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Isolation and identification of *Bacillus velezensis* RTS-M11 and assessment of its antifungal activity

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Abstract

The selection of putative antagonists for the biological control of plant diseases usually involves collecting and screening large numbers of microbial isolates so as to increase the probability of discovering highly effective strains. Different strains of *Bacillus velezensis* produce secondary antifungal metabolites that could control plant diseases. The ability to form spores makes this bacterium an ideal candidate for biological control. Isolation, characterization, and identification of *B. velezensis* (native to Iran) from soil and its antifungal activity against *Fusarium* sp. have been reported in the present study. Eight out of 75 isolates showed antifungal activity against three main species of *Fusarium* under standard conditions. The morphological and biochemical characteristics, along with the 16S rRNA and *gyrB* genes sequences of the selected isolate, indicated that it belongs to the *B. velezensis* species. The results showed that sucrose as a carbon source and peptone as a nitrogen source in a culture medium at pH 7 and an agitation speed of 200 rpm led to the maximal growth rate and antifungal activity in the *B. velezensis* sp.RTS-M11 selected strain. This isolate seems potentially useful as a biological agent against a few *Fusarium* sp. but needs more study in the future.

1. Introduction

Plant pathogenic fungi are a major threat to crops and food production. One of the most important fungal diseases of plants is Fusarium head blight caused by the fungal pathogen *Fusarium* sp. (Haile et al., 2019). Contamination of plants with the *Fusarium* genus causes wilt, plantlets death, and yield and quality losses of products. *Fusarium* sp. is usually controlled using fungicides such as Orthocide and Metalaxyl-Mancozeb (Sardrood et al., 2018). However, their intensive use in conventional crop management has led to many problems, such as side effects on the environment and living beings and the emergence of pathogens resistant to chemicals, especially if the resistance is genetically-based (Khan & Ahmad, 2019). Therefore, biological control is now regarded as an alternative control tool.

In integrated pest management (IPM), biological control means the use of various organisms, especially bacteria and fungi, to directly or indirectly prevent the growth and development of plant pests by producing metabolites with

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Table 4. Effect of carbon sources on antifungal activity of selected isolates (measured clear zones)

Bacterial isolates	***F. solani***						
	Carbon sources						
	Glucose	Xylose	Arabinose	Sucrose	Maltose	Lactose	Starch
PTTC 1023	7.5	6.7	6.8	8.2	7.1	5.2	6.5
B_1	8.7	7.6	8.1	9.5	8.5	5.9	7.2
B_2	10.5	11.2	10.8	11.6	11	8.3	9.9
B_3	7.9	6.8	7.1	8.4	7.6	5.1	5.9
B_4	11.3	10.3	10.5	11.8	11.1	8.6	9.8
B_7	10.2	9	9.2	10.4	9.7	7.6	8.2
B_8	12.6	11.8	12	13	12.5	10.5	11.1
B_9	9.4	8.9	8.9	9.8	9.3	8	8.4
B_{28}	14.5	13.5	13.8	**14.9**	14.2	12.4	13
Bacterial isolates	***F. graminearum***						
	Carbon sources						
	Glucose	Xylose	Arabinose	Sucrose	Maltose	Lactose	Starch
PTTC 1023	7.3	6.3	6.5	7.8	6.9	5.1	5.8
B_1	15.4	14.3	14.7	15.9	15	13.2	13.8
B_2	8.8	7.5	7.9	9.2	8.5	6	6.8
B_3	5.6	4.8	5.1	6.1	5.3	3.9	4.5
B_4	11.1	10.1	10.5	11.5	10.7	8.7	9.4
B_7	16.2	15	15.3	16.5	15.9	12.4	4.2
B_8	9.8	8.6	9	10.4	9.5	7.4	8.1
B_9	10.8	9.2	9.5	11.1	10.3	7.5	8.6
B_{28}	16.5	14.8	15.2	**16.9**	15.8	13	13.9
Bacterial isolates	***F. oxysporrum***						
	Carbon sources						
	Glucose	Xylose	Arabinose	Sucrose	Maltose	Lactose	Starch
PTTC 1023	9	7.8	8.1	9.2	8.5	6.1	6.7
B_1	12.5	11	11.3	12.5	12.1	9.7	10.6
B_2	8.4	7.2	7.5	8.8	8.1	5.8	6.4
B_3	5	3.8	4.2	5.1	4.3	2.9	3.2
B_4	11.5	10.5	10.8	11.9	11	9.3	9.9
B_7	8.3	7.5	7.5	8.5	7.8	6.4	7.2
B_8	11.9	10.3	10.5	12	11.2	9.1	9.6
B_9	9	8.4	8.2	9.6	9	6.5	7.6
B_{28}	9.5	8.5	8.6	**10.1**	9.2	7	7.8

The effect of ammonium nitrate, yeast extract, and peptone as the sole sources of nitrogen was also investigated. The results showed that peptone was the most favorable nitrogen source, providing the maximum antifungal activity for the eight bacterial isolates (Table 5).

Table 5. Effect of different nitrogen sources on antifungal activity of selected isolates (measured clear zones)

Bacterial isolates	*F. solani*			*F. graminearum*			*F. oxysporrum*		
	Nitrogen sources								
	Peptone	Yeast extract	Ammonium nitrate	Peptone	Yeast extract	Ammonium nitrate	Peptone	Yeast extract	Ammonium nitrate
PTTC 1023	8.5	8.2	5.3	7.9	7.6	5.4	9.5	9.4	7.3
B_1	9.8	9.5	5	16.4	15.9	13.8	12.8	12.5	9.9
B_2	11.6	11.3	8.6	9.5	9.2	6.8	9.3	8.8	7.5
B_3	8.6	8.4	5.6	6.3	6.1	4.2	5.5	5.1	4.2
B_4	12	11.6	9.1	11.7	11.3	9.2	12.8	12	10.3
B_7	10.5	10.5	7.3	16.5	16.4	14.6	9.1	8.7	7.5
B_8	13.4	12.8	9.4	11.1	10.5	8.8	12.5	11.9	9.2
B_9	9.8	9.7	5.1	11.3	10.9	8.9	9.9	9.5	7.3
B_{28}	15.3	15	11.8	17.3	16.5	14.2	10.5	10	8.1

3.7. Optimization of shaker agitation rate

Bacterial cells were shaken at 150, 200, and 250 rpm to find the best shaking rate (revolutions per minute (rpm)) for the highest antifungal activity. Maximum antifungal activity was obtained at 200 rpm (Table 6).

3.8. Molecular identification

The amplified 16S rDNA and *gyrB* genes of the selected isolate were first sequenced by the Sanger sequencing method. Then, sequences of the 16S rDNA and *gyrB* genes from different isolates were compared using the Basical Local Alignment Search Tools (BLAST) available at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As a results, the 16S rDNA sequences of the strain had 99.87% similarity to *B. velezensis* FZB42. Finally, the assembled sequence for the 16S rDNA fragment belonging to the RTS-M11 strain was submitted to the NCBI database (https://www.ncbi.nlm.nih.gov/) under accession number MH628438.1.

The phylogenetic trees constructed from the 16S rDNA gene sequences of different species of *Bacillus*, including the *B. velezensis* RTS-M11 from this study, are shown in Fig. 2. The phylogenetic tree based on the 16S rDNA gene sequences clearly delineated four distinct clusters: cluster 1 contained strains of *B. atrophaeous, B. amyloliquefaciens, B. ciamensis, and B. nakamuria*; cluster 2 contained strains of *B. vallismortis*; cluster 3 contained the *B. velezensis* strains, including the RTS-M11 strain and *B. nematocida;* and cluster 4 contained *B. subtilis, B. mojavensis, B. halotolerans*, and *B. tequilensis* strains. *B. velezensis* RTS-M11 shared 99.86-99.87% similarity with other *B. velezensis* strains, 99.73% similarity with *B. vallismortis*, and 98.42-99.8% similarity with *B. subtilis*. Moreover, *B. velezensis*, including the RTS-M11 strain, shared 95.47-99.54% similarity with other *B. velezensis* strains present in the phylogenetic tree, based on 16S rDNA gene sequences.

Table 6. Effect of shaker agitation rate on antifungal activity of selected isolates (measured clear zones)

Bacterial isolates	*F. solani*			*F. graminearum*			*F. oxysporrum*		
	Revolutions per minute (RPM)								
	150	**200**	**250**	**150**	**200**	**250**	**150**	**200**	**250**
PTTC 1023	8.7	10.6	7.6	8.8	9.1	8.1	10.8	11.4	10.1
B_1	10.8	11.3	9.9	18.1	19	17.2	13.8	15.1	12.6
B_2	12.7	13.3	12.3	9.1	10.5	8.6	9.8	11.3	9.2
B_3	9.8	10.2	9.1	6.9	8.1	6.8	6.8	7.6	5.8
B_4	13.3	14.6	13.1	12.6	13.5	12.3	13.8	15	13.9
B_7	11.1	11.8	10.5	17.3	18.6	16.9	10.8	11.6	10.2
B_8	14.4	15.3	13.6	11.5	12.9	11.3	13.9	15.2	13.3
B_9	8.6	10.8	7.3	12.2	13.5	11.6	9.9	10.7	9.7
B_{28}	17.6	**18.1**	16.3	18.8	**20.1**	17.9	11.2	**12**	10.8

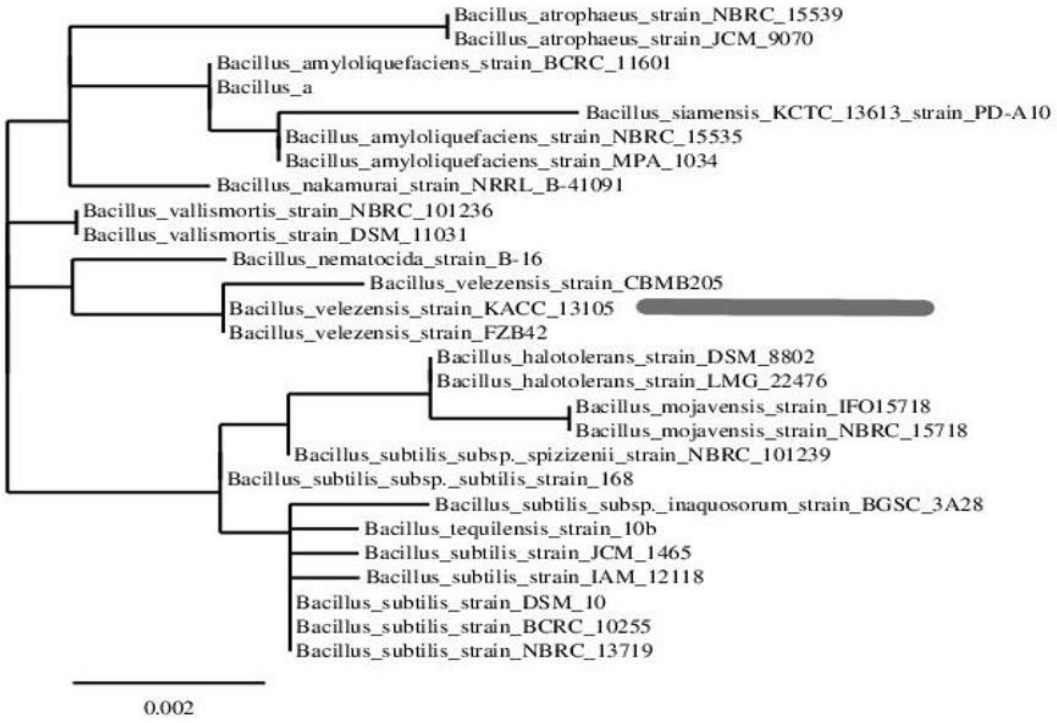

Figure 2. Phylogenetic trees of *B. velezensis* M11-RTS (KACC_13105) based on 16S rDNA. The ClustalW method was used for multiple sequence alignment and the trees were constructed using the neighbor-joining method. Genetic distances were computed by Kimura's two-parameter model.

4. Discussion

The selection of putative antagonists for the biological control of plant diseases usually involves collecting and screening large numbers of microbial isolates to increase the probability of discovering highly effective strains. Therefore, in the present study, the rhizosphere soil of forest plants in Tehran was collected and used for the isolation, identification, and analysis of antifungal activity of the resulting *B. velezensis* strains as biocontrol agents are known to improve the growth and productivity of crops. From a collection of 171 colonies obtained from the rhizospheric soil, we found 75 *Bacillus*-like colonies, eight of which were effective in biological control.

The use of biological control to manage agricultural pests and diseases is an effective

alternative to pesticides. Chemical pesticides that accumulate in plants can be lethal to humans, and beneficial organisms present in the soil may develop resistance. Many studies have reported the prevalence of different strains of *Bacillus* in the rhizosphere. The spore-forming *Bacillus* group is predominantly present in the rhizosphere of healthy chickpea plants. *Bacillus* species have the ability to form endospores and synthesize a vast number of metabolites and, with the exception of toxin-producing *B. anthracis* and *B. cereus*, they are often considered beneficial and safe for plants and the ecological environment (Phelan, 2019). These properties of the *Bacillus* species make them good biocontrol agents and suitable alternatives to chemical fungicides. *Bacillus* species, especially *B. subtilis* and *B. amyloliquefaciens*, play a prominent role in protecting plants from pathogens and promoting plant growth based on their capacity to colonize plant roots (Fira et al., 2018). In a previous study, 905 strains of *B. subtilis* were isolated from the rhizosphere of an avocado plant in Spain. Four strains showed significant antifungal activity against *Rosellina necartix* and *F. oxysporum* (Cazorla et al., 2007). From 205 *Bacillus* spp. isolated from the soil, 23 strains showed antagonistic activity against *Penicillum digitatum*, and nine strains demonstrated more than 80% antifungal activity (Leelasuphakul et al., 2008).
In one study, 69 strains of *B. subtilis* were isolated from the salty soil of north Tunisia; one strain designated SRT46 showed high antifungal activity of 82.85% against *F. solani* (Rebib et al., 2012). The effectiveness of *B. methylotrophicus* in reducing root-knot-disease was also shown in another study (Zhou et al., 2016). Antagonistic *Bacillus* strains might be useful in formulating new inoculants, offering an alternative environmental-friendly biological control for plant diseases.

The present study collected the strain that exhibited the strongest antifungal characteristics against *F. solani*, *F. graminearum*, and *F. oxysporum* from the Koohsar Forest Park in Tehran. The selected isolate was identified as *B. velezensis* using molecular techniques. The new strain was deposited in Iran's Persian Type Culture Collection (PTCC), and the 16S rDNA sequence was registered in the Genbank under accession no. MH628438.

Growth patterns are highly sensitive to conditions including temperature, pH, oxygen and nutrient concentrations, growth medium content, and the number of bacterial cells used for seeding cultures. Two parameters, proper temperature and aeration, can be controlled by orbital shakers (Bates et al., 2016). For the *B. velezensis* in this study, maximal growth was obtained using sucrose as the carbon source, peptone as the nitrogen source, a medium pH of 7, and an agitation rate of 200 rpm. In a study, the highest *B. subtilis* biomass was achieved when using sucrose and xylose (Kumari and Khanna, 2014). High levels of levan and exo-polymeric substances of *B. subtilis* have been observed in sucrose-containing mediums (Shih et al., 2005). Also, peptone has been used as a nitrogen source for the production of α-amylase by *B. subtilis* (Aiyer, 2004). Organic nitrogen sources such as peptone are also better than the inorganic sources for amylase production by *B. licheniformis* (Lal et al., 2016). The optimal pH for growth of this bacterium is around 7, and antibiotics produced by *B. subtilis* are only active at pH values of 5.6 and above (Moita et al., 2005). 16S rDNA analysis is a reliable method in the field of microbiology; it is used to explore microbial diversity and identify new strains. In this study, a 1500 bp fragment of the 16S rDNA gene was sequenced and used to identify isolated bacterial strains. Subsequently, a 16S rRNA gene sequence-based phylogenetic tree was constructed, showing high sequence homology (>99%) between the studied isolate and other *B. velezensis* strains. In a previous study, a bacterium isolated from rice rhizosphere soil was identified using 16S rDNA sequencing and phylogenetically classified as a novel *B. methylotrophicus* species of the genus *Bacillus* (Grover & Dadarwal, 1997). The latter sequence was very similar to the 16S rDNA sequence of this study. In another research, the association of bacteria with chickpea was studied, and the results showed that out of 150 isolates, 40 isolates belonged to the genus *Bacillus*, according

to the physiological, morphological, and biochemical characteristics outlined in Bergey's *Manual of Systemic Bacteriology* (Kumari and Khanna, 2014). In the past 20 years, sequencing of the gene coding 16S ribosomal RNA (rRNA) has become the most valuable tool for the identification of bacteria. However, other genes can also be used for PCR-based strain identification and have been successfully employed to differentiate between bacterial species.

It is broadly accepted that the identification of bacteria at the species or strain level based on physiological and biochemical features is very ambiguous, complicated, and unreliable. Even the 16S rDNA sequence analysis, which is considered to be rapid and reliable, might yield confusing results. Therefore, the use of multiple strategies is necessary to identify bacterial species or strains. Besides the 16S rDNA sequence analysis, the *gyrB* gene may be useful for the identification and phylogenetic analysis of members of the *Bacillus* group at the species and subspecies level, with some exceptions (Mercier & Lindow, 1996). Our analysis of the *gyrB* sequence confirmed the results obtained from the 16S rRNA gene sequence analysis. *gyrB* gene sequence analysis has been used in studies of *Salmonella*, *Shigella*, *Escherichia coli* (Fukushima et al., 2002), and the *Bacillus anthracis–cereus–thuringiensis* group (La Duc et al., 2004). Comparative analysis of 16S rDNA and *gyrB* sequences demonstrated excellent correlation for identifying bacteria belonging to *B. velezensis*.

Conflict of Interest

The authors declare that there is no conflict of interests.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors.

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