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Hanseniaspora uvarum enhanced with trehalose induced defense-related enzyme activities and relative genes expression levels against *Aspergillus tubingensis* in table grapes

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ABSTRACT

In this study, the activitiesof defense-related enzymes andthose involved in reactive oxygen species (ROS) and their corresponding genes coding for *PPO*, *APX*, *CHI*, *PAL* and *CAT* in response to *Hanseniaspora uvarum* and *Hanseniaspora uvarum* harvested from NYDB supplemented with 2% w/v trehalose in nutrient yeast dextrose broth (NYDB) were investigated. Conventional methods and qRT-PCR were used to perform defense-related enzyme activities and relative genes expression respectively. The results indicated that *H. uvarum* enhanced with 2% w/v trehalose demonstrated biocontrol efficacy against *Aspergillus tubingensis*in grapes.Furthermore, it was noticed that *H. uvarum* supplemented with 2% w/v trehalose in NYDB had a significantly effect on the induction of defense-related genes expression andenzyme activities compared to the control, 2% w/v trehalose and *H. uvarum*after storage at 20 °C for 72 h. *CAT* relative gene expression level in grapes treated with *H. uvarum* supplemented with 2% w/v trehalose was the highest with 23-fold increase compared to the control. These results suggested that *H. uvarum* pretreated with trehalose up-regulatedthe defense-relatedgenes expression and enzyme activitiesin the grapes. These findings indicated that, the application of *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose enhanced the defense-related mechanism of grapes against *A. tubingensis*.

1. Introduction

Increasing consumer awarenesson the benefits associated with biological control over synthetic fungicides and other methods of disease control makes biocontrol a promising method (Sanzani et al., 2016). Moreover, restrictions on the permitted active ingredients and consumers' demand on the reduction of chemical residues in fruits and vegetables makes biocontrol the preferred method for disease control (Castoria et al., 2011; Yu and Lee, 2015). Credence is given to biological control due to its track record on human safety and ecological friendliness. Biological control of postharvest diseases of fruits and vegetables is therefore gaining worldwide attention and a lot of progress has been made over the last 30 years (Droby et al., 2016). Pursuance to this, different biological control agents (BCAs) (Cao et al., 2013; Zhang et al., 2009), bioactive compounds (Sharma et al., 2009; Zhang et al., 2015), food additives (Lai et al., 2015; Sharma et al., 2009) as well as the mechanisms involved in biological control have been explored to explicate and develop biocontrol products. Therefore, understanding the mechanism of actions of BCAs is imperative in order to improve their viability and efficacy against disease control (Di Francesco et al., 2016).

Trehalose is a non-reducing disaccharide, though it is not an antioxidant it has successfully been revealed to act against oxidative stress, dehydration, nutrient starvation, heat shock, and harmful chemicals (Argüelles, 2000; Estruch, 2000). Gancedo and Flores (2004) reported that, trehalose can protect enzymes, antibodies, liposomes and microorganisms during drying or storage. However, despite the significance of this bioactive compound in augmenting microbial activities, very little has been done in this direction.

The genus *Hanseniaspora* is an ascomycete characterized to bipolar budding, which confers its typical apiculate form under microscopic observation (Cadez et al., 2002). Typically, this genus is among the dominant non-saccharomyces yeasts. *H. uvarum* is one of the species of this genus commonly found on grape berries. They can also be foundon the surface of apples as well as onindustrial machineriesuse in harvesting and processing these fruits (López et al., 2015). *H. uvarum* displayed industrially relevant antagonistic properties against the development of molds responsible for fruits spoilage (Albertin et al., 2015). Previously, *H. uvarum* combined with phosphatidylcholine has

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been used to control green mold in orange fruit (Li et al., 2016). Also, Liu et al. (2010) reported that *H. uvarum* in combination with ammonium molybdate improved the biocontrol efficacy against gray mold.

Grape berry is one of the most important economic fruits which supports the economies of many countries. The fruit is commonly used for the production of wine and in lesser amounts the production of juice. Some grapes are also dried and consumed afterwards as raisins. However, grape berry is susceptible to many diseases which results in economic losses. Besides, some of the pathogens that cause these diseases produce secondary metabolizes (mycotoxins) which are harmful to human health.

During the infection process and pathogenesis, enzymes and defense-related genes play important roles. For instance, the induced production of some enzymes such as cellulase, pectinase, polyphenoloxidase, chitinase, among others play important roles in plant defense. Most often, theses enzymes occur in many isoforms and mainly involved in thesynthesis of defense metabolize which have direct antimicrobial activities(Lebeda et al., 2001). For instance, PR-proteins with the features of enzyme activity such as chitinase and β-l,3-glucanase play vital role by cleaving fungal cell wall (Tuzun et al., 1989).Chitinase and β-l,3-glucanase are antimicrobial being able to degrade glucan and chitin of the cell wall of fungi.

In this study, we investigated the efficacy of *H. uvarum*Y3 harvested from NYDB supplemented with trehalose against *A. tubingensis*. *A. tubingensis* is one of the species that belongs to black aspergilli (*Aspergillus* section *Nigri*) commonly found on grapes (Merlera et al., 2015). Medina et al. (2005) reported that *Aspergillus* section *Nigri* are prevalent in Spanish grapesand are found to produce ochratoxin A. Most varieties of grapes are susceptible to black mold and if not controlled, the grapes within a cluster can easily get rotten especially at postharvest stage. To the best of our knowledge, there is paucity of information on defense-related genes expression in table grapes enhanced with BCAs and the mechanisms involved in disease control. This study therefore tested the biocontrol efficacy of *H. uvarum* supplemented with trehalose in the control of *A. tubingensis* in table grapes. Moreover, the relative genes expression and enzyme activities of grape berries treated with *H. uvarum* Y3 supplemented with 2% w/v trehalose in NYDB and the putative mechanisms involved in the control of *A. tubingensis* were investigated. It is imperative to unravel the molecular mechanisms of enzymes and the genes involved in defense response of table grapes induced with *H. uvarum* harvested from NYDB supplemented with or without trehalose 2% w/v against *A. tubingensis*.

2. Materials and methods

2.1. Fruit material

Grape (*Vitis vinifera*) berries of the “Hongti” cultivars were harvested from a commercial organic vineyard in Zhenjiang, Jiangsu Province, one of the grapes producing areas in China. For reliability and accuracy of results, all berries were randomly selected based on uniformity of size, commercial maturity and absence of visible blemishes. Berries were surface disinfected using 0.1% sodium hypochlorite for 1 min and rinsed with tap water. Afterwards, the berries were air-dried at room temperature for further experiments.

2.2. Trehalose

Trehalose a natural alpha-linked disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units was of analytical grade and purchased from Sangon Co., Shanghai, China.

2.3. Microbial antagonist

The yeast antagonist *H. uvarum* Y3 (CGMCC 2.5414) was isolated from the surface of grape berries. The isolation was carried out according to the method described by Kurtzman et al. (2011) with some modifications. Five berries were randomly selected from bunches. The epiphytic yeasts were isolated by washing the 5 grapes with 50 mL of sterile distilled water (SDW) (containing 0.02% (v/v), Tween-20) into a 250 mL Erlenmeyer flask. The flask was shaken for 3 min and 10-fold serial dilutions were made using SDW. After incubation on nutrient yeast dextrose agar (NYDA) for 48 h at 28 °C, the developed yeast colonies were subcultured on NYDA to obtain a pure culture. Subsequently, the yeast was identified through sequence analysis of 5.8S internal transcribed spacer rDNA region, β-tublin gene and RNA polymerase II second largest subunit (Li et al., 2010). Acute toxicity studies, Ames and physiological tests were conducted on male and female rats to authenticate the safety of the yeast to human (data not published).The yeast isolate wasthen cultured in 250 mL Erlenmeyer flasks containing 50 mL of nutrient yeast broth (8 g nutrient broth, 5 g yeast extract, 10 g glucose and 1000 mL of SDW). The *H. uvarum* was then incubated in a rotary shaker (180 × *g*) for 20 h at 28 °C. Afterwards, the samples were centrifuged(TGL–20 M centrifuge Xiangyi Co., Changsha, China) at 7000 × *g* for 10 min at 4 °C. Yeast cells were washedtwice with SDW in order to get rid of the growth media, re-suspended in SDW and the microbial concentration determined (1×10^8 cells/mL) with a hemocytometer (Hongtia, China). Thereafter, 1 mL of the *H. uvarum* cell suspensions was eachaddedintoNYDB and NYDB supplemented with 2% w/v trehalose in 250 mL Erlenmeyer flasks. The samples were incubated in a rotary shaker (200 × *g*) for 20 h at 28 °C, after which cell pellets were centrifuged at 7000 × *g* for 10 min and washed twice with SDW. The cell pellets were re-suspended in SDW and adjustments made to a concentration of 1×10^8 cells/mL.Subsequently, the treatments used were as follows: (1) SDW as a control, (2) 2% w/v trehalose, (3) *H. uvarum*and (4) *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose. All the treatments were chosen based on our previous experiments(data unpublished).

2.4. Fungi and culture conditions

The fungus *Aspergillus tubingensis* M1was isolated from infected grape berries in our laboratory and identified according to the method of (Barnett and Hunter, 1972). The conserved genes of *A. tubingensis* were amplified using PCR (ITS, β-tublin and calmodulin genes) (Visagie et al., 2014). The strain has been registered in a GenBank and the accession numbers are MF327718, MF327716 and MF327717 for ITS, benA and CMD respectively. Subsequently, the fungus was maintained on potato dextrose agar (PDA) wort made of 200 g of boiled potato, 20 g of glucose, and 20 g of agar in 1000 mL of distilled water. Then, the fungus was cultured (activated) in fresh petri dish containing PDA at 25 °C prior to the experiment. After 7 days of culturing, the spores in the suspensions were removed from the sporulating edges of the petri dishes with a bacteriological loop and suspended in SDW. A hemocytometer was then used to compute the concentration of the fungus and adjusted with water to 1×10^5 spores/mL.

2.5. Effect of H. uvarum Y3 supplemented with trehalose on mycelial growth of A. tubingensis

Initially, the grapes samples were prepared as mentioned above and wounds (3 mm deep x 3 mm diameter) created on each lot of grapeswith a sterile cork borer. Then, (1)30 μLof SDW as a control, (2) 30 μLof 2% v/w trehalose (3) 30 μL of *H. uvarum* (1×10^8 cells/mL) and (4) 30 μL of *H. uvarum*Y3 (1×10^8 cells/mL) harvested from NYDB supplemented with 2.0% w/v trehalose wereinoculated into the grapes. Subsequently, 30 μL of *A. tubingensis* (1×10^5 spores/mL) was inoculated into each group of grapes. Two hours after the inoculation, the grapes in the baskets were wrapped with polyethylene film to prevent contamination and to maintain relatively high humidity (95%). The grapes were later stored for 5 days at 28 °C and the lesion diameter of

each grape was determined using a slide rule to measure the decay extension of the wounds of each berry minus the initial diameter (3 mm). The formula: (number of infected berries/total number of berries × 100) was used to calculate the decay incidence and the results expressed in (%). Each group of treated samples composed of 12 grapes and each treatment was replicated.

2.6. Crude extract and enzyme assay

2.6.1. Crude extract

Uniform wounds(3 mm deep × 3 mm diameter) were made on the equator region of all grape berries using a sterile cork borer. This was to assess the effect(s) ofthe treatmentson the enzymatic activities of the grapes. Afterward, SDW, 2% w/v trehalose, 30 µL of the *H. uvarum*suspension (10^8 cell/mL) obtained from NYDB and 30 µL of *H. uvarum* (10^8 cell/mL) harvested from NYDB supplemented with 2% w/v trehalose were pipetted into each wound. Then, 30 µL *A. tubingensis* (1×10^5 spore/mL) was inoculated into the wounds. The grapes were air-dried in plastic baskets, wrapped with plastic filmand incubated at 20 °C for 5 d at95%relative humidity.Samples were taken at 0, 24, 48 and 72 h for further analysis. Subsequently, wound tissues of each lot of 5 berrieswere excised using a sterile cork borer at 5 mm diameter from the center of the wound and 6 mm deep. Then, 10 mL of water (4 °C) was added to 3 g of the excised fresh tissues and ground. Afterwards, 50 mM buffer consisting of 1% polyvinyl pyrolidone (PVPP) and 1.33 mM was added to the sample.

2.6.2. Enzyme assay

2.6.2.1. Polyphenoloxidase. PPO activity was evaluated using the method (González et al., 1999) with slight modifications. Firstly, crude enzyme extract (0.1 mL) was added to 2.9 mL of 0.1 M catecholprepared in 50 mM sodium phosphate (pH 6.4) after which sample was kept in a water bathfor 5 min at 30 °C. Then, for every 30 s the change in absorbance at 398 nm was monitored and recorded over a period of 3 min. The activity of the enzyme was expressed as unit g kg^{-1}fresh weight of the sample.

2.6.2.2. Ascorbate peroxidase (APX). The APX activity was assessed based on the method of (Vicente et al., 2006) with slight modifications. Briefly, 10 mL of ice-cold sodium phosphate buffer (50 mM, pH 7.8) was added to 3.0 g of thecrude enzyme extract and mixed thoroughly. The homogenate was then centrifuged at 10,000 × *g* at 4 °C for 15 min. A 3 mL of the supernatant was collected and its absorbance read at 290 nm. A unit was defined as the change in 0.001 absorbance units per minute at 290 nm. The specific enzyme activity was expressed as unit g kg^{-1}fresh weight.

2.6.2.3. Chitinase (CHI). CHI assay was conducted using the method described byIppolito et al. (2000) with slight modifications. Briefly, 3 g of the crude enzyme extract was homogenized with 5 mL of a working solution containing 50 mM sodium acetate buffer (pH 5.2), 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM β-mercaptoethanol. The sample was thoroughly mixed and centrifuged at 11000 × *g* for 35 min at 4 °C after which the supernatant was used for the enzyme assay. CHI was determined based on the amount of N-acetyl-D-glucosamine (NAG) that was released from the colloidal chitin and expressed as the amount of enzyme required to catalyze the production of 1 µg NAG per hour at 37 °C. The specific activity was expressed as the unit g kg^{-1}fresh weight.

2.6.2.4. Phenylalanine ammonia lyase (PAL) activity. The assay PAL activity was conducted according to the method described by Assis et al. (2001) with modifications. In brief, 3 mL of borate buffer (50 mM, pH 8.8) containing 10 mMphenylalanine was preincubated at 37 °C for 10 min. One milliliter of crude enzyme extract was added and mixed thoroughly. The mixture was then incubated at 37 °C for 60 min after which absorbance was read at 290 nm using distilled water as blank (no component). The difference in the absorbance before and after the experiment was calculated. One unit of the PAL was defined as the formation of 1 µg of cinnamic acid equivalents per hour, and the specific activity was expressed as the unitg $kg^{-1}$of fresh weight.

2.6.2.5. Catalase (CAT). The CAT activity was performed according to the method of (Chance and Maehly, 1955).Initially, 10 mL of ice-cold sodium phosphate buffer (100 mM, pH 7.8) was added to 3.0 g of the crude enzyme extract and mixed. The homogenate was then centrifuged at 1000 × *g* for 15 min at 4 °C. The decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm of a reaction mixture consisting of 2 mL of sodium phosphate buffer (50 mM, pH 7.0), 0.5 mL enzyme extract and 0.5 mL H_2O_2 (40 mM).The specific activity of the enzyme was expressed in unit g kg^{-1}fresh weight, where one unit of catalase converts one µmol of H_2O_2 per min.

2.7. RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Wounds measuring 3 mm diameter by 3 mm deep were created on each grapeberry with the aid of a sterile cork borer and the following treatments applied to each lots of grapes: (1) SDW as control, (2) 2% w/v trehalose, (3) 30 µL of the antagonist *H. uvarum* (10^8 cell/mL) harvested from the NYDB and (4) 30 µL*H. uvarum* (10^8 cell/mL) harvested from NYDB supplemented with 2% w/v trehalose. Then, 30 µL *A. tubingensis* (1×10^5 spore/mL) were inoculated into the wounds. The grape berries were subsequently placed in plastic baskets, enclosed with polyethylene and incubated at 20 °C and 95% relative humidy. After 0 (1 h after treatment), 24, 48 and 72 h, 3 g of the tissues of the wounds were excised into a mortar and liquid nitrogen added and ground.RNA was extracted as described by Liu et al. (2012)with slight modifications. The solution used was Diethyl pyrocarbonate (DEPC) treated water. The extraction buffer composed of β-mercaptoethanol, 25 mM EDTA, 2.0 M NaCl, 2% CTAB, 2% polyvinylpyrrolidone (PVP) K-30 (soluble) and 100 mM TrisHCl (pH8.0) were mixed,sterilized and kept at room temperature before use. Then, the prepared samples were transferred into 50 mL polypropylene tubes with a chilled sterilized spatula and placed in ice for further analysis. Afterward, 10 mL of the extraction buffer preheated to 65 °C in a water bath was added to each sample,after which each tube was vortexed and heated at 65 °C for 10 min. The samples were vortexed every 2 min to obtain a homogenized mixture-ofchloroformand isoamyl alcohol(24:1 v/v),then transferred into a 50 mL tubes homogenized for 5 min. Afterward, the samples were centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant carefully transferred into new test tubes. The supernatant was re-extracted with the same volume and ratio of chloroform and isoamyl and then centrifuged at 10,000 × *g* for 10 min at 4 °C. Thereafter, the supernatant was transferred into a 2 mL tube and Isoamylalcohol added and vortexed. The samples were stored for 10 min in ice and then centrifuged at 12,000 × *g* for 15 min at 4 °C and the supernatant discarded. One mL of ethanol (75%) was used to wash the samples after which they were vortexed for 5 s, and centrifuged at 7500 × *g* for 1 min. The liquid was cautiously pipetted and the pellets air-dried for 5 min, after which DEPC-treated water was used to redissolve the pellets. The RNA was kept at −80 °C. RNA contamination and degradation were examined on 1% agarose gel. Spectrophotometer (Thermo Scientific, CA, USA) was used to check the RNA purity and quantityat wavelengths of 230, 260, and 280 nmThe concentrationand integrity of RNA was evaluated using RNA Nano 6000 Assay Kit of Bioanalyzer 2100 system (Agilent Technologies, CA, USA).Subsequently, 1000 ng RNA was used for cDNA by conducting reverse transcript using reverse transcriptase and oligodT primer. The specific primers used for the relative gene expression assays are tabulated in Table 1. Real-time qRT-PCR was conducted withBio-Rad CFX-96 Real-Time PCR System (Bio-Rad, USA) according to the method described by Zhang et al. (2015).

Table 1
Primers used in qRT-PCR reactions of defense-related relative gene expressions in table grapes.

Gene Name	Accession number	Primer	Primer Sequence (5′ → 3′)	T_m (°C)	Product Length (bp)
PPO	NM_001281116.1	qRT-PPO-F	5′ GTCCCCGTAGCCAGAAACC 3′	59.5	171
		qRT-PPO-R	5′ AGCAGCACCATACAGCCCT 3′	62.4	
APX	XM_010655137.1	qRT-APX-F	5′ TTCAAAAAATCCAACGGTCG 3′	52.4	170
		qRT-APX-R	5′ TTCTGGTACTCCTCGCTCACA 3′	58.9	
CHI	Z54234.1	qRT-CHI-F	5′ GACACCCCAGTCACCCAA 3′	54.6	143
		qRT-CHI-R	5′ CTTTCCCACATTCAACACCTC 3′	54.3	
CAT	KP271927.1	qRT-CAT-F	5′ ATGTGCTGATTTCCTTCGTACC 3′	55.1	251
		qRT-CAT-R	5′ GTCAACAACTCTCCAATACTCCTG 3′	56.7	
PAL	KU162977.1	qRT-PAL-F	5′ AACCGAATCAAGGAGTGC 3′	54.1	151
		qRT-PAL-R	5′ ACTGAGACAATCCAGAAGAG 3′	53.9	
ACTIN	XM_002277287.3	ACTIN-F	5′ TTCAATAAGGAGAAGATGGTGGA 3′	53.8	232
		ACTIN-R	5′ TTGGTGAGGTAGTCTGTGAGGTC 3′	54.8	

Polyphenoloxidase (*PPO*), Ascorbate peroxidase (*APX*), Chitinase (*CHI*), Catalase (*CAT*),Phenylalanine ammonia lyase (*PAL*) and ACTIN.

The conditions of the PCR were set as follows: 95 °C for 30 s then 40 cycles of 95 °C for 5 s and 60 °C for 15 s. The amplified products were determined at the end of the PCR reaction using Melting Curve Analyses. The melting cycle was 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s.The grape *actin*gene served as an internal control to normalize the expressed data (Liu and Ekramoddoullah, 2006). The relative expression levels of the identified genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The RT-qPCR assay was repeated twice and standard error was determined among three biological replicates.

2.8. Statistical analysis

All the results represent the mean of three independent replicates and the experiments were repeated. One-way ANOVA ($p < 0.05$) was performed usingOriginPro 2015 software (OriginLab Corporation, Northampton, United States).

3. Results

3.1. Effects of 2% w/v trehalose, H. uvarum and H. uvarum supplemented with 2% w/v trehalose on A. tubingensis

The results in Fig. 1a showed that the decay incidence of the grape samples treated with *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose exhibited the best effect. A significant ($p < 0.05$) reduction of rotten grapes was observed in *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose compared to 2% w/v trehalose, *H. uvarum* and the control. The control was found to have the highest (70%) rotten grapes after the 5 days of storage at 20 °C. Similarly,the findings on the lesion diameter of the treated grapes showed that*H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose significantly inhibited the growth of the *A. tubingensis* throughout the storage period (Fig. 1b).There were significant differences among all the treatments ($p < 0.05$) (Fig1aand b).From the findings both *H. uvarum* and *H. uvarum* harvested from the NYDB supplemented with 2% w/v trehalose significantly reduced the disease severity of *A. tubingensis*in the treated grapes.

3.2. Effects of 2% w/v trehalose, H. uvarum and H. uvarum supplemented with 2% w/v trehaloseon defense-related enzyme activities

In Fig. 2a, the results indicated that PPO activity in the grapestreated with 2% w/v trehalose, *H. uvarum*and*H. uvarum* harvested from NYDB supplemented with 2% w/v trehaloseincreased significantly compared to the control after inoculation and storage at 20 °C. PPO activity in the treated grapes increased gradually at the lag phase from 0 to 24 h after inoculation. There was no significant difference ($p < 0.05$) observed between 2% w/v trehalose and *H. uvarum* at 48 hHowever, PPO activity in grapes treated with*H. uvarum*harvested from NYDB supplemented with 2% w/v trehalosereached 45 g kg^{-1} at 48 h. It was found that 2% w/v trehalose, *H. uvarum* and *H. uvarum*pretreated with 2% w/v trehalose significantly impacted on PPO activity in the grapes comparedto the control. Moreover, *H. uvarum*pretreated with 2% w/v trehalose in the NYDB showed a remarkable effect on grapes PPO activity afterapplication throughout the entire storage period (Fig. 2a). This demonstrated that*H. uvarum*enhanced with trehalose has the ability to activate PPO activity in table grapes.

The APX activity results in the table grapes showed that the control, 2% w/v trehalose and *H. uvarum*suppressed APX activity from 1 h after treatment (0 h) to 24 h. Afterwards, APX activity in the grapes increased gradually and then declined at 72 h (Fig. 2b). As shown in Fig. 2b, APX activity inthe grapes inoculated with *H. uvarum* and *H. uvarum*pretreated with 2% w/v trehalose both revealed significant effects. As can be seen, both the control and 2% w/v trehalose had a negative effect on APX activity as it decreased from 32 g kg^{-1} to 28 and 29 g kg^{-1} respectively.

The CHI activity in the grapes treated with both *H. uvarum* and *H. uvarum*harvested from NYDB pretreated with 2% trehalose increased exponentially from 23 g kg^{-1} to 42 g $kg^{-1}$and 47 g^{-1} respectively. The control remained all time low throughout the entire duration of the experiment. Although, the effect of 2% w/v trehalose on CHI activity in the grapes was higher than the control however, there was no significant difference ($p < 0.05$) between them. Furthermore, a significant difference was observed between*H. uvarum*and *H. uvarum* +2% w/v trehaloseat 24–72 h (Fig. 2c). This finding demonstrated that both *H. uvarum* and *H. uvarum*+ 2% w/v trehalosehad positive effects in inducing CHI activity in the treated grapes.

As indicated in Fig. 2d, all the treatments positivelyinduced the PAL activity after the grape berries were inoculated and stored at 20 °C for 72 h. The findings revealed that*H. uvarum* triggered the highest PAL activity in the grapes compared to the control, 2% w/v trehalose and *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose. PAL activity in the grapes treated with *H. uvarum* harvested from NYDB supplemented with 2w/v trehalose also resulted in a significant effect at all the tested times compared to the control and 2% w/v trehalose. These suggest that *H. uvarum* and *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose have theability to elicit PAL activityin table grapes.There was however a marginal difference between the control and 2% w/v trehalose at 48 and 72 h.

With regards to CAT activity, from the start (0 h) of the assay to 24 h, CAT activity in the grapes increased rapidly, then decrease in the case of the control, 2% w/v trehalose and *H. uvarum*.However, *H. uvarum*harvested from NYDB pretreated with2% w/v trehalose stabilized and then declined after 48 h (Fig. 2e). The results showed that, there were significant differences among all the treatments at 48 h of storage. At the end, it was noticed that the control, 2% w/v trehalose and *H. uvarum* decreased below the initial CAT activity of 25 g kg^{-1}.

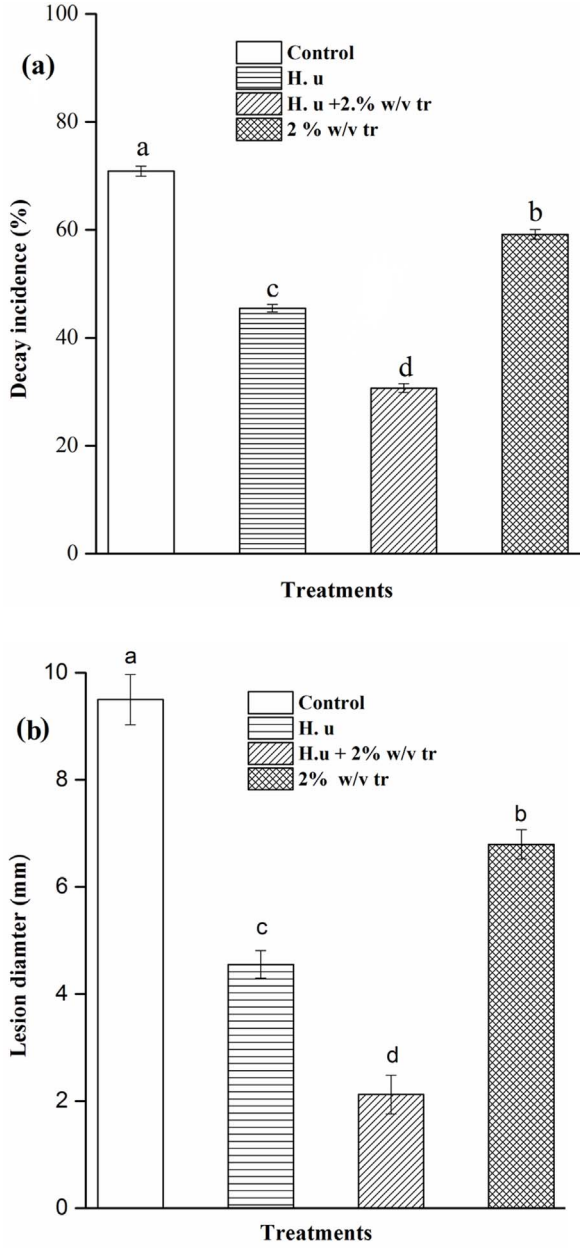

Fig. 1. Effect of trehalose on the biocontrol efficacy of *H. uvarum* on lesion diameter and decay incidence against *A. tubingensis in vivo* stored for 5 d at 20 °C. **(a)** decay incidence **(b)** lesion diameter Treatments: (1). control (sterile distilled water), (2) H u (*H. uvarum* (1×10^8 cells/mL) harvested from NYDB), (3) H u + 2% w/v tr (*H. uvarum* (1×10^8 cells/mL) harvested from NYDB supplemented with 2% w/v trehalose. (4) 2% w/v tr (2% trehalose alone). *A. tubingensis* (1×10^5 spores/mL) was inoculated 2 h after the treatments. Bars represent the mean lesion diameter expressed in (mm) from three independent experiments (± standard deviation). Letters on each bar indicate the results of the Tukey's test ($p < 0.05$); values with the same letters are not significantly different.

3.3. qRT-PCR assay on relative gene expression of table grapes treated with 2% w/v trehalose, H. uvarum and H. uvarum supplemented with 2% w/v trehalose

The results in Fig. 3a indicated that table grapes treated with 2% w/v trehalose, *H. uvarum* and *H.uvarum*harvested from 2% w/v trehalose significantly impacted on the *PPO* relative gene expression levelscompared to the control. The relative gene expressions of *PPO* in the berries treated with *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose increased progressively from 0 to 72 h. The *PPO* relative expression level was significantly up-regulated. Although there was no significant difference in *PPO* expression level between *H. uvarum* and the control at 0 h, it was however, observed that the expression levels of *PPO* in the grapes treated with *H. uvarum* was significantly different from the control at 24, 48 and 72 h Fig. 3a. At 48 h,*PPO*ingrapes treated with *H. uvarum*increased by 1.5-fold and while those treated with *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose increased by 3.5-fold with respect to the control. In Fig. 3a, *PPO*relative gene expression showed a 9.5-fold increase compared to the control.

With regards to *APX,*similar trend was observed. *APX* relative gene expression levels in the grape berries treated with both *H. uvarum* and *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose markedly increased at 24–72 h with a significant effect between both treatments. (Fig. 3b).*APX* relative gene expression levels of 2% w/v trehalose, *H. uvarum*and *H. uvarum* harvested from NYDB pretreated with 2%w/v trehalose showed an enhancement compared to the control. A maximum increase of *APX*relative gene expression level was observed in grapes treated with *H. uvarum*harvested from NYDB supplemented with 2% trehalose at 48 h, with a 7-fold increase compared to the control. Contrarily, it wasfound that at 0 h there were no significant difference ($p < 0.05$) in *APX* gene expression level among all the treatments. The findings also revealed that at 72 h, grapes treated with *H. uvarum* and *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose increased 3.90-fold and 5.79-fold respectively (Fig. 3b).

Additionally, the findings obtained from the qRT-PCR regarding *CHI*relative gene expression levels in the grapes revealed that at 48 h of incubation at 20 °C, the expression level of *CHI* in the grapes treated with *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose reached the highest phase and decreased thereafter. Interestingly however, at 0 h *CHI* relative gene expression level in grapes treated with 2% w/v trehalose was observed to be significantly lower than the control ($p < 0.05$) with a 0.4-fold decrease (Fig. 3c). The highest *CHI* relative gene expression level was observed in grapes treated with *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose at 48 h. At the end of the incubation period, *CHI* relative gene expression level in *H. uvarum*treated grapes was 1.54-fold while that of *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose was 2.20 relative to the control.

The findings (Fig. 3d)on *PAL*relative gene expression levels in the grapes was not different from that of the *APX*. It was observed that there was no significant difference between the control and 2% w/v trehalose at 0, 48 and 72 h. *PAL* relative gene expression level was significantly enhanced in the grapes treated with *H. uvarum*and *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose at 48 and 72 h. There was a progressive increase of *PAL* relative gene expression in grapes treated with *H. uvarum* and *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose from 0 to 48 h.*PAL* relative gene expression level was 12.15-fold increase in grapes treated with *H. uvarum* compared to the control. However, *PAL* relative gene expression level in grapes treated with *H. uvarum*supplemented with 2% w/v trehalose was 7.97-fold increase compared to the control. In Fig. 3e, theresults revealed that*CAT*relative genes expression levels in the grapes were significantly ($p < 0.05$) up-regulated. Unlike *PAL,* maximum *CAT*increment was observed at 24 h for 2% w/v trehalose, *H. uvarum* and *H. uvarum* + 2% w/v trehalose treated grapes with 18-fold increase, 23-fold increase and 23-fold increase respectively compared to the control.It was also noticed thatall treatments differed significantly ($p < 0.05$) from the control.

4. Discussion

The results showed that *H. uvarum* and *H. uvarum* harvested from the NYDBsupplemented with 2% w/v trehalose had positive effects in the enhancement of the grapes resistance to the *A. tubingensis*. The successful biocontrol efficacy of the *H. uvarum*in this finding, may be as a result of the *H. uvarum* interference with the pathogenicity enzymes of

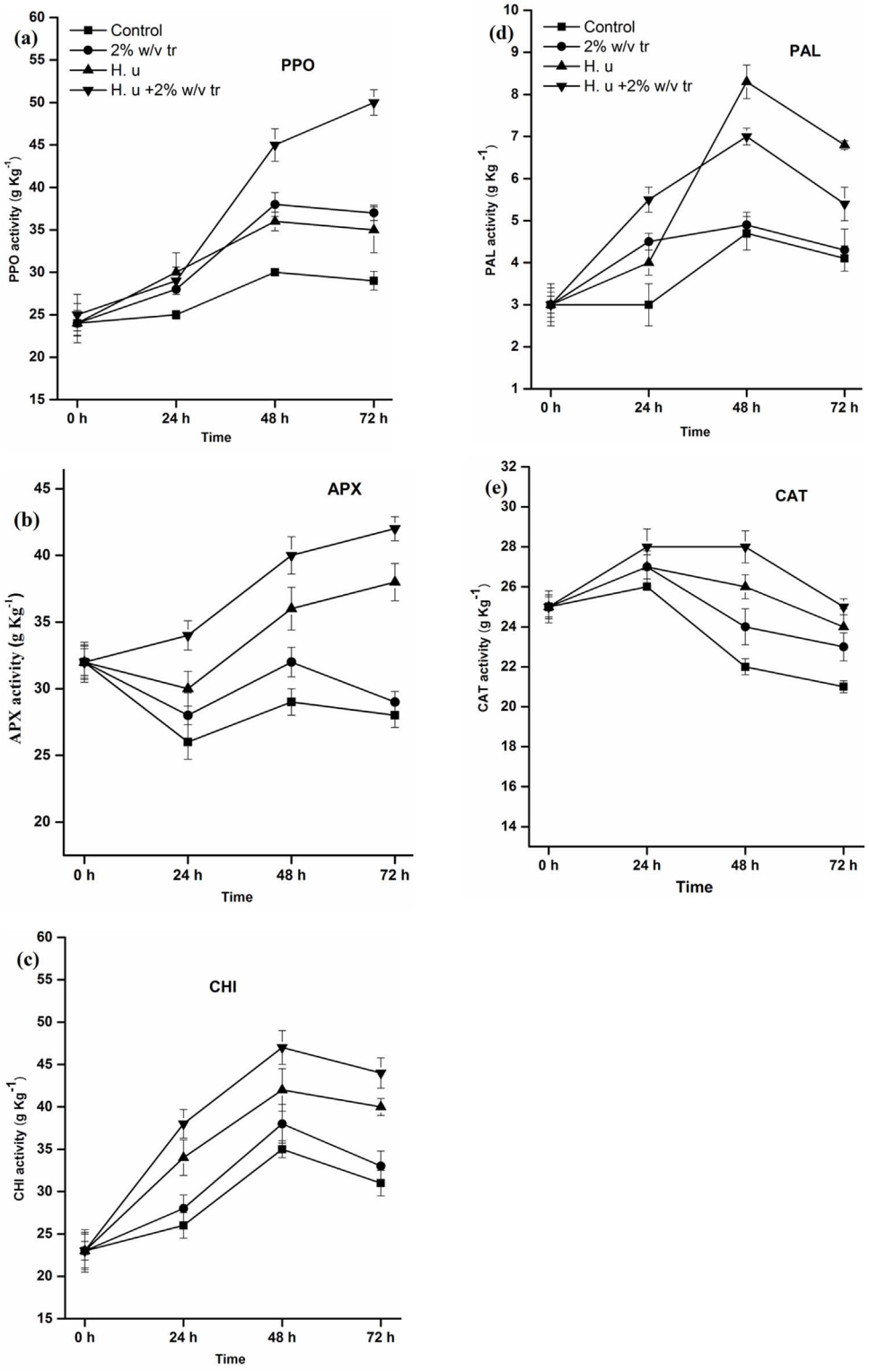

Fig. 2. Time course change of defense-related enzymes activities in table grapes. (a) PPO, (b) APX, (c) CHI, (d) PAL and (e) CAT in grape berry incubated at 20 °C. Treatments: control (sterile distilled water), 2% w/v tr (2% w/v trehalose), H. u (*H.uvarum*) and H. u + 2% w/v (*H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose) The results represent the mean of three independent replicates. Error bars indicate the SE (n = 3). Vertical bars represent the standard errors of the means.

the *A. tubingensis*. This finding suggests that the *H. uvarum* and *H. uvarum*harvested from NYDB supplemented with2% w/v trehalosemay have triggered the defense-related enzyme activity and genes of the grapes. Furthermore, the significant difference in the lesion diameter and decay incidence observed in the grapes treated with the *H. uvarum*supplemented with the trehalose compared to the other treatments may be due to theability of the activated *H. uvarum*cells to enhance the grapes resistance against the stress imposed by the *A. tubingensis*. These results are in line with those of Li and Tian (2006) who reported that trehalose enhanced the biocontrol efficacy and stress tolerance of *C. laurentii*. Similar reports by Liu et al. (2010) demonstrated that the biocontrol efficacy *H. uvarum* was improved against gray mold due totreatment with ammonium molybdate. Likewise, the improvement noted in the lesion diameter and the decay incidence following the treatment with the *H. uvarum* harvested from NYDB pretreated with 2% w/v trehalose, suggest that the*H. uvarum* population may have increased resulting in theout-competitionof the *A. tubingensis* in the grapes. This could possibly be due to the abilityof the induced *H.*

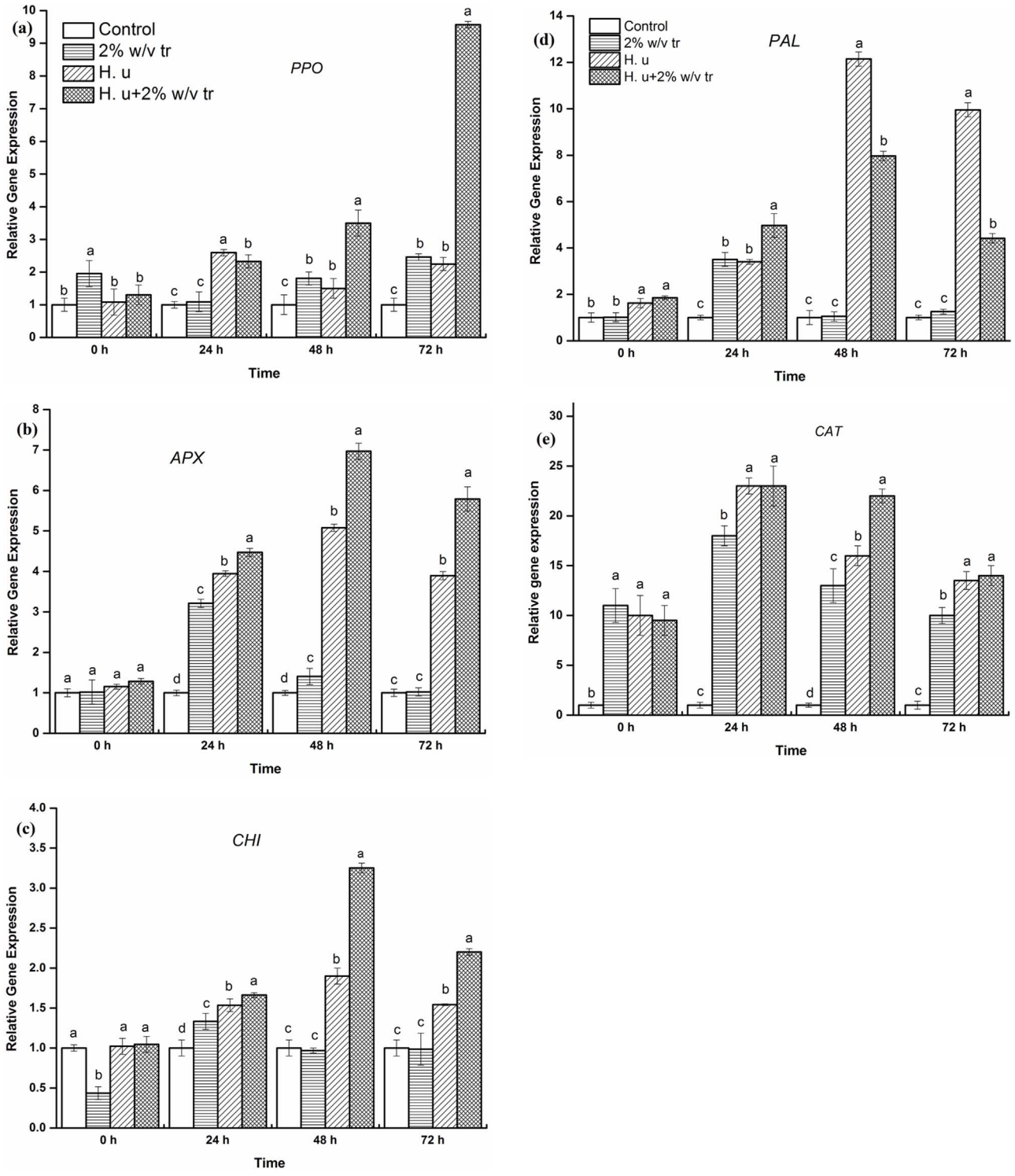

Fig. 3. Time course change of defense-related genes expression in table grapes. The results were obtained using qRT-PCR to determine relative gene expression levels of table grape berries incubated at 20 °C from 0 to 72 h (h). Treatments: control (sterile distilled water), 2% w/v tr (2% w/v of trehalose), H. u (H. *uvarum*) and H. u + 2% w/v (*H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose). After the incubation, total RNA of the grape berries were extracted and transcript levels of the genes determined. (a) *polyphenoloxidase (PPO),* (b) *Ascorbateperoxidase* (*APX*) (c) *chitinase* (*CHI*), (d) *phenylalanine ammonialyase* (*PAL*) and (e) *catalase* (*CAT*) were measured. qRT-PCR was performed using Actin as the internal control.The results represent the mean of the three independent replicates. Error bars indicate the SE (n = 3). Different letters on each bar indicate statistical significance at $p < 0.05$. 0 h means 1 h after the treatments were carried out.

*uvarum*cells to suppress the *A. tubingensis* in the completion for space and nutrients.

PPO, APX, CHI, PAL and CAT activities have previously been studied(Awad et al., 2015; Zhang et al., 2015), nevertheless, the use of qRT-PCR to examine the relative genes expressionsof table grapes treated with *H.uvarum*has not been studied. Yeast effectively adapts to environmental stress by altering their physiology and fine-tuning metabolism. This phenomena is achieved by regulation of both protein activities and genes expression. In this study, we tested the extent to which *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose enhanced defense-relative genes expressions and/or enzyme activities in table grapes using both the conventional methods and qRT-

PCR.

From the investigation, it was observed that *H. uvarum* harvested from NYDB pretreated with 2% w/v trehalose induced defense-related enzyme activities and relative gene expression levels of the grapes compared to the other treatments.These results suggest that the addition of the trehalose to the yeast in the NYDB prior to inoculation into the grapes may have contributed to the viability *H. uvarum*which impacted positively in the up-regulation of both the enzymes and genes activitiesin the grapes.The vulnerability of plant tissues to fungal attack is basically connected to the stimulation of reactive oxygen species (ROS) (Wojtaszek, 1997). Hydrogen peroxide (H_2O_2)which is a type of ROS, posesdeleteriouseffects on plant defense (Torres, 2010). It has also been reported that H_2O_2 can be destroyed by APX and CAT (Apel and Hirt, 2004). CAT ameliorates the damaging effect of H_2O_2 by decomposing it into O_2 and H_2O resulting in the production of benign molecules. Therefore the outburst of CAT activity (Fig. 2e)and relative gene expression (Fig. 3e) in *H. uvarum*and *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose in the grapes compared to the control showed its remarkable ability to curtail H_2O_2.Previous work has documented the effectiveness of CAT and APX capacities to scavenge excess ROS (Landberg and Greger, 2002). Qin et al. (2015) reported that salicylic acid (SA) trigger the production of H_2O_2 content which is mediated by an inhibition of APX and CAT in many plants.

Additionally, Mayer (2006) reported that, peroxidase rapidly increased PPO level which is genotypically resistant to pathogens infection. Moreover, Qin et al. (2015) stated that PPO has the ability to produce antimicrobial phenolic substances by oxidizing phenolic compounds. This suggests that PPO inhibits the advancement of diseases in plant. This is in agreement with Zhao et al. (2009) who revealed that PPO activity was induced by antagonistic yeast in peach fruit, making the fruit resistant toinfection. Our findings provide compelling evidence that *H. uvarum* alone and *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose significantly enhanced the defense-related genes of the table grapes. These findings extend those of Li and Tian (2006) who used trehalose to improve *Cryptococcus laurentii* which enhanced defense-related enzymes activity in apple fruit against *P. expansum* confirming that the internal defense enzyme activities of the apple fruit were up-regulated.

The improvements noted in our results on both PAL enzyme activity (Fig. 2d) and *PAL* relative gene expression (Fig. 3d) of *H. uvarum* and *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose is an indication that the table grapes defense system were enhanced. Similar results were obtained by Li and Tian (2006) in which *Cryptococcus laurentii* pretreated with trehalose up-regulated the PAL activity in apple. Phytoalexin, *p*-coumaric acid derivatives and lignin are biosynthesized by PAL and these compounds contribute to the defense of plant. Beyond these, PAL is actively involved in the biosynthesis of salicylic acid, a defense hormone required for plant defense. Phenylpropanoid metabolic pathway involves mainly the synthesize of phenolic compounds, where PAL is the limiting enzyme while stilbene synthase is the key enzyme responsible for the production of stilbene (Sun et al., 2013). The up-regulated of *PAL* genes expression levels may have been through the biosynthesisof the phenylpropanoid pathway.

Our findings also depicts, that CHI activities and relative gene expressions were significantly up-regulated in the grapes (Figs. 2 c and 3 c) respectively. The up-regulation of *CHI* in the grapes treated with *H. uvarum* and *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose compared to the control revealed that CHI was enhanced suggesting that the defense mechanism of the grapes was been improved. *H. uvarum* harvested from NYDB supplemented with 2% w/v trehalose reached a peak at 48 h after cultivation. Similar kinetics was observed after stimulation of strawberry, where chitinase activity increased in the treated samples compared to the control at 48 h (Hashmi et al., 2013). In a previous report,Yu et al. (2012) revealed that chitinase activity in peach fruit was induced after treatment with yeast saccharide. Some investigations have been conducted on chitinase produced from yeasts, (Chan and Tian, 2005) which was successful against fungal infections. Chitinase combine with β-1,3-gluconases hydrolyze chitin which is the main cell wall component of fungal resulting in the inhibition. This improved resistance to pathogens may be linked to the increased activity of β-1,3-glucanaseand chitinase in the SA pathway. The level of defense-related enzymeprotection in plants is variable andmaybe affected by specificity of the enzyme activity, enzyme concentration in the cell, enzyme localization, characteristics of the pathogen and the host-pathogen interaction. The results suggest that both the enzymes and genes of the grapes were significantly improved against *A. tubingensis*.

5. Conclusion

In conclusion, the findings revealed that *H. uvarum* pretreated with trehalose in NYDB demonstrated biocontrol efficacy against *A. tubingensis* which causes black mold in grapes. The biological significance of defense-related enzymesand relative genes expressions are important components in plants against pathogens. Ostensibly, our study demonstrated thatboth the defense-related enzyme activities and the relative genes expression were up-regulated.In this study, it was noticed that *H. uvarum*harvested from NYDB supplemented with 2% w/v trehalose, *H. uvarum* and 2% w/v trehalose up-regulated the enzyme activities and their corresponding genes that were investigated. It was observed that in most cases,both the enzyme activities and gene expressions in the grapes were significantly different at 24, 48 and 72 h after inoculation. These findings suggested that trehalose could be involved in the enhancement of *H. uvarum's* viability and population growth which subsequently activated enzyme and gene expression levels in the grapes. Future experiment should thus include follow-up work designed to evaluate whether there was a corresponding protein accumulation/up-regulation since proteomics has been revealed to be another mechanism of action through which antagonist/host inhibit fungal growth.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

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