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EFFICIENCY *OF BACILLUS POLYMYXA* FOR NITROGEN FIXATION THROUGH MUTATION AND CONJUGATION

Samia F.M. Ahmed
Agric.Microbiol.Dept.Fac.of Agric. Minia Univ.; Minia Egypt

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ABSTRACT

The present study aimed to improve the ability of *Bacillus polymyxa* nitrogen fixation through mutation induction which may be considered as a high quality biofertilizer. The study included two parts. The first part was conducted to induce mutants from the wild type of *B. polymyxa* strain using UV mutagen. Data revealed that number and percentage of mutant increased with increasing time of exposure to UV light. However, out of 775 colonies tested 19 proved to be mutants: one required glutamine, seven required alanine; one required aspartic, one required serine, one required histidien and eight revertant.

In the second part, a pots experiment was performed to examine the efficiency of induced *Bacillus polymyxa* mutants conjugats and wild type in nitrogen fixation and thereafter on growth of maize plant. Results indicated significant increase in fresh weight of plants inoculated with *Bacillus polymyxa* wild type and some mutants compared with the control and the later were more effective than the wild type. The highest increase in fresh and dry weight were recorded in plants inoculated by some hybrids of mutants. Inoculation with Glu requiring mutant of *Bacillus polymyxa* and hybrids (Glu×Alan, Alan × Alan, Alan ×Asp, and Ser×His) significantly increased phosphorus content in maize plants and the maximum increase was attained in plants inoculated with Glu requiring mutants and hybrid (Ser×His). Some mutants and conjugants significantly increased nitrogen fixation compared with the wild type strain and the control.

INTRODUCTION

Nitrogen fertilizers are commonly applied to the soil for agricultural crops. However, some of N-fertilizers are expected to be lost via nitrate reduction, denitrification and/or ammonia volatilization. Also, some N-fertilizer could be leached to the surface and underground water, causing environmental pollution. From the economical point of view, the high price of these fertilizers may increase production costs of agricultural crops (Abdel Ati *et al.*, 1996).

Vinay and Bhadwaj (1994) found that inoculation of maize with *Azotobacter* numerically increased the plant biomass and grain yield.

Gopal *et al.* (2000) found that, inoculation of wheat with *Azotobacter* plus increasing **N** rate led to significant improvement in yield and yield attributes and N uptake by grain and straw.

Okon and *labander* (1994) reported that the free N-fixing rhizobacteria of genus *Azospirillum* live in close association with plant roots, where they exert beneficial effect on plant growth and yield of many crops of agronomic importance. Balota *et al.*, (1995) found that Cassava root exudates stimulated growth of *Klebsiella* and *Azospirillum Lipoferum* in vitro. Both *Klebsiella* and *A. lipoferum* produced indoleacetic acid in vitro.

Different mutagens were found to be effective in inducing nutritionally different mutants in Rhizobia species e.g. UV (Schwinghammer, 1969), X-rays (Lokk and Tokhrer, 1969 and Ali and Younis, 1975).

The main object of the present study was to improve the ability of *Bacillus polymyxa* for nitrogen fix through mutation induction which may be considered as a high quality biofertilizer.

MATERIALS AND METHODS

Materials:

(A)- Strain: a wild type strain of nitrogen fixing bacteria was isolated from the rhizosphere of wheat plants cultivated in the experimental farm, Faculty of Agric. Minia University. This isolate was identified according to Bergey's Manual (1984) to be a strain of *Bacillus polymyxa.*

(B)- Mutagen: was achieved by using UV light (lamp 50 cm, 220 V) and distance from lamp to samples was 25 cm.

(C)- Media

Complete medium (CM): was prepared according to Allen (1959).

Minimal medium (MM): was consisted of: NH_3Cl(20.0 g) , NH_3NO_3 (4.0 g) , Na_2SO_4 (8.0 g) , K2HPO4 (12.0 g), KH2PO4 (4.0 g) , $MgSO4.7H_2O$ (0.4 g) and distilled water to 1l. (Abdel-Rahem *et al* 1995).

Isolation and characterization of mutants:

Cell suspensions were prepared in liquid MM and incubated at 30 °C for 24 h. Portions of 0.1 ml from suitable diluted culture were plated on 54 Petri dishes containing CM media. These dishes were exposed to UV light for different intervals (0.0, 2.0, 6.0 and 8.0 min.). After incubation for three day, surviving colonies were recorded. Single colonies were tested on MM and CM plates incubated for four days. After growth the mutants were selected and re-tested. Those which proved to be mutants were streaked on CM and incubated for 3 days after that they were stored at 5^0C until used. Mutants were identified according to Holliday (1960).

Conjugation experiments:

Intrasepecific hybridization identified mutants was carried out by mixing two mutants in 5 ml sterile distilled water. After incubation for two hours, 0.1 ml from this suspension were plated on 4 plates MM incubated for four days at 30^0C. Ten single colonies were isolated on slant complete media and incubated for three days at 30^0C. Cell suspensions were prepared from each isolate and plated on five plates CM and incubated for three days at 30^0C. Single colonies were tested on MM and CM plates.

Table 1: Mechanical and chemical properties of the used soil.

Coarse sand %	Fine sand %	Silt %	Clay %	Texture grade	Total N%	$CaCO_3$ %	Available P, ppm	Organic matter %	pH
2.6	26.0	31.0	40.4	Clay loam	0.14	2.14	18.4	1.51	8.2

Testing of mutants and conjugants on zea maize (Giza 3):

The efficiency of isolated wild type, mutants and conjugants were grown in flasks, each containing 100 ml liquid CM, sterilized and then inoculated with the wild type, mutants and their conjugants after incubation for 4 days at 30^0C. Grains were planted in pots (v 30 cm in diameter and 25 cm in depth) containing 2.5 kg soil after sterilization of the soil used in these pots (three grains for each pot). For each treatment three pots were used as replicate pots. After 5 days of planting, the soil was inoculated with wild type strain mutants and their conjugants. Three non inoculated pots were used as control, each of which was inoculated only with the same volume of sterilized medium. After 40 days from sowing, the plants were removed, fresh weight and dry weight of the plants were recorded and the total Nitrogen in plants was determined according to **AOAC** (1980).

Statistical analysis:

Data were subjected to the statistical analysis and means were calculated according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Effect of UV on survival and mutant type transformed from *Bacillus polymyxa* cells

Table 2 shows the cells number of *Bacillus polymyxa* treated with UV light for different exposure time. No cells were survived after exposure to UV for eight min. However, number and percentage of mutant increased with increasing time of exposure to UV light. Out of 775 colonies tested, 19 proved to be mutants one required glutamine, seven required alanine, one are required aspartic, one required serine required histidine and eight revertant. These results are consistant with those reported by Abdel-Rahem *et al.* (1995) who used diethyl sulphate as a mutagenic for mutants' induction in *Azotobacter vinelandii* wild type strain. Four mutants were induced by treating the wild.type strain with different concentrations and incubation periods. These mutants were identified as,2 argininless. 1 histidineless and 1 threonineless.

Table 2: Effect of UV on survival and mutant type transformed from *Bacillus polymyxa* cells:

time exposure (min.)	Number and percentage of survivals		No. of colonies tested	No. of mutants	mutants %	No. and requirement of single mutants					
	No.	%				Glu	Alan	Asp	Ser	His	rev.
Cont.	1150000	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	99000	8.61	220	2.0	0.91	0.0	1.0	0.0	0.0	1.0	0.0
4	12000	1.04	390	7.0	1.79	1.0	2.0	0.0	1.0	0.0	3.0
6	2600	.023	265	10.0	3.77	0.0	4.0	1.0	0.0	0.0	5.0
8	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Effect of inoculation of *Bacillus polymyxa*, mutants and conjugation growth and nitrogen fixation in maize plant

Data shown in Table 3 represent the effect of inoculation of soil by *Bacillus polymyxa* on maize growth and nitrogen fixation in plant. A significant increase in fresh weight of plants inoculated by *Bacillus polymyxa* wild type and some mutants which were more effective than the wild compared to control. The highest increases in fresh weight were recorded in plants inoculated by some hybrids of mutant and the same is true for dry weight levels (Table 3). These results agree with the findings of Eweda and Vlassak (1981).

Jagnow *et al,* (1991) and Abdel-Rahem *et al*, (1995) reported a remarkable increase in plant fresh and dry weight when the soil inoculated either by *A. vinelandii* wild type strain or the mutants. The mutants increased the fresh weight of plants by 25.4 to 49.7 % while the hybrids between mutants increased the fresh weight by 49.8 to 60.3 %.

Nitrogen in plants:

Data presented in Table 3 indicated that inoculation with Glu requiring mutants of *Bacillus polymyxa* and hybrids (Glu×Alan, Alan × Alan, Alan × Asp, and Ser × His) and (Alan × Asp and Ser × His) significantly increased nitrogen content in maize plants and the maximum increase was recorded in plants inoculated by Glu requiring mutant and hybrid (Ser × His).

Some mutants and conjugants significantly increased nitrogen fixation in plants compared with the control and the wild type strain. Mutant Glu requirement and conjugant (Ser × His) were the best strains for increasing nitrogen in the plant (4.12)

These results confirmed the results of others (Kass *et al.*, 1971: Badawy and Amer, 1974: Monib *et al.* 1997 and El-shehaby, 1981).

Table 3: Effect of *Bacillus polymyxa* mutants and conjugants on growth characters and nitrogen uptake in plants.

Isolates and mutant requirements	Fresh weight (gm)	Dry weight (gm)	N In plants
Cont.	3.50	1.17	0.86
W.T	4.28	1.93	2.38
Glu (1)	3.77	1.27	4.45
Alan (2)	4.91	1.67	2.02
Alan (3)	4.61	1.54	3.14
Asp (4)	3.73	1.24	2.05
Ser (5)	4.14	1.47	2.50
His (6)	3.20	1.07	2.02
Glu × Alan 1 (1×2)	4.90	1.63	3.48
Glu × Alan 2 (1×3)	5.29	1.74	2.52
Glu × His (1×6)	4.58	1.57	2.89
Alan 1 × Alan 2 (2×3)	5.44	1.78	3.59
Alan 1 × Asp (2×4)	6.03	2.03	3.01
Alan 1 × Ser (2×5)	7.12	2.32	2.84
Alan 2 × Asp (3×4)	4.22	1.26	2.90
Alan 2 × Ser (3×5)	7.03	2.11	3.45
Alan 2 × His (3×6)	5.17	0.99	3.22
Asp × Ser (4×5)	5.14	1.71	3.70
Asp × His (4×6)	6.73	1.83	3.65
Ser × His (5×6)	6.02	1.75	4.12
L.S.D	1.47	0.56	0.75

On the other hand, Ali (2003) reported that inoculation of different plants with *Azotobacter* and/or *Azospirillum* markedly increased N content in plant leaves. Elsharouny (2007) found that inoculation of maize plant with wild type or muants of either *Azotobacter* lead to a significant increase in nitrogen uptake, fresh and dry weight of the plants as compared to the uninoculated ones. Such

results indicated that the applied micorganisms were well adapted to the conditions in the rhizosphere zone of the studied plants and were able to colonize at the root surface, hence, high amounts of fixed nitrogen were supplied to the tested pants.

REFERENCES

Abdel-Ati, Y. Y.: Hammad, A. M. M. and Ali, M. Z. H. (1996). Nitrogen-fixing and phosphate solubilizing bacteria as biofertilizers for potato plant under Minia condition. First Egyptian- Hungarian Hort Conf., Kafr El-Shekikh, Egypt. 15-17 sept.

Abdel-Rahem, A. T.: R.A. Ragab: O. F. Dakhly and R. A. Eid (1995). Improvement of Azotobacter vinelandii efficiency for nitrogen fixing through mutation induction and conjugation. Egypt J. Appl. Sci., 10 (8), 753-771.

Ali, M.D. (2003). Response of sugar beet to biofertilization. M. Sc. Thesis. Fac. Agric. Minia Univ., Minia, Egypt.

Ali, A. M. M. and Younis, S. E. A. (1975). Gamma rays induced mutants in R. meliloti. Egypt. J. Genet. Cytol., 4, 68.

Allen, O. N. (1959). Experimentes in soil Bacteriology. 3rd ed. Burgess Publishing Co., Minnieapolis, Minnesota, USA.

A.O.A.C. (1980). Association of official Analytical Chemists. Official Methods of Analysis, 16 th Ed. Washington, DC. Ch 4, p. 17.

Badawy, Farida. H. and S. B. Amer (1974). The effect of inoculation with Azeotobacter on the growth of wheat and tomato plants. Libyan. J. Agric., 3:141-143

Balota, E. L.,: lopes, E. S.: Hungria, M. and Dobereiner, J. (1995). Interactions and physiological effects of diazotrophic bacteria and arbuscula mycorrhizas on cassava. Pesquisa Agropecuaria Brasileira., 30 (11): 1335-1345.

Bergey's Manual of Systematic Bacteriology, (1984). Volume 1, Kreig, N.R. (ed.) Villiam & Wilkins, Baltimore, U.S.A. Rhizosphere of different plants. lll-inoculation with

phosphate- dissolving microorganism. 261 Bakt. ll., 132:245-267.

Elsharouny,T.H.M. (2007). Studies on bacterial viruses (Bacteriophages) of certain bacteria contributing to soil ertility.M.Sc. Thesis. Fac. Agric. Minia Univ., Minia, Egypt.

El-Shehaby, A. F. E. (1981). N_2-fixing bacteria in the rhizosphere of certain plants M.Sc. Thesis, Fac. Agric. Cairo Univ.

Eweda, Wadad and K. Vlassak (1981). Effect of inoculation with some a symbiotic nitrogen fixing bacteria on maize roots. Univ., Cairo, Egypt 33 (1) : 89 – 104

Gomez, K.A and A.A Gomez (1984). Statistical procedures of Agricultural Research Jehon Wily & Sons. New York, USA.

Gopal, Singh; L.L; Totawat, K.L. and Singh, G. (2000). Effect of integrated nitrogen management on yield attributing characters and yield of wheat. Research on crops., 1 (2) : 123 – 127

Holliday, R. (1960) A new method for the identification of biochemical mutants of micro – organism in " Mycogenetics " John Wiley* Sons London, P. 41.

Janow, G; G. Hoffich and K. H. Hoffmann (1991): Inoculation of non- symbiotic rhizosphere bacteria. Possibilities increasing and stabilizing yields. Angew Botanik, 65: 97-126

Kass, K.L; M. Drosdaff and M Alexander (1971). Nitrogen fixation by A paspali in association with bahiagress. Soil Sci. Amr Proc. 35:286-289.

Lokk, E and Tokhrer, V. (1969) Symbiotic efficiency of amino acid auxotrophic mutants of the vetch and soybean rook nodule bacteria. Eesti Nay Tead Akad Toim Biol., 20, 321.

Monib, M; Y Abdel-Malek; l. Hosny and M Fayez (1997) Associative symbiosis of Azotobacter chroococcum and higher plants Abl Bakt. Ll Bbt 134 : 133 – 139.

Okon, Y. and Labander-Cozalez, C.A. (1994) Agronomic application of Azospirillum: An evaluation of 20 years worldwide field inoculation.Soill Biol Biochem, 26: 1591-1601.

Schwinghammer, Ae (1969) Mutation to auxotrophy and protoprophy as related to symbiotic effectiveness in R. leguminosarum and R. trifolii. Carad J Microbiol. 15, 611.

Vinay, Gandotra, R.D. and Bhadwaj, K.K. R. (1998). Abundance of Azotobacter in great soil groups of north – west Himalayas. Journal of the Indian Society of Soil Sci., 46 (3): 379-38

Samia F.M. Ahmed

دراسة على فاعلية الباسيلس بولي مكسا لتثبيت أزوت الهواء الجوى خلال استحداث الطفرات والاقتران.

سامية فرحات محمد أحمد

قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة المنيا – مصر.

تهدف الدراسة إلي تحسين مقدرة بكتيريا الباسيلس بليومكسا على تثبيت آزوت الهواء الجوى من خلال استحداث الطفرات حتى يمكن الحصول على سماد حيوى عالى الجودة.

هذه الدراسة اشتملت على جزئين :

الجزء الأول يتضمن استحداث الطفرات من الطراز البرى للبكتريا محل الدراسة وذلك باستعمال الأشعة فوق البنفسجية كمطفر.

تشير النتائج إلى أن عدد ونسبة الطفرات تزيد مع زيادة وقت التعريض للأشعة فوق البنفسجية. ولقد حصلنا على ٣٧ طفرة من بين ١٧٩٠ مستعمرة مختبرة أى بمعدل (٢٠٧%) ولقد تم تعريف هذه الطفرات وكانت النتائج كالتالى : ١ينقصها الجلوتامين ؛ ٧ ينقصها الألانين ؛ ١ ينقصها الاسبارتيك ؛ ١ ينقصها السرين ؛ ١ ينقصها الهستدين ؛ ٨ مرتدة .

فى الجزء الثانى من الدراسة تم دراسة واختبار فعالية الطفرات المستحدثة فى الجزء الأول والسلالات الناتجة من الاقتران بالاضافة للطراز البرى على نمو محصول الذرة التى زرعت فى الاصيص وأوضحت النتائج زيادة معنوية فى الوزن الطازج للنباتات عندما قورنت بالكنترول (النباتات التى لم يتم تلقيحها) وذلك بالطراز البرى. وأعلى زيادة فى الوزن الطازج سجلت على النباتات الملقحة ببعض الهجن ونفس النتائج كانت على الوزن الجاف.

ولقد أوضحت النتائج أن التلقيح بالطفرة جلوتامين من الباسيلس بولي مكسا والهجن (الجلوتامين × الألانين ؛ الألانين × الألانين ؛ الألانين × الاسبارتيك ؛ السرين ×الهستدين) أدى إلى زيادة معنوية فى محتوى النيتروجين فى نباتات الذرة وأعلى زيادة سجلت كانت بالتلقيح بالسلالة التى تحتاج الى الجلوتامين والهجن (السرين× الهستدين).

بعض الطفرات ونواتج الاقتران زادت زيادة معنوية فى محتوى النيتروجين في البيئة مقارنة بالكنترول وكذلك عزلة الطراز البرى.