REPLACEMENT OF THE TRADITIONAL FERTILIZER WITH MICROBIAL TECHNOLOGY: ISOLATION AND CHARACTERIZATION OF BENEFICIAL NITROGEN FIXING RHIZOBACTERIA

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Scopul lucrării a fost izolarea şi selectarea unor bacterii din sol şi din rizosfera plantelor, cu caractere benefice multiple asupra plantelor în scopul de a contribui la aplicarea lor ca biopreparate, o strategie alternativă pentru evitarea folosirii în exces a îngrăşămintelor chimice.

Microorganismele benefice au un rol important în transformarea, mobilizarea şi solubilizarea nutrienților aflate în sol. 50 de tulpini bacteriene benefice fixatoare de azot au fost izolate din nodozitățile şi din rizosfera diferitelor plante leguminoase. O parte din aceste bacterii izolate au fost caracterizate prin teste clasice biochimice şi testate in vitro pentru caracterelor benefice cum ar fi producerea de amoniac, de siderofori şi solubilizarea fosfaților anorganici.

The aim of our study was the isolation and screening of plant and soil bacteria with multiple beneficial effects on plants in order to contribute to the application of these isolates as biofertilizers, an alternative strategy to avoid the application in excess of mineral fertilizers.

The beneficial microbes play an important role in the transformation, mobilization and solubilization of the soil nutrients content. 50 beneficial diazotrophic rhizobacteria were isolated from different leguminous plant nodules and rhizosphere. A part from these isolated bacteria was characterized by classical biochemical tests and was tested also in vitro for their beneficial traits like ammonia and siderophore production and inorganic phosphate solubilization.

Keywords: rhizobacteria, siderophore production, phosphate solubilization

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1. Introduction

Rhizobia are well known as the microbial symbiotic partners of legumes, forming N_2-fixing nodules. However these bacteria also share many characteristics with other plant growth promoting rhizobacteria [1].

The availability of sufficient soluble C, N and P nutrients is indispensable for plant growth, but soluble N and P nutrients are often limiting factors in agricultural soils [2]. The use of beneficial microbes for biological control and to improve crop production became important in the sustainable agriculture. The diazotrophic bacteria play an important role in the transformation, mobilization and solubilization of the soil nutrient content and their ability to fix N_2 in symbiosis with legumes plays central role in the N recycling of most natural ecosystems [3]. Beneficial effects of these bacteria have been attributed to mechanisms such as production of ammonia and siderophore, solubilization of phosphates.

Phosphorus is one of the major essential macronutrient for biological growth and development. Most of the agricultural soils contain a large amount of insoluble phosphates. The phosphorus applied as chemical fertilizers, after application rapidly transform in insoluble form and becomes unavailable to plants [4]. Some microorganisms such as nitrogen fixing bacteria convert these insoluble phosphates into soluble forms due to organic acid production. Production of organic acids results in acidification of the microbial cell and its surroundings. Orthophosphates may be released from insoluble phosphate compounds (hydroxiapatite, tricalcium phosphate) with the substitution of Ca^{2+} [5].

The role of siderophores is to scavenge iron from complexes and to mineralize making available them to the microbial cell, which is almost always essential [6]. Siderophores chelate iron and other metals contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats. Siderophores may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria and may function as a stress factors or including local and systematic host resistance.

The production of siderophores occurs only under iron-limited conditions. Such conditions are likely to prevail in the rhizosphere, and competition for iron through the production of siderophores is one of the mechanisms of bacterial antagonism against soil-borne pathogens. Siderophores play a role not only in microbial antagonism in the rhizosphere, but also stimulates the plant defense capacity [1].

These beneficial microorganisms can be considered as a significant component of the management practices in order to achieve an attainable yield, which has been defined as crop yield, determined only by the natural physical environment of the crop and its innate genetic potential [7].

The aim of our study was to isolate and characterize nitrogen fixing nodule bacteria and rhizobacteria and to screen these isolates for ammonia production, phosphate solubilization and siderophore production.

2. Experimental

2.1. Isolation of rhizobacteria

50 bacteria were isolated from different leguminous plant roots nodule and rhizosphere soil from Transylvanian place. 11 of the cultures originated from *Lupinus polyphyllus* Scop. nodules and six from rhizosphere soil of this plant, eight from *Lathyrus laevigatus* Gren. soil, eight from *Trifolium hybridum* L., and 17 from *Vicia grandifloras* Scop. rizosphere.

The nodules were disinfected in a 6% sodium hypochlorite solution, then washed in sterilized water and smashed in a sterile mortar, homogenized with 9 ml distilled water. From the homogenized extract that contains the rhizobia an amount of 0.1 ml was spread on the surface of yeast extract mannitol agar (YEM medium). The YEM medium contained per liter of distilled water: 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 1.0 g yeast extract, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeCl_3 \cdot 6H_2O$, 20 g agar, 25 μg/ ml bromothymol blue, pH 6.7–7.0.

From the soil samples serial dilutions were prepared, and a volume of 0.1 ml was spread on YEM medium. The inoculated Petri-dishes were incubated 48 h on 28°C.

The isolates were maintained on YMA agar slants, with the following composition per liter of distilled water: 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g yeast extract, 15 g agar.

2.2. Morphological and physiological characterization of rhizobacteria isolates

Bacterial isolates were characterized by biochemical tests (utilization of lactose and glucose, oxidase test, nitrate reduction) cell and morphological characteristics (Gram's reaction, determination of the presence of spores) and growth in thioglycolate agar [8].

2.3. Production of ammonia

Bacterial cultures were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water and incubated for 48–72 h at 30°C. After incubation Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color was a positive test for ammonia production.

2.4. Siderophore production

Siderophore production was detected by the universal method of Schwyn and Neilands (1987) using blue agar plates containing the chrom azurol S (CAS) dye. Orange halos around the colonies on blue were indicative for siderophore production.

2.5. Phosphate solubilization

The ability of isolates to solubilize phosphate was assessed using Pikovskaya's agar containing $Ca_3(PO_4)_2$. Each bacterial culture was spot inoculated in the centre of the plate. After incubation for 48 hour at 28°C a clear zone around the colony was the indication for inorganic phosphate solubilization [9].

The isolated bacteria were classified on the basis of the examined characteristics with Principal Component Analysis (PCA).

3. Results and Discussions

50 isolates from different leguminous plants and rhizosphere soil were characterized by classical biochemical tests and screened in vitro for their beneficial traits like ammonia and siderophore production, and inorganic phosphate solubilization.

84 % of the isolated bacteria were found to be Gram-negative and at no one of the isolates could be determined the presence of spores. Some of the microorganisms have the property to decompose carbohydrates into aldehydes, acids and CO_2. All the tested isolates were able to utilize glucose as carbon source and only one was able to oxidize lactose.

A lot of bacteria have the capacity to utilize oxygen from nitrate as electron acceptor in anaerobic respiration, a reaction controlled by the enzyme nitratase. From the isolated bacteria 96% are capable to reduce the nitrate, a trait characteristic of rhizobia.

The strains were tested for the presence/absence of cytochrome oxidase enzyme, that catalyses the electron transport among different substrates from bacterial cell and tetramethyl-p-phenylenediamine. The lack of this enzyme is characteristic for rhizobia. 84 % of the isolated bacteria were negative for the oxidase test.

The results of the oxygen use showed that 26 of the isolates were strictly aerobic, 11 facultative anaerobic, 3 microaerephilic and 10 bacterial isolates aerobic and facultative anaerobic.

` 82% of the isolates were able to produce ammonia.

The production of siderophore was detected in 32% of isolates that means 16 bacterial isolates had the capacity to produce these compounds.

The ability of bacteria to solubilize inorganic phosphate was observed at 27 isolates. The solubilization of P increases the phosphorous availability to the host plant. The low availability in natural environments is caused by the insoluble forms of the soil phosphate. The plants are able to absorb the monobasic and the dibasic phosphate ions that are converted from insoluble forms by the bacteria.

The results of the PCA (Fig.1) showed that the isolates can be classified into four main groups.

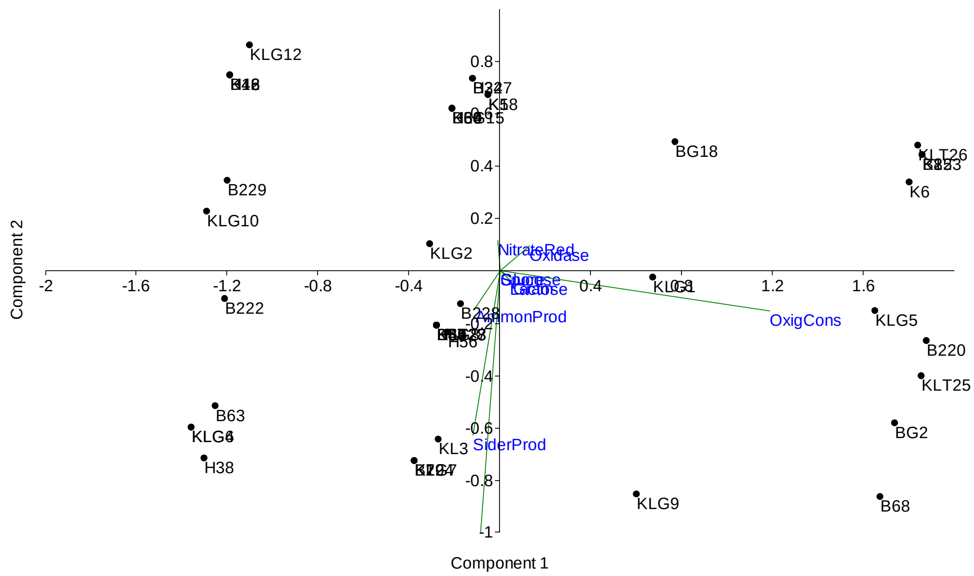

Fig.1. Classification of the isolates with PCA

In the first part of the diagram are grouped isolates with no phosphate solubilization capacity and they differ from the others in oxygen utilisation and in beneficial traits. Isolates grouped in the second part of the PCA graph are not able to produce ammonia. The third class the isolates differ in nitrate-reduction capacity and possess at least two beneficial traits. The last group may divide in two subgroups, where the isolates can be differentiated in oxygen consumption. In the second subgroup are classified bacteria isolates that are aerobic and facultative anaerobic.

The bacterial isolates marked as B63, KLG4, KLG6, H38, KL3, K7 forms same group based on they common ability to produce ammonia and siderophore and also to solubilize phosphate. The bacterial isolates marked as KLG5, B220, KLT25, BG2, B68, KLG9 were found to be similar from point of view of their oxygen utilization, lactose and glucose fermentation, Gram's reaction, spore formulation. These bacterial isolates are facultative anaerobic, with ability to utilize glucose but not lactose.

4. Conclusions

On the basis of our results, 22 of the 50 isolates had similar characteristics with *Rhizobium* species and almost all strains of the isolated bacteria posses at least one beneficial trait that promote plant growth. A number of 10 bacterial isolates originated from *Lupinus polyphyllus* Scop., *Trifolium hybridum* L, *Vicia grandiflora* Scop. plants were found to be positive for the three beneficial traits ammonia and siderophore production and phosphate solubilization ability.

These isolates could be a promising source for plant growth promoting biofertilizers in sustainable agriculture.

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