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# Stimulation of androgenesis in white cabbage (*Brassica oleracea* var. *capitata*) anthers by low temperature and anther dissection

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**Abstract**

Anther culture was performed on two local cultivars, Ljubljansko and Varaždinsko, and the $F_1$ cv. Krautman (Bejo-Zaden). The effects on androgenesis of hot and cold temperature treatments and different dissections of anthers were evaluated. In contrast to cv. Krautman, cvs. Ljubljansko and Varaždinsko produced more embryos after cold pretreatment of flower buds (4°C, 48 h) than after standard treatment (35°C, 24h). Simultaneous cutting of the anther tip and removal of the filament gave the best results in comparison to other tested dissections. Microscopical observations of sectioned anthers revealed enhanced embryo development near the cut ends of the anthers. Ploidy analysis revealed the presence of haploids among embryos resulting from cold treatment (4°C, 48 h), treatment at elevated temperature (35°C, 24 h), and among embryos resulting from dissections of anther tips.

## Introduction

Mass production of homozygous lines by anther culture could enhance breeding programmes. Although good results have been obtained in most forms of *Brassica oleracea*, namely Brussels sprouts (Ockendon 1984; Ockendon & Sutherland 1987), cauliflower (Ockendon 1988), broccoli (Orton & Browers 1985; Arnison et al. 1990) and cabbage (Chiang et al. 1985; Lelu & Bollon 1985; Lillo & Hansen 1987; Doré & Boulidard 1988; Roulund et al. 1990; Arnison & Keller 1990) genotypic differences and low level of embryo production present serious barriers to routine application in breeding programmes (Roulund et al. 1990). Preselected genotypes are often used for the achievement of higher rates of induction (Keller et al. 1987; Roulund et al. 1990). Among the most studied factors affecting androgenesis of cabbage have been high temperature treatments (Chiang et al. 1985; Lelu & Bollon 1985) and genotype (Arnison & Keller 1990; Roulund et al. 1990).

The present study attempted to evaluate the effects of alternative temperature treatments and anther dissections on embryo production.

The beneficial effect of cold pretreatment of flower buds was first studied in *Datura* (Nitsh & Norreel 1973) and later demonstrated in many plant species, while in the genus *Brassica*, treatment with elevated temperatures has routinely been used since being reported by Keller & Armstrong (1977). In the presented study, we compared the most common treatment of anthers (35°C, 24 h) with different cold pretreatments of flower buds or a combination of cold pretreatment of flower buds followed by anther treatments at elevated temperatures.

The effect of different dissections of anthers has only been studied in the anther culture of

broccoli (Arnison et al. 1990), in which anthers with detached or attached filaments were compared and it was found that levels of embryo formation were significantly lower if filaments were left attached to the anthers. In our preliminary studies, we observed that the majority of embryos were formed on the side of anthers from which the filaments had been detached. We studied this effect in more detail, including treatment in which the anther tips were cut off. Our experiments included three differently dissected anther types, including in addition to the removal of the filament also the cutting of the anther tip.

The three cultivars used were selected on the basis of breeding values in our region, with special regard to leaf tenderness and colour after pickling.

## Materials and methods

The genotypes used for anther culture were two local open pollinated cvs., Ljubljansko and Varaždinsko, and the $F_1$ cv., Krautman (Bejo Zaden). Donor plants were grown from seed in a greenhouse and vernalized for 4 months at 4°C in a cold room or left in an unheated greenhouse over the winter. They were then raised in a greenhouse with additional lighting (Phillips SON T, 400 W bulbs, illumination 220 $W/m^2$). Buds with anthers containing microspores at the late uninucleate stage were harvested from the beginning of March to mid April. In experiments in which cold pretreatment was used, buds were cut and stored in containers with moist cotton in a refrigerator (4°C for 2, 4 or 6 days) prior to inoculation. Cultures subjected to high temperature treatment were cultured at 35°C for 24 h before being transferred to 25°C; the experiment also included a combined treatment in which flower buds were first pretreated at 4°C for 48 h and, after inoculation, the anthers were treated at 35°C for 24 h. The anthers of each flower bud were plated in 36 mm petri dishes with 5 ml of medium and cultivated in darkness until embryo emergence.

The basal medium used was Gamborg's $B_5$ (Gamborg et al. 1968) as modified by Keller & Armstrong (1977). The induction medium contained 1 mg $l^{-1}$ α-naphthaleneacetic acid (NAA) and 1 mg $l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) as reported by Lillo & Hansen (1987) and Arnison & Keller (1990). The induction medium was supplemented with 10% sucrose, 800 mg/l L-glutamine, and 100 mg $l^{-1}$ L-serine. Gellan Gum – Gelrite (2 g $l^{-1}$) was used in the temperature treatment experiment and Difco-Bacto agar (8 g $l^{-1}$) in all other experiments. All media were sterilized by autoclaving. Plant regeneration was carried out on the medium described by Keller et al. (1975). Root tips were taken from embryos during the first subculture on hormone free media and after regeneration of plantlets. Chromosome counts on root tips were performed as described by Chiang et al. (1979) Sectioning of anthers was performed according to Gerlach (1978).

Statistics were executed by genstat 5 (the numerical algorithms limited). The embryo yields were logarithmically transformed, ln (no. of embryos per 100 anthers cultured + 1). Treatment means were tested by analysis of variance one way classification, for each cultivar separately.

## Results

### *Temperature pretreatment of flower buds and anthers*

Our experiments were designed to evaluate several combinations of cold pretreatment of buds in comparison to standard treatment of anthers at elevated temperatures. Studies included:

- hot treatment (standard) of anthers for 1 day at 35°C,
- cold pretreatment of flower buds at 4°C for 2 days (for 'Krautman' also 4 and 6 days)
- cold pretreatment of flower buds at 4°C for 2 days followed by another hot treatment at 35°C for 1 day.

Embryos were scored between the third and sixth week after cultivation; the results are presented in Table 1, and Table 2.

Analysis of variance showed that significant differences exist ($p = 0.001$) between treatments at 4°C (2 days) and 35°C (1 day) for cvs.

*Table 1.* The effect of cold and hot temperature treatments on embryo formation in anther cultures of white cabbage.

| Cultivar | No. of plants tested | No. of anthers cultured | Flower bud or anther treatment | No. of embryos formed | Mean embryo yield/100 anthers untransformed | Mean embryo yield/100 anthers transformed |
|---|---|---|---|---|---|---|
| Varaždinsko | 2 | 622 | 1 | 62 | 9.97 | 1.502 |
| | | 709 | 2 | 35 | 4.94 | 0.661 |
| Ljubljansko | 2 | 966 | 1 | 113 | 11.70 | 1.717 |
| | | 870 | 2 | 35 | 4.02 | 0.668 |
| Krautman | 3 | 1845 | 1 | 33 | 1.79 | 0.310 |
| | | 1677 | 2 | 33 | 1.97 | 0.340 |
| | | 619 | 3 | 3 | 0.48 | 0.080 |
| | | 1379 | 4 | 16 | 1.16 | 0.200 |
| | | 1371 | 5 | 12 | 0.88 | 0.150 |

| Treatment no. | Flower bud pretreatment | Anther treatment |
|---|---|---|
| 1 | 4°C, 2 days | – |
| 2 | – | 35°C, |
| 3 | 4°C, 2 days | 35°C |
| 4 | 4°C, 4 days | – |
| 5 | 4°C, 6 days | – |

*Table 2.* Standard error for differences between treatments

| Cultivar | Between treatments | Transformed mean difference | SED | d.f. |
|---|---|---|---|---|
| "Varaždinsko" | 1–2 | 0.841*** | 0.197 | 197 |
| "Ljubljansko" | 1–2 | 1.049*** | 0.160 | 304 |
| "Krautman" | 1–2 | 0.030 | 0.066 | 1127 |
| | 1–3 | 0.230* | 0.094 | 1127 |
| | 1–4 | 0.110 | 0.069 | 1127 |
| | 1–5 | 0.160* | 0.069 | 1127 |
| | 2–3 | 0.260** | 0.095 | 1127 |
| | 2–4 | 0.140 | 0.072 | 1127 |
| | 2–5 | 0.190** | 0.072 | 1127 |
| | 3–5 | 0.070 | 0.097 | 1127 |
| | 4–5 | 0.050 | 0.074 | 1127 |

| Treatment no. | Flower bud pretreatment | Anther treatment |
|---|---|---|
| 1 | 4°C, 2 days | – |
| 2 | – | 35°C, |
| 3 | 4°C, 2 days | 35°C, |
| 4 | 4°C, 4 days | – |
| 5 | 4°C, 6 days | – |

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Ljubljansko and Varaždinsko. For cv. Krautman, treatment of anthers at 35°C (1 day) was superior to combined treatment at 4°C followed by 35°C and to pretreatment at 4°C for 6 days ($p = 0.01$). Pretreatment at 4°C (2 days) was superior ($p = 0.05$) to the same treatment followed by anther treatment at 35°C (1 day).

*Dissection of anthers*

Preliminary studies revealed a tendency to enhanced embryo development near the cut ends of the anthers. To study this effect on the subsequent development of embryos, we included three differently dissected anther types: anthers with filament attached, anthers with filament removed and anthers with filament removed and anther tip cut off (Fig. 1 A, B, C). Data are presented in Table 3, and Table 5.

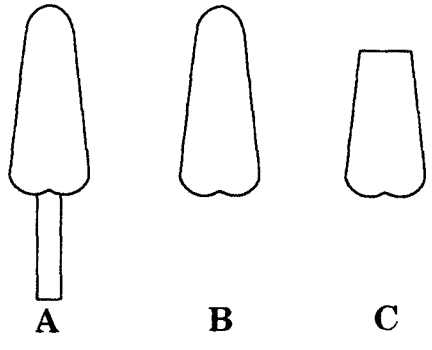


*Fig. 1.* Scheme of different dissections: *A* anther with filament attached, *B* anther with filament removed, *C* anther with filament removed and tip cut off.

*Table 3.* The effect of different dissections on embryo formation in anther culture of white cabbage.

| Cultivar | No. of plants | Dissection | No. of anthers | No. of embryos | Mean embryo yield/100 anthers untransformed | Mean embryo yield/100 anthers transformed |
|---|---|---|---|---|---|---|
| Krautman | 3 | A | 833 | 12 | 1.44 | 0.204 |
| | | B | 1023 | 19 | 1.86 | 0.321 |
| | | C | 932 | 54 | 5.79 | 0.952 |
| Varaždinsko | 1 | A | 144 | 5 | 3.47 | 0.51 |
| | | B | 159 | 3 | 1.89 | 0.31 |
| | | C | 180 | 26 | 14.44 | 1.38 |

A: filament attached
B: filament removed
C: filament removed and tip cut off

*Table 4.* The effect of different dissections on embryo formation in anther culture of white cabbage.

| Cultivar | No. of plants | Dissection | No. of anthers | No. of embryos | Mean embryo yield/100 anthers untransformed | Mean embryo yield/100 anthers transformed |
|---|---|---|---|---|---|---|
| Ljubljansko | 2 | B | 1110 | 42 | 3.78 | 0.567 |
| | | C | 1214 | 294 | 24.17 | 2.693 |
| Varaždinsko | 3 | B | 1712 | 73 | 4.26 | 0.592 |
| | | C | 2212 | 406 | 18.35 | 1.973 |

B: filament removed
C: filament removed and tip cut off

*Table 5.* Standard error for differences between dissections.

| Cultivar | Between dissections | Transformed mean difference | SED | d.f. |
|---|---|---|---|---|
| Krautman | A–B | 0.12 | 0.123 | 447 |
| | A–C | 0.75*** | 0.130 | 447 |
| | B–C | 0.63*** | 0.115 | 447 |
| Varaždinsko | A–B | 0.20 | 0.388 | 79 |
| | A–C | 0.87* | 0.368 | 79 |
| | B–C | 1.07** | 0.347 | 79 |
| Ljubljansko | B–C | 2.126*** | 0.1295 | 401 |
| Varaždinsko | B–C | 1.381*** | 0.1179 | 654 |

A: filament attached
B: filament removed
C: filament removed and tip cut off
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

For cv. Krautman the removal of filaments and cutting of anther tips was superior to other studied dissections at $p = 0.001$. The same type of dissection proved to be best for cv. 'Varaždinsko' at $p = 0.01$ and $p = 0.05$.

Because of low embryo yields, anthers with filament attached were omitted in the third experiment (Table 4 and Table 5). For both cvs., the simultaneous removal of filaments and cutting of tips was superior to the removal of filaments ($p = 0.001$).

All dissections were subjected to standard treatment at 35°C for 24 h.

*Evaluation of regenerated embryos*

Our investigation did not establish morphological differences among embryos resulting from

cold pretreatments, or those formed on the cut ends of anthers and the standard treatments. The gametophytic origin of these embryos was confirmed by cytogenic analysis of the chromosome number on the root tips of those embryos which formed roots during the first subculture on hormone free media. Chromosome counts performed on root tips of 34 embryos revealed comparable percentages of haploids between cold and hot treatments. The overall frequency of haploids was 39%.

## Discussion

In the genus *Brassica*, treatment of inoculated anthers at elevated temperatures (for cabbage 35°C for 24 h) is most frequently adopted. There is no report on the beneficial effect of cold pretreatment, as is the case in several other crops.

Our experiment was focused on different temperature treatments of flower buds and anthers. Domestic local cultivars seemed to favour cold pretreatment, in contrast to cv. Krautman, which gave lower embryo yields in all treatments.

It may be that cvs. Varaždinsko and Ljubljansko are more acclimatized to a warm climate than cv. Krautman and cold pretreatment would thus for them be a more adequate thermal shock. Another possible explanation is that the temperature regime at which the donor plants were grown was higher than that usually employed, so cold pretreatment of buds had beneficial effects.

It is well documented that the anther wall plays an important role in embryo development. However, most studies have been focused on anther orientation on the medium, anther density and studies of abnormal pollen development according to the position within the locule (Arnison et al. 1990). Opening the locules by removing the anther tip is therefore a new approach to studies of the relation between the anther wall and the medium and its influence on pollen development.

Our experiments showed that cutting of the anther tip and removing the filament contributed to embryo development.

Opening the locules seems to stimulate androgenesis. There are several possible explanations, one of which is that by cutting the anther tip, the intake of medium substances to the interior of the locules is accelerated, so the quantity of hormones that reach the pollen grains at the opened anther positions is more adequate.

Cytological analysis has confirmed the gametophytic origin of embryos, regardless of whether cold, hot, combined temperature treatments are used or embryos are generated in anthers without tip and filament.

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