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MUTATION BREEDING OF *CHRYSANTHEMUM MORIFOLIUM* RAM. USING IN VIVO AND IN VITRO ADVENTITIOUS BUD TECHNIQUES

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SUMMARY

During experiments, which are being carried out to study the factors which control the process of adventitious bud formation in vivo on detached leaves of *Chrysanthemum morifolium* RAM, adventitious shoots were produced from leaves, irradiated with 500 rad of X-rays. The most important but disadvantageous result was that the majority of the adventitious shoots proved to be of a chimeral nature and obviously developed from more than one cell.

An in vitro adventitious bud technique was developed using different types of explants. Pedicel segments regenerated the highest number of adventitious shoots and, moreover, they developed faster as compared to explants of young flower heads or leaves. The mutants produced by irradiating the various explants were almost exclusively of a solid (non-chimeral) nature. In addition, histological observations suggest that single epidermal cells are involved in the initiation of the adventitious shoot apices.

The optimum dose for mutant production is approximately 800 rad X-rays. Rather often, more than one phenotypically identical mutant was found, which was always derived from the same explant. They could for instance originate from a multi-apical meristem formed by a single mutated cell.

INTRODUCTION

Mutation breeding is of great potential value for vegetatively propagated plants, such as potatoes, sweet potatoes, cassava, sugar cane, numerous fruit crops, forest trees, ornamentals, peppermint and various apomicts (*Poa;* other grasses). The main advantage is the possibility to improve one or a few important characters of an otherwise excellent cultivar, without basically altering the remaining genotype. Thus, outstanding cultivars, often being the result of a time-consuming and painstaking cross breeding programme, can be further perfected within a relatively short time-period. Moreover, it is the only way to induce variation in sterile plants and in apomicts. In *Chrysanthemum*, as in many other ornamental species, an additional advantage is the fact that selection of visible changes generally offers no serious problem and a favourable one soon may lead to the commercialization of the mutant. This holds true also for visible characters in other crops, like fruit colour and spurtype in apples and pears, skin colour of potato tubers as well as growth pattern, size, form and many other directly perceptible characters in various crops.

The main stumbling-block of mutation breeding in vegetatively propagated species is the phenomenon that the irradiation of multi-cellular apices of plants, rooted

cuttings, tubers, rhizomes or bulbs in most cases results in the formation of chimeras. Since, moreover, a mutated cell is subjected to intrasomatic selection and may also get lost as a consequence of chimera formation and of the structure of the apex, the result is a low frequency of unstable mericlinal chimeras which by repeated pruning (fruit trees; *Chrysanthemum*) or repeated asexual propagation (potatoes, 2 or 3 years) have to be transformed into stable periclinal chimeras and occasionally solid mutants (the so-called bud sports) before selection can be carried out. The difficulties related to chimera formation can be overcome by growing plants from single cells, in vivo or in vitro, which automatically would lead to a high(er) percentage of solid, non-chimeral mutants.

For the plant breeders a promising in vivo method is the adventitious bud technique, using detached leaves. This rests on the phenomenon that (the apex of) adventitious buds, formed at the base of the petiole, ultimately originate from a single (epidermal) cell. This has been demonstrated in *Saintpaulia* (BROERTJES, 1968, 1972b; SPARROW et al., 1960), *Streptocarpus* (BROERTJES, 1969), tobacco (DE NETTANCOURT et al., 1971), *Achimenes* (BROERTJES, 1972a), *Kalanchoë* (BROERTJES & LEFFRING, 1972), *Begonia* (DOORENBOS & KARPER, 1975) as well as in *Lilium* and *Peperomia* (BROERTJES, unpublished). In bulb-crops modified leaves (bulb-scales) are widely used. In *Lilium* exclusively solid, non-chimeral mutants are obtained when bulb-scales are irradiated immediately after scaling (BROERTJES, unpublished). The use of wounded bulbs or of artificially made bulb-scales may also result in wholly mutated plants (BROERTJES & ALKEMA, 1970).

In *Achimenes* and *Streptocarpus* hundreds and in *Begonia* even many more non-chimeral mutants have been produced. Several of these, generally completely stable mutants, were introduced into commerce in no more than approximately three years after the very beginning of the project.

Many plants can be propagated via adventitious plantlet production on detached leaves. BROERTJES et al. (1968) list over 350 species, covering a number of families, reported in the literature to belong to that group. This does not mean that plants not listed cannot be propagated that way: many have been tried without success but many more have never been tried. The breeder therefore should, with today's knowledge, always make an attempt with the cultivar(s) he is interested in. Many variables, however, are involved such as leaf-factors (age, position, absence or presence of the petiole, leaf-length (monocots), etc.), rooting medium, mineral nutrition, growth regulators and environmental conditions (BROERTJES & LEFFRING, 1972; ROEST & BOKELMANN, 1976).

Since in vitro culture have been developed for a great number of plant species and is increasingly used for a rapid multiplication, it seemed worthwhile to examine the regeneration ability of different types of *Chrysanthemum* explants and to investigate, by mutation induction, their significance for mutation breeding.

MATERIAL

Chrysanthemum has been selected for studying in vivo and in vitro propagation methods and their value for mutation breeding since detached leaves produce (few) adventitious shoots after rooting and because it was expected that in vitro propaga-

tion, using various explants, could be developed within a relatively short time-period. Moreover, the plant is easily handled and propagated whereas flower induction under short day conditions can be carried out throughout the year.

We used the pink flowered cultivar Bravo, of which numerous flower colour mutations can be obtained (JANK, 1957), in order to be able to decide whether or not a mutant has a chimeral structure. Furthermore, it was known that cv. Bravo occasionally developed adventitious plantlets on rooted detached leaves (most cultivars produce fewer plantlets or no plantlets at all).

METHODS AND RESULTS

Adventitious shoot formation in vivo. Under the most optimum conditions 100% of the detached, rooted leaves developed adventitious shoots, on callus at the base of the petiole or occasionally on callus formed on the upper part of roots, within a period of 2–6 months after leaf excision and with an average of 3–4 shoots per leaf (ROEST & BOKELMANN, 1976).

The next step was to grow adventitious plantlets from irradiated material. Just mature, detached leaves were irradiated with 500 rad X-rays and then rooted. The rooted leaves were potted 3 weeks later and started to produce the first adventitious shoots approximately 3 months after potting. During a few months all shoots were cut off, after having reached a certain size (25 cm) and then rooted and potted. When the lateral shoots of the adventitious plant(s) on the rooted leaf had reached a length of approximately 15 cm they were transferred to short day conditions together with the rooted cuttings (by taking cuttings and thus forcing the original adventitious plantlet to produce side-shoots it was expected to obtain more information about the possible chimeral structure of the adventitious shoots). The results were very complex and confusing and attempts, to order the data in such a way that a clear picture of the process of adventitious bud formation could be obtained, failed.

Of the 400 adventitious shoots, produced on 247 irradiated leaves (125 leaves with one shoot, 93 leaves had two shoots, 25 three shoots and 4 produced four or five shoots), and their rooted topshoots, 185 plants showed mutations: from (part of) a single petal to complete plants. All kinds of intermediate situations could be observed, such as part of the inflorescence mutated, only one flower head mutated, whereas also mutations were scored in the rooted cutting and not in the lateral shoots of the original adventitious plantlet or vice versa. Of the 185 plants (either the rooted topshoots or the original adventitious plants) carrying a mutation, 103 were solid (looking). But in a number of cases the rooted top-shoot, being a 'solid' mutant, was derived from an adventitious plant which was not or only partly mutated for the same character. It also occurred that the original adventitious plant was (partly) mutated whereas the top-shoot did not show the mutation. With other words, the number of adventitious plantlets, carrying the mutation in both the original plantlet and its rooted top-shoot, was restricted and amounted to approximately 10% of the total number of mutated adventitious plantlets. The 'complete' mutants were not checked upon possible periclinal chimerism.

It is obvious that the in vivo production of adventitious shoots on callus, which is developing at the base of the petiole or on root callus of *Chrysanthemum* is not the

method of propagation which we are looking for to be used in mutation breeding. Such an *indirect* regeneration of plantlets is too slow and does not produce enough 'complete' mutants, since apparently more than one callus cell is involved in the formation of the apex of an adventitious plantlet.

Adventitious shoot formation in vitro. The cultivar Bravo, as was experienced, rather easily regenerates shoots on various explants, such as petals, flower heads, tiny leaves and pedicels. On all explants, excluding pedicels, the earliest adventitious shoots appear after three weeks and usually on callus. Explants of pedicels, however, produce in a *direct* way of regeneration the first adventitious shoots 10 days after incubation. The shoots are developing over the whole length of the explant, which is placed on the medium with the outer side of the half-cylinder up and the wounded side in contact with the medium. As histological studies have revealed, the adventitious shoots develop from epidermal tissue (Fig. 1), like has been described in *Saintpaulia* (BROERTJES, 1972b). The development of the shoots was almost completed 2–3 months after incubation in vitro. Shoots with a length of at least 0.4 cm were then excised from the explants and sub-cultured on another medium to induce root formation. Adventitious roots were initiated 2 weeks after transfer and thus plantlets were produced within approximately 3 months after incubation of the pedicel explants in vitro (ROEST & BOKELMANN, 1975). Various types of explants were, before incubation, irradiated with a series of X-ray doses to determine the radiosensitivity, the mutation frequency and thus the optimum dose.

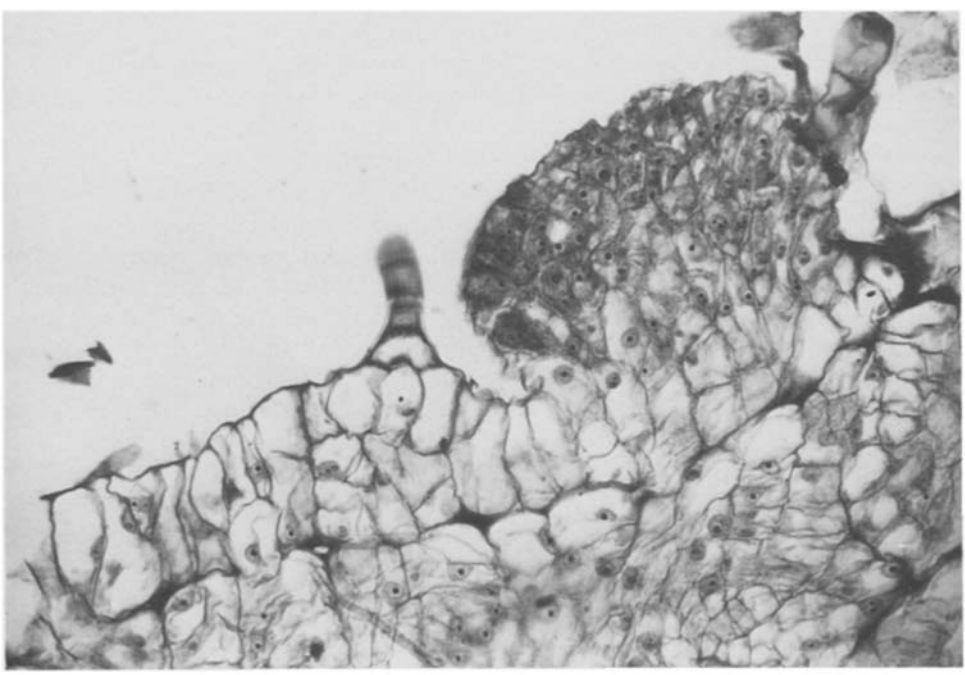

Fig. 1. Adventitious meristem formed by epidermal cells on pedicel explants of cv. Bravo, cultivated in vitro (12 days after incubation).

Table 1. Results of in vitro propagation of various types of irradiated explants of *Chrysanthemum morifolium* cv. Bravo.

Explant type; X-ray dose	% of uncontaminated explants producing adventitious shoots	Total number of adventitious shoots (> 0.4 cm)	Number of adventitious shoots per shoot-forming explant	Number of plantlets potted[2]	Number of mutated plantlets (mutant frequency)	Number of different mutant genotypes (mutation frequency)	Number of solid (looking) non-chimeral mutated plantlets (in % of mutated plantlets)
Tiny leaves							
control	86	132	11.0	43	0	–	–
400 rad	77	58	5.8	30	1(3.3%)	1(3.3%)	0(0%)
600 rad	92	85	7.1	46	2(4.3%)	2(4.3%)	1(50%)
800 rad	60	33	5.5	26	17(65.4%)	6(23.1%)	17(100%)
1000 rad	27	49	12.3	38	19(50.0%)	2(5.3%)	19(100%)
1200 rad	40	15	3.8	11	2(18.2%)	2(18.2%)	2(100%)
Pedicels							
control	100	580	30.5	161	0	–	–
400 rad	100	470	27.6	179	0	–	–
600 rad	100	390	21.7	157	8(5.1%)	3(1.9%)	8(100%)
800 rad	100	264	14.7	144	50(34.7%)	11(7.6%)	50(100%)
1000 rad	100	144	10.3	74	45(60.8%)	10(13.5%)	45(100%)
1200 rad	100	110	6.1	73	24(32.9%)	8(11.0%)	24(100%)
Flowers heads[1]							
control	80	16	4.0	13	0	–	–
400 rad	100	15	3.0	11	0	–	–
600 rad	55	15	2.5	12	0	–	–
800 rad	25	0	–	0	0	–	–
1000 rad	40	5	2.5	2	0	–	–
1200 rad	33	3	1.5	3	0	–	–

[1] Figures are not very significant because of a high contamination percentage.

[2] Only part of the rooted shoots were potted.

From the results, summarized and presented in Table 1, the following conclusions can be drawn:

1. The highest production of adventitious shoots and plantlets is obtained when using pedicel segments, as compared with tiny leaves and flower heads.
2. The optimum dose considering the number of shoots per shoot-forming explant, the number of mutants and the mutation frequency, lies around 800–1000 rad of X-rays.
3. Except one, all mutants were solid (looking) (Plate 1).
4. Unexpectedly, rather frequently apparent identical mutant genotypes were found, always originating from the same explant (Plate 2).

A second experiment was carried out in which tiny leaves were irradiated with a series of high X-ray doses. As can be seen in Table 2 doses of and above 1250 rad are very heavy or even lethal. Although the mutant frequency (second part of the table, second row between brackets) as well as the mutation frequency (second part of the table, third row between brackets) are rather high, such doses must be considered as supra-optimal since shoot production is too slow and impracticable. A dose of 1000 rad, being approximately the LD_{50}, or 750 rad seems to be the best choice. In this experiment (only) two chimeras were found. Again, many identical mutant genotypes were found, its frequency hardly or not being correlated with dose.

In a final experiment approximately 450 adventitious plantlets were produced, using the best in vitro propagation method (pedicel explants) and a dose of 800 rad of X-rays. As can be seen from Table 3 the results are in conformity with the ones found before, namely a high percentage of solid (looking) mutants and several cases of identical mutant genotypes which always were derived from one explant.

In the meantime 5 flower colour mutants, obtained from irradiated explants, were propagated asexually in two ways, namely by rooting shoot cuttings and by propagation in vitro, using young pedicel explants, to investigate whether solid (looking) mutants are genetically homogeneous or are periclinal chimeras.

It was shown that in both cases the mutant genotype can be propagated clonally true to type and without difficulties, except for one of the mutants which hardly developed adventitious shoots in vitro. Radiation of plants of both groups with a high dose (2000 rad of X-rays) produced a few mutated sectors, such as for flower colour, but revealed no sign of uncovering the Bravo-genotype. With other words, it has been proven that mutants produced by in vitro techniques, using explants of pedicels or tiny leaves, are genetically homogeneous and consequently originate from a single mutated cell.

DISCUSSION

Although the idea of producing solid mutants, using in vitro techniques, is not new (SPIEGEL-ROY & KOCHBA (1973) in *Citrus* and HEINZ (1973) in sugarcane) the results presented before clearly demonstrate the usefulness of in vitro techniques for mutation breeding (as well as for clonal propagation) of *Chrysanthemum*. A vast production of non-chimeral mutants in a relatively short time is achievable for anyone who has the simple equipment needed for in vitro production of plants. A further large scale propagation of interesting mutants or of commercial cultivars furnishes no

Table 2. Results of in vitro propagation of irradiated tiny leaves of *Chrysanthemum morifolium* cv. Bravo.

Explant type; X-ray dose	% of uncontaminated explants producing adventitious shoots	Total number of adventitious shoots (> 0.4 cm)	Number of adventitious shoots per shoot-forming explant	Number of plantlets potted[1]	Number of mutated plantlets (mutant frequency)	Number of different mutant genotypes (mutation frequency)	Number of solid (looking) non-chimeral mutated plantlets (in % of mutated plantlets)
Tiny leaves							
control	84	280	17.5	82	0	–	–
750 rad	100	232	11.6	66	11(16.7%)	6(9.9%)	11(100%)
1000 rad	94	154	9.1	54	16(29.6%)	5(9.3%)	16(100%)
1250 rad	79	51	3.4	38	25(65.8%)	10(26.3%)	24(96%)
1500 rad	73	26	3.3	26	17(65.4%)	7(26.9%)	17(100%)
1750 rad	55	9	0.8	7	7(100%)	4(57.1%)	6(85.7%)
2000 rad	10	0	0	0	0	–	–

[1] Only part of the rooted shoots were potted.

Table 3. Results of in vitro propagation of irradiated pedicels of *Chrysanthemum morifolium* cv. Bravo.

Explant type; X-ray dose	Number of explants	% of explants producing adventitious shoots	Number of plantlets potted[1]	Number of mutated plantlets (mutant frequency)	Number of different mutant genotypes (mutation frequency)	Number of solid (looking) non-chimeral mutated plantlets (in % of mutated plantlets)
Pedicels						
Control	20	100	44	0	–	–
800 rad	100	100	453	95 (21%)	36 (7.9%)	32 (88.9%)

[1] Only part of the rooted shoots were potted.

problems either. The question, however, arises whether mutants can be produced which can meet the requirements of commercial production. It is, namely, a fact that most mutants are not only mutated for flower-colour, for instance, but in addition also for (many) other characteristics. In periclinal chimeras, which generally are produced when rooted cuttings are irradiated for mutant production, most of these accompanying mutations are harmless, because they cannot express themselves (mutations for flower-size, plant-height, production, etc. generally are not located in the L_1). When, however, mutants are genotypically homogeneous (solid; non-chimeral) the situation is completely different. All mutations can be expressed and it depends on the combination of mutations what the final result will be. With other words, the in vitro technique provides us with an easy method for producing mutants, an early and rapid selection and a problemless propagation but probably a relatively low percentage of commercially useful mutants. Experiments on a practical scale are the only way to decide which way under certain circumstances will have to be preferred. A similar problem could come up when existing cultivars, which are often periclinal chimeras are being propagated via the in vitro method described. As a consequence of the fact that regeneration in this case takes place from L_1-tissue only, they may, and often will be, different as compared to the original plant and probably frequently in a negative sense. Cultivars being seedlings or mutants (produced, using the in vitro technique) can be propagated without any problems by the in vitro method.

In many cases more than one phenotypically identical mutant plant was found, always originating from the same explant. In a few cases, even all plantlets, regenerated on a given piece of explant, were identical mutants, 22 mutants per 22 plantlets being the most extreme case. One of the explanations could be that a given cell very rapidly grows out into a multi-apical meristem. However, a positive correlation between the number of such cases and increasing dose (when by radiation-damage the number of cells which are able to regenerate shoots is reduced) could not be demonstrated with certainty.

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