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SELECTION AND CHARACTERIZATION OF NEW ENDOPHYTIC BACTERIAL STRAINS ISOLATED FROM POTATO TUBER USEFUL IN BIOCONTROL STRATEGIES

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Abstract

Endophytic bacteria are plant-associated bacteria colonizing the internal plant tissue, living in symbiotic association with their host. Such microorganism could contribute to plant growth promotion and defence against biotic and abiotic stress. Our study aimed to describe new endophytic bacteria from potato tubers. Therefore, a group of 20 endophytic bacteria was isolated from seven Romanian varieties of healthy potato tubers. Four of the isolated strains revealed antifungal activity against three important pathogens of potato, Fusarium solani involved in tubers dry rot, Rhizoctonia solani involved in stem canker and black scurf of potato, and Alternaria solani causing early blight of potato plants. Among all twenty isolates obtained, 13 were Gram positive bacteria. Most of the newly isolated endophytes (65%) expressed phosphatase and protease activity, and 55% presented amylases, however only 10% revealed cellulose degrading enzymes. Based on preliminary laboratory analysis, the isolate 6T4 identified as *B. atrophaeus/subtilis* revealed promising perspectives for biocontrol strategies.

Key words*: bacterial endophytes, antifungal activity.*

INTRODUCTION

The environmental friendly approaches of plant growth promotion and plant protection, sustain the use of microorganisms as an alternative to chemicals. The most studied plant beneficial microorganisms are the rhizobacteria, but in the meantime the microbial endophytes gained a special attention, due to their understudied benefits for their hosts, including plant defence against biotic and abiotic stress. The particular interest for endophytes, is due to their attributes regarding plant growth promotion (Abbamondi et al., 2016; Liaqat and Eltem, 2016), and plant defence against biotic and abiotic stress (Choudhary and Johri, 2009; Rania et al., 2016; Akbari et al., 2016).

This study was focused on the endophytic bacteria from Romanian potato tubers, as potato culture is an important crop for Romanian agriculture. The aim of the study was to describe new endophytic strains with plant beneficial attributes.

MATERIALS AND METHODS

Isolation of bacterial endophytes

Seven varieties of potato tubers were used for bacterial endophytes isolation: Tâmpa 5, Zamolxis 5, Kronstad 5, Rustic 5, Christian 5, Cumidava 5 and Roclas 5, kindly provided by INCDCSZ Braşov (Romania). In order to remove the adhering soil particles and decrease the microbial load of epiphytic microorganisms, the potato tubers were vigorously washed with NaOCl 0.4% based detergent and rinsed with tap water (Anjum and Chandra, 2015). For surface disinfection they were immersed in 70% ethanol and flamed (Zinniel et al., 2002).

Using aseptic procedures the tubers were sliced with a sterile scalpel in laminar air flow cabinet and approximately 1 cm^3 of potato pulp was harvested and crushed in sterile phosphate saline buffer using a sterile mortar and pestle. After 15 minutes of diffusion, 100 µl of homogenate was plated on Luria Bertani (LB) agar and incubated at 28±0.5°C in order to

recover bacterial endophytes. Bacterial cultures were observed after 18, 24 and 40h of incubation, however morphologically different bacterial colonies were selected only after 48h of growth. To achieve pure bacterial isolates the selected bacteria were subcultured on the same solid medium. All selected isolates were stored in glycerol at -80°C. Before use the bacterial isolates were two times cultured in LB agar and, if necessary, maintained at 4°C till further used.

Characterization of bacterial isolates

Endophytic bacterial isolates were characterized based on colony morphology, Gram reaction, swimming and swarming motility, King B fluorescent growth in UV light, enzymes production (amylase, cellulase, protease, and phosphatase) and Voges-Proskauer reaction.

Antifungal activity evaluation

Isolated bacterial endophytes were analyzed for their antifungal activity against three fungal pathogens of stored potato tubers (*Fusarium solani*) or potato plants *(Rhizoctonia solani and Alternaria sp.).* The test was performed *in vitro*, on PDA medium, by dual culture technique (Soria et al., 2012).

Bacterial identification

Four of the studied endophytic bacterial isolates were identified using the Biolog GEN III technique according to the manufacturer protocol.

The identification was made using the semi-automatic Biolog Microbial Identification System, by analyzing 71 carbon source utilization capacity and 23 chemical sensitivity assays.

RESULTS AND DISCUSSIONS

Endophytic bacteria

All seven varieties of potato tubers hosted endophytic bacteria, from which twenty isolates were obtained (table 1). Most of them (13 isolates) are Gram positive bacteria.

Table 1. Endophyte bacterial isolates

Bacterial isolate	Hosting potato variety	Gram reaction	Motility		Enzymes production				
			swimming	Swarming	phosphatase	amylase	cellulase	protease	VP reaction
1T1	Tâmpa 5	–	±	–	+	–	–	–	+
1T2		+	+	+	+	–	+	+	+
1T3		+	–	–	±	+	–	+	–
2T1	Zamolxis 5	–	–	–	±	+	–	+	–
2T2		+	–	–	+	+	–	+	–
3T1	Kronstad 5	+	–	–	+	+	–	+	–
3T2		+	–	–	±	+	–	+	–
3T3		–	–	–	+	+	–	+	–
4T1	Rustic 5	+	–	–	+	+	–	+	–
4T2		+	–	–	–	–	–	–	–
4T3		+	±	–	+	–	–	–	–
5T1	Christian 5	+	–	–	–	+	–	+	–
5T2		+	–	–	–	–	–	–	–
6T1	Cumidava 5	–	+	+	+	–	–	+	+
6T2		–	+	+	–	–	–	–	–
6T3		+	–	–	+	+	–	+	–
6T4		+	+	+	+	+	+	+	–
7T1	Roclas 5	–	+	–	–	–	–	–	–
7T2		–	–	–	–	+	–	+	–
7T3		+	–	–	–	–	–	–	–

One of the isolated strains 6T1 was remarked for its orange pigmentation, abundantly synthesized and diffused on various nutritional substrates. This strain revealed swimming and swarming motility, protease activity, and positive VP reaction. Another strain, 6T4 was also highly distinctive among the other isolates especially due to its rapid colonization of soaked agar media. This Gram positive strain also presented swimming and swarming

motility on soft agar plates, and produced various hydrolytic enzymes such as amylase, cellulose, protease and phosphatase.
The bacterial strain 6T2 was the only one producing fluorescent pigment on King B medium, exposed in UV light.
This strain was not able to express any of the studied enzymes, was negative to VP and Gram reactions, but revealed very good swimming and swarming motility.
Among phosphate solublilizing bacteria, 1T1 isolate was the first initializing the hydrolysis, but 1T2 isolate expressed the largest and most clear zone of phosphate solubilization of all isolates (3mm).

Antifungal potential

The antifungal properties of the endophytic strains was analyzed *in vitro* against three important pathogens of potato: *Fusarium solani*, involved in tubers dry rot, *Rhizoctonia solani* involved in stem canker and black scurf of potato, and *Alternaria* spp. causing early blight of potato plants. Among all twenty selected isolates only 1T2, 6T1, 6T2 and 6T4 expressed inhibitory activity against the mentioned fungi.
The 6T1 and 6T4 strains were active against all three pathogenic fungi. However, 1T2 inhibited only *Rh.solani* and 6T2 reduced *Alternaria* sp. and *Rh.solani* growth.
Analyzing the microbial interactions, it was noticed that 6T1 maintained clear inhibiting zones, restraining each fungal growth at minimum 5mm (*F.solani* and *Rh.solani*), or 2mm distance (*Alternaria* sp.) from the bacterial colony edge, indicating diffusible antifungal compounds (figure 1).

Figure 1. Bacterial interaction of 6T1 strain with *Fusarium solani* hyphae reveal clear inhibition zone of the fungal growth.

Analyzing the interactions of 6T1 strain under the optical light microscope alterations in the morphology of the fungal hyphae was observed. The main alterations of *Alternaria* sp. growth consisted of curly growth of hyphae and irregular cells formations (figure 2).

Figure 2. Bacterial interaction of 6T1 strain with *Alternaria* sp. hyphae observed under the optical light microscope. Details illustrate irregular cells formations in the presence of 6T1 bacterial exudates.

Curly growth of the mycelia was also seen on *F.solani* towards the 6T1 colony (figure 3).

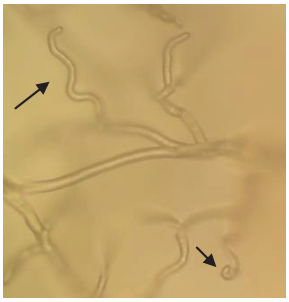

Figure 3. Curly growth of *Rh.solani* hyphae in the presence of 1T2 bacterial strain

On *Rh.solani*, the main alterations consisted of an increased number of vacuoles in the fungal cell, increased vacuole size, disruption of the tonoplast membrane or cytoplasmic coagulation within the hyphae (figure 4). Similar changes of cytoplasmic coagulation were also reported on eugenol treated fungal cultures of *Botrytis cinerea* (Wang et al., 2010). Moreover, fungal growth alterations with increased number of vacuoles in the fungal cell, and increased vacuole size were also reported in *Rosellinia necatrix* exposed to the biocontrol bacteria *Pseudomonas chlororaphis* PCL1606 (Calderón et al., 2014).
Rh.solani fungal growth was visible affected also by the presence of 6T4 bacterial strain. The

main alterations of fungal growth consisted of an increased number of vacuoles present within the hyphae; and an increase thickness of part of the mycelia (figure 5). Similar symptoms were also described by Giorgio et al. (2015) in *Sclerotinia sclerotiorum* exposed to *Bacillus* spp. USB2103 strain, and by Calderón et al. (2014) in other microbial interactions.

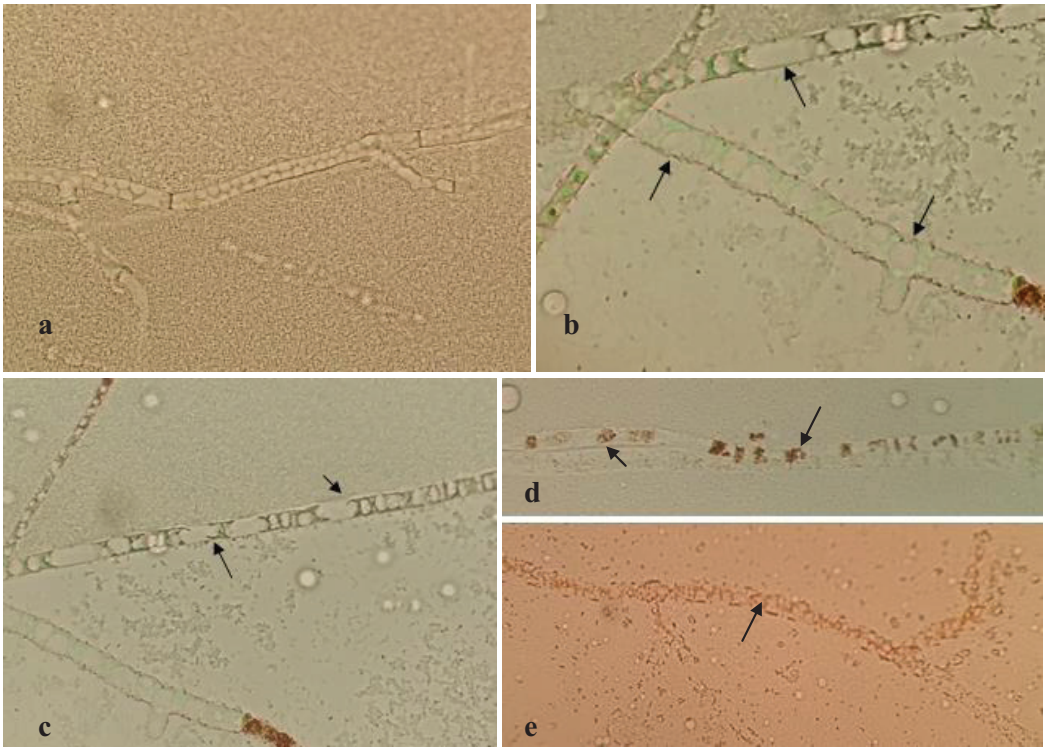

Figure 4. Bacterial interaction of 6T1 strain with *Rhizoctonia solani* hyphae observed under the optical light microscope. Details in a) illustrate an increased number of vacuoles in the fungal hyphae due to 6T1 bacterial exudate. The black arrows in picture b) indicate some of the vacuoles with an increased size. In picture c) there are indicated several internal disruptions in the fungal cells. Cytoplasmic coagulation within the hyphae is illustrated in d) and e) images revealing severely affected fungal hyphae due to bacterial exposure.

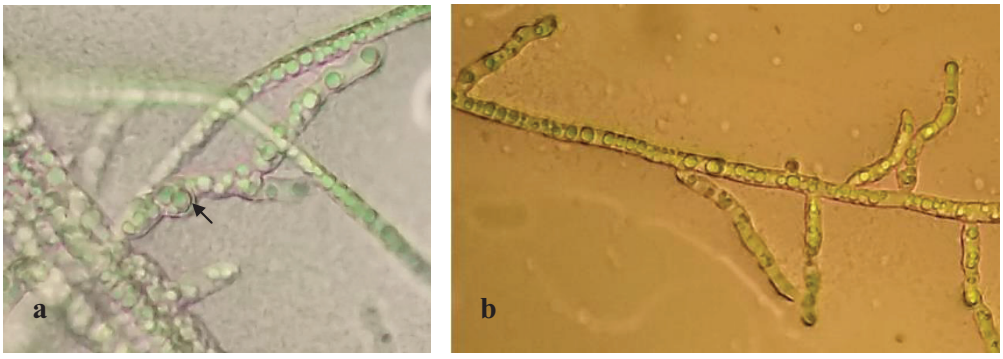

Figure 5. Bacterial interaction of 6T4 strain with *Fusarium solani* hyphae observed under the optical light microscope. Details in a) illustrate clear inhibition zone of the fungal growth. Details in b) reveal an increase number of vacuoles in the fungal hyphae due to 6T1 bacterial exudate exposure.

The fungal vacuoles are dynamic organelles which undergo extensive expansion and remodeling during morphogenetic transitions and moreover, they are involved in several cellular functions, contributing to the cellular homeostasis and storage of irons and molecules (Richards et al., 2012), and also act as a recycling centre for worn-out organelles and macromolecules especially under nutrient starvation (Raven et al., 1999; Klionsky et al., 1990). Therefore, the increased number of vacuoles in the fungal cells or their increased volume could be a correlated to a fungal stress reaction, which might be generated by the antifungal metabolites released by the endophytic bacterial cells which repress mycelia growth and fungal access to the nutritional substrate. Several studies describing similar perturbations of the fungal growth correlated these disturbances with the presence of different antifungal metabolites such as 2-hexyl,5-propyl resorcinol produced by *Ps. chlororaphys* (Calderón et al., 2014) and various volatile organic compounds produced by *Pseudomonas* spp. *and Bacillus* spp. strains (Giorgio et al., 2015). Based on these data it could be assumed that the modifications detected in the new bacterial isolates from potato are related to similar compounds.

Identification

Five of the newly isolated endophytic bacteria were selected for Biolog GEN III identification, 1T1 due to its rapid activation of phosphatase; 1T2 for its high phosphatase activity and inhibitory action against *Rh.solani*; 6T1 for its antifungal potential against the three analysed potato pathogens and orange pigmentation; 6T2 due to UV fluorescence on King B medium, correlated to siderophore production, and antagonistic activity against *Alternaria* spp. and *Rh.solani*; 6T4 due to its rapid colonisation potential and wide spectrum of antifungal activity. The Biolog GEN III identification revealed bacterial species commonly found as endophytes (table 2).

Table 2. Identification of selected endophytic isolates

Endophytic bacterial isolates	Biolog GEN III identification
1T1	*Klebsiella oxytoca*
1T2	*Bacillus endophyticus*
6T1	*Pseudomonas marginalis*
6T2	*Ps. Viridilivida*
6T4	*B. atrophaeus/subtilis*

Based on the biochemical identification, some of the isolates were identified as potential plant or human pathogens, like: *Pseudomonas viridilivida* reported as pathogenic to lettuce (De Vos et al., 1985), *Pseudomonas marginalis* described as post-harvest pathogen able to cause soft rot in many legumes (Achbani et al., 2014), and *Klebsiella oxytoca* sometime involved in human bacteremia (Lin et al., 1997), however highly appreciated for its potential of nitrogen fixation in cereals (Bao et al., 2013).

Although 6T1 and 6T2 were identified as potential plant pathogenic species, they did not induce any disease symptoms in the potato tubers used for endophytes isolation.

However, the data obtained with the isolate 6T4 identified as *B. atrophaeus/subtilis* are very promising for biocontrol strategies. Further molecular analyses are necessary for confirmation the identification of bacterial isolates performed by Biolog system.

CONCLUSIONS

The endophytic bacteria isolated from Romanian potato tubers were symptomless colonizers, harvested from healthy plant material.

Out of twenty newly isolated endophytic bacteria, five strains (1T1, 1T2, 6T1, 6T2, and 6T4) presented beneficial properties for plant protection and/or growth promotions.

The identification of these strains revealed bacterial species commonly found as endophytes: *Bacillus endophyticus, B. atrophaeus/subtilis, Pseudomonas marginalis, Ps. Viridilivida and Klebsiella oxytoca.*

The *B. atrophaeus/subtilis* 6T4 strain was selected due to its biocontrol potential, high colonization competitivety and enzymatic activity, which could bring several benefits for the host plant.

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