Psychotropic endophytes of cereals as potential bioagents

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Abstract. Creating new biopesticides for protecting agricultural crops and developing their application methods, which are among the priority areas of the agro-industrial complex, are inextricably linked with continuous observation of soil microbiocenosis, growing plants, and changes in agro-climatic conditions in a specific region. Plant endophytes are potentially active bioagents for creating new microbiological preparations for protecting winter crops, as well as agricultural produce during its storage period. In connection with this, the morphophysiological characteristics of six isolates of endophytic bacteria, isolated at low temperatures from winter cereal plants, were studied. The species affiliation of the strains was determined - five isolates belonged to the genus Pseudomonas and one to the genus Chryseobacterium. An assessment of the antagonistic activity of endophytes in relation to phytopathogens causing rot of agricultural produce was conducted.

1 Introduction

Endophytes are facultative or obligate symbiotic microorganisms that inhabit the internal tissues of plants (endosphere), without causing diseases or negative effects. [1] Unlike rhizosphere microorganisms, which colonize the surface of roots, and phyllosphere organisms, which inhabit aboveground parts, endophytes do not form specific structures. However, they can have a beneficial impact on the host organism. Research indicates that endophytic microorganisms can regulate the level of phytohormones, produce vitamins, increase plant resistance to stress, participate in the regulation of osmotic pressure, stomatal function, modification of plant root system development, and regulation of nitrogen and phosphorus nutrition. Endophytic bacteria can reduce or prevent the negative effects of phytopathogenic microorganisms, particularly phytopathogenic micromycetes. [2] Endophytes are promising biocontrol agents against phytopathogens, as they inhabit the same ecological niches as the phytopathogenic microorganisms. Plant inoculation with endophytic bacteria can significantly reduce the damage caused by pathogenic fungi, bacteria, viruses, insects, and nematodes. Inoculation of seeds or seedlings with unique strains of endophytic bacteria results in active colonization of the internal tissues, thus reducing the negative influence of biotic and abiotic factors and increasing positive biochemical and physiological effects on the plant. Endophytic microorganisms have significant advantages over those inhabiting the rhizosphere and phyllosphere. Being in the endosphere, they have stable conditions: pH, humidity, sufficient nutrients, and lack of competition from a large number of microorganisms. With such competitiveness of endophytic bacteria and the high specificity of this plant-microbial symbiosis, very small amounts of inoculum are sufficient for plant inoculation. Endophytes can serve as the basis for bio-preparations, reducing the need for mineral fertilizers and chemical pesticides in agricultural practice, and consequently reducing the negative impact on soil fertility, biodiversity, and human health [3]. Such techniques can be highly successful for modern biotechnological productions seeking alternatives to traditional ones. Thus, endophytic microorganisms can be promising agents for producing bio-preparations for combating phytopathogens and stimulating plant growth. Studying their properties and potential uses to increase the efficiency of crop production is one of the current directions in the development of agrobiotechnology. [1] [2, 3].

The purpose of this study was to identify and describe the isolated endophytic psychrophilic bacteria, investigate the activity of the isolated strains against psychrophilic phytopathogenic micromycetes that cause rot of agricultural produce.

2 Materials and methods

We used 6 isolates of endophytic bacteria isolated from cultivated and weed cereal plants– WAC1, WAC4, WACA, RX38, RX39, RX42, provided by the laboratory of the Center for Agroecological Research of the Kazan State Agrarian University.

Bacteria were cultivated on liquid and agar LB medium. Cell morphology was studied using an AXIO Imager M2 universal research biological light microscope, staining a daily culture grown on LB solid medium with methylene blue. Gram staining was performed using a Deltalab Gram stain kit, Spain.

When determining whether endogenous strains belong to psychrophilic microorganisms, the isolates were grown at a temperature +4ºC, +25ºC и +37ºC on LB medium for 48 hours. The growth rate of cultures was assessed by their optical density determined at a wavelength of 590 nm and a cuvette thickness of 10 mm in a UV Specord spectrophotometer (Carl Zeiss, Germany).

The antagonistic activity of the cell suspension of the studied isolates was evaluated by the well-diffusion method during co-cultivation with seven different strains of phytopathogenic micromycetes: *Fusarium solani, Alternaria alternate, Alternaria solani, Alternaria alternate, Bipolaris sp., Fusarium oxysporum, Fusarium solani, Fusarium oxysporum*. Fungal strains were grown on Czapek-Dox agar medium (sucrose, 30 g; NaNO3 – 2 g; KH2PO4 – 0.7 g; MgSO4 * 7H2O – 0.5 g; K2HPO4 – 0.3 g; FeSO4* 7H2O – 0.01g; agar - 2 g; distilled water - 1l).

Determination of the species affiliation of the strains was carried out using the analysis of 16S RNA according to the Sanger method. A bacterial suspension was prepared from a culture of bacteria grown overnight in LB medium at 25 ± 1 °C. Each culture of the bacterial isolate was centrifuged at 4000 rpm for 5 min at 4°C. The pellets obtained were washed twice with sterile phosphate-buffered saline (PBS) (140 mM NaCl, 5 mM KH 2 PO 4 , 1 mM NaHCO 3 , pH 7.4) and resuspended in the same solution to an optical density (OD) of 0.1 at 600 nm.

The production of a sixverse reaction was carried out using the General-specific primmers and a set of BigdyTerminator v3.1CYCECECEQUENCINGKIT (AppliedbiosyStyMS, USA) in the amount of 10 μl 3.2picomol of the primer, 0.8 mkl Readyreactionmix, 1 , 6 mkl 5x sequencingbuffer, 2-5 μl of purified PCR product in an amount of 10 to 50 ng, depending on the length of the PCR product, water up to a volume of 10 μl. To confirm the mutation, the reading was carried out from both primers (forward and reverse). The reaction was carried out using a Veriti amplifier (Applied Biosystems, USA) according to the temperature protocol: preliminary denaturation 96°C1 min; 26 cycles 96°C 10 sec, 50°C 5 sec, 60°C 4 min.

Purification of the sequence reaction was carried out according to the following protocol. 40 µl of a mixture of ammonium acetate with ethanol was added to the tube with the PCR product for every 10 µl of the PCR mixture. The tubes were vortexed and the mixture was incubated at room temperature in the dark for 30 minutes. The samples were then centrifuged for 30 minutes at 2500 rpm. After that, the supernatant was carefully removed and the pellet was washed with 150 µl of chilled 80% ethanol, followed by centrifugation for 10 minutes at 2500 rpm. Then ethanol was removed, the precipitate was dried at a temperature of 55°C in an amplifier and dissolved in 10 µl of Hi-Di-formamide. Before sequencing, the samples were denatured for 2 minutes at 95°C.

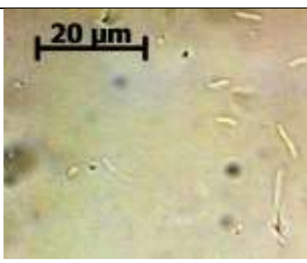
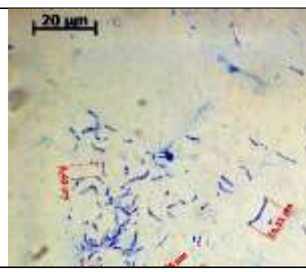
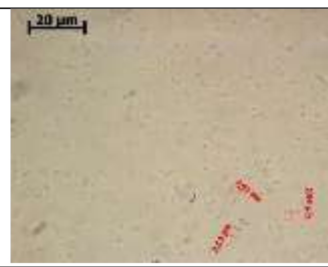
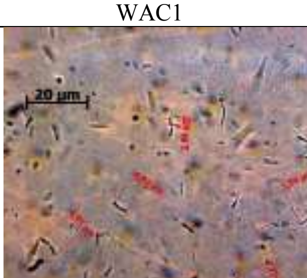
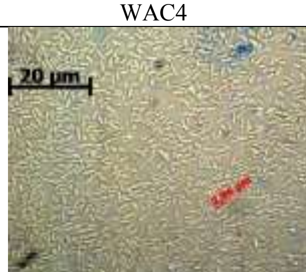
Detection of sequencing products was carried out automatically by capillary electrophoresis on an ABI PRISM 3730 instrument (Applied Biosystems, USA) at the Multidisciplinary Center for Collective Use of Kazan Federal University.

3 Research results

3.1 Physiological and biochemical properties

Microscopic examination of preparations prepared from cultures of endophytic bacteria revealed that all 6 isolates are aerobic rod-shaped, non-spore-forming bacteria with a gram-negative morphotype (Table 1). All isolates, with the exception of one, WAC4, had varying degrees of mobility.

Table 1. Cell morphology of six isolates grown on LB agar medium.

		
WAC1	WAC4	WACA
		
RX 38	RX 39	RX 42

Study of growth activity of isolated isolates at different temperatures.

Cultivation of isolates under various temperature conditions showed that they grow in the temperature range of 4–37°C with an optimum at 25–28°C, which allowed

refer them to psychrotrophic organisms (Fig. 1). In all bacteria at a temperature of +25°C and at a temperature of +37°C, colony growth was observed after 20 hours, at a temperature of +4°C, growth was delayed, appearing only on the second day, but then all strains showed good growth at a given temperature. This allows us to say that the obtained endophytic bacteria can be attributed to psychrotrophs. Psychrotrophs are cold-resistant bacteria, but their maximum growth temperature fluctuates around 20°C, and in many cases their optimal growth temperature is even higher than 20°C [4].

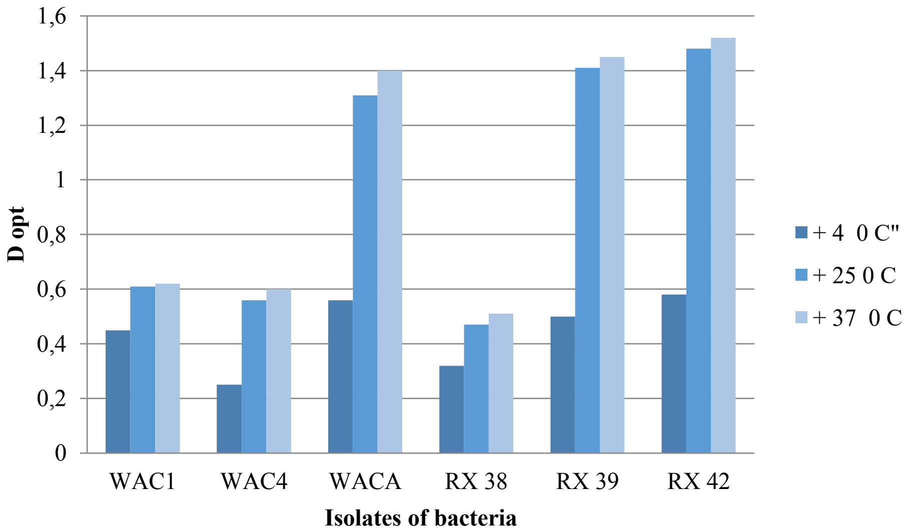


Fig. 1. Growth rate of endophyte isolates at different temperatures.

All strains had a pronounced ability to form exopolysaccharides, especially under growth conditions at low temperatures (+4°C), which is noticeable on the glossy surface of the colonies and on the fuzzy, spreading edge of the colonies. Since bacteria lack thermoregulatory mechanisms, the production of exopolysaccharides also appears to play an important role in the cryoprotection of psychrophiles and psychrotrophs [4].

3.2 Molecular identification of isolates

Based on the data on the nucleotide sequence of the 16S rRNA gene and phenotypic properties, the WAC4 isolate was assigned to Gram-negative bacteria of the genus Chryseobacterium. It is now known that the genus Cryseobacterium includes both saprotrophic and pathogenic forms isolated from various habitats: soils, fresh water bodies, plants, insects, and Antarctic soils [5.6]. Representatives of all species of this genus described in the literature are gram-negative, aerobic, heterotrophic, mesophilic, and psychrotolerant bacteria [6, 7]. The growth of the strains of Chryseobacterium sp. NF4 and NF5 were observed at lower temperatures (up to 5°C), with growth optima at 28°C.

Based on the data on the nucleotide sequence of the 16S rRNA gene and phenotypic properties, the remaining 5 isolates were identified as different representatives of Gram-negative bacteria of the genus Pseudomonas: WAC1 and WACA were identified as Pseudomonas simiae strain YL-192, RX39 and RX42 were identified as Pseudomonas synxanta strain NCTC10696. Isolate RX38 was assigned to Pseudomonas lini strain CP DC20.

Since the 1980s, numerous mutualistic strains of Pseudomonas spp. have been used in studies of the biology of rhizobacteria that stimulate plant growth and their interaction with host plants [8,9,10]. .

Psychrophilic strains of Pseudomonas, having fungicidal and bactericidal activity, growth rate in the range of low temperatures, and the ability to produce auxin-like phytohormones, can be used to increase the safety and optimize biochemical processes in potato tubers during low-temperature storage [3]. It has been established that the introduction of Pseudomonas sp. RF13H, due to the action of antioxidant defense enzymes (catalase, peroxidase, superoxide dismutase), contributes to the activation of redox processes, the induction of its own immunity and the creation of a barrier to the penetration of a pathogen, thus increasing the resistance of tubers to infection.

4 Conclusion

Endophytic bacteria colonize the internal tissues of their host plant and have been found in virtually all plants studied, where they can form a number of different relationships, including symbiotic, mutualistic, commensalistic, and trophobiotic [11]. Endophytes can also be beneficial to the host plant by producing metabolites that could be considered for potential use in medicine, agriculture, or industry. Endophytic bacteria can promote plant growth and yield and can act as biocontrol agents. Psychrophilic endophytes with fungicidal and bactericidal activity at low temperatures can be promising bioagents for ensuring the safety of agricultural products during low-temperature storage.

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