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# Biosynthesis of Phenolic Compounds in First Internodes of Sorghum: Lignin and Related Products[1]

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*Summary.* Lignin biosynthesis in excised tissues of *Sorghum vulgare* variety Wheatland milo incubated in air with and without prior infiltration with $H_2O$ was presumably limited by $H_2O_2$ production and was dependent upon an endogenous substrate, probably starch. In solution culture without shaking, this conversion of endogenous material was partially blocked at some step prior to *p*-hydroxycinnamic acid. The synthesis was light independent and continued protein synthesis was not required. The accumulation of lignin products was paralleled by an increase in dhurrin, alkaline sensitive esters of *p*-hydroxycinnamic, ferulic and sinapic acids, and flavin coenzymes, especially flavin-adenine dinucleotide. There was no detectable evidence of competition for substrates with other phenols such as anthocyanins or with the growth of adventitious roots. There was evidence, however, of mechanisms limiting lignification in the first internode in the intact seedling. Comparisons are made with lignin production in comparable tissues of *Phleum*.

The major phenolic compounds synthesized in the first internode of *Sorghum*, identified in this and previous papers (2, 8, 9, 13, 14), can be divided into 4 major groups. In group I, containing dhurrin, lignin, and esters of $C_6$-$C_3$ phenolic acids except caffeic acid, the compounds are present in intact internodes after 4 days of germination in the dark. They continue to accumulate upon incubation of the excised internodes (2, 9, 13). In group II, consisting of the anthocyanins apigeninidin and luteolinidin, the compounds are accumulated in the dark or light only after excision from the rest of the seedling (13). In group III, including a third anthocyanin, a cyanidin glycoside, and an ester of caffeic acid, accumulation occurred predominantly only after light treatment of excised internodes (13). Protein synthesis in the excised internodes was necessary for accumulation of compounds in group II and III, but not for those in group I (8, 14).

This paper is concerned with the accumulation of compounds in group I, with emphasis on lignin production in excised internodes. Relationships with other biosyntheses and growth processes are discussed.

## Materials and Methods

Seeds of *Sorghum vulgare* var. Wheatland milo (13, 15) were germinated at 25° on moist filter paper in the dark. After 4 days, the internodes were excised in the laboratory or under a green safe light (15). Light treatment (about 2000 ft-c of white fluorescent light) when used was for a 12 hour light-dark cycle. For culture in solution (table I,A), 5 internodes were surfaced sterilized and placed in 25 ml of sterile solution (14). For infiltrated internodes (table I,C), 5 internodes were placed in a 5 ml volume containing 0.01 ml Aerosol, a commercial wetting agent, and were infiltrated by repeated evacuations and slow releases of the vacuum until tissues were translucent. Internodes were placed on small glass rods on moist filter paper in petri dishes (15). Internodes incubated in air (table I,D), were placed directly on rods.

Lignin analyses, based on the ionization difference spectrum at 340 mμ and on phenol groups reacting with the quinoneimine reagent, have been described before (14).

Alkaline sensitive esters of $C_6$-$C_3$ hydroxycinnamic acids soluble in 70 % methanol were estimated by fluorometric analyses after separation via paper chromatography (14).

Dhurrin was estimated by its absorption at 330 mμ after conversion to *p*-hydroxybenzaldehyde (1). Tissues were extracted in methanol containing HCl to stabilize anthocyanins (15). After 2-dimensional chromatography in acetic acid-HCl-water (10:3:87, v/v), followed by butanol-acetic acid-water-concentrated HCl (30:5:10:0.5, v/v), dhurrin was detected as a dark area under UV (266 mμ) at $R_F$ values of about 0.5 and 0.6 respectively in the above solvents. After elution with 95 % ethanol,

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an aliquot was diluted with water and extracted with ether. The absorbancy at 330 mμ of the aqueous phase was determined before and 3 hours after the addition of enough NaOH to make the solution 0.1 N. The amount of *p*-hydroxybenzaldehyde formed was calculated from a standard of *p*-hydroxybenzaldehyde. No correction was made for percent recovery.

Flavins were determined by fluorometric analysis with internal standards (3). Internodes were ground in 10 % trichloracetic acid. Flavin mononucleotide (FMN) was determined before hydrolysis and flavin-adenine dinucleotide (FAD) by the increase in FMN after hydrolysis at 100° for 15 minutes. No correction was made for possible riboflavin. The trichloracetic acid extracts were neutralized to pH 6.8 with $K_2HPO_4$, and the fluorescence measured in a Turner fluorometer (primary filter 7-60; secondary #58).

Analyses of $^{14}C$ experiments were made in a scintillation counter either directly on areas cut out of paper chromatograms or on aliquots dried on paper discs (15). Washed precipitates or cell wall residues were dried directly in the vials. Ten ml of a mixture containing 4 g 2,5-diphenyloxazole plus 50 mg *p-bis* [2-(5-phenyloxazolyl)]-benzene per liter of toluene was used. No correction was made for the efficiency of counting which ranged from 60 to 70 %. Radioactive areas were detected with Xray film.

All values reported are based on dry weights of internodes before incubation, calculated from fresh weights. Dry weight was about 7 to 10 % fresh weight. Tissues for determination of esters were dried at 50°; all other tissues were extracted directly or after freezing.

## Results

*Lignin Production in Solution Culture.* (Table I,A). There was only a small endogenous production of lignin in excised internodes incubated in solution culture without shaking. Differences from 4-day-old controls (zero time) were statistically significant at the 5 % probability level in the UV analysis, but only between 5 to 10 % in the phenol test. Neither sucrose, phenylalanine or tyrosine supported any additional net synthesis. Ferulic acid and *p*-hydroxycinnamic acid, on the other hand, induced considerable accumulation of lignin, while cinnamic and chlorogenic acids were inactive or inhibited the endogenous production. The presence of azaguanine or ethionine produced no statistically significant (at the 5 % probability level) inhibition of the products formed by ferulic acid. Light had no detectable effect on this lignin synthesis. The addition of $H_2O_2$ along with ferulic acid (table I,B) increased the amount of lignin to the levels found in mature plants grown under artificial light (table I,E).

Table II. *Analyses of Alkaline Sensitive Esters of $C_6$-$C_3$ Hydroxycinnamic Acid Esters and of Dhurrin in First Internodes Before and After Excision and Incubation in Air*

| Product | After 4 days germination = 0 time | Excision + 2 days incubation | |
|---|---|---|---|
| | | Dark | Light |
| N = 3 | μmoles per g initial dry wt | | |
| *p*-OH-Cinnamic acid | 0.2 | 1.8 | 1.8 |
| Caffeic acid | Trace | 0.03 | 0.2 |
| Ferulic acid* | 0.4 | 1.1 | 1.1 |
| Sinapic acid* | 0.2 | 0.7 | 0.9 |
| Dhurrin | 3.3 | 10 2 | 10.0 |

* Cross contamination due to overlapping areas on chromatograms; identification of hydroxy acids based on chromatographic and fluorescence characteristics.

*Lignin Production in Infiltrated Tissues Subsequently Incubated in Air.* (table I,C). Considerable endogenous synthesis of lignin products occurred under these conditions. All values differed significantly from zero time controls at the 5 % probability level or better (t-test). The addition of sucrose, ferulic acid or inhibitors of nucleic acid and protein synthesis had no detectable effect on this endogenous synthesis.

*Lignin Production in Tissues Incubated in Air.* (table I,D). The amount of lignin produced in air in non-infiltrated internodes was consistently slightly greater than in infiltrated internodes, indicating that gas transport was still a limiting factor in infiltrated tissues. Light did not alter the endogenous synthesis of lignin products.

According to histochemical tests with phloroglucinol-HCl, lignified cells of the stele were restricted to the non-elongating base of 4-day-old internodes. After incubation of the excised internodes for 2 days in air, the phloroglucinol test was positive to the tip and the color in the lower portion was intensified.

Factors necessary for lignification are sufficient within each section of the internode. When internodes were cut into equal top, middle and basal sections before incubation, the total lignification of the parts estimated by the phloroglucinol test was similar to that in excised internodes incubated in one piece. This was not true with cyanidin synthesis and growth of adventitious roots, where approximately 50 % of the synthesis occurring in the intact excised internode was inhibited if the internodes were incubated as 3 sections (unpublished data).

*Starch Patterns in the First Internode.* Histochemical starch tests (IKI) indicated that at least some of the endogenous substrate being metabolized upon excision was starch. Numerous small starch grains were visible in the dark-grown internode at 4 days. Although scattered throughout the cortex

and stele, they were concentrated in the outer cortex and just outside the endodermal layer, and were prominent in the immature metaxylem vessels of the stele. The starch test was negative in all parts of the internode after incubation of the excised internodes for 2 days in dark or light. The R.Q. ($CO_2/O_2$) for 4-day-old internodes was about 1, indicating that sugar was being oxidized.

*Changes in Other Synthetic Systems Related to Lignin.* Aside from lignin, the following compounds accumulated in excised internodes in the dark or light; anthocyanins, hydroxycinnamic acids, dhurrin and flavin coenzymes. In addition, adventitious roots appeared in the basal half of the internodes. Rapid growth of the latter in air in contrast to their poor growth after infiltration had no detectable effect on lignin synthesis. No lignin, however, was detected histochemically within the young adventitious roots. Alterations in amount of anthocyanin biosynthesis also had no detectable effect on lignification.

The hydroxycinnamic acids of the internode accumulated as alkaline sensitive esters (table II). All but caffeic acid were present in detectable amounts in the intact growing internodes at 4 days. Upon incubation in air after excision, all increased in amount, but only the accumulation of the caffeic ester was strongly dependent upon light. The

Table I. *Lignin Analyses of First Internodes at 4 Days After Germination and After Excision and Incubation Under Different Conditions*

Solutions in part A & C contained 10 mM neutralized solutions of phenolic substrates, 20 mg/ml sucrose, 1 mM 8-azaguanine, and 10 mM ethionine. Solutions in part B contained 50 mM $KH_2PO_4$ at pH 4.5, 20 mM $H_2O_2$ and 10 mM ferulic acid dissolved in alcohol (12). Four day incubation, except 1 day in B. Values (X) expressed as per mg original dry weight ± $S_x$ in some cases. N = 3 except where noted in parentheses with 5 internodes per analysis. Statistical symbols used as in (15).

| Treatment | UV Analysis OD @ 340 mμ | Quinoneimine test μg phenol |
|---|---|---|
| Controls - 0 time (N = 12) | 0.19 ± 0.02 | 0.88 ± 0.06 |
| A-Solution culture | | |
| Dark | | |
| $H_2O$ | 0.29 ± 0.04 | 1.10 ± 0 02 |
| Sucrose | 0.33 ± 0.08 | 1.26 ± 0.17 |
| Ferulic acid acid | 0.59 ± 0.14 | 3.49 ± 0 44 |
| Sucrose + ferulic acid | 0.42 ± 0.05 | 2.64 ± 0.36 |
| *p*-OH-Cinnamic acid | 0.64 ± 0.11 | 2.31 ± 0 85 |
| Chlorogenic acid | 0.24 ± 0.04 | 0.97 ± 0.04 |
| Cinnamic acid | 0.19 ± 0.01 | 0.63 ± 0 04 |
| Tyrosine | 0.29 ± 0.03 | 1.03 ± 0.06 |
| Phenylalanine | 0.30 ± 0 02 | 1.11 ± 0.13 |
| Ferulic acid | 0.62 ± 0.11 | 2.99 ± 0.63 |
| Ferulic acid + azaguanine | 0.63 ± 0.10 | 2.82 ± 0.62 |
| Ferulic acid + ethionine | 0.41 ± 0.09 | 2.22 ± 0.56 |
| Light | | |
| $H_2O$ | 0.28 ± 0.02 | 1.20 ± 0 01 |
| Sucrose + ferulic acid | 0.54 ± 0.06 | 2.72 ± 0.46 |
| B-Ferulic acid + $H_2O_2$ | 2.15 ± 0.02 | 6.57 ± 0 34 |
| C-Infiltration + air | | |
| Dark | | |
| $H_2O$ (N = 6) | 0.55 ± 0.04 | 1.48 ± 0.14 |
| Azaguanine* | 0.53 ± 0.02 | 1.48 ± 0 20 |
| Ferulic acid + azaguanine | 0.56 ± 0.04 | 1.44 ± 0.06 |
| Sucrose + azaguanine | 0.52 ± 0.04 | 1.41 ± 0 19 |
| Ferulic acid + sucrose + azaguanine | 0.47 ± 0.02 | 1.66 ± 0.18 |
| Ethionine* | 0.62 ± 0.23 | 1.61 ± 0 07 |
| D-Air | | |
| Dark (N = 6) | 0.73 ± 0.05 | 1.79 ± 0.22 |
| Light | 0.71 ± 0.01 | 1.75 ± 0.15 |
| E-Intact plants (N = 2) | | |
| Young lamina | 0.55 ± 0.01 | 2.6 ± 0.1 |
| Bases of mature lamina | 1.4 ± 0.14 | 6.5 ± 0 5 |
| Mature sheaths | 2.2 ± 0.4 | 5.5 ± 1.1 |
| Mature stems | 2.8 ± 0.01 | 5.9 ± 0 07 |

* Growth of adventitious roots strongly inhibited.

amount of caffeic ester is still relatively small; it becomes much more prominent in green shoots (unpublished data). The ratio of monophenolic to diphenolic acids changed upon excision from 1:3 to 1:1.

Dhurrin, a cyanogenic glucoside derived from tyrosine (2,8), was a dominant phenolic compound in the internodes of 4-day-old etiolated seedlings. After excision, the amount was approximately trebled (table II). In contrast to data for elongating coleoptiles, internodes showed no light dependent increase (1).

Table III. *Flavin Coenzymes in Excised First Internodes*

| Treatment | FMN | FAD | Total |
|---|---|---|---|
| | N = 6 | μg per g initial dry wt | |
| 0 time – after 4 days germination | 56 | Trace | 56 |
| Excision + incubation | | | |
| 2 days dark | 74 | 45 | 119 |
| 2 days light | 84 | 42 | 126 |
| 0 time – | | | |
| Tip 3 mm section* | 96 | 74 | 170 |
| Basal 2.7 mm section | 72 | 10 | 82 |
| Sum: tip + base | 74 | 13 | 87 |
| Excision + incubation 2 days light | | | |
| Tip section | 193 | 231 | 424 |
| Basal section | 87 | 43 | 130 |
| Sum: tip + base | 91 | 49 | 140 |

* Approximately 6 % fr wt of whole internode.

FMN and FAD increased upon incubation of the excised internodes in either dark or light. The increase in FMN occurred mainly in the top 3 mm segment, while there was a 4-fold increase in FAD throughout the internode. FAD, however, was still more concentrated in the tip section (table III).

*Experiments with $^{14}C$ Tracers.* (table IV). The site of the partial block in the conversion of endogenous substrates in solution culture was investigated with $^{14}C$ tracers. In the methanol soluble fraction (A), a much larger proportion of the total $^{14}C$ incorporated into the fractions analyzed (A+B+C) was still present as either phenylalanine or tyrosine in solution culture in contrast to air. Phenolic acid esters, observed as blue fluorescing areas on chromatograms, were also much less evident in extracts of internodes incubated in solution culture in contrast to air. Data from internodes incubated with sucrose-$^{14}C$, on the other hand, indicated that the percent distribution of label within the sugar fraction was similar in the 2 types of culture. Incorporation of sucrose-$^{14}C$ into the area identified as dhurrin, however, was inhibited in solution culture. A similar inhibition was observed with tyrosine-$^{14}C$.

A partial block in the incorporation of label into possible lignin products in the acidified ether fraction and acid precipitate of alkaline hydrolyzates of cell walls (table IV,C) was evident after incubation with $^{14}C$-phenylalanine in solution culture. With tyrosine-$^{14}C$, an inhibition was detectable only in the acid precipitate.

Table IV. *Percent Incorporation of $^{14}C$ into Fractions and Compounds Isolated from Internodes Infiltrated with Labelled Compounds and Subsequently Incubated for 2 Days in Solution or Air Culture*

Ten internodes were infiltrated with 10 ml solution containing 1 mM azaguanine and 10 μc U-$^{14}C$-sucrose, L-phenylalanine or L-tyrosine, s.a of 270, 297, or 4 mc/mmole respectively. After one-half hour aeration, internodes were placed in 25 ml of solution or in air culture for 2 days. After extraction in methanol-HCl (A), a water and ether extracted wall fraction was hydrolyzed with NaOH, forming an insoluble (B) and soluble fraction (C). The above fractions and lignin products were obtained as previously described and compounds were isolated via 2-dimensional chromatography (11, 12, 15). The acid precipitate (12) was redissolved in ethanol.

| | Sucrose $^{14}C$ | | Phenylalanine $^{14}C$ | | Tyrosine $^{14}C$ | |
|---|---|---|---|---|---|---|
| | Soln. | Air | Soln. | Air | Soln. | Air |
| A-Methanol-HCl soluble (15) | 64 | 60 | 50 | 41 | 29 | 37 |
| Phenylalanine | ... | ... | 15 | 2 | ... | ... |
| Tyrosine | ... | ... | ... | ... | 9 | 2 |
| Sugars | 16 | 21 | ... | ... | ... | ... |
| Dhurrin | 2 | 5 | ... | ... | 7 | 15 |
| B-NaOH insoluble of wall | 13 | 10 | 0.5 | 1 | 0.7 | 1 |
| C-NaOH soluble of wall | 23 | 28 | 50 | 59 | 70 | 62 |
| Acid ether soluble (11) | 1 | 2 | 12 | 20 | 14 | 11 |
| Ferulic acid | ... | ... | 2 | 5 | 2 | 2 |
| *p*-OH-Cinnamic acid | ... | ... | 0.4 | 1.5 | 1 | 1 |
| Vanillin-syringaldehyde | ... | ... | 0.1 | 0.3 | 0.1 | 0.1 |
| Acid precipitate (12) | 0 5 | 1 | 2 | 7 | 5 | 9 |
| Total incorporated: A+B+C = 100 %* | | | | | | |
| cpm × $10^{-3}$ | 20 | 42 | 102 | 127 | 85 | 128 |

* Water and ether soluble components of the methanol insoluble residue were not analyzed in these studies. Other unpublished data indicate that about 7 % of the activity in the methanol insoluble fraction was soluble in water.

## Discussion and Conclusions

Lignification probably occurs only after elongation in a particular area of a cell wall has ceased. In the first internode of a 4-day-dark-grown seedling, lignin was limited to the vascular stele in the non-elongating lower half of the internode, and none was detected in the young elongating adventitious roots of excised internodes. Upon incubation after excision, growth in length ceased, and lignification was completed in the xylem along the whole internode. The upper active meristems of the intact seedling may control the transport, consumption and storage of the carbohydrate supply from the endosperm. The relatively high concentration of indoleacetic acid (IAA) maintained by the upper meristems could inhibit lignification either directly as an antioxidant (4) or indirectly by a promoting effect on cell wall elongation. Excision may lessen the level of endogenous IAA, and the growth processes controlling the carbohydrate supply in the upper part of the internode would cease. Storage starch would then be mobilized. This change in IAA level may be closely related to the growth of adventitious roots which begins about 24 hours after excision. But the growth of aventitious roots, which arise opposite the protoxylem points of the stele, varied independently of lignification in the internode proper.

In excised internodes incubated in air with or without prior infiltration, $H_2O_2$ may be the major limiting factor because none of the intermediates supplied increased the endogenous rate of lignification. While both blue and red-far red light systems are still operative in excised internodes (5, and unpublished data), there was no effect of light on lignification and inhibitor studies indicated that protein synthesis was not required.

In excised internodes in solution culture, however, some step in the conversion of the endogenous substrate to *p*-hydroxycinnamic acid required a relatively aerobic atmosphere or a more effective gas exchange. Preliminary experiments with $^{14}C$-labelled compounds indicated that a major block may be at the level of conversion of phenylalanine and tyrosine, presumably via their respective ammonia lyases. In the case of phenylalanine, subsequent hydroxylation to *p*-hydroxycinnamic acid might also be a limiting factor at lower $PO_2$. The inhibition of incorporation of $^{14}C$ into dhurrin with sucrose or tyrosine was an indication of an oxygen lack (9).

Although some of these phenolic biosyntheses occurring in excised internodes use the same substrates or are formed from common enzyme-substrate complexes (6), there was no evidence that competition for these substrates limited lignification. There may be separate metabolic pools, since anthocyanins and hydroxycinnamic esters may accumulate only in vacuoles of cortical cells, while lignin is produced only within the xylem walls in the stele (6). However, there appears to be a regulatory control that prevents the cells of the cortex from becoming lignified in the excised first internode. Cortical cells of upper internodes can become lignified in grasses (11).

Since flavoprotein enzymes may be responsible for the production of $H_2O_2$ required for lignification, and some of the hydroxylating enzymes require FAD as coenzyme, the increase in lignification upon excision could be related to the increase in FAD upon excision. The flavin values obtained for the first internode are in the same order of magnitude as those reported for oat and barley coleoptiles (3) and wheat leaves (10), but in these still elongating tissues FMN was increased by light treatment.

In all of these studies, the amount of product present at any one time may be the result of an equilibrium between synthetic and degradative processes. For instance, the increase in dhurrin, generally associated only with growing tissues of the seedling (2, 9), could be due to a lessened rate of degradation or conversion to other products such as asparagine (1). Lignin is probably not re-metabolized, and alcohol soluble hydroxycinnamic esters may only re-enter certain metabolic pathways (6, 7).

Factors limiting or controlling lignification in the first internode of *Sorghum* can be compared with those observed for internodes of *Phleum* (14). A major difference in *Phleum* was the lack of any endogenous production, and the ability of exogenous sucrose to serve as a substrate under the more anaerobic conditions of solution culture. It is possible that this latter difference is due to the smaller size of *Phleum* internodes and the subsequent shorter path of gas diffusion. The actual amount and type of lignin products formed with appropriate substrates were similar, and in neither case was the synthesis of enzymes a limiting factor. In both tissues there was evidence of control mechanisms limiting lignification in the intact plant, and lignification in excised tissues was generally inhibited by substrate amounts of cinnamic and caffeic acid derivatives. The 2 tissues differed in that the endogenous levels of caffeic ester were much higher in internodes of *Phleum* than in *Sorghum,* but the ratio of monophenols to diphenols in etiolated seedlings was similar.

The relationship between the alcohol insoluble hydroxycinnamic acids and lignin is not clear. I consider these alkaline sensitive esters as part of the lignin complex because they appear to arise from an initial peroxidation of the acids themselves (forming acid lignin) in a manner similar to the peroxidation of their aldehydes and alcohols (forming classical lignin) (12). Neish and co-workers consider these ethanol insoluble esters apart from the lignin complex (7, fig 4). They have also demonstrated that a small fraction of esters, in-

soluble in acetone but soluble in aqueous buffers, are metabolically active (6). Preparative methods in the 2 laboratories differ. The insoluble ester fraction of Neish et al. would include alcohol insoluble compounds that are also water soluble. In my procedures, any water soluble components of the initial alcohol-insoluble fraction were removed before alkaline hydrolysis of the residue. This would have eliminated most cytoplasmic proteins and any associated phenolic compounds, but probably not proteins firmly bound to the cell walls. A study of the turnover of label in this possible protein fraction of the walls will be necessary to determine whether the labelled compounds in the acid ether soluble part of the wall (C in table IV) are partly intermediates or mainly end products of the acid fraction.

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