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PHYTOCHEMICAL ANALYSIS AND MINERAL ELEMENTS COMPOSITION OF *DATURA STRAMONIUM* FOLLOWING BIOFERTILIZERS APPLICATION

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***Azospirillum brasilense* or/ and *Azotobacter vinelandii* are employed in agriculture as biofertilizers. The main target of the present study is to assess the effect of nitrogen fixing microorganisms to improve and boost medicinal plants therapeutic effectiveness and better growth using *Datura stramonium* as an indicator plant. The inoculation of *Datura stramonium* with *Azospirillum* or/and *Azotobacter* partially or completely counteracted the improvements on some minerals content and secondary metabolic mechanisms. Generally, the application of *Azospirillum* or/ and *Azotobacter* as biofertilizers enhance the metallic contents of *Datura stramonium* and improve therapeutic effectiveness.**

Key words: *Azotobacter*, *Azospirillum*, Biofertilizers, *Datura stramonium*, secondary metabolite pathway, alkaloids, total phenols, flavonoids, saponins and tannins

INTRODUCTION

Soil is a habitat for several microorganisms, complex and interactive community of soil organisms, whose activities largely determine the chemical and physiological properties of the soil and growth of the plant. From seed germination until a plant reaches maturity, it lives in close association with soil organisms. The vast majority of plant associated soil organisms inhabit the rhizosphere, which is stimulated by the release of nutrients (1). Members of the genus *Azospirillum* associate with plant roots and have been shown in some cases to improve the nitrogen nutrition of several agriculturally important crops (2). *Azotobacter* lives as free-living saprophyte in soil, fresh water, marine environments and many other natural habitats and have been used as an effective inoculum to enhance plant growth and pest control (3 and 4).

Datura stramonium L. (Solanaceae) is a plant species distributed throughout most parts of temperate regions of the world (5) and is a noxious weed of cultivated cereal crops (6). Phytochemical composition of *Datura* species makes them attractive for conventional medicine (7).

Alkaloids are highly reactive substances with biological activity in low doses (8). Flavonoids are well recognized as antioxidant with protective effect against several diseases (9). Saponins play essential roles in medicine including serving as expectorant and emulsifying agents (10 and 11). Tannins are reported to inhibit pathogenic fungi (12).

Major salts and minerals such as nitrates (13) calcium (14 and 15) and potassium (16) are among the most essential ingredients of the nutrient medium known to affect the growth and metabolism of hair root cultures, such as alkaloids.

Species belonged to *Azospirillum* and *Azotobacter* are free-living, surface colonizing, sometimes endophytic diazotroph and plant growth promoting rhizobacteria (17 and 18). The presence of *Azospirillum* in the rhizosphere was reported to elicit or activate the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus bringing about the release of compounds in their active forms (19). Applying biological control agents to infected plants increase mineral levels [(nitrogen (N), phosphorous (P), potassium (K) and magnesium (Mg)], which in turn leads to the accumulation of metabolites (20).

The present study was, therefore, conducted with the view to identify efficient strain of *Azotobacter* or *Azospirillum* or a combination of two strains for enhancing *Datura* therapeutic effectiveness and better growth.

MATERIALS AND METHODS

Bio-inoculants isolation

Azospirillum brasilense isolation

Two media were employed: nitrogen-free semisolid malate (NFb) as an enrichment medium (21) and Rojo Congo (Congo red; RC), as an isolation medium. On the basis of the chemical composition of this medium (22), several carbon sources for *A. brasilense* and concentrations of yeast extract (0 to 1 g per liter-) were used in RC medium, which also contained the following (grams per liter of distilled H_20): K_2HPO_4, 0.5; $MgSO_4.7H20$, 0.2; NaCl, 0.1; yeast extract, 0.5; $FeCl_3.6H_20$, 0.015; DL-malic acid, 5; KOH, 4.8; and agar, 20. The pH was adjusted to 7.0 with 0.1 N KOH then the medium sterilized. Addition of 15 ml of a 1:400 aqueous solution of Congo red (autoclaved separately) aseptically to each liter of the melted medium just before tubing or use.

I-Samples of soil from corn (*Zea mays* L.) were collected from areas of South Valley University, or root from the same species (washed with sterile water or treated with 1% chloramine T (23) were placed into flasks, each of which contained 50 ml of NFb medium. These enrichment cultures were incubated at 37°C for 72 h. *Azospirillum* colonies were easily recognized.

II-Positive cultures were serially diluted 10-fold in sterile distilled H_2O to 10^{-4} and 10^{-5}. Loopfuls of the dilutions were streaked on plates of RC medium, which were incubated at 37°C for 72 h. Light-pink and colorless colonies were observed after 48 h. After 72 h, the light-pink colonies became scarlet. Small scarlet colonies were observed in the first streaks. Colonies were examined microscopically.

Azotobacter vinelandii isolation

The *Azotobacterium* isolation; 10 g collected soil sample were added to 90 ml of sterile distilled water in a sterile conical flask (250 ml), shaken well by vortex then allowed to stand for 30 minutes. 1 ml of sample suspension was then transferred to a sterile 9 ml distilled water containing bottle and shaken well and again allowed to stand for 30 minutes. In this way, samples were diluted up to 10^{-5} dilution fraction. One ml of sample suspension (from 10^{-1} to 10^{-5} fractions) were taken in a sterilized Petri Plates contained approximately 15 ml melted (45ºC) Ashby's medium nitrogen-free agar medium supplemented with sucrose (15 g/l), comprising (g/liter): Agar, 17.0; Mannitol, 15.0; $CaCl_2 \cdot 2H_2O$, 0.2; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; Na_2MoO_3 (10% solution), 0.1ml; $FeCl_3$ (10% solution), 0.05ml, pH7.2 ± 0.2 at 25 °C (24) and then incubated at 28±2ºC temperature for about 3 days. Growing colonies were purified through repeated plating. Media used for the purpose were Ashby's media. The purified cultures were examined. According to morphological and physiological characteristics, by referring to the Bergey's Manual of Determinative Bacteriology (25), the strains could be tentatively identified as *Azotobacter vinelandii* and *Azospirillum brasilense.*

Culture technique:

Soil was collected from the research field in South Valley University campus, sieved and sterilized at 2 atm., 121 °C for 2 h and then air dried for two weeks at room temp.

Datura stramonium L. seeds were collected from locations around Faculty of Science, in South Valley University camus. The seeds were surface sterilized using 70% ethyl alcohol for 2 min., rinsed several times with sterilized water. The seeds were soaked for 2 h in the dark at room temp. into the bacterial suspensions (*Azotobacter vinelandii*, *Azospirillum brasilense* or mixed suspension of both (1:1 v:v)) then the seeds were left to dry. Eight seeds were sowed in each pot. Plants inoculated with bacterial suspension only were the reference control whereas plants inoculated with sterilized distilled water were used as absolute control. The pots were daily irrigated with sterilized water until appearance of seedlings.

The experiment was carried out in completely randomized design with three replicate. The experimental data were analyzed using the least significant difference test.

Qualitative phytochemical analysis

Test for phenols and tannins

Crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for flavonoids

Alkaline reagent test: Crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow colour was formed which turned colourless on addition of few drops of diluted acid which indicating the presence of flavonoids

Test for saponins

Crude extract was mixed with 5ml of distilled water in a test tube and was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for alkaloids

Crude extract was mixed with 2ml of 1% HCl and heated gently. Mayer's And Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Quantitative phytochemical analysis

Determination of total phenols content and total flavonoids content

Extraction: This was done as described by (26). 40 g of 62.5 % aqueous methanol was added to 0.5 g of dried sample; 10 ml of 6 M HCl was added. The mixture was stirred carefully and the extraction mixture was refluxed in a water bath at 90 ^{o}C for 2 h. After cooling, the extract was filtered.

Determination of total phenols

Total phenols of aqueous methanol extract from each plant were determined according to the Folin-Cioalteu's reagent method (27). 20 µl of sample (1 mg ml^{-1}) and 930 µl of 2 % $NaCO_3$ were seeded in a tube, and then 50 µl of Folin-Cioalteu's reagent was added. The reduction of Folin-Cioalteu's by phenolic compounds under alkaline conditions resulted in the development of blue color, which was measured spectrophotometercally at 760 nm, after inculation at 40 ^{o}C for 60 min. Quantification was done on the standard curve of gallic acid. Results were expressed as mg of gallic acid equivalent (GAE) g^{-1} dry mass (28).

Determination of total flavonoids

Total flavonoids were estimated using the method of (29) To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoids content were calculated using a calibration curve. The total flavonoids content are reported as mg quercetin equivalent/g of extract powder by reference to standard curve ($y = 0.0071x + 0.0137$, $r^2 = 0.979$).

Determination of total alkaloids

Quantitatively, alkaloids were determined using the procedure put forward by (30) as described by (10). Five grames (5 g) of the powdered sample were weighed into 250 ml beaker. 100 ml of 10% acetic acid in ethanol was added. The mixture was covered and allowed to stand for 4 h. The mixture was filtered and the extract concentrated on a water bath to ¼ the original volume. Thereafter, concentrated ammonium hydroxide added dropwise until precipitation was completed. The solution was then allowed to settle and the precipitate collected, washed with diluted ammonium hydroxide and filtered. The residue that was dried and weighed was the alkaloid.

Determination of Saponin

The saponin content in the plant extracts was estimated as described by (31). Ten grams of the powdered sample was placed in 200 ml of 20% ethanol. The suspension was heated in a water bath at 55°C for 4 hours with continuous stirring. The mixture was filtered and the residue was re-extracted as above. The combined extracts were reduced to 40 ml over a water bath at 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml diethyl ether was added and shaken vigorously. The ether layer was discarded, while the purification process was repeated. 60 ml of n-butanol was added and the extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven to a constant weight. The saponin content was calculated according to the equation: amount of saponin (mg/100g) = weight of residue/ weight of sample* 100.

Determination of tannins

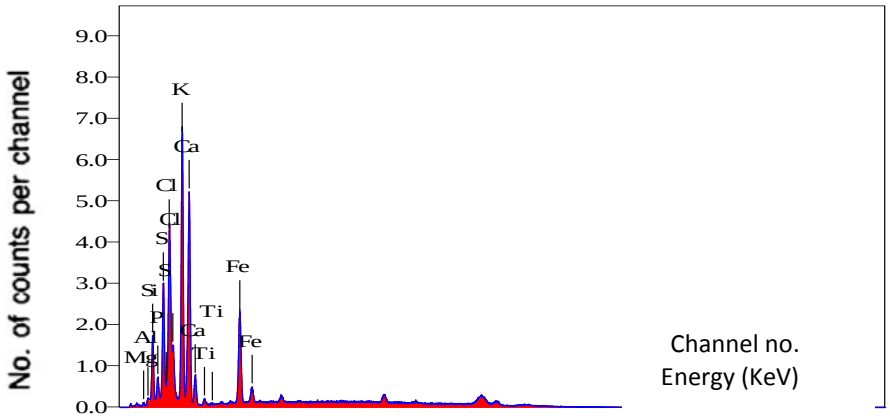
Okeke and Elekwa (32) method was used for tannin determination 0.5 g of the sample was shaken with 10 ml of 2M HCl in a test tube for 5 min. The content was then transferred into a volumetric flask and diluted up to 50 ml and 5 ml of the filtrate was introduced into a test tube and 3 ml of 0.008 M of K_3Fe (CN_3) were added. The absorbance was measured at 720 nm within 10 min.

Determination of some minerals

Mineral content of datura shoots were clustered by Central Laboratory, Faculty of Science, South Valley University, and Qena, Egypt. Potassium, calcium, magnesium, chlorine, phosphorous, silicon, sulfur and iron… concentrations were determined for undigested dry samples as loose powder. One gram of ground leaves was packed into a polyethylene cup of 20 mm internal diameter and covered with 6-µm-thick polypropylene film (Mylar®) (**33**). The samples were irradiated in triplicates under vacuum using an energy dispersive X-ray fluorescence spectrometer with an X-ray elemental analyzer (JEOL JSX 3222 Element Analyzer with energy Dispersive X-Ray Fluorescens system (EDXRF) (JOEL, Japan)

RESULTS AND DISCUSSION

The profiles of medicinal plants used in the analyses are shown in Tables (1, 2 &3) and Figures (1).



Figures 1: Mineral analysis of *Datura* shoots with energy dispersive X-ray fluorescence spectrometry (EDXRF).

Table 1: Minerals content (K, Mg, Ca, P, Fe, Al, Ti S, Si, Sr and Cl ms%) datura (*Datura stramonium* L.) shoots in presence and absence of bacteria inocula (*Azotobacter vinelandii* or *Azospirillum brasilense* or dual inoculum of both).

Treatment		Shoots mineral contents									
		K	**Ca**	**Mg**	**Al**	**S**	**Cl**	**Si**	**P**	**Ti**	**Fe**
Control		39.07	29.09	2.63	0.74	4.85	13.05	3.93	--	0.73	5.91
Bacterial inoculants											
Azotobacter		32.64**	32.35**	2.64	1.15**	5.09**	10.05**	6.37**	--	0.86**	7.66**
Azospirillum		37.24**	29.07	2.53*	0.80*	4.57**	13.53*	4.70**	--	0.72	6.85**
Dual inoculum		34.37**	28.67*	2.53*	0.88**	4.88*	14.91**	4.62**	1.37*	0.71*	7.04**
LSD	0.05	0.15	0.23	0.03	0.04	0.08	0.10	0.09	0.06	0.01	0.14
	0.01	0.23	0.33	0.04	0.07	0.12	0.15	0.14	0.09	0.02	0.20

*= Significant differences **= Highly significant differences from the absolute control.

Table 2: Phytochemical screening of the datura plant

Phytochemical	Shoots	Roots
Total phenols	+	+
Alkaloids	+	+
Flavonoids	+	+
Tannins	++	+++
Saponins	++	+

Table 3: Total phenols (mg/g dry mass), alkaloids content (mg/100g dry mass), flavonoids content (mg/g dry weight), tannins content (mg/g dry weight) and saponin content (mg/100g dry weight) of *Datura stramonium* L. shoots and roots in presence and absence of bacteria inocula.

Treatment		Shoots					Roots				
		Total phenols	**Alkaloid**	**Flavonoid**	**Tannin**	**Saponin**	**Total phenol**	**Alkaloid**	**Flavonoid**	**Tannin**	**saponin**
Control		85.00	14.20	6.40	17.60	6.70	27.33	11.84	8.60	13.60	4.90
Bacterial inoculants											
Azotobacter		77.99**	15.00**	8.00**	22.0**	7.60**	31.5**	12.4**	9.40**	12.2**	5.50**
Azospirillum		65.34**	18.00**	7.80**	22.0**	19.3**	44.0**	7.26**	7.40**	11.2**	7.50**
Dual inoculum		81.00**	13.00**	8.20**	22.4**	5.80**	39.0**	19.56**	6.00**	13.2**	5.80**
LSD	**0.05**	1.33	0.18	0.16	0.19	0.16	1.36	0.95	0.18	0.17	0.18
	0.01	1.94	0.27	0.23	0.27	0.23	1.98	1.38	0.27	0.27	0.27

*= Significant differences **= Highly significant differences from the absolute control .

Nutritionally valuable minerals analysis has shown that the plants are rich sources of K, Ca and Cl with datura shoots having K (39.07ms%), Cl (13.05ms%) and Ca (29.09 ms%). Those concentrations more accumulated with bacterial application having higher ones with application (Ca, 32.35ms% and Cl, 14.91ms %). K and Ca are essential for contribute to some of the traditional medicinal influences of the plants (34). Trace elements such

as manganese, iron and sulfur are essential in enzymes metabolism. The concentrations of these elements in the plants are quiet important. Iron contents of datura shoot (7.66 ms %) with *Azotobacter* treatment were higher than those reported for absolute control (5.91 ms %). The amount of aluminum (1.15ms %) was highest in *Azotobacter* inoculant and less in *Azospirillum* treatment (0.80 ms %). Other trace elements detected in higher concentrations include Ti (0.86 ms%) and S (5.09 ms%) in *Azotobacter* application. These data agreed with previously reported results on the effects of *Azotobacter vinelandii* or *Azospirillum brasilense* on the mineral nutrition of sorghum, wheat, non-nodulated soybeans and zea mays (35, 36, 37and 3). With respect to this, it has been reported that plant growth promoting rhizobateria to exert an effect on the membrane activities and proton efflux of inoculate plant roots, which are involved in the enhancement of mineral uptake of plants by these bacteria (38). The synthesis and excretion of plant-growth regulators in the rhizosphere explain the ability of these strains to promote growth, as well as the effect on plant nutrition reported here and in previous studies (39 and 40).

The results of the phytochemical screening and quantitative estimation of the chemical constituents of plant samples with *Azotobacter vinelandii* or *Azospirillum brasilense* inocula have indicated high content of flavonoids presented in roots especially with *Azospirillum* application alone (7.52 mg/g dry wt)(table 3).The abundance of flavonoids which are hydroxylated phenolics substances might be responsible for their therapeutic effectiveness against wide array of microorganisms, probably due to their ability to complex with extracellular and soluble proteins and to complex with the bacterial cell wall (41). Flavonoids and other phenolic compounds are potent water soluble antioxidants and free radical scavengers, which prevent oxidative cell damage, have strong anti-cancer activity (42).

In the present study, all bacterial suspensions enhanced the production of total phenols and tannins in both tested organs of datura plant. Dardanelli *et al.* (41) reported that bacterial exudates had key roles in different aspects of plant growth and development and they were also signaling molecules which induce biosynthesis of defense compounds such as phytochemical production. Alkaloids are very important in medicine and constitute most of the valuable drugs. They have marked physiological effect on animals (10). The plenty levels of *Datura* shoots (22.90 mg/100g dry wt.) with coinoculation indicates potential source for useful drugs. Saponin concentration was appreciable (7.50 mg/100g dry wt.) under each of individual inoculants in datura roots. These might contribute to some medicinal properties the plants. In agreement with previous study by (43), inoculation with *Azospirillum* favorably affected the production of more

species of flavonoids in *phaseolus vulgaris* cv. Plant growth promoting rhizobacteria correlated with change in the chemical composition of the root exudates and the quality of the phytochemicals of inoculated plants (43).
Presently, plant growth promoting rhizobacteria-based products are considered as relatively novel biological control agents which can help farmers to increase plant growth. Even, its use in the farming system needs to be confirmed by more field experiments.

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التركيب الكيميائي النباتى وتركيب العناصر المعدنية لنبات الداتورا سترامونيوم بعد اضافة المخصبات البيولوجية

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يعتبر التسميد الحيوى من اهم التقنيات الحياتيه المستخدمه، اذ يتم بعزل وتنقية وتوصيف احياء مجهريه مختلفه تضاف على شكل انوع من البكتريا كلقاح للنباتات بهدف زيادة امتصاص العناصر الغذائيه. تعد البكتريا المثبته للنيتروجين الغير تعايشية من اهم انواع البكتريا المستعمله فى مجال التسميد الحيوى وتعد الازوتوبكتر و الازوسبايريليم من اكثر الاحياء حرة المعيشه المثبته للنيتروجين كفاءه من حيث مقدرتها على تثبيت النيتروجين الجوى وانتاج منشطات النمو وزيادة امتصاص العناصر الغذائيه.

لذلك أجرى هذا البحث الذي يتناول دراسة مدى تأثير المعامله بمخصبات بيولوجيه مثل جنسى البكتريا سالفة الذكر او المعامله بكلاهما على معدل تراكم الأملاح المعدنيه والمركبات الكيميائيه الفعاله لنبات الداتورا سترامونيم كنبات طبى هام.

عند معاملة نبات الداتورا سترامونيم بمعلق بكتريا الازوتوبكتر بكتيريا الازوسبايريلم او المعامله بكلاهما و الذى صاحبه او قد يكون صاحبه تحسن ملحوظ فى كل من محتوى بعض الاملاح المعدنيه وكذلك تراكم بعض المواد الكيميائيه الفعاله بمقارنتها بتلك التى لم تعامل بالمخصبات البيولوجيه .