Plant Physiol. (1985) 78, 338–342
0032-0889/85/78/0338/05/$01.00/0

# Effects of Fungal Elicitor on Lignin Biosynthesis in Cell Suspension Cultures of Soybean[1]

Received for publication October 24, 1984 and in revised form February 19, 1985

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## ABSTRACT

**Soybean (*Glycine max* L.) cells cultured in B5 medium produce extremely low amounts of lignin. However, modification in the growth medium, by lowering the concentration of $NO^-_3$ and $PO^{2-}_4$, results in the lignification of these cells without affecting levels of cell wall-esterified 4-coumaric and ferulic acid. The production of an extracellular, macromolecular complex by the cultured soybean cells (Moore TS Jr 1973 Plant Physiol 51: 529–536) allows a rapid, nondestructive solubilization of the lignin which can be estimated by reaction with phloroglucinol in free solution. This system has been used to study the effects of fungal elicitor on the synthesis of lignin in soybean cells. The inclusion of very low levels of an elicitor fraction from the cell walls of *Phytophthora megasperma* in the medium in which lignification of the soybean cells occurs suppressed both the accumulation of extracellular lignin and phloroglucinol staining of the cell walls without affecting the levels of bound hydroxycinnamic acids. The activity profiles of phenylalanine ammonia-lyase (EC 4.3.1.5) and isoenzymes of 4-coumarate:CoA ligase (EC 6.2.1.12) were compared in lignifying and elicitor-treated cell cultures as was the activity of chalcone synthase, an enzyme of flavonoid biosynthesis. The measured activities of these enzymes in cell cultures treated with elicitor were considerably lower than in untreated cells.**

Cell suspension cultures provide excellent systems for the study of secondary metabolism; simple alterations of growth media often result in the expression of a specific metabolic pathway. One such system studied in this way is phenylpropanoid metabolism, a branched pathway leading to the biosynthesis of a variety of compounds including flavonoids and lignin. Phenylpropanoid metabolism has been well studied in soybean cell suspension cultures by Hahlbrock *et al.* (10). Although detailed studies of lignification in soybean cell suspension cultures have not been published previously, Hösel *et al.* (12) were able to induce lignification in these cells by modification of the hormone content of the culture medium.

Soybean cells in culture have been shown to produce very low quantities of a hardwood-type lignin (21). Additionally, these cells produce an extracellular complex which was shown to contain a lignin-like material (19). The present study describes a system for inducing lignification in cultured soybean cells and studying changes in the activities of enzymes of lignin biosynthesis.

[1] Supported by a Royal Society (Great Britain) European Fellowship and by the Deutsche Forschungs-gemeinschaft (SFB 206).

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Due to the simplicity of inducing lignin biosynthesis in cultured soybean cells, lack of differentiation in lignifying cells, and the development of a rapid, sensitive lignin detection method, the system described is of use as a model system in the study of the effects of fungal elicitor on the lignin content of the soybean cell wall. Such studies have relevance because it has long been argued that lignin may play an important role in host-pathogen interaction (see review by Vance *et al.* [25]) for example by acting as a mechanical barrier to pathogen invasion. Many previous studies (reviewed in 25) have failed to distinguish lignin from related hydroxycinnamic acids known to exist in the cell wall (6). Such compounds could play a role in host-pathogen interactions in a similar manner to that proposed for lignin. In the present study, both lignin and cell wall-esterified hydroxycinnamic acids are estimated. Biosynthesis of the former is affected by both the nature of the growth medium and treatment of the soybean cell cultures with fungal elicitor.

## MATERIALS AND METHODS

**Cell Cultures.** Cell suspension cultures of *Glycine max* L. cv Mandarin were maintained in the dark in B5 growth medium (8) containing 0.05 mM $FeSO_4$-EDTA (15). Cells were subcultured each 6 to 7 d when the conductivity of the medium reached 1.5 ± 0.5 mS (9).

**Medium for the Induction of Lignification.** B5 medium used to maintain the soybean cells was modified by lowering the levels of two salts, $KNO_3$ and $NaH_2PO_4 \cdot H_2O$, without any alteration of hormone concentrations. $KNO_3$ level was lowered from 2.5 g $L^{-1}$ (24.7 mM) in B5 medium to 0.94 g $L^{-1}$ (9.26 mM) and $NaH_2PO_4 \cdot H_2O$ levels lowered from 0.15 g $L^{-1}$ (1.1 mM) in B5 medium to 0.0225 g $L^{-1}$ (0.165 mM). The new medium was designated LSB5 medium.

Lignification was induced by transferring cells from B5 to LSB5 medium. In the case of 40-ml cultures, a larger inoculum of cells than was normally used to maintain the culture was transferred to LSB5 medium. Routinely, 7-d-old, 40-ml cell cultures (conductivity, 1.5 ± 0.5 mS) were poured into 400 ml of B5 culture medium in 1 L Erlenmeyer flasks. When this culture had reached conductivity of 1.5 to 2.0 mS, usually between 6 and 7 d, cells were allowed to settle and the spent B5 medium withdrawn under axenic conditions. LSB5 medium (400 ml) was then poured onto the settled cells which were further cultured for 5 to 6 d during which time lignification occurred.

**Isolation of Cells and Extracellular Complex.** Spent growth medium (either B5 or LSB5) was removed from the cells by filtration on a sintered glass funnel. The cells were then immediately frozen in liquid $N_2$ before storage at −70°C. The filtrate (spent growth medium) was retained for conductivity measurement and lignin determination.

**Preparation and Use of Elicitor.** *Phytophthora megasperma* f. sp. *glycinea* (Pmg) race 1, was cultured as described by Erwin

and Katznelson (5). Elicitor was released from the mycelia of 5-week-old cultures by autoclaving and purified according to Ayers *et al.* (1). It contained 24% glucose equivalent in the anthrone test (3). In some experiments, elicitor was added to freshly made culture medium before autoclaving or, alternatively, an autoclaved solution of elicitor in water was added to the medium at the time of inoculation with soybean cells. In the latter case, autoclaved water was added to another flask as control.

**Lignin Measurement.** Phloroglucinol staining (24) of whole cells or isolated cell wall material was routinely used to identify lignification. Additionally, phloroglucinol reaction in free solution was perfomed on lignin solubilized from the extracellular complex present in spent growth medium. In this case, spent growth medium (1 ml) in an Eppendorf tube was centrifuged at 10,000*g* for 15 min, the supernatant was discarded and the pellet (*i.e.* extracellular complex) washed twice with water and once with ethanol. The pellet was resuspended in ethanol/dioxan (2:3, 800 μl) and shaken for 2 h at 70°C. After centrifugation (10,000*g* for 10 min), 700 μl of the supernatant was withdrawn and transferred to a new Eppendorf tube. Phloroglucinol solution (0.1% in ethanol, 20 μl) and $H_2SO_4$ (20 μl) were added to the supernatant and the tube was shaken vigorously. Color was allowed to develop at room temperature and, after 20 min, spectra were recorded between 600 and 400 nm against a blank containing no lignin. This procedure did not extract all lignin from the extracellular complex since, after dioxan/ethanol extraction, the complex still showed some phloroglucinol staining. Washing the pellet (extracellular complex) with water and ethanol, prior to dioxan/ethanol extraction, did not solubilize detectable amounts of lignin.

Cell walls that had not been subject to alkaline hydrolysis, were prepared as detailed under 'isolation of cell walls and alkaline hydrolysis.' Cell wall samples (20 mg) were subject to the nitrobenzene oxidation method of Whitmore (26). Vanillin was localized on TLC plates by spraying with acidic phloroglucinol (0.1% in ethanol).

**Isolation of Cell Walls and Alkaline Hydrolysis.** Frozen cells were thawed at room temperature and thoroughly washed with water, methanol, and finally ether, on a fine sintered glass funnel. Cell walls were then dried on a tray at 70°C for 1 h before storage at room temperature. Dried walls were partially hydrolyzed in degassed 0.1 M NaOH, under $N_2$, for 2 h at 90°C. The solution was then made slightly acidic by the addition of 6 M HCl and extracted twice with ethyl acetate which was then removed by rotary evaporation.

**Thin Layer Chromatography (TLC).** Extracts from NaOH-treated cell walls were taken up in a small volume of ether/isopropanol/acetic acid (9:1:0.05) and run through a silica SEP-PAK cartridge (Waters Associates). Nonbinding material was collected and dried by rotary evaporation before application, in ethyl acetate, to Kieselgel 60 TLC plates (Merck, Darmstadt). Plates were developed in benzene/acetic acid (9:1, 6) without UV illumination. Freshly made solutions of various cinnamic acid derivatives in ethanol were applied as reference compounds and in this system migrated largely as *trans*-isomers.

**Enzyme Extraction and Protein Determination.** Buffer used for the extraction of enzymes was 0.15 I[3] Tris-HCl, pH 7.8, containing 25 g $L^{-1}$Tris, 0.5 mM PMSF, 10 mM 2-mercaptoethanol, 1 mM $Na_2EDTA$, and 20% glycerol, titrated to pH 7.8 with HCl. All stages of extraction were carried out at 5°C or when possible on ice. Frozen cells (0.4 g) were shaken with extraction buffer (0.6 ml) for 30 min and then centrifuged at 10,000*g* for 30 min. The supernatant was transferred to an Eppendorf tube containing preequilibrated Dowex 1 × 2 (50 mg) and shaken intermittently for 30 min before centrifugation at 10,000*g* for 30 min. This supernatant was normally used directly for assay but, in the case of data presented in Figure 2, was further purified by passage through a 1-ml column of Sephadex G-50 (fine). Protein was estimated after Bensadoun and Weinstein (2) with ovalbumin (Sigma) as a standard.

**Enzyme Assays.** PAL (EC 4.3.1.5) activity was measured by continuous spectrophotometric determination of cinnamic acid production (27).

4 CL (EC 6.2.1.12) was measured by the continuous spectrophotometric assay of Knobloch and Hahlbrock (16) using the following values for long-wave absorption maxima and millimolar extinction coefficients of various cinnamoyl:CoA esters from Luderitz (17); 4-coumaryl CoA, 333, 21; sinapoyl CoA, 352, 17.8; 3,4-dimethoxycinnamoyl CoA, 346, 21; feruloyl CoA, 347, 17.8. Relative activities of the two soybean 4 CL isoenzymes were assessed using 4-coumaric acid as a substrate for both isoenzymes and 3,4-dimethoxycinnamic acid as a substrate for isoenzyme 1 (15).

CHS activity was determined according to Schröder *et al.* (23). TLC of the reaction products on silica in benzene/acetic acid/$H_2O$ (125/72/3) and subsequent analysis of the plate by scanning and autoradiography revealed one spot of radioactivity migrating identically to authentic naringenin. Addition of more than 10 μl of cell extract to the assay resulted in nonlinearity of the assay.

## RESULTS

With very few exceptions (<0.01% of cells), cells grown in B5 medium were not stained red by phloroglucinol treatment. By contrast, cells cultured for several days in LSB5 medium stained cherry-red with phloroglucinol, as did authentic Björkman lignin. Addition of Pmg elicitor to the LSB5 medium resulted in cells that stained weakly orange with phloroglucinol. Phloroglucinol staining of the cells was paralleled by the staining of the extracellular complex. Vanillin, a characteristic oxidation product of lignin, was detected after nitrobenzene treatment of the cell walls from cells grown for 5 d in LSB5 medium but not those grown for 5 d in B5 medium. Vanillin was only detected when cells stained cherry-red with phloroglucinol and when lignin could be detected in the extracellular medium. Quantitative estimation of vanillin was not made. Attempts to use an established method for lignin determination (20) gave unsatisfactory results possibly due to interference from cell wall-bound hydroxycinnamic acids.

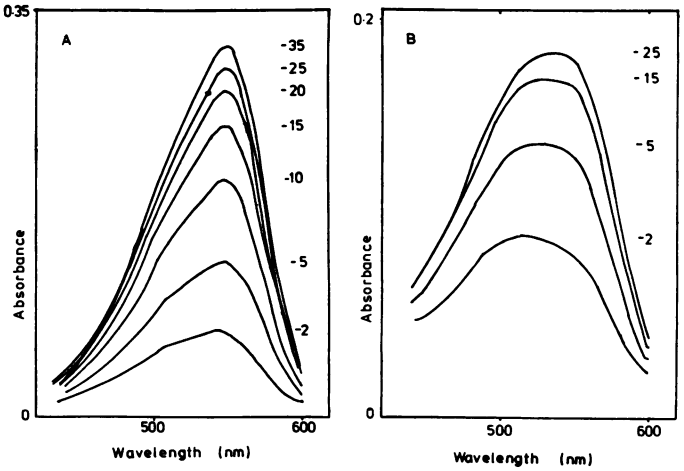


FIG. 1. Color development in the reaction of phloroglucinol with (A) coniferylaldehyde (1 μg) and (B) with wheat lignin (200 μg). Numbers inside the panels are minutes.

[3] Abbreviations: I, ionic strength; PMSF, phenylmethanesulfonylfluoride; Pmg, *Phytophthora megasperma* f. sp. *glycinea;* 4CL, 4-coumarate:CoA ligase; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; NAA, naphthaleneacetic acid.

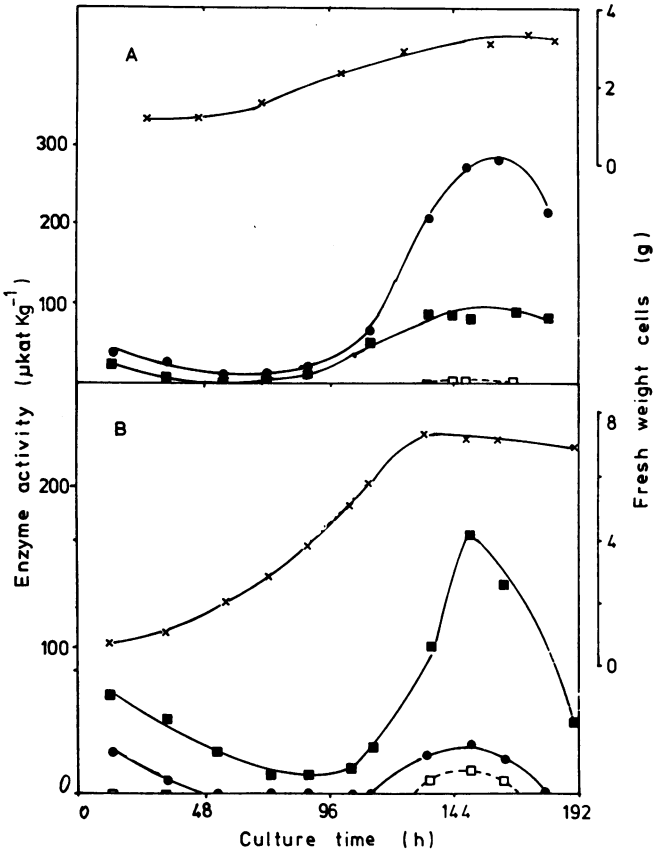


FIG. 2. Changes in enzyme activity during the growth of soybean cells in (A) LSB5 medium and (B) B5 medium. Forty-ml cultures were inoculated with 1.5 ± 0.1 g wet weight cells. One flask was harvested for each time point given. (■——■), PAL activity; (□- - -□), CHS activity; (●——●), 4CL activity with 4-coumaric acid as substrate; (×——×), fresh weight cells. Enzyme activity points are the average of duplicate measurements.

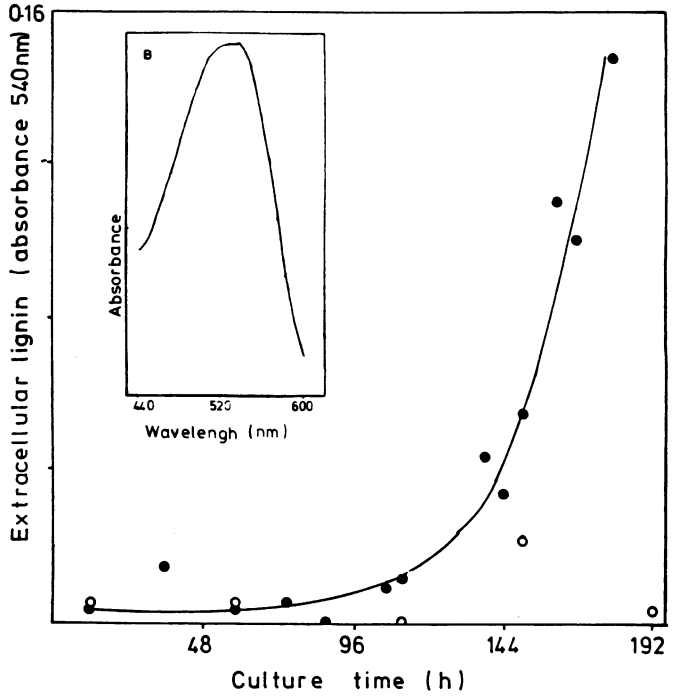


FIG. 3. Changes in the lignin content of the culture medium, as estimated by reaction with phloroglucinol, during the growth of soybean cells. (○), From spent B5 medium; (●——●), from spent LSB5 medium. Culture medium was taken from the experiment illustrated in Figure 2. Inset, Spectrum of the soybean-lignin-phloroglucinol reaction product from cells grown for 5 d in LSB5 medium.

Additionally, it was found that soybean cell wall lignin was extremely difficult to solubilize in organic solvents. In the present study, lignin was routinely extracted and determined from the extracellular complex found in the spent growth medium of these cells. A solvent mixture was sought in which lignin from the extracellular complex produced by soybean cells was soluble and in which its reaction product with phloroglucinol was stable long enough to be measured spectrophotometrically. Initially, different mixtures of dioxan, water, and ethanol were investigated, and the solvent mixture finally chosen was dioxan/ethanol (3:2). Coniferylaldehyde reacted with phloroglucinol in this solvent, under conditions stated in "Materials and Methods." Maximum color development took 35 min at room temperature (Fig. 1A), the colored product was stable for several hours and had a λ max of 546 nm and a ε mol of 57,000. A Björkman wheat lignin preparation (a gift of H. Nimz to this laboratory) reacted with phloroglucinol and gave maximum color development after 25 min; the spectrum (Fig. 1B) was somewhat broader than that observed with coniferylaldehyde having maximum absorption between 534 and 540 nm. Color was stable for only 10 min but was directly proportional to the amount of lignin in the assay up to 300 μg. Lignin extracted from the extracellular complex of cultured soybean cells reacted similarly with phloroglucinol. Maximum color development, stable for 10 min, was reached after 20 min at room temperature. Maximum absorption was between 535 and 540 nm. For routine determination of lignin in the spent culture medium of soybean cells, absorption at 540 nm of the phloroglucinol reaction product was recorded. A spectrum of the soybean lignin-phloroglucinol reaction is given in the inset to Figure 3.

Some lignin could be solubilized (dioxan/ethanol, 3:2) from the soybean cell wall and gave a measurable spectrum with phloroglucinol. This spectrum was similar to that obtained by extraction of the extracellular complex.

**Development of LSB5 Medium.** A variety of growth regulators (NAA, BA, and kinetin), added in various combinations and concentrations (between 10 and 0.1 μM $L^{-1}$) to B5 medium in which 2,4-D (a normal component of B5 medium) had been omitted, did not result in strong lignification of the cells (data not given). When a small amount of lignification was observed, it was invariably associated with a late stage of growth of the culture. Lowering the levels of $KNO_3$ alone, or of $NaH_2PO_4 \cdot H_2O$ alone, did not result in lignification of the soybean cells. Lignification did occur if the levels of both of these salts were reduced; strongest phloroglucinol staining occurred at the levels of $KNO_3$ and $NaH_2PO_4 \cdot H_2O$ chosen for use in LSB5 medium. Although the induction of lignification in cells grown in either 40- or 400-ml LSB5 cultures was basically similar, a far greater degree of lignification occurred in those cells grown in 400-ml cultures. This is apparent from comparison of Figures 3 and 5 illustrating the amount of extracellular lignin detectable in these cultures.

In size and shape, almost all cells grown in LSB5 medium appeared very similar to those grown in B5 medium. On one occasion, a large, elongate cell with some spiral thickenings—possibly a partially differentiated tracheid—was seen. The cell walls of some cells grown in LSB5 medium appeared somewhat thicker than those of cells from B5 medium and showed more intense phloroglucinol staining on the cytoplasmic side of the cell wall. Cells grown in B5 and LSB5 medium stained well with fluorescein diacetate during and beyond the duration of the

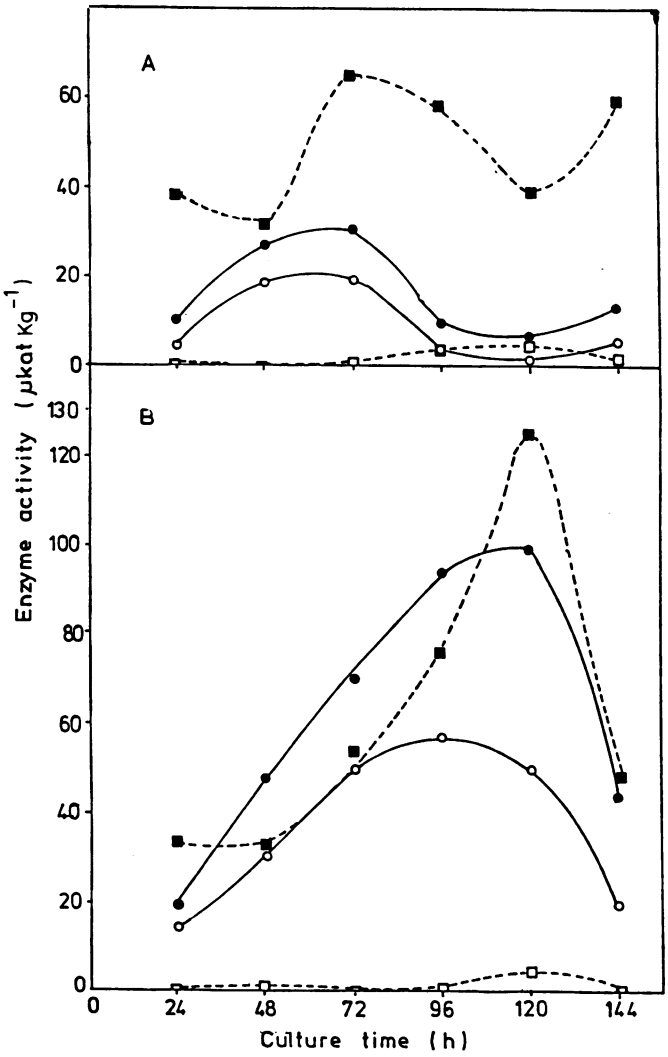


FIG. 4. Changes in enzyme activity during the growth of soybean cells in (A) LSB5 medium containing Pmg elicitor (5 μg ml$^{-1}$) and (B) LSB5 medium with no additions. Four hundred ml growth medium was inoculated with approximately 36 g wet weight cells. Some cells and medium were withdrawn, under axenic conditions, at each time point given. (■--■), PAL activity; (□--□), CHS activity; (●——●), 4CL activity with 4-coumaric acid as substrate; (○——○), 4CL activity with 3,4-dimethoxycinnamic acid as substrate. Enzyme activity points are the average of duplicate measurements.

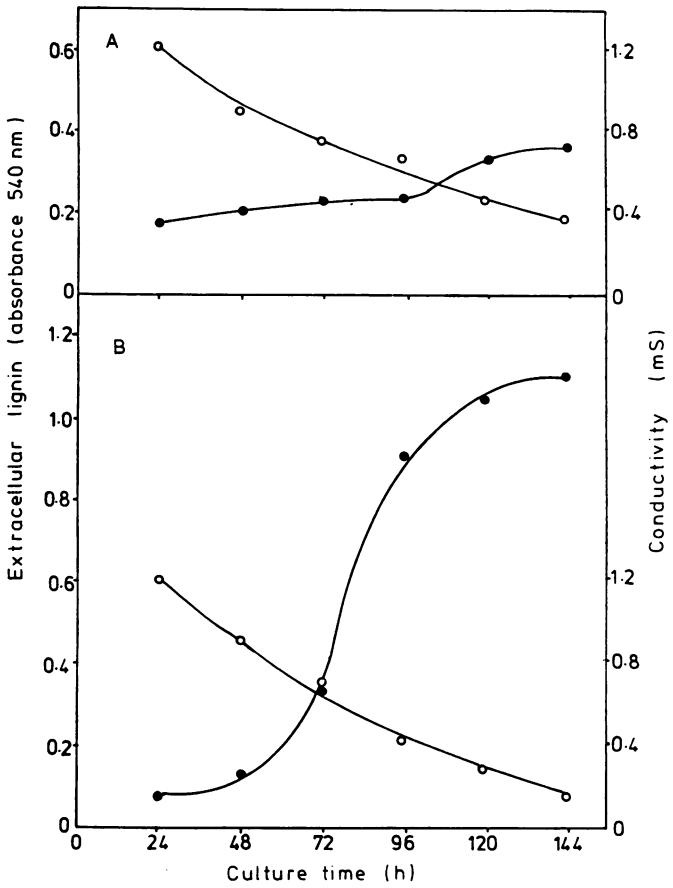


FIG. 5. Changes in the lignin content (●——●) and conductivity (○——○) of the culture medium during the growth of soybean cells in (A) LSB5 medium containing Pmg elicitor (5 μg ml$^{-1}$) and (B) LSB5 medium with no additions. Culture medium was taken from the experiment illustrated in Figure 4. Lignin was estimated by reaction with phloroglucinol.

experiments described. Cultures grown in the presence of Pmg elicitor showed some browning after 4 to 5 d, although they stained well with fluorescein diacetate at this stage and beyond; in the presence of elicitor, cells continued to grow and take up nutrients from the medium (as seen from the conductivity profile shown in Fig. 5). The results presented are all highly reproducible.

**Correlation of Enzyme Activities with Lignification.** The induction of lignification in LSB5 medium was studied in both 40- and 400-ml cultures. In both cases (Figs. 2 and 3, and 4 and 5), the induction of 4CL corresponded to the time at which cells stained with phloroglucinol and to the detection of lignin in the growth medium. In 40-ml cultures, the activity of PAL relative to 4CL was lower than in 400-ml cultures where a greater extent of lignification was observed. Enzyme activities from cells grown in B5 medium were comparable to those previously published by Ebel *et al.* (4). Low levels of Pmg elicitor (5 μg ml$^{-1}$) in the LSB5 medium altered the observed enzyme activity profile (Fig. 4A) with respect to all three enzymes assayed. In cells grown in LSB5 medium (Fig. 4B), levels of PAL, 4CL, and CHS reached a maximum around a culture time of 120 h. Cells grown in the same medium in the presence of Pmg elicitor (5 μg ml$^{-1}$) showed a very different profile (Fig. 4A), 4CL and PAL activity maxima occurring at approximately 72 h and CHS activity reaching a maximum around 120 h.

**Cell Wall Hydroxycinnamic Acids.** TLC revealed that 4-coumaric acid was a major phenolic component released by mild alkaline hydrolysis of the soybean cell walls; a smaller amount of ferulic acid was also detected. HPLC analysis (A. Heitrich, Freiburg) confirmed the presence of 4-coumaric acid at approximately 5 μg g$^{-1}$ fresh weight cells. The quantities of 4-coumaric and ferulic acids detected were similar whether the cells were grown in B5 medium or in LSB5 medium with or without elicitor. No sinapic, caffeic, 5-hydroxyferulic, cinnamic, or vanillic acids, or substances reacting with phloroglucinol, were detected in the cell wall hydrolysate. Similar assays of the hydroxycinnamic acid content of the extracellular complex were not carried out.

## DISCUSSION

In many previous studies of lignification, changes in growth medium hormone content have resulted in the lignification of plant tissue (18, 22), callus culture (22), and cell suspension culture (7, 12). Hösel *et al.* (12) screened many cell suspension cultures, including soybean, for lignification in the presence of

NAA and BA; in a large proportion of the cultures screened, including soybean, positive staining was recorded. In the present study, soybean cells transferred to B5 medium containing both NAA and BA (both at 5 $\mu M$ $L^{-1}$) showed only weak phloroglucinol staining at a late stage of growth. Furthermore, Knobloch and Berlin (14) have shown that growth medium composition is important in the production of secondary metabolites (alkaloids and phenolics) in suspension-cultured *Catharanthus* cells; low levels of nitrogen and phosphate-containing salts led to the enhanced accumulation of these compounds. These observations might suggest that the nutritional status of the suspension culture is an important factor in the induction of lignification and led to the development of the present system for soybean cells where no alteration of growth hormone content is necessary. Rapid, transient increases in the measurable activity of a number of enzymes of phenylpropanoid metabolism occur towards the end of the growth phase in soybean cells grown in B5 medium, a stage at which the medium is depleted of nitrogen (9). In this case, measurable PAL activity usually exceeds that of the 4CL isoenzymes. Interestingly, soybean cells grown for several days in LSB5 medium exhibit very high 4CL activity, exceeding that of PAL (Fig. 4). The measured activities of both 4CL isoenzymes invariably followed congruent profiles during growth of soybean cells in LSB5 medium (whether or not the cells had been elicitor treated), questioning the suggestion (15) that these isoenzymes play unique roles in lignin and flavonoid biosynthesis, respectively. The high specific activity of 4CL isoenzymes in LSB5-grown soybean cells might facilitate purification of these unstable enzymes. Addition of Pmg elicitor to LSB5 medium alters the observed enzyme activity profile, most notably in that elicitor-treated cells show a peak of CHS activity which coincides with a trough in 4CL activity. Additionally, both PAL and 4CL activities are lower in elicitor-treated cells than in untreated cells. This is consistent with the measured suppression of lignification.

Hille *et al.* (11) conducted experiments on soybean cells cultured in B5 medium. They were able to measure a rapid, transient induction of PAL and CHS enzymes by the addition of fairly high levels (routinely, 40 $\mu$g/ml) of Pmg elicitor. The present study utilized a similar elicitor preparation to that used by Hille *et al* (11). However, in this study, lower concentrations of Pmg elicitor (5 $\mu$g/ml) were used and the experiments were carried out in a medium which induces soybean cells to lignify. The observed differences in response to elicitor between the experiments of Hille *et al.* (11) and the present study can be attributed, at least in part, to the fact that different growth media were utilized. Thus, alteration of the growth medium not only affects lignin biosynthesis but also the response of the soybean cells to elicitor.

Köhle *et al.* (13) have recently investigated the effects of chitosan, a $\beta$-1,4-glucosamine polymer, on suspension-cultured soybean cells. The approach they developed differed in a number of ways from that presented here; soybean cells treated with chitosan rapidly synthesized callose and the authors also noted an increase in cell wall phenolics. These phenolics were not identified but appear not to have been lignin. In the present study, the implementation of a spectrophotometric lignin determination based on reaction with phloroglucinol, in addition to phloroglucinol staining of the cells, confirms that addition of Pmg elicitor to the soybean cells suppressed lignification without altering levels of 4-coumaric and ferulic acids.

Advantages of the methods presented herein are that modification of the growth medium to induce lignification is extremely simple and does not require alteration of growth-regulating substances in the medium. Carrying out the phloroglucinol reaction in free solution allows a rapid confirmation of the presence of lignin and is useful where simply staining cells does not give unequivocal evidence for lignin.

*Acknowledgments*—I gratefully acknowledge the provision of laboratory facilities and much help and encouragement from Professor H. Grisebach and Professor K. Hahlbrock. I am indebted to H. Banholzer, Ch. Feser, M-L. Hagmann, A. Heitrich, and W. Heller for much practical assistance and to J. P. Helgeson for helpful suggestions. I also wish to thank the referees for constructive criticism.

Part of this work was presented as a talk at the 3rd Cell Wall Meeting, March 1984, Fribourg, Switzerland.

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