Plant Physiol. (1979) 63, 1022–1028
0032-0889/79/63/1022/07/$00.50/0

Tissue Distributions of Dhurrin and of Enzymes Involved in Its Metabolism in Leaves of *Sorghum bicolor*[1]

Received for publication November 27, 1978 and in revised form January 29, 1979

MINEO KOJIMA[2], JONATHAN E. POULTON, SUSAN S. THAYER, AND ERIC E. CONN
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

ABSTRACT

The tissue distributions of dhurrin [*p*-hydroxy-(S)-mandelonitrile-β-D-glucoside] and of enzymes involved in its metabolism have been investigated in leaf blades of light-grown *Sorghum bicolor* seedlings. Enzymic digestion of these leaves using cellulase has enabled preparations of epidermal and mesophyll protoplasts and bundle sheath strands to be isolated with only minor cross-contamination. Dhurrin was located entirely in the epidermal layers of the leaf blade, whereas the two enzymes responsible for its catabolism, namely dhurrin β-glucosidase and hydroxynitrile lyase, resided almost exclusively in the mesophyll tissue. The final enzyme of dhurrin biosynthesis, uridine diphosphate glucose:*p*-hydroxymandelonitrile glucosyltransferase, was found in both mesophyll (32% of the total activity of the leaf blade) and epidermal (68%) tissues. The bundle sheath strands did not contain significant amounts of dhurrin or of these enzymes. It was concluded that the separation of dhurrin and its catabolic enzymes in different tissues prevents its large scale hydrolysis under normal physiological conditions. The well documented production of HCN (cyanogenesis), which occurs rapidly on crushing *Sorghum* leaves, would be expected to proceed when the contents of the ruptured epidermal and mesophyll cells are allowed to mix.

The biosynthesis and distribution of various cyanogenic glucosides within higher plants have been closely investigated during the past decades (8). Among such plants, the *Sorghum* genus which possesses the cyanogenic glucoside dhurrin [*p*-hydroxy-(S)-mandelonitrile-β-D-glucoside] has received special attention (1, 18, 24). This secondary product may constitute 5% or even more of the dry weight of young *Sorghum bicolor* seedlings (1, 22). When this tissue is crushed or otherwise disrupted, a large amount of free HCN is rapidly released, as has been reported for other cyanogenic plants (7). Under these conditions, the dhurrin is hydrolyzed by an endogenous β-glucosidase to yield *p*-hydroxymandelonitrile. This intermediate then dissociates to produce free HCN and *p*-hydroxybenzaldehyde (Fig. 1). Although this dissociation can occur nonenzymically, *Sorghum* seedlings also possess an hydroxynitrile lyase, which catalyzes this reaction.

Since free HCN can be detected only when the tissue becomes damaged, several possibilities regarding compartmentation under normal physiological conditions may be envisaged. First, dhurrin and its degradative enzymes may be sequestered in different tissues. Alternatively, dhurrin and the β-glucosidase may exist within the same cell, but in separate intracellular compartments. Finally, all components could exist even within the same intracellular compartment (*e.g.* the vacuole), if the activity of the β-glucosidase could be controlled by endogenous inhibitors.

The above alternatives have been investigated here using epidermal and mesophyll protoplasts and bundle sheath strands isolated from light-grown *Sorghum* leaves. The level of dhurrin and the activities of UDPG[3]:*p*-hydroxymandelonitrile glucosyltransferase, dhurrin β-glucosidase, and hydroxynitrile lyase have been determined. A clearer understanding about the role of each tissue in dhurrin metabolism in *Sorghum* seedlings has thereby been obtained.

MATERIALS AND METHODS

CHEMICALS

Uridine diphospho-D-[U-^{14}C]glucose (NH_4 salt) was obtained from Amersham/Searle and diluted with unlabeled uridine diphosphoglucose from Sigma Biochemicals, Inc. Dhurrin was isolated from 4-day-old etiolated *Sorghum* shoots and purified as described (19). *p*-Hydroxy-(R,S)-mandelonitrile was synthesized according to the method of Ladenburg *et al.* (13). Cellulase (Onozuka R-10) was purchased from All Japan Biochemicals Co., Ltd., and almond emulsin was obtained from Sigma Biochemicals, Inc. Hydroxynitrile lyase was purified from *Sorghum* as described by Bové and Conn (5). The 20-μm nylon net was purchased from Tetko Inc., Elmsford, N. Y., and the 44-μm and 149-μm nets were obtained from Cistron, Lebanon, Pa.

BUFFERS

The following solutions were commonly used: medium A: 0.5 M mannitol, 25 mM K-phosphate-citrate (pH 5.5); medium B: 10 mM Na-phosphate (pH 7.5); medium C: 10 mM Na-phosphate (pH 7.5), containing 1 mg/ml BSA.

PLANT MATERIALS

Seeds of *Sorghum bicolor* (Linn.) Moench, var. Redland × Greenleaf, were soaked in aerated water for 24 h at room temperature. They were then allowed to germinate under fluorescent light (1,700 ft-c) at 26 C in water-saturated Vermiculite, employing a 16:8 h (light:dark) photoperiod. Leaf blades were digested or extracted 3 to 4 h after the beginning of the light period. After 6 days, the *Sorghum* shoot was 9 to 10 cm tall and possessed two expanded leaves.

PREPARATION OF TISSUE FRACTIONS

Mesophyll and Epidermal Protoplasts. Approximately 1.5 g of leaves were excised at the base of the leaf blades and abraded with

[1] This work was supported in part by National Science Foundation Grant PCM 77-25769 and National Institutes of Health Grant GM-05301-21.

[2] Permanent address: Institute for Biochemical Regulation, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464 Japan.

[3] Abbreviations: UDPG: uridine diphospho-D-glucose; C_4 plants: plants having C_4 pathway and reductive pentose phosphate pathway.

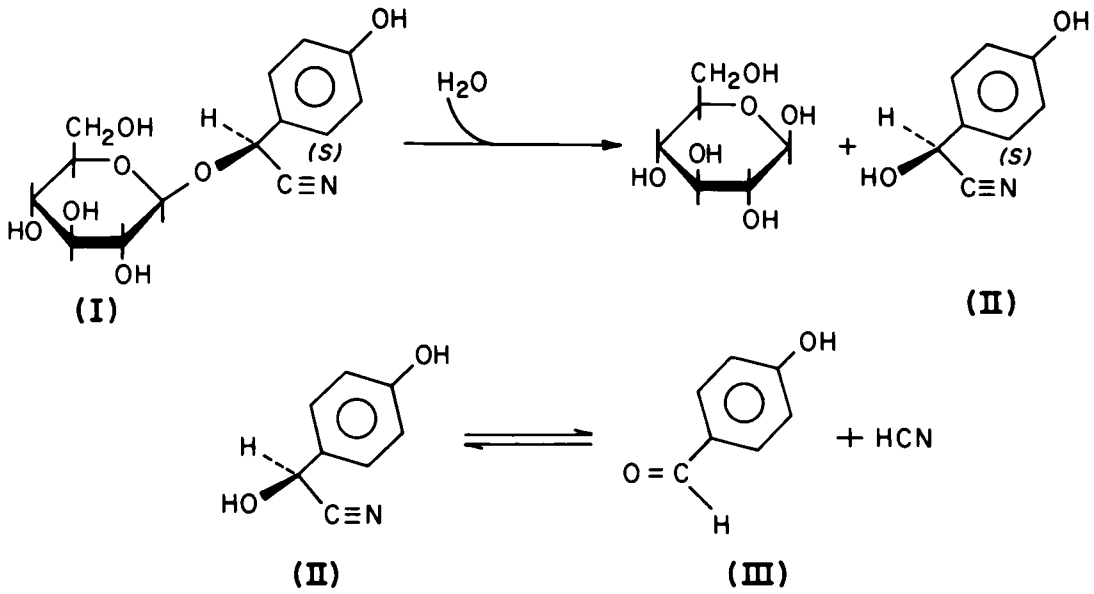

FIG. 1. Catabolism of dhurrin in injured *S bicolor* seedlings. Dhurrin (I) is hydrolyzed by dhurrin β-glucosidase to yield glucose and *p*-hydroxy-(S)-mandelonitrile (II). The latter then dissociates either enzymically in the presence of hydroxynitrile lyase or nonenzymically to release HCN and *p*-hydroxybenzaldehyde (III).

150 grit carborundum using a small artist brush according to the method of Beier and Bruening (3). After the abraded leaves had been rinsed in distilled H_2O, they were incubated for 2 to 2.5 h in 18 ml medium A, containing 1.5% (w/v) cellulase, in a covered 9-cm Petri dish. The incubation was carried out at 30 C in a shaking water bath at 50 oscillations/min. The partially digested leaves were filtered using a 44-μm nylon net and were rinsed with medium A to maximize the yield of protoplasts. The released protoplasts were harvested by centrifuging the filtrate at 220*g* for 3 min in a bench-top centrifuge (swing-out type). The pellet, which contained a mixture of mesophyll and epidermal protoplasts, was gently resuspended in 2 ml medium A. Following the procedure of Saunders and Conn (21), 1 or 2 drops of neutral red (dissolved in medium A) were added to the suspension. The dye was quickly taken up by the epidermal protoplasts and became sequestered in their vacuoles. By contrast, dye could be observed within the mesophyll protoplasts only after much longer time periods. Since the average diameter of the epidermal protoplast population significantly exceeded that of the mesophyll, their partial separation could be effected as follows. The protoplast suspension, after treatment with neutral red, was filtered without applied suction through one layer of 20-μm nylon mesh, which rested upon a single layer of Miracloth in a Büchner funnel (75 mm, i.d.). The mesophyll protoplasts passed unimpeded through the filtration system and were collected by centrifugation at 500*g* for 3 min in a bench-top centrifuge. The pellet was resuspended in medium A. The epidermal protoplasts were preferentially retained on the 20-μm net and were therefore collected by rinsing the latter with a small volume of medium A.

In addition to filtration, the cross-contamination of the mesophyll and epidermal protoplast fractions could be reduced as follows. Protoplast preparations were allowed to stand for 15 to 30 min at either 4 C or at room temperature. Since the epidermal protoplasts sedimented more quickly, the overlaying mesophyll protoplasts could be removed using a Pasteur pipette. Fractions were then concentrated by centrifugation. This procedure was repeated several times as necessary.

Bundle Sheath Strands. Bundle sheath strands were obtained using a method modified from that of Kanai and Edwards (10, 11). The leaves were cut across the veins into 0.5-mm segments using a Gilson gel slicer. Approximately 0.8 g of leaf segments was placed in a covered 9-cm Petri dish with 18 ml medium A, containing 2% cellulase, and the tissue was quickly evacuated twice (40 mm Hg) with a vacuum pump. Incubation was then undertaken at 31 to 32 C in a shaking water bath at 60 oscillations/min. After 2 h, the cellulase medium was replaced by fresh medium, and digestion was continued for a further 2 h. The digestion process was accelerated at intervals by drawing the tissue fragments up and down through a Pasteur pipette. The contents of the Petri dish were then transferred to two 12-ml centrifuge tubes and allowed to settle for approximately 5 min. The overlaying medium, which contained mainly cuticular fragments, was removed using a Pasteur pipette. This procedure was repeated after resuspending the strands in 12 ml of medium A. The strands were then redispersed in medium A, collected by filtration on a 149-μm nylon net and were thoroughly washed using large volumes of this medium. The strands were then recovered by washing the net with medium A and by centrifugation at 300*g* for 1 min. The supernatant fluid was discarded and the pellet was taken up in 12 ml medium A. Further purification by sedimentation (settling), as described above, was undertaken to remove cuticular contaminants. Finally, the bundle sheath strands were collected by centrifugation at 100*g* for 3 min and were redispersed in 3 ml medium A.

ENZYME ASSAYS

UDPG: Aldehyde Cyanohydrin β-Glucosyltransferase. Glucosyltransferase activity was assayed using a modified method of Reay and Conn (20). Mesophyll (or epidermal) protoplasts were sedimented by centrifugation at 370*g* for 3 min, and the supernatant liquid was carefully removed. The pellet was resuspended in 100 μl 0.15 M Tris-HCl (pH 8.0) containing 100 μg BSA, 0.2 μmol DTT, and 10 μmol *p*-hydroxy-(R,S)-mandelonitrile. The protoplasts were ruptured by freezing the suspension three times using liquid N_2. After the addition in 20 μl of 0.6 μmol UDP-[U-^{14}C]-glucose, containing 1.6 μCi, the mixture was incubated at 30 C for 25 min. The reaction was terminated by addition of 30 μl 20% acetic acid, and the precipitated material was removed by centrifugation at 1,050*g* for 10 min. The supernatant liquid was applied to a 4-cm-wide strip of Whatman No. 1 chromatography paper and developed with butanone-acetone-water (15:5:3, v/v). Radioactive dhurrin (R_F = 0.74) was located using a Packard radiochro-

matogram scanner. The area on the chromatogram was cut up into a vial, and the radioactivity was determined by liquid scintillation counting.

In the case of the bundle sheath strands, homogenization could not be successfully effected by freezing and thawing. The strands were homogenized in a TenBroeck homogenizer with 650 μl 0.15 M Tris-HCl (pH 8.0) containing 500 μg BSA, 1.0 μmol DTT, 50 μmol *p*-hydroxy-(R,S)-mandelonitrile. The subsequent addition of radioactive UDPG and the incubation conditions were as described above. The enzyme activity is expressed as the amount of dhurrin synthesized during incubation for 60 min at 30 C.

Preparation of Extracts for β-Glucosidase and Hydroxynitrile Lyase Assays. Mesophyll protoplasts were washed with four 10-ml aliquots of medium A and were finally pelleted by centrifugation at 500*g* for 3 min. After discarding the supernatant liquid, the protoplasts were resuspended in a small volume (3–6 ml) of medium B and ruptured using a sonicator (model W185F, Heat Systems-Ultrasonics, Inc.) at 4 C for periods of 3 × 10 s. The sonicate was centrifuged for 1.5 min in a Beckman microfuge B centrifuge, and the supernatant liquid was used as enzyme source. Epidermal protoplasts were washed carefully using medium A and were then pelleted by centrifugation at 500*g* for 3 min. The pellet was resuspended in 200 to 500 μl medium B or medium C, and the protoplasts were ruptured by drawing them up and down through a narrow micropipette tip (Eppendorf, 200 μl).

The bundle sheath strands were washed and then pelleted by centrifugation as described above. The strands were resuspended in 2 to 6 ml medium B and thoroughly homogenized at 4 C using a tightly fitting TenBroeck homogenizer. After removal of an aliquot for Chl and protein determinations, the remainder was centrifuged for 1.5 min in a Beckman microfuge B centrifuge. The supernatant liquid was assayed for enzyme activity.

Dhurrin β-Glucosidase Activity. Dhurrin β-glucosidase activity was assayed by measuring the production of *p*-hydroxybenzaldehyde from dhurrin in the presence of extract and exogenous hydroxynitrile lyase (17). The enzyme extract was added to a quartz cuvette containing 1.8 μmol dhurrin, 55 milliunits purified hydroxynitrile lyase, and 80 μmol Na-acetate (pH 5.5) in a total volume of 1.2 ml. Incubation was carried out at 37 C and the *A* of the liberated *p*-hydroxybenzaldehyde was measured at 285 nm.

Hydroxynitrile Lyase Activity. The hydroxynitrile lyase activity of isolated fractions was followed by measuring the decomposition of the cyanohydrin of *p*-hydroxybenzaldehyde (5). The assay was carried out at 37 C in a total volume of 1.2 ml which contained enzyme, 2 μmol *p*-hydroxy-(R,S)-mandelonitrile, and 80 μmol Na-acetate (pH 5.5). The reaction was initiated by addition of cyanohydrin, and the *A* of the liberated *p*-hydroxybenzaldehyde was measured at 285 nm. Since the cyanohydrin also decomposes slowly at pH 5.5 in the absence of enzyme, the rate of the apparent enzymic reaction had to be corrected for nonenzymic decomposition.

Ribulose-DiP Carboxylase Activity. The ribulose-diP carboxylase activity of the bundle sheath strands was assayed as described (11, 15). The strands were broken by grinding them in the "activation" buffer of Lorimer *et al.* (15) in a tightly fitting TenBroeck homogenizer at 4 C. After filtering the homogenate through a 37-μm nylon net, the filtrate was assayed for carboxylase activity (15).

OTHER METHODS

Estimation of Dhurrin. Dhurrin was assayed using a modification of the Lambert procedure (14). The mesophyll and epidermal protoplasts (in medium A) were pipetted into small incubation vials (45 × 15 mm i.d.) and centrifuged at 540*g* for 3 min. After carefully removing the supernatant liquid, the protoplasts were quickly resuspended in 0.5 ml 0.1 M Na-phosphate (pH 6.8) initially incubated at 37 C for 3 h. The protoplast mixture was containing 0.3 g emulsin. A container holding 300 μl 1 N NaOH was suspended inside the vial above the protoplast mixture, before the vial was closed off using an air-tight rubber seal. The vial was initially incubated at 37 C for 3 h. The protoplast mixture was then frozen twice using liquid N_2 and incubation was continued at 37 C for a further 19 h. In order to promote HCN release from the mixture and its subsequent absorption by the NaOH "trap," 75 μl 5 N H_2SO_4 was injected into the mixture and incubation was continued for 4 h. The subsequent analysis of the liberated HCN was undertaken as previously reported (14).

This method had to be modified in the case of the bundle sheath strands, since the latter were not efficiently disrupted by freezing and thawing procedures. Instead, a bundle sheath strand preparation was centrifuged at 500*g* for 3 min, and the supernatant liquid was removed. The strands were lyophilized and then quickly ground using a pre-cooled pestle and mortar. The resultant fine powder was weighed and a sample thereof was retained for protein determination. The remainder was introduced into a small incubation vial and treated as described above for the HCN analysis of mesophyll and epidermal protoplasts.

Determination of Protein and Chl. The protein content of isolated fractions was determined by the Lowry method (16) after precipitating twice from solution by 10 ml of 10% (w/v) trichloroacetic acid. BSA treated similarly served as a standard. Chl was estimated by the technique of Arnon (2).

Determination of Cell Number and Microscopic Appearance. Mesophyll and epidermal protoplasts were counted using an AO Spencer Bright Line hemocytometer. This technique was facilitated by staining the preparation with neutral red as described above. The degree of contamination of the various fractions was estimated visually using an AO Spencer microscope at low (× 100) and high (× 450) power magnification. Photographs were taken on a Zeiss universal photomicroscope.

RESULTS

INTACT LEAVES: HCN ANALYSIS AND ENZYME ACTIVITY DETERMINATION

The two expanded leaf-blades of 6-day-old light grown *Sorghum* seedlings were analyzed to determine if they differed significantly with respect to dhurrin content or metabolism. As seen in Tables I and II, the first (older) leaf blades contained somewhat larger amounts of dhurrin and higher activities of the dhurrin β-glucosidase and hydroxynitrile lyase than the second (younger) leaf blades. Inasmuch as no major differences were observed in these preliminary experiments, both leaf blades were utilized in the production of protoplasts and bundle sheath strands.

ISOLATION OF TISSUE FRACTIONS USING DIGESTIVE ENZYMES

Digestion of six-day-old *Sorghum* leaf blades (Fig. 2A) as described under "Materials and Methods" allowed the isolation of large numbers (5–10 × 10^6/g tissue) of mesophyll protoplasts (Fig. 2C). These were spherical in shape, ranging from 10 to 30

Table I. The distribution of dhurrin within leaf blade sections derived from six-day-old sorghum shoots.

The following sections of the first and second leaves of six-day-old light-grown sorghum seedlings were analyzed for dhurrin content using previously described methods (15).

Leaf age	Section of expanded leaf	Dhurrin content (μmol/g fresh weight)
First	Upper half (older)	81.1
First	Lower half (younger)	68.4
Second	Upper half (older)	58.6
Second	Lower half (younger)	55.9

Table II. The distribution of dhurrin β-glucosidase and hydroxynitrile lyase activities in the leaf blades of six-day-old sorghum shoots.

Twenty leaf blades (from either first or second leaf) were weighed, washed and blotted dry. They were then cut into approximately 0.5 cm strips using a razor blade and homogenized at 4 C in a mortar with 2 ml of medium B and a little sand. The homogenate was centrifuged for 1.5 min in a Beckman Microfuge B centrifuge to remove the larger debris. The activities of β-glucosidase and hydroxynitrile lyase were determined as described in the Methods section.

Leaf age	Dhurrin β-glucosidase activity (μmol/h /g fresh weight)	Hydroxynitrile lyase activity (μmol HCN/ h /g fresh weight)	Ratio (hydroxynitrile lyase/ β-glucosidase)
First leaf	313	929	3.0
Second leaf	217	617	2.9

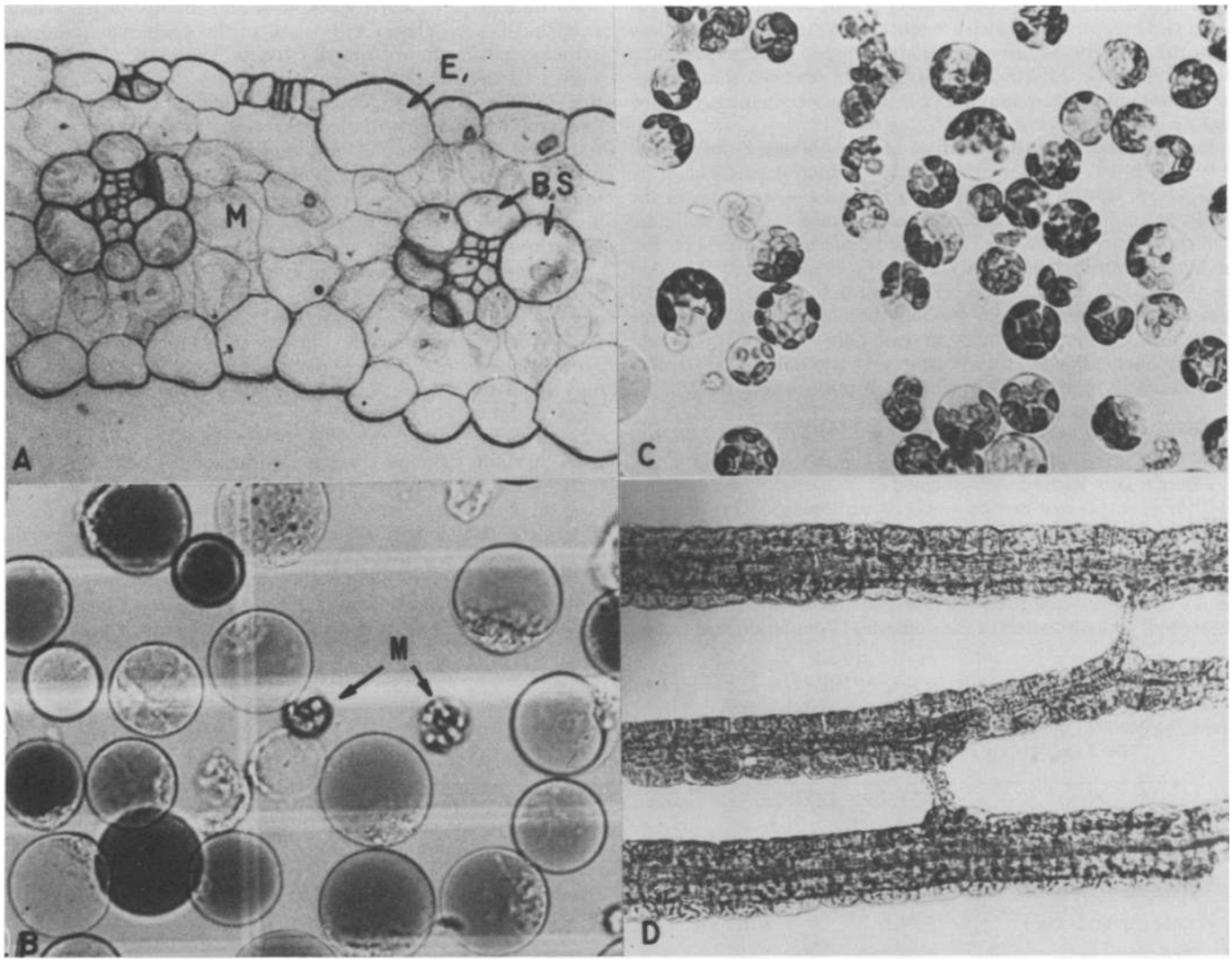

FIG. 2. A: transverse section of a 6-day-old *Sorghum* leaf blade fixed in 4% glutaraldehyde, dehydrated in ethanol, embedded in Spurr's plastic (23), and stained with toluidine blue. Lower epidermis is toward the top of the photograph. m, mesophyll; E, epidermis; BS, bundle sheath cells (× 420). B: preparation of purified epidermal protoplasts, stained with neutral red (× 540). M, mesophyll. C: preparation of purified mesophyll protoplasts (× 540). D: preparation of purified bundle sheath strands, showing three veins with interconnections (× 370).

μm in diameter, and consisted of one central vacuole surrounded by 10 to 20 chloroplasts and the remainder of the cytoplasm. In contrast, the accompanying epidermal protoplasts (Fig. 2B) were larger, having diameters ranging from 25 to 50 μm, and did not contain visible plastids. The yield of epidermal protoplasts was only about 2 to 3 × 10^4/g tissue. The separation procedure for the two protoplast populations, achieved by filtration and sedimentation techniques, produced preparations having a 5 to 10% cross-contamination within each fraction.

Accumulated evidence proved that the colorless protoplasts were indeed derived from the epidermal layer of the leaf. First, these protoplasts were actually observed being released from the

epidermal layers of leaf segments during cellulase digestion. In other experiments, the anthocyanin content of the *Sorghum* seedlings was increased specifically in the epidermal cells by growing the plants under continuous illumination (5,000 ft-c). No anthocyanins were visible in the mesophyll or vascular bundles in hand-cut sections of these seedlings. Upon digestion of the leaves, the epidermal protoplasts were easily distinguishable from the green mesophyll protoplasts by their larger size and anthocyanin content; moreover, they behaved identically with those colorless protoplasts which were routinely obtained from the control seedlings. Additional microscopic evidence demonstrated that the epidermal protoplasts were not vacuoles, as described by Saunders and Conn (21). The presence of nuclei was indicated using the nuclear stain acetocarmine, while ER and other cytoplasmic organelles were recognized within the protoplasts using electron microscopy.

Bundle sheath strands (Fig. 2D) were prepared in high yield from thin slices of leaf blades as described by Kanai and Edwards (10, 11). These strands, which are resistant to the action of cellulase (Onozuka), were almost completely stripped of the adjoining mesophyll tissue. Microscopic examination revealed some mesophyll contamination only in those fragments originating from the leaf tip and the leaf margins.

Before undertaking biochemical analysis of these three tissue fractions, it was necessary to determine which precautions were compulsory in order to avoid contamination by enzymes in the digestion mixture. Trial experiments indicated that the cellulase (Onozuka) preparation itself possessed dhurrin β-glucosidase but no hydroxynitrile lyase activity. However, the latter activity could be detected in the medium after digestion of the leaves and presumably originated from mesophyll protoplasts which became broken during the incubation process. This contamination could be completely eliminated by washing the protoplasts and bundle sheath strands four times with medium A prior to homogenization.

ISOLATED FRACTIONS: HCN ANALYSIS AND ENZYME ACTIVITY DETERMINATIONS

Dhurrin Distribution. As indicated in Table III, large amounts of dhurrin (expressed here as μmol/10^6 protoplasts) were detected within epidermal protoplasts. By contrast, less than 10% of this value was found in the mesophyll protoplast preparations. Since these two fractions were not completely homogeneous, simultaneous equations were used to correct for the approximately 10% mesophyll contamination of the epidermal population and for the 5 to 10% epidermal contamination of the mesophyll protoplast population. Such calculations suggest that the epidermal protoplasts should possess about 47 times more dhurrin than the mesophyll protoplasts. A ratio of 35:1 was indeed observed experimentally with the purest fractions obtained; even in this case, the dhurrin content of the almost homogeneous mesophyll preparation could be accounted for by the epidermal protoplast contamination. The dhurrin content of the bundle sheath strands was only 0.02% of that of the epidermal cells (expressed here as μmol/mg protein). We therefore concluded that the dhurrin within the 6-day-old leaf-blade is localized almost exclusively in the epidermal cells.

Distribution of UDPG:Aldehyde Cyanohydrin β-Glucosyltransferase. The activity of this enzyme was monitored as a possible marker of dhurrin synthesis. Table III shows that no glucosyltransferase activity was detectable in the bundle sheath extracts. About eight times greater activity (expressed as μmol dhurrin synthesized/h·10^6 protoplasts) was found in the epidermal protoplast extracts than in the mesophyll extracts. Simultaneous equations were used as described above to correct for the cross-contamination of the protoplast preparations, yielding an activity ratio (epidermal to mesophyll) closer to 17:1. By observation of thin transverse and longitudinal sections of *Sorghum* leaves, the estimation was made that the ratio of mesophyll to epidermal cells in the whole leaf blade is approximately 8:1. At least 68% of the total glucosyltransferase activity of the leaf blade is localized in the epidermal layer.

Dhurrin β-Glucosidase Activity. High dhurrin β-glucosidase activity was detectable in extracts from mesophyll protoplasts, whereas epidermal extracts exhibited much lower activities (Table III). After correction for the 5 to 10% contamination of the latter extracts by mesophyll protoplasts, epidermal protoplasts possessed only approximately 6.5% of the activity (expressed as enzyme units/protoplast) of the mesophyll protoplasts. Assuming that the ratio of mesophyll to epidermal cells in the leaf blade is 8:1, one may conclude that the epidermis possesses less than 1% of the total β-glucosidase activity present in the mesophyll tissue. The activity of the bundle sheath preparation could not be expressed in terms of cell number. Kinetic analysis showed that the bundle sheath strand fraction contained only 6% and 7% of the dhurrin β-glucosidase activity of the mesophyll fraction, when Chl and protein contents, respectively, were used as basis for comparison.

Hydroxynitrile Lyase Activity. Extracts from mesophyll proto-

Table III. The distribution of dhurrin and enzymes involved in its metabolism in different fractions from six-day-old sorghum leaf blades.

The figures in this table are the mean values of several experiments (3-8).

Component	Epidermal protoplasts		Mesophyll protoplasts			Bundle sheath strands	
	per 10^6 protoplasts	per mg protein	per 10^6 protoplasts	per mg protein	per mg chlorophyll	per mg protein	per mg chlorophyll
Dhurrin (μmol)	5.3	26.5	0.47	1.27	10.4	0.006	0.054
Chlorophyll (mg)	N.D.[1]	N.D.	0.045	0.12	--	0.12	--
Protein (mg)	0.200	--	0.370	--	8.2	--	8.55
UDPG:aldehyde cyanohydrin β-glucosyltransferase (μmol/h)	0.420	2.1	0.055	0.149	1.22	0	0
Dhurrin β-glucosidase (μmol/h)	0.55	2.75	8.5	23	191	1.65	11.6
Hydroxynitrile lyase (μmol/h)	0.99	4.95	19.7	53	441	2.23	13.4

[1] N.D., not determined.

plasts exhibited high hydroxynitrile lyase activity (Table III). In contrast, identical experiments using extracts from epidermal protoplasts demonstrated that they possessed relatively low activities. After correction had been made for 5 to 10% mesophyll contamination, the epidermal protoplasts exhibited only 5% of the hydroxynitrile lyase activity (expressed as units/protoplast) shown by the mesophyll protoplasts. Because of the 8:1 ratio of mesophyll to epidermal cells in the leaf blade, one may conclude that the epidermis possesses less than 1% of the total lyase activity present in the mesophyll tissue. The bundle sheath strand fraction contained only approximately 3 and 4% of the lyase activity of the mesophyll fraction, when activities were compared using Chl and protein content as basis, respectively.

Ribulose-DiP Carboxylase Activity. The observed carboxylase activities of the bundle sheath strands (about 170 μmol CO_2 fixed/hr·mg Chl) are comparable with those reported elsewhere (10) and, in addition to measured shikimate oxidoreductase activities (data not shown), provide an indication that the isolated strands are still biochemically functional.

ADDITIONAL CONTROLS AND CONFIRMATORY EVIDENCE

Confirmation of Localization of Dhurrin and Degradative Enzymes in Different Tissues. The release of HCN from mixtures of epidermal and mesophyll protoplasts was also undertaken in the absence of exogenous emulsin. Table IV demonstrates that the production of HCN was much smaller when the two protoplast populations were incubated separately than when incubated together. These data may be easily explained by the foregoing sections, which demonstrated the specific localization of dhurrin in the epidermal protoplasts and of the degradative enzymes in the mesophyll protoplasts.

Investigation of Possibility of Enzyme Inhibitors. Control experiments were undertaken in which extracts from the three tissue fractions were mixed and subsequently analyzed for enzymic activity. Such "mixing" experiments failed to reveal the presence of any inhibitors in the bundle sheath strands, which could inhibit the hydroxynitrile lyase of β-glucosidase activities of the mesophyll protoplasts or the UDPG-cyanohydrin glucosyltransferase activity of the epidermal protoplasts.

Table IV. The release of HCN from epidermal and mesophyll protoplasts in the absence of exogenous emulsin.

The determination of cyanide, as described in the Methods section, was modified as follows. Protoplasts were centrifuged at 540 g for 3 min, and the supernatant liquid was removed. The pellet was resuspended in 0.3 ml of 0.1 M sodium phosphate buffer, pH 6.8, which contained no emulsin. A cyanide trap, consisting of 0.2 ml of N NaOH, was suspended in the vial, before the latter was sealed off. The protoplasts were ruptured by freezing them using liquid N_2, and the vials were incubated at 37 C for 4 hr. After injection of 20 μl of N H_2SO_4, incubation was continued for another 13 hr. The released HCN was determined as described earlier (15).

Epidermal protoplasts (number added)	Mesophyll protoplasts (number added)	HCN released (nmol)
7.5×10^3	--	0.4
7.5×10^3	7.5×10^3	3.0
7.5×10^3	7.5×10^4	15.9
--	7.5×10^3	0.4
--	7.5×10^4	1.3

Effect of Gel Filtration on Activities of Degradative Enzymes. The possibility that the enzyme extracts possessed an endogenous inhibitor was further investigated using gel filtration chromatography. Passage of a mesophyll extract (in buffer C) through a Sephadex G-25 column (preequilibrated and eluted with buffer C) resulted in the recovery of 113 and 105% of the hydroxynitrile lyase and β-glucosidase activities, respectively. In identical experiments employing bundle sheath strand extracts, 100% of both activities was recovered in the column eluate. These data indicate the absence of an inhibitor in the bundle sheath strands, but suggest that the enzymic activities given in Table III for the mesophyll extracts may be 5 to 15% lower than the real values.

DISCUSSION

In recent years, extensive use has been made of digestive enzymes in isolating protoplasts and bundle sheath strands from plant tissues (6, 9, 10). Biochemical analysis of such preparations has elucidated how various cell types cooperate in certain aspects of primary metabolism. For example, this approach has established the complementary roles of mesophyll and bundle sheath cells during photosynthesis in C_4 plants (12). In this paper, the isolation of epidermal and mesophyll protoplasts from leaves of 6-day-old *Sorghum* seedlings has been described. These fractions, in addition to bundle sheath strands isolated from the same source, were used to investigate the location and metabolism of the cyanogenic glucoside dhurrin, which is found in high concentrations in these leaves.

It is generally assumed that dhurrin is physically separated within *Sorghum* seedlings from the degradative enzymes (β-glucosidase and hydroxynitrile lyase), which catalyze its decomposition. Several modes of compartmentation have been described in the Introductory section, proposing how this might be achieved. Analysis of the three tissue fractions from *Sorghum* leaves clearly demonstrated that dhurrin and the degradative enzymes are localized in different tissues. Essentially all of the dhurrin contained in leaf-blades of 6-day-old *Sorghum* is located in the epidermis. This localization pattern resembles that of several other secondary products derived from L-phenylalanine and L-tyrosine (25–28). In contrast, the β-glucosidase and hydroxynitrile lyase are found in the mesophyll tissue. This mode of compartmentation provides a mechanism under normal physiological conditions, whereby dhurrin may be protected from large scale enzymic breakdown. The rapid release of HCN, which is noted on crushing *Sorghum* leaves, would proceed when the contents of the ruptured epidermal and mesophyll cells were allowed to mix.

The synthesis of dhurrin in etiolated *Sorghum* seedlings is carried out by a microsomal fraction, which catalyzes the conversion of L-tyrosine to *p*-hydroxy-(S)-mandelonitrile via *p*-hydroxyphenylacetaldoxime (18). A soluble glucosyltransferase then catalyzes the glucosylation of *p*-hydroxy-(S)-mandelonitrile using UDP-glucose as a glucosyl donor (18, 20). The demonstration of the multistep conversion of L-tyrosine to *p*-hydroxymandelonitrile by microsomes isolated from light-grown seedlings has so far been unsuccessful (Kojima, unpublished). It was therefore not possible to determine which cell types catalyze this complex reaction sequence. However, it was demonstrated that 68 and 32% of the total UDPG:aldehyde cyanohydrin glucosyltransferase activity of the leaf blade were found in the epidermal and mesophyll tissues, respectively. If these data indicate that both tissues are participating in dhurrin synthesis, one must conclude that dhurrin synthesized in the mesophyll layer must subsequently be translocated into the epidermal cells.

Bough and Gander (4) reported that dhurrin undergoes metabolic turnover in etiolated *Sorghum* seedlings with rates of approximately 0.05 μmol/h. Since the β-glucosidase and hydroxynitrile lyase are presumed to take part in such turnover, dhurrin

would have to be translocated into the mesophyll cells to accomplish this degradation. Alternatively, the data presented here indicate the presence of low levels of the degradative enzymes within epidermal cells, which might be sufficient to account for the turnover rates observed within etiolated tissue.

Recently in this laboratory, Saunders and Conn (21) compared the dhurrin content of protoplast and vacuole preparations isolated from light-grown *Sorghum* leaves. In that study, protoplasts obtained primarily from mesophyll cells, as evidenced by their chloroplasts (Fig. 1 of ref. 21), were lysed in hypotonic solutions and the release of vacuoles from such protoplasts was observed. When the vacuoles were subsequently collected and analyzed for dhurrin, it was concluded that all of the dhurrin in the original protoplast preparation was located in the vacuolar space of those protoplasts.

The demonstration in this paper that only epidermal cells contain dhurrin means that the protoplast preparations used by Saunders and Conn contained protoplasts of epidermal origin as well as mesophyll. Structures similar to the epidermal protoplasts described in the present work (Fig. 2B) were indeed present (Fig. 1 of ref. 21) and presumably are the source of dhurrin in those preparations. In our experience, it is often difficult to distinguish between a mesophyll vacuole contaminated by adhering cytoplasm and a small epidermal protoplast, which contains a vacuole comprising over 90% of its volume. It is therefore possible that a low level of contaminating epidermal protoplasts, or even epidermal vacuoles, could account for the dhurrin content of the vacuolar preparations in that study. Saunders and Conn did note cytoplasmic contamination in their vacuoles and were able to reduce but not eliminate it by including 1% BSA in their resuspension medium.

One can estimate (Fig. 2A) that epidermal cells constitute approximately 40% of the volume of the *Sorghum* leaf blades used in these experiments. From the dhurrin concentration (Table I) of such tissue, one can calculate that the concentration of dhurrin in the epidermis is approximately 0.2 M. (This same value is obtained if one assumes that epidermal protoplasts have a dhurrin concentration of 5 μmol/10^6 protoplasts and an average spherical diameter of 35 μm.) If the cytoplasm occupies 5% of the epidermal cell and the dhurrin were confined to that volume, the dhurrin content would have to be 4 M. Such figures obviously support the earlier conclusion (21) that dhurrin is a vacuolar constituent.

Acknowledgments—The authors appreciate the help of Mr. J. E. Dunn in the isolation of dhurrin. Our sincere thanks are due to Dr. L. Peterson, Shell Development Co., Modesto, Calif. for providing seedlings grown under high light intensities, and to Dr. T. Rost and the Facility for Advanced Instrumentation, University of California, Davis, for the use of the Zeiss universal photomicroscope.

LITERATURE CITED

1. AKAZAWA T, P MILJANICH, EE CONN 1960 Studies on the cyanogenic glucoside of *Sorghum vulgare*. Plant Physiol 35: 535–538
2. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
3. BEIER H, G BRUENING 1975 The use of an abrasive in the isolation of cowpea leaf protoplasts which support the multiplication of cowpea mosaic virus. Virology 64: 272–276
4. BOUGH WA, JE GANDER 1971 Exogenous L-tyrosine metabolism and dhurrin turnover in sorghum seedlings. Phytochemistry 10: 67–77
5. BOVÉ C, EE CONN 1961 Metabolism of aromatic compounds in higher plants. II. Purification and properties of the oxynitrilase of *Sorghum vulgare*. J Biol Chem 236: 207–210
6. COCKING EC 1972 Plant cell protoplasts, isolation and development. Annu Rev Plant Physiol 23: 29–50
7. CONN EE 1969 Cyanogenic glucosides. Agric Food Chem 17: 519–526
8. CONN EE 1973 Biosynthesis of cyanogenic glucosides. Biochem Soc Symp 38: 277–302
9. CONSTABEL FC 1975 Isolation and culture of plant protoplasts. *In* OL Gamborg, LR Wetter, eds, Plant Tissue Culture Methods. National Research Council of Canada, Ottawa, pp 11–21
10. KANAI R, GE EDWARDS 1973 Enzymatic separation of mesophyll protoplasts and bundle sheath cells from C_4 plants. Naturwissenschaften 60: 157–158
11. KANAI R, GE EDWARDS 1973 Separation of mesophyll protoplasts and bundle sheath cells from maize leaves for photosynthetic studies. Plant Physiol 51: 1133–1137
12. KELLY GJ, E LATZKO, M GIBBS 1976 Regulatory aspects of photosynthetic carbon metabolism. Annu Rev Plant Physiol 27: 181–205
13. LADENBURG K, K FOLKERS, RT MAJOR 1936 The synthesis of 3-hydroxy-2-(3)-benzofuranone and of 4-hydroxymandelic acid. J Am Chem Soc 58: 1292–1294
14. LAMBERT JL, J RAMASAMY, JV PAUKSTELIS 1975 Stable reagent for the colorimetric determination of cyanide by modified Konig reactions. Anal Chem 47: 916–918
15. LORIMER GH, MR BADGER, TJ ANDREWS 1977 D-Ribulose-1,5-bisphosphate carboxylase-oxygenase. Improved methods for the activation and assay of catalytic activities. Anal Biochem 78: 66–75
16. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
17. MAO CH, L ANDERSON 1967 Cyanogenesis in *Sorghum vulgare*. III. Partial purification and characterization of two β-glucosidases from *Sorghum* tissue. Phytochemistry 6: 473–483
18. MCFARLANE IJ, EM LEES, EE CONN 1975 The *in vitro* biosynthesis of dhurrin, the cyanogenic glucoside of *Sorghum bicolor*. J Biol Chem 250: 4708–4713
19. REAY PF 1969 An improved procedure for the isolation of dhurrin. Phytochemistry 8: 2259–2260
20. REAY PF, EE CONN 1974 The purification and properties of a uridine diphosphate glucose: aldehyde cyanohydrin β-glucosyl transferase from *Sorghum* seedlings. J Biol Chem 249: 5826–5830
21. SAUNDERS JA, EE CONN 1978 Presence of the cyanogenic glucoside dhurrin in isolated vacuoles from *Sorghum*. Plant Physiol 61: 154–157
22. SAUNDERS JA, EE CONN, CH LIN, CR STOCKING 1977 Subcellular localization of the cyanogenic glucoside of *Sorghum* by autoradiography. Plant Physiol 59: 647–652
23. SPURR AR 1969 A low viscosity epoxy embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43
24. STAFFORD HA 1969 Changes in phenolic compounds and related enzymes in young plants of *Sorghum*. Phytochemistry 8: 743–752
25. STEINITZ B, H BERGFIELD 1977 Pattern formation underlying phytochrome-mediated anthocyanin synthesis in the cotyledons of *Sinapis alba L.* Planta 133: 229–235
26. TISSUT M 1972 La synthèse flavonique dans les tuniques en survie de bulbe d'oignon. Physiol Vég 10: 381–391
27. TISSUT M 1974 Etude de la localisation et dosage *in vivo* des flavonols de l'oignon. CR Acad Sci Paris Ser D 279: 659–662
28. WELLMAN E 1974 Gewebespezifische Kontrolle von Enzymen des Flavonoidstoffwechsels durch Phytochrom in Kotyledenen des Senfkeinlings (*Sinapis alba* L.). Ber Deutsch Bot Ges 87: 275–279