Phytoalexinlike Compounds Apparently Involved in Strawberry Resistance to Phytophthora fragariae

Harry W. Mussell and Richard C. Staples

Assistant Plant Pathologist and Plant Biochemist, respectively, Boyce Thompson Institute for Plant Research, Inc., 1086 North Broadway, Yonkers, New York 10701.

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ABSTRACT

Inoculation of the red stele-resistant strawberry cultivars Surecrop and Stelemaster with either race A-2 or A-4 of *Phytophthora fragariae* resulted in the formation of two materials inhibitory to the growth of *Cladosporium cucumerinum* in a standard bioassay. These materials have not been detected in extracts from healthy roots, but appear in resistant cultivars within 48 hr after infection. Both of these compounds were partially inhibitory to mycelial growth of *P. fragariae* on either lima-bean agar or a synthetic medium. Inoculation of the susceptible cultivar Blakemore resulted in the appearance of only one of these compounds, and this compound was not detected until 5-8 days after infection. Phytopathology 61:515-517.

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In several resistant plant species, invasion by a plant pathogen triggers production of phytoalexins believed to be responsible for resistance (5, 6). Although this relationship has been extensively characterized for several legume diseases (6), evidence for phytoalexin production by nonleguminous plants has not been thoroughly examined. In fact, only one example of phytoalexin production in nonleguminous root tissue has been reported (2).

Growth of *Phytophthora fragariae* Hickman in susceptible strawberry root tissues results at first in a reddening of the stele, then a blackening of root tissues (13). This suggests that susceptible host tissues produce polyphenols and melanins in response to fungal irritation, perhaps in place of effective phytoalexins. Eventually, roots slough off at the crown and the plant succumbs. Although the fungus penetrates resistant root tissues, no discoloration occurs, and several days after inoculation, the fungus cannot be isolated from resistant root tissue (13). The inability of the pathogen to colonize resistant root tissues in the absence of any discernible hypersensitive response on the part of the resistant host implies the presence of phytoalexinlike materials in the challenged, resistant tissue. The present study was initiated to search for phytoalexins after inoculation of susceptible and resistant strawberry cultivars with *P. fragariae*.

MATERIALS AND METHODS.—Runner plants of strawberry cultivars resistant and susceptible to *P. fragariae* were grown in nutrient solution (13). Vigorous 3-week-old runner plants were transferred to stainless steel trays (30 × 20 × 10 cm) at a density of 40 plants/tray. The plants were supported over nutrient solution by a Transite (Johns-Manville) sheet. Groups of 40 plants were placed in environmental chambers programmed for 10-C night temp and 12-C day temp, with a 16-hr photoperiod generated by 1,000 ft-c of fluorescent light. Plants were acclimated to this regime for 2-5 days prior to inoculation.

Zoospores of race A-2 or A-4 of *P. fragariae* were produced by flooding 14-day-old cultures of the fungus growing on red kidney bean agar (13), and incubating the flooded cultures at 10 C for 48 hr. The cultures were then reflooded and incubated for 1 hr at 20 C, 1 hr at 4 C, and 4 hr at 20 C (13). Zoospore suspensions were then pooled, counted with a haemocytometer, diluted to a titer of 10,000-12,000 motile spores/ml, and used for inoculation.

Inoculation was accomplished by removing the nutrient solution from the steel trays and replacing it with 4 liters of zoospore suspension from either race A-2 or A-4. The spore suspension remained in contact with the roots for 48 hr, after which the plants were returned to nutrient solution and incubated at the above temperatures.

Root extracts were prepared by cutting the roots from 40 plants into 1-2 cm pieces in 90% ethanol, boiling the ethanol-root brei for 5 min, then blending the brei for 5 min at top speed in a Sorvall Omni-Mixer. Solids were removed by filtering the extract through Whatman No. 2 filter paper, and the volume of the extract was reduced by rotary evaporation in a partial vacuum at 45 C. Condensed extracts were partitioned against 4 volumes of diethyl ether, and the ether phase was reduced to dryness by rotary evaporation. Resulting materials were redissolved in 20 ml diethyl ether and extracted with 4 volumes of 5% NaOH. The NaOH extract was adjusted to pH 2 with HCl and extracted with several half-volumes of diethyl ether until the ether phase appeared colorless. The ether phase was reduced to dryness by rotary evaporation, and the resulting solids were dissolved in 10 ml of absolute ethanol. This ethanol solution was evaporated to 1 ml and used for chromatography.

Thin-layer chromatography (TLC) plates of Adsorbosil-1 were prepared and spotted with 10 µliters of test extract. The plates were developed in a solvent consisting of the organic phase of a mixture of ethyl acetate:benzene:50% ethanol (4:1:1, v/v). The developed chromatograms were dried and sprayed with a spore suspension of *Cladosporium cucumerinum* Ell. & Arth. in nutrient media (4), and incubated at 100%

relative humidity and room temp for 4 days (8).

Inhibitors were removed from the white areas on assayed TLC plates and eluted from the silica gel by repeated extraction with ethanol. These ethanol extracts were spotted on 1-cm discs of Whatman No. 1 filter paper, dried, and placed on lima bean agar or synthetic medium that had been seeded 24 hr previously with a zoospore suspension of *P. fragariae*. All cultures were maintained at 20 C in darkness.

Our lima bean agar was prepared by adding 71 g of blended frozen lima beans to 1 liter of 1.5% agar solution. The synthetic medium used was that of Davies (7) supplemented with 75 ppm cholesterol or β-sitosterol, 5×10^{-3} M $CaNO_3$, 10^{-5} M kinetin, and 10^{-4} M glutathione. Growth inhibition was determined by microscopic observation of the cultures at intervals after application of the inhibitors. Controls received filter papers containing the dried residue from 1 ml of absolute ethanol.

Phenylalanine-ammonia lyase was assayed according to the methods described by Zucker (14). Aliquots of ^{14}C-labeled phenylalanine were incubated with root extracts prepared by grinding frozen roots in 0.01 M barbital buffer pH 8.5. After 1 hr of incubation at 30 C, the resulting cinnamic acid was extracted with toluene, and the activity in each sample was counted in a Beckman LS 100 scintillation counter. Results are presented as millimicromoles phenylalanine converted to cinnamic acid per μg root protein per hr. Protein in the root extracts was determined according to Lowry et al. (11), using crystalline bovine serum albumin as a reference standard.

RESULTS.—Inoculation of the susceptible cultivar Blakemore with either race A-2 or A-4 of the pathogen resulted in development of typical symptoms of red stele disease. Roots first developed a brown coloration, then a reddening of the stele, followed by a progressive decline and eventual death of the aerial portion of the plants. The only symptom observed on the roots of the resistant cultivars Surecrop and Stelemaster was a slight browning of root epidermal cells 48-72 hr after inoculation.

Typical TLC plates of root extracts from Blakemore and Surecrop, when chromatographed and assayed with *C. cucumerinum*, contained at least three inhibitors (Fig. 1). These same three inhibitors have been observed in extracts of both Surecrop and Stelemaster when inoculated with either race A-2 or A-4 of *P. fragariae*. One of these inhibitors (C, R_F 0.84) was observed in extracts of both susceptible and resistant roots regardless of whether or not they were inoculated. The other two inhibitors (A, R_F 0.52 and B, R_F 0.39) appeared only after inoculation. Inhibitors A and B were detectable in inoculated resistant roots 24-48 hr after inoculation. While it was not possible to measure amounts of the inhibitors present, visual observation of the TLC plates suggested that the apparent quantities of inhibitors A and B increased for 5-8 days, then declined. Small quantities of inhibitor A were detectable in extracts from susceptible roots 5-8 days after infection (Fig. 1), while inhibitor B was never detected in extracts from susceptible roots. Inhibitors A and B were detectable by bioassay using less pure extracts than described, and inhibitor A has been detected in ethyl acetate extracts from the nutrient solution used for postinoculation incubation of resistant roots. However, the presence of the inhibitors in these less purified extracts was frequently masked by the presence

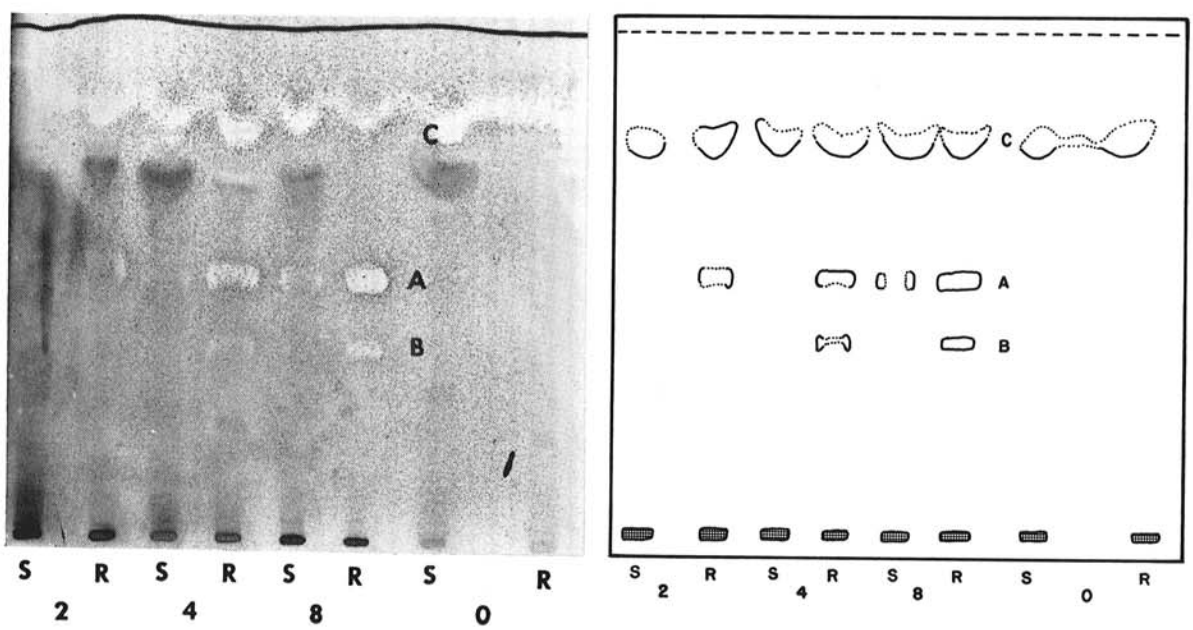

Fig. 1. Inhibitors (designated A, B, & C) in root extracts of the susceptible strawberry cultivar Blakemore (S) and the resistant cultivar Surecrop (R) 2, 4, and 8 days after infection with race A-4 of *Phytophthora fragariae* and in healthy roots (0). (Left) Assayed thin-layer chromatogram. (Right) Line drawing location of inhibitors.

of other materials apparently stimulatory to the growth of the test organism used in the bioassay.

Phenylalanine-ammonia lyase activity was detectable in healthy and infected strawberry roots, but the levels of lyase activity did not change after infection in either resistant or susceptible plants (Table 1).

Inhibitors A and B, extracted from thin-layer plates and introduced into cultures of *P. fragariae*, proved to be inhibitory to mycelial growth but not lethal. Inhibitor A retarded the growth of young sporelings of *P. fragariae* for 48-72 hr, after which the paper discs were overgrown by the mycelium. Inhibitor B alone retarded the development of the fungus for 24-36 hr, after which the discs were overgrown. In combination, A and B were capable of retarding development of the fungus for up to 96 hr; then the discs were overgrown. Inhibitor C was not effective in retarding growth of sporelings of *P. fragariae*, nor did the addition of inhibitor C to a mixture of A and B enhance the inhibitory powers of these materials.

DISCUSSION.—Red stele of strawberries is a highly specialized disease relationship involving physiological races of the pathogen and differential resistance in the host (3, 12). If there is a general mechanism of resistance operative in this disease, it should be detectable in more than one race-cultivar combination. Therefore, in order to examine strawberries for a general mechanism of resistance to red stele, we have chosen two virulent races of the pathogen, two cultivars resistant to these races, and one cultivar susceptible to both races of the fungus.

The data suggests that strawberry resistance to the races of *P. fragariae* studied may be related to the ability of resistant roots to generate two inhibitors in response to invasion by the pathogen. It appears that resistance relates to the rate of production of inhibitor A coupled with the ability to generate inhibitor B. The detection of inhibitor A in extracts from susceptible roots late in the infection process implies that this material must be generated early during the infection sequence in sufficient quantities to be effective in resistance. As discussed by Kuć (10), the ability of a plant tissue to synthesize a given inhibitor does not necessarily indicate that this tissue will be resistant. The amount and/or time of synthesis are also critical if the material is to be effective in a resistance response. Bell (1) has described a similar situation relating to *Verticillium* wilt resistance in cotton. In this system, both resistant and susceptible plants are capable of generating phytoalexinlike materials (gossypol and precursors), but the susceptible plants do not generate sufficient amounts of these materials fast enough to restrict the spread of the pathogen. Possibly inhibitor A in strawberry roots has the same function postulated by Bell for gossypol in cotton; i.e., to retard the spread of the pathogen during the time required for synthesis of other materials which play a role in eradication of the pathogen.

Although phenylalanine-ammonia lyase increases during the synthesis of other phytoalexins (9), activation of this enzyme is apparently not essential for synthesis of the inhibitors observed in resistant strawberry roots after infection.

Characterization of these inhibitors and elucidation of the pathways involved in their biosynthesis are essential for a precise definition of the role of these materials in resistance to *P. fragariae*. However, it seems that resistance to *P. fragariae* in strawberries can be added to the increasing list of resistance situations in which phytoalexin-like materials are involved.

TABLE 1. Phenylalanine-ammonia lyase activity in root extracts from susceptible and resistant strawberry roots after infection with *Phytopthhora fragariae*

Cultivar	Time after infection (days)				
	0	1	2	3	4
	Millimicromoles cinnamic acid/ μg protein/hr				
Blakemore	52	44	43	48	39
Surecrop	44	46	41	48	46

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