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Endosperm responses to irradiated pollen in apples

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Summary. The cytological effects of pollen γ-irradiation at 50 and 100 krad on both embryo and endosperm development were studied in *Malus* × *domestica*. Fruit and seed set were reduced by increasing doses of pollen irradiation, while embryo sacs resulting from the treatments differed in number and morphology of endosperm nuclei and in the presence or absence of an embryo. Nuclear abnormalities, distinguished from normal nuclear behaviour in embryo sacs derived from unirradiated pollen, included enhanced numbers of polyploid restitution nuclei, bridges between nuclei, excluded metaphase chromosome fragments and disrupted mitotic synchrony. Generally, a high dose of pollen irradiation (100 krad) generated an all-or-nothing response in the embryo sac, either creating highly abnormal embryos and/or endosperms which aborted, or showing relatively normal development. Callus produced from excised cellular endosperm differed in average genome size, that derived from 100 krad pollen being smaller than that from unirradiated pollen.

Key words: Endosperm – Pollen irradiation – *Malus* – Fluorometry

Introduction

Pollen irradiation disturbs double fertilisation, and subsequently the development and interactions of embryo and endosperm, in a dose-related manner.

Genetic and developmental phenomena associated with irradiated pollen include mutational damage (Werner et al. 1984), selective gene transfer (Shizukuda et al. 1983; Borrino et al. 1985); "egg transformation" (via incorporation of fragments of male DNA after high pollen irradiation doses, Pandey 1978, but see Chyi and Sanford 1985); and the mentor pollen effect used to overcome incompatibility barriers (Wolf and van Tuyl 1984). The degree of matromorphy of the mature progeny is inferred from the frequency of transfer of marker genes carried by the irradiated pollen. In contrast to earlier descriptive work (Brewbaker and Emery 1962; Nishiyama and Uematsu 1957; Vassileva-Dryanovska 1966a, b, c) the cytological events after fertilisation of the egg and fusion nucleus have not been followed, and the endosperm largely ignored despite its potential as a matromorphic or transformed tissue.

This paper combines evidence from embryo sac dissection techniques (Allington 1985) and fluorescence microscopy to outline a new approach to the study of endosperm in exalbuminous seeds. Apple (*Malus* × *domestica* Borkh.) was chosen as the model system since it has 1) binucleate pollen (so irradiation occurs prior to division of the generative nucleus, Marcucci et al. 1984); 2) a large accessible embryo sac with a prolonged free nuclear stage of the endosperm; 3) seed parts, including endosperm, which are readily cultured in vitro (James et al. 1984) and 4) shown a possible 'Hertwig' response, as James et al. (1985) noted the reappearance of a small number of embryos after high pollen irradiation doses following a decline in embryo numbers at medium level irradiations.

Materials and methods

1 Pollen collection, irradiation and pollination

Pollen was collected from *Malus* × *domestica* cv. 'Baskatong' which carries the dominant homozygous gene for anthocyanin production in all tissues (*RR*). Pollen was irradiated in Petri dishes using a ^{60}Co source of γ-irradiation (Meat Research Institute, Langford) at a dose rate of 96.679 krad/h to give final doses of 0, 50 and 100 krad. Pollinations were carried out on orchard trees of 'Cox's Orange Pippin' after emasculation of flowers and their reduction to four per cluster. Clusters were bagged after pollination to eliminate contamination by foreign pollen.

2 Fruit sampling and dissections

Fruitlets were sampled at 13–35 days after pollination when the endosperm is mostly free-nuclear, and at 57–65 days after pollination when it is cellular and the embryo is heart-shaped. At the first sampling, most swollen seeds were fixed in ethanol:acetic acid (3:1) for 24 h, then transferred to 70% ethanol for storage. Fixed or fresh embryo sacs were dissected out intact under a stereo dissecting microscope into 0.2 M phosphate buffer (pH 8). Seeds from fruitlets remaining 57–65 days after pollination were removed aseptically. The contents of each seed (nucellus, embryo and endosperm) were separated and placed next to one another on Linsmaier-Skoog hormone-free media (James et al. 1984). Cultures were incubated at 22 °C in the dark, and subcultured every four weeks.

3 Staining and photometry

After incubation in phosphate buffer for 10 min fixed embryo sacs were stained with the DNA specific fluorochrome 4′-6-diamidino-2-phenylindole (DAPI) (10 µg/ml in 0.2 M phosphate buffer, pH8) for 2 h in the dark. They were transferred to buffer alone for 10 min then gently mounted in glycerol with phenylenediamine to reduce dye fading, and the coverslip sealed with nail varnish. Embryo sacs from ovules between 19 and 22 days after pollination were found to be most suitable for fluorometric evaluation of endosperm nuclear DNA contents. For this, each replicate consisted of one embryo sac from each pollen irradiation treatment, 0, 50 and 100 krad, taken from comparably sized ovules, and, when possible, a diploid tissue standard (apple root tips or integuments, fixed concurrently with embryo sacs). Relative DNA values of endosperm nuclei were measured using a Zeiss SF photometer attached to an epifluorescence microscope. Readings were daken on a transect of the embryo sac from the embryo zone to the chalazal end.

Relative DNA values of endosperm callus nuclei were also determined using DAPI fluorometry. Small pieces of callus were fixed overnight in 3:1 ethanol:acetic acid, hydrolysed for 5 min in 45% acetic acid at 60 °C, squashed and then stained as above.

Results

Intact embryo sacs were isolated from ovules from about 13 to 35 days after pollination, before and after fixation, and their nuclei counted with their relative positions in the endosperm noted after DAPI staining (Fig. 1). The normal, untreated apple embryo sac is of the Polygonum type (Maheshwari 1950) and its three antipodal cells degenerate soon after fertilisation (Wanscher 1939). In this study, the free-nuclear stage of the endosperm of 'Cox' persisted for approximately 30 days after pollination, by which time waves of mitoses had produced up to 1,500 free endosperm nuclei and the embryo was globular. The embryo sac elongated with the ovule so it extended the length of ovule. Endosperm cell wall formation commenced at the micropylar end when the ovule was from 5–6 mm long. The haustorial (but unbranched) chalazal end of the endosperm did not become cellular but disintegrated.

Growth of fruitlets, ovules and embryo sacs derived from 0 krad and 50 krad pollen lagged behind that of tissues derived from 0 krad and 50 krad pollen but differences only became marked more than 16 days after pollination. The slower rate of ovule elongation was associated with a delayed onset of endosperm cell wall formation in embryo sacs derived from irradiated pollinations. Pollen irradiation effects on endosperm development were pronounced, but natural variation in nuclear number, size and morphology within and between normal endosperms derived from unirradiated pollen must be considered when examining treated endosperms from 50 krad and 100 krad pollen.

Number of endosperm nuclei

Numbers of nuclei in endosperms from ovules of comparable size and age differed within and between treatments (Table 1, Fig. 1). Endosperms resulting from 50 and 100 krad pollen irradiations contained, on average, less than half the number of nuclei of normal (0 krad) endosperms. However, the range in number was greatest after the 100 krad pollen dose than after 50 krad, as several sacs from the 100 krad treatment possessed fewer than 25 nuclei, and two contained more than 200, 20 days after pollination.

Morphology of endosperm nuclei

In normal embryo sacs up to eight large, lobed chalazal nuclei with diffuse chromatin were always observed, frequently densely packed together, making DNA determination difficult. These were not antipodal nuclei as they lay within the central cell without their own cell walls. These were never observed in division, but waves of mitosis were occasionally observed proceeding from the centre of the embryo sac towards the chalazal and micropylar ends, resulting in many endosperm nuclei in synchronous mitotic stages (Fig. 1). Spherical metaphase nuclei with highly condensed chromosomes and persistent (unstained) nucleoli were often observed in mitotically active sacs, in the chalazal zone, and nuclei of different ploidy levels were often found adjacent to one another. Their chromosome counts in one normal sac ranged from 36 to 108 (in apple, $2n = 34$).

No nuclear abnormalities were observed in normal sacs as nuclei were regularly spaced and evenly sized, irregular lobing occurring only in the large chalazal nuclei. However, 50 krad endosperm generally contained highly lobed nuclei, chromatin bridges between nuclei at telophase and persisting into interphase, and acentric fragments excluded from metaphase plates (Figs. 1 and 2a, b, c, and Table 1). What were presumed to be nuclear fusions were more common in endosperms derived from 50 krad pollen than in those from 0 krad pollen (Fig. 2d). Embryo sacs from

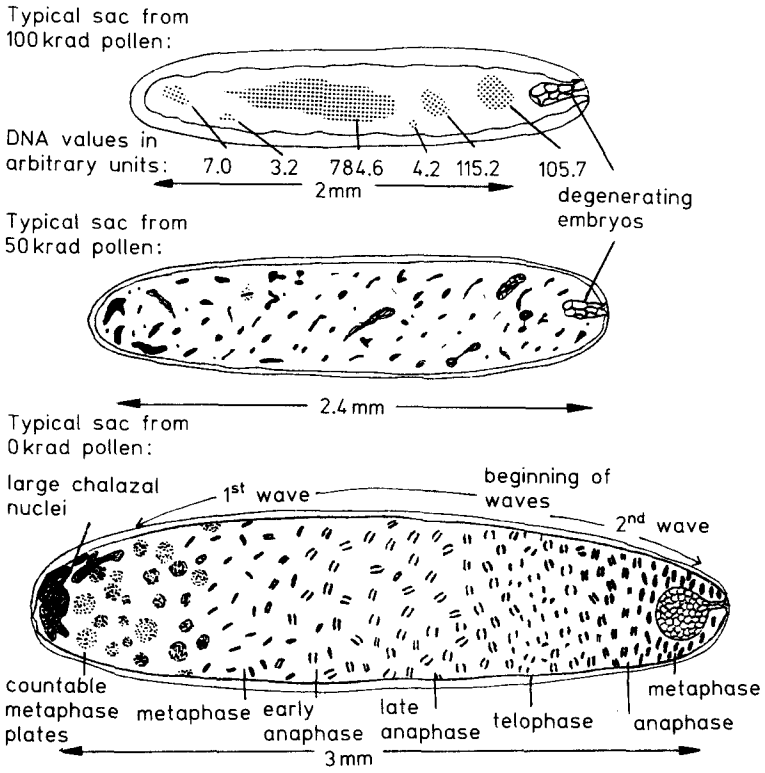

Fig. 1. Representative embryo sacs, showing free nuclear endosperm and embryos, 20 to 22 days after pollination with 0, 50 and 100 krad irratiated pollen. Drawn from life after staining with the DNA-specific fluorochrome DAPI

Table 1. Relationship between endosperm size, embryo size and occurrence of nuclear bridges in apple embryo sacs after pollen irradiation at 0, 50, and 100 krad. – : missing data

Days post pollination		No. endosperm nuclei	No. of bridges	No. of embryo cells
0 krad	19	119	0	30
	19	140	–	–
	19	134	–	–
	20	236	–	–
	20	106	0	24
	20	176	0	12
	20	108	0	18
	22	500	0	33
	22	550	0	48
	22	367	0	Many
50 krad	20	75	–	–
	20	109	5	10
	20	101	2	–
	20	65	0	0
	20	79	4	0
	20	220	2	–
	20	131	0	20
	22	106	1	12
100 krad	20	110	0	–
	20	7	0	5
	20	16	0	5
	20	6	0	12
	22	205	0	8
	22	24	1	0
	22	340	1	0

100 krad pollinations possessed either grossly abnormal endosperms, with very few nuclei of which at least one was enormous with many lobes and nucleoli (Figs. 1 and 2e), or relatively normal endosperms with evenly sized and spaced nuclei. This "all-or-nothing" growth of endosperm from 100 krad pollen differs from that of endosperm from 50 krad pollen, many of which developed to an intermediate size despite nuclear abnormalities (Table 1, Fig. 1).

DNA levels of endosperm nuclei

Relative nuclear DNA contents varied within normal and treated endosperms and the spread of DNA values increased with increasing pollen irradiation dose (Fig. 3). Frequency distributions of nuclear DNA contents from normal endosperms were generally more peaked than those from irradiated pollinations, indicating a greater degree of synchrony of the nuclear cycling as well as less ploidy variation within the endosperm. Rarely, distributions of nuclei from endosperms derived from irradiated pollen resembled those of normal (0 krad) endosperms (Fig. 3).

The location of polyploid nuclei within the endosperm varied between treatments (Fig. 4). Generally, DNA contents were relatively constant around an embryo from an unirradiated pollination in the zone which undergoes cell wall formation first. In most endosperms from 50 krad pollen polyploid nuclei were

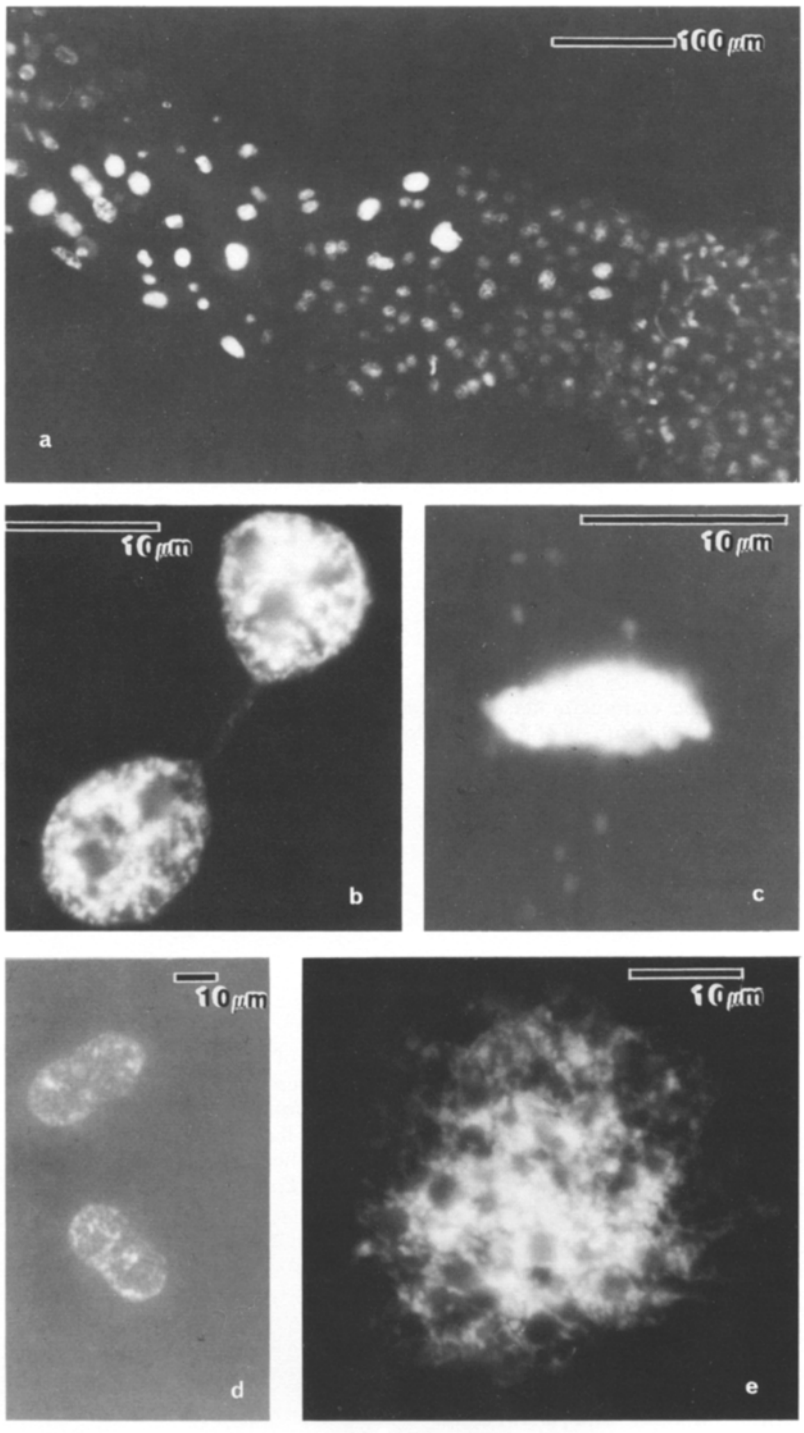

Fig. 2. Nuclear abnormalities in endosperm derived from 50 krad (**a, b, c, d**) and 100 krad (**e**) irradiated pollen. **a**) general view of section of 50 krad-derived endosperm, showing nuclear abnormalities and DNA content variation, measured from intensity of DAPI fluorescence; **b**) chromatin bridge between two nuclei; **c** acentric fragments excluded from a metaphase plate; **d** probably nuclear fusions; **e** abnormally large centrally-located nucleus from 100 krad-derived endosperm. Note many lobes and unstained nucleoli

distributed randomly through the endosperm and often occurred close to the embryo (Fig. 4). Endosperms derived from 100 krad pollen were usually highly abnormal but, as with those from 50 krad pollen, the occasional exception possessed a similar ploidy distribution to that along most normal sacs.

Embryo and endosperm persistence

Two 50 krad and two 100 krad embryo sacs were found lacking embryos 20 days after pollination (Table 1). Later, after endosperm cell wall formation, greater differences in endosperm and embryo persistence were

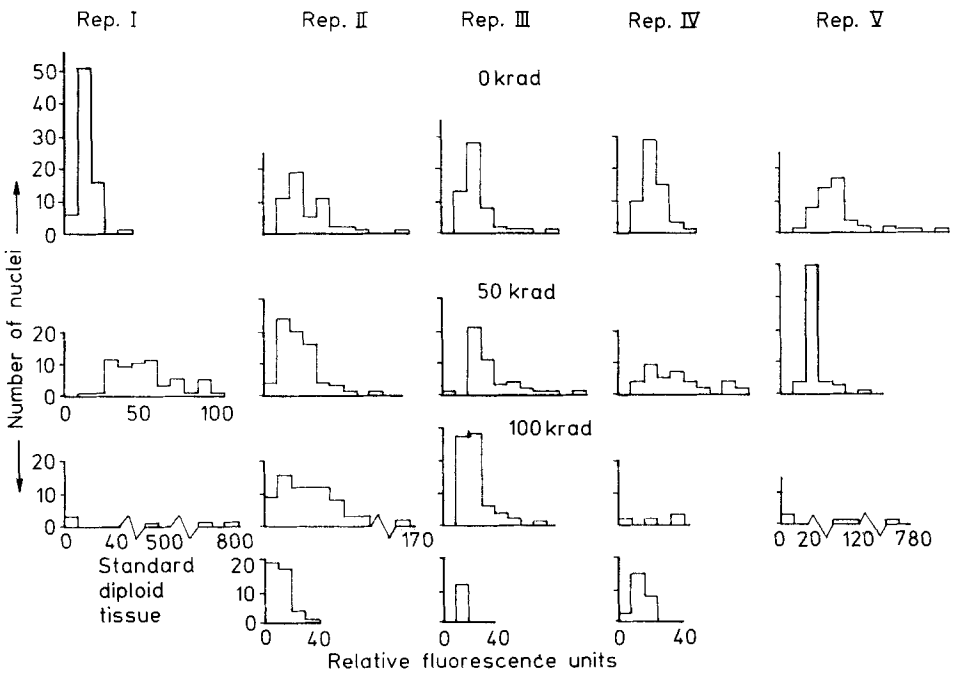

Fig. 3. Frequency distribution of relative DNA values of apple endosperm nuclei derived from three pollen irradiation treatments: 0, 50, and 100 krad; determined by microfluorometry after DAPI staining of five replicates of each treatment, with three controls of standard diploid tissue

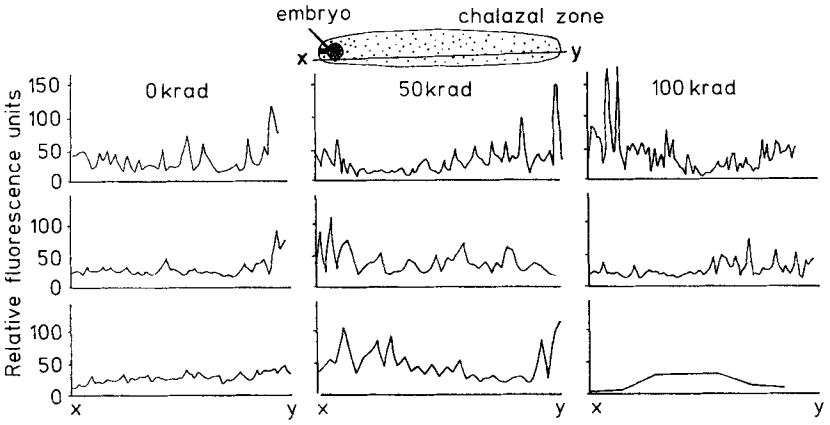

Fig. 4. Plots of relative DNA values of apple endosperm nuclei against their relative distance from the embryo on a transect (x–y) across the free-nuclear endosperm; with three pollen irradiation treatments: 0, 50, and 100 krad. DNA values shown in relative fluorescence units after DAPI microfluorometry of three replicates of each treatment

apparent (Table 2). By this time, some selection against highly abnormal embryo sacs will have occurred. However, more than 30% of swollen 100 krad seeds showed normal development of both embryo and endosperm, but only a quarter of swollen 50 krad seeds were normal in this respect. Very few 100 krad seeds possessed a poorly developed embryo, they either contained a normal embryo or none at all. This contrasts with the response of 50 krad seeds of which one-fifth contained weak embryos.

Endosperm callus

While the same proportion of endosperms from 0 krad and 50 krad pollen irradiations formed callus, the 100 krad pollen treatment resulted in endosperms less prone to callus formation (Table 2). Initial vigour of the endosperm callus, indicated by the number of repli-dish wells filled by callus four months after endosperm excision, decreased with increasing pollen irradiation dose (Table 2). Fluorometric determination showed

Table 2. Seed development 57 to 65 days after pollination with unirradiated and irradiated pollen

Irradiation level (krad)	Mean no. seeds/fruit (±SE)	Mean no. swollen seeds/fruit (±SE)	Total no. seeds dissected	% of total seeds dissected				% endosperms callused	Mean no. wells filled[b] (±SE)
				Components normally developed[a]	Poorly developed[a] embryo and endosperm	No embryo but endosperm	No embryo or endosperm		
0	6.1±1.4	6.0±1.5	58	96.6	0	3.4	0	54.4	4.0±1.41
50	5.9±1.5	5.0±1.3	50	26.0	20.0	50.0	4.0	54.2	2.5±1.63
100	4.4±1.9	1.8±1.9	38	31.6	2.6	50.0	15.8	43.8	1.3±0.65

[a] Poor development = not beyond heart-shaped (embryo); small and thin (endosperm). Normal development = cotyledons and radicle discernable (embryo); plump and creamy-coloured (endosperm)

[b] Number of 1 cm² wells filled by endosperm callus in a 25-well repli-dish, over three months with 3 subculturings

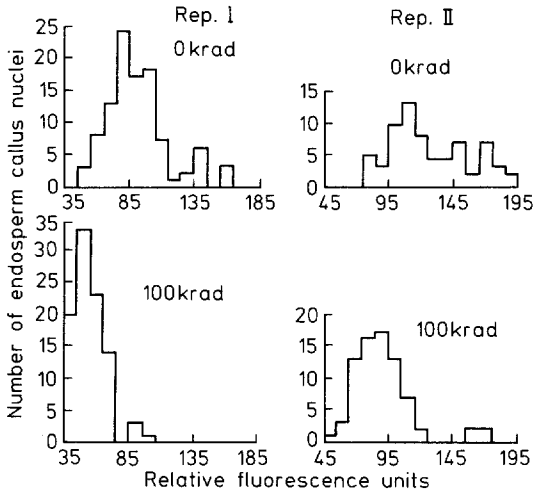

Fig. 5. Frequency distributions of relative DNA values of nuclei from apple endosperm callus, derived from 0 and 100 krad irradiated pollen. DNA values shown in relative fluorescence units after DAPI staining

that for endosperms arising after the 100 krad pollen treatment nuclear DNA contents had declined (Fig. 5) implying a smaller genome than that of callus derived from 0 krad pollen.

Discussion

Although the 3n-6n-12n-6n-3n gradient of ploidies observed by Wanscher (1939) in the apple cv. 'Woldike Pigeon' endosperm was not seen here, polyploidy or polyteny does occur in the normal 'Cox' endosperm. Structures interpreted as nuclear fusions and proposed as the primary cause of ploidy variation in maize endosperm (Lin 1977) were seen in the current study. However, frequent observation of metaphase nuclei within which the chromosomes were arranged spherically suggests that restitution cycles (endomitoses) are common in the normal endosperm of apple. In these endocycles, chromosomes do not complete anaphase but return to interphase within the same nucleus (Nagl 1979). Endoreduplication leading to polytene nuclei, rather than nuclear fusion giving polyploid nuclei, has been postulated recently for maize endosperm (Kowles and Phillips 1985). In the apple this could account for the large sizes and DNA contents of the chalazal nuclei which were never seen in metaphase. One or more of these mechanisms may operate in apple endosperm and this is the background on which pollen irradiation effects are superimposed.

The present study focused on post-fertilisation events, but the disturbance of these will be greatly influenced or even caused by disruption of earlier

embryological events. Pollen germination and tube growth in vitro and in vivo are only slightly inhibited by the irradiation doses used in the present study (James et al. 1985; Nicoll, unpublished data), so the very poor seed set achieved probably reflects the failure of fertilisation and subsequent events rather than an earlier rejection of unfit pollen by the stigma, or simply the inability of irradiated pollen to reach the embryo sac.

A simple dose-related response to pollen irradiation in the embryo sac is unlikely. Since half the generative nuclei in apple pollen at maturity are at G1 and half are at G2 (Forino and Avanzi 1981) the consequences of irradiation need not be exactly the same for a given dose. Whether irradiation occurs before or after DNA synthesis in a generative nucleus may influence its division into two male gametes, a process which often fails after doses of 150 krad in apple (E.C. Menhinick, pers. commun.) and sometimes after 100 krad (Nicoll, unpubl. data). This would result in only one male 'gamete' per tube being delivered to the female gametophyte, so (excepting heterofertilisation) only the egg or fusion nucleus would be fertilised. The unfertilised component of the embryo sac might die or it might attempt parthenogenesis as a haploid (egg) or diploid (fusion nucleus), or spontaneously double become dihaploid or didiploid, concomitantly homozygous at all loci.

Several factors – irradiation dose, state of the generative nucleus at irradiation, ability of the nucleus to divide, which embryo sac nucleus is fertilised, which can develop without fertilisation, which can double spontaneously without fertilisation and the crucial genomic balance between maternal, endosperm and zygotic tissue, as well as the degree of abnormal fusion which can be regarded as fertilisation – can account for their being no simple dose-related response to irradiated pollen.

Abnormal fertilisation results from a failure of the gamete(s) to fuse completely with the egg or fusion nucleus. Vassileva-Dryanovska (1966a) observed 'pycnotic' (highly condensed) male chromatin adhering to these nuclei in *Tradescantia* after pollen X-irradiation, in some cases remaining 'pycnotic' while the female nucleus entered division. The later stages of free nuclear endosperm development observed in the present study revealed many nuclear aberrations after use of irradiated pollen. Processes which could produce these bridges and/or aberrant polyploid nuclei in endosperms could include a) break-fusion-bridge (BFB) cycles; b) late replication of some chromatin; c) spindle malfunction and d) sister chromatid exchange.

Cell wall formation and subsequent callusing of endosperm are also affected by pollen irradiation in a dose dependent manner. Growth of a mixoploid tissue such as endosperm, from irradiated or non-irradiated pollinations, must involve considerable cell line competition and selection. Callus fromation in vitro prolongs the life of the normally ephemeral apple endosperm and must accentuate this competition, so the callus tissue inspected several months after endosperm excision may not have the same mixoploid constitution as its progenitor. However, despite a wide range of ralative DNA contents, both endosperm callus genotypes derived from 100 krad pollen investigated here had smaller nuclei overall than corresponding genotypes from unirradiated pollen, suggesting that incorporation of male DNA into the fusion nucleus after pollen irradiation was minimal or that male DNA was excluded during endosperm division. As no cases of excluded chromosome fragments were noted from the few endosperms from 100 krad pollen which were developing apparently normally, the exclusion must occur very early in endosperm development.

The implications of this situation deserve comment. Callus derived from irradiated endosperm could be maternal, diploid homozygous tissue, and each genotype will descend from a single meiotic product (the original megaspore which divided mitotically to form the eight-nucleate embryo sac). Preliminary work has examined the possibility of using endosperm callus derived from irradiated pollen for the segregation of isozyme marker genes through the determination of meiotic ratios. A number of enzymes are expressed in endosperm callus (S. Maganaris, pers. commun.) and if crosses and enzyme systems are planned so that each parent carries a recognisably distinct isozyme(s), contamination by male DNA, even after irradiation, could be detected. This is because the hybrid endosperm will contain a double dose of the maternal marker, but only a single dose of the male gene. Endosperm has been suggested as a suitable tissue for electrophoretic determination of meiotic ratios before (Schoen 1979), but the present system incorporating pollen irradiation has the added advantage of using potentially entirely maternal endosperm, rather than hybrid triploid endosperm. Again, the use of callus allows the system to be extended to species without persistent endosperm, and may permit regeneration of plants, as has been done already in apple endosperm (Mu et al. 1977). If endosperm-derived plants are available many more genes which are not expressed in callus may be used for analysis.

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References

Allington PM (1985) Micromanipulation of the cereal embryo sac. In: Chapman GP, Mantell SH, Daniels RW (eds) Experimental manipulation of ovule tissues. Longman, London, pp 39–51

Borrino EM, Caligare PDS, Powell W, MacNaughton IH, Hayter AM (1985) Cytological observation on the effects of pollen irradiation in diploid and polyploid crops. Heredity 54:165–170

Brewbaker JL, Emery GC (1962) Pollen radiobotany. Radiat Bot 1:101–154

Chyi YS, Sanford JC (1985) "Egg transformation" induced by irradiated pollen in *Nicotiana*: a re-examination. Theor Appl Genet 70:433–439

Forino LMC, Avanzi S (1981) Stage heterogeneity of generative and vegetative cells in pollen grains of dehiscent anthers of two cultivars of *Malus domestica* Borkh.: 'Golden Delicious' and 'Starkrimson'. Protoplasma 106: 205–210

James DJ, Passey AJ, Deeming DC (1984) Adventitious embryogenesis and the in vitro culture of apple seed parts. J Plant Physiol 115:217–229

James DJ, Passey AJ, MacKenzie KAD, Menhinick EC (1985) The effects of pollen irradiation of the development of the post-fertilisation ovule of apple (*Malus pumila* Mill.). In: Chapman GPC, Mantell S, Daniels R (eds) Experimental manipulation of ovule tissues. Longman, London, pp 210–224

Kowles RV, Phillips RL (1985) DNA amplification patterns in maize endosperm nuclei during kernel development. Proc Natl Acad Sci USA 82:7010–7014

Lin B-Y (1977) Ploidy amplification in maize endosperm. J Hered 68:143–149

Maheshwari P (1950) An introduction to the embryology of angiosperms. McGraw-Hill, London New York

Marcucci MC, Ragazzini D, Sansavini S (1984) The effects of gamma and laser rays on the functioning of apple pollen in pollination and mentor pollen experiments. J Hortic Sci 59:57–61

Mu S, Liu S, Zhan Y, Qian N, Zhang P, Xie H, Zhang F, Yan Z (1977) Induction of callus from apple endosperm and differentiation of the endosperm plantlet. Sci Sin 20: 370–376

Nagl W (1978) Endopolyploidy and polyteny in differentiation and evolution. North Holland, Amsterdam New York Oxford

Nishiyama I, Uematsu S (1967) Radiobiological studies in plants 13. Embryogenesis following X-radiation of pollen in *Lycopersicon pimpinellifolium.* Radiat Bot 7:481–489

Pandey KK (1978) Gametic gene transfer in *Nicotiana* by means of irradiated pollen. Genetica 49:53–69

Schoen DJ (1979) An angiosperm analogue to gametophyte analysis. J Theor Biol 79:543–546

Shizukuda N, Yamamoto K, Nakajim T (1983) Sexual transfer of an incomplete chromosome complement from *Nicotiana tabacum* L. to *N. rustica* L. Jpn J Breed 33:15–22

Vassileva-Dryanovska OA (1966a) Fertilisation with irradiated pollen in *Tradescantia.* Radiat Bot 6:469–479

Vassileva-Dryanovska OA (1966b) Development of embryo and endosperm produced after irradiation of pollen in *Tradescantia.* Hereditas 55:129–148

Vassileva-Dryanovska OA (1966c) The induction of haploid embryos and tetraploid endosperm nuclei with irradiated pollen in *Lilium.* Hereditas 55:160–165

Wanscher JH (1939) Contributions to the cytology and life history of apple and pear. R Vet Agric Coll Yearb Copenhagen, pp 21–70

Werner CP, Dunkin IM, Cornish MA, Jones GG (1984) Gene transfer in *Nicotiana rustica* by means of irradiated pollen. II. Cytogenetical consequences. Heredity 52:113–119

Wolf ST, van Tuyl JM (1984) Hybridization of Liliaceae: Overcoming self-incompatibility and incongruity. Hort Sci 19:696–697