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Induction of resistance in apple fruit inoculated with antagonistic *Candida membranifaciens* isolates and *Botrytis mali*

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Abstract: The biocontrol activity of two isolates of *Candida membranifaciens* against grey mold of apple fruit caused by *Botrytis mali* and their ability to induce biochemical defense responses in apple tissue were investigated. Apple fruit (*Malus domestica*) wounds were inoculated with 50 µl yeast suspension (1×10^7 CFU/ml) of *C. membranifaciens* followed 4 h later by 20 µl of conidial suspension of *B. mali* (1×10^5 conidia/ml). The apples were then incubated at 20 ºC for 8 days. Lesion diameter sizes were measured 4 and 8 days after pathogen inoculation. In addition to controlling grey mold, these two isolates of *C. membranifaciens* caused increases in peroxidase and β-1, 3-glucanase activities. These isolates also caused inhibition in catalase activity. The accumulation of phenolic compounds was increased in apple fruit treated with antagonists and inoculated with *B. mali* and reached its highest level 6 days after treatment. The ability of *C. membranifaciens* to affect H_2O_2-metobolizing enzymes and increase levels of β-1, 3-glucanase activity and phenolic compounds may be some of mechanisms responsible for its biocontrol activity.

Keywords: Catalase, Peroxidase, Phenolic compounds, β-1, 3-glucanase

Introduction

Grey mold diseases are probably the most common and widely distributed diseases of vegetables, ornamentals, fruits and even field crops throughout the world (Barka *et al*., 2002). They are caused by *Botrytis cinerea* Pers.: Fr and *B. mali* Ruehle.

Due to the development of fungicide-resistant isolates of postharvest pathogens, and the possible deregistration of some of the more effective postharvest fungicides (Eckert *et al*., 1994), considerable attention has been placed on postharvest application of antagonistic agents for the inhibition of plant disease (Cook *et al*., 1999; Castoria *et al*., 2001; El Ghaouth *et al*., 2003).

Information on the mechanisms of action of most of the antagonists is still incomplete. Nevertheless it is essential to develop appropriate formulations and methods of application, in order to obtain registration and to select new and effective microorganisms. Several mechanisms have been reported to play a significant role in the biocontrol activity of antagonistic yeasts. The main mode of action of the yeast biocontrol agents is believed to be competition for nutrients and space (Droby and Chalutz, 1994). Additional modes of action such as mycoparasitism, induced resistance and the production of lytic enzymes such as chitinases and β-1, 3-glucanase have been suggested (Wisniewski *et al*., 1991; El Ghaouth *et al*., 1998).

Considerable attention has been focused on induced resistance as an important management form of crop protection (El-Ghaouth *et al*., 1994; Droby *et al*., 2002). Antagonistic yeasts induce several defense responses including an increase in

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β-1, 3- glucanase, chitinase, and peroxidase activities in apple fruit (Ippolito *et al*., 2000).

The objective of this study was to confirm the ability of two isolates of *Candida membranifaciens* (Lodder & Kreger) Wick. & K. A. Burton 1954 to control postharvest grey mold rot caused by *B. mali* in apple fruit. Other objectives were to determine whether the activities of the peroxidase (POX), catalase (CAT), β-1, 3-glucanase and total phenolic compounds would be affected in apple fruit tissue, following the application of the two isolates of *C. membranifaciens* alone or in combination with *B. mali*.

Material and Methods

Grey mold pathogen

Bm_2 isolate of *Botrytis mali* (Mikani *et al*., 2008) was obtained from plant pathology lab of Aburayhan College, University of Tehran. All cultures were derived from single spore isolates and maintained on potato dextrose agar at 4 ºC in darkness until needed.

Biological control agents

Two isolates of yeast (A2 and A5) were obtained from apple fruits with serial dilution method on Yeast Malt extract agar medium. The isolates were identified by Centraalbureau voor Schimmelcultures (CBS), Fungal Biodiversity Centre at the Institute of the Royal Netherlands Academy of Arts and Science Uppsalalaan 8, 3584 CT Utrecht The Netherlands, as *Candida membranifaciens.*

Fruit samples

Apple fruits (*Malus domestica* cv. Golden Delicious) were harvested at commercial maturity and kept at 1.0 ± 0.5 ˚C in cold storage and used in this study. The apples fruits were washed in 70 % ethanol for 30s followed by dipping in 0.1 % sodium hypocholorite solution, and rinsed three times with sterile distilled water (SDW).

In vivo biological control studies

Inoculum of each yeast was prepared in nutrient yeast dextrose broth (NYDB) on a rotary shaker at 200 rpm for two days at room temperature. The yeast cells were collected by centrifugation at 3000 × g for 10 min and resuspended in SDW (El Ghaouth *et al*., 1998).

The fruits were wounded in triplicate with a 3 mm diameter nail to a depth of 5 mm. Fifty µl aliquots of each yeast suspension (1×10^7 CFU/ml) or sterile distilled water were dispensed in each wound.

B. mali Bm_2 was grown on potato dextrose agar (PDA) (Merck, Darmstadt, Germany) plates (90 mm diameter) for 10 days. Conidia were harvested by pouring a few milliliter of SDW containing 0.05 % Tween 20 on the plates. The conidial suspensions were adjusted to 1.0×10^5 conidia/ml using a haemacytometer. A 20 µl aliquot of conidial suspension or SDW was dispensed to each wound by a micropipette 4 h after inoculation with *C. membranifaciens* A2 or *C. membranifaciens* A5. The treated apples were placed on cardboard trays that were then enclosed in plastic bags. The inside of the bags was sprayed with SDW to maintain high relative humidity in the bags. The apples were incubated at 20 ºC for 8 days.

The lesion sizes were measured 4 and 8 days after inoculation with pathogen using calipers and lesion area was calculated.

Induction of defense responses by *C. membranifaciens*

Tissue samples from different treatments were collected at various times (2, 4, 6 and 8 days) after pathogen treatment. At each sampling time, tissue samples containing the wounds were removed with a cork borer (10 mm in diameter by 10 mm deep) from four apples that were randomly selected from each treatment.

Extraction and Assay of peroxidase activity

The method of extraction as described by Gong *et al*. (2001) was used in this experiment. Fresh apple tissue (5 g) was homogenized with 15 ml of 0.05 M phosphate buffer (pH 7.0) containing 10 % polyvinyl pyrrolidone (Merck, Darmstadt, Germany) and 0.1 M Ethylene diamine tetra acetic acid (EDTA) (Merck, Darmstadt, Germany).

The homogenate was centrifuged at 14000 × g for 16min at 4 ºC. The supernatant was used for

the peroxidase assay. Peroxidase activity was measured by the method of Vetter *et al*. (1958) as modified by Gorin and Heidema (1976). The reaction mixture contained 0.1 ml sample extract, 1.35 ml 100 mM MES buffer (2-morpholinoethanesulfonic acid, monohydrate) (Sigma, St. Louis, USA) (pH 5.5), 0.05 % H_2O_2 (Merck, Darmstadt, Germany) and 0.1 % ρ-phenylenediamine (Merck, Darmstadt, Germany). Changes in absorbance were recorded at 485 nm for 1 min with a spectrophotometer (Milton Roy, Spectronic 501, Unterfoehring-Germany). The activity of peroxidase was presented as ΔOD_{485nm} /min/mg protein.

Extraction and Assay of catalase activity

The method of extraction as described by Gong *et al*. (2001) was used in the experiment. Fresh apple tissue (5 g) was homogenized in 15 ml of Tris-HCl buffer (pH 8.5) including 2 mM EDTA (Merck, Darmstadt, Germany), 10 % (w/v) PVPP (Merck, Darmstadt, Germany). The homogenate was centrifuged at 16,000 × *g* for 14 min at 4 ºC. Supernatant was used for the activity measurement. CAT activity was measured by following the disappearance of H_2O_2 in the enzyme reaction mixture (Brennan and Frenkel 1977; Du and Bramlage 1995). The sample extract (0.25 ml) was added to 2 ml assay mixture (50 mM Tris-HCl buffer pH 6.8, containing 5 mM H_2O_2). The reaction was stopped by adding 0.25 ml 20 % titanic tetrachloride (in concentrated HCl, v/v) after 10 min at 20 ºC. A blank was prepared by addition of 0.25 ml 20 % titanium tetrachloride at zero time to stop the enzyme activity. The absorbance of the reaction solutions was measured at 415 nm against water. CAT activity was determined by comparing absorbance against a standard curve of H_2O_2 from 0.25 to 2.5 mM. The activity of CAT was presented as H_2O_2 mM /min/mg protein.

Extraction and Assay of β-1, 3-glucanase activity

The method of extraction as described by Ippolito *et al*. (2000) was used in this experiment. From each fruit, tissue samples were taken from the wounds and individually homogenized at 4 ºC in two volumes (w: v) of 50mM sodium acetate buffer, pH 5.0, and the homogenate was centrifuged at 4 °C (15 min, 10000 × *g*). Proteins in the supernatant were precipitated in 60 % acetone (v: v) at –20 ºC and the resulting pellet, following centrifugation (30 min, 12000 × *g* at 4 ºC), was washed three times with 60 % acetone. The pellet was suspended in 2 ml of 50 mM sodium acetate buffer (pH 5.0) and assayed for β-1, 3-glucanase activities. β-1, 3-glucanase was determined following the method of Abeles and Forrence (1979). β-1, 3-glucanase activity was assayed by incubating 62.5 ml of enzyme preparation for 2 h at 40 ºC in 62.5 ml of 4 % laminarin. The reaction was terminated by heating the sample in boiling water for 10 min and the amount of reducing sugars was measured spectrophotometrically at 492 nm after reaction with 372 ml of 3,5-dinitrosalicilate. Final activity values are reported as nmol glucose/min/mg of total protein.

Assay of protein content

Total soluble protein was determined based on the method of Bradford (1976). Bovine serum albumin (BSA) fraction V was used as a standard.

Determination of total phenolic compounds

The method of extraction as described by Yamomoto *et al*. (1977) was used in the experiments. Apple fruits (1.0 gr fresh weight) were ground in a mortar with 8 ml of 80 % methanol (Merck, Darmstadt, Germany) and filtered through double layers of gauze. The residue was washed twice with 80 % methanol (each time with three ml). The filtrate and washing were combined and centrifuged at 1000 g for 5 min at room temperature and the supernatant was assayed.

Total phenol was measured with Folin-Ciocalteu s reagent (Merck, Darmstadt, Germany). 0.5 ml extracts were diluted with distilled water to 7 ml in a test tube. The contents were well mixed. 0.5 ml Folin-Ciocalteu s reagent was added and the tubes were thoroughly shaken again. Exactly 3 min later 1 ml of saturated sodium carbonate

solution was added and the mixture made up to 10 ml with good mixing. After leaving the samples for one hour at room temperature, the absorbance was measured at 725 nm. Caffeic acid (Fluka, Germany) was used as a reference phenolic compound. The total phenolic compounds of samples were expressed as mg caffeic acid per g of fruit fresh weight.

Data analysis

Enzyme assays were carried out twice for each sample and a mean of two assays were used for statistical analysis as value of each replicate.

The completely randomized design was used for all experiments. Analysis of variance was performed on the data and means were separated using Duncan s Multiple Range Test at $P < 0.01$ (Little and Hills, 1978).

Results

Biocontrol activity

Both isolates of yeast were effective in controlling decay of apple fruit caused by *Botrytis mali*. They reduced *B. mali* decay area from 0.4 cm^2 in control to 0.06 and 0.09 cm^2 in A_5 and A_2 respectively 4 days after pathogen inoculation at 20 ºC.

Lesion size was from 1.77 to 2.01 cm^2 for antagonistic treatment and 17.56 cm^2 for the control treatment after 8 days (Table 1).

Table 1 Decay area of Golden delicious apples inoculated with *Botrytis mali* (Bm_2) after treatment with *Candida membranifaciens* and incubated for 4 and 8 days at 20 ºC.

Treatment	Decay area (cm^2) after 4 days	Decay area (cm^2) after 8 days
B.mali Bm_2	0.40a*	17.56a
C.membranifaciens A2	0.09b	1.77c
C.membranifaciens A5	0.06c	2.01c

*Each treatment was replicated 4 times with each lesion diameter the average of 2 independent measurements. Means within columns followed by the same letter do not differ significantly at $p \leq 05$ according to Duncan's Multiple Range

Peroxidase activity

POX activities in treated fruit with antagonist alone or with pathogen showed an approximately 1.5-2 fold increase as compared with healthy control after two days of storage and then declined and remained at a relatively low level in treatment without pathogen, but in treatment of yeast-pathogen again showed a little increase on the 6th day and then decreased. POX activities in *B.mali*-inoculated fruit showed a marked increase after 4 days and then decreased slowly (Figure 1).

Fruit treated with yeast in combination with pathogen expressed lower POX activity than fruit treated with pathogen alone at 4, 6 and 8 days. In treated fruit with yeast POD activities declined after 4 days and its level compared with the pathogen-inoculation control was lower. An increase in POD activity was observed also in healthy control fruit after 4 days, but the level of increase was markedly lower than that detected in treated fruit (Figure 1).

Catalase activity

CAT activities in all treated fruit compared with the water control increased first, however yeast alone did not increase CAT activity, significantly. Then CAT activities in treated fruit with yeast alone and yeast in combination with pathogen decreased and its level was lower than healthy control at 4 and 6 days. CAT activities in *B. mali*-inoculated fruit reached maximum level after 6 days and then decreased (Figure 2).

β-1, 3-glucanase activity

β-1, 3-glucanase activity in apples fruit treated with yeast and with pathogen alone increased, reaching maximum levels 6 days after treatment and then decreased. β-1, 3-glucanase activity in combination of antagonist-pathogen increased as compared with water control and showed a maximum increase after 4 days of storage and then decreased slowly (Figure 3).

Determination of total phenol

Total phenolic compounds content in all treated fruit with antagonist and pathogen increased slowly, reaching maximum levels 6 days after treatment and then decreased. Phenolic contents

in treated fruits with antagonist were maximal in the presence of pathogen challenge and reached a peak at 4 days after inoculation Figure 4).

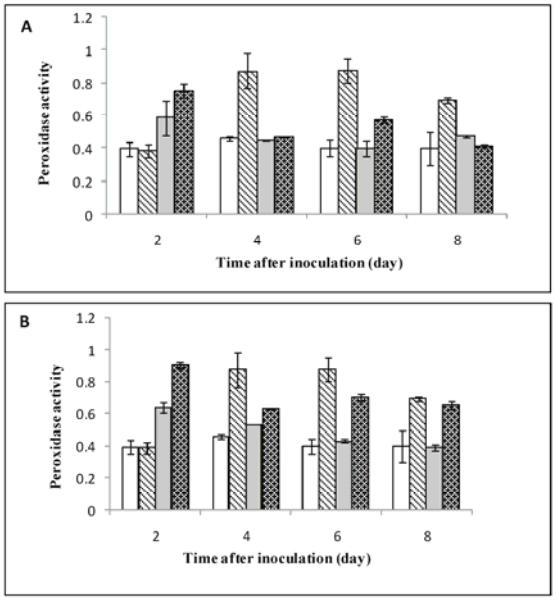

Figure 1 Peroxidase activity in apple tissue in presence or absence of *C. membranifaciens* or *B. mali* Bm_2.
Peroxidase activity in apple fruit treated with sterile water ☐ *B. mal*i ▨ *Candida membranifaciens* ▩ *Candida membranifaciens + B. mali* ▩
Isolates of *C. membranifaciens* are A2 (A) and A5 (B). The activity of peroxidase was presented as Δ $OD_{485\ nm}$ /min/ mg protein and values are averages of 4 replicates.

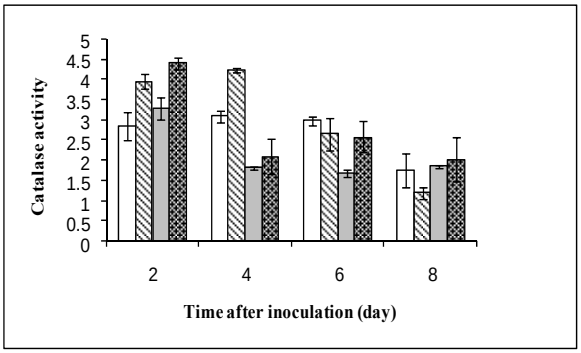

Figure 2 Catalase activity in apple tissue in presence or absence of *C. membranifaciens* A2 or *B. mali* Bm_2
Catalase activity in apple fruit treated with sterile water ☐ *B. mal*i ▨ *Candida membranifaciens* ▩ *Candida membranifaciens + B. mali* ▩
The activity of Catalase was presented as mM H_2O_2/min/mg protein and values are averages of 4 replicates.

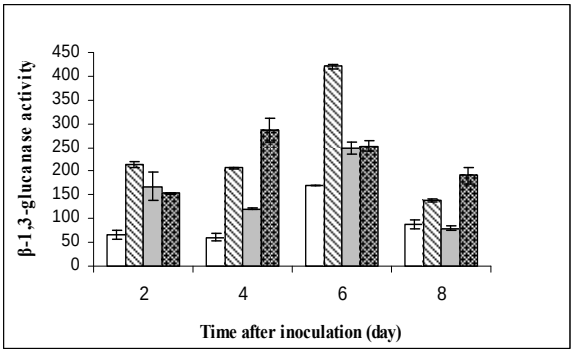

Figure 3 β-1, 3-glucanase activity in apple tissue in presence or absence of *C. membranifaciens* A2 or *B. mali* Bm_2
β-1, 3-glucanase activity in apple fruit treated with sterile water ☐ *B. mal*i ▨ *Candida membranifaciens* ▩ *Candida membranifaciens +B. mali* ▩
The activity of *β*-1, 3-glucanase was presented as Glucose/min/µg protein and values are averages of 4 replicates.

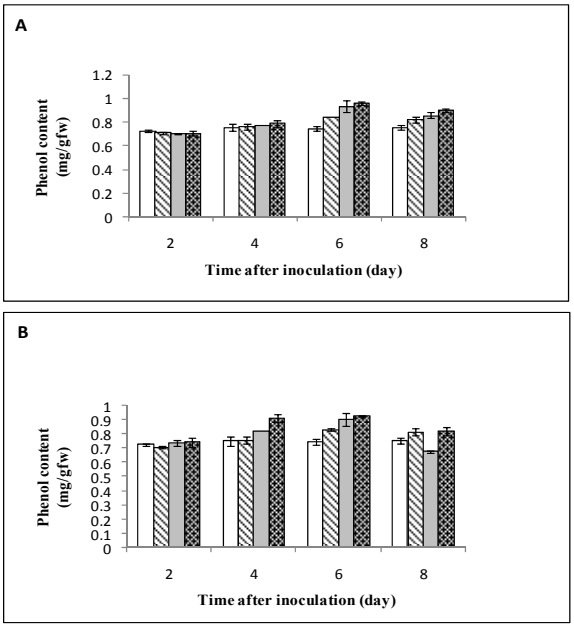

Figure 4 Total phenol content of apple tissue treated with *Candida membranifaciens* or *Botrytis mali* Bm_2
Total phenol content of apple fruit treated with sterile water ☐ *B. mali* ▨ *Candida membranifaciens* ▩ *Candida membranifaciens +B. mali* ▩
Isolates of *C.membranifaciens* are A2 (A) and A5 (B).
Total phenolic contents of the roots were expressed as mg caffeic acid g^{-1} of fruit weight and values are averages of 4 replicates

Discussion

The present data show that these two isolates of *Candida membranifaciens* when applied as a wound treatment were effective in controlling postharvest decay of apple fruit caused by *B. mali*. Comparable levels of decay control have been reported with other microbial antagonists (Roberts, 1990; Wilson and Wisniewski, 1994; Bull *et al*., 1997; Chand-Goyal and Spotts, 1997; Droby *et al*., 1998; El Ghaouth *et al*., 1998; Janisiewicz, 1998; Nigro *et al*., 1999)

Treatment with yeast affected H_2O_2-metabolizing enzymes in harvested apple, which indicated yeast treatment could directly or indirectly activate antioxidant enzymes. In comparison with water control, yeast treatment significantly increased activities of POX and inhibited CAT activity. The results supported the view of Chan and Tian (2006). It also induced the accumulation of β-1, 3-glucanase. Furthermore phenolic accumulation was increased in apple fruit treated with either of the two isolates of *C. membranifaciens* when inoculated in combination with *B. mali*. Caruso *et al*. (1999) stated that plants develop a complex variety of events that involve synthesis and accumulation of new proteins which have direct or indirect roles during pathogenesis. Antagonistic yeasts could compete for space and nutrients with pathogens or directly attach to the hyphae of pathogens (Cook *et al*., 1999; Castoria *et al*., 2001; Chan and Tian, 2005). Others have shown that host induced resistance may be one of the mechanisms by which yeasts inhibit pathogens (Ippolito *et al*., 2000; Droby *et al*., 2002). Ippolito *et al.* (2000) showed that *Aureobasidium pullulans* (de Bary) Arnaud caused a transient increase in chitinase, β-1, 3-glucanase and peroxidase activities in apple fruit. Antifungal glucanohydrolases and peroxidases are considered potentially important in host resistance mechanisms. Peroxidase is involved in lignin formation (Hammerschmidt *et al*., 1982; Kuc, 1990), while β-1, 3-glucanase is capable of hydrolyzing fungal cell walls, and it has been shown to inhibit the growth of several pathogenic fungi *in vitro* (Schlumbaum *et al*., 1986; Sela-Buurlage *et al*., 1993). These observations, together with the fact that the induction and accumulation of glucanohydrolases and peroxidases are often correlated with the onset of induced resistance, suggest an active role for these enzymes in defense against pathogenic fungi (Kuc, 1990; Sticher *et al*., 1997; Van Loon *et al*., 1998). Catalase (CAT) dismutases H_2O_2 to oxygen and water, and peroxidase (POX) decomposes H_2O_2 by oxidation of phenolic compounds (Chan and Tian, 2006). These enzymes associated with super oxide dismutase (SOD catalyses the dismutation of O_2^- to H_2O_2) are considered to be the main enzymatic systems for protecting cells against oxidative damage (Tommasi *et al*., 2001; Wang *et al*., 2005). The balance between SOD and POX or CAT activities in cells is crucial for determining the steady-state level of O_2^- and H_2O_2. Results of Castoria *et al*. (2003) clearly showed that ROS (Reactive oxygen species) generation occurs in apple wounds immediately after wounding. Sustained accumulation of ROS could cause lipid peroxidation, aggravate oxidative damage, and accelerate senescence (Hariyadi and Parkin, 1991). Yeast interaction with the above mentioned enzymes leads to low levels of H_2O_2 accumulation in apple fruit, which activates protective enzymes and then induces fruit resistance against postharvest pathogens. Castoria *et al*. (2003) have also found that the combined application of biocontrol yeasts and ROS-deactivating enzymes in apple wounds enhances colonization and antagonistic activity of biocontrol yeasts against *B. cinerea* and *P. expansum* Link. This indicates that biocontrol efficiency of antagonistic yeasts may be related to ROS metabolism.

In our experiment in treated fruit with yeast in presence of pathogen POX activities declined after 4 days and its level compared with the pathogen-inoculation control was lower. Perhaps antagonist by prevention of pathogen penetration and retarding pathogenesis prevents the induction of POX by the pathogen. Cytological study of El Ghaouth

et al. (1998) on the infection process of apple tissue by *B. cinerea* showed that the growth pattern of *B. cinerea* is mainly necrotrophic. The massive colonization of apple tissue by *B. cinerea* resulted in extensive host cell wall degradation and tissue collapse (El Ghaouth *et al*., 1998). Similar wall alterations have also been reported in other *Botrytis* sp.-host interactions and were attributed to a synergistic action between cell wall-degrading enzymes and organic acids, namely oxalic acid (Johnston and Williamson, 1992; Mansfield and Richardson, 1981; Rijkenberg *et al*., 1980). El Ghaouth *et al*. (1998) reported that the observed restriction of fungal ingress and preservation of host wall integrity strongly indicates that *C. saitoana* Nakase & Suzuki may have affected the ability of *B. cinerea* to degrade host tissue and establish a nutritional relationship.

Phenolic compounds are secondary metabolites produced by most members of the plant kingdom and are major part of the chemical component of plants. Phenols have been suggested to play a role in plant resistance against many diseases. In addition to direct effects of phenols on fungal pathogens, phenolic compounds are oxidized by peroxidases to form the more toxic, quinines (Gogoi *et al*., 2001). Phenolic compounds and flavanoids may also serve as preformed phytoanticipins or inducible phytoalexins in plant to slow down the growth of the pathogen (Shadle *et al*., 2003). Guleria and Kumar (2006) reported higher activity of phenylalanine ammonialyase (PAL) and peroxidase and increase in level of phenolic compounds in sesame (*Sesamum indicum*) leaves treated with leaf extract of neem (*Azadirachta indica*) and inoculated with Alternaria leaf spot pathogen (*Alternaria sesami*). It is suggested that neem leaf extract induces activity of PAL and peroxidase enzymes in sesame leaves, and increases biosynthesis and metabolism of phenol that might in turn have protected the sesame plant from *Alternaria sesami*. Vivekananthan *et al*. (2006) found that the pre-harvest application of biocontrol agents may help overcome pre and post-harvest infections by promoting levels of defense-related enzymes and phenolic substances.

In conclusion, the present results show that two isolates of *C. membranifaciens* have potential as biocontrol agent for the control of postharvest decay of apple caused by *B. mali* and are capable of inducing the accumulation of peroxidase, β-1, 3-glucanase and boosting the level of phenolic compounds thereby decreasing in CAT activities. These results suggest that postharvest treatment with *C. membranifaciens* holds promise as a new technology, that could be substituted for fungicidal control of postharvest diseases in apple fruit.

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القای مقاومت در میوه سیب مایه‌کوبی شده با جدایه‌های آنتاگونیستی *Candida membranifaciens* و *Botrytis mali*

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چکیده: فعالیت بیوکنترلی دو جدایه *Candida membranifaciens* بر علیه کپک خاکستری میوه سیب با عامل *Botrytis mali* و نیز توانایی آنها برای القای پاسخ‌های دفاع بیوشیمیایی در بافت سیب مورد بررسی قرار گرفت. زخم‌های میوه سیب (*Malus domestica*) با ۵۰ میکرولیتر سوسپانسیون مخمر *C. membranifaciens* (1×10^7 CFU/ml) و ۴ ساعت بعد با ۲۰ میکرولیتر سوسپانسیون کنیدیایی *B. mali* (1×10^5 conidia/ml) مایه‌کوبی شدند. سپس سیب‌ها ۸ روز در دمای ۲۰ درجه سلسیوس نگهداری شدند. قطر زخم‌ها ۴ و ۸ روز بعد از مایه‌کوبی با بیمارگر اندازه‌گیری شد. علاوه بر کنترل کپک خاکستری این دو جدایه *C. membranifaciens* موجب افزایش فعالیت پراکسیداز و β-1, 3-glucanase گردیدند. این دو جدایه همچنین موجب کاهش فعالیت آنزیم کاتالاز شدند. تجمع ترکیبات فنلی در میوه سیب تیمار شده با آنتاگونیست‌ها و مایه‌کوبی شده با *B. mali* افزایش یافت و بالاترین میزان آن ۶ روز پس از تیمار بود. توانایی *C. membranifaciens* برای تحت تأثیر قرار دادن آنزیم‌های متابولیزکننده H_2O_2 و افزایش سطح فعالیت β-1, 3-glucanase و ترکیبات فنلی ممکن است برخی از مکانیسم‌های فعالیت بیوکنترلی آن باشد.

واژگان کلیدی: سیب، کاتالاز، پراکسیداز، ترکیبات فنلی، β-1, 3-glucanase