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EFFECT OF NITROGEN FIXING ASSOCIATIVE DIAZOTROPHS ENCAPSULATED IN ALGINATE OR FREE CELL SYSTEMS INOCULATION ON SEED GERMINATION AND GROWTH OP SOME VEGETABLE PLANTS

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ABSTRACT

A laboratory experiment was carried out to study the effect of inoculation with some non-symbiotic nitrogen fixing diazotrophs (*Azospirillum liopferum, Azospirillum brasilense, Azotobacter chroococcum)* in addition to *Bacillus polymyxa* either each in free or encapsulated in alginate beads on seed germination and growth of some vegetable plants such as lettuce, onion and carrot. Results revealed that all the tested bacterial strains, when entrapped in alginate had significantly higher nitrogenase activity and in turn higher nitrogen fixation efficiency than that achieved by the free bacterial cells. When the encapsulated alginate bacteria were inoculated to any of the tested vegetable seeds they surpassed those of inoculated free cells in affecting their seeds germination and growth. However, encapsulated *Azospirillum brasilense* was superior for lettuce, encapsulated *Azospirillum liopferum* was superior for onion and encapsulated *Azotobacter chroococcum* was superior for carrot, all in increasing seed germination per cent, the radical and epicotyl lengths, seedlings fresh and dry weights and leaves chlorophyll contents compared to their corresponding free bacterial cells.

Keywords: alginate, associative diazotrophs, nitrogenase activity, nitrogen fixation, lettuce, onion, carrot plants.

INTRODUCTION

Intensive efforts are being done to minimize the amounts of nitrogen fertilizers to decrease production cost and the environmental pollution without major grain yield reductions. Therefore, many workers studied the possibility of using N_2-Fixing bacteria to supply plants with a part of their N requirements and consequently to reduce the amount of N chemical fertilizer.

Numerouse investigations have dealt with the role of nitrogen fixing associative diazotrophs in plants (Eid *et al*., 1986 and Hegazi *et al*., 1986). However, few attempts are made to define the methods of large scale application of such micro-residents into soil.

El-Shanshoury (1995) reported that inoculation of the soil with *Azotobacter chroococcum, Azospirillum brasilense* and *Streptomyces mutabilis* as biofertilizers could improve early plant growth as wheat biofertilizers due to their rhizospher intensification, N_2-fixing potentiality, plant growth regulators production, and antimicrobial substance production that could be useful against pathogenic microorganisms.

Fallik and Okon (1996) studied the response of field-grown maize (*Zea mays*) to *Azospirillum brasilense* inoculation under various soil types, they reported that the application of peat-based powder inoculant of *Azospirillum brasilense* as well as a granular inoculant (each containing 0.5-1.0x10^7 cells g^{-1} moist peat), in the furrows of zea *mays* resulted in a significantly higher grain yield (11-14%) in light soils at low rates of N-fertilization.

A major problem in reducing the effectiveness of introduced bacteria, is the poor inoculant survival after introduction into soil (Thampson *et al*., 1990), furthermore, if bacteria introduced into soil are to be used to supply plants with fixed nitrogen, these bacteria must be able to colonize the plant roots. The use of liquid cell suspensions, in nutrient or buffer solutions, as inoculant may lead to poor survival in soil whereas the use of carrier materials such as clay-peat, K-carrageenan or alginate can protect cells initially when added to soil (Deluca *et al.*, 1990). Van Elsas *et al*., (1992) reported that survival of and root colonization by *Pseudomonas fiuorescens* cells in soil was promoted by encapsulation in alginate.

The present study was undertaken to obtain some information about the activity of some diazotrophs in addition to *Bacillus polymyxa*, all were separately encapsulated in alginate beads as compared to those of free (unencapsulated) cells. In particular, alginate beads were used to deliver the most efficient strain to some vegetable crops such as onion, lettuce and carrot planted in bottles contain semi agarized nutrient solution in comparison to those received free bacterial cells.

MATERIALS AND METHODS

Bacterial strains:

A number of microorganisms were isolated from different Egyptian soils cultivated with various crops. Based on microscopic, cultural and nutritional characteristics the isolates were defined as N_2-fixing diazotrophs viz *Azospirillum liopferum, Azospirillum brasilense* and *Azotobacter chroococcum* in addition to *Bacillus polymyxa* as non-diazotrophic N_2-fixing bacteria. Both diazotrophic bacteria and B. *polymyxa* were grown in its respective broth culture medium with continuous shaking and /or aeration to reach a population density of 10^8 cells ml^{-1}. Equal portion of each of the isolated bacteria strain was separately shaked to have homogenous culture suspension. The prepared bacterial culture suspensions were then activated by growing each strain in its specific medium for 5 days at 30 ˚C in 250 ml Erlenmeyer flasks containing 100 ml of modified Ashby's liquid medium for *Azotobacter* (Abd El-Malek and Ishac, 1968), nitrogen free malate medium (D◌bereiner *et al*., 1976) for *Azospirillum* and Hino and Wilson (1958) for *Bacillus polymyxa*. The viable cell count in this suspension before encapsulated was10^8 Cells / ml for each.

Alginate encapsulation:

Alginate bacteria encapsulation process was carried out according to the method described by Zayed, (1999).

An appropriate volume of the above mentioned cell suspension was mixed with an equal volume of a sterilized cool sodium alginate (2%w/v). The final alginate concentration upon dilution with the cell suspension was 1% (w/v). The mixture was extracted through a sterile Pasteur pipette into sterile 0.1M $CaCl_2$ thus forming beads about 2 mm in diameter. After hardening for one hour, $CaCl_2$ solution was removed, the beads were washed with several rinses of distilled water. Before use, beads were blotted dry on filter paper. Viable cell counts in the beads before use were 2x10^8Cells / g beads.

Measuring immobilized or free cells activity:

Erlenmeyer flaks 250 ml each contained 100 ml sterilized medium were inoculated with 4 ml cell suspension or a calculated weight of alginate beads (4g) containing the same number of bacteria in the 4 ml of cell suspension. The cells for each bacterial strain were grown at 30°C to the exponential phase (5 days for the free cells and 3 days for the immobilized cells), then the nitrogenase activity (nmoles C_2H_4 $ml^{-1}h^{-1}$) was measured as described by Turner and Gibson (1980). The most efficient strains were used for vegetable seeds inoculation experiment.

Vegetable seeds of onion (*Alluim cepa*), Lattuce *(Lactuca sativa* L.) and carrot (*Daucus carota* L)] were used in this experiment to study the effect of inoculation with the nitrogen fixing free cell bacteria and the encapsulated ones on their germination per cent and seedlings growth. The surface sterilized seeds (0.01 mercuric chloride) of each vegetable crop were planted in 500 ml glass bottles, each containg 200 ml sterilized semi agarized Hogland solution (Hoagaend and Arnon, 1938). The bottles containing the planted vegetables seeds were then incubated at the ambient room temperature for day and night in the laboratory till 4 weeks. After one week of the incubation, the germination percent was evaluated, at the end of the incubation period (4weeks) the seedling of each crop was subjected to determine the lengths of both radical and epicotyls (cm), fresh and dry of roots (g), total chlorophyll (a+b) in leaves ($\mu g\ g^{-1}$ leaves) (Talling and Drivers 1963).

Data obtained were statistically analyzed as described by Snedecor and Cochran (1982).

RESULTS AND DISCUSSION

Data presented in Table (1) show the seed germination as well as the formation of radical, a epicotyls, fresh and dry weight of lettuce seedlings, the leaves chlorophyll and the nitrogenas activity of alginate encapsulated and free cells of three diazotrophic bacteria in addition to *Bacillus polymyxa*. Results show that all tested strains encapsulated in alginate beads exhibited excellent results as compared with free cells.

Based on the tested strains, *Azospirillum brasilense* can be selected as the most efficient one, since the nitrogenase activity (N-ase) of it either in free cells or in alginate encapsulated form recorded significantly the highest values compared to the other tested bacterial strains in both forms. The corresponding (N-ase) was 90.00 and 311.60 nmoles C_2H_4 $ml^{-1}h^{-1}$ for both free cells and the encapsulated form, respectively. This excellent enhancement of encapsulated cells due to the advantage of encapsulation system. The major advantage of encapsulated cells is the high concentration of biocatalyst entrapped into support matrixes resulting in faster processing expressing very higher activity than that of the conventional system. The immobilized cells remain alive, able to multiply, can carry out multistep enzymatic reactions and regenerate cofactors (Zayed and Hunter, 1991).

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Therefore, strain of *Azospirillum brasilense* was used for lettuce inoculation experiment. On the other respect, regarding all the measured parameters, the inoculation with the encapsulated bacteria recorded significantly higher values than those obtained in case of inoculation with free bacterial cells. For instance, encapsulated *A. brasilense* gave 12.33 cm (radical length), 6.13 cm (epicotyl length), 1450.00 mg (seedling fresh weight), and 126.67 mg (seedling dry weight), 100% (germination per cent) and 78.20 μg g leaves^{-1} (leaves total chlorophyll). While inoculation with free bacterial cells recorded lower values of 10.23 cm (radical length), 3.27 cm (epicotyl length), 1146.60 mg (seedling fresh weight), and 83.30 mg (seedling dry weight), 86.70% (germination per cent) and 54.77 μg g leaves^{-1} (leaves total chlorophyll).

The improvement of germination and growth of lettuce plants inoculated with encapsulated *A. brasilense* cells is in accord with findings from previous studied, which have shown that alginate beads are efficient inoculant carrier for bacteria (Sougufara *et al*., 1989). In addition, Bashan, (1986) indicated that *Azospirillum* sp. cells encapsulated in alginate beads released slowly into soil and \ or the surrounded environment during degradation of the beads, and the librated cells then colonize plant roots. This in turn encourages the plant growth through the increase of the nitrogen fixed amount.

Same as in case of lettuce, inoculation of Onion plants with alginate encapsulated and free cells of the tested bacteria showed that *Azospirillum liopferum* was the superior for all determined parameters (Table 2). However, this behavior of onion plants towards the encapsulated *A. liopferum* may due to that alginate encapsulated cells ensured excellent levels of occurrence of viable cells released from alginate beads in rhizospher area as compared to those free cells. The plant rhizospher is considered as relatively a rich nutrient environment, in which inoculant can grow and multiply. Similar effects have been described by Breland and Bakken (1991). The rhizospher plants contain cells at levels exceeding the counts in the rhizospher of plants inoculated with unencapsulated cells. These results suggest that migration of *A. liopferum* cells to developed seedling roots was highly enhanced by their presence at higher population and higher activity levels in alginate beads as compared to free cells. However, Mixing bacteria and alginate prior to inoculation enhanced bacterial survival. This enhanced survival most likely was the key mechanism resulting in satisfactory colonization of the onion rhizospher (Zayed, 1999).

Data presented in Table (3) indicate that inoculation of carrot seeds with some diazotrophs in addition to *B. polymyxa* either in free cells or in encapsulated form showed that *Azotobacter chroococcum* was the superior strain and resulted in marked significant increases in values of seedling dry weight and total chlorophyll content in comparison with the values recorded for the other strains. However, the highest values of theire measurements were achieved in seeds inoculated with alginate encapsulated *Azotobacter chroococcum* cells. The dry weight of carrot seedling increased by 41.6% compared to those obtained by the free cells of the same bacterial strain.

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Such high significant increase in seedling dry weight may due to the effect of encapsulated cells which resulted in a very high density of nitrogen fixing bacteria (*Azotobacter*) in rhizospher of carrot seedlings. Consequently, the plant growth was support by increasing availability of N_2 due to the high levels of N_2- fixation. *Azotobacter* inoculants were also reported to produce compounds of auxin type and increase plant resistance to disease (Ishak *et al*., 1990). Also Zayed, (1999) reported that several microorganisms such as the genus *Azotobacter* exert beneficial effects on plant growth when used in alginate encapsulated inoculant form.

In conclusion, all tested crops in the present study, it is obvious that application of alginate bacterial beads lead to higher bacterial population densities in the rhizosphere of lettuce, onion and carrot plants, significantly exceeding those of the uncapsulated cells showing that the superior survival of the inoculant in beads could overcome any putative restriction to cell movement to developing roots paced by the presence inside an alginate matrix.

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تأثير التلقيح بالبكتيريا المثبتة للنتروجين الجوى لاتكافليا فى صورة حرة أوفى صورة كبسولات من مادة الجينات الصوديوم على إنبات ونمو بعض محاصيل الخضر

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أجريت تجربة معملية لدراسة أثر التلقيح بالبكتيريا المثبتة للنتروجين الجوى لا تكافليا على إنبات ونمو بعض محاصيل الخضر مثل الخس والبصل والجزر.

استخدمت سلالتين من الآزوسبيريللم بالإضافة إلى كل من الأزوتوباكتر كروكوكم واالباسيلس بوليمكسا فى صورة كبسولات من مادة الجينات الصوديوم وأيضا فى الصورة الحرة، حيث تم إضافتها كلقاحات في إحدى الصورتين السابقتين لثلاثة أنواع من محاصيل الخضرهى (الخس- البصل-الجزر). وقد اوضحت النتائج أن الميكروبات في صورة كبسولات من مادة الجينات الصوديوم كانت قدرتها على تثبيت النيتروجين أكبر منها في الصورة الحرة حيث كان نشاط إنزيم النيتروجينيز للميكروبات في الحالة الأولى أكبر منها في الحالة الثانية. وعندما لقحت الميكروبات في صورة كبسولات لبذور الخس كانت أفضل السلالات هى السلالة *Azospirillum brasilense* وفى حالة البصل كانت أفضل السلالات هى السلالة *Azospirillum liopferum* ومع الجزر كانت أفضل السلالات هى السلالة *Azotobacter chroococcum* وذلك من حيث تأثيرها على الوزن الطازج و الجاف للبادرات ونسبة الانبات وطول البادرة والجذير ومحتوى الأوراق من الكلوروفيل وذلك بالمقارنة مع البذور الملقحة بالميكروبات فى الصورة الحرة.

Table (1): Nitrogenase activity of free or alginate encapsulated bacterial cells and their effect on radical and epicotyl lengths, seedling fresh and dry weight, seed germination, chlorophyll content of leaves of lettuce plants.

Treatments	Length (cm)				Seedling fresh weight (mg)		Seedling dry weight (mg)		Germination %		Leaves chlorophyll ug.g^{1} leaves Chlorophyll type						Nitrogenase activity (n moles $C_2H_4ml^{-1}$ h^{-1})	
	Radical		Epicotyl								Chl. a		Chl. b		Total			
	F	En	F	En	F	En	F	En	F	En	F	En	F	En	F	En	F	En
Azospirillum liopferum	9.86	11.83	2.77	5.00	1080.00	410.00	80.00	110.00	86.47	95.53	26.82	41.83	15.59	28.49	42.41	70.3	71.40	241.7
Azospirillum brasilense	10.23	12.33	3.27	6.13	1146.60	450.00	83.30	126.67	86.70	100.00	34.69	44.86	18.08	33.32	54.77	78.2	90.42	311.6
Azotobacter chroococcum	7.83	10.63	2.03	3.83	736.67	213.30	56.67	90.00	57.57	88.90	16.32	37.48	10.81	22.03	27.13	60.2	35.38	115.9
Bacillus polymyxa	9.57	11.1	2.57	4.30	976.67	306.66	73.33	100.00	71.13	93.30	17.75	40.87	1068	24.67	28.42	65.5	55.41	173.4
S.D. at 0.05																		
eatment	0.313		0.198		86.66		5.692		5.008		2.251		1.896		2.859		14.036	
oculation	0.443		0.275		122.56		8.050		7.083		3.184		2.683		4.043		17.851	
eraction	Ns.		0.433		Ns.		Ns.		14.12		5.521		Ns.		7.830		34.961	

F = Free cells
En = Encapsulated cells

Table (2): Nitrogenase activity of free or alginate encapsulated bacterial cells and their effect on radical and epicotyl lengthes, seedling fresh and dry weight, seed germination, chlorophyll content of leaves of onion plants.

Treatments	Length (cm)				Seedling fresh weight (mg)		Seedling dry weight (mg))		Germination %		Leaves chlorophyll ug.g^{-1} leaves Chlorophyll type						Nitrogenase activity (n moles C_2H_4 ml^{-1} h^{1}) Epicotyl	
	Radical		Epicotyl								Chl. a		Chl. b		Total			
	F	En	F	En	F	En	F	En	F	En	F	En	F	En	F	En	F	En
Azospirillum liopferum	10.90	13.83	7.96	10.00	436.66	683.3	20	46.0	100.0	100	39.75	53.91	19.58	30.79	59.34	84.71	125.5	323.6
Azospirillum brasilense	9.86	12.17	7.33	9.03	310.00	490.0	20	30.0	90.0	100	19.22	44.27	11.56	26.73	30.79	71.00	72.7	171.5
Azotobacter chroococcm	10.23	13.06	7.70	9.43	340.00	570.0	20	33.3	93.3	100	29.06	45.32	16.88	30.87	45.95	76.19	98.3	222.2
Bacillus polymyxa	9.07	11.47	6.70	8.47	260.00	456.6	10	30	76.7	100	17.68	39.32	11.70	23.86	29.39	65.18	54.1	141.2
S.D. at 0.05																		
eatment	0.1469		0.111		14.785		4.218		4.871		2.444		2.055		3.097		33.093	
oculation	0.208		0.157		20.909		5.966		6.889		3.455		2.906		4.381		46.810	
eraction	0.351		0.282		36.010		Ns.		11.212		5.910		Ns.		7.574		Ns.	

F = Free cells
En = Encapsulated cells

Table (3): Nitrogenase activity of free or alginate encapsulated bacterial cells and their effect on radical and epicotyl lengths, seedling fresh and dry weight, seed germination, chlorophyll content of leaves of carrot plants.

Treatments	Length (cm)				Seedling fresh weight (mg)		Seedling dry weight (mg)		Germination %		Leaves chlorophyll ug.g^{-1} leaves Chlorophyll type						Nitrogenase activity n moles C_2H_4 m^{-1} h^{1}) Epicotyl	
	Radical		Epicotyl								Chl. a		Chl. b		Total			
	F	En	F	En	F	En	F	En	F		En	F	En	En	F	En	F	En
Azospirillum liopferum	10.9	13.86	6.23	7.4	416.6	570.0	70.0	90.0	66.7	77.76	20.70	47.67	12.45	28.78	33.16	76.46	52.90	146.10
Azospirillum brasilense	11.6	14.83	6.56	7.66	450.0	646.6	80.0	100.0	71.1	82.23	31.29	48.80	14.85	33.25	49.47	82.05	76.88	181.80
Azotobacter chroococcum	11.9	16.30	6.77	8.16	476.7	823.3	80.0	113.3	73.3	88.90	42.81	52.34	21.09	38.87	63.91	91.22	94.66	250.40
Bacillus polymyxa	10.13	12.77	5.63	6.93	323.3	503.3	63.3	90.0	66.7	73.30	19.04	44.50	12.61	25.70	31.65	70.20	37.87	113.29
S.D. at 0.05 eatment	1.064		0.149		22.567		2.340		2.001		2.629		3.808		3.334		23.198	
oculation	1.505		0.211		31.915		3.309		2.831		3.709		5.385		4.715		32.807	
eraction	Ns.		Ns.		54.761		5.749		4.920		6.531		Ns.		8.741		Ns.	

F = Free cells
En = Encapsulated cells