Investigating the cultural-morphological features of rhizobacteria and allocating it from the cotton plant (Gossypium hirsutum): in the example of irrigated meadow soils of Uzbekistan

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Abstract. This paper investigates the cultural-morphological, physiological and biochemical properties of bacterial isolates with stimulating properties isolated from the rhizosphere of cotton (Gossypium hirsutum) grown in saline soils of Uzbekistan. The cells of the isolated culture No. 12 were found to be rod-shaped, 2-3x0.5-0.6 μm in size, single or chain-linked, Gram-positive aerobic bacteria producing spores. In studying the culture No. 146, its cells were in the form of thick, less mobile rods, 1-2x0.6-0.8 μm in size, forming thermostable spores. The spores were located in the center of the cell; the Gram-stained colonies in meat-peptone agar were round, bulging; the edges were flat, consistency oily, smooth and, mucous; and, the upper part was found to be shiny. Studies have shown that the isolates appertained to the genus Bacillus sr.

1. Introduction
Biological control agents based on microorganisms are a viable alternative to the use of widely used pesticides, which are life-threatening and costly to accumulate in plants [1, 2]. Chemical pesticides have a destructive effect on beneficial organisms living in the soil and have a short-term inhibitory effect on phytopathogens that are resistant to fungicides. Biological agents, on the other hand, are characterized by long-term, that is, exposure throughout the growing season [2, 3].

The technologically promising aspect of the bacteria of the genus Bacillus is that they are widely and competitively resistant due to the formation of thermostable endogenous spores against adverse environmental factors [4, 5].

The purpose of this study was to study some cultural-morphological, physiological-biochemical properties of rhizosphere microorganisms isolated from the saline soils of the Syrdarya province, Uzbekistan. Besides that the object of the study was representatives of rhizobacteria isolated from the rhizosphere of cotton (Gossypium hirsutum) grown in saline soils of different levels in the Syrdarya province, Uzbekistan.
2. Materials and Methods

Separation of samples from the rhizosphere of cotton root for research was carried out in the T. Gulomov massif of Saykhunabad administrative district of Syrdarya province, Uzbekistan (Figure 1) [6].

![Figure 1. Map of the study area](image)

In the distribution of salts in the profile, the degree of salinity was taken into account the percentage of dense residues of Cl\(^-\) and SO\(_4^{2-}\) ions. According to the agrochemical parameters of the soils of T. Gulomov massif of Saykhunabad district of Syrdarya province, the salinity of the studied soils according to the content of sulfate and chloride ions (mg/eq) is as follows (Tables 1-2).

To isolate microorganisms from the rhizosphere of the cotton plant, the young roots were separated from the soil monoliths using 5.0 g using sterile scissors and tweezers, and the young roots with the soil adhering to them [7]. The roots were placed in a flask containing 100 ml of sterile saline solution and shaken for 2.0 min. The fluid inside the flask was taken from 1 ml with a sterile pipette, transplanted into a sterile nutrient medium, and incubated at 280°C for 480 h [8].

The method of sequential washing of the roots was used to isolate the microorganisms in the rhizoplane [9, 10]. The washed material was transferred 3 times to a test tube containing saline, and then washed 4 times with sterile water. The washed roots were crushed with sterile scissors and transferred to sterile Petri dishes [10, 11]. Afterwards, 5 g of root was taken and grinded in a sterile jar. The crushed roots were then transferred to a flask filled with a sterile saline solution, in which the total volume of the suspension was 50 ml. Thereafter, 5 g of sterile quartz sand was added to the flask and shake for 5 minutes [11, 12]. The 5 ml suspension was then removed from the flask and transferred to another 45 ml sterile water flask, then the 5ml suspension was sequentially transferred to the second, third, fourth, fifth, sixth flask, and the root in each flask containing the same 45 ml sterile water was washed for 5 min. Thus, 7 consecutive root washes were taken and then grown in a sterile nutrient medium at 28°C [12, 13].
Table 1. Description of salinity levels of irrigated meadow soils of T. Gulamov massif of Saykhunabad district of Syrdarya province

| Depth, cm | Total dissolved solids | Total HCO₃ | Cl⁻ | SO₄²⁻ | Ca²⁺ | Mg²⁺ | Various Na⁺ | Toxic salt content, % | Non-toxic salt content, % | Reserves of salts, % | Salinity type and level |
|-----------|------------------------|-------------|-----|--------|------|------|-------------|------------------------|------------------------|------------------------|-------------------------|
| 0-29      | 0.345                  | 0.024       | 0.014 | 0.205  | 0.065 | 0.012 | 0.019       | 0.113                  | 0.226                  | 0.339                  | Sulphur Weak            |
| 29-44     | 0.755                  | 0.027       | 0.021 | 0.491  | 0.085 | 0.061 | 0.046       | 0.437                  | 0.295                  | 0.731                  | Sulphur Weak            |
| 44-79     | 0.555                  | 0.024       | 0.035 | 0.347  | 0.065 | 0.048 | 0.032       | 0.325                  | 0.226                  | 0.551                  | Sulphur Weak            |
| 79-110    | 0.420                  | 0.030       | 0.035 | 0.236  | 0.055 | 0.024 | 0.038       | 0.225                  | 0.193                  | 0.418                  | Ch.sulphur Moderate     |

13 cross-section: Irrigated meadow soil

| Depth, cm | Total dissolved solids | Total HCO₃ | Cl⁻ | SO₄²⁻ | Ca²⁺ | Mg²⁺ | Various Na⁺ | Toxic salt content, % | Non-toxic salt content, % | Reserves of salts, % | Salinity type and level |
|-----------|------------------------|-------------|-----|--------|------|------|-------------|------------------------|------------------------|------------------------|-------------------------|
| 0-31      | 1.360                  | 0.021       | 0.087 | 0.839  | 0.135 | 0.110 | 0.103       | 0.832                  | 0.463                  | 1.295                  | Sulphur Moderate        |
| 31-59     | 0.895                  | 0.027       | 0.063 | 0.520  | 0.090 | 0.051 | 0.100       | 0.540                  | 0.312                  | 0.851                  | Sulphur Weak            |
| 59-85     | 0.335                  | 0.030       | 0.042 | 0.164  | 0.040 | 0.015 | 0.043       | 0.191                  | 0.142                  | 0.334                  | Ch.sulphur Moderate     |
| 85-117    | 0.300                  | 0.030       | 0.031 | 0.150  | 0.035 | 0.015 | 0.035       | 0.171                  | 0.125                  | 0.296                  | Ch.sulphur Weak         |

65 cross-section: Irrigated meadow soil

| Depth, cm | Total dissolved solids | Total HCO₃ | Cl⁻ | SO₄²⁻ | Ca²⁺ | Mg²⁺ | Various Na⁺ | Toxic salt content, % | Non-toxic salt content, % | Reserves of salts, % | Salinity type and level |
|-----------|------------------------|-------------|-----|--------|------|------|-------------|------------------------|------------------------|------------------------|-------------------------|
| 0-29      | 0.640                  | 0.021       | 0.154 | 0.261  | 0.085 | 0.030 | 0.078       | 0.336                  | 0.293                  | 0.629                  | Ch.sulphur Moderate     |
| 29-45     | 0.275                  | 0.027       | 0.059 | 0.119  | 0.035 | 0.012 | 0.043       | 0.170                  | 0.125                  | 0.295                  | Ch.sulphur Weak         |
| 45-88     | 0.200                  | 0.024       | 0.031 | 0.082  | 0.045 | 0.003 | 0.011       | 0.048                  | 0.148                  | 0.196                  | Ch.sulphur Weak         |
| 88-120    | 0.160                  | 0.027       | 0.024 | 0.061  | 0.035 | 0.003 | 0.037       | 0.122                  | 0.159                  | Ch.sulphur Weak         |

102 cross-section: Irrigated meadow soil
Table 2. Agrochemical indicators of soils of T. Gulamov massif of Saykhunabad district of Syrdarya province

| Cross-section | Depth, cm | Humus, %  | P₂O₅, mg/eq | K₂O, mg/eq |
|---------------|-----------|-----------|-------------|------------|
| 13            | 0-29      | 1.24      | 25.06       | 165        |
|               | 29-44     | 1.46      | 23.06       | 123        |
|               | 44-79     | 1.18      | 12.80       | 108        |
|               | 79-110    | 1.43      | 7.33        | 128        |
| 65            | 0-31      | 1.61      | 21.60       | 240        |
|               | 31-59     | 1.38      | 20.26       | 275        |
|               | 59-85     | 1.23      | 10.80       | 88         |
|               | 85-117    | 1.06      | 7.33        | 88         |
| 102           | 0-29      | 1.15      | 20.26       | 275        |
|               | 29-45     | 1.37      | 14.93       | 240        |
|               | 45-88     | 1.14      | 8.67        | 215        |
|               | 88-120    | 1.13      | 9.47        | 200        |

In the next stage, 5 g of nystatin was added to the nutrient medium in Petri dishes to inhibit fungi in general and species identification of bacteria [14, 15]. Growing was carried out for 5 days at a temperature of 28°C. After the total calculation, the first microscopic preparation of the colonies was prepared. The drugs were visualized under a MIKMED-5 microscope. The type of cultures was determined using biochemical properties [16, 17]. The results of identification were analyzed using a mathematical method of classification.

The following tests were performed to determine the generation of microorganisms [18, 19]:
1. Test for oxidase. A few drops of a freshly prepared 1% solution of tetramethylphenylenediamine were added to the filter paper. The grown culture was rubbed on a damp filter paper and waited 10 seconds when reflected in purple or red with a transient reaction;
2. An oxidative-enzymatic test (Hugh Leifson test) was used to study the glucose degradation reaction. Bacterial suspensions were placed in solutions containing a semi-liquid medium. A layer of 10 mm sterile mineral oil (anaerobic solution) was poured over one test tube containing Hugh Leifson medium. The second test tube containing the nutrient medium was not covered with anything.

The results of the experiment were explained as follows: if the acid is formed in an aerobic solution, then the catabolism of carbohydrates in the cells takes place using O₂. The increase in this solution was to be significant. If acid is formed in both tubes, then the cells must be capable of fermentation.

The species affiliation of isolated bacteria was determined using the Berg determinant according to morphological, cultural, and chemotaxonomic characteristics. Cultural markers of bacteria were described in solid feed (e.g. MPA (meat peptone agar), potato slices and liquid feed media MPB (meat peptone broth) [20].

Bacterial colonies in solid nutrient media were characterized by the following characteristics: the size of the colonies (colonies large — 10 mm in diameter) averaged -1~10 mm; small, dotted -1 mm; profile of colonies (colony bulging, conical, flat, crater-shaped); the edge of the colonies (smooth, feathery, wavy, toothed, fluffy, convex) the color of the colony; gloss and clarity; consistency (doughy, dry, mucous, elongated, liquid, sticky), the appearance of colonies under a microscope (granular, uniform, dashed), shape - round, oval, etc. described according to the results of such studies [21, 22].

The colonies were seen in a small magnifying section (x8) of the microscope. The morphology of living bacterial cells was seen in “crushed drop” preparations and seen on a large magnifying lens
(x90) under a microscope. The size of the cells was determined and measured with an ocular micrometer, a 12–14-hour culture was used to study cell mobility, and the shape was recorded. Fixed and stained drugs were prepared to study cell structure. Bacterial preparations for fixation and staining (smear) were prepared on clean degreased glass [23].

A drop of bacterial suspension was added to the finished glass, made into a thin layer, spread with a bacterial slurry along the glass surface, and dispersed. The smears were dried at room temperature. The most common method of drug fixation (fixation) was heat fixation [24]. The dried mazak glass was carried over the burner three or four times. To study the delicate structure of bacteria, bacterial preparations were treated with chemicals - anhydrous methyl alcohol (5 minutes), 96% ethyl alcohol (5 minutes), equal amounts of ethyl alcohol and ether (2 minutes), and corneal fixator (ethyl alcohol - 60 ml, chlorophore - 30 minutes, hydrochloric acetic acid-10 ml, fixation period-15 minutes) [8].

After fixation, the drug was stained. Dyes in dyeing: methyl blue (alcohol or aqueous solution); based fuchsin (a mixture of alcohol or water-alcohol mixture), using several dyes, and stained by the Gram method. A quick and simple method was used to determine whether the bacteria were stained with gram-positive and gram-negative (these two groups are related to the cell wall properties of the bacteria) [12, 20].

Bacterial cells (1–2 days) were taken with a bacterial sieve and 3% KOH was taken into the drip pan, rotated in a circular motion, and after 5 - 6 s, the sieve was suddenly raised. The suspension of gram-negative bacteria was viscous and elongated to form a belt using a suture. Gram-negative bacteria are evenly distributed in the alkali droplet. The reaction was considered negative if no mucous membranes were formed within 600°C [8, 10].

The formation of a viscous suspension of alkaline by gram-negative cells is associated with the dissolution of the cell wall and the release of DNA [7]. This method was used for initial diagnosis. In the study, the physiological biochemical properties of bacteria were studied according to their relationship to carbon and nitrogen sources.

3. Results and Discussion

During the research, rhizobacterial samples were isolated from Aqdaarya 6 cultivars of cotton grown in soils with different amounts of sulfate and chloride ions, and cultural morphological and some physiological biochemical properties were analyzed.

Studies have shown that the cells of the isolated No. 12 culture are rod-shaped, 2.3x0.5-0.6 μm in size, single or chain-linked, Gram-positive aerobic bacteria that produce spores. Meat peptone agar (MPA) was noted to form dense colonies, the color of the colonies was fuzzy, round-shaped capillaries were uncurled, and abundant growth was observed along the agar surface.

The optimum growth temperature for these bacteria was 18-36°C rN6.8-7.0. The cultures were found to be tolerant of salinity and could grow in nutrient media up to 7.5% sodium chloride. It was noted that sucrose is weakly alkaline, glucose is acidic, xylose is alkaline, arabinose is weakly acidic, rhamnose is alkaline, maltose is weakly alkaline, lactose is alkaline, mannitol is alkaline, sorbitol is alkaline, and glycerin cannot be absorbed. Respiration was found to be aerobic. The study of physiological biochemical properties revealed activity on enzymes such as amylase, catalase. Dissolves gelatin in layers, hydrolyzes starch, assimilates urea, increases in nitrates, KNO₃, lacks denitrification properties, peptonizes milk, does not assimilate casein, but assimilates sodium citrate. However, it does not grow well in phenylalanine and in tryptose.

Culture No. 146, its cells are in the form of thick low-mobility rods, size 1.2 x 0.6-0.8 μm thermostable spores, spores are located in the center of the cell, Gram staining, colonies in MPA are round, bulging, flat edges, consistency oily, smooth, mucous, the upper part was found to be shiny. MPB was noted to grow to form a white non-wrinkled film, the clarity of the broth. When the physiological biochemical properties are studied, they are aerobic, the optimum temperature for growth is 20-36°C, can grow at a maximum of 41°C, have catalase activity, sucrose is weakly acidic, D-glucose is alkaline, does not assimilate xylose, rhamnose is weakly alkaline, lactose is acidic, mannitol assimilation in a weakly alkaline environment, inability to assimilate glycerin at all,
assimilation of sorbitol in a weakly acidic environment. D-mannitol was found to be assimilated into acids. Hydrolysis of starch, layered dilution of gelatin, peptonization of milk was noted.

Table 3. Cultural-morphological and physiological biochemical properties of isolated No. 12 rhizobacterial isolate

| Morphological features | 2-3x0.5-0.6 μm, the cells are thin, mobile, with spores at the end of the cell |
|------------------------|--------------------------------------------------------------------------------|
| Gram staining          | Gram positive                                                                  |
| Growth in MPA         | Colonies are round, fuzzy-white, abundant growth                               |
| Growth in MPB         | White thin film, broth clear                                                   |
| Growth temperature (°C)| Optimum 18-36, maximum 41                                                      |
| Breathing              | Aerobic                                                                       |
| Assimilation of gelatin| Decomposes to form a layer                                                     |
| Assimilation of sucrose| Weakly alkaline                                                                |
| D-glucose              | Sour                                                                           |
| Xylose                 | Alkaline                                                                       |
| Arabinoza              | Weakly acidic                                                                  |
| Maltose                | Weakly alkaline                                                                |
| Lactose                | Alkaline                                                                       |
| Mannitol               | Alkaline                                                                       |
| Glycerin               | -                                                                              |
| Sorbitol               | Alkaline                                                                       |
| Starch                 | Hydrolyzes                                                                    |
| Milk                   | Peptonizes                                                                     |
| Casein                 | -                                                                              |
| Urea                   | +                                                                              |
| Catalase               | +++                                                                            |
| Nitrates, KNO₃        | +                                                                              |
| Denitrification        | -                                                                              |
| Na citrate             | +++                                                                            |
| Phenylalanine          | ++                                                                              |
| Trypton                | +                                                                              |
| Lysotsim               | -                                                                              |
| Reaction with NaCl     | Grows from 7.5%                                                                |
| Voges-Proskauer reaction| +                                                                              |

Note: +++ grows or assimilates very well; ++ assimilates or grows well; + positive result; - does not grow or assimilate.

The culture has high catalase activity, very good urea uptake, growth from nitrates - KNO₃, non-denitrification, very good uptake of casein, good uptake of sodium citrate, good uptake of phenylalanine and tiptonin, and very good growth in lysozyme medium (Tables 3-4).
Table 4. Cultural-morphological and physiological biochemical properties of isolated No. 136 rhizobacterial isolate

| Morphological features | 1-2x0.5-0.6 μm, cells thick rods, less mobile, forms spores at the end of the cell |
|------------------------|---------------------------------------------------------------------------------|
| Gram staining          | Gram positive                                                                   |
| Growth in MPA          | Colonies round, gray-white, round, bulging, edges smooth, oily consistency, smooth, slimy, upper part glossy |
| Growth in MPB          | White thin uncurled film, broth clear                                           |
| Growth temperature (°C) | Optimum 20-36, maximum 41                                                       |
| Breathing              | Aerobic                                                                         |
| Assimilation of gelatin| Decomposes to form a layer                                                       |
| Assimilation of sucrose| Weakly acidic                                                                   |
| D-glucose              | Alkaline                                                                        |
| Xylose                 | -                                                                               |
| Arabinose              | Sour                                                                            |
| Maltose                | -                                                                               |
| Lactose                | Acidic                                                                          |
| Mannitol               | Weakly alkaline                                                                 |
| Glycerin               | -                                                                               |
| Sorbitol               | Weakly acidic                                                                   |
| Starch                 | +                                                                               |
| Milk                   | Peptonizes                                                                      |
| Casein                 | +++                                                                             |
| Urea                   | +++                                                                             |
| Catalase               | +++                                                                             |
| Nitrites, KNO3         | +                                                                               |
| Denitrification        | -                                                                               |
| Na citrate             | +                                                                               |
| Phenylalanine          | +++                                                                             |
| Trypton                | +++                                                                             |
| Lysostim               | +++                                                                             |
| Reaction with NaCl     | Grows from 3%                                                                    |
| Voges-Proskauer reaction| -                                                                              |

Note: +++ grows or assimilates very well; ++ assimilates or grows well; + positive result; - does not grow or assimilate.

4. Conclusions

All over the world, salinity is one of the main detrimental factors for agricultural production. Nevertheless, the issue of using saline lands to meet the food needs of the growing population is relevant.
Despite the physical and chemical methods used in conjunction with a number of traditional methods to prevent salinization in irrigated soils, there are serious difficulties in finding a solution to the problem. The ability of soil rhizobacteria to accelerate the growth and development of plants under conditions of salinity stress is used around the world in the field of sustainable agricultural development, including recultivation of soils by phytoregulation, bioremediation.

As a result of the study, it was determined that the bacterial isolates isolated from the saline soil conditions belonged to the genus Bacillus and were selected as the basis for the development of stimulant drugs for further research.

References
[1] Chebotar VK 2015 Agrobiology 50(5) 648-654.
[2] Olfa KF, Saoussen BK, Mouna D, Amel K, Hayfa JK, Majda DR, Slim T 2016 Biol. Control 26 73-82.
[3] Mnif I, Ghibri D 2015 Biopolymers 104(3) 129-147.
[4] Lirong Y, Quan X, Xuea B, Goodwinb PH, Lua S, Wang J, Dua W, Wua C 2015 Biol. Control 85 52-58.
[5] Ines M, Dhouha G 2015 Peptides 71 100-112.
[6] Netrusov AI, Egorova MA, Zakharchuk LM 2005 Practical Course on Microbiology, Akademiya, Moscow 608. (in Russian)
[7] Lysak LV, Dobrovolskaya TG, Skvortsova IN 2003 Methods of Bacterial Differentiation of Soil and Identification of Soil Bacteria, Max Press, Moscow 120. (in Russian)
[8] Kreig NR, Holt JG 1994 Berge’s Manual of Determinative Bacteriology 787.
[9] Borris R 2015 Principles of Plant-Microbe Interactions, Springer-Berlin 379-392.
[10] Brimecombe MJ, De Leij FAAM, Lynch JM 2010 Plant growth and health promoting bacteria, Springer-Heidelberg 21-43.
[11] Glik B 2015 Principles of Plant-Microbe Interactions, Springer-Berlin 257-265.
[12] Lugtenberg B 2015 Principles of Plant-Microbe Interactions, Springer-Berlin 7-15.
[13] Nakkeren A 2005 Biocontrol and biofertilization, Springer-Dordrecht 257-296.
[14] Thomashow LS 2013 Microbial phenazines, Springer-Berlin 199-216.
[15] Ahmad F, Husain FM, Ahmad I 2011 Microbes and Microbial Technology, Springer-New-York 363-391.
[16] Antoun H, Kloeper JW 2001 Encyclopedia of Genetics 1477-1480.
[17] Timmusk S, Grantcharova N, Wagner EG 2005 Appl. Environ. Microb. 71 7292-7300.
[18] Wang T, Liang Y, Wu M, Chen Z, Lin J, Yang L 2015 Chinese J. Chem. Eng. 23(4) 744-754.
[19] Jasques P 2011 Microbiology monographs, Springer-Berlin 20 3-10.
[20] Nagorska K, Bikowski M, Obuchowski M 2007 ActaBiochim. Pol. 54(3) 495-508.
[21] Lopez D, Fischbach MA, Chu F, Losick R, Kolter R 2009 PNAS USA 106 280-285.
[22] Fickers P, Guex LS, Damblon C, Leclérel V, Béchet M, Jacques P, Joris B 2009 Appl. Environ. Microb. 12 4636-4640.
[23] Kino K, Kotanaka Y, Arai T, Yagasaki M 2009 Biosci. Biotech. Bioch. 73(4) 901-907.
[24] Isaev S, Begmatov I, Goziev G, Khasanov S 2020 In IOP Conference Series: Materials Science and Engineering 883(1) 012080.