Association of Cyanide with Infection of Birdsfoot Trefoil by Stemphylium loti

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ABSTRACT

Cyanide was released from excised shoots and leaves of birdsfoot trefoil plants infected with *Stemphylium loti* regardless of whether they were cyanogenic (containing both cyanogenic substrate and β-glucosidase) or not cyanogenic (containing substrate but lacking β-glucosidase). Enzymes elaborated by the pathogen in vivo effected the release of HCN from plants not cyanogenic, and presumably acted together with enzymes of host origin in liberating HCN from cyanogenic plants. Infection by *S. loti* resulted in a degradation of host cyanogenic glucoside, and symptom severity was correlated with the amount of HCN released. *S. loti* was markedly more tolerant of HCN than were eight other fungi tested. All of eleven fungi when grown on autoclaved trefoil shoots or leaves produced enzymes that were effective in releasing HCN from trefoil cyanogenic substrate or from amygdalin. Phytopathology 60:104-110.

In New York, copperspot caused by *Stemphylium loti* Graham is the principal disease of birdsfoot trefoil (*Lotus corniculatus* L.). Symptoms consist of brick-red to dark brown leaf spots and stem cankers, and extensive premature defoliation of affected leaflets (5). These are the consequence of injury to cells during invasion of the tissues by the pathogen.

Injury to healthy tissues of trefoil results in the release of hydrogen cyanide (HCN) (1, 6). HCN is liberated from the cyanogenic glucosides linamarin and lotaustralin presumably as a two-step sequence that involves the enzymes β-glucosidase and oxynitrilase (16).

These considerations led us to investigate (i) whether HCN liberation might be involved in pathogenesis by *S. loti;* (ii) whether *S. loti* might be relatively tolerant of HCN; and (iii) whether HCN might be involved in resistance of trefoil to nonpathogens of the crop. A preliminary report of the investigation has been published (10).

MATERIALS AND METHODS.—The *Stemphylium* spp. used in this study were obtained originally from J. H. Graham, USDA, Beltsville, Md., and bore the following designations: 520 and 757, *S. loti* isolated from birdsfoot trefoil; 653, *S. botryosum* Wallr. (from alfalfa); and 627B, *S. sarcinaeforme* (Cav.) Wilt. (from red clover). The virulence of the isolates was maintained by regular passage through their respective suscepts. Other organisms were obtained in New York as follows: *Thielaviopsis basicola* (Berk. & Br.) Ferr. and *Rhizoctonia solani* Kühn (isolate RB) from *Phaseolus vulgaris* (courtesy of D. F. Bateman, Cornell University); *Helminthosporium turcicum* Pass. from corn; *Fusarium oxysporum* Schlecht., *F. roseum* Link, and *F. solani* (Mart.) Appel & Wr. from alfalfa roots; *Monilinia fructicola* (Wint.) Honey from peach; and *Colletotrichum lagenarium* (Pass.) Ell. & Halst. from cucumber.

Stock cultures of *Stemphylium* spp. were maintained at 20-23 C under fluorescent lights on V-8 juice agar (11). Stock cultures of other organisms were maintained similarly, except that potato-dextrose agar was used.

Birdsfoot trefoil (*Lotus corniculatus* L. 'Viking') plants were obtained originally from R. R. Seaney, Department of Agronomy, Cornell University. Rooted stem pieces from these plants provided additional plants that were genetically homogeneous. Two types of trefoil were used. One type was characteristic of the commercial variety, Viking, and was cyanogenic; it therefore was designated hydrogen cyanide-positive or HCN^+. The second type, which contained cyanogenic substrate but not β-glucosidase, was designated hydrogen cyanide-negative or HCN^-; this type was obtained by Dr. Seaney as a selection from Viking. The foliage was harvested at 4- to 5-week intervals from greenhouse-grown plants. The plant material used in this study was 6- to 8-week-old regrowth of 4- to 18-month-old plants. Each plant was tested to determine its cyanogenic properties. Care was taken to maintain plants of each type under comparable conditions for growth.

Determination of cyanide released.—Hydrogen cyanide released as a result of infection or by enzymatic activity in vitro was investigated in disposable Conway microdiffusion dishes with two chambers (60 × 35 mm diam). Test material was placed in the outer chamber. Three ml of alkaline sodium picrate solution (picric acid, 5 g; Na_2CO_3, 25 g; H_2O, 2 liter) (2) were added to the inner chamber. The lid was sealed to the dish with vaseline. Absorption of HCN by the picrate solution produces a colored product that absorbs maximally at 480 mμ. At appropriate intervals, therefore, absorbance of the picrate solution was determined at 480 mμ in a Spectronic 20 colorimeter. HCN concentration was determined from a standard curve prepared by plotting the KCN concentration of a solution against the absorbance at 480 mμ.

When the test material was trefoil shoots, the shoots were excised from the plants and divided into 10 samples (2 or 3 shoots/sample) that appeared uniform with respect to leaf area. Spores of *S. loti* from V-8 juice agar cultures were collected in sterile distilled water containing Tween 20 (polyoxyethylene sorbitan monolaurate) at 1 drop/100 ml, and the resulting suspension was strained through two layers of cheesecloth. The shoots of five samples (1 sample/dish) were dipped

into the spore suspension and then placed in the outer chamber. Shoots of five samples dipped in water plus Tween 20 served as plant controls. Three days later, the absorbance of the picrate solution in each dish was determined. Picrate solution from two dishes prepared similarly but which did not receive trefoil shoots was used as the reagent control.

The procedure was modified as follows for excised leaves. Three ml of picrate solution were added to the outer chamber of a microdiffusion dish. One ml of water was added to the inner chamber. Leaves were excised and trimmed by removal of the outermost pair of leaflets to three leaflets, and were floated temporarily on distilled water in a large glass tray. When sufficient leaves were collected, three leaves (i.e., nine leaflets) were transferred to each dish and floated on the water in the inner chamber. A drop of spore suspension or of water plus Tween 20 was placed on each leaflet. When the spores had settled onto the leaf surface, most of the liquid in the drops was removed by means of weak suction, and the dishes were sealed. At appropriate intervals thereafter, absorbance of the picrate solution in each dish was determined.

The cyanogenic activity of host or pathogen enzyme preparations was tested in a manner similar to that used for shoots, except that the enzyme reaction mixture was added in place of the shoots to the outer chamber of the microdiffusion dish. The reaction mixture, unless indicated otherwise, contained 2 ml enzyme preparation, 1 ml substrate solution (either 0.003 M amygdalin or host substrate preparation), and 1 ml 0.1 M phosphate buffer at pH 6.0. Reaction mixtures used as controls were prepared similarly, except that the enzyme preparations were autoclaved to inactivate the enzymes.

Enzyme preparations were tested for cyanogenic activity with either 0.003 M amygdalin or extracts of trefoil leaves or shoots as substrates. The plant material was harvested, autoclaved immediately for 20 min at 120 C, and homogenized in distilled water (1:2 or 1:4, w/v). The mixture was strained through two layers of cheesecloth to remove debris and centrifuged for 30 min at 3,300 *g*. The supernatant fluid was collected and stored at −20 C.

Determination of the relative amounts of cyanogenic glucosides in healthy and in *S. loti*-infected HCN− plants involved collection of leaflets with lesions from inoculated plants and a comparable number of leaflets from healthy noninoculated plants. Fresh and dry wt were determined for both lots of leaves. Equivalent amounts of healthy and of diseased leaf tissue (based on dry wt) were homogenized in water (1:2, w/v). The resulting extracts were centrifuged at 3,300 *g*. The extracts were tested in microdiffusion dishes as substrates for an enzyme preparation from cultures of *S. loti* grown on autoclaved alfalfa roots. Reaction mixtures consisted of 1 ml substrate preparation, 1 or 2 ml enzyme extract, 1 ml 0.1 M phosphate buffer at pH 6.0, and water to a total volume of 4 ml.

Enzyme production in vivo and in vitro.—Preparations of *S. loti* enzyme produced in vivo were obtained as follows. Spores from 5- to 7-day-old cultures of *S. loti* were prepared as a suspension in Tween 20 solution (1 drop/100 ml water) at a concentration of approximately 75,000 spores/ml. The suspension was sprayed on leaves and shoots of HCN+ or HCN− plants grown in soil in the greenhouse. The plants then were enclosed in polyethylene bags and held 3-4 days at 20-25 C in the greenhouse. Leaflets that developed lesions were harvested and stored at −20 C until they were extracted. The leaves were homogenized in 0.25 N NaCl (1:2, w/v), and the resulting mixture was strained through cheesecloth to remove debris, centrifuged for 30 min at 3,300 *g*, and dialyzed in a large volume of distilled water 24-48 hr at 4 C.

Host enzyme preparations were obtained by the same extraction procedure from healthy HCN+ plants.

Comparisons of activities of enzymes from healthy or infected trefoil plants were made with preparations made equivalent with respect to protein concentration. Protein was measured in dialyzed extracts by the Folin-Ciocalteu method of Lowry et al. (8) with bovine albumin as the standard.

A solid culture medium composed of trefoil shoots from HCN+ or HCN− plants also was used for enzyme production by *S. loti* as well as for other fungi. The shoots were excised, autoclaved 20 min at 120 C, and stored at −20 C. The shoots were cut into small pieces, transferred in 10-g lots to 250-ml Erlenmeyer flasks plugged with cotton, then autoclaved 30 min at 120 C. The test fungi were added aseptically to the flasks either as spore suspensions prepared in sterile distilled water (1 ml/flask) when the fungi sporulated profusely or as mycelium on a small square of agar when the fungi sporulated poorly or produced only mycelium. Flasks with sterile shoot material to which was added 1 ml sterile distilled water or a small square of agar served as controls. The cultures were incubated at 23 C, usually for 7 days, then extracted by homogenizing the culture in 0.25 N NaCl (1:2, w/v) in a blender. The mixture was squeezed through cheesecloth and the liquid was collected, centrifuged for 20 min at 3,300 *g*, and dialyzed against distilled water 24-48 hr at 4 C.

Roots of alfalfa plants (*Medicago sativa* L.) collected from field plots on November 1963 and stored at −20 C were used as a solid culture medium in the same manner, except that 15-g lots of small pieces of the roots were added to the flasks, and 30 ml of 0.25 ml NaCl was used for extraction of each 15 g culture material. Alfalfa was tested because Colotelo & Ward (2) reported an association of cyanogenic substrates with *M. sativa*.

Liquid culture media also were used for enzyme production in vitro. These included potato-dextrose broth (PDB) and Czapek's broth (Difco) tested alone or supplemented with 0.003 M amygdalin, and trefoil leaf extract. The trefoil leaf extract was prepared by excising the leaves, autoclaving them 20 min at 120 C, and then homogenizing them in distilled water (1:3, v/v). The resulting mixture was filtered through two layers of cheesecloth, and the filtrate was used as the medium. Each medium was adjusted to pH 6.4 with 0.1 N NaOH or 0.1 N HCl before it was sterilized in the autoclave.

Sensitivity of several fungi to HCN.—The sensitivity to HCN of several fungi was tested in microdiffusion dishes with three chambers (80 × 65 × 40 mm diam). A suspension of spores of the test fungus was prepared in sterile distilled water at a concentration of about 10 spores/field at low-power magnification of the microscope. Two ml of the suspension was distributed uniformly over the surface of potato-dextrose agar in a petri dish, and about 5 min later when the spores had settled, the surplus water was poured from the dish. Two agar discs bearing spores of the test fungus were cut with a sterile No. 9 cork borer and transferred on a sterile spatula to the inner well of a microdiffusion dish. One ml of water or KCN solution was added to the middle chamber. Water was added to the outer chamber, and provided an air-tight seal when the lid of the dish was fitted into the outer chamber. Replicate dishes were prepared for several concentrations of KCN solution. Three replications were prepared for the water control, and one of these was used to follow germination and development of the test fungus to a stage at which lengths of the germ tubes of spores could be readily measured. The dishes were held at 24-25 C until germ tube growth was satisfactory for measurement, at which time the agar discs for each treatment, i.e., per KCN concentration tested, were transferred to petri dishes, and one or two drops of lactophenol (with or without cotton blue) were added to each disc to halt development of the fungus. The longest germ tubes of each of the first 25 spores examined per disc were measured with an ocular micrometer. The mean germ tube lengths for each of the four discs were averaged to give the mean germ tube length for the treatment. The data for a treatment are expressed as the percentage of the mean germ tube length of spores in the water control treatment.

RESULTS.—*Cyanogenesis in response to infection.*—Inoculation of shoots of HCN+ trefoil plants with *S. loti* 757 resulted in the release of HCN. This was clearly indicated by the values recorded in one experiment for five replications (6, 12, 12.6, 9.6, 18 μmoles × 10^{-2}). Noninoculated and apparently undamaged shoots did not release HCN over a period of 3 days.

Infection of excised leaflets resulted in the release of HCN. In these experiments, excised leaves of HCN+ or HCN− plants inoculated or noninoculated with *S. loti* were incubated either in the presence of picrate solution or water. There was an increase in the amount of HCN released as infection of the leaves proceeded and symptoms increased in severity (Table 1). *Stemphylium loti* was pathogenic on both HCN+ and HCN− plants, and HCN was released from both plant types. This finding is significant because it indicates that in HCN− leaves the pathogen produced the enzymes that effected the release of HCN. When HCN released as a result of infection was removed from the atmosphere in the dish by absorption in the picrate solution, symptom severity was greater than when HCN was allowed to accumulate (Table 2). This result was less definite for HCN+ leaflets inoculated with *S. botryosum,* a nonpathogen of trefoil.

Cyanogenesis as a consequence of infection results in a depletion of cyanogenic glucoside in diseased tissues. This was determined by comparing healthy and *S. loti*-infected HCN− plants for the amount of cyanogenic glucosides they contained. Cyanide was released from the substrate from healthy leaves in the amounts of 10.8 and 28.2 μmoles × 10^{-2} for 1 and 2 ml, respectively, of enzyme extract. Amounts of cyanide released from substrate from diseased leaves were only 2.4 and 3.6 μmoles × 10^{-2} for 1 and 2 ml, respectively, of enzyme extract. These results show, based on the amount of HCN released, that there is a depletion of

TABLE 2. Symptom severity for two types (HCN+ and HCN−) of birdsfoot trefoil inoculated with *Stemphylium loti* and incubated under conditions that provided for removal or accumulation of HCN released

S. loti-infected trefoil type[a]	Symptom severity[b] Days after inoculation							
	+ picrate				— picrate			
	1	2	3	4	1	2	3	4
HCN+	0	.64	1.33	2.72	0	.18	.76	.90
HCN−	0	.28	1.65	1.67	0	.21	.64	.67

[a] HCN+ indicates healthy plants cyanogenic (i.e., contained both cyanogenic substrate and β-glucosidase); HCN− indicates healthy plants not cyanogenic (i.e., contained cyanogenic substrate but lacked β-glucosidase).

[b] Values are averages for four replications each with nine leaflets; inoculated leaflets incubated in presence of alkaline picrate solution (to remove HCN) or in presence of water (to allow HCN to accumulate). Symptom severity rated on 0-5 scale with 0 = no necrosis, and 5 = entire leaflet affected.

TABLE 1. Symptom severity and HCN released for two types (HCN+ and HCN−) of birdsfoot trefoil at intervals after inoculation with *Stemphylium loti*

S. loti-infected trefoil type		Days after inoculation			
		1	2	3	4
HCN+[a]	μMoles CN− × 10^{-2}[b]	0	0	9	7.5
	Symptom severity	0	1.29	3.55	3.04
HCN−	μMoles CN− × 10^{-2}	0	6	7.8	22.2
	Symptom severity	0	2.62	3.57	4.21

[a] HCN+ indicates healthy plants cyanogenic (i.e., contained both cyanogenic substrate and β-glucosidase); HCN− indicates healthy plants not cyanogenic (i.e., contained cyanogenic substrate but lacked β-glucosidase).

[b] Values are averages for four replications each with nine leaflets. Cyanide released was trapped in alkaline picrate solution. Symptom severity rated on 0-5 scale with 0 = no necrosis, and 5 = entire leaflet affected.

cyanogenic substrate in diseased leaves. Comparable results were obtained when an enzyme preparation from cultures of *S. botryosum* grown on alfalfa roots was used.

Production and characteristics of enzyme with cyanogenic activity.—All of the fungi tested, when grown in vitro on autoclaved trefoil shoots, produced enzymes capable of effecting the release of HCN both from trefoil substrate or amygdalin. When grown on other media, however, the ability to produce enzymes with cyanogenic activity on these substrates varied greatly with the fungus and the medium on which it was grown (Table 3). The enzymes apparently are produced adaptively in response to trefoil substrate or amygdalin, and are not produced in media in the absence of these substrates. If a fungus grown on a particular medium produced enzymes active on trefoil substrate, then the same enzyme preparation was active on amygdalin. However, an enzyme preparation that was active on amygdalin in some instances was inactive when tested on trefoil substrate.

S. loti, S. botryosum, and *F. solani* when grown in vitro on autoclaved alfalfa roots each produced enzymes that were active on either trefoil substrate or amygdalin.

pH optima were established for enzyme preparations from the three sources: *S. loti*–trefoil shoot culture, *S. loti*-infected–HCN$^-$ trefoil plants, and healthy-HCN+ trefoil plants (Fig. 1). Reaction mixtures tested at pH 3, 4, and 5 were buffered with 0.1 M NaOH-citric acid buffer, at pH 6 and 7 with 0.1 M phosphate buffer, and at pH 8 and 9 with Tris-HCl buffer. Replicate dishes were prepared for each treatment. Maximum activity for the shoot culture enzyme preparation was at pH 6.0. The pH optimum for the preparation from *S. loti*-infected HCN$^-$ plants appeared to be between pH 6 and 7. Activity for the preparation from healthy HCN+ plants was highest at pH 7, but was almost as high over the range pH 6-9. The absorbance for the control (heated) enzyme preparations was negligible for the *S. loti*-shoot culture and *S. loti*-infected HCN$^-$ preparations, but ranged from 0.38 at pH 3.0

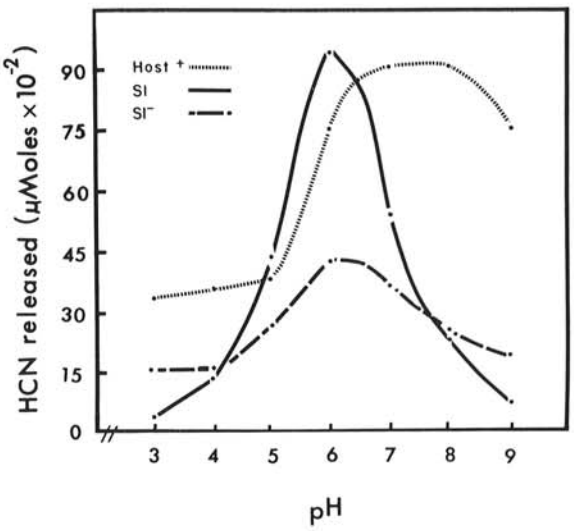

Fig. 1. Relation of pH to relative activity of crude enzyme preparations from *Stemphylium loti*-trefoil shoot cultures (S1), *S. loti* infected-HCN$^-$ trefoil plants (S1$^-$), and healthy- HCN+ trefoil plants (Host+). Reaction mixtures tested at pH 3, 4, and 5 were buffered with 0.1 M NaOH-citric acid buffer, at pH 6 and 7 with 0.1 M phosphate buffer, and at pH 8 and 9 with Tris-HCl buffer, and contained 2 ml enzyme preparation, 1 ml trefoil extract as substrate, and 1 ml buffer solution. Values for control preparations of S1 and S1$^-$ were negligible; those for Host+ ranged from 0.38 at pH 3.0 to 0.51 at pH 9.0.

TABLE 3. Beta-glucosidase production in vitro by several plant pathogenic fungi as influenced by growth on various media

Test fungus	Trefoil extract		PDB		PDB + amygdalin[a]		Czapek		Czapek + amygdalin		Trefoil shoots	
	T[b]	*A*[b]	*T*	*A*	*T*	*A*	*T*	*A*	*T*	*A*	*T*	*A*
Fusarium oxysporum	++[c]	++	—	tr	—	++	—	—	—	—	++	++
F. roseum	—	++	—	—	tr	tr	—	—	—	—	++	++
F. solani	—	tr	—	—	++	++	—	—	—	—	++	++
Colletotrichum lagenarium	tr	++	—	—	—	++	—	—	—	—	++	++
Monilinia fructicola	++	++	—	tr	tr	tr	—	tr	—	—	++	++
Stemphylium botryosum	+	++	—	—	++	++	—	—	—	—	++	++
S. sarcinaeforme	++	++	—	—	++	++	—	—	tr	tr	++	++
S. loti	—	++	—	—	—	++	—	—	—	—	++	++
Rhizoctonia solani	++	++	—	++	++	++	—	—	++	++	++	++
Helminthosporium turcicum	++	++	—	—	—	tr	—	—	—	++	++	++
Thielaviopsis basicola	++	++	—	—	—	—	—	—	—	tr	++	++

[a] PDB = potato-dextrose broth; medium supplemented with 0.003 M amygdalin.

[b] Enzyme preparations tested on substrate T (trefoil leaf extract) or A (0.003 M amygdalin); reaction mixtures contained 1 ml substrate, 2 ml enzyme preparation, and 1 ml 0.1 M phosphate buffer at pH 6.0.

[c] + Or ++, slight to strongly positive test for HCN; tr or —, trace or negative test.

to 0.51 at pH 9.0 for the healthy HCN+ preparation. The values for the healthy HCN+ control preparation were obtained regularly; the reason for their relatively high levels was not determined. Enzyme activity at pH 6.0 was directly proportional to the amount of enzyme preparation tested when the source of the enzyme was *S. loti*-infected HCN− trefoil plants and the enzyme was tested on trefoil substrate.

The activities of enzyme preparations from healthy HCN+ or HCN− trefoil plants and from *S. loti*-infected-HCN+ or -HCN− trefoil plants were compared on trefoil substrate and on amygdalin (Fig. 2). The results for two levels (0.5 or 1 ml) of enzyme concentration were in close agreement, but only the values obtained for the lower concentration are shown in Fig. 2. The extracts containing pathogen enzyme were more active on amygdalin than on trefoil substrate, whereas trefoil or host enzyme was relatively more active on trefoil substrate than on amygdalin.

Relative tolerance of S. loti and other fungi to HCN.—Both isolates of *S. loti* were markedly more tolerant of HCN than was *S. botryosum, S. sarcinaeforme, H. turcicum, T. basicola, C. lagenarium, F. oxysporum, F. solani,* or *F. roseum.* The nonpathogens were essentially equal in their sensitivity to HCN regardless of the KCN concentration tested (Fig. 3). Comparable results were obtained for the other fungi tested. Except for *S. loti,* none of the fungi germinated when KCN concentrations higher than 74×10^{-2} μmoles/ml were tested. However, *S. loti* germinated at KCN concentrations as high as 185×10^{-2} μmoles/ml.

Discussion.—Cyanogenesis has been recognized for many years as a property of various plant species (6, 14), but the possibility that cyanogenesis may be a factor in pathogenesis (4, 7, 12, 16) or in resistance of plants to pathogens has received little consideration (15). The findings of the present investigation suggested that cyanogenesis in birdsfoot trefoil may have a significant role both in the development of copperspot caused by *S. loti* and in the resistance of trefoil to a variety of fungi. That cyanogenesis in trefoil is associated with pathogenesis by *S. loti* was indicated by (i) release of HCN as a result of infection; (ii) correlation of symptom severity with the amount of HCN released; (iii) release of HCN effected by enzymes elaborated by the pathogen in infected leaves; and (iv) degradation of cyanogenic glucoside of trefoil as a result of infection. The possibility that cyanogenesis serves as a defense mechanism was indicated by the determinations that (i) *S. loti* was markedly more tolerant of HCN than were any of the other fungi tested; (ii) the amount of infection caused by *S. loti* and *S. botryosum* (a non-pathogen of trefoil) increased when HCN was removed from the atmosphere of the leaves, but infection by both fungi was retarded when HCN was allowed to accumulate in the atmosphere; and (iii) all of the fungi tested produced enzymes that hydrolyzed the cyanogenic substrates from trefoil.

Drake (3) reported that *S. loti* upon gaining ingress into a leaflet grows intercellularly and apparently produces a chemical that affects cells in advance of the

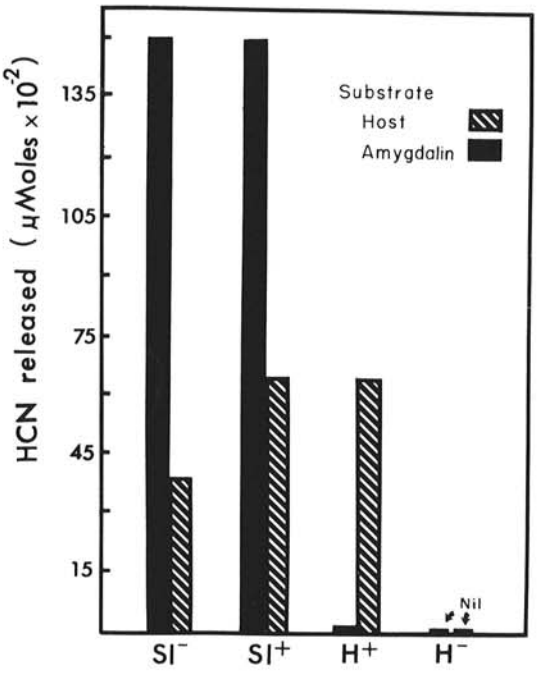

Fig. 2

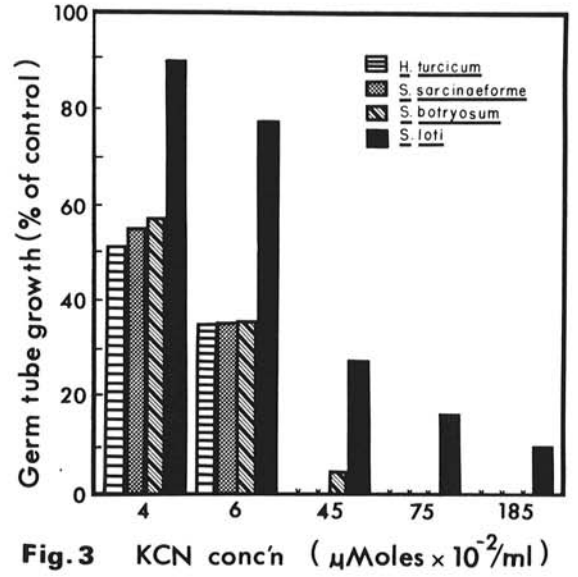

Fig. 3

Fig. 2-3. **2)** Comparison of activities of enzyme preparations from healthy HCN+ (H+) or healthy HCN− (H−) trefoil plants and from *Stemphylium loti* infected-HCN+ (S1+) or -HCN− (S1−) trefoil plants on trefoil extract or 0.003 M amygdalin as substrates. Enzyme preparations were made equivalent with respect to protein concentration. Reaction mixtures contained 0.5 ml enzyme preparation, 1 ml substrate, 1 ml 0.1 M phosphate buffer at pH 6.0, and 1.5 ml water. **3)** Comparison of germination of conidia of *Helminthosporium turcicum, Stemphylium sarcinaeforme, S. botryosum,* and *S. loti* on water agar discs held 5 hr in an atmosphere of cyanide provided by KCN solutions at several concentrations.

fungus. Subsequent development of the pathogen is both inter- and intracellular, and is associated with increasingly more violent reactions and ultimate collapse of the cells. These observations and our findings suggest the following hypothetical interpretation of events in development of copperspot. *Stemphylium loti,* on gaining ingress to the tissues, elaborates enzymes, or as a result of injuring the host tissues causes the release of host enzymes which either alone or in combination with pathogen enzymes act on the cyanogenic substrates linamarin and lotaustralin to liberate HCN. The HCN that is volatile and highly reactive affects cells in advance of the hyphae. This action in advance may facilitate continued invasion of the tissues by *S. loti,* which, owing to its marked tolerance of HCN, can function as a pathogen and eventually cause death of the cells. On the other hand, if nonpathogens gain ingress they also are capable of elaborating enzymes that hydrolyze linamarin and lotaustralin to release HCN, or by injuring cells at the point of ingress they may effect a reaction between host enzymes and the cyanogenic substrates to release HCN. However, owing to the nonpathogen's marked sensitivity to HCN, further development is halted, and thus resistance is expressed.

Respiration and spore germination studies (5) that involved *S. loti* and *S. sarcinaeforme* also demonstrated that *S. loti* is relatively tolerant of HCN, and that inhibition of germination of *S. sarcinaeforme* by cyanide was correlated with a marked stimulation in respiration of the spores. This strongly suggested that the action of cyanide may be due to an uncoupling of respiration from oxidative phosphorylation. Thus, should these conditions actually prevail, even low cyanide concentrations likely to occur in leaflets in response to the initial interaction between trefoil cells and a fungus that is less tolerant of HCN than is *S. loti* may prevent further development of the fungus.

Reynolds (13) suggested that part of the resistance of certain flax varieties to *Fusarium oxysporum* f. *lini* might be due to their linamarin content. Support for this idea was provided by Timonin's report (17) that roots of a variety resistant to flax wilt liberated significant quantities of HCN, whereas those of a susceptible variety liberated only a trace. On the other hand, Trione (18) found that the linamarin content of several flax varieties was not correlated with resistance to wilt. Moreover, he was unable to detect any liberation of HCN from flax roots although he used extremely sensitive methods. Lüdtke and Hahn (9) could not demonstrate that the resistance of flax lines to *Colletotrichum lini* was determined by their linamarin content. Lebeau (7) has reported that there is no quantitative relationship between resistance of a plant and its cyanogenic glycoside content. In view of our results, we think it possible that it is not the total amount of cyanide potentially available for release from a variety that is important, but rather the extent to which a fungus invading the tissues is capable of effecting a release of HCN and the relative sensitivity or tolerance of the fungus to the HCN released. The mode of ingress and the localization of the cyanogenic compounds in the tissues also may be significant factors.

Although cyanide has received principal consideration here, cyanogenesis may be only a secondary factor in pathogenesis and resistance. For example, the additional products of hydrolysis of linamarin and lotaustralin (acetone and methylethyl ketone, respectively) may be significant factors that have not yet been examined.

Our studies did not clearly indicate the nature or relation of the β-glucosidases. They apparently were produced adaptively by the fungi in response to a given substrate. Yet induction of β-glucosidase in response to a given substrate sometimes but not always resulted in production of an enzyme capable of hydrolyzing both trefoil cyanogenic substrate and amygdalin. In addition, pathogen β-glucosidase produced in vivo appears to differ from host β-glucosidase, but the extent of the differences was not determined. The system provided by HCN^+ and HCN^- types of trefoil should prove especially valuable for comparing pathogen β-glucosidase produced in vitro or in vivo and host β-glucosidase from healthy or diseased plants.

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