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IS *p*-HYDROXYBENZALDEHYDE A MAJOR CONSTITUENT OF EPICUTICULAR WAX FROM *SORGHUM BICOLOR* SEEDLINGS?*

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Key Word Index—*Sorghum bicolor*; Gramineae; epicuticular wax; *p*-hydroxybenzaldehyde; hydrocyanic acid; dhurrin.

Abstract—Free *p*-hydroxybenzaldehyde was not present in appreciable quantity on the surface or in the interior of week-old *Sorghum bicolor* shoots that had been heated to inactivate hydrolytic enzymes, nor was *p*-hydroxybenzaldehyde detected in epicuticular wax of greenhouse-grown sorghum *ca* 4.5 months old.

We have made extensive use of a procedure [1, 2] for determining the hydrogen cyanide potential of young sorghum (*Sorghum bicolor*) seedlings, in which the cyanogenic glucoside, dhurrin [(*S*)-*p*-hydroxymandelonitrile β-D-glucopyranoside] is extracted and simultaneously hydrolysed by autoclaving leaf tissue in water, the extract diluted in 0.1 M sodium hydroxide, and the *A* at 330 nm then determined to provide a measure of the liberated *p*-hydroxybenzaldehyde (**1**). This procedure provides a reliable estimate of hydrogen cyanide potential only if all **1** in the seedling extracts actually comes from hydrolysed dhurrin. Thus, the recent report of Woodhead *et al.* [3] that significant quantities of **1** occur in the epicuticular wax of week-old sorghum seedlings casts serious doubt on the validity of our cited procedure. We did the following experiments in an effort to learn more about the possible occurrence of **1** in the epicuticular wax of sorghum.

Chloroform extraction of fresh shoots

Week-old, chamber-grown (27°, continuous cool white fluorescent light at *ca* 140 μE/m²·sec) sorghum (cv Redlan B) shoots weighing *ca* 100 mg/shoot were excised just above the soil surface, and weighed samples consisting of two shoots were subjected to the following four treatments. (a) Shoots were immersed in 15 ml chloroform for 20 sec at room temperature immediately after which they were dried in a 75° oven for 2.5 hr. The chloroform extract was evaporated just to dryness in an air stream at 65° and the residue was taken up in 10 ml water. The oven-dried tissue was pulverized and then extracted with 20 ml water at room temperature for 3 hr. The aqueous extract was freed of tissue residue by filtration. (b) Identical to (a) except that the shoots were immersed in chloroform for 5 min. (c) Identical to (a) except that the shoots were not immersed in chloroform. (d) Fresh shoots were immersed in 20 ml water and autoclaved for 30 min at 117°. This is the procedure used for the extraction and hydrolysis of dhurrin from seedling leaves in the hydrogen cyanide assay cited above [1].

Extracts resulting from the foregoing treatments were diluted in 0.1 M sodium hydroxide (1:10 for aqueous extracts, 1:5 for reconstituted chloroform extracts), and spectra were scanned immediately from 400 to 240 nm against a blank of 0.1 M sodium hydroxide. For the aqueous extracts resulting from treatments (a–c) scans were repeated after the basic solutions had stood at room temperature for 2 hr to permit the hydrolysis of dhurrin [4].

We interpret the results (Table 1) of this experiment as follows. The 20 sec immersion in chloroform (treatment a) removed little if any **1** from the shoots. In as much as the procedure of Ebercon *et al.* [5] specifies an immersion time of 15 sec for extraction of sorghum epicuticular wax, the 20 sec immersion should be ample to remove the wax as well as any **1** from the shoots if the latter is present in the wax as reported by Woodhead *et al.* [3]. Although the

Table 1. Spectral characteristics of extracts of Redlan sorghum seedlings

Treatment*	Extract	λ_{max} (nm)	A_{255}†	A_{330}†
a	$CHCl_3$, 20 sec	None	0.006	0.003
	H_2O, initial	255, 330	0.441	0.266
	H_2O, 2 hr	330	0.260	0.562
b	$CHCl_3$, 5 min	330	0.011	0.158
	H_2O, initial	255, 330	0.376	0.108
	H_2O, 2 hr	330	0.211	0.384
c	H_2O, initial	255	0.705	0.071
	H_2O, 2 hr	330	0.310	0.700
d	H_2O, autoclaved	330	0.330	0.565

*See text for description.

†Listed A_{225} and A_{330} values for $CHCl_3$ extracts are observed values divided by four, to provide dilution equivalence with H_2O extracts.

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20 sec chloroform extract did not contain appreciable **1** the aqueous extract of oven-dried tissue remaining after chloroform treatment contained both free **1** (λ_{max} at 330 nm) and dhurrin (λ_{max} at 255 nm). The enzymes dhurrinase and hydroxynitrile lyase are responsible for dhurrin catabolism and are physically separated from their substrate in the intact shoot [6, 7]. We suggest that the 20 sec chloroform treatment caused sufficient membrane disruption to make dhurrin accessible to these enzymes, but this brief treatment did not permit appreciable **1** to be released into the chloroform. However, some free **1** was formed after removal of the shoots from chloroform and before these enzymes were inactivated in the 75° oven. The 255 nm peak initially seen in the aqueous extract was not present after the basic solution had stood for 2 hr. The loss in A_{255} was *ca* 60% as great as the gain in A_{330}, a result that was expected in view of the molar absorbance values of dhurrin and **1** at 255 nm and 330 nm, respectively [4].

The 5 min immersion in chloroform (treatment b) allowed substantial liberation of **1**, and the spectrum of the reconstituted chloroform extract indicated very little contamination with other materials absorbing in the 400–240 nm range. The duration of tissue immersion used by Woodhead *et al.* [3] was not specified, but it was stated that the period was sufficiently brief to prevent contamination of the chloroform with chlorophyll. We have noted no readily visible diffusion of chlorophyll from shoots allowed to stand in chloroform for 24 hr and we suggest that the immersion period used by Woodhead *et al.* [3] may have been sufficiently long to permit the hydrolysis of substantial amounts of dhurrin resulting in release of **1** into the chloroform. The aqueous extract of the dried tissue resulting from treatment (b) contained appreciable amounts of both **1** and dhurrin, but these amounts were less than those observed following treatment (a) as expected in view of the quantity of **1** extracted with chloroform in treatment (b).

Heating fresh shoots at 75° without a prior chloroform immersion (treatment c) apparently prevented dhurrin hydrolysis. Extraction of the dry tissue with water yielded intact dhurrin which, on standing in basic solution, was hydrolysed to yield **1**. It appears that little if any free **1** was present either on the surface or in the interior of the intact shoots.

The only spectral peak observed in extracts of shoots subjected to treatment (d) was the 330 nm maximum characteristic of **1**. As previously noted [1], autoclaving the tissue in water effectively extracted, and also hydrolysed, dhurrin.

Shoots of six sorghum entries, in addition to Redlan, were subjected to treatments (b) and (c). Entries included the grain sorghums CK60R normal, CK60R bloomless (the bloomless trait results in a reduction of *ca* 50% in the content of epicuticular wax [5]) and Wheatland, and the forage sorghums White Collier, Rancher and Leoti. In each case results were similar to those obtained with Redlan, that is, the 5 min chloroform (treatment b) extracts of fresh shoots contained substantial amounts of **1**, but shoots subjected to heating prior to water extraction (treatment c) yielded no more than trace amounts of **1**.

Chloroform extraction of dried shoot tissue

Ca 90 week-old Redlan shoots were dried at 75° for 3 hr, the dry tissue was chopped and a portion was extracted with chloroform (1 ml/4 mg) for 1.25 hr at room temperature. The chloroform was evaporated, and the extract was reconstituted with an equal vol. of water. The tissue residue was dried and then extracted with water. (1 ml/4 mg) for 1 hr at room temperature. Extracts were diluted in 0.1 M sodium hydroxide and spectra were scanned immediately and after the basic solutions had been standing for 2 hr. Results indicated that neither the chloroform nor the aqueous extract contained appreciable amounts of **1**. A substantial amount of dhurrin was found in the aqueous extract; alkaline hydrolysis of this dhurrin produced **1**. Based on the 2 hr spectral scans, the chloroform extract contained less than 1% as much **1** as the aqueous extract.

Spectrum of epicuticular wax

With a small spatula, epicuticular wax was gently collected from the leaf sheaths of greenhouse-grown sorghum plants that were *ca* 4.5-months-old and were nearing the flowering stage. Plants at this stage, in contrast to seedlings, bore wax as a readily visible white powder, especially on the leaf sheaths. The collected wax was dissolved in chloroform and the solution was diluted with methanol and then with 0.1 M sodium hydroxide to a final concentration of 40 μg wax/ml. The spectrum of this solution rose steadily from an A of 0.03 at 400 nm to 0.10 at 260 nm, with no discernible peaks. Because there was no 330 nm peak, the A_{330} reading clearly overestimated the content of **1** in solution, but even if the A_{330} reading (0.055) had been due solely to **1**, a content of only 0.6% of the wax would have been indicated.

On the basis of the foregoing observations, we suggest that **1** did not constitute a significant fraction of any wax present on the surface of the sorghum seedlings or plants used in these experiments. We conclude that chloroform can rapidly disrupt sorghum membranes with the result that dhurrin is hydrolysed and **1** is released. This effect of chloroform is not surprising—a common qualitative test for hydrogen cyanide in sorghum involves treating plant tissue with a small amount of chloroform and testing for liberated hydrogen cyanide with picrate paper [8]. If hydrogen cyanide is produced, the liberation of **1** also would be expected.

We continue to believe that the assay of **1** in autoclaved extracts of sorghum seedlings provides a reliable measure of the hydrogen cyanide potential of these seedlings.

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REFERENCES

1. Gorz, H. J., Haag, W. L., Specht, J. E. and Haskins, F. A. (1977) *Crop Sci.* **17**, 578.
2. Akazawa, T., Miljanich, P. and Conn, E. E. (1960) *Plant Physiol.* **35**, 535.
3. Woodhead, S., Galeffi, C. and Bettolo, G. B. M. (1982) *Phytochemistry* **21**, 455.
4. Mao, C.-H. and Anderson, L. (1965) *J. Org. Chem.* **30**, 603.
5. Ebercon, A., Blum, A. and Jordan, W. R. (1977) *Crop Sci.* **17**, 179.
6. Kojima, M., Poulton, J. E., Thayer, S. S. and Conn, E. E. (1979) *Plant Physiol.* **63**, 1022.
7. Thayer, S. S. and Conn, E. E. (1981) *Plant Physiol.* **67**, 617.
8. Hogg, P. G. and Ahlgren, H. L. (1942) *J. Am. Soc. Agron.* **34**, 199.