Elucidation of Seed Failure and Breeding of F₁ Hybrid in Reciprocal Crosses between *Iris ensata* Thunb. and *I. laevigata* Fisch.

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The reciprocal crosses were made between two Japanese representative *Iris* species, *I. ensata* (2n=24) and *I. laevigata* (2n=32), which are highly devoid of affinity each other, and the development of seeds was embryologically traced from 3 to 33 days after pollination to elucidate the cause of seed failure in process of development.

Owing to the seed failure, no viable seed was obtained in either of the reciprocal crosses. In both crosses, the initial symptom of the seed failure was the start of degeneration of endosperms, which appeared around 21 and 27 days after pollination in *I. ensata* × *I. laevigata* and *I. laevigata* × *I. ensata*, respectively. The degeneration of endosperm always occurred prior to that of embryos and was not accompanied with any abnormality in such maternal tissues as nucellus and integument. Moreover, it was substantiated by the success of embryo culture in *I. laevigata* × *I. ensata* that embryos could develop normally when put on a suitable medium in place of abnormal endosperm. These findings drew a conclusion that the decisive cause for seed failure appearing in these reciprocal crosses is the degeneration of endosperm.

The limit of development of embryos was clearly different between the two reciprocal crosses, namely, the hybrid embryos of *I. ensata* × *I. laevigata* failed to reach the stage of differentiation, while those of *I. laevigata* × *I. ensata* could produce some organ primordia as in the case of parental species.

A F₁ hybrid plant (2n=28) was obtained from *I. laevigata* × *I. ensata* by embryo culture. This new plant was scored for its morphological characteristics and discussed on its utility.

Introduction

In the previous paper (YABUYA and YAMAGATA 1978), the authors pointed out that the accumulation of detailed studies on the failure of hybrid seeds as well as that of the exploration of effective means for acquiring hybrids are essentially required for interspecific cross breeding of iris. From the experimental results with reciprocal crosses between *I. sanguinea* and *I. laevigata*, they drew a line of conclusion that the seed failure depends upon the degeneration of endosperm, and that artificial cultures of embryos or ovules may be effective means for acquiring hybrids.

In the present paper, the authors studied the seed failure and tried to rear F₁ hybrids, using another pair of reciprocal crosses involving *I. ensata* and *I. laevigata*. In consequence, they obtained results almost consistent with, but delicately different from those of the previous paper. Besides, they succeeded actually in acquiring a new hybrid plant by means of embryo culture, whereas all the efforts to obtain a viable seed ended in failure.

The results from observations on the seed development after the reciprocal cross pollination and the morphological characteristics of the F₁ plant are reported here.

Materials and Methods

Two Japanese representative irises, *Iris ensata* Thunb. var. *ensata* (= *Iris Kaempferi* Sieb. var. *hortensis* Makino, $2n=24$, two garden varieties "Kachô" and "Tatsutagawa" were used.) and *I. laevigata* Fisch. ($2n=32$, a garden variety "Yukidôrô" and a wild type were used.) were reciprocally crossed after the control of flowering time: in some plants of *I. ensata* to be used as pollen parents flowering was quickened by growing at 30°C in a phytotron with natural daylight. After pollination, embryos were traced for their development until 33 days. The methods of pollination and embryological observation which included measurement of endosperm nuclear volume and was conducted through paraffin work, were the same as described in the previous paper (YABUYA and YAMAGATA 1978).

Embryo culture could be applied only to the cross *I. laevigata* $\times$ *I. ensata*, because the hybrid embryos of this cross developed better than those of the reciprocal cross *I. ensata* $\times$ *I. laevigata*, and attained to nearly mature stage without any artificial treatment, whereas the latter stopped the development at earlier stages. Hybrid embryos of the relevant cross were excised about 60 days after pollination from ovules, whose surface was sterilized with 70% ethanol for 30 seconds and 10% antiformin for 20 minutes prior to embryo isolation, and placed on the nutrient medium in test tubes under dark conditions at 28°C . The medium was composed of Nitsch's solution (NITSCH 1951), 5% saccharose and 0.8% agar, and its pH was adjusted to 6.0 before autoclaving, which was operated at a pressure of $1.2\text{kg}/\text{cm}^2$ for 20 minutes. Ten days later, test tubes in which the embryos were germinated were transferred into the phytotron with daylight and 20°C . When the seedlings came to the age of 4 or 5 leaves, they were transplanted to flower pots with vermiculite. Thus, one of these seedlings grew into a mature plant, that is, F_1 hybrid.

Morphological characteristics of flower and leaf, pollen fertility and somatic chromosome number of the F_1 hybrid were examined and compared with those of the parental species. Somatic chromosome number was determined with Feulgen squash method for cells of root-tips, which had been fixed in Farmer's fluid after pretreatment with cold water (0°C) for 24 hours. The root-tips were hydrolysed in N HCl at 60°C for 10 minutes and stained with leuco-basic fuchsin. Squashing was carried out in a drop of aceto-carmine to increase the intensity of chromosome staining. Pollen fertility of the F_1 hybrid was observed using preparation stained with aceto-carmine.

Results

Seed development of the parental species

As shown in Table 1 and Figs. 1~4, *I. ensata* (Kachô) and *I. laevigata* (wild type) exhibited a similar pattern of seed development, excepting that the rapid development of embryos as well as the cell-wall formation in endosperms began 15 days after pollination in *I. laevigata* and 21 days in *I. ensata* (Table 1, Fig. 1). In both species, the formation of cell walls in endosperm was commenced in the peripheral region of embryo sac, soon followed by vigorous development of endosperm and embryo (Figs. 3 and 4). The initiation of embryonic differentiation into leaf and root primordia was recognized 27 days after pollina-

Table 1. Pattern of seed development in *I. ensata*, *I. laevigata* and their reciprocal crosses

Cross	Days after pollination						
	0	3	9	15	21	27	33
<i>I. ensata</i> Self. <i>I. laevigata</i> Self. { <i>Embryo</i> (See Fig. 1) { <i>Endosperm</i>	Initiation of differentiation						
	Division of cells						
	Division of free nuclei						
	Cell-wall formation ↑ 1 ↑ e						
<i>I. ensata</i> × <i>I. laevigata</i> { <i>Embryo</i> { <i>Endosperm</i>	Accumulation of nutritive substances						
	* Degeneration proceeds						
	Normal growth						
	* Degeneration proceeds						
<i>I. laevigata</i> × <i>I. ensata</i> { <i>Embryo</i> { <i>Endosperm</i>	Cell-wall formation						
	Accumulation of nutritive substances (Partially)						
	Initiation of differentiation						
	* Degeneration proceeds						
	Normal growth						
	Cell-wall formation						

1, e; The initiation of cell-wall formation in *I. laevigata* and *I. ensata*, respectively.

* The initiation of degeneration.

Fig. 1. Embryo growth in *I. ensata*, *I. laevigata* and their reciprocal crosses Fig. 2. Embryo-sac growth in *I. ensata*, *I. laevigata* and their reciprocal crosses. Embryo-sac size was calculated from (length + width)/2.

Fig. 3. Seed development in *I. ensata*. Ovules of 3(a), 9(b), 15(c), 21(d), 27(e) and 33(f) days after pollination (a~e: $\times 175$, f: $\times 70$)

Mp: Micropylar part, Cp: Charazal part, Nu: Nucellus, In: Integument, Em₁: Proembryo, Em₂: Differentiating embryo, En₁: Endosperm free nuclei, En₂: Endosperm nuclei with cell wall, Ns: Nutritive substances.

Fig. 4. Seed development in *I. laevigata*. Ovules of 3(a), 9(b), 15(c), 21(d), 27(e) and 33(f) days after pollination. (a~e: $\times 175$, f: $\times 70$)

Fig. 5. Seed development in *I. ensata* × *I. laevigata*. a~h(×175): Ovules of 3(a), 9(b), 15(c), 21(d, e), 27(f) and 33(g, h) days after pollination.

a~c: Normal development of ovules, d: Normal development of embryo and endosperm in micropylar part, e: Degenerating endosperm in charazal part, f: Degenerating embryo, g: Degenerating embryo only exists in micropylar part, h: Some endosperm cells are filled up with nutritive substances, En: Endosperm, De: Degenerating embryo, Ns: Nutritive substances.

tion.

Seed development in *I. ensata* × *I. laevigata*

Hybrid embryos of *I. ensata* (Kachô) × *I. laevigata* (wild type) were scarcely different from those of the parental species until 21 days after pollination, but were in a striking contrast to them from 27 to 33 days (Figs. 1 and 5). Namely, they exhibited relatively slight development from 21 days after pollination downward (Fig. 1), and showed the initial symptom of degeneration at 27 days (Fig. 5f), proceeding to the degeneration at 33 days (Fig. 5g). Thus, they could never differentiate such leaf and root primordia as observed in the parental species.

Hybrid endosperms, on the other hand, developed normally at least till 15 days after pollination (Figs. 5a~d), then began to show symptoms of degeneration such as disintegration of nuclei and cytoplasm, which were observed first at 21 days (Fig. 5e). These symptoms became intensified rapidly from 27 to 33 days, as deduced also from the decreased size of embryo sacs (Fig. 2). The degeneration of endosperms was more remarkable in the micropylar part than in the charazal part, and the nutritive substances, which were recognized at 27 days, finally remained only in the charazal part (Figs. 5g and h). Such degeneration could not be ascribed to an abnormality of endosperm nuclei, because an abnormal increase of nuclear volume took place neither in micropylar nor charazal part as seen in Table 2a, which shows the average nuclear volume of endosperms from 9 to 27 days.

From 33 days after pollination downward, all of survived endosperms hardened themselves, and seeds finally turned shrivelled and non-viable.

Table 2-a. Observed and expected values of endosperm nuclear volume (e. n. v., μ^3) in *I. ensata* × *I. laevigata*

Days after pollination	Observed value ¹⁾		Expected value ²⁾	
	Micropylar part	Charazal part	Micropylar part	Charazal part
9	1545.9	1690.1	2947.7	2689.1
15	3651.1	4252.3	3677.3	3466.4
21	1169.3	2083.1	1421.9	1919.5
27	759.4	1428.4	485.4	1024.6

1) 60~120 nuclei randomly chosen from each part were measured.

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

Table 2-b. Observed and expected values of endosperm nuclear volume (e. n. v., μ^3) in *I. laevigata* × *I. ensata*

Days after pollination	Observed value ¹⁾		Expected value ²⁾	
	Micropylar part	Charazal part	Micropylar part	Charazal part
9	1379.9	2018.9	2429.5	2082.4
15	963.8	1426.4	3185.3	3187.6
21	476.8	1443.5	984.3	1481.1
27	563.0	1115.6	538.2	1075.0

1) 60~120 nuclei randomly chosen from each part were measured.

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

Seed development in *I. laevigata* × *I. ensata*

Hybrid embryos of *I. laevigata* (wild type) × *I. ensata* (Kachô) showed quite a normal development through the observation period, or grew rather better than one of the parental species, *I. ensata* (Table 1, Figs. 1 and 6). They initiated the differentiation of leaf and root primordia by 27 days after pollination like the embryos of the parental species.

Hybrid endosperms were scarcely different in development from endosperms of the parental species until 21 days after pollination, and the symptoms of degeneration such as disintegration of nuclei and cytoplasm were recognized first at 27 days (Table 1, Figs. 6a~e). As in the case of the reciprocal cross, this degeneration was also substantiated by the decrease of embryo-sac size from 27 to 33 days (Fig. 2), and an abnormal increase of nuclear volume in endosperm was recognized neither in micropylar nor chalazal part (Table 2b). One of the characteristic features in the present cross was that no nutritive

Fig. 6. Seed development in *I. laevigata* × *I. ensata*. Ovules of 3(a), 9(b), 15(c), 21(d), 27 (e, f) and 33(g) days after pollination. (a~f: ×175, g: ×70)

a~d: Normal development of ovules, e: Degenerating endosperm in chalazal part, f: Normal development embryo and endosperm in micropylar part, g: Differentiating embryo, En: Endosperm.

substance was reserved in endosperms at least until 33 days (Fig. 6g).

From 33 days after pollination downward, survived endosperms did never harden themselves at all, and seeds finally became empty owing to the collapse of embryos and endosperms.

Rearing of F₁ hybrid by embryo culture

Embryo culture, which was applied to the cross *I. laevigata* (Yukidôrô) × *I. ensata* (Tatsutagawa), resulted in successful yielding of hibrid seedlings as shown in Table 3. However, those seedlings were possessed of light yellowish green leaves and of poor vigor, dying except two soon after transplanting from test tubes to flower pots (Table 3). The only one of the survivors grew into a mature plant and,

Table 3. Result of embryo culture in *I. laevigata* × *I. ensata*

No. of embryos planted	No. of seedlings obtained	Percent of germination	No. of plants survived
14	7	50	2

Fig. 7. Somatic chromosomes in root-tip cells (×2500) and flowers of *I. laevigata*, *I. ensata* and *I. laevigata* × *I. ensata*.

a and b: *I. laevigata* (2n=32), c and d: *I. ensata* (2n=24), e and f: *I. laevigata* × *I. ensata* (2n=28)

from the observation of somatic chromosomes in the root-tip cells, was confirmed to be the very F_1 hybrid ($2n=28$) between two species, *I. laevigata* ($2n=32$) and *I. ensata* ($2n=24$) (Figs. 7a, c and e).

Characteristics of F_1 hybrid

Morphological comparison of the F_1 hybrid (Yukidôrô×Tatsutagawa) with the parental species is given in Table 4 and Figs. 7b, d and f. As seen in the Table and Figures, the F_1 hybrid bore resemblance to *I. laevigata* in shape of outer perianth and did to *I. ensata* in pollen colour and leaf-midrib condition, but other characters, such as colour of outer and inner perianths, were peculiar to the F_1 hybrid. Besides, the anthers of the F_1 hybrid contained few pollen, and all of 654 pollen grains examined were abortive.

Table 4. Traits of F_1 plant (*I. laevigata*×*I. ensata*)

Character	<i>I. laevigata</i>	<i>I. ensata</i>	F_1
1. Outer perianth			
a. Size ¹⁾	79.5×40.8	91.2×86.2	94.0×64.0
b. Colour	Light purple spots on white ground, rimmed with light purple at the base	Dark reddish purple with white veins	Bluish purple
c. Shape	Not waved	Waved	Not waved
2. Inner perianth			
a. Size ²⁾	50.2×13.3	43.7×17.3	51.0×21.0
b. Colour	White, rimmed with light purple	White, rimmed with dark reddish purple	Bluish purple
3. Pistil colour	Light purple, crested with white	White, rimmed with dark reddish purple	Pale purple, rimmed with light purple
4. Anther colour	Pale purple	Pale purple	Pale purple
5. Pollen colour	Cream	Yellow	Yellow
6. Midrib condition in leaf	Not upheaved	Upheaved	Upheaved

1), 2): Length×width (mm)

Discussion

The present embryological observation in both the reciprocal crosses showed that the degeneration of endosperms always took place prior to embryo degeneration and was not accompanied by any abnormality of maternal tissues such as nucellus and integument. The success of embryo culture in *I. laevigata*×*I. ensata*, on the other hand, suggested that hybrid embryos kept their normality throughout the whole development, if they were released from endosperms at suitable time. From these results, it comes to a conclusion that the most important cause for seed failure observed in the present reciprocal crosses is the degeneration of endosperm per se. This is really consistent with our previous conclusion drawn from the reciprocal crosses between *I. sanguinea* and *I. laevigata* (YABUYA and YAMAGATA 1978). In contrast with our conclusion, SAWYER (1925) and SMITH and CLARKSON (1956) reported from the cross *I. pseudacorus*×*I. versicolor* and the cross *I. tenax*×*I. tenuis*, respectively, that the seed failure was caused by the abortion of embryo. Such discrepancy of conclusions may suggest that the cause of seed failure varies with cross combination of species. For elucidating this problem, embryological information

on this genus, which has scarcely been obtained up to date, should be further accumulated.

Although many efforts have been directed towards clarifying the cause of endosperm degeneration (BRINK and COOPER 1947, TANDON and KAPOOR 1962, NISHIYAMA and YABUNO 1978), the mechanism of the degeneration is still beyond full understanding. The present authors pointed out in the previous paper (YABUYA and YAMAGATA 1978) that the endosperm degeneration was ascribed to the cytological abnormality of endosperm nuclei, such as abnormal enlargement of nuclei and high frequency of mitotic aberrations involving chromosome bridges and lagging chromosomes. In the present reciprocal crosses, however, such types of cytological abnormality as pointed above were not recognized, and, as naturally gathered from that, a prolonged duration of endosperm development was observed. Namely, the endosperms normally developed over free-nuclear division stage, which was the developmental limit in the reciprocal crosses of the previous paper (*I. sanguinea* × *I. laevigata*), and finally attained to the stage of cell-wall formation or a little more advanced level, i. e., the early stage of nutritive-substance accumulation. These results may indicate that the poor ability for accumulation of nutritive substances also is an important cause of endosperm degeneration after interspecific crosses in *Iris* as well as the cytological abnormality described before. Some delicate unbalance in genomic constitution of hybrid endosperms may possibly be concerned with such poor ability.

The present reciprocal crosses were clearly different from each other in the developmental limit of embryos. Namely, the hybrid embryos of *I. laevigata* × *I. ensata* differentiated the primordia of shoots and roots, but those of *I. ensata* × *I. laevigata* failed to reach the stage of such organ differentiation. This difference seems to depend upon whether the degeneration of endosperm precedes the time of organ differentiation as in the latter or not as in former. On the other hand, the embryonic organ differentiation was first recognized 27 days after pollination in the former cross, as in both the parental species. Putting those facts together, it is naturally considered that the endosperm development up to around 27 days after pollination is indispensable for the differentiation of embryonic organ and required also for the success of embryo culture. To substantiate this consideration, of course, factors directly affecting the organ differentiation should be detected through further studies.

There exists in iris a high cross-incompatibility not only between Japanese and other species but also among Japanese species (TOMINO 1963, SAKURAI and TOMINO 1969, 1970, YABUYA and YAMAGATA 1978), and this barrier has ever checked the release of new interspecific hybrids. Thus, the F₁ hybrid derived from *I. pseudacorus* × *I. ensata* (TOMINO and SAKURAI 1972) has so far been only one instance of success in producing interspecific hybrid in Japan. The new hybrid raised in the present study is, therefore, really the second. The results obtained here may indicate that embryo culture provides high possibilities for acquiring hybrids from other incompatible crosses.

One of the practical objects of hybridization between *I. laevigata* and *I. ensata* consists in combining the perpetual anthesis of *I. laevigata* with the high horticultural value of *I. ensata*. Though the F₁ hybrid plant obtained here is not yet of practical use because of its poor vigour and pollen sterility, this defect may be overcome by its amphidiploidy.

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ハナショウブ (*Iris ensata* Thunb.) とカキツバタ (*I. laevigata* Fisch.) の正逆交雑における種子崩壊の解明と F₁ の育成について

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わが国の代表的なアイリスであり、かつ互いに交雑不和合性が極めて高いハナショウブ (*I. ensata* Thunb. var. *ensata*, 2n=24) とカキツバタ (*I. laevigata* Fisch., 2n=32) を供試し、両種の自家受粉後並びに正逆交雑後の種子発育を追跡調査して雑種種子崩壊の原因を探るとともに、胚培養による雑種植物の育成を試みた。

正逆両交雑とも充実不良でかつ発芽不能の種子のみを生じたが、いずれの場合も胚乳は常に胚にさきがけて退化した。即ち、ハナショウブ×カキツバタでは受粉 21 日後には胚乳、ついで 27 日後には胚の退化が観察され、一方カキツバタ×ハナショウブでは受粉 27 日後に胚乳の退化が認められたが、胚は観察期間中(受粉 33 日後まで) 退化には至らなかった。また両交雑とも珠心や珠皮などの母本組織には何ら異常が認められなかった。さらに後述するごとく、適切な胚培養を行えば F₁ 植物を獲得し得ることが明らかになった。これらのことから、両種の正逆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、胚の発育限度に関して正逆交雑間に差異が認められた。即ちハナショウブ×カキツバタでは器官原基の分化が全くみられなかったが、カキツバタ×ハナショウブでは葉や根の原基が分化した。この差異は胚乳の発育限度の差異に基づくものとみられる。

カキツバタ×ハナショウブの交雑後約 60 日目の種子より胚を取り出してこれを培養し、F₁ 雑種 (2n=28) を育成することに成功した。この植物について形態的特性を明らかにし、育種の意義を検討した。