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BIOLOGICAL CONTROL OF *BOTRYTIS CINEREA* AND *ALTERNARIA ALTERNATA* WITH BIOAGENT BACTERIA AND FUNGI UNDER *IN VITRO* CONDITIONS

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ABSTRACT

There are many fungal diseases observed in pre and post harvest in plants. *Botrytis cinerea* and *Alternaria alternata* are belong to these fungal diseases. For a long time fungicides are used to prevent these fungal diseases. Because fungicides have some negative effects on human and environmental health, cause resistance and give rise to the problem of residues in agricultural products, the use of environment-friendly biological preparations has gained importance. In this study; The effect of 3 different isolates (ET 4, ET 14 ve NT 1) of *Trichoderma harzianum*, and 9 different isolates of *Bacillus megaterium* (TV 3D), *Bacillus substilis* (TV 6F, TV 17C, CP 1), *Paenibacillus polymxa* (TV 12E), *Bacillus cereus* (TV 85D), *Pseudomonas fluorescens* (AR 9), *Pantoea agglomerans* (RK 79) and *Pseudomonas fluorescens* Biotip F (MF 3) were tested against 3 different isolates (ET 59, ET 62 ve ET 63) of *B. cinerea* and 1 isolate of *A. alternata* (ET 61) by dual culture method in *in vitro* conditions. According to the results of the study; the most effective fungal isolate was ET 4 against *B. cinerea* (85.73%) and *A. alternata* (70.47%). The percentage inhibition rate of *T. harzianum* isolates against *B. cinerea* is determined to be higher compared with *A. alternata* in *in vitro* conditions. The antagonistic effect of bioagent bacteria has been shown at different levels, too. The most effective bacterial isolates were TV 6F (95.24%) for ET 59, TV 85D for ET 61 (64.29%), ET 62 (80.36%) and ET 63 (71.43%). It will be planned to these quite promising bioagents bacteria and fungi and their formulations tested on fruits in storage conditions, too.

KEYWORDS:
Biological control, Bioagent bacteria, *Botrytis cinerea, Alternaria alternata*, *Trichoderma harzianum.*

INTRODUCTION

While the world population is getting increase, the agricultural land and other important natural resources is getting decrease. This will be the one of the most important problem in the future. For that reason developed and developing countries are taking a set of precautions. As the foremost of these precautions come to increase the yield per unit and prevention of the losses resulting from biotic/abiotic factors. In plant production, annual losses rate is %36,5 on average [1]. According to a report prepared by Food and Agriculture Organization of the United Nations, 1/3 of food production is stated to get lost after the harvest [2]. Contamination with pathogen can occur during storage, marketing and after the purchase of the consumer as it is during and after the harvest as well [3]. Most of the fungus causing an post harvest disease are among the Ascomycotina and Deuteromycotina (*Botrytis*, *Alternaria*, *Penicillium*, *Aspergillus*, *Geotrichum*, *Fusarium*, *Colletotrichum* and *Phomopsis*) [4].

Botrytis cinerea Fr. (telemorph: *Botryotinia fuckeliana* (de Bary) Whetzel) and *Alternaria alternata* (Fr.) Keissl. among these pathogens cause a significant loss in fruits and vegetables post harvest. Additionally, according to a recent research *B. cinerea* is stated to rank second on the list of 10 most important fungal plant pathogen in the world [5]. To reduce the lost of these 2 common and destructive fungus should be provided suitable conditions during pre and post harvest for the storage.

The producer can provide an effective protection by using external and internal protective synthetic fungicides relatively pre and post harvest. However the widespread use of chemicals in agriculture has been a subject of public concern and scrutiny due to their potential harmful effects on the environment, their undesirable effects on non-target organisms, development of resistant races of pathogens and possible carcinogenicity of some chemicals [6, 7].

It becomes more and more difficult to clean world in future. For these reasons, the researchers focused environmentally safe, long lasting and effective biological control methods against the chemical control [8, 9]. In biological control antagonist and hyperparasitic bacterial and fungal microorganisms or their metabolites are use [10]. The species of *Trichoderma* are used intensively and effectively in biological controls [11, 12, 13]. Among the bacterial

bioagents *Bacillus* and *Pseudomonas* genera show promising biocontrol characteristics [14].

In this study carried out; 3 different isolates (ET 4, ET 14 ve NT 1) of an important fungal bioagent *Trichoderma harzianum* and 9 different bacteria isolates (TV 3D, TV 6F, TV 12E, TV 17C, TV 85D, AR 9, CP 1, RK 79, MF 3) efficiency to 3 different isolates (ET 59, ET 62 ve ET 63) belonging to *B. cinerea* and 1 isolate *A. alternata* (ET 61) species which is isolated according to Koch postulates from diseased plant materials are tested *in vitro* conditions.

MATERIALS AND METHODS

Material. Pathogen fungi isolates were obtained from diseased fruit materials (black mulberry, orange and strawberry). Pathogen fungi were isolated and purified. Pathogenicity test and molecular diagnosis were made and code given [ET 59 (black mulberry), ET 61 (orange), ET 62 (orange), ET 63 (strawberry)]. These isolates were kept slant Potato dextrose agar (PDA: Difco) medium for long term storage at +4ºC in Atatürk Univercity, Agriculture Faculty, Plant Protection Department Plant Clinic Laboratory.

Bioagent bacteria isolates (TV 3D, TV 6F, TV 12E, TV 17C, TV 85D, AR 9, CP 1, RK 79, MF 3) and bioagent fungal isolates (ET4 and ET14) were obtained from the culture collection in the Department of Plant Protection, Faculty of Agriculture at Atatürk University, Erzurum, Turkey. Bioagent fungal isolate, NT 1, was obtained soil in this study. Diagnosis and host information about these isolates are given in Table 1.

Bioagent bacteria isolates had been identified Microbial Identification System (MIS) [15] and pathogen/bioagent fungi had been identified 18SrDNA gene sequencing [16]. Bioagent bacteria isolates were grown on nutrient agar (NA) for routine use, and maintained in Luria Bertani (LB) Broth with 30% glycerol at -80°C for long-term storage. Fungal bioagents were maintained on PDA slant cultures at +4°C in the refrigerator until use.

Method. Isolation of pathogen and bioagent fungi. The diseased fruits were brought to the laboratory from local market and washed with tap water. These materials were subjected to surface sterilization with %70 ethanol for 3 minutes and were dried for 3-5 minutes in laminar cabin. 1 cm pieces were cut to contain both healthy and diseased tissue were placed in petri plates containing PDA and left to incubation at 25-27°C. After 4 days, the fungus developed on PDA and was transferred to a new petri dishes to obtain pure culture. These isolates were then transferred to slant PDA and were maintained at 4°C.

NT 1 bioagent fungal isolate was obtained from the soil by dilution method. 1 g of soil was placed on 9 ml of sterile distile water and was shaken on hematological shaker for 2-3 hour. Consequently, was prepared a serial dilution up to 10^{-8}. 100 µL sample was placed on PDA with streptomycin. 24 hours later fungi developed on PDA medium and then this fungus pure culture were obtained. This fungus was maintained at +4°C.

TABLE 1
Pathogen/bioagent fungi and bioagent bacteria isolates in this study.

Fungi	Identification Results	Isolated from	Pathogenicity	References
ET 59	*Botrytis cinerea*	Black mulberry	+	In this study
ET 62	*Botrytis cinerea*	Orange	+	In this study
ET 63	*Botrytis cinerea*	Strawberry	+	In this study
ET 61	*Alternaria alternata*	Orange	+	In this study
ET 4	*Trichoderma harzianum*	Horse chestnut	-	[17]
ET 14	*Trichoderma harzianum*	Yellow pine	-	[17]
NT 1	*Trichoderma harzianum*	Soil	-	In this study
Bacteria	**Identification Results**	**Isolated from**	**Pathogenicity**	**References**
TV 3D	*Bacillus megaterium*	Rye	-	[18]
TV 12E	*Paenibacillus polymyxa*	Wheat	-	[19]
AR 9	*Pseudomonas fluorescens*	Soil	-	[20]
RK 79	*Pantoea agglomerans*	Apple	-	[21]
MF 3	*Pseudomonas fluorescens*	Soil	-	[22]
TV 6F	*Bacillus substilis*	Wheat	-	[19]
TV 17C	*Bacillus substilis*	Raspberry	-	[23]
TV 85D	*Bacillus cereus*	Sugar beet	-	[19]
CP 1	*Bacillus subtilis*	*Ricania simulans*	-	[24]

+: Positive reaction, **-:** Negative reaction

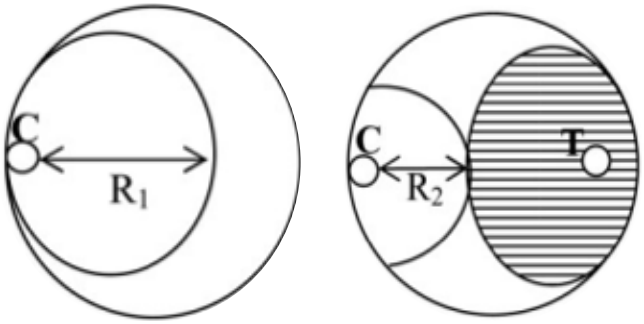


FIGURE 1
Pathogen and bioagent fungi dual culture test application.

The pathogenicity of fungi. ET 59, ET 61, ET 62 and ET 63 fungi were tested for pathogenicity on the parts of the plant tissue from which the pathogen fungus were isolated black mulberry, orange and strawberry. The fruit materials were washed with tap water, then surface sterilization with %70 ethanol for 3 minutes and were dried for 3-5 minutes in laminar cabin. A 6 mm mycelial disc of these fungi, which was previously developed in PDA medium for 5-7 days at 25-27ºC, was applied to the fruit by wounding and the wound surface is wrapped with parafilm and placed in plastic boxes (7L) where sterile moist whatman paper is placed on the base, the growth of the fungi was monitored for 7 days in a 12 hour light / 12 hour dark environment at room temperature. The same procedure was applied to the control application, but blank PDA disc was placed in the wound parts of the plant. Each application was carried out in randomized plot design with 3 replications. At the end of 7 days, deterioration and mycelial development of fruit surface were evaluated as positive and Koch postulates were completed by re-isolation from these regions.

Identification of the pathogen and bioagent fungi. The genomic DNA of each pathogen and bioagent fungi isolates were extracted using the method described by Möller *et al*., (1992) to make molecular identification of pathogen and bioagent fungi isolates. ITS region was used and universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′0) used for the Polymerase Chain Reaction (PCR) (White *et al*., 1990). After PCR samples were sent to the Refgen Co., Ltd., Turkey for sequencing and all sequences submitted to GenBank.

Development of bioagent fungi in different media. Among the bioagent fungi ET 4 and ET 14 were made in previous study [17] and NT 1 was made in this study. The 6 mm mycelial disks taken from the tips of the cultures of NT 1 isolate of *T. harzianum* which was developed in PDA were placed on petri dishes (90 mm) containing 20 ml Malt Extract Agar (MEA), Nutrient Agar (NA), PDA, Sabourd Dextrose Agar (SDA) and Water Agar (WA) media. Its development was followed for 5-7 days on these medium at 25-27°C.

Bioassay. Petri dishes (90 mm) containing 20 ml PDA were used in bioassay, bioagent bacteria isolates were grown on Nutrient agar (NA) at 25-27°C for 24 h, pathogen and bioagent fungi isolates were grown on PDA at 27°C for 3-5 days for *in vitro* dual culture method. Bioagent bacteria were applyed around PDA petri plate with sterile swap and the discs measuring 6 mm obtained from pathogen fungal isolates were placed in the middle of the petri dishes. Petri dishes were wrapped with parafilm and allowed to incubate at 27°C. As control, only mycelial disks of pathogen fungi were placed in the middle of petri dishes and they were incubated at 25-27°C in incubator untill pathogen fungi isolates myclium cover the surface of PDA completely. Each application was carried out in randomized plot design with 3 replications. Finally, pathogen fungi growth was determined by measuring the diameter of colony radial growth in mm. The percentage inhibition of pathogen fungi by bioagent bacterial isolates were calculated by using the formula [26].

Inhibition (%) = (C−T)×100/(C−6)
C: the diameter of the pathogen colony of control group
T: the diameter of pathogen colony after treatments
6: the diameter of pathogen disk.

Three isolates of *T. harzianum* were tested for *in vitro* antagonistic activity against the pathogens by using the dual culture method on PDA petri plate. The pathogen and bioagent fungi were placed in the petri dish as in Fig. 1. As the controls, only pathogen fungus and bioagent fungus isolates were placed in petri dishes. Each application was carried out in randomized plot design with 3 replications. The mycelium of the bioagent fungi isolates cover the PDA surface completely incubated at 25±3°C.

The hyperparasitic effect of bioagent fungi isolates were determined against pathogen fungi were expressed as percentage interference rate (%) was calculated according to formula [27]. The percentage

interference of the radial development were calculated.

PIRG (%) = (R_1-R_2)/R1x100
R_1: The semi-diameter of the pathogen in the control petri
R_2: The semi-diameter of the pathogen mycelium in the dual culture petri
PIRG > 75%: Very high 60%<**PIRG**≤75%: High
50%<**PIRG** ≤60%: Medium **PIRG**≤50%: Low
-: Ineffective

***In vitro* chitinase enzyme plate assays.** Bioagent fungi chitinase enzyme activities were investigated by petri plate assays according to Abd-Aziz [28] and the colloidal chitin was prepared as described by Örtücü [29]. Among the bioagent fungi ET 4 and ET 14 was made [17], NT 1 was made in this study.

Statistical analyses. The obtained values were analyzed by using JMP 5.0.1 statistical software and the differences between the means were compared at the significance level of p <0.01 according to the LS Means Student test.

RESULTS

Isolation result from diseased fruits four fungi were obtained. Pathogenicity tests of pathogen isolates (ET 59, ET 61, ET 62 and ET 63) isolated from different fruits were carried out, and the results were determined to be positive (Fig. 2).

The identification of pathogen and bioagent fungi isolates were made according to 18rDNA sequence analysis ET 59, ET 62 and ET 63 were determined *Botrytis cinerea*, ET 61 *Alternaria alternate* and NT 1 *Trichoderma harzianum*. Molecular diagnostic results of the pathogen and bioagent fungi were given in Table 2.

The growth and spore formation of NT 1, in different culture media were shown in Fig. 3. When the daily growth of NT 1 was examined, the fastest growth was observed in SDA medium, followed in order by MEA, PDA, NA, and WA.

9 bacterial isolates were determined according to fatty acid methyl esters in previous study (Table 1). Of the bacterial isolates applicated in this study: 4 of bacterial isolates were *B. subtilis* (TV 6F, TV 17C, CP 1), *P. polymyxa* (TV 12E), *B. megaterium* (TV 3D), *P. fluorescens* (AR 9), *B. cereus* (TV 85D), *P. fluorescens* (MF 3) and *Pantoea agglomerans* (RK 79).

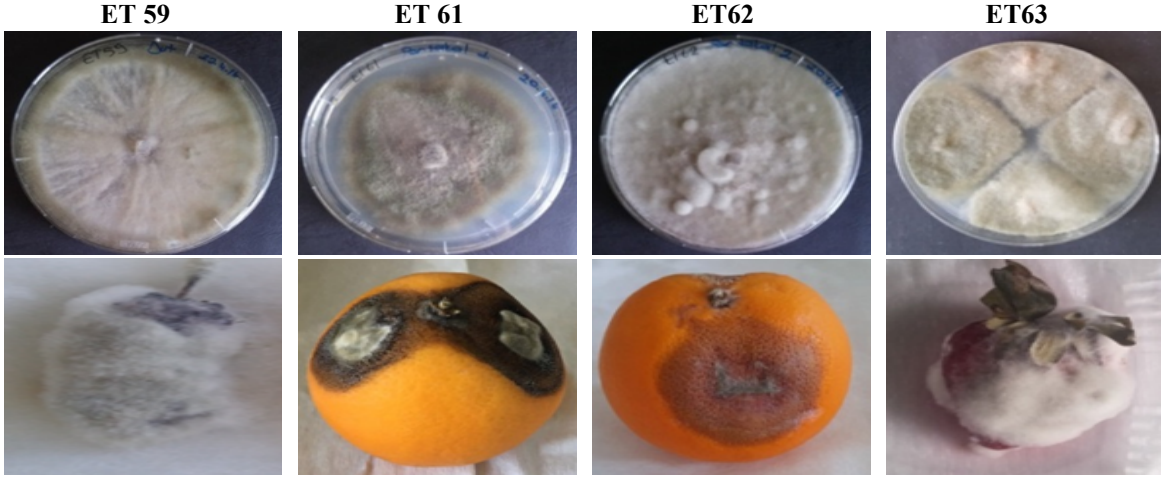


FIGURE 2
Development of pathogen fungi in PDA medium and pathogenicity test results.

TABLE 2
Molecular identification of pathogen and biogent fungi isolates.

Isolates	Molecular identification	Accession Number	Reference
ET 59	*Botrytis cinerea*	2153969	In this study
ET 61	*Alternaria alternata*	2153880	In this study
ET 62	*Botrytis cinerea*	2153898	In this study
ET 63	*Botrytis cinerea*	2153903	In this study
NT 1	*Trichoderma harzianum*	MF038806	In this study
ET 4	*Trichoderma harzianum*	KT897696	[17]
ET 14	*Trichoderma harzianum*	LN864822	[17]

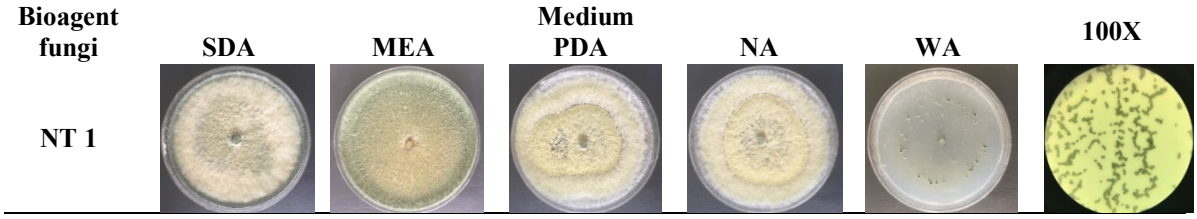


FIGURE 3
Bioagent fungi growth different media and microscopic view.

TABLE 3
Antagonistic effects of bioagent bacteria isolates tested against the pathogens in *in vitro* conditions.

Isolates	Percentage Inhibition Rates (%)*			
	ET 59	ET 61	ET 62	ET 63
TV 6F	**95.24 A**	59.52 C	74.40 C	66.67 B
TV 85D	64.29 C	**64.29 A**	**80.36 A**	**71.43 A**
TV 17C	62.5 CD	61.9 B	78.57 B	60.11 C
CP 1	68.45 B	62.5 B	65.48 D	60.71 C
MF 3	71.43 B	43.45 E	59.52 E	54.76 D
RK 79	64.29 C	62.5 B	41.67 F	11.9 E
TV 3D	59.52 DE	47.62 D	26.19 G	11.9 E
AR 9	56.55 E	47.62 D	23.81 H	11.9 E
TV 12E	47.62 F	42.86 E	11.9 I	11.9 E
Control	0 G	0 F	0 J	0 F
CV	0.03	0.01	0.01	0.02
LSD	3.01	0.9	1.05	1.19

* In the same column by the same letter are not significantly different to the test of LS Means Differences Student's ($p<0.01$)

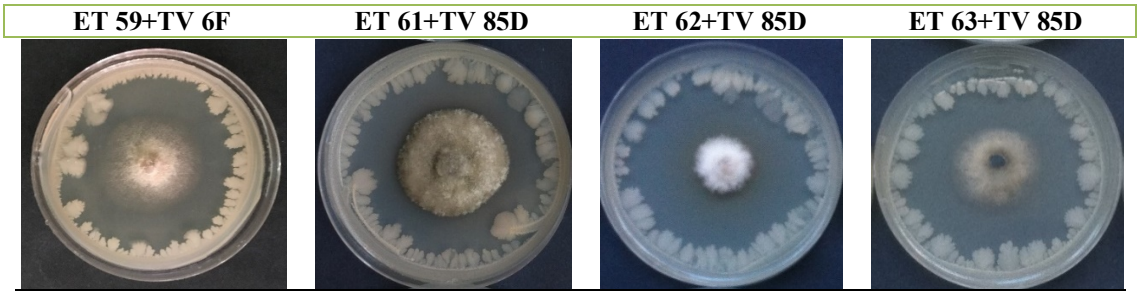


FIGURE 4
Effective bioagent bacteria.

It was observed that bioagent bacteria isolates examined in the present study had antifungal effects at different levels (Table 3). According to the results of antifungal analysis percentage inhibition rate of bacterial isolates ranged from 47.62 to %95.24 for ET 59, % 43.45 to 64.29 for ET 61, %11.9 to 80.36 for ET 62 and %11.9 to 71.43 for ET 63. TV 85D bioagent bacteria isolate the highest effect on ET 61, ET 62 and ET63 at different levels. TV 6F bioagent bacteria isolate the highest effect on ET 59. TV 12E had the lowest antifungal activity among the other bioagent bacteria isolates (Table 4). All of bioagent bacteria isolates were different group from control application ($p<0.01$%). The antifungal activities of the most effective bacterial isolates were given on Fig. 4.

It was observed that *T. harzianum* isolates examined in the present study had hyperparasitic effects at different levels. According to the average results, hyperparasitic effect against *B. cinerea* and *A. alternata* was highest in ET 4 (85.73%, 70.47%), followed by ET 14 (80.96%, 61.91%) and NT 1 (78.00%, 60.95%), respectively (Table 4).

All *T. harzianum* isolates showed very high effect agains *B. cinerea* ET 59, ET 62 and ET 63 isolates (87.23%, 85.65% and 84.31% for ET 4; 83.08%, 83.33% and 76.47% for ET 14; 83.08%, 75.93% and 75.00% for NT 1, respectively) and *A. alternata* ET 61 isolate (70.47% for ET 4; 61.91% for ET 14 and 60.95% for NT1, respectively) (Table 4).

In vitro test results of pathogen and bioagent fungi were presented in Fig. 5.

In vitro, chitin test results of NT 1 isolate was presented in Fig. 6. It was qualitatively determined that ET 4 isolate produced the highest amount of chitin, followed by NT 1 and ET 14 isolates in decreasing order of chitin production [17].

TABLE 4
Hyperparasitic effects of *Trichoderma harzianum* isolates tested against the pathogens in *in vitro* conditions.

PATHOGEN FUNGI		BIOAGENT FUNGI					
		ET 4		ET 14		NT 1	
		*PIRG (%)	HL	PIRG (%)	HL	PIRG (%)	HL
Botrytis cinerea	ET 59	87.23	++++	83.08	++++	83.08	++++
	ET 62	85.65	++++	83.33	++++	75.93	++++
	ET 63	84.31	++++	76.47	++++	75.00	+++
	Average	**85.73**	**++++**	**80.96**	**++++**	**78.00**	**++++**
Alternaria alternata	ET 61	70.47	+++	61.91	+++	60.95	+++
	Average	**70.47**	**+++**	**61.91**	**+++**	**60.95**	**+++**

***PIRG:** Percentage interference rate (%), **HL:** Hyperparasitic level

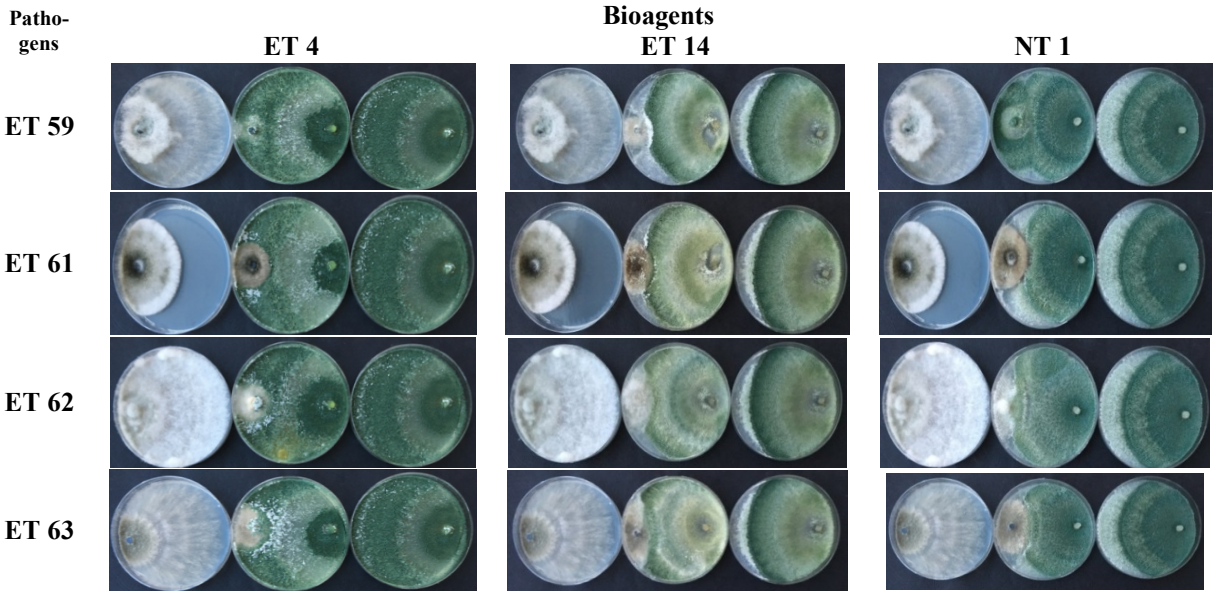


FIGURE 5
The hyperparasitic effects of *T. harzianum* isolates of dual culture method
(left: only pathogen; middle: pathogen and bioagent isolate; right: only bioagent isolate).

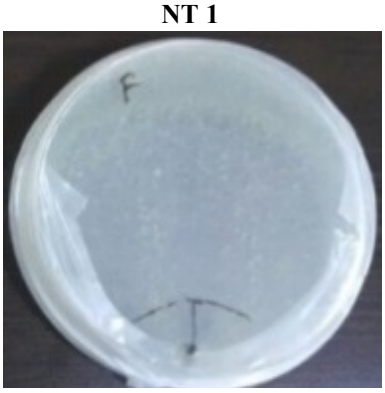


FIGURE 6
Chitin test result.

DISCUSSION

Fungal pathogens cause many damages to agricultural products in terms of both quality and quantity. Producers use chemicals for minimize these problems as best control method. However, many long-term problems associated with their use have led producers to search for biological alternatives. Recent studies show that products of microbial origin don't have the disadvantages that arise from the use of chemicals [30].

Several postharvest diseases can now be controlled by microbial antagonists [31]. Among the microbial products that are obtained from microorganisms or their products. Use of the microbial antagonists like yeasts, fungi, and bacteria is promising, important and gaining popular. The most common biocontrol agents are *Bacillus* and *Pseudomonas* genera in bacteria and *Trichoderma* genera in fungi [32, 14]. Researchers are note that although the mechanism(s) by which microbial antagonists suppress the postharvest diseases is still unknown. Biocontrol agents use various mechanisms (competition for nutrients and space, production of antibiotics, direct parasitism, and induced resistance) for suppress the activity of postharvest pathogens in fruits and vegetables [31].

Different levels of protection were observed between strains belonging to biocontrol agents. This is probably due to the fact that several different

mechanisms can be used to combat the pathogen. The alteration of the plant cell wall causing more protection against pathogens has been found to occur with both *B. subtilis* and *Pseudomonas aeruginosa.* In addition, many biocontrol products originating from *Bacillus* and *Pseudomonas* are commercially produced against fungal diseases [33-37].

There are many study about biological control against *B. cinerea* [38] and *A. alternata* [17, 39, 40] by bacteria. *B. subtilis* TV-6F isolate used in this study was the most effective isolate against *Sclerotinia sclerotiorum* [37]. Similar results were obtained with this study.

Recent studies have shown that more than 1100 isolates belonging to 75 *Trichoderma* species identified with molecular analysis had mycoparasitic potential against *B. cinerea* [41]. Necrotrophic pathogens like *B. cinerea* usually require exogen nutrients before penetration in order to germinate and reproduce on plant surface [42], and they enter the host through wounds that are rich in nutrients, aging tissues, and natural openings where sugar and amino acids are exudated. As a result of the competition between *Trichoderma* species and *B. cinerea* for nutrients, the reduced amount of nutrients causes reduced germination and slower growth of the pathogen's germination tube. Recent studies have demonstrated that the competition between the bioagent *Trichoderma* species and *B. cinerea* resulted in a reduced rate of necrosis caused by the pathogen [43, 44].

In addition to this mechanism, several researchers noted that enzymes produced by bioagents, such as chitinase and glucanase, contributed to the elimination of pathogens by destructing fungal cell wall which is composed of substances including polysaccharides, chitin, and β-glucan [45] Tokimoto (1982) [46] and Chet (1987) [47] suggested that chitinase-producing fungi could be used as bioagents against plant pathogens, and they reported that biological control activity was directly proportional to chitinase production. In the present study, we also determined that *Trichoderma* isolates that were found effectively produced chitin.

There are many study about biological control of *B. cinerea* [48, 49] and *A. alternata* [50, 51, 52] by *T. harzianum. T. harzianum* is used in the biological control of *B. cinerea* [48, 49] and *A. alternata* [50, 52].

In conclusion, it is seen that the results of the present study are in agreement with the previous related studies. 3 isolates of T. *harzianum,* 2 isolates of *B. substilis* and *B. cereus* obtained from different sources were found to be effective against the isolates of *B. cinere*a and *A. alternata* in *vitro* conditions.*T. harzianum* isolates were found to produce chitin in the culture medium, qualitatively. It is thought that the inhibition of the pathogens growth may be attributed to mycoparasitism and chitin production. It is probable that this effect observed in vitro conditions may be showed changes in field conditions with different temperature and humidity levels.

Field studies including these fungal and bacteria bioagent isolateseffective against these important diseases will be important in future. However, more work needs to be done to clarify the mechanisms between effective and ineffective isolates. Moreover, formulation tests are required for the wider application of these new sources.

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