DEVELOPMENT OF ENDOSPERM AND EMBRYO IN RECIPROCAL CROSSES BETWEEN 2X AND 4X IN *RAPHANUS SATIVUS*¹⁾

NOBUMICHI INOMATA

Faculty of Agriculture, Kyoto University, Kyoto 606

Received January 16, 1970

Collapse of immature seed is a common cause of sterility in crosses between diploids and their autotetraploid plants, *i.e.* *Brassica* (Howard 1939; Nishiyama and Inamori 1952, 1953; Nishiyama and Inomata 1966), *Datura* (Sansome *et al.* 1942), *Lycopersicon* (Cooper and Brink 1945), *Secale* (Håkansson and Ellerström 1950), *Zea mays* (Cooper 1951), *Raphanus* (Nishiyama 1952a), *Hordeum* (Håkansson 1953), etc.

Håkansson (1956) made embryological studies of seed development in reciprocal crosses between diploids and their autotetraploids using *Brassica oleracea* and *B. rapa*, and their interspecific crosses. Usually no viable seeds were produced, because of the failure of endosperm and embryo to develop normally. In autotetraploid *Brassica pekinensis* and diploid *B. chinensis*, no viable hybrid seeds were ordinarily obtained in their crosses, but hybrid embryos appeared to develop normally during the early developmental stage. In $2x \times 4x$, cell wall formation of endosperm was completely inhibited but in the $4x \times 2x$ cross, endosperms were smaller and cell wall formation took place earlier than in the parents (Nishiyama and Inomata 1966).

In *Raphanus sativus*, no hybrid seeds were obtained in the cross, $2x(\text{♀}) \times 4x(\text{♂})$, while small seeds were produced from the reciprocal cross, $4x \times 2x$ (Nishiyama 1952a, b). However, no embryological studies on seed development had been made until now. Cause of the failure of seed development in reciprocal crosses between diploid and autotetraploid *Raphanus sativus* was studied. The results are reported in this paper.

MATERIALS AND METHODS

Diploid *Raphanus sativus* L. cultivar Shogoin-Daikon and its autotetraploid were used. The autotetraploid was produced by the colchicine treatment of Nishiyama. The following crosses were made in 1965.

Raphanus sativus ($2n=18$) $\times$ *Raphanus sativus* ($2n=18$)

Raphanus sativus ($2n=18$) $\times$ *Raphanus sativus* ($2n=36$)

Raphanus sativus ($2n=36$) $\times$ *Raphanus sativus* ($2n=36$)

Raphanus sativus ($2n=36$) $\times$ *Raphanus sativus* ($2n=18$)

Flower buds were emasculated and pollinated with fresh pollen grains two days afterward in the greenhouse. The capsules 3, 6, 9, 12, 15, 18 and 21 days after pollination were treated with a mixture of 3 parts 95% ethanol and one part glacial acetic

1) Contribution from Laboratory of Genetics, Faculty of Agriculture, Kyoto University, Japan, No. 355.

acid for about 10 minutes, then fixed with Bouin's fluid for 24 hours. After embedding in paraffin, the sections were cut 11-15 micra thick and stained with Heidenhain's iron alum haematoxylin. Embrological observations of preparations were made using a microscope. Camera lucida was used to sketch embryos and endosperm.

A small number of hybrid seeds was obtained from reciprocal crosses and were sown in a greenhouse. The chromosome numbers of their plants were determined by the acetocarmine squash method, using PMC's.

RESULTS

Results of crosses

The percentage of seed set, estimated from the number of seeds obtained and the total number of ovules, in different crosses is shown in Table 1. Seed fertilities of $2x \times 2x$ and $4x \times 4x$ were above 85% and 75%, respectively, and that of $4x(\varphi) \times 2x(\sigma)$ was 70%. In the reciprocal cross, $2x(\varphi) \times 4x(\sigma)$, 35 capsules were examined and many ovules showed slight development, yet most seeds collapsed in the young embryonic stages. One good seed was obtained in $2x \times 4x$, its size similar to that of $2x \times 2x$. Morphological characteristics of the germinated plant were completely the same as the diploid parent, and its diploid nature was confirmed by chromosome count. It was probably raised by

Table 1. Results of crosses between diploid and tetraploid *R. sativus*

Cross combination	No. of flowers pollinated	No. of capsules examined	No. of seeds obtained	Seed set (%)	Average capsule length (mm)
$2x \times 2x$	37	32	148	85.2	57.0
$2x \times 4x$	36	35	1*	0.5	45.8
$4x \times 4x$	6	6	9	75.0	38.3
$4x \times 2x$	7	7	7**	70.0	33.9

* One seed was diploid

** All seven seeds were hybrid

Fig. 1. Seeds produced by artificial pollination.

A: normal seeds from the cross, $2x \times 2x$. B: hybrid seeds from the cross, $2x \times 4x$, extremely shrivelled. C: normal seeds from the cross, $4x \times 4x$. D: viable hybrid seeds from the cross, $4x \times 2x$.

Fig. 2. Embryo and endosperm in various developmental stages.

a: $2x \times 2x$, 6 days after pollination; 4-celled proembryo. b: $2x \times 2x$, 9 days after pollination; Embryo at globular stage. c: $2x \times 2x$, 12 days after pollination; Heart-shaped embryo and cell wall formation in the endosperm. d: $2x \times 2x$, 15 days after pollination; Partial cell wall formation of basal endosperm. e: $4x \times 4x$, 6 days after pollination; 4-celled proembryo. f: $4x \times 4x$, 9 days after pollination; Embryo at globular stage. g: $4x \times 4x$, 12 days after pollination; Pre-heart-shaped embryo and cell wall formation occurred in endosperm near the embryo. h: $2x \times 4x$, 6 days after pollination; 4-celled proembryo. i: $2x \times 4x$, 9 days after pollination; Degeneration occurring in endosperm. j: $2x \times 4x$, 12 days after pollination; Conspicuous degeneration occurring in endosperm. k: $2x \times 4x$, 12 days after pollination; Extremely large nuclei observed in endosperm near embryo. l: $2x \times 4x$, 12 days after pollination; Abnormal nuclei found in other parts of endosperm.

selfing. Seven capsules were examined in the cross, $4x \times 2x$, and seven good seeds were obtained. Their sizes were about one-fourth those of the parents (Fig. 1). All these seeds germinated and their plants were triploid. Capsule length was longest in $2x \times 2x$ and shortest in $4x \times 2x$.

Comparison of embryo growth rates

The developmental rate of embryos from reciprocal crosses was compared to that of the parents ($2x$ and $4x$) in various stages (Fig. 2). The number of ovules examined is shown in Table 2. Length of the whole embryo was measured up to the globular stage. After this embryo length, except the cotyledonous part, was measured. The developmental rates of embryos in different crosses showed no differences until 12 days after pollination (Fig. 3).

Table 2. Number of ovules used for growth rate measuring embryo

Cross	Days after pollination						
	3	6	9	12	15	18	21
$2x \times 2x$	0	4	7	7	3	3	4
$2x \times 4x$	1	3	7	10	2	1	2
$4x \times 4x$	1	6	6	4	4	2	2
$4x \times 2x$	1	8	9	5	5	3	1

Embryos of $2x \times 2x$ and $4x \times 4x$ grew markedly from the 12th day after pollination, while development of $2x \times 4x$ embryos stopped 15 days after pollination, some were already disintegrating. The developmental rate of $4x \times 2x$ embryos was less than that of $2x \times 2x$ and $4x \times 4x$ at the 15th day after pollination. After 18 days, growth of the $4x \times 2x$ embryos almost stopped, though embryos of the parents ($2x$ and $4x$) were growing normally. Twenty-one days after pollination, development of embryos in three crosses ($4x \times 2x$, $2x \times 2x$, $4x \times 4x$) was complete, though the sizes of $4x \times 2x$ embryos were about a half that of either parent ($2x$ or $4x$). Differences in embryo development clearly appeared 12 days after pollination in the cross, $2x \times 4x$, and 15 days after in the cross, $4x \times 2x$.

Embryology of hybrid seeds

I. $2x \times 2x$ and $4x \times 4x$: Each ovary contained four to six ovules, of which one to four had been fertilized three days after pollination. Of all the ovules fertilized in these crosses, a one-celled proembryo was found in only one.

Within six days, the number of cells in the proembryo ranged from three to eight

m: $2x \times 4x$, 15 days after pollination; Collapsed endosperm. n: $2x \times 4x$, 15 days after pollination; Nuclei gathering in a particular part. o: $2x \times 4x$, 15 days after pollination; Nuclei varying in size. p: $2x \times 4x$, 18 and 21 days after pollination; Collapse of endosperm in all parts of the ovule. q: $4x \times 2x$, 3 days after pollination; One-celled proembryo. r: $4x \times 2x$, 6 days after pollination; Vacuoles in endosperm near embryo. s: $4x \times 2x$, 9 days after pollination; Embryo at globular stage and cell wall formation in endosperm. t: $4x \times 2x$, 12 days after pollination; Embryo at heart-shaped stage and cell wall formation in endosperm.

Fig. 3. Growth rates of embryos in reciprocal crosses between $2x$ and $4x$ of *Raphanus sativus*, —: 95% fiducial interval.

in the cross, $2x \times 2x$, and from four to eight in the cross, $4x \times 4x$. Development of endosperm was poor in all parts of the embryo-sac, except in the region near the embryo, specifically near the suspensor (Fig. 2a and 2e).

Nine days after pollination, embryos became globular shaped and the number of cells in a transverse section of an embryo at the central axis ranged from four to eight in the cross, $2x \times 2x$, and from four to seven in the cross, $4x \times 4x$. The number of cells in longitudinal section was from five to nine in $2x \times 2x$ and from four to 12 in $4x \times 4x$. Endosperm also showed an increase in volume and endosperm nuclei were arranged in one to two layers throughout the whole embryo-sac, in which, a large central cavity was observed. Cell wall formation was not observed in any part of the endosperm, even near the suspensor (Fig. 2b and 2f).

Within 12 days after pollination, embryos of both $2x \times 2x$ and $4x \times 4x$ crosses began to change to a heart shape. When differentiation of the embryo proceeded from the globular to the heart-shaped stage, cell wall formation in endosperm began near the embryo (Fig. 2c and 2g). Some advanced embryos in crosses, $2x \times 2x$ and $4x \times 4x$, were early in the "torpedo" stage and endosperm in ovules with these embryos formed cell wall throughout except for the basal part.

Fifteen days after pollination, further development of embryos was conspicuous in both crosses; these were mostly in a late "torpedo" or "upturned U" stage. Cell wall formation in the basal part of the endosperm occurred partly in the ovule having well developed embryos (Fig. 2d).

Eighteen and 21 days after pollination, development of embryos was also conspicuous, especially, in the cotyledonous part. Development proceeded from the "upturned U" almost to the matured stage. Cell wall formation which existed poorly was observed in the basal endosperm.

II. $2x \times 4x$ and $4x \times 2x$: Three days after pollination, one to five fertilized ovules were observed in each pollinated ovary. It was difficult to observe embryo development in this stage, but one-celled proembryos were recognizable in both crosses (Fig. 2q).

Six days after pollination, the number of cells in an embryo varied from two to three in the cross, $2x \times 4x$, and from four to eight in the cross, $4x \times 2x$. As compared with development of embryos in other crosses, development of embryos in the cross, $2x \times 4x$ was somewhat late. Development of endosperm in the ovule of this cross was poor throughout except near the embryo and suspensor (Fig. 2h). In $4x \times 2x$, some vacuolization of endosperm near the embryo was observed (Fig. 2r), but no cell wall formation in endosperm occurred in any ovules.

Nine days after pollination, development of embryos in two crosses, $4x \times 4x$ and $4x \times 2x$, was better than in $2x \times 2x$ and $2x \times 4x$ (Fig. 3). The number of cells in transverse and longitudinal sections through the central axis of the embryo varied from four to nine and from five to nine, respectively, in the cross, $2x \times 4x$, it also varied from 14 to 15 and from 12 to 15 in the cross, $4x \times 2x$. Degeneration of endosperm in the cross, $2x \times 4x$, began near the suspensor. No endosperm nuclei existed in degenerating endosperm (Fig. 2i). In the cross, $4x \times 2x$, the embryo was in the globular stage, but cell wall formation was observed in all parts of the endosperm (Fig. 2s).

Twelve days after pollination, some embryos in the cross, $2x \times 4x$, were in a late heart stage. In some ovules, embryos, which did not develop well, remained in the globular stage. The number of cells in transverse and longitudinal sections through the central axis was eight in both. Embryos of the cross, $4x \times 2x$, were globular or "torpedo" in shape. Conspicuous vacuolization of endosperm due to degeneration was observed near the embryo (Fig. 2j) and abnormally large nuclei were found in endosperm near the embryo (Fig. 2k) in some other parts where endosperm development was poor (Fig. 2l). However, in the cross $4x \times 2x$, cell wall formation in endosperm was observed in all ovules (Fig. 2t).

Fifteen days after pollination, development of embryos stopped completely in the cross, $2x \times 4x$ (Fig. 3). From histological observations, embryos in the cross, $2x \times 4x$, appeared arrested at the globular or heart-shaped stage. Embryos of the reciprocal cross,

$4x \times 2x$, however, were smaller than those of the control, though they were already in the "upturned U" to maturing stage. Their developmental stage was similar to that of embryos in the control material 18 and 21 days after pollination. As the ovule was small, it was filled with developed embryo. No cell wall formation in the endosperm occurred in the cross, $2x \times 4x$, and different types of endosperm degeneration were observed; *i.e.* partial but conspicuous collapse of endosperm nuclei (Fig. 2m), gathering of the nuclei in a specific part (Fig. 2n) and variable size of endosperm nuclei (Fig. 2o). In the cross, $4x \times 2x$, embryo fully packed the ovule and little cell walled endosperm was left in some ovules, also cell wall formation occurred in basal endosperm at the charazal part.

Eighteen and 21 days after pollination, some globular or heartshaped embryos of the cross, $4x \times 2x$, were almost matured, though their size was about half that of the control (Fig. 3). Collapse of endosperm in the cross, $2x \times 4x$, was observed throughout (Fig. 2p), and no endosperm tissue remained in some ovules. Matured embryos almost filled their ovule in the cross, $4x \times 2x$.

These results are summarized in Table 3.

Table 3. Embryo and endosperm development 3-21 days after pollination in the $2x$ and $4x$ parents and their reciprocal crosses

Cross	Days after pollination						
	3	6	9	12	15	18 and 21	
<i>Embryo</i>							
$2x \times 2x$	globular shape		globular~ early "torpedo"		"walking stick"	"upturned U"	
$4x \times 4x$	"		"		late "torpede"	"	
$2x \times 4x$	"		globular~ heart shape		globular~ early "torpedo" (almost disintegrated)		
$4x \times 2x$	"		globular~ early "torpedo"		"upturned U"		
<i>Endosperm</i>							
$2x \times 2x$	developing endosperm		initiating cell wall formation		cell wall formation throughout	digested	
$4x \times 4x$	"		"		"	"	
$2x \times 4x$	developing endosperm		initiating abnormal vacuoles	begining to collapse	abnormalities of nuclei and cytoplasm		
$4x \times 2x$	"		cell wall formation throughout				digested

DISCUSSION

Triploid hybrid seeds of *Raphanus sativus* obtained from the cross, $4x \times 2x$, were viable and gave hybrid plants. Sizes of seeds were about one-fourth those of the controls ($2x \times 2x$ and $4x \times 4x$). Histological observation clearly showed that this small seed

size was mainly due to the small size of the hybrid embryos. No particular abnormalities were observed throughout the development of both embryo and endosperm, except that cell wall formation in the endosperm that took place a little earlier than in the control.

In the reciprocal cross, $2x \times 4x$, embryos developed normally like those of the other crosses until 12 days after pollination. After this stage, development ceased completely. The genome constitution of the embryo of this cross was the same as that of the cross, $4x \times 2x$. However, embryological study revealed that abnormalities in endosperm development appeared 9 days after pollination. Consequently, stoppage of embryo development in the cross, $2x \times 4x$, seems to be caused by abnormal development of the endosperm.

In the $4x \times 2x$ cross, cell wall formation in the endosperm took place earlier than in the parents ($2x \times 2x$ and $4x \times 4x$). In a similar $4x \times 2x$ cross of *Brassica*, the same phenomenon was observed (Nishiyama and Inomata 1966). The developmental rate of hybrid embryos in *Raphanus* was the same as that in *Brassica*, in the early stages, though in later stages development of the former was better than that of the latter (Nishiyama and Inomata 1966). Development of endosperm in *Raphanus* was poorer than in *Brassica* throughout all the stages, and it was difficult to count the number of nuclei in developing endosperm.

From a study of reciprocal crosses between *Avena strigosa* ($2n=14$) and *A. fatua* ($2n=42$), Kihara and Nishiyama (1932) assumed that the "stimulative strength" of the male gamete was excessive for initiation of mitosis of fertilized egg and polar nuclei, resulting in abnormally rapid nuclear division in both the endosperm and embryo, when a species of higher chromosome number was used as the male parent. Conversely, male gametes with a lower chromosome number than females, *i.e.* in the cross, *A. fatua* $\times$ *A. strigosa*, caused retarded growth of embryo and endosperm because of the less "stimulative strength" of the male nuclei. In reciprocal crosses of *Raphanus* studied here, however, no such phenomenon was found so far as early development of the embryo was concerned. Differences in growth rates of embryos 15 days after pollination, or later, might have been secondarily affected by abnormal development of the endosperm.

A phenomenon called somatoplastic sterility (Brink and Cooper 1939), in which seeds collapse followed by abnormal growth or overgrowth of the maternal tissue surrounding the embryo-sac, was assumed to be a primary cause of degeneration of seed development in crosses between diploid and tetraploid races of *Lycopersicon pimpinellifolium* (Cooper and Brink 1945) and *Medicago* (Brink and Cooper 1939), in interspecific crosses of *Primula* (Woodel 1960a, b), and in certain crosses of *Datura* (Satina *et al.* 1950; Sansome *et al.* 1942). In this last case, ovular tumors resulted. In this study of the four crosses of *Raphanus*, no overgrowth of maternal tissue surrounding the embryo-sac nor formation of ovular tumours in the endosperm was found.

Spontaneous chromosome breakage, which resulted in degeneration of the endosperm and abortion of the embryo, occurred frequently in the endosperm of hybrids, and occasionally in the self fertile species of *Lilium* and *Hyacinthus* (Brock 1954a, b, 1955). In endosperm of the hybrid, *Hordeum jubatum* ($n=14$) $\times$ *Secale cereale* ($n=7$), Cooper and Brink (1944) described occurrences of chromosome bridges, dumb-bell-shape nuclei and high-polyploid nuclei. Their occurrence was the cause of endosperm degeneration. In

the present investigation, no mitotic abnormalities in the endosperm of the cross, $2x \times 4x$, were observed in the early degenerating stage, but soon after abnormal vacuolization took place in this stage, abnormally large nuclei varying in size and their collapse were found in all parts of the endosperm.

In general, development of hybrid embryos was better in *Raphanus* than in *Brassica*. Degeneration of the endosperm which followed abnormal vacuolization progressed more slowly in *Raphanus*, as compared to *Brassica*.

SUMMARY

Embryological studies were made to clarify developmental processes, leading to seed collapse in crosses between diploid *Raphanus sativus* ($2n=18$) and its autotetraploid ($2n=36$).

Hybrid embryos of reciprocal crosses appeared to develop normally in the early developmental stage, but in the cross, $2x \times 4x$, they failed in general to develop beyond the globular stage because of degeneration of the endosperm. In the reciprocal cross, $4x \times 2x$, viable hybrid seeds were produced, though their sizes were only one-fourth those of their parents.

In the $2x \times 4x$ cross, cell wall formation in the endosperm was inhibited, and abnormal vacuolization of endosperm occurred in all ovules, resulting in various nuclear irregularities and degeneration of ovules.

In the $4x \times 2x$ cross, the amount of endosperm was smaller but cell wall formation took place in it earlier than in parental endosperm. Development of the embryo was faster than in the parents.

A possible cause for the failure of viable seed formation was discussed in relation to the various abnormalities observed in various developmental stages.

ACKNOWLEDGEMENT

The author is greatly indebted to Dr. K. Tsunewaki for his valuable suggestions on the manuscript.

LITERATURE CITED

- Brink, R. A., and D. C. Cooper, 1939 Somatoplastic sterility in *Medicago sativus*. *Science* **90**: 545-546.
- Brock, R. D., 1954a Fertility in *Lilium* hybrids. *Heredity* **8**: 409-420.
- Brock, R. D., 1954b Spontaneous chromosome breakage in *Lilium* endosperm. *Ann. Bot.* **18**: 7-14.
- Brock, R. D., 1955 Chromosome balance and endosperm failure in hyacinths. *Heredity* **9**: 199-222.
- Cooper, D. C., 1951 Caryopsis development following matings between diploid and tetraploid strains of *Zea mays*. *Am. J. Bot.* **38**: 702-703.
- Cooper, D. C., and R. A. Brink, 1944 Collapse of the seed following the mating of *Hordeum jubatum* $\times$ *Secale cereale*. *Genetics* **29**: 370-390.

- Cooper, D. C., and R. A. Brink, 1945 Seed collapse following mating between diploid and tetraploid races of *Lycopersicon pimpinellifolium*. *Genetics* **30**: 376-401.
- Håkansson, A., 1953 Endosperm formation after $2x$, $4x$ crosses in certain cereals, especially in *Hordeum vulgare*. *Hereditas* **38**: 57-64.
- Håkansson, A., 1956 Seed development of *Brassica oleracea* and *B. rapa* after certain reciprocal pollinations. *Hereditas* **42**: 373-396.
- Håkansson, A., and Ellerström, 1950 Seed development after reciprocal crosses between diploid and tetraploid rye. *Hereditas* **36**: 256-296.
- Howard, H. W., 1939 The size of seeds in diploid and autotetraploid *Brassica oleracea* L. *J. Genet.* **38**: 325-340.
- Kihara, H., and I. Nishiyama, 1932 Different compatibility in reciprocal crosses of *Avena*, with special reference to tetraploid hybrids between hexaploid and diploid species. *Japan. J. Bot.* **6**: 245-305.
- Nishiyama, I., 1952a Polyploid-studies in the *Brassicaceae*, I. The seed development in reciprocal crosses between diploid and tetraploid *Raphanus*. *Mem. Res. Inst. Food Sci. Kyoto Univ.* **3**: 1-7.
- Nishiyama, I., 1952b Polyploid-studies in the *Brassicaceae*, II. Free crossing between $2x$ - and $4x$ varieties in *Raphanus sativus*. *Mem. Res. Inst. Food Sci. Kyoto Univ.* **3**: 8-14.
- Nishiyama, I., and Y. Inamori, 1952 Studies on artificial polyploid plants, XVII. On the occurrence of unexpected $4x$ -hybrids from the cross $2x \times 4x$ in *Brassica*. *Bull. Res. Inst. Food Sci. Kyoto Univ.* **10**: 99-106.
- Nishiyama, I., and Y. Inamori, 1953 Polyploid studies in the *Brassicaceae*, IV. Hybridization between diploid *Brassica* species ($2n=20$) and their autotetraploids. *Mem. Res. Inst. Food Sci. Kyoto Univ.* **5**: 1-13.
- Nishiyama, I., and N. Inomata, 1966 Embryological studies on cross-incompatibility between $2x$ and $4x$ in *Brassica*. *Japan. J. Genet.* **41**: 27-42.
- Sansome, E., S. Satina, and A. F. Blakeslee, 1942 Disintegration of ovules in tetraploid-diploid and in incompatible species crosses in *Datura*. *Bull. Torrey Bot. Club.* **69**: 405-420.
- Satina, S., J. Rapaport, and A. F. Blakeslee, 1950 Ovular tumours connected with incompatible crosses in *Datura*. *Am. J. Bot.* **37**: 576-586.
- Woodel, S. R. J., 1960a Studies in British primulas, VII. Development of normal seed and of hybrid seed from reciprocal crosses between *P. vulgaris* Huds. and *P. veris* L. *New Phytologist* **59**: 302-313.
- Woodel, S. R. J., 1960b Studies in British primulas, VIII. Development of seed from reciprocal crosses between *P. veris* and *P. elator* (L.) Hill. *New Phytologist* **59**: 314-325.