Nuclear Size and DNA Content of the Embryo and Endosperm During their Initial Stages of Development in Glycine max (Fabaceae)

Author(s): Mark A. Chamberlin, Harry T. Horner and Reid G. Palmer

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NUCLEAR SIZE AND DNA CONTENT OF THE EMBRYO AND ENDOSPERM DURING THEIR INITIAL STAGES OF DEVELOPMENT IN *GLYCINE MAX* (FABACEAE)[1]

MARK A. CHAMBERLIN,[2] HARRY T. HORNER,[2,4] AND REID G. PALMER[3]

[2] Department of Botany and Bessey Microscopy Facility; and
[3]USDA-ARS FCR, Departments of Agronomy, and Zoology and Genetics,
Iowa State University, Ames, Iowa 50011-1020

A technique was developed for isolating embryo sacs from ovules of soybean and for separating embryo from endosperm. Image analysis and cytophotometry were used to determine the relative mass of DNA and size of nuclei of endosperm and embryo cells. Analyses were done at the globular through late heart-shaped embryo stages to correlate ploidy level or nuclear size, and differentiation in these tissues. Mean size of embryo nuclei was fairly constant through all stages studied. Ploidy condition of the embryo was stable, 95%–99% of the nuclei were distributed in a bipolar pattern by relative mass at 2C and 4C. Few embryo nuclei (3%) had ploidy levels above 4C at the late heart-shaped embryo stage. Variability in size of endosperm nuclei seemed correlated with the morphological state of these nuclei (free-nuclear vs. cellular). Most endosperm cells did not show significant polyploidy with 84%–92% of nuclei in the expected 3C–6C range, but some nuclei with elevated ploidy levels were noted during endosperm cellularization. Endosperm senescence was correlated with nuclear DNA loss over time. Polyploidy seems to have no direct role in the early differentiation of the soybean embryo and endosperm, but these stable conditions may be necessary for the early establishment of the embryo.

The endosperm is a unique tissue formed during double fertilization. As the endosperm develops, its nuclei may become polyploid and have maximum levels of 384C in maize (Tschermak-Woess and Enzenberg-Kunz, 1965), 96C in *Phaseolus vulgaris* (Nagl, 1970), 24C in *Triticum aestivum* (Chojecki, Bayliss, and Gale, 1986), 96C in *Borassus flabellifer* (Stephen, 1974) and 3,072C in *Echinocystis lobata* (Turala, 1966). Not all nuclei within a given endosperm exhibit these high ploidy levels inasmuch as cytological differentiation within this tissue exists. The aleurone endosperm of *Oryza sativa* exhibited no polyploidization of its nuclei, retaining the base 3C–6C ploidy level, but greater than 20% of the central endosperm cells attained the 24C level (Ramachandran and Raghavan, 1989). A similar pattern existed in *Zea mays,* in which the central endosperm cells evidenced a mean DNA content up to 20-fold greater than that of peripheral endosperm cells (Kowles and Phillips, 1985, 1988). Numerous examples also have been reported in the differential polyploidization of the endosperm proper and the haustoria of various taxa (Chopra and Sachar, 1963; Turala, 1969).

The differentiation of the young angiosperm embryo into distinct organs may suggest a cytological differentiation of its tissues similar to that seen in the endosperm. The nuclei of the embryo proper of *Phaseolus coccineus* contained only two base levels of DNA (2C and 4C), whereas the nuclei of the suspensor proper and the large suspensor basal cells contained large polytene chromosomes with ploidy levels of 2C–64C and 256C–8,192C, respectively (Brady, 1973). Other taxa have demonstrated cytological distinction between embryo proper and suspensor such as in *Tropaeolum majus* (Nagl, 1976), various species of *Phaseolus* (Nagl, 1974), and also, between cotyledons and embryo axes in *Brassica napus* (Silcock et al., 1990), *Phaseolus vulgaris* (Johnson and Sussex, 1990), and other papilionaceous legumes (Bryans and Smith, 1985). These results suggest a division of labor of embryo and endosperm tissues that may be a result of the increased ploidy level of their cells. D'Amato (1952) suggested further that polyploidy in the embryo may directly affect differentiation and, therefore, function of these tissues. The gradient ploidy levels in the epidermal cells (2C), hypodermal cells (8C–32C), and central storage cells (64C) of the cotyledons of *Pisum sativum* (Scharpe and Van Parijs, 1973) seem to support this premise.

Therefore, polyploidy in endosperm and embryo tissues is likely to play an important functional role due to its prevalence in a number of diverse genera. In this study we report on the relative mass of DNA and the size of nuclei in soybean endosperm and embryo at the globular through the late heart-shaped embryo stages of development (6–35 d postfertilization, dpf), to determine whether polyploidy has any relevance to morphogenesis in these soybean tissues. The small size of the soybean ovules at these early stages and difficulties in obtaining a sufficient number of endosperm cells from this "nonendospermic" legume have challenged our abilities to study the endosperm and embryo tissues for ploidy changes. Because of these difficulties, we have developed a technique for the isolation of embryo sacs that allows us to obtain homogeneous samples of embryo and endosperm nuclei for cytophotometric analysis. In the present study, we utilize this new technique to analyze the embryo and endosperm nuclei in a normal system to serve as a model for understanding ploidy and morphogenesis in these tissues.

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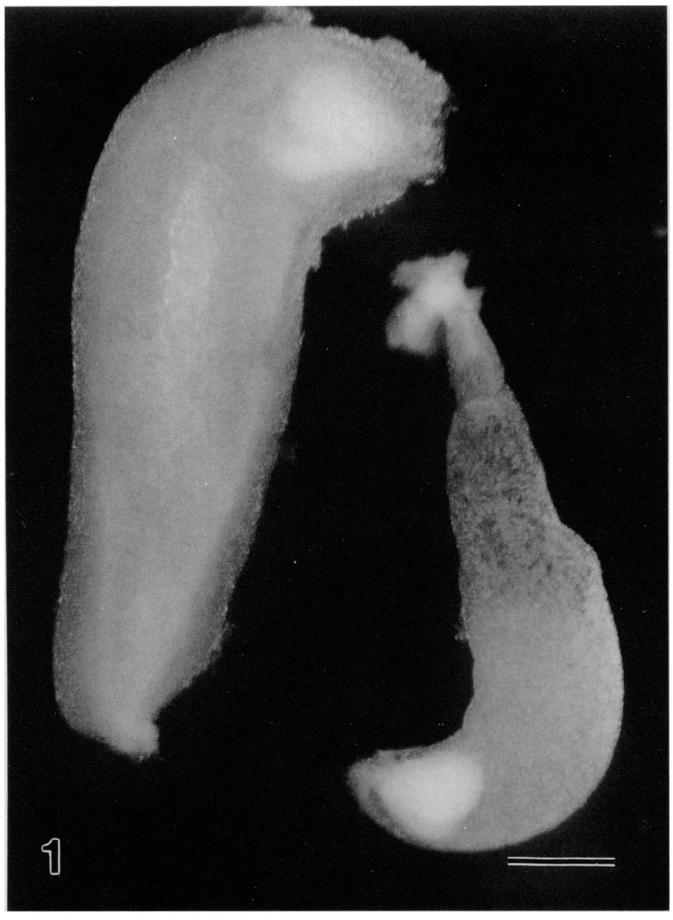
[4] Author for correspondence.

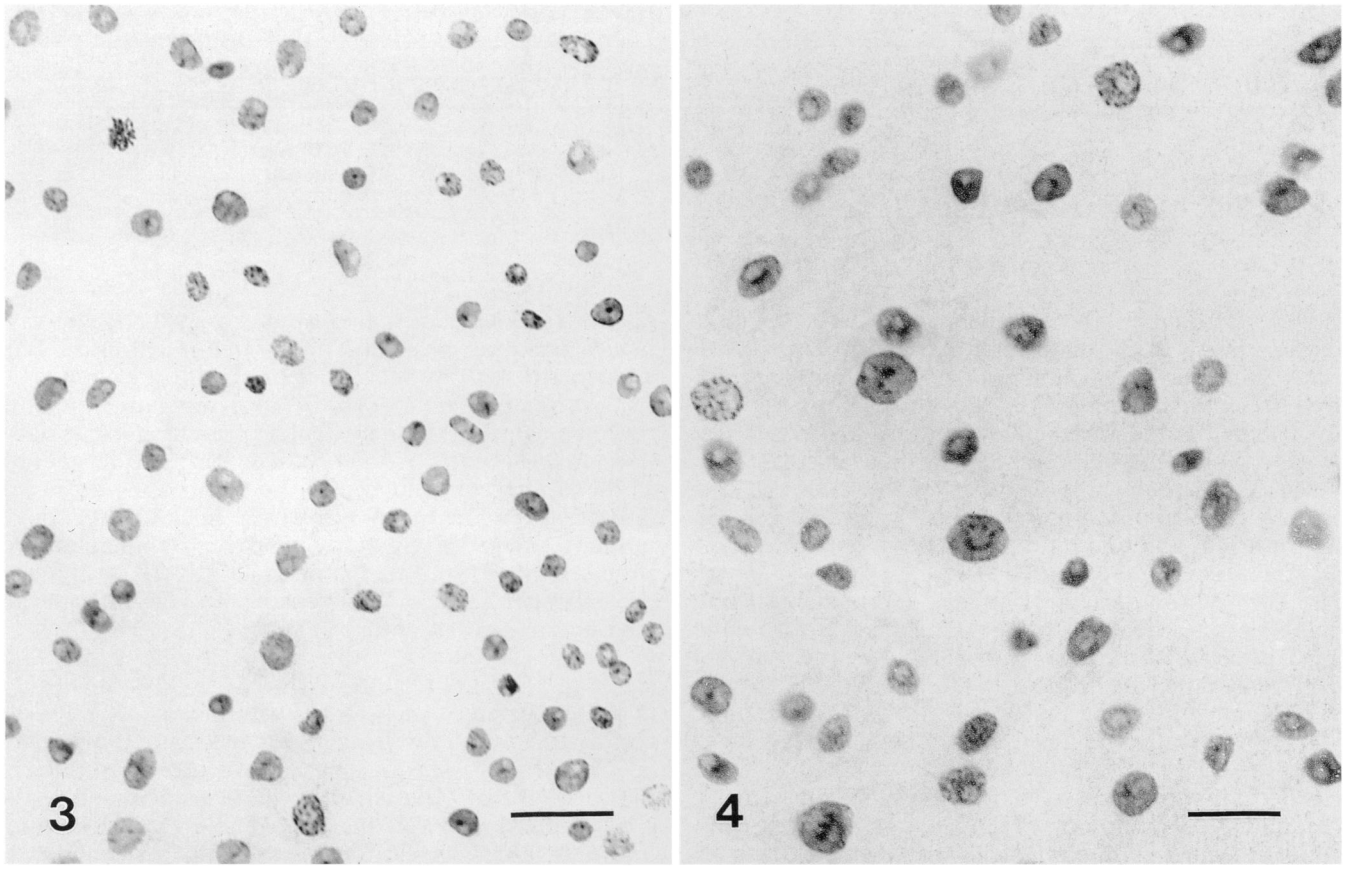


Figs. 1, 2. Photomicrographs of immature *Glycine max* ovules. Bars = 100 μm. **1.** Early heart-shaped embryo stage, ovule at left with integuments intact and embryo sac at right with integuments removed by enzymatic digestion. **2.** Midheart-shaped embryo stage embryo sacs with integuments removed by enzymatic digestion.

MATERIALS AND METHODS

Plants of *Glycine max* (L.) Merr. cv. Harosoy were germinated and grown in the USDA-ARS FCR glasshouse at Iowa State University, Ames. Ovules were dissected at various stages 6–35 dpf. Fertilization occurs approximately 24 hr after pollination in soybean. Ovules were fixed in fresh 10% formalin for 12 hr at room temperature. Ovules were washed in distilled water (2 hr), and the integuments superficially nicked. Ovules were placed in 5% cellulase (Cellulysin-Calbiochem, La Jolla, CA) plus 5% pectinase (Macerase-Calbiochem) in 5% mannitol solution for 3 hr at 37 C. They were washed in 5% mannitol (1 hr), and the integuments were dissected away to expose the embryo sacs (Figs. 1, 2). A modified Feulgen technique (Berlyn and Miksche, 1976) was used to stain the embryo and endosperm nuclei as follows: embryo sacs were hydrolyzed in 1 N HCl at 60 C for 10 min, and the hydrolysis reaction was stopped by a quick rinse in cold distilled water (4 C). The embryo sacs were placed in Schiff's reagent for 2 hr at 4 C in the dark, bleached in fresh 4% sodium meta-bisulfite for 30 min, and postwashed for 20 min in distilled water. Removal of the integuments allowed for consistent staining of the endosperm and embryo nuclei. No variability of staining was noted among ovules at the same stage of development. After staining, embryos were dissected from the endosperm tissue, and both tissues were squashed under coverslips on separate glass slides (Figs. 3, 4). The slides were frozen with liquid CO_2, and the coverslips removed. Slides were dehydrated in a graded ethanol series, then placed in xylene, and a coverslip was mounted with Permount. The nuclei were observed with a Leitz MPV microscope photometer fitted with a Leeds and Northrup 2430 DC galvanometer, and a transmitted illuminating system using a Leitz in-line mirror monochromator and a stabilized 150 W pressure xenon lamp. The two-wavelength method was used to determine the relative mass (RM) of the nuclear DNA (Ornstein, 1952; Patau, 1952). As a standard, mature leaf tissues were digested, the nuclei stained, and the cells squashed, as described. Because cells of the leaf were nondividing, the nuclei of this tissue were used to determine relative DNA mass of a resting diploid nucleus. The 2C value (diploid amount of DNA) was equal to a relative mass of 125.0.

A Leitz microscope fitted with a video camera (COHU model 4815-5000) connected to a video digitizer (Colorado Video, Inc., Boulder, CO, model 270A) was used to digitize bright-field images to determine nuclear size of embryo and endosperm tissues. The digitized images were saved and manipulated with a Kevex Delta IV system



Figs. 3, 4. Light micrographs of *Glycine max* embryo and endosperm squashes at midheart-shaped embryo stage; nuclei stained in Feulgen reaction. Bars = 20 μm. **3.** Population of embryo cells. **4.** Population of endosperm cells.

and a Kevex Automated Image Analysis software program.

RESULTS

Embryo and endosperm morphology—The embryo and endosperm passed through distinct morphological changes in the first 35 dpf. The globular embryo (glob) was formed 6–10 dpf, and was composed of an undifferentiated embryo proper and a short multiseriate suspensor. The bulk of the endosperm was free-nuclear at this stage of development, but cellularization of the endosperm began at the micropylar end of the embryo sac. The early heart-shaped embryo (early ht) was marked by the formation of the two cotyledon primordia at 11–16 dpf, and the cellular endosperm occupied greater than one-half the volume of the embryo sac.

At 17–25 dpf, the midheart-shaped embryo (mid ht) developed its polar meristems, and the cotyledonary cells began to fill with storage reserves. The endosperm was completely cellular at this stage, except for the chalazal process, which remained free-nuclear. These latter nuclei were generally hypertrophied in comparison with the nuclei of central and peripheral endosperm cells. The embryo enlarged rapidly to occupy approximately 75% of the embryo sac at 26–35 dpf, and its cotyledons continued to fill with storage reserves at the late heart-shaped embryo stage (late ht). At this stage of development, the embryo had encroached upon the central endosperm cells, and only the peripheral and the chalazal free-nuclei of the endosperm remained within the embryo sac.

Embryo nuclei—The mean size (area) of the embryo nuclei varied little through all stages studied (Table 1). Embryo mean nuclear size at the late ht stage (19.5 μm^2) was slightly greater than at earlier stages which ranged from 14.7 to 16.1 μm^2. Within each developmental stage, size of individual nuclei varied considerably. The largest

TABLE 1. Mean size (area in μm^2) of embryo and endosperm nuclei at various stages of embryo development.[a]

	Embryo nuclei				Endosperm nuclei			
Stage	Ovules	*N*	Mean	SD	Ovules	*N*	Mean	SD
Globular	5	330	16.1a	6.8	7	265	37.1c	25.8
Early heart	4	364	14.7a	5.5	4	248	20.8d	7.9
Midheart	6	1,047	15.7a	7.1	6	845	25.3e	14.4
Late heart	4	1,035	19.5b	9.8	4	873	34.1f	15.8

[a] Values of means followed by the same lowercase letter are not significantly different at the $P < 0.01$ level, according to *t*-test.

TABLE 2. Mean DNA relative mass and C-value of embryo and endosperm nuclei at various stages of embryo development.[a]

Stage	Embryo nuclei					Endosperm nuclei				
	Ovules	*N*	Mean	SD	C-value	Ovules	*N*	Mean	SD	C-value
Globular	5	179	145.0a	39.3	2.3C	7	228	213.1c	49.7	3.4C
Early heart	4	155	142.5a	46.5	2.3C	4	183	237.3c	89.5	3.8C
Midheart	6	306	152.9a	42.7	2.4C	6	296	206.2d	75.0	3.3C
Late heart	4	202	167.1b	50.6	2.7C	4	203	223.5c	64.8	3.6C

[a] Values of means followed by the same lowercase letter are not significantly different at the $P < 0.01$ level, according to *t*-test.

nuclei were noted in the late ht stage (64.5 μm^2); the maximum sizes in the preceding stages were 35.4–47.8 μm^2. A minimum nuclear size of 4.0–4.4 μm^2 was observed at each developmental stage, a size comparable to that of the smallest nuclei in the mature leaf tissue. In general, the extreme values noted for nuclear size made up less than 2% of the total nuclei in a given embryo. The mean nuclear size of the mature leaf tissue was 9.6 μm^2, with minimum and maximum areas of 4.8 μm^2 and 13.9 μm^2, respectively.

The embryo ploidy levels (Table 2) were stable from the glob stage to the mid ht stage (2.3C, 2.4C), with only a slight increase in mean nuclear DNA relative mass to 167.1 (2.7C) at the late ht stage. In all stages studied, 95%–99% of all nuclei were in the expected 2C–4C range. Significant amplification of DNA above the 4C level (Table 3) was noted only at the late ht stage, at which 3% of the nuclei had a maximum of 5.7C. Nuclear DNA mass levels lower than 2C were noted at each stage of development, but only in a small number of nuclei (Table 3). The embryo nuclei were distributed in a bipolar pattern by RM in all stages reported (Fig. 5). The peak distribution of nuclei at 125.0 RM was equal to 2C, as determined from nondividing nuclei of mature leaf tissue. The 4C level was represented by the second distribution peak of nuclei at approximately 250.0 RM. A curious population of embryo nuclei at approximately 180.0 RM was intermediate in ploidy between 2C and 4C at the late ht stage (Fig. 5). This intermediate peak (probably nuclei in S-phase) indicated the high level of mitotic activity observed at this stage of development.

Endosperm nuclei—The mean size (area) of the endosperm nuclei varied considerably among the different stages of development (Table 1). At the glob stage, the embryo sac was filled predominantly with large free-nuclei, although some cellular endosperm was observed at the micropylar end. These endosperm nuclei had a mean size of 37.1 μm^2 and reached a maximum size of 164.0 μm^2. The endosperm nuclei at the early ht and mid ht stages were significantly smaller than those at the glob stage (Table 1). The endosperm at the early ht stage was a heterogeneous tissue consisting of free-nuclei and cellular endosperm with a mean nuclear size of 20.8 μm^2. Cellularization of the endosperm was nearly complete at the mid ht stage, and these nuclei had a mean nuclear size of 25.3 μm^2. A large portion of the endosperm degenerated as the embryo expanded into the embryo sac during the late ht stage. The mean size of the nuclei at this developmental stage was 34.1 μm^2, and very large nuclei with a maximum of 195.0 μm^2 were also observed. At all stages, the endosperm nuclei were significantly larger than their counterparts in the embryo (Table 1).

The ploidy level of the endosperm nuclei was fairly stable at all stages (Table 2), with 84%–92% of all nuclei in the expected 3C–6C range. The mean DNA RM for endosperm nuclei at all stages ranged from 206.2 RM to 237.3 RM (3.3C–3.8C). Only a few endosperm nuclei at the early ht (7.1%) and mid ht (2.4%) stages showed increased ploidy, greater than 6C (Table 3), and the maximum C-value was 9.7. The mean RMs of the endosperm nuclei were statistically (*t*-test) greater than those of the embryo (Table 2).

Surprisingly, the endosperm nuclei did not show a bipolar RM distribution at 3C and 6C (Fig. 5). In all stages a distinct peak representing the 3C condition was seen at 190–200 RM, but the expected 6C peak at 380.0 RM was conspicuously absent at each stage in the endosperm. Instead, the peak distribution of endosperm nuclei at the glob, early ht, and mid ht stages had a smaller than expected nuclear DNA RM at 350.0 (5.5C). This trend continued into the late ht stage, during which there was further reduction in the nuclear DNA RM of this tissue. A broad distribution peak at 280–325 (4.4C–5.1C) was observed at this stage. An increasing number of endosperm nuclei exhibited DNA levels below 3C as well, during the development of the endosperm (Table 3). At the glob stage, 7.9% of the endosperm nuclei had C-values below the expected ploidy level, and this trend continued through the late ht stage (12.8%).

TABLE 3. Incidence (%) of embryo and endosperm nuclei with greater than or less than normal ploidy levels.

Stage	Embryo			Endosperm		
	N	<2C	>4C	*N*	<3C	>6C
Globular	179	2.2%	0.0%	228	7.9%	0.0%
Early heart	155	1.9%	0.0%	183	8.7%	7.1%
Midheart	306	0.7%	0.7%	296	10.5%	2.4%
Late heart	202	2.0%	3.0%	203	12.8%	0.0%

DISCUSSION

Previous studies have found a direct correlation between the incidence of polyploidy and increased nuclear volume in endosperm and embryo tissues (D'Amato, 1984; Bryans and Smith, 1985; Silcock et al., 1990). Because polyploid nuclei were rare in the present study, no direct correlation could be made between nuclear size and DNA content. Nevertheless, it can be stated that an increase in the endosperm mean nuclear size did not reflect elevated ploidy levels. The endosperm mean nuclear size showed distinct variation at each stage of development (Table 1),

but the mean RM of these nuclei (Table 2) did not reflect this variation in size.

The variation in size of the endosperm nuclei likely reflects the morphological status of this tissue, and not the DNA content of its nuclei. The large value for the mean nuclear size observed at the glob stage reflects a predominance of acellular endosperm with its free-nuclei. The mean size of the endosperm nuclei decreased as cellularization of the endosperm proceeded, and the latter was nearly completed between the early ht and mid ht stages. It is unclear why individual nuclei are reduced in size during cellularization of the endosperm. In previous work (unpublished data), we noted that the bulk of the endosperm cells rarely divided after cellularization. In the absence of mitosis, it is probable that much of the biosynthetic machinery necessary for division may be lost and, as a consequence, the nuclei become more compact.

Most of the central endosperm is eventually los due to the encroachment of the embryo into the embryo sac during the late ht stage. Only the large free-nuclei of the chalazal process and the rapidly dividing peripheral layer remained. Therefore, the increase in mean nuclear size of the endosperm observed at this stage (Table 1) likely reflects a disproportionate reduction in the number of small nuclei of the central endosperm, and not a general increase in nuclear size for the endosperm.

The embryo passed through a number of distinct morphological changes in the early stages of its development, but this was not reflected cytologically. The RM distribution of embryo nuclei at 2C (G1-phase) and 4C (G2-phase), and the reduced distribution at the intermediate S-phase, suggests a normal pattern for mitotically dividing diploid tissues (Fig. 5). The ploidy level of these tissues was stable, with a large percentage of the embryo nuclei in the 2C–4C range (Table 3). Only at the late ht stage was there any indication of increased ploidy, with a small number of nuclei (3%) above the expected 4C value (Table 3). This increase coincided with a slight elevation in mean ploidy (2.7C); (Table 2). These latter results are similar to those noted in *G. max* cv. Dare (Dhillon and Miksche, 1983). They observed 5%–7% of the cotyledon nuclei above the 4C level and a mean DNA content of 2.7C at 15 days postanthesis through the "very early maturation" stage. The mean DNA content and the number of polyploid nuclei increased as the cotyledons continued to mature. These researchers found a direct correlation between DNA content in the cotyledon nuclei and increased RNA and protein synthesis in these cells. In the present study, the cotyledons began to accumulate storage reserves at the mid ht stage, and this continued through the late ht stage. It is possible that the presence of embryo nuclei with DNA content greater than 4C marks the initiation of protein synthesis and its accumulation in the cotyledons that is similar to that found in *G. max* cv. Dare. The incidence of polyploidy and its relation to protein accumulation were also observed in the cotyledonary cells of *Pisum sativum* by Scharpe and Van Parijs (1973).

As a result of the variation in mean nuclear DNA content of the endosperm nuclei (Table 2), no distinct correlation could be made between ploidy level and endosperm differentiation. Although mean nuclear RM was fairly constant over time, the extreme examples of nuclei with C-values greater than 6C (9.7C instead of expected

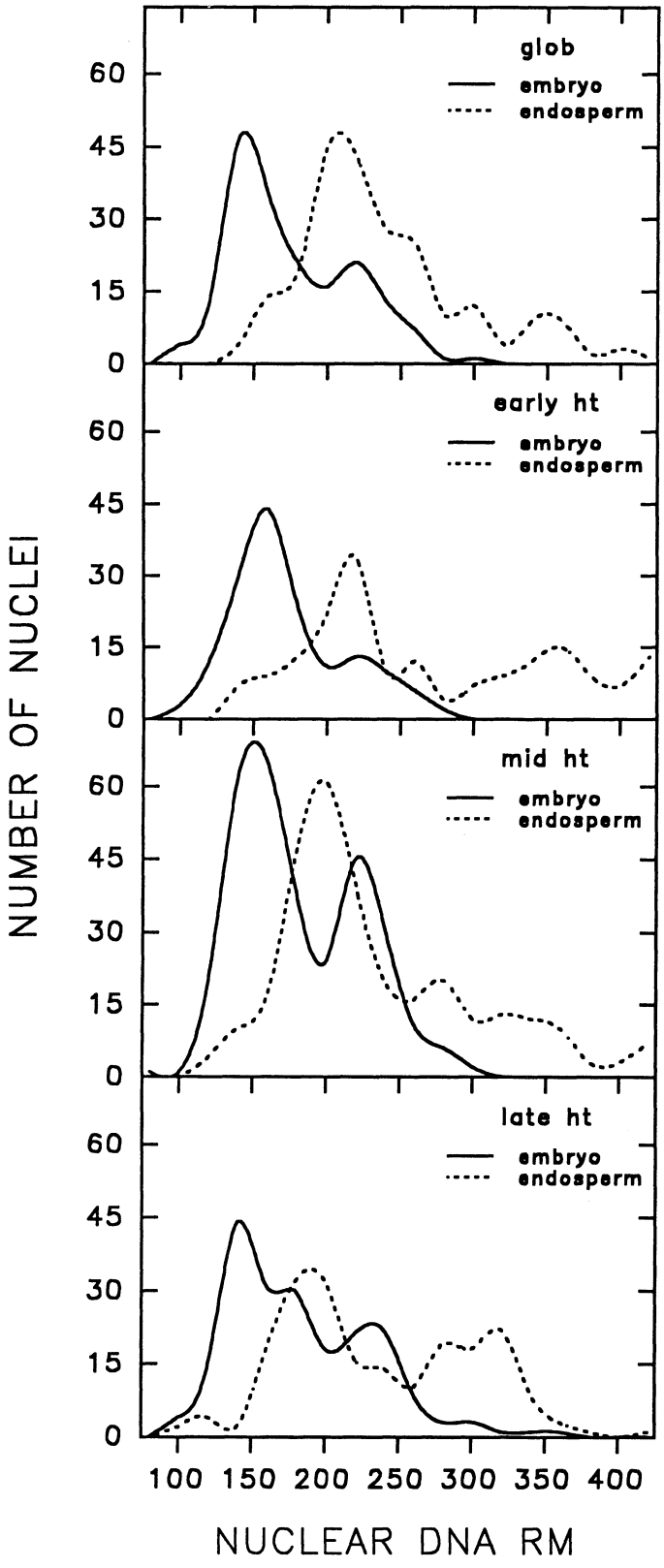


Fig. 5. Frequency distribution of embryo and endosperm nuclei by DNA relative mass (RM) at four stages of embryo development in *Glycine max*: glob = globular; early ht = early heart-shaped; mid ht = midheart-shaped and late ht = late heart-shaped.

12C; possibly due to selective DNA amplification) and less than 3C may be indicative of some morphological or physiological change in this tissue. Cellularization of the bulk of the endosperm seemed to coincide with the presence of nuclei with increased DNA content at the early ht (7.1%) and mid ht (2.4%) stages (Table 3). These results and the near absence of such endosperm nuclei (above 6C) at the glob stage (initial stage of cellularization) and at the late ht stage (completed cellularization) suggest that a small population of endosperm cells may have a functional role in cellularization.

An increasing number of endosperm nuclei exhibited some loss of DNA content below the expected 3C level during development (Table 3). This loss was reflected in the RM frequency distribution of endosperm nuclei (Fig. 5). A loss of DNA was indicated by a shift in the RM of the endosperm nuclei in the G2-phase of DNA synthesis (375 RM or 6C) toward lower levels of DNA content (Fig. 5). After cellularization, the central endosperm cells seemed to degenerate with a loss of cytoplasm and organelles. Senescence of these cells likely included the loss of nuclear DNA and, therefore, an increased incidence of nuclei with reduced ploidy levels. A similar correlation between cellular degradation and DNA loss has been noted in the endosperm of maize (Kowles, Srienc, and Phillips, 1990) and wheat (Chojecki, Bayliss, and Gale, 1986) and in the senescent cotyledons of soybean (Chang, Miksche, and Dhillon, 1985).

Another possible explanation for the decline in DNA content of the endosperm nuclei is nutritive. Supplying nutrients necessary for growth of the embryo is a typical function of the endosperm of angiosperms. The loss of DNA may form a pool of free bases possibly serving as a ready source of deoxynucleosides and phosphates for the developing embryo (Dhillon and Miksche, 1983). Further research is necessary to determine the exact nature and consequence of DNA loss in this tissue.

In conclusion, the stable ploidy condition predominated in the early stages (6–35 dpf) of embryo and endosperm development in soybean. Thus, polyploidy had no direct role in the early differentiation of these tissues. The limited number of nuclei with elevated ploidy levels observed in this study was likely a consequence of differentiation, and not its cause. The absence of polyploidy in the early stages of zygote/embryo and endosperm development may be a necessary condition for establishment and success of the angiosperm embryo. Variability in ploidy of one or both of these tissues may result in a lack of embryo/endosperm compatibility and subsequent embryo abortion, as suggested by other researchers (White and Williams, 1976; Williams and White, 1976). These former conditions seem to be fulfilled in normal soybean, as evidenced in this study by the stable ploidy levels and the near constant ratio of DNA content between embryo and endosperm (2C:3C). Once the embryo is established, polyploidization of embryo and endosperm tissues may be a necessary requirement for the continued development of the embryo. This latter statement is a possibility, due to the prevalence of polyploidy in endosperm and embryo tissues of a number of diverse genera. The majority of studies observing polyploidy in these tissues have concentrated on stages of development during which the embryo is well established or nearly mature. Further research seems warranted, in these genera, to determine whether there is a continuum of polyploidy in the early stages of zygote/embryo and endosperm development as well. The technique presented here, for the isolation of embryo sacs, gives us the tool for analyzing these tissues at early stages of development.

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