Fire Blight Resistance in Pyrus: Involvement of Arbutin Oxidation

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ABSTRACT

Arbutin was readily oxidized by commercial polyphenol oxidase (PPO) and tissue homogenates of pear. Greater oxidation occurred with homogenates of old leaves than with young ones. Hydroquinone was oxidized by homogenates (independently of arbutin) but not by PPO. Oxidation products of arbutin were toxic to *Erwinia amylovora*. More were formed by older leaves. Hydroquinone stimulated the oxidation of arbutin by PPO, but the degree of stimulation depended upon when the hydroquinone was added to the reaction mixture. Chromatographic evidence indicated that hydroquinone reacted with one of the oxidation products of arbutin. It is suggested that the oxidation pathway of arbutin degradation may be involved in fire blight resistance through both the formation of toxic substances and an interaction with the hydrolytic pathway. Phytopathology 60:337-340.

The degradation of arbutin may be part of the biochemical processes that operate as defense mechanisms against bacterial invasion in *Pyrus* sp. The process receiving the most attention in relation to fire blight resistance in pear has been the hydrolytic degradation of arbutin by β-glucosidase yielding hydroquinone, a substance toxic to many bacteria. Certain observations suggest that there may be other pathways leading to the formation of toxic substances from arbutin in *Pyrus* sp. For example, β-glucosidase was not detected in the exterior receptacle tissue of pear blossoms (5), although these tissues exhibited considerable antibiotic activity when bioassayed on media containing arbutin (4). Other observations have indicated that concurrent processes may be present that interfere with the β-glucosidase system. Little β-glucosidase could be detected histochemically in the woody part of pear stems compared with the bark (5), although large amounts of hydroquinone were formed in aqueous extracts of the former tissues and little was formed in extracts of the latter (6).

The direct oxidation of arbutin could conceivably be responsible for these results. Oxidative pathways such as described by Hattori & Sato (3) in *Pyrus* sp. might influence fire blight resistance by forming toxic compounds directly from arbutin, or compounds which alter the toxicity of arbutin hydrolysis products or which interfere with the hydrolysis of arbutin. Knowledge of such pathways may bear on the biochemical resistance mechanisms of pear to *Erwinia amylovora*. The purpose of this investigation was to determine if the oxidative pathway might be involved in fire blight resistance by forming toxic substances or substances which interact with the arbutin-hydroquinone complex.

MATERIALS AND METHODS.—*Respirometry.*—Polyphenol oxidase (PPO) activity was determined in a Gilson Differential Respirometer at 27.5 C. The flasks contained 0.2 ml of 1 N NaOH soaked into filter paper in the center well, 0.5 mg of commercial mushroom PPO (Sigma Chemical Co.) in 1 ml of 0.01 M acetate buffer at pH 5.6 in the sidearm, and 1 ml of 0.02 M arbutin in the same buffer in the main compartment. At time zero, the PPO solution was tipped from the sidearm into the main compartment. Control solutions consisted of (i) 0.5 mg commercial β-glucosidase (Sigma Chemical Co.) with arbutin; (ii) the complete reaction mixture plus 0.001 M sodium diethyldithiocarbamate (DIECA); (iii) 0.01 M hydroquinone with PPO; and (iv) PPO; (v) emulsin; (vi) hydroquinone; and (vii) arbutin alone.

Homogenates consisted of 0.05 g of ground blossom or leaf tissue in 1 ml acetate buffer with 1 ml of 0.02 M substrate. Oxygen uptake was read directly from the calibrated respirometer in μliters of O_2.

Chromatography.—Ascending paper chromatography was performed using a 61:10:26:3 mixture of butanol-acetic acid-water-ethanol. The dried chromatograms were sprayed with 0.1 M Na_2CO_3, followed by a second spray with 0.2% *p*-diazobenzenesulfonic acid.

Antibiosis.—Potato-dextrose-peptone (PDP) agar plates were seeded with washed cells from a 24-hr broth culture of *E. amylovora,* and the plates maintained at 40 C until the surface was dry. Test fluid was drawn up into a standard antibiotic assay disc (SS-740E), and the discs were dried and placed onto the agar surface. Results were read after 24 hr, and are presented as the average diameter (mm) of the inhibition zones, excluding the diameter of the discs.

RESULTS.—*Respirometric measurement of arbutin oxidation.*—The respirometric studies indicated that arbutin is readily oxidized by commercial PPO (Fig. 1). Nearly 200 μliters of O_2 uptake occurred over a 7-hr period; the reaction mixture turned brown. None of the controls, including hydroquinone, were oxidized, as they did not change color and no O_2 uptake was measured.

Similar results were obtained when D'Anjou blossom tissue homogenates were incubated with arbutin (Fig. 2). Glucose, the sugar moiety of arbutin, had no effect on gas uptake, but hydroquinone, the aglycone of arbutin, was appreciably oxidized by the pear tissue, although not to the same extent as arbutin. Oxidation of hydroquinone appears to be independent of the arbutin oxidation, as an additive effect was obtained when both were added to the reaction mixture. As with commer-

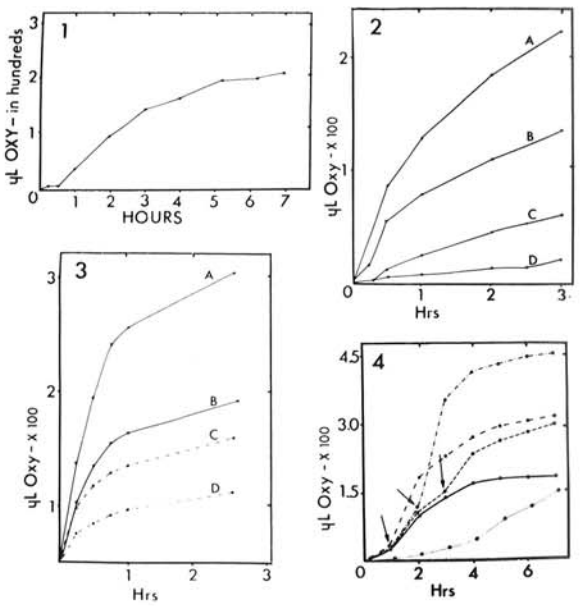

Fig. 1-4. **1)** Polyphenol oxidase (PPO) oxidation of arbutin (μliters O_2 taken up/mg PPO). **2)** Oxygen uptake by tissue homogenates of D'Anjou blossoms: A = arbutin + hydroquinone as substrate; B = arbutin; C = hydroquinone; and D = no substrate (μliters O_2 taken up/mg tissue). **3)** Oxygen uptake by tissue homogenates of old and young leaves of D'Anjou: A = arbutin and old leaves; B = old leaves alone; C = arbutin and young leaves; and D = young leaves alone (μliters O_2 taken up/mg tissue). **4)** The effect of the addition of hydroquinone on O_2 uptake after the start of the tissue homogenate reactions (●———● = no hydroquinone added; ●·····● = hydroquinone added at 0 hr; ●— — —● = hydroquinone added at 1 hr; ●-·-·-● = hydroquinone added at 2 hr; ●– – – – –● = hydroquinone added at 3 hr) (μliters O_2/mg tissue). Arrows indicate time of hydroquinone addition.

cial PPO, brown products were formed with arbutin only.

Old and young leaves of variety D'Anjou were also examined for their ability to oxidize arbutin. The homogenates of old leaves consistently showed more oxidative ability than those from young leaves (Fig. 3) when compared on a fresh wt basis.

Chromatography of reaction products.—In the first series, an arbutin-PPO reaction was stopped after varying periods of time by immersion into boiling water for 1 min. One hundred and fifty μliters of the reaction fluid was then separated chromatographically. An unknown compound (or compounds) with R_F 0.26 was formed early in the reaction (Table 1). Later in the reaction, a compound was formed which was tentatively identified as isopyroside (1) on the basis of R_F (0.71) and color. The unknown decreased in quantity during this period when isopyroside was being formed. None of these compounds was formed in the PPO control.

An analysis of reaction products resulting upon pear tissue disruption was achieved by mashing 0.5 g of pear tissue on filter paper and placing this filter paper on 2% water agar with or without 1% arbutin. After 24 hr, only the arbutin agar had turned dark brown around

TABLE 1. Chromatographic analysis of the products of an arbutin-polyphenoloxidase reaction after varying incubation periods

Compounds	R_F value	Reaction time (hr) 0	0.25	2	3
Hydroquinone	0.84	—[a]	—	—	—
Isopyroside (?)	0.71	—	—	±	+
Arbutin	0.57	+++	+++	++	++
Unknown	0.26	—	±	+	±

[a] Degree of size and intensity of spots (+++ = large, dense spot; — = no spot).

and beneath the filter paper. The agar was then removed and extracted with water, and the extract concentrated in a vacuum evaporator.

D'Anjou blossom tissue treated in this way showed evidence of the formation of an unknown at R_F 0.26. Furthermore, old leaves formed much more of this compound than did young leaves, which was consistent with the respirometer data presented previously. No evidence of either the unknown or hydroquinone was found in extracts of control plates which did not contain arbutin.

Antibiosis of reaction products.—Toxicity studies of the reaction mixtures containing arbutin and PPO, emulsin, or a D'Anjou blossom tissue homogenate, were conducted to evaluate the possibility of a direct effect of such a pathway on the fire blight organism. The reaction mixtures were tested for bacterial growth inhibition after 4- and 30-hr incubation. In an additional test, 0.5 g emulsin was added to the reaction mixtures after 4 hr, and toxicity readings were made after 30 hr. Whereas the initial emulsin-arbutin reaction mixture produced toxic products very quickly, the PPO-arbutin mixture did not show toxicity until it had incubated for 30 hr (Table 2). This was long after measurable oxidation had ceased. The addition of emulsin to reaction mixtures after 4 hr of oxidation did not have an effect on the buildup of toxicity upon subsequent standing of emulsin-arbutin or PPO-arbutin. This later addition of emulsin, however, reduced toxicity buildup in the PPO-emulsin.

The D'Anjou tissue-arbutin mixture contained hydroquinone immediately after preparation, which probably accounts for the initial level of toxicity. However, this substance did not appear to be responsible for the increased toxicity upon standing.

The oxidation products and hydroquinone formed in arbutin agar by homogenates of old and young D'Anjou leaves were separated by chromatography, eluted and brought to the same volume, and checked for toxicity. The inhibition zone average for the oxidation products of old leaves was 16.0, as compared to 7.2 mm for the products in reaction mixtures containing homogenates of young leaves. Conversely, more hydroquinone was formed by young leaves, as reflected by the inhibition zone average of 25.2 mm as compared to 12.8 for old leaves.

Hydroquinone interaction.—The influence of hydroquinone upon the oxidation of arbutin was investigated to determine if products of the hydrolytic pathway of

TABLE 2. Antibiotic activity of reaction mixture containing 0.01 M arbutin when assayed against *Erwinia amylovora* on potato-dextrose-peptone agar plates

Reaction time (hr)	Reaction mixture				
	Arbutin	Polyphenol oxidase	Emulsin	Tissue homogenate	Polyphenol oxidase + emulsin
	diam inhibition zone (mm)				
4	0.0	0.0	6.8	3.6	4.4
30	0.0	5.2	13.4	9.4	14.4
30 (emulsin added after 4 hr)		5.6	11.8	0.0	8.6

arbutin degradation could interact with the oxidative pathway. The uptake of O_2 was stimulated when 0.01 M hydroquinone was added to the standard reaction mixture of arbutin and PPO (Fig. 4). The degree of stimulation depended upon when the addition was made. Much more O_2 was taken up when hydroquinone was added at 2 hr than when added prior to or after this time (Fig. 5). Hydroquinone appeared to be reacting with a temporary oxidation compound derived from arbutin.

To further examine this possibility, chromatograms were made of the products at the termination of all reactions (7 hr). Although changes in the amount of the oxidized unknown were not detected (Table 3), a diminution of hydroquinone coupled with an increase in what appeared to be isopyroside could be observed correlating with the time of greatest O_2 uptake stimulation by hydroquinone (Fig. 4).

In addition to the possible interaction of hydroquinone with the oxidation products of arbutin, one further effect was noted. When hydroquinone was added to a PPO-arbutin mixture at the outset, a lag in O_2 uptake was observed (Fig. 4).

Gluconolactone interference.—Tests were conducted to determine which of the two metabolic pathways, oxidative or hydrolytic, was predominant in blossom tissue macerates given exogenous arbutin. α-Gluconolactone, an inhibitor reported to selectively diminish β-glucosidase activity, was added at 0.1 M to eliminate the formation and subsequent oxidation of hydroquinone.

Gluconolactone decreased the rate of O_2 uptake by two pear varieties (D'Anjou, Bartlett) considered to be susceptible to fire blight (Fig. 6). The significance of this observation is not known, however, because gluconolactone stimulates hydroquinone oxidation (Table 4). Nevertheless, the different effects noted between the resistant and susceptible varieties may have some importance.

DISCUSSION.—The occurrence of systems capable of direct oxidative catabolism of β-glucosides has been reported in pear (2, 3) and other plants (8, 10). Several workers (9, 10) have suggested that such pathways may play a role in the resistance of plants to plant pathogens through the formation of toxic substances. In pear, toxic substances are formed from arbutin via at least two processes, hydrolytic and oxidative.

Although the toxicity to *E. amylovora* of the hydro-

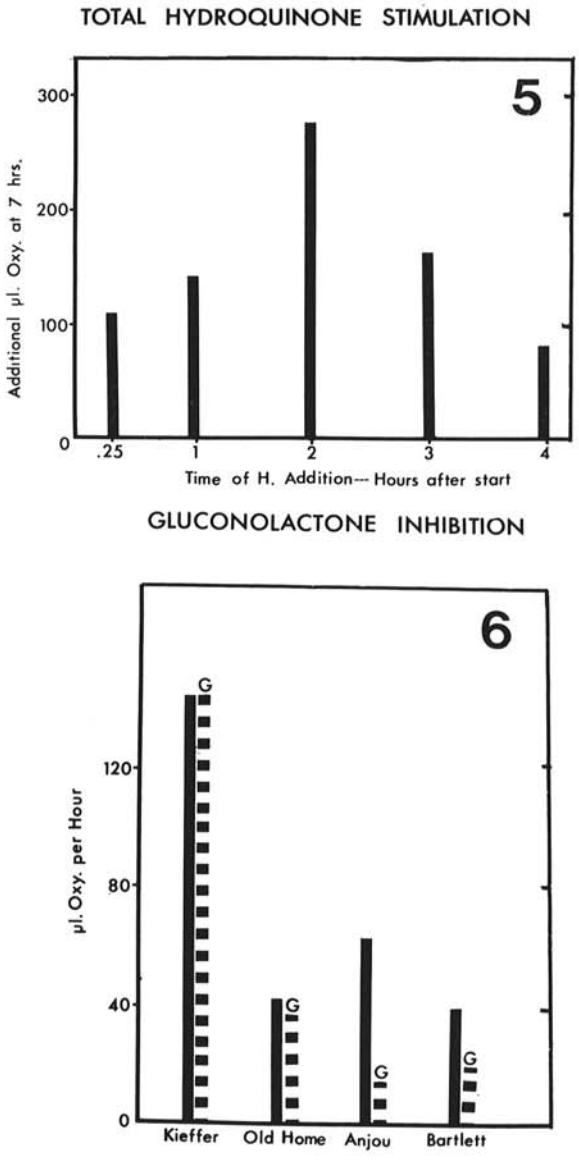

Fig. 5-6. **5)** Total O_2 uptake above that of the control when hydroquinone is added at varying times after the start of the tissue homogenate reactions (measured after 7 hr). **6)** Comparison of rates of O_2 uptake with and without added gluconolactone (µliters O_2 taken up/hr/mg tissue).

TABLE 3. Chromatographic analysis of the products of an arbutin-polyphenol oxidase reaction formed after 7 hr when hydroquinone is added at varying times after the start of the reaction

Compounds	Hydroquinone added (hr)					
	0	0.25	1	2	3	4
Hydroquinone	++[a]	+	±	++	++	++
Isopyroside (?)	±	+	++	±	±	±
Arbutin	+++	+++	++	++	++	+
Unknown	+	+	+	+	+	+

[a] Degree of size and intensity of spots (+++ = large, dense spots; ± = small, light spot).

TABLE 4. Gluconolactone stimulation of hydroquinone oxidation in tissue homogenates from pear blossoms

Pear variety	Reaction time					
	15 min		30 min		60 min	
	Control[a]	+0.1 M Gluconolactone	Control	+0.1 M Gluconolactone	Control	+0.1 M Gluconolactone
Kieffer	105[b]	195	175	240	245	270
D'Anjou	10	230	55	240	100	240
Old Home	10	215	15	265	35	280
Bartlett	5	225	10	265	35	265

[a] Control contained 0.05 g leaf tissue homogenate in 1 ml 0.01 M acetate buffer pH 5.6 and 1 ml of 0.02 M arbutin.
[b] Oxygen uptake (μliters/mg tissue).

lytic and oxidative products may be important in resistance to fire blight, other possibilities must be considered. Oxidative products or hydroquinone might effect pathogenesis by altering metabolites or inactivating enzymes of the host or pathogen. Additional effects of these pathways might prevent the formation of toxic materials by the host, or might interact with previously formed host toxins. The evidence we presented that hydroquinone interacts with the products of the oxidative pathway, that the initial addition of hydroquinone to the oxidative reaction mixtures reduces the subsequent arbutin oxidation, and that treatment of the combined reaction products with emulsin reduces toxicity, would support the latter two effects. The oxidation products of phloridzin are known to react with the hydrolysis products of the glucoside in apple (10).

The presence of such interacting pathways makes extremely difficult any extrapolation of in vitro work to reactions occurring in nature in response to an infection of pear tissue by *E. amylovora*. Nevertheless, the oxidative pathway of arbutin catabolism, in addition to the hydrolytic pathway, may play a role in fire blight resistance. This seems plausible in view of the toxicity of the oxidation products and their interactions with the hydrolytic pathway. Comparisons of arbutin oxidation in old and young tissues correlated well with the manifestation of resistance of pear trees in the field (7, 11). However, a more detailed investigation of the interactions of this pathway with the hydrolytic pathway as well as a comparative study of several pear varieties must be conducted before the full significance of this pathway as a resistance factor can be evaluated.

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