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BRIEF COMMUNICATION

The effect of a short heat treatment on the *in vitro* induced androgenesis in *Silene latifolia* ssp. *alba*

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Abstract

The effect of a short heat treatment in combination with different culture medium composition on the efficiency of *in vitro* induced androgenesis in *Silene latifolia* ssp. *alba* was studied. The heat shocks (33 and 37 °C) were applied for 1, 3, and 5 d. The best androgenic response was observed at 25 °C and after a one-day treatment at 33 °C. All other treatments reduced androgenic response. Among different media compositions tested, the most satisfactory results were obtained on BMS medium supplemented with 6-benzylaminopurine (0.5 mg dm^{-3}) and sucrose. The green, albino and chimeric, only female, plants were regenerated. Flow cytometry of 110 regenerants identified haploids, mixoploids (n+2n and 2n+4n) and dihaploids.

Additional key words: campion, green regenerants, ploidy analysis.

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Silene latifolia subsp. *alba* (campion*)* is a dioecious plant that is a suitable model for cytological, cytogenetic and molecular studies of sex determination (Westergaard 1958, Grant *et al.* 1994, Farbos *et al.* 1997, Monéger 2001). Its sex determination is fully dependent on the presence or absence of a sex chromosome Y. However, the control of sexual dimorphism is still not understood. Because of the simple chromosomal sex determination system, the species appears to be a good model for androgenesis. Published reports on the *in vitro* induced androgenesis in *S. latifolia* dealt primarily with the factors affecting androgenic ability and on attempts to regenerate plants of different sex and different ploidy levels, preferably haploid (Ye *et al.* 1990, Vagera *et al.* 1994) and intended to study the role of genes located on sex chromosomes and the critical function of X chromosomes.

Despite numerous studies, induction of androgenesis in campion is still difficult. Many factors affecting androgenesis in *Silene* have been studied but not the temperature stress.

Seeds of *Silene latifolia* ssp. *alba* were obtained from the Botanical Garden in Olomouc, Czech Republic. Donor plants were grown in the experimental field. The flower buds were collected between April and September 2001.

Immature anthers with microspores in the uninuclear stage were selected for the *in vitro* culture. These were usually found in flower buds of 3 - 4 mm in length. Buds of such lengths were surface sterilised by 70 % ethanol for 1 min, followed by an immersion in 2.5 % solution of sodium hypochlorite for 20 min, and rinsed three times in sterile double distilled water. Anthers, ten per bud, were aseptically excised and placed on the surface of culture medium in a Petri dish (with 100 anthers per dish, 6 cm in average) and were cultured under controlled

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Abbrevitations: BAP - 6-benzylaminopurine, IAA - indole-3-acetic acid, KIN - kinetin, DAPI - 4,6-diamidino-2,5-phenylindole.
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temperature in the dark. Anthers on the medium were exposed to short heat shocks at the following levels and duration: 33 °C for 1 or 3 or 5 d, and 37 °C for 1 or 3 d. Following the temperature treatment and the control (no heat shock treatment) anthers were cultured at 25 °C. The responsive anthers were identified after four weeks in culture. The obtained embryoids, plantlets or calli were transferred to a cultivation chamber and grown at 22 ± 2°C under 16-h photoperiod. Regenerating embryoids and calli were grown on the regeneration or hormone-free media, and plants were grown in non-axenic conditions after the acclimatisation process.

All anthers were grown on a medium based on the BMS formula (Vagera *et al*. 1994). This was supplemented with sucrose or maltose and different concentrations of IAA and/or BAP, and solidified with agar. The following media compositions were used: S1 - BMS + 0.5 mg dm^{-3} IAA + 0.5 mg dm^{-3} KIN + 10 g dm^{-3} sucrose, S2 - BMS + 0.5 mg dm^{-3} IAA + 0.5 mg dm^{-3} KIN + 10 g dm^{-3} maltose, S3 - BMS + 1 mg dm^{-3} BAP + 10 g dm^{-3} sucrose, S4 - BMS + 1 mg dm^{-3} BAP + 10 g dm^{-3} maltose; regeneration medium was: BMS + 0.1 mg m^{-3} IAA + 0.1 mg dm^{-3} KIN + 10 g dm^{-3} sucrose or maltose.

The ploidy levels of the regenerants were estimated by flow cytometric measurements of the DNA content of isolated nuclei according to Doležel and Göhde (1995). The nuclei were released from cells by chopping young leaves with a razor blade in 1 cm^{-3} Otto I buffer; the suspension was filtered through a 42 μm nylon mesh, diluted with 2 cm^{-3} of the Otto II buffer supplemented with DAPI (4 μg cm^{-3}). The fluorescence of the DAPI-stained nuclei was analysed with the *Partec Pas II* flow cytometer (*Partec GmbH*, Münster, Germany). Histograms were registered and evaluated with the *Flowstar* software (Doležel 1989).

Anther responsiveness on all medium variants tested was generally very low. This is in line with our previous experiments and may be typical in of campion randomized populations (Paulíková and Vagera. 1993, Vagera *et al.* 1994). Anther responsiveness is strongly influenced by the genotype of the donor material and perhaps could be improved by selection (Ye *et al.* 1990).

Across all media combinations tested, the best androgenic response was observed in anthers continuously cultivated at 25 °C that is without any heat pre-treatment. Medium combinations S2 and S3 had similar androgenic reactions (1.56 and 1.5 % of responding anthers, respectively). Combinations S1 and S4 produced lower response rates (0.89 and 0.54 %), but the difference was not statistically significant ($P = 0.05$). A one-day heat treatment at 33 °C produced a comparable androgenic response. The 1.35 % of responsive anthers was noted on S3 medium, this response was significantly different from the others ($P = 0.05$). The heat shocks applied to isolated anthers or microspores are considered an effective way to change the microspore development pathway from gametic to sporophytic. In wheat and tobacco (Touraev *et al.* 1997) or in rape (Smýkal 2000) the heat shocks stimulated androgenesis, on the other side in horse chesnut it was not necessary (Ćalić *et al.* 2003/4). Unfortunately, they were ineffective in campion. Only the shortest treatment at the lowest chosen temperature did not negatively impact the androgenic response of the anthers. Longer or higher temperature treatments significantly depressed the response (Table 1).

Table 1. Androgenic responsivity upon different cultivation conditions. Within the androgenic responses each media variant upon the treatment followed the same capital letter (A-I) and the same media of each treatment followed the same small letter (a-h) are not significantly different at $P = 0.05$ (using Student *t*-test).

Treat-ment [°C/d]	Medium	Number of cultured anthers	Number of responsive anthers[%]	Number of regenerants green	albino
25	S1	900	0.89Aa	8	4
	S2	900	1.56Ab	24	3
	S3	800	1.50Ae	7	8
	S4	560	0.54Ag	0	0
33/1	S1	1807	0.61Ca	27	5
	S2	1781	0.56Cc	29	2
	S3	1848	1.35Be	58	12
	S4	1810	0.44Cg	21	3
33/3	S1	675	0.15Da	4	0
	S2	665	0.45Dcd	9	1
	S3	775	0.26Df	1	1
	S4	665	0.15Dgh	0	0
33/5	S1	400	0.00Ea	0	0
	S2	400	0.00Ecd	0	0
	S3	400	0.00Ef	0	0
	S4	400	0.00Egh	0	0
37/1	S1	820	0.24Fa	3	0
	S2	830	0.00Fd	0	0
	S3	668	0.00Ff	0	0
	S4	728	0.28Fgh	5	0
37/3	S1	975	0.72GIa	6	4
	S2	1083	0.09Hd	1	0
	S3	1070	0.47GHf	6	0
	S4	955	0.00Hh	0	0

The sucrose supplement (S1/S3) generally promoted androgenic response more than maltose (S2/S4). Maltose is used to increase the androgenic response in recalcitrant genotypes; it positively affected the callus induction and embryogenesis in cereals (Lentini *et al.* 1995, Gonzales and Jouve 2000), but it did not impact the frequency of androgenesis in our experiment. The androgenic response in this study was affected by interactions between growth regulators and the type of sugar in the culture medium. Androgenic response on medium with BAP and sucrose

was higher than on medium with BAP and maltose (S3/S4). The three-fold higher yield of responsive anthers (medium S3 versus S4 at 33 °C for 1 d) clearly shows its stimulating effect. The effects of BAP and IAA (S1/S2) were not clear. Ye *et al.* (1990) did not observe any difference in yield and quality of the embryo produced and of the androgenic plants regenerated on medium containing either BAP or IAA: both hormones improved the overall condition or responsiveness of the anthers.

Most of the observed embryoids continued development into shoots; these are transferred onto regeneration medium and were grown up into mature plants. The 70 regenerants (of which 12 were albino) that originated from anthers treated at 33 °C for one day (S3) had higher numbers of shoots. It is possible that a short mild heat treatment increases the regeneration ability of androgenic embryoids but decreases the yield (frequency) of responsive anthers relative to non-treated anthers.

Among regenerated plants, 209 were green, 49 were albino, and four were chlorophyll chimeras (with *albina* or *lutea* sectors on green leaves). Among 103 plantlets tested for their ploidy levels by flow cytometry, 20.4 % were haploid, 38.8 % were mixoploid n+2n, 27.2 % dihaploid and 13.6 % were mixoploid 2n+4n. High frequency of mixoploids n+2n in this experiment suggests a strong tendency for spontaneous polyploidization, especially dihaploidization.

Sex of the regenerants was determined by the visual observation. Flow cytometry could not be employed because the difference in the nuclear genome size between sexes in *Silene* is on the resolution limit of the technique (Dolezel and Göhde 1995). All flowering androgenic plants were females; no individuals of any other sex were noted. It was contrary to the expectation as a regenerated dihaploid androgenic male plant has been observed (Vagera *et al.* 1994). In *Silene latifolia* ssp. *alba* chromosome Y plays a crucial role in sex determination, but chromosome X is necessary for survival and its absence is lethal (Negrutiu *et al.* 2001). Clearly, X must carry genes essential for viability of the developing embryos (Westergaard 1958, Ye *et al.* 1990, Grant *et al.* 1994). The exception of this rule is male AY gametophyte, which seems less viable than the AX gametophyte (Taylor 1994). Taking into account the absence of males among a reasonably large population of regenerants in this study, the repeatedly verified AAYY male in the previous experiment could only be explained by a translocation or mutation in male promoting/female suppressing genes on chromosome Y or on one of the autosomes. Chromosome X, and genes localized on it, are necessary for the viability of embryos and plants, so the only the AX microspores have a chance for sporophytic development.

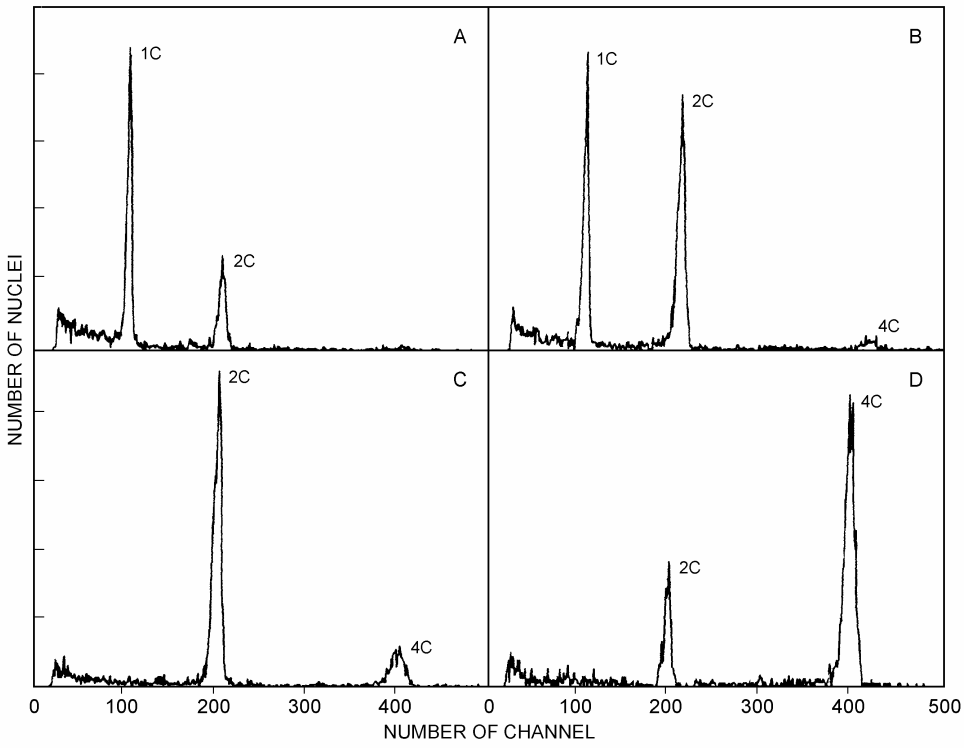

Fig. 1. Distribution of relative DNA contents of nuclei isolated from leaf tissues of regenerated plants: *A* - haploid regenerant (n); *B* - mixoploid regenerant (n+2n); *C* - diploid control plant; *D* - mixoploid regenerant (2n+4n).

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