SIDEROPHORES AND INDOLE-3-ACETIC ACID PRODUCTION BY BACTERIAL STRAINS ISOLATED FROM SOYBEAN RHIZOSPHERE

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Keywords: siderophores; indole-3-acetic acid, soybean, rhizosphere

Abstract: Rhizobacteria with siderophores and IAA-producing ability may be useful in growth promotion of crop plants or in growth suppression of weeds. Considering the benefits of intensive agriculture practice and the negative environmental impact of chemical fertilizers and pesticides, usage of rhizobacteria as biofertilizers is one of the most promising biotechnologies. Therefore, the present study was performed in order to select several bacterial strains isolated from soybean rhizosphere and rizoplane with siderophores and IAA producing capabilities. Our results showed that soybean rhizosphere represent a very important source for isolation of bacteria with plant growth promoting capabilities. The majority of these microorganisms live in the soil surrounding the roots, but it can be also found in the rizoplane. Many of the isolated rhizobacteria present both siderophores and IAA producing capabilities, proving that the promoting plant growth effect is the result of synergic relations established between different rhizospheric microorganisms.

INTRODUCTION

In an aerobic environment, iron is virtually insoluble at biological pH, where it exists in the trivalent state as oxyhydroxide (Schwyn and Neilands 1987). In response, microorganisms produce and secrete siderophores to sequester and transport iron. These compounds can be defined typically as small molecules (often <1000 Da, although some siderophores are bigger) that are rapidly assembled through short, well-defined metabolic pathways (Perez-Miranda et al. 2007). Siderophores appear to be confined to microbes and are not products of the metabolism of plants or animals, which have their own pathways for uptake of iron (Neilands 1995).

Typically, microbial siderophores are classified as catecholates, hydroxamates, and α-carboxylates, depending on the chemical nature of their coordination sites with iron (Winkelmann 2002). Hydroxamates are produced by fungi and bacteria, whereas catecholates are produced exclusively by bacteria and comprise catechol and hydroxy groups as ligands. α-carboxylates are produced by the group of fungal zygomycetes (*Mucorales*) and a few bacteria, such as *Rhizobium meliloti* and *Staphylococcus hyicus*, and coordinate iron through hydroxy and carboxyl groups (Baakza et al. 2004).

Capacity to form different siderophores type has been associated with improved plant growth either through a direct effect on the plant, through control of noxious organisms in the soil or via some other route (Neilands 1995).

Auxins, a class of plant hormones, are known to affect plant growth throughout ontogeny. Auxins are produced by plants and several microorganisms including bacteria and fungi (Arshad and Frankenberger 1991). Certain bacteria can be very prolific producers of the indole-3-acetic acid (IAA - a phytohormone called auxin), when provided with L-tryptophan (L-TRP) as a precursor (Loper and Schroth 1986).

Determination of siderophores and IAA-producing ability of microorganisms is useful for identification and also serves as a valuable indicator of physiological roles and ecological significance of these microorganisms in the environment. Rhizobacteria selected for siderophores and IAA production may be useful in growth promotion of crop plants (Kloepper et al. 1991) or in growth suppression of weeds (Lynch 1990). Considering the benefits of intensive agriculture practice and the negative environmental impact of chemical fertilizers and pesticides, usage of rhizobacteria as biofertilizers is one of the most promising biotechnologies for growing the primary production with less quantity of fertilizers (Bashan 1998). In this line, the present study was performed in order to identify siderophores and IAA producing capabilities of several bacterial strains isolated from soybean rhizosphere (a narrow zone of soil subject to the influence of living roots, where root exudates stimulate or inhibit microbial populations and their activities) (Pinton et al. 2007) and rizoplane (thin soil layer intimately adherent to plant roots (Sylvia et al. 1999). The main goal is to isolate rhizobacteria with plant growth promoting capabilities which can be used in different agricultural biotechnological applications.

MATERIALS AND METHODS

Microorganisms and growth conditions

Bacteria were isolated from the rhizosphere and rizoplane of field-grown soybean crop from Ezareni Farm, Iasi. Plants and roots were collected by removing 20-cm^2 blocks of soil, which were kept in plastic bags at 4 °C for 12 h until processing. Roots were separated from bulk soil. Soil remained on the surface roots after moderate shaking was used for

isolation of rhizospheric microorganism. After the separation of soil, roots were soaked in sterile phosphate-buffered saline (PBS, pH 7.2, 10 mM K_2PO_4-KH_2PO_4, 0.14 M NaCl) for 10 min, chopped into 5 g pieces and suspended in 45 ml PBS. Rhizospheric soil and root samples were blended in a sterile Waring blender at high speed for 1 min and serial dilutions (1/10) were made in PBS. Aliquots (0.1 ml) were plated on Bunt Rovira nutrient medium and incubated at 28°C for 7 days. 26 rhizobacteria isolates were selected to represent distinct types based on differences in colony morphology including: colony form, elevation, opacity and pigment production. Isolates were re-streaked on Bunt Rovira nutrient medium, checked for purity, and stored on slants at 4° C.

O-CAS assay

CAS medium was prepared according to Schwyn and Neilands (Schwyn and Neilands 1987). The medium for a liter of overlay was as follows: Chrome azurol S (CAS)-60.5 mg, hexadecyltrimetyl ammonium bromide (HDTMA)-72.9 mg, Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)-30.24 g, and 1 mM$FeCl_3 \cdot 6H_2O$ in 10 mM HCl-10 mL. Agarose (0.9%, w/v) was used as gelling agent. Siderophore detection was achieved after 10 mL (standard, 100 mm diameter Petri dishes) overlays of this medium were applied over those LB plates containing cultivated rhizobacteria to be tested for siderophore production. After a maximum period of 15 min, a change in color will be observed in the overlaid medium, exclusively surrounding producer microorganisms, from blue to purple (as described in the traditional CAS assay for siderophores of the catechol type) or from blue to orange (as reported for microorganisms that produce hydroxamates). All these experiments were made at least three times with three replicates for each one.

Colorimetric IAA assay

For colorimetric IAA assay, a 24-h tryptic soy agar (TSA) culture of each isolate was suspended in sterile water to an optical density (O.D.) of 0-5 at 500 nm. The suspension (2 ml) was added to 28 ml of growth medium in a 50-ml tube. The growth medium contained (in g/l): glucose-5; yeast extract-0.025; L-TRP-0.204. Controls were prepared by substituting sterile water for bacterial suspension. Tubes were capped, vortexed and statically incubated in the dark at 28° C for 72 h. Prior to analyses for auxins, individual cultures were adjusted to 10^8 cells ml^{-1} at 500 nm absorbance with sterile water and filtered through 0.2 µm membranes. Triplicate tubes of each isolate were used for the assays.

Samples were assayed for production of auxins (IAA equivalents) using standard method of Gordon and Weber (Gordon and Weber 1951) in which auxin present in the culture filtrate (3 ml) was reacted with Salkowski reagent (2 ml) to yield a pink-coloured product after 30 min incubation, which was quantitatively measured on a Beckman-Coulter DU 730 Life Sciences UV-VIS spectrophotometer at 530 nm.

RESULT AND DISCUSSIONS

Siderophores production

After macro- and micromorphological characterization, 26 strains isolated from soybean rhizosphere and rizoplane were tested for siderophores producing capabilities based on the O-CAS assay. 17 strains were found positive for siderophore production. There were no significant qualitative differences among strains isolated from rhizosphere and from rizoplane, although most of the positive strains were isolated from the rhizospheric soil (10 strains).

Several microorganisms produced changes in medium coloration that are in agreement with previously reported studies (Perez-Miranda et al. 2007; Baakza et al. 2004). Hence, strain R2.7 changed the color to orange (which corresponds to hydroxamates, as mentioned above), whereas other strains changed the color to purple (which corresponds to catechol-type siderophores) - Fig. 1 and Table 1.

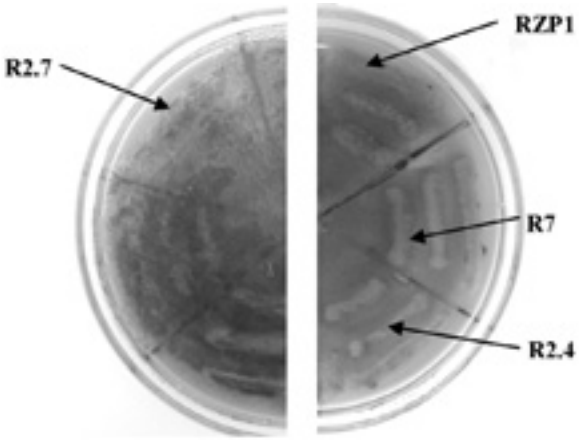

Fig. 1 – O-CAS assay performed using different rhizobacterial strains. Different changes in the color of the medium can be appreciated: left – R2.7 strain that produce hydroxamates; right - strains that produce siderophores of the catechol type

Table 1 – Siderophores production by rhizobacterial isolates

Strains	Results	O-CAS medium color
R1	+	purple
R2	-	-
R2.1	+	purple
R2.3	-	-
R2.4	+	purple
R2.5	+	purple
R2.6	+	purple
R2.7	+	orange
R6	+	purple
R7	+	purple
R8	+	purple
R9	-	-
R10	+	purple
RZP1	+	purple
RZP2	-	-
RZP2.1	+	purple
RZP2.3	+	purple
RZP2.5	-	-
RZP2.6	+	purple
RZP3	-	-
RZP4	+	purple
RZP5	+	purple
RZP6	+	purple
RZP8	-	-
RZP9	-	-
RZP10	-	-

+: positive reaction
-: negative reaction

Indoleacetic acid production by rhizobacteria

The selection of indoleacetic acid producing microorganisms was based on colorimetric IAA assay. The overall results are presented in Table 2. Only 13 strains were found positive for IAA production with different IAA synthesis capabilities. The vast majority of the positive strains were isolated from the soil surrounding soybean roots (9 strains), although the best IAA producers were found in the rizoplane (RZP2.3 – 3.92 μg IAA/ml and RZP1 – 3.14 μg IAA/ml) – Fig. 2.

Table 2 – Indoleacetic acid production by rhizobacterial isolates

Strains	IAA production
R1	nd
R2	nd
R2.1	+
R2.3	nd
R2.4	+
R2.5	nd
R2.6	+
R2.7	+
R6	+
R7	+
R8	+
R9	+
R10	+
RZP1	+
RZP2	nd
RZP2.1	nd
RZP2.3	+
RZP2.5	nd
RZP2.6	nd
RZP3	nd
RZP4	+
RZP5	+
RZP6	nd
RZP8	nd
RZP9	nd
RZP10	nd

+: positive reaction
nd: not detected

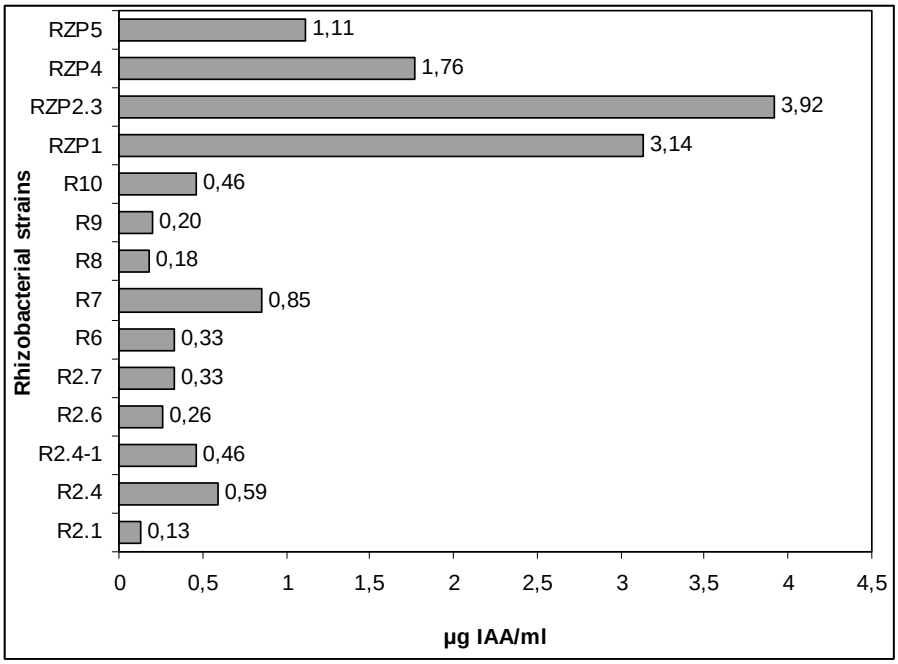

Fig. 2 – IAA producing capabilities of different bacterial strains isolated from soybean roots

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms (Vessey 2003). These mechanisms includes siderophores and phytohormones production (Saleh and Glick 2001; Bashan 1998). As our study revealed, more than 65 % of the bacterial strains isolated from soybean roots produced siderophores of different types (mainly catechol and hydroxamates type) as well as more than 50 % presented IAA producing capabilities. The majority of these strains were isolated from the rhizosphere (Fig. 3). This is not an unusual situation since rhizosphere is defined as the soil directly influenced by root secretions with intensive microbial activities (Lynch 1990). Although the rhizosphere can be a great source of bacteria with plant growth promoting potential (Zahir et al. 2003), the strains with the highest IAA producing capability were isolated from the rizoplane. This observation can be related with the role of IAA in plant development. Indole-3-acetic acid is a phytohormone which is known to be involved in root initiation, cell division, and cell enlargement (Salisbury 1994). Most commonly, IAA-producing bacteria are believed to increase root growth and root length, resulting in greater root surface area (Vessey 2003). Therefore, the presence of such microorganisms at the interface between root tissues and surrounding soil enables the plant to access more nutrients from soil, influencing directly plant growth.

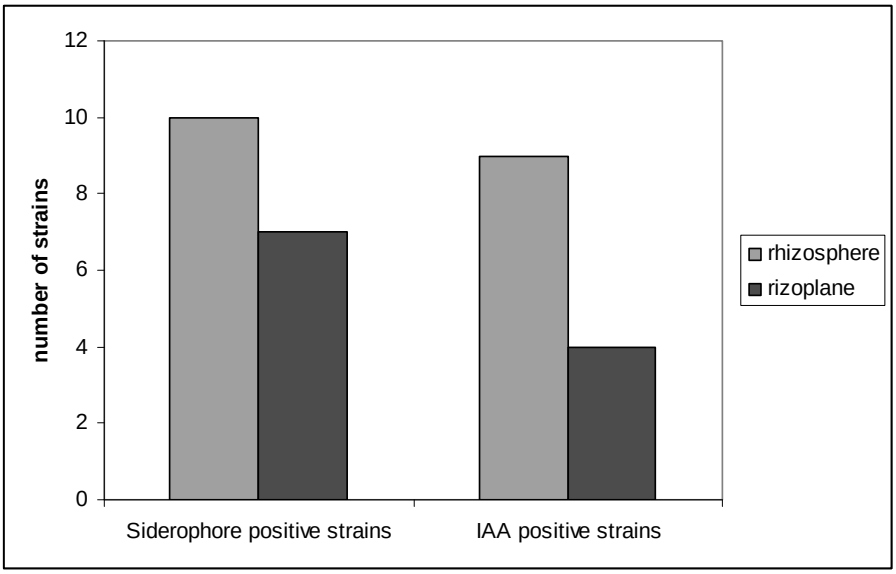

Fig. 3 – Isolation sources for tested bacterial strains

In normal conditions, it is not uncommon to see the promoting plant growth effects of rhizobacteria via a synergism of the rhizospheric microorganism (Vessey 2003). Our study revealed a similar situation since more than 12 bacterial strains isolated from rhizosphere and rizoplane presented both siderophores and IAA producing capabilities.

CONCLUSIONS

This study has shown that soybean rhizosphere represent a very important source for isolation of bacteria with plant growth promoting capabilities. The majority of these microorganisms live in the soil surrounding the roots, but it can be also found in the rizoplane. Many of the isolated rhizobacteria present both siderophores and IAA producing capabilities, proving that the promoting plant growth effect is the result of synergic relations established between different rhizospheric microorganisms. Further tests are necessary to be employed in order to specify if those strains present also antibacterial activity. This type of bacteria can be used successfully as biofertilizing agents in different agricultural applications.

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Acknowledgements. This research was supported by the National Council of Scientific Research and University Education (CNCSIS, Grant IDEI, cod 440, nr. 249/01.10.2007), Romania.

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