Hydrogen Cyanide Potential During Pathogenesis of Sorghum by Gloeocercospora sorghi or Helminthosporium sorghicola

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ABSTRACT

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The production of hydrogen cyanide (HCN) may be important in diseases of cyanogenic plants such as sorghum (*Sorghum bicolor*, *S. sudanense*). We measured the hydrogen cyanide potential (HCN-p) during pathogenesis of sorghum leaves by *Gloeocercospora sorghi* or *Helminthosporium sorghicola*. Different methods of measuring HCN-p were evaluated. A new, improved method combining an enzymatic and a nonenzymatic degradation of the cyanogenic glycoside dhurrin was used to estimate HCN-p. A decrease in HCN-p in diseased primary leaves was detected first between 24 and 48 hr after inoculation. Although plants of cultivar Grazer contained about twice as much HCN-p as those of cultivar Piper, the infection of either cultivar by either pathogen reduced the HCN-p to about 10% of the original level within 3-4 days after inoculation. The HCN that volatilized from nondisrupted primary leaves of cultivar Grazer infected by *G. sorghi* accounted for about 14% of the original total HCN-p. Efficiency of enzymatic dhurrin degradation in sorghum primary leaves increased 2- to 4-fold between 12 and 24 hr after inoculation. The results provide a basis for relating changes in HCN-p to specific events in pathogenesis of sorghum by *G. sorghi* or *H. sorghicola*.

Sudangrass and sorghum-sudangrass hybrids are important forage and grain crops because of their drought tolerance and high productivity (11). However, because they can produce toxic amounts of hydrogen cyanide (HCN), these forages are potentially dangerous to livestock (12). Because of the toxicity of HCN to most organisms, its production may be an important factor in disease resistance, pathogenesis, or both (16, 18, 21). Initial efforts to determine a specific role for HCN in the diseases of cyanogenic plants have not been successful. Investigators who have attempted to associate high hydrogen cyanide potential (HCN-p) in cultivars to high levels of disease resistance have not found consistently positive correlations (21, 22, 23). This lack of consistent, positive correlations could be due to HCN detoxification by pathogens (7, 8, 9). If a compatible pathogen can detoxify HCN, the total amount of potentially available HCN in a leaf is unlikely to be correlated with disease resistance. Alternatively, Millar and Higgins (16) suggested that HCN may injure host cells and facilitate pathogen development. However, specific toxic effects of HCN on the cells of cyanogenic host plants have not been examined.

All of these considerations presume that HCN is produced in toxic concentrations from cyanogenic glycosides, and some researchers have indicated that HCN-p may decline as a result of disease (3). However, the relative amount and timing of cyanogenic glycoside metabolism (or decline in HCN-p) have not been defined carefully. Therefore, the purpose of this study was to determine whether HCN-p declined upon pathogenesis and if so, to describe the kinetics of the decline relative to pathogenesis. Because the classic method used to determine HCN-p was not adequate in our studies, we also evaluated different methods for measuring HCN-p.

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MATERIALS AND METHODS

Pathogens.—*Gloeocercospora sorghi* D. Bain & Edg. was isolated from diseased sorghum provided by R. A. Frederiksen, Texas A & M University, College Station, TX 77840. *Helminthosporium sorghicola* Lefebvre & Sherwin was obtained from K. J. Leonard, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27607. Both fungi were maintained on V-8 juice agar (17) under continuous fluorescent light at 22 to 25 C, and remained pathogenic during our studies.

Hosts.—Piper sudangrass (*Sorghum sudanense* 'Piper' Stapf.) and Grazer, a sorghum [*Sorghum bicolor* (L.) Moench] × sudangrass hybrid were seeded in a sandy loam soil contained in metal flats, and maintained, prior to inoculation, under supplemental light at about 30 C in a greenhouse.

Inoculation and incubation.—Inoculum was prepared by washing conidia from 7- to 13-day-old cultures into sterile distilled water and filtering the suspended spores through three layers of cheesecloth. The spore concentration was adjusted to 4-6 × 10^5 spores/ml in 0.05% Tween-20 (polyoxyethylene sorbitan monolaurate). This inoculum concentration insured that a large area of the inoculated leaves was affected at the same time. Seedlings were sprayed to runoff with the spore suspension when the first leaf had fully elongated—usually 5 to 7 days after planting. Inoculated seedlings were incubated in a moist chamber at 27 C in

100% relative humidity for 4 to 5 days. Seedlings were easy to manipulate, and provided leaves of relatively uniform initial HCN-p.

Estimation of hydrogen cyanide potential (HCN-p).—*Chloroform method.*—Hydrogen cyanide potential (HCN-p) refers to the amount of HCN that is volatilized from leaves that have been physically disrupted (often by chloroform or less often by some type of homogenization). Because dhurrin is the only known source of HCN in sorghum (1, 5), the amount of HCN trapped is assumed to reflect the concentration of dhurrin in the sample (19). Five or 10 excised leaves were weighed, placed on filter paper in the center chamber of solvent resistant, polypropylene Conway microdiffusion dishes (Bel-Art Products, Inc., Peguannock, NJ 07440; there were five replicates per treatment. Cells were disrupted by saturating each set of leaves with chloroform (0.5 - 1.0 ml). The dish was closed with a water seal. Error due to loss of volatilized HCN by absorption in the water seal was not significant. The HCN volatilized from chloroform-treated leaves was trapped in 4 ml alkaline sodium picrate (16) in the outer chamber of the microdiffusion dish. The amount of HCN volatilized during the 2-day interval after chloroform treatment was determined colorimetrically (16).

We observed an initial increase in the HCN-p in diseased primary leaves as measured by the chloroform method (see Results). To determine whether this increase was due to an increase in the synthesis of cyanogenic glycoside or to increased efficiency in the extraction of HCN from diseased primary leaves, we evaluated three additional methods for the determination of HCN-p.

Chloroform - base method.—Two days after the initial chloroform treatment, the original picrate solution was replaced with fresh picrate solution (4 ml), and 1.0 ml of 0.2 N glycine buffer, pH 9.0 or pH 10.0, was added to the leaves in the center wall–a modification of earlier procedures (4, 6). Leaves so treated were incubated 2 more days and the additional HCN volatilized was determined colorimetrically. Presumably this HCN was volatilized at a slower rate than at lower pH (pK_a of HCN = 9.31), but the 2-day incubation was sufficient to allow diffusion of about the same amount of HCN detectable by other procedures.

Chloroform - β-glucosidase method.—To test the hypothesis that β-glucosidase may be limiting in chloroform-disrupted primary leaves, some samples (10 primary leaves) were treated after chloroform treatment with commercial β-glucosidase (β-D-glucoside glucohydrolase from almonds; EC 3.21.21, Sigma Chemical Co., St. Louis, MO 63178) in 0.1 N citrate-phosphate buffer, pH 5.7. One unit was added to 10 primary leaves in each diffusion dish. (One unit will liberate glucose from salicin at the rate of 1 μmole/min at pH 5.0 at 37 C). The HCN produced as a consequence of this treatment was trapped and determined colorimetrically as above.

Reflux method.—To measure HCN-p by a method not involving disruption of leaves by chloroform, a modification of Harrington's (11) method was used. A weighed sample of 50 primary leaves was refluxed for at least 4 hr in 50 ml of glass distilled water at atmospheric pressure. The evolved gases were removed in a slow stream of air through two absorption towers in series charged with 1% KOH. At the end of the experiment, the HCN trapped in the solution was determined colorimetrically as before.

Volatile hydrogen cyanide (HCN) from nondisrupted leaves.—The HCN volatilized from nondisrupted healthy or diseased leaves also was determined colorimetrically in sodium picrate. Healthy and diseased leaves were excised at 12-hr intervals through 108 hr after inoculation, weighed, and placed on distilled water-moistened filter paper in the center chamber of diffusion dishes. After a 12-hr incubation, the leaves were removed from the dishes and dried at 60 C for dry weight determination. The diffusion dishes were resealed quickly and incubated for an additional day to allow maximum chromogen development.

All HCN concentrations were expressed as μmoles HCN/g leaf dry wt.

All experiments were performed at least twice. The experiment for HCN-p of healthy and *G. sorghi*-infected Grazer seedlings (summarized data presented in Fig. 3) was repeated seven times.

RESULTS

Methods for determine hydrogen cyanide potential (HCN-p).—In tests with the first leaf of cultivar Grazer, the chloroform method produced a low estimate of the HCN-p relative to estimates obtained with other methods in comparable samples (Table 1). The chloroform-β-glucosidase method, the chloroform-base (pH 9) method, and the reflux method all were efficient and their respective measurements were not significantly different ($P = 0.05$). The chloroform-base (pH 10) method was slightly less efficient. The chloroform-base (pH 9) method was efficient, applicable to many samples, inexpensive,

TABLE 1. The hydrogen cyanide potential (HCN-p) in primary leaves of sorghum cultivar Grazer estimated by various treatments

Treatment[x]	HCN-p detected (μmoles HCN per gram leaf dry wt)
Chloroform + buffer, pH 5.7	115.1 a[z]
Chloroform + buffer, pH 10.0 after 48 hr	503.9 b
Chloroform + buffer, pH 9.0 after 48 hr	533.7 bc
Reflux[y]	510.4 bc
Chloroform + β-glucosidase after 48 hr	553.8 c

[x]Leaves were treated with chloroform and incubated in microdiffusion dishes containing alkaline sodium picrate. After 48 hr the picrate was removed and HCN concentration was analyzed colorimetrically. Fresh picrate was added to the outer chamber of the diffusion dish and buffer or β-glucosidase added to the leaves. The HCN in the fresh picrate was analyzed after an additional 48 hr of incubation. The pH 5.7 buffer was 0.1 N citrate-phosphate, and the pH 9.0 and 10.0 buffers were 0.2 N glycine.

[y]Leaves were extracted in boiling distilled water and HCN was trapped in KOH and estimated colorimetrically.

[z]Means followed by the same letter are not significantly different according to the Duncan's multiple range test, $P = 0.05$.

and did not rely soley on endogenous β-glucosidase activity. Therefore, this procedure was used to determine HCN-p in both healthy and diseased sorghum.

Hydrogen cyanide potential (HCN-p) in sorghum leaves.—We measured the HCN-p from the time of leaf expansion to leaf senescence for the first leaf and second leaf of each cultivar. The first leaf contained more HCN-p than the second, but the pattern of HCN-p disappearance was similar in both leaves (Fig. 1 and 2). The HCN-p (amount/g leaf dry wt) decreased during the early stages of leaf development. The increase in leaf dry weight was sufficient to account for the decrease in HCN-p (amount/g leaf dry wt). In the first and second leaves of Grazer, HCN-p was most stable between 7 and 19 days after planting, although small fluctuations were detected (Fig. 1 and 2). A decrease in HCN-p was evident in each leaf immediately before and during leaf senescence (23 days after planting). Changes in HCN-p in leaves of Piper were similar to those in Grazer, although those of Piper contained about half as much HCN-p as Grazer. These values of HCN-p (about 400 μmoles/g leaf dry wt for Grazer and about 175 μmoles/g leaf dry wt for Piper) are similar to other values reported for these cultivars (11, 13).

The chloroform method enabled detection of between 20 and 50% of the HCN-p in the first leaf of Grazer (Fig. 1) depending on the age of the leaf. However, chloroform treatment enabled detection of about 90% of the HCN-p in the second leaf (Fig. 2), regardless of leaf age. The same pattern was obtained for Piper.

Disease development.—Lesions produced by *G. sorghi* or *H. sorghicola* first were observed at 12 to 18 hr after inoculation. These initial lesions appeared as chlorotic, water-soaked areas 1-3 mm in diameter. The lesions enlarged and became reddish-brown by 48 hr after inoculation. Because of the large number of lesions, nearly 100% of the leaf area was affected with *G. sorghi* within 48-96 hr after inoculation, and with *H. sorghicola* within 72-96 hr after inoculation. Cultivar differences had no noticeable effect on pathogen development.

Effect of pathogenesis on hydrogen cyanide potential (HCN-p).—The two pathogens caused a similar decrease in HCN-p in each cultivar (Table 2). Both pathogens caused approximately 90% of the initial HCN-p in both cultivars to be lost or degraded by 96 hr after inoculation. In a typical experiment, a decrease in the HCN-p of Grazer infected by *G. sorghi* was first detected between 24 and 48 hr after inoculation and continued at a linear rate until at least 84 hr after inoculation (Fig. 3).

The chloroform treatment enabled a better estimation of HCN-p in infected leaves than in healthy ones. Although less than 50% of the HCN-p was detected after chloroform treatment of healthy leaves, 70% was detected

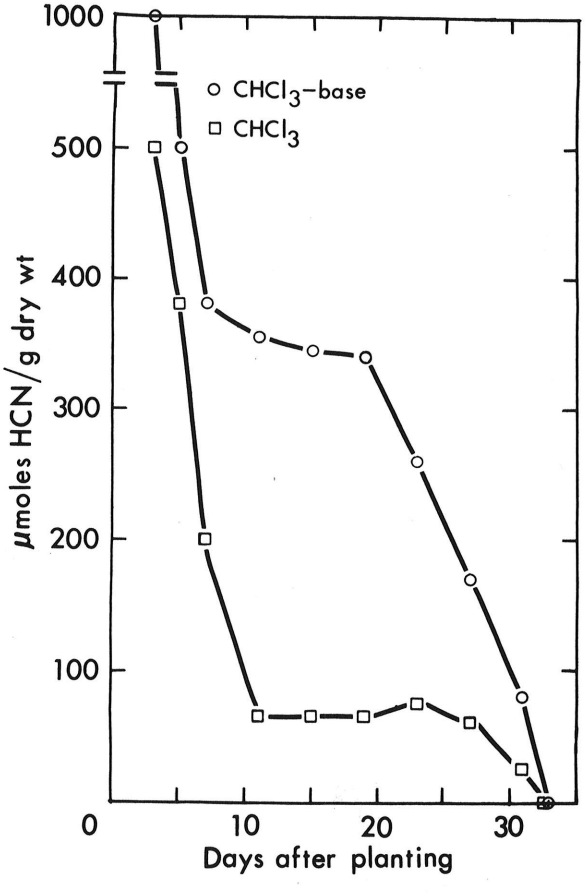

Fig. 1. Hydrogen cyanide in primary leaves of sorghum cultivar Grazer as determined by the chloroform method ($CHCl_3$) or by the chloroform-base method ($CHCl_3$-base). Estimations were made at intervals from the first appearance of the leaf until senescence.

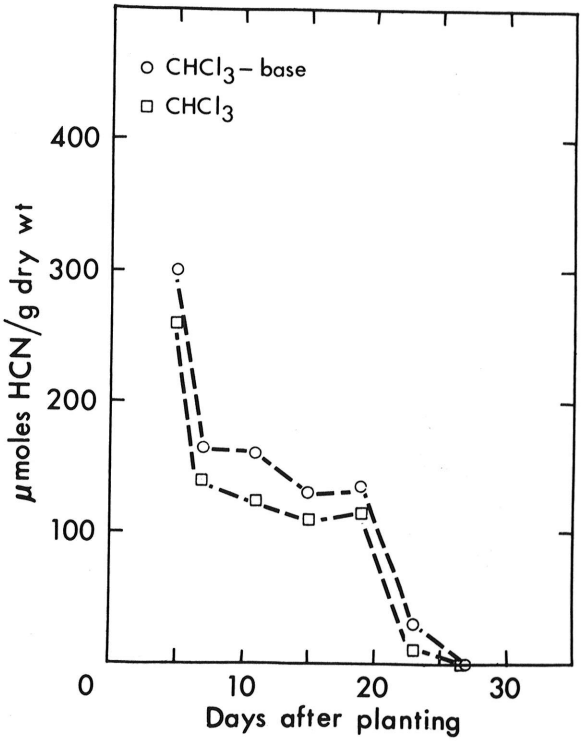

Fig. 2. Hydrogen cyanide in the second leaf of sorghum cultivar Grazer as determined by the chloroform method ($CHCl_3$) or by the chloroform-base method ($CHCl_3$-base). Estimations were initiated when the leaf first appeared and continued at intervals until leaf senescence.

at 12 hr after inoculation and 90% by 24 hr in diseased leaves (Fig. 3). The kinetics of the decline in HCN-p from diseased leaves were similar for all host-pathogen combinations.

Volatilization of hydrogen cyanide from diseased leaves.—The decline in HCN-p from diseased leaves is probably the result of dhurrin degradation. As a result, HCN may have been released into the tissues and perhaps into the atmosphere surrounding diseased leaves. To estimate the amount of HCN volatilized into the atmosphere, we measured the HCN volatilized from detached, nondisrupted (not treated with chloroform) diseased leaves (Fig. 4). The HCN volatilized from these leaves was detected first 24 hr after inoculation and continued to be detected until at least 84 hr. Only 14% of the HCN-p lost from nondisrupted diseased leaves was detectable as volatile HCN.

TABLE 2. The hydrogen cyanide potential (HCN-p) estimated by the chloroform-base method[a] in primary leaves of sorghum cultivars Grazer and Piper at inoculation (0 hr) and 96 hr after inoculation with either *Gloeocercospora sorghi* or *Helminthosporium sorghicola*

Pathogen and incubation time	HCN-p			
	Grazer		Piper	
	(μmole HCN/g leaf dry wt)	(% of total)	(μmole HCN/g leaf dry wt)	(% of total)
G. sorghi				
0 hr	430	(100)	189	(100)
96 hr	60	(14)	28	(15)
H. sorghicola				
0 hr	467	(100)	191	(100)
96 hr	51	(11)	10	(5)

[a]Leaves were placed in microdiffusion dishes containing alkaline sodium picrate, treated with 0.5 ml chloroform, and incubated 48 hr. Then they were treated with 1.0 ml of 0.2 N glycine buffer (pH 9.0) and incubated an additional 48 hr. The trapped HCN was measured colorimetrically.

DISCUSSION

Apparently the chloroform method was more efficient for assessing HCN-p in the second leaf of each cultivar. The method may be ineffective for primary leaves because of insufficient β-glucosidase activity. Some researchers (4, 10) have found it necessary to add the commercially available β-glucosidase, emulsin, to leaf extracts to hydrolyze all dhurrin for determining HCN-p. Since addition of β-glucosidase did increase the HCN detected after chloroform treatment in our experiments, low β-glucosidase activity may reduce the effectiveness of the chloroform treatment during some stages in primary leaf development. However, chloroform also may inhibit β-glucosidase activity in treated leaves or not be completely efficient in making the cyanogenic substrate available to endogenous β-glucosidase.

We used the chloroform-base (pH 9) method for routine estimates of HCN-p in this study for two reasons. First, this method enabled us to detect levels of HCN-p which were equivalent to values extracted via the chloroform-β-glucosidase and the reflux methods.

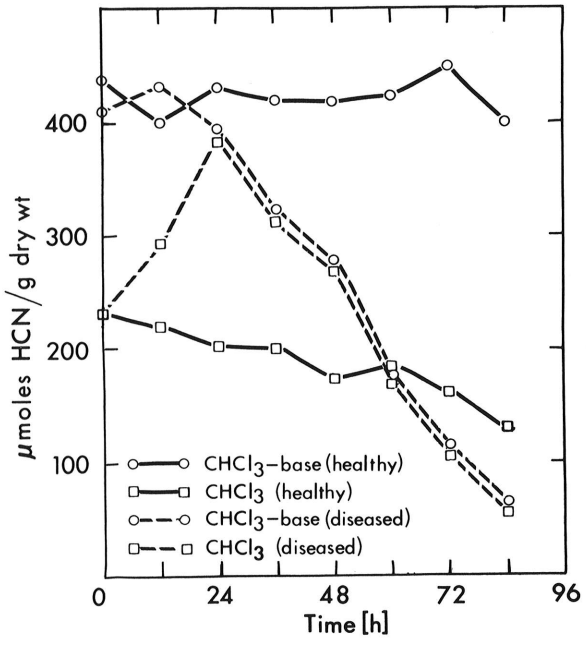

Fig. 3. Hydrogen cyanide in the primary leaf of healthy and *Gloeocercospora sorghi*-infected sorghum cultivar Grazer, determined by the chloroform ($CHCl_3$) or by the chloroform-base method ($CHCl_3$-base). Estimations were made at 12-hr intervals from inoculation at 0 time until 84 hr after inoculation.

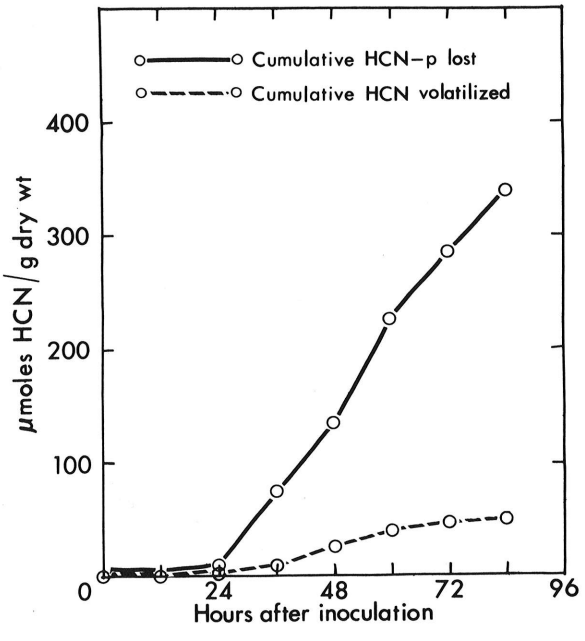

Fig. 4. Cumulative hydrogen cyanide potential (HCN-p) lost and cumulative HCN volatilized from primary leaves of the sorghum cultivar, Grazer, infected by *Gloeocercospora sorghi*. Estimations were made at 12-hr intervals from inoculation at 0 time until 84 hr after inoculation. Values are accumulative and expressed as μmoles HCN per gram of leaf dry wt.

Second, it is simple, inexpensive, and easily performed.

In diseased primary leaves, chloroform treatment was efficient for estimating HCN-p. Presumably chloroform allows the dhurrin in the vacuole (20) and free or bound enzymes, which probably are not in the vacuole (2), to interact. Any pathogen-mediated activity that would affect the relative localization of dhurrin to its degrading enzymes, or degrading-enzyme activity, may increase the efficiency of the chloroform treatment in diseased leaves. The activity of one of the dhurrin-degrading enzymes, β-glucosidase, may be low in primary leaves. The most direct explanation for the increased enzymatic degradation in diseased leaves is a pathogen effect on β-glucosidase activity. Increases in β-glucosidase activity are common in many plant diseases (14, 15). The activity of β-glucosidase active against dhurrin increases 20-fold in leaves of sorghum infected by *G. sorghi* during the first 24 hr after inoculation (Myers and Fry, *unpublished*). This is also the interval during which increases in the efficiency of the chloroform treatment were first observed (Fig. 3).

The function of dhurrin or its toxic degradation product HCN in diseased *Sorghum* spp. is still undefined. At least 14% of the HCN-p lost from diseased sorghum was volatilized as HCN. The fate of the remaining HCN-p is unknown. There are at least four possibilities: (i) HCN is bound to host or pathogen cell constituents; (ii) HCN is destructively metabolized by host or pathogen enzymes; (iii) HCN is incorporated by host or pathogen enzymes into metabolically active product(s); (iv) some dhurrin is metabolized by a pathway that does not produce HCN. Knowledge of the fate of this dhurrin during pathogenesis would be a major contribution to the understanding of the role of HCN in sorghum plant disease.

If HCN has some protective function against pathogens or potential pathogens, its effectiveness may be reduced as the leaf ages and HCN-p decreases below the minimum toxic concentration. The rapid development of *G. sorghi* and *H. sorghicola* in sorghum of high HCN-p is an indication that these pathogens can readily cope with HCN or have evolved a mechanism to release HCN in host cells prior to colonization.

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