Methanol, pectin and pectinesterase changes during soybean seed maturation

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Abstract

Methanol accumulates in maturing seeds, correlating with preharvest deterioration. Since the source of methanol may be from pectin de-methylation, methanol, cell wall uronic acid, pectin methyl esterification, pectinesterase (PE; EC 3.1.1.11) activity, and neutral sugar composition and partitioning of cell wall polysaccharides were determined during soybean (*Glycine max* [L.] Merrill) seed development, maturation, and desiccation *in planta*. Axis cell wall polysaccharides were more easily solubilized, richer in uronic acid, rhamnose, and xylose, and less rich in galactose than cotyledon cell wall polysaccharides. Methanol accumulated to 9.7 μg per two cotyledons and 0.5 μg per axis; total methanol decreased to 3 μg per two cotyledons during loss of green color. Total uronic acid increased from 0.12 to 0.27 mg per axis and 0.9 to 4 mg per cotyledon between 24 and 50 days after flowering (DAF). After loss of green color, pectin methyl esterification in axes increased from 7 to 24 mole% between 50 and 60 DAF but decreased to 14 mole% by 62 DAF in latter stages of seed desiccation. In cotyledons, methyl esterification ranged from 25 to 40 mole% and was 31 mole% after desiccation. PE activity increased 100 fold in axes, including a 30-fold increase in activity after loss of green color at 46 DAF. Cotyledon PE activity was 40-fold higher than in axes at 24 DAF, declined 75% by 56 DAF, and then increased 5 fold during desiccation. Pectin methyl de-esterification by PE is sufficient to be the sole source for methanol accumulation in seed tissues during development and maturation.

Keywords: cell wall, *Glycine max*, methanol, pectin, pectinesterase (EC 3.1.1.11), seed maturation, soybean

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Introduction

Methanol is a major volatile released from maturing tomato (*Lycopersicon esculentum* L.) fruits (Frenkel *et al.*, 1998), soybean (*Glycine max* (L.) Merrill) seeds (Honig and Rackis, 1975) and expanding soybean leaves (Nemecek-Marshall *et al.*, 1995). Methanol accumulation in soybean seed axis tissue correlates with preharvest deterioration, and exposure to exogenous methanol vapors during precocious maturation of immature soybean seeds grown *in planta* decreased subsequent seedling vigor and germination (Obendorf *et al.*, 1990). The mechanism by which methanol may enhance seed deterioration is unknown, but recent research provides some clues. Methanol inhibits alcohol dehydrogenase (ADH, EC 1.1.1.1) (Frenkel *et al.*, 1998). ADH appears to utilize volatile substrates in dry seeds during storage (Zhang *et al.*, 1995a). If methanol inhibits ADH in soybean seeds, methanol accumulation may result in less accumulation of ethanol and elevated acetaldehyde (Obendorf *et al.*, 1990; Zhang *et al.*, 1995b). Increased acetaldehyde and acetaldehyde-protein adducts correlate with slow deterioration of soybean seeds during long term storage (Zhang *et al.*, 1995b, 1997), suggesting a possible linkage between methanol accumulation and seed deterioration. During the *in vitro* growth of immature soybean seeds, methanol accumulated to 50 μg seed^{-1} in the liquid medium, while methanol decreased from 37 to about 3 μg g^{-1} fresh weight in cotyledon tissues (Obendorf *et al.*, 1990). By contrast, methanol concentration increased 20-fold in axis tissues to 90 μg g^{-1} during 20 d in culture. Pectin methyl esters are the most likely source of methanol released by pectinesterase (PE, EC 3.1.1.11) activity. Genetic evidence demonstrates that PE regulates methanol accumulation in ripening tomato fruits (Frenkel *et al.*, 1998). The formation of intercellular spaces early during cotyledon development (Bils and Howell, 1963; Kollöffel and Linssen, 1984) and the association of de-esterified pectin with the primary cell wall and intercellular spaces (Moore and Staehelin, 1988; Knox *et al.*, 1990)

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suggest that pectin methyl esters may be sufficient to account for the observed accumulation of methanol (Obendorf *et al.*, 1990). However, an analysis of pectin and pectin methyl esters in developing and maturing soybean seeds has not been reported.

The objectives of this study were to document the amount of methanol, uronic acids in cell walls, degree of methyl esterification of pectin and PE activity, and partitioning of cell wall polysaccharides during soybean seed growth *in planta*. Changes in methanol, uronic acids, methyl esterification, and PE activity were characterized at 17 defined seed growth stages during seed development, maturation and desiccation.

Materials and methods

Plant materials

Soybean (*Glycine max* [L.] Merrill cv. Chippewa 64) plants were grown in a greenhouse at 27/21°C (day/night) as previously described (Obendorf *et al.*, 1983). Natural sunlight was supplemented 14 h daily with approximately 740 μmol m^{-2} s^{-1} incandescent light from 1000-W metal halide lamps. Seeds were harvested at 17 stages during seed growth and maturation corresponding to 24, 30, 34, 36, 38, 40, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, and 70 days after flowering (DAF). Color (green to yellow) of axis and cotyledons was estimated visually and expressed as percent of green surface area.

Methanol extraction and assay

Axis and cotyledon tissues were separated, frozen at −85°C, and pulverized while frozen. Methanol in the "dry-ice powder" was extracted with sterile glass-distilled water containing 100 mM $HgCl_2$ in a ground-glass homogenizer and assayed on a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame-ionization detector, a Hewlett Packard 3396A integrator, and a stainless steel column (2 × 1830 mm) packed with 80–100 mesh Porapak Q (Supelco, Bellefonte, PA, USA) (Obendorf *et al.*, 1990). The column was operated isothermally at 130°C with helium as the carrier gas at 30 ml min^{-1}. Detector and injection port temperatures were 250 and 200°C, respectively.

Cell wall preparation

Axis and cotyledon tissues were separated, intact tissues were placed in five volumes of ethanol:water (60:40, v/v) for 1 to 3 h, and then endogenous enzymes were heat-inactivated by refluxing at 80°C for 20 min (Koch and Nevins, 1989). Heat-inactivated tissues were washed in water to remove soluble sugars, washed in acetone to remove lipid-soluble materials, and rinsed in water to remove the acetone. Uronic acid (Blumenkrantz and Asboe-Hansen, 1973) was not detected in the ethanol and water fractions. Soluble sugars were assayed by the phenol-sulphuric acid method (Dubois *et al.*, 1956). All aqueous solutions contained Thimerosal (ethyl(2-mercaptobenzoato-S) mercury sodium salt; 0.2 g l^{-1}) to reduce microbial contamination.

The heat-inactivated, defatted tissue residues of a single axis or cotyledon were pulverized and suspended in 1 (axis) or 2 ml (cotyledon) of 20 mM phosphate buffer, pH 7.0, containing 100 mM NaCl. Endogenous starch in the pulverized cell wall fraction was digested with *Porcine* pancreas α-amylase for 48 h (Kato and Nevins, 1984). Total solubilized starch was estimated from the buffer fraction by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Uronic acid (Blumenkrantz and Asboe-Hansen, 1973) was not detected in the solubilized fraction nor in water washes. Cell wall residues were washed extensively in water, dehydrated in acetone, dried at 35°C for 48 h, and weighed to determine cell wall dry weight.

Approximately 20 mg of finely pulverized dry cell wall material from a cotyledon, or a single axis, was hydrolysed with sulphuric acid and assayed for total uronic acids (Ahmed and Labavitch, 1977). A single axis or 20 mg of cotyledon wall powder was assayed for pectin methylesterification after saponification with 0.5 N NaOH (Wood and Siddiqui, 1971). Protein was estimated by ninhydrin analysis (Moore and Stein, 1954) of amino acids after sulphuric acid hydrolysis of cell walls.

Carbohydrate composition of cell walls was determined following hydrolysis with enolase (Sigma) for 12 h. Cell wall residue was washed four times with water, two times with acetone, and dried at 60°C. Preweighed samples of cell walls were placed in Teflon-lined screw-cap tubes with *myo*-inositol added as an internal standard. Walls were hydrolyzed with 2 N trifluoroacetic acid for 3 h at 100°C. Carbohydrate monomers were converted to their alditol acetates and analyzed by GC on a DB-225 column (0.25 mm diameter, 15 m length, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) operated isothermally at 210°C with 3 ml min^{-1} nitrogen as carrier gas and an FID detector (Nishitani and Nevins, 1988). Individual sugars were identified by retention time, corrected for relative recovery of internal standard, and expressed as mole ratios. Mole ratios of uronic acids were estimated by colorimetry (Blumenkrantz and Asboe-Hansen, 1973) using galacturonic acid as a standard.

Pectinesterase activity

Axis or cotyledon tissues (250 mg fresh weight) were frozen and pulverized in a mortar imbedded in dry

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ice. All subsequent procedures were at 4°C. The "dry ice powder" was suspended in 20 ml of 400 mM sucrose and centrifuged at 3000 × *g* for 15 min. The pellet was resuspended in 20 ml of sucrose and centrifuged. Resuspension was repeated with 600, 800 and 1000 mM sucrose to reduce cytoplasmic contamination (Ricard *et al.*, 1981). Cell wall residues were resuspended in 20 ml of 20 mM phosphate buffer, pH 7.0, and centrifuged at 10,000 × *g* for 15 min to reduce sucrose. PE activity was not detected in 600–1000 mM sucrose washes nor in the phosphate buffer wash. Cell wall residues were incubated in 1 ml of 1 M NaCl at 4°C for 18 h. After rinsing with 1 M NaCl, a second overnight extraction resulted in no additional extractable PE activity from axis or cotyledon cell wall residues. The NaCl extract was dialyzed against 20 mM phosphate buffer, pH 7.0, and assayed for PE activity (Wood and Siddiqui, 1971) using DD Slow Set pectin (Hercules Chemical Co., Inc.) with 66 mole% methyl ester content, a natural substrate. Activity represented slightly-bound PE (Markovič and Obendorf, 1998).

Cell wall fractionation

Total polysaccharide and total protein content of the crude cell wall fraction was determined after hydrolysis with 72% sulphuric acid (Kato and Nevins, 1984). Total protein was estimated by ninhydrin assay of amino acids (Moore and Stein, 1954), and total polysaccharides were estimated by phenol-sulphuric acid analysis (Dubois *et al.*, 1956) of the cell wall hydrolysate. Cell wall fractions were solubilized sequentially (Kato *et al.*, 1982). At each step, the extraction was repeated until no carbohydrate was solubilized as determined by the phenol-sulphuric acid method. Crude cell walls from tissues were extracted sequentially with water at 80°C for 3 h, 2.5 g kg^{-1} ammonium oxalate-oxalic acid at 80°C for 1 h, 1 N NaOH at 22°C for 24 h, and 6 N NaOH at 22°C for 24 h (Kato *et al.*, 1982). Total polysaccharide (Dubois *et al.*, 1956), protein (Moore and Stein, 1954), and uronic acid (Blumenkrantz and Asboe-Hansen, 1973) content of cell wall fractions were determined after hydrolysis of each fraction in 2 M sulphuric acid. The residue fraction was analyzed following digestion with (72%) sulphuric acid.

Results

Seed development and maturation

Soybean seeds were harvested at 17 synchronous developmental stages (Fig. 1). Maximum seed fresh weight was at 46 DAF (Fig. 1A) corresponding to the cessation of cell wall accumulation (Fig. 1B).

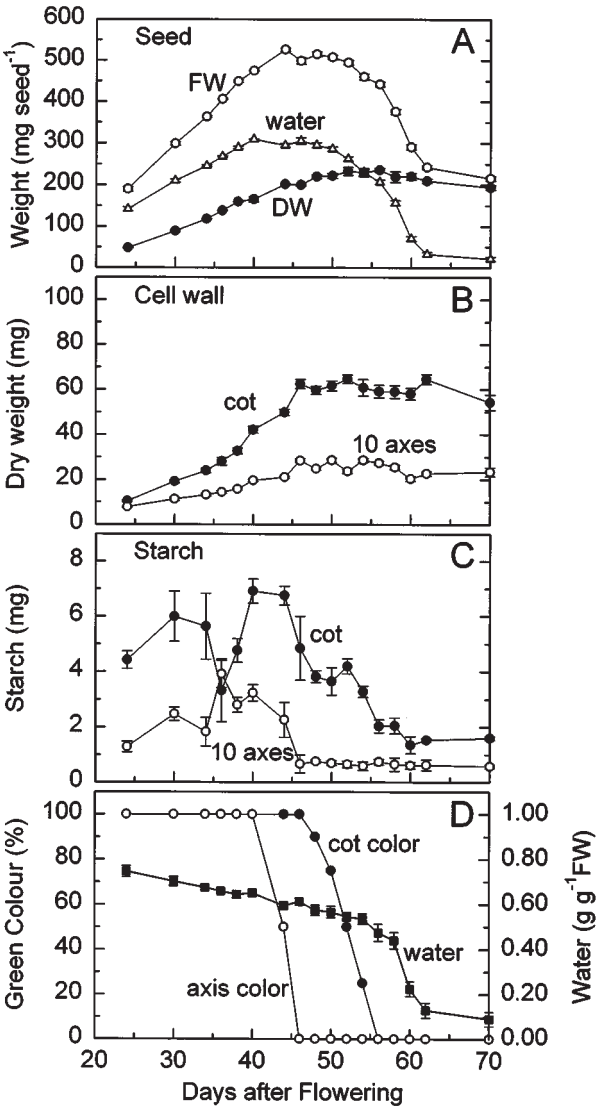

Figure 1. A–D. Soybean seed development as a function of days after flowering. Fresh weight (○, FW), dry weight (●, DW) and water content (Δ) of whole seeds (**A**). Wall dry weight of one cotyledon (●) or 10 axes (○) (**B**). Starch content of one cotyledon (●) or 10 axes (○) (**C**). Colour changes of cotyledon (●) or axis (○) tissues and relative water concentration (■) of whole seed tissues on a fresh weight (FW) basis (**D**). Vertical bars represent ± S.E. of the mean at each growth stage for at least three replicate experiments; not shown if smaller than symbol.

Maximum water content was at 40 DAF (Fig. 1A) after which the axis tissues began to loose green color (Fig. 1D). Physiological maturity (maximum dry weight) was at 52 DAF (Fig. 1A) when seed water concentration was 0.55 g of water g^{-1} fresh weight (Fig. 1D) and water content had started to decline (Fig. 1A). Starch was maximal (Fig. 1C) before axis and

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cotyledon tissues began to lose green color (Fig. 1D). Starch declined during the period of yellowing of axis and cotyledon tissues and continued during seed desiccation.

Methanol accumulation

The patterns and amount of methanol accumulation in cotyledon and axis tissues were distinctly different (Fig. 2). Methanol concentration in cotyledons was 48.3 μg g^{-1} fresh weight at 24 DAF and declined to 7.4 μg g^{-1} at 48 DAF (Fig. 2B), coincident with the cessation of dry matter accumulation (Fig. 2A). The amount of methanol was maximum at 38 DAF and declined to 3.0 μg per cotyledon at 48 DAF and 2.0 μg in the mature dry seed (Fig. 2C). By contrast, highest concentrations and accumulation of methanol in axis tissues occurred between 36 and 60 DAF (Fig. 2E,F), roughly 10 days before to 16 days after the cessation of dry matter increase (Fig. 2D). Methanol concentration in axis tissues reached 62 μg g^{-1} fresh weight *in planta* (Fig. 2E) compared to 90 μg g^{-1} during maturation *in vitro* (Obendorf *et al.*, 1990). Cultured embryos retain high moisture concentrations after maximum mass, whereas methanol evaporates during desiccation of embryos matured *in planta*. The amount of methanol

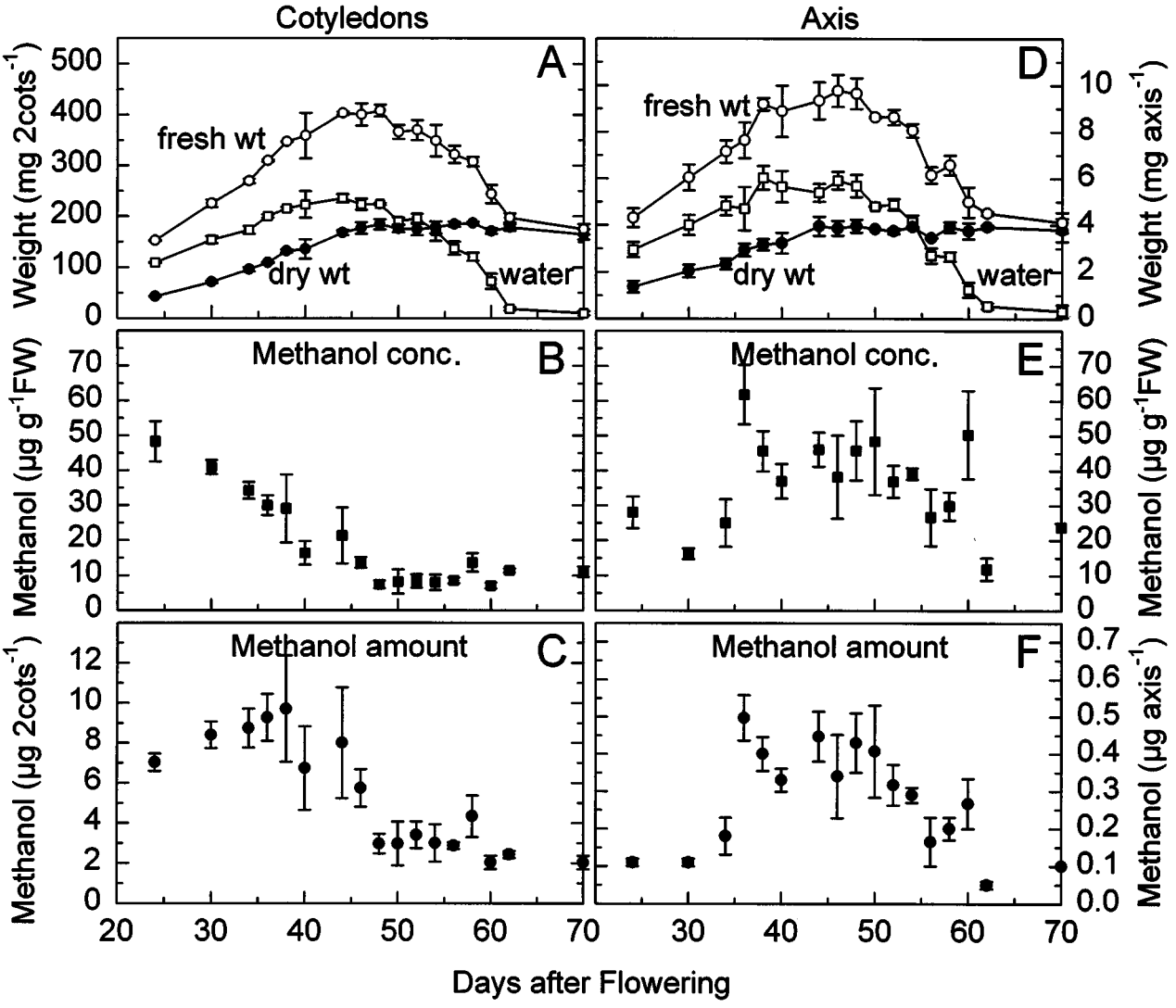

Figure 2. A–F. Methanol accumulation in cotyledons and axes of developing soybean seeds as a function of days after flowering. Fresh weight (○), dry weight (●), and water content (□) per two cotyledons (**A**) or one axis (**D**). Methanol concentration in cotyledon tissues (**B**) or axis tissues (**E**) on a dry weight (DW) basis. Methanol amount per two cotyledons (**C**) or one axis (**F**). Vertical bars represent ± S.E. of the mean at each growth stage for three replicate experiments; not shown if smaller than symbol.

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in axis tissues *in planta* was highest late in seed development (Fig. 2F); in contrast, cotyledons had lowest amounts of methanol late in seed development (Fig. 2C), a pattern similar to that for embryos matured *in vitro* (Obendorf *et al.*, 1990).

Pectin content and methyl esterification

Total pectin in cell walls, measured as galacturonic acid equivalents (Ahmed and Labavitch, 1977; Blumenkrantz and Asboe-Hansen, 1973), was determined throughout seed growth and maturation. Total uronic acids increased four fold in cotyledons and doubled in axis cell walls between 24 and 50 DAF (Fig. 3A). Uronic acid accumulation (Fig. 3A) and cell wall dry weight (Fig. 1B) reached a maximum in axis and cotyledon tissues at the same developmental stage. The degree of methyl esterification of pectin, estimated from methanol released by 0.5 N NaOH hydrolysis of ester linkages, increased slightly and continuously from 25 to 33 mole% in cotyledon tissues (Fig. 3C). Axis tissues maintained a low degree of esterification at about 10 mole% or less from 24 to 50 DAF. After complete yellowing of axis tissues, the degree of esterification increased to 24 mole% by 60 DAF but dropped to 13.6 mole% during the next 2 days as water concentration declined to 0.13 g g^{-1} fresh weight. The decline in methyl esterification occurred after cessation of growth and initial loss in water associated with a decrease in seed volume, and may signify an osmotic or desiccation control of PE activity, regulation, or release into cell walls.

Pectinesterase activity

Developmental changes in PE activity were tissue specific. At 24 DAF, PE activity in cotyledon tissues was 40-fold higher than in axis tissues (Fig. 3D). PE activity of axis tissues increased 100 fold by 62 DAF, including a 30-fold increase after axis tissues lost green color at 46 DAF (Fig. 3D). In cotyledons, PE activity increased 4 fold after loss of green color at 56 DAF.

Partitioning of cell wall polysaccharides

Release of uronic acid at elevated temperatures was completely inhibited by presence of ethanol during the heat treatment, demonstrating that pectin degradation was effectively controlled during the extraction procedure. This inactivation step significantly reduced the release of water-soluble and oxalate-soluble pectins (data not shown). It has been proposed that this is due to the inactivation of endogenous hydrolytic enzymes that would normally make the pectins soluble (Koch and Nevins, 1989).

Eighty to 100% of the cotyledon crude-cell-wall fraction was recovered after fractionation by step-wise

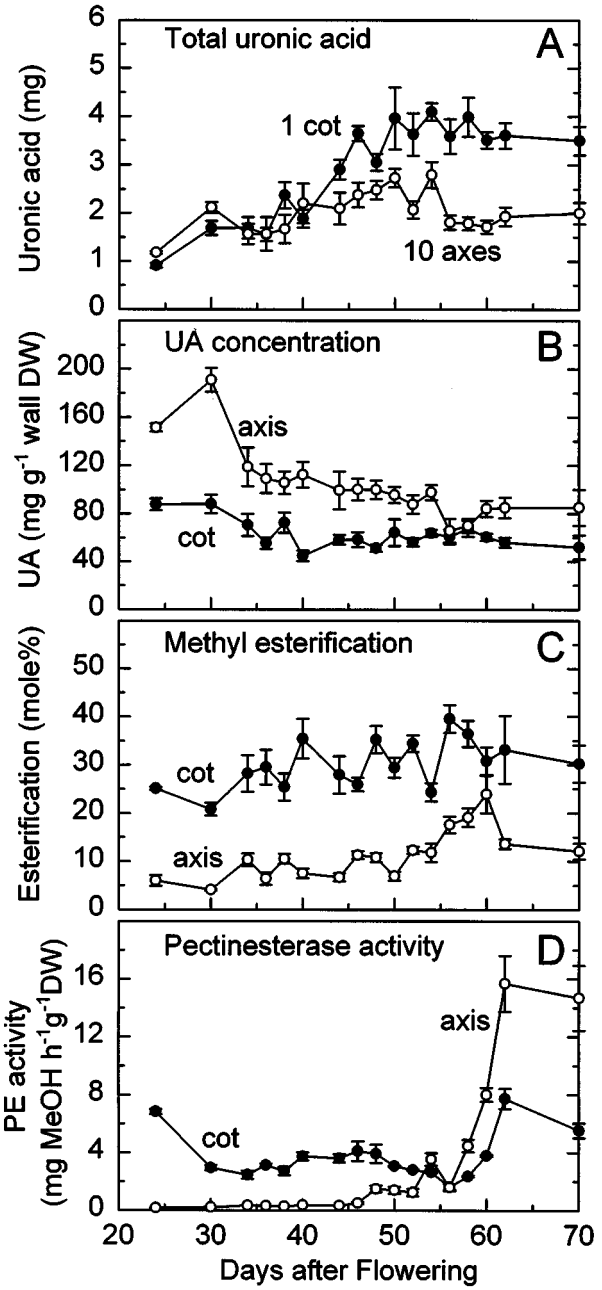

Figure 3. A–D. Total uronic acids, pectin methyl esterification, and pectinesterase (PE) activity in cell walls of soybean seeds as a function of days after flowering. Total uronic acid content in cell walls of one cotyledon (●) or 10 axes (○) (**A**). Relative concentration of uronic acids (UA) in the cell wall fractions (dry weight basis) from cotyledons (●) or axes (○) (**B**). Degree of methyl esterification of pectin (mole%) in the cell wall fractions of cotyledons (●) or axes (○) (**C**). Slightly-bound PE activity expressed as mg methanol h^{-1} mg^{-1} dry weight (DW) of tissue after extraction from cotyledon (●) or axis (○) cell walls with 1 M NaCl (**D**). Vertical bars represent ± S.E. of the mean at each growth stage for at least three replicate experiments; not shown if smaller than symbol.

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solubilization (Fig. 4A). One-half of the dry matter in the cotyledon cell wall fraction was polysaccharide and one-half was protein indicating contamination of the crude cell wall fraction with cotyledonary seed storage proteins. About 48% of total polysaccharides were solubilized by 6 N NaOH (Fig. 4B). Other major polysaccharide fractions included insoluble residue and the fraction soluble in 1 N NaOH (Fig. 4B). With increasing DAF, the proportion of polysaccharide solubilized by NaOH decreased slightly while polysaccharide in the insoluble fraction increased. Thirty-six to 52% of the protein was solubilized with 6 N NaOH (Fig. 4C). Seventy-six to 88% of the uronic-acid-containing polysaccharides were solubilized with 6 N NaOH (Fig. 4D) with 8 to 21% in the insoluble residue.

Solubility of axis cell walls differed from solubility of cotyledon cell walls. Eighty-eight to 100% of the axis crude-cell-wall fraction was recovered after fractionation by step-wise solubilization (Fig. 4E). Sixty-eight to 86% of the cell wall fraction was polysaccharide and 17 to 25% was protein. At 24 and 38 DAF during growth of axis cell walls, 31 and 39% of the cell wall polysaccharides were solubilized in 1 N NaOH (Fig. 4F) in contrast to the higher concentration (6 N NaOH) required to solubilize cotyledon cell wall polysaccharides (Fig. 4B). After cessation of cell wall growth, solubility of polysaccharides in either NaOH or oxalate declined while the fraction of polysaccharides in the insoluble fraction increased. Over half of the protein in the axis cell wall fraction was solubilized with ammonium oxalate (Fig. 4G), indicating that proteins in the axis cell wall fraction were different than proteins in the cotyledon cell wall fraction. Solubility of uronic-acid-containing polysaccharides was strikingly different in cell walls from axis tissues. At 24 and 38 DAF, 64 and 56% of the total uronic acids were solubilized with 1 N NaOH (Fig. 4H). Polysaccharides containing uronic acids became more soluble upon seed maturation with 33 and 43% of the total uronic acid polymers being solubilized with hot water at 56 and 70 DAF (Fig. 4H).

Axis tissues maintain a constant mole% of Rha, Ara, and Man (3, 14, and 1.5, respectively) throughout development (Table 1). Xyl and Gal residues were low during the linear growth phase (8.4 and 9.5 mole%, respectively), increased to maximum at physiological maturity (26 and 22 mole%, respectively), and remained constant during desiccation. Glucose residues were high (7–13 mole%) during the growth phase, but declined to 3 mole% by maturity. Ratios were unchanged after digestion of cell wall fractions with β-glucanase from *Bacillus subtilis* (Sigma) or with glucoamylase, suggesting the glucose residues are cellulosic or callosic in nature (data not shown). By contrast, cotyledon tissues maintained constant ratios of UA, Rha, Man and Gal residues throughout development (Table 1). Ara and Xyl residues were low (8 and 6.5 mole%, respectively) during the linear growth phase but increased to ~20 and 10 mole%, respectively, by maturity. Glucose mole% was similar to axis tissues.

Discussion

This is the first report of changes in pectic polysaccharides in cell walls of developing soybean seed. The relatively constant concentration of total uronic acids in the cell wall fraction during seed growth is consistent with conservation of cell wall composition during growth (Labavitch, 1981). A

Table 1. Uronic acid and neutral sugar composition of cell wall polysaccharides from soybean seed axis and cotyledon tissues as a function of days after flowering (DAF)

DAF	Mole%[a]						
	UA[b]	Rha	Ara	Xyl	Man	Gal	Glc
Axis cell wall fraction							
24	51.0 ± 0.9	8.0 ± 4.3	14.6 ± 2.5	8.4 ± 2.5	1.3 ± 0.5	9.5 ± 2.4	7.2 ± 0.9
38	36.7 ± 3.2	3.4 ± 1.5	9.9 ± 0.3	17.5 ± 2.9	1.4 ± 0.6	16.9 ± 1.1	13.8 ± 5.6
48	35.0 ± 2.2	3.4 ± 0.5	12.4 ± 1.4	25.7 ± 1.2	1.8 ± 0.8	18.7 ± 1.2	3.1 ± 1.2
56	26.0 ± 3.8	3.6 ± 0.1	14.0 ± 0.7	26.3 ± 0.8	2.3 ± 1.0	22.1 ± 2.5	3.6 ± 1.2
70	27.3 ± 4.2	2.3 ± 1.2	17.7 ± 1.4	24.4 ± 3.1	1.6 ± 0.9	28.6 ± 2.6	3.0 ± 1.0
Cotyledon cell wall fraction							
24	28.7 ± 2.1	1.3 ± 1.1	8.0 ± 2.5	6.5 ± 1.9	1.1 ± 0.5	45.3 ± 8.4	8.9 ± 3.3
38	23.3 ± 2.4	1.3 ± 0.8	5.8 ± 4.7	4.3 ± 3.4	0.6 ± 0.5	52.9 ± 10.7	2.5 ± 2.1
48	18.3 ± 1.7	1.4 ± 1.1	21.5 ± 2.5	10.8 ± 3.8	0.5 ± 0.1	46.7 ± 2.6	0.6 ± 0.5
56	20.7 ± 2.0	1.4 ± 0.9	18.2 ± 2.9	9.1 ± 0.6	0.2 ± 0.2	48.6 ± 1.6	1.5 ± 0.7
70	17.3 ± 2.7	1.9 ± 1.1	22.6 ± 1.8	9.0 ± 2.7	0.4 ± 0.1	47.6 ± 3.6	1.0 ± 0.1

[a] Values are mean ± S.E. of the mean for three replications.
[b] Uronic acid.

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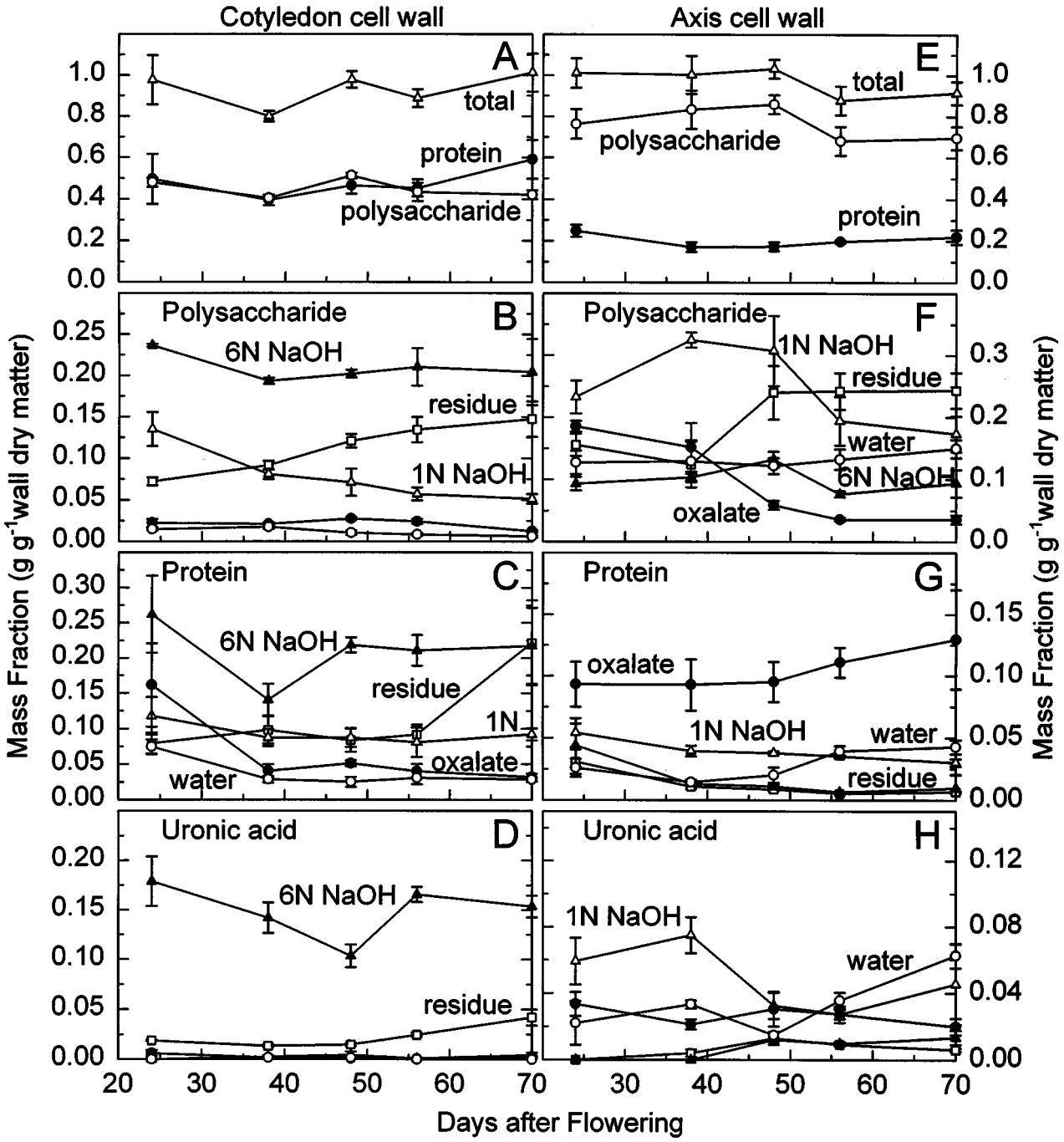

Figure 4. A–H. Cell wall extracts as a function of days after flowering. Fraction of total crude cell wall fraction as polysaccharide (○) and protein (●) (**A**, **E**). The crude cell wall fractions from cotyledon (**B–D**) or axis (**F–H**) tissues were fractionated by sequential extraction with hot water (○) at 80°C for 3 h, 2.5 g kg^{-1} ammonium oxalate-oxalic acid (●) at 80°C for 1 h, 1 N NaOH (Δ) at 22°C for 24 h, and 6 N NaOH (▲) at 22°C for 24 h. Insoluble residue (□) remained after extractions. Total polysaccharide (phenol-sulphuric acid method) (**B**, **F**), protein (ninhydrin method) (**C**, **G**), and uronic acid (Blumenkrantz method) (**D**, **H**) content of cell wall fractions determined after hydrolysis of each fraction with 2 M sulphuric acid. The residue fraction was analyzed following digestion with 72% sulphuric acid. Vertical bars represent ± S.E. of the mean at each growth stage for three replicate experiments; not shown if smaller than symbol.

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homeostasis may exist during growth if pectin is involved in wall turnover during cell expansion and may provide a lubricant or temporary adhesive similar to xyloglucan in pea or glucan during maize coleoptile elongation (Labavitch, 1981; Huber and Nevins, 1979).

PE activity appears to be induced upon yellowing of axis tissues, and PE activity of axis tissues continues to increase until cotyledon tissues are yellow and desiccated. Gene expression was not determined, but PE activity and methanol concentration patterns are clearly tissue specific and appear to be developmentally regulated.

The increase in PE activity corresponds with an increase in methyl esterification of the axis cell wall pectin while a net decrease in esterification occurs at maximum PE activity. Several situations could explain these observations.

(1) Although PE is present, its activity may be inhibited.
(2) The enzyme may be sequestered in the cytoplasm away from its substrate, but this is unlikely since soluble PE decreases during maturation. Only slightly-bound PE was assayed herein. The proportion of total PE as slightly-bound PE is constant at about 60% of total PE activity during seed development (Markovič and Obendorf, 1998). The proportion of soluble PE declines from 33 to 20% and the proportion of strongly-bound PE increases from 8 to 20% between 25 and 50 DAF (Markovič and Obendorf, 1998).
(3) The substrate may be bound to the cell wall or embedded in thickened cell wall and not accessible to the enzyme.
(4) A rapid turnover of pectin may be occurring which permits an increase in methyl esterification without increasing pectin accumulation or concentration. Changes occurred after cessation of cell wall growth indicating considerable metabolism of cell wall polysaccharides after cessation of growth. Maximum PE activity occurred during desiccation.
(5) During desiccation, the enzyme protein may be secreted into the cell wall, post-translational processing may be completed, or the inhibition may be reduced permitting de-esterification of the cell wall pectin. Additional experimentation is required to distinguish among the various situations.

Pectin de-esterification may function to increase the flexibility of the cell wall (including folding), allowing the axis tissue to rapidly change its water volume without damage to tissues during dehydration and subsequent hydration (imbibition). While rapid desiccation occurs over a few days, seed volume more than doubles during 12 h of hydration, requiring that flexibility be in place in the desiccated seed. Folding of cotyledon cell walls occurs during seed desiccation and unfolding occurs upon re-hydration (Lott, 1974; Webb and Arnott, 1982). Folding of cell walls has been described as a means of coordinating the shrinkage/expansion of cell wall and protoplasm during seed desiccation/re-hydration and preserving the integrity of the plasmalemma-cell wall interface and plasmodesmatal connections (Webb and Arnott, 1982). The species-specific pattern of cell wall folding may result from a mechanism related to the chemical structure of the cell wall. While cell contacts are undisturbed by desiccation, some enlargement of intercellular spaces may occur. Site specific changes in pectin esterification may facilitate cell wall folding during desiccation.

Carbohydrate composition of axis tissues during the linear growth phase are similar to those reported for vegetative development in other dicot species (Labavitch, 1981 and references therein). The relative increase in Gal and Xyl residues, components of gums, in axis tissues at physiological maturity suggests that these wall polymers may be important in preparation for desiccation, since they appear relatively low during the linear growth phase. Uronic acid residues account for 50 mole% of the carbohydrate residues in axis cell walls, much higher than in cotyledon cell walls and probably the reason for a higher concentration of accumulated methanol in axis tissues. In addition to rhamnogalacturonans (pectins), primary cell walls of legume seed cotyledons contain arabinogalactans, arabinans, galactans, and galactoxyloglucans (Al-Kaisey and Wilkie, 1992). Arabinogalactans are the major fraction, especially in thickened primary cell walls that serve as storage carbohydrates. Galactomanans are usually in cell walls of the endosperm, a minor fraction mostly adhering to the seed coat in mature soybean seeds. Composition of cotyledon cell walls is consistent with previous analyses of mature soybean cotyledons (Aspinall *et al*., 1967; Brillouet and Carré, 1983; Huisman *et al*., 1998). Further structural analysis is necessary to identify specific cell wall polymers that change during seed maturation.

During *in vitro* growth of a single soybean seed, up to 50 μg of methanol accumulates in the liquid medium (Obendorf *et al*., 1990), a value which is 5 to 7 fold higher than methanol initially in the tissues. Based on the uronic acid level and the degree of methyl esterification in the present study, pectin methyl esters in the cotyledon and axis tissues contain the equivalent of 70 μg of methanol per seed in addition to newly synthesized pectin and turnover of pectin methyl esters during growth *in vitro*. The 10% decrease in methyl esterification of cell walls in axis tissues between 60 and 62 DAF during desiccation can account for 3 μg of methanol axis^{-1} which is 6 to 30

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times the amount of methanol measured in axis tissues *in planta* (Fig. 2) and three times that measured in axes during *in vitro* culture (Obendorf *et al.*, 1990). These observations indicate that pectin methyl de-esterification, especially if pectin turnover occurs, is sufficient to be the sole source for methanol accumulated in seed tissues during development and maturation *in planta* and *in vitro*.

While metabolism of methanol by soybean seeds or methylotropic microbes cannot be ruled out, methanol is evaporated during *in planta* seed development and desiccation. Removal of methanol corresponded to removal of water (Honig and Rackis, 1975), which may explain the decline in methanol in seed tissues during maturation while PE activity increases. Methanol in axis tissue of soybean embryos matured *in vitro* declined from 920 to 4 ng per axis during desiccation (Obendorf *et al.*, 1990). During *in vitro* development and maturation, significant amounts of methanol are trapped in liquid medium and moist tissues, especially the axis (Obendorf *et al.*, 1990). Similarly, enhanced accumulation of methanol may be expected under hot, humid field conditions during which seed desiccation may be slower. If methanol inhibits ADH activity (Frenkel *et al.*, 1998) resulting in increased acetaldehyde concentration, enhanced seed deterioration may be expected (Zhang *et al.*, 1995b, 1997). Further research is needed to confirm the relationship of methanol accumulation to seed deterioration, either pre-harvest or post-harvest.

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