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In vitro plant regeneration via somatic embryogenesis from root culture of some rhizomatous irises

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Abstract

A method for plant regeneration of *Iris* via somatic embryogenesis is described. Root and leaf pieces from in vitro-grown plants of several genotypes of rhizomatous *Iris* sp. were cultured in vitro. Callus induction occurred only on root cultures incubated under low light intensity (35 μmol m^{-2} s^{-1}) on two induction media containing 2,4-D (4.5 or 22.5 μM), NAA (5.4 μM) and kinetin (0.5 μM). Somatic embryos developed after transfer of callus onto four regeneration media containing 9 or 22 μM BA, or 5 μM kinetin and 2 μM TIBA or 9 μM BA and 4 μM TIBA. Plantlets could be obtained from these somatic embryos. Genotypic differences were found both in callus induction and somatic embryo formation, with *I. pseudacorus* responding better than *I. versicolor* or *I. setosa*. Cytological analysis performed on root tips of 80 regenerated plants revealed that two of the *I. pseudacorus* regenerants were tetraploid.

Abbreviations: 2,4-D – dichlorophenoxy acetic acid, NAA – naphthaleneacetic acid, BA – 6-benzyladenine, TIBA – 2,3,5-triiodobenzoic acid, IBA – indolebutyric acid

Introduction

The genus *Iris* includes over 300 species, many of which are valuable and horticulturally important. Flowers are either bearded or beardless types and are composed of parts in sets of three. The species may differ in shoot structure, some being bulbous, some rhizomatous and a few stoloniferous (Lenz 1978). Like other ornamental monocotyledonous species with bulbs or rhizomes, irises are generally propagated vegetatively. In recent years, a number of studies have appeared regarding in vitro propagation of some *Iris* species using shoot apices (Reuther 1977), rhizomes (Kromer 1985), young inflorescences (Meier et al. 1975), mature embryos (Randolph & Cox 1943; Stoltz 1977), shoots (Radojevic et al. 1987) and bulb scales (van der Linde et al. 1988a, b). With one exception (Reuther 1987), these studies have, to a variable degree, stressed the fact that irises respond poorly in tissue culture. In a comparative study evaluating the regeneration capacity of some monocotyledonous ornamental species, members of the Iridaceae ranked last, after Amaryllidaceae, Araceae and Liliaceae, respectively (Hussey 1975). Among the responding species, only *I. hollandica*, a very important flower-bulb crop, exhibited sufficient regenerative capacity to permit mass propagation in vitro (van der Linde et al. 1988a, b).

Our research has been focussed on water garden irises such as *I. setosa* Pall. (2n = 38), *I. pseudacorus* L. (2n = 34) and *I. versicolor*. Among these, our primary interest is in *I. versicolor* (2n = 108), the large blue flag, native of the northeastern North America, which carries the highest chromosome number known in the genus *Iris*. To the best of our knowledge, no

reports are available on in vitro culture of these or any other species belonging to the Tripetalae and Laevigatae series, which are all of the rhizomatous, beardless type. In the present paper we describe how regeneration of plants was achieved via somatic embryogenesis from in vitro-derived root segments in several genotypes of *I. pseudacorus* and *I. versicolor*. Somatic embryogenesis and plant regeneration from root culture has not yet been reported in irises.

Materials and methods

Plant material used in the present study was kindly provided by the research group of W.H. Perron & Co. Ltd, Montréal, Canada. In order to establish in vitro stock material, mature seeds from several genotypes belonging to *I. setosa*, *I. pseudacorus* and *I. versicolor* were rinsed for 60 s with 95% ethanol, surface disinfested in 2.5% sodium hypochlorite for 10 min and washed three times in distilled water. Embryos were dissected from seeds and cultured in 90 mm petri dishes containing 20 ml of growth regulator-free Murashige & Skoog's (1962) nutrient medium (MS) solidified by 7.0 $g\,l^{-1}$ Difco Bacto-agar. The pH was adjusted to 5.8 with 0.5 N NaOH before adding agar, and the medium was autoclaved at 1.2 $kg^{-1}\,cm^{-2}$ for 20 min at 121°C. Cultures, sealed with Parafilm®, were incubated at 25°C under a 16-h photoperiod provided by cool-white fluorescent lights (Sylvania) at 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 2 weeks the resulting shoots reached a 3–4 cm height and were transferred into Magenta GA-7 vessels (Magenta Corp. Chicago, I11.), one plant per vessel, containing 50 ml of MS medium supplemented with 5 μM of IBA to induce rooting. The material was multiplied by subculturing newly formed basal shoots every 5–6 weeks on the same medium. The culture conditions were identical to those described above. Approximately 1 cm long root pieces and 5 × 2 mm leaf pieces from vigorously growing material were thereafter isolated and cultured on 2 induction media (20 pieces per petri dish).

Induction medium A, containing MS nutrients, was supplemented with 4.5 μM 2,4-D, 5.4 μM NAA and 0.5 μM kinetin; Medium B was identical except for 22.5 μM 2,4-D. All cultures were sealed with Parafilm® and kept under either high (80 $\mu mol\,m^{-2}\,s^{-1}$) or low light intensity (35 $\mu mol\,m^{-2}\,s^{-1}$). After 10–12 weeks, responsive root pieces had clusters of nodular callusses on their surface. When these discrete structures reached 3–4 mm diameter, they were transferred onto one of 4 regeneration media containing MS medium supplemented with 9 μM BA (medium C), 2 μM TIBA and 5 μM kinetin (medium D), 22 μM BA (medium E) or 4 μM TIBA and 9 μM BA (medium F).

On these media, the surface of nodular calluses became covered with white structures resembling young zygotic embryos. Four to six weeks later, structures at different developmental stages could be observed on the same callus. Less mature embryos (globular or elongating) were then transferred onto fresh medium for further growth, while the most advanced ones (notch-stage or beyond) were transferred onto the rooting medium. Two or three weeks later, chromosome counts were performed on somatic cells of the root meristems in a number of regenerated plants. The root tips were treated with a freshly prepared saturated solution of 1-bromonaphthalene for 90 min, stained with Schiff's reagent and squashed in 1% acetocarmine. Five to ten metaphases were studied per selected individual. For histological studies, cultured root pieces and developing calluses were fixed in Craf type III (Berlyn & Miksche 1976) for 24 h, dehydrated using an ethanol-tertiary butanol series, and embedded in paraffin (Paraplast). Embedded tissues were sectioned at 8 μm thickness with a rotary microtome, the paraffin removed in a xylene-ethanol series and the sections stained with blue Astra and safranin according to Gerlach (1977) modified by Morisset (unpublished) by using anilined, alcoholic safranin and by reversing the order of the stains.

Results

In the present study only root pieces were found to respond positively to our culture conditions, in spite of substantial efforts to induce callus from leaves. In preliminary experiments several callus induction media were tested (data not

Table 1. Comparison of two culture media for callus induction from root pieces of different genotypes of *Iris* sp.[z] cultured under low light intensity (35 μmol m^{-2} s^{-1}).

Genotype	Medium A[y]		Medium B[y]		χ^2
	No. of roots cultured	% of roots that produced callus	No. of roots cultured	% of roots that produced callus	
I. pseudacorus, gen.-1	160	37.5	160	26.3	4.663**[x]
I. pseudacorus, gen.-2	120	36.7	120	18.3	10.115***
I. versicolor, gen.-1	120	21.7	120	8.3	8.366**
I. versicolor, gen.-2	80	32.5	80	5.0	24.935***
I. setosa, gen.-1	80	11.3	80	5.0	NS
χ^2		24.602***[w]		38.490***	

[z] Based on the number of root pieces capable of callus formation rather than on the number of calluses produced by responding root pieces.
[y] Medium A and B contained 4.5 μM and 22.5 μM 2,4-D, respectively, and were identical in other components.
[x] χ^2 for comparison within each row.
[w] χ^2 for comparison within each column.
*, **, and *** denote significant at 5, 1 and 0.1% level, respectively. NS, not significant.

shown), two of which (Medium A and B) were retained for further evaluation. More than 4000 leaf pieces were cultured on both media. Sporadic callus initiation was observed in cultures incubated under low light intensity; further proliferation, however, never occurred. Roots cultured under high light intensity never formed any callus on either medium, and turned brown in about 4–6 weeks. The results obtained with root pieces cultured under low light intensity are reported in Table 1. Medium A was consistently better than medium B in inducing callus formation and in sustaining faster callus growth. It was, therefore, retained for further experiments. With regard to genotypic differences, *I. setosa* responded poorly on both media (Table 1). In contrast, genotypes 1 and 2 of *I. pseudacorus* were particularly responsive. In these genotypes, callus appeared sooner (after only 8–9 weeks), in higher numbers (10–20 vs 2–3 on other genotypes), and proliferated more rapidly. These observations held true for both media tested. Histological sections showed that callus generally developed from lateral root primordia at the peripheral region of the central cylinder, and ruptured the root exodermis during later growth (Fig. 1). A single root usually produced several calluses, which were yellowish, nodular and compact (Fig. 2). Sometimes one or more calluses also appeared on a lateral root. Histological examination of nodular callus revealed large vacuolated parenchymatous cells at the inner core,

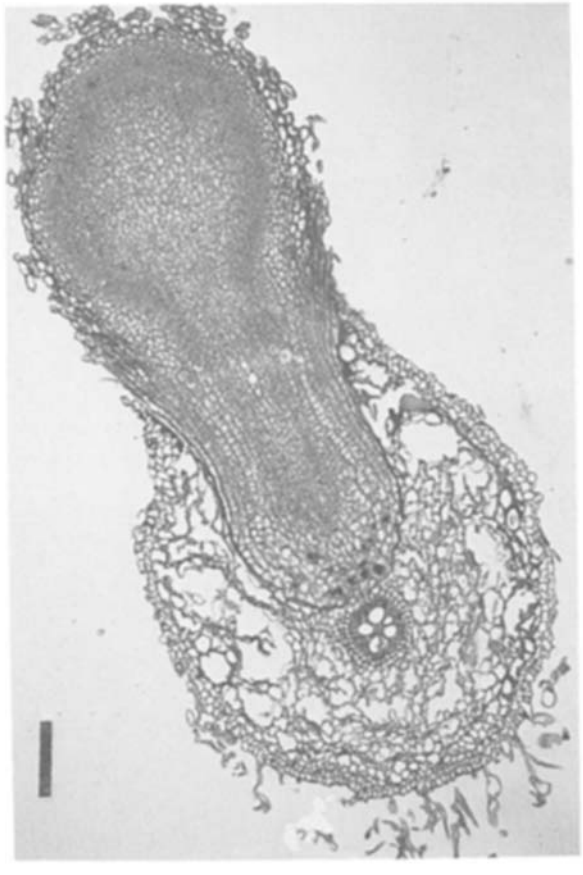

Fig. 1. Transverse root section of *I. pseudacorus* genotype 1 showing callus initiation after culture on induction medium A for 8 weeks (bar = 260 μm).

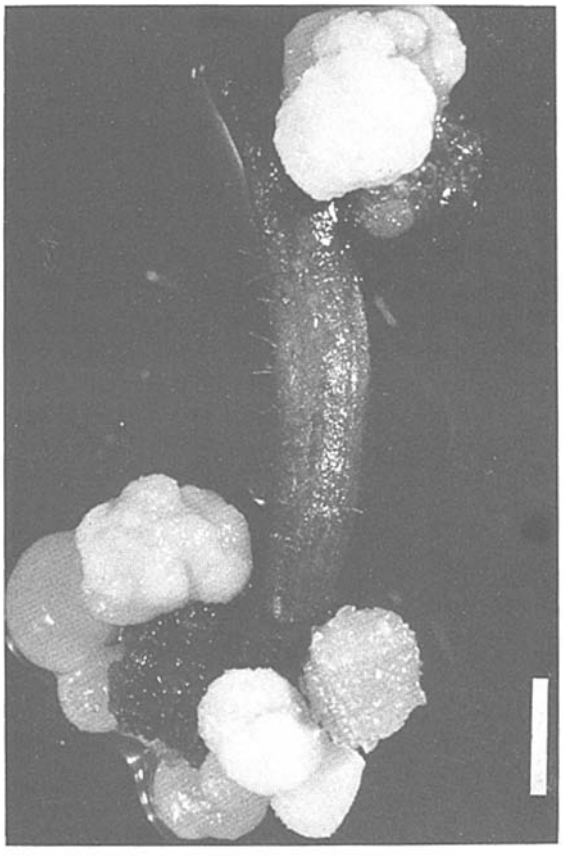

Fig. 2. Callus formation on root explants of *I. pseudacorus* after culture on medium A for 10 weeks (bar = 1.5 mm).

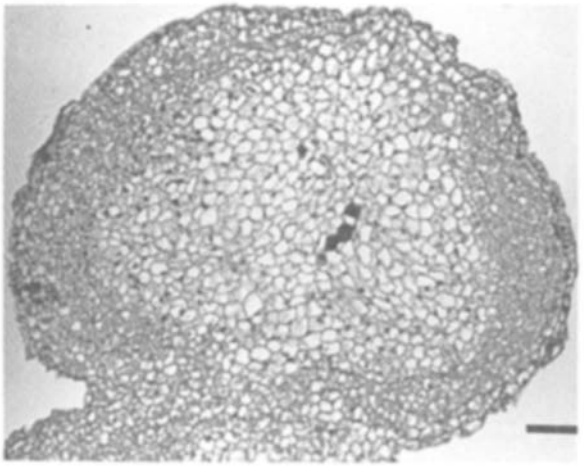

Fig. 3. Section of nodular callus from *I. pseudacorus* cultured for 13 weeks on medium A (bar = 180 μm).

while the peripheral region consisted of small, densely stained, highly cytoplasmic, meristematic cells (Fig. 3). On the basis of these results, additional root pieces from more genotypes were cultured only on medium A (Table 2). As in the previous experiments, genotypes 1 and 2 of *I. pseudacorus* produced the fastest growing calluses. Genotypic variability was observed within *I. versicolor*, where 3 genotypes responded well, two were intermediate and one gave a poor response. Six additional *I. versicolor* genotypes were also tested, but callus induction was not obtained (data not shown).

After the individual calluses had reached a diameter of 3–4 mm, they were transferred onto one of 4 regeneration media. Within 2 weeks of transfer to auxin-free media, white embryo-like globular structures started to appear (Fig. 4). These grew rapidly while new ones continued to appear on the callus surface, as well as on previously formed structures, so that within 3 more weeks, clusters of structures at various stages of development could be found on the same callus. These structures were very similar to the somatic embryos obtained on other *Iris* species and previously described by Reuther (1977). At early stages of development they appeared separated from the callus tissue by a protoderm-like cell layer. In the course of further development they were surrounded by a well-defined protoderm and elongated to form bipolar structures. These

Table 2. Callus induction from root pieces of various *Iris* genotypes on culture medium A[z].

Genotype	No. of cultured root pieces	% of roots that produced callus
I. pseudacorus, gen.-1	845	38.3
I. pseudacorus, gen.-2	440	39.1
I. pseudacorus, gen.-3	100	21.0
I. versicolor, gen.-4	150	37.3
I. versicolor, gen.-5	375	38.9
I. versicolor, gen.-6	175	4.6
I. versicolor, gen.-7	75	38.7
I. versicolor, gen.-8	50	30.0
χ^2		91.253***

[z] Based on the number of root pieces capable of callus formation rather than on the number of calluses produced by responding root pieces. See Table 1 for medium A composition.

*** Significant at 0.1% level.

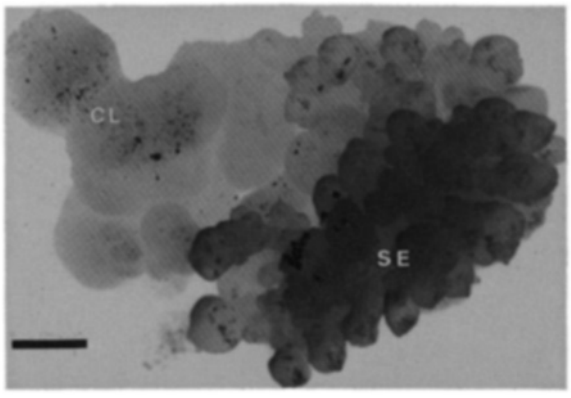

Fig. 4. Young somatic embryos of *I. pseudacorus* growing on responsive calluses subcultured for two weeks on regeneration medium C (bar = 1.2 mm). CL = callus; SE = somatic embryos.

remained attached to the embryogenic callus through a sort of proembryonal complex characteristic of indirect embryogenesis (Gray 1990). Gradually, the somatic embryos assumed the morphology typical of monocotyledonous zygotic embryos and displayed, at maturity, a characteristic pyriform bipolar configuration (Fig. 5). During the elongation process, a slight depression, or notch, was formed on one side of the developing embryos. This concavity, where shoot apex growth would occur, was deeper and

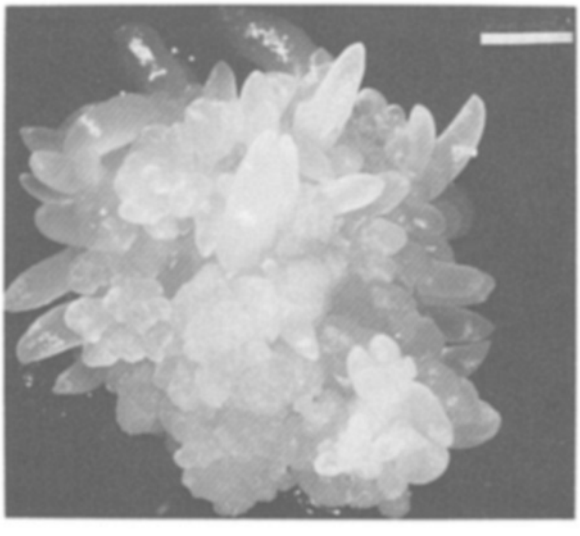

Fig. 5. Somatic embryos of *I. pseudacorus* at late stages of development, after subculture on medium C for 4 weeks (bar = 2.5 mm).

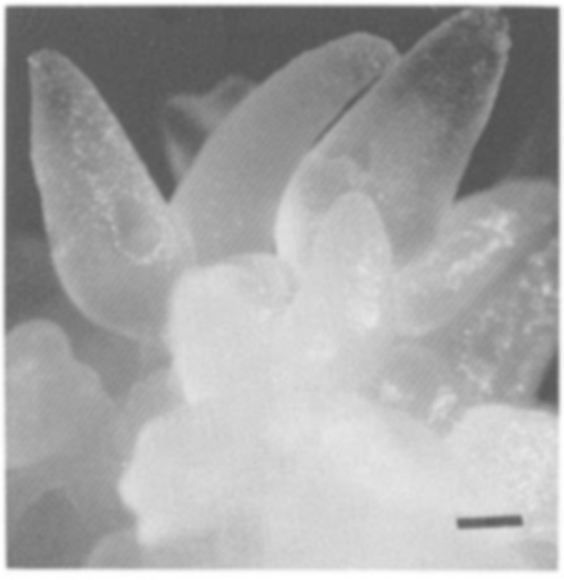

Fig. 6. Near mature somatic embryos of *I. pseudacorus* showing the notch (bar = 650 μm).

quite evident on the most advanced embryos (Fig. 6). In comparison, mature sexual embryos were characterised by a cylindrical shape, and the concavity was not as visible. Further details on anatomical and ultrastructural features of the somatic embryos will be reported later (Laublin et al. in preparation).

Frequency of somatic embryo formation differed among genotypes and media (Table 3). *Iris pseudacorus* genotype 1 and *I. versicolor* genotype 1 responded equally well on all 4 media. These genotypes, however, differed in plant regeneration ability; over 500 plants have been regenerated from the former as compared to less than 50 from the latter (data not shown). *Iris pseudacorus* genotype 2 responded similarly, but only on media D and F. Over 200 plants have been regenerated from this genotype. Chromosome counts were obtained from a random sample of 40 vigorously growing regenerants of *I. pseudacorus* genotype 1 and 20 each of *I. pseudacorus* genotype 2 and *I. versicolor* genotype 1. Cytological analysis did not reveal any variation in chromosome number except for 2 plants from *I. pseudacorus* genotype 1, where chromosome doubling had occurred (2n = 4x = 68).

Table 3. Effects of different regeneration media (see Materials and methods) and genotype on the development of somatic embryos on the surface of calluses obtained from root pieces[z].

Genotype	% calluses that produced somatic embryos (total number of pieces cultured)[y]				
	Medium C	Medium D	Medium E	Medium F	χ^2
I. pseudacorus, gen.-1	85.0 (20)	76.2 (21)	81.8 (22)	78.9 (19)	NS[x]
I. pseudacorus, gen.-2	27.8 (18)	55.6 (9)	0 (14)	53.8 (13)	11.979**
I. versicolor, gen.-1	66.7 (3)	50.0 (4)	75.0 (4)	60.0 (5)	NS
I. versicolor, gen.-4	0 (6)	0 (8)	0 (6)	0 (9)	–
I. versicolor, gen.-5	0 (6)	0 (10)	0 (9)	0 (10)	–
I. versicolor, gen.-7	14.3 (7)	0 (9)	0 (3)	0 (7)	–
I. versicolor, gen.-8	0 (4)	0 (4)	0 (3)	0 (4)	–
χ^2	17.207***[w]	NS	NS	NS	

[z] Based on the number of calluses capable of somatic embryos/plant formation rather than on the number of somatic embryos or plants produced each responding callus.
[y] χ^2 was calculated only for the genotypes or the media where somatic embryos were observed.
[x] χ^2 for comparison within each row.
[w] χ^2 for comparison within each column.
** significant at 1% level, *** significant at 0.1% level, NS not significant.

Discussion

Somatic embryogenesis from callus tissue derived from root pieces has not been previously reported in *Iris*. Plant regeneration from root cultures has been reported only in a few species (for review see Tisserat 1985). In the Poaceae, the first example of somatic embryogenesis from root culture was reported by Abe & Futsuhara (1984) in rice. In that study, two pathways for plant regeneration, i.e. organogenesis and somatic embryogenesis, were observed, and it was found that the predominant mode of regeneration was genotype-dependent. In the present report only somatic embryogenesis has been observed in all responding genotypes. Our observation that *Iris* callus usually developed from lateral root primordia was similar to those of Abe & Futsuhara (1985) for rice. Histology showing calluses growing from peripheral cells of the central cylinder did not clarify whether or not the callus originated from pericycle cells. Direct formation of somatic embryos or shoot differentiation from root tissues, as reported in alfalfa (dos Santos et al. 1983) and aspen (Ahuja 1983), respectively, was never observed in the present study.

It is frequently observed in plant tissue culture that the steps leading to plant regeneration are genotype dependent. Wenzel & Uhrig (1982) working with potatoes observed that a responsive genotype would regenerate regardless of the medium used, whereas a non-responding genotype would fail to regenerate on practically any media tested. Our findings support their observations except for a lack of response of *I. pseudacorus* genotype 2 on medium E, and a positive response with *I. versicolor* genotype 7 on medium C (Table 3).

In the present study, the mature somatic embryos quickly germinated on the regeneration media but did not form roots, despite the presence of a well-defined root meristem, unless they were transferred to an IBA-containing medium. In this regard our results appear in contrast with observations on *Iris* made by Reuther (1977) and Radejevic et al. (1987), who reported normal germination of their somatic embryos. The failure of somatic embryos to properly germinate or root has been reported several times in other plants, for example bahiagrass (Marousky & West 1990), asparagus (Kunitake & Mii 1990), wheat (Ozias-Akin & Vasil 1983), and rice (Abe & Futsuhara 1985). This has been generally associated with embryo malformations, competition for resources and abundant secondary embryogenesis. The rooting problems, in particular, could be overcome after water treatment and transfer to medium with IBA (Kunitake & Mii 1990) or transfer to growth regulator-free media (Marousky & West 1990). In the present study, transfer of somatic embryos to IBA-containing

medium allowed rapid development of roots. It is pertinent to point out that in preliminary experiments (data not shown) we had found that even isolated sexual embryos, capable to germinate rapidly, were unable to form roots for up to 4 weeks, unless transferred to a rooting medium.

Finally, cytological analyses of selected regenerants revealed a normal chromosome number in all but 2 of the 80 plants investigated, although the occurrence of cryptic changes such as translocations, inversions, deletions, etc. cannot be excluded. Since the species we used here generally flower at the second year, the data concerning the phenotypic expression of characters related to the plant at maturity are not yet available, but will be collected at the proper time. Among reports dealing with plant regeneration in irises, only Radojevic et al. (1987) performed some genetic analyses on seven regenerants of *I. pumila* produced via somatic embryogenesis. The analyses revealed that the regenerants had retained the genetic traits of the donor plant.

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