Amphidiploids between *Iris laevigata* FISCH. and *I. ensata* THUNB. Induced through *in vitro* Culture of Embryos Treated with Colchicine¹⁾

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To overcome the sterility of the F_1 hybrids of *Iris laevigata* ($2n=32$) $\times$ *I. ensata* ($2n=24$), *in vitro* culture of embryos treated with colchicine was evaluated as a means to induce fertile amphidiploids ($2n=56$). Treatment of hybrid embryos with 0.05% level of aqueous colchicine for 1, 2 and 4 days enabled to induce the amphidiploids. The colchicine-induced amphidiploids bore a strong resemblance to the F_1 hybrids ($2n=28$) in the colour of the outer and inner perianths, midrib configuration of leaves, pollen colour and flowering date. However, the amphidiploids showed a taller scape and larger outer and inner perianths than the F_1 hybrids. Moreover, the amphidiploids exhibited high pollen and seed fertility. Such amphidiploids thus appear to be suitable for use as breeding materials for interspecific cross-breeding of *I. ensata*.

KEY WORDS : Induced amphidiploids, *Iris laevigata* $\times$ *I. ensata*, embryo culture, colchicine.

Introduction

Most efforts for the breeding of Japanese garden iris, *Iris ensata*, have so far been directed primarily to the utilization of intraspecific variation due to the high degree of cross-incompatibility of the plant with other species (TOMINO 1963, SAKURAI and TOMINO 1969, YABUYA and YAMAGATA 1980 a, b). This naturally suggests that the success of interspecific hybridization would enable to make great strides in the improvement of *I. ensata* through increased genetic variation. Only two types of interspecific hybrids between *I. ensata* and other species have been obtained : *I. laevigata* $\times$ *I. ensata* (YABUYA and YAMAGATA 1975, 1980 b, YABUYA *et al.* 1983) and *I. pseudacorus* $\times$ *I. ensata* (TOMINO and SAKURAI 1972, YABUYA 1984 a), but both hybrids were sterile. The previous paper (YABUYA 1984 b) indicated that the former hybrids lacked pollen and seed fertility because of the partial homology of the chromosomes between *I. ensata* and *I. laevigata*. An approach to overcome such sterility would be the amphidiploidization of interspecific hybrids.

This study was conducted to obtain amphidiploids between *I. laevigata* and *I. ensata* through *in vitro* culture of embryos treated with colchicine and to analyze the characteristics of the amphidiploids thus induced.

Materials and Methods

In vitro culture of hybrid embryos treated with colchicine

The garden variety 'Kachō' of *Iris ensata* THUNB. var. *ensata* (MAKINO) NAKAI (= *I. kaempferi* SIEB. var. *hortensis* MAKINO, $2n=24$) and the garden variety 'Shikizaki' of *I. laevigata* FISCH. ($2n=32$) were used as materials. The cross *I. laevigata* $\times$ *I. ensata* was made by the same method as that described by YABUYA and YAMAGATA (1978).

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On the 40th day after pollination, hybrid ovules of *I. laevigata* X *I. ensata* were excised from the capsules, whose surface was rinsed with benzalkonium chloride and tap water, and were sterilized by 20 min treatment with 50% purelox (=3% sodium hydrochlorite solution), followed by rinsing with sterile distilled water. Then the excised embryos were treated with 0.05% of aqueous colchicine in flasks for 1, 2 and 4 days. The colchicine level was that proposed by TOMINO (1967). The colchicine treatments were performed under shaking at ca. 25°C, and the colchicine solution was previously autoclaved at 120°C, under a pressure of 1.2 kg/cm² for 20 min. After the treatment, the embryos were washed with sterile distilled water and explanted onto the culture medium in test tubes under aseptic conditions. NITSCH's inorganic medium (NITSCH 1951) containing 2% sucrose and 0.6% agar was used as the culture medium. The medium was adjusted to pH 5 with 1 N HCl and 1 N NaOH and sterilized by autoclaving.

The explanted embryos were grown at ca. 25°C under continuous illumination, and the number of materials exhibiting leaf, root and seedling formation was recorded at about four months after inoculation. After the above examination, the seedlings obtained were transplanted to flower pots containing peat moss and sand and grown in a glass-house. Somatic chromosome numbers of the plants that survived after transplantation were determined using the aceto-carmin squash method for root-tips, which had been fixed in FARMER's fluid after pretreatment with cold water (0°C) for 24 hours. Such examination was also applied to the parental plants, *I. laevigata* 'Shikizaki' and *I. ensata* 'Kachô', and their F₁ hybrids (*I. laevigata* 'Shikizaki' X *I. ensata* 'Kachô').

Observation of the characteristics of the colchicine-induced amphidiploids

The garden variety 'Kachô' of *I. ensata* (2n=24), the garden variety 'Shikizaki' of *I. laevigata* (2n=32), F₁ hybrids (2n=28) and colchicine-induced amphidiploids (2n=56) of *I. laevigata* X *I. ensata* ('Shikizaki' X 'Kachô') were used as materials.

The characteristics of amphidiploids of *I. laevigata* X *I. ensata* were examined in comparison with those of the parental species, *I. laevigata* and *I. ensata*, and their F₁ hybrids (*I. laevigata* X *I. ensata*). Characters investigated were size and colour of outer and inner perianths, scape length, midrib configuration of leaves, pollen colour, flowering date, pollen and seed fertility, size and weight of seeds. Pollen fertility was determined by the same method as that described in the previous paper (YABUYA 1984b). The size and weight of seeds of the F₁ hybrids could not be examined due to sterility.

Results and Discussion

Table 1 shows the results of *in vitro* culture of the embryos of *I. laevigata* X *I. ensata* treated with 0.05% of aqueous colchicine for 1, 2 and 4 days. In the three cases, the colchicine treatments of hybrid embryos inhibited leaf and root formation (Table 1). Among the three kinds of colchicine treatments, the 4 day-treatment induced the most remarkable inhibition of such formation.

Cytogenetic examination of root-tip cells of the parental species, *I. laevigata* and *I. ensata*, and F₁ hybrids of *I. laevigata* X *I. ensata* revealed somatic chromosome numbers of 2n=32, 2n=24 and 2n=28, respectively (Fig. 1 A~C). Therefore, somatic chromosome numbers of amphidiploids of *I. laevigata* X *I. ensata* should be regarded as 2n=56,

Table 1. Results of culture of embryos treated with colchicine in *Iris laevigata* × *I. ensata*¹⁾

Duration of colchicine treatment(Days)	No. of embryos cultured	No. of embryos with leaf formation (%)	No. of embryos with root formation (%)	No. of embryos with seedling formation (%)
0	32	24(75.0)	26(81.3)	24(75.0)
1	34	14(41.2)	15(44.1)	13(38.2)
2	35	15(42.9)	21(60.0)	15(42.9)
4	28	8(28.6)	11(39.3)	8(28.6)

¹⁾ Colchicine in 0.05% aqueous solution.Table 2. Observation of somatic chromosome numbers in colchicine-treated plants of *Iris laevigata* × *I. ensata*

Duration of colchicine treatment(Days)	No. of plants examined	Somatic chromosome number			% of amphidiploids (2n=56) obtained ¹⁾
		2n=28	2n=56	2n=84	
1	10	8	2	0	5.9
2	7	2	5	0	14.3
4	6	0	5	1	17.9

¹⁾ [Number of amphidiploids/number of embryos treated with colchicine (See Table 1)] × 100.

and chromosome counts of colchicine-treated plants are shown in Table 2 and Fig. 1 D. In all of the colchicine treatments, the effect of the drug was manifested by the induction of the amphidiploids (Table 2). Longer period of the colchicine treatment resulted in a higher percentage of induced amphidiploids, i.e., 5.9% for the 1 day-treatment, 14.3% for the 2 day-treatment and 17.9% for the 4 day-treatment (Table 2). Consequently, the treatments of hybrid embryos for 2 and 4 days at the 0.05% level of aqueous colchicine were useful in inducing the amphidiploids.

From the above results, *in vitro* culture of embryos treated with colchicine appears to be an effective means for inducing amphidiploids of *I. laevigata* × *I. ensata*, and this is the first report of *in vitro* colchicine chromosome doubling in interspecific hybrids of the genus *Iris*. Such treatment is more advantageous than that in which hybrid plants are used as described in WATANABE (1982), because the former enable to shorten the time required for raising the amphidiploids of interspecific hybrids.

To induce the diploidization of *Hordeum vulgare* (2n=14) × *H. jubatum* (2n=28), ORTON and STEIDL (1980) used colchicine-treated callus cultures derived from immature embryos of the hybrids. Ten out of the 50 plants regenerated in this manner exhibited variable chromosome numbers with a mean of approximately 37, and these plants were considered to have originated from *in vitro* doubling of chromosome numbers by colchicine. However, these authors were not able to induce pure diploidization (2n=42) of the hybrids. Hence, tissue culture may be an effective tool for the diploidization of interspecific hybrids provided the variability in the chromosome numbers in the regenerated plants can be overcome.

The morphological and physiological characteristics of colchicine-induced amphidiploids of *I. laevigata* × *I. ensata* were examined and compared with those of the parental species, *I. laevigata* and *I. ensata*, and F₁ hybrids of *I. laevigata* × *I. ensata*. The results obtained are shown in Tables 3~6 and Figs. 2~4. As seen in Table 3 and Fig. 2, the colour

Fig. 1. Somatic chromosomes in root-tip cells of *Iris laevigata*, *I. ensata*, their F_1 hybrid and amphidiploid (*I. laevigata* × *I. ensata*). (× ca. 680)

A : *I. laevigata* ($2n=32$), B : *I. ensata* ($2n=24$), C : F_1 hybrid ($2n=28$),
D : Amphidiploid ($2n=56$).

Table 3. Comparison of traits among *Iris laevigata*, *I. ensata*, F_1 hybrids and amphidiploids of *I. laevigata* × *I. ensata*

Plants	No. of scapes examined	Outer perianth		Inner perianth	
		Size (Length × width, mm)	Colour	Size (Length × width, mm)	Colour
<i>I. laevigata</i>	4	$84.3 \pm 1.1 \times 48.9 \pm 0.7$	Purple	$61.9 \pm 0.9 \times 16.2 \pm 0.3$	Purple
<i>I. ensata</i>	12	$96.4 \pm 2.0 \times 75.6 \pm 2.9$	Light purple with white veins	$52.5 \pm 1.6 \times 20.0 \pm 1.2$	Light purple
F_1 hybrid	9	$91.9 \pm 1.3 \times 59.4 \pm 0.9$	Bluish purple	$56.2 \pm 0.5 \times 20.1 \pm 0.3$	Purple
Amphidiploid	6	$103.1 \pm 1.8 \times 65.9 \pm 1.3$	Bluish purple	$64.9 \pm 1.4 \times 22.9 \pm 0.9$	Purple

Table 3. (continued)

Plants	Pollen colour	Midrib configuration of leaves	Scape length (mm)	Flowering date (Month/day)
<i>I. laevigata</i>	Cream	Not heaved up	62.7± 4.6	April/26
<i>I. ensata</i>	Yellow	Heaved up	81.6± 5.1	June/2
F ₁ hybrid	Yellow	Heaved up	92.3± 4.8	May/22
Amphidiploid	Yellow	Heaved up	96.3±10.6	May/18

Fig. 2. Flowers of *Iris laevigata*, *I. ensata*, their F₁ hybrid and amphidiploid (*I. laevigata* × *I. ensata*).

A : *I. laevigata*, B : *I. ensata*, C : F₁ hybrid, D : Amphidiploid.

of the inner perianth of the amphidiploids was similar to that in *I. laevigata* while the pollen colour and leaf-midrib configuration were similar to those of *I. ensata*. Other characters, such as the size of the outer and inner perianths, the colour of the outer perianth and scape length, were different from those of the parental species. Besides, the flowering date was intermediate between that of the parental species (Table 3). This fact was considered to be important for the breeding of *I. ensata* with early flowering, since in the amphidiploids the flowering date proceeded by 15 days that of

Fig. 3. Pollen grains of *Iris laevigata*, *I. ensata*, their F_1 hybrid and amphidiploid (*I. laevigata* $\times$ *I. ensata*). ($\times$ ca. 50)

A : *I. laevigata*, B : *I. ensata*, C : F_1 hybrid, D : Amphidiploid.

I. ensata. The amphidiploids bore strong resemblance to the F_1 hybrids in the colour of the outer and inner perianths, leaf-midrib configuration, pollen colour and flowering date (Table 3, Fig. 2 C, D). However, the amphidiploids showed a taller scape and larger outer and inner perianths compared with the F_1 hybrids (Table 3).

Though the parental species exhibited a very high pollen fertility, i. e., 98.0% for *I. laevigata* and 91.1% for *I. ensata*, the F_1 hybrids of *I. laevigata* $\times$ *I. ensata* had no fertile pollen grains (Table 4 and Fig. 3 A~C). On the other hand, the pollen fertility of the colchicine-induced amphidiploids of *I. laevigata* $\times$ *I. ensata* was also very high, and the mean value and range of fertility were 80.6% and 73.3~88.3%, respectively (Table 4, Fig. 3 D).

Table 5 shows the results of self-fertility of the parental species, *I. laevigata* and *I. ensata*, F_1 hybrids and colchicine-induced amphidiploids of *I. laevigata* $\times$ *I. ensata*. The F_1 hybrids naturally exhibited no self-fertility due to pollen sterility (Table 4, 5). In contrast, the amphidiploids were endowed with a high self-fertility (Table 5), and the size and weight of their seeds were larger and heavier than those of *I. laevigata* and *I. ensata*, as shown in Table 6 and Fig. 4.

The restoration of pollen and seed fertility in the colchicine-induced amphidiploids of *I. laevigata* $\times$ *I. ensata*, as described above, is due to the doubling of the number of

Table 4. Comparison of pollen fertility among *Iris laevigata*, *I. ensata*, an F₁ hybrid and amphidiploids of *I. laevigata* × *I. ensata*

Plants	No. of pollen grains observed	Pollen fertility (%)
<i>I. laevigata</i>	759	98.0
<i>I. ensata</i>	715	91.1
F ₁ hybrid	1000	0
Amphidiploid-1	1000	73.3
-2	1000	73.8
-3	1000	80.6
-4	1000	82.2
-5	1000	85.5
-6	1000	88.3
Total (Mean)	6000	(80.6)

Fig. 4. Seeds of *Iris laevigata*, *I. ensata* and their amphidiploid (*I. laevigata* × *I. ensata*).
A : *I. laevigata*, B : Amphidiploid, C : *I. ensata*.

Table 5. Comparison of self-fertility among *Iris laevigata*, *I. ensata*, F₁ hybrids and amphidiploids of *I. laevigata* × *I. ensata*

Plants	No. of flowers pollinated	No. of capsules obtained	No. of seeds obtained per flower	
			Total	Normal seeds (%)
<i>I. laevigata</i>	5	5	66.4	64.4 (97.0)
<i>I. ensata</i>	5	5	89.2	83.8 (93.9)
F ₁ hybrid	18	0	—	—
Amphidiploid-1 ¹⁾	2	1	12.5	10.5 (84.0)
-2	2	2	64.0	53.5 (83.6)
-3	2	2	42.0	34.5 (82.1)
-4	3	1	9.3	8.7 (93.5)
-5	4	4	53.8	39.3 (73.0)
-6	2	2	43.0	18.0 (41.9)
Total (Mean)	15	12	(37.7)	(27.7)(73.5)

¹⁾ Strain No. of amphidiploids corresponds to that in Table 4.

Table 6. Comparison of seed size and weight among *Iris laevigata*, *I. ensata* and an amphidiploid of *I. laevigata* × *I. ensata*

Plants	Seed size (mm) ¹⁾			100 seeds weight (g)
	Length	Width	Thickness	
<i>I. laevigata</i>	7.35 ± 0.10	5.94 ± 0.07	3.22 ± 0.09	2.4
<i>I. ensata</i>	7.82 ± 0.13	8.99 ± 0.14	2.07 ± 0.07	1.5
Amphidiploid ²⁾	10.42 ± 0.21	7.76 ± 0.12	3.49 ± 0.12	3.0

¹⁾ Mean value of 50 seeds, ²⁾ Amphidiploid-2 was used (See Table 5).

chromosomes of the F₁ hybrids by the colchicine treatment. Therefore, such restoration implies that meiosis proceeds regularly. This aspect should be clarified by further experiments.

The successful achievement of interspecific hybridization may contribute to the promotion of interspecific cross-breeding and to the clarification of phylogenetic relationships between species. One of the objectives of hybridization between *I. ensata* and *I. laevigata* is the transfer of useful genes such as those for early and perpetual flowering from *I. laevigata* to *I. ensata*. Although it was possible to obtain F_1 hybrids of *I. laevigata* × *I. ensata* by embryo culture, their sterility prevented successful interspecific cross-breeding of *I. ensata* (YABUYA 1984 a, b). In this study since high pollen and seed fertility was restored in the amphidiploids of *I. laevigata* × *I. ensata* induced by the culture of embryos treated with colchicine, it can be considered that these amphidiploids are bridge plants which play an important role in the interspecific cross-breeding of *I. ensata*.

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カキツバタ (*Iris laevigata* FISCH.) × ハナショウブ (*I. ensata* THUNB.)
におけるコルヒチン処理胚の培養による人為複二倍体の育成

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我が国におけるハナショウブ [*Iris ensata* THUNB. var. *ensata* (MAKINO) NAKAI, $2n=24$] の育種は、他種との交雑不和合性が極めて高いためにもっぱら種内変異の利用に頼ってきたが、育種効果の飛躍的な増大のためには種間交雑の導入による変異の拡大が必要である。相互の交雑不和合性が高いハナショウブとカキツバタ (*I. laevigata* FISCH., $2n=32$) との間では、既に胚培養によりカキツバタ × ハナショウブの F_1 雑種 ($2n=28$) が育成されているが、両親種染色体間の相同性が低いため、これまで F_1 雑種の花粉および種子稔性は認められていない。このような F_1 雑種の不稔性を克服するために、コルヒチン処理胚の培養を実施し、カキツバタ × ハナショウブの人為複二倍体 ($2n=56$) の獲得に成功した。本報では、この複二倍体について育成の経過ならびに特性を明らかにした。

カキツバタ × ハナショウブの交雑 40 日後の胚珠から無菌的に摘出した胚をオートクレーブで滅菌済の 0.05% コルヒチン水溶液で 1, 2 および 4 日間の 3 区で処理後, NITSCH (1951) の無機培地 (蔗糖 2%, 寒天 0.6% および pH 5 に調整) を用いて胚培養を行った。その結果、雑種胚へのコルヒチン処理はいずれの区も葉および根の形成を阻害し、とくに 4 日間の処理区ではそのような阻害が顕著であった。

約 4 か月間の培養後、幼苗はピートモスと砂のポットに移植し、ガラス室内で生育させた。移植後、生存した植物体について根端の体細胞染色体数を観察した結果、いずれの処理区も複二倍体の獲得に関するコルヒチンの有効性が認められた。その中でも、2 日および 4 日間の両処理区において複二倍体の獲得が比較的良好であり、それぞれ 14.3% および 17.9% であった。このことから、カキツバタ × ハナショウブのコルヒチン処理胚の培養は複二倍体の獲得に有効な手段であり、しかも本研究はアヤメ属の種間交雑における胚培養を利用した染色体倍化の最初の報告である。さらに、このような胚培養を利用した複二倍体の獲得法は従来の雑種植物体へのコルヒチン処理法より複二倍体の育成期間を短縮する上からも有利であると思われる。

獲得した複二倍体は内花被の色がカキツバタに、また花粉の色および葉の主脈における突出の有無がハナショウブに類似していた。一方、複二倍体の開花日は両親種の間中型であり、外花被の大きさと色、内花被の大きさおよび花茎長は複二倍体に独特であった。これらの形質のうち開花日に注目すると、複二倍体の開花は花粉親のハナショウブよりも 15 日も早いことは興味深い。さらに、複二倍体の形質は花茎が長く、かつ外花被と内花被が大きい点を除けば F_1 雑種とよく類似していた。

次に花粉および種子稔性についてみると、 F_1 雑種は両稔性とも全く認められなかった。これに対して、複二倍体は花粉および種子稔性とも両親種と比べてかなりの頻度で回復していた。このような稔性の回復は染色体の倍化によっており、しかも複二倍体の減数分裂が規則的に行われていることを示唆するものである。

以上の結果、カキツバタ × ハナショウブのコルヒチン処理胚の培養により獲得した人為複二倍体は F_1 雑種の不稔性を克服することが明らかになり、しかもこの複二倍体にはカキツバタの有用形質である早咲性や四季咲性をハナショウブに導入する際に橋渡し植物としての効用が期待されている。