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ORIGINAL PAPER

Nutrient and hormone levels in cotton ovules during embryony

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Abstract In vitro zygotic and somatic embryogenesis protocols rely on nutrient and hormone levels from media to satisfy the physiological and developmental requirements of embryony. To better understand these requirements for cotton, we quantified levels of major and minor elements, carbohydrates, NH_4^+, free amino acids and six hormones in whole cotton ovules (with fibers removed), nucelli (ovules with integuments removed), or ovule fluid (extracted from the endosperm region). Samples were collected from field-grown cotton at 1–18 days-past-anthesis (DPA) during each of three growing seasons. Replication across 2 years was obtained for carbohydrates, NH_4^+, free amino acids and hormones from nucellus samples. The year effect was large primarily for hormones only. The most abundant minerals across tissue types and years were K, P, Mg and S. Potassium was the most abundant at 260, 600 and 1,660 mmol kg^{-1} dry mass (DM) in nucelli, whole ovules and ovule fluid, respectively. Magnesium, Ca, Zn and Mn levels were 2–8-fold higher in ovule fluid compared to whole ovules or nucelli. In the free amino acid plus NH_4^+ category, NH_4^+, alanine, serine, glycine, asparagine (plus aspartic acid), glutamine (plus glutamic acid), leucine, threonine and arginine predominated in nucelli and ovule fluid, and levels tended to be higher in the older samples across years and tissue types. Fructose and glucose levels also increased with age with very high levels being found in late DPA ovule fluid. Arabinose, inositol and melibiose were also prominent sugars. Indole-3-acetic acid levels were similar between nucelli and ovule fluid and ranged from 10 to 80 μmol kg^{-1} DM. An abscisic acid spike, from 15 to 400 μmol kg^{-1} DM, occurred in nucelli and whole ovules from 2 to 8 DPA. Thereafter, abscisic acid levels remained between 5 and 10 μmol kg^{-1} DM. Zeatin and zeatin riboside were the most abundant cytokinins, and levels of these hormones fluctuated between 1 and 4 μmol kg^{-1} DM in both nucelli and ovule fluid.

Keywords ELISA · Embryogenesis · Metabolites · Plant growth regulators · Seed fill

Abbreviations

Ala	Alanine
Ara	Arabinose
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Asx	Asparagine and aspartic acid
Cys	Cystine
DHZ	Dihydrozeatin
DHZR	Dihydrozeatin riboside
DPA	Days past anthesis
DM	Dry mass
ELISA	Enzyme linked immunosorbent assay
FM	Fresh mass
Fru	Fructose
Fu	Fucose
GA	Gibberellic acid

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Glu	Glutamic acid
Gluc	Glucose
Gln	Glutamine
Glx	Glutamine and glutamic acid
Gly	Glycine
His	Histidine
HPLC	High pressure liquid chromatography
IAA	Indole-3-acetic acid
Ino	Inositol
IP	Isopentenyl adenine
IPA	Isopentenyl adenosine
Ise	Isoleucine
Leu	Leucine
Lys	Lysine
Mel	Melibiose
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Suc	Sucrose
Thr	Threonine
Tyr	Tyrosine
Val	Valine
Z	Zeatin
ZR	Zeatin riboside

Introduction

Plant embryos develop in a chemical environment initially regulated by maternal tissues. The extent to which this environment is responsible for normal embryo development is not fully understood. An approach for elucidating important interactions between embryos and maternal tissues is to chemically define tissues and fluids adjacent to embryos, from anthesis through embryo differentiation, and to conduct embryo culture experiments that simulate the chemical and physical parameters of ovules (Carman 1990, 1995; Triplett 2000; Raghavan 2006).

The nutrient and hormone content of fluids that nourish cotton (*Gossypium hirsutum* L.) embryos during development (ovule fluid) have not been reported. However, some data for more generalized tissues have been published. For example, NO_3^- reductase activity was found to be high in whole cotton bolls, and this caused <5% of the N in cotton bolls to occur as NO_3^- (Radin and Sell 1975). Mauney et al. (1967) documented high levels of malic acid (52.2–74.6 mmol kg^{-1} DM) in cotton endosperm, and Tarczynski et al. (1992) determined that 90% of the total carbohydrate composition of cotton phloem sap, which flows into cotton ovules, is sucrose. Chen et al. (1996, 1997) quantified hormone levels of whole cotton ovules from a few days before anthesis to 8 DPA. Levels of free amino acids in cotton bolls, ovules, embryo sacs or phloem sap have not been reported.

The present paper is part of a larger project designed to characterize physical and chemical parameters of angiospermous and gymnospermous megagametophytes important to embryony in situ and to improve zygotic and somatic embryogenesis of plants in vitro. Herein we describe fluctuations in mineral nutrients, NH_4^+, free amino acids, carbohydrates and hormones that occur in whole cotton ovules (minus fibers), nucelli (whole ovules with integuments removed) or ovule fluids (fluid extracted from the endosperm region) from 1 to 2 DPA through early embryo maturation (16–18 DPA). We have used these results to develop tissue culture media that normalize development of early stage globular embryos in vitro.

Materials and methods

Plant materials and extraction procedures

Conventional dryland cotton production procedures were used to produce plants of the cotton cultivar HS-26. The plants were grown near Lubbock, Texas, in 1994, 1995 and 1996. Bolls were tagged at anthesis. In 1994, the bolls were collected at 2, 8 and 16 DPA (±2 DPA) from July 21 to August 29 and shipped overnight on ice to Utah State University, Logan. These collection dates roughly correspond to the zygote, early heart, and torpedo stages of embryo development, respectively. From 20 to 30 ovules were excised per boll. Using microdissection tools and dissecting microscopes, the integuments were removed from each ovule, and intact (turgid) nucelli were transferred to silanized 1.7 ml Eppendorf tubes. Nucelli were immediately frozen in liquid N_2 for storage. The 1995 bolls were collected at 2, 5, 8, 11, 14 and 17 DPA (±2 DPA) from August 8 to September 15 and sent overnight on ice to Logan. Nucelli were collected for the 2, 5 and 8 DPA samples as in 1994. Ovules from the 11, 14 and 17 DPA samples were cut in half horizontally and fluid, primarily from the endosperm region, was collected from each ovule using a 0.25 ml syringe (29 gauge needle) and frozen in liquid N_2. In 1996, bolls were harvested and dissected the same day in Lubbock, TX, from August 20 through August 31. The bolls ranged from 2 to 18 DPA. Samples from 2 to 7 DPA consisted of whole ovules with few fibers. Samples from 8 to 18 DPA consisted of ovules with the epidermal fibers removed. The samples were collected on ice in silanized 2 ml Eppendorf tubes, frozen in liquid N_2 and lyophilized to dryness. Dry matter values were recorded

prior to extraction and analysis. Lyophilized samples for hormone analyses were homogenized in 80% methanol supplemented with 8.0 mmol ammonium acetate l^{-1}, 2.6 mmol citric acid l^{-1}, and 0.05 mmol 2,6-di-tert-butyl-4-methylphenol l^{-1} (modified from Banowetz et al. 1994) using a Brinkmann Polytron homogenizer (Metrohm, Riverview, FL, USA). These samples were lyophilized again prior to analysis. For convenience, samples for mineral analyses were processed using the same procedures as for hormone analyses. These procedures resulted in powdered samples that could be accurately weighed prior to hormone or mineral analyses. Two to four biological replications were prepared for all sample categories except for the 1995 13–18 DPA mineral sample (ovule fluid) and the 1996 1–3 DPA mineral sample (whole ovules). The DM collected for these two samples was sufficient for a single mineral analysis only.

Mineral analyses

Homogenized and lyophilized samples for elemental analyses were digested using nitric and perchloric acids (Jones et al. 1991) and analyzed by inductively coupled plasma spectrometry using a Thermo Jarrell Ash Spectrometer (model ICAP 9000). NO_3^- was analyzed by the chromotropic acid method (Sims and Jackson 1971).

Carbohydrate analyses

High performance anion exchange chromatography with pulsed amperometric detection was used to quantify water soluble carbohydrates (Chatterton et al. 1989a). Briefly, lyophilized samples for carbohydrate analyses were ground in $AgNO_3$ (2.5 mmol) extraction buffer, to stop invertase activity (Chatterton et al. 1989b), and lyophilized again. Five microgram samples were then double extracted in boiling water. Samples were deionized by passage through Dowex-50 and Dowex-1 ion exchange resins. Hexoses were separated from short chain carbohydrates in 10 µl samples using anion exchange resin and an isocratic sodium hydroxide gradient (75–500 mmol). A Dionex Series 4000 ion exchange chromatograph was used for separation and pulsed amperometric detection, and the data were electronically captured and analyzed.

Free amino acid and NH_4^+ analyses

Lyophilized tissue samples (4–15 mg) were homogenized (Brinkmann Polytron) on ice in 5 ml tubes for 15–60 s at medium to high speed using a ratio of 15 mg sample to 1.0 ml diluted buffer (Beckman 7300/6300 high performance amino acid dilution buffer diluted 50% with deionized water), passed through a 0.2 µm filter, and collected in a second 5 ml tube. The homogenizer bit was rinsed once using diluted buffer (same volume for the rinsate as was used for the initial grinding), and the rinsate was passed through the 0.2 µm filter. The sample was then dried in a rotary evaporator and brought to volume using diluted buffer according to the following ratio: 15 mg original tissue sample to 1.0 ml diluted buffer. Vortexing and sonication were used to dissolve the pellet. Sample solutions were then frozen until analysis. Sample aliquots (50 µl) were injected in a Beckman 6300 High Performance Amino Acid Analyzer, and data were electronically captured and analyzed. These procedures did not discriminate between Gln and Glu (reported as Glx) or Asn and Asp (reported as Asx).

Hormone analyses

Hormones were extracted and purified as in Hess et al. (2002). Briefly, the homogenized and lyophilized samples were extracted in 80% methanol and spiked with DL-*cis, trans*-[G-^{3}H]ABA, specific activity of 4.25 TBq mmol^{-1} (TRK.644); 3-[5(n)-^{3}H]IAA, specific activity of 1.04 TBq mmol^{-1} (TRK.781) (Amersham, Arlington Height, IL); and ^{3}H-ZR, ^{3}H-DHZR and ^{3}H-IPA. We synthesized the latter cytokinins using nucleoside phosphorylase (12.3 units mg^{-1}, N8264, EC 2.4.2.1, Lot #31H0322, Sigma) to catalyze the transfer of ^{3}H-labeled ribose from [2,5′, 8-^{3}H]adenosine (specific activity of 2.07 TBq mmol^{-1} [TRK.609], Amersham) to the cytokinin free bases Z, DHZ, and IP (Sigma). Extracted hormones were purified by C_{18} octadecyl silica solid phase chromatography and ODS C18, 250 × 4.6 mm, 5 µm column (Alltech Associates, Inc) HPLC. Fractions (0.5 min) for HPLC were collected, and a 20 µl aliquot was taken from each HPLC fraction for scintillation counting. Fractions containing tritiated hormone were pooled, and fractions containing the cytokinins Z, DHZ and IP, which did not have corresponding tritiated tracers, were located by reference to the respective cytokinin riboside tritiated tracers and pooled. Collected and pooled hormones were lyophilized to dryness. Sample and standard IAA were methylated at the carboxyl site using diazomethane as in Cohen (1984).

Hormone levels were quantified by non-competitive indirect ELISA (Hess et al. 2002). In brief, tissue culture grade hormones were HPLC-purified prior to use as standards, and the following antibodies were used: +ABA monoclonal (Banowetz et al. 1994), methylated IAA monoclonal (provided by John Caruso, University of Cincinnati, OH; prepared as in Leverone et al. 1991), anti-*trans*-ZR#3 monoclonal (Trione et al. 1985), anti-DHZR#4 monoclonal (Banowetz et al. 1997), and anti-IPA#3 monoclonal (Trione et al. 1987). Hormone levels were corrected for losses by determining recovery of

^{3}H-hormone prior to conducting the ELISA. Twelve aliquots were analyzed from each biological replication, and aliquot values falling within the appropriate standard curves were averaged to yield a single value for each biological replication. Means and standard errors were calculated for each DPA category.

Results and discussion

The goal of this project was to identify levels of minerals, NH_4^+, free amino acids, carbohydrates and hormones in cotton ovules and in the fluid adjacent to developing cotton embryos for the purpose of refining media for in vitro cotton embryogenesis. Three tissue types were collected: whole cotton ovules (least likely to represent embryo nourishing fluids), turgid nucelli (more likely to represent such fluids), and ovule fluid (most likely to represent such fluids). Amounts of tissue collected were not sufficient to conduct analyses on all tissue types and all collection dates for each year. In this respect, only the 2 and 8 DPA nucellus samples for 1994 and 1995, collected for NH_4^+, amino acids, sugars and hormones, were comparable for determining a year effect on nutrient and hormone levels. Values obtained for the remaining sample types and or DPA categories represent a single year of data only. While these limitations prevent strict comparisons across tissue types and years for most samples, the overall goal of identifying ranges of nutrient and hormone levels appropriate for testing with regard to cotton embryo growth and development in vitro was accomplished. Nutrient and hormone levels that remain constant across the environments sampled in the present study may reflect a physiological regulation of such nutrient and hormone levels appropriate for normal embryo development.

The cotton plants used in this study were grown near Lubbock, TX, during three consecutive years. Rainfall near Lubbock differed substantially across years (Table 1). Notably, the 1995 growing season was dry, and many cotton bolls aborted, while the 1996 season was exceptionally wet. These differences also affected timing of flowering. In 1994 and 1995, cotton bolls were collected over extended periods (see "Materials and methods"), but in 1996 they were collected over a brief period. Given these additional environmental differences, we suspect that nutrient and hormone level trends observed to be similar across years and tissue types may reflect underlying metabolic processes that maintain nutrient and hormone levels within ranges appropriate for cotton embryony. Such trends are identified and discussed below.

Minerals

Regardless of year or sampling date, >95% of the total molar concentration of minerals in the three types of plant samples (whole ovules, excised nucelli and ovule fluid)

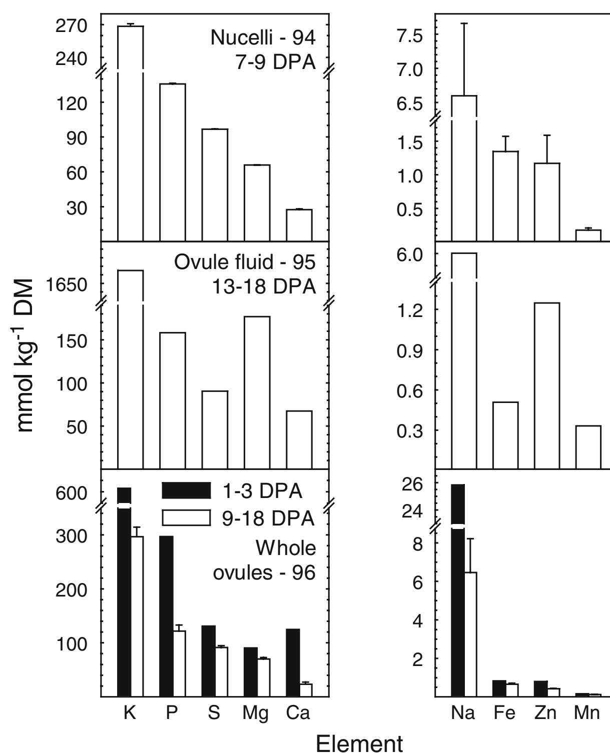

Fig. 1 Elements in cotton nucelli, ovule fluid and whole ovules. Values are means ± SE

Table 1 Average maximum and minimum temperatures and rain fall near the location where the cotton plants for this project were grown (U.S. weather station data for Levelland, TX)

Days	Temperature max (°C)			Temperature min (°C)			Rain fall (cm)		
	1994	1995	1996	1994	1995	1996	1994	1995	1996
Jun (all)	37.1	33.4	34.1	17.6	15.5	17.1	3.3	3.8	9.9
Jul (all)	36.2	36.9	33.8	18.0	18.1	18.4	3.8	0.3	4.7
Aug (1–14)	33.3	33.7	34.4	16.7	17.3	17.9	0.5	0.6	6.0
Aug (15–27)	34.2	33.8	30.9	17.2	18.1	17.6	0.6	4.3	1.2

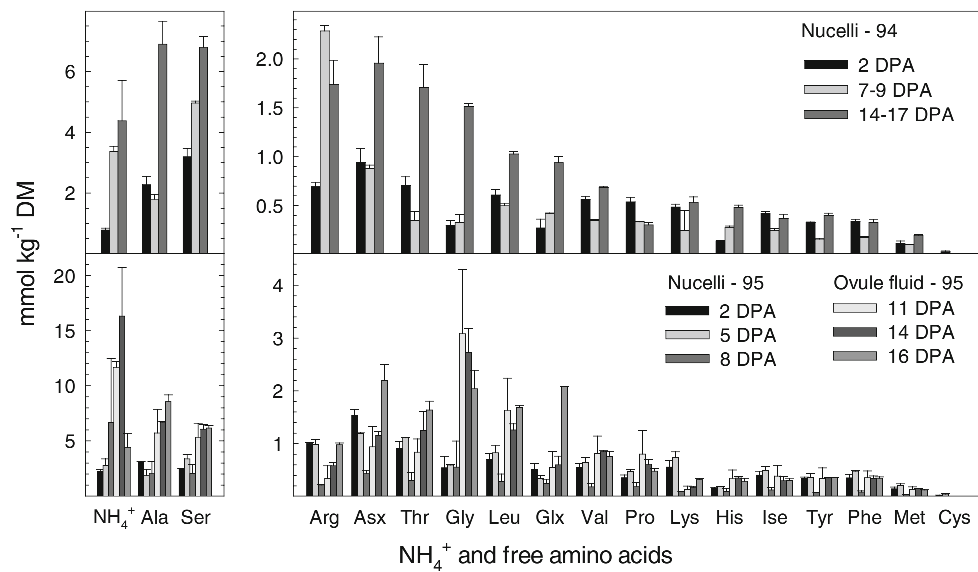

Fig. 2 Free amino acids and NH_4^+ in cotton nucelli and ovule fluid. Values are means ± SE

consisted of K, P, Mg, S and Ca (Fig. 1). Likewise, Hocking and Pate (1977) found that K, Ca and Mg are the three most abundant mineral cations in embryo sac liquid of various legumes. Levels of Cl were not determined in our study, and levels of essential elements not shown were below detectable limits. Levels of Mg and Zn in ovule fluid were disproportionately high relative to other minerals when compared to Mg and Zn in nucelli or whole ovules (Fig. 1). Also, ovule fluid levels of K, Mg, Ca, Zn and Mn were >2-fold higher than in similarly staged nucelli and whole ovules (Fig. 1). Ovule fluid levels of NH_4^+, amino acids and sugars did not show these multi-fold difference (Figs. 2, 3; discussed below), which suggests that the >2-fold higher concentrations of certain mineral elements

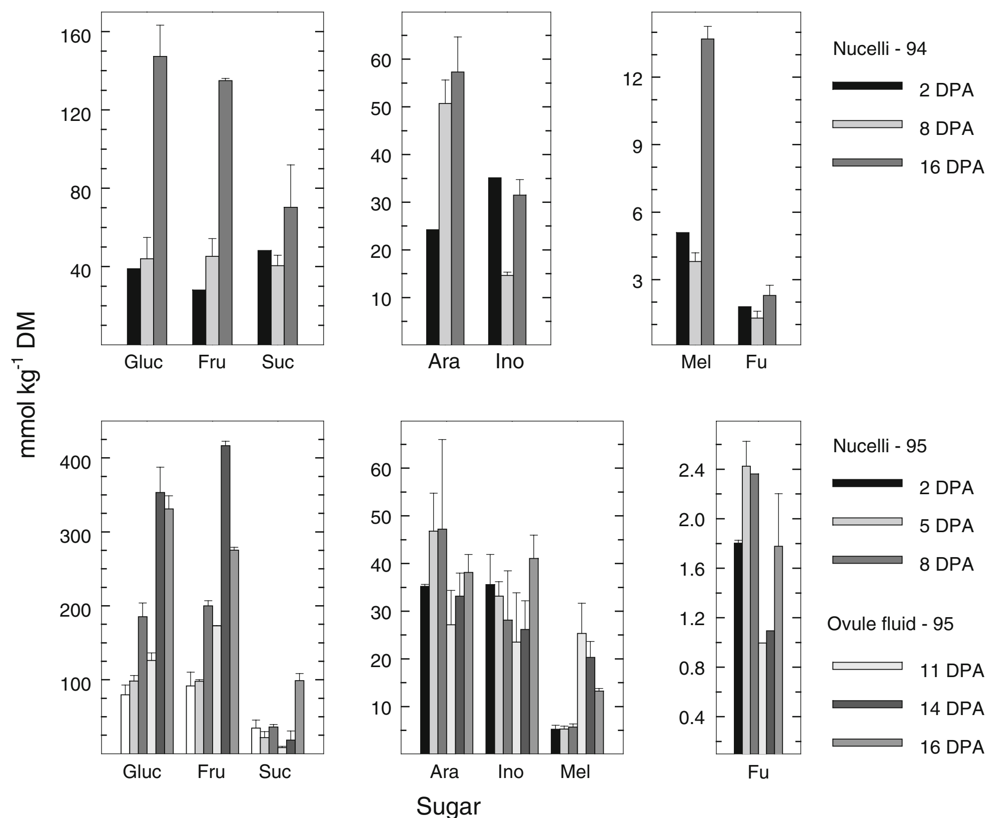

Fig. 3 Carbohydrates in cotton nucelli and ovule fluid. Values are means ± SE

in ovule fluid compared to nucelli or whole ovules may be a characteristic of the ovule fluid near developing embryos rather than an anomaly caused by environmental variables.

The ovule fluid samples studied herein were derived primarily from endosperm, which supplies embryos with nutrients and hormones for growth and development. Similar elemental analyses have been obtained for ovule fluids collected throughout wheat embryony (Carman et al. 1996), a monocot, and for megagametophyte corrosion cavity fluids throughout embryony of the gymnosperm Douglas fir (Carman et al. 2005). Mineral levels in cotton ovule fluid largely paralleled those obtained for wheat ovule fluid. However, in fluids obtained from Douglas fir megagametophytes (corrosion cavity fluid), Zn and Fe levels were several fold higher and Mn levels were nearly 10-fold higher compared to the cotton ovule fluids reported herein. These differences suggest that mineral nutrition regimes necessary for normal development of cultured zygotic or somatic embryos of gymnosperms may differ substantially from those required for normal development of cultured embryos of angiosperms.

Free amino acids, NH_4^+ and NO_3^-

Concentrations for several of the more prominent free amino acids, e.g. Ala, Ser, Asx, Thr, and Gly, increased from 2-fold to several-fold between 2 and 17 DPA (Fig. 2, 1994 nucelli), and the concentration of all amino acids combined doubled between 8 and 17 DPA (early heart to early torpedo stage embryos) (Fig. 2). A similar trend was observed in 1995 between early-staged nucelli and later-staged ovule fluids. Levels of NH_4^+, Gly, Leu and Glx tended to be higher in ovule fluids (14 and 16 DPA samples) than in other samples from similar DPA categories (e.g. Fig. 2, 14–17 DPA nucelli). Similar trends have been reported in other plants, e.g. amino acid levels in phloem exudate from barley were found to be distinctly higher than in the cytosol (Winter et al. 1992). However, concentration values for most amino acids were similar among tissues for similar DPA categories. In most samples, >70% of the free amino acid component consisted of Ser, Glx, Ala, Asx, Leu and NH_4^+. In wheat (Fisher and Macnicol 1986), tobacco (Hocking 1980), barley (Winter et al. 1992), spinach (Riens et al. 1991) and *Pisum sativum* (Lewis and Pate 1973), the same group of five amino acids (Ser, Glx, Ala, Asx and Leu) plus NH_4^+ comprised 57, 64, 92, 88 and 77% of the total free amino acid plus NH_4^+ component of phloem sap, respectively. Carman et al. (1996) also found similarly high levels of amino acids in the ovule fluid of immature wheat kernels, with Ser and Ala also being the most abundant. Interestingly, free amino acid levels in corrosion cavity fluids of Douglas fir (Carman et al. 2005) tended to be 10–50-fold higher than those observed herein for cotton. This again emphasizes the potentially large difference in nutrient requirements of Douglas fir embryos (and possibly embryos of other gymnosperms, see Carman et al. 2005) compared to nutrient requirements of angiospermous embryos.

The 1996, whole ovule samples were also tested for NO_3^-. At 0–3, 4–7, 8–11, 12–15 and 16–18 DPA, NO_3^- levels were 2.1, 4.3, 9.1, 4.9 and 4.6 mmol kg^{-1} DM, respectively, which indicates that NO_3^- may also be an important nutrient component for embryony compared with other N sources in ovules (Fig. 2).

Levels of amino acids in ovule fluids depend on phloem unloading in nucellar cells, facilitated transfer from nucellar cells into fluids of the endosperm cavity, facilitated uptake into endosperm cells, and release of the temporarily stored amino acids (and other nutrients) from endosperm cells upon their degradation adjacent to developing embryos (Donovan et al. 1983; Murray 1988; Carman 1995; Carman et al. 1996). Hence levels of free amino acids and other nutrients in nucellus, integument and even endosperm cells may not reflect nutrient availability at the embryo surface.

Carbohydrates

Sucrose, Gluc, Fru and Ara were the major carbohydrates observed in nucelli and ovule fluid (Fig. 3). Concentrations of Gluc and Fru in the 1994 nucelli, like many amino acids (Fig. 2), increased with boll age (Fig. 3, 2–16 DPA). Likewise, levels of these sugars in ovule fluid were particularly high as ovules and embryos matured (Fig. 3, 11–16 DPA). Arabinose levels in the 1994 nucelli also increased with time (Fig 3, 2–16 DPA). Inositol and Mel were also important constituents. Inositol levels remained fairly constant across time and tissue types, but Mel levels, like those of the other sugars, increased in the 1994 nucelli 2–3-fold by 16 DPA (Fig. 3, 2–16 DPA). Interestingly, Mel levels decreased in ovule fluid during the later phase of ovule growth (Fig. 3, 11–16 DPA), a tendency that was also observed for Fru. Fucose was also detected, but levels of this sugar were relatively low in all samples (Fig. 3).

The prominence of sucrose and sucrose equivalents (fructose and glucose) has also been observed in ovule fluids of *Phaseolus vulgaris* (Smith 1973), *Pisum sativum* (Murray 1988) and wheat (Carman et al. 1996; Carman and Bishop 2004) and in corrosion cavity fluids of Douglas fir (Carman et al. 2005). However, the molar ratio of amino acids to water soluble carbohydrates in Douglas fir corrosion cavities was much higher than in fluids from wheat and cotton ovules. This suggests that amino acids may play a major nutritional role during Douglas fir embryony, and possibly during embryony of other gymnosperms (Carman et al. 2005), compared to angiosperms. The low molar ratios of amino acids to water soluble carbohydrates, as

seen in fluids from cotton and wheat ovules, have also been observed in barley phloem sap (Winter et al. 1992).

Hormones

Hormone levels were determined for nucelli (1994, 1995), ovule fluid (1995), and whole ovules minus fibers (1996). Sufficient quantities of ovule fluid for ELISA analyses were obtained only from the 11, 14 and 16 DPA samples. Because these fluids were derived primarily from the embryo sac, we suspect they represent the most direct measurement of hormone levels to which cotton embryos are exposed during embryony. Following this line of reasoning, isolated nucelli, which contained ovule fluid plus cells of the nucellus, endosperm and embryo, would provide a less direct measurement of hormone levels near embryos. Finally, whole ovules, which contained nucelli plus integument cells would provide the least direct measure of hormone levels near embryos.

A spike in ABA level was detected shortly after anthesis in each year (Figs. 4, 5). This time period corresponds with boll abortion, which occurs to a greater or lesser extent each year depending on weather conditions. As boll abortion occurs over several days, it is possible that some bolls selected for analysis during the 1–7 DPA period were, without visual indication, undergoing the abortive process. Levels of ABA in such bolls may be excessive compared to healthy bolls. In this respect, levels of ABA remained relatively low in nucelli and ovule fluids after the initial ABA spike (Figs. 4, 5).

The ABA spike in 1995 nucelli was twice as high as the spike observed in 1994 nucelli (Fig. 4). This may reflect a drought-induced increase in ABA during the 1995 season. In 1994 and 1995, 7.6 and 4.7 cm of rain, respectively, were reported from June 1 to August 14 near the Lubbock, TX, production field (Table 1). Nan et al. (2002) observed a 4-fold increase in ABA levels in wheat leaves within 48 h of applying a water deficient treatment, which indicates ABA levels can fluctuate rapidly in response to drought. However, in 1996, 20.6 cm of rain fell during the same 75 days time interval (Table 1), and the ABA spike (whole ovules) was 10-fold higher than in 1995 (Figs. 4, 5). The nucellus samples of 1994 and 1995 differed from the whole ovule samples of 1996 only by removal of the integuments from the ovules in 1994 and 1995. There is no precedent for integuments containing 10-fold or higher levels of ABA than adjoining nucellus tissues. Hence, the large spike in ABA observed in 1996 was probably induced by stresses associated with either the unusually high rainfall or possibly by a lack of rainfall prior to sampling. In this respect, rainfall in 1996 was minimal during the 2 weeks prior to sampling (later half of August, 1996; Table 1).

Guinn et al. (1990) reported that water stress increases cotton boll ABA levels, but not IAA levels, and that increased ABA levels are closely associated with boll abscission. Altman (1988) found that gibberellic acid applications to floral bracts of cotton shortly after pollination prevents boll abscission in wide crosses, possibly by countering the effects of ABA. In this respect, Chen et al. (1996) observed high levels of gibberellic acid in whole

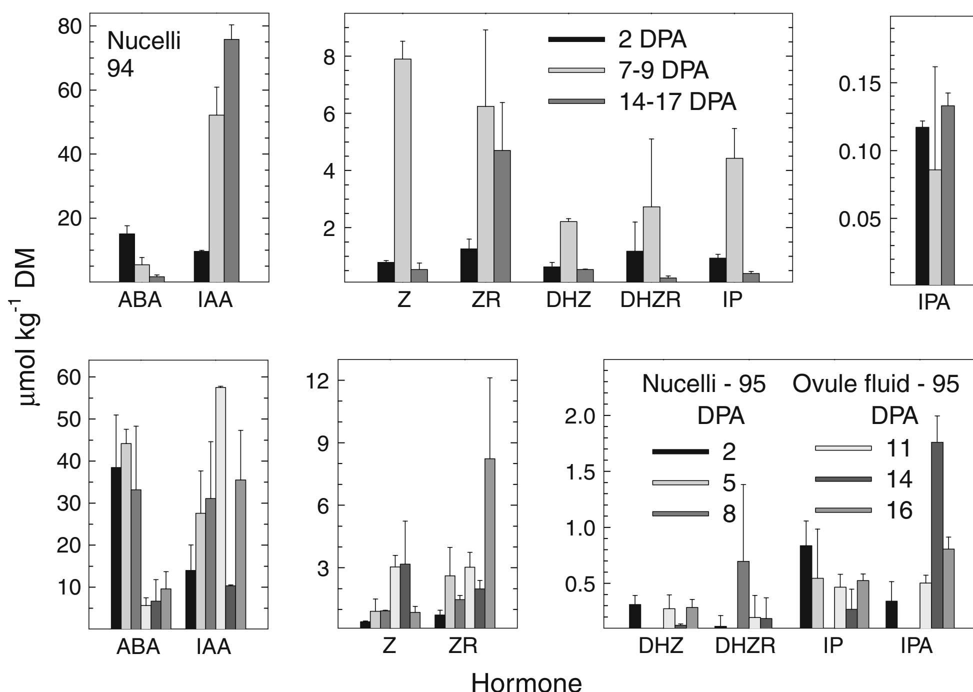

Fig. 4 Hormones in cotton nucelli and ovule fluid. Values are means ± SE

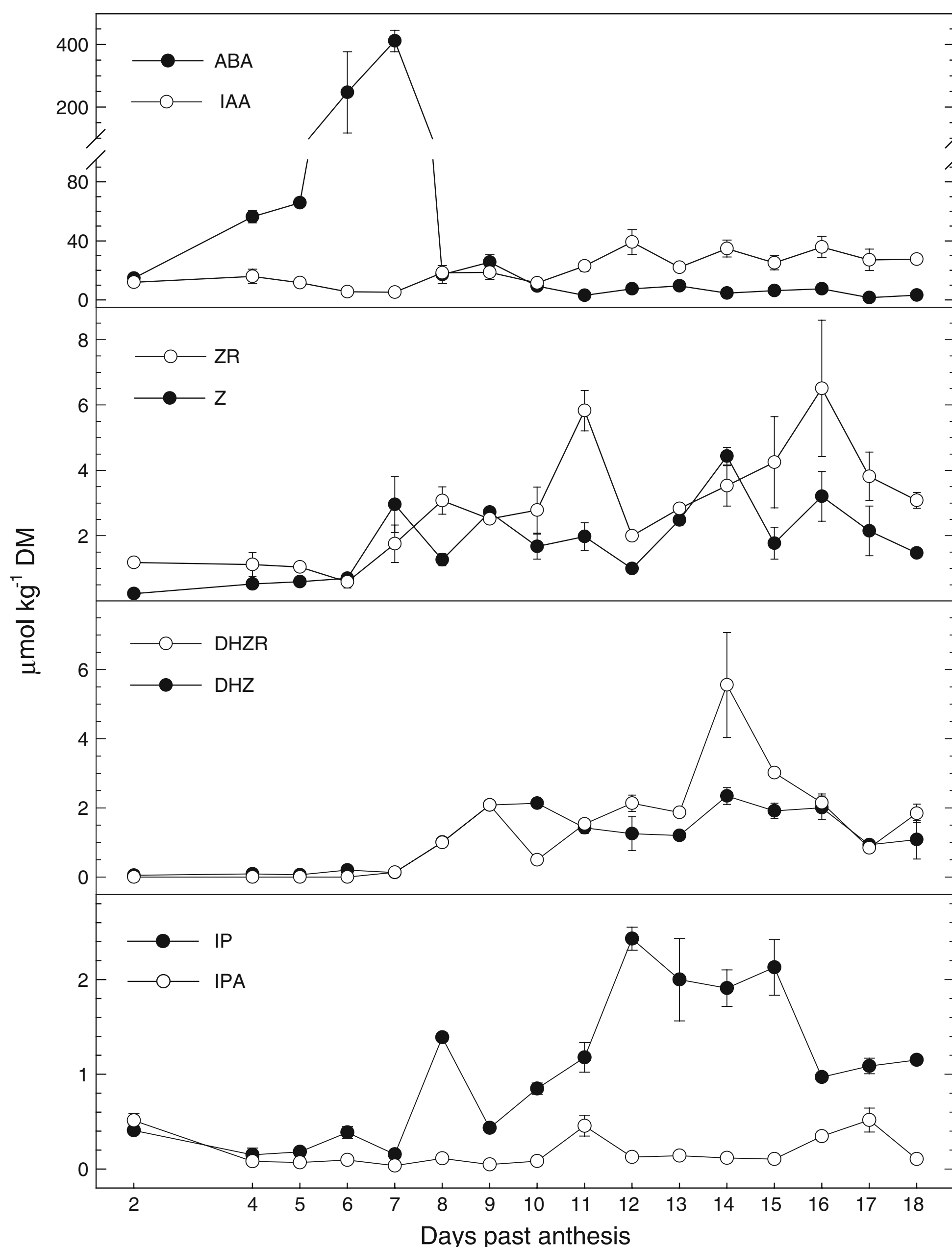

Fig. 5 Hormones in whole ovules (1996). Values are means ± SE

cotton ovules at 1 DPA. Hence, endogenous gibberellic acid in cotton bolls shortly after pollination may counteract the effects of ABA in preventing boll excision under low to moderate stress conditions. The ABA spike in cotton ovules studied by Chen et al. (1996) occurred at 2 DPA (10.0 µmol kg^{-1} FM), i.e. shortly after the GA spike. Levels of ABA then decreased to 0.09 µmol kg^{-1} FM by 8 DPA. An ABA spike was also observed in corrosion cavity fluids of Douglas fir seeds obtained from field nurseries early in embryony (Carman et al. 2005) but not in wheat ovules grown under ideal greenhouse conditions (Hess et al. 2002).

Levels of IAA increased over time in the 1994 nucelli ranging from 10 µmol kg^{-1} DM at 2 DPA to between 40 and 80 µmol kg^{-1} DM at 16 DPA (Fig. 4). A similar trend was observed for whole ovules (Fig. 5). Chen et al. (1996) observed a peak in IAA levels (4.0 µmol kg^{-1} FM) in cotton ovules at 2 days prior to anthesis. However, they did not sample ovules after 8 DPA. Levels of IAA in wheat ovule fluids (Hess et al. 2002) also accumulated throughout active embryo growth and remained within a similar range as observed for cotton nucelli and whole ovules. In the 1996 data set, IAA levels in nucelli decreased as ABA levels increased (5–7 DPA, Fig. 5). This tendency was not

observed in nucelli from 1995 (Fig. 4). Similarly, short durations of drought increased levels of ABA in wheat leaves but not levels of IAA (Nan et al. 2002).

The two most abundant cytokinins found in whole ovules, nucelli and ovule fluid were Z and ZR (Figs. 4, 5). In whole ovules (Fig. 5), levels of these cytokinins tended to increase initially through 8–16 DPA and then decrease by 18 DPA. Similar patterns were observed for other cytokinins in the 1994 nucelli (Fig. 4). In general, cytokinin levels in cotton ovules paralleled those in wheat ovules (Hess et al. 2002) and Douglas fir corrosion cavities (Carman et al. 2005) with peaks occurring during embryo histodifferentiation followed by decreases in cytokinin levels. As high cytokinin levels coincide with high mitotic indices (Rock and Quatrano 1995), it is likely that the decline in Z levels observed at 16–18 DPA (Fig. 5) reflects a decrease in the mitotic index of maturing cotton embryos. Chen et al. (1997) also studied cytokinin levels in cotton ovules shortly before and after flowering. In their study, IPA levels peaked at 1 day prior to anthesis and DHZR and ZR peaked at 1 and 8 DPA, respectively.

Implications for in vitro culture systems

Large discrepancies exist between the nutrient levels we identified in cotton ovules and the nutrient levels of plant tissue culture media used to culture cotton zygotic and somatic embryos. According to our findings, levels of K, P, Ca, Mg and S are much higher in cotton ovule fluids than in conventional tissue culture media, e.g. Murashige and Skoog (1962) medium, and NO_3^{-1} levels are much lower. The primary source of N in cotton ovule fluid is organic (NH_4^+ and free amino acids), and this contrasts sharply with conventional tissue culture media where the primary source of nitrogen is NO_3^-. Other major differences involve carbohydrate requirements, which are met in conventional plant tissue culture media primarily by sucrose, but in cotton ovules, glucose, fructose, arabinose and melibiose may also be important to embryony. Likewise, endogenous IAA levels in ovule fluid were 2–3 times higher than levels used in conventional cotton embryo culture media (Triplett 2000). Types and levels of cytokinins also differ with a mixture of cytokinins occurring with abrupt peaks in vivo but often only one cytokinin being used in vitro. Based on the nutrient and hormone levels reported herein, we have developed cotton embryo culture media that simulate those observed in ovules and have compared, through a series of embryo culture experiments, our simulation media to conventional media. In these experiments, simulation media were identified that are superior to conventional media in terms of supporting rapid growth and development in vitro of pre-heart-staged (globular) embryos.

As levels of amino acids, minerals, carbohydrates and hormones in tissue culture media are made to coincide with those that occur in vitro, many of the abnormalities and problems currently encountered with zygotic and somatic embryo culture will likely diminish. There remain many in vivo parameters to study that may improve cotton embryo culture. These include inorganic acids, other hormones, vitamins, small proteins, and organic minerals such as phytate. By simulating such parameters, media and procedures that normalize embryo development, in vitro, from globular staged embryos and possibly zygotes should be obtainable.

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