Degradation of Alfalfa Phytoalexin by Stemphylium loti and Colletotrichum phomoides

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ABSTRACT

Stemphylium loti, a weak pathogen of alfalfa, induced production of phytoalexin by alfalfa, but only small amounts of the phytoalexin were detected in infection drops. The phytoalexin was rapidly degraded by S. loti in vitro; degradation proceeded concomitantly with the production of a compound (λ max 285 mμ) that inhibited germ tube growth of S. loti. Colletotrichum phomoides, a nonpathogen of alfalfa, effected slow degradation of alfalfa phytoalexin in vitro and concomitant production of a compound that inhibited germ tube growth of C. phomoides and had an ultraviolet absorption spectrum similar to that of the phytoalexin, but differing in its solubility in CCl$_4$. Phytopathology 60:269-271.

Stemphylium botryosum Wallr., a pathogen of alfalfa (Medicago sativa L.), and Helminthosporium turcicum Pass. [= Bipolaris turcicum (Pass) Shoem.] and Colletotrichum phomoides (Sacc.) Chester, two nonpathogens of alfalfa, each induce production of a phytoalexin by alfalfa leaves (4, 5). S. botryosum immediately degrades the phytoalexin to a phenolic compound designated compound I (5, 6). Compound I is degraded to a second phenolic compound designated compound II, which in turn either is further degraded or assimilated. H. turcicum, apparently, is unable to degrade the phytoalexin; consequently, the phytoalexin accumulates in infection drops containing only H. turcicum spores (5). The capacity of C. phomoides to degrade alfalfa phytoalexin was heretofore unknown.

In addition, results of previous investigations (4) on Stemphylium loti Graham, which is weakly pathogenic on alfalfa, left unclear whether the minute amount of an antifungal compound associated with infection drops that contained only S. loti spores was actually phytoalexin; if this antifungal compound was phytoalexin, it was not clear whether the small amount present reflected weak ability of S. loti to induce the phytoalexin, or strong ability of this fungus to degrade the phytoalexin.

The investigations reported here were undertaken, therefore, (i) to determine by chromatographic and spectrophotometric assays if S. loti induces production of phytoalexin; (ii) to determine if C. phomoides and S. loti degrade the phytoalexin in vitro; and (iii) providing C. phomoides and S. loti were found capable of degrading phytoalexin, to compare these fungi with S. botryosum in terms of the rates and mechanisms by which each effects degradation of the phytoalexin.

RESULTS.—The fungal isolates and procedures used have been described previously (4, 5, 6). An isolate of Stemphylium consortiale (Thum.) Groves & Skolko isolated by S. W. Braverman, New York State Agriculture Experiment Station, Geneva, N.Y., was used in one experiment, and was grown in the same manner as the other Stemphylium spp. Rapid adjustment of spore suspensions to desired concentrations was done by means of a standard curve that related optical densities of suspensions at 540 mμ (for Stemphylium spp. or H. turcicum) and 420 mμ (for C. phomoides) to either spores/ml or to dry wt of spores/ml.

S. loti was tested for its ability to induce phytoalexin production by incubation of a spore suspension (5×10^4 spores/ml) as drops on excised alfalfa leaves for 24 hr. The diffusate solutions were harvested, extracted, and assayed for phytoalexin by spectrophotometry and by Silica Gel G thin-layer chromatography (4, 5). Small but detectable amounts of phytoalexin were found; the amount of phytoalexin present in S. loti-diffusate solutions was approximately 50 times less than that in H. turcicum-diffusate solutions.

In a preliminary degradation experiment, phytoalexin (obtained from H. turcicum-diffusate solutions) was added to 5-ml portions of spore suspensions of C. phomoides, S. loti, or S. consortiale (0.2 mg dry wt spores/ml) in Czapek-Dox broth buffered at pH 6.0 with phosphate buffer. These spore suspensions were incubated for 10 hr prior to and for 15 hr after the addition of the phytoalexin. The cultures then were filtered. The filtrates were extracted with CCl$_4$, and the CCl$_4$-extracts, after they had been adjusted to provide a concentration that was 2.5 times that of the filtrates, were scanned for absorbance. The absorbance at 285 mμ for the control (Czapek's medium plus phytoalexin), C. phomoides, S. loti, and S. consortiale treatments was 0.52, 0.24, 0.02, and 0.41, respectively. To determine if the mycelium and spores had taken up phytoalexin, the mycelium and spores retained on the filter papers when the cultures were filtered were shaken in 2 ml of 95% ethanol in a test tube for 30 min, after which 2 ml of water was added, the ethanol was evaporated, and the aqueous solution was extracted twice with 2 ml of CCl$_4$. The CCl$_4$ was evaporated, the residue was taken up in 1 ml of 95% ethanol, and the resulting ethanol solution was scanned for absorbance. Phytoalexin (absorbance of 0.07 at 285 mμ) was recovered only from S. consortiale mycelium and spores.

The rates and mechanisms of degradation of the phytoalexin by S. botryosum, S. loti, C. phomoides, and H. turcicum were compared in a time study that involved spore suspensions (0.2 mg dry wt spores/ml)

of each fungus. The procedure used to follow degradation was like that used in the previous experiment, except that the spore suspensions were harvested at 0, 4, 8, 12, and 24 hr after the addition of the phytoalexin, and the filtrates were extracted first with CCl_4 and then with ethyl acetate (EA). Spectrophotometric analysis was used to assay for phytoalexin in the CCl_4-extracts and for degradation products in the EA-extracts; chromatography was used to test for the presence of phytoalexin and of degradation products in both the CCl_4- and EA-extracts.

The added phytoalexin had almost completely disappeared from spore suspensions of *S. botryosum* or *S. loti* by 4 hr, and from comparable spore suspensions of *C. phomoides* by 24 hr, whereas no significant loss occurred in spore suspensions of *H. turcicum* (Fig. 1, above). In the EA-extracts for *S. botryosum*, there was, as expected, an increase in Compound II as measured by its absorbance at 260 mµ (Fig. 1, above). Comparable extracts for *S. loti* contained a compound, designated as compound SLI, that had an ultraviolet absorption spectrum with a maximum at about 285 mµ (Fig. 1, below) but whose spectrum was not that characteristically obtained for phytoalexin. The concentration of compound SLI appeared to increase as the concentration of phytoalexin decreased (Fig. 1, above). In a second trial of this experiment a 2-hr harvest was included, and by means of this additional time interval it was shown more clearly that compound SLI increased in concentration as the phytoalexin concentration decreased. Chromatography of the extracts and subsequent elution of various fractions of the chromatogram revealed that compound SLI remained at the origin on silica gel plates irrigated with *n*-pentane:ethyl ether: acetic acid (75:25:1, v/v).

The EA-extracts of the *C. phomoides* treatments contained a compound, designated as compound CPI, with an ultraviolet absorption spectrum (Fig. 1, below) similar to that of the alfalfa phytoalexin. The amount of compound CPI increased as the amount of phytoalexin decreased (Fig. 1, above). Chromatography of the EA-extracts showed that compound CPI appeared at R_F 0.12 on silica gel plates and reacted with *p*-nitroaniline to give a bright orange spot in contrast to the yellow color given by the phytoalexin. There were no compounds present in EA-extracts for *H. turcicum* that absorbed in the ultraviolet range or reacted with *p*-nitroaniline (Fig. 1, above).

Aliquots of the CCl_4- and EA-extracts for each fungus and for each incubation interval were spotted together on silica gel plates (i.e. the CCl_4-extracts were overspotted with the EA-extracts). These chromatograms illustrated the differences in the rates of degradation of the phytoalexin by the four fungi and the various degradation products that appeared coincidently with the disappearance of the phytoalexin. Chromatograms of CCl_4- and EA-extracts spotted separately showed that, whereas the CCl_4-extracts contained small amounts of the degradation products (compound I, compound SLI, and compound CPI), the major portion of these compounds was in the EA-extracts.
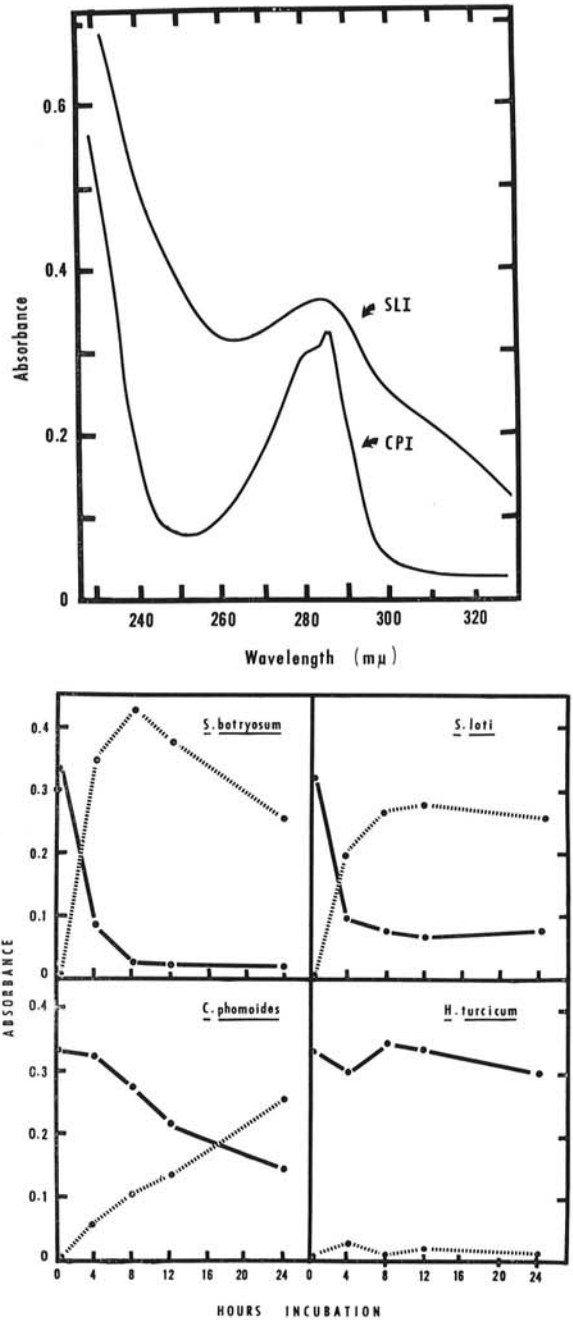

Fig. 1. Above) Ultraviolet absorption spectra of products formed during the degradation of alfalfa phytoalexin by *S. loti* (SLI), and *C. phomoides* (CPI). **Below)** Rate of disappearance of phytoalexin and appearance of other phenolic compounds in filtrates of *Stemphylium botryosum*, *S. loti, Colletotrichum phomoides*, and *Helminthosporium turcicum* spore suspensions to which phytoalexin was added. The filtrates were obtained at various incubation times after the addition of phytoalexin, and extracted first with carbon tetrachloride, which removed the phytoalexin, and then with ethyl acetate, which removed other phenolic compounds. Absorbance for the phytoalexin (solid line) and the phenolic products (broken line) was determined at 285 mµ, except for that of the phenolic product in *S. botryosum*-extracts, which was determined at 260 mµ.

To determine whether compounds SLI and CPI inhibited germination and germ tube growth of *S. loti* and *C. phomoides,* respectively, the EA-extracts of filtrates that were obtained from treatments incubated for 24 hr after the addition of the phytoalexin were bioassayed. Fifty-μliter aliquots of aqueous solutions of the EA-extracts, concentrated to 8, 16, and 40 times the concentration of the EA-extractable substances in the original filtrates, were added to agar discs seeded with ungerminated spores of either *S. loti* or *C. phomoides.* The controls were EA-extracts of filtrates from spore suspensions to which no phytoalexin was added. The discs with *S. loti* spores were incubated for 7 hr, and those with *C. phomoides* spores for 24 hr, prior to measurement of the longest germ tube of each of 25 spores/disc.

The EA-extracts of filtrates of both the *S. loti* and *C. phomoides* treatments were inhibitory to germination or germ tube growth of these fungi (Table 1). The substances in the EA-extracts appeared to be as inhibitory as the phytoalexin.

DISCUSSION.—The pathogenicity of four fungi on alfalfa, each of which induces phytoalexin, and their ability to degrade the phytoalexin from alfalfa, is correlated. *S. botryosum,* a vigorous pathogen of alfalfa, degraded the phytoalexin rapidly to compounds that were not inhibitory to germ tube growth of that fungus (6). *S. loti,* which is weakly pathogenic on alfalfa, also degraded the phytoalexin rapidly, but the loss of phytoalexin coincided with the appearance of a compound (or compounds) that inhibited the growth of *S. loti.* *C. phomoides,* a nonpathogen of alfalfa, degraded the phytoalexin slowly (possibly partly as a result of its slower growth rate) and, as occurred with *S. loti,* an inhibitory compound appeared coincident with the disappearance of the phytoalexin. Another nonpathogen, *H. turcicum,* did not degrade the phytoalexin in 24 hr.

S. botryosum, S. loti, and *C. phomoides* apparently

degrade alfalfa phytoalexin by different mechanisms, since the degradation products differ for each fungus; it is possible, however, that the same pathway was operative with each fungus, but that different intermediates accumulated. That the ability to degrade this phytoalexin was not a characteristic of all *Stemphylium* species, but instead was related to their pathogenicity on alfalfa, was shown by the inclusion of *S. consortiale* in one experiment; *S. consortiale* caused only slight loss of phytoalexin from the culture medium in 15 hr, and a portion of the phytoalexin lost was recovered upon extraction of the mycelium.

The results of this study are comparable to those obtained with pisatin, a phytoalexin from peas. Pathogens of peas degrade pisatin in vitro, whereas nonpathogens of peas either do not degrade the pisatin (7, 8, 9, 10) or do so more slowly than the pathogens (1). This difference in the ability to degrade pisatin was suggested (9) as an explanation for the different sensitivities of fungi to pisatin in bioassays. Deverall (2) and Deverall et al. (3) have suggested, on the basis of in vivo studies, that the difference in pathogenicity between *Botrytis fabae* and *B. cinerea* on broad bean may be due to a difference in their abilities to degrade the phytoalexin from broad bean. Very little phytoalexin accumulated in tissue of the lesions caused by *B. fabae* as compared to the amount found in the small delimited lesions caused by *B. cinerea.* The ability of fungi to degrade phytoalexins is a factor that must be considered in the interpretation of the role of phytoalexins in resistance to plant disease.

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TABLE 1. Inhibitory effect on germination of compounds appearing in ethyl acetate (EA) extracts of filtrates of *Stemphylium loti* or *Colletotrichum phomoides* spore suspensions incubated for 24 hr after the addition of phytoalexin to the suspensions

Filtrate tested	Concn. of EA-soluble substances in extracts[a]		
	8×	16×	40×
	Bioassayed with *C. phomoides* spores		
C. phomoides	51[b]	46	0[c]
	Bioassayed with *S. loti* spores		
S. loti	55	18	0

[a] The concentration of EA-soluble substances added in assay to agar disc as a 50-μliter aliquot is expressed in terms of the concentration of these substances in the original filtrates.

[b] Germ tube length as % of control. Controls were EA-extracts of filtrates of spore suspensions to which no phytoalexin was added, but which otherwise were treated similarly. Agar discs seeded with *C. phomoides* spores incubated 24 hr, *S. loti* incubated 7 hr.

[c] No germination.