EFFECTS OF TREATMENT WITH *TRICHODERMA HARZIANUM* AND SOME PLANT ACTIVATORS ON POST-HARVEST DECAY OF APPLE BLUE MOLD *(PENICILLIUM EXPANSUM* LINK.) AND BROWN ROT (*MONILINIA FRUCTIGENA* HONEY EX WHETZEL)

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Abstract. The post-harvest application of *Trichoderma harzianum* proved to be effective in the control of blue mold and brown rot on apple. The possibilities of separately application of harpin protein (hp) and *Lactobacillus acidophilus*'s fermentation product (Lafp) and boscalid+pyraclostrobin fungicide were evaluated to the shelf-life of apples. TRIC8 and the plant activators applied to the fruits one hour before pathogens inoculations. The apples were inoculated with a micropipette via the artificially created wounds with the pathogens *P. expansum* and *M. fructigena* with suspensions of 1×10^6 conidia/mL^{-1}. After one hour the pathogens inoculations, the fungicide was sprayed onto the fruits at recommended dosage. The apples were stored at (22±2°C) for ten days. TRIC8 was reduced lesion diameter of *P. expansum* and *M. fructigena* by 69.73% and 97.13% respectively. Significant results on brown rot was provided by the application of Lafp (100%) but not had same success on blue mold (29.59%). Hp was provided to reduction of lesion diameter on blue mold and brown rot at 29.76% and 41.65%, respectively. The fungicide demonstrated 37.22% and 100% control of blue mold and brown rot on the apples in the shelf-life study, respectively.
Keywords: *apple, biological control, blue mold, brown rot, harpin protein, Lactobacillus acidophilus, post-harvest*

Introduction

Apple (*Malus domestica*) is an economically important crop in Turkey. According to 2017 data, there were produced 3.032164 tons of apple in the field of 175.357 ha in Turkey (FAOSTAT, 2017). The rate of loss of fresh fruits and vegetables after harvest is 25% in developed countries; however, it can increase to 50% in developing countries (Salunkhe and Kadam, 1998). Fruits and vegetable continue to physiological events such as respiration, sweating, and ethylene production after the harvest. In storage as well, fruits that continue physiological activities like respiration, transpiration, and so on, are exposed to activities of fungal pathogens present in the environment, as well as to biochemical and physical changes. Pathogen fungi cause postharvest disease are usually passive pathogens that can only entrance into wounds on fruit. The fruit are susceptible to the decay caused by several pathogenic fungi including *Penicillium* sp., *Geotrichum* sp., *Rhizopus* sp., *Phytophthora* sp., *Monilinia sp.* and *Botrytis* sp. (Barad, 2016; Benli, 2003; Errampalli et al.,2004; 2005; Fiori et al., 2008; Chávez et al., 2014).

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More than 90 fungal pathogen species have been reported as causal agents in apple decay during storage (Jones and Aldwinckle, 1990).

To control blue mold disease caused by *P. expansum*, is the most common wound pathogen during harvest, transport and storage. So, store should be cleaned, ventilated, disinfected before the fruits are stored, and excessive humidity should be avoided (Ozgonen and Kilic, 2013). Little is known about to mechanism used to decay of caused by *P. expansum* on apple. The secretion of pectolytic enzymes have an important role on pathogenicity. On the other hand patulin known as secondary toxic metabolite that produce by blue mold (Morales et al., 2010; Barad et al., 2016; Snini et al., 2016). *M. fructigena* is a common species of *Monilinia* found on apples and pears in Europe, and is commonly referred to as "apple brown rot" (Jones and Aldwinckle, 1990; Leeuwen et al., 2002). Of pome fruits, apple and pear are sensitive to brown rot disease caused by *M. fructigena*, and of stone fruits, plum is sensitive to brown rot disease (Byrde and Willets, 1977). *M. fructigena* has been reported to infect in more than 40 hosts. These include apples, pears, quince, apricots, peaches, plums and cherries (Sagasta, 1977). Post-harvest disease control is achieved by the use of synthetic fungicides such as sodium *ortho*-phenylphenate, imazalil or thiabendazole (Penrose et al., 1989; Yildiz et al., 2005). Use of the fungicides provides satisfactory control against mold infection. But fungicidal residues can have harmful effect on people and environment. The global trend appear to reduced use of fungicides on produce and demand for reducing disease loss in the harvested commodities (Sharma et al., 2009). So, development of alternative control possibilities has become very important because of the need to reduce the use of fungicides, their residues, concerns about human health and environmental pollution, and development of fungicide-resistant strains for pathogenic fungi (Capdeville et al., 2002; Calvo et al., 2017). Nowadays the researches on using biocontrol agents and alternative chemicals and hot-water treatments on postharvest diseases have been increasing (Falconi and Mendgen, 1994; Janisiewicz et al., 1994; Hong et al., 1998; Ippolito, 1998; Benli, 2003; Li and Yu, 2000; Karabulut et al., 2002; Capdeville et al., 2002, 2003; Irina et al., 2006; Wang et al., 2008; Fiori et al., 2008; Manso and Nunes, 2011; Amiri and Bompeix, 2011; Haıssam et al., 2011; Mari et al., 2012; Zhao et al., 2012; Li et al., 2015; Yaseen et al., 2015; Zhu et al., 2016; Kabelitz et al., 2018). Some antagonistic yeasts such as *Candida sake* Satio & Ota, *C. oleaphila* Montrocher, *C. saitona* Nakase & Sutuki, were used to controlling decay of apple and pear caused by *B. cinerea* Pers.: Fries and *P. expansum* Link. (Sharma et al., 2009). Bacteria as antagonist biological control agents such as *Bacillus amyloliquefaciens* BUZ-14 and *Aureobasidium pullulans* and *Pseudomonas fluorescens* have been used against blue mold in apples (Vinas et al., 1998; Ippolito et al., 2000; Mari et al., 2012; Calvo et al., 2017; Wang et al., 2018).

The aim of the study the control possibilities of blue mold disease caused by *P. expansum* and brown rot caused by *M. fructigena*, with *T. harzianum* (TRIC8), harpin protein (hp), *Lactobacillus acidophilus*'s fermentation product (Lafp), and with boscalid+pyraclostrobin was evaluated to extend the self life of the apples.

Materials and methods

Fruit

Granny Smith apple cultivars not treated with pesticides were harvested in Tekirdag Viticulture Research Institute orchards, Turkey. Healthy fruits were selected without

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physical injuries. The apples were surface disinfected with 70% ethanol and rinsed in sterile distilled water (SDW), dried at room temperature (24 °C).

Pathogens and antagonist isolates

In the study, *P. expansum* was isolated from decayed apples and other pathogen isolate *M. fructigena* (MON14) and biological control antagonist isolate *T. harizanum* (TRIC8, Accession number: MH351669) were provided by Prof. Dr. Nuray Ozer, Tekirdag Namik Kemal University, Agricultural Faculty, Department of Plant Protection.

Postharvest treatments of apples

P. expansum isolate and *T. harzianum* TRIC8 antagonist isolate were grown on potato dextrose agar (PDA) and incubated at 24 °C for 7 days. Petri dishes containing vegetable juice agar (V8 agar) was inoculated with *M. fructigena* isolate (MON14) and incubated at 24 °C for 10 days. The concentration of the *P. expansum* and *M. fructigena* were adjusted to 1×10^6 conidia/mL^{-1} with the aid of a thoma slide. Artificial wounds were performed using sterile needle to make 2 mm deep and 2 mm wide wounds (two wounds for each the apple) along the fruit equatorial areas. Spore suspension of *T. harzianum* (TRIC8) was counted with a thoma slide and adjusted to concentration 1×10^8 conidia/mL^{-1}. Firstly, each wound on apple was inoculated with 20 µL drop of 1×10^8 conidia/mL^{-1} of TRIC8 and left for incubation at room temperature (22 ± 2 °C) for one hour. After one hour, the apples treated with TRIC8 were inoculated with a micropipette into the wounded points with 20 µL drop of 1×10^6 conidia/mL^{-1} of the pathogens.

1% Harpin protein (Hp) (WDG formulation, Eden Bioscience Co.; Messenger Gold, 12 g/100 l water) (Wang et al., 2008). *Lactobacillus acidophilus'*s fermentation product (Lafp) (Alltech Crop Science; ISR 2000; 100 ml/da) (Boyraz et al., 2006). Hp and Lafp plant activators were dissolved in SDW and then sprayed with commercial dosage to fruits one hour before pathogens inoculations.

However, to compare the results of chemical reactions with *T. harzianum* and plant activators, the test fungicide 25.2% boscalid + 12.8% pyraclostrobin (Basf®, Bellis WG; 50 g/100 l water) was applied with the commercial dose by spraying one hour after the inoculation of the pathogens. To maintain humidity environment, two wet papers were placed in each plastic box was wrapped in an unsealed 0.05 mm polyethylene bag. The development of the diseases were determined by measuring the lesion diameters of decay on apples inoculated with 10^6 spores mL^{-1} pathogens inoculum and incubated for 10 days at 24 °C (22 ± 2 °C). Mean lesion diameter was calculated at the end of the study. Non-inoculated with the pathogens: 20 µl drop Sterile Distilled Water (SDW) was used only for each wound on apple in our study as negative control. Each treatment was independently performed two times with five replicate fruit; in each experiment twenty fruit per treatments were included.

We used to the effectiveness of the applications was calculated using Abbott's formula (Abbott, 1925; *Eq 1*):

$$\% \text{ effectiveness} = [(Ic - It) / Ic] \times 100 \qquad \text{(Eq 1.)}$$

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Where: Ic is the disease diameter of the untreated control (+), It is the disease diameter of the treatment.

Statistical analysis

At the end of the storage period, the severity of the diseases was determined by the mean lesion diameter in millimetre on the rotted apples. Statistical significance was judged at level $P \leq 0.05$. When the analysis was statistically significant, the Duncan multiple comparison test was used for means separation. SPSS (Statistical Package for Social Sciences, Inc., 2001, Model 11.0 Chicago) was used analysis program.

Results

The results of this study demonstrated that post-harvest applications of the TRIC8 significantly controlled blue mold caused by *P. expansum* on apple at the rate of 69.73%. This effect was found to be significant at $P \leq 0.05$ level. While the mean lesion diameter of *P. expansum* growth of untreated apples was 5.90 cm, the apples treated with TRIC8 had 1.79 cm, at 10^{th} day. The mean lesion diameters of the hp and Lafp treated apples were 4.14 cm and 4.16 cm, respectively, with both plant activators being equally effective (□29.00%) in reducing decay. The control of blue mold by boscalid+pyraclostrobin indicates that is not highly effective (37.22%) against the sporulation of *P. expansum* in the self-life condition (*Table 1*; *Fig. 1*).

Table 1. *Effects of treatments on lesion development of P. expansum on apple fruits*

Treatments	Lesion diameter (cm)	Effect (%)
Inoculated control	5.90 a	0.00 c
T. harzianum TRIC8	1.79 c	69.73 a
hp	4.14 b	29.76 b
Lafp	4.16 b	29.59 b
B+p (fungicide)	3.56 b	37.22 b

Means of treatment within the same column followed by different letters are significantly (P≤0.05) different according to Duncan's multiple range test.

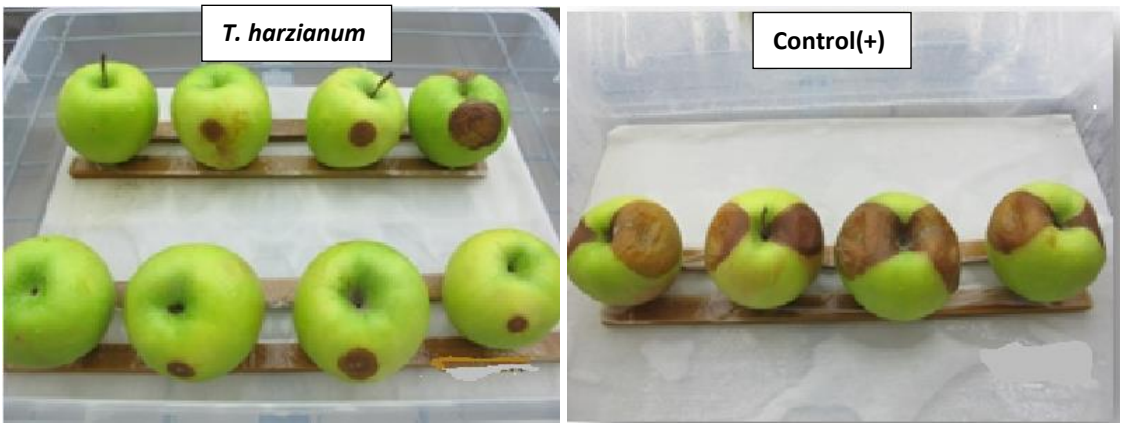

Figure 1. *Biocontrol activity of T. harzianum TRIC8 in inhibiting blue mold caused by P. expansum on apples*

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The results show that TRIC8 is also effective on the mean lesion diameter of *M. fructigena* on apple. While the mean lesion diameter of *M. fructigena* growth of untreated apples was 7.50 cm, the apples treated with TRIC8 had 0.21 cm, at 10^{th} day. The effect of TRIC8 treatment on brown rot was calculated as 97.13%. This value was significantly as statistically ($P \leq 0.05$). The mean lesion diameters of the hp and Lafp treated apples were 4.23 cm and 0.00 cm, respectively. Lafp treatment had a significantly greater effect (100%) in reducing decay than hp (41.65%) treatment (*Table 2*) (*Fig. 2*) ($P \leq 0.05$).

Table 2. *Effects of treatments on lesion development of M. fructigena on apple fruits*

Treatments	Lesion diameter (cm)	Effect (%)
Inoculated control	7.50 a	0.00 c
T. harzianum TRIC8	0.21 b	97.13 a
hp	4.23 b	41.65 b
Lafp	0.00 c	100.00 a
B+p (fungicide)	0.00 c	100.00 a

Means of *M. fructigena* lesions diameter within each column followed by different at ($P \leq 0.05$) according to Duncan's multiple range test.

Figure 2. *Plant activator of Lafp in inhibiting brown rot caused by M. fructigena on apples*

Significant differences ($P \leq 0.05$) were obtained in *M. fructigena* lesion diameters on wounded the apples when treated with the B+p compared to the treatment with only inoculated *M. fructigena* on the apple (*Table 2*).

Discussion

T. harzianum TRIC8 treatment had a significantly greater effect in reducing decay caused by *M. fructigena* than either decay caused by *P. expansum*. The effectiveness of TRIC8 against both blue mold and brown rot of apple were significantly statistically ($P \leq 0.05$). TRIC8 isolate of *T. harzianum* proved its antagonistic potential in controlled reducing blue mold and brown rot on Grany Smith apple cultivars. When the apple treated with TRIC8, demonstrated postharvest pathogens of fruit has the ability to be efficient at 1×10^8 conidia/mL^{-1} concentration. In apples, Batta (2004) observed low effect (48.8%) of formulated *T. harzianum* against the blue mold on apple is

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significantly attributed to the antagonistic effect of *T. harzianum* but not to because of the formulation ingredients.

Preliminary studies show that different active ingredients with fungicide; such as benomyl, captan, carbendazim, iprodione, mancozeb, myclobutanil, procymidone, thiram, thiophanate methyl, triforin, vinclozolin, and prochloraz etc., were effective to control of *P. expansum* during preharvest and postharvest. Since noticed of thiabendazole resistant blue mold in apple packing houses, study for alternative chemical control strategies have increased greatly (Eckert and Ogawa, 1988; Biyk et al., 1994; Li and Yu, 2000; Moreira and Mio, 2007; Feliziani et al., 2012).

Boscalid + pyraclostrobin active ingredients with fungicide against *M. fructigena* on apples that is only applied postharvest. Also, brown rot was reduced by 100% in apple fruit postharvest with this fungicide in this study. The same fungicide was able to mycelial growth of *P. expansum* on apple. But, this fungicide was effective when applied 7 or 14 days before harvest and reduced blue mold incidence by 41 to 70% (Xiao and Boal, 2009).

Harpin protein and *L. acidophilus* fermentation product elicitors of systemic acquired resistance in the host tissue that could indirectly help to protect apples from pathogens. The effect of application of harpin protein on the apples was less effective against blue mold and brown rot in this study. Capdeville et al. used to Hp in pre and postharvest treatments of apples induced resistance to *P. expansum* on apple. When they inoculated with the concentration 1×10^4 conidia/mL^{-1} *P. expansum* on apple in hp treatment a few days (4-8 days) before harvest was even more effective than the presented study (Capdeville et al., 2002, 2003).

Lafp had successfully controlled the brown rot caused by *M. fructigena* on apple in the present study. Similarly, the control of blue mold and grey mold on apple with Acibenzolar-S- methyl was reported before (Spadaro et al., 2004). β -aminobutyric-acid (BABA) on the activation of resistance responses in apple was investigated by Quaglia et al. (2017). Conversely, BABA resistance was not effective in the control of blue mold on apple such as Lafp.

Successful results similar to our study were obtained concerning the prevention of disease with *Trichoderma* spp. against *M. fructicola* on peaches and plums in the study of Hong et al. (1998) and with *Trichoderma polysporum* against *M. fructigena* on apples. The antagonist fungus *T. polysporum*, which was isolated from the apple leaves, was found to inhibit *B. cinerea* at 93%, *M. fructigena* at 80%, and *P. expansum* at 87% in postharvest apples (Falconi and Mendgen, 1994).

Aureobasidium pullulans, *Candida infirmominiatus*, *C. laurentii*, *Rhodotourula spp*, *M. pulcherrima*, and *Pichia angust*, from the yeasts isolated from the surface of the fruit, leaves and flowers, were found to be successful to prevent the disease, preharvest and postharvest in addition to *T. harzanium* that we applied in the biological control of post-harvest brown rot (Falconi and Mendgen, 1994; Chand and Spots, 1995; Hong et al., 1998; Irina et al., 2006; Fiori et al., 2008). As for our study, a similar effect was achieved (~70%) as the biological control agent *T. harzianum* successfully controlled *P. expansum* (Falconi and Mendgen, 1994). When the lesions were measured for the different postharvest fruits (pear, grape and kiwi) that were treated with the *T. harzianum* emulsion was found to support our research results (Batta, 2006).

In the biological control of *P. expansum* in the postharvest apples, different *Trichoderma* spp. (*T. atroviде*), apart from *T. harzianum*, also were found to have successful decay inhibition ranged of 35-50% while *Pseudomonas syringae* isolates and

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P. agglomorates were found to be 100% and 81% effective, respectively. This may be attributed to the different action mechanisms of antagonist bacteria (Nunes et al., 2002; Quaglia et al., 2010).

Yeast-based and bacteria antagonist isolates isolated from the fruit surface of apples, leaves, flowers and soils such as *Sporobolomyces roseus*, *Aureobasidium pullulans*, *Metschnikowia andauensis*, *Candida sake*, *Cryptococcus in firomo-miniatus*, *C. laurentii*, *Hanseniaspora uvarum*, *Rhodotourula spp.* and *Bacillus amyloliquefaciens* BUZ-14 have also been successfully applied to the postharvest biologic control of *P. expansum* (Janisiewicz et al., 1994; Leibinger et al., 1997; Ippolito et al., 2000; Nunes et al., 2002; Karabulut et al., 2002; Turkekul, 2003; Spadaro et al., 2004; Manso and Nunes, 2011; Calvo et al., 2017).

There was no study to treatment of the apples with Lafp against *P. expansum* postharvest. However, 14 days and 21 days before the harvest, Lafp plant activator was applied to oranges, followed by the inoculation of *P. digitatum* (1×10^6 conidia/mL^{-1}). The reported 50% success rate as a result of the application of plant activator to the fruit 21 days before pathogen application was more effective than our work. In this study as well, in stimulating durability against disease, it is inferred that the time period after the application of the plant activator should be long (Bower, 2007).

The effective results obtained in this research will contribute to the expansion of the usage areas of plant activators that do not harm human health and the environment. It is thought that promising results can be obtained for different pathogens with different combinations of applications of Lafp and *T. harzianum*, which have been successfully used individually.

Conclusion

Biological control with antagonist fungi have been a promising alternative, with lower environmental effect, either alone or as part of integrated pest management to reduce synthetic fungicide usage in postharvest diseases of fruit and vegetables for many years. The present results indicate that *T. harzianum* (TRIC8) has potential as a biocontrol agent for the control of postharvest decay of apple caused by *P. expansum* and *M. fructigena*. TRIC8 treatment was used to control of *M. fructigena* more effective than *P. expansum* on apple and could become an alternative to fungicide in postharvest decay control of brown rot on apple, but registration and development investigations to acquire a commercial product are needed.

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