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Isolation and Characterization of Bacterial Endophytes of *Chelidonium majus* L.

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A b s t r a c t

The aim of this study was to isolate and identify endophytic bacteria from stems of *Chelidonium majus* L. (greater celandine) and to evaluate their antifungal properties. In total, 34 bacterial endophyte strains were isolated. The fungistatic effects of these bacteria on the growth of five moulds (*Alternaria alternata*, *Chaetonium* sp., *Paecilomyces variotti*, *Byssochlamys fulva*, *Aureobasidium pullulans*) and one species of black yeast (*Exophiala mesophila*) were tested. The majority of the bacterial isolates were found to inhibit the growth of fungi and those with the strongest antifungal properties were further characterized. Of the twelve isolates examined, 11 were species of *Bacillus thuringiensis* and one was *Bacillus amyloliquefaciens.*

K e y w o r d s: *Bacillus amyloliquefaciens, Bacillus thuringiensis, Chelidonium majus* L., endophytes, fungistatic properties

Introduction

Plant tissues are not sterile – spaces within them are inhabited by different species of fungi and bacteria known as endophytes. Most of these microorganisms are not pathogenic to the host plant. Moreover, the association between the plant and its endophytes is very often mutualistic. Endophytic bacteria have been isolated from many different plants including trees (pine, yew), fodders (alfalfa, sorghum, clover), vegetables (carrot, radish, tomatoes, sweet potatoes, lettuce, soybean), fruits (banana, pineapple, citrus), cereal grains (maize, rice, wheat), and other crops (sugarcane, marigold, coffee) (Rosenblueth and Martínez-Romero, 2006). Experiments with soybean have demonstrated that different species of endophytic microorganisms colonize this plant, with the species composition depending on the plant's age, genotype, the tissue sampled, the soil type and the season of isolation (Rosenblueth and Martínez-Romero, 2006). The endophytes of leguminous plants have been studied for many years and bacteria belonging to a number of different genera have been isolated: *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavimonas*, *Pseudomonas* and *Sphingomonas* (Hung and Annapurna, 2004). The tissues of a single plant can be colonized by microorganisms from different species of separate genera, but it is not known whether individual endophyte groups cooperate with each other (Rosenblueth and Martínez-Romero, 2006). Numerous studies have demonstrated that endophytes synthetize bioactive compounds which can, for example, stimulate plant growth and increase resistance to plant pathogens (Rosenblueth and Martínez-Romero, 2006; Ryan *et al.*, 2008). It is considered that some part of of all the metabolites detected in plant tissues originates from colonizing bacteria.

Chelidonium majus L. is a plant that produces bioactive substances. This herb is toxic but has many therapeutic uses. It contains alkaloids which inhibit the growth of fungi, bacteria, viruses, protozoans and also have anticancer properties.

The aim of this study was to examine whether the internal tissues of celandine are colonized by endophytic bacteria, to isolate and identify any endophytes and to test their fungistatic properties.

Experimental

Materials and Methods

Material. The plant material used in this study was the above ground parts of *Chelidonium majus* plants collected at the flowering stage between

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August-November 2007 from three locations in Warsaw, Poland. A total of 15 plants were tested – 5 from each location.

Isolation of endophytic bacteria. The collected plants were briefly washed under running tap water and the stems were cut into 2–3 cm long pieces. These pieces were rinsed in sterile water and then surface-disinfected by soaking in 70% ethanol for 30 sec and 0.1% mercuric chloride ($HgCl_2$) solution for 2 min. The disinfected stem pieces were then rinsed extensively in sterile water and drained. Next, they were cut longitudinally with a sterile scalpel and laid, with the exposed inner surface facing downwards, on plates of sterile nutrient agar (NA) or potato dextrose agar (PDA) (Hung and Annapurna, 2004). As controls, uncut, surface-disinfected stem pieces and non-disinfected pieces were also placed on the same agar. All plates were incubated for several days at 25°C.

Fungistatic properties of endophytic bacteria. The moulds and black yeast used as test strains were obtained from the collection of the Department of Microbial Biology (Faculty of Agriculture and Biology, Warsaw University of Life Sciences, Poland). The fungistatic activity of endophytic bacteria was examined using 24 h cultures of the separate strains grown in nutrient broth at 28°C. Fungal test cultures were prepared on plates of PDA medium. They were inoculated with three separate 20 µl drops of each bacterial culture spotted in rows on the agar. The plates were incubated at room temperature for seven days and any fungal growth inhibition was scored (Owen and Hundley, 2004).

Species identification of endophytic bacteria

Biochemical tests. Taxonomic identification of the bacterial isolates was made on the basis of the results of API-20E and -50CHB tests (BioMérieux). These data were supported by morphological observations: shape and size of cells, Gram staining, motility, spore formation, presence of intracellular protein crystals (visualized by microscopy); and by the results of a number of physiological tests including starch hydrolysis, acetoin production, citrate utilization, growth in 6.5% sodium chloride, growth at 5°C, and the ability to ferment sugars (glucose, xylose, arabinose) and mannitol. The tests were carried out followed standard protocols (Kędzia and Koniar, 1974; Burbianka and Pliszka, 1983; Sneath, 1986; Slepecky and Hemphill, 2006).

Molecular analysis. The extraction of genomic DNA from 24 h cultures of bacterial endophytes grown in tryptic-soya broth at 28°C was performed according to the procedure of Hung and Annapurna (2004) with slight modifications. The isolated DNA was diluted in sterile water and stored at 4°C. PCR with oligonucleotide primers p13B (5'-AGGCCCGGGAAGGCGTATTCAC-3') and PCR-1 (5'-AGTTTGATCCTGGCTCAGGA-3') was used to amplify a 1300-bp fragment of the 16S rDNA gene from the of bacterial DNA templates (Hung and Annapurna, 2004). Reaction mixtures of 25 µl contained 100 ng bacterial DNA, 10 pM of each primer, 1×Taq pol buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1 U of Taq DNA polymerase. The PCR thermal cycle, performed in a PTC-1148 MJ Mini Gradient Thermal Cycler (Bio-Rad), consisted of an initial denaturation step of 3 min at 94°C, then 30 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C, followed by a final extension of 4 min at 72°C. The amplified products were then examined by electrophoresis on 1.5% agarose gels in TAE buffer, purified using a Clean up kit (A@A Biotechnology) and sequenced with primers p13B and PCR-1 using an ABI 3730 Genetic Analyzer (Applied Biosystems). The 16S rDNA sequences were compared with the NCBI GenBank database.

Results and Discussion

Following the procedure of surface disinfection of plant tissues and bacterial isolation described by Hung and Annapurna (2004), 34 bacterial endophyte strains were isolated from internal stem tissues of *Chelidonium majus* L. (Fig. 1). The efficiency of disinfection method was checked. There was no growth of bacteria on the plates containing surface-disinfected, uncut stem pieces.

To examine which of the isolated bacterial endophytes have fungistatic properties, six fungal species were used as reporters: *Alternaria alternata*, *Paecilomyces variotti*, *Aureobasidium pullulans*, *Byssochlamys fulva*, *Chaetomium* sp. and *Exophiala mesophila*.

Most of the moulds are cosmopolitan fungi that are common in the environment, but may cause in-

Fig. 1. Growth of endophytic bacteria from cut pieces of *Chelidonium majus* L. stem on nutrient agar.

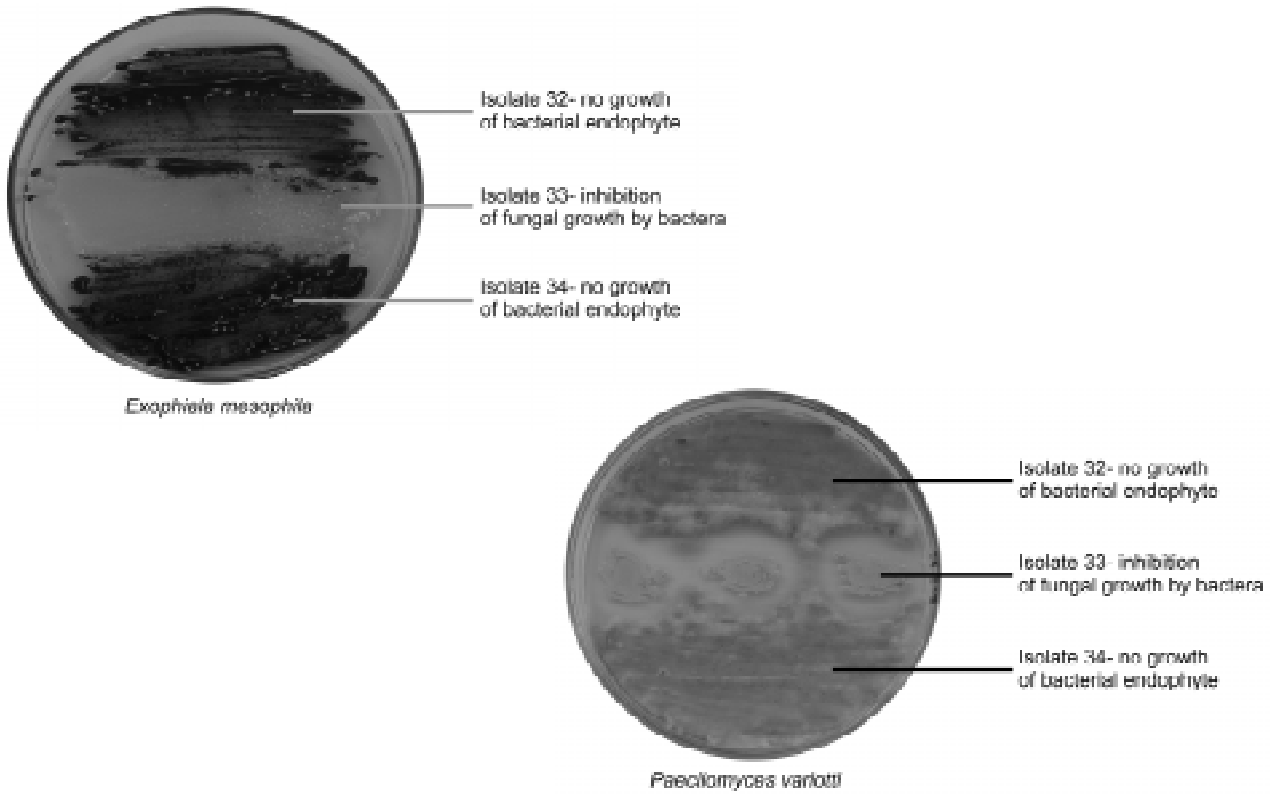

Fig. 2. Growth of fungal strains in the presence of different endophytic bacterial isolates.

fections in humans, animals or plants. *P. variotti* causes paecilomycosis, *A. alternata* produces mycotoxin, *A. pullulans* may damage fruit, while *B. fulva* causes fruit rots (Fassiatová, 1983).

The strain *Exophiala mesophila*, used in this study, belongs to the group of black yeasts, which characteristically produce the pigment melanin. Black yeasts from the *Exophiala* genus are common in the environment and are often isolated from oligotrophic waters and from soil. Few species are known to be pathogenic to humans. *E. mesophila* is a relatively new species and has not been extensively studied. It was isolated from dental unit waterlines but it is not known if it is pathogenic (Porteous *et al.*, 2003).

Examples of the results of the test for fungistatic properties of the bacterial endophytes isolated from celandine stems are presented in Fig. 2. Fungistatic activity is indicated by a zone of growth inhibition in the area where the bacteria were applied to the agar plate.

Of the 34 bacterial isolates, 19 exhibited antifungal properties (Table I). Only one strain, isolate number 33, demonstrated activity against all the tested fungi. Eleven isolates (5–13, 26 and 27) inhibited the growth of all fungi except *B. fulva*. Isolate 23 showed fungistatic properties against *Chaetonium* sp., *P. variotti*, *A. pullulans* and the black yeast strain. Isolates 2 and 19 affected the growth of both *A. pullulans* and *E. mesophila*, while the remaining isolates inhibited only one of the tested fungal strains.

Among 19 bacterial isolates with antifungal properties 12 that inhibited the majority of the tested fungal strains were selected for species identification: 5, 6, 7, 8, 9, 10, 11, 12, 13, 26, 27 and 33. All of these

Table I
Influence of bacterial endophytes isolated from *Chelidonium majus* L. on the growth of the tested mould and yeast strains

Bacterial isolate	Inhibition of microscopic fungal growth caused by the bacterial isolates					
	A. alternata	*Chaetonium* sp.	*P. variotti*	*B. fulva*	*E. mesophila*	*A. pullulans*
33	+	+	+	+	+	+
5, 6, 7, 8,9, 10, 11, 12, 13, 26, 27	+	+	+	–	+	–
23	–	+	+	–	+	+
2, 19	–	–	–	–	+	+
3, 14	–	–	–	–	+	–
28, 32	–	–	–	–	–	+
1, 4, 15, 16, 17, 18, 20, 21, 22, 24, 25, 29, 30, 31, 34	–	–	–	–	–	–

"+" – zone of inhibition of fungal growth caused by endophytic strain
"–" – absence of zone of inhibition of fungal growth caused by endophytic strain

Table II
Selected biochemical features of bacterial endophytes isolated from *Chelidonium majus* L. stems

Feature	Bacterial isolate											
	5	6	7	8	9	10	11	12	13	26	27	33
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Endospore formation	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	+
VP test	–	–	–	–	–	–	–	–	–	–	–	+
Cell diameter >1µm	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	–	–	–	–	–	–	–	–	–	–	–	+
Growth in 6.5% NaCl	–	–	–	–	–	–	–	–	–	–	–	+
Growth at 5°C	–	–	–	–	–	–	–	–	–	–	–	–
Glucose fermentation	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose fermentation	–	–	–	–	–	–	–	–	–	–	–	–
Xylose fermentation	–	–	–	–	–	–	–	–	–	–	–	+
Mannitol fermentation	–	–	–	–	–	–	–	–	–	–	–	+

"+" – positive test result; "–" – negative test result

isolates are Gram-positive, motile, spore forming, aerial rods that were classified within the genus *Bacillus*. Further physiological-biochemical characterization divided the isolates into two groups (Table II). Eleven shared the same features and one, isolate 33, exhibited different properties in seven of the tests. Isolate 33 did not ferment xylose or mannitol, produced acetoin (VP test), could grow on citrate and in 6.5% sodium chloride, and was able to hydrolyze starch.

The results of the API-20E and -50CHB tests permitted the classification of eleven of the isolates as species of *Bacillus cereus* (probability 98.2%), while isolate 33 was closest to *B. amyloliquefaciens* and *B. subtilis* (probability 81.5% and 17.6%, respectively). It is known that these species are extremely similar physiologically and genetically and it is difficult to distinguish between them (O'Donnell *et al.*, 1980). To confirm the species identification of these strains, molecular analysis involving the amplification and sequencing of their 16S rDNA genes was performed.

PCR using isolated bacterial genomic DNA as template and primers p13B and PCR-1 produced 16S rDNA fragments of 1300-bp (Fig. 3). The amplified fragments from isolates 6 and 33 were purified and sequenced using the same primers and the sequences compared with the NCBI GenBank database. The sequences from strain number 6 shared 99% similarity with 16S rDNAs of two species: *Bacillus cereus* and *Bacillus thuringiensis* (Table III). The sequence from isolate 33 with primer P13B was 100% identical to 16S rDNAs of three species: *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*. The sequence produced using the second primer PCR-1 shared 99% similarity with the same *Bacillus* species (Table III).

The problematic identification of *B. cereus* is due to the fact that this species is very closely related to three other species belonging to the *B. cereus* group: *B. cereus* var. *mycoides*, *B. thuringiensis* and *B. anthracis*. The results of this study confirm the inadequacy of commonly used identification protocols.

Table III
Comparison of 16S rDNA sequences of bacterial endophytes to sequences in the GenBank database

Bacterial isolate	Primer	Closest 16S rDNA sequence match (BLASTN)		
		Organism	Bits	% homology
6	P13B	*Bacillus cereus* *Bacillus thuringiensis*	1802	99
	PCR1	*Bacillus thuringiensis* *Bacillus cereus*	1867	99
33	P13B	*Bacillus subtilis,* *Bacillus amyloliquefaciens* *Bacillus licheniformis*	1820	100
	PCR1	*Bacillus subtilis* *Bacillus amyloliquefaciens* *Bacillus licheniformis*	1774 1770 1768	99 99 99

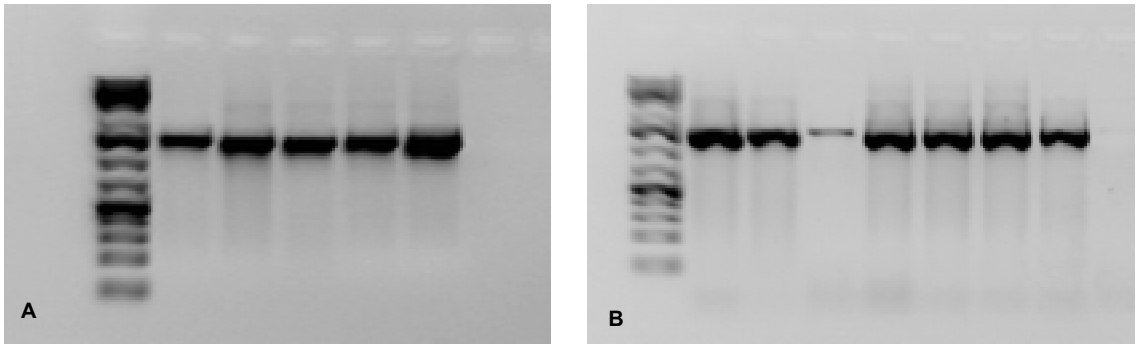

Fig. 3. Agarose gel electrophoresis of 16S rDNA fragments amplified by PCR from the genomic DNA of endophytic bacterial isolates.

(A) Lane 1 – 1kb DNA Ladder Plus molecular size marker (Fermentas); lanes 2–6 – isolates 5, 6, 7, 8, 9; lane 7 – control reaction;
(B) Lane 1 – 1kb DNA Ladder Plus molecular size marker; lanes 2–8 – isolates 10, 11, 12, 13, 26, 27, 33; lane 9 – control reaction.

For isolate 6, the API test results indicated a strain of *B. cereus*, but molecular analysis indicated either of two species: *B. cereus* or *B. thuringiensis*, with the same probability. This problem was resolved by the detection of crystal bodies inside cells producing spores which permitted firm classification of isolate 6 as a species of *B. thuringiensis* (Fig. 4).

Sequence analysis of the 16S rDNA of isolate 33 demonstrated very high homology to three bacterial species: *Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis.* The results of the API tests supported the classification of this isolate as a species of *Bacillus amyloliquefaciens*.

Many previous studies have demonstrated antagonistic reactions between bacteria and fungi. In the 1980s, antibiotics produced by *Pseudomonas fluorescens* were shown to inhibit the growth of *Rhizoctonia solani* and *Pythium ultimum*: both fungal species are pathogenic to cotton (Howell and Stipanovic, 1980; Howell and Stipanovic, 1987). Phenazine produced by *P. fluorescens* can inhibit the growth of *Gaeumannomyces graminis*, a serious fungal pathogen of cereal crops (Thomashow and Weller, 1988). *Pseudomonas cepacia* and *P. fluorescens* inhibit the growth of fungi from genera *Pythium* and *Aphanomyces*, that cause rotting of pea roots (Parke *et al.*, 1991). *Erwinia herbicola* shows antagonistic properties to *Fusarium culmorum* and *Puccinia recondita*, two fungal pathogens of wheat (Kempf and Wolf, 1989). *Bacillus pumilus* inhibits the growth of *Rhizoctonia solani*,

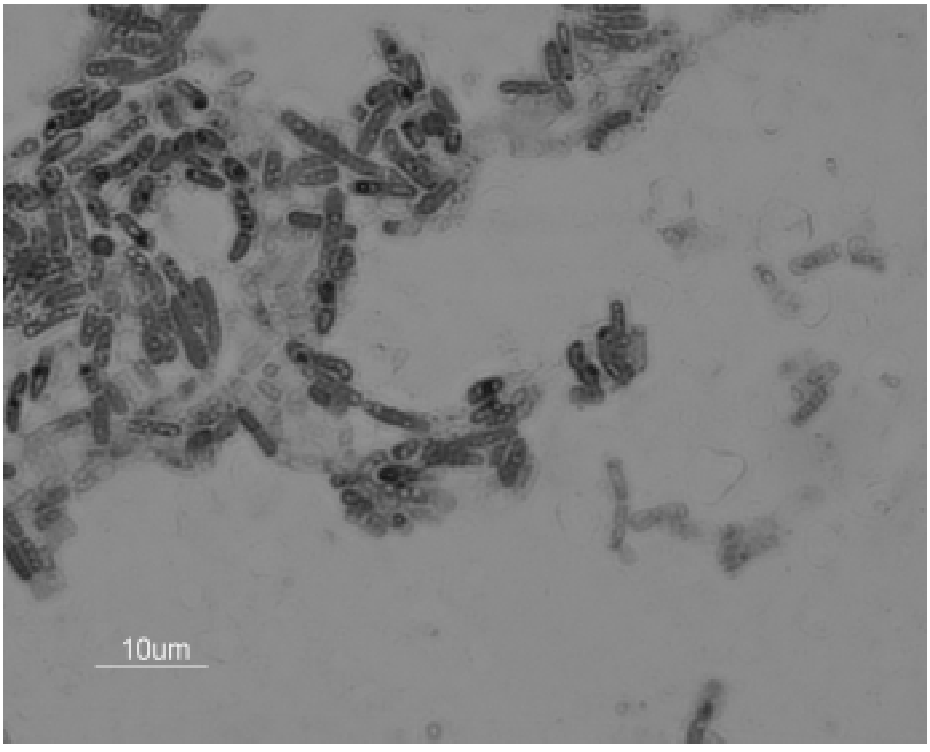

Fig. 4. Cells of *Bacillus thuringiensis* containing endospores and crystal bodies.

a fungus causing diseases of soybean and other economically important plants (Domingos da paz, 2008). The species *Propionobacterium* can inactivate mycotoxins produced by various fungal species (Gwiazdowska *et al.*, 2008).

Numerous investigations have reported the beneficial influence of *Bacillus thuringiensis* and *B. amyloliquefaciens* on cultivable plants. *Bacillus thuringiensis* is a common environmental bacterium found in soil and on the surface of plants. The characteristic feature of this species is the production of parasporal crystalline inclusions during its sporulation phase. The proteins forming these parasporal bodies are encoded by plasmid-borne *cry* genes. They are the Bt (*Bacillus thuringiensis*) toxins and are toxic to insect larvae, especially within the orders *Lepidoptera*, *Coleoptera* and *Diptera* (Frederiksen *et al.*, 2006). Once the Bt toxin has been ingested by a larva it disturbs the functioning of the insect's digestive system causing death. However, Bt toxin is harmless to humans, animals and other insects. For more than 30 years *Bacillus thuringiensis* has been used in agriculture for the biological control of the larvae of insect pests. Furthermore, the genes encoding Bt toxin have been transferred by molecular methods into crop plants to produce transgenic varieties that are resistant to pests. The first genetically modified plant carrying Bt genes was tobacco produced by the Belgian company Plant Genetic Systems (Broderick *et al.*, 2006). To guard against the development of insects resistant to commonly used plant protection products based on *Bacillus thuringiensis* there is an ongoing worldwide program searching for new strains producing proteins with novel toxicity. In 2008 scientists from Spain and Brazil isolated endophytic *Bacillus thuringiensis* strains from sugar cane (Suzuki *et al.*, 2008).

Bacillus amyloliquefaciens is a free-living soil bacterium which promotes the growth of plants by antagonistic activity against plant pathogens. This bacterium produces antifungal chitinases which can inhibit the growth of *Fusarium oxysporum* (Wang *et al.*, 2002). Chitinases are produced by many bacterial species including *Pseudomonas aeruginosa, Enterobacter agglomerans*, *Rhizobacteria* sp., *Aeromonas capia*, *Serratia marcescens*, *Vibrio harveyi* and *B. circulans* (Chernin *et al.*, 1995; Chet *et al.*, 1990; Inbar and Chet, 1991; Ordentlich *et al.*, 1988; Folders *et al.*, 2001). *B. amyloliquefaciens* also produces secondary metabolites that inhibit the growth of plant pathogens: bacillomycin D, surfactin, bacillaene, the polyketides and difficidin. The majority of the genome of this species is engaged in the production of secondary metabolites, with more than 8.5% committed to the synthesis of siderophores and antibiotics (Chen *et al.*, 2007). In the closely related species *B. subtilis* there are five gene clusters engaged in the production of secondary metabolites. Chen *et al.* (2007) identified four additional gene clusters in *B. amyloliquefaciens* that are not present in *B. subtilis*.

Bacteria producing chitinases or other substances with biological activity against plant pathogens (fungi, insects) may find applications in agro-biotechnology. Biological control of plant pests and diseases is much more attractive than chemical treatment methods due to its greater specificity and less harmful impact on the environment (Wang *et al.*, 2002). Bacteria from the *Bacillus* genus, such as those isolated and characterized in this present study, possess a number of characteristics that are desirable in biological control agents: they are naturally abundant in soil, produce many biologically active substances and are persistent, forming endospores that are resistant to unfavorable environmental conditions.

The research has shown that internal tissues of *Chelidonium majus* are settled by endophytic microorganisms and that they produce biologically active substances. In the nearest future the isolation and identification of the biologically active substances, from liquid cultures of *Bacillus thuringiensis* and *Bacillus amyloliquefaciens*, is planned. The use of natural products obtained from endophytic microbes in the pharmaceutical and agrochemical areas will be considered. An interesting question is whether the metabolites from endophytes are the same as those identified in *Chelidonium majus*.

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