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Phenolic Content During Healing of 'Valencia' Orange Peel Under High Humidity[1]

M. A. Ismail and G. Eldon Brown[2]

University of Florida, IFAS Agricultural Research and Education Center Lake Alfred

Abstract. Free phenolic constituents increase more than 2-fold in injured peel of oranges (*Citrus sinensis* Osbeck) cv. Valencia after 48 hr at 30°C and 96 to 98% relative humidity (rh). Concomitantly, conjugated phenolic compounds decrease to near depletion as a heavily lignified layer forms on injured cells. At 5°C all wound healing processes slow down. Infection of injured tissue by *Penicillium digitatum* Sacc. at 27°C inhibits lignin synthesis and the disappearance of conjugated phenolic compounds, but does not interfere with the usual increase in free phenolics. Mycelium of *P. digitatum* contributes little to the level of phenolic compounds of decayed fruit tissue. Extracts of free phenolic substances from healed tissue do not exhibit fungistatic activity on *P. digitatum* spores. Lignin formation provides a mechanical barrier which retards or inhibits penetration of injured tissue by *P. digitatum*.

In 1948, Hopkins and Loucks (9) reported that citrus fruit degreened with ethylene at 86°F (30°C) and 90% rh for 60 to 72 hr exhibited less green mold (caused by *Penicillium digitatum* Sacc.) than nondegreened fruit. Recently, Brown (3) confirmed these findings and noted that under such degreening conditions a layer of lignified tissue is formed on injured flavedo.

Since lignin constitutes the major portion of the post-wounding scar tissue (3) and phenylpropanoid compounds are known to be the building blocks of lignin (16), changes in phenolic content of injured 'Valencia' orange flavedo as influenced by temp, rh, and the decay organism *P. digitatum* were studied.

Materials and Methods

Mature, fully colored 'Valencia' orange (*Citrus sinensis* Osbeck) fruit were collected from trees on rough lemon rootstock. They were washed, dried, sorted for uniformity of size and external appearance, and randomly divided into as many groups as required in each experiment. In all trials, 20 and 1 or 2 g of flavedo tissue were collected at 24-hr intervals for phenolic substances and dry wt determinations, respectively. Histological evaluation of lignification was made on tissue sections stained with phloroglucinol-HCl (3).

Effect of humidity on wound healing and phenolic content of 'Valencia' orange flavedo. Two samples, each consisting of 120 fruit, were injured by rubbing fruit surfaces in 4 locations along the equatorial plane against #60 coarse sandpaper. One sample was stored at 30 ± 2°C and 96 to 98% rh; the other sample was held at similar temp, but at ambient humidity (55–75% rh). Both temp and rh were continuously monitored throughout the duration of the experiment.

Temperature effect on phenolic content of injured 'Valencia' orange flavedo. 'Valencia' oranges were injured as above, divided into 2 lots (80 fruit per lot), and placed at 90 to 96% rh. One lot was held at 5 ± 2°C, the other at 30 ± 2°C for 72 hr.

Effect of inoculation with Penicillium digitatum and storage temperature on the amount of phenolic compounds in injured 'Valencia' orange flavedo. 'Valencia' oranges were injured, divided into 2 lots, one of which was inoculated with dry spores of *P. digitatum* (3); the other group was not inoculated. All fruit were stored at 30 ± 2°C and 96 to 98% rh. The same experiment was repeated with fruit held at 25 ± 2°C and 96 to 98% rh. Fruit were also checked for decay.

Concn of phenolic compounds in in vitro-grown mycelium of P. digitatum. In order to determine the extent of fungal contribution to phenolic content of infected tissue, twelve 30 ml centrifuged orange juice samples were inoculated with *P. digitatum* spores and held in a shaker at 25°C (78°F) for 5 days. Nineteen g of mycelial tissue was collected, washed thoroughly with distilled water, and extracted for free and conjugated phenolic substances.

Effect of free phenolic extracts on germination of spores of P. digitatum. Spores of *P. digitatum* were germinated in the presence of free phenolics on a medium composed of 10 ml filtered and centrifuged orange juice, 1 g dextrose, 1 g yeast extract, and 30 g Bacto agar in one l of distilled water. A 10-ml aliquot of sterile medium was poured into a petri dish and cut after solidification to form 4 islands 7 mm in diam. An aliquot containing 10 µl of a free phenolic extract in 95% ethanol was placed on each of the 4 islands in a dish. After evaporation of ethanol, spores of *P. digitatum* suspended in 1000 ppm Tween 20 were placed on each island and incubated for 18 hr at 25°C. They were then killed with cotton blue-lactophenol. Percent germination was determined using 50 spores in each island.

Extraction of free and total phenolic constituents from injured 'Valencia' orange flavedo. The method used was a modified version of those described by Ibrahim et al. (10) and Biehn et al. (2). Tissue (20 g) was homogenized in a Lourdes mixer with 200 ml boiling 95% ethanol. The homogenate was boiled for 1 hr in a water bath and filtered on Whatman no. 42 filter paper. The filtrate was divided into 2 equal portions, each of which was evaporated to dryness under vacuum at 45° ± 2°C.

a. Extraction of free phenolic constituents. The dry residue remaining after evaporation of 1 of the ethanol fractions was suspended in 200 ml boiling distilled water and extracted twice with 0.5 vol n-hexane to remove peel oil and carotenoid pigments. Active acidity of the aqueous layer was then reduced to pH 2 using 2N HCl and extracted 3 times with 0.33 vol distilled ethyl acetate. Ethyl acetate extracts were combined and evaporated to dryness. The dry residue was subjected to a stream of N to remove residual ethyl acetate and then dissolved in 10 ml 95% ethanol.

b. Extraction of total phenolic constituents. The other half of dried ethanolic filtrate was dissolved in 50 ml boiling distilled water and subjected to 2 successive washes with equal volumes of n-hexane. The remaining aqueous layer was evaporated to dryness at 48 ± 2°C and

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[2] Plant Physiologist II and Plant Pathologist III, FL Dept. of Citrus.

suspended in 100 ml 2N HCl. The acidic solution was hydrolyzed by boiling for 1 hr in a water bath (1), filtered, and the residue was washed with distilled water. After cooling, the filtrate was extracted 3 times with 0.33 vol distilled ethyl acetate. The extracts were then handled as described for the free phenolic fractions. This fraction, referred to as total phenolic fraction, contains both free and acid hydrolyzed phenolic constituents.

Phenolic compounds were assayed in aqueous dilutions using the method described by Keith et al. (11) with Folin-Ciocalteau reagent and caffeic acid as a standard. Conjugated phenolic compounds were calculated by subtracting the free fraction from the total.

Results

Concentration of free phenolic compounds in injured flavedo tissue of 'Valencia' oranges stored at 30°C and 96 to 98% rh was doubled within 48 hr (Table 1). Only a slight increase in free phenolics occurred in injured fruit held at ambient relative humidity. The change in free phenolics was accompanied by a sharp decline in conjugated phenolics of injured fruit held at high relative humidity, while those held at ambient humidity showed only a moderate decrease (Table 1). Injured fruit, held at high humidities, developed a much thicker layer of lignin-type material compared to those maintained at ambient rh (Fig. 1).

Free phenolic compounds increased 100% in 72 hr when injured fruits were held at 30°C compared to only 25% increase in fruits held at 5°C (Table 2). Concomitantly, conjugated phenolics declined by 75 and 2% at 30°C and 5°C, respectively (Table 2).

Changes in free and conjugated phenolic constituents were not altered to any great extent by the presence of the decay organism *P. digitatum* on injured fruit held at high humidity and 30°C (Table 3). Levels of phenolic compounds were essentially identical in both inoculated and noninoculated tissue except for the higher level of conjugated phenolics in inoculated tissue 72 hr after injury. Under these environmental conditions, microscopic examination of inoculated tissue slices showed that the spores had germinated but the epidermal cells were lignified so that germ tube penetration and infection apparently had not taken place. Decay occurred in all injured and inoculated fruit held at 25°C, a temperature favorable for fungal growth. Free phenolics increased sharply in both inoculated and noninoculated fruit (Table 4), while conjugated phenolics declined only in noninoculated healed fruit.

The pattern of relative change in free and conjugated phenolic constituents in inoculated and noninoculated fruit tissue held under various environmental conditions was found reproducible despite variations in initial and post-storage levels. When initial levels were low, subsequent levels were proportionately low and vice versa.

Washed mycelium of *P. digitatum* contained 0.071 mg free phenolics and 0.005 mg conjugated phenolics/g dry wt.

When extracts of free phenolics were tested against spores of *P. digitatum*, no significant reduction in spore germination or germ tube elongation was observed with 10 μl aliquots containing 1 to 7 μg phenolic compound.

Discussion

High humidity is essential for natural injury healing in 'Valencia' orange peel. The increase in free phenolics, decrease in conjugated phenolics, and lignin formation following injury may be interrelated since phenylpropanoid compounds are known to be lignin precursors (16). These changes are apparently enzymatically regulated as evidenced by the retarding effect of low temperature on lignification, healing, and accumulation of free phenolics. The accelerating effect of high relative humidity is apparently caused by retardation of tissue desiccation, thus allowing normal cellular activities to continue in adjacent uninjured cells.

High temp (30°C) accelerates the healing process and retards decay, apparently by delaying spore germination and germ tube elongation of *P. digitatum*, thus preventing penetration before formation of the lignified protective layer. This was demonstrated when injured, inoculated fruit held at 25°C decayed within 72 hr after injury, while similarly treated fruit held at 30°C healed.

The decline in conjugated phenolic constituents appears to be essential for lignin formation in injured tissue since the 2 processes occur simultaneously as healing proceeds. When inoculated fruit are held at 25°C, lignification and degradation of conjugated phenolics are disrupted by the infecting fungus. Decaying tissue exhibits an

Table 1. Effect of relative humidity and time on free and conjugated phenolic compounds in injured flavedo of mature 'Valencia' orange fruit.

Time after injury (hr)	Phenolic concn (mg/g dry wt)			
	Free		Conjugated	
	55–75% rh	96–98% rh	55–75% rh	96–98% rh
0	1.47	1.47	6.48	6.46
24	1.40	1.59	3.54	3.95
48	1.98	4.06	2.35	1.16
72	2.48	5.13	2.22	0.68

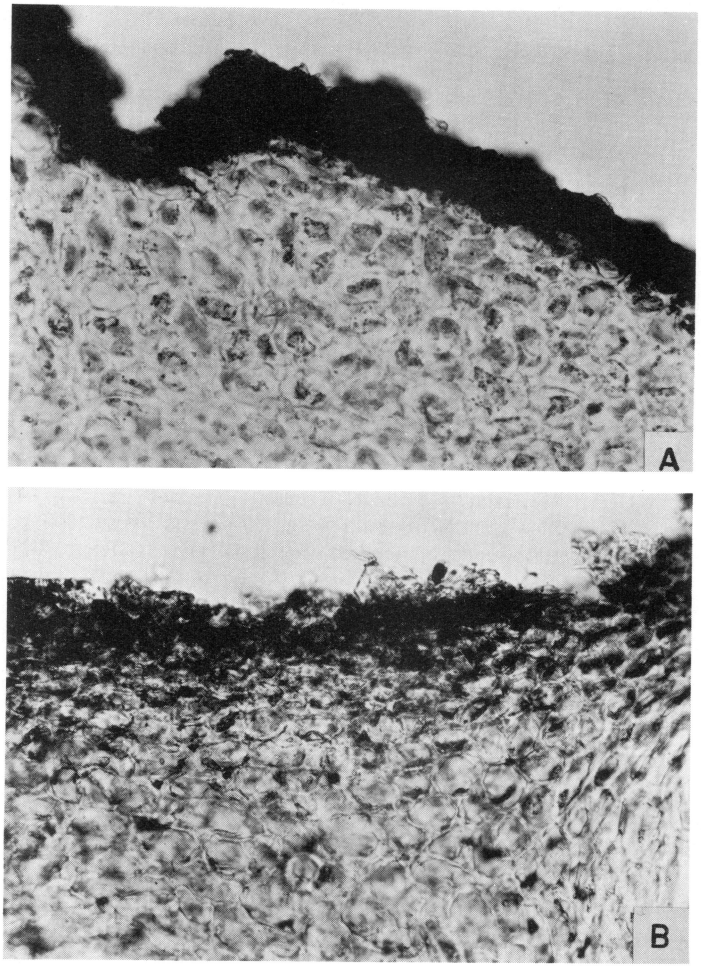

Fig. 1. Effect of 96 to 98% rh (A) vs. 55 to 75% rh (B) on lignification of injured 'Valencia' orange flavedo (×160).

Table 2. Effect of temperature on free and conjugated phenolic constituents of mature 'Valencia' orange flavedo.

Time after injury (hr)	Phenolic concn (mg/g dry wt)			
	Free		Conjugated	
	5°C	30°C	5°C	30°C
0	1.82	1.82	5.03	5.03
72	2.29	3.64	4.91	1.25

increase in free phenolics without the usual decline in conjugated phenolics or lignin formation. Failure of conjugated phenolics to hydrolyze while free phenolics increase indicates that formation of free phenolics is not entirely dependent on hydrolysis of conjugated phenolics. In this case, *de novo* synthesis of free phenolic compounds may be taking place in injured tissue via deamination of phenylalanine and/or tyrosine (16) or via acetate condensation (14).

The role of phenolic compounds which form in injured or infected plant tissue is not fully understood. Some plant phenolics (15, 19) possess fungistatic activity and are produced by plant tissue in direct response to invading pathogens. Clarke (4) reported scopolin to accumulate in greater quantities in potato tissue only in response to infection with virulent isolates and little or no accumulation occurred in response to physical injury. Feldman and Hanks (7) were able to increase tolerance of roots of susceptible grapefruit cultivars to burrowing nematodes (*Radopholus similis* Cobb) by exogenous application of vanillic acid. Earlier, however, they (6) reported no consistent pattern of accumulation or reduction in phenolic compounds in roots of citrus cultivars, susceptible or tolerant to burrowing nematodes. In soybean, increased levels of the isoflavonoid phaseolin (5) and hydroxyphaseolin (12, 13) in response to fungal or viral infections were reported. Biehn et al. (2) obtained 70 to 90% inhibition of germ tube growth by phenolic extracts from soybean seedlings inoculated with *Helminthosporium carbonum* Ullstrup or *Alternaria sp.* compared to those from noninoculated seedlings. There are, however, reports which suggest that phenolic compound formation after infection is merely the result of mechanical injury caused by the pathogen gaining entry into plant tissue (14). Piattelli and Impellizzeri (17) found no significant differences in the levels of fungistatic flavones in citrus cultivars susceptible or resistant to *Deuterophoma tracheiphila*. Furthermore, Rathwell and Bendall (18) demonstrated that general increases in phenolics after tissue inoculation were independent of phaseolin production and may only be related to cell necrosis.

Table 3. Effect of inoculation, at 30°C, with *Penicillium digitatum* spores on concn of free and conjugated phenolic compounds in injured 'Valencia' orange flavedo.

Time after injury and inoculation[z]	Phenolic concn (mg/g dry wt)			
	Free		Conjugated	
	noninoculated	inoculated	noninoculated	inoculated
0	1.21	1.21	5.21	5.21
24	1.53	1.81	3.37	2.73
48	3.10	3.17	1.56	2.02
72	3.86	3.67	0.32	3.11
96	4.23	5.13	0.02	0.0

[z] Fruit was injured, inoculated, and held at 30°C and 96 to 98% rh.

Table 4. Effect of inoculation, at 25°C, with spores of *Pencillium digitatum* on concn of free and conjugated phenolic compounds in mature 'Valencia' orange flavedo.

Time after inoculation and injury (hr)	Phenolic concn (mg/g dry wt)			
	Free		Conjugated	
	noninoculated	inoculated	noninoculated	inoculated
0	1.29	1.29	6.04	6.04
24	1.42	1.43	4.78	4.16
48	2.79	2.40	3.68	5.44
72	3.99	4.56	2.46	5.79
96	5.30	4.43	0.57	6.57

Failure of phenolic extracts from 'Valencia' flavedo tissue to exhibit fungistatic activity may have been due to the use of low concn of extracts. However, these findings lend support to the concept that in some plant tissue the change in phenolic compounds following injury is simply a biochemical response associated with scar formation. The newly formed free phenols may provide the necessary intermediates for lignin formation which provides a mechanical barrier against possible infection by microorganisms. Findings of Friend et al. (8), that more rapid and uniform lignin formation occurs in potato slices taken from a tolerant potato cultivar than in slices from a susceptible cultivar, support the latter concept.

Thus, it appears that free phenolic compounds formed in injured flavedo tissue of 'Valencia' oranges may not contribute to retardation of decay and that lignin produced following injury plays the major role in healing and subsequent prevention of infection.

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