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Embryo Development in *Phaseolus vulgaris*

II. ANALYSIS OF SELECTED INORGANIC IONS, AMMONIA, ORGANIC ACIDS, AMINO ACIDS, AND SUGARS IN THE ENDOSPERM LIQUID[1,2]

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ABSTRACT

Endosperm liquid bathes the embryo of *Phaseolus vulgaris* from the heart stage through the late cotyledon stage. This liquid was aspirated from many ovules of the same stage, pooled, and analyzed for the following constituents and parameters:

1. Osmolarity and pH, using freezing point depression and Hydrion paper.

2. The elements Ca, K, P, and Mg, using the electron microprobe, atomic absorption spectrophotometry, and the Bartlett modification of the Fiske-SubbaRow P determination method.

3. NH_4^+, using the Conway diffusion procedure.

4. Organic acids, using paper and two directional thin layer chromatography for qualitative measures, and the enzymatic oxidation and reduction of NADH for quantitative measures of citrate and malate.

5. Amino acids, using two directional thin layer chromatography and an amino acid analyzer.

6. Sugar, using thin layer chromatography, the Somogyi-Nelson method, with and without prior hydrolysis, and the Glucostat reagent.

These analyses revealed the following:

1. Osmolarity decreased from 0.7 in the heart stage to 0.5 in the late cotyledon stage and pH remained at 5.2 to 5.4 throughout.

2. Ca was not detected, at 0.5 mM sensitivity, K decreased from 34.4 mM to 20.8 mM, P decreased from 5.3 mM to 3.2 mM, and Mg decreased from 4.1 mM to 2.5 mM.

3. NH_4^+ decreased from 110 mM in the early cotyledon stage to 80 mM in the late cotyledon stage.

4. A pool of five organic acids was dominated by malate, which decreased from 23 mM in the early cotyledon stage to 17 mM in the late cotyledon stage, and citrate, which remained at about 5 mM.

5. At least 18 amino acids were present for a total concentration of about 42 mM, including 3 nonprotein amino acids.

6. Most of the sugar was in the form of sucrose, glucose, and fructose. Sucrose increased, from 20 mM in the early cotyledon stage to 60 mM in the late cotyledon stage, whereas the total reducing sugar remained approximately constant. These results are discussed in relation to the culture of bean embryos *in vitro*. Comparisons are made with the compositions of other media which have been used for plant embryo or tissue culture.

The ovule of the snap bean, *Phaseolus vulgaris* L., is filled with liquid during much of the development of the embryo, *i.e.*, from the end of the globular stage to the end of the late cotyledon stage (19). This liquid is contained within a thin sack of endosperm cytoplasm and bathes the embryo, separated from it by only a thin layer of cellular endosperm. Bean embryos cultured *in vitro* should grow and develop best in a culture environment most nearly like that *in vivo*. Thus, analyses of this liquid were made for several stages, *i.e.*, the heart, early cotyledon, and later cotyledon stages. This paper reports the analyses of K, Ca, Mg, P, and NH_4^+ ions, organic and amino acids, and sugars, which together comprise about 76% of the solutes in the late cotyledon stage on an osmolar basis. It does not cover analyses of protein, hormones, sugar alcohols, and an unknown acid component. Somewhat similar analyses have been reported for the endosperm liquids of coconut (22) and cotton (10).

MATERIALS AND METHODS

Fresh beans of *Phaseolus vulgaris* L. cv. "Black Valentine" and "Topcrop" were obtained from plants grown in flats in the greenhouse throughout the year. The mean day temperature of the greenhouse was 26 C, and the mean night temperature was 22 C. Additional material was grown in field plots and the Matthaei Botanical Gardens during the summer or purchased from local stores. Materials purchased commercially were used only for the verification of ionic composition and for the preliminary investigation of organic acid content.

Osmolarity and pH. Osmolarity was determined by the freezing point depression method of Prosser (14). Endosperm liquid from globular stage, heart stage, early cotyledon stage, and late cotyledon stage ovules was drawn into melting point capillary tubes. These were frozen in a Dry Ice-salt water slurry and visualized with polarized light, as were NaCl standards. Time to melting was used to compute osmolarity. Three different sets of samples were used, and four determinations were run on each set.

Because the volume of endosperm liquid was very small,

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p-Hydrion paper (range 3.0–5.5) was used by placing drops of the freshly aspirated liquid onto the paper. The determined color was several shades from the end point color, and accuracy was about 0.2 pH units. Liquid was measured from several pods at each developmental stage. Measurements were taken on sets of samples collected at three different times including field-grown and greenhouse material.

Ca, K, Mg, and P Concentrations. The *relative* amounts of Ca, K, Mg, and P were measured with an electron microprobe (Model EMX-SM Applied Research Labs, operated at 20 kev, with a sample current of 0.15 μamp). For these measurements samples of liquid endosperm from heart stage ovules and from late cotyledon stage ovules were ashed to provide a homogeneous, anhydrous sample. Ashing was accomplished by heating in a covered crucible. The ash was compressed tightly into shallow holes (1 $\times$ 3 mm) in a polished graphite block. Another hole was filled with a pulverized standard mixture of sucrose, KH_2PO_4, KNO_3, and $Mg(NO_3)_2 \cdot 6H_2O$ such that the mole fractions of K, Mg, and P were 0.033, 0.030, and 0.008 respectively. Another hole was filled with pulverized $Ca(NO_3)_2$. A series of 10 point counts at 10 locations, in each sample and in the standards, was taken for Ca, K, Mg, and P. The mole fraction of each element in the samples was calculated by proportionality with the mole fraction and average point count of that element in the standards. The above procedure was employed for two sets of samples. The sensitivity limit was 3×10^{-5} mole fraction, and the standard error of the point counts averaged 4.66%.

The absolute concentrations of Ca and Mg were measured with an atomic absorption spectrophotometer (Jarrell-Ash Model 82-516). Samples were prepared by refluxing 0.31 ml of endosperm liquid from early cotyledon stage and late cotyledon stage ovules in 2 ml of H_2SO_4 for 50 hr. Each sample was then diluted to 5 ml in 1% lanthanum chloride in double glass distilled water, and this solution was run in the instrument. Standards of $MgSO_4$ and $Ca(NO_3)_2$ were made up in 40% sulfuric acid and 1% lanthanum chloride at concentration ranges of 2×10^{-5} to 2×10^{-2} M and 4×10^{-7} to 4×10^{-2} M respectively. The lanthanum ion prevents phosphorus interference with the calcium readings.

Phorphous was measured as P_i with the Bartlett modification of the Fiske-SubbaRow method (2). Bean endosperm liquid was used directly for P_i determination, compared to a concentration series (0.25–2 μM) of Na_2HPO_4, and two sets of samples were tested. Although there was some variation in the standard curves, the determinations from them were consistent from one experiment to another.

Ammonium Ion and Amino Acids. Ammonium ion was measured directly in the liquid endosperm using the Conway diffusion procedure (4). Two sets of samples were analyzed with three replicates each.

Amino acids were assayed with two directional chromatography on thin layer plates of Silica Gel G (Warner-Chilcott Labs) on glass (21). Spots of 15 μl of endosperm liquid were chromatographed alone or with added known amino acids. The solvents used were phenol-water (80:20) v/v for the first direction and ethyl alcohol-acetic water-water (90:10:25) v/v/v for the second direction (21). Seventy plates were run in various combinations and repetitions. A quantitative measure of the amino acids, as well as a check on the qualitative measures above, was obtained from a single sample assayed in an amino acid analyzer.

Organic Acids. Citric acid cycle acids were analyzed qualitatively by TLC[5] and by descending paper chromatography in a solvent of n-butanol-water-formic acid (60:50:1) v/v/v (21). Cellulose thin layer plates of two sets of samples (MN 300, Brinkman Instruments) were developed in ether-formic acid-water (7:2:1) v/v/v in the first direction and phenol-water-formic acid (75:25:1) v/v/v (11) in the second direction. After drying at 100 C, the plates were sprayed with bromophenol blue (pH 6.5) to reveal the acids.

The two predominant acids, citrate and malate, were measured quantitatively. Citrate was measured enzymatically with citrate lyase and NADH oxidation according to the method of Williamson and Corkey (23). Their method was also used to measure malate with malate dehydrogenase and NAD reduction. Absorbance was measured at 340 nm with a Zeiss spectrophotometer, PMQII. Samples were prepared for enzymatic analysis by diluting the endosperm liquid immediately upon collection from freshly harvested pods with distilled water to one hundredth of the original concentration. They were then immediately frozen until analyzed. Analyses were made of three replicates for each stage of embryo development for citrate measurement and four each for malate.

Sugars. Reducing sugars were evaluated quantitatively with the Somogyi-Nelson test (12, 20) and with Glucostat reagent (method II, Worthington Biochemical Corp.). Endosperm liquid from freshly harvested pods was used directly for these measurements. For measurement of total sugar, samples were digested, using HCl hydrolysis, prior to application of the Somogyi-Nelson test. Three separate samples were analyzed for each stage with all of the above methods. Thin layer plates of Kieselguhr, developed in acetone-ethyl acetate-water (20:20:3) v/v/v, were utilized for a qualitative assay of endogenous free sugars. Color identification was achieved with 1% naphthoresorcinol in ethyl alcohol (1). Assay samples were spotted on the plates, in 1- to 5-μl spots, as the liquid endosperm was collected from the bean ovules.

RESULTS

Osmolarity and pH. The osmolarity of the bean endosperm liquid was 0.7 osmolar in heart stage and 0.5 osmolar in late cotyledon stage. The 0.7 osmolar value is more than double the osmolarity of any medium reported for the *in vitro* culture of plant embryos. The pH was found to be constant, between 5.2 and 5.4 in all of the stages examined. This value is consistent with those reported for plant tissue or embryo culture media.

Inorganic Components. Ca, K, P, and Mg were measured in the bean endosperm liquid at early cotyledon stage and at late cotyledon stage. The relative contribution, or mole fraction, of each was measured with the electron microprobe, as described above. The results of these determinations are presented in Table I. No Ca was detected at a lower limit of detection of 0.00003 mole fraction. The mole fraction for K was 0.0143, for P, 0.0022, and for Mg, 0.0017. To quantify this ratio, the P was measured, using the Bartlett modification of the Fiske-SubbaRow method; 55 μl of late cotyledon stage fluid was determined to have 175 nmoles of P_i or a concentration of 3.2 mM. For late heart stage endosperm liquid, 15 μl contained 80 nmoles of P_i, or a concentration of 5.3 mM.

These concentrations of P were combined with the ratio from the electron microprobe to calculate the approximate concentrations of K and Mg. For K this was 34.4 mM for heart stage endosperm liquid and 28 mM for late cotyledon stage endosperm liquid. For Mg the heart stage concentration was 4.1 mM, and the late cotyledon stage concentration was 2.5 mM. These calculated concentrations of Mg, as well as the lack of detectable Ca, were checked by means of atomic absorption flame spectrophotometry. By this method Ca was

[5] Abbreviation: TLC: thin layer chromatography.

Table I. *Ca, K, Mg, and P Concentrations in Bean Liquid Endosperm*

Stage	Element	Concentration		
		From the microprobe[1]	Bartlett modification of Fiske-SubbaRow method	Atomic absorption spectrophotometry
			mM	
Heart	Ca	ND[2]		
	K	34.4		
	Mg	4.1		
	P	5.3	5.3	
Early cotyledon	Ca			ND[3]
	Mg			5.4
Late cotyledon	Ca	ND		ND
	K	20.8		
	Mg	2.5		
	P	3.2	3.2	

[1] The concentrations listed for the elements from the electron microprobe analysis were calculated by taking the ratio of the elements given by the microprobe and setting the phosphorus concentration equal to that determined by the Fiske-SubbaRow method.

[2] ND: not detectable.

[3] Not detectable at 0.5 mM.

Table II. *Ammonia Content of the Endosperm Liquid in the Early and Late Cotyledon Stages*

Sample	Sample Size	NH_4^+ in Sample	Concn in Endosperm NH_4^+ (calculated)
	µl	*µmoles*	*M*
Early cotyledon	15	2.0	0.13
	25	2.3	0.09
Late cotyledon	25	2.0	0.08
	25	2.1	0.08

not detectable at a level, in the endosperm liquid, of 0.5 mM (the instrument limit was 0.04 mM, but the sample was diluted). The Mg concentration in young cotyledon stage endosperm liquid was 5.4 mM. It is clear that this figure corresponds closely with that calculated for Mg from the electron microprobe.

The results of the measurements of NH_4^+ concentrations are presented in Table II. The average concentration of NH_4^+ in early cotyledon stage endosperm liquid was 110 mM; in late cotyledon stage endosperm liquid it was 80 mM. This compares with a concentration of about 1 mM in Norstog's medium J (13), 20 mM in Linsmaier and Skoog's revised medium (8), and 30 mM in Mauney's medium for cotton embryos (10).

Organic Nutrients. Qualitative determinations of the organic acids in heart stage to young cotyledon stage endosperm liquid (pooled together) indicated five spots. The two spots which were nearest the front had R_F values similar to those for fumarate and succinate, respectively. The next spot, which was by far the largest, was positioned near the spots for citrate and malate. Although the R_F of this spot more precisely matched the R_F of citrate, it may have included malate which did not separate due to overload drag.

That possibility was verified with two-dimensional TLC on cellulose. The volume of bean endosperm liquid which was chromatographed was reduced from 5 µl to 2 µl. This reduction cause the succinate and fumarate spots to disappear, but the two spots visualized coincide in R_F values in both dimensions with those for the authentic spots of malate and citrate.

Enzymatic procedures yield quantitative determinations as well as verification of qualitative measures of the organic acids. Applied to citrate and malate, they indicated that heart to young cotyledon stage endosperm liquid contained 4.7 mM citrate and 23 mM malate. For late cotyledon stage endosperm liquid the concentrations were 5 mM citrate and 17 mM malate. Although these concentrations are more than 100 times the concentration of malate estimated in the liquid endosperm of cotton (10), they approximate the range observed in coconut water, namely 4.5 to 17 mM malate for various stages of young coconuts (22).

Qualitative analyses of the amino acids of the liquid endosperm were made by two-dimensional chromatography. Seventy plates were run, using 5 to 15 µl of endosperm liquid each. Spots were identified by co-chromatography with standard samples. Qualitative and quantitative measures of the amino acids were also obtained with an amino acid analyzer. The results shown in Table III indicate at least 18 amino acids; 10 of these were at concentrations greater than 1 mM, the most prevalent being alanine at 10.2 mM.

TLC of bean endosperm liquid, alone and in combination with various sugar standards, revealed that most of the sugar could be accounted for as sucrose, glucose, and fructose. Quantitative measures of each were obtained by combining the results of the Somogyi-Nelson determination of reducing sugar and total sugar with the results of the Glucostat test (Table IV). The Somogyi-Nelson determination for hydrolyzed *early cotyledon stage* endosperm liquid indicated 105 mM of total sugar. The qualitative analyses indicated that these are composed almost entirely of sucrose, glucose, and fructose. The Somogyi-Nelson determination of fresh early cotyledon stage endosperm liquid indicated a value of 65 mM. Thus, 105 minus 65 or 40 mM represented hydrolyzed sucrose. The sucrose concentration in early cotyledon stage endosperm liquid was therefore 20 mM. The glucose concentration in early cotyledon stage endosperm liquid, as given by the Glucostat

TABLE III. *Free Amino Acid Components of the Liquid Endosperm of Heart Stage Bean Ovules*

Amino Acid	Concentration	
	mM	*mg/l*
Alanine	10.2	907.8
Methionine	6.5	968.5
Glutamine or glutamic acid	5.2	759.0
Threonine	4.4	523.6
Serine	3.6	378.0
Glycine	2.0	150.0
Valine	1.9	222.0
α-Aminoadipic acid	1.5	
Isoleucine	1.1	
Pipecolic acid	1.1	
Citrulline	1.1	
Arginine	0.9	189.9
Histidine	0.85	131.8
Asparagine or aspartic acid	0.6	90.0
Leucine	0.6	78.6
α-Aminobutryic acid	0.4	
Lysine	0.3	
Proline	trace	
Total molarity	42.25	

test, was 40 mM. The fructose concentration in early cotyledon stage endosperm liquid was then 65 minus 40 or 25 mM.

For *late cotyledon stage* endosperm the determination before hydrolysis was 50 mM, and after hydrolysis 170 mM. Thus, following the reasoning of the previous paragraph, the concentration of sucrose was half the difference between the above two figures or 60 mM. The concentration of glucose plus fructose, 50 mM, was not further differentiated on this material via the Glucostat test.

As seen in Table V, a major contribution toward the very high osmolarity is reduced N in the form of NH_4^+ and amino acids. Another major component is sugar, with the proportion of reducing sugar being highest in earlier stages. A third major component is organic acid, particularly malate. Of the salts, K dominates and Ca is very low if present at all.

DISCUSSION

An embryo culture medium composed in accordance with the results presented here would be at variance with most of the plant tissue and embryo culture media reported in the literature. Several correlations with the analyses of other endosperm liquid and with some successful culture media may be important, however. Two obvious differences are the high osmolarity in the bean endosperm liquid and the very low concentration of Ca. Similarities are found in the high proportion of K and the presence of ammonium malate, amino acids, and sugar.

Ryczkowski (18) reported a general rise in the osmolarity of endosperm liquid (central vacuolar sap) in seven species up to a stage comparable to the globular stage in bean embryos. The osmolarity at this stage varied from 0.285 M in *Asparagus officinalis* to 0.6 M in *Leucojum aestivum*. In progressively older ovules the osmolarity decreased, the low varying from 0.169 M in *Asparagus officinalis* to 0.45 in *Crambe tataria*. Data from embryo cultures suggest, moreover, that high osmolarity may be important for normal plant embryo development, especially of the earlier stages, to suppress "precocious germination." Rijven (17) reported that higher osmolarity was essential for the normal culture of younger embryos of several species. Some disagreement with this finding has been voiced, notably by Raghavan and Torrey (15), but there is some doubt that their *Capsella* embryos are free of the precocious germination syndrome. Norstog (13) found that high osmolarity was required to prevent the precocious germination of young barley embryos in the dark. In the light, however, high K concentration together with ammonium malate sufficed. Ammonium malate could also replace part of the osmolarity requirement of young cotton embryos (10). In all cases the immediate response was seen in cell turgor and the long term response was the blocking of precocious germination.

Ammonium malate and relatively high K concentration have been reported to be important for the maintenance of turgor and perhaps for morphogenetic control as well, for in coconut endosperm liquid the K to P and K to Mg ratios are about 10:1 as they are in bean, and the malate concentration is also comparable in both endosperm liquids (NH_4^+ was not measured) (22). In cotton endosperm also ammonium malate was found to be present, although at lower concentration (10). Furthermore, NH_4^+ stimulates carrot embryoid growth (6, 16) and tissue cultures of a number of species (5). Cultures of soybean, wheat, and flax would grow on NH_4^+ as the sole N source, if the anion were citrate, malate, succinate, or fumarate, but not shikimate, tartrate, acetate, carbonate, or sulfate. The observation that high K concentration, NH_4^+, and malate maintain turgor only in the light, coupled with the observations of light stimulated K uptake in stomata (24) and of malate synthesis accompanying K uptake in roots (3), suggests the possibility that these ions might be active in a light driven K pump. The very low concentration or absence of Ca was unexpected. This low level in the endosperm liquid might have been due to a very rapid uptake rate by the embryo, in which case one would expect to find elevated levels of Ca in the embryo. Atomic absorption spectrophotometry of H_2SO_4 digests of early cotyledon stage and late cotyledon stage embryos failed to indicate any Ca however. Moreover, Ca is not listed in the elements detected in coconut endosperm liquid (22). The physiological reason for this low calcium concentration in unclear. At some point in development, however, Ca must become available to the embryo, for an important storage compound in the mature ovule is probably the Ca salt of phytic acid.

Table IV. *Levels of Glucose, Reducing Sugar, and Total Sugar in Bean Endosperm Liquid*

Sample Stage	Sugar in Sample			Reducing Sugar (calculated)	Sucrose (calculated)
	Glucose[1]	Glucose and fructose	Glucose, fructose and sucrose		
µl	*µmoles*			*mM*	
Early cotyledon					20
25, fresh		1.63		65	
25, fresh	0.20			40	
25, hydrolyzed			2.63	105	
Late cotyledon					60
25, fresh		1.25		50	
25, hydrolyzed			4.25	170	

[1] Glucostat determined.

Table V. *Composition of the Endosperm Liquid of Phaseolus vulgaris L.*

Component	Concentration		
	Globular-heart	Early cotyledon	Late cotyledon
		mM	
NH_4^+		110	80
Ca	<0.5		<0.5
K	34.4		20.8
P	5.3		3.2
Mg	4.1	5.4	2.5
Malate		23	17
Citrate		5	4.8
Amino acids	42		
Reducing sugar		65	50
Total sugar		105	170
Total osmolarity	700		500

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