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Assay of *p*-Hydroxybenzaldehyde as a Measure of Hydrocyanic Acid Potential in Sorghums[1]

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ABSTRACT

A method of assessing the hydrocyanic acid potential (HCN-p) of sudangrass [*Sorghum sudanense* (Piper) Stapf] and sorghum [*S. bicolor* (L.) Moench] seedlings is described. This procedure is based on the determination of p-hydroxybenzaldehyde (p-HB), which is released upon hydrolysis of dhurrin, the cyanogen normally present in plants of *Sorghum* species. Extraction and hydrolysis of dhurrin are accomplished by autoclaving young leaf tissue in water. The content of p-HB in the aqueous extract is then determined by spectrophotometric assay in alkaline solution at 330 nm. Uniform samples for the comparison of widely divergent genotypes are obtained by using the first leaf of young, chamber-grown, green seedlings. Relative ranks of a wide variety of sorghum germplasm assayed for HCN content by this technique are in good agreement with those obtained by other methods. The procedure shows promise of providing a rapid and precise tool for conducting genetic and breeding studies with sorghums.

Additional index words: **Dhurrin, Prussic acid, Cyanide, Sudangrass, Forage sorghum, *Sorghum sudanense*, *Sorghum bicolor*.**

VARIOUS methods for the determination of cyanogenic glucosides in plants have been reported. Among these are colorimetric (8, 11, 15, 18), fluorometric (10, 28), potentiometric (4, 5, 12), and titrimetric (1) procedures, all of which are based on the assay of hydrocyanic acid (HCN) released when the cyanogenic glucoside is chemically or enzymatically hydrolyzed to yield HCN, glucose, and aglycone. In most of the published procedures, hydrolysis of the cyanogen is accomplished enzymatically, using either endogenous or exogenous glucosidases. For colorimetric and fluorometric assays, the hydrolyzed sample is subjected to diffusion, distillation, or aeration, and the volatilized HCN is trapped in an alkaline solution for subsequent assay. These time-consuming procedures help to reduce the effects of interfering compounds, but they may result in erroneous values due to incomplete hydrolysis of the cyanogen or incomplete recovery of the released HCN.

Much of the early published work on the determination of the cyanogen content or HCN potential (HCN-p) of sorghum [*Sorghum bicolor* (L.) Moench] and sudangrass [*S. sudanense* (Piper) Stapf] was based on variations of the sodium picrate procedure (9, 15, 24). Because these techniques usually depended upon endogenous glucosidases for hydrolysis of the cyanogen, they sometimes permitted erroneously low readings resulting from the incomplete release of HCN from the tissues. In more recent years, the cyanide-specific electrode has been utilized by many investigators to assess the HCN-p of various cultivars. Although the continuous potentiometric method reported by Easty et al. (5) involved volatilization of the HCN prior to measurement, other workers (4, 7, 12) have used the electrode for direct measurement of the cyanide content of tissue extracts without volatilization. Blaedel et al. (4) reported that most constituents of biological samples do not interfere significantly with the response of the electrode, but they noted that, because of unidentified interfering compounds, the electrode was not satisfactory for direct assay of low-cyanide tissue extracts. In spite of this possible weakness, the cyanide-specific electrode has been used extensively in recent investigations (4, 6, 7). The direct potentiometric assay of HCN-p, however, has certain drawbacks for screening large numbers of tissue samples because each sample must be homogenized, and low-HCN samples may require extensive equilibration with the electrode. In addition, "poisoning" of the electrode membrane during extended use may further increase the lag in electrode response, and erroneous readings may result (2).

In assaying for plant cyanogenesis, the investigator also has the problem of obtaining uniform and comparable tissue samples from the lines or cultivars among which comparisons may be desired. In the case of dhurrin (*p*-hydroxy-L-mandelonitrile β-D-glucoside), the cyanogen of sorghum species, the choice of an optimum sampling time and tissue is complicated by the observations that: a) dhurrin content in plants declines as the plant matures (1, 16, 19, 22, 30); b) distribution of dhurrin is not constant throughout the plant but is generally highest in new growth, such as newly emerged seedlings, young leaves, and tillers (3, 19, 22); and c) environmental variables such as water stress, (23), high N fertility (13, 17, 26), and frost (29) can increase dhurrin content. It has been suggested (16) that tillers 13 to 18 cm in length could be used to provide uniform samples for assay. However, it is difficult to obtain tillers of uniform size and development from all genotypes, because many genotypes exhibit differential dates for the onset of tillering and rate of tiller growth (19). A sampling procedure that would minimize these difficulties has been greatly needed.

Dhurrin is one of the few alkali-labile cyanogenic glucosides. Its aglycone, p-hydroxybenzaldehyde (p-HB), exhibits strong absorption at 330 nm in alkaline solution (1, 20, 21). Akazawa et al. (1) demonstrated that the contents of HCN and p-HB in alkaline hydrolysates of young sorghum seedlings were equiva-

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lent on a molar basis, indicating that HCN-p could be estimated by assaying either HCN or p-HB. Although these authors measured p-HB by the 2,4-dinitrophenylhydrazone method, they also observed that measurement of p-HB in alkaline extracts of young seedlings simply by absorbance at 330 nm gave essentially equivalent values.

Two other observations made in our laboratory suggested a method for uniformly and efficiently sampling plant tissue. First, it was found that dhurrin could be efficiently extracted from plant tissue, and simultaneously hydrolyzed, simply by autoclaving the plant tissue in water. Although the lability of dhurrin in alkaline solutions is well-known (20, 21), and its hydrolysis in hot, acidic solutions has been documented (1), no reports of dhurrin hydrolysis in hot water were found in the literature. Second, various preliminary studies revealed that the HCN-p of the first leaf to emerge from the coleoptilar sheath (hereafter referred to simply as the first leaf) not only remained relatively constant for several days, but also appeared to be higher than the HCN-p of other parts of the seedling.

The present paper describes a rapid spectrophotometric method of determining the HCN-p of young, chamber-grown sorghum and sudangrass seedlings. The method is nondestructive to the seedling, and does not involve enzymatic hydrolysis of dhurrin.

MATERIALS AND METHODS

Plant Culture. Plastic trays, 5.5 cm deep, containing a mixture of three parts soil, one part sand, one part vermiculite, and one part peat moss, were used for growing the seedlings. Moisture was supplied by subirrigation with water, except in comparisons of cultivars and strains where subirrigation was accomplished with Hoagland's Nutrient Solution No. 1 (14) without trace elements. This solution was used to minimize differences in fertility among different batches of soil. Seeds were planted at a depth of about 0.5 cm, covered with sand, and incubated in growth chambers at 27 C. Continuous light was supplied by cool-white fluorescent tubes delivering about 10 kilolux at the level of the seedlings.

Sampling. For the general assay procedure, only the first leaves of young seedlings were sampled except as otherwise noted. Samples were taken when the second leaf was one-and-one half to two times as long as the first leaf. Sampling at this stage of growth, (which usually occurred 7 or 8 days after planting) rather than at a predetermined age, helped to reduce possible effects of inherent differences in growth rate. The first leaf was excised at the leaf collar, and the leaf blade was weighed and assayed as a single sample. In the split-leaf sampling procedure, the excised leaf was divided lengthwise along the midrib and each half was treated as an individual sample. For the comparison of various genotypes, first leaves were excised from 10 seedlings selected at random from those that were at the proper stage of growth. These 10 leaves were divided at random into two groups of five, and each group was then assayed as a single unit.

In the study of the variation of dhurrin content with seedling age, sampling was initiated on the 4th day after planting and continued on a daily basis, to the 15th day after planting. In this study, seedlings were excised at the coleoptilar node, the portion of the coleoptile adhering to the seedling was removed and discarded, the first leaf blade was separated from the remainder of the seedling, and the two portions (first leaf blade and seedling remainder) were weighed and assayed separately. In the early stages of growth (4th and 5th days), when no leaf collar was visible, the first leaf was unfolded and the blade and sheath were excised and assayed as a unit.

Potentiometric Measurements. Weighed individual first leaves (usually 10 to 20 mg fresh weight), or other samples as previously described, were homogenized in a glass tissue grinder (Pyrex[3] brand, no. 7725, 16 × 150 mm) containing 5 ml of 0.1 *N* NaOH. The homogenate was diluted with an additional 5 ml of 0.1 *N* NaOH and allowed to stand at room temperature for 2 hours to permit alkaline hydrolysis of the dhurrin.

Following hydrolysis, the cyanide content of the unfiltered homogenate was determined with a cyanide-specific electrode (Orion Model 94-06, Orion Research, Inc., Cambridge, Mass.) coupled with a single junction reference electrode (Orion Model 90-01 with Orion no. 90-00-01 filling solution). Potentials were read to the nearest 0.1 mV with an expanded scale pH meter (Corning Model 12, Corning Glass Works, Medfield, Mass.). The sample reservoir consisted of a 50-ml beaker clamped to a ring stand and positioned over a magnetic stirring motor (covered with a 1-cm thick sheet of polyurethane to minimize heat transfer from the motor to the sample solution). Sample volumes were restricted to 10 ml, and a constant stirring rate (provided by a small stirring bar) was applied to all samples. All readings were made at ambient temperature (22 to 25 C). Calibration curves were prepared daily, and were based on the potentials of several KCN standard solutions, ranging in concentration from 10^{-4} to 10^{-6} *M*, freshly prepared in 0.1 *N* NaOH. After each reading, the sample was aspirated from the reservoir and the electrodes, stirring bar, and reservoir were rinsed thoroughly with 0.1 *N* NaOH before introduction of the next sample.

Spectrophotometric Measurements. Each fresh leaf sample was weighed and placed in a dry 20 × 150 mm test tube. A small glass marble was placed on top of the leaf to keep it submerged during the subsequent extraction procedure. Ten milliliters of distilled water was added to each tube (20 ml with five-leaf samples) and each was capped with a large glass marble to reduce evaporative loss. The samples were then autoclaved for 30 min at 120 C to achieve extraction and hydrolysis of dhurrin. After autoclaving, the tubes were cooled immediately in a cold water bath. From each tube, an aliquot was removed and diluted with 0.1 *N* NaOH. Absorbance values were then determined at 330 nm (dilutions were generally adjusted to register an absorbance in the range of 0.2 to 0.5).

To convert these absorbance readings to HCN-p values in ppm, the following equation was used:

$$\text{ppm HCN} = \frac{(A330)\,(DF)\,(VE)\,(27.03)}{(\text{fr wt})\,(27.9)}$$

where,

- A330 is the absorbance of the base-diluted sample at 330 nm,
- DF is the dilution factor obtained when the sample was diluted for assay,
- VE is the volume (ml) of distilled water used to extract the tissue.
- 27.03 is the formula weight of HCN (μg/μmole),
- fr wt is the fresh weight (g) of the extracted tissue, and
- 27.9 is the extinction coefficient (ml/μmole) at 330 nm of p-HB in a 0.1 *N* NaOH solution.

The HCN-p values calculated in this way are on a fresh weight basis. First leaves of sorghum and sudangrass grown as indicated contain approximately 12% dry matter. Thus, approximate values on a dry weight basis could be obtained by multiplying the fresh weight HCN-p vaules by 8.3.

RESULTS AND DISCUSSION

The spectra of an alkaline solution of p-HB and an alkaline dilution of an autoclaved extract of the first leaf of a seedling of 'White Collier' forage sorghum are reproduced in Fig. 1. The similarity of the two spectra indicated that the constituent of the extract that was primarily responsible for the absorbance at the 330 nm peak was p-HB. The spectrum of an extract obtained from the first leaf of a sudangrass seedling was essentially identical to that of the forage sor-

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

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ghum extract. These spectra from the first leaf extracts of sudangrass and sorghum seedlings remained essentially unchanged until seedlings were at least 15 days old. Similar spectra were obtained for extracts of complete seedlings, except that beginning on about the 11th or 12th day after planting these extracts displayed an increase in absorbance (relative to the absorbance at 330 nm) in the wavelength regions below 330 nm and near 375 nm. The identity of the components giving rise to this interference is not known, but with older seedlings or mature plant tissue, the interference cannot be ignored. Therefore, we have restricted the use of the spectrophotometric assay primarily to seedlings less than 2 weeks old.

Several studies were conducted to determine an optimum autoclaving time for the extraction and hydrolysis of the dhurrin contained in the first leaf tissue. In initial experiments, it was observed that a 10^{-4} *M* aqueous solution of purified dhurrin was completely hydrolyzed when heated on a water bath at 85 to 90 C for 45 min. Extraction and hydrolysis by autoclaving, however, was much more convenient than use of a hot water bath. When the efficiency of the procedure described in Materials and Methods was investigated by extending the time of autoclaving (or by repeated extractions of leaf tissue with added portions of water), it was found that these additional treatments accounted for only 2 to 4% of the total p-HB detected. Thus, the single, 30-min autoclaving appeared to be highly satisfactory for dhurrin extraction and hydrolysis.

For comparing the spectrophotometric assay and the direct potentiometric assay, seedlings of 'Piper' and 'Tift' sudangrass and 'Atlas' forage sorghum were used. Results of these comparisons are shown in Table 1. Values of HCN-p obtained by the direct potentiometric assay were, in most cases, higher than those obtained by the spectrophotometric assay. Although differences between means were not statistically significant in any of the comparisons, the uniformity in direction of these differences suggested the possibility of loss of p-HB during the extraction and assay procedures. This possibility was investigated in recovery experiments in which a known amount of p-HB was added before autoclaving. No appreciable loss of p-HB was detected.

If there was no loss in the spectrophotometric assay, the possibility of inflated values in the direct potentiometric assay values must be considered. Substances which interfere with the cyanide-specific electrode have been reported (25), but these substances are normally absent or are present only in negligible amounts in plant tissue. We have found no reports of the effect of protein on the cyanide-specific electrode, but the effect of protein on ion-selective electrodes containing silver sulfide membranes has been documented (2). The cyanide-specific electrode does not contain silver sulfide, but it does contain a silver halide (25). Since the potential developed by these silver salt-containing electrodes depends on the silver ion concentration, and since sulfhydryl groups of protein molecules, like cyanide, react with silver ions, the effect of protein on the cyanide-specific electrode was investigated. Electrode potentials of several standard cyanide solutions, with and without various levels of bovine serum albumin, were determined with the cyanide-specific electrode. It was observed that protein concentrations of 10 μg/ml or greater resulted in significant shifts of the calibration curve, resulting in "apparent" cyanide values that were significantly higher than the known values. Protein contents of the tissue homogenates used in the potentiometric assays of Table 1

Table 1. Comparison of the spectrophotometric (S) and potentiometric (P) assay techniques using different sampling procedures for Piper and Tift sudangrass and Atlas forage sorghum. Values shown are the means and standard errors of 10 determinations.

Sampling procedure	HCN content (fresh weight basis)					
	Piper		Tift		Atlas	
	S	P	S	P	S	P
	ppm					
Whole leaf†	431 ± 31	530 ± 46	613 ± 56	679 ± 45	1398 ± 68	1531 ± 52
Split leaf A‡	487 ± 34	498 ± 31	653 ± 41	644 ± 54	1777 ± 84	1793 ± 74
Split leaf B§	491 ± 30	594 ± 32	698 ± 29	795 ± 20	1597 ± 92	1900 ± 106

† Twenty first leaves from each cultivar were excised and assayed on an individual leaf basis with ten leaves selected at random for each technique. ‡ Ten first leaves were excised and divided along the midrib. One half of each of the 10 leaves was assayed by the S technique, the other half of each by the P technique. § Ten first leaves were excised and divided along the midrib. Both halves of five leaves were individually assayed by the S technique, both halves of the other five by the P technique.

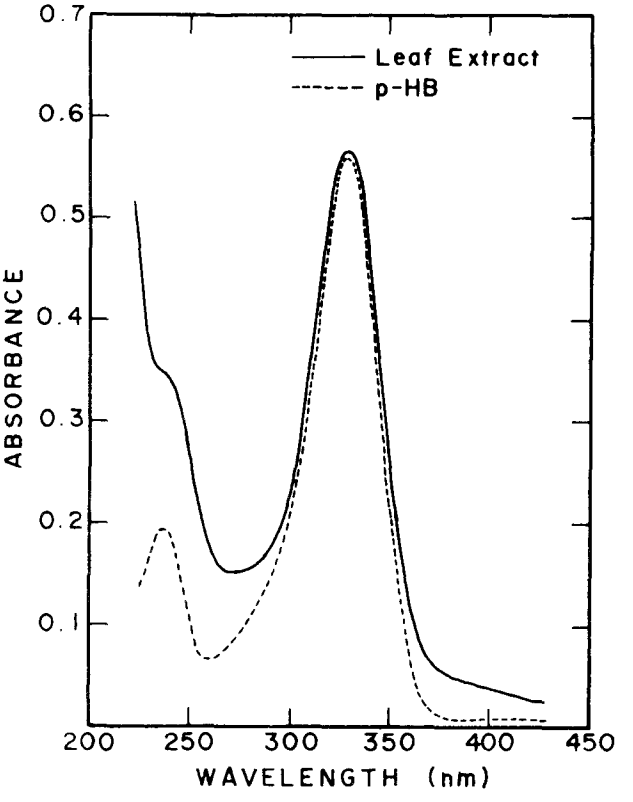

Fig. 1. Absorption spectra of p-hydroxybenzaldehyde (p-HB, concentration 2.45 μg/ml) and an extract of the first leaf of a 6-day old seedling of White Collier forage sorghum. The extract was diluted so that its absorbance was similar to that of the p-HB standard at the 330 nm maximum. Solvent: 0.1 *N* NaOH.

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Table 2. Fresh weight and HCN-p of the first leaf and complete seedling with increasing seedling age for White Collier forage sorghum and two inbred experimental lines of sudangrass. Each value represents the mean of ten seedlings assayed individually on a daily basis.†

Cultivar or line	Days after planting	First leaf		Complete seedling	
		Fresh weight	HCN	Fresh weight	HCN
		mg	ppm	mg	ppm
White Collier	4	13.6	1,334	19.9	1,288
	5	12.2	1,469	25.5	1,246
	6	13.8	1,285	57.4	826
	7	15.1	1,221	77.4	676
	8	13.6	1,284	93.3	527
	9	13.7	1,221	117.4	468
	10	13.8	1,304	132.8	417
	11	14.1	1,199	156.4	371
	12	12.5	1,123	158.4	315
	13	13.6	997	215.9	286
	14	13.6	1,007	254.7	253
	15	12.6	1,004	286.2	225
N74-9308-2	4	14.0	1,010	17.4	976
	5	18.9	871	32.8	755
	6	20.2	794	54.6	519
	7	19.5	769	66.9	440
	8	20.5	788	92.0	337
	9	18.4	770	76.6	319
	10	19.7	753	117.2	248
	11	19.9	735	129.1	227
	12	20.7	776	168.3	198
	13	20.3	690	188.6	167
	14	19.7	638	186.7	158
	15	21.2	493	297.0	127
N74-9827	4	11.6	429	5.5	416
	5	19.2	294	36.6	217
	6	18.3	280	50.3	150
	7	18.6	268	58.1	123
	8	18.8	296	78.4	117
	9	17.2	294	91.0	99
	10	17.7	290	100.5	99
	11	17.4	278	110.1	102
	12	17.7	226	133.7	97
	13	18.2	193	147.8	102
	14	19.1	204	178.5	110
	15	19.1	112	198.1	95

† Standard errors for both fresh weight and HCN-p were usually about 5% of the means.

were not determined. However, based on reasonable assumptions, they probably were at least 10 μg/ml. Thus, protein in the homogenates may well have inflated the direct potentiometric values. Such interference also may explain the observation of Blaedel et al. (4) that direct potentiometric determination on low-cyanide tissues gave high readings in comparison with volatilization procedures. No attempt was made to correct the potentiometric assay values shown in Table 1 for protein interference.

The decline in dhurrin content with increasing plant age was verified in several studies employing the spectrophotometric assay. The results of one such study are summarized in Table 2. Although the HCN-p of the complete seedling declined with time, the HCN-p of the first leaf maintained a fairly level "plateau" between the 6th and 12th day. The fresh weight of the first leaf also was quite uniform during this period. Similar effects were not observed for leaves emerging after the first leaf (i.e., individually assayed 2nd, 3rd, and 4th leaves); these leaves exhibited the typical decline in HCN-p and the rapid fresh weight increase similar to that already observed for complete seedlings. It is noteworthy that the HCN-p of complete seedlings or other leaves never exceeded the HCN-p of the first leaf; thus, for the time period studied, the HCN-p of the first leaf probably represents the maximum cyanogenic potential of a particular seedling. This observation, the relative freedom of first leaf extracts from interfering constituents, and the relative uniformity of first leaf HCN-p values led us to use first leaves in routine comparative assays. An added advantages of this procedure is that it is nondestructive to the seedling.

Table 3. HCN potentials of various sorghum and sudangrass cultivars and lines as determined by the spectrophotometric assay of p-HB.

Cultivar or line	HCN potential (fresh weight basis)				
	Sampling date A	Sampling date B	Sampling date C	Mean	± SE†
	ppm				
Grain sorghum A lines:					
Martin A	1,192	1,267	1,166	1,208	33
Redlan A	1,048	1,183	1,160	1,130	32
CK60 A	1,045	1,153	1,060	1,087	24
Wheatland A	932	915	858	902	18
Grain sorghum R lines:					
Redbine 60	1,100	1,230	1,240	1,190	31
Texas 414	1,093	1,097	968	1,053	37
Plainsman	915	889	1,011	939	26
KS-53	900	896	843	880	18
WD-16R	818	922	845	861	30
Sweet sorghums:					
Ramada	1,395	1,354	1,274	1,341	37
Roma	1,258	1,185	1,224	1,223	16
Tracy	1,091	1,059	1,195	1,115	29
Brawley	1,202	1,103	1,031	1,112	33
Brandes	945	1,055	1,038	1,013	26
Rio	1,038	942	949	976	20
Forage sorghum A lines:					
KS9A	1,167	1,164	1,139	1,157	16
N4692A	1,198	1,106	1,091	1,132	29
KS5A	895	907	898	900	20
KS8A	826	744	769	780	18
Forage sorghums:					
Atlas	1,621	1,638	1,419	1,560	64
Early Sumac	1,405	1,425	1,243	1,358	39
Rancher	1,279	1,241	1,102	1,208	42
Rox	1,203	1,250	960	1,138	64
Ellis	842	1,049	954	948	42
White Collier	1,029	846	917	931	42
Leoti	921	936	827	895	39
Early Hegari	766	858	725	783	32
N6229	707	708	663	693	25
Sudangrasses:					
GA 337	647	568	627	614	21
Sweet	549	545	485	527	29
Greenleaf	575	495	511	527	20
Tift	517	536	514	522	22
Wheeler	484	473	475	477	22
Nebr. 7035	413	415	465	431	16
Piper	344	436	320	367	28
N74-9386-1	280	257	290	276	10
N74-9348-1	276	270	253	266	10

† SE is based on two determinations per sampling date or a total of six determinations.

The sampling procedure now used in this laboratory for the rapid and routine estimation of the HCN-p of sorghum and sudangrass genotypes is as follows: The first leaf is excised from 10 seedlings of each genotype when the second leaf is one-half to two times as long as the first. The first leaves are then randomly grouped into two samples of five leaves each for extraction and assay. This procedure provides two determinations of HCN-p per genotype on each sampling date. Table 3 presents the HCN-p values of several cultivars and lines of sorghum and sudangrass that were assayed in this manner on three separate dates over a period of several months. The data indicate that satisfactory repeatability can be achieved with the spectrophotometric assay. The ranking of the genotypes by HCN-p

(Table 3) agrees well with the rankings obtained by other investigators using different assay techniques (7, 13, 16, 19, 27, 29). Thus, Piper sudangrass is acknowledged to be the lowest in HCN-p among the widely used cultivars and it also occupies that rank with our procedure. Similarly, all sudangrasses are considered to be lower in HCN-p than either forage or grain sorghums, and this ranking also is found in our results in Table 3. Note that there are differences in HCN-p within types of sorghum, but differences of a few hundred ppm HCN at such high HCN levels are of little practical importance because all plants at those levels could be lethal to livestock under optimum conditions for HCN formation. Two low-HCN-p experimental sudangrass lines, N74-9386-1 and N74-9348-1, have been developed by use of the spectrophotometric assay. Both lines are lower in HCN-p than the cultivar, Piper.

As a further check on the precision and repeatability of the assay procedure, two inbred experimental lines (one high and one low in HCN-p), replicated five times each, were always included with each new planting and assay. In one series of 10 such tests covering a period of 6 weeks, the mean HCN-p contents of the high and low lines were 703 and 315 ppm, respectively. An analysis of variance of these data (not shown) indicated a highly significant difference between the HCN-p of the two lines, but differences among dates of sampling or replications within dates were not significant. Thus, the procedure described permits direct comparison of HCN-p values obtained on different sampling dates.

The use of HCN-p values obtained from chamber-grown grain sorghum seedlings to predict the HCN-p values of corresponding field-grown material has been seriously questioned (6). It is true that the HCN-p of field-grown material can be accurately assessed only in the field because of the large number of environmental and other variables which influence HCN-p. We believe, however, that the reliability of the seedling assay described in this report in ranking a wide array of sorghum germplasm with respect to HCN-p has been adequately demonstrated. Therefore, we suggest that this spectrophotometric procedure can be used as an efficient tool in screening for low-cyanide lines of sorghum and sudangrass.

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