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Full Length Research Paper

Potential Activity of Some Biofertilizer Agents on Antioxidant and Phytochemical Constituents of Faba bean Plant

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Faba bean (*Viciafabae* L.,) is an annual legume crop which is consumed as plant foods for human and animal nutrition. So that, *Rhizobium leguminosarum* was used as nitrogen fixer with *Aphanocapsaalpida* and *Laurenciaobtusa* as biofertilizer agents to enrich faba bean plants with primary and secondary phytochemical content especially: nitrogen, protein, flavonoids, anteoxidants and polyphenolics contents. The results in this study showed that application of *Aphanocapsaalbida* caused the highest increase of polyphenolic content in the root of faba bean plants followed by the treatment of *Rhizobium leguminosarum* var*., faba*e*+ Laurenciaobtusa* (10.78,7.97 and 7.54 mg Gallic acids /gm. dry wt. respectively), while the treatment of *Laurenciaobtusa + Aphanocapsa albida* gave the highest increase in polyphenolic content in the shoots of faba bean plants in compared with the other treatments and control (6.11 mg Gallic acids/gm. Dry wt.). All the treatments caused a significant increase in the shoots and roots antioxidants activity but *Aphanocapsaalbida* was the superior one. *Rhizobium leguminosarum* var. *faba*, caused the highest increase in flavonoids contents in the shoots of faba bean plants followed by *Rhizobium leguminosarum* var. *Faba + Laurenciaobtusa* whereas the combination of different treatments caused the highest amounts of flavonoidsin the roots followed by the treatment of *Rhizobium leguminosarum* var. *faba+Laurenciaobtusa*. All the treatments caused increase in the both of shoots and roots protein content. The treatment *Laurenciaobtusa + Aphanocapsaalbida* caused the best result which reached 12.5% and 11.87% in roots and shoots respectively followed by the treatment of *Aphanocapsaalbida* which caused 11.87% in the both of roots and shoots of faba bean plants.

Keywords: faba bean, phytochemicals content, antioxidant, polyphenole, Tannic acid, flavonoids, protein contents.

INTRODUCTION

Grain legumes are major crops cultivated in the Northern and the River Nile Governorates of Egypt. For example, faba bean (*Viciafaba* L.) is produced in an average area of 69720 ha. with an average yield of 1896 kg/ha (AOAD, 2007). Grain legumes play an essential role in human nutrition balancing the deficiencies of cereal-based diet (Dartand Krantz, 1977). Faba bean (*V. faba* L.) is an annual legume that is consumed as plant foods for human

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and animal nutrition, because it is rich in protein. Nitrogen is one of the most important elements for growth of plants, so that nitrogen deficiency is one of the most important limited factors in agricultural production (Dashadi *et al*., 2011). Nitrogen is one of the most consumed chemical fertilizers in the world in other hand these fertilizers are considered as a major environmental pollutant (McIsaac, 2003). Faba bean has a nitrogen fixation symbiosis in relation with *Rhizobium Leguminosarumbv. Viciae* (Dashadi *et al*., 2011). Inoculation with Rhizobium increased the seed yield of faba bean in six areas in Australia. Biofertilizers are likely to assume greater significance as complement and/or supplement to chemical fertilizers in improving the nutrient supplies to cereal crops because of high nutrient turn-over in cereal production system, exorbitant cost of fertilizers and greater consciousness on environmental protection(Ahmed, 2009). Manach *et. al.,* (2004) reported that nowadays, emphasis multi strains biofertilizer has already been tidied. Biofertilizers are biological preparations embodying, essentially, sufficient densities of potent strains of microorganisms, having a tangible beneficial role in filleting a proper rhizosphere for plant growth (Saber, 2001). Organically grown cabbage, spinach, welch, union, green pepper generally had higher levels of flavonoids and antioxidants activity (Ran *et al*., 2001).). Dinitos (2006) showed that the health benefits of fruits and vegetables are largely due to the antioxidants and vitamins supported by the large number of phytochemicals, some with greater antioxidant properties. Also, Asami, *et al.,* (2003) mentioned that phenolic and ascorbic acids are presented in higher levels in organic corn, strawberry and marine berry than in conventional. Dave *et al.,* (2013) found that there was quantitative increase in total phenol, total protein and major three fatty acids after treatment. Abd El-Moniem, and Abd-allah, (2008) reported that algae extract is a new biofertilizer containing N, P, K, Ca, Mg, and S as well as Zn, Fe, Mn, Cu, Mo, and Co, some growth regulators, polyamines, natural enzymes carbohydrates, proteins and vitamins applied to improve vegetative growth and yield. Safinaz and Ragaa (2013) reported that, using marine algae as biofertilizers improved the vegetative characters of maize plants. Al-Shakankery *et. al*., (2014) stated that there is a significant increase in total phenol, ascorbic acids and nitrogen content of maize grains of plants treated with algae as a biofertilizer compared to that of the control maize plants. Using combination of marine algae and cyanobacteria as bioferrtilizers agents improved the growth and phenol content of faba bean (*V. faba* L.) (Hamouda and Farfour, 2013). Cyanobacteria are one of the major components of the nitrogen fixing biomass in paddy fields, due to the important characteristic of nitrogen fixation. Cyanobacteria plays an important role to build up soil fertility consequently increasing in the yield, (Sahu *et al*., 2012 and Song *et al*., 2005).

The aim of the present study was to evaluate the effects of individual and combined *Rhizobium leguminosarum* var., *faba*, *Aphanocapsaalbida* (cyanobacteria) and *Laurenciaobtusa* (red algae) applications on the protein, flavonids, tannic acids phenolic content and antioxidant of faba bean (*Viciafaba* L.).

MATERIALS AND METHODS

Commercial formula known as "Okkadeen" biofertilizer contained *Rhizobium leguminosarum*var*. Faba* was obtained from Legume Crops Dept., Field Crops Research Institute, A R C, Giza, Egypt. One gm of the Okkadeen biofertilizer was suspended in 100 ml sterilized distilled water and shacked well. Serial dilutions were made by taking a loop of each of them to 100 ml sterilized distilled water. A loop from 10-9 dilution was transferred to 100 ml Yeast Extract Manitol "YEM" medium and incubated in a water bath shaker at 25°C±1 for 72 hrs. Seeds were immersed in sugar solution; as an adhesive material; prepared by dissolving 20 gm of sugar in 100 ml water. Treated seeds were then mixed thoroughly with the "Okadeen" biofertilizer and left for 30 min. in a shadow place for drying before cultivation.

Algae collection and preparation

Laurenciaobtusa was collected in May, 2011 from shallow water beside the shore of Red Sea at Safaga. After collection, algae were washed with fresh sea water to remove the epiphytes, sand and other extraneous matter then they were dried in shadow open air and completing the drying process in the oven at 60ºC for 5 hours. Then, dried algae were ground to fine powder by mechanic grinder. The algae were applied as a soil treatment at the rate of 3 gm. powdered algae/Kg soil seven days before planting and watered twice daily. The blue green algae, *Aphanocapsaalbida* were isolated from soil. Isolation and purification of algae were done according to the method described by Rippka, (1988). Algae were isolated after repeated light migrations on solid BG11, medium, (Zarrouk, 1966 and Stainer *et al*., 1971). They were grown in Erlenmeyer flasks (500 ml) in axenic conditions.

The cultures were incubated in the room temperature of approx., 25±2^{o}C and a light intensity 2500 lux. Provided by cool, white, fluorescent tubes under continuous illumination for 15 days. Two hundred ml. from the culture was added to the pots after planting.

Plant material

Seeds of faba bean (*Viciafa*ba L.) cv. Sakha1 were distilled water, then dried in shadow open air. The seeds were planted in 30 cm diameter earthen pots containing mixture

of 1:1 autoclaved peat and sand soil. Every pot contained 2 seeds. They watered every week.

The treatments

1-Control, 2- Rhizobium leguminosarumvar., faba, 3- Aphanocapsaalbida, 4- Laurenciaobtusa + Aphanocapsaalbida, 5- Rhizobium leguminosarumvar., faba + Aphanocapsaalbida,6- Rhizobium leguminosarumvar., faba+ Laurenciaobtusa+ Aphanocapsaalbida.

Determination of tannins (Vanillin - HCL assay)

Samples (0.2 g) of ground parts (shoot and root) were extracted with 10 ml of methanol for 24 hrs.at 30º C. One milliliter of the resulting extract was reacted with 5 ml of vanillin reagent (50:50 mixtures of 1% vanillin/ 8% HCl in methanol) for 20 min at 30º C, and absorbance was read at 500 nm. For blanks, 4% HCl in methanol instead of vanillin reagent was added to the extract, and absorbance was also read at 500 nm. Blank values were subtracted from experimental values to give adjusted data. Tannic acid standard curve from 0.0-1.0 mg/ml was used in calculating tannin levels.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) was determined by the Folin Ciocalteu method (Singleton & Rossi, 1965) using spectrophotometer (UV-200-RSLW scientific). Distilled water (3.16ml) was mixed with the 40 µl of sample, and then 200 µl of Folin Ciocalteu reagent was added. After 5 min, 600 µl of 20 % sodium carbonate solution was added and solutions were mixed again. The solution was left at room temperature for 2 hrs. The color intensities were measured at wave length 750nm. TPC expressed as grams of Gallic acid equivalents per 100g plant.

Antioxidant capacity (DPPH Assay)

The free radical scavenging activity was estimated by 1, 12-picryl-diphenyl-hydrazyl (DPPH) assay. The reaction mixture contained 100 µl of test extracts (100-500 µg/ml) and 1 ml of methanol solution of 0.1 mM DPPH radical. The mixture was then vigorously shaken and incubated at 37oC for 30 min. The absorbance was measured at 517 nm using ascorbic acid (100-500 µg/ml) as positive control. Lower absorbance of the reaction mixture +indicated higher free radical scavenging activity which was calculated using the following equation: DPPH scavenging effect (%) = 100 x $(A_o - A1)/(A_o)$.

Where: A_o is the absorbance of the control reaction and A1 is the absorbance of reaction mixture containing DPPH and extract at 517 nm.

Determination of flavonoid contents

The flavonoid contents of roots and shoots extracts of faba bean samples were determined according to the aluminum chloride colorimetric method described by (Barku *et al*., 2013) with some modifications. Sample solution (1ml, 10 mg/ml) of each plant extract was added to 0.5ml of distilled water. Sodium nitrite solution (0.075ml, 5%) was then added to the mixture followed by incubation for 6 minutes after which 0.15ml of 10% aluminum chloride was added, shaken, and was left to stand for 6 min. at room temperature before 0.5ml of 1M sodium hydroxide was finally added and the mixture diluted with 0.275ml distilled water, shaken and left to stand for 15 min before determination using the sample solution without coloration as reference solution. The absorbance of the reaction mixture was measured at 500 nm with a UV/VIS spectrophotometer. Quercetin was used as the standard for the standard curve. Flavonoid contents were expressed as mg quercetin equivalent (QE)/g dry weight.

Determination of protein-content

Total protein content: Protein content was determined by the Kjeldahl method for the calculation of all proteins which equal nitrogen content multiplied by 6.25.**(**A.O.A.C.,1990).

Statistical analysis

The responses of the treatments were compared by analysis of variance (ANOVA) (Sokal and Rohlf, 1995). Significant differences between the means of parameters were determined using Duncan's multiple range tests ($P \leq 0.05$). All analysis was carried out with SPSS software.

RESULTS AND DISCUSSION

Effect of some biofertilizer agents on total phenolic contents of faba bean plants

Phenolic compounds are secondary metabolites have repeatedly been implicated as natural antioxidants in fruits, vegetables, and other plants (Larson, 1988). Polyphenols play a vital part in the protection of plant against UV radiation, pathogens and herbivores, and help maintain structural integrity for the cell wall (Klepacka *et al*., 2006 and Inglett *et al.,*2011). Data in table 1 shows that application of *Aphanocapsaalbida* caused the highest increase of polyphenol compounds in the roots of faba bean plants followed by the treatment of *Rhizobium leguminosarum*var*., faba*e *+ Laurenciaobtusa* (10.78 and 7.97 mg gallic acid /gm. dry wt. roots, respectively), while the treatment of *Laurenciaobtusa + Aphanocapsaalbida*

Table 1. Effect of some biofertilizer agents on total phenolic content (mg equivalent of gallic acids /gm. Drymaterial) in shoot and root of faba bean plants

Treatments	Root	Shoot
Control	1.6 **a**	1.47**a**
Rh	2.98**a**	3.64**b**
Aph	10.78**c**	3.06**b**
Lu+ Aph	2.41**a**	6.11**c**
Rh + Lu	7.97**b**	1.87**a**
Rhi+ Lu + Apha.	7.45**b**	1.04**a**

Rh.,:*Rhizobium***Lu.,:***Lurancia***Aph.,:***Aphenocasa*

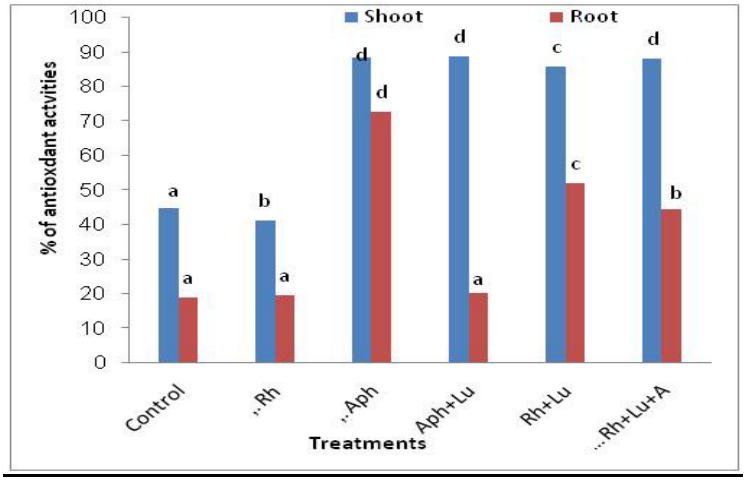

Figure 1. Effect of some biofertilizer agents on antioxidant capacity (DPPH assay) in shoot and root of faba bean plants
Rh.,:*Rhizobium***Lu.,:***Lurancia***Aph.,:***Aphenocasa*

gave the highest increase in total phenolic contents in the shoots of faba bean plants in compared with the other treatments and control (6.11 mg gallic acid/gm. Dry wt. shoots). This result is in agreement with Houssien *et al*. (2011) who reported that marine bioactive compounds extracted from marine algae as a biocontrol agents are used in agricultural and horticultural crops as a biofertilizers to improve their yield and quality and moreover to reduce the negative environmental impact. Also, Al-shakankery *et al*., (2014) reported that there is a significant increase in polyphenol content of maize grains of plants treated with algae as a biofertilizer compared to that of the control maize plants.

Effect of some biofertilizer agents on antioxidant capacity of faba bean plants by DPPH assay

The preservative effect of many plant spices and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues (Hirasa & Takemasa, 1998). Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu *et al*., 1998). Applications of *Rhizobium leguminosarum* var., *faba*, *Laurenciaobtusa* and *Aphanocapsaalbida* and their combinations as a biofertilizers significantly increased the antioxidant activity in the shoots and roots of faba bean plants except of the treatment of *Rhizobium leguminosarum* var. *faba* in the comparison with the blank control. There was a significant increasing ($p \leq 0.05$) with the application of *Aphanocapsaalbida* followed by the treatment of *Aphanocapsaalbida* + *Laurenciaobtusa* and *Rhizobium leguminosarum* var*, faba* + *Aphanocapsaalbida* + *Laurenciaobtusa* in the antioxidant activities of shoot compared with control. It is clear that the scavenging effect of different parts (shoot and rood) of faba bean plants on the DPPH radical increased and the highest values in shoot and root were (88.53 and72.89%, respectively)observed when treated with *Aphanocapsaalbida* (Cyanophyta) followed by *Aphanocapsaalbida* + *Laurenciaobtusa* (Figure 1).These observations were reported by Bhaskar and Miyashita,(2005) who found that seaweeds provide an excellent source of bioactive compounds such as

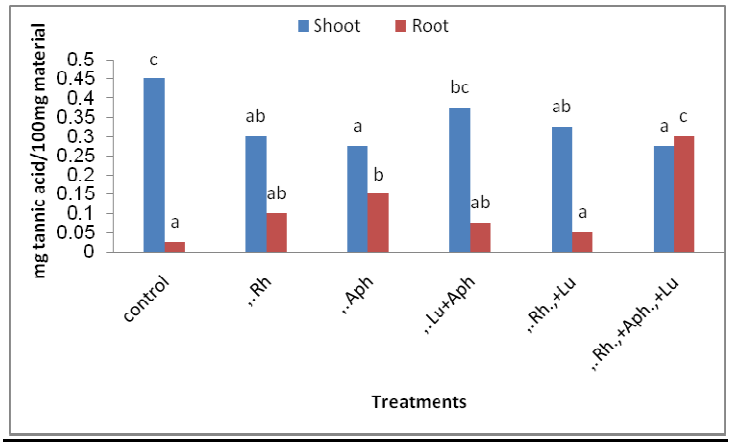

Figure 2. Effect of some biofertilizer agents on tannic acid content in shoot and root of faba bean plants Rh.,:*Rhizobium* **Lu.,:***Lurancia* **Aph.,:** *Aphenocasa*

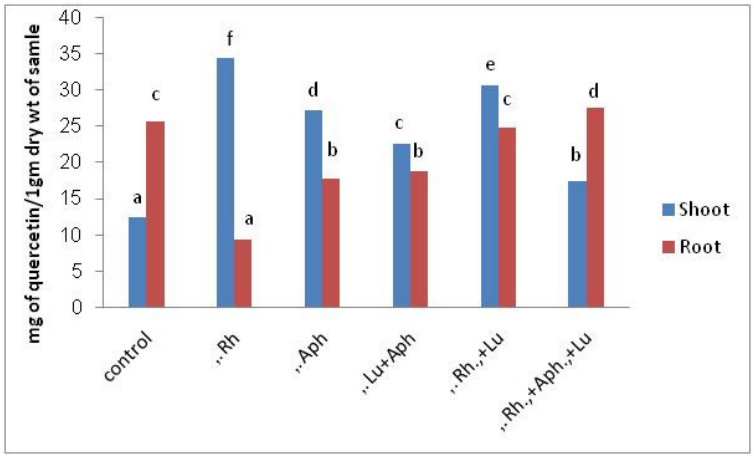

Figure 3. Effect of some biofertilizer agents on Flavonids content in shoot and root of faba bean plants Rh.,: *Rhizobium***Lu.,:** *Lurancia***Aph.,:** *Aphenocasa*

carotenoids, dietary fiber, protein, essential fatty acids, vitamins and minerals. Also, Verkleij (1992) and Turan and Köse (2004) reported that seaweeds enhance the antioxidant properties.

Tannins are polyphenols compounds that bind to and precipitate proteins, found in leaf buds, seeds, roots and stem tissues. An example of the location of the tannins in the stem tissues is that they are often found in the growth areas of trees, such as the secondary phloem and xylem and the layer between the cortex and epidermis. Tannins may regulate the growth of these tissues (Hemingway and Karchesy, 1989). Tannins act as a barrier for microorganisms and protect the tree (Ashok and Upadhyaya, 2012).

Figure (2) showed that the application of *Rhizobium leguminosarum* var. *faba*, *Laurenciaobtusa* and *Aphanocapsaalbida* and their combinations as a biofertilizers were non-significant on the tannin contents in the shoots of faba bean plants compared with the control (without any treatments), whereas they cause significant effect on the tannin contents in the roots of faba bean plants. The highest concentration of tannin as tannic acid in root of faba bean plants was obtained with the treatment of *Rhizobium leguminosarum* var*, faba* + *Aphanocapsaalbida* + *Laurenciaobtusa* (0.3 mg tannic acid/100gm dry material). This result is in agreement with Ruiz (1977) found that the beans contained tannin (0.48%) and Perez-Maldonado *et al.* (1999) measured the condensed tannin in beans.

The antioxidative effect is mainly due to phenolic components, such as flavonoids (Pietta, 1998). Data presented in figure (3) indicated that there are significant differences among the total flavonoid contents of faba bean plants treated with different biofertilizer agents. *Rhizobium*

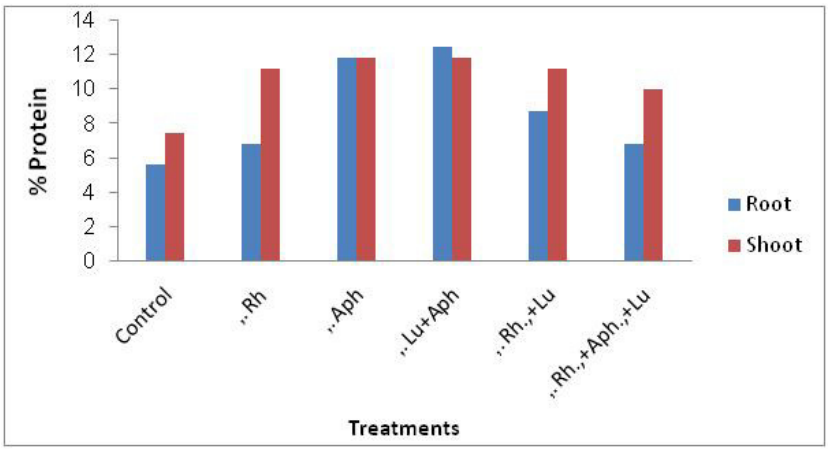

Figure 4 Effect of some biofertilizer agents on protein content in shoot and root of faba bean plants Rh.,:*Rhizobium* **Lu.,:***Lurancia* **Aph.,:***Aphenocasa*

leguminosarum var. *faba*, caused the highest increase in the total flavonoid contents in the shoots of faba bean plants followed by *Rhizobium leguminosarum* var. *Faba* + *Laurenciaobtusa* whereas the combination of different treatments caused the highest amounts of total flavonoidin the roots followed by the treatment of *Rhizobium leguminosarum* var. *Faba* + *Laurenciaobtusa*.

Duc, (1997) reported that the nutritional value of faba bean has always been traditionally attributed to its high protein content, which ranges from (27-34%).

Figure (4) illustrates the mean percentage of protein contents in the shoots and roots of faba bean plants. The obtained results indicate that all the treatments caused increase in the both of shoots and roots protein content. The treatment *Laurencia obtuse* + *Aphanocapsaalbida* caused the best result which reached 12.5% and 11.87% in roots and shoots respectively followed by the treatment of *Aphanocapsaalbida* which caused11.87% in the both of roots and shoots of faba bean plants. This result was reported by Sahu *et al*., (2012) who found that cyanobacteria provide inexpensive nitrogen to plants besides increasing crop yield by making the soil fertile and productive. Cyanobacteria play an important role in maintenance and build-up of soil fertility, consequently increasing rice growth and yield as a natural biofertilizer (Song *et al*., 2005). Also, Adam (1999); Lozano *et al*.,(1999) and Subramaniyan and Malliga (2011) stated that Nitrogen content was increased in response to seaweed fertilizers compared to those of control treatment.

CONCLUSION

The results of this study indicated that using of *Rhizobium leguminosarum* var., *fabae*, *Aphenocapsaalpida* and *Lauranciaobtusa* as biofertilizer gents enhanced some phytochemicalfor example polyphenols, antioxidants, flavonoids, tannins and protein in faba bean plants.

Aphenocapsaalpida enhanced both of polyphenol contents in the roots and antioxidant contents in the shoots. *Rhizobium leguminosarum* var., *fabae* increased the shoots flavonoide contents. The treatment of *Lauranciaobtusa* + *Aphenocapsaalpida* gave the best increase of the phenolic compounds in the shoots while the combination of the three agents gave the best result of tannins in the roots.

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