DEVELOPING NEW CHRYSANTHEMUM CULTIVARS BY COMBINING RADIATION MUTAGENESIS AND *IN VITRO* TISSUE TECHNIQUES

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Abstract: Chrysanthemums are flowers with great economic significance worldwide as there is a large number of cultivars to be used as cutf lowers, potted plants, and garden ornamentals. For commercial purposes, chysanthemum is commonly propagated in vitro using stem nodal explants with lateral meristems or shoot tip explants. This paper presents a breeding scheme for chrysanthemums based on a combination of gamma ray irradiation and in vitro propagation. In vitro propagation is achieved by direct organogenesis from pedicels as explants and from stem nodal explants, followed by in vivo selection of valuable mutants. This scheme was successfully applied to develop two original chrysanthemum cultivars which have been licensed. Several other promising mutants were also selected. When chrysanthemums were micropropagated by pedicels and stem nodes without irradiation only true-to-type plants were obtained from the cultivars Escapade, Rivalry, Westland Dark, etc. in our experiments. Mainly non-chimeric mutants in flower colour were obtained from irradiated pedicels. Irradiated nodes produced mostly mutants with chimeric structure.

Key words: adventitious buds, gamma rays, micropropagation, mutation, pedicel, stem node.

Chrysanthemum x grcmdiflorum Lapeyr. (synonyms: *Chrysanthemum x morifolium* Ramat., *Dendranthema x morifolia* (Ramat.) Tzvelev, *Dendranthema x grandiflora* (Ramat.) Kitam., *Chrysanthemum sinense* Sabine) belongs to the family Asteraceae and is also known as «florist's chrysanthemum» or «mum». Chrysanthemums are flowers with great economic significance worldwide as there is a large number of cultivars to be used as cut flowers, potted plants, and garden ornamentals [2]. Chrysanthemums are typically vegetatively propagated plants because they do not produce seeds. Increased variability for the development of new cultivars of chrysanthemumis is achieved through induced mutations. The techniques for propagating ornamental plants and laboratory equipment used for relevant tissue culture experiments are being continuously improved to meet the demand of the floriculture breeding and industry [12]. In commercial *in vitro* multiplication of chrysanthemum, stem nodal explants with lateral meristems are commonly used [4, 5].

The combination of mutagen treatment with *in vitro* techniques has successfully been applied in breeding of many plant species [2, 13, 14].

Material and methods

The mutation breeding programme for chrysanthemum was started in the Institute of Ornamental Plants in 1996. Three cultivars used for cut flower production: Westland Dark (with deep red ray flowers), Fred Shosmit (with white hemispheric flowers) and Escapade (with violet hemispheric flowers) served as research objects.

Pedicel and nodal explants of these cvs. were irradiated with 8,11, and 14 Gy gamma rays (^{60}Co) and cultured on Murashige & Skoog (MS) medium including MS vitamins and supplemented with 2 mg.l^{-1} benzilaminopurine (BAP), 1 mg.l^{-1} naphtylacetic acid (NAA), and 100 mg.l^{-1} myoinozitol, 3% sucrose and 0.7% agar, pH 5.7. Controls were not exposed to irradiation. The pedicels were taken at a stage before flowering when the plants have already formed well-shaped green buds. When nodal explants were used, the chrysanthemums were grown in long-day conditions. The shoots were removed from the explants and then propagated *in vitro* to create individual clones on MS solid medium [9]. Ten plants per clone were acclimatized to *in vivo* conditions and grown in a non-heated greenhouse. The clones with phenotypically changed individuals were selected, propagated and cultivated again in the next two seasons when the induced mutations were counted and the valuable stable mutants were selected.

The frequency and spectrum of the phenotypic changes were assessed during the vegetation and flowering periods in two successive years. The changed plants were micropropagated again and tested for their homogeneity and stability during the next two seasons. Plants and clones with unstable changes and/or those of low significance for practice were eliminated by negative selection.

In the next stage of the programme, the newly created cultivar Milka (with large yellow ray flowers) was used as an initial material. Pedicel explants were irradiated with 5, 10 and 15 Gy gamma rays (^{60}Co) and the same methods as before were applied for cloning and selection of new mutants.

Results

Pedicels and stem nodal explants of chosen cultivars were irradiated with 8, 11 and 14 Gy of gamma-rays (^{60}Co) directly before *in vitro* incubation. For each option, 20 explants were used. After 7 to 8 weeks of *in vitro* cultivation, when most of the shoots reached a height of 5 mm, they were micropropagated and 10 plants per clone were acclimatized and planted in greenhouse conditions. The number of plants obtained during the flowering period decreased significantly with the increase of the irradiation dose - from 2349 for non-irradiated ones to 305 for those irradiated with 14 Gy (Table 1).

As the dose of irradiation was being increased, the total frequency of mutations grew from 2.46% at 8 Gy to 44.26% at 14 Gy (Table 1). In cv. Fred Shosmit, only 2% of mutants were found, particularly with morphological changes but flower colour was not altered. In contrast, in Dark Westland (6.88%) and Escapade (6.67%), the mutations in flower colour predominated.

The total frequency of induced mutations in plans originating from pedicels was 3.54%, which was about 3 or 4 times less than that in plants from nodal explants (13.02%). The irradiation of pedicel explants from cv. Westland Dark induced stable non-chimeric changes in flower colour from red to bronze, orange, yellow, purple or pink. Stable non-chimeric mutations in flower colour (pale violet, pale pink) were obtained mainly from adventitious shoots formed *in vitro* from irradiated pedicels of cv. Escapade. The adventitious shoots usually appeared on the surface of pedicel explants in 3 to 5 weeks of *in vitro* cultivation.

Irradiated nodal explants of these three cultivars were found to produce mainly chimeric mutants, and unstable changes were induced in flower or leaf colour as well as in some desirable morphological traits such as petal shape (from ray to flat in Dark Westland), flower diameter and flowering time (Table 1). Most of observed mutants from nodal explants, however, had poor ornamental characteristics, e.g. dwarfs, and plants lacking flowers, or with deficient flowers, etc.

Table 1

Frequency of mutations induced after irradiation by gamma-rays (^{60}Co) in *Chrysanthemum* cultivars and *in vitro* propagation of different explants, %. The mutations were observed during flowering

Type of changed traits (Mutations)	Frequency depending on the genotype, %			Frequency depending on the type of explant, %		Frequency depending on the irradiation dose, %			
	Dark Westland	Fred Shosmit	Esca-pade	Pedi-cel	Node	0 Gy	8 Gy	11 Gy	14 Gy
Flower colour, non-chimeric	3.08	0	2.54	2.64	0	0	2.21	3.39	12.46
Flower colour, chimeric	0.24	0	0.49	0	4.20	0	0.17	0.48	3.63
Chlorophyll, chimeric	0	0	0.34	0	1.68	0	0.08	0.16	1.45
Petal shape, non-chimeric	0.49	0	0	0.42	0	0	0	0	4.35
Flower size, non-chimeric	0.49	0.67	0.98	0.48	0	0	0	0	7.97
Earlier or later flowering	1.21	0.27	0.34	0	6.30	0	0	2.26	2.17
Dwarfs and without flowers	1.37	1.06	1.97	0	0.84	0	0	0	12.17
Total % of mutants	6.88	2.00	6.67	3.54	13.02	0	2.46	6.29	44.20
Number of plants observed	2717	1650	2251	2838	3780	2349	2600	1364	305

Non-irradiated pedicel and nodal explants produced only true-to-type plants. In our experiments for micropropagation by pedicels and stem nodes without irradiation, only true-to-type plants were obtained from cvs. Westland Dark, Escapade, Rivalry, etc.

In the next step of the mutation-based breeding programme, cv. Milka previously developed by us was used. It was chosen because of its proven attractive large yellow flowers suitable for high quality cut flower production in a non-heated greenhouse. The aim was to induce mutations and select mutants with different ornamental traits (colour and/or size and shape of inflorescence). For this purpose, only pedicel explants were irradiated. The frequency and range of resultant phenotypic changes were assessed during the vegetation and flowering periods in two successive years (Table 2).

Table 2

Frequency of induced mutations in cv. Milka after irradiation by gamma-rays (^{60}Co) and ***in vitro*** micropropagation of pedicel explants, %. The mutations were observed during flowering

Type of changed characteristics (Mutations)	Frequency depending on the dose of irradiation, %			
	0 Gy	5 Gy	10 Gy	15 Gy
Flower colour, non-chimeric	0	2.15	3.57	7.62
Flower colour, chimeric	0.12	0.31	0.24	0.89
Flower size, non-chimeric	0	0	0.24	3.39
Total % of mutants	0.12	2.46	3.95	11.9
Number of plants observed	820	650	420	118

As the dose of gamma rays increased, the number of both shoots *in vitro* and plants *in vivo* decreased and the frequency of induced mutations grew. In our experiments, doses from 5 to 15 Gy of gamma rays appeared suitable for the induction of mutations and allowed to obtain a sufficient quantity of plants *in vitro.* The most suitable doses for irradiating pedicel and nodal explants of chrysantemums usually depend on the genotype and have to be determined experimentally.

In case of our experimental mutation breeding programme, four new mutants with valuable ornamental characteristics, low temperature tolerance and unchanged flowering time were selected: № 1806 with white flowers; № 509 with salmon-coloured flowers of larger diameter; № 306Y with bright red-orange flowers, smaller in comparison with Milka, and № 206 with smaller pink flowers.

Discussion

In our experiments, the number of plants obtained during the flowering period decreased significantly as a function of the irradiation dose. This could most probably be considered a result of the decrease in the number of shoots formed from explants *in vitro* [10].

With the increase of the irradiation dose, an increase in the total frequency of mutations was observed. The type and number of mutations differed depending on the cultivar. In cv. Fred Shosmit, the percentage of obtained mutants did not appear to be very high (only 2%) and flower colour was not altered. Using this cultivar, mainly plants with morphological changes were scored. In contrast, in Dark Westland and Escapade, a higher percentage of mutants (~6-7%) was shown and the mutations in flower colour predominated. These results could be explained by the fact that white colour of Fred Shosmit was determined by a dominant gene inhibiting the synthesis of pigments in chrysanthemum flowers [6]. Hence, cultivars with white flowers are not recommended for inducing flower colour mutations.

In general, our data demonstrated the induction of non-chimeric mutations in flower colour in plants originating from irradiated pedicels. It is found to be a result of direct organogenesis of each adventitious bud from a single epidermal cell [1,3].

Following the irradiation of nodal explants of the three cultivars used in these experiments, mainly chimeric mutants were produced and unstable changes in flower or leaf colour were induced. Although mutations in some desirable morphological traits (e.g. petal shape, flower diameter, flowering time) were observed, most of the mutants obtained from nodal explants showed poorer ornamental characteristics (dwarfs, plans lacking flowers or with deficient flowers). These results might be helpful in choosing the appropriate micropropagation procedure in future experiments in order to better address the specific breeding purposes. The propagation by nodal explants is based on proliferation of already existing multicellular axillary buds in the axil - the junction of the stem and the petiole. Nodal explants readily develop axillary shoots *in vitro.* Proliferation of axillary buds is the most common method of *in vitro* propagation for chrysanthemum and many other plant species [7, 8, 11]. It is based on the activation of axillary bud growth. This is achieved through the removal of the stem apical part - this action helps to eliminate the apical meristem dominance and results in the development of lateral meristems. In short, if the aim of breeding is to create cultivars with various different flower colours, most suitable sources are irradiated pedicel explants. In case the objective is to change some morphological, physiological or quantitative traits, the irradiation of stem nodal explants would be more suitable.

To combine several mutations in one individual plant, a nodal explant from an already selected mutant with changed colour (originating from an irradiated pedicel) should be irradiated and new mutant(s) with altered morphological or other different characteristic(s) but with the same colour might be selected. E.g., this mutation breeding scheme was successfully applied by us and resulted in developing two presently licensed cultivars: Milka (License № PD 09-1396/1998) and Mitko (License № 33-00-221/2000). Their colour, shape and inflorescence size as well as the flowering time were altered. The cultivars demonstrate outstanding ornamental characters and ensure the production of high quality cut flowers which meet the demands of Bulgarian flower producers.

The second stage of our experiments, involving the irradiation of cv. Milka pedicel explants, resulted in production and selection of four new mutants (№ 1806, № 509, № 306Y, № 206). They were characterized by unchanged flowering time, low temperature tolerance, and valuable ornamental characteristics however different from those of cv. Milka. Such good and attractive original ornamental traits, together with low temperature tolerance are very likely to turn these new mutants into promising future cultivars suitable for growing in non-heated greenhouses in Sofia region.

Conclusions

An original mutation breeding scheme for developing new chrysanthemum cultivars was elaborated. It combines radiation mutagenesis (1st step) and two *in vitro* techniques: for direct adventitious shoots formation from pedicel explants and for the axillary bud proliferation nodal explants (2nd step), *in vitro* cloning (3rd step), *in vivo* selection of valuable mutants (4th step), and irradiation of selected mutant genotypes (5th step) again followed by the 2nd, 3rd and 4th step.

Using this scheme, we were able to develop two now licensed cultivars (''Milka'' and "Mitko") for cut flower production of chrysanthemum in non-heated greenhouses; other four mutants were selected as promising future cultivars.

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ВЫВЕДЕНИЕ НОВЫХ СОРТОВ ХРИЗАНТЕМ С ИСПОЛЬЗОВАНИЕМ ГАММА-ИЗЛУЧЕНИЯ В СОЧЕТАНИИ С МИКРОКЛОНАЛЬНЫМ РАЗМНОЖЕНИЕМ *IN VITRO*

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Аннотация: хризантемы имеют большое значение во всем мире благодаря множеству сортов для использования на срезку, а также в качестве декоративных горшечных и садовых растений. В промышленных масштабах хризантемы обычно размножаются in vitro при помощи нодальных (узловых) эксплантантов с боковыми меристемами или верхушечных экс-

плантантов. В статье представлена селекционная программа для хризантем, основанная на сочетании облучения гамма-лучами и размножении in vitro. Размножение in vitro достигается путем прямого органогенеза из эксплантантов цветоножек или нодальных эксплантантов, с последующим отбором мутантов с ценными признаками. Данная программа была успешно применена для выведения двух сортов, впоследствии лицензированных. Было также отобрано несколько других мутантов с высоким потенциалом. В результате микроклонального размножения с использованием эксплантантов цветоножек и нодальных эксплантантов без облучения в эксперименте были получены только типичные растения на основе сортов Escapade, Rivalry, Westland Dark и др. При облучении эксплантантов цветоножек были получены в основном мутанты по окраске цветка нехимерной природы. При облучении нодальных эксплантантов были получены преимущественно химерные мутанты.

Ключевые слова: придаточные почки, гамма-лучи, микроклональное размножение, мутация, цветоножка, узел стебля.

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