Proc. Fla. State Hort. Soc. 103:234-237. 1990.

CHANGES IN PHENYLALANINE AMMONIA-LYASE, SOLUBLE PHENOLICS AND LIGNIN IN INJURED ORANGE EXOCARP

G. ELDON BROWN
Florida Department of Citrus
Citrus Research and Education Center
700 Experiment Station Road
Lake Alfred, Florida 33850

Additional index words. wound healing, *Penicillium digitatum,* ethylene, postharvest decay

Abstract. **The accumulation of phenolic compounds and lignin and the activity of phenylalanine ammonia-lyase (PAL), the major enzyme involved in their synthesis, were measured daily for 3 days in injured exocarp tissue removed from the rind of oranges stored at 30C near 100% relative humidity. PAL was not usually present in freshly injured tissue, but was detected by 1 day and was more active at 2 than 3 days. Levels of phenolics in fresh injuries of Hamlin oranges harvested in October, December, and February decreased with maturity, but lignin deposition increased. PAL activity and phenolic and lignin levels in injured tissue after 2 or 3 days were greater in fruit of the last 2 harvests than the first harvest. Treatment of oranges with ethylene before injury stimulated PAL activity and lignin accumulation. A reduction in PAL activity and an increase in soluble phenolics were measured in injured tissue that was resistant to infection by *Penicillium digitatum*. Ethylene treatments before inoculation with *P. digiatum* reduced the susceptibility of immature, but not mature, Valencia oranges to green mold.**

Injuries to citrus rind are required for the entry of the fungal wound pathogens, *Penicillium digitatum, P. italicum,* and *Geotrichum candidum* (3). As wounds heal following injury, they become less susceptible to fungal infection (5). Major physiological changes after injury that are detected in the cells adjacent to the injured tissue include the increase in phenylalanine ammonia-lyase (PAL) activity (11), and the accumulation of phenolic and lignin materials (5, 10). Cinnamic acid produced by the activity of PAL is a precursor to many of these materials, some of which may play a role in resistance of the injured tissue to fungal penetration (4).

Ethylene has been shown to enhance PAL activity (16), and the accumulation of phenolic and lignin compounds in plant tissues (2). With oranges, treatments with 1000 ppm of ethylene for 6 days before inoculation with *P. italicum* increased phenolic levels in the rind, and suppressed the rate of subsequent lesion development (8).

These experiments were designed to measure the activity of PAL and the consequential accumulation of phenolics and lignin in injured orange rind as influenced by maturity, ethylene and *P. digitatum.* Treatments to enhance fruit resistance to infection by *P. digitatum* were evaluated at different maturity dates using ethylene applications before injury and inoculation.

Florida Agricultural Experiment Station Journal Series No. N-00291. The author wishes to thank M. W. Chambers and M. T. Davis for their assistance in conducting these studies.

Materials and Methods

Fruit and injuries. Hamlin and Valencia oranges [*Citrus sinensis* (L.) Osbeck] were washed and then injured by rubbing the fruit against fine (220 grit) waterproof silicon carbide sandpaper at 2 areas on opposite sides of the fruit equator. The sandpaper was submerged in running tapwater during the injury process to remove the peel oil and minimize damage to the exocarp from the phytotoxic oil. The fruit were washed in additional tapwater and then dried with cheesecloth.

Ethylene treatments. Ethylene was applied to fruit in small cabinets in a continuous flow-through system at 30C near 100% relative humidity (RH). Ethylene was maintained within ± 10% of the desired concentration by monitoring levels with a gas chromatograph equipped with a flame ionization detector.

Sample preparation for soluble phenolics. Thin slices of the injured rind were removed from each of 30 fruit and minced with a scalpel. A 1 g portion of the sample was used for dry weight determinations and 2 g were extracted to measure soluble phenolics. The tissue was extracted in a homogenizer for 5 min with hot (70C) 0.1 N HCL in methanol (12). The homogenate was boiled for 1 hr in a water bath at 70C, stored overnight at 4C, and filtered with Whatman #42 filter paper. The residue was rinsed with additional hot 0.1N HCL in methanol. The filtrate was washed 3 times with n-hexane and the phenolics remaining in the methanol soluble fraction were assayed using Folin-Ciocolteau reagent with caffeic acid as a standard (12).

Sample preparation for PAL analysis. A 5 gm portion of the rind was prepared into an acetone powder by macerating it in 100 ml of cold acetone in a homogenizer cup submerged in an ice bath. The tissue was homogenized for 5 min, filtered, resuspended in cold acetone, and homogenized again for an additional 5 min. Acetone was again separated from the powder by filtration and evaporation, and the powder was then stored at −17C until analysis. Extraction and assay of PAL from 200 mg of the powder was completed following previously published methods (14). A small amount (50 mg) of polyvinylpolypyrrolidone was added during extraction to minimize degradation of the enzyme by phenolic materials. The reaction mixture composed of 0.3 ml of enzyme preparation and 4.7 ml of borate buffer (pH 8.8) with L-phenylalanine (60 μM) was incubated at 40C for 1 hr. Enzyme activity was determined by measuring the increase in absorbance at 290 nm with a spectrophotometer. Enzyme activity was expressed as μm cinnamic acid produced hr^{-1} mg^{-1} of protein. Protein content of the enzyme preparation was determined with the Bio-Rad Laboratories procedure (1).

Sample preparation for lignin analysis. Protein-extracted acetone powder was dried at 60C for 24 hr and 50 mg were used to measure lignothioglycolic acid (LTGA) content using the thioglycolic acid method (9, 17). The final pellet was dissolved in 10 ml of 0.5N NaOH, centrifuged at 10,000 g for 5 min, and the absorbance was measured

at 280 nm. Concentrations of LTGA (mg/g dwt) were determined using a standard curve developed with LTGA derived from lignin in orange rind (6).

Fruit inoculations. Fruit were inoculated with *P. digitatum* using 2 different procedures. Rind of injured fruit was immediately inoculated by covering the injured area with an aqueous suspension of spores (10^6/ml) dispersed with 0.05% Triton X-100 surfactant. Injured areas of control fruit were covered only with water and surfactant. Fruit were stored near 100% RH for 1 week at 27C for decay studies, and for 3 days at 29C for physiological studies. With the other procedure, fruit were inoculated with a syringe and needle to a depth of 1.5 or 2.5 mm with 5 µl of spores at 4 equidistant sites around the fruit equator. Five treatments were used with spore concentrations site^{-1} of log 1.7, 2.7, 3.7, 4.7, and 5.7. The number of infected sites were counted after 4 days at 24C. The log spores required to cause 50% infection was determined by the linear relationship between the probit of the percentage infection and the logarithm of the spore concentration.

Results

Fruit maturity. Activity of PAL and the accumulation of soluble phenolics and lignin in injured orange exocarp were affected by the maturity of the fruit and the age of the injury (Table 1). Relatively little PAL activity was noted in freshly injured tissue of Hamlin orange while the highest level of activity usually occurred at 48 hr after injury. PAL was more active in injured tissue of mature than immature fruit. Initial phenolic levels in fresh injuries decreased with maturity (Table 1), but increased with time from injury. The rate of increase, however, was greater as the fruit became more mature. An increase in soluble phenolics over the initial level at time 0 was evident by 72, 72 and 24 hr, respectively, in harvests 1, 2, and 3. Significant increases in lignin accumulation were observed as the injuries increased in age (Table 1), and as the fruit became more mature.

Ethylene. The application of ethylene to Valencia fruit for 3 continous days before making the injury caused some detectable effects (Table 2). Ethylene did not affect PAL activity in freshly injured tissue, but it did cause a stimulation of activity following injury that was observed in samples taken at 24, 48, and 72 hr. No differences were noted between the 2 levels of 100 and 300 ppm of ethylene. Levels of soluble phenolics were enhanced in fresh injuries by ethylene, but the effect did not remain consistent with time. However, the highest level of soluble phenolics was detected at 72 hr in tissue taken from fruit treated with 300 ppm of ethylene (Table 2). Lignin accumulation was enhanced by ethylene pretreatment at time 0, as well as at other sampling times. A concentration of 300 ppm of ethylene was more effective than the treatment at 100 pm.

Penicillium digitatum. The presence of the fungus caused a significant reduction in the activity of PAL in injuries which were resistant to infection (Table 3). Initial levels of soluble phenolics were lower in inoculated fruit, but by 48 hr these levels exceeded those found in tissue recovered from the uninoculated fruit. *Penicillium digitatum* did not appear to enhance lignin accumulation. The higher levels of lignin recovered from inoculated fruit at 24 and 48 hr could be accounted for by the higher lignin

Table 1. Activity of phenylalanine ammonia-lyase (PAL) and accumulation of soluble phenols and lignin in exocarp of Hamlin oranges after harvest at 3 maturity dates, injury, and storage for 3 days at 30C near 100 percent relative humidity.

	Time after injury (hr)					
Maturity[v]	0	24	48	72	Contrasts	P>F[w]
	PAL (µm Cin A[x]/mg protein/hr)					
1	0.00	0.11	0.18	0.11	1 vs 2	0.000
2	0.00	0.23	0.30	0.16	2 vs 3	0.324
3	0.02	0.19	0.32	0.19	24 vs 48	0.000
					48 vs 72	0.000
	Soluble phenolics (mg Caf A[y] equiv./g dwt.)					
1	11.20	9.26	10.36	12.69	1,0 vs 2,0	0.006
2	9.98	9.81	10.14	12.09	1,0 vs 3,0	0.000
					1,0 vs 1,72	0.001
3	9.42	11.81	14.09	16.57	2,0 vs 2,48	0.708
					2,0 vs 2,72	0.000
					3,0 vs 3,24	0.000
	Lignin (mg LTGA[z]/g dwt.)					
1	2.18	4.18	8.04	9.28	1 vs 2	0.000
2	2.71	5.46	10.25	14.72	2 vs 3	0.000
					0 vs 24	0.000
3	4.26	7.00	10.17	15.07	24 vs 48	0.000
					48 vs 72	0.000

[v]Harvest dates: 1 = 10/21/85; 2 = 12/16/85; 3 = 2/3/86
Brix/acid: 1 = 7.56; 2 = 10.93; 3 = 13.22
Rind color a/b: 1 = –3.70; 2 = –0.51; 3 = 0.36
[w]Probability, using orthogonal contrasts, that values are different due to chance.
[x]Cinnamic acid.
[y]Caffeic acid.
[z]Lignothioglycolic acid.

Table 2. Effect of treating Valencia oranges with ethylene for 3 days before injury on activity of phenylalanine ammonia-lyase and accumulation of soluble phenols and lignin in injured exocarp during storage for 3 days at 30C near 100% relative humidity.

Ethylene (ppm)	Time after injury (hr) 0	24	48	72	Contrasts	P>F[w]
	PAL (μm Cin A[x]/mg protein/hr)					
0	0.00	0.13	0.27	0.27	0 vs 100	0.000
100	0.00	0.30	0.47	0.38	0 vs 300	0.000
300	0.00	0.34	0.50	0.39	100 vs 300	0.000
	Soluble phenolics (mg Caf A[y] equiv./g dwt.)					
0	9.50	11.02	12.37	12.49	0,0 vs 0,100	0.000
100	12.00	11.00	11.95	12.26	0,0 vs 0,300	0.000
300	11.88	10.43	12.21	13.25	0,72 vs 300, 72	0.005
	Lignin (mg LTGA[z]/g dwt.)					
0	3.64	4.29	8.53	9.67	0 vs 100	0.000
100	4.44	7.08	12.96	15.37	0 vs 300	0.000
300	5.58	7.59	14.89	20.05	100 vs 300	0.000
					0,0 vs 100,0	0.015
					0,0 vs 300,0	0.000

[w]Probability, using orthogonal contrasts, that values are different due to chance.
Harvest date 4/4/85.
[x]Cinnamic acid.
[y]Caffeic acid.
[z]Lignothioglycolic acid.

content of the inoculated tissue taken in the initial sample at time 0 (Table 3).

Fruit susceptibility. Ethylene applied to fruit before inoculation with *P. digitatum* did reduce susceptibility at some harvests (Table 4). Green mold in Valencia oranges was reduced by treatment with 100 ppm of ethylene at the 2 early harvests, but not at the last one. In later harvested fruit, in the following season, higher levels of inoculum were needed to cause 50% infection in ethylene than in nonethylene-treated fruit (Table 4). At the 2 harvests, spore concentrations of 708 and 1413 were required for ethylene-treated fruit while 501 and 708 were needed for non-treated fruit, respectively. These differences, however, were not large enough in this study to be statistically significant.

Discussion

Phenylalanine ammonia-lyase catalyzes the deamination of phenylalanine to trans-cinnamic acid which is utilized in the synthesis of numerous phenolic and lignin-like compounds associated with secondary plant metabolism. The enzyme is active in uninjured immature citrus fruit (7), but only a trace can be found as the fruit becomes more mature. Activity of the enzyme is induced, however, upon injury to the exocarp of mature fruit (11).

Table 3. Activity of phenylalanine ammonia-lyase and accumulation of soluble phenols and lignin in exocarp of injured Valencia oranges infected by *Penicillium digitatum.*

Inoculation	Time after injury and inoculation (hr) 0	24	48	72	Contrasts	P>F[w]
	PAL (μm Cin A[x]/mg protein/hr)					
Control	0.02	0.31	0.44	0.24	C vs I	0.000
Inoculated	0.00	0.27	0.25	0.15		
	Soluble phenolics (mg Caf A[y] equiv./g dwt.)					
Control	9.81	8.52	8.49	8.96	C,0 vs I,0	0.000
Inoculated	7.77	8.86	12.31	11.11	C,48 vs I,48	0.000
					C,72 vs I,72	0.000
	Lignin (mg LTGA[z]/g dwt.)					
Control	1.50	4.00	13.09	18.90		
Inoculated	2.38	5.38	16.35	18.88		

[w]Probability, using orthogonal contrasts, that values are different due to chance. C = control; I = inoculated. Harvest date 3/25/85.
[x]Cinnamic acid.
[y]Caffeic acid.
[z]Lignothioglycolic acid.

Table 4. Susceptibility of Valencia oranges to green mold after treating fruit with ethylene before inoculating with *Penicillium digitatum*.

Ethylene (ppm)	Dates of harvest				
	Green mold (%)[v]			Log spores (50% infection)[w]	
	1/21/85	2/14/85	2/28/85	3/3/86	3/24/86
0	14 a[y]	49 a	28 a	2.70 a[z]	2.85 a
100	4 b	13 b	31 a	–	–
1000	–[x]	–	–	2.85 a	3.15 a

[v]Fruit were treated with ethylene for 72 hours, and inoculations were made to treatments of eight replications, each with 25 fruit.
[w]Fruit were treated with ethylene for 96 hours, and inoculations at each spore concentration were made to 25 fruit.
[x]No data taken.
[y]Mean separation in columns by Duncan's multiple range test, 5% level.
[z]Separation of log values in columns by Student's method of paired comparisons, 5% level.

In this study, activity of the enzyme in injured tissue reached its highest point at 48 hr after injury, and was most active in the more mature injured fruit. The reactivation of the enzyme in mature fruit occurs apparently because of the injury and the subsequent healing process that involves the synthesis and accumulation of phenolic compounds and lignin under conditions of high RH.

In previous studies (11, 13), activity of PAL was suppressed and eventually eliminated by *P. digitatum* in susceptible responses where the tissue was invaded by the fungus. Suppression even occurred in healthy tissue taken from an infected and decayed fruit (13). The authors postulated that suppression of the enzyme was an example where the pathogen was able to neutralize the defense mechanisms of the host and induce susceptibility. Similarly, a reduction in PAL activity due to the presence of *P. digitatum* was also noted in this study. However, in this case, the fruit were resistant to fungal infection, and a stimulation in the production of soluble phenolics by the presence of the fungus was even indicated. Apparently, the activity of PAL in the resistant response studied here was not reduced sufficiently by *P. digitatum* to prevent or retard healing to the extent necessary to induce susceptibility.

The healing process, measured by PAL activity and the accumulation of phenolics and lignin, was enhanced by treating fruit with ethylene before injury. This response was similar to that observed with maturity, where more mature fruit contained higher levels of PAL, phenolics and lignin. Ethylene is known to enhance senescence, and therefore the ethylene treatments may be causing physiological changes, such as enhanced sensitivity to injury, that are normally associated with more mature fruit.

Increased resistance to *P. digitatum* was observed only with ethylene treatments to immature fruit. Once certain threshold levels of phenolics and lignin are deposited in injuries, such as may occur in more mature fruit, additional deposits induced by ethylene may have little effect on resistance to decay. The rate of accumulation and deposition may play an important role in resistance. In the maturity study, deposits of phenolics and lignin were more rapid in the mature fruit.

Besides ethylene, other specific fungal or plant constituents have enhanced the accumulation of phenolics and lignin in injured tissue (15). These rather innocuous compounds, such as fatty acids, polysaccharides, chitin, chitosan, cellulose, pectin, and polygalacturonic acid (3), could possibly enhance resistance of injured tissue if they were applied in postharvest treatments.

Literature Cited

1. BIO-RAD Laboratories. 1977. Bio-rad protein assay. Tech. Bull. 1051. Richmond, CA. 4 p.
2. Boller, T. 1982. Ethylene-induced biochemical defenses against pathogens. Pages 303-312 in: Plant Growth Substances. P. F. Waring, ed. Academic Press, London.
3. Brown, G. E. 1989. Host defenses at the wound site on harvested crops. Phytopathology 79:1381-1384.
4. Brown, G. E., and Barmore, C. R. 1983. Resistance of healed citrus exocarp to penetration by *Penicillium digitatum*. Phytopathology 73:691-694.
5. Brown, G. E., Ismail, M. A., and Barmore, C. R. 1978. Lignification of injuries to citrus fruit and susceptibility to green mold. Proc. Fla. State Hort. Soc. 91:124-126.
6. Doster, M. A., and Bostock, R. M. 1988. Quantification of lignin formation in almond bark in response to wounding and infection by *Phytophthora* species. Phytopathology 78:473-477.
7. Dubery, I. A. and Schabort, J. C. 1986. Phenylalanine ammonia-lyase from *Citrus sinensis*: Purification by hydrophobic interaction chromatography and physical characterization. Biochemistry International 13:579-589.
8. El-Kazzaz, M. K., Chordas, A., and Kader, A. A. 1983. Physiological and compositional changes in orange fruit in relation to modification of their susceptible to *Penicillium italicum* by ethylene treatments. J. Amer. Soc. Hort. Sci. 108:618-621.
9. Hammerschmidt, R. 1984. Rapid deposition of lignin in potato tuber tissue as a response to fungi non-pathogenic on potato. Physiological Plant Pathology 24:33-42.
10. Ismail, M. A., and Brown, G. E. 1975. Phenolic content during healing of 'Valencia' orange peel under high humidity. J. Amer. Soc. Hort. Sci. 100:249-251.
11. Ismail, M. A., and Brown, G. E. 1979. Postharvest wound healing in citrus fruit: Induction of phenylalanine ammonia-lyase in injured 'Valencia' orange flavedo. J. Amer. Soc. Hort. Sci. 104:126-129.
12. Keith, R. W., Le Tourneau, D. and Mahlum, D. 1958. Quantitative paper—chromatographic determination of phenols. J. Chromatog. 1:534-536.
13. Lisker, N., Cohen, L., Chalutz, E. and Fuchs, Y. 1983. Fungal infections suppress ethylene-induced phenylalanine ammonia-lyase activity in grapefruits. Physiological Plant Pathology 22:331-338.
14. Rahe, J. E., Kuc, J. and Chueng, C. 1970. Cinnamic acid production as a method of assay for phenylalanine ammonia-lyase in acetone powders of *Phaseolus vulgaris*. Phytochemistry 9:1009-1015.
15. Ride, J. P., and Barber, M. S. 1987. The effects of various treatments on induced lignification and the resistance of wheat to fungi. Physiological and Molecular Plant Pathology 31:349-360.
16. Riov, J., Monselise, S. P., and Kahan, R. S. 1969. Ethylene-controlled induction of phenylalanine ammonia-lyase in citrus fruit peel. Plant Physiol. 44:631-635.
17. Whitmore, F. W. 1978. Lignin-carbohydrate complex formed in isolated cell walls of callus. Phytochemistry 17:421-425.