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Changes in Peroxidase and Phenols Activity in Apple Fruit Inoculated with Antagonistic *Pseudomonas fluorescens* Isolates and *Botrytis mali*

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Abstract: The biocontrol activity of three isolates of *Pseudomonas fluorescens* against gray mold of apple fruit caused by *Botrytis mali* and their ability to induce biochemical defense response in apple tissue were investigated. Apple fruit (*Malus domestica*) wounds were inoculated with 20 μL bacterial suspension (10^8 CFU mL^{-1}) of *Pseudomonas fluorescens* followed 24 h later by 20 μL of conidial suspension of *B. mali* (10^5 conidia mL^{-1}). The apples were then incubated at 20°C for 11 days. Lesion diameters were evaluated 6 and 10 days after pathogen inoculation. In addition to controlling gray mold, these three isolates of *P. fluorescens* caused increase in peroxidase activities that reached maximum levels 2-6 days after pathogen inoculation. Phenolic accumulation was increased in apple fruit treated with antagonists and inoculated with *B. mali* and exhibited the highest level 6-8 days after treatment. The ability of *P. fluorescens* to increase activities of peroxidase and levels of phenol compounds maybe one of mechanism responsible its biocontrol activity.

Key words: Apple fruit, *Pseudomonas fluorescens*, *Botrytis mali*, peroxidase, phenolic compound

INTRODUCTION

Gray mold diseases are probably the most common and widely distributed diseases of vegetables, ornamental, fruit and even field crops throughout the world (Barka *et al*., 2002). They are caused by *Botrytis cinerea* Pers: Fr and *Botrytis mali* Ruehle. *Botrytis mali* was recently described by O'Gorman *et al.* (2005) as a separate species from *B. cinerea* based on morphological characteristics and DNA sequence difference.

Control of gray mold through chemical sprays has been successful (Barka *et al*., 2002) but the development of fungicide-resistant strains and the public demand to reduce pesticide use have increased the search for alternative control strategies (Wilson *et al*., 1993).

As an alternative to fungicides, treatment of fruit with microbial agents has shown promise for the control of several post harvest fruit diseases (Etebarian *et al*., 2005; Etebarian and Holberg, 2006; McLaughlin *et al*., 1990; Roberts, 1990; Wilson and Wisniewski, 1994; Bull *et al*., 1997; Chand-Goyal and Spott, 1997; Droby *et al*., 1998; El-Ghaouth *et al*., 1998). Currently at least three formulated microbial antagonists such as Aspire, Biosave-100 and Biosave-110 are now commercially available for use of post harvest treatment (Ippolito *et al*., 2000). The main mode of action of yeast biocontrol agents is believed to be competition for nutrient and space (Droby and Chalutz, 1994).

Bacteria such as fluorescent pseudomonads have the potential to suppress fungal as well as bacterial plant pathogens (Dunne *et al*., 1996; Weller, 1988). *P. fluorescens* produces pyrrolnitrin, a secondary metabolite formed from tryptophan that has strong antifungal activity (Kirner *et al*., 1998). Benizri *et al*. (1995) demonstrated that antagonist activity of *Pseudomonas* sp. was due to the production of antibiotics, volatile compounds and siderophores. Additional modes of action such as induced resistance have been suggested (Droby and Chalutz, 1994). In recent years, considerable attention has been placed on induced resistance as an important form of plant protection and several active microbial and chemical elicitors have been identified and shown to protect a variety of plants (Kloepper *et al*., 1992). Upon infection or treatment with elicitors, plant tissue often reacts by activating a highly coordinated biochemical and structural defense system that helps ward off the spread of pathogens (Kloepper *et al*., 1992; Ryals *et al*., 1996; Sticher *et al*., 1997).

The elicitors are such as a virulant fungi, bacteria, viruses, degradation products of fungal cell wall and some chemical substance. Recently, some physical elicitors are reported to have remarkable effects on induced resistance, which include UV-ray, high or low temperature, electromagnetic waves and mechanical wounds (Zhao *et al*., 2005).

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Guleria and Kumar (2006a) showed that treatment of mustard (*Brassica juncea* (L.) *Czern* and Cross) cv. varuna with Benzothiadiazol (BTH) induced changes in the qualitative profile of total phenols and acid soluble extra cellular protein. There was a temporal increase in the level of total soluble phenolics after BTH treatment and maximum content was observed 72 h after treatment. It was suggested that changes in specific phenols and proteins as a result of BTH treatment might be useful markers of induced resistance in mustard.

Zhao *et al*. (2005) showed that a stress stimulus can efficiently induce the resistance of cucumber against fungal pathogens. After the treatment of stress stimulus on leaves, the activities of resistance-related enzymes were increased significantly. They included Phenylamine Ammonialyase (PAL), peroxidase and polyphenol oxidase, which are strongly associated with plant diseases resistance. The data showed that one of the mechanisms of stress stimulus induction of plant resistance may act via eliciting the metabolism related disease resistance within plant, which can produce many suppressing and antimicrobial compounds against pathogens infection.

Many microbial elicitors are reported to have remarkable effects on induced resistance. For example, Honty *et al*. (2005) showed that in susceptible pear fruits, symptoms of *Erwinia amylovora* infection at the inoculation point have developed within 48 h, with a likely consequence of a gradual decrease in peroxidase synthesis. During the time period peroxidase activity increased continuously in tissues neighboring infection, which could indicate mobilization of plant defense processes in healthy neighboring tissue, in response to disease symptoms at the inoculation site.

El-Ghaouth *et al*. (2003) showed that treatment of apple fruit with *Candida saitoana* induces systemic resistance to *Botrytis cinerea* and increase in chitinase and β, 1, 3-glucanase activity with time. In fresh apples, the onset of systemic resistance to *B. cinerea* coincided with the increase in chitinase and β, 1, 3-glucanase activity in systemically protected tissue. Wang *et al*. (2004) showed that treatment of peach fruit with *Cryptococcus laurentii* or the pathogen *Penicillium expansum* induces activities of Polyphenol Oxidase (PPO), Peroxidase (POD) and Superoxide Dismutase (SOD), whereas only *P. expansum* had a significant effect on the inducing of Malondialdehyde (MDA) content of the fruit. *C. laurentii* showed more efficiency in the promotion of PPO, POD and SOD activities of peach fruit than *P. expansum*.

Yu *et al*. (2006) used *C. laurantii* and gibberellic acid in *Penicillium expansum* control and indicated that combination of *C. laurantii* with gibberellic acid at 2000 μg mL^{-1} resulted in a rapid induction of peroxidase activity in pear fruit. Phenolic compounds act as substrates for number of oxidoreductase such as polyphenol oxidase and peroxidase. The first step of plant defense activities involves a rapid accumulation of phenols at the infection site, which restricts or slows the growth of pathogen (Robards *et al*., 1999).

The primary objective of this study was to confirm the ability of three isolates of *Pseudomonas fluorescens* to control post harvest gray mold rot caused by *B. mali* in apple fruit.

Another objective was to determine whether the activities of the Peroxidase (POD) and phenol compound would be affected in apple fruit tissue, following the application of three isolates of *Pseudomonas fluorescens* alone or in combination with *Botrytis mali*.

MATERIALS AND METHODS

Gray mold pathogen: Isolate of *Botrytis* sp. identified as *B. mali* Rheule (O'Gorman *et al*., 2005) designated Bm1 from *Malus domestica* Golden delicious from Ghazvin, Iran was used in this study. All cultures were derived from single spore isolate and maintained on potato dextrose agar at 4°C in darkness until needed.

Biological control isolates: Two hundred and ten *Pseudomonas* isolates were obtained from leaf surfaces and apple fruit with serial dilution methods on King's B medium (Scharlau, Barcelona, Spain). *Pseudomonas fluorescens* was identified by conventional biochemical bacterial tests (Schaad, 2001). Primary tests were Levan, Oxidase, Pectat lyase and Argenin dehydrolase.

These isolates were screened *in vitro* against *B. mali*. Three promising isolates were selected and evaluated as potential biological control agents for the control of gray mold in apple.

Fruit samples: *Malus domestica* Golden delicious apples that were harvested at commercial maturity and kept at 1.0±0.5°C in cold storage were used in this study. The apples were washed in 70% ethanol for 30 sec followed by dipping in 0.1% sodium hypochlorite solution and rinsed with Sterile Distilled Water (SDW).

***In vivo* biological control studies:** The bacterial inoculum was prepared in potato dextrose broth on a rotary shaker at 150 rpm min^{-1} for two days at room temperature. The bacterial cells were collected by centrifugation at 6500×g for 5 min and resuspended in water.

The fruits were wounded in triplicate with a 2.5 mm diameter nail to a depth of 3 mm.

Twenty microliter aliquots of each bacterial suspension (10^8 CFU mL^{-1}) or sterile distilled water were dispensed in each wound. The treated apples were placed on cardboard trays that were then enclosed in plastic bags. The inside of the bags was sprayed with Sterile Distilled Water (SDW) to maintain high relative humidity in the bags.

B. mali was grown on Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) plates (90 mm diameter) for 8 days. Conidia were harvested by pouring a few mililitre of Sterile Distilled Water (SDW) containing 0.05% Tween 20 on the plates. The conidial suspensions were adjusted to 1.0×10^5 conidia mL^{-1} using a hemacytometer. A 20 μL aliquot of conidial suspension or Sterile Distilled Water (SDW) was applied to each wound 24 h after inoculation with *P. fluorescens*. The apples were incubated at 20°C for 11 days.

Lesion diameters were measured after 6 and 10 days after pathogen inoculation, using calipers and lesion area was calculated.

Peroxidase activity: The method of extraction as described by Yiping *et al.* (2001) was used in the experiment.

Tissue samples from different treatments were collected at various times (2, 4, 6, 8 and 10 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 three apples randomly selected from each treatment.

Fresh apple tissue (5 g) was homogenized with 15 mL of 0.05 M phosphate buffer (pH 7.0) containing 10% polyvinil 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 16 min at 4°C. 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 assay mixture contained 0.1 mL enzyme extract, 1.35 mL 100 mM MES buffer (2-morpholino ethane sulfonic acid, monohydrate) (Sigma, St.Louis, USA) (pH 5.5), 0.05% H_2O_2 (Merck, Darmstadt, Germany) and 0.1% p- phenylenediamine (Merck, Darmstadt, Germany). Changes in absorbance were recorded at 485 nm for 3 min with a spectrophotometer (Milton Roy, Spectronic 501, Unterfoehring-Germany). The activity of peroxidase was presented as ΔOD_{485nm} min^{-1} mg^{-1} protein.

Assay of protein content: Total protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Determination of total phenol: The method of extraction as described by Yamomoto *et al.* (1977) was used in the experiments. Apple fruits (1.0 g fresh weight) were ground in a mortar with 8 mL of 80% methanol (Merck, Darmstadt, Germany) and filtered through a double layer of gauze. The residue was washed twice with 80% methanol (each time with 3 mL). The filtrate and washing were combined and centrifuged at 4000 g for 5 min at room temperature and the supernatant was used in the assayed.

Total phenol was determined with Folin-Ciocalteu's reagent (Merck, Darmstadt, Germany). Five milliliter 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 one hour, the absorbance was measured at 725 nm. Caffeic acid (Fluka, Germany) was used as a reference phenolic compounds. The total phenolic compounds of samples were expressed as mg caffeic acid per g of fruit fresh weight.

Statistical analysis: Enzymatic 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 $p<0.05$ (Little and Hills, 1978).

RESULTS

Biocontrol activity: Three isolates of *Pseudomonas fluorescens* were effective in controlling decay of apple fruit caused by *Botrytis mali*. They prevented *B. mali* decay from 0.5 to 4.4 cm^2 compared with 12 cm^2 in control 10 days after pathogen inoculation at 20°C.

Lesion size was from 0 to 0.6 cm^2 for antagonistic treatment and 2.4 cm^2 for the control treatment after 6 days (Table 1).

Peroxidase activity: Peroxidase activity in bacterial inoculated fruit in the presence of the pathogen showed

Table 1: Decay area of Golden delicious apples inoculated with *Botrytis mali* (Bm_1) after treatment with *Pseudomonas fluorescens* and incubated for 6 and 10 days at 20°C

	Decay area (cm^2)	
Treatment	After 6 days	After 10 days
Bm_1	2.4a	12a
$P.f_1$+Bm1	0c	0.5d
$P.f_7$+Bm1	0.6b	4.4b
7a+Bm1	0.1c	1.3c

Each treatment was replicated 4 times. Means followed by the same letter within a column do not differ significantly at $p<0.05$ according to Duncan's Multiple Range Test. Isolates of *P. fluorescens* are: $P.f_1$(A), $P.f_7$(B) and 7a (c)

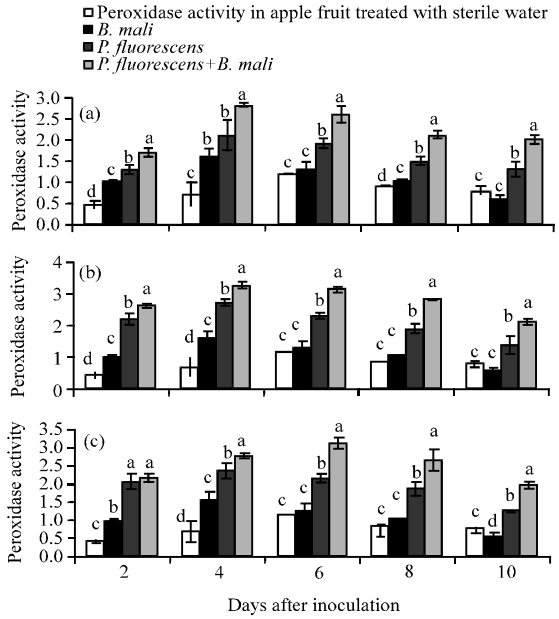

Fig. 1(a-c): Peroxidase activity in apple tissue in presence or absence of *Pseudomonas fluorescens* or *Botrytis mali*. Peroxidase activity in apple fruit treated with sterile water Isolates of *P. fluorescens* are :P.f_1 (a), P.f_7 (b) and 7a (c). The activity of peroxidase was presented as $\Delta OD_{485\ nm}$ /min/ mg protein and values are averages of 4 replicates. Treatments with the same letters, at 2, 4, 6, 8 and 10 days separately, do not differ significantly ($p<0.05$) according to Duncan' s Multiple Range Test Bars represent standard deviations

an increase, reaching maximum levels 4-6 days after treatment. At 96 h after treatment, peroxidase activity increased by more than 1.75-fold, above that observed in pathogen inoculated control tissue.

An increase in peroxidase activity was observed also in healthy control fruit but the level of increase was markedly lower than detected in treated fruits. In healthy control fruit, a comparatively small increase in peroxidase activity with time was detected.

Peroxidase activity of the bacterial inoculated fruits were elevated and reached the highest level 4-6 days after inoculation.

Peroxidase activity increased and reached a maximum 4 and 6 days after treatment in pathogen and healthy control, respectively.

At the end of the storage, peroxidase decreased rapidly in all treatments. Although peroxidase activity was decreased in inoculated fruits with combination of antagonist and pathogen, the level of peroxidase remained over 2.5-fold higher than the healthy control level. There was significant difference in peroxides activity between bacterial inoculated fruits in the presence of pathogen and each of other treatments at 2, 4, 6, 8 and 10 days separately (Fig. 1).

Phenolic compounds: Phenolic compounds in the bacterial inoculated fruit in the presence of the pathogen showed an increase and exhibited the highest level 6 days after pathogen treatment.

The phenolic contents of bacterial inoculated fruit reached a maximum level 2-6 days after inoculation.

Although Phenolic compounds decrease rapidly in all treatments 10 days after pathogen inoculation, they remained at least 1.9-fold higher than the healthy control level in bacterial inoculated fruit in presence of pathogen. There was a significant difference in phenolic contents between bacterial inoculated fruits in the presence of pathogen and each of the other treatments at 2, 4, 6, 8 and 10 days separately (Fig. 2).

DISCUSSION

The present data show that these three isolates of *Pseudomonas fluorescens* when applied as a wound treatment were effective in controlling post harvest decay of apple fruit caused by *B. mali*.

They also induced the accumulation of peroxidase and phenol compounds in apple fruit over and above the stimulation of them in wounded, non-inoculated fruits.

Comparable levels of decay control have been reported with other microbial antagonists (Ippolito *et al*., 2000; Wilson and Wisniewski, 1994; Bull *et al*., 1997; Chand-Goyal and Spotts, 1997; El-Ghaouth *et al*., 1998; Nigro *et al*., 1999) and attributed to a complex mechanism that may involve nutrient competition, site exclusion, direct parasitism, production of lytic enzymes and possibly induced resistance (Droby and Chalutz, 1994).

Here we used three isolates of *Pseudomonas fluorescens* and assayed the changes of peroxidase and total phenolic compounds which contribute to enhance the ability against pathogen infection. Peroxidase participates in wall-building processes such as oxidation of phenol, suberization and lignification of host plant cells during the defense reaction against pathogenic agents (Chittor *et al*., 1999).

Also peroxidase and antioxidant enzymes (superoxide dismutase and catalase) convert the potentially dangerous O_2 and H_2O_2 to water through their combined action. A balance of these enzymes provides

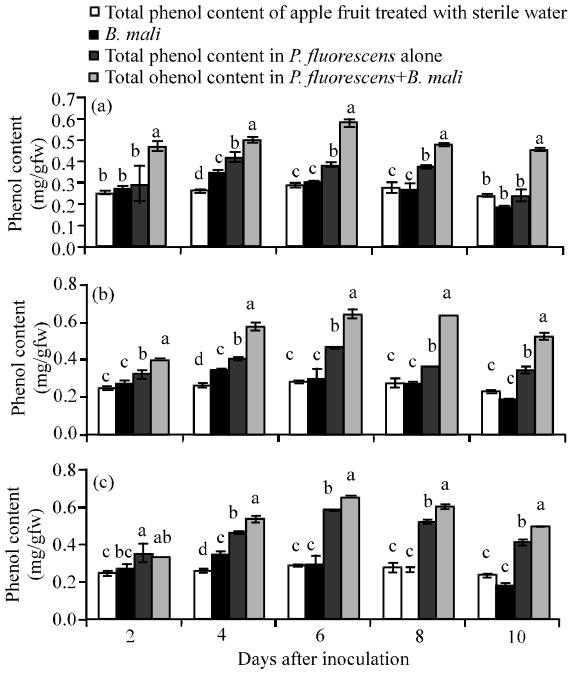

Fig. 2(a-c): Total phenol content of apple tissue treated with *Pseudomonas fluorescens* or *Botrytis mali*. Total phenol content of apple fruit treated with sterile water Isolates of *P. fluorescens* are Pf_1 (a), Pf_7 (b) and 7a (c). Total phenolic contents of the roots were expressed as mg caffeic acid g^{-1} of fruit weight and values are average of 4 replicates. Treatments with the same letters at 2, 4, 6, 8 and 10 days separately, do not differ significantly ($p<0.05$) according to Duncan' s Multiple Range Test Bars represent standard deviations

an efficient system to prevent oxidative damage (Yiping *et al*., 2001). High peroxidase activities are associated with the onset of induced resistance (Lurie *et al*., 1997; Prusky, 2003; Jung *et al*., 2004; Silva *et al*., 2004).

Ippolito *et al*. (2000) indicated that *Aureobasidium pullulans* caused a transient increase in chitinase, β-1,3-glucanase and peroxidase activity in apple fruit. Similarly in this study three isolates of *Pseudomonas fluorescens* caused increases in peroxidase activity, in apple fruit at 20°C.

The fact that the induction and accumulation of peroxidase is often correlated with the onset of induced resistance suggests an active role for this enzyme in defense against pathogenic fungi (Sticher *et al*., 1997; Van Loon *et al*., 1998). It is not possible, however, to determine the extent of the role played by host defense responses in the observed protection because of the antagonistic activity of *Pseudomonas fluorescens* at the wound site. The accumulation of peroxidase can be expected to retard fungal growth (Ippolito *et al*., 2000).

Induction of these defense-related reactions might indicate the recognition by the host tissue of *Pseudomonas fluorescens* isolates which might facilitate the formation of the appropriate defense mechanism in the fruit against the potential attacking pathogen because the success in the defense response depends on the speed at which the plant recognizes the attacking pathogen and on the intensity with which the appropriate defense mechanism is activated. If the plant fails to respond in time, the appropriate defense will be activated too late so that the pathogen can colonize the plant tissue (Kuc, 2001; Conrath *et al*., 2002; Ton *et al*., 2005).

7a isolate had the most peroxidase activities 2 days after pathogen inoculation.

Kagale *et al*. (2004) reported higher activity of Phenylalanine Ammonialyase (PAL), peroxidase, chitinase, β-1,3-glucanase and increase in level of phenol in rice leaves treated with *Datura metel* leaf extract and inoculated with *Rhizoctonia solani* or *Xanthomonas oryzae pv. oryzae*.

Similarly, Guleria and Kumar (2006b) reported higher activity of Phenylalamine 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 sesame*). It is suggested that neem leaf extract induced activity of PAL and peroxidase enzymes in sesame leaves, which resulted in increased biosynthesis and metabolism of phenol might have protected the sesame plant from *Alternaria sesami*.

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 pathogen, phenolic compounds are oxidized to form more toxic, quinines by peroxidases (Gogoi *et al*., 2001).

In this study, phenolic accumulation was increased in apple fruit treated with three isolates of *P. fluorescens* and inoculated with *B. mali*.

Peroxidase activity and level of phenolic compound in all treated fruit decreased in 11 days after bacterial inoculation, meanwhile, lesion diameter of fruit inoculated by *B. mali* and *P. fluorescens* expanded. This fact may

suggest that disease development is associated with decrease in disease resistance of fruit (Wang *et al*., 2004).

In conclusion, the present results show that three isolates of *P. fluorescens* have potential as a biocontrol agent for the control of post harvest decay of apple caused by *B. mali* and are capable of inducing the accumulation of peroxidase and increase in level of phenolic compound and suggests that post harvest treatment with *P. fluorescens* hold promise as a new technology, substituting for fungicidal control of post harvest diseases in apple fruit.

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