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Segmentations in Microspores of *Nicotiana sylvestris* and *Nicotiana tabacum* which Lead to Embryoid Formation in Anther Cultures

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With 2 Figures

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Summary

It is shown that an equal division in uninucleate microspores, giving two cells with similar staining nuclei, can initiate embryogenesis in anther cultures of *Nicotiana sylvestris* and *N. tabacum* var. Wisconsin 38 and evidence is presented that this pathway is quantitatively important and may be the only one leading to successful pollen embryos. Neither an initial asymmetric division in the uninucleate microspore nor the formation of a "suspensor" are essential to the development of embryo polarity.

1. Introduction

Starting with the pioneer work of GUHA and MAHESHWARI (1967) on *Datura innoxia*, solanaceous plants have proved particularly amenable to anther culture and in the case of several solanaceous species attention has been directed to tracing the segmentations in the microspores which lead to embryogenesis. Work with *Nicotiana tabacum* (SUNDERLAND and WICKS 1971, BERNARD 1971) and *Datura metel* (IYER and RAINA 1972) has pointed to embryo development from the larger (presumptive vegetative) cell arising from an initial asymmetric division in the microspore. However, in both these species an alternative route in which both cells, arising from a more symmetrical division, take part in embryo development has been considered as a less frequent occurrence, and as possibly occurring even from anthers excised at a stage when most of the microspores have undergone the initial, normal, asymmetric division.

Previous work in our laboratory (RASHID and STREET 1973) involving the culture of anthers of *Atropa belladonna* excised when the microspores were

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predominantly uninucleate has indicated that the normal pathway of embryo development is initiated by an equal division and that both the daughter cells are involved.

To trace the pathway(s) leading to successful embryo development from microspores it is necessary not only to observe early divisions in the microspores but to observe as complete sequences as possible between these early divisions and the emergence of the normal bipolar proembryo. Further, whenever divisions are observed in microspores which have initially differentiated a "vegetative" and a "generative" cell, there is the difficulty of being able to recognize the division products of these cells on the basis of a contrast in the size and density of their nuclei. In all anther cultures only a small proportion of the microspores embark on cell division and in only a very much smaller proportion do these divisions continue in the organized way which results in embryo development. With these three considerations in mind we have endeavoured to assess the relative importance in embryogenesis of the two alternative segmentation pathways using anthers of *Nicotiana sylvestris* Speg. and Comes and *N. tabacum* L. var. Wisconsin 38 cultured in a synthetic medium lacking both an auxin and cytokinin and containing the ferric salt of ethylenediamine-di-O-hydroxyphenylacetic acid (Fe-EDDHA).

2. Materials and Methods

The general technique of anther culture was as previously described (RASHID and STREET 1973). The culture medium contained 2% sucrose, and the following in mg/l: $Ca(NO_3)_2 \cdot 4\,H_2O$ 250, KNO_3 150, $MgSO_4 \cdot 7\,H_2O$ 150, KH_2PO_4 150, $(NH_4)NO_3$ 250, $MnSO_4 \cdot 4\,H_2O$ 25, H_3BO_3 10, $ZnSO_4 \cdot 7\,H_2O$ 0.5, Fe-EDDHA 15, *meso*-inositol 100, thiamine HCl 1.0, and was solidified with 0.6% Difco bacto-agar.

Microspores were stained in acetocarmine and, after 8–12 hours, dehydrated overnight in 1 : 1 butanol/glacial acetic acid, transferred to butanol for 4–6 hours and mounted in Euparol.

Anthers were excised both at the late uninucleate stage and when most were binucleate. Cultured anthers were examined microscopically at intervals over 4 weeks, and for embryoid formation for up to 6 weeks. 15% of anthers from *N. sylvestris*, and 50% from *N. tabacum* gave rise to embryoids. Embryo formation was only observed in anthers from *N. sylvestris* excised at the uninucleate microspore stage.

3. Results

3.1. Nicotiana sylvestris

In preparations made after 6 days of culture from anthers excised at the uninucleate microspore stage, most of the microspores had undergone an asymmetric division to form vegetative and generative nuclei distinguishable by the large size and more diffuse and less dense staining of the vegetative nucleus. In anthers excised at the binucleate microspore stage, many of the microspores had become abnormally enlarged and full of starch. However,

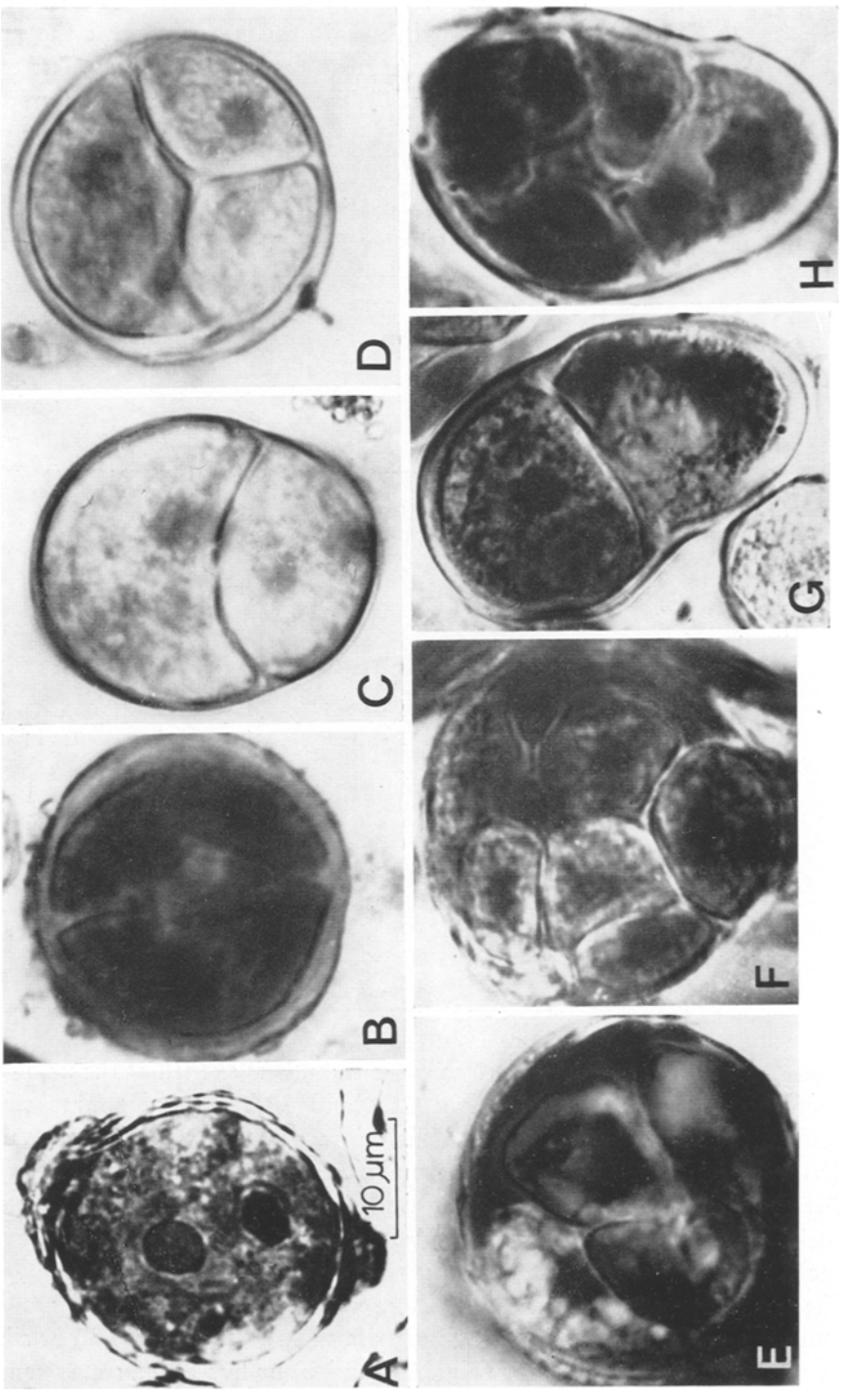
in both cases some microspores were observed containing two similar diffusely staining nuclei (Fig. 1 *A*). No further divisions occurred until the anthers had been in culture for 12–15 days. At this time, most of the anthers excised at the binucleate microspore stage had dehisced prematurely and were found to contain swollen, starch-filled grains and grains germinating to give pollen

Table 1. *Frequencies of Different Stages in the Development of Pollen Embryos Observed in Cultured Anthers of Nicotiana sylvestris*

Extent of cell division and embryo development and whether distinction between vegetative and generative type nuclei observed (+) or not observed (–)		Frequency of stages in five anthers derived from different buds					Totals
2–5 celled	–	6	1	2	16	15	40
	+	0	0	0	1	0	1
6–15 celled	–	9	1	1	9	3	23
	+	4	0	0	3	0	7
Early globular	–	2	0	0	4	0	6
	+	0	0	0	0	0	0
Globular	–	6	1	1	0	1	9
	+	1	0	1	1	0	3
Late globular	–	4	0	6	4	7	21
	+	0	0	0	1?	0	1?
Multinucleate apparently from division of generative nucleus		4	0	0	0	2	6
Presence of "suspensor" to early globular or globular embryo		6	–	1	2	1	10

tubes. Further observations were confined to anthers excised at the uninucleate microspore stage and between the 21st and 28th day of culture selected anthers (derived from different buds) were scored for various extents of division and embryo development and for whether there was or was not distinction into large, relatively diffuse and weakly staining, vegetative-type nuclei and smaller, denser and more intensively staining, generative-type nuclei (Table 1). At this stage many of the grains appeared empty.

The most frequent patterns of division encountered were such as would be consistent with the first division giving rise to two equal (Fig. 1 *B*) or slightly unequal (Fig. 1 *C*) cells. Four (Figs. 1 *D* and *E*) or five-celled stages consisted of similar sized cells, four-celled stages often taking the form of a typical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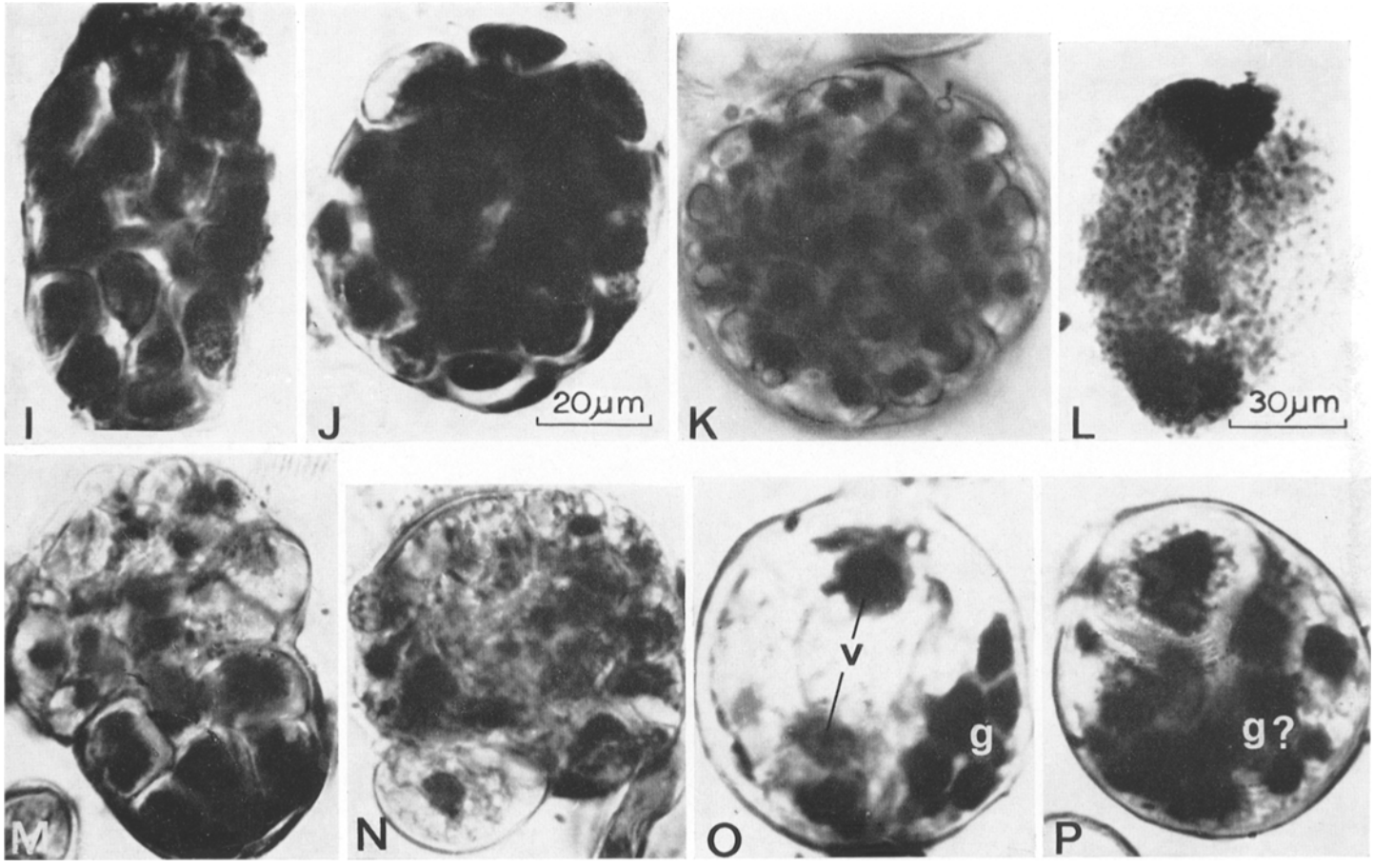
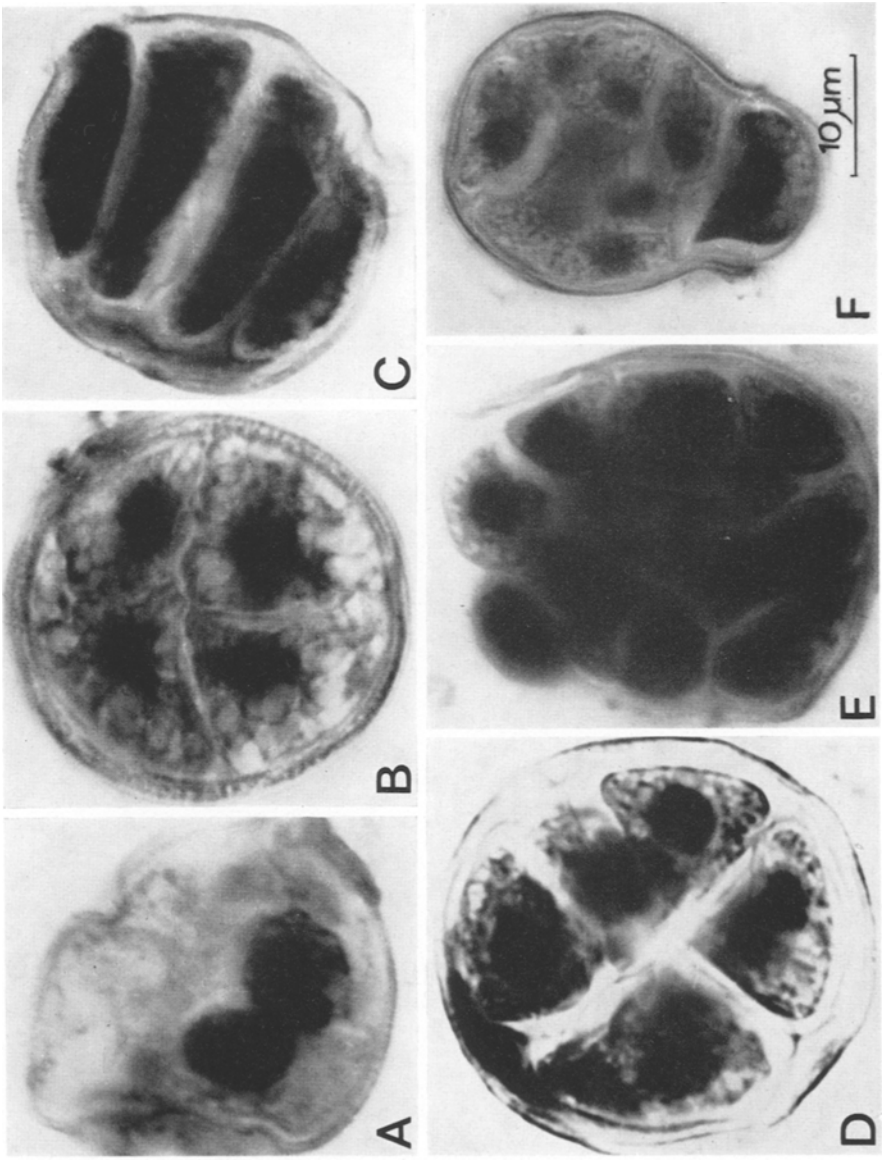


Fig. 1. Stages in pollen embryoid development in *Nicotiana sylvestris*. *A* = microspore containing two similar staining nuclei. *B*, *C* = bicellular microspores. *D*, *E* = 4-celled microspores. *F* = 7–8 celled stage. *G*, *H*, *I* = elongated embryoids at various stages. *J* = early globular stage showing emergence of epidermal layer. *K* = late globular stage. *L* = released embryoid showing root and shoot poles. *M* = premature dehiscence of globular embryoid. *R* = globular embryoid associated with a "suspensor" cell. *O*, *P* = microspores containing vegetative (*v*) nuclei (in division in *O*) and generative nuclei (*g*). Note absence of cell walls between the generative nuclei. Scale marked in *A* also applies to *B–H*, *O*, and *P*. Scale marked in *J* also applies to *I*, *K*, and *N*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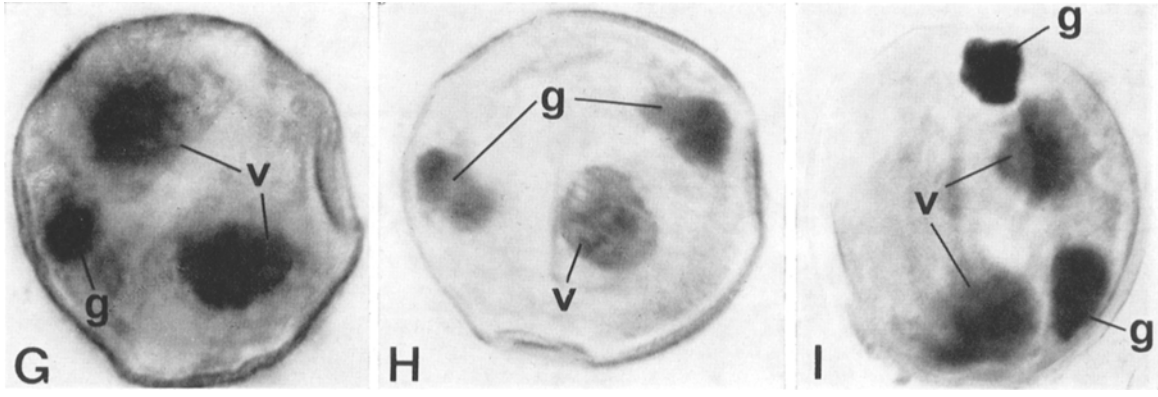

Fig. 2. Stages in pollen embryoid development in *Nicotiana tabacum*. *A* = microspore containing two similar staining nuclei. *B*, *C* = 4-celled microspores. *D* = microspore as *B* above in which cells are undergoing next division. *E* = microspore with young globular embryoid undergoing premature dehiscence. *F* = early globular embryoid associated with a "suspensor" cell. *G*, *I* = microspores with one generative (*g*) and two vegetative (*v*) nuclei. *H* = microspore with two generative and one vegetative nucleus. Scale in *F* applies to all photographs

quadrant (Fig. 1 *D*). These early stages of presumptive embryo development characteristically contained equal sized cells with similar staining nuclei (Fig. 1 *F*). Typically, the microspores remained spherical as they enlarged but occasionally they became elongated (Figs. 1 *G*, *H*, and *I*). At the globular stage, the outer cells became organized into an epidermal layer (Fig. 1 *J*). Up to a late globular stage the embryos normally remained enclosed by the microspore wall (Fig. 1 *K*), although instances were observed of earlier rupture of the microspore wall (Fig. 1 *M*). Occasionally it appeared that one or more cells formed early in division did not further participate in the formation of the globular embryo (Fig. 1 *N*); the effect was that these appeared as a "suspensor" of one or more larger cells. Embryos showing clear distinction into root and shoot poles and with a central vascular strand were formed following release from the microspore wall (Fig. 1 *L*). Many grains containing a vegetative and a generative cell retained their contents but did not divide further. A few grains were observed in which the generative nucleus had apparently undergone successive divisions without formation of intervening cell walls; such grains contained one or two "vegetative"-type cells (Fig. 1 *P*). No later stages were observed to indicate this as a pathway leading to embryo development. In rare instances ruptured grains appeared to be embarking on callus formation from a group of uniform cells such as observed in organized globular embryos.

3.2. Nicotiana tabacum var. Wisconsin 38

Under the conditions here used many of the anthers excised at the binucleate microspore stage dehisced prematurely and contained numerous enlarged, starch-filled grains. As with *N. sylvestris* our detailed observations were, therefore, confined to anthers excised at a stage when most of the microspores were uninucleate. Divisions commenced after 7 days of culture, and such anthers were scored for various extents of division and embryo development (Table 2) as described above. A considerable number of grains showed equal division into two cells with similar diffusely staining nuclei (Fig. 2 *A*). Variable planes of division were observed in 4-celled stages derived from such bicellular grains (Figs. 2 *B* and *C*) although the predominant pattern was the formation of a typical quadrant (Fig. 2 *B*). Globular embryos were predominantly composed entirely of similar cells of "vegetative" type (Fig. 2 *D*). Rupture of the microspore wall occurred over a wider range of extents of embryo development than in *N. sylvestris* and in some anthers rupture at an early globular stage (Fig. 2 *E*) occurred in about half the microspores generating embryos. Occasionally a single cell, presumably formed early in the division sequence, failed to divide further and simulated a single-cell suspensor (Fig. 2 *F*).

A high proportion of the microspores proceeded through the normal asym-

metric division to give rise to microspores containing a vegetative and a generative cell and when such microspores showed further divisions this was predominantly by division in the vegetative cell (Fig. 2 *G*) as described by SUNDERLAND and WICKS (1971). The generative nucleus rarely divided more

Table 2. *Frequency of Different Stages in the Development of Pollen Embryos Observed in Cultured Anthers of Nicotiana tabacum var. Wisconsin 38*

Extent of cell division and embryo development and whether distinction between vegetative (V) and generative (G) type nuclei observed (+) or nor observed (−)		Frequeny of stages in seven anthers derived from different buds							Totals
2–5 celled	−								
2 nuclei		43	3	4	5	2	8	1	66
3 nuclei		6	4	0	5	1	6	0	22
4 nuclei		0	7	2	4	3	12	3	31
	+								
1G + 2V		13	8	7	4	2	2	1	37
1G + 3V		1	0	3	0	1	0	0	5
1G + 4V		0	2	0	1	0	0	1	4
2G + 1V		2	2	0	0	0	0	0	4
2G + 2V		0	0	1	0	1	0	0	2
2G + 3V		0	1	0	0	0	1	1	3
3G + 2V		0	1	0	0	0	0	0	1
6–15 celled	−	0	21	27	8	30	45	28	159
	+	0	4	—	1	—	9	2	16
Early globular	−	0	10	10	3	6	11	0	40
	+	0	5	6	2	1	0	0	14
Globular	−	0	21	8	1	0	0	0	30
	+	0	0	0	0	0	0	0	0
Late globular	−	0	23	13	1	5	31	1	74
	+	0	0	0	0	0	0	0	0
Multinucleate apparently from division of generative nucleus		0	5	4	0	0	10	0	19
Presence of "suspensor" to early globular or globular embyro		0	6	2	1	0	3	1	13

than once and the derived nuclei were recognized by their dense staining and by becoming elliptical (Figs. 2 *H* and *I*). These nuclei did not appear to organize cells. Apparently normal early globular stages of embryo development were observed in microspores which contained one, two or, in one instance, three "generative"-type nuclei (Table 2). However, in microspores containing more advanced globular stages, no such nuclei could be observed so that no evidence was obtained that microspores containing persistent generative nuclei continued embryo development.

4. Discussion

The observations reported indicate that the pathway of pollen grain embryogenesis in *Nicotiana sylvestris* is predominantly, and possibly exclusively, initiated by an equal division in mononucleate microspores. The fate of microspores which divide to give a vegetative and generative cell appears to be that they either become swollen and starch-filled, or embark upon pollen grain germination or undergo a number of divisions short of full embryo development.

The situation in *N. tabacum* is less clear but certainly does not exclude that successful embryogenesis is normally (or always) initiated by an equal division in uninucleate microspores (categorized as the B pathway by SUNDERLAND 1973). Microspores containing two equal cells appear to be formed in sufficient numbers to account for the embryo yield of the anthers. Furthermore, no distinction between vegetative and generative-type nuclei can be recognized in most of the microspores containing 6–15 cells or early globular embryos. The observations on more advanced globular embryos might be interpreted as indicating that only where no generative cell is formed does embryo development continue its normal progress. Such an interpretation is, however, very controversial because, by this stage, the generative nucleus (or derived nuclei) originally present may have broken down or have failed to be recognized.

It is clear that divisions, other than that involved in the normal formation of the male nuclei, do occur in microspores of *N. tabacum* which have differentiated a vegetative and a generative cell and that in this the predominant pathway involves continuing division in the derivatives of the vegetative cell and no or only limited division of the generative nucleus (this is the A pathway as described by SUNDERLAND 1973). The present data, however, calls into question whether these divisions only proceed in a relatively small number of the microspores and suggests that in only a still smaller number does a globular embryo form and that this may be the ultimate limit of development in all or almost all of these grains. The concept of an initial equal division in uninucleate microspores initiating embryogenesis in anther cultures is supported by previous work in our laboratory with *Atropa belladonna* (RASHID and STREET 1973) and by the recent work of NITSCH and NORREEL (1973) on *Datura innoxia* which showed that a temperature shock applied at the time of the first haploid mitosis altered, in a proportion of the microspores, the axis of division resulting in two-celled microspores with no differentiation into vegetative and generative cells, that many of these grains remained viable in culture and that their formation was correlated with a significant enhancement in embryogenesis.

The evidence that degradative changes and subsequent establishment of a new pattern of cytoplasmic organization must occur in the vegetative cell

of tobacco microspores before this can give rise by division to meristematic-like cells (work of M. J. DUNWELL cited by SUNDERLAND 1973) would strongly suggest that the mitosis which gives rise to the vegetative and generative cells is a determinative step in normal pollen grain development which needs to be reversed if embryogenesis is to occur. Hence, it might be expected that microspores which had not taken this step would more readily embark upon embryogenesis. The existence of the B pathway indicates that this asymmetric division is in no way implicated in the determination of some polarity essential to a cell acquiring the capacity for embryogenesis.

The yield of plantlets following the culture of anthers excised at different stages does not pinpoint any very precise stage in microspore development essential to embryogenesis in tobacco. Successful cultures have been obtained with anthers excised within the time which elapses from the separation of the tetrads to several days after the peak of the first haploid mitosis (NITSCH 1969, SUNDERLAND and DUNWELL 1971). The synchrony of this mitosis is not tight so that uninucleate microspores are always present in anthers classed as at the binucleate microspore stage. In *A. belladonna, N. sylvestris,* and *D. innoxia* the anthers are, however, most productive of plantlets if excised whilst the majority of the microspores are still uninucleate.

The occurrence of suspensor-like cells in developing microspore embryos reported in tobacco by NITSCH (1969) and here for both this species and *N. sylvestris* is at so low a frequency that formation of such "suspensors" cannot be regarded as essential to successful embryogenesis. This is a further example of the extent to which embryology can depart from the classic embryology based upon studies of zygote segmentation and yet give rise to a perfectly normal embryo (MCWILLIAM, SMITH, and STREET 1974). It would, however, be of interest to know, when such "suspensors" are formed, whether they determine the axis of polarity of the developing embryo.

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