Low Temperature Metabolism of Apple Phenolics and Quiescence of *Phlyctaena vagabunda*

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The content of chlorogenic acid, (+)-catechin, (−)-epicatechin, phloretin glycosides, and quercetin glycosides in fresh and stored Golden Delicious apples (*Malus domestica* Borkh) was determined. The relative amount of phenolics in the peel, with the exception of chlorogenic acid and (−)-epicatechin, was higher than that in the flesh. In addition, quercetin glycosides were detected only in the skin. These compounds were tested for fungicidal activity against *Phlyctaena vagabunda* Desm., the causal agent of a postharvest rot. Chlorogenic acid only inhibited *P. vagabunda* spore germination and mycelial growth in vitro. Changes of apple phenolics and polyphenol oxidase activity during cold storage and the biological activity of these phenolics have also been analyzed with reference to the development of quiescent infections during cold storage plus shelf life at room temperature. The results obtained suggested that phloridzin and chlorogenic acid in combination with polyphenol oxidase activity could function to arrest *P. vagabunda* in quiescent infections associated with immature and ripening apple fruit.

Keywords: *Postharvest rot; phenolics; polyphenol oxidase; quiescent infections; disease resistance; storage*

INTRODUCTION

Rotting of stored apples (*Malus domestica* Borkh) by *Phlyctaena vagabunda* Desm. (syn. *Gloeosporium album* Osterw.) is an important source of wastage at the present time. Postharvest losses of fruits, depending upon the varietal degree of susceptibility to attack by the pathogen, the length of storage, and the efficiency of the marketing systems, have been estimated to be more than 30% when environmental conditions promote microorganism diffusion (*1*). Conidia of *P. vagabunda* are produced by small infections on the wood of the tree throughout the year and are spread by rain and dew on the fruit, which is thus exposed to infection during the whole of the growing season. There are conditions, depending on the fungus and the nature of vegetable tissue, in which infections, which take place in lenticels, can develop in storage to produce lesions. Normally, the susceptibility to attack by *P. vagabunda* is directly related to the length of the storage period (*2*−*4*).

The term "quiescent infection" is used to describe a limited development of the infection. No lesions are produced while the fruit is on the tree. When picked, however, the fruit, depending on the physiological stage and the storage conditions, can undergo decay quite quickly. To be successful, a pathogen must also be able to overcome host defenses and initiate attack under prevailing physiological and environmental conditions (*4*). The problem of the nature of the resistance then lies in determining which factors are responsible for arresting the development of the fungus at the time of the initial infection and, in addition, finding which changes taking place prior to the onset of rotting enable the fungus to resume growth (*5*).

In view of the research dealing with the fungistatic properties of phenolics (*6*−*8*) and, in particular, with the role of these substances against fungal pathogens such as *Venturia* sp., *Gloeosporium* sp., *Sclerotinia fructigena*, and *Botrytis cinerea* in apple (*3*, *9*, *10*), it was decided to examine the phenolics of apples in relation to susceptibility to attack by *P. vagabunda*. It is, in fact, well-known that apple fruits contain, besides quercetin glycosides, catechins and chlorogenic acid, a dihydrochalcon-*O*-glucoside, phloridzin, whose oxidation products may be involved in the defense mechanism of apple leaves against the scab fungus *Venturia inaequalis* (*11*−*17*). During the maturation of Golden Delicious apples, an increase of internal ethylene levels and phenylalanine ammonia lyase (PAL), the key enzyme of phenolic biosynthesis, has been observed: the onset of ethylene production did occur at approximately the same time as an increase in PAL activity (*18*, *19*). It has been suggested that ethylene produced by the host specifically at ripening may act as a signal to terminate pathogen quiescence on the fruit surface (*20*). Finally, it has been observed that low storage temperature

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stimulates PAL activity during the storage of fruits and vegetables, thus increasing their antibiotic phenolic content (*21*−*23*). When developmental changes in the phenolic content of apple skin are considered, there is a general agreement that phenolic levels decrease during the fruit development and remain relatively constant during fruit maturation (*24*, *25*). On the contrary, literature data concerning changes in phenolic compound during cold storage are much more contradictory (*12*, *24*, *26*, *27*).

The objectives of this study were (i) to describe the effect of low nonfreezing temperatures on the fate of apple phenolics during cold storage, (ii) to evaluate the antifungal activity of preformed and oxidized phenolics against *P. vagabunda*, and, finally, (iii) to discuss these results with reference to the development of infection during storage, occurring after a latent infection period, to improve the knowledge of defense mechanisms against fungal pathogens, and, consequently, to improve the postharvest management of apple fruits.

MATERIALS AND METHODS

Plant Material. Apple fruits of the cv. Golden Delicious (*Malus domestica* Borkh), which is considered a cultivar susceptible to *Phlyctaena* postharvest rot, harvested at the beginning of October, were stored at 2 °C. Analysis for cinnamic acid derivatives and flavonoids were carried out on fresh and stored samples. Stored samples comparable with the ones analyzed were inoculated with spores of *P. vagabunda* 95 days after the harvest, a period long enough to increase the susceptibility to rotting, and then analyzed for phenolics from time to time. While stored inoculated samples were analyzed, at the same time a comparable sample was transferred at 24 °C for 8−11 days and then analyzed.

Analysis of Phenolic Compounds. Chromatographic analyses were carried out on apple "peel" and flesh. The term peel in this context means tissue prepared from the fruit peeled with a household potato peeler. The strips of peel were about 1 mm thick and consisted of cuticle, epidermis, and subepidermal chlorenchyma. Thirty grams of plant material were homogenized with hot MeOH−EtOH (1:1) and then refluxed under nitrogen for 1 h. After centrifugation, the solution was first concentrated under vacuum, depigmented with petroleum ether (bp 40−70 °C), and then qualitatively and quantitatively analyzed for phenolics by HPLC.

HPLC analyses were performed with a Perkin-Elmer series 4 liquid chromatograph, which was equipped with a fluorimetric detector PE-LS3 and a computer aided Hewlett-Packard spectrophotometric photodiode array detector 1040-A, following the method of Lattanzio and Van Sumere (*22*). In all cases, phenolics were subjected to UV spectroscopy and chromatographic comparison against authentic samples by means of an HP-K3 software postrun analysis coupled with PE-Chromatographics 2 software. The pilot signal to the spectrophotometric detector was set at 280 nm and the fluorimetric detector was set at 285 nm (excitation) and 320 nm (emission). An analytical Waters (Milford, MA) column (300 × 4 mm i.d.) packed with μBondapak C18 (10 μm) was used throughout this work. The solvent system consisted of (A) MeOH and (B) acetic acid−water (5/95; v/v). The elution profile was 15−40% A in B for 0−25 min; 40% A in B (isocratic) for 25−30 min; 40−63% A in B for 30−45 min; 63% A in B (isocratic) for 45−47 min; 63−99% A in B for 47−51 min. The flow rate was always 1 mL/min. Samples of 6 μL were applied to the column by means of a 6 μL loop valve.

Apple Polyphenol Oxidase. A crude extract of apple polyphenol oxidase (PPO) was prepared by macerating apple tissues in a blender for 10 min with (2.5 mL/g) cold acetone (−15 °C). The suspension was centrifuged, and the residue was twice homogenized with cold acetone. The acetone powder was extracted, in the presence of insoluble poly(vinylpyrrolidone), with Mc Ilvaine's buffer pH 4.5 (5 mL/g of original tissue wt). Enzyme activity was determined polarographically according to Lattanzio et al. (*23*).

Bioassays. A virulent strain of *P. vagabunda* isolated from infected apple fruits was maintained on potato-dextrose agar (PDA: 2% dextrose, 1.5% agar) under light at 21 °C. Phenolic compounds used for bioassays were purchased from Extrasynthèse (Genay, France).

The quantification of mycelium growth in agar-glucose culture was used to determine antifungal activity of phenolics. The nutrient solution contained 1.5% agar, 2% glucose, and solvent for phenolics (MeOH 0.5%). Medium (10 mL), containing an appropriate amount (10^{-3} M) of the particular phenolic compound, was "filter sterilized" using a Millipore filter (0.45 μm), transferred to a Petri dish, and then inoculated with a small mycelial plug (6 mm in diameter), obtained from the actively growing margin of the fungal culture on PDA medium, and placed in the center of the test plate. Plates were incubated in the dark at 21 °C until the mycelium of the control plate (containing no test phenolics) had almost filled the Petri dish (about 10−15 days). Growth rate was evaluated as the average diameter of the mycelial mat following incubation.

For spore germination studies, 5-day-old cultures of *P. vagabunda* maintained on PDA plates was used to prepare a conidial suspension (6×10^5 conidia/mL) in sterile 0.1 M sodium citrate buffer, pH 5. One milliliter of the conidial suspension was diluted in a 200-mL Erlenmeyer flask with 20 mL of 0.1 M citrate buffer, pH 5, containing 1% glucose and 10^{-3} M of phenolic compound to be tested. Samples were incubated in the dark at 21 °C for 48 h. After 24 h of incubation, 1 mL of the crude apple polyphenol oxidase was added to the sample (1 mL of buffer in the control flask). Following incubation, 100 μL of spore suspension was seeded in PDA plates to evaluate the percentage of germinated spores.

The bioassays were repeated twice with three duplicates for each test compound, and the results were averaged.

RESULTS AND DISCUSSION

The phenolics in apple flesh, in good agreement with previous literature data (*24*, *25*, *27*), consisted of (+)-catechin, chlorogenic acid, (−)-epicatechin, phloridzin (phloretin 2′-*O*-glucoside), and an unidentified phloretin 2′-*O*-glycoside. This latter phloretin derivative was provisionally identified because of its spectra characteristics. In addition, their lower retention time indicated a more polar structure, which was identified as phloretin 2′-*O*-xylosylglucoside. Apple peel contained an additional five or six quercetin glycosides (3-*O*-galactoside (hyperin), 3-*O*-glucoside (isoquercitrin), and/or 3-*O*-rutinoside (rutin), 3-*O*-xyloside, 3-*O*-arabinoside, and 3-*O*-rhamnoside (quercitrin)). Apple peel also contains ferulic acid and *p*-coumaric acid derivatives.

During the first 60 days of cold storage, when fruits are rot-free, the concentrations of phenolics in the skin of Golden Delicious apples showed a relevant biosynthetic increase (Figure 1). Afterward, fruit stored for a longer period had a gradually decreasing content of phenolics, particularly phloridzin. This decrease was more pronounced after 10 days of shelf life at room temperature, and these quantitative differences are due to the effect of the temperature on the enzyme systems of the phenolic metabolism. However, at the end of the storage period the content of phenolic compounds in apple skin was comparable or higher than that found at the harvest. From these results, it is clear that even with fruit changed in storage, thus becoming quite susceptible to fungal attack, a significant decrease in phenolic content in the skin of the fruit was not recorded. In addition, among the phenolic compounds identified in apple peel, only chlorogenic acid was found to be an in vitro inhibitor of spore germination or

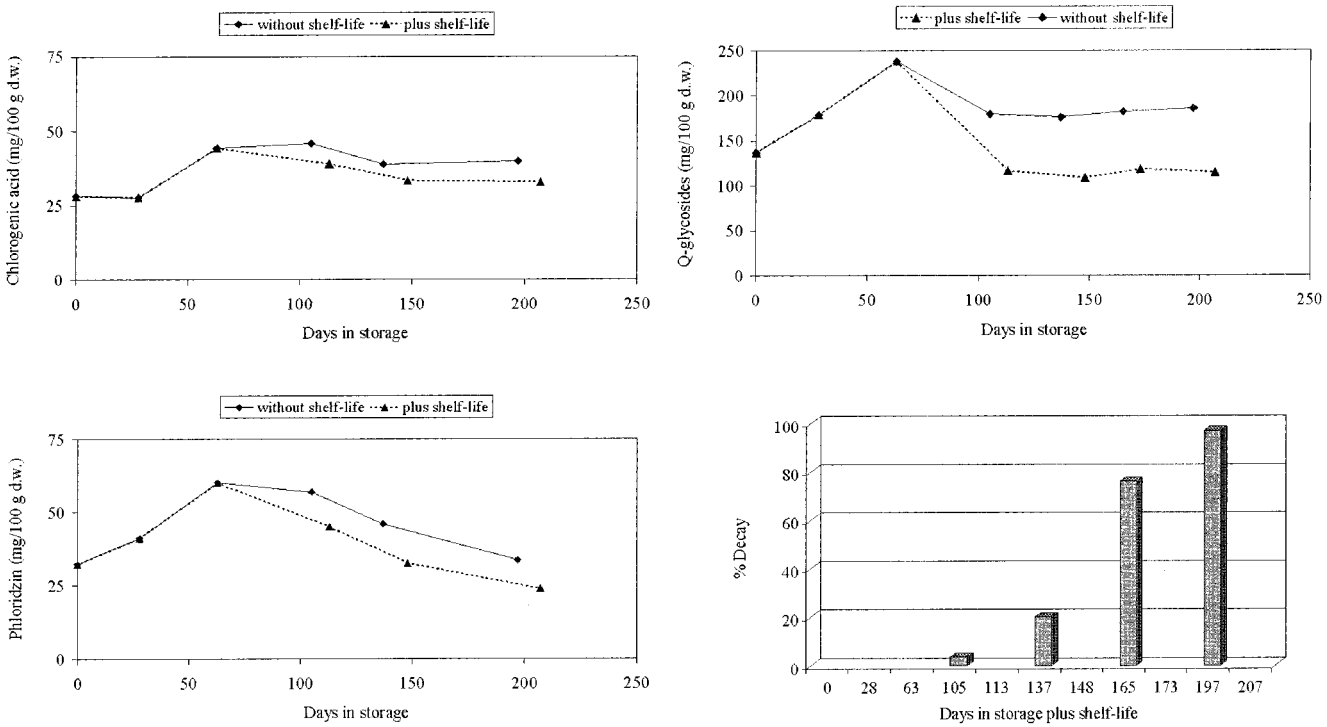

Figure 1. Changes in preexisting phenolics and decay losses in Golden Delicious apples stored at 2 °C plus shelf life at room temperature.

Table 1. Changes in Phenolic Compounds (mg/100 g d.w.) in Infected Tissues Surrounding the Rotten Zone of Apple Peel

compound	healthy tissue	infected tissue
(+)-catechin	31.1	49.4
chlorogenic acid	22.5	47.2
(−)-epicatechin	39.7	66.6
phloretin 2′-*O*-xylosylglucoside	27.1	44.4
quercetin 3-*O*-galactoside	26.7	42.6
quercetin 3-*O*-glucoside + quercetin 3-*O*-rutinoside	11.6	18.0
quercetin 3-*O*-xyloside	8.1	13.0
phloridzin	22.6	37.9
quercetin 3-*O*-arabinoside	17.2	27.6
quercetin 3-*O*-rhamnoside	12.4	22.1

mycelial growth of *P. vagabunda*, while (+)-catechin, (−)-epicatechin, phloridzin, and quercetin glycosides showed no activity. It has been suggested (*16*, *28*) that phloridzin can be hydrolyzed in vivo by various fungi such as *Venturia inaequalis*, *Pennicillium* spp., *Fusarium* spp., and *Aspergillus* spp. to give phloretin, which, in turn, is degraded to phloroglucinol, phloretic acid, and *p*-hydroxybenzoic acid which inhibits the development of the fungus. However, in vitro bioassays of these catabolic phloridzin derivatives showed no inhibitory activity on fungal mycelial growth of *P. vagabunda*.

These data concerning changes in phenolic compounds and rotting of cold stored apples are in good agreement with that of Nevoroske and co-workers (*14*, *15*) concerning the relationships between phloridzin content and resistance to *Venturia inaequalis* in apple leaves. These authors suggested the hypothesis that phloridzin oxidation in infected tissues, instead of preexisting phenols, plays an important role in host resistance and that the mechanism of resistance may be governed by the hypersensitive responses linked to the host−pathogen interaction. Therefore, it was decided to examine the phenolic metabolism in cold stored

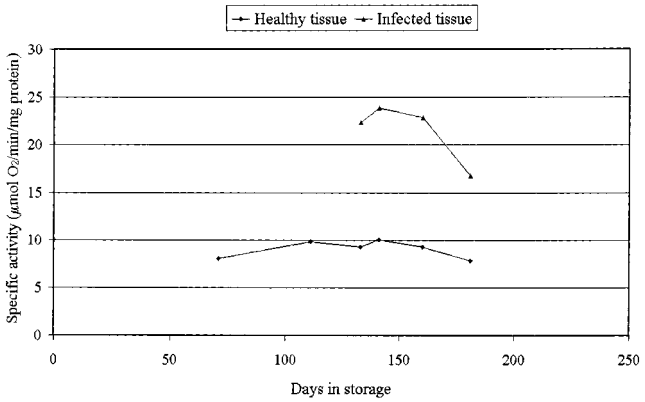

Figure 2. In vivo changes of PPO activity in healthy and infected tissues during cold storage of apple fruits.

apples when rotting became evident. For phenolic and polyphenol oxidase activity analysis, the skin was separated into two samples: infected tissues around (2 mm) the rotten zone and healthy tissues on the opposite side of the apple.

Table 1 shows that when rot appears in infected tissues surrounding the rotten zone a general increase in phenolic levels was observed, as compared to healthy tissue of the same fruit. PPO activity also increased in these tissues, 2−3 times that in healthy tissues (Figure 2). These changes in phenolic content and PPO activity may be considered as a part of the so-called hypersensitive reaction of the host cells to pathogen that is useful for arresting the development of fungus without causing further damage to the surrounding tissue (*18*, *29*, *30*). Postinfectional accumulation of preexisting phenolics, especially phloridzin and chlorogenic acid which are the better substrates of apple PPO (K_M = 1.351 and 1.139 mM, respectively), provides an adequate substrate to the increased PPO activity. Polyphenol oxidase, consuming oxygen and producing fungitoxic quinones,

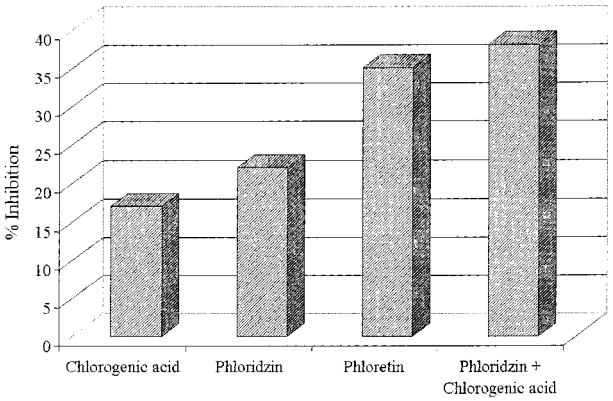

Figure 3. In vitro inhibition of fungal spore germination of *P. vagabunda* by apple oxidized phenolics.

makes the medium unfavorable to further development of pathogens.

In fact, in vitro bioassays show that, when a crude extract of apple PPO was added to a spore suspension of *P. vagabunda* that contains 10^{-3} M of each apple phenolics, an inhibition of fungal spore germination was observed. Figure 3 shows in vitro inhibition of fungal spore germination of *P. vagabunda* by some oxidized apple phenolics. This figure also shows a potential synergistic effect of phloridzin and chlorogenic acid, in good agreement with the results of Oszmianski and Lee (*31*), demonstrating that the simultaneous presence of chlorogenic acid and catechin in a model system increases the oxidation rate of phloridzin in the presence of polyphenol oxidase. This synergistic effect should probably be considered in the overall defensive strategy of apple against fungal attack. Finally, when in long stored apples these primary responses (increased phenolic level and activation of apple PPO in infected tissues), which counteract the further development of the pathogen, fall during the storage, a simultaneous increase in apple rot is observed.

We can conclude that over the past few years the importance of phenylpropanoid metabolism in fruit and vegetable tissue under biotic and abiotic stress has been well studied. In this connection, it is a well-known fact that exposure of some plant tissues to low nonfreezing temperatures can stimulate phenolic metabolism (*21*, *23*, *32*). However, there is relatively little information on the effect of storage conditions on the fate of inducible and preformed phenolic compounds frequently associated with postharvest disease resistance. Optimizing storage conditions for maintaining this biochemical potential can help improve the shelf life of plant commodities. These conditions (pretreatments, refrigeration, controlled/modified atmospheres) must be selected with the aim of inducing an increase of phenylpropanoid/flavonoid levels in apple fruits during cold storage.

Data presented in this research showed that a low temperature of 2 °C caused a metabolic increase of phenolics, especially chlorogenic acid and phloretin glycosides, in stored apples. When phenolic levels decreased during long-term storage, a concomitant decline in resistance to latent infections was observed. This drop is associated with the appearance of symptoms caused by *P. vagabunda*. As far as the resistance mechanism to decay development is concerned, it must be remembered that, among the phenolic compounds identified in apple peel, chlorogenic acid only inhibited *P. vagabunda* spore germination or mycelial growth in vitro. Therefore, changes in phenolic content and PPO activity in infected tissues surrounding the rotten zone, i.e., an efficient hypersensitive response, must be considered in the overall defensive mechanism of apple against fungal attack.

The results obtained suggested that phloridzin and chlorogenic acid in combination with polyphenol oxidase activity could function to arrest *P. vagabunda* in quiescent infections associated with immature and ripening apple fruit. According to Noveroske et al. (*15*) and Oszmianski and Lee (*31*), it appears that infection of apple tissue elicited an active glycosidase and polyphenol oxidase capable of converting phloridzin to phloretin, which was subsequently oxidized. This metabolism of phloridzin initiated by cell decompartmentation at the site of fungal infection and the synergistic effect of chlorogenic acid that accelerated the oxidation of phloridzin in the presence of polyphenol oxidase could play a prominent role in host resistance to further pathogen development.

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