BENEFICIAL CHARACTERISTICS OF SOME BACTERIAL STRAINS ISOLATED FROM RHIZOSPHERE

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Abstract

The present study refers to selection in steps of beneficial bacterial strains for cropped plants, based on important biological traits which are involved in the induced systemic resistance (ISR) and therefore are key elements in plant defense mechanisms.
A total of 85 cropped plants rhizosphere isolates were analyzed for antagonistic activity *in vitro* against soil borne fungi, like *Fusarium graminearum, Alternaria* spp., *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Botrytis cinerea* and *Sclerotium bataticola*, for enzymes production, like cellulase, amylase and lactonase, for mobility, biofilm formation and plant growth promotion effect on wheat seedlings. Five strains were selected, coded with Usa2, Cpb6, Mzb4 (Gram positive, isolated from garlic, onion and pea plants rhizosphere), Salc2 and Rdb2 (Gram negative, isolated from lettuce and radish rhizosphere). The strains Usa2, Mzb4, Cpb6 belong to *Bacillus* spp. and the strains Salc2 and Rdb2 to *Pseudomonas* spp. based on their fatty acid profile and to Bergey's Manual of Systematic Bacteriology and showed high antagonistic activity *in vitro* against the mentioned soil borne fungi. All of them produced amylase, the strains Usa2 and Rdb2 produced cellulase and the strains Rdb2, Cpb6, Mzb4, Usa2 produced lactonase. All strains showed both swimming and swarming motility and formed biofilm in the tested growing media.

***Key words*:** biocontrol, beneficial bacteria, plant growth promotion.

INTRODUCTION

Crop loss due to plant diseases was estimated at 9.7% to 15.7% worldwide (www.apsnet.org).

Biological control by using beneficial microorganisms is considered an alternative way of reducing the use of pesticides in agriculture. In the last decades, the increasing use of agrochemicals lead to several negative effects, like for example development of pathogen resistance to the applied pesticides, harmful impact on non-target organisms, soil and water pollution.

There are several mechanisms which might be involved in biocontrol, like antibiosis, induced systemic resistance, predation and parasitism and competition for nutrients and niches. Activity is not restricted to only one of these, and, indeed, an efficient biocontrol agent may affect pathogens by a combination of mechanisms (Chet, 1987).

The rhizosphere region is heavily populated by different microorganisms including both beneficial and deleterious ones. The rhizosphere is the first-line defense for roots against attack by pathogenic fungi (Weller, 1988). Therefore, there is an excellent opportunity to find rhizosphere-competent bacteria in the rhizosphere which are potential biocontrol agents. A successful biocontrol agent efficiently suppresses the pathogen and reduces disease incidence (Chet, 1990).

Soil borne fungi are a serious threat for the vegetables crops as tomatoes, pepper, cucumber and onion, produced in greenhouse conditions, due to the important economically losses of 10-30% of the seedlings.

The aim of this study was to isolate, characterize and select beneficial rhizobacteria based on their biological traits, like antagonistic activity *in vitro*, enzymes production, quorum quenching response, mobility, biofilm formation and plant growth promotion.

MATERIAL AND METHOD

Microorganisms and growth conditions

The microorganisms were isolated by distributing 1 g of vegetal sample from rhizosfere in 15 ml Nutrient Broth (NB) media in Erlenmeyer flasks follow by incubation at 28°C and 150 rpm for 24 hours. After, 50-100 µl from the culture were distributed with a Drigalsky ooze on nutrient agar (NA) or Luria Bertani agar (LBA) media and incubated at 28°C. To identify the spores producing strains, 2 ml from each culture were placed in sterile glass tubes and treated at 100°C on water bath for 15 minutes and 100µl were placed on NA and incubated at 28°C.

The plates were incubated for 24- 48 hours and then single colonies were picked up and purified on fresh NA media. Routinely, the strains were grown on LBA media at 28^0C.

Antagonistic activity

The antagonistic activity of the isolates was tested *in vitro* against the soil borne fungi: *Fusarium graminearum, Alternaria* spp., *Fusarium oxysporum* f. sp. *radicis-lycopersici, Botrytis cinerea* and *Sclerotium bataticola*.

The fungi were refreshed on potato-dextrose-agar (PDA) media and incubated at 28°C for 5 days.

The bacterial strains were grown on Luria-Bertani agar (LBA) at 28°C for 24 hours.

Briefly, the test was performed on PDA media and the distance between the bacteria and the calibrated fungal plug (5 mm) was 2 cm. The plates were incubated at 28°C and analyzed at 24, 48 and 72 hours for the inhibition of the mycelia growth. The test was repeated 3 times.

Identification of the selected strains

Selected strains, Usa2, Cpb6, Mzb4, Salc2 and Rdb2, were subjected to identification as per Bergey's Manual of Systematic Bacteriology (Juni, 1984) and in addition, two of the strains (Salc2, Usa2) by fatty acid profile analysis.

Enzymes production: cellulase, amylase and lactonase

Cellulase activity was determined by the breakdown of the substrate carboxymethylcellulose. Bacterial strains were inoculated on plates with minimal media supplemented with 1% carboxyl-methyl-cellulose (CMC) and grown for 5 days at 28°C. After, the plates were stained for 30 min with 0.3% Congo Red and subsequently rinsed with excess tap water and the dye was fixed by incubation with a 10% acetic acid solution for 15 minutes. The presence of a clear halo indicated the presence of cellulose activity.

Amylase activity was tested by growing the strains on nutrient agar (NA) media supplemented with 0,4% soluble starch and incubated at 28°C for 48-72 hours and then treated with iodine solution. The presence of a clear halo around the bacterial strains indicated the presence of amylase activity.

For the ***lactonase*** assay, the strains were inoculated in 2 ml of LB broth containing 5µM of C6-hexanoyl homoserine lactone (C6-HHL) and grown overnight (ON) at 28°C and 150 rpm. As a negative control the same media without bacteria was also incubated under these conditions. The next day a LC agar plate containing 50µg/ml of kanamicin was overlaid with Cv026 by spreading 250µl of an overnight culture. Wells were punctured into the plate (5 mm in diameter) and filled with 100µl of

bacterial culture. The plates were incubated overnight at 28°C and scored for the presence or absence of purple halos. Absence of purple halos indicates that all of the C6-HHL was degraded.

Motility

The strains were tested for their swimming and swarming motility. All strains were refreshed on LB agar plates supplemented with 1,8% agar and grown ON at 28 °C. LB agar plates (25 ml) containing 0,3% (swimming) or 0.5% (swarming) bacto-agar were prepared fresh and were dried for 30 min in the laminar flow cabinet. Each plate was toothpick inoculated and scored for swimming and swarming motility after 18 h incubation at 28°C.

Biofilm formation

The strains were checked *in vitro* for their ability to form biofilm.

Briefly, the strains were grown over night in LB media at 28^0C and 150 rpm. Ten microliters from each culture were used to inoculate 0,5 ml CM (Fall et al., 2004) and 0,5 ml M63 (O'Toole et al., 1998) media distributed in polypropylene Eppendorfs tubes. Together with the tested strains we took along in the experiment two control strains, *Pseudomonas fluorescens* WCS 365 (Simons et al.1996) as a positive control and *Bacillus subtilis* B168 (Burkholder et al., 1947) known as a week biofilm producer.

The inoculated tubes were incubated overnight at 37^0C without shaking. In order to analyze the biofilm formation phenotype, after discarding the medium and rinsing the tubes with water, adhering cells were stained with 1% w/v crystal violet for 15 minutes at room temperature, and then rinsed with water.

Crystal violet stained, surface- attached cells were quantified by solubilizing the dye in ethanol and determining the absorbance at 540 nm.

Plant growth promotion on seedlings

The bioassay was performed *in vitro* by using wheat seeds. Seeds were surface sterilized with 0.1% HgCl2 solution and washed three times with sterile distilled water. Subsequently seeds were placed on sterile filter paper discs (lodged in Petri plates Φ 9 cm) and wetted with 1.5 ml sterile water. After 24 hours of incubation in the dark at 25°C, the seeds were inoculated by imersion with 0.1 ml bacterial suspension in PBS. The control variant consisted in seeds treated with distilled water. The root length was measured after 72 hours of incubation in the dark at 25^0C.

RESULTS AND DISCUSSIONS

Beneficial rhizosphere bacteria are known to be used as inoculants for improving the growth and yield of agricultural crops and not at least to protect the plants against phytopathogens.

This study focuses on the selection of beneficial bacterial strains based on their potential to produce antifungal metabolites, enzymes that may play an important role in their biocontrol activity, mobility and biofilm formation. A total of 85 isolates were obtained from cropped plants rhizosphere.

Table 1

Antagonistic activity of the selected strains *in vitro*, by double culture method, inhibition zone (mm) after 72 hours incubation at 28^0C

Phytopathogenic fungus/ Strain code	*Alternaria* spp.	*Botrytis cinerea*	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	*Fusarium graminearum*	*Sclerotium bataticola*
Usa2	7	6	7	8	4
Salc2	10	8	5	5	5
Cpb6	7	8	7	7	5
Mzb4	8	3	3	6	8
Rdb2	9	6	3	6	6

Out of 85 isolates, 5 strains Usa2, Cpb6, Mzb4 (Gram positive, isolated from garlic, onion and pea plants rhizosphere), Salc2 and Rdb2 (Gram negative, isolated from lettuce and radish rhizosphere) showed the highest antagonistic activity *in vitro*, were mobile on semisynthetic media, formed biofilm in *in vitro* conditions, produced important enzymes and some of them promoted the growth of wheat seedling, therefore being selected for further studies.

Screening for the antagonistic activity of the isolates against the soil borne fungi: *Fusarium graminearum*, *Alternaria* spp., *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Botrytis cinerea* and *Sclerotium bataticola*, highlighted five strains Usa2, Cpb6, Mzb4, Salc2 and Rdb2 which were antagonistic against all the tested phytopathogenic fungi (table 1).

The highest inhibition zone against *Alternaria* spp. was induced by the strain Salc2 (10 mm) followed by the strain Rdb2, against *Botrytis cinerea* by the strains Salc2 and Cpb6 (with equal size of 8 mm), against *Fusarium oxysporum f. sp. radicis-lycopersici* by the strains Usa2 and Cpb6 (with equal size of 7 mm), against *Fusarium graminearum* by the strain Usa2 (8 mm) follow by the strain Cpb6 (7 mm) and against *Sclerotium bataticola* was induced by the strain Mzb4.

The selected strains were identified as *Bacillus* spp., Usa2, Mzb4, Cpb6 and as *Pseudomonas* spp., Salc2 and Rdb2 as per Bergey's Manual of Systematic Bacteriology (table 2). The strains Salc2 and Usa2 were identified based on their fatty acid profile and the results indicated that Salc2 belong to *Pseudomonas* spp. and Usa2 is a *Bacillus subtilis*.

Table 2

Biochemical properties of strains Usa2, Mzb4 and Cpb6

Biochemical test	**Results**
Gram Stain	+
Voges-Proskauer test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
$NO_3 \rightarrow NO_2$ reduction	+
Anaerobic growth	–
Catalaze production	+
Carbon sources:	
malonate	+
citrate	+
propionate	–
tartrate	+
trehalose	+
glucose	+
Acidify:	

xylose	+
glucose	+
arabinose	±
mannitol	+
raffinose	+
cellobiose	+
Tolerance at NaCl 7%	+

All 85 isolates were characterized for the production of compounds which might play a role in their biocontrol activity. Amylase allow bacteria to degrade extracellular starches, cellulase aids in root colonization and lactonase can act against gram negative phytopathogens that use AHL for their quorum sensing. From the selected five strains, all of them produced amylase, two strains, Usa2 and Rdb2 produced cellulase and four strains, Rdb2, Cpb6, Mzb4, Usa2 produced lactonase.

Motility plays a major role in root colonization. The selected strains showed both swimming and swarming after 24 h incubation at 28^0C.

The results from the biofilm formation test showed that all strains formed biofilm in both growing media, the highest amount being produced in CM media.

The strain Mzb4 produced the highest amount of biofilm (A_{540}=3,270), higher than in our positive control WCS365 (A_{540}=0,826). This strain was followed by the strain Usa2 (A_{540}=1,472) and Salc2 (A_{540}=0,856).

On the minimal media M63 all strains produced less biofilm than on the CM media (table 3).

Table 3

Biofilm quantification for the selected strains at A540nm, grown in CM and M63 media

Strain	Absorbance 540 nm – Grown on CM media	Absorbance 540 nm – Grown on M63 media
WCS365	0,826	0,206
B168	0,273	0,096
Mzb4	3,270	1,109
Rdb2	0,234	0,125
Cpb6	0,707	0,253
Salc2	0,856	0,204
Usa2	1,472	0,803

Biofilm formation is an important characteristic of the beneficial bacterial strains, this mechanisms being involved in plant protection by substrate colonization and competition for nutrients and nishes.

The plant growth promotion test on seedlings revealed that all strains promoted the wheat seedlings root growth comparing with the untreated check. The highest length of the roots and total length of the seedlings was registered in the variants were the seeds were inoculated with the strain Mzb4 (4,88 cm) and with the strain Salc2 (3,93 cm).

It was noticed that the inoculation of the seeds with the bacterial strains stimulated the germination comparing with the non-inoculated control variant. The highest percent of germinated seeds was reached after 72 hours in the variant were the seeds were immersed in the Mzb4 strain suspension (97%).

The results showed that, all strains promoted the growth of seedlings, the total length of the plants beeing of 5,45 – 6,77 cm.

There was no difference between the treatments concerning the total number of the adventitious roots developed, in all variants, except variant Salc2 (3 roots) the total number of roots being 4.

Further studies will focus on indole 3-acetic acid (IAA) production by the selected strains and also the strains will be tested in greenhouse conditions against soil borne phytopathogens.

Table 4

Plant growth promotion test on wheat plants (results at 3 days after inoculation of the seeds with 10^8cfu/ml)

Treatment/ Strain code	Germination		Number of roots	Root lenght (cm)	Wheat stem lenght (cm)	Total length of the plants (cm)
	From 30 seeds	%				
Control (distilled water)	25	83	4	2,87	2,53	5,40
Mzb4	29	97	4	4,88	1,89	6,77
Rdb2	26	87	4	3,78	2,45	6,23
Cpb6	26	86	4	3,05	1,25	4,30
Salc2	26	86	3	3,93	2,27	6,20
Usa2	26	86	4	3,45	2,00	5,45

CONCLUSIONS

- The selected strains proved high antagonistic activity *in* vitro against important soil borne fungi, like *Alternaria* spp., *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Fusarium graminearum* and *Sclerotium bataticola.*
- The strains were identified based on their fatty acid profile and according to Bergey's Manual of Systematic Bacteriology and the results proved that the strains Usa2, Mzb4, Cpb6 belong to *Bacillus* spp. and the strains Salc2 and Rdb2 to *Pseudomonas* spp.
- Biochemical characterization of the strains showed that all of them produced amylase, two strains, Usa2 and Rdb2 produced cellulase and four strains, Rdb2, Cpb6, Mzb4, Usa2 produced lactonase.
- All strains showed both swimming and swarming motility and formed biofilm in both growing media, M63 and CM, the highest amount being produced in CM media. The highest amount of biofilm was produced by the strain Mzb4 followed by the strain Usa2 and Salc2.

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