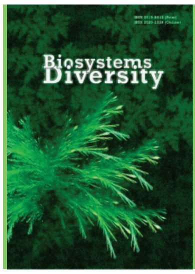

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Antifungal properties of rhizobacterial strains in relation to fungi of agricultural crops

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On earth, pathogenic fungal infections are considered one of the most common crop problems, accounting for more than 80% of all plant diseases. The production of biologics and the fight against pathogens are highly relevant priorities. In this article, rhizosphere cultures isolated from wheat root tubers have been studied in detail for the development of pathogenic fungi. In the course of research, the inhibitory properties of rhizobacteria against common pathogenic fungi were studied. The main mechanism of antagonism is that bacteria directly affect phytopathogens or increase plant resistance to diseases by enhancing immunity. The antagonistic activity of rhizospheric microorganisms was studied and the level of their action was determined. It has been established that wheat rhizospheric bacteria *Escherichia hermannii*, *Enterobacter cloacae* and *Rahnella aquatilis* affect the development of pathogens that cause *Alternaria* and *Fusarium* wilt. In the experiments, 46 strains of rhizobacteria of the genus *Enterobacter* (strain *E. cloacae* CCIM1022), *Rahnella* (*R. aquatilis* CCIM1023), *Pantoea*, *Pseudomonas*, *Bacillus*, *Escherichia* were isolated and the effectiveness of their antifungal properties on the reproduction of pathogenic fungi was tested. Pathogenic fungi *Alternaria*, *Fusarium*, causing diseases of vegetable and melon crops, were isolated from host plants. It is noted that rhizobacteria have a negative impact and block the development of pure cultures of fungi isolated from tomatoes, zucchini, figs, melons, causing fungal diseases on cups. The 46 isolated and tested strains of rhizobacteria effectively inhibited the development of pathogenic fungi. Of these, *Rahnella* is the first bacterium studied for this purpose, and has been given great importance in experiments to block the development of *Alternaria* (*A. alternata* CCIM 1021). The causative agents of alternariosis are widely open. However, large-scale analysis of their presence and control of pathogens has always been difficult. In experiments to combat the pathogen in the laboratory, a new agent was used to spread nitrogen-fixing bacteria.

Keywords: alternariosis; wheat; rhizobacteria mobilization of phosphates; potassium and nitrogen fixation; antagonistic activity.

Introduction

In recent years, the number of diseases caused by bacterial, fungal and viral infections has been increasing globally. Infections affect plants at different stages of agricultural production. Depending on weather conditions and the phytosanitary condition of crops, the spread of diseases can reach 70–80% of the total number of plants, and the yield can decrease in some cases by 80–98%. Plants have their own immunity, but phytopathogens have the ability to evade this immunity (Nazarov et al., 2020).

It is known in the scientific literature that many strains of rhizosphere microorganisms have antagonistic properties with respect to a wide range of phytopathogenic fungi and bacteria (Loginov, 2005). The most studied antagonist bacteria isolated from the root zone of plants are bacteria belonging to the genera *Pseudomonas*, *Bacillus*, and *Azotobacter* (Boronin, 1998; Gurevich, 2012; Malanichiva, 2012). Most strains with antagonistic activity against pathogens of root rots of cereals are classified as bacteria belonging to the genus *Pseudomonas*. Under laboratory conditions, *Pseudomonas* sp. strain V–6798 is *Fusarium oxysporum* var. *lini*, which causes root rot in cereals. *Fusarium oxysporum* var. *lini* and *F. oxysporum* var. *gladioli* a strong inhibitory effect on pathogenic cultures was found (Minayeva et al., 2008).

Alternariosis is the most common disease of agricultural plants in Uzbekistan. *Alternaria* can reduce yields by 20% to 50% due to the disease. *Alternaria* infects leaves to fruit of tomato, pepper, melon, watermelon, figs and various berries and fruits. *Alternaria* is a ubiquitous fungal genus that includes saprobic, endophytic, and pathogenic species and a wide variety of substrates. In recent years, DNA-based studies have identified several non-monophyletic genera within the *Alternaria* assemblage and a clade of *Alternaria* species that do not always correlate with species groups based on morphological characteristics. The *Alternaria* complex currently includes nine genera and eight sections of *Alternaria* (Woudenberg et al., 2013).

However, most representatives of the genus *Alternaria* (*A. brassicae*, *A. brassicicola*, *A. solani*, *A. porri*, etc.) cause plant diseases, which frequently affect the most common crops. As a rule, fungi of this type infect leaves, but can also infect seeds and fruit (Thomma, 2003).

A number of *Alternaria* spp. have been isolated from potatoes worldwide but only *A. solani* and *A. alternata* have been described as pathogenic to this host in the United States. These taxa are easily differentiated based on conidial morphology but species delimitation among the small-spored *Alternaria* spp. associated with potato are much more challenging. Accurate identification methods for small-spored *Alternaria* spp. are necessary so that a more thorough understanding of *Alternaria* epidemiology can be obtained (Tymon et al., 2015).

Fighting alternariosis is quite difficult. In experiments, we tested the antifungal properties of the bacteria *Enterobacter*, *Rahnella*, *Pantoea*, *Pseudomonas*, *Bacillus*, and *Escherichia*, which were isolated from the root surface of wheat.

The purpose of our experiment was to identify the antifungal properties of rhizobacteria. The results showed that, out of 100 isolated bacteria, 46 had fungicidal properties, and 14 of them blocked *Alternaria*.

Materials and methods

Isolation and taxonomic analysis of local rhizobacteria, and the mobilization of phosphate and potassium, nitrogen fixation activity, and vegetation experiments were carried out in a microbiological thermostat at a temperature of 25 °C. The study of the antagonistic activity of rhizobacteria against phytopathogenic fungi was carried out under laboratory conditions. Rhizobacteria belonging to the genera *Enterobacter*, *Rahnella*, *Pantoea*, *Pseudomonas*, *Bacillus* and *Escherichia* isolated from the rhizosphere and the root surface of wheat served as the material for the study.

Pikovskaya medium (ingredients per g/L): yeast extract 0.5, dextrose 10.0, calcium phosphate 5.0, ammonium sulfate 0.5, potassium chloride 0.2, magnesium sulfate 0.1, manganese sulfate 0.0001, agar 15. Ashby mannitol broth was used to isolate *Azotobacter* species from soil (g/L): mannitol 20.0, dipotassium phosphate 0.2, magnesium sulfate 0.2, sodium chloride 0.2, potassium sulfate 0.1, and calcium carbonate 5.0 were adjusted according to operating parameters. Final pH (at 25 °C): 7.4 ± 0.2.

Isolation and cultivation: *Enterobacter*, *Rahnella*, *Pantoea*, *Pseudomonas*, *Bacillus*, and *Escherichia* were grown on Pikovskaya nutrient media, and 46 bacteria were selected on the basis of molecular nitrogen uptake and the mobilization of phosphorus and potassium. To isolate phosphate-mobilizing and nitrogen-fixing local rhizobacteria, four-month-old winter wheat plants were brought from the rhizosphere and roots of wheat grown in the agricultural regions of the Tashkent, Syrdarya, Andijan, and Kashkadarya regions and the Republic of Karakalpakstan. To isolate bacteria from the root surface of the plant, the root was thoroughly washed with water and then rinsed in sterile distilled water. To obtain enrichment cultures of rhizobacteria from plant roots and rhizosphere soils, samples of nitrogen-free Dobereiner's were placed in a liquid nutrient medium and incubated at 28 °C for 7 days.

A week later, a bacterial film formed on the surface of the nutrient medium. The microorganisms of the enrichment cultures formed by the film were examined under a light microscope. Enrichment cultures with high bacterial motility were streaked onto Petri dishes with 2% agar-peptone medium and grown at 28 °C. After sowing bacteria in 3–5 days, bacterial colonies formed from individual bacterial cells appeared on the surface of the nutrient medium.

Next the resulting bacterial colonies were studied under a light microscope and it was determined that they belonged to only one species, more than 100 rhizobial bacterial isolates were placed in a peptone nutrient medium. To select nitrogen-fixing bacteria from these isolates, nitrogen-free semiliquid 0.2% agar was cultured in Dobereiner's medium. Bacterial growth was observed within a week. It was noted that nitrogen-fixing bacteria grow down from the surface of the nutrient medium due to the property of aerotaxis, i.e., they move toward a low oxygen content. Bacterial growth was not observed in the sample inoculated with *Bacillus subtilis* as a control. Thus, more than 70 visually nitrogen-fixing isolates were selected, depending on the growth of bacteria in a nitrogen-free semiliquid 0.2% Dobereiner agar nutrient medium. These bacteria were found to have strong motility.

The fungicidal properties of native rhizobacteria isolated from the rhizosphere and rhizoplane of wheat against pathogenic fungi were studied under laboratory conditions. *Alternaria alternata*, *Fusarium solani*, *Verticillium dahliae* and *Aspergillus niger* were grown on the surface of a 2% MPA agar medium to form a lawn. Then, grooves 5 mm in diameter were made on the surface of the agar nutrient medium in which these fungi were planted. In the pits of the agar medium, 0.2 mL of a suspension of local strains of *Escherichia*, *Rahnella*, *Enterobacter*, *Pantoea*, and *Pseudomonas* bacteria, grown in the GPB liquid medium for 3 days, was poured. Samples were grown at 28 °C for 3 days.

Phytopathogenic fungi were isolated according to the Dudka method (Dudka et al., 1982). Parts of plants from the study sites with signs of disease were washed with clean distilled water. Then, the surface was sterilized with 2% sodium hypochlorite solution for one minute, washed in distilled water, and air dried. Pieces that were 0.1–0.3 mm in size from the studied parts of the diseased plant (trunk, leaves, fruit) were cut out and placed on a sterilized Petri dish, covered with filter paper and poured with distilled water. Tablets were placed in desiccators and stored in thermostats at a temperature of 27–28 °C. The subsequent stages of their development were studied on a nutrient medium, potato agar with dextrose (PDA).

Results

Those spots begin as small black corners, which eventually become necrotic. Dark brown spots appear on leaves, stems, twigs, and leaf stripes. Stem damage usually appears as black streaks 3 cm long. The pathogenic type causes streaked spots on the stems and moist, sunken sores on the back of the sunflower head. Sometimes, there are yellow borders around the spots. Infection leads to the death of flowers and premature drying (Fig. 1). The spores of these pathogenic fungi overwinter in damaged plant debris, but the wild-growing alien sunflower (*Carthamus tinctorius*) *Xanthium strumarium* can also support overwintering (Keinath, 2011). All species, including *Alternaria alternata*, also can be stored in seeds.

Pathogenic spores are spread through the lower leaves of the sunflower by wind or water (Udayashankar et al., 2012).

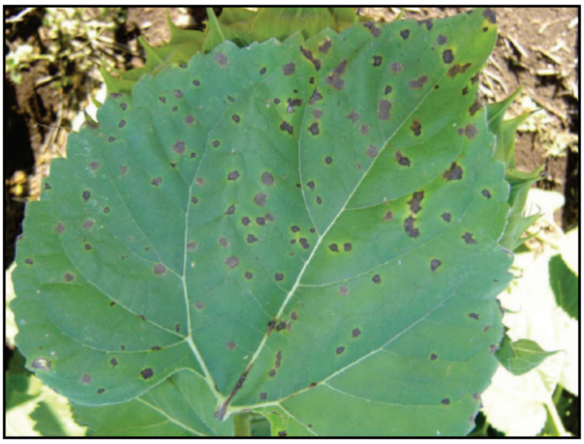

Fig. 1. External signs of sunflower alternariosis

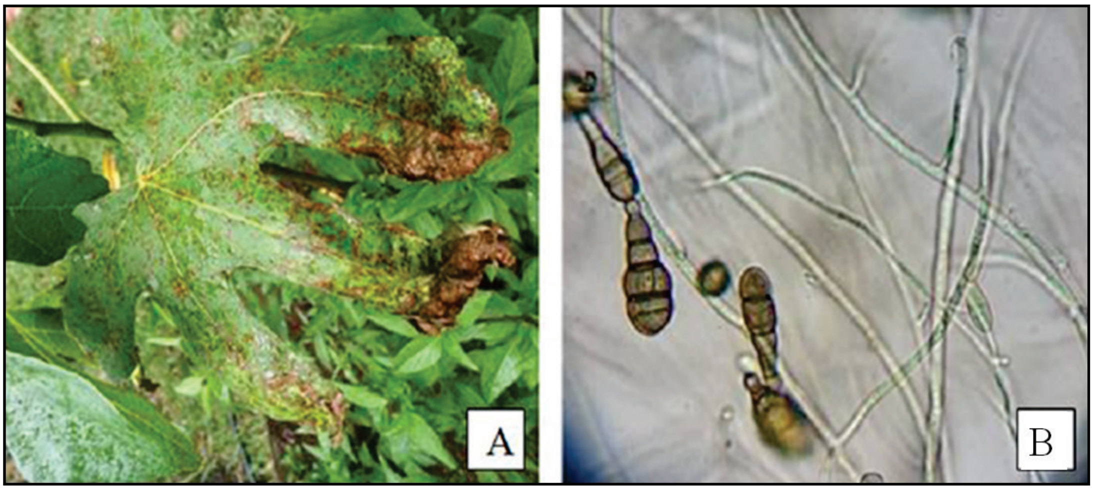

Fig. 2. Alternariosis of figs: *A* – diseased leaf, *B* – fungal conidia

On the upper and lower parts of fig leaves, spots form first. Then, the edges of the leaves gradually turn reddish-brown and begin to dry out (Fig. 2). In gourds, alternariosis causes severe damage to stem leaves and even fruit (Fig. 3). This leads to a decrease in the yield and quality of the crop. Alternariosis causes damage to the stems, leaves, and unripe fruit of tomatoes. Unripe fruit changes shape and turns brown. Heavy mortality from alternariosis causes a sharp decrease in the yield of the fruit and makes it inedible. This has a significant impact on the quality of the exported crop (Fig. 4).

Based on laboratory data, studies were carried out on the fungicidal properties of local rhizobacteria isolated from the rhizosphere and rhizoplane of wheat against pathogenic fungi. Fungi *Alternaria alternata* (GEPB 1021), *Fusarium solani*, *Verticillium dahliae*, and *Aspergillus niger* were planted on the surface of agar medium with 2% MPA to form a lawn. Holes 5 mm in diameter formed on the surface of the agar medium into which these fungi were planted. A suspension of local bacterial strains of *Escherichia*, *Rahnella*, *Enterobacter*, *Pantoea*, and *Pseudomonas* grown for 3 days in liquid GPB medium was poured into 0.2 mL of agar medium. Samples were grown at 28 °C for 3 days.

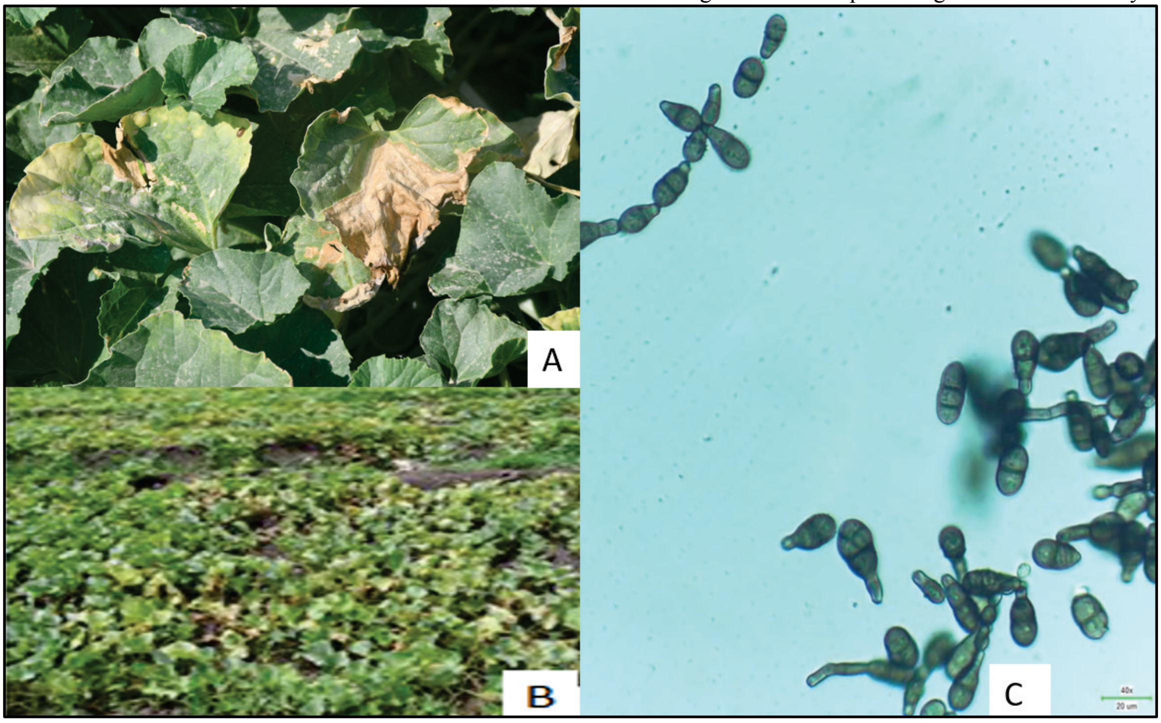

Fig. 3. *Alternaria* in melons: *A* and *B* – affected leaves and field view; *C* – fungal conidia

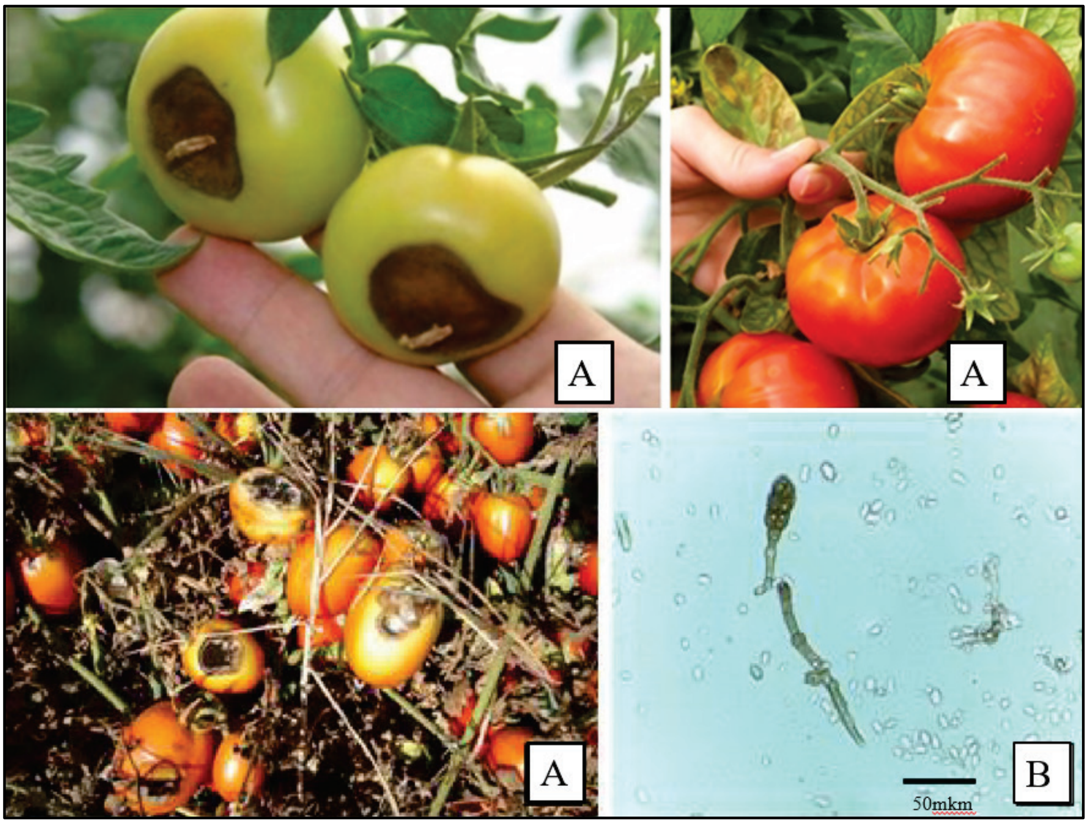

Fig. 4. Alternariosis of tomatoes: *A* – fruit, leaves, and twigs of tomatoes affected by *Alternaria*; *B* – fungal spores

It was noted that the fungicidal activity of rhizobial bacteria against the fungus *Alternaria alternata* (GEPB 1021) was higher than against other fungi (Fig. 5, 6).

The causative agents of alternariosis are widely described in the literature. However, a large-scale analysis of their presence and control over pathogens has always been difficult. In this study, a new tool with various types of nitrogen-fixing bacteria was used to control the pathogen in the laboratory (Fig. 5, 6).

Discussion

Microorganisms that protect plants from pathogenic bacteria and fungi are called "biocontrol agents". Plant growth-promoting rhizobacteria (PGPR) have a positive effect on the growth and development of plants, and they biologically control the growth and development of phytopathogenic microorganisms. A major mechanism of antagonism is that bacteria have a direct effect on phytopathogens, or they increase plant resistance to diseases by strengthening immunity. The antagonistic activity of rhizospheric microorganisms is associated with the production of secondary metabolites that inhibit the growth and reproduction of phytopathogenic bacteria and fungi (Shafikova & Omelichkina, 2015).

Studies have shown that many strains of rhizospheric microorganisms have properties that are antagonistic to wide range of phytopathogenic fungi and bacteria (Loginov, 2005). The most widely studied antagonist bacteria isolated from the root zone of plants belong to the genera *Pseudomonas*, *Bacillus*, and *Azotobacter* (Loginov, 2005; Smolin et al., 2022). Most strains with antagonistic activity against root rot pathogens of cereals belong to the genus *Pseudomonas* (Loginov, 2005).

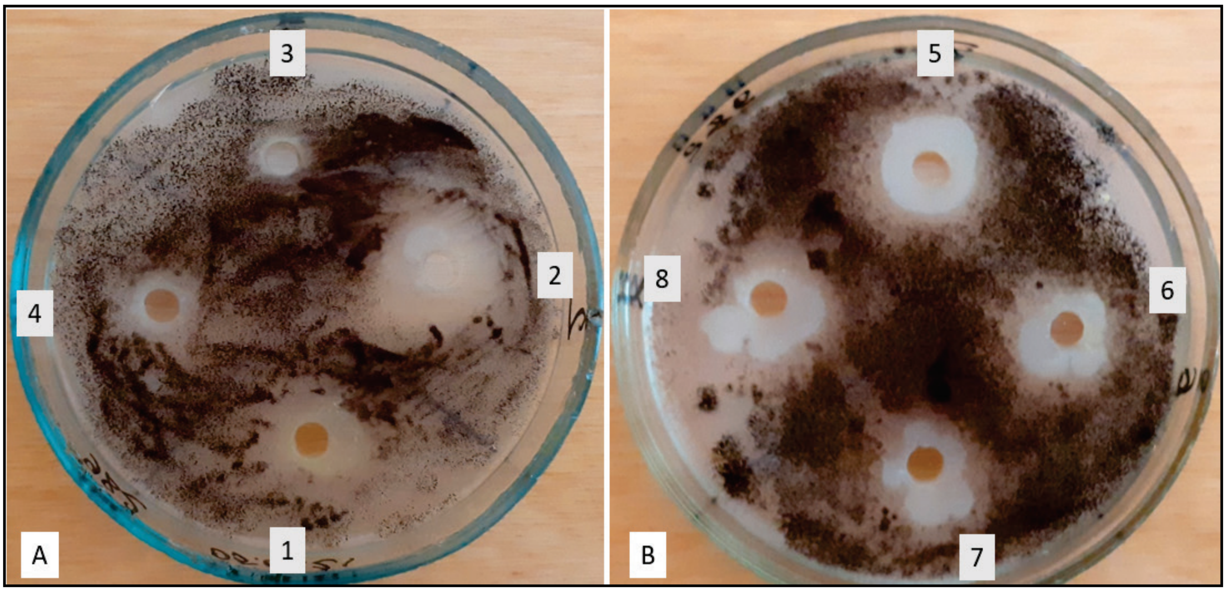

Fig. 5. Antifungal activity of rhizobacteria against strain *Alternaria alternata* (GEPB, 1021): *A*: *1 – Escherichia hermannii*, *2 – Enterobacter cloacae* (GEPB 1022), *3–4 – Rahnella aquatilis* (GEPB 1023); *B*: *5–8 – Enterobacter cloacae*

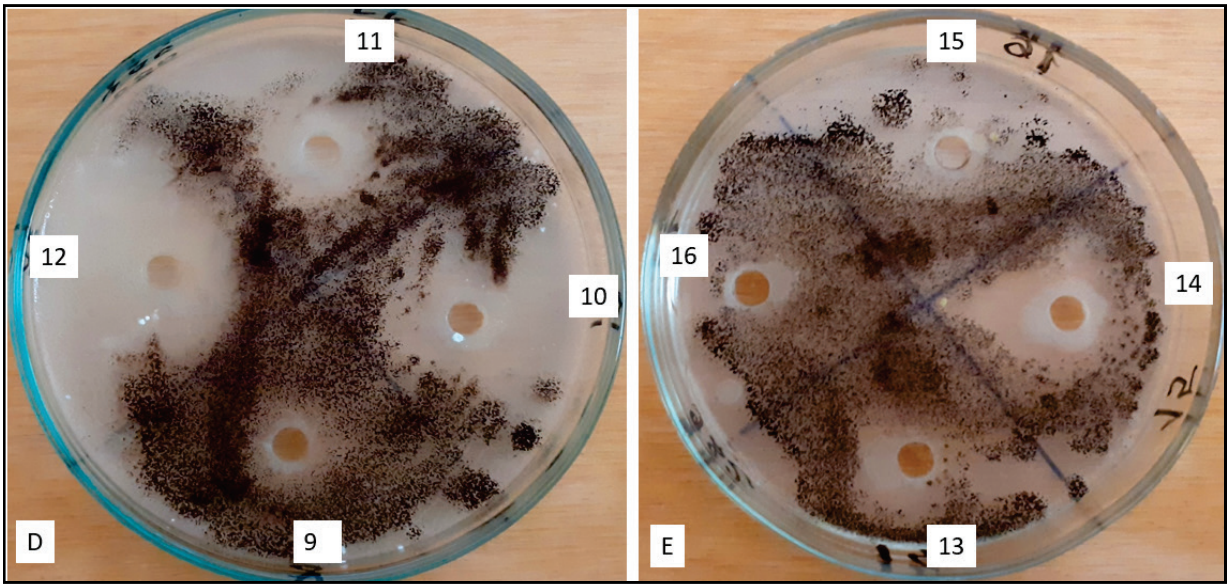

Fig. 6. Antifungal activity of rhizobacteria against *Alternaria alternata* GEPB 1021: *D*: *9–10 – Rahnella aquatilis* (GEPB 1022), *11–12 – Enterobacter cloacae* (GEPB 1023); *E*: *13*, *15–16 – Rahnella aquatilis*, *14 – Enterobacter cloacae*

Lukatkin et al. (2006) showed that inoculating tomato seeds with strains of *Pseudomonas aureofaciens* and introducing bacteria into the soil by irrigation completely stopped the growth of the fungal pathogens *Fusarium culmorum* and *Botrytis cinerea*. In the Bukhara and Namangan regions of Uzbekistan, where agriculture is well developed, samples of pathogenic fungi common on arable lands were studied.

Alternaria is a genus of fungi belonging to the class Dotideomycetes. Dark-coloured species are mainly parasitic on plants. They usually form chains of dictyoconidia or phragmoconidia. The diseases they cause are called altenarioisis. The anamorphic stage forms widely growing dark grey, brown-black, or blackish colonies. Most species are plant saprotrophs or facultative-specific parasites that occur in all plant organs.

Alternaria is a ubiquitous genus of fungi that includes saprobic, endophytic, and pathogenic species that live on a wide variety of substrates. In recent years, DNA-based studies have identified several non-monophyletic genera in the *Alternaria* community and *Alternaria* species clade that do not always correlate with species groups based on morphological characteristics. The *Alternaria* complex currently includes nine genera and eight sections of *Alternaria* (Woudenberg et al., 2013).

It was previously reported that *Alternaria alternata* has a high frequency of post-harvest losses (Akhtar et al., 1994). Among 35 isolates of *A. alternata* collected from rotten fruit from fields and markets, only one isolate from the field caused symptoms of leaf spot. This shows that *A. alternata*, which causes the spotting in the present manifestation, is a separate pathotype. It is believed that this is the first report of *A. alternata* causing a tomato leaf spot in a natural environment (Akhtar et al., 2004). During the study, many diseases of cultivated plants and trees with alternariosis were revealed. Expeditions across Bukhara and Namangan in Uzbekistan have identified around 10 species of agricultural plants that suffer from *Alternaria*.

Antifungal properties of rhizobacteria (*Enterobacter cloacae*, *Rahnella aquatilis*, etc.), mainly to *Alternaria* have been little studied. This study investigated the antipathogenic properties of rhizobacteria isolated from wheat roots such as *Rahnella aquatilis* GEPB1023, *Enterobacter cloacae* GEPB1022 against the fungi *Alternaria alternata* GEPB1021, *Fusarium solani*, *Verticillium dahlia* and *Aspergillus niger*.

Plant growth-promoting rhizobacteria (PGPR) enhance the adaptive capacity of their hosts through a variety of mechanisms, including mobilization of hard-to-absorb soil nutrients, molecular nitrogen fixation, and phytohormone synthesis. PGPRs also have several properties that allow them to act as biocontrol agents: they produce siderophores, antibiotics, and various enzymes that can be used to limit plant damage caused by fungal pathogens (Meen et al., 2014).

Kadyrova et al. (2022) analyzed the antifungal and antibacterial activity of wheat (*Triticum aestivum* L.) rhizobacteria: *Pseudomonas aeruginosa* C10, *P. aeruginosa* C23, *P. stutzeri* C4, *Bacillus pumilus* C16, *B. cereus* C19, and *B. subtilis* C27. The experimental results indicate a more effective suppression of phytopathogenic fungi of the genus *Fusarium* by rhizobacteria *P. aeruginosa* C10, *P. stutzeri* C4 and *B. pumilus* C16. It was shown that rhizobacteria of the genus *Bacillus* demonstrate the highest antagonistic activity against the pathogen *Alternaria alternata*. It was established that the *P. aeruginosa* C10 strain has the highest bactericidal activity against pathogenic bacteria: *Bacillus badius*, *B. mesentericus*, *B. mojavensis* (Kadyrova et al., 2022).

Mageshwaran et al. (2022) investigated the antagonism of endophytic bacteria. 255 bacterial endophytes were isolated from the leaves, stems and roots of seven different cultivated plants (chickpea, tomato, wheat, bersem, mustard, potato and green peas). The researchers identified potential endophytic bacteria antagonistic to three soil-borne fungal pathogens: *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp. *ciceri* causing root rot, neck rot and fungal wilt. The results showed endophytic isolates to possess strong inhibition (>50%) against all three tested pathogens.

Carole Balthazar et al. (2022) studied the antifungal activity of twelve *Bacillus* and *Pseudomonas* strains, which were first screened with *in vitro* confrontational assays against 10 culturable cannabis pathogens, namely *B. cinerea*, *Sclerotinia sclerotiorum*, *Fusarium culmorum*, *F. sporotrichoides*, *F. oxysporum*, *Nigrospora sphaerica*, *N. oryzae*, *Alternaria alternata*, *Phoma* sp., and *Cercospora* sp. Six strains displaying the highest inhibitory activity, namely *Bacillus velezensis* LBUM279, FZB42, LBUM1082, *Bacillus subtilis* LBUM979, *P. synxantha* LBUM223, and *P. protegens* Pf-5, were further assessed in planta where all, except LBUM223, significantly controlled grey mould development on cannabis leaves (Carole et al., 2022).

Siahmoshteh et al. (2018) studied the antifungal activity of culture filtrates of soil strains of the bacterium *Bacillus subtilis* and *B. amyloliquefaciens* against some common field pathogenic fungi, with particular reference to a possible mechanism of action against *Aspergillus parasiticus* NRRL 2999. Fungal growth and aflatoxin production were determined by microbiological analysis. Overall, the results of this study indicate that both *B. subtilis* and *B. amyloliquefaciens* can influence fungal growth (Fatemeh et al., 2018).

In our study, the diameter of the zones of antagonistic activity of various rhizobacteria against pathogenic fungi was measured. As can be seen from Table 1 and Figures 5 and 6, the fungicidal activity of the strains *Enterobacter cloacae* (2, 8, 12, 15) (Fig. 6) and *Rahnella aquatilis* (10), against *Alternaria alternata* is high. Bacteria showed antifungal activity against *Alternaria alternata* GEPB WDCM 1228 No. 1021, *Fusarium solani*, *Verticillium dahlia*, and *Aspergillus niger* for zones with diameters of 2–11, 12–20, and 4–14 mm, respectively.

The antagonistic activity of various rhizobacteria against pathogenic fungi has been studied. The fungicidal activity of strains *E. cloacae* (2, 8, 12, 15), *R. aquatilis* (10), and *Pantoea agglomerans* (22) was high and stopped the growth of the pathogenic fungus *Alternaria alternata* in a zone with a diameter of 30–42 mm. The fungicidal activity of rhizobacteria against fungi *Fusarium dahliae*, *Verticillium dahliae*, and *Aspergillus niger* was 2–11, 12–20, and 4–14 mm, respectively.

In our experiments, for the first time, the antifungal properties of *Rahnella aquatilis*, which was isolated from the wheat root, were studied. As a result, it can be seen from Figure 6 that *R. aquatilis* also blocks advanced alternariosis quite well. One of the alternative methods of combating pathogenic fungi that cause diseases of agricultural plants is the introduction of bacteria with high biofungicidal properties into the soil and into the rhizosphere of plants. In the plant rhizosphere, these microorganisms act as stimulators of plant growth and development, and exercise biocontrol phytopathogens that cause plant death.

Conclusion

The results show that among the studied rhizobacteria, *Rahnella aquatilis* (strain R. aquatilis CCIM1023), and *Enterobacter cloacae* (strain *E. cloacae* CCIM1022) can be used as biofungicides to protect plants from pathogenic fungi.

Another way to combat pathogenic fungi that cause diseases in agricultural plants is to introduce bacteria with high biofungicidal properties into the soil and rhizospheres of plants. In the rhizospheres, these microorganisms act as stimulators of plant growth and development while they exercise biocontrol against phytopathogens that cause crop death. Therefore, creating local highly effective biofungicides on their basis and their use in agriculture is a guarantee of environmentally friendly high yields.

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No potential conflict of interest was reported by the authors.

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