J. Crop Prot. 2016, 5 (3): 349-357

Research Article

Involvement of protective enzymes and phenols in decay (*Penicillium expansum*) resistance in apple

Tahmineh Naeem-Abadi[1] and Mansureh Keshavarzi[2*]

1. Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran.
2. Horticultural Research Institute, Karaj, Iran.

Abstract: Blue mold disease caused by *Penicillium expansum* is a major post-harvest disease of apples. In this research, the biochemical basis of apple resistance to this pathogen was studied in two relatively resistant and susceptible cultivars, Granny smith and Mashhad, respectively. The activities of catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and polyphenol oxidase (PPO) enzymes and polyphenol content were compared at different time intervals of 0 to 7 days. Based on the results, fruit polyphenol content of Granny smith was higher than that of Mashhad PPO, SOD and CAT activity was higher in Granny smith than Mashhad but CAT activity decreased three days post-treatment. No detectable difference was found in POX activities in the two cultivars. It is concluded that polyphenols contribute in apple resistance to blue mold. Activation of PPO and SOD, lack of POX activity and decrease of CAT activity, all together, could lead to a toxic environment around the blue mold fungus.

Keywords: *Penicillium expansum*, blue mold, apple, pathogenesis-related protein

Introduction

Some reported values of disease losses show that approximately 10-30% of harvested fresh horticultural crops is lost due to post-harvest spoilage in developed countries, whereas losses are even greater, up to 10-50% in developing countries in which sanitation and refrigeration are lacking or minimal (Eckert and Ogawa, 1985). More than 90 fungal species have been described as causal agents of apple decay during storage (Jones and Aldwinckle, 1990). Blue mold decay caused by *Penicillium expansum* Link. is the most important post-harvest diseases of apple worldwide (Pierson *et al*., 1971). It also produces Patulin, a mutagenic, immunotoxic and neurotoxic mycotoxin which is particularly noxious in apple juice industry (Bracket and Marth, 1979).

Control of post-harvest pathogens relies on the use of synthetic fungicides, biological control, sanitation and various physical treatments (*e.g*., heat, UV radiation) (Janisiewicz and Korsten, 2002; Lurie, 1998; Wilson and Wisniewskim, 1994). Current reports also indicate some differences in relative susceptibility levels of apple cultivars against post-harvest decays, and opening a new venue to use decay resistant cultivars as a control measure (Cappellini *et al*., 1987; Janisiewicz and Peterson, 2004; Spotts *et al*., 1999; Janisiewicz *et al*., 2008, Naeem Abadi *et al*., 2014).

Obviously, it is valuable to study the interactions between post-harvest decay agents and apple fruit during post-harvest storage.

Handling Editor: Vahe Minassian

[*] **Corresponding author**, e-mail: kmansureh@gmail.com
Received: 9 August 2015, Accepted: 1 April 2016
Published online: 28 May 2016

Although harvested products also possess inducible defensive responses, this potential has not received the attention it deserves. Activating biochemical defense responses in harvested tissue through prestorage treatment with UV light (Mercier e*t al*., 1993; Rodov *et al*., 1994, Lu *et al*., 1999) and antagonistic yeasts (Droby *et al*., 1994; El Ghaouth *et al*., 2003) suggest that intensification of defense mechanisms has potential in reducing post-harvest decay. Results, which are consistent with the temporal and spatial induction of pathogenesis-related (PR) proteins (Sticher *et al*., 1997), show that apple fruits are capable of responding to microbial attack as seen in other crops. In several plant-pathogen interactions, the induction and accumulation of PR proteins are often correlated with the onset of induced resistance (Ryalls *et al*., 1996; Sticher *et al*., 1997). Polyphenol oxidase (PPO) is one of PR proteins abounding with and accounting for resistance in a variety of plant taxa (Zhu *et al.,* 2008; Raj *et al*., 2006). They act in defense by generating toxic quinones and forming physical barriers to pathogen by cross-linking plant cell wall proteins. Peroxidase (POX) can also contribute to plant defense by decomposing H_2O_2 in lignification and suberization processes, leading to strengthening plant cell wall around invading pathogen (Zhulong and Shiping *et al*., 2006). Superoxide dismutase (SOD) catalyses dismutation of O_2^- to H_2O_2, leading to H_2O_2 accumulation around pathogen (Torres *et al*., 2003). Changes in phenolic content is also considered as part of plant defense responses. They may be readily polymerized by oxidation and the oxidized forms can restrict lesion formation associated with invading pathogen (Lattanzio *et al*., 2001). The objective of this research was to study contribution of PPO, SOD, CAT and POX enzymes in resistance of apple fruit to *P. expansum*.

Materials and Methods

P. expansum isolate and inoculum

A local *P. expansum* strain already isolated from a decaying apple fruit was used (Naeem-Abadi *et al*., 2014). The isolate was maintained on acidified potato dextrose agar (1.5 ml per litre lactic acid) slants at 25 ºC. Spore suspension was prepared from a 7-day-old culture with sterile distilled water containing 0.5% Tween-80 and mixed for 1 min to break spore chains. Spore concentration was determined with a haemocytometer and adjusted to obtain 5000 spores per ml to be used as inoculum (Spotts *et al*., 1999).

Fruit inoculation

Fruits were harvested from Apple Collection Orchard of seed and Plant improvement Institute, Karaj, at commercial maturity dates. Based on our previous survey, Mashhad genotype and Granny smith cultivar were used as susceptible and resistant control, respectively (Naeem Abadi *et al.*, 2014). Mature fruits were surface disinfected with 70% ethanol and rinsed twice with sterile water, each fruit was wounded with a metal device (3 mm in diameter, 3 mm depth) at the equatorial region. The pores were filled with 20 µl inoculums, placed in humid polyethylene bags at 25 °C and lesion diameter was recorded after 10 days (Spotts *et al*., 1999). Control fruits were not inoculated. The experiment was conducted in a completely randomized design in 3 replicates.

Polyphenol measurement

For Polyphenol extraction, fruit flesh tissue (10.0g) was homogenized in 100ml of ice-cold 80% acetone. The homogenate was filtered through two layers of microcloth and centrifuged at 6,000 rpm for 30 min. The extraction was repeated twice and the supernatants combined and used as polyphenol source. The total phenolic contents were measured using colorimetric Folin-Ciocalteu method (Singleton *et al*., 1999). A volume of 1.58 ml deionized water and 20 µl extract were mixed with 100µl Folin-Ciocalteu reagent (Merck) and allowed to react for 3 min. Then, 500 µl 20% sodium carbonate solution was added and the color developed after 120 min was read at 650 nm. The measurement was compared to a standard curve prepared for gallic acid solutions (50, 100,

150, 250 and 500 mg/l) and expressed as milligrams of gallic acid equivalents per 100 g fruit flesh for the triplicate extracts.

Enzyme extraction

Approximately 10 g fruit, 2 mm away from infection site, was sampled using a knife at various time intervals (1, 3, 5 and 7 days after treatment), homogenized in 25 ml ice-cold 100 mM phosphate buffer pH 6.4 containing 0.5 g polyvinyl pyrrolidone (PVP). The homogenate was then centrifuged at 6000 rpm for 50 min in a refrigerated device and the supernatant used for determining activities of POX, PPO and SOD enzymes. For CAT extraction, the same method was used in 0.05 M phosphate buffer (pH 7.0).

Enzyme activity

For POX assessment, 0.5 ml crude extract was added to 2 ml of 100 mM phosphate buffer (pH 6.4) containing 8 mM guaiacol and after 5 min incubation at 30º C, 1 ml of 24 mM H_2O_2 was added to solution. The POX activity was determined by measuring the increase in absorbance at 460 nm for 4 min and the specific activity was expressed as ΔOD_{460}/min/mg protein (Jiang *et al*., 2002).

For CAT activity Beers and Sizer (1952) method was used. Based on this method, 0.5 ml crude extract was added to 2 ml of 50mM phosphate buffer (pH7.0) and mixed with 0.5 ml of 40 mM H_2O_2 and the decline in absorbance at 240 nm was determined for 2 min. The specific activity was expressed in units per mg protein, where one unit of catalase converts one µmol of H_2O_2 per min. Wang *et al.* (2004) method was used for PPO assessment. Briefly, 0.5 ml of enzyme preparation was incubated in 3.0 ml 100 mM phosphate buffer (pH 6.8) containing 500 mM catechol (standard analytical grade) at 30 ºC and the change in absorbance at 398nm was determined for 10 s. The specific activity was expressed as ΔOD_{398}/min/mg protein. For SOD estimation, 0.1 ml crude extract was added to 3 ml 50 mM phosphate buffer (pH 7.8) containing 13 mM methionine, 75 µM nitro-blue tetrazolium (NBT), 10 µM EDTA and 2 µM riboflavin (Wang *et al*., 2004). The reaction was illuminated by placing the solutions in 30 cm distance from a 15W fluorescent lamp for 10 min and the absorbance was then determined at 560 nm. Identical solutions held in the dark served as blanks. The specific activity was expressed as units per mg protein where one unit of SOD was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibitable NBT reduction. All enzymatic activity data represent the mean of three individual fruits at each time course. Fruit-soluble protein content was determined according to Bradford method (1976) with bovine serum albumin (Sigma) as standard.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) with SAS 9.0 software. Duncan's Multiple Range Test ($P \leq 0.05$) to separate means was applied to compare treatments.

Results and Discussion

Polyphenol content

Polyphenol content of the challenged fruits by *P. expansum* was generally higher than the control (healthy) fruits (mean 46.5 and 36.2 mg gallic acid equivalents/100g fruit, respectively, $P \leq 0.01$), indicating *P. expansum* inoculation of apple increased phenolic amounts. Higher polyphenol content in healthy tissues than infected grapevines tissues with *Uncinula necator* has been reported by Baruah and Chowfla (1994), while Kumar (1991) and Sharma and Chowfla (1991) reported higher amounts of polyphenols in virus-infected than in healthy *Amaranthus caudatus* L. tissues. In Golden delicious apples, phenolic content was higher in infected tissues surrounding the rotten zone caused by *Phlyctema vagabunda* compared to healthy tissue of the same fruit (Lattanzio *et al*., 2001). Apparently, the first stage of apple defense involves rapid accumulation of phenols at infection site, which slow down the growth of the pathogen.

Lattanzio *et al*. (2001) showed that in cold stored Golden Delicious apples, when rot caused by *P. vagabunda* appears in infected tissues surrounding the rotten zone, a general increase in phenolic levels was observed, compared to a healthy tissue of the same fruit.

In *P. expansum*-infected fruits, polyphenol content differed between the two cultivars studied. It has been already demonstrated that apple phenolic contents is dependent on many factors including cultivar, maturity stage, environmental conditions and the part of the fruit used (Drogoudi Wolfe *et al*., 2003; D'Abrosca *et al*., 2007).

The polyphenol content was higher in resistant (Granny smith) than the susceptible cultivar (Mashhad) (mean 58.2 and 35.3 mg gallic acid equivalents/100g fruit, respectively, $P \leq 0.01$) suggesting polyphenols acontribute to apple protection against invading stimuli. Polyphenols are involved in many defense responses. They play an important role in restricting lesion formation associated with the brown rot disease of resistant apple varieties to *Sclerotinia fructigena* (Byrde *et al*., 1960) and in the defense of apple leaves against the scab fungus *Venturia inaequalis* (Mayer *et al*., 1997).

CAT, SOD, POX and PPO activities

Activity of all enzymes in healthy non-inoculated fruits remained unchanged and was lower than those of the *P. expansum*-infected fruits, indicating activation of apple defense reponses upon encountering external stimulus via inoculation. PPO activity was higher in Granny smith than Mashhad (Fig. 1). It continuously increased in Granny smith but declined after five days post-inoculation in Mashhad, indicating it possibly was a key element in apple defense against blue mold. Contribution of PPO enzyme in defense responses has been described in a variety of plant taxa (Liu *et al*., 2005; Cao *et al*., 2005; Zhu *et al.,* 2008). Direct evidence for its role in defense comes from PPO-overexpresing transgenic tomato plants with reduced *Pseudomonas syringae* pv. *tomato* growth whereas PPO antisense-suppressed lines supported greater bacterial numbers (Li and Steffens, 2002). PPOs are ubiquitous copper-containing enzymes which use molecular oxygen (O_2) to oxidize common *ortho*-diphenolic compounds such as caffeic acid and catechol to their respective quinones. They can function in defense through: (1) general toxicity of PPO-generated quinones to pathogens and plant cells, (2) alkylation and reduced bioavailability of cellular proteins to the pathogen, (3) cross-linking of quinones with protein or other phenolics, forming a physical barrier to pathogens in the cell wall, and (4) quinone redox cycling leading to H_2O_2 and other reactive oxygen species (via Li and Steffens, 2002).

Changes in phenolic content and PPO activity may be considered as a part of defense response of apple cells to blue mold. There are many phenolic compounds present in plant tissues with no anti-microbial activity *per se* but the oxidation products of these pre-existing phenolics might have antimicrobial activity through inhibition of the cell wall degradation by extracellular enzymes produced by pathogens (Lattanzio *et al*., 2006). In addition, many simple, low molecular phenolic compounds present in plants may be readily polymerized by oxidation and the oxidized phenolics products, can restrict lesion formation associated with pathogen invasion (Lattanzio *et al*., 2001). Post-infectional accumulation of preexisting phenolics, provides an adequate substrate to the increased PPO activity. PPO, consuming oxygen and producing fungitoxic quinones, makes the medium unfavorable for further development of pathogens. Phenolic oxidation products could have an antifungal action by polymerizing and forming a protective seal on cell wall and decreasing nutrients essential for the fungal development. Oxidized phenolics in resistant varieties of apple play an important role in the restricted lesion formation associated with the brown rot disease of fruits caused by *Sclerotinia fructigena* Aderh. (Byrde *et al*., 1960) or with rotting of stored Golden Delicious apples caused by *Phlyctaena vagabunda* (Lattanzio *et al*., 2001).

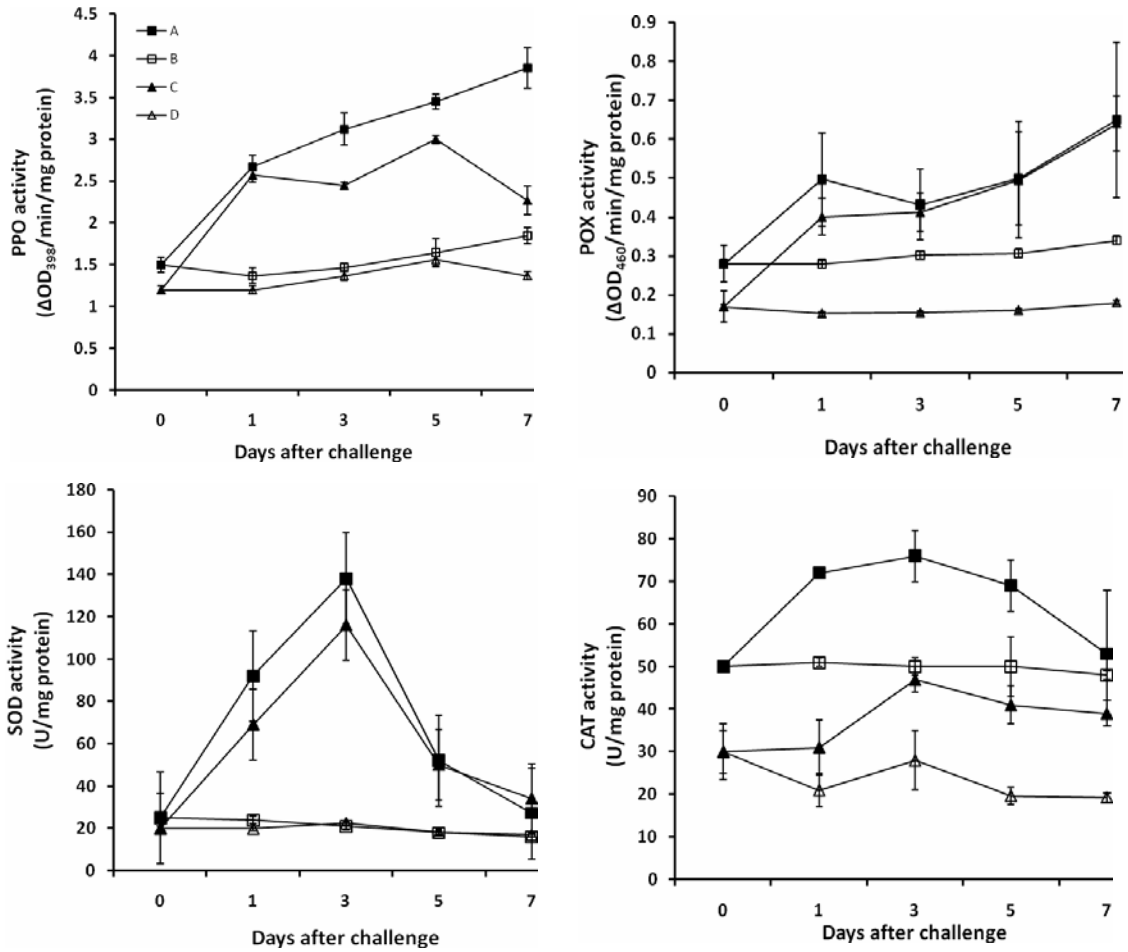

Figure 1. Changes in PPO, POX, CAT and SOD activities, during time course of 7 days, in apple fruits inoculated with *Penicillium expansum*. Data means ± S. E. of three replicates. A: Granny smith fruit inoculted with *P. expansum*; B: healthy Granny smith (control); C: Mashhad fruit inoculted with *P. expansum*, D: healthy Mashhad fruit (control).

POX activity remained unchanged during the time course of the study and was not different between the two cultivars indicating it was not a key element in apple defense against blue mold. In a number of studies, it has been demonstrated that POX contributed to plant defense by decomposing H_2O_2 in lignification and suberization processes, leading to strengthening of plant cell wall around pathogen (Zhulong and Shiping *et al*., 2006). On the other hand, lack of POX activity can lead to H_2O_2 accumulation and creation of a toxic environment around invading pathogen (Mittler, 2002).

Although CAT activity was higher in Granny smith than Mashhad, it started to decrease three days post-treatement. CAT cleaves H_2O_2 to oxygen and water. It is possible that early elicitation of CAT activity was blocked in successive stages of disease development, leading to accumulation of toxic H_2O_2 around the pathogen. Similarly, a defense elicitor (BTH), acted in part by inhibiting CAT activity in peach, pear and mango fruits leading to accumulation of toxic H_2O_2 against development of invading pathogens (Liu *et al*., 2005, Zhu *et al*., 2008; Cao *et al*., 2005).

SOD activity was generally higher in Granny smith than Mashhad. It increased during the time course of the study, reached its peak value three days after treatment and started to decrease afterward. SOD catalyses dismutation of O_2^- to H_2O_2. In a disease-resistant apple fruit, elevation of SOD activity and H_2O_2 level was observed and was suggested that SOD activation leads to accumulation of H_2O_2 around pathogen. In apple cv. Golden delicious challenged with blue mold, an increased level of SOD activity was associated with a significant increase in H_2O_2 while POX and CAT activities remained unchanged (Torres *et al*., 2003).

Overall, comparison of activities of four defensive enzymes between resistant and susceptible aaple cultivars indicated that activation of PPO and SOD and accumulation of phenolic compounds are involved in apple defense against blue mold. Our result are consistent with those of Torres *et al*. (2003) indicating a role of H_2O_2 in apple defense against blue mold through activation of SOD and PPO concomitant with CAT inactivation. H_2O_2 is therefore suggested as being a key element in apple defense against *P. expansum* (Torres *et al*., 2003).

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مشارکت آنزیم‌های دفاعی و فنل کل در مقاومت سیب علیه کپک آبی (*Penicillium expansum*)

تهمینه نعیم‌آبادی[1] و منصوره کشاورزی[2*]

۱- دانشگاه ولیعصر رفسنجان، رفسنجان، ایران.
۲- مؤسسه تحقیقات باغبانی، کرج، ایران.
* پست الکترونیکی نویسنده مسئول مکاتبه: kmansureh@gmail.com
دریافت: ۱۸ مرداد ۱۳۹۴؛ پذیرش: ۱۳ فروردین ۱۳۹۵

چکیده: کپک آبی با عامل *Penicillium expansum* بیماری انباری مهمی در سیب است. در این تحقیق، مبنای بیوشیمیایی مقاومت به این قارچ در دو رقم سیب مقاوم و نسبتاً حساس، به‌ترتیب گرانی اسمیت و مشهد، بررسی شد. فعالیت آنزیم‌های کاتالاز، سوپراکسیددسموتاز، پراکسیداز و پلی‌فنل اکسیداز و محتوای پلی‌فنلی میوه دو رقم در طول یک دوره مطالعاتی بررسی شد. براساس نتایج، محتوای پلی‌فنلی میوه رقم گرانی اسمیت بیش از مشهد بود. فعالیت پلی‌فنل اکسیداز، سوپراکسید دسموتاز و کاتالاز در گرانی اسمیت بیش از مشهد بود اما فعالیت کاتالاز از روز سوم پس از آلوده‌سازی شروع به کاهش کرد. تفاوتی در فعالیت پراکسیداز بین دو رقم مشاهده نشد. بر این اساس نتیجه‌گیری می‌شود که پلی‌فنل‌ها در دفاع سیب علیه کپک آبی نقش دارند. فعالیت پلی‌فنل اکسیداز و سوپراکسیددسموتاز، عدم فعالیت پراکسیداز و کاهش فعالیت کاتالاز همگی در ایجاد محیطی سمی در اطراف کپک آبی نقش دارند.

واژگان کلیدی: کپک آبی، سیب، پروتئین مرتبط با بیماریزایی، *Penicillium expansum*