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STUDIES IN IRIS EMBRYO CULTURE

I. Germination of embryos of the subsection
Hexapogon Benth. (sect. *Regelia* sensu Dykes)

LEE W. LENZ

Since the pioneer work of Werckmeister (1936) in Germany and of Randolph and Cox (1943) and Randolph (1945) in the United States the use of embryo culture has become a common procedure for overcoming delayed germination in *Iris*. Thus it is possible to cut the time between generations from a minimum of two to three years for soil-sown seed to as little as ten months for embryo-cultured seed. The saving of time is especially great for breeders using *Oncocyclus* species which have been known to take up to eighteen years to germinate. More recently Lenz (1954) has shown the usefulness of the technique for growing immature iris embryos of certain hybrids which otherwise would have perished in the developing ovule due to a failure of the endosperm.

The early tall-bearded iris, nearly all of which were diploids ($2n=24$), are believed to have arisen through hybridization between *I. pallida* Lam. and *I. variegata* L., both diploids themselves. Toward the end of the last century several new species were brought into cultivation among them being *I. mesopotamica* Dykes, *I. trojana* Kerner, and *I. cypriana* Baker and Foster, species which later were shown to be tetraploids ($2n=48$). From the hybridization of these new tetraploids with the then existing garden forms there were developed the large-flowered modern-day varieties, nearly all of which are tetraploids or near tetraploids. It was seeds from some of these varieties that Randolph used in his embryo culture studies.

More recently representatives of several other groups, principally species of the subsections *Oncocyclus* and *Hexapogon* (section *Regelia* of Dykes), have been crossed with the tall-bearded iris to produce a series of beautiful and unusual hybrids. Hybrids between the tall-bearded iris and *Oncocyclus* species are often called Oncobreds. Interspecific crosses between species of the subsections *Hexapogon* and *Oncocyclus* have also been made and these hybrids are known variously as *Regliocyclus*, *Oncoregelias*, or *Oncogelias*, all names which are confusing if Lawrence's (1953) recent reclassification of the genus is to be used.

At the Rancho Santa Ana Botanic Garden numerous species representing various sections and subsections of the genus have been grown by the embryo culture technique during recent years. In 1952 it became apparent that members of the subsection *Hexapogon* did not behave in the same manner as those of the other sections which had been grown. At about the same time Werckmeister (1952) reported very much the same thing. Excised embryos of *I. boogiana* Dykes, *I. korolkowii* Regel, and *I. stolonifera* Maxim. when grown on nutrient agar under conditions favorable for the growth of tall-bearded iris embryos usually failed to germinate at all and instead became enlarged and malformed (Fig. 1). After several weeks in diffused light in the laboratory some of the embryos produce chlorophyll and become partially green while others, in addition to the chlorophyll, may produce small amounts of anthocyanin. Only very occasionally will an embryo germinate normally and those that do are often weak and die within a short period of time. According to Werckmeister this characteristic holds true in the case of interspecific hybrids where the female parent is a member of the subsection *Hexapogon* and the male parent a member of any other group. He further says that this peculiarity is partially true for

hybrids involving species of the section *Oncocyclus* and that from nearly one thousand embryos obtained from *Regliocyclus* crosses, where the *Regliocyclus* hybrid was used as the seed parent, only about three hundred grew.

MATERIALS AND METHODS

The methods used in this laboratory for embryo culturing of iris are essentially the same as those described by Randolph (1945) with the exception that instead of 7 g. of agar per liter of medium the amount has been cut to 5 g. The reason for the use of the softer agar is that it has been found that the young roots penetrate it

Fig. 1. Embryos and seedling of *Iris boogiana* 'Bronze Beauty' $\times$ *I. stolonifera* 'Decorated Delight' at the end of three months at room temperature. Seedling at right showed normal germination and growth.

more easily than they do the harder medium. Another deviation is that ordinary bacteriological test tubes (16 x 150 mm) are used in place of 2 oz. bottles. The test tubes have been found to be superior to the bottles for several reasons: (1) contamination is cut almost to nil, (2) the test tubes require smaller amounts of nutrient agar than do the bottles and, (3) there is a great saving of space by using the test tubes since large numbers of them can be stored in a relatively small area.

If the planted tubes are to be placed in the greenhouse, or if they are to be stored

for long periods of time, it has been found that if small squares of aluminum foil are folded down over the cotton plugs the amount of evaporation from the tubes is cut down and there is also a lower percentage of contamination of the tubes when they are placed where dust and dirt might otherwise get into them.

The young seedlings are easily removed from the tubes by use of ordinary ten-inch forceps whose tips have been covered with some material such as adhesive tape so that the seedlings are not damaged as they are pulled from the agar.

EXPERIMENTAL

In order to determine whether the germination peculiarity reported in the *Hexapogons* might also be found in other sections of the genus, embryos were cultured using species distributed in as many different sections of the genus as possible. Examination of Table I. shows that, of the species available for study, this peculiarity is apparently restricted to the one subsection.

TABLE I

	Germination normal	No germination or abnormal
I. Subgen. <i>Iris</i>		
1. Sect. <i>Pogoniris</i> Tausch.		
a. Subsect. <i>Pogoniris</i> (Spach) Benth.		
series <i>Pumilae</i> Lawr.		
<i>I. pumila</i> L.	X	
<i>I. chamaeiris</i> Bert.	X	
series <i>Elatae</i> Lawr.		
<i>I. cengialtii</i> Abrosi	X	
<i>I. cypriana</i> Baker and Foster	X	
<i>I. mesopotamica</i> Dykes	X	
<i>I. trojana</i> Kerner	X	
<i>I. variegata</i> L.	X	
<i>I. pallida</i> Lam.	X	
b. Subsect. <i>Hexapogon</i> Benth.		
<i>I. hoogiana</i> Dykes		X
<i>I. korolkowii</i> Regel		X
<i>I. stolonifera</i> Maxim.		X
c. Subsec. <i>Oncocyclus</i> (Siemss.) Benth.		
<i>I. acutiloba</i> Mey.	X	
<i>I. gatesii</i> M. Foster	X	
<i>I. sustiana</i> L.	X	
d. Subsect. <i>Pseudoregelia</i> (Dykes) Lawr.		
2. Sect. <i>Spathula</i> Tausch. emend. Lawr.		
a. Subsect. <i>Pardanthopsis</i> (Hance) Lawr.		
b. Subsect. <i>Foetidissima</i> Diels		
<i>I. foetidissima</i> L.	X	
c. Subsect. <i>Apogon</i> Benth.		
series <i>Sibiricae</i> (Diels) Lawr.		
<i>I. chrysographes</i> Dykes	X	
<i>I. forrestii</i> Dykes	X	
<i>I. sibirica</i> L.	X	
series <i>Californicae</i> (Diels) Lawr.		
<i>I. bracteata</i> Wats.	X	
<i>I. chrysophylla</i> Howell	X	
<i>I. douglasiana</i> Herb.	X	
<i>I. fernaldii</i> R. C. Foster	X	
<i>I. bartwegii</i> Baker	X	
<i>I. innominata</i> L. F. Henderson	X	
<i>I. macrosiphon</i> Torr.	X	
<i>I. munzii</i> R. C. Foster	X	
<i>I. pinetorum</i> Eastw.	X	

series <i>Californicae</i> (Diels) Lawr. <i>Continued</i>	
<i>I. purdyi</i> Eastw.	X
<i>I. tenax</i> Dougl.	X
<i>I. tenuissima</i> Dykes	X
<i>I. thompsonii</i> R. C. Foster	X
series <i>Syricae</i> (Diels) Lawr.	
series <i>Chinenses</i> (Diels) Lawr.	
series <i>Ruthenicae</i> (Diels) Lawr.	
series <i>Unguiculares</i> (Diels) Lawr.	
<i>I. unguicularis</i> Poir.	X
series <i>Spuriae</i> (Diels) Lawr.	
<i>I. graminea</i> L.	X
<i>I. kerneriana</i> Aschers	X
<i>I. spuria</i> L.	X
<i>I. crocea</i> Jacquem (= <i>I. spuria</i> L. ?)	X
<i>I. ochroleuca</i> L. (= <i>I. spuria</i> L. ?)	X
<i>I. monnieri</i> DC (= <i>I. spuria</i> L. ?)	X
series <i>Laevigatae</i> (Diels) Lawr.	
<i>I. pseudacorus</i> L.	X
series <i>Prismatica</i> Lawr.	
series <i>Hexagonae</i> (Diels) Lawr.	
<i>I. fulva</i> Ker-Gawl.	X
<i>I. hexagona</i> Walt.	X
'Louisiana hybrids' hort.	X
series <i>Longipetalae</i> (Diels) Lawr.	
<i>I. longipetala</i> Herb.	X
<i>I. missouriensis</i> Nutt.	X
series <i>Tripetalae</i> (Diels) Lawr.	
series <i>Vernae</i> (Diels) Lawr.	
d. Subsect. <i>Evansia</i> Benth.	
<i>I. confusa</i> Sealy	X
<i>I. japonica</i> Thunb.	X
<i>I. milesii</i> M. Foster	X
<i>I. tectorum</i> Maxim.	X
II. Subgen. <i>Nepalensis</i> (Dykes) Lawr.	
III. Subgen. <i>Xiphium</i> (Mill.) Spach	
1. Sect. <i>Xiphium</i>	
<i>I. filifolia</i> Boiss.	X
<i>I. fontanesii</i> Godr.	X
<i>I. xiphium</i> L.	X
2. Sect. <i>Reticulata</i> Spach	
IV. Subgen. <i>Scorpiris</i> Spach	
<i>I. alata</i> Poir.	X
<i>I. bucharica</i> M. Foster	X
V. Subgen. <i>Gynandiris</i> (Parl.) Lawr.	

In 1953 Mr. Lloyd Austin of the Rainbow Hybridizing Gardens, Placerville, California, made, in the course of his breeding program, numerous crosses between various species and horticultural varieties of different subsections of the bearded iris. Among these crosses were those of: (1) *Oncocyclus* × *Oncocyclus*, (2) *Oncocyclus* ♀ × *Hexapogon* ♂, (3) *Hexapogon* ♀ × *Oncocyclus* ♂, and (4) *Oncogelia* ♀ × *Oncocyclus* ♂. Seeds from a number of these matings were embryo cultured at the Rancho Santa Ana Botanic Garden during the winter of 1953. Table II summarizes the results.

TABLE II
Cross*

	Embryos Excised	Embryos Germinated
<i>Oncocyclus</i> × <i>Oncocyclus</i>		
1. <i>I. gatesii</i> × <i>I. auranitica</i>	30	30
2. <i>I. gatesii</i> × <i>I. nigricans</i>	58	49
4. <i>I. gatesii</i> × Mx-8	5	5
<i>Hexapogon</i> ♀ × <i>Oncocyclus</i> ♂		
6. <i>I. boogiana</i> 'Bronze Beauty' × <i>I. belenae</i>	5	2
8. <i>I. boogiana</i> 'Bronze Beauty' × <i>I. nigricans</i>	10	0
10. <i>I. boogiana</i> × <i>I. auranitica</i>	6	2
11. <i>I. korolkowi</i> 'Brown and Green' × <i>I. auranitica</i>	4	1
14. <i>I. korolkowi</i> 'Brown and Green' × <i>I. lortetii</i>	22	1
15. <i>I. korolkowi</i> 'Brown and Green' × <i>I. nigricans</i>	3	0
16. <i>I. korolkowi</i> 'Brown and Green' × <i>I. sofarina</i>	6	1
17. <i>I. korolkowi</i> 'Brown and Green' × <i>I. susiana</i>	3	2
20. <i>I. korolkowi</i> 'Violaceae' × <i>I. auranitica</i> 'Spotted'	3	0
22. <i>I. korolkowi</i> 'Violaceae' × <i>I. hauranensis</i>	2	0
23. <i>I. korolkowi</i> 'Violaceae' × <i>I. hermona</i>	2	0
24. <i>I. korolkowi</i> 'Violaceae' × <i>I. susiana</i>	2	0
<i>Oncocyclus</i> ♀ × <i>Hexapogon</i> ♂		
30. <i>I. nigricans</i> × <i>I. boogiana</i> 'Bronze Beauty'	1	1
31. <i>I. nigricans</i> × <i>I. boogiana</i> 'Late Amethyst'	1	1
<i>Oncogelia</i> ♀ × <i>Oncocyclus</i> ♂		
35. <i>I. 'Parthenope'</i> × <i>I. auranitica</i> 'Spotted'	2	2
36. <i>I. 'Sirona'</i> × <i>I. susiana</i>	6	6
37. <i>I. 'Teucros'</i> × <i>I. gatesii</i>	4	4
38. <i>I. 'Teucros'</i> × <i>I. haynei</i>	2	2
39. <i>I. 'Teucros'</i> × <i>I. lortetii</i>	2	2
41. <i>I. 'Theseus'</i> × <i>I. auranitica</i> 'Spotted'	28	28
42. <i>I. 'Theseus'</i> × <i>I. gatesii</i>	8	8
43. <i>I. 'Theseus'</i> × <i>I. nigricans</i> 'Black'	1	1
44. <i>I. 'Theseus'</i> × <i>I. nigricans</i>	1	1
47. <i>I. 'Theseus'</i> × <i>I. sofarina</i>	5	5
48. <i>I. 'Theseus'</i> × <i>I. susiana</i>	14	13
50. <i>I. 'Ulysses'</i> × <i>I. auranitica</i> 'Spotted'	12	12

The number of embryos obtained from some of the crosses is very small, however, totals for groups gives some indication of germination percentages.

TABLE III

Cross	Total Seeds Collected	Seeds with Embryos	Embryos Germin.	% Germin.
<i>Oncocyclus</i> × <i>Oncocyclus</i>	136	93	84	90%
<i>Hexapogon</i> ♀ × <i>Oncocyclus</i> ♂	323	48	9	19%
<i>Oncocyclus</i> ♀ × <i>Hexapogon</i> ♂	19	2	2	100%
<i>Oncogelia</i> ♀ × <i>Oncocyclus</i> ♂	201	85	84	99%

Causes for delayed seed germination are many and varied and the reader's attention is directed to the recent publications of Barton and Crocker (1948) and Crocker and Barton (1953) for a comprehensive survey of the literature. In studying the cause of delayed germination in tall-bearded iris Randolph and Cox (1943) were able to show that the endosperm had an inhibiting effect on the growth of the embryo. When excised embryos of the tall-bearded iris were placed on nutrient agar they germinated within a few days and in a few weeks the seedlings were large enough to transplant. However, if the embryo was left in contact with even a por-

*Botanical names are those under which the plants were imported and are being grown. A careful taxonomic study might show that certain changes should be made.

tion of the endosperm the growth of the embryo was inhibited or progressed slowly and in an atypical manner.

Experiments designed to remove or inactivate the inhibiting substances were only partially successful; and Randolph and Cox concluded that the inhibiting substances were highly stable compounds, relatively insoluble in water and not readily diffusible from the region of the embryo.

Work at this laboratory has shown that when seeds of *I. douglasiana* were soaked for 48 hours in only sufficient water to cover them and the water then removed and used to prepare nutrient agar that the resulting medium was highly inhibitory to excised embryos up to a dilution of 1 part filtrate to ten parts of water. The principal effect shown by the seedlings was the inhibition of root formation at the higher dilutions and complete inhibition of the embryos at high concentrations. As suggested by Randolph and Cox the inhibiting substance or substances are apparently highly stable compounds and are not inactivated by sterilizing at 15 pounds pressure in an autoclave. An attempt is now being made to identify the material which is responsible for this inhibiting effect.

Failure of the embryos from seeds of *Hexapogons* to germinate when removed from the influence of the endosperm clearly demonstrates that a different inhibitor is present in these species than that that is operating in other species of the genus that were tested.

In an attempt to find out if there was some substance within the embryo itself which was preventing germination, one hundred embryos from the cross *I. stolonifera* 'Decorated Delight' $\times$ *I. korolkowii* 'Brown and Green' were excised and macerated in 10 cc. of water. The material was then filtered and the filtrate used to prepare 10 cc. of nutrient agar, the filtrate being used in place of water. As a check 10 cc. of standard nutrient agar was also prepared. Petri plates were poured and each was then planted with fifteen embryos of *I. longipetala*. The plates were incubated at 80° F. for one week and then photographed (Fig. 2). None of the embryos planted on the agar prepared with the embryo extract showed normal germination and only two showed an abnormal type of growth. The remaining embryos had merely elongated somewhat. In the case of the check a number of the embryos had already germinated and had produced roots up to a centimeter long. Another difference noted was that many of the embryos planted on the embryo filtrate agar showed a yellowish or brown color, quite unlike the checks which were ivory white.

In order to determine whether the inhibiting effect shown here was due to some substance peculiar to *Hexapogon* embryos or whether it was to be found in other species as well, a second experiment was run using embryos of *I. longipetala* and the resulting medium then planted with embryos of the same species. Here growth of the embryos was similar to that of the embryos grown on the standard nutrient agar except that growth was slightly slower. Thus it would appear there is some substance in the embryos of the *Hexapogon* species which can inhibit germination of embryos of other species. The slight growth depression shown in the case of the *longipetala* embryos growing on *longipetala* embryo extract agar could be due either to some substance normally present in the embryos or possibly to a certain amount of the inhibiting substance from the endosperm which might have entered the embryos during the time that they were soaking preparatory to excision.

One of the oldest known and commonest methods for breaking seed dormancy is stratification where the seeds are stored in a moist condition at low temperatures for varying lengths of time after which they are brought into warm temperatures favorable for germination. Barton and Crocker (1948) report that *Iris versicolor*

reacted favorably after 75 days at 33-50° F. with the optimum temperature given as 41° F. They do not, however, give the percentage of germination. At the Rancho Santa Ana Botanic Garden it has been found (Lenz, unpubl.) that seeds of the Pacific Coast species of iris germinate well without treatment of any kind, and seeds planted in flats and placed in a cool greenhouse in October will, by early February, show from 90-100% germination. Randolph and Cox (1943) on the other hand report that in tall-bearded iris, over a period of four years, that only 14-50% of the seeds planted in the fall germinated the following spring. Furthermore they did not find that storage of seed either in a dry condition, or stored in moist sand at -2° C., 0° C., or +4° C. gave satisfactory results.

Justice (1940) working with *Polygonum scandens* L. found that less than 10% of the excised embryos from mature untreated seeds germinated when placed on nutrient agar, while 76% of those from stratified seeds grew when excised and grown under similar conditions. He also found that the low temperature necessary for after-ripening could be given the embryos after they had been excised and that five weeks at 2-4° C. resulted in 85-95% germination when placed in the germinator.

In order to determine whether cold treatment might be effective in bringing about germinations of *Hexapogon* embryos, test tubes containing standard nutrient agar and planted with *Hexapogon* embryos were placed in the refrigerator maintained at approximately 38° F. where they were allowed to remain for varying lengths of time. Preliminary tests with small numbers of embryos showed that ten weeks at 38° F. gave the highest percentage of germination when the embryos were removed and placed in the germinator.

Table IV shows germination percentages of embryos after ten weeks refrigerator storage compared with checks which were kept at room temperature for the same period of time. Since seedlings from these crosses were wanted for breeding, in the earlier experiments only a very few tubes were kept in the laboratory to serve as checks. In later experiments an equal number of tubes were maintained at room temperature so that more accurate germination percentages could be made.

TABLE IV
Embryo Germination at the end of Ten Weeks

CROSS NUMBER	CROSS	ROOM TEMP.			REFRIGERATOR		
		No. embryos excised	No. embryos germin.	% Germina- tion	No. embryos excised	No. embryos germin.	% Germina- tion
51	<i>I. hoogiana</i> 'Bronze Beauty' × self	14	0	0%	14	11	79%
53	<i>I. hoogiana</i> 'Bronze Beauty' × <i>I. stolonifera</i> 'Decorated Delight'	29	1	3%	27	21	78%
54	<i>I. hoogiana</i> 'Blue Joy' × self	25	0	0%	24	19	79%
57	<i>I. hoogiana</i> No. 2 × <i>I. stolonifera</i> 'Leichtlini'	5	1	20%	72	54	75%
58	<i>I. hoogiana</i> No. 2 × <i>I. korolkowi</i> 'Pink'	5	1	20%	27	25	93%
59	<i>I. hoogiana</i> 'Late Amethyst' × <i>I. korolkowi</i> 'Brown and Green'	3	0	0%	4	1	25%
62	<i>I. hoogiana</i> 'Late Amethyst' × <i>I. stolonifera</i>	5	1	20%	82	70	85%

68 <i>I. stolonifera</i> 'Decorated Delight' × <i>I. korolkowi</i> 'Pink'	10	1	10%	29	23	79%
69 <i>I. stolonifera</i> 'Decorated Delight' × self	12	2	17%	11	7	64%
				Room Temperature		Refrigerator
Number of embryos				108		290
Number of embryos germinated				7		231
% germination				6.4%		79.6%

At the conclusion of the germination studies reported in Table II the ungerminated embryos of numbers 6-24 (*Hexapogon* ♀ × *Oncocylus* ♂) were placed in the refrigerator and allowed to remain from March 5th until May 15th when they were removed and placed in the germinator. Table V shows the results of this experiment. It is interesting to note that these embryos had all remained at laboratory temperature from the date of excision on December 28th until March 5th before being given the cold treatment and all of them showed the characteristic malformed swelling shown in Figure 1.

TABLE V

	Embryos Excised	Number Germin. Room Temp.	Number Ungermin. end of 10 weeks Room Temp.	Number Germin. after Cold Treatment
6. <i>I. boogiana</i> 'Bronze Beauty' × <i>I. helena</i>	5	2	3	2
8. <i>I. boogiana</i> 'Bronze Beauty' × <i>I. nigricans</i>	10	0	10	2
10. <i>I. boogiana</i> × <i>I. auranitica</i>	6	2	4	2
11. <i>I. korolkowi</i> 'Brown and Green' × <i>I. auranitica</i>	4	1	3	0
12. <i>I. korolkowi</i> 'Brown and Green' × H-1	5	1	4	4
13. <i>I. korolkowi</i> 'Brown and Green' × H-7	7	2	5	2
14. <i>I. korolkowi</i> 'Brown and Green' × <i>I. lortetii</i>	2	1	1	1
15. <i>I. korolkowi</i> 'Brown and Green' × <i>I. nigricans</i>	3	0	3	3
16. <i>I. korolkowi</i> 'Brown and Green' × <i>I. sofarina</i>	6	1	5	3
17. <i>I. korolkowi</i> 'Brown and Green' × <i>I. susiana</i>	3	2	1	1
18. <i>I. korolkowi</i> 'Brown and Green' × W-189	2	1	1	1
20. <i>I. korolkowi</i> 'Violaceae' × <i>I. auranitica</i> 'Spotted'	3	0	3	1
22. <i>I. korolkowi</i> 'Violaceae' × <i>I. hauranensis</i>	2	0	2	1
23. <i>I. korolkowi</i> 'Violaceae' × <i>I. hermona</i>	2	0	2	1
24. <i>I. korolkowi</i> 'Violaceae' × <i>I. susiana</i>	2	0	2	2

DISCUSSION AND CONCLUSIONS

Cause of delayed germination in iris is apparently of at least two types: (1) an inhibitor or inhibitors present in the endosperm and, (2) dormant embryos. Of the species tested, the later type has been found only in the subsection *Hexapogon* of the Section *Pogoniris*, one of the three divisions of the bearded iris. All other species tested germinated and grew normally when planted on Randolph's nutrient agar and kept at room temperature after the initial period in the germinator.

Fig. 2. *Iris longipetala* embryos after one week on: (1) standard nutrient agar; (2) *Hexapogon* embryo extract agar.

The dormant embryos of the *Hexapogons* and their interspecific hybrids were found to react favorably to cold treatment administered after the embryos had been excised and planted on nutrient agar. Ten weeks at $38^{\circ}\text{F.} \pm$ was found to produce up to 93% germination of the embryos when they were later placed in the germinator. Thus from the plant breeding standpoint the cold treatment is a useful method for overcoming the long delayed germination so characteristic of this group of irises. However, even after the cold treatment, many of the embryos still react differently from the majority of the species in the genus that have been tested. During the time that the embryos are undergoing the cold treatment they change very little except to elongate somewhat. When they are removed from the cold and placed in the germinator, they increase in size very rapidly and some germinate within a matter of 2 to 3 days while others will become rather deformed and will even take on some of the characteristics of the untreated embryos. However, most of the embryos that are going to germinate will do so within ten days to two weeks and once germinated the seedlings grow normally though slowly. It would therefore appear that there may be factors other than the need for cold treatment that are involved in the germination of these irises.

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