POLLEN ULTRASTRUCTURE IN ANTHER CULTURES OF DATURA INNOXIA

II. THE GENERATIVE-CELL WALL

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SUMMARY

In young pollen grains of *Datura innoxia*, a wall of the usual hemispherical type separates the 2 gametophytic cells initially and, in the electron microscope, appears as an electron-translucent matrix which is contiguous with the intine. Before detachment of the generative cell from the intine, the matrix decreases in thickness and in places is dispersed altogether leaving the plasmalemmae on either side of it in close apposition. A particularly prominent zone, triangular in profile, is left where the wall joins with the intine. After detachment of the cell, remnants of the matrix can be seen distributed irregularly around the cell and it is supposed that these are partly derived from material in the triangular zone as the cell is drawn away from the intine. The wall residues persist throughout the maturation phase of the pollen and are considered to be either callose resulting from incomplete digestion of the initial wall, or some other polysaccharide material which is unevenly laid down along the wall and concentrated at the junction with the intine.

In pollen induced into embryogenesis by anther culture, wall material is also distributed irregularly around the detached cell in a series of discrete zones, but these are more extensive than in *vivo*, closer together and in many instances highly dilated. The wall profiles thus have a beaded appearance, the 'beads' being connected together by short links of the 2 apposed plasmalemmae. The contents of the swollen zones have a similar electron density to that of the matrix in *vivo* but also show traces of a fibrillar component. It is postulated that this unusual swelling is a prelude to dispersal of the wall by disruption of the plasmalemmal links and to the establishment of cytoplasmic continuity between the 2 cells. The significance of such binucleate pollen grains in the formation of non-haploid embryos is discussed.

INTRODUCTION

It is generally considered that androgenic embryos arise by division of one of the male gametes in the cytoplasm of a non-functioning egg cell and genetic evidence has recently been provided in maize (Kermicle, 1974). In pollen induced into embryogenesis by anther culture, however, the generative cell has so far been observed to function as an embryo mother cell only in *Hyoscyamus niger* (Raghavan, 1976). In *Datura innoxia*, the generative cell can function in embryogenesis by entering into a series of complex fusion events with the vegetative cell, the so-called C pathway of pollen embryogenesis (Sunderland, Collins & Dunwell, 1974). The fusion products give rise to non-haploid embryos. The process of nuclear fusion envisaged demands that the generative-cell wall is in some way dispersed to allow cytoplasmic continuity between nuclei. In examining the ultrastructural features of embryogenic pollen grains in *D. innoxia*, therefore, we paid particular attention to the generative cell and its wall and...
searched for structural changes that might be associated with the proposed fusion process.

**MATERIALS AND METHODS**

*In vivo* observations were made on sectioned anthers of *Datura innoxia* Mill. in which the generative cell was in the process of detachment. Anthers were fixed and sectioned in the manner previously described (Dunwell & Sunderland, 1976a). *In vitro* observations were made on the anther cultures described in the latter paper.

**RESULTS**

*In vivo observations*

As indicated in the preceding paper (Dunwell & Sunderland, 1976a), the hemispherical wall that separates the two gametophytic cells partitions the cytoplasm of the microspore unequally, the smaller portion being assigned to the generative cell. In *D. innoxia*, this cytoplasmic distribution is most clearly seen in pollen grains that have the vegetative nucleus in a peripheral position close to the generative cell. Tangential sections cut through both nuclei in the median plane include little of the vacuole and thus display the bulk of the cytoplasm in each cell (Fig. 1). The generative cell is seen to be rich in ribosomes and to have a full complement of organelles. Plastids are mostly elongate, and, like those in the vegetative cell, have little internal structure beyond occasional lamellae, and ribosomes which fill the stroma (Fig. 2). Mitochondria are numerous and more electron-dense than the plastids; they are also mostly spherical and contain few cristae.

The disparity in cytoplasmic distribution to the 2 gametophytic cells appears to be less marked in *D. innoxia* than in some other species, for example *Dactylorchis fuchsi* (Heslop-Harrison, 1968) and *Nicotiana tabacum* (Vazart, 1971), in which the amount of cytoplasm assigned to the generative cell is so small that plastids are totally excluded and the number of mitochondria severely restricted. Participation of the generative cell in the C pathway of pollen embryogenesis in *D. innoxia* may possibly be related to the amount of cytoplasm assigned to the cell at the microspore division.

At first the hemispherical wall is a relatively thick barrier (Figs. 1, 3). The plasma-lemmae bounding the 2 cytoplasms are separated by an electron-translucent matrix

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Fig. 1. Tangential section through the generative and vegetative cells in a pollen grain of *D. innoxia* before detachment of the generative cell from the intine (stage 5 anther). The vegetative nucleus lies at the periphery of the grain. Portions of the vacuole (v) which is carried over from the microspore can be seen. Both the generative (gn) and vegetative nuclei (vn) have prominent nucleoli at this stage. The wall provides an uninterrupted barrier between the 2 cells and shows traces of electron-dense elements. × 12 000.

Fig. 2. Enlarged portion of the generative cell (gc) in *D. innoxia* after its detachment from the intine. Traces of the electron-translucent material present in the attached wall can be seen (arrows). Elsewhere the plasmalemmae of the 2 cytoplasms lie in close apposition. Note the elongated plastid (p) and the numerous mitochondria (m) with electron-dense cristae. × 38 500.
which contains elements of a more electron-dense nature. These latter may correspond with inclusions reported in other species, for example, the ‘discontinuous electron-dense core’ of Angold (1968) in *Endymion nonscriptus*, and the ‘middle lamella of heterogeneous material’ of Vazart (1969) in *Linum usitatissimum*. The wall at this stage reacts positively to tests for callose (Góraska-Brylass, 1967; Heslop-Harrison, 1968).

As reported in other species (Angold, 1968; Mepham & Lane, 1970; Echlin, 1972), the hemispherical wall starts to decrease in thickness while the generative cell is still attached to the intine. In places, the wall matrix and other elements disappear altogether and leave the 2 plasmalemmatae in close apposition (Fig. 4). The thinning is due to dissolution of callose (Góraska-Brylass, 1967; Heslop-Harrison, 1968). In *D. innoxia*, dissolution appears to be incomplete and a distinctive zone, triangular in profile, is left at the junction of the wall with the intine (Fig. 4).

After detachment of the cell, residual wall material can still be seen and is distributed irregularly around the cell (Fig. 2). Some of this material may be derived from the triangular zone as the cell is drawn away from the intine. In more mature pollen grains, the generative cell is compressed and its outline obscured by the dense cytoplasm that develops in the vegetative cell (see fig. 3 in Dunwell & Sunderland, 1976a). At high magnification, however, traces of wall material can still be observed. In the retention of isolated zones of wall material around the detached generative cell, *Datura* pollen resembles the pollens of *Linum usitatissimum* (Vazart, 1969) and *Nicotiana tabacum* (Dunwell & Sunderland, 1974).

**In vitro observations**

Structural changes associated with the detachment process in the pollen of cultured anthers is less easily studied owing to the low frequency of viable pollen grains present and to the rapid onset of division in the vegetative cell. However, after culture for 72 h, pollen grains can be observed in which the generative cell is completely detached from the intine (checked by serial sections) and the vegetative cell still in interphase (Fig. 5). These correspond with grains about to enter the C pathway of embryogenesis as assessed in the light microscope. The vegetative cell in the type C grains shows only a thin layer of parietal cytoplasm and a large vacuole, which indicates that little cytoplasmic synthesis occurs during detachment of the generative cell from the intine. This is the converse of the situation in vivo. The generative cell has the usual complement of organelles but a different wall profile. As in vivo, wall material is distributed irregularly around the cell in a series of discrete zones, but the zones are more extensive and closer together, so that the profiles have a distinct beaded appearance (Fig. 6).

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**Fig. 3.** Enlarged portion of the wall (gw) that separates the generative cell (gc) from the vegetative cell in a young pollen grain of *D. innoxia* before dissolution of callose. Electron-dense elements can be seen in the wall. gc, generative nucleus. ×44,000.

**Fig. 4.** Enlarged portion of the wall as in Fig. 3 after dissolution of callose. Two small zones of residual wall material can be seen (arrows) and a more extensive zone (tz), triangular in profile, at the junction with the intine. Electron-dense elements are prominent in this zone. ×26,000.
The zones occupied by wall material are connected by short links of the two apposed plasmalemmae. The wall material itself has a similar electron density to that of the matrix seen in vivo except that at high magnification fibrillar elements are visible (Fig. 7). The wall zones vary in size between grains and also in the same grain; some are exceedingly large and give the impression that they are dilating.

The presence of constrictions in the wall of the detached generative cell, composed only of the 2 apposed plasmalemmae, suggests that dissolution of callose does occur prior to detachment of the cell, as in vivo. However, after detachment of the cell, there appears to be a swelling of the residual wall material. Since fusion of the generative and vegetative nuclei is known to occur in these grains it seems highly probable that the beaded appearance of the generative-cell wall is in some way connected with the fusion process. It is suggested, therefore, that the swelling of the wall zones is a prelude to disruption of the tenuous connecting plasmalemmal links and to dispersal of the wall material in double-membraned vesicles. Confirmation of this hypothesis must await the finding, in the electron microscope, of type C grains with the 2 nuclei actually in mitosis.

While definite evidence of the disruption of the plasmalemmal links is lacking, evidence of membrane disruption can be seen elsewhere in the type C grains. As in the type A grains described in the preceding paper, the tonoplast is coated by a deposit of highly osmiophilic material associated with a vesicular component (Figs. 8, 9). There appears to be an aggregation of vesicles to give large osmiophilic droplets. At the same time the tonoplast itself is disrupted (Fig. 5) and the vacuole contains ribosomes and other unidentified materials which pass out through the disrupted membrane. The osmiophilic droplets resemble lipid but are clearly different in composition from the spherical lipid droplets visible elsewhere in the cytoplasm and which are less electron-dense (Figs. 6, 8). The deposits on the tonoplast may represent some form of storage centre for membrane lipids during the transformation of the pollen's developmental programme, and might be analogous to the 'lipid centres' observed in embryogenic pollen grains of *Nicotiana tabacum* (Dunwell & Sunderland, 1975).

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**Fig. 5.** Tangential section through the generative and vegetative cells in an embryogenic pollen grain of *D. innoxia* after detachment of the generative cell from the intine. The vegetative nucleus lies at the periphery of the grain as in Fig. 1. The vacuole (v) carried over from the microspore is still present and a vacuole (gv) has developed in the generative cell. Cytoplasmic density in the vegetative cell is similar to that in Fig. 1. The tonoplast is broken in places (arrows) and ribosomes can be seen in the vacuoles in both cells. The tonoplast is also associated with osmiophilic droplets (od). Stage 3 anther cultured for 72 h at 28°C in darkness. H medium. ×10000.

**Fig. 6.** Enlarged view of the generative cell after detachment from the intine in an embryogenic pollen grain of *D. innoxia*. Note the beaded appearance of the generative-cell wall. Connexions between the beads cannot always be traced. Lipid droplets (l) are present in the vegetative-cell cytoplasm. ×13000.
DISCUSSION

Although many references have been made in the past to thinning of the generative-cell wall before and after detachment of the cell from the intine, the processes involved are not fully understood. The data of Górska-Brylass (1967, 1970) and of Heslop-Harrison (1968, 1972) suggest that the wall is composed wholly of callose and that this material is completely dissolved by the time the cell is detached. The presence of residual wall material in the detached generative cell of *Datura innoxia* is not easily reconciled with this view unless the residues consist of callose resulting from incomplete digestion. The possibility cannot be excluded that the residual material comprises some other polysaccharide material which is laid down unevenly along the wall in conjunction with callose and possibly concentrated at the junction with the intine (Fig. 4). Information on the extent of the surface area of the cell before and after detachment is also lacking. Stretching of the existing structure may occur as the cell is drawn away from the intine, but it is equally possible that extra membrane units are intercalated into the existing structure, especially in respect of the plasmalemma of the vegetative-cell cytoplasm. If detachment of the cell is accompanied by *de novo* membrane formation then this too will influence the distribution of the wall residues.

In the case of the embryogenic pollen, because the switch in developmental programme is effected when the generative cell is still attached to the intine (Dunwell & Sunderland, 1976a), it can reasonably be argued that the switch arrests the whole process of detachment and the accompanying dissolution of callose. Certainly the data presented on type A embryogenesis in the preceding paper are consistent with this interpretation. When the vegetative cell is dividing for the first time, there is a relatively thick barrier of electron-translucent material between the two cells (see fig. 4 of Dunwell & Sunderland, 1976a), and this is retained as the cell divides further. Retention of such a barrier can be seen as a determining factor in the quiescence of the generative cell and its non-functioning in embryo formation. Yet the detachment process is not halted in all the embryogenic pollen. Because the pollen in an anther is slightly asynchronous, it could be that some of the grains (the most advanced) have already received instructions for detachment to proceed, although there is no manifestation of it, when the switch to embryogenesis takes effect. If this is so, detachment must then be able to proceed to completion despite the arresting influence of the switch in programme. That the switch does become effective before there is any

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Fig. 7. Higher magnification of part of the generative-cell wall illustrated in Fig. 6. Traces of fibrillar elements are visible in the electron-translucent wall material. gc, generative cell; vc, vegetative cell. ×54,000.

Fig. 8. View of the tonoplast in the vegetative cell of an embryogenic pollen grain of *D. innoxia* and the osmiophilic material (arrows) deposited upon it. Vesicles are interspersed among the osmiophilic droplets. Note the difference in electron density between the tonoplast-coating and the spherical lipid droplets (l) in the cytoplasm. ×20,000.

Fig. 9. Tangential section giving a surface view of the osmiophilic coating on the tonoplast. ×62,000.
visible sign of detachment is indicated by the lack of cytoplasmic synthesis in the vegetative cell.

With regard to the unusual beaded appearance of the generative-cell wall in cultured anthers, further work is needed to determine the nature of the mechanism involved. De novo synthesis of new wall material seems unlikely since this might be expected to lead to joining up of the existing zones to form a continuous wall instead of the larger separate zones observed. A passive process of swelling would seem a more plausible explanation. We have suggested that the swelling might disrupt the plasmalemmata linking the wall zones together and thus establish conditions appropriate for fusion of the two adjacent nuclei when they enter mitosis simultaneously. Dispersal of the wall could also be aided by the atypical enlargement of the generative nucleus which occurs in these grains by endoreduplication (Sunderland et al. 1974).

A point not answered by the present data but which has bearing on observations discussed in the other 2 papers of this series is the angle of orientation of the spindle upon which the generative and vegetative chromosomes divide. At present this can only be deduced from observations made in the light microscope. These show that, after 4–5 days of culture, the anthers contain, in addition to multicellular proembryos derived by the A pathway, bicellular proembryos in which the nuclei are unusually large and identical in both size and staining properties. The size of the nuclei, and the absence of a generative cell, identifies these proembryos as products of the C pathway. In general, these bicellular non-haploid proembryos have straight dividing walls, and in many instances, though not all, the wall partitions them into 2 equal cells. It follows that the type C grains divide relatively consistently in a similar plane. This plane, however, is different from that in which the vegetative cell divides when it functions as the embryo mother cell in the A pathway. As shown previously (Dunwell & Sunderland, 1976a), the first division of the vegetative cell takes place invariably in the same plane as the preceding microspore division. The first division is thus unequal and the bulk of the vacuole present in the vegetative cell is distributed to the larger of the 2 daughter cells. In the C pathway, on the other hand, the vacuole appears to be partitioned equally from the start and this suggests that the common spindle on which the generative and vegetative nuclei divide is oriented at right angles to that of the microspore division. The cell plate thus develops across the vacuole in a plane perpendicular to the pollen wall and in some cases partitions the vacuole equally. A similar situation is probably involved in the formation of bicellular proembryos from the anomalous pollen grains described in the next paper of this series (Dunwell & Sunderland, 1976b).

REFERENCES

Pollen ultrastructure in anther cultures. II


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