Biological Control of Walnut Brown Apical Necrosis Disease *In Vitro* Conditions

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ABSTRACT: Walnut (*Juglans regia* L.) is widely grown in the world and in our country, economic and medical importance, belonging to the Juglandaceae family which is one of the hard-shelled fruits. Fungal diseases, one of the biotic factors, significantly limit the production of walnut plants. Fungicides are used in the control of fungal diseases. However, the use of bacterial bioagents as an alternative to fungicides has gained importance in recent years. In this study, it was aimed to investigate the effects of some bacterial strains against the brown apical necrosis causative *Fusarium* sp. under *in vitro* conditions, the efficacy of twenty bacterial strains (16 *Bacillus* sp., 3 *Pseudomonas* sp., 1 *Brevibacillus* sp.) in terms of antagonistic properties against agent was determined by dual culture test. In the study, it was determined that the percent inhibition rate values changed between 69.80% and 90.10%. The most effective results in *in vitro* conditions were obtained from *Brevibacillus choshinensis* (TV 53D, 90.10%), respectively by *Pseudomonas fluorescens* (FDG 37, 83.7%) and *Bacillus pumilus* (TV 73F, 82.57%). As a result, it was noted that these isolates are promising for controlling walnut fruit rot disease *in vitro* caused by *Fusarium* sp. It is important to test the promising bacterial strains that are effective against the pathogen in different environmental *in vivo* conditions, in terms of determining their usability as a biopesticide.

Keywords: Biological control, Brown apical necrosis, *Fusarium* sp., *Pseudomonas fluorescens,* Walnut

INTRODUCTION

The Persian walnut tree (*Juglans regia* L. or English walnut) is a widespread monoecious tree species of the Juglandaceae family and the Juglans genus hard-shelled fruit (Germain et al., 1999). Walnut is the most produced and highly economically important nuts globally (Hayes et al., 2015; Khir and Pan, 2019). *J. regia* L. native to the mountain valleys of Central Asia, is grown worldwide in temperate areas (Bernard et al., 2018). World walnut production is estimated to exceed approximately three million tons (in-shell basis) by 2022-23 and is mostly supplied by China, the USA, Iran, and Turkey (Anonymous, 2023). Walnut is in high demand due to their long-term storage, use in the pharmaceutical industry, and valuable wood in Turkey (Bayazit et al., 2016). While our country was at the top of the world in walnut production in the 1970s, it fell to second place in the 1980s, third in the 1990s, and fourth in the 2000s (Aslansoy, 2012). One of the reasons for this decline in walnut production fungi from biotic factors is fungal diseases and Brown Apical Necrosis (BAN) disease significantly affects walnut production (Belisario et al., 2002).

BAN is the most recently described (Belisario et al., 2002) and a critical disease complex of walnut in France, Italy, Spain, and Turkey (Belisario et al., 2004; Moragrega and Özaktan, 2010). It began early walnut fruit drop in our country (Akat et al., 2016). The presence and the economic importance of BAN are related to the occurrence of conducive cultural and environmental conditions promoting the dissemination and colonization of fungal pathogens.

BAN derives the name from its typical symptomology that produces brown to dark-brown spots exclusively at the blossom end of walnut fruit, then the spot enlarges to a patch. Sometimes lesions extend over two-thirds of the whole fruit's inner tissue and surface however, the extent of external and internal lesions are not parallel (Belisario et al., 2002). BAN is caused by present everywhere and polyphagous fungi among which Alternaria and Fusarium were the main genera (Belisario et al., 2010). The commonly isolated species are *A. alternata*, *A. arborescens*, *A. tenuissima*, *F. avenaceum*, *F. graminearum*, *F. semitectum*, *F. culmorum*, and *F. oxysporum* (Belisario et al., 2002; Hong et al., 2006; Scotton et al., 2015; Akat et al., 2016; Moya-Elizando et al., 2021).

Different control methods such as pesticide application, use of resistant varieties, cultural methods, and biological control methods are applied to prevent losses due to fungal diseases (Tekiner et al., 2019). However, BAN disease epidemiological aspects are not fully known, and limited information is available (Moragrega and Özaktan, 2010; Moya-Elizondo et al., 2021) therefore, there is a need for improved phytosanitary programs to control this disease.

The present work aimed to identify the biological control agents bacteria that can be used against BAN disease *in vitro* conditions.

MATERIALS AND METHODS

The fungus was isolated and purified from diseased walnut (*Juglans regia* L.) fruits taken from the Banaz district of Uşak. Pathogenicity test and classical diagnosis of the fungus were made (Figure 1).



Figure 1. Diseased plant samples and fungi in Petri dishes

As the biological control agent bacteria, isolates determined to be effective against different disease agents in previous biological control studies were used (Table 1).

Table 1. Biological control agent bacteria used in the study

Isolate	Host	Microbial Identification System (MIS)	Similarity Index	References
FDG 37	Soil	*Pseudomonas flourescens*	0.222	Güneş et al., 2015
RK 103	Apple Branch	*Bacillus pumilus*	0.626	Kotan R., 2000
TV 3A	Rye	*Pseudomonas flourescens*	-	**In this study**

TV 6F	Wheatgrass	*Bacillus substilis*	0.831	Erman et al., 2010
TV 7F	Wheatgrass	*Bacillus mycoides*	-	**In this study**
TV 13A	Raspberry	*Bacillus megaterium*	-	**In this study**
TV 13C	Raspberry	*Bacillus megaterium*	0.595	Erman et al., 2010
TV 17C	Raspberry	*Bacillus substilis*	0.677	Çakmakcı et al. 2010
TV 20D	Raspberry	*Bacillus cereus*	-	**In this study**
TV 42A	Wheatgrass	*Pseudomonas putida*	0.113	Erman et al., 2010
TV 53D	Taraxacum	*Brevibacillus choshinensis*	0.689	Erman et al., 2010
TV 59D	Taraxacum	*Bacillus megaterium*	-	**In this study**
TV 67C	Raspberry	*Bacillus pumilus*	0.630	Erman et al., 2010
TV 73F	Sedum	*Bacillus pumilus*	-	**In this study**
TV 77B	Kekre	*Bacillus substilis*	-	**In this study**
TV 89E	Gladiolus onion	*Bacillus substilis*	-	**In this study**
TV 93B	Sugar beet	*Bacillus substilis*	-	**In this study**
TV 95A	Peas	*Bacillus megaterium*	-	**In this study**
TV 103B	Wheatgrass	*Paenibacillus polymyxa*	0.514	Tozlu et al., 2016
TV 112G	Wheatgrass	*Bacillus atrophaeus*	-	**In this study**

Pathogenicity Test

The pathogenicity of *Fusarium* sp. was tested on walnut fruit. Walnuts were washed under tap water and subjected to surface sterilization with 70% ethyl alcohol in a sterile cabinet. A 6 mm mycelial disc from the fungus, which was previously developed in PDA medium for 7 days, was applied to the fruit by making a wound and the wound surface was wrapped with parafilm. The samples were placed in plastic boxes (7 l) on the bottom of which sterile moist blotting paper was laid, and the growth of the fungus was monitored for 21 days in a 12-hour light/12-hour dark environment at room temperature. In the control application; a sterile PDA disc was placed on the wound site and wrapped with parafilm. The study was carried out in a randomized plot design with 3 replications. Deterioration and mycelial growth on the fruit surface were evaluated as positive and Koch postulates were completed by reisolation from these areas.

Identification of Bioagent Bacterial Strain by Microbial Identification System (MIS)

Bacteria (TV 3A, TV 7F, TV 13A, TV 20D, TV 59D, TV 73F, TV 77B, TV 89E, TV 93B, TV 95A, TV 112G) were identified according to their fatty acid methyl esters (FAME) profiles using Sherlock Microbial Identification System (Miller 1982). These tests were repeated 3 times, and the highest diagnostic result in terms of percentage was considered the definitive result.

Hypersensitivity Response (HR) Testing of Bacterial Biocontrol Agents

The hypersensitivity test of the bioagent bacteria used for the first time in the study in tobacco was carried out on the fresh leaves of the 5-6 week-old *Nicotiana tabacum* var Samsun variety. The bacterial suspensions (10^8 cfu/ml) were prepared in sterile distilled water and infiltrated into the intercostal area

of the leaves of tobacco plants by using a 3-cc syringe without a needle. The inoculated plants were incubated in a completely randomized design on the greenhouse bench for 24-48 h at 20-28°C. The presence of rapid tissue necrosis at the inoculation site was recorded within 24-48 h after infiltration. This test was repeated at least three times for each strain. Sterilized distilled water (sdH_2O) was used as a negative control. The tobacco plants' symptoms were examined. The bacterial isolates which weren't developed any symptoms were evaluated as nonpathogenic, but developed were pathogenic (Klement et al., 1964).

Dual Culture Tests

Petri dishes (90 mm) containing 20 ml of PDA were used in dual culture tests, bioagent bacterial isolates were grown on Nutrient Agar (NA) for 24 hours and pathogenic fungus isolate was grown on PDA for 7 days. Then, bioagent bacterial culture was drawn with a sterile stick around the Petri dishes containing PDA, and a 6 mm fungal disc was placed in the middle of the petri dish. Petri dishes were wrapped with parafilm and incubated at 27°C. As a control, a mycelial disc of pathogenic fungus was placed in the middle of the Petri dish. In the control Petri dish, the radial growth of the fungus was measured in mm when the pathogenic fungus covered the entire surface of the Petri dish. The study was carried out in a randomized plot design with 3 replications and 3 Petri dishes were used for each application. The percent inhibition rate (YEO) of the pathogenic fungus colony was calculated using the percent inhibition of radial growth formula specified by Mari et al. (1993).

$$\text{Percent inhibition rate (\%)} = (C-T)\times 100/(C-6)$$

C: Diameter of control plate pathogen

T: Colony diameter of the pathogen in bacterial application

6: Diameter of the pathogen disc

Statistical Analysis

Statistical analysis was performed on the obtained values using JMP 5.0.1 statistical software and the differences between the means were compared at the $P<0.01$ significance level according to the LS Means Student test.

RESULTS AND DISCUSSION

The pathogenicity of the fungus isolated from the walnut fruit was determined and the result was positive (Figure 2).

Pathogen	Control	Re-isolation Petri

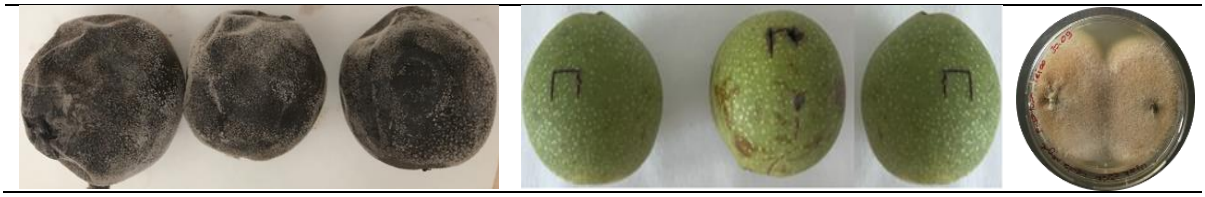

Figure 2. Pathogenicity test on walnut fruit and reisolation fungi Petri dish

According to the classical diagnostic results, it was determined that the pathogenic fungus isolate belonged to the genus *Fusarium*. ET 107 code was given and added to the fungus culture collection. Information on the bioagent bacterial isolates that were tried for the first time in this study and the HR test result are given in Table 2. Fatty methyl ester analyzes of bioagent bacterial isolates applicated in this study: 1 of bacterial isolate was *P. fluorescence* (TV 3A), 1 was *B. mycoides* (TV 7F), 3 were *B. megaterium* (TV 13A, TV 59D, TV 95A), 1 was *B. cereus* (TV 20D), 1 was *B. pumilus* (TV 73F), 3 were *B. substilis* (TV 77B, TV 89E, TV 93B) and 1 was *B. atrophaeus*. All bioagent bacteria HR test results were negative.

Table 2. Microbial Identification System and hypersensitivity test results of bioagent bacteria

Isolate	Host	Microbial Identification System (MIS)	Similarity Index	HR
TV 3A	Rye	*Pseudomonas flourescens*	0.562	-
TV 7F	Wheatgrass	*Bacillus mycoides*	0.575	-
TV 13A	Raspberry	*Bacillus megaterium*	0.124	-
TV 20D	Raspberry	*Bacillus cereus*	0.466	-
TV 59D	Taraxacum	*Bacillus megaterium*	0.504	-
TV 73F	Sedum	*Bacillus pumilus*	0.594	-
TV 77B	Kekre	*Bacillus substilis*	0.483	-
TV 89E	Gladiolus onion	*Bacillus substilis*	0.690	-
TV 93B	Sugar beet	*Bacillus substilis*	0.732	-
TV 95A	Peas	*Bacillus megaterium*	0.502	-
TV 112G	Wheatgrass	*Bacillus atrophaeus*	0.421	-

The results of the antifungal activities of the bioagent bacteria tested against the ET 107 isolate in the dual culture tests are given in Table 3 and the Petri dishes views of the most effective bacteria are given in Figure 3.

Table 3. Percent inhibition rates of bioagent bacteria tested against ET 107 isolate

	Bacteria	MIS	PIRG*	
1	TV 53D	*Brevibacillus choshinensis*	A	90.10
2	FDG 37	*Pseudomonas fluorescens*	B	83.70
3	TV 73F	*Bacillus pumilus*	BC	82.57
4	RK 103	*Bacillus pumilus*	BCD	81.37
5	TV 67C	*Bacillus pumilus*	BCDE	80.57
6	TV 7F	*Bacillus mycoides*	BCDE	80.57

7	TV 42A	*Pseudomonas putida*	BCDE	80.17
8	TV 13A	*Bacillus megaterium*	CDE	79.00
9	TV 112G	*Bacillus atrophaeus*	DE	78.60
10	TV 13C	*Bacillus megaterium*	DE	78.20
11	TV 17C	*Bacillus subtilis*	DEF	77.80
12	TV 95A	*Bacillus megaterium*	DEF	77.80
13	TV 6F	*Bacillus substilis*	EF	77.40
14	TV 93B	*Bacillus subtilis*	EF	77.40
15	TV 59D	*Bacillus megaterium*	FG	74.20
16	TV 77B	*Bacillus subtilis*	GH	72.60
17	TV 103B	*Bacillus megaterium*	GH	71.80
18	TV 3A	*Pseudomonas flourescens*	H	70.20
19	TV 20D	*Bacillus cereus*	H	70.20
20	TV 89E	*Bacillus subtilis*	H	69.80
	Control		I	0.0
		CV	**0.032**	
		LSD	**3.86**	

*There is no statistical difference between the values expressed with similar letters in the same column ($p<0.01$).

The percent inhibition rates of bioagent bacterial isolates varied between 69.80% and 90.10%. The most effective results in *in vitro* conditions were obtained from *B. choshinensis* (TV 53D, 90.10%), respectively by *P. fluorescens* (FDG 37, 83.7%) and *B. pumilus* (TV 73F, 82.57%). The lowest inhibition rate was obtained from TV 89E (69.80%) (Table 3).

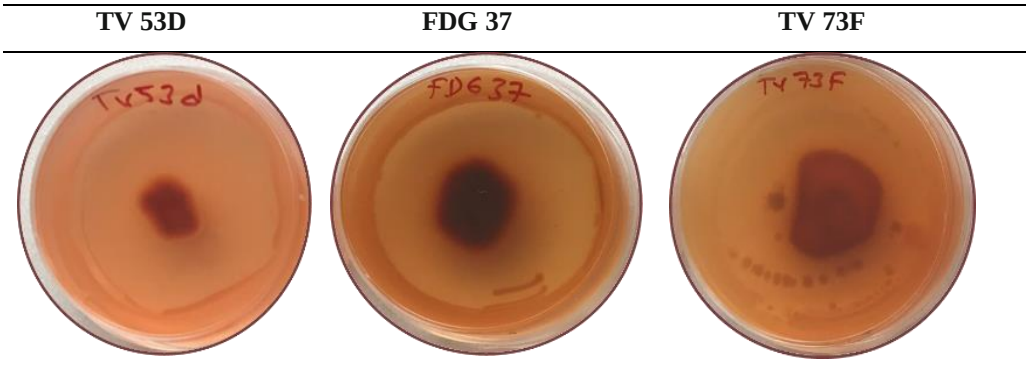

Figure 3. Petri dishes of bacteria most effective against pathogenic fungi *in vitro* conditions

The efficacy of 11 bacterial isolates used for the first time in this study ranged from 69.80% (TV 89E) to 82.57% (TV 73F). The percent inhibition rate in the control, in which only the pathogen isolate ET 107 was applied, was statistically ($p<0.01$) different from all other tested bacteria. In this study, bioagent bacteria belonging to 1 *Brevibacillus*, 3 *Pseudomonas*, and 16 *Bacillus* genera were used as biological control agents and these bioagents prevented the growth of pathogenic fungi at different rates. Biological control is an effective method of controlling plant diseases in an

environmentally friendly way by using microorganisms and bacterial biocontrol agents are used to prevent fungal diseases successfully (Kotan et al., 2009; Abdalla et al., 2014). The effectiveness of bacterial bioagents may be due to various factors (genus of the bioagent bacteria, the competitiveness of the bioagent, the aggressiveness of the pathogen, the host susceptibility and environmental conditions) (Frances et al., 2006). Additionally, there are many studies showing that species belonging to *Pseudomonas* sp., *Pantoea* sp., *Bacillus* sp. and *Brevibacillus* sp. genera can be used as potential bioagent against fungal and bacterial diseases (Kotan et al., 1999; Panwar et al., 2013; Pane and Zaccardelli, 2015; Tekiner et al., 2019; Tozlu and Akça 2022). Studies have shown that bacterial bioagents inhibit the development of the pathogen by producing antibiotic-like substances and hydrolytic enzymes and one of the most important mechanisms for biocontrol of pathogenic fungi (Elshafie et al. 2012; Güneş et al., 2015). It is thought that the bioagent bacteria that were effective in this study inhibited the growth of the fungus by using one of these mechanisms.

In conclusion, TV 53D, FDG 37 and TV 73F bacteria had the potential as bioagents for the control of *Fusarium* sp. under *in vitro* conditions. This study is the first *in vitro* biological control study against BAN disease agent *Fusarium* sp. In future studies, it is of great importance to focus on the determination of the working mechanisms of these bioagents and their *in vivo* applications against *Fusarium* sp. and making them into biological preparations.

Statement of Conflict of Interest

The authors should declare that they are no conflicts of interest.

Authors' Contributions

All authors contributed equally.

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