

Full Length Research

Importance of pH modification for Disease Development in Apple fruits and Bio-control of Apple Decay

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Apple fruits are usually stored after harvest. During cold storage losses of economic importance are produced by several post-harvest fungal pathogens. Pathogens modify the pH of their host tissue to ease for infection and disease development. The present study was intended to show the importance of pH modification for decay development on fruits of two commercial apple cultivars (Fuji and Elstar) caused by four fungal pathogens (*Penicillium expansum*2020, *Penicillium expansum*21525, *Botrytis cinerea* and *Colletotrichum acutatum*). Besides, it was intended to highlight the use of bio-control products as one of the management options against blue mold. The study pointed out that *P. expansum*2020, *P. expansum*21525 and *B. cinerea* acidified the host tissues by lowering the pH, where as *C. acutatum* experienced alkalisation by raising the pH during decaying on both apple cultivars. Among the pathogens, *B. cinerea* was more virulent on both cultivars followed by *P. expansum*2020, *P. expansum*21525 and *C. acutatum* respectively. The enzyme activity of the pathogens in the infected apple tissues was examined using AZCL-Azurine-Crosslinked Polysaccharide plates using different substrates and variation was obtained. *P. expansum*2020 inoculated samples showed better enzyme reaction in all of the substrates except in Beta-Glucan. The strongest enzyme reaction was obtained in substrate Xyloglucan followed by Xylan on both apple cultivars while *C. acutatum* showed better enzyme reaction in the substrate Casein. In the experiment involving bio-control on the blue mold fungus, Bio-Save gave the highest effect by significantly decreasing the size of the fungal lesions on both apple cultivars.

Key words: Apple decay, Bio-Save, Cell-wall degrading enzymes, Post-harvest, Thyme oil, pH

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INTRODUCTION

Some fruits, apples among them, are usually stored after harvest. During cold storage losses of economic

importance are produced by several decays due to fungal rots. *Penicillium expansum*, *Botrytis cinerea* and *Colletotrichum acutatum* are among well-known post-harvest fungal pathogens (Calvo et al., 2007) in apples and several other fruits in storages. These pathogens use different strategies during infection and disease development on their hosts although the fungal virulence is influenced by the combined effect of the host and the pathogen on ambient pH. Host pH can be modified in response to signals including acidification by secretion of organic acids as observed in *Penicillium* and *Botrytis* or alkalization by ammonification of the host tissue as observed in *Colletotrichum* (Prusky and Lichter, 2007; Prusky et al., 2014). The pathogens can dynamically alter the local pH at the infection site on the host to suit the increased expression of pathogenicity factors and the enzymatic arsenal (Denison, 2000; Yakoby et al., 2000; Prusky et al., 2001; Eshel et al., 2002; Prusky and Yakoby, 2003).

The blue mold fungus (*P. expansum*) is one of one of the most prevalent post-harvest rots that infect apples. Although it is a major economic problem in apples, this pathogen can cause rots on a wide host range, including pears, strawberries, tomatoes, corn, and rice (Ashizawa and Eunice, 2000; Morales et al., 2006; Prusky et al., 2014). *C. acutatum* is also a widespread pathogen which causes bitter rot on apple fruits and has numerous hosts and is adapted to different climatic conditions around the world (Cabi.org, 2014; Børve1 and Stensvand, 2015). Like the latter two pathogens, *B. cinerea* also known as 'Grey mold fungus', is well known for its broad host range and can cause serious pre- and post-harvest diseases in at least 200 plant species (Jarvis, 1977). It mainly causes damages on agronomically important crops and harvested commodities, such as grapevine, tomato, strawberry, cucumber, bulb flowers, cut flowers and ornamental plants. The fungus often invades senescent or damaged plant tissues and it causes many problems in post-harvest storage of plant products at a temperature down to 2°C (Ten Have et al., 1998).

Hence, these rots causing post-harvest pathogens are economically important in apple storages and hence it is necessary to investigate the nature of pH modification in the host tissue of different apple cultivars during infection and disease development. Besides, it is important to test some control measures against the rots so as to minimize the losses. Different control measures are registered to take care of the pathogens with different efficacy. The use of synthetic chemicals such as fungicides has been a primary method of control of post-harvest fungal decay of apple fruit. However, several fungicides are not used anymore for post-harvest treatment or have been removed from the market due to possible toxicological risks (Calvo et al., 2007). Besides, due to the negative public perceptions about the use of pesticides,

development of resistance to fungicides among fungal pathogens, and high development costs of new chemicals, biological control of post-harvest diseases of fruits has become an important field for research. A number of bacteria, yeasts and fungi have been reported to inhibit post-harvest decay of fruits effectively (Janisiewicz and Korsten, 2002).

This study was conducted to understand decay development on apple fruits due to four potential post-harvest fungal pathogens and investigate pH modification in the infected host tissues as strategies for disease development. Besides, the enzyme activity of the pathogens in the infected host tissues was also examined on different substrates to understand the ability of the pathogens to produce cell wall degrading enzymes during decaying. We also tested two biological control products as alternative methods for controlling blue mold fungus on apple fruits.

MATERIALS AND METHODS

Three sets of experiments were conducted in the study:

1. **Evaluation of disease development and measurement of pH in tissues of apple fruits infected with different fungal pathogens**

In this experiment, fruits of two European commercial hybrid apple cultivars (Elstar and Fuji) were used as hosts. The fruits were inoculated with four fungal pathogen isolates (*P. expansum*₂₀₂₀, *P. expansum*₂₁₅₂₅, *B. cinerea*, *C. acutatum*) and water as control. Ten matured apple fruits from each cultivar were washed with tap water and wounded with sterile tooth-picks at 2mm diameter and 3mm depth and 10µl of the spore suspension (1×10^6 spores/ml) of each pathogen or water was added to the wound. Two apples from each cultivar were inoculated with each of the pathogens and placed in storage boxes wrapped in plastic bags and stored for 7 days at room temperature. Each apple and pathogen inoculation treatments were replicated 5 times and a total of 50 apples from each cultivar were used. The infected apples were assessed 7 days after storage. The size of the fungal lesion developed on the apples was evaluated and pH of the infected tissues was measured with a pH meter and a glass electrode (Orion, Thermo Scientific model 8163BNWP). The size of the fungal lesions and pH changes in the infected tissues were compared among the pathogens and the two apple cultivars and presented in the result.

2. **Assessment of enzyme production in infected apple tissues on different substrates**

Following the first experiment of host inoculation with

fungal pathogens, an enzyme assay was set up to study the enzyme activity of the fungal pathogens in the infected tissues of the apples. The enzyme activity was measured using AZCL-plates (Azurine-Crosslinked Polysaccharides) in which 6 substrates including Arabinoxylan, β -glucan, Xylan, Xyloglucan, Casein and HE-cellulose were placed in. AZCL-plates are used as substrates to locate enzyme activity in the gel and enzyme producing organisms in the material (<http://secure.megazyme.com/>).

About 1 cm³ pieces were cut from the infected part of the fruit tissues and chopped into small pieces in the 24-well plates. About 500 μ l of distilled water was added in each well and incubated at 30°C by shaking for one hour. Then 15 μ l of the suspension from each well was transferred into wells of the AZCL-plates containing the substrates and incubated at 30°C overnight and then stored at 4°C before assessment for the enzyme activity. The enzyme activity of the pathogens was evaluated by measuring the diameter of the blue pigmentation zones released into the medium (mm) on the centre of the wells on the AZCL-plates. The extent of the enzymes produced by the pathogens and its activity on different substrates were evaluated by measuring the size and intensity of the blue pigmentation zones on the plates and presented in the result.

3. **Evaluation of Bio-Save and Thyme oil as bio-control products against blue mold caused by *P. expansum***

In this experiment, two biological control products: Bio-Save (*Psydomonas syringae*) and Thyme oil were evaluated against blue mold fungus on the two apple cultivars infected with *P. expansum2020* isolate. Three apples from cultivar Elstar and Fuji were washed with tap water and wounded with a cork borer (7mm diameter x 3mm depth). Ten μ l of water, Bio-Save (suspension with 5x10⁷ CFU/g of *P. syringae*) or Thyme oil (5 % w/v solution) were applied around the edges of the wounds followed by inoculation with 10 μ l of *P. expansum2020* isolate with spore suspension of 1x10⁴ spores/ml. The inoculated apples were marked, packed and incubated at room temperature for 7 days. After incubation, the apples were assessed for the size of the lesions developed on the infected area of the fruits. The efficacy of the two bio-control products over the control of the decay was compared and presented in the results.

Data were analysed using CoHort statistical software (Version 6.204, 2003, USA) (<http://www.cohort.com>) and statistical significance was tested at p <0.05.

RESULTS

Relationship between disease development on apple fruits and host pH modification

The size of the lesions on fruits of the two apple cultivars caused by infection of the four fungal pathogens was significant (p <0.05) after 7 days of storage except that no significant variation was found between the two isolates of *P. expansum* (*P. expansum2020* and *P. expansum21525*) (Figure 1). The pathogens showed variation in their virulence on both apple cultivars in that *B. cinerea* tended to be more virulent as compared to the other three pathogens followed by *P. expansum2020*, *P. expansum21525* and *C. acutatum* (Figure 1). In reaction to pathogen infection, pH modification occurred in infected apple tissues during decay development and significant difference (p <0.05) was obtained among the pathogens and the cultivars (Figure 2). Alkalinization was experienced by *C. acutatum* by increasing the pH values of the fruit tissues during decaying on cultivar Fuji and Elstar by 1.92 and 3.06 units respectively in contrary to the other pathogens (*P. expansum2020*, *P. expansum21525* and *B. cinerea*) which experienced acidification by decreasing the pH values from 0.14 to 0.39 units (Figure 2). The results depicted that the pH of a healthy fruit tissue of cultivars Fuji (pH=4.03) and Elstar (pH=3.60) increased to 5.95 and 6.66 respectively after infected with *C. acutatum*. However, these pH values decreased to 3.70, 3.69, 3.64 and 3.46, 3.40, 3.32 after fruits of the two cultivars were infected with the other three pathogens *P. expansum2020*, *P. expansum21525* and *B. cinerea* respectively (Figure 2). Besides, the relationship between host pH at inoculation and decay development on the two apple cultivars after 7 days was examined and found that fruits of cultivar Fuji with initial pH of 4.03 got more decay development when infected with *P. expansum2020* and *P. exapnsum21525* than cultivar Elstar with relatively lower initial pH of 3.60 (Table 1).

Assessment of enzyme production in infected apple tissues

In the enzyme activating substrates, we found that *P. expansum2020* reacted with all of the substrates except with Beta-glucan. The Xyloglucan substrate gave the highest blue coloured diffusion zone indicating that there was strong enzyme activity by *P. expansum2020* in fruit tissues of both apple cultivars. The pathogen *P. expansum21525* also did well on the substrates Xyloglucan and HE-cellulose but only on cultivar Fuji. *C. acutatum* enzymatic reaction was high in Casein substrate on both cultivars. *B. cinerea* did not show any reaction at all in any of the substrates tested while any of the pathogens did not show enzyme reaction in Beta-

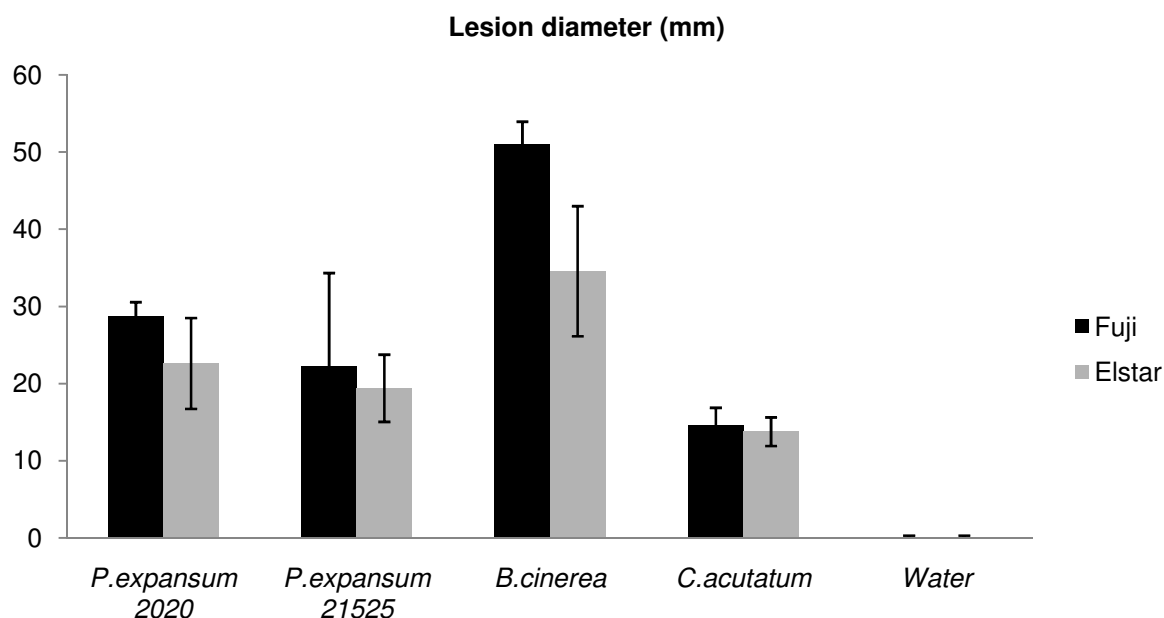


Figure 1. Lesion development on fruits of cultivars Fuji and Elstar infected with *P. expansum*2020, *P. expansum*21525, *B. cinerea* and *C. acutatum* after 7 days of inoculation at room temperature.

Table 1. Relationship between host pH at inoculation and development of fungal lesion on the two apple cultivars infected with *P. expansum*2020 and *P. expansum*21525.

Cultivar	pH at inoculation	pH after infection	Change unit	Lesion diameter (mm)	
				<i>P. expansum</i> 2020	
Fuji	4.03	3.70	0.33	28.732	
Elstar	3.60	3.46	0.14	22.597	
				<i>P. expansum</i> 21525	
Fuji	4.03	3.69	0.34	22.252	
Elstar	3.60	3.40	0.20	19.393	

glucan substrate (Figure 3).

Use of Bio-Save and Thyme oil as bio-control products against apple decay caused by *P. expansum*2020

The results showed that Bio-Save had better control than Thyme oil extract over the blue mold decay caused by *P. expansum*2020 on both apple cultivars ($p < 0.05$) (Figure 4). The bio-control product Thyme oil was not significantly different from the control (untreated treatment) in

suppressing the decay as shown for both lesion diameter (Figure 4a) as well as lesion depth (Figure 4b).

DISCUSSION

The study showed the variation in pathogenicity and mode of host pH modification among the four post-harvest fungal pathogens: *P. expansum*2020, *P. expansum*21525, *B. cinerea* and *C. acutatum* on two hybrid apple cultivars: Fuji and Elstar. Besides, the study

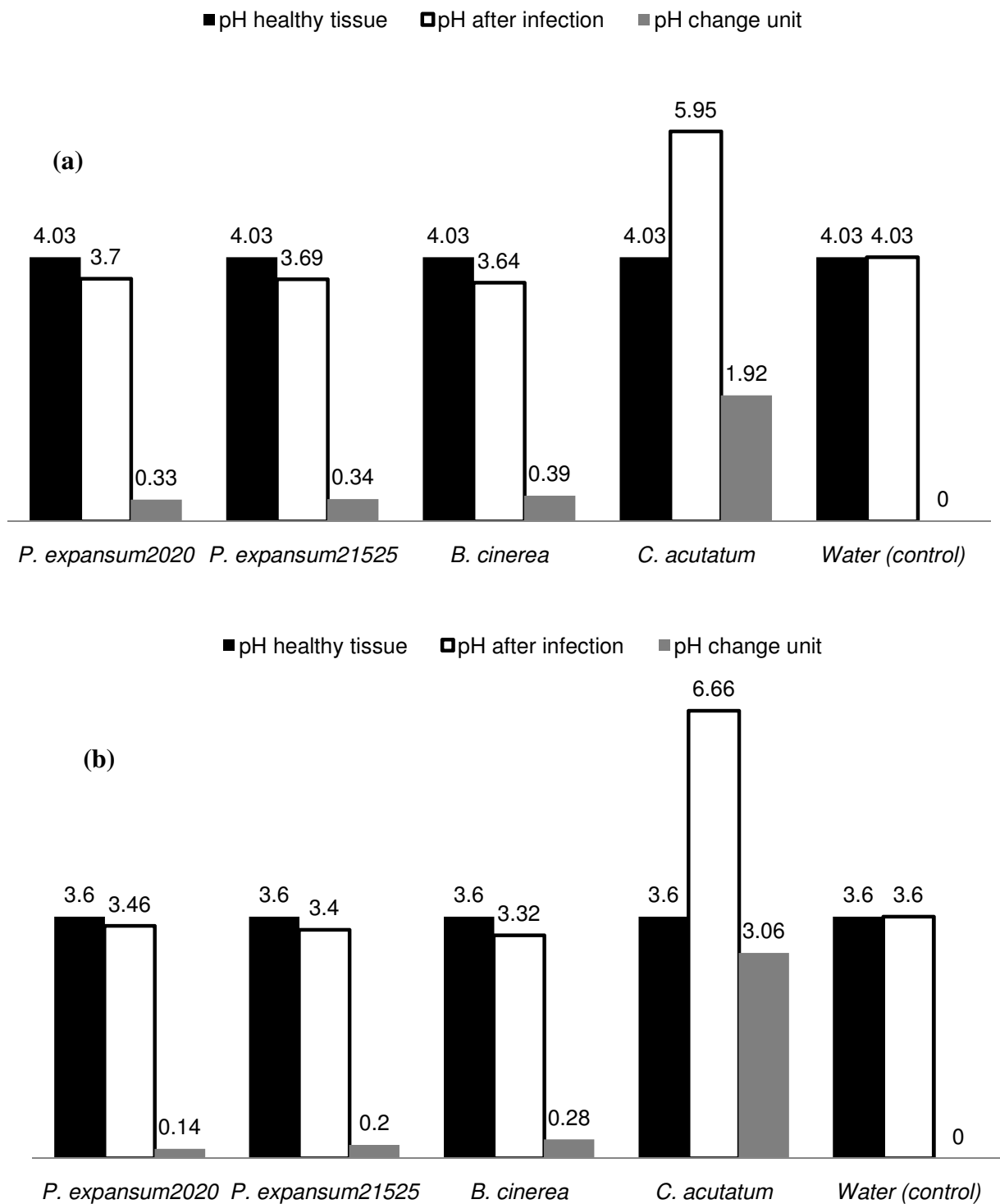


Figure 2. pH values of fruit tissues of the two apple cultivars (Fuji-a) and (Elstar-b) before and after infection with the four fungal pathogens.

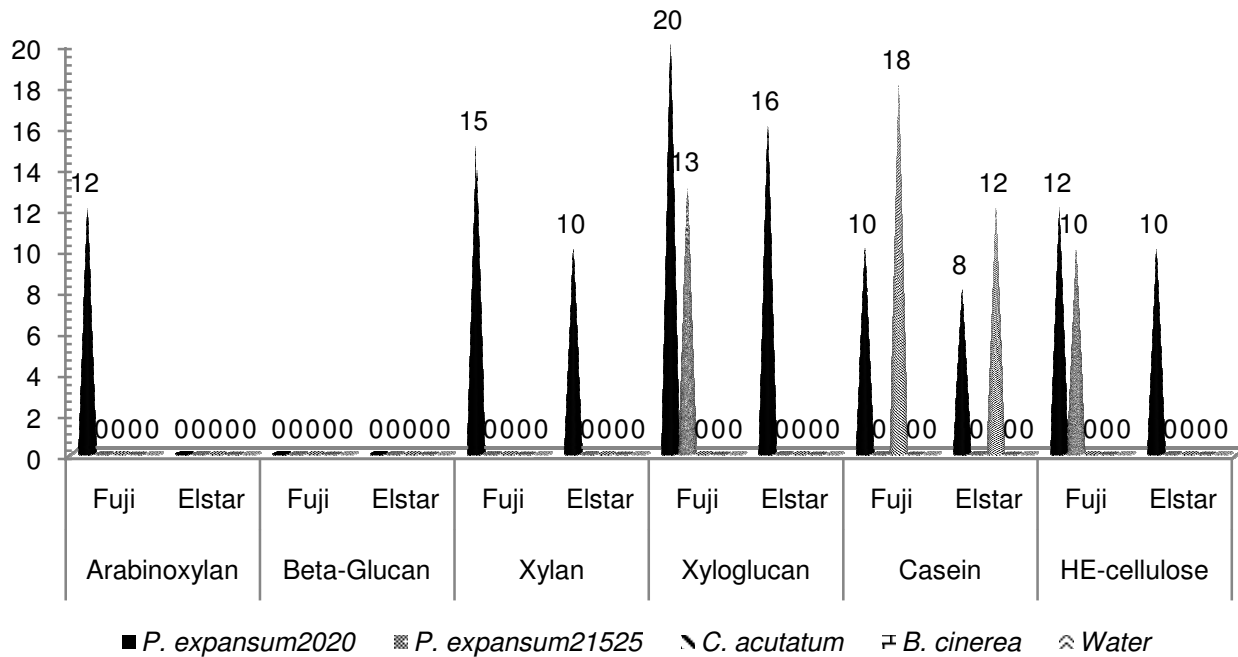


Figure 3. Size of coloured diffusion zone (mm) measured on AZCL-plates depicting the enzymatic activity of the four fungal pathogens in infected tissues of the two apple cultivars on different substrates.

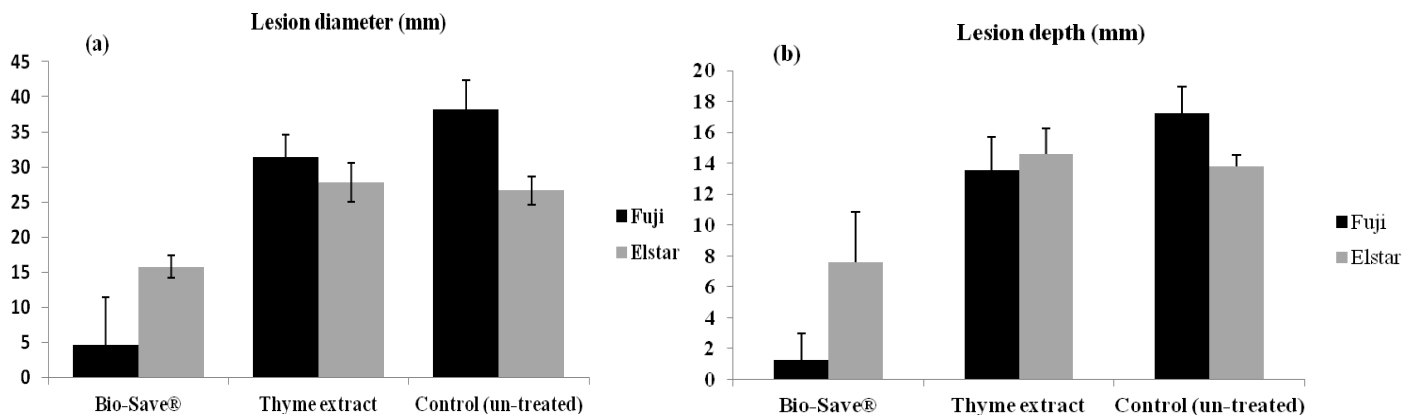


Figure 4. Efficacy of Bio-Save and Thyme oil on apple decay (lesion diameter (a) and lesion depth (b)) on two apple cultivars (Fuji and Elstar) infected with *P. expansum2020*.

confirmed that the two *P. expansum* isolates (*P. expansum2020* and *P. expansum21525*) and *B. cinerea* used mechanism of host acidification during tissue decaying by lowering the pH of the fruit tissues of both cultivars. *P. expansum* colonization increases when the pH of an apple fruit becomes more acidic (Prusky et al., 2004). Although the real cause for the lowering of the pH

in the host tissues was not examined in the present study, other investigations showed accumulation of organic acids such as gluconic acid, fumaric acid, oxalic acid, citric acid and ammonium in the tissues of *P. expansum* and *P. digitatum* decayed apple fruits decreased the pH during decaying (Rollins and Dickman, 2001; Manteau et al., 2003; Prusky et al., 2004). In the

same study, other mechanism of acidification was tested in *P. expansum* and other *Penicillium spp.* decayed tissues of three apple cultivars and found that ammonia concentration was found depleted in the decayed tissues, which lowered the pH. Therefore, it is likely to say that accumulation of those organic acids and depletion of ammonia in the decayed fruit tissues could be the causes of the acidification in our study. According to Prusky et al. (2004), not only *P. expansum* infection acidifies the host via the secretion of organic acids, but also that the acidification of potential hosts increased *P. expansum* development, indicating a link between environmental acidity and *P. expansum* virulence. In addition, the capability of those pathogens to acidify the environment has resulted in the expression of genes and the secretion of many hydrolytic enzymes which are encoding genes, such as polygalacturonase (PG) (Prusky et al., 2004). Conversely, *C. acutatum* used mechanism of host alkalinisation by significantly increasing pH of the fruit tissues during decaying in both apple cultivars. Prusky et al. (2001) studied the mechanism of *C. acutatum* and other *Colletotrichum spp.* in apple, avocado and tomato fruits and found that the rise in pH in the decayed fruit tissues was due to accumulation of ammonia produced by the pathogen using the available nitrogen in the fruit tissues during decaying. In contrary to *P. expansum*, when ammonia is secreted locally in the host tissue by the *Colletotrichum* species, it results in a pH increment that enables enzymatic secretion and enhanced virulence. A research showed ammonia secretion by *Colletotrichum spp.* increased the medium pH by producing one pectate lyase (PL), which is a key virulence factor in disease development (Prusky et al., 2001).

The relationship between host pH at inoculation (on healthy fruits) and severity of decay development due to the two *P. expansum* strains showed variations between the two cultivars and which was in contrary to what was observed by Prusky et al. (2004). Prusky et al. (2004) found that the size of the decay lesions caused by *P. expansum* infection in apple cultivars of Fuji, Rome and Granny Smith (the latter two cultivars were not used in our study) increased as the pH of the untreated tissue decreased from 4.5 to 3.8. In case of our investigation, bigger sized lesion was obtained on cultivar Fuji at a higher pH (4.03) than on cultivar Elstar at a lower pH (3.6) (Table 1) although the difference was not statistically significant between the two pathogen strains (Figure 1). This could be due to some other reasons in that cultivar Elstar could be less susceptible to the *P. expansum* strains used at lower pH as compared to Rome and Granny Smith and could retard the growth of the pathogens. Probably the pathogen strain used in Prusky et al. (2004) might also be different from the ones used in our study.

In the enzyme activity reaction, the pathogens did not

perform equally in all of the substrates in which *P. expansum*2020 showed the highest enzymatic reaction in all of the substrates except in Beta-glucan followed by *P. expansum*21525 and *C. acutatum* which performed in Xyloglucan, and HE-cellulose and Casein respectively (Figure 3). However, *B. cinerea* did not show any enzyme reaction in any of the substrates although a larger lesion size was obtained on the infected fruits compared to that of the rest of the pathogens (Figure 1). This implies that *B. cinerea* may need different substrates other than the ones tested in the present study to show the enzymatic activity in the decayed fruit tissues. Moreover, studies confirmed that, in apple tissues, the *B. cinerea* may cause a latent infection and wait for the wounding to appear before attacking (Ten Have et al., 1998). It is therefore called a weak pathogen and this could support our result in that, *B. cinerea* may need more time to degrade the substrates and show the enzyme activity. So, we suggest testing the same substrates with more incubation period or different substrates if any to increase the chance to see the enzyme activity of this particular pathogen.

In controlling the blue mold due to *P. expansum*2020, we obtained a significant difference between the Bio-Save agent and Thyme oil extract. Both apple cultivars (Fuji and Elstar) had a highly reduced infection from the pathogen when treated with Bio-Save as compared to that of Thyme oil extract. *P. syringae* grows on wounded apple tissue and it prevents pathogens such as *P. expansum* or *B. cinerea* from entering the fruit through openings caused by wounding (<http://www.nysaes.cornell.edu/>). Bio-Save is a broad spectrum biological control which has a wide range of actions against fungal pathogens on apple surfaces. *P. syringae* (strain L-59-66 renamed as strain ESC- 11) can control blue mold caused by *P. expansum*, gray mold caused by *B. cinerea*, and *Mucor* rot caused by *Mucor* spp. on apple and pear. It can also control blue mold caused by *P. italicum*, and green mold caused by *P. digitatum* on citrus fruit (<http://www.nysaes.cornell.edu/>). However, we did not see a significant result in using Thyme oil extract as a control agent against blue mold caused by *P. expansum* on both apple cultivars. The thyme oil is originally considered to have antibacterial and antimicrobial qualities and hence, it has been tested on pathogens affecting food. Since we did not see a good result on *P. expansum*, there might be a chance where Thyme oil would react better on other strains of pathogens. A research using Thyme oil extract stated that there was a total inhibition of the mycelia growth on the food borne bacteria: *Aspergillus niger* and *A. flavus* (Paster et al., 1990). Hence, although our results on Thyme oil were not significant, the future for use of essential oil like Thyme oil in bio-control might look promising. Since bio-control agents are living organisms, to be more efficient, the post-harvest environment should

undergo strict control, by constant checking of temperature, relative humidity and other components required by the organisms. Besides, other factors like fruit maturity and cultivar have to be taken into account while using the bio-control methods to become highly consistent and commercially acceptable (<http://www.nysaes.cornell.edu/>).

From the results, we reiterated that *P. expansum*2020, *P. expansum*21525, *B. cinerea*, and *C. acutatum* can cause decay in apple fruits. Besides, the study proved that host pH modification through acidification or alkalisation has significant contribution for the pathogens to ease for the onset of infection as well as decay development. We were also able to realize variations between apple cultivars in their reaction to pathogen infections in spite of differences in pH levels during infection. Besides, the study confirmed that there are differences among the pathogens for substrate preferences to show their enzymatic activity. In the biological control experiment, the Thyme oil extract failed to suppress decay development on apples infected with *P. expansum*2020, however Bio-Save significantly retarded the growth of the decay. However, further investigation is needed to see the efficacy of Bio-Save on the rest of the pathogens (*B. cinerea*, and *C. acutatum*) using more number of apple cultivars as well as enzyme reacting substrates for comprehensive conclusion and recommendation.

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