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RESEARCH ARTICLE

Determination of Some Biological Control Agents Against Alternaria Fruit Rot in Quince

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ABSTRACT

The use of bioagents has become important as an alternative to fungicides to prevent postharvest losses in recent years. In this context, it is aimed to investigate effective of some bacterial and fungal biocontrol agents for control against *Alternari alternata*, which has a wide range of hosts, leading to losses pre and postharvest. In this aim, dual culture of nine bacterial bio-agents isolate [*Bacillus megaterium* (TV 3D), *Bacillus subtilis* (TV 6F, TV 17C, CP 1), *Bacillus cereus* (TV 85D), *Paenibacillus polymxa* (TV 12E), *Pantoea agglomerans* (RK 79, RK 92), *Pseudomonas fluorescens* (MF 3)] and 3 fungal bioagents [*Trichoderma harzianum* (ET 4, ET 14, NT 1)] were tested for antagonistic properties against *Alternari alternata* under *in vitro* conditions. It has been determined that all bio-agents have an inhibitory effect on the growth of pathogen fungus under *in vitro* conditions. RK 79 (79.76%) was the most effective isolate in bio-agent bacteria isolates. All of the bio-agent fungal isolates showed a high hyperparasitic effect and the most effective isolate was ET 4 (67.74%). Consequently, promising results were obtained from these bio-agent bacteria and fungi. It is important to carry out studies *in vivo* bioassays in order to control postharvest decay with bacterial and fungal bio-agents which are determined to be effective.

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Introduction

Quince (*Cydonia oblonga* Mill), among soft-seeded fruits, is a fruit grown in walled gardens in almost every region of Türkey (Büyükyılmaz, 1999). Total quince production in the world is 630,325 tons and Türkey is the first with 139,311 tons in the world (Şirikçi and Gül, 2017). Although fruit production areas show a steady increase, losses during pre and post harvest storage have a significant impact on yield and quality (Acarsoy and Mısırlı, 2010). In developed countries, approximately 20-25% of post-harvest fruits are estimated to be spoiled by pathogens (El-Ghaouth et al., 2004; Droby, 2006; Singh and Sharma, 2007). *Botrytis cinerea*, *Alternaria alternata*, *Monilinia linhartiana*, *Monilinia fructigena*, *Diplocarpon mespili*, *Penicillium* spp., *Mucor* spp., *Aspergillus* spp. fungal pathogens cause diseases pre and post harvest (Wan and Tian, 2005). *Alternaria* diseases are among the most common diseases of many plants in the world among these diseases (Agrios, 1997). *Alternaria alternata* (Kessler) in *Alternaria* genus is an important pathogen that develops during cold storage of fruits in quince, becomes visible during marketing period and thus causes major post harvest losses (Troncoso-Rojas and Tiznado-Hernández, 2014). Synthetic fungicides are used today to prevent post harvest losses, but development of resistance to fungicides, people's awareness of the harmful effects of synthetic pesticides, view of leaving a cleaner world for future generations becoming more widespread have led to the search for environmentally friendly alternative control strategies (Mari et al., 2003; Jayapradha and Yesu, 2016). These new strategies include natural compounds (chitosan, essential oils, elicitors of natural defense mechanism) and biological control (Troncoso-Rojas and Tiznado-Hernández, 2014). Biological control method is a

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suitable for post harvest applications (Mari and Guizzardi, 1998) due to storage conditions where are controlled many parameters and biological control agents can confront with the pathogens, directly (Wilson and Pusey, 1985). In order to prevent post harvest losses, different researchers were determined fungi, bacteria and yeasts used successfully in *in vitro* and *in vivo* assay in biological control of dry and wet rot in fruits (Schisler et al., 1997; Kotan et al., 1999, 2002; Roco and Perez, 2001; Sadfi et al., 2002; Sempere and Santamarina, 2007; Panwar et al., 2013; Pane and Zaccardelli, 2015, Ghosh et al., 2016; Ahmed, 2017).

The aim of this study is to test 9 bacterial bio-agents and 3 fungal bio-agents under *in vitro* conditions against *A. alternata* causing disease in quince in storage conditions.

Materials and Methods

Bio-agent Bacteria

In this study, bacterial bio-agents which different researchers determined to be effective in biological control studies were used. Bacterial isolates, MIS results and their hosts were given Table 1.

Table 1. Bio-agent bacteria used in the study

Isolate	MIS diagnosis	Isolated host	References
TV 3D	*Bacillus megaterium*	Rye	Ekinci et al., 2014
TV 6F	*Bacillus subtilis*	Wheat	Erman et al., 2010
TV 12E	*Paenibacillus polymyxa*	Wheat	Erman et al., 2010
TV 17C	*Bacillus subtilis*	Raspberry	Ekinci et al., 2014
TV 85D	*Bacillus cereus*	Sugarbeet	Erman et al., 2010
RK 79	*Pantoea agglomerans*	Apple	Karakurt et al., 2010
RK 92	*Pantoea agglomerans*	Pear	Ekinci et al., 2015
CP 1	*Bacillus subtilis*	*Ricania simulans*	Tozlu et al., 2018c
MF 3	*Pseudomonas fluorescens*	Soil	Güneş et al., 2015

Bio-agent Fungi

The three isolates of *T. harzianum* isolated from different hosts and stored in Fungal Culture Collection, Plant Protection Laboratory of Atatürk University Faculty of Agriculture were used. The fungal bio-agents, molecular diagnosis and their hosts were given in Table 2.

Table 2. Bio-agent fungi used in the study

Isolate	Isolated host	Molecular diagnosis	Accesion number
ET 4	*Aesculus hippocastanum*	*Trichoderma harzianum*	KT897696*
ET 14	*Pinus sylvestris*	*Trichoderma harzianum*	LN864822*
NT 1	Soil	*Trichoderma harzianum*	MF038806**

*Tozlu et al., 2018a
**Tekiner et al., 2018

Isolation of Fungi

Pathogen fungi was isolated from the infected quince fruits taken from greengrocery (Figure 1). Small pieces of diseased fruit tissue were then surface sterilised with 70% ethanol for 3 minutes and rinsed with sterile distilled water. They were then dried on a sterile Whatman filter paper and placed in 90 mm petri dish containing 20 ml Potato Dextroz Agar (PDA) (Merck, Darmstadt, Germany). Petri dishes were incubated at 25-27°C for 4 days in the incubator and small fungal hyphae were transferred to new PDA to obtain pure culture. The fungal isolate "ET 86" was kept in agar plates in Fungal Culture Collection, Plant Protection Laboratory of Atatürk University Faculty of Agriculture.

Figure 1. Infected quince fruit

Pathogenicity of Fungus

The pathogenicity of the ET 86 isolate was tested on quince fruit. Quince fruits were washed under tap water and then surface sterilised with 70% ethanol and inoculated at the center with a 6 mm PDA plug from 5 day old mycelial cultures growth at 26^0C. Inoculated fuits were maintained at 26^0C, 95% relative humidity in 12 hours light / 12 hours dark in growth chamber. Fruits inoculated only with PDA plug were used as control. The fungal pathogen was re-isolated from the diseases fruits. The re-isolated pathogen exhibited the same morphological characteristics as those original isolates. Koch postulates were completed. Each application was performed with 3 replications.

Molecular Diagnosis of Pathogen Fungus

In order to identify fungal pathogen at species level, molecular sequence was performed. Genomic DNA was isolated from the micelles of the fungus using the protocol prepared by Moller et al. (1992). Using the rDNA of the fungal pathogen, Internal Transcribed Spacer (ITS), region was amplified using ITS1-ITS4 primers. The amplified PCR product was sent to Refgen Co. Ltd. for sequencing, and the result of the sequence was stored in Genbank.

In vitro Tests

In dual culture tests, 20 ml PDA containing petri dishes (90 mm) were used, and bacterial bio-agent isolates were developed in Nutrient Agar (NA) for 24 hours, whereas fungal pathogen isolate was developed in PDA for 5 days. Then, the bacterial bio-agent culture was spread with a sterile swap around petri dishes containing PDA, while a 6 mm fungal disc was placed in the middle part of the petri dishes. Petri dishes were wrapped with parafilm and then incubated in a 27^0C incubator until the entire surface of the control petri dish was covered with the fungal pathogen. As a control, only the pathogenic fungal micelle disc was placed in the middle of the petri dish. Radial development of the fungal pathogen was recorded in mm. Each application was performed with 3 replications, and the bio-agent's inhibition rate on the development of pathogenic fungal colony was calculated using the inhibition rate of radial growth formula stated by Mari et al. (1993).

$$\text{Inhibition}(\%) = (C-T)\times 100/(C-6) \quad (1)$$

C: the diameter of the pathogen colony of control group
T: the diameter of pathogen colony after treatments
6: the diameter of pathogen disk.

In testing of fungal bio-agent isolates, pathogens and fungal bio-agents were developed in PDA at ^{270}C for 3 days. 6 mm discs obtained from the fungal pathogen and bio-agent isolates were placed in petri dish as in Figure 2, and the *T. harzinum* isolate was incubated in the incubator until the entire surface of control petri dish was covered. Each application was performed with 3 replications.

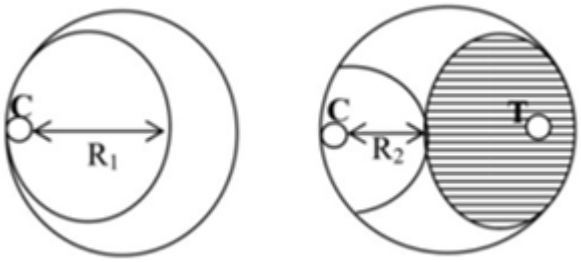

Figure 2. Measurement of radial development of the pathogen mycelia

Inhibition rate (%) of *T. harzianum* isolates on *A. alternata* was calculated according to Skidmore and Dickinson (1976) Formula:

$$\text{PIRG}(\%) = R_1\text{-}R_2/R1\text{x}100 \quad (2)$$

PIRG= Percentage interference rate (%)
R_1= The semi-diameter of the pathogen mycelium in the control petri
R_2= The semi-diameter of the pathogen mycelium in the double culture petri
PIRG>75%: Very high effective (++++),
60%<PIRG≤75%: High effective (+++),
50%<PIRG≤60%: Medium effective (++),
PIRG≤50%: Low effective (+)
Ineffective (-)

Statistical Analyses

The obtained values were analyzed by using JUMP 5.0.1 statistical software, and the difference between the means was compared according to LSMeans Student's test at the significance level of $p<0.01$.

Results

The fungal isolates obtained from the quince were tested for pathogenicity and the result was positive (Figure 3).

Figure 3. Pathogenicity test result and reisolation petri

Table 3 shows the results of the sequencing of pathogenic fungi obtained from the molecular identification, and the sequence was found to be similar to *A. alternata* by 99% as a result of screening using the BLAST program. The result of the molecular sequence was uploaded to Genbank and given an access number of MH992152.

Table 3. ET 86 isolate molecular sequence

1	CCTTCCCCTGTGGTATCCCTAACCTAGATCCGAGGTCAAAGTTGAAAAAAGGCTCTAATGGATGCTAGACCTT
81	TGCTGATAGAGAGTGCGACTTGTGCTGCGCTCCGAAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGAGTC
161	TCCAGCAAAGCTAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGC
241	ATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACA
321	CTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAATTATT
401	AATTTGTTACTGACGCTGATTGCAATTACAAAAGGTTTATGTTTGTCCTAGTGGTGGGCGAACCCACCAAGGA
481	AACAAGAAGTACGCAAAAGACAAGGGTGAATAATTCAGCAAGGCTGTAACCCCGAGAGGTTCCAGCCCGCCT
521	TCATATTTGTGTAATGATCCCTCCGCAGGTTCACCTACGGAGACCTTGTTACGACTTTTACTTCCTTAAAATGA
594	CCAAGA

The results of the antifungal activity of bacterial bio-agent tested against ET 86 isolates in dual culture tests were given in Table 4 and petri dish views were given in Figure 4. All bio-agent bacteria prevented the development of ET 86 at different levels. The inhibition rates of bacterial bio-agent isolates ranged from 14.28% to 79.76%. The highest inhibition rate was observed in RK 79 (79.76%) isolates, followed by RK 92 (73.21%) and MF 3 (62,50) isolates. The lowest inhibition rate was obtained from TV 3D (14.28%) (Table 4). The inhibition rate of the control application was found to be statistically different from all other tested bacteria ($p \leq 0.01$).

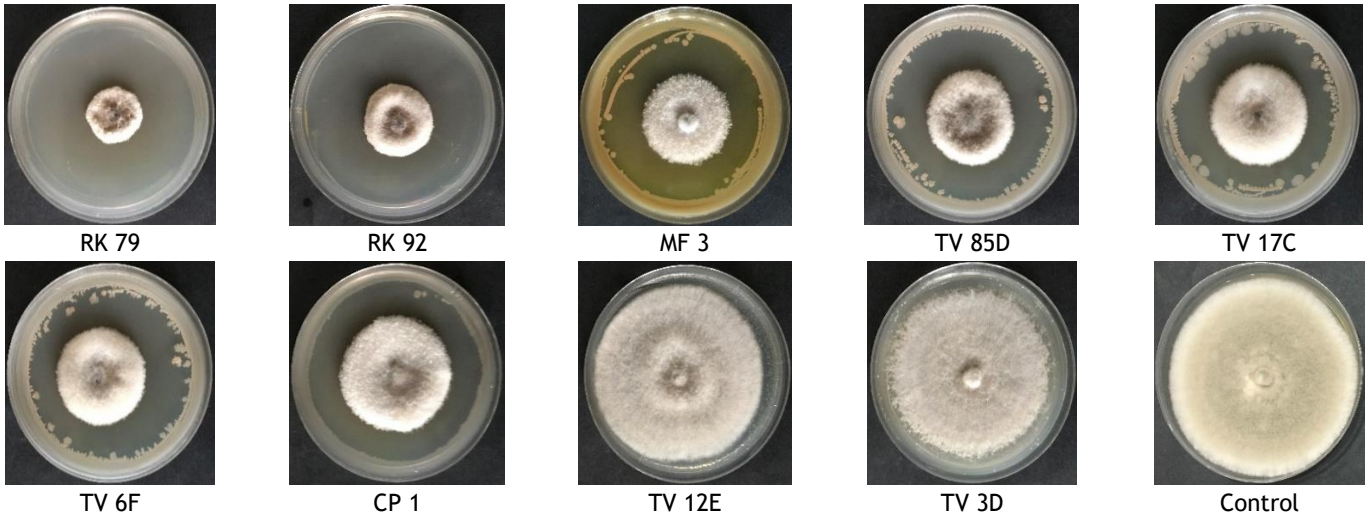

Figure 4. The results of the antifungal activity of bacterial bio-agents tested against ET 86 isolate

Table 4. Percentage inhibition rate of ET 86 with bio-agent bacteria in dual culture test

Bio-agent bacteria	PIRG (%)	
RK 79	79,76	A
RK 92	73,21	B
MF 3	62,50	C
TV 85D	61,31	CD
TV 17C	60,71	CD
TV 6F	59,52	D
CP 1	51,19	E
TV 12E	20,83	F
TV 3D	14,28	G
Control	0.00	H
LSD	3,95	
CV	0,02	

Efficacy of *Trichoderma* isolates tested against ET 86 isolate was tested *in vitro* and the hyperparasitic effects of *T. harzianum* isolates were shown in Table 5. All isolates were highly effective. Among *Trichoderma* isolates, ET 4 (67.74%) had the highest inhibition rate, which was followed by ET 14 and NT 1 (61.29%). ET 14 and NT 1 had an equal effect (Table 5). Petri dish views of the hyperparasitic effects of *T. harzianum* isolates under *in vitro* conditions are given in Figure 5.

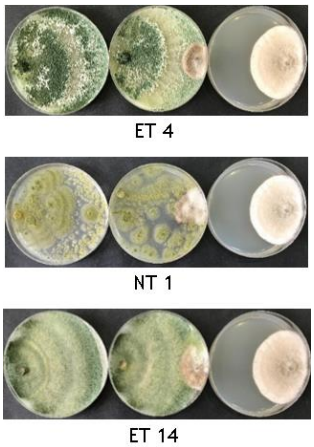

Figure 5. Petri dishes of bio-agent fungi tested against ET 86 (top to bottom: *T. harzianum*, *T. harzianum*+*A. alternata*, *A. alternata*).

Table 5. The hyperparasitic effects of *T. harzianum* isolates against ET 86 *in vitro* condition

Pathogen Fungi	Bio-agent fungi					
	ET 4		ET 14		NT 1	
ET 86	PIRG (%)	HL	PIRG (%)	HL	PIRG (%)	HL
	67.74	+++	61,29	+++	61.29	+++

*PIRG, Percentage inhibition rate (%); HL, Hyperparasitic level

Discussion

Bacterial and fungal bio-agents are used to prevent post harvest diseases successfully. Because storage conditions such as temperature and humidity are controlled conditions. Bacterial and fungal bio-agents used in this study were prevented pathogenic fungi development at different levels under *in vitro* conditions in previous studies.

The effectiveness of bio-agents may be due to various factors. These factors are the genus of the bio-agent, the competitiveness of the bio-agent, the aggressiveness of the pathogen, the host susceptibility and environmental conditions (Frances et al., 2006). 9 bacterial bioagents tested against *A. alternata*, *P. agglomerans* (RK 79) was the most effective bacterial bio-agent isolate, *T. harzianum* (ET 4) was the most effective fungal bio-agent isolate, too. It was determined by different researchers that this bacterial and fungal bio-agent isolate was used effectively against some pathogenic fungi and bacterial plant pathogens (Beer et al., 1984; Kearns and Hale, 1995; Montesinos et al., 1996; Voland et al., 1999; Usall et al., 2001; Kotan et al., 2009; Begum et al., 2010; Tozlu et al., 2018a; Tozlu et al., 2018b).

In addition, there are many studies showing that species belonging to *Pseudomonas* sp., *Pantoea* sp., *Bacillus* sp., *Trichoderma* sp. genera can be used as potential bio-agent against *Alternaria* fruit rot (Roco and Perez, 2001; Pandey, 2010; Pastor et al., 2012; Abbo et al., 2014; Arzanlou et al., 2014; Zhang et al., 2014) These fungal and bacterial bio-agents inhibit the development of pathogens by producing enzymes or antibiotics, rapidly colonizing and competing strongly. Some researchers have determined that *P. agglomerans* prevents the development of pathogens by the antibacterial substances it produces (Chernin et al., 1995; Wright et al., 2001; Kotan et al., 2009). *T. harzianum* effects against post harvest pathogens both direct parasitizing of the pathogen (Goldman and Goldman, 1998; Monte, 2001) and the production of some enzymes (Ulhoa and Peberdy, 1991; Harman, 1993).

Chitinolytic enzymes have an important role in the biological control of post harvest pathogens because they can destroy the structure of chitin in the cell wall of pathogenic fungi. *P. agglomerans* (Kotan et al., 2009) and *T. harzianum* isolates (Tozlu et al., 2018b) used in this study produce chitin degrading enzymes and prevent the development of the pathogens in this way.

In conclusion, RK 92 and ET 4 had the potential as bio-agents for the control of *A. alternata* under *in vitro* conditions, as well as against other fungal pathogens (Kotan et al., 2009, Tozlu et al., 2016, 2018a). Furthermore, it is great importance to test this fungal and bacterial bio-agent in different storage conditions with different temperature and humidity.

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