BIOLOGIA PLANTARUM 49 (2): 181-186, 2005

The use of mutagens to increase the efficiency of the androgenic progeny production in *Solanum nigrum*

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Abstract

Pollen embryogenesis was successfully induced in *Solanum nigrum* L. (2n=6x=72). Stimulation of androgenesis expressed as the frequency of androgenic responsive anthers was observed after 10 and 20 mM ethyl methanesulphonate (EMS), 10 and 20 mM sodium azide (NaN_3) and 0.2 mM N-nitroso-N-methylurea (MNU) treatment applied on seeds for 24 h. The frequency of androgenesis on the medium with sucrose was higher than on the medium with maltose. Androgenic regenerants originated also in the anthers collected from donor plants where survival after mutagenic treatment was lower than 50 %. Green haploid (3x), aneuploid (to 8x) and dihaploid (6x) plants were obtained. The high frequency of aneuploids among androgenic plants is explained by cell division irregularities in microsporial calli.

Additional key words: enhanced androgenesis, mutagenesis, induced haploidy, anther culture, black nightshade.

Introduction

In vitro androgenesis in *Solanum* species is a complicated method of obtaining haploid plants affected by numerous factors in spite of the fact that the first reports were published in the early 70s of the 20th century (Irikura and Sakaguchi 1972, Dunwell and Sunderland 1973). In the past 3 decades there have been many reports describing *in vitro* successful androgenesis in genus *Solanum* (*S. acaule*: Rokka *et al.* 1998, *S. carolinense*: Reynolds 1990, *S. phureja*: Veilleux *et al.* 1985, *S. tuberosum*: Veilleux 1996, *etc.*), and also in hybrids of various *Solanum* species (*S. brevidens* × *S. tuberosum*: Rokka *et al.* 1995, *S. melongena* × *S. aethiopicum* group *gilo*: Rizza *et al.* 2002, *etc.*). However, there has been no other report dealing with successful *in vitro* androgenesis in *Solanum nigrum* since the paper by Irikura (1975).

The most important factors for successful haploid plant production comprise a genotype, culture conditions, and culture media composition. Optimization of all these factors may be inefficient not only in recalcitrant genotypes, but also in those characterized by a low frequency of responsive anthers. Although induced mutagenesis as an enhancer of androgenic response has been reported, it is not yet a common method to increase the quantity of green plants regenerated from anther culture. Chemomutagens and/or ionising radiation have proven practical, but induced mutations are mainly recessive, and therefore, it is hard to detect them in the heterozygous stage (Forster and Powell 1997). The increase in the inner genetic heterogeneity through recessive mutations has a stimulatory effect on induced androgenesis as reported in tobacco. Vagera *et al.* (1976) and Vagera (1978) used butyl methanesulphonate (BMS) and N-nitroso-N-methyl urea (MNU) to increase the number of androgenic regenerants in *Nicotiana tabacum* and other tobacco species. In this case, the stimulatory effect of mutagens contributed to increasing the amount of androgenic responsive anthers, but the number of haploid plants per androgenic responsive anther was lower than in the untreated control. Przewozny *et al.* (1980) also described the increase in microspore-derived structures in dihaploid potato anther cultures following mutation treatment, namely radiation and chemical

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Received 10 May 2004, *accepted* 27 October 2004.
Acknowledgements: This work was supported by research grants of Grant Agency of the Czech republic (No. 521/01/1383) and Grant Agency of Ministry of Agriculture (No. 521/03/0113).

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mutagens. MNU was found the most efficient agent to stimulate responses ranging from 126 to 165 % over control. Aldemita and Zapata (1991) studied the effect of mutagenic treatment more precisely in rice cultivars. Even if they found the androgenic response in recalcitrant cultivars, the responsive genotypes exhibited a decreased anther culture productivity. The only review concerning the relationship between mutation techniques and haploidy production was published by Maluszynski *et al.* (1996). Recently, Shtereva and Atanassova (2001) have succeeded in increasing the production of calli in tomato anther culture using γ-radiation. Castillo *et al.* (2001) displayed two ways of mutagen application. The first procedure starts with growing plants from mutagen treated seeds, and the second is based on the mutagenic treatment applied directly to haploid cells (*i.e.* anthers with microspores).

Mutagenic treatment can be detected by counting chlorophyll deficiencies in somatic tissues in M_0. Within the population of plants grown from the treated seeds, the plants with chlorophyll deficiencies are characterized as chlorophyll chimeras. Their colour is classified according to Lamprecht (1960). Nevertheless, this method is not completely applicable for species with a greater number of chromosome sets, because of the fact, that the induced mutations are mainly recessive, and the phenotype effect of mutation may be suppressed by chromosomes of other sets.

The aim of this paper was to compare the effects of different mutagens on the frequency of androgenesis in *Solanum nigrum*.

Materials and methods

Seeds of *Solanum nigrum* L. were obtained from wild plants growing on the locality of Olomouc-Klášterní Hradisko (Czech Republic). After imbibition for 48 h in water, soaked seeds were treated with 10 and 20 mM solution of sodium azide (NaN_3), 10 and 20 mM solution of ethyl methanesulphonate (EMS) and 0.2 and 0.4 mM solution of N-nitroso-N-methylurea (MNU). They were kept in dark at room temperature for 24 h. The volume of mutagen solution or distilled water (control variant) was ten times larger then that of the treated seeds. Solution of NaN_3 had pH 6.3, solution of MNU had pH 4.5, solution of EMS had pH 4.8 and redistilled water had pH 5.5. The buffer was not used. Being treated with the chemomutagens, the seeds were washed three times in sterile distilled water and surface sterilized with 70 % ethanol and 5 % solution of sodium hypochloride. Seedlings were cultured on Murashige and Skoog (1962; MS) medium under 12-h photoperiod with irradiance of 500 μmol(photon) m^{-2} s^{-1} at 24 °C. The seedlings were transplanted into 15-cm pots and grown under standard greenhouse conditions. The number of chlorophyll chimeras was counted during the first month after potting.

Immature anthers with microspores preferably in the uninucleate stage were used for *in vitro* cultivation. Flower buds were collected, surface sterilized with 70 % ethanol for 1 min followed by 5 % solution of sodium hypochlorite for 10 min, and rinsed three times in sterile distilled water. Isolated axenic anthers at the rate of 50 anthers per 60 mm Petri dish were cultured on the surface of the induction medium at 24 °C in dark. MS medium supplemented with 4 mg dm^{-3} 2,4-D (2,4-dichlorophenoxyacetic acid), 1 mg dm^{-3} zeatin and 60 g dm^{-3} sucrose or maltose was used as induction medium.

Regenerated calli were replaced on the regeneration medium (MS with 0.5 mg dm^{-3} benzylaminopurine, BAP) in 100 cm^3 Erlenmeyer flasks. All the media used were solidified with 3.5 g dm^{-3} of *Phytagel* and sterilized by autoclaving (121 °C per 20 min).

The amount of responsive anthers was recorded during ten weeks after initiation of *in vitro* cultivation, and the numbers of responsive calli were recorded in the course of further ten months.

Shoots regenerated from microsporial calli were transferred onto the basal MS medium to promote root induction. After two weeks, the plants with a fully-developed root system were put into the soil and kept in the greenhouse.

Ploidy of regenerants was then screened by means of a flow cytometer (*Pass II, Partec GmbH*, Münster, Germany) according to DNA content in the nuclei isolated from young leaves (Doležel and Göhde 1995). The detected aneuploids were screened for their chromosome constitution by standard acetocarmine staining of roots (Singh 2003). The significance of differences was assessed from the ratios among numbered values (Myslivec 1957) using *t*-test.

Results

The mutagenic treatment influenced donor material (M_0 plants) seeds viability. The survival of treated plants was strongly depressed in the variants 20 mM EMS (84 % lethality), 20 mM NaN_3 (76 % lethality) and 10 mM EMS (69 % lethality), in the other variants it was influenced moderately (Table 1).

Some leaves of mutagen-treated plants contained sectors with chlorophyll deficiencies referred to as *albina*, *xantha* and *chlorina.* The frequency of chimerical plants varied from 0 to 51 % (Table 1). The area of chlorophyll deficiency was changed through plant ontogenesis because it was overgrown by non-deficient tissue.

Table 1. Donor plants in M_0 generation (110 seeds were sown). Values followed by the same small letter are not significantly different at the $P < 0.05$.

Variants	Number of survived plants	Number of plants with chlorophyll deficiencies
0.2 mM MNU	90 gaf	46
0.4 mM MNU	86 fa	22
10.0 mM EMS	34 eb	4
20.0 mM EMS	18 db	0
10.0 mM NaN_3	62 c	4
20.0 mM NaN_3	26 b	0
Control	96 a	0

Pollen embryogenesis evaluated according to the frequency of androgenic anthers was induced in all the variants, *i.e.,* control and treated (Table 2). The frequency of the responsive anthers cultured on the sucrose medium was significantly higher compared to the medium with maltose (55 - 85 % and 24 - 49 % of the cultured anthers, respectively). The greatest differences between the media with sucrose and maltose in the frequency of androgenic anthers were found out in the control and EMS variants, smaller (but still significant) in NaN_3 and MNU variants. There was a significant increase in the number of the responsive anthers on both sucrose and maltose media in the treated variants with 10 and 20 mM EMS, 10 and 20 mM NaN_3 and on maltose only with 0.2 mM MNU as compared to the control, whilst the variant with 0.4 mM MNU exhibited a non-significant increase.

Table 2. Induced androgenesis in anthers collected from donor plants. Values followed by the same small letter are not significantly different at the $P < 0.05$.

Variants	Medium sugar	Number of cultured anthers	Number of androgenic responsive anthers	Number of embryo-like structures forming regenerants
0.2 mM MNU	sucrose	505	329 nb	5
	maltose	522	224 mce	2
0.4 mM MNU	sucrose	503	278 lb	3
	maltose	501	172 kag	1
10.0 mM EMS	sucrose	506	395 jh	15
	maltose	503	123 i	4
20.0 mM EMS	sucrose	510	415 hd	9
	maltose	504	182 ge	0
10.0 mM NaN_3	sucrose	505	360 f	7
	maltose	505	206 e	0
20.0 mM NaN_3	sucrose	507	431 d	8
	maltose	500	243 c	1
Control	sucrose	502	306 b	4
	maltose	626	186 a	0

The frequencies of androgenic anthers on the medium with sucrose were significantly stimulated in 10 and 20 mM EMS, 10 and 20 mM NaN_3, whilst there was no significant shift in 0.2 and 0.4 mM MNU. On the maltose medium the frequencies of androgenic anthers were significantly stimulated in 20 mM EMS, 10 mM NaN_3 and 0.2 mM MNU, with no significant shift in 0.4 mM MNU.

The androgenic response was significantly improved with increased concentrations of EMS and NaN_3. However, the higher concentration of MNU (0.4 mM) decreased the androgenic response compared to 0.2 mM.

The frequency of plants regenerated from anther cultures (per cultured anther) was lower than was the frequency of androgenic anthers per cultured anther. Although the real number of regenerated plants was expressively higher because of a high rate of shoot multiplication, it could not be used for objective statistics. Therefore the amount of plant regenerants was quantified according to numbers of microsporial calluses or embryo-like structures from which the androgenic plants originated.

In all the mutagen-treated variants the frequency of plants regenerated from anthers on the sucrose medium was higher than on the medium with maltose in all mutagenically treated variants. Significant differences were found out in the control, 10 and 20 mM EMS, 10 and 20 mM NaN_3 variants, non significant in both 0.2 and 0.4 mM MNU.

A significant increase in the frequency of regenerants in the treated variants contrary to the control was recorded in 10 mM EMS, whilst in the other variants the enhancement was not significant. In 10 mM EMS the increase was observed on the sucrose as well as on the maltose media.

There were significant differences in the production of androgenic plants per androgenic anther between the sucrose and maltose media in 20 mM EMS and 10 mM NaN_3 in comparison with the control. The effect of the sucrose medium was better. Across the treated variants, significant stimulation was also observed in 10 mM EMS, but the growth in the other treated variants was non significant.

The induction of pollen embryogenesis and regeneration of androgenic plants took place slowly. The cultured anthers developed calluses during ten weeks after inoculation, but these calluses rarely gave rise to secondary embryos. The calluses and embryos produced the majority of shoots in the next ten weeks in succession, although organogenesis proceeded for ten months after inoculation of isolated anthers on the culture medium.

Fifty-nine plant regenerants were evaluated by flow cytometry and standard caryology: 56 % of the evaluated androgenic plants were haploids, 24 % were diploids and 20 % were aneuploids at various levels of ploidy (Figs. 1 and 2).

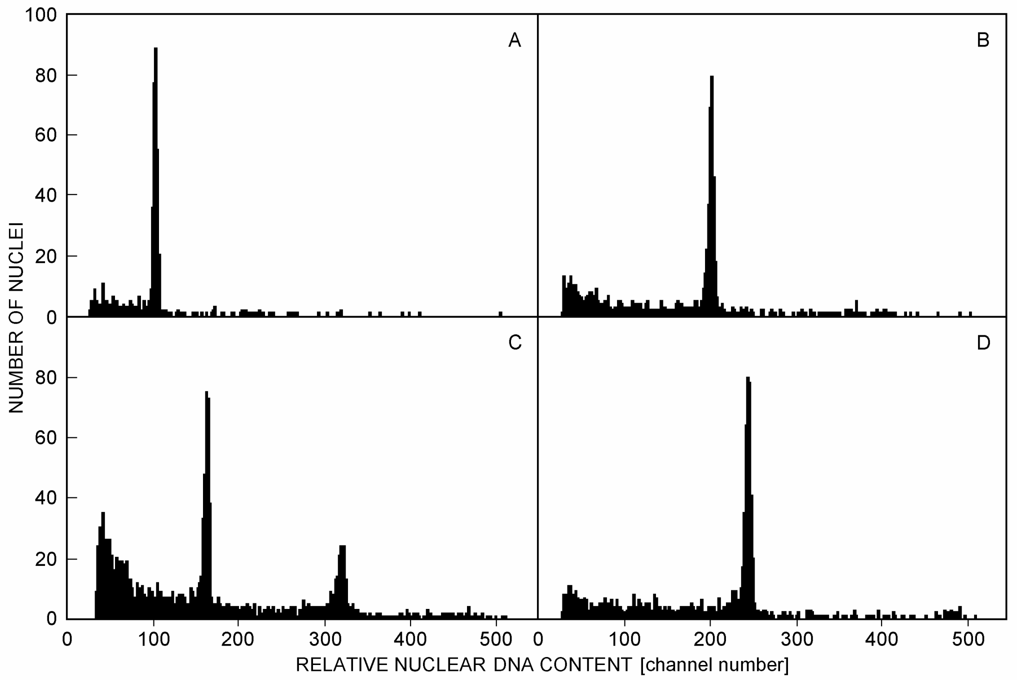

Fig. 1. The histograms of relative nuclear DNA content obtained by analysis of cell nuclei isolated from leaves of haploid (*A*), dihaploid (*B*) and aneuploid (*C*, *D*) androgenic regenerants. The flow cytometer was calibrated so that peak of haploid nuclei in G_1 phase was on channel 100. The peak of diploid nuclei in G_1 phase was on channel 200.

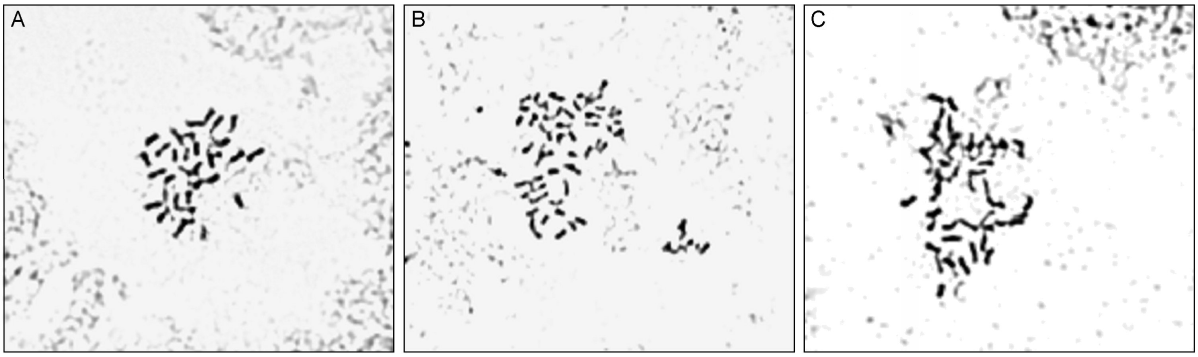

Fig. 2. The chromosomes in root cells of haploid (*A*), dihaploid (*B*) and (as an example) aneuploid (*C*) androgenic regenerants in *Solanum nigrum*.

Discussion

The only successful experiments with induced androgenesis in *S. nigrum* were published as late as thirty-two years ago (Harn 1972, Irikura 1975). These experiments did not continue, although *Solanum nigrum* has long been a widely used species in plant tissue cultures as a model plant not only in family *Solanaceae* (Hassanein and Soltan 2000). It is well-known that induction of androgenesis is dependent on many nutritive, physical and genetic factors and their effects are often superimposed (González and Jouve 2005). The other manipulations can change the ploidy of regenerants (Burun and Emiroglu 2003). In our experiments evaluation was made of the effects of chemomutagens and two culture media with different sugars.

The first step in androgenesis *in vitro* is induction of sporophytic development in microspores inside cultured anthers. This sporophytic pathway can be realized by creation of microsporial calluses or embryo like structures. The second step is regeneration of intact plants. The effects of mutagenic treatments and/or the impact of sugar in the culture medium on the androgenesis induction level were strongly pronounced in our experiment. The frequency of androgenic anthers was increased in all the treated variants as compared to the control. Although the androgenic response on the maltose media was lower, all the mutagen-treated variants on both the culture media exhibited the same tendency, in particular improvement of the production of androgenic anthers in the treated variants.

Our experiment demonstrated that the frequency of induced androgenesis *in vitro* could not be necessarily reduced in the variants where survival of donor plants after mutagenic treatment was below 50 %. It was also described in barley (Vagera *et al*. 2004). It is evident, that immediate physiological influence of microsporogenesis in anthers of plants developed from mutagen-treated seeds is not expressive.

The changes in numbers of regenerated plants were not so pronounced as the changes in the quantity of androgenic anthers. The frequency of plant regenerants from anther culture on the sucrose medium was higher than on the maltose medium. A significant increase in the frequency of plant regenerants in the treated variants compared to the control was observed in 10 mM EMS variant, whilst the increase in the remaining treated variants was not significant, but the tendency to the changes supported significance of the results.

It is evident that the genotype affects the frequency of androgenic anthers, but the androgenic response does not correlate with the frequency of regenerated androgenic plants. It can be related to the fact that the number of androgenic anthers represents the diploid level of androgenesis, while the sporophytic development of microspores takes place on haploid or spontaneously polyhaploid (*i.e.* isogenic) levels. The development of haploidy can be easily suppressed by recessive lethal mutations (induced or spontaneous). According to this opinion, it seems realistic that the survival of androgenic plants induced in species with high autopolyploidy will be higher than in plants with high allopolyploidy.

Flow cytometry evaluation of nuclear DNA content in young androgenic regenerants and standard caryology demonstrated that the majority of haploid and dihaploid regenerants also comprised aneuploid androgenic plants. The higher frequency of aneuploids can be explained either by irregularities in cell divisions in the first step of androgenesis (induction of microsporial calluses or embryo like structures) or by formation of regenerants from gametes with irregular numbers of chromosomes. Larger amounts of aneuploids were also obtained from populations of androgenic regenerants in potato (unpublished) and tomato (Zagorska *et al.* 1998) where we can suppose the similar mechanisms of their origin.

In our experiments, we explain the stimulation of induced androgenesis after mutagenic treatment by increased genetic heterogeneity in tissues of the anthers promoting better sporophytic development of immature microspores.

The majority of the regenerated plants were fertile, nevertheless in some of these there were abnormalities of the reproductive organs. Our experiment has clearly shown that chemomutagen treatment is able to remarkably enhance induced androgenesis *in vitro* in *Solanum nigrum.*

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