IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 11, Issue 10 Ver. III (October. 2017), PP 66-71
www.iosrjournals.org

Phytosimulating Action of Associated Diazotrophs on Growth And Development of Rice

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Abstract: *In the logarithmic phase, the development of Azospirillium UT13-8 strain cells number was $2.3x10^8$ cfu/ml, and in Azotobacter sp.12 – $1.8x10^7$ cfu/ml. As the results of the experiments showed, a day after inoculation the seeds of rice of varieties "Laser", "Mustakillik" and "Iskander" germination was 95-100%, the root length was in the range 0.2-0.3 cm. The biomass of rice of varieties "Lazer" in variants inoculated with Azotobacter sp. 12 and Azospirillium UT13-8 were 58-61% higher than the control variant, whereas the Mustakillik biomass increases by 51-55% when inoculated with Nostoc calcicola 25 and Azospirillium UT13-8 cultures as compared with non-inoculated plants in microvegetation experiments.The maximum efficacy in both rice varieties was revealed by inoculation with mixed cultures of diazotrophs (Azospirillium UT13-8 + Azotobacter sp.12 + Nostoc calcicola 25).*

Keywords: *association, Azospirillum, Azotobacter, Cyanobacteria, diazotroph, rice.*

Date of Submission: 12-09-2017 Date of acceptance: 06-10-2017

I. Introduction

It is known that soil microorganisms actively interact with plants and can have a positive effect on their nutrition and growth. In recent years, there has been an increased interest in microorganisms living in the rhizosphere, which do not realize close bonds of the type of legume-rhizobia symbiosis, but are in associative symbiosis with plants. Among them, a lot of attention is attracted to bacteria of the genus *Azospirillum*, *Azotobacter* and *Cyanobacteria*. This is due to their ability to nitrogen fixation [1], production of phytohormones [2, 3] and nitrite [4], iron accumulation [5], improvement of mineral nutrition [6] and water balance of inoculated plants [7], as well as synthesis of antifungal metabolites , competition for nutrient substrates and root surfaces, as well as induction of plant protective systems [8]. Numerous studies have shown that these bacteria do not exhibit specificity to the plant and successfully colonize many species of higher plants [9, 10, 11]. Large-scale inoculation with azospirilla plants may represent a means of enhancing growth-development and ultimately increasing the yield of non-leguminous plants, without the use of expensive, environmentally unfavorable mineral fertilizers.

The purpose of this work was to study the growth dynamics of diazotrophs during periodic cultivation and their influence on the growth and development of rice plants.

II. Materials And Methods

2.1 Growing diazotrophs

Azospirillium UT13-8 was grown on a potato medium of the following composition (g/l): 50 g potatoes were boiled for 40-45 min and potato broth was used to prepare the medium, sodium malate – 2.5; unrefined sugar 2.5, biotin – 100 µg/l, pyridoxine, 200 µg/l, pH 7.0 [12]. *Azotobacter* sp.12 and mixed culture (*Azospirillum* UT13-8 + *Azotobacter* sp.12) were grown on Ashby's medium with the following composition (g/l): mannitol, - 20,0; K_2HPO - 0,2; $MgS0_4$ x $7H_2O$ - 0.2; NaCl - 0,2; K_2SO_4 - 0,1; $CaCO_3$ - 5,0; pH 7,3. Growth of diazotrophs was carried out at a temperature of 28°C for 2 days. Every 4 hours was measured the optical density of the cultures at 590 nm. *Nostoc calcicola* 25 were cultured on BG-11 medium without addition of nitrogen [13] in the following conditions: light illumination with an intensity of 3500 lux, 2% CO_2, temperature 26°C, cultivation was performed strictly under sterile conditions for 7 days.

2.2 A study of the influence of diazotrophs on the germination of rice seeds

For the sterilization of rice seeds of varieties "Laser", "Mustakillik" and "Iskander" was treated with 3% H_2O_2 for 20 seconds and after that the seeds were washed several times with sterile water. 20 seeds of rice were added to each Petri dish containing 20 ml 1000 times diluted cultures of *Azospirillium* UT13-8, *Azotobacter* sp.12, *Nostoc calcicola* 25 and their mixed suspensions. Germination of the seeds was carried out at 3,500 lux of illumination, at a temperature of 26°C for 3 days. The repetition of the experiments was threefold.

2.3 Microvegetation experiments

To study the effect of inoculation of diazotroph cultures on the growth and development of rice plants in microvegetation experiments, the following strains were used: *Azospirillium* UT13-8, *Azotobacter* sp.12 and *Nostoc calcicola* 25. Varieties of rice "Laser" and "Mustakillik" served as plant objects in experiments. Sterilization of the seeds was carried out with 3% H_2O_2 for 20 seconds, followed by multiple washing of the treated seeds with sterile water. Further, the seeds were sterile germinated in Petri dishes containing 20 ml of sterile water for 24 hours. Two seedlings of rice were placed in each 60 ml vial containing 0.3% agar medium of the following composition: $MgSO_4$ x $4H_2O$ - 5 mM, K_2SO_4 - 10 mM, $CaCl_2$ x $2H_2O$ - 1 mM, phosphate buffer (NaH_2PO_4 + Na_2HPO_4, pH 6.5) - 15 mM, Fe-Sequestrene 138 (Fe-EDDHA) - 5 mM, microelements - 0.05 ml/L of medium. Microelements composition (g/L): H_3BO_3 - 17.16, $MnSO_4$ - 7.2 , $ZnSO_4$ - 1.32, $CuSO_4$ - 1.65, Na_2MoO_4 - 0.12 [14].

For the inoculation of seeds, dasotroph cultures of 2-day age grown on potato agar were used [12]. Plants were grown at a temperature of 25^{o}C in a greenhouse, the light regime was -16 h, illumination - 3500 lux. After 1 month from inoculation of plants, the tubes were sealed with rubber stoppers and acetylene was injected to a concentration of 10% by volume. A day later nitrogen fixation of associative symbiosis of plants was determined [15]. Plant biomass was dried at room temperature for 7 days. The repetition of the experiments was threefold.

III. Results and discussion

3.1 Dynamics of growth of diazotrophs

The results of the study of the growth dynamics of *Azospirillium* UT13-8 and *Azotobacter* sp.12 cells with the periodic method of cultivation are shown in Fig. 1. At the initial titer of 7.6x10^{5} cfu/ml in the *Azospirillium* strain UT13-8 and in the *Azotobacter* sp.12 strain 2.5x10^{5} cfu/ml, the lag phase lasted up to 4 hours. The logarithmic (exponential) growth phase of *Azospirillium* UT13-8 cells lasted from 8 to 20 hours and the growth of bacteria stopped (stationary phase) after 24 hours of cultivation. The number of cells of *Azotobacter* sp.12 increased by 4-28 hours of cultivation, then at the 36th hour was observed the fall growth of bacteria. The titer of the cells of *Azospirillium* UT13-8 + *Azotobacter* sp.12 mixed cultures gradually increased to 28 hours and further cultivation led то slowed and stopped the growth of the cultures. In the logarithmic phase, the development of *Azospirillium* UT13-8 cells number was 2.3x10^{8} cfu/ml, and in *Azotobacter* sp.12 – 1.8x10^{7} cfu/ml, i.e. the number of cells of *Azospirillium* UT13-8 cultures increases by three orders, and cells *Azotobacter* sp.12 by two orders of magnitude in relation to the initial number of cells of cultures.

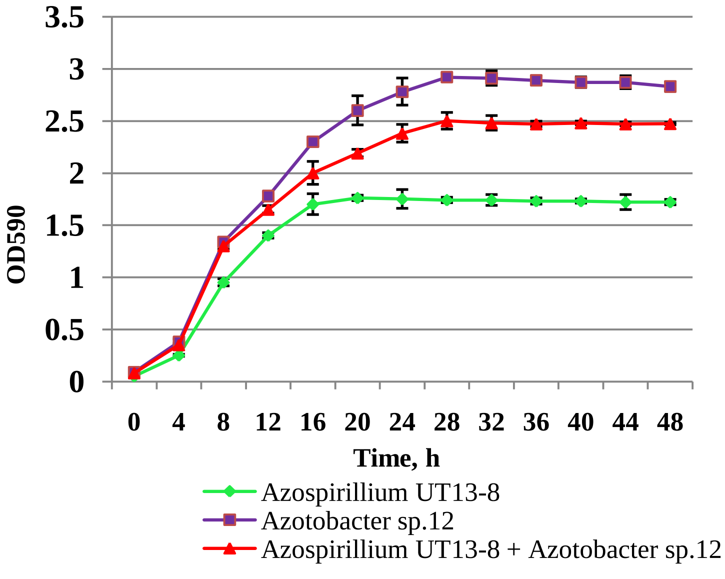


Fig. 1. Dynamics of changes in the optical density of diazotrophs during periodic cultivation.

3.2 The influence of associative symbiosis on germination and growth-development rice

Diazotrophs refers to the so-called PGPB-bacteria (plant-growth-promoting bacteria) on the proposal expressed in [11]. The ability of diazotrophs for phytostimulation is based on a number of mechanisms [16], realized successively or in parallel [17]. The production of phytohormones [18, 19, 20], in the first place of auxins [21], has been studied the most. In our studies, the effect of the bacterial suspension of diazotrophs on germination of seeds and the development of seedlings of various rice varieties was used by suspensions of *Azospirillium* UT13-8 cultures in the amount of 7.6 x 10^5 cfu/ml, *Azotobacter* sp.12 - 2.5x10^5 cfu/ml and *Nostoc calcicola* 25 -1.3x10^5 cfu/ml. As the results of the experiments showed, a day after inoculation the germination of rice seeds of varieties "Laser", "Mustakillik" and "Iskander" was 95-100% and the root length was in the range 0.2-0.3 cm (Table 1). On the 3rd day of cultivation, the roots of the seedlings, regardless of the type of rice, increased more than 10 times as compared to the first day of cultivation. It should be emphasized that *Azospirillium* UT13-8 and mixed cultures of diazotrophs more effectively influenced the growth of roots and the appearance of rice leaves. At the inoculation of plants with *Azospirillum*, in the roots was observed changes of morphology, which was the result of bacterial growth products of plant regulatory substances [22]. The increase in the number of lateral roots and their hairs increases the root area (volume) of plants available for nutrients and, as a result, ensures the optimal absorption of nutrients by the roots of plants, which is the main factor that enhances plant growth [23]. As our studies on light microscopy have shown, that when seeds of rice "Laser" with *Azospirillium* UT13-8 are inoculated, in the roots of seedlings long deformed lateral root hairs are identified as compared to the control (Fig. 1). With the association between bacteria and plants, there are formed no any structures similar to nodules or tumors in the roots of plants, however, deformation of the root hairs is observed, which correlates with the ability of azospirill to colonize plant roots [24]. Single and aggregated bacterial cells were densely absorbed on the surface of the root hairs (Fig. 2). The process of attachment of bacteria of the genus *Azospirillum* with the roots non-legume plants is similar to the initial stages of the legume-rhizobia symbiosis. The effect of azospirill on the growth and development of rice was studied in sterile micro vegetation experiments. Seedlings of rice grown in a 0.3% semi-liquid environment, after inoculation with

Table 1 influence of diazotrophs on the germination of seeds of various varieties of rice

Varieties of rice	Diazotrophs	Germination of seeds			
		24 h		72 h	
		Germination, pcs.	The average root length, cm	The average root length, cm	Height of plants, cm
Lazer	Control	18	0.15	2.0	1.0
	Azospirillium UT13-8	20	0.2	4.0	2.15
	Azotobacter sp. 12	19	0.2	3.5	2.25
	Nostoc calcicola 25	20	0.2	2.5	2.25
	Azospirillium UT13-8 + *Azotobacter* sp.12 + *Nostoc calcicola* 25	20	0.25	2.25	2.75
Mustakillik	Control	17	0.2	3.5	1.25
	Azospirillium UT13-8	20	0.3	4.5	2.0
	Azotobacter sp. 12	19	0.3	3.75	1.6
	Nostoc calcicola 25	20	0.25	4.5	1.45
	Azospirillium UT13-8 + *Azotobacter* sp.12 + *Nostoc calcicola* 25	20	0.25	2.25	2.25
Iskander	Control	18	0.17	3.0	1.25
	Azospirillium UT13-8	20	0.25	3.25	1.5
	Azotobacter sp. 12	19	0.27	3.5	1.35
	Nostoc calcicola 25	19	0.25	4.0	1.74
	Azospirillium UT13-8 + *Azotobacter* sp.12 + *Nostoc calcicola* 25	20	0.25	2.5	1.75

strains of azospirill for 3-4 days around the roots of rice, visual-visible colonies of bacteria were formed. As can be seen from Table. 2, when inoculating different rice varieties with diazotrophic bacteria, plant biomass increases in different degrees. The biomass of rice "Lazer" in variants inoculated with *Azotobacter* sp. 12 and *Azospirillium* UT13-8 were 58-61% higher than the control variant, while variety "Mustakillic" was increased by 51-55% when inoculated with *Nostoc calcicola* 25 and *Azospirillium* UT13-8 cultures compared to non-inoculated plants. The maximum efficacy in both rice varieties was revealed by inoculation with mixed cultures of diazotrophs (*Azospirillium* UT13-8 + *Azotobacter* sp.12 + *Nostoc calcicola* 25). In general, inoculation of rice seedlings, positively influenced the growth and development of rice roots in all variants. Nitrogen-fixing activity of the associative symbiosis of the variety "Laser" varied in the range from 166 to 253 nmoles of C_2H_4/plants/day. In the variety "Mustakillik", the nitrogen fixing activity was significantly higher and amounted to 223-320 nmoles of C_2H_4/plants/day.

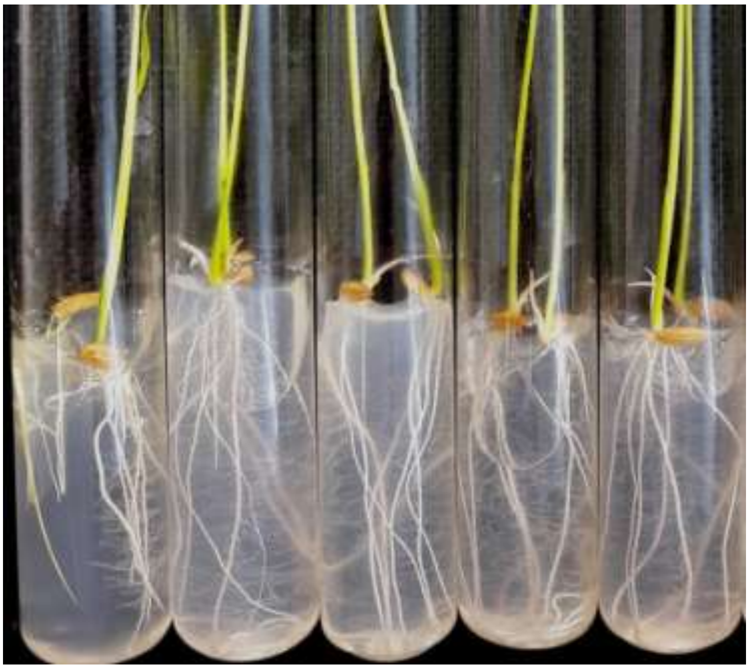
Table 2 influence of diazotrophs on the growth and development of various varieties of rice in microvegetation experiments

Varieties of rice	Diazotrophs	Shoot DW, mg	Root DW, mg	ARA C_2H_4 nomoles/ plants/day	Effectiveness, %
Lazer	Control	4.1 ± 0.23	2,6 ± 0,35	-	-
	Azospirillium UT13-8	6.6 ± 0.76	3,5 ± 0,28	207 ± 4.0	60
	Azotobacter sp. 12	6.5 ± 0.5	3,5 ± 0,3	235 ± 3.0	58
	Nostoc calcicola 25	6.1 ± 0.28	3,1 ±0,28	166 ± 5.5	48
	Azospirillium UT13-8 + *Azotobacter* sp.12 + *Nostoc calcicola* 25	7.1 ± 0.57	4.6 ± 0,5	253 ± 8.0	73
Mustakillik	Control	4.3 ± 0.28	2.3 ± 0.28	-	-
	Azospirillium UT13-8	6.8 ± 0.76	2.8 ±0.15	267 ± 7.3	58
	Azotobacter sp. 12	6.0 ± 1.32	2.8 ± 0.12	320 ± 7.7	39
	Nostoc calcicola 25	6.5 ± 0.86	3.0 ± 0.1	223 ± 7.4	51
	Azospirillium UT13-8 + *Azotobacter* sp.12 + *Nostoc calcicola* 25	7.1 ± 1,04	3.1 ± 0.2	290 ± 9.1	66

Note: $P<0.05$, ARA – acetylene-reductase activity, DW – dry weight

Fig. 2. Root hairs of three-day seedlings of rice of variety "Laser" : a - inoculated seedlings with strains of *Azospirillium* UT13-8; b - not inoculated seedlings.

Fig. 3. Adsorption of the strain *Azospirillium* UT13-8 on the surface of the root hairs of a three-day seedlings of rice. Arrows indicate the associative symbiosis of bacteria and plants.



a b c g d

Fig. 4. Development of roots and root hairs of rice when inoculated with diazotroph strains in microvegetation experiments: a - control (uninoculated plants), b - *Azospirillium* UT13-8; c - *Azotobacter* sp. 12; g - *Nostoc calcicola* 25; d - *Azospirillium* UT13-8 + *Azotobacter* sp.12 + *Nostoc calcicola* 25

IV. Conclusions

The most noticeable effects of inoculation of diazotroph cultures on plants of rice were observed in the form of various morphological changes in the root system of plants. In figure 3, visually it is evident that the strains of *Azospirillium* UT13-8 and the mixed cultures of diazotrophs increased the number and length of the lateral hairs of the root of rice in comparison with the control. More dense development and increase in the length of root hairs of inoculated plants is explained by the fact that the bacterial strains used are potential producers of phytohormones necessary for plant root stimulation. Large volume, and good root growth more efficiently provides plant nutrients. In the works of Okon and Kapulnik [25] it was shown that the association of *Azospirillum* with the roots of cereal plants increased the volume of roots and the patency of the N, P, and K ions in the roots of the host. Thus, the results obtained make it possible to conclude that the investigated strains of diazotrophs are the most effective for using them as an inoculum of cereal plants. At the same time, the harvest of cereal crops increases by a natural bio-technological route, especially in low fertile soils.

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Shakirov Zair Saatovich Phytosimulating Action of Associated Diazotrophs on Growth And Development of Rice.” IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT), vol. 11, no. 10, 2017, pp. 66-71.