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Model of associative symbiosis of bacteria of the genus *Azospirillum* with microalgae

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ABSTRACT

We developed an express–method of the formation process study of associative symbiosis of bacteria of the genus *Azospirillum*, which can be observed by light microscopy during in situ experiment. *Chlorella sorokiniana* were used as higher plants. During 2 hours incubation of bacteria *Azospirillum brasilense* UZ A13-6 with *Chlorella sorokiniana* gradual formation of bacterial associations with microalgae was revealed. During eight-hour incubation, we have found that the bacteria cells have accumulated around microalgae cells, forming associative symbiosis with microalgae.

KEYWORDS

Azospirillum;
Chlorella;
Associative symbiosis.

INTRODUCTION

The Gram-negative nitrogen-fixing a-proteobacterium *Azospirillum brasilense* lives in association with the roots of many agriculturally important crops. Upon inoculation of cereals, the bacteria significantly promote plant growth and crop yields[1,2]. The beneficial effect on plant growth has been attributed to the production of phytohormones by the associated bacteria, rather than nitrogen fixation[3]. Although much has been learnt about the beneficial effects of inoculated azospirilla on host plants, little is known about the molecular mechanisms underlying the successful establishment of *Azospirillum brasilense* in the rhizosphere, probably because of the lack of a distinctive plant phenotype.

In associative symbiosis with the roots of non-leguminous plants, presence of polar flagella of bacteria is of paramount significance, that involved in attaching bacteria on the root surface[4], whereas bacterial synthesis exopolysaccharide defines dense interaction of bacteria with the surface of the roots, that leads to the aggregation of bacteria and the formation of large morphological lumps and corresponding loss of bacterial motility[5].

In *Azospirillum*, physiological responses to starvation have been studied in relation to changes in the cell surface, which lead to the attachment of *Azospirillum brasilense* to roots[6]. External root colonization is apparently not associated with any morphological or structural changes in the cortex cells in wheat[7]. The mode of root colonization by *Azospirillum* may vary, depending on the bacterial strain, plant species, environmental conditions, and other unidentified factors. The interaction between these entire variable creates different degrees and patterns of root colonization, different population

sizes, and different colonization sites. The main colonization sites, for most plant species studied, are the elongation and root-hair zones. Root surface colonization supported by fibrillar anchoring is one of the proposed features of *Azospirillum* root colonization[8].

Despite intensive study of the association formation of *Azospirillum* with plants still are not yet fully understood, as well the study of the formation of associative symbiosis of bacteria with leguminous plants is laborious, as visually noticeable morphological changes, during association of bacteria of the genus *Azospirillum* with not legumes plants on the roots, are not observed, as it happens on the roots of legumes plants (root nodules)[8].

Based on the above mentioned, the objective of this work is to study of the formation of an association between *Azospirillum* and microalgae, as a model of associative symbiosis.

MATERIALS AND METHODS

Strains and culture conditions

The object of our research was associative bacteria of the genus *Azospirillum brasilense* UZ A13-6 isolated from the surface of wheat roots[9] and microalga *Chlorella sorokiniana* (University of Minnesota, St. Paul, Minnesota, USA). *Azospirillum brasilense* UZ A13-6 were cultivated in Potato medium prepared on the basis of a broth obtained by boiling for 1 h 200 g of potato in 1 L of water and containing the following (g/L): sodium malate 2.5, unreûned sugar 2.5, biotin 0.001, pyridoxine, 0.002, pH 7.0[10]. *Chlorella sorokiniana* were grown under sterile conditions in a modified "Chu-13" medium[11] for 7 days at 25°C, with the supply of carbon dioxide by blowing air containing 1% CO_2 and continuously illuminated by fluorescent white light (200 μmol photons $m^{-2} \cdot s^{-1}$).

Light microscopy

Formation of associative symbiosis between *Azospirillum brasilense* UZ A13-6 and *Chlorella sorokiniana* was observed and photographed with a digital video camera to the video-microscope "Olympus" (Japan).

Electron Microscopy

Bacterial preparations were grown in TY hard medium during 24 hours. After growing up the bacterial cells 3-4 times were rinsed by 0.025 M potassium hosphate buffer (pH 7.2) and centrifuged at 6000 g during 20 min, and then they were fixed by 3% formalin solution (preliminarily neutralized by K_2CO_3 beforehand 24 hours). Before their transfer to special metallic grids the bacterial cells were fixed (contrasted) by 2% phosphorous-tungstic acid (pH 7.2) during 15 min and with help of sprinkler the cells were dusted to metallic grids. All electron microscopic samples were viewed in electron microscope "JEM (Jeol)-10" (Japan).

RESULTS AND DISCUSSION

Previously isolated bacteria A13-6 morphological-physiological properties by nucleotide sequence of 16S rRNA belonged to the genus *Azospirillum*, type *Azospirillum brasilense* UZ A13-6. Electron microscopic examination of cells *A. brasilense* UZ A13-6 showed that growing crops on TY liquid medium, cells formed amphitrichate flagella Figure 1a. Bacterial cell aggregation formation was observed on 2-3 day of cultivation Figure 1b. Many properties as nitrogen fixation, production of phytohormones, biocontrol, motility, shemotaxis, aerotaxis, cell aggregation of bacteria genus *Azospirillum* positively affect on growth, development and yield of non-legumes[4,13,14,15,16,17]. At the present time the study of formation of associative symbiosis of non-leguminous plants, is very relevant, both from fundamen-

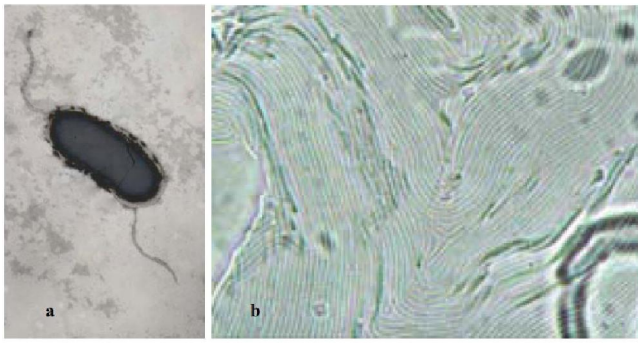

Figure 1 : Electron - microscopic microphotograph of *Azospirillum brasilense* UZ A13-6 (a) - and light microscopy - cell aggregation of *Azospirillum brasilense* UZ A13-6 when grown in a liquid potato medium (b)

tal and applied aspects.

Due to the above mentioned, we have developed an express-method for the study of formation of associative symbiosis of bacteria of the genus *Azospirillum in situ*. In place of higher plants were used unicellular green alga *Chlorella sorokiniana*. Proposed method is quite simple and opportune to visually observe the formation of associative symbiosis between plants and bacteria *in situ* during the experiment. 24 hour culture biomass *Azospirillum brasilense* UZ A13-6 was grown on potato medium, harvested by centrifugation at 6000 g for 30 min, precipitate biomass *Azospirillum* cultures was washed three times with sterile distilled water. 1 ml of the bacterial suspension (10^8 cells/ml) *A. brasilense* UZ A13-6 was added to 5 ml cultures of unicellular green algae *Ch. sorokiniana* (10^5 cells/ml). Mixed cultures incubated at 25 ± 1°C illuminated by fluorescent white light (200 μmol photons $m^{-2} \cdot s^{-1}$). Every 30 min. samples were taken from mixed cultures to study of interactions between bacteria and microalgae. Formation associative symbiosis was observed by light microscopy. At the beginning of the incubation of mixed cultures, interaction between bacteria and microalgae was seldom. After two hours of incubation was revealed gradual formation of associative symbiosis of bacteria and microalgae. At eight-hour incubation, for the first time we have shown, that the bacteria cells amassed around the microalgae cells and had formed associative symbiosis Figure 2a.

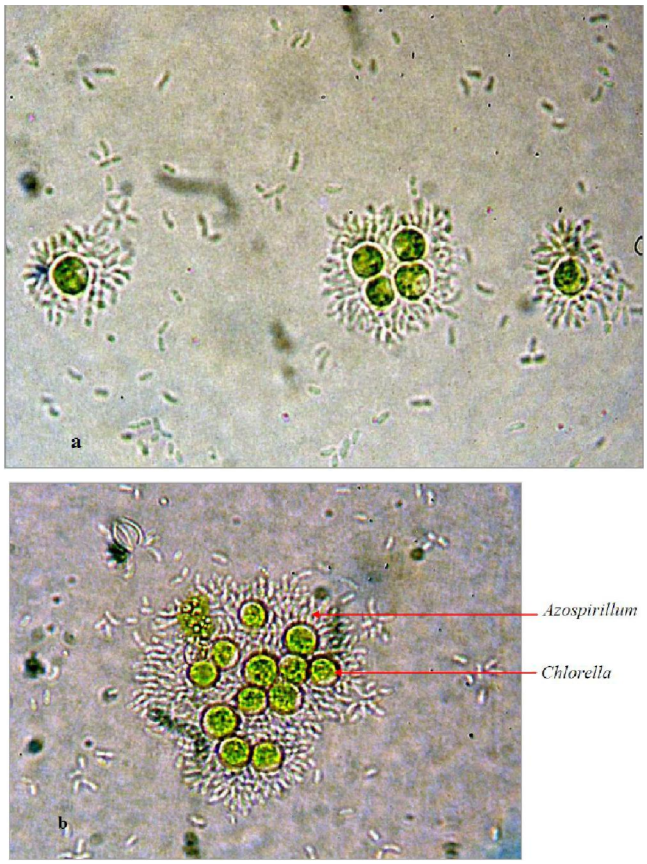

Figure 2 : Model of associative symbiosis formation between the cells of bacteria *Azospirillum brasilense* UZ A13-6 and unicellular green algae *Chlorella sorokiniana*: association of *Azospirillum* cultures with *Chlorella* after 8 hour (a) and 24 hour (b) incubation in laboratory conditions

On https://www.youtube.com/watch?v=eTz6An1P7 ro video clip is posted, which shows the formation of associative symbiosis of bacteria *Azospirillum brasilense* UZ A13-6 at 8 hours of incubation. At further incubation (24 hours) of mixed cultures led to the formation of a durable association of bacteria *Azospirillum brasilense* UZ A13-6 with unicellular green algae *Chlorella sorokiniana*, as well as among themselves (cell aggregation) (Figure 2b). The results prove that bacteria of the genus *Azospirillum*, is genuine associative bacteria that have a whole arsenal of properties providing quick formation of associations with plants. From these data, one can assume that *Azospirillum* exist in nature mainly in the form of associations with the roots of plants.

CONCLUSION

Indisputably the number of properties of bacteria (the presence of flagella, chemotaxis, aerotaxis, cell aggregation among themselves, immobilization of cultures on plant's root surface, synthesis of phytohormones) is a determining factor in the formation of associative symbiosis "bacterium + plant" and its sustainability aims to protect plants from adverse environmental factors. Thus, we developed a method which can be used in microbiology to determine the ability of formation of associative symbiosis of different rhizosphere bacteria.

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