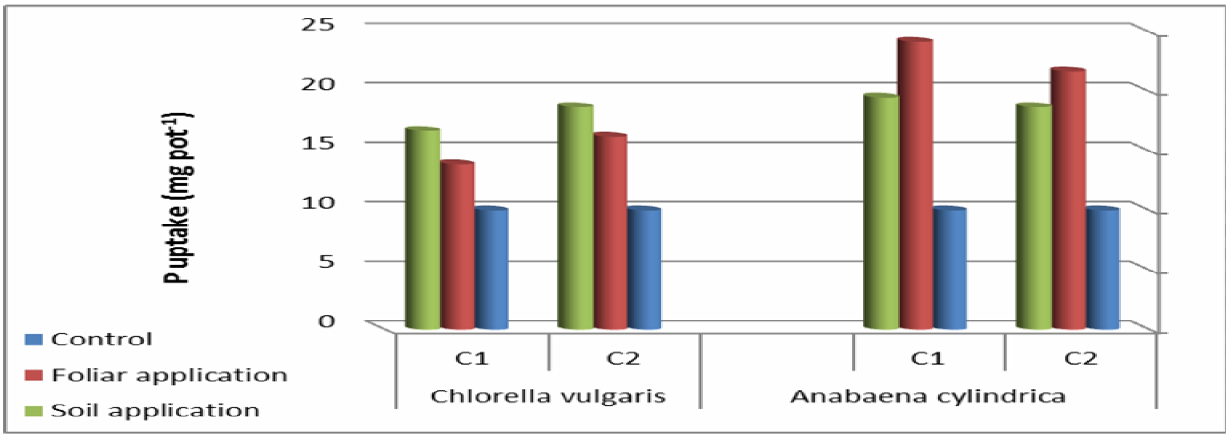

Fig. 3 : Effect of algae species, concentration and method of application on P uptake in spinach plants.

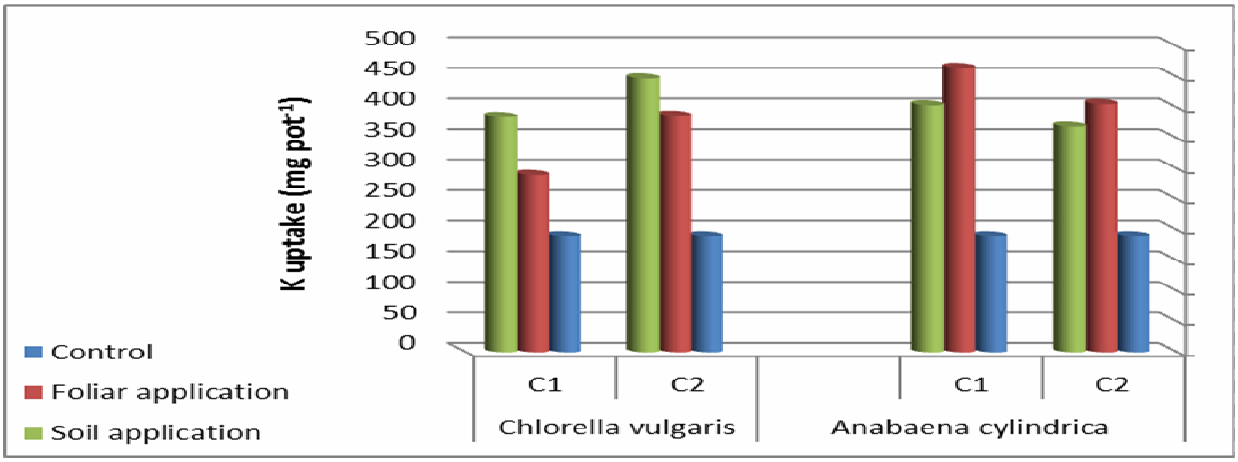

Fig. 4 : Effect of algae species, concentration and method of application on K uptake in spinach plants.

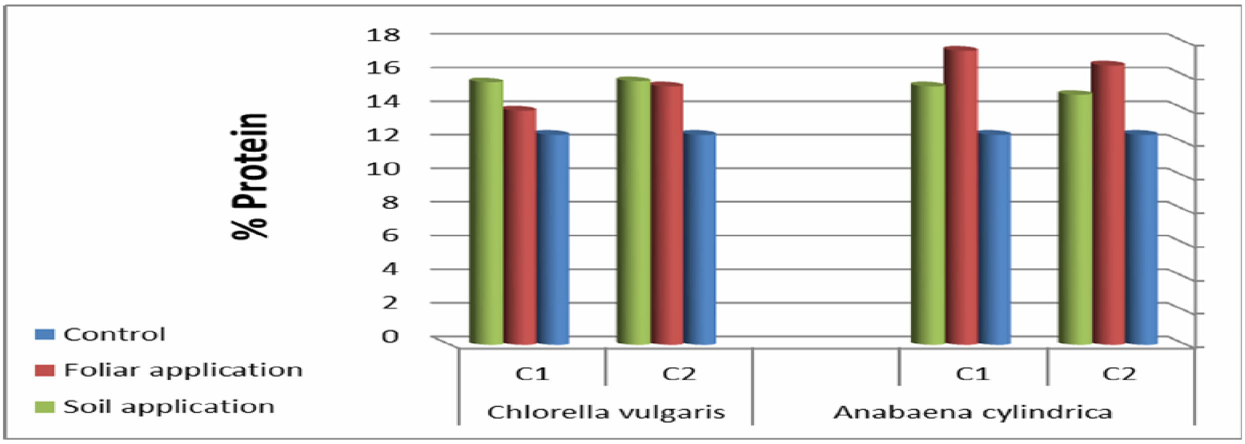

Fig. 5 : Effect of algae species, concentration and method of application on protein content in spinach plants.

Table 5 : Effect of algal species, concentration and method of addition on heavy metals content (ppm) in spinach plants.

Treatment	Heavy metal content (ppm)											
	Chlorella vulgaris						*Anabaena sphaerica*					
	Cd		Pb		Cu		Cd		Pb		Cu	
	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2
control	1.00	1.00	4.30	4.30	10.6	10.6	1.0	1.0	4.3	4.3	10.6	10.6
Foliar applied	0.82	0.77	4.00	3.91	10.5	10.4	0.8	0.7	4.0	4.1	10.0	10.2
Soil applied	0.70	0.73	3.83	3.70	10.4	10.1	0.9	0.9	4.1	4.1	10.3	10.5

Protein content: Data in Figure (5) show the effect of algal species, concentration and method of addition on protein content for spinach plant. Protein percentage increased about 11.52% - 40.0% at foliar application and between 19.04% - 25.52% at soil application. Also, protein content is affected by algal strain where *Chlorella vulgaris* increase protein percentage from 11.52% to 25.52% and *Anabaena sphaerica* from 19.04% to 40.0%. This may attributed to protein content of the algal extract which split into natural plant amino acids involved directly in the metabolism (Shaaban 2001a). These results are in accordance with those obtained by Schreber *et al.* (2018) who describe the potential of green algae *chlorella vulgaris* to accumulate phosphorus and other elements such as N and P in shoots and roots of wheat plant.

Heavy Metal Content: Available results represented in Table (5) revealed the ability of biofertilizers to reduce heavy metals accumulation in spinach plants. Cadmium was reduced to (18%-30.0%), (10%-30%), Pb reduced about (6.9%- 13.9%), (2.32%- 6.97%) and Cu reduced about (1% - 4.7%), (1%-5.6%) when treated by *Chlorella vulgaris* and *Anabaena* in foliar and soil applied, respectively.

This may be algae show high capacity for accumulation of heavy metals as results of tolerance mechanism and many algae synthesize phytochelatins that can form complexes with heavy metals and translocate them into vacuoles (Suresh *et al*., 2004). Generally, foliar application more enhanced in reduced of heavy metals concentration in plant when used *Anabaena sphaerica* compared soil application while *chlorella vulgaris* has positive effect in soil applied. Such results coincide with that obtained by Ali *et al.* (2009) they found that foliar application recorded the highest effect on quality of sesame plants.

The highest ability of *chlorella vulgaris* and *Anabaena sphaerica* to accumulate metal within their tissues found at Cd element compared with other elements. Recently, the use of aquatic plants especially micro and macro algae has received much attention due to their ability to absorb the metals and toxic elements from the environment or rendering them less harmful (Mitra *et al*., 2012).

Conclusion

This study revealed that micro algal extracts enhanced the growth of spinach plants and play role in plant nutrition and can used the algae to improve soil fertility. The best result of spinach growth and quality found at algae extract concentrate 2% with *Chlorella vulgaris* under soil application while the highest resulted of spinach plants was found at *Anabaena sphaerica* under foliar application with extract concentrate 1%. Generally, *Anabaena sphaerica* strain gave better results for spinach growth, macronutrients, and protein contents than *Chlorella vulgaris* strain. Also, all biofertilizers treatment reduced heavy metals contents in spinach plants. Such results indicated that algae can be used to improve soil fertility

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