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Antagonistic Potential of *Bacillus* and *Pseudomonas* Isolates from Wheat Rhizosphere against *Gaeumannomyces graminis* var. *tritici* and *Bipolaris sorokiniana*

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ABSTRACT

Take-all and common root rot diseases are the important diseases of wheat that are caused by two fungi, *Gaeumannomyces graminis* var. *tritici* and *Bipolaris sorokiniana*, respectively. In this study, we aimed to isolate rhizobacteria *Bacillus* and *Pseudomonas* from wheat rhizosphere and evaluate their antagonistic effect against *G. graminis* var. tritici and *B. sorokiniana*. Thirty six soil samples from wheat rhizosphere were cultured and isolates were identified by bacteriological and biochemical tests. Using the dual culture method antagonistic effects of all the *Bacillus* and *Pseudomonas* isolates were tested against the target fungal pathogens. The isolates were also evaluated for volatile metabolites and sidrophore production. The polymerase chain reaction assay was performed for accurate identification of the isolates. Fifty seven *Bacillus* and *Pseudomonas* strains were isolated. Of the isolates, 19 strains had antagonistic effect against the tested pathogens. *Bacillus* isolates had a greater antagonistic effect against the tested fungi than *Pseudomonas* isolates. In addition, *Bacillus* isolates showed a greater antagonistic effect against *B. sorokiniana* than *G. graminis* var. *tritici*. In this study, only 7 isolates were able to produce volatile metabolites. Sidrophore production was detected in 3 strains of *Pseudomonas* isolates. Based on the 16S rRNA analysis, the 3 strains of *Bacillus* and *Pseudomonas* were identified as *Bacillus megatrium*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. According to majority of the isolates belonged to the *Bacillus* strains and some of them had a good antagonistic activity against *G. graminis* var. tritici and *B. sorokiniana*, they are promising for biocontrol of these important pathogens.

1. Introduction

Wheat is the most abundant crop in the world and is the staple food of many countries (Delcour and Hoseney, 2010). Every year, a significant amount of this crop is lost in the field and storage due to various factors, including diseases. About 200 diseases of wheat have been reported that the most important causes of these diseases are fungi (Akbarpour & behbodi, 2019).

Among the important diseases of wheat are take-all and common root rot diseases, caused by two fungi, *Gaeumannomyces graminis* var. *tritici* and *Bipolaris sorokiniana*, respectively. The common root rot disease causes severe

damage to wheat and barley crops in hot and humid regions of the world every year (Gupta et al., 2018). In recent years, this disease has become one of the most important factors limiting wheat production in various parts of Asia. It is observed in countries such as Pakistan, India and Central Asia with an area of 12 million hectares and the global area of infection of wheat fields with this disease is estimated about 25 million hectares (Acharya et al., 2011).

The take-all is a root and crown rot disease of wheat that has been reported from warm regions of the world such as Australia, Japan, parts of Europe, North and South America, as well as temperate regions, the Middle East and warm tropical heights (Khezri, 2017) and is referred to as the global wheat disease (Kwak & Weller, 2013). In Iran, the disease was first reported in the spring of 1989 from wheat fields in Dasht-e Naz, Sari and other areas of Mazandaran province and Gorgan by Forootan et al. After that, wheat Take-all disease has been reported from most provinces of the country (Rajabi & Behrozin, 2003).

The primary approach for control of plant fungal diseases is use of chemical fungicides but they increase the cost of production, promote resistance, and lead to environmental pollution (Wang et al., 2018). These days due to the lack of public acceptance of these chemicals, resistance of some fungal pathogens and the high cost of producing new chemicals, they are being phased out from use (Heydari & Pessarakli, 2010). *Rhizobacteria* are an alternative to chemical fungicides because of playing a key role in crop production by means of siderophore and antibiotic production, antagonism to soilborne root pathogens, phosphate and potassium solubilization (Wang et al., 2018). There have been many reports of promising biocontrol potentials of *rhizobacteria* such as *Bacillus subtilis*, *Pseudomonas fluorescens* and *Streptomyces* spp. (Quecine et al., 2008, Khezri, 2017, Lagzian et al., 2013). Therefore, according to Golestan province is one of the most important areas for wheat cultivation and common root rot and take-all diseases are problems of wheat growers in this region, in this study, we aimed to isolate rhizobacteria *Bacillus* and *Pseudomonas* from wheat rhizosphere and evaluate their antagonistic effect against *G. graminis* var. *tritici* and *B. sorokiniana*.

2. Materials and Methods

2.1. Sample Collection

Thirty six soil samples from wheat rhizosphere of farming fields around Kalaleh, Gulikesh, Minoodasht, Gonbad Kavous, Azadshahr, Khanbebein, Aliabad and Fazelabad (located in Golestan province) were collected randomly and transferred to a sterile zip lock bag. The samples were labeled and sent to laboratory of Gorgan Branch of Islamic Azad University.

2.2. *Bacterial isolation and identification*

Then 100 g of each soil sample was poured into 200 cc of sterile distilled water and placed on a shaker for 15 min with a slow motion, then left motionless for 5 min and after the sediments rest, 100 µl of the suspension was cultured on nutrient agar medium containing 1% sucrose and King B. The plates were incubated at 25°C for 5 days (Schaad et al., 2001). All isolates were evaluated by Gram staining, oxidase, sporulation and examined also for fluorescence under UV light. Standard biochemical tests given in Bergey's manual of determinative bacteriology and laboratory guide by Schaad et al. were used to identify the bacterial isolates (Bergey's Manual of Determinative Bacteriology, 2000, Schaad et al., 2001).

2.3. Detection of Antagonistic Effect

2.3.1 Dual Culture Method

Antagonistic effects of all the *Bacillus* and *Pseudomonas* isolates were tested against *G. graminis* var. *tritici* and *B. sorokiniana* using the dual culture method. The isolates were streaked as a streak line with a loopfull of 2 day-old culture on potato dextrose agar (PDA) plates, and incubated for 48 h. A mycelial disc (5 mm in diameter) of the tested fungus was placed at a distance close the other edge of the plate and incubated at 30°C for 5 days. After that, inhibition zones (the distance among the edge of antagonistic bacterial growth and the edge of fungal growth) were measured. The experiment was repeated three times for each fungus (Abo-Zaid et al., 2020) (Figure 1).

2.3.2. Screening for volatile metabolites production

The bacterial isolate was cultured uniformly on PDA plate. A mycelial disc (5 mm in diameter) of the tested fungus was placed in another plate containing the PDA medium. Under sterile condition, the plates containing bacteria and fungi were placed on top of each other in such a way that the plate containing the fungus is placed on top. The area around the plates is completely surrounded by parafilm. In the control plate instead of bacteria, a drop of sterile distilled water was placed. The plates were incubated at 25° C for 7 days, after that the fungal colony in control and treatment petri dishes were compared (Fiddaman & Rossal, 1993) (Figure 2).

2.4. *Screening for Sidrophore production*

To perform this test, all *Pseudomonas* isolates were checked for their ability to produce siderophores. Detection of siderophores was achieved on CAS agar plates, the color changes from greenish blue to orange indicated a positive siderophore production. The *Pseudomonas* isolates were grown in succinic medium contained 4 g succinic acid; 6 g KH_2PO_4; 4 g K_2HPO_4; 0.2 g $MgSO_47H_2O$; 1 g $(NH_4)_2SO_4$ and dH_2O up to 1000 mL for 24 h at 200 rpm and 30 °C. After that, 10 mL of each culture was centrifuged at 10,000 rpm for 10 min and then the supernatant was filtrated throughout a 0.2 µ syringe filter. Then, 80 µL of the culture filtrate was poured in each 5-mm-deep wells punched into CAS agar plate. The plates were incubated at 30 °C for 24 h and the presence of siderophores was detected visually (Abo-Zaid et al., 2020) (Figure 3).

2.5. Molecular Identification of Bacterial Isolates

Preliminary identification of the isolates was carried out according to standard cultural and biochemical tests. The PCR assay was used for accurate identification of the isolates possessing higher antagonistic activity. To do PCR, the bacterial isolate was cultured in nutrient broth overnight. Then, the genomic DNA from overnight culture of the isolate was extracted using Geno Plus TM Genomic DNA Extraction Miniprep System (Viogene, China). The qualification and quantity of the extracted DNA was determined by agarose gel electrophoresis (1%). The genomic DNA was used as a template for 16S rDNA gene amplification using universal primers. Amplification was carried out in 25 µL volumes consisting of 0.5-µLTaq DNA polymerase, 5 µL of × 5 reaction buffer (Bioline, UK), 1 µL of each primer, 1 µL of template DNA, and 16.5 µL of DEPC water. The PCR amplification was performed with 30 cycles of denaturation at 94˚C for 30 s, annealing at 56 ˚C for 30s, and extension at 72˚C for 30 s. The initial denaturation and final extension were 94 ˚C for 3min and 72˚C for 10min respectively. The primers used in this study are summarized in Table 1. The 16S rDNA genes were successfully amplified with universal primers and expected fragments with 1500 bp were observed on 1% agarose gel. Then, the purified PCR products were sent to Macrogen (South Korea) for sequencing. The obtained nucleotide sequences were searched for homology in the NCBI nucleotide database using BLAST tool. The 16S rRNA gene sequences were aligned against the reference nucleotide sequences retrieved from GenBank (Ebrahimi and Ahani Azari, 2016).

Figure 1. Inhibitory effect of the antagonistic isolate against *B. sorokiniana*

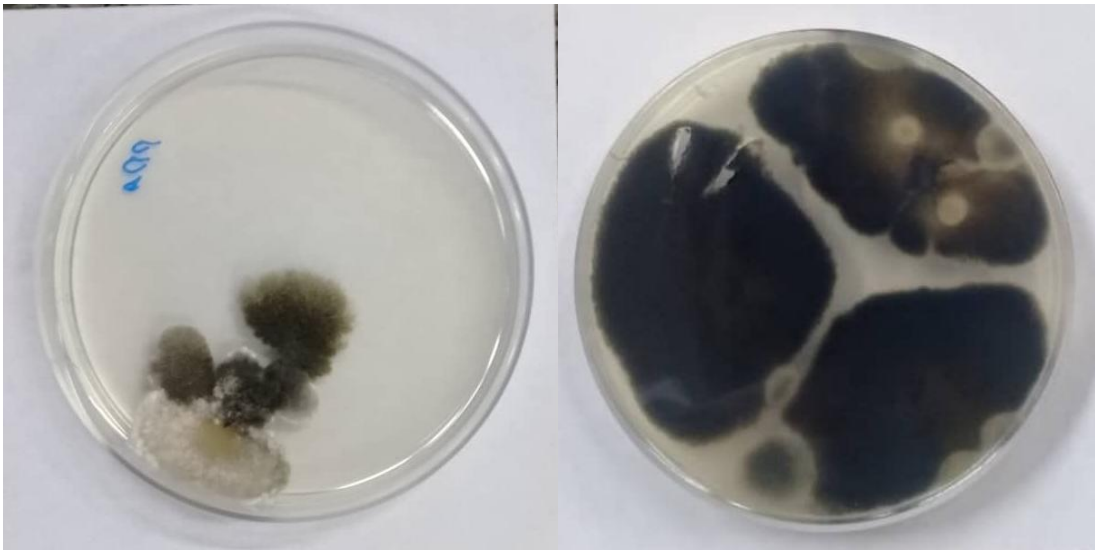

Figure 2. Qualitative assay of volatile metabolites production of the isolates. Right: control, Left: treatment petri

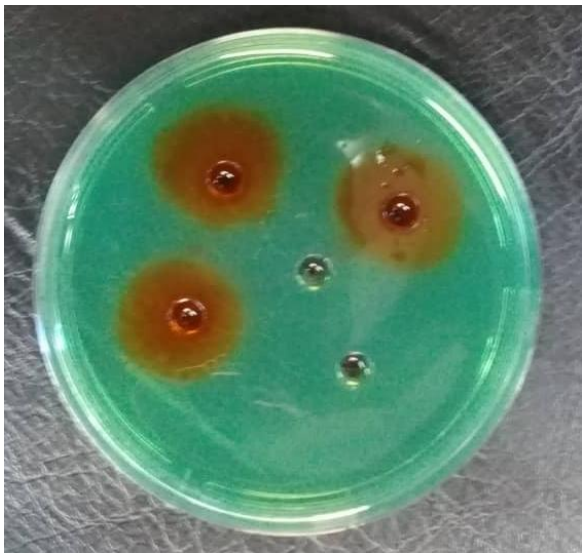

Figure 3. Qualitative assay of siderophore production on CAS agar plates using supernatant of *Pseudomonas* isolates

Table 1. Primers used in this study (Weisburg et al., 1991).

Primer name	Primer sequence	PCR product
16S-Forward	5'-AGAGTTTGATCCTGGCTCAG 3'	1500bp
16S-Reverse	5'-ACGGCTACCTTGTTACGACTT3'	

3. Results

Based on the preliminary identification, 57 *Bacillus* (43) and *Pseudomonas* (14) strains were isolated from the samples. Of the isolates, 19 strains (12 *Bacillus* and 7 *Pseudomonas*) showed inhibition zones and had antagonistic effect against the *G. graminis* var. *tritici* and *B. sorokiniana*. Overall, *Bacillus* isolates had a greater antagonistic effect against the tested fungi than *Pseudomonas* isolates. In addition, *Bacillus* isolates showed a greater antagonistic effect against *B. sorokiniana* than *G. graminis* var. *tritici*. In this study, only 7 isolates (2 *Bacillus* and 5 *Pseudomonas* strains) were able to produce volatile metabolites and inhibit the growth of fungi by this mechanism. In addition, sidrophore production was detected in 3 strains of *Pseudomonas* isolates (A1, A2 and A3) that showed a higher antagonistic effect against each of the target fungal pathogens.

Among the isolates, the 3 strains of *Bacillus* (R1 and R10) and *Pseudomonas* (A2) with a higher antagonistic effect against each of the tested fungus were selected and identified based on the 16S rRNA gene sequence. After sequencing of 16S rRNA gene, isolates R1, R10 and A2 were identified as *Bacillus megatrium*, *Bacillus subtilis* and *Pseudomonas aeruginosa* strains, respectively.

Table 1. Characteristics and mean diameter of growth inhibition zone (mm)

Location	Isolates	*G. graminis* var. *tritici*	*B. sorokiniana*
Kalaleh	*Bacillus* spp. R1	19.8	22.5
Kalaleh	*Bacillus* spp. R2	16.5	17.2
Azadshahr	*Bacillus* spp. R3	16.6	17.5
Aliabad	*Bacillus* spp. R4	17.4	19.2
Aliabad	*Bacillus* spp. R5	19.6	20.7
Minoodasht	*Bacillus* spp. R6	14.4	15.2
Fazelabad	*Bacillus* spp. R7	12.4	13.8
Gulikesh	*Bacillus* spp. R8	14.7	15.4
Gonbad Kavous	*Bacillus* spp. R9	18.7	19.6
Gonbad Kavous	*Bacillus* spp. R10	20.3	22.1
Gonbad Kavous	*Bacillus* spp. R11	17.1	18.2
Khanbebein	*Bacillus* spp. R12	16.2	16.8
Gonbad Kavous	*Pseudomonas* spp. A1	13.5	13.8
Gonbad Kavous	*Pseudomonas* spp. A2	15.6	15.1
Gonbad Kavous	*Pseudomonas* spp. A3	13.6	14
Kalaleh	*Pseudomonas* spp. A4	12.8	13.2
Fazelabad	*Pseudomonas* spp. A5	12.4	12.6
Minoodasht	*Pseudomonas* spp. A6	11.6	12.9
Gulikesh	*Pseudomonas* spp. A7	11.8	12.4

4. Discussion

According to take-all and common root rot diseases are the main diseases of wheat and many wheat fields are affected by these diseases in the country, we aimed to isolate and identify *Bacillus* and *Pseudomonas* from wheat rhizospher in Golestan province and evaluate their potential for antifungal activity against *G. graminis* var. *tritici* and *B. sorokiniana*. Nineteen *Bacillus* and *Pseudomonas* isolates showed antagonistic effect that further examined for volatile metabolites and sidrophore production. Among the isolates, the 3 strains of *Bacillus* (2) and *Pseudomonas* (1) with a higher antagonistic effect against each of the tested fungus were analyzed based on the 16S rRNA and showed more than 90% relatedness to *Bacillus megatrium*, *Bacillus subtilis* and *Pseudomonas aeruginosa*.

In consistent with our study, Joulideh et al. reported that the strong antagonist bacteria against *G. graminis* var. *tritici* mainly belonged to the genera *Erwinia* and *Bacillus*, *Pseudomonas* (Joulideh et al., 2012). In addition, in Khezri research all 27 studied strains of *Bacillus subtilis* were able to inhibit the growth of *G. graminis* var. *tritici* (Khezri, 2017). In a research by Babaeipoor et al. antagonistic effect of *Bacillus* (*B. subtilis* and *B. pumilus*), *Pseudomonas* (*P. fluorescent*, *P. putida* and *P.aeruginosa*) and *Chromobacteria* sp., separated from wheat rhizosphere against *G. graminis* var. *tritici* was reported that is in line with the present study (Babaeipoor et al. 2011). Alvani et al. isolated 130 strains of *Pseudomonas fluorescent* from wheat rhizosphere in different parts of Khorasan province, of which 21 isolates were growth inhibitors of *G. graminis* var. *tritici* (Alvani et al., 2012). Gajari Mohammad Abadi et al. showed biological control of take-all disease under greenhouse condition by *Pseudomonas* sp, *Xanthomonas* sp, *Ralstonia* sp and *Pseudomonas fluorescens* (Gajari Mohammad Abadi et al. 2016). Akbarpour and Behboudi also reported the antagonistic effect of wheat rhizosphere *Streptomyces* on *B. sorokiniana* (Akbarpour and Behboudi, 2019).

There are similar studies about antifungal effect of rhizospheric *Bacillus* and *Pseudomonas* in and out of the country. In agreement with our study, Ehab saleh et al. reported biological control activity of *Bacillus megaterium* BM344-1 against toxigenic fungi (Ehab saleh et al., 2021). In a study from Bangladesh, biocontrol potential of rhizospheric *Pseudomonas aeruginosa* against *Fusarium oxysporum* f. sp. *Cucumerinum* was reported (Ariful Islam et al., 2018). Zhang et al. also reported antifungal effects of *Bacillus subtilis* against *Alternaria solani* in potato (Zhang et al., 2020). In similar studies, antifungal activity of *Bacillus* species against *Fusarium* and other phytopathogenic fungi has been reported (Khan et al., 2018, Mokabber &Ahani Azari, 2021). In a study by Abo-Zaid et al. antagonistic activity of *Pseudomonas aeruginosa* F2 and *Pseudomonas fluorescens* JY3 on the in vitro growth of *Fusarium oxysporum* and *Rhizoctonia solani* was shown (Abo-Zaid et al., 2020).

Consistent with the results of Jolideh et al. in this study, 7 isolates (2 *Bacillus* and 5 *Pseudomonas* strains) produced volatile metabolites and their inhibitory effect was more pronounced with the *Pseudomonas* strains than with the *Bacillus* strains (Joulideh et al., 2012). Sidrophore production was detected in 3 strains of *Pseudomonas* isolates that were more effective in inhibiting the mycelial growth of the tested fungi compared to the other isolates. Similar to our findings, in a study by Abo-Zaid the isolates with high siderophore production had a more inhibitory effects against the tested fungi (Abo-Zaid et al., 2020).

Based on the result of the present study and other researches rhizospheric *Bacillus* and *Pseudomonas* strains have a potential to produce antifungal metabolites against fungal pathogens and may be used as a biological control of plant diseases. However, for better conclusions about the antagonistic activity of *Rhizobacteria*, it is recommended to perform experiments in laboratory and greenhouse conditions.

Refereces

Abo-Zaid, G., Soliman, N., Abdullah, A., El Sharouny, E., Matar, S., Sabry, S. (2020). Maximization of Siderophores Production from Biocontrol Agents, Pseudomonas aeruginosa F2 and Pseudomonas fluorescens JY3 using

batch and exponential fed-Batch fermentation. Processes. 8(4), 455.

Acharya, K., Dutta, AK. , Pradhan, P. (2011). Bipolaris sorokiniana (Sacc.) Shoem: the most destructive wheat fungal pathogen in the warmer areas. Australian Journal of Crop Science. 5(9): 1064–1071.

Akbarpour, R., & behbodi, K. (2019). Evaluation of antagonistic effect of wheat rhizosphere Streptomyces on biocontrol of common root rot caused by Bipolaris sorokiniana. Biological Control of Pests and Plant Diseases. 8(2): 113-126.

Alvani, S., Rouhani, H., Falahati Rastegar, M., Ahmadzadeh, M. (2012). Detection phenazine-1-carboxylic acid antibiotic in forescent Pseudomonads of wheat rhizosphere and its effect on bicontrol of Take-all disease. Journal of Plant Protection. 26(2): 116-126.

Ariful Islam, Md., Nain, Z., Khasrul Alam, Md., Akhter Banu, N., Rezuanul Islam, Md..(2018). In vitro study of biocontrol potential of rhizospheric Pseudomonas aeruginosa against Fusarium oxysporum f. sp. cucumerinum Islam et al. Egyptian Journal of Biological Pest Control.28 (90): 1-11.

Aya Ehab Saleh, Zahoor Ul-Hassan, Randa Zeidan, Noora Al-Shamary, Thoraya Al-Yafei, Hajer Alnaimi, Nayla Salah Higazy, Quirico Migheli, and Samir Jaoua. (2021). Biocontrol Activity of Bacillus megaterium BM344‑ 1 against Toxigenic Fungi. ACS Omega. 6:10984-10990.

Babaeipoor, E., Mirzaei, S., Rezaee Danesh, Y., Arjmandian, A, Chaichi, M. (2011). Evaluation of some antagonistic bacteria in biological control of Gaeumannomyces graminis var tritici causal agent of wheat take-all disease in Iran. African Journal of Microbiology Research. 5(29): 5165-5173.

Bergey's Manual of Determinative Bacteriology. (2000). 9th ed. Philadelphia: Lippincott Williams & Wilkins, Baltimore, Maryland. 384-426.

Delcour, J., Hoseney, RC. (2010). Principles of cereal science and technology authors provide insight into the current state of cereal processing. Cereal Foods World. 55(1): 21-22.

Ebrahimi, M. and Ahani Azari, A. (2016) Study on Lead Biosorption by Lead Resistant Bacteria Isolated from Industrial Wastewater of Plants in AqQala Industrial Park. Journal of Microbial World. 9(3): 258-267.

Fiddaman, P.J. & Rossal, S. (1994). Effect of substrate on the production of antifungal volatiles from Bacillus subtilis. Journal of Applied Bacteriology. 76: 395-405.

Foroutan, A., Rahimian, H. & Alizadeh, A. (2005). Effect of antagonistic bacteria on Fusarium head blight. Iranian Journal of Plant Pathology. 41: 455- 473. (In Persian).

Gajari Mohammad Abadi, M., Khodakaramian, GH. , Zafari, D. (2016). Biological control of take-all disease under greenhouse condition by bacteria isolated from wheat in Hamedan province. 2nd International Conference on Agricultural Engineering and Natural Resources. 16-17 February, Tehran, Iran.

Gupta, PK., Chand, R., Vasistha, N., Pandey, SP., Kumar, U., Mishra, VK. , Joshi, AK. (2018). Spot blotch disease of wheat: the current status of research on genetics and breeding. Plant pathology. 67(3): 508-531.

Heydari, A., & Pessarakli, M. (2010). A review on biological control of plant pathogens using microbial antagonists. Journal of Biological sciences. 10(4): 273-293.

Joulideh, F., Maaref, A., Naseri, B. (2012). Occurrence of wheat take-all disease caused by Gaeumannomyces Graminis var. tritici in Zanjan province and evaluation of inhibitory effect of wheat rhizosphere bacteria on the causal agent of the disease. Plant protection (Scientific Journal of Agriculture). 35(3): 19-30.

Khezri, M. (2017). Biological control of wheat take-all disease using some biofilm-forming Bacillus subtilis strains. Biocontrol in Plant Protection. 5 (1): 15-30.

Kwak, Y.S. & Weller, D.M. (2013). Take-all of Wheat and Natural Disease Suppression:

A Review. The Plant Pathology Journal, 29: 125-135.

Lagzian, A., Saberi Riseh, R., Khodaygan, P., Sedaghati, E. & Dashti, D. (2013). Introduced Pseudomonas fluorescens VUPf5 as an important biocontrol agent for controlling Gaeumannomyces graminis var. tritici the causal agent of take-all disease in wheat. Archive of Phytopathology and Plant Protection. 46: 2104-2116.

Mokabber, SH., &Ahani Azari, A. (2021). Antagonistic activity of cellulose-degrading bacteria isolated from soil and bovine waste against some phytopathogenic fungi. International Journal of Molecular and Clinical Microbiology, 11(1):1431-1436.

Quecine, M.C., Araujo, W.L., Marcon, J., Gai1, C.S. Azevedo, J.L. & Pizzirani-Kleiner, A.A. (2008). Chitinolytic activity of endophytic Streptomyces and potential for biocontrol. Letters in Applied Microbiology. 47:486-491.

Rajabi, G. H., & Behrozin, M. 2003. Pests and Diseases of Wheat in Iran. Agricultural Education Publications, 186 pp. (In Persian).

Schaad, N.W., Jones, J.B., and Chun, W. (2001). Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd edn. The American Phytopathological Society: St. Paul MN, USA, 396 p.

Wang, X., Wang, CH., Ji, CH., Li, Q., Zhang, J., Song, X., Kang, S., Liu, Zh., Du, J., Liu, X. (2018). Isolation and characterization 1 of antagonistic bacteria with the potential for biocontrol of soil-borne wheat diseases. 27 pp.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology. 173(2): 697–703.

Zhang D, Yu S, Yang Y, Zhang J, Zhao D, Pan Y, Fan S, Yang Z and Zhu J (2020). Antifungal effects of volatiles produced by Bacillus subtilis against Alternaria solani in potato. Frontiers in Microbiology. 11:1196.