Embryological and Cytological Studies on Seed Development
after Reciprocal Crosses between *Iris sanguinea*
Hornem. and *I. laevigata* Fisch.

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The development of seeds from the reciprocal crosses between two Japanese representative species, *Iris sanguinea* Hornem. ($2n=28$) and *I. laevigata* Fisch. ($2n=32$), was examined embryologically and cytologically, and the following results were obtained: 1) Any viable seeds were not obtained in either of the reciprocal crosses, and there was found an obvious difference of the time of seed failure between the crosses, namely, initial symptoms of seed failure appeared on around 6 and 15 days after pollination in *I. laevigata*×*I. sanguinea* and *I. sanguinea*×*I. laevigata*, respectively. 2) In the reciprocal crosses, the decisive cause for seed failure was degeneration of endosperm, and the endosperm degeneration was ascribed to cytological abnormality of endosperm nuclei, such as abnormal enlargement of nuclei and high frequency of mitotic aberrations involving chromosome bridges and lagging chromosomes. 3) Abnormal growth of maternal tissue, i.e., hypertrophy of nucellus was recognized in *I. laevigata*×*I. sanguinea*. Such hypertrophy was attributable to the early degeneration of endosperm. 4) The developmental stages of embryos suitable for initiating the artificial culture were on around 6 and 15 days after pollination for *I. laevigata*×*I. sanguinea* and *I. sanguinea*×*I. laevigata*, respectively.

Introduction

In Japan, most efforts for breeding of horticultural iris, one of the representative ornamental plants, have so far been directed only to the utilization of intraspecific variation due to natural mutations and varietal crossings. As the consequence, Japanese iris is still short of the charm of variety in color and type of flower as compared with some foreign irises such as bearded iris.

The genus *Iris* includes 200 or more species (RANDOLPH 1959) and therefore, it should hold true also for iris that interspecific cross prepares the effective way to increase genetic variation inclusive of synthetic amphidiploids. In *Iris*, however, there stands a strong barrier which hinders the development of hybrid seed and thus blocks the way of interspecific-cross breeding. This is, of course, the case also for Japanese iris: no viable seed can be obtained in most of the interspecific crosses not only between Japanese and other species but also among Japanese species (TOMINO 1963, SAKURAI and TOMINO 1969, 1970). Such being the case, interspecific hybrids involving Japanese iris have so far barely bred from *I. pseudacorus*×*I. ensata* (TOMINO and SAKURAI 1972) and *I. laevigata*×*I. ensata* (YABUYA and YAMAGATA 1975). For taking this barrier away, detailed studies are to be accumulated on the development of hybrid seed. But unfortunately, very few studies (SAWYER 1925, SMITH and CLARKSON 1956) have been made from this viewpoint up to now.

In the present study, the development of seed from the reciprocal crosses between two

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Japanese representative species, *I. sanguinea* Hornem. and *I. laevigata* Fisch., was examined embryologically and cytologically to clarify the cause of seed failure. In addition, the ovular age suitable for embryo culture was determined from the results obtained.

Materials and Methods

Two species of *Iris*, *I. sanguinea* Hornem. ($2n=28$) and *I. laevigata* Fisch. ($2n=32$), were self-pollinated respectively and cross-pollinated reciprocally. In crossing, flowers were emasculated one or two days before flowering and pollinated with fresh or stored pollen grains one or two days after emasculation in glass house or in the open air. The following method was applied for pollen storage. Anthers required for pollination were plucked from the buds shortly before dehiscence and pollen grains were gathered from each anther. They were packed with paraffin papers and stored in bottles with silica gel at 0°C , because the iris pollen stored under this condition had been proved to function as well as fresh ones for three months (SUZUKI 1969, unpublished).

On 0, 3, 9 and 27 days after pollination, capsules were measured for their width to evaluate their growth. On 3, 6, 9, 15, 18, 21, 27 and 36 days after pollination, on the other hand, ovules were taken out from capsules and fixed, being submitted to embryological and/or cytological observations to clarify their seed development.

Embryological observation

Ovules of 3, 6, 9, 15, 21 and 27 days after pollination were microscopically observed for the development of embryo, endosperm and maternal tissue. They were fixed in a mixture of 60%-ethanol, formalin and acetic acid (18:1:1). Then, the materials were stored in 70%-ethanol at 0°C . Their microtome sections with 12 or 15μ in thickness were made through paraffin work and stained with safranin and fast green FCF.

Of above-mentioned ovules, those of 9 to 27 days after pollination, except the case of *I. laevigata* $\times$ *I. sanguinea*, were measured for the sizes of embryo and endosperm-nuclei with ocular micrometer. The reason for the exception is that the embryos and endosperms degenerated too early to be examined in that cross. As for endosperm nuclei, those of both micropylar and chalazal part were observed for examining also the variation due to position. A nucleus was regarded as a spheroid and the nuclear volume was calculated from $4/3\pi ab^2$ (a =length of major axis, b =length of minor axis).

Cytological observation

Ovules of 9, 15, 18, 21, 27 and 36 days after pollination, except those from *I. laevigata* $\times$ *I. sanguinea*, were mainly put to the observation of the inner situation of their embryo and endosperm-nuclei. The exception is based on the same reason as mentioned above. They were fixed in 1:3 acetic alcohol or modified Navashin's fluid, then the embryos and endosperms were cytologically examined with iron-aceto-carmin squash method.

Of above-mentioned ovules, only those of 15 days after pollination were available for examining the mitotic aberrations of endosperm nuclei, because any such mitotic stages suitable for detecting aberrations as late anaphase and early telophase could not be found out in other ovules. Ovules of 36 days after pollination were too hard to be examined cytologically.

Results

1. Capsule growth and fertility

Figure 1 shows the growth of capsules in the two species (parents) and their reciprocal crosses. As seen in this figure, the capsules of *I. sanguinea* × *I. laevigata* grew almost normally, but those of *I. laevigata* × *I. sanguinea* stopped the growth on 9 days after pollination, degenerating and turning brown before 27 days after pollination. This bad growth of the latter seemed to be closely connected with the lower fertility (Table 1).

Fertilization of ovules was judged from embryological situation of the ovule of 6 days after pollination. As shown in Table 1, different fertility was found between reciprocal crosses, namely, considerably higher when *I. sanguinea*, the self-fertility of which was far higher than that of *I. laevigata*, was used as the female parent. This fact may suggest that the fertility of hybrid seed reflects the fertilization ability of female parent. In any case, the hybrid seeds obtained from the crosses were entirely shrivelled and inviable, independently of cross combination.

2. Seed development

Results obtained from embryological and cytological observations of seed development after pollination are shown in Tables 2~6 and Figs. 2~6. For convenience of discussion, these results will be described here on each of the following divided periods of observation, i. e., 3~9, 15~18 and 21~36 days after pollination for *I. sanguinea* × *I. laevigata*, and 3~

Table 1. Fertility of *I. sanguinea*, *I. laevigata* and their reciprocal crosses

Cross	No. of ovules observed	No. of fertilized ovules ¹⁾	Fertility (%)
<i>I. sanguinea</i> Self.	49	48	98.0
<i>I. sanguinea</i> × <i>I. laevigata</i>	55	28	50.9
<i>I. laevigata</i> × <i>I. sanguinea</i>	83	26	31.3
<i>I. laevigata</i> Self.	66	37	56.1

1) Fertilization was judged from embryological observation of the ovule of 6 days after pollination.

Fig. 1. Capsule growth in *I. sanguinea*, *I. laevigata* and their reciprocal crosses

Table 2. Pattern of seed development in *I. sanguinea*, *I. laevigata* and their reciprocal crosses

Cross	Day of observation (Days after pollination)														
	0	3	6	9	15	18	21	27	36						
<i>I. sanguinea</i> Self. <i>I. laevigata</i> Self.	{ Embryo (See Fig. 2) Endosperm										Slow growth	Rapid growth			
											Division of free nuclei		Cell wall formation		Condensed cytoplasm, accumulation of numerous granules
<i>I. sanguinea</i> × <i>I. laevigata</i>	{ Embryo Endosperm										Normal growth	↑ ↓		* Degeneration proceeds	
											Normal growth	* Degeneration proceeds		↑ Large nuclei begin to appear	
<i>I. laevigata</i> × <i>I. sanguinea</i>	{ Embryo Endosperm										Normal growth	* Degeneration proceeds		Collapsing	
											Normal growth	Degeneration proceeds		Collapsing	
<i>I. sanguinea</i>	{ Maternal tissue ¹⁾										Normal growth	↑ *		Hypertrophy of nucellus	
											Normal growth	Collapsing		Collapsing	

1) Maternal tissue of *I. sanguinea*, *I. laevigata* and *I. sanguinea* × *I. laevigata* developed normally.
l, s; The initiation of cell wall formation in *I. laevigata* and *I. sanguinea*, respectively,
 * ; The initiation of degeneration.

Fig. 2. Embryo growth in *I. sanguinea*, *I. laevigata* and *I. sanguinea* × *I. laevigata*

Fig. 3(1). Seed development in *I. sanguinea*.
a~f(×175): Ovules of 3(a), 6(b), 9(c), 15(d), 21(e) and 27(f) days after pollination.

Fig. 3(2). Seed development in *I. laevigata*.

g~l($\times 175$): Ovules of 3(g), 6(h), 9(i), 15(j), 21(k) and 27(l) days after pollination. Mp: Micropylar part, Cp: Charazal part, Nn: Nucellus, Em: Embryo, En₁: Endosperm free nuclei, En₂: Endosperm nuclei with cell wall, Su: Suspensor.

6 and 9~21 days after pollination for *I. laevigata* $\times$ *I. sanguinea*, because these respective periods proved to stand for the representative pattern of seed development in each combination.

(a) *I. sanguinea* and *I. laevigata*

These two species exhibited a similar pattern of seed development. The embryos developed slowly until 15 days after pollination and rapidly after that time (Figs. 2 and 3). The pattern of endosperm development was also similar except for the time of initiation of cell wall formation (Table 2). In both species, cell wall formation commenced in the periphery of embryo sac and was soon followed by vigorous development of endosperm and embryo.

(b) *I. sanguinea* $\times$ *I. laevigata*

From 3 to 9 days after pollination Both embryo and endosperm presented normal development throughout this period (Table 2, Figs. 2 and 4a~c), and large nuclei, such as

Fig. 4. Seed development in *I. sanguinea* × *I. laevigata*
 a~f(×175): Ovules of 3(a), 6(b), 9(c), 15(d), 21(e, f) and 27(g, h) after pollination.
 a~d: Normal development of ovules. e, g: Degenerating embryo and endosperm
 in micropylar part. f, h: Degenerating endosperm in chalazal part.
 Cs: Collapsing suspensor, En: Endosperm, Av: Abnormal vacuole.

fused nuclei (Fig. 5 a) and nuclei involving multi-strand chromosomes (Fig. 5 b), appeared sporadically in the endosperms of 9 days after pollination. Fused nuclei, however, similarly appeared also in the parental endosperms of the same days after pollination,

Fig. 5. Cytological abnormality of endosperm nuclei in *I. sanguinea* × *I. laevigata* a~f(×2500): Fused nuclei (a), nucleus with multi-strand chromosomes (b), chromosome bridge (c), lagging chromosome (d), chromosomal stickiness (e) and disintegration of chromatin (f).

where no cell wall was formed yet as in those of the hybrid (Table 2). Hence, nuclear fusion in endosperm was considered to be concerned closely with the state of free nuclei.

From 15 to 18 days after pollination In embryo development, the hybrid made little difference from the parents during this period (Figs. 2 and 4 d).

In endosperm development, however, the hybrid differed considerably from the parents in this period: no cell wall was formed and apparent mitotic abnormality was revealed. Table 3 and Figs. 5 c, d, which show the mitotic aberrations on 15 days after pollination, indicate the extremely high frequency of mitotic aberrations, especially chromosome bridges in the hybrid endosperm. Chromosomal stickiness was recognized in the endosperms that exhibited high frequency of such mitotic aberrations (Fig. 5e).

As in the endosperms of 9 days after pollination, large nuclei were observed also in those of this period. The occurrence of these nuclei was considerably higher than in the preceding period. The increased frequency of large nuclei was due mainly to the high

Table 3. Mitotic aberrations at late anaphase and early telophase in endosperm nuclei of 15 days after pollination

Cross	No. of ovules examined	No. of nuclear plates examined	Normal (%)	Mitotic aberration		
				Bridge (%)	Laggard (%)	Bridge+Laggard (%)
<i>I. sanguinea</i> Self.	2	109	89 (81.7)	15 (13.8)	3 (2.8)	2 (1.9)
<i>I. sanguinea</i> × <i>I. laevigata</i>	2	84	24 (28.6)	53 (63.1)	6 (7.2)	1 (1.2)
<i>I. laevigata</i> Self.	2	128	114 (89.1)	6 (4.7)	8 (6.3)	0 (0)

Table 4. Observed and expected values of endosperm nuclear volume (e. n. v., μ^3) in *I. sanguinea* × *I. laevigata*

Days after pollination	Observed value ¹⁾		Expected value ²⁾	
	Micropylar part	Charazal part	Micropylar part	Charazal part
9	1109.9	1188.6	1649.3	1689.4
15	1810.1	3470.6	1813.9	1853.8
21	3490.1	3340.5	521.6	838.2
27	3122.0	1161.1	562.9	731.6

1) 67~126 nuclei randomly chosen from each part were measured.

2) $\frac{2}{3}(\text{e. n. v. of } I. \text{ sanguinea}) + \frac{1}{3}(\text{e. n. v. of } I. \text{ laevigata})$.

frequency of the occurrence in charazal part, resulting in the abnormal increase of average nuclear volume in charazal part as shown in Table 4.

In addition to those described above, it was another distinctive feature of this period that some hybrid endosperms had begun to develop such symptoms of degeneration as disintegrated chromatins and abnormal vacuoles in endosperm.

From 21 to 36 days after pollination The embryo growth of hybrid was in striking contrast to that of parents. Namely, the observation of ovules of 21 and 27 days after pollination revealed that hybrid embryo had made only slight development since 15 days after pollination (Fig. 2). Furthermore, this period was characterized by manifest degeneration of embryos as shown in Table 5 and Figs. 4e, g: about 40% of embryos had

Table 5. Embryological observation of the ovules of 21 days after pollination (*I. sanguinea* × *I. laevigata*)

Condition of ovule	No. of ovules observed	Frequency (%)
Normal embryo, degenerating or collapsing endosperm	11	61.1
Embryo at beginning of degeneration, degenerating endosperm	2	11.1
Degenerating embryo and endosperm	2	11.1
Degenerating embryo and collapsing endosperm	2	11.1
Embryo isolated from micropylar part, collapsing endosperm	1	5.6
Total	18	100.0

already shown the symptoms of degeneration on 21 days after pollination (Table 5), and the great majority of embryos had entirely degenerated on 36 days after pollination.

On the other hand, the endosperm of hybrid also developed poorly and continued in the state of free nuclei without cell walls through this period (Table 2). There was not observed such condensed cytoplasm with accumulated numerous granules as recognized in parental endosperms. On 21 days after pollination, enlargement of endosperm nuclei occurred not only in charazal part but also in micropylar part (Table 4). From 21 and 27 days after pollination, the frequency of large nuclei increased markedly, and the degeneration of endosperm represented in terms of disintegration of chromatin and vacuolization of cytoplasm became more notable (Figs. 4 h, 5 f). Such degeneration appeared to proceed from charazal part to micropylar part (Figs. 4 e~h). It should be noted that

Fig. 6. Seed development in *I. laevigata* × *I. sanguinea*

a~f: Ovules of 3(a, ×175), 6(b, ×175), 9(c, d, ×175), 15(e, ×175) and 21(f, ×70) days after pollination. a~c: Normal development ovules. d: Degenerating embryo, collapsing endosperm and hypertrophying nucellus. e: Collapsing embryo and endosperm, hypertrophying nucellus. f: Collapsing ovule.

Ce: Collapsing endosperm, De: Degenerating embryo, Hn: Hypertrophying nucellus, Do: Destroyed ovule.

Table 6. Embryological observation of the ovules of 9 days after pollination (*I. laevigata* × *I. sanguinea*)

Condition of ovule	No. of ovules observed	Frequency (%)
Normal	3	8.8
Normal embryo, collapsing endosperm	6	17.6
Normal embryo, poorly developed endosperm and hypertrophied nucellus	2	5.9
Degenerating embryo and collapsing endosperm hypertrophied nucellus	20	58.8
Degenerating embryo and collapsing endosperm	3	8.8
Total	34	99.9

both the enlargement of nuclei and the degeneration originated thus in charazal part. Hybrid endosperms almost or entirely collapsed on 36 days after pollination.

(c) *I. laevigata* × *I. sanguinea*

From 3 to 6 days after pollination Until 6 days after pollination, most of ovules developed normally (Table 2, Figs. 6 a~c), while some ovules gave slight development of endosperm in spite of normal growth of embryo.

From 9 to 21 days after pollination Already on 9 days after pollination, various kinds of seed development were observed. The vast majority of ovules revealed abnormal development of embryo, endosperm and nucellus (Table 2). In about 65% of the ovules of 9 days after pollination, there was found hypertrophied nucellus, by which a feature of this cross was characterized (Table 6, Fig. 6 d). Such hypertrophy did not take place in any normally growing ovules (Figs. 6 b, c). This may suggest that the occurrence of hypertrophy depends upon the collapse or poor development of endosperm, whereas the collapse of endosperm does not necessarily induce the hypertrophy (Table 6). On 15 days after pollination, every embryo and endosperm collapsed while nucellus went on hypertrophying (Fig. 6 e). On 21 days after pollination, all ovules *per se* collapsed completely (Fig. 6 f).

Discussion

Cause of seed failure

It was made clear in the reciprocal crosses that the degeneration of endosperm always occurred prior to the degeneration of embryo and the span of embryo life depended upon the time of endosperm degeneration. It may be concluded from these results that the most important cause for seed failure appearing in these reciprocal crosses is the degeneration of endosperm *per se*. This conclusion agrees with many other observations (COOPER and BRINK 1944, THOMPSON and JOHNSTON 1945, COOPER 1951, HÅKANSON 1952, BROCK 1954, WEAVER 1957, INOMATA 1970, KISHI and FUJISHITA 1970 and etc.).

In addition to degeneration of embryo and endosperm, an abnormally grown maternal tissue, i.e., hypertrophied nucellus was found in *I. laevigata* × *I. sanguinea*. Although several workers (BRINK and COOPER 1939, COOPER and BRINK 1940, 1945, WOODELL 1960 a, b) reported that the cause of seed failure could be attributed to such growth of maternal

tissue, yet it is not the case for the nucellus which appeared in this cross, because such an abnormal nucellus was not recognized in the ovules until 9 days after pollination, which still showed normal development of embryo and endosperm. Thus the hypertrophy of nucellus, as well as the degeneration of embryo, can be regarded as the result of inhibited development of endosperm and not the cause. The fact that no hypertrophy of maternal tissue was recognized in *I. sanguinea* × *I. laevigata*, where endosperm degenerated considerably late as compared with the reciprocal cross, also may support the conclusion.

Mechanism of endosperm degeneration

Elucidation of the mechanism of endosperm degeneration, which causes seed failure, appears to be highly significant also from the standview of breeding. As to such the mechanism, many evidences acquired in various plants have drawn a conclusion that all the endosperm degeneration is ascribed to the cytological abnormality of endosperm nuclei represented in terms of polyploidization and/or mitotic aberration (KIHARA and NISHIYAMA 1932, COOPER and BRINK 1944, BROCK 1954, 1955, KATO 1957, TANDON and KAPOOR 1962, KAPOOR and TANDON 1963 a, b, DOWRICK and BRANDRAM 1970 and etc.). However, the cytological abnormality does not always cause endosperm degeneration (TANDON and KAPOOR 1963, KAPOOR and TANDON 1964, KAPOOR 1966). Therefore, it may safely be said that the development of endosperm reflects the tolerance of endosperm nuclei against cytological abnormality.

In spite of a clear-cut difference of the time of degeneration, both the endosperms developed from the two crosses in the present study were apparently characterized by the presence of cytological abnormality of endosperm nuclei. In the cross *I. sanguinea* × *I. laevigata*, where detailed observation of fertilized seed could be continued for longer period of time, the cytological abnormality was recognized as abnormal enlargement of nuclei and high frequency of mitotic aberration, such as chromosome bridges and lagging chromosomes. The abnormal enlargement of nuclei is readily inferred to have originated in the polyploidization of endosperm nuclei, because fused nuclei and nuclei with multi-strand chromosomes due to endomitosis were frequently observed. Anyway, it was no doubt the case that cytological abnormality like the above was the initial symptom of endosperm degeneration. Therefore, it can be concluded for this cross, presumably also for the reciprocal one, that endosperm degeneration is caused by polyploidization and mitotic aberrations of endosperm nuclei. This accords with the general conclusion introduced before.

The factor inducing such cytological abnormality may have to be analyzed next. As described above, abnormal enlargement of endosperm nuclei, which reflects the polyploidization, results from nuclear fusion or endomitosis. The former, however, should be excluded from this discussion, because this was concerned only with the free-nuclei state of endosperm and, in fact, recognized also in parental endosperms. That is, the factor responsible for polyploidization due to hybridization is safely considered the latter, endomitosis. Of the above-mentioned mitotic aberrations, on the other hand, chromosome bridges, the presence of which is one of the particular features of the hybrid endosperms, may play an important part in this discussion. These chromosome bridges seem to be attributed to chromosomal stickiness as has been pointed with hyacinth endosperm (Brock

1955) and to some other unknown factors, if any.

Thus, it can be concluded that the endomitosis and chromosomal stickiness are important and direct factors causing the cytological abnormality, and they should be clarified for their origin through further detailed studies.

Proposal for artificial culture

From the foregoing discussion, it is clear that seed failure in the present reciprocal crosses is not caused by embryo degeneration but endosperm one. This means that hybrid plants can be obtained by artificial culture of embryo. The present results suggest that the developmental stages of embryos appropriate for initiating the artificial culture are on around 6 and 15 days after pollination for *I. laevigata* × *I. sanguinea* and *I. sanguinea* × *I. laevigata*, respectively. It is, however, almost impossible for embryos to be removed from ovules on 6 or 15 days after pollination, because the embryos are still too small to be handled. For obtaining hybrid plants, therefore, ovule or ovary culture should be adopted in place of embryo culture.

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アヤメ (*Iris sanguinea* Hornem.) と カキツバタ (*I. laevigata* Fisch.) の
正逆交雑後の種子発育に関する胚発生学および細胞学的研究

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我国における *Iris* 属の育種は、その高い種間交雑不親和性のため、もっぱら種内変異の利用に頼ってきた。今後 *Iris* 属の育種に種間交雑を取り入れて遺伝的変異の拡大をはかるためには、交雑種子の崩壊原因を探り、交雑不親和性の打破に関する知見を蓄積することが必要である。本研究では、相互の交雑不親和性が特に高いアヤメ (*I. sanguinea* Hornem. 2n=28) およびカキツバタ (*I. laevigata* Fisch. 2n=32) を供試し、両種の自家受粉ならびに正逆交雑後の種子発育に関して胚発生学的ならびに細胞学的な追跡調査を行った。得られた結果は次のとおりである。

1. カキツバタ × アヤメでは受粉 6 日後にはすでに胚乳退化の徴候が現われ、9 日後には胚の退化が観察された。またアヤメ × カキツバタでは受粉 15 日後には胚乳の退化、21 日後には胚の退化が認められた。このように胚および胚乳退化の時期は正逆交雑間で著しく異なったが、両交雑とも胚乳の退化は胚の退化に先行しており、胚の寿命が胚乳の退化時期に依存することは明らかであった。このことから、両交雑における種子崩壊の原因は胚乳の退化にあると結論される。なお、両交雑間における退化時期の差異の原因については今後の解明にまたねばならない。
2. カキツバタ × アヤメにおいては、胚および胚乳の退化以外に珠心の異常肥大が認められた。このような異常肥大は、胚や胚乳がまだ正常な発育を続けている受粉 6~9 日後までの胚珠には出現しなかったことから、胚乳退化の原因となるべきものではなく、胚乳退化の結果生じたものと思われる。
3. アヤメ × カキツバタにおいて、受粉 15 日後に異常な核体積の増大および高頻度の分裂異常 (特に染色体橋) が認められた。このような核体積の増大は、主として核内有糸分裂による倍数化で生じたものと推測される。これらの結果から、この交雑における胚乳の退化は、胚乳核の細胞学的異常すなわち倍数化や分裂異常に起因すると結論される。
4. アヤメとカキツバタの正逆交雑においては、以上のように種子崩壊の原因が胚乳にあるので、雑種植物獲得には胚の人工培養が有効であると結論される。なお、培養の適期はカキツバタ × アヤメでは受粉 6 日目頃、アヤメ × カキツバタでは 15 日目頃と推測される。