

Attempts to induce haploids in anther cultures of sugar, fodder and wild species of beet

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Abstract

In the present investigation, aimed at obtaining beet haploids from anthers, the effect of mineral media, potato and sugar beet extract and p-fluorophenylalanine (PFP) in combination with growth substances was tested. Nutrient-starved plants as anther-donors, anther-starvation, cold treatment and photoperiod were also analysed. On all mineral media the anthers produced callus and roots; however, the percentage depended on the combination of growth substances used. The best medium for differentiation was that of Linsmaier and Skoog with 25 μM zeatin or 6-(3-methyl-2-butenylamino)purine with 5 μM naphthalene-1-acetic acid (25.5%). The addition of PFP caused an increase in the percentage of anther differentiation (41.6%). Besides callus and roots on one of the anthers (in ca. 140000 tested), vegetative buds were formed from which numerous plants were obtained (2n). Plant and anther nutrient starvation did not improve the anther response to differentiation, nor did it induce haploid development, similarly as cold treatment of inflorescences or isolated anthers. The anthers of wild species showed lower ability to differentiate than those of sugar or fodder beets. Cytological analyses showed formation of multicellular structures until ca. the 12-th day of anther culture; afterwards, they degenerated.

INTRODUCTION

Studies on obtaining beet haploids from anthers grown *in vitro* have not hitherto been successful. Beet anthers rather easily form callus and roots (Atanassov 1973, Rogozińska and Gośka 1976) but extremely rarely buds (Banba and Tanabe 1972, Rogozińska et al. 1977). Also, the development of microspores into haploid plants does not take place. The present report is a continuation of earlier work on beet haploids.

MATERIAL AND METHODS

The anthers of polygerm sugar beets (*Beta vulgaris* L. provar. *altissima* Döll), fodder beets (*Beta vulgaris* L. provar. *crassa* Alef.) and wild species (*Beta macrorhiza*, *B. lomatogona*, *B. corolliflora* and *B. trigyna*) were taken from plants grown in the greenhouse (February, March, April) or in the field (June, July).

After inflorescence surface-sterilization the anthers (with tetrads and young uninucleate microspores) were isolated from the florets and cultured on agar media of Linsmaier and Skoog (LS), (1965), Nitsch and Nitsch (NN), (1969) and White (W), (1943). The media were supplemented with: naphthalene-1-acetic acid (NAA) in combination with 6-benzylaminopurine (BAP), zeatin (Z), kinetin (K), or 6-(3-methyl-2-butenylamino)purine (2iP). Moreover, the effect of p-fluorophenylalanine (PFP) was investigated. Besides synthetic media, potato and beet extract prepared according to Schaeffer et al. (1979) were tested. The influence of starvation on field-grown plants as anther-donors was investigated. The nutrient-starved sugar beets were grown in sand and watered only with tap water. The isolated anthers were grown on complete or diluted 1:1 LS medium. Cold stress of 4, 7 and 15°C was applied during 7, 14 and 21 days to the detached inflorescences from which the anthers were isolated or to the anthers incubated on the LS media.

The cultures of sugar beet anthers were maintained at a temperature of ca. 25°C in a photoperiod of 16h light (ca. 1500 lux) and 8h dark. The anthers of fodder beets were also grown in continuous dark or light. In experiments with potato and sugar beet extract the anthers were placed in dark at 4°C for 64h, followed by a 5 day period at 25°C and later were transferred to the 16h photoperiod. Growth of the anthers was observed during the 3 month culture period.

For cytological studies anthers were taken every three days during three weeks of growth on media with 20 µM BAP, Z or 2iP with 5 µM NAA. The anthers were stained with orcein. Anthers with enlarged microspores were fixed for investigation by electron microscope (Młodzianowski and Idzikowska 1978). Cytological analyses of the plantlets were performed on root and stem tips, fixed in Carnoy mixture and stained with orcein for 48h, macerated and analysed in 45% acetic acid.

RESULTS AND DISCUSSION

Various attempts at haploid induction in anthers of beets cultured *in vitro* were undertaken, and the effect of stress on anther-donor plants was studied. It was observed that after about 14 days incubation, anthers began to proliferate small amounts of callus and, later, to produce roots.

The frequency of differentiation varied and callus formation was rather slow but abundant enough when the cultures were incubated on appropriate media.

In studies on the influence of the mineral composition of Linsmaier and Skoog (1965), Nitsch and Nitsch (1969) and White (1943) media, the highest percentage of sugar beet anthers forming callus and roots was obtained on LS medium (Fig. 1). This percentage depended on the auxin and cytokinin concentration. On LS medium containing NAA and zeatin, the highest percentage of anthers producing callus amounted to 25.4%, whereas 2.1% produced roots. On NN medium, the highest percentage of anthers forming callus was 16.6%, and 2.9% produced roots. On W medium the anthers produced

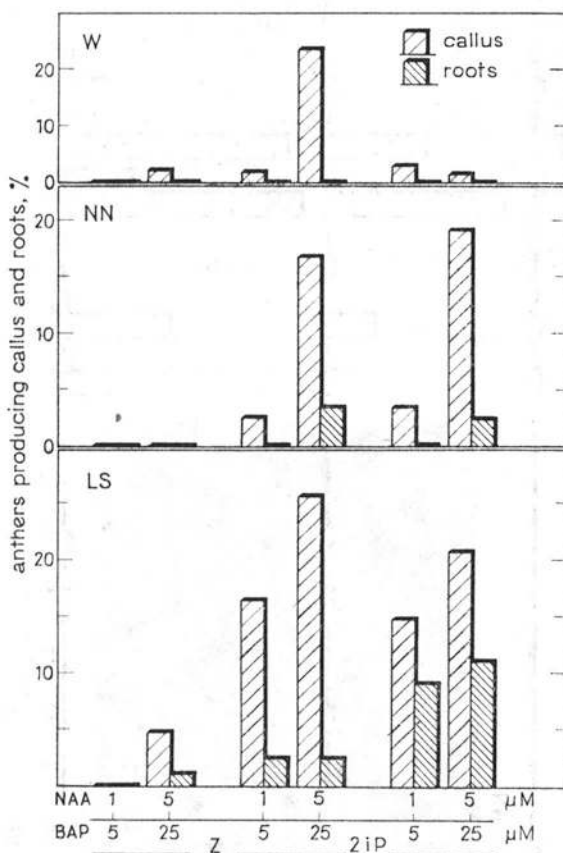


Fig. 1. Effect of mineral media (White — W, Nitsch and Nitsch — NN, Linsmaier and Skoog — LS) and growth substances on the differentiation of sugar beet anthers. NAA — naphthalene-1-acetic acid, BAP — 6-benzylaminopurine, Z — zeatin, 2iP — 6-(3-methyl-2-butenylamino)purine. The results represent the mean from 240 anthers for each combination

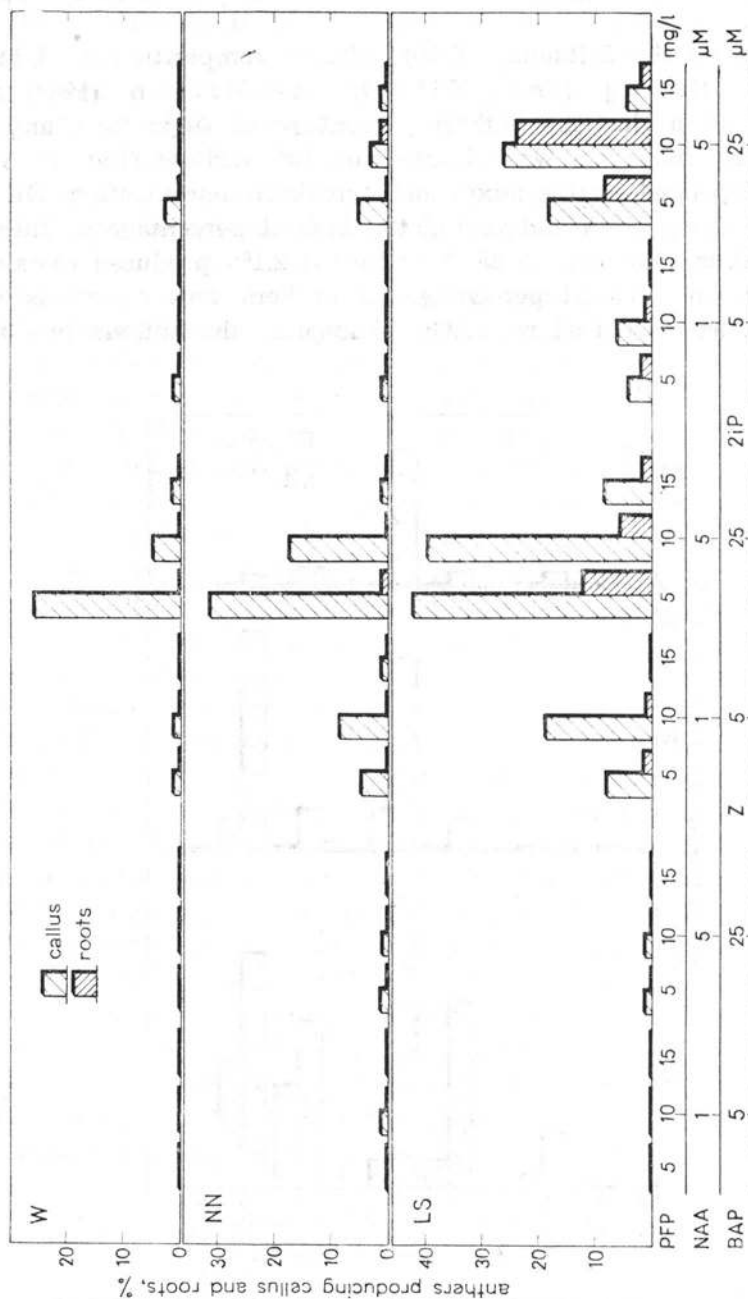


Fig. 2. Effect of mineral media (White — W, Nitsch and Nitsch — NN, Linsmaier and Skoog — LS), p-fluorophenylalanine (PFP) and growth substances on the differentiation of sugar beet anthers. Abbreviations as in Fig. 1. The results represent the mean from 240 anthers for each combination

only callus — 11.3%. The percentage of anthers forming callus and roots on media with 2iP was somewhat lower than on media with zeatin. On LS medium 20.8% of the anthers produced callus and 12.5% roots; on NN medium — 19.1% callus and 2.1% roots; on W medium like on that with zeatin, the anthers produced only callus — 1.3%. Very feeble callus formation by the anthers was obtained on media with BAP. On LS medium it amounted to 4.4%, on W medium to 0.8%, whereas on NN medium the anthers failed to form callus.

The addition of p-fluorophenylalanine which is used for haploidization of fungi and callus (Reinert and Bajaj 1977) greatly improved the ability of the anthers to form callus (Fig. 2). In the presence of 5 or 10 mg/l PFP the highest percentage of anthers which formed callus and roots was obtained on LS medium with zeatin followed by 2iP. This percentage of anthers forming callus and roots obtained with PFP was the highest achieved hitherto by us and amounted to 41.6. On W medium with 25 μ M Z + 5 μ M NAA + 15 mg/l PFP, vegetative buds were formed on one of the anthers only from which, after transferring to a medium with 1 μ M BAP + 5 μ M NAA, numerous plantlets were produced (Fig. 3). Cytological analyses revealed that they were diploids (18n); whether they are diploids of somatic origin or diploids from spontaneously doubled haploid cells, is as yet not known and is now being determined by standard genetic methods. The results indicate that PFP greatly modifies the percentage of anthers forming callus and roots; however, it did not induce the development of haploids. Thus, direct formation of buds was obtained by us for the first time during



Fig. 3. Plantlets differentiated from anther after the first subculture on regeneration medium (1 μ M BAP + 5 μ M NAA). Growth period: May 10 — June 20, 1980

the 6 year research period. Bud formation was obtained in our earlier studies (Rogozińska et al. 1977), albeit not directly from anther but from the subcultured callus which differentiated from anther. The frequency of bud formation is extremely low, taking into consideration the amount of about 140000 anthers cultured from 1974 to 1980.

The positive results of Schaeffer et al. (1979) with potato extract in obtaining wheat haploids stimulated us to replace the mineral media by natural extracts. However, the potato and beet extract used did not induce anther differentiation.

As shown by Sunderland (1978), batch to batch variation in culture yield, the age of the plant, and the environmental conditions of the anther-donor plants are constant problems because they affect

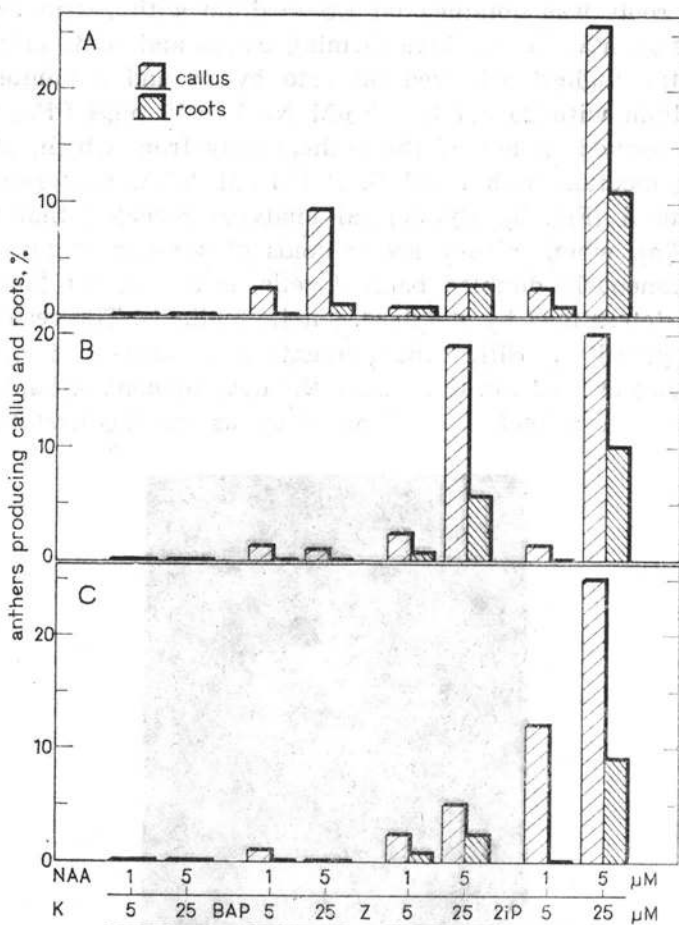


Fig. 4. Effect of nutrient-starvation of donor-plants and anther starvation on the differentiation of sugar beet anthers. A — nutrient-starved donor-plants; B — anthers cultivated on nutrient-starved medium; C — nutrient-starved donor-plants, anthers of which were cultured on nutrient-starved medium. K — kinetin, other abbreviations as in Fig. 1

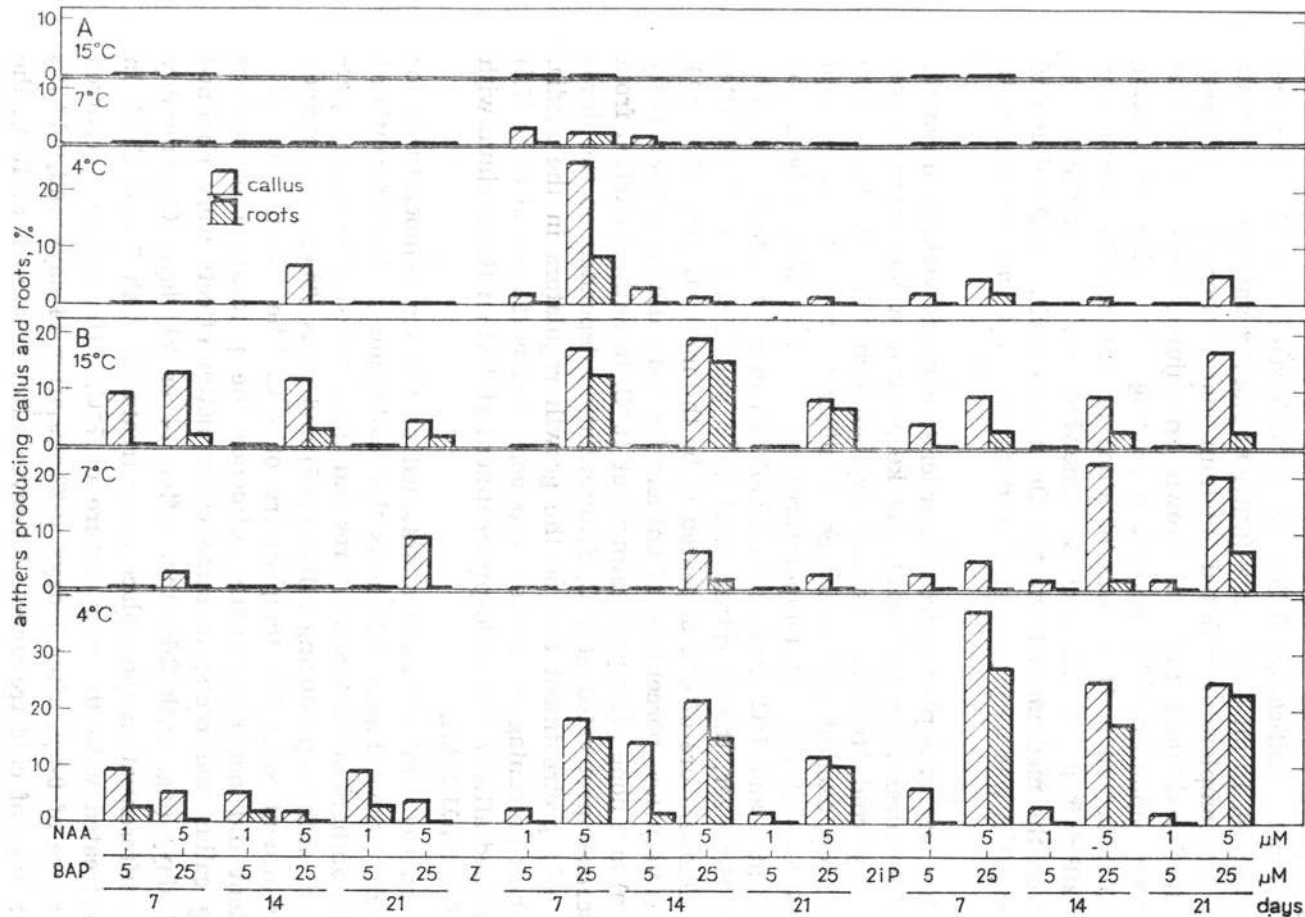


Fig. 5. Effect of growth substances and cold-treatment on the differentiation of sugar beet anthers. A — detached inflorescences as anther-donors were placed at temperatures of 4, 7 and 15°C for 7, 14 and 21 days; B — excised anthers were incubated at temperatures of 4, 7 and 15°C for 7, 14 and 21 days. Abbreviations as in Fig. 1

the embryogenic potential of the anthers. The demonstration of a positive effect with nitrogen-starved tobacco plants led us to test nutrient-starved sugar beets as anther-donors. The results showed that these factors did not significantly influence the differentiation of sugar beet anthers (Fig. 4). In all three cases (nutrient-starved anther-donor plants cultured on complete or diluted medium and anthers derived from normal fertilized field material grown on diluted medium) similar results were obtained. The highest percentage of anthers producing callus (23.7) and roots (11.2) was obtained on the medium containing $25 \mu\text{M}$ 2iP + $5 \mu\text{M}$ NAA and a somewhat lower percentage was obtained on the medium with zeatin. On medium with BAP callus and root production was very poor and no differentiation was obtained with kinetin.

Another way of applying stress in order to enhance yield is to remove the inflorescences from the plant and keep them at low temperatures or to apply cold treatment to the incubated anthers. Anthers were isolated from excised inflorescences of sugar beets which were kept for 7, 14 and 21 days at temperatures of 4, 7 and 15°C . The inflorescences at 4 and 7°C showed no visible changes in appearance after 21 days. However, those pretreated at 15°C yellowed and wilted after 7 days. Temperature of 4°C appeared to be the most effective (Fig. 5A). At 7°C a very low percentage of anthers formed callus and roots (only on zeatin) and none after pretreatment at 15°C . In anthers excised from inflorescences pretreated at 4°C , differentiation depended on the duration of cold pretreatment and on the growth regulators in the media. The highest percentage of anthers producing callus (23.7) and roots (7.5) was obtained after 7 days cold-pretreatment at 4°C on the medium with $25 \mu\text{M}$ Z + $5 \mu\text{M}$ NAA.

The excised anthers were maintained at the low temperatures for 7, 14 and 21 days. Figure 5B shows that callus and roots differentiated from the anthers at all temperatures and times tested. The highest percentage of anthers producing callus (37.5) and roots (25.0) was obtained after treatment with the temperature of 4°C . The duration of cold-treatment did not significantly influence the percentage of anthers forming callus and roots; in some cases, better effects were obtained after 7 days (e.g. with 2iP) than after 14 or 21 days. Considerably weaker differentiation took place after treatment with 7 and 15°C . In anthers treated with the temperature of 7°C , differentiation occurred in the range of 0-5% after 7 days, 0-22.5% after 14 days, and 0-13.7% after 21 days of cold treatment. However, after treatment at 15°C , the percentage of anthers forming callus and roots amounted to 0-12.5% after 7 days, 0-13.7 after 14 days, and 0-12.5 after 21 days. Thus, the differentiation of callus and roots as a function of the duration of cold

treatment on isolated anthers was analogous though somewhat better results were obtained after 7 and 14 days.

Cold pretreatment of inflorescences before excision of the anthers was considerably less effective (0-23.7%) than cold treatment of anthers after incubation on the media (0-37.7%). Hence, cold treatment which increases the percentage of haploids in many plant species (Nitsch and Cacco 1978, Sunderland 1978, Sunderland and Roberts 1979) is more complex in sugar beet and failed to bring about the induction of haploids.

Experiments on obtaining haploids from fodder beet anthers were carried out on less numerous field material (ca. 10000). The effect of growth regulators, light conditions, as well as agar and liquid media were analysed. On control LS media without growth substances and on media containing individual cytokinins (BAP, Z or 2iP) or auxin (NAA) the anthers failed to grow or underwent insignificant enlargement. The combination of cytokinin with auxin induced on some anthers some small production of callus and roots. The highest percentage of

Table 1

Effect of growth substances on callus and root formation in fodder beet anthers
(*Beta vulgaris* L. provar. *crassa* Alef.)

Growth substances, μM				Anthers producing callus, %	Anthers producing roots, %
BAP	Z	2iP	NAA		
0	0	0	0	0	0
5	0	0	0	0	0
10	0	0	0	0	0
20	0	0	0	0	0
0	5	0	0	0	0
0	10	0	0	0	0
0	20	0	0	0	0
0	0	5	0	0	0
0	0	10	0	0	0
0	0	20	0	0	0
0	0	0	5	0	0
0	0	0	10	0	0
0	0	0	20	0	0
5	0	0	5	13.3	13.3
10	0	0	5	14.6	13.7
20	0	0	5	8.7	7.9
0	5	0	5	2.5	2.1
0	10	0	5	2.9	2.5
0	20	0	5	5.4	1.7
0	0	5	5	1.2	0.8
0	0	10	5	0.8	0.8
0	0	20	5	0	0

The number of anthers in each combination amounted to 240. Growth period: April 27—August 27, 1978. BAP—6-benzylaminopurine, Z—zeatin, 2iP—6-(3-methyl-2-butenylamino) purine, NAA—naphthalene-1-acetic acid

anthers producing callus and roots obtained with $5 \mu\text{M}$ NAA in combination with cytokinins amounted to: with $10 \mu\text{M}$ BAP — 14.6; with $20 \mu\text{M}$ Z — 5.4; and with $25 \mu\text{M}$ 2iP — 1.2 (Table 1, Fig. 6).

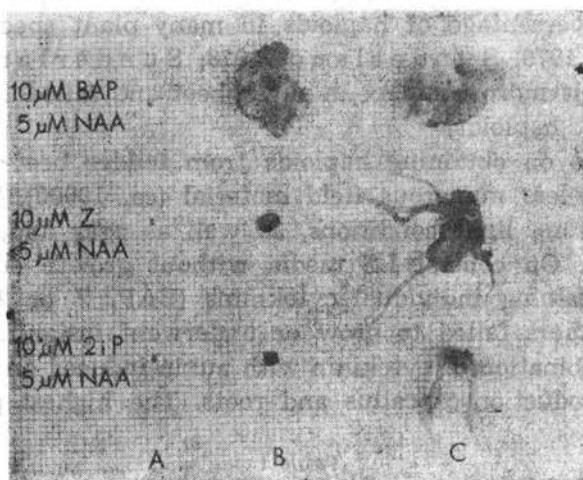


Fig. 6. Effect of growth substances on the differentiation of fodder beet anthers. A — isolated anthers; B — anthers producing callus; C — anthers producing callus and roots. LS medium. Growth period: May 20 — July 3, 1978

In experiments on the influence of light conditions as well as agar and liquid media on anther differentiation, NAA ($1 \mu\text{M}$) in combination with BAP, Z and 2iP was used. On agar media, the highest percentage of anthers producing callus and roots was obtained in the 16h photoperiod (11.7) followed by continuous light (9.2), and the smallest in dark (1.2), (Fig. 7). On liquid media, however, the highest percentage of anthers producing callus and roots was obtained in continuous light (10.4) followed by dark (5.0), and the smallest in the 16h photoperiod (0.8). Thus, the application of various light conditions and other factors did not induce haploid production in fodder beet anthers and the percentage of anthers producing callus and roots was smaller than that observed for the sugar beet.

Analogical experiments were performed on anthers of wild species. The anthers were incubated on media with those auxin and cytokinin combinations which were optimal for sugar and fodder beet anther differentiation. The anthers of *B. lomatogona* produced callus and roots (0-2.1%) and the anthers of other species callus only: *B. trigyna* — 0-6.6%, *B. corolliflora* — 0-1.3% and *B. macrorhiza* — 0-0.4% (Table 2). The best results concerning callus and root differentiation from the anthers of the investigated wild species of beet were obtained on media

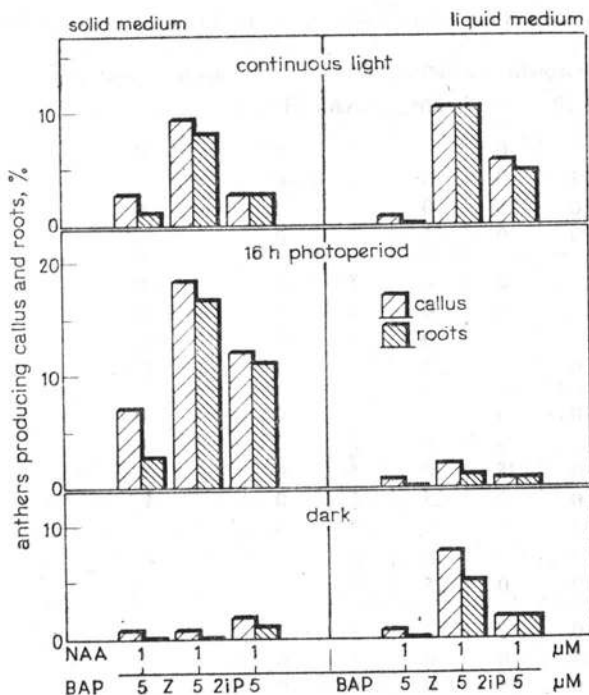


Fig. 7. Effect of light conditions as well as agar and liquid LS medium on the differentiation of fodder beet anthers. Abbreviations as in Fig. 1. The results are the mean from 240 anthers for each combination

containing 25 μM 2iP + 5 μM NAA with the exception of *B. trigyna* anthers which differentiated better with 10 μM BAP + 10 μM IBA (indole-3-butyric acid). Thus the anthers of wild species of beet, similarly as those of fodder beets, exhibited a lower ability to form callus and roots than anthers of the sugar beet.

The calluses which differentiated from the anthers of sugar beet, fodder beet and wild species of beet were transferred to regeneration medium. They exhibited different appearance and various growth rates. The calluses derived from anthers of sugar and fodder beets grew rather slowly, attaining after 6 weeks of growth a weight of ca. 2 grams. They did not produce buds but showed a weak ability of rhizogenesis. After ca. 3 weeks of growth the callus cultures began to turn brown presumably reflecting the formation and accumulation of phenolic compounds and their oxidation products which are also present in beets grown *in situ* (Trzebiński 1957). As the number of consecutive subcultures increased, the growth rate of the calluses was insignificantly reduced and they finally lost the ability of rhizogenesis (after 5 subcultures). The calluses derived from the anthers of wild species exhibited

Table 2

Effect of growth substances on callus and root formation in anthers of wild species of beet

Wild species	Growth substances, μM						Anthers producing callus, %	Anthers producing roots, %
	K	BAP	Z	2iP	NAA	IBA		
<i>B. macrorrhiza</i>	25	0	0	0	5	0	0	0
	0	25	0	0	5	0	0	0
	0	0	25	0	5	0	0	0
	0	0	0	25	5	0	0.4	0
<i>B. corolliflora</i>	25	0	0	0	5	0	0	0
	0	25	0	0	5	0	0	0
	0	0	25	0	5	0	0.4	0
	0	0	0	25	5	0	1.3	0
<i>B. lomatogona</i>	25	0	0	0	5	0	0	0
	0	25	0	0	5	0	0	0
	0	0	25	0	5	0	0	0
	0	0	0	25	5	0	1.3	0
	0	25	0	0	0	5	0	0
	0	0	25	0	0	5	1.3	0
	0	0	0	25	0	5	2.1	2.1
<i>B. trigyna</i>	25	0	0	0	5	0	0	0
	0	25	0	0	5	0	0	0
	0	0	25	0	5	0	0	0
	0	0	0	25	5	0	0	0
	0	5	0	0	0	5	3.3	0
	0	10	0	0	0	10	6.6	0
	0	15	0	0	0	5	1.6	0
	0	15	0	0	0	10	1.3	0

The number of anthers in each combination amounted to 240. Growth period: June 3—September 9, 1980. IBA—indole-3-butyric acid, other abbreviations as in Table 1

a somewhat slower growth rate and different appearance. E.g. the callus derived from the anthers of *B. lomatogona* reached a ca. 50% lower weight than the callus of sugar beet but maintained for a longer time its ability to form roots (it still maintains this ability in its 13 sub-culture). This was connected with differences in morphology and texture. During the whole period of its culture the callus maintained its dark green color; its structure was nodular, and it grew flat on the surface of the medium.

Cytological analyses were performed on anthers of polygerm sugar beets, excised in the stage of tetrads and uni- and binucleate microspores. In the 3 day old cultures an enlargement of the tetrads was observed and later after 6 days a multiplication of microspore number. The number of microspores in the tetrads increased from 4 to 8 or 16. The layer surrounding the nuclei in the microspore (Fig. 8A) consist probably of a mixture of callose and cellulose. Similar changes were observed

in the anthers after 9 days of culture. In 12 day cultures besides enlarged tetrads multicellular structures were present (Fig. 8B). Simultaneously, initial stages of degeneration of these structures took place. In anthers after 15, 18 and 21 days of incubation a further degeneration of multicellular structures was observed accompanied by the presence of shapeless masses of dying cells (Fig. 8C). In anthers excised in the stage of uni- and binucleate pollen, growth changes were not noticed during 21 days of culture.

The present investigations showed a very small number of anthers with growth changes in the initial time after inoculation and their almost complete absence later on. This makes the analysis of this material difficult. Further experiments are necessary in order to obtain further developmental stages of the multicellular structures into haploid embryoids.

For interpretation of the changes in the cultured anthers and because of the lack of further developmental stages of the multicellular structures, further analyses were performed by electron microscopy. The electron micrographs revealed the presence of collapsing tetrads (Fig. 9A) and degenerated microspores. Symptoms of degeneration were especially distinct in the nuclear envelope (Fig. 9B). In the tapetum, however, cell division occurred and numerous mitochondria indicative of cell activity were present (Fig. 10A). Chloroplasts were also observed and, in some of them crystalline bodies were present (Fig. 10B). In anthers inoculated in the stage of uni- and binucleate pollen, flattened and degenerated pollen grains were present (Fig. 11A, B). Whereas Hoefert (1969) has given a description of the ultrastructure of *Beta* pollen *in situ*, the present study disclosed initial stages of androgenesis when culturing sugar beet anthers *in vitro*.

The application to beet anthers of factors inducing haploids in plants of the *Solanaceae*, *Gramineae*, *Cruciferae*, *Ranunculaceae* families and others (Sunderland 1978) proved unsuccessful. Media, growth regulators and culture conditions were determined for anther differentiation into callus and roots. Bud formation occurred very rarely and was obtained through callus formation (Rogozińska et al. 1977) or directly from the anther (Banba and Tanabe 1972 and present paper). In our studies, bud formation occurred in both cases at 25 μ M cytokinin (BAP or Z) with 5 μ M NAA. The significance of the present results for plant breeding is not yet clear because there is doubt regarding the origins of the plantlets. All the stem and root tip preparations studied by us revealed chromosome numbers of 18.

Cold treatment did not improve the percentage of anther differentiation though anther treatment after incubation on the media was more effective than pretreatment of the inflorescences before excision. Also

the use of nutrient-starved plants as anther-donors and culturing the anthers on diluted medium did not increase their ability to differentiate nor led to the development of haploid embryoids.

However, on applying PFP, the percentage of anther differentiation into callus and roots increased considerably but haploids were not obtained. Though initial stages of microspore development into multicellular structures were observed after a dozen or so days, the structures degenerated. These symptoms appeared particularly distinctly in the nucleus envelope of the microspores.

Beside the continuation and modification of anther culture *in vitro* there remain other methods of obtaining haploids used hitherto in beet breeding (Yüce 1973) which are similarly difficult and not very successful.

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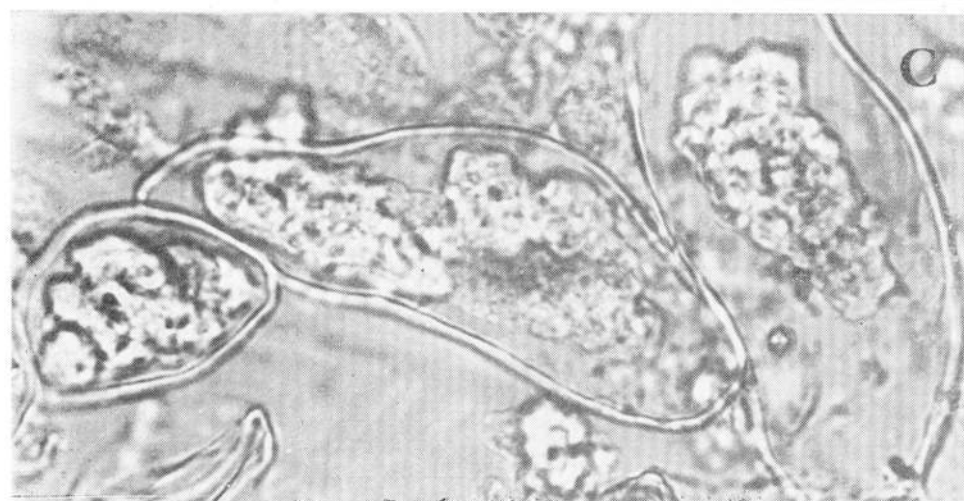


Fig. 8. Consecutive stages of microspore division and degeneration. A — development of multicellular structures; B — initial stages of fusion and degeneration; C — final stages of degeneration. 800X

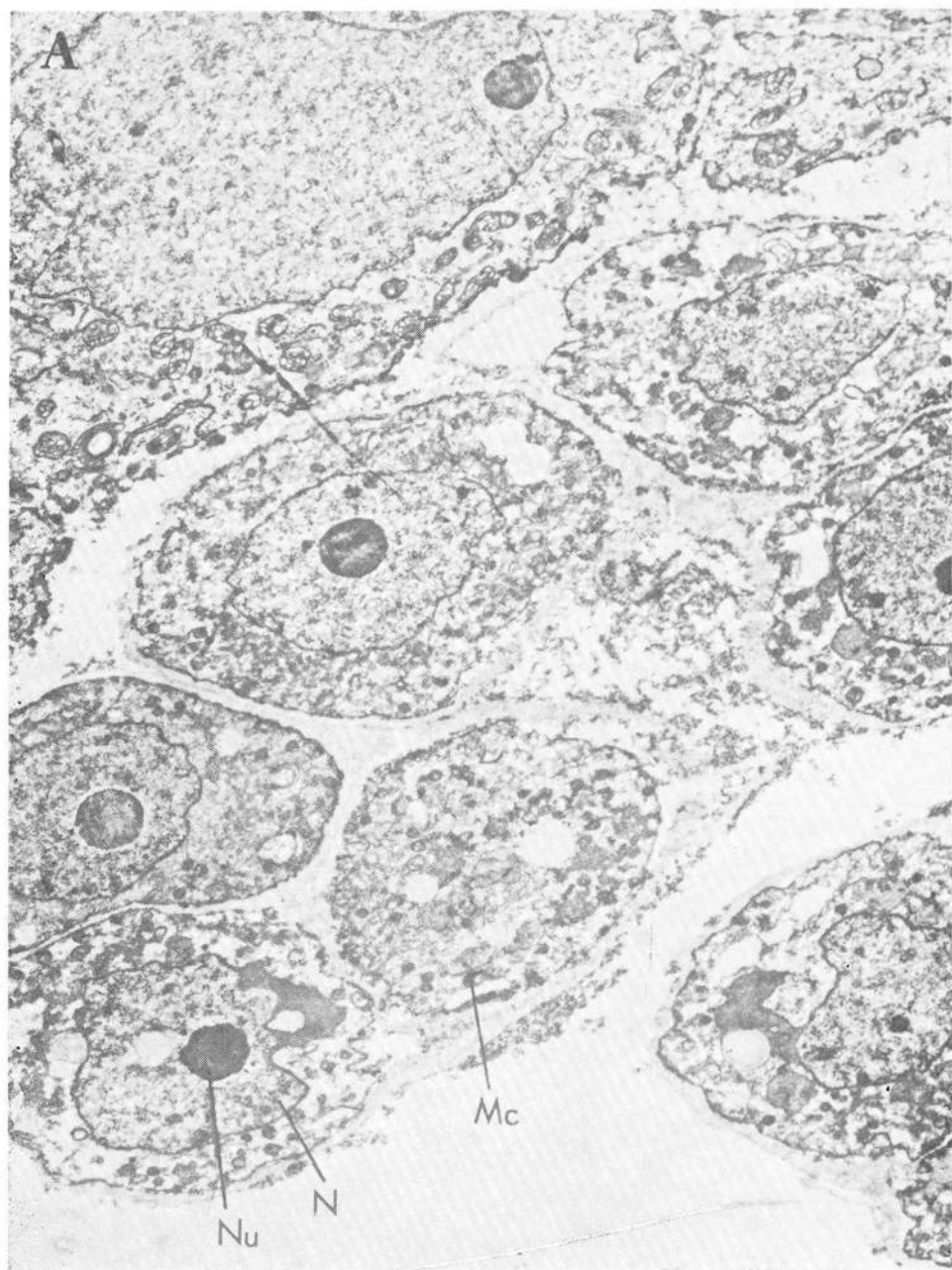


Fig. 9A. Desintegrating tetrad with degenerating microspores (Mc), nucleus (N), nucleolus (Nu). Anthers were fixed after 6 days culture on LS medium with $20\text{ }\mu\text{M}$ BAP + $5\text{ }\mu\text{M}$ NAA. $6200\times$

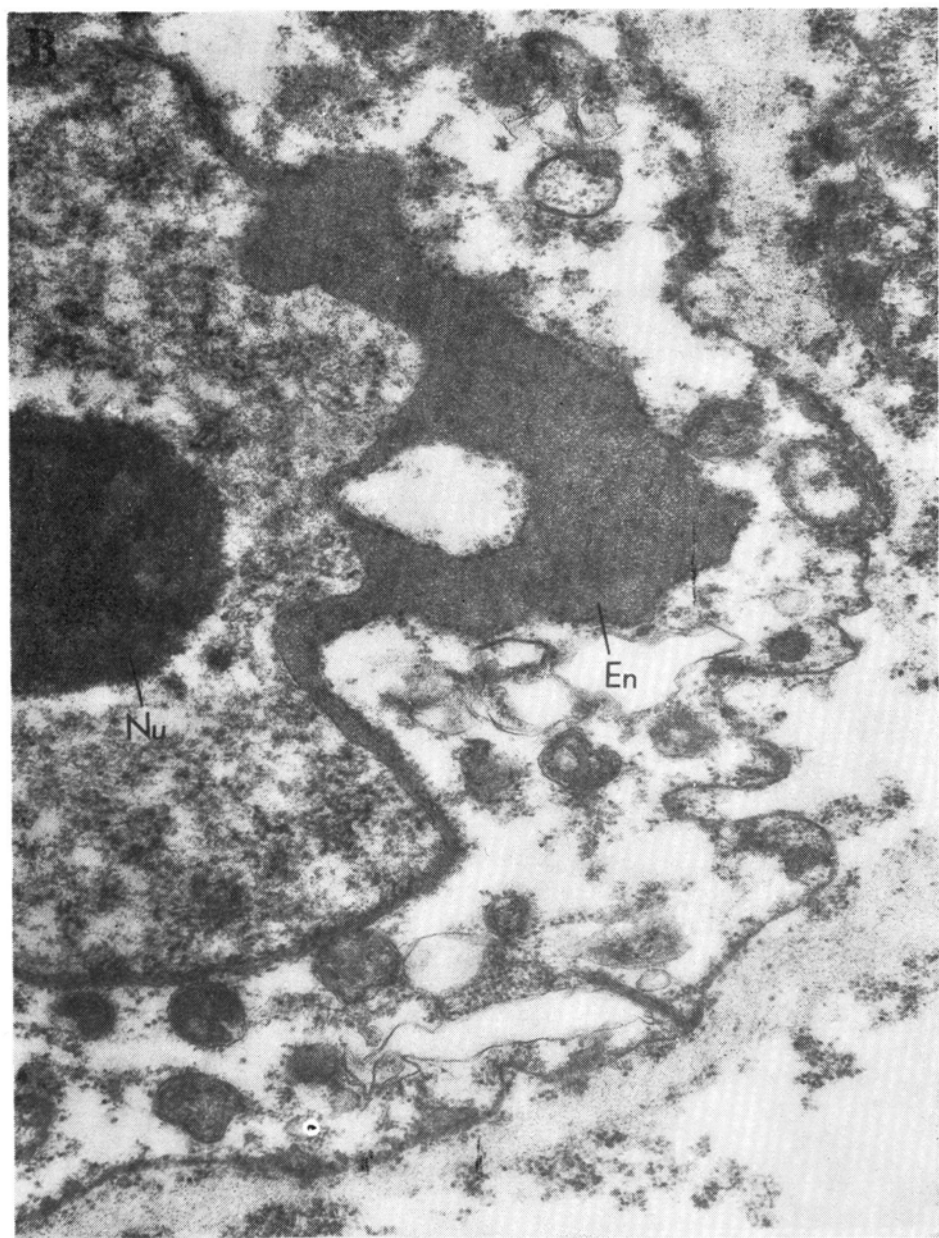


Fig. 9B. Fragment of microspore with changes in the nucleus envelope (En). Anthers were fixed after 6 days of culture on LS medium with 20 μM BAP + 5 μM NAA. 29 700 \times

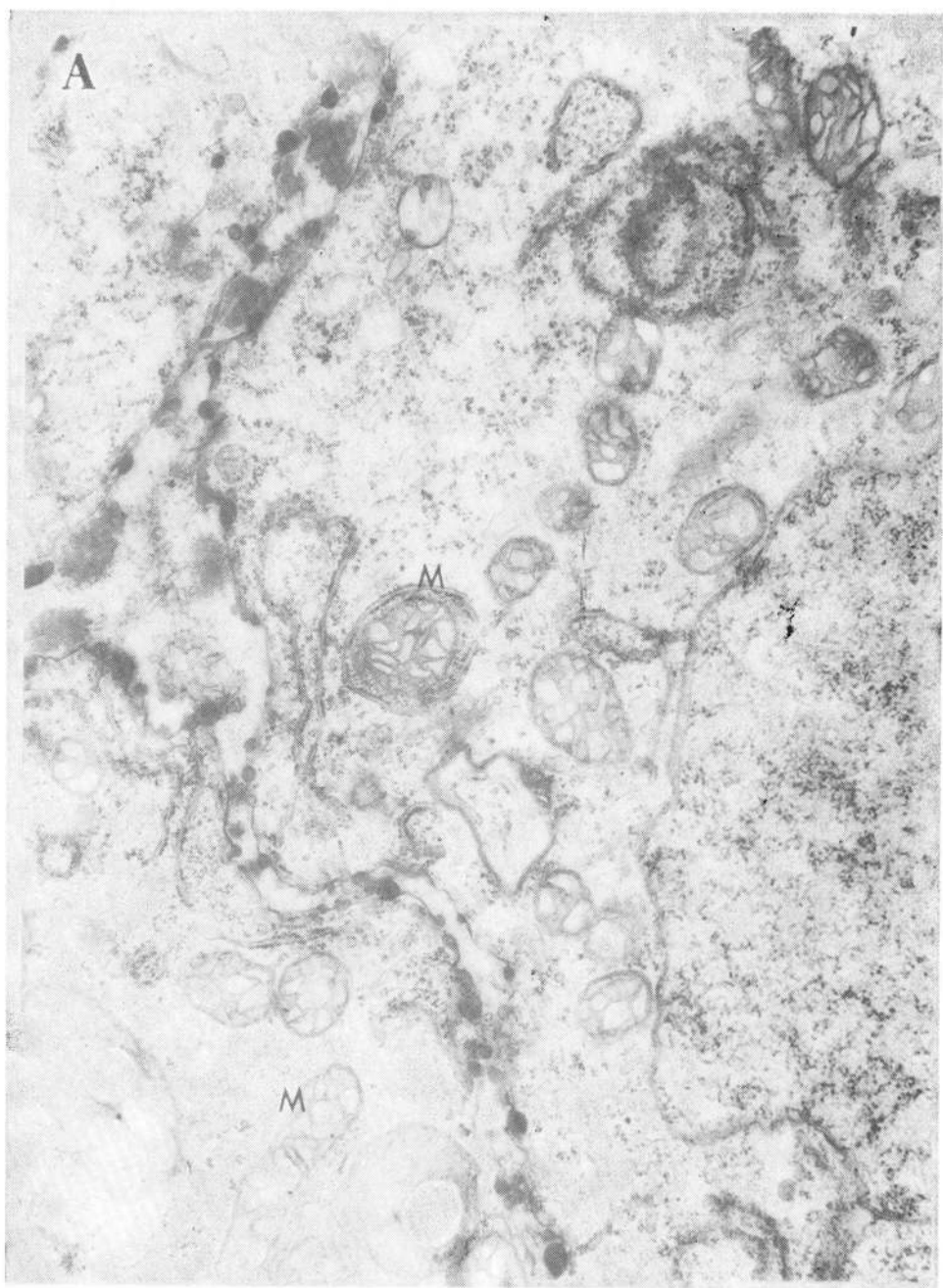


Fig. 10A. Cross section through dense cytoplasm of tapetum cells with numerous mitochondria (M). Anthers were fixed after 9 day culture on LS medium with $20\text{ }\mu\text{M}$ BAP + $5\text{ }\mu\text{M}$ NAA. $16\text{ }200\times$

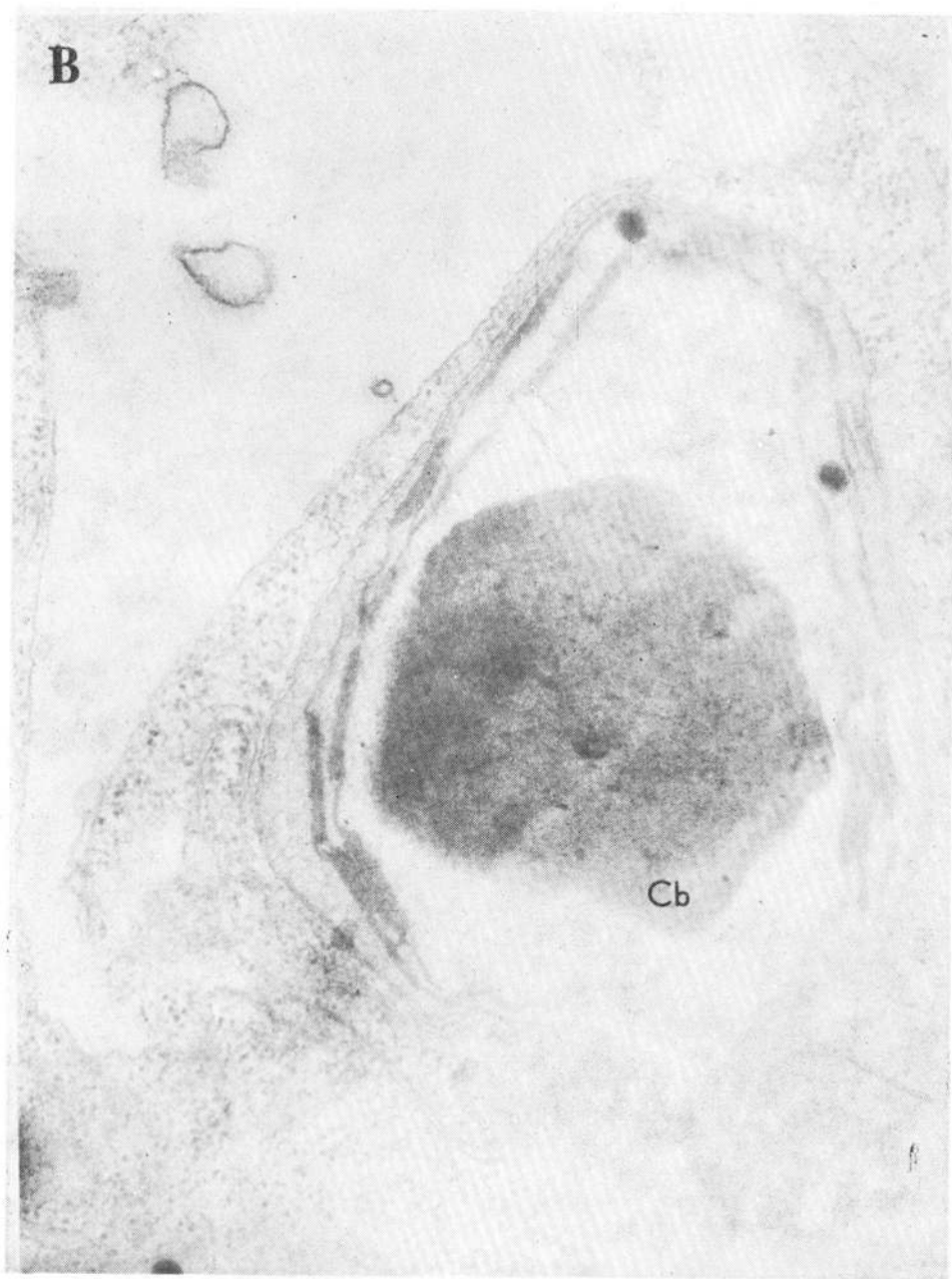


Fig. 10B. Crystalline bodies (Cb) within chloroplast of tapetum cells. Anthers were fixed after 9 day culture on LS medium with 20 μ M BAP + 5 μ M NAA. 45 000 \times



Fig. 11A. Cross section of anther fragment with flattened and degenerated pollen grains (P). Anthers were fixed after 15 day culture on LS medium with 20 μ M BAP + 5 μ M NAA. 14 500 \times

B

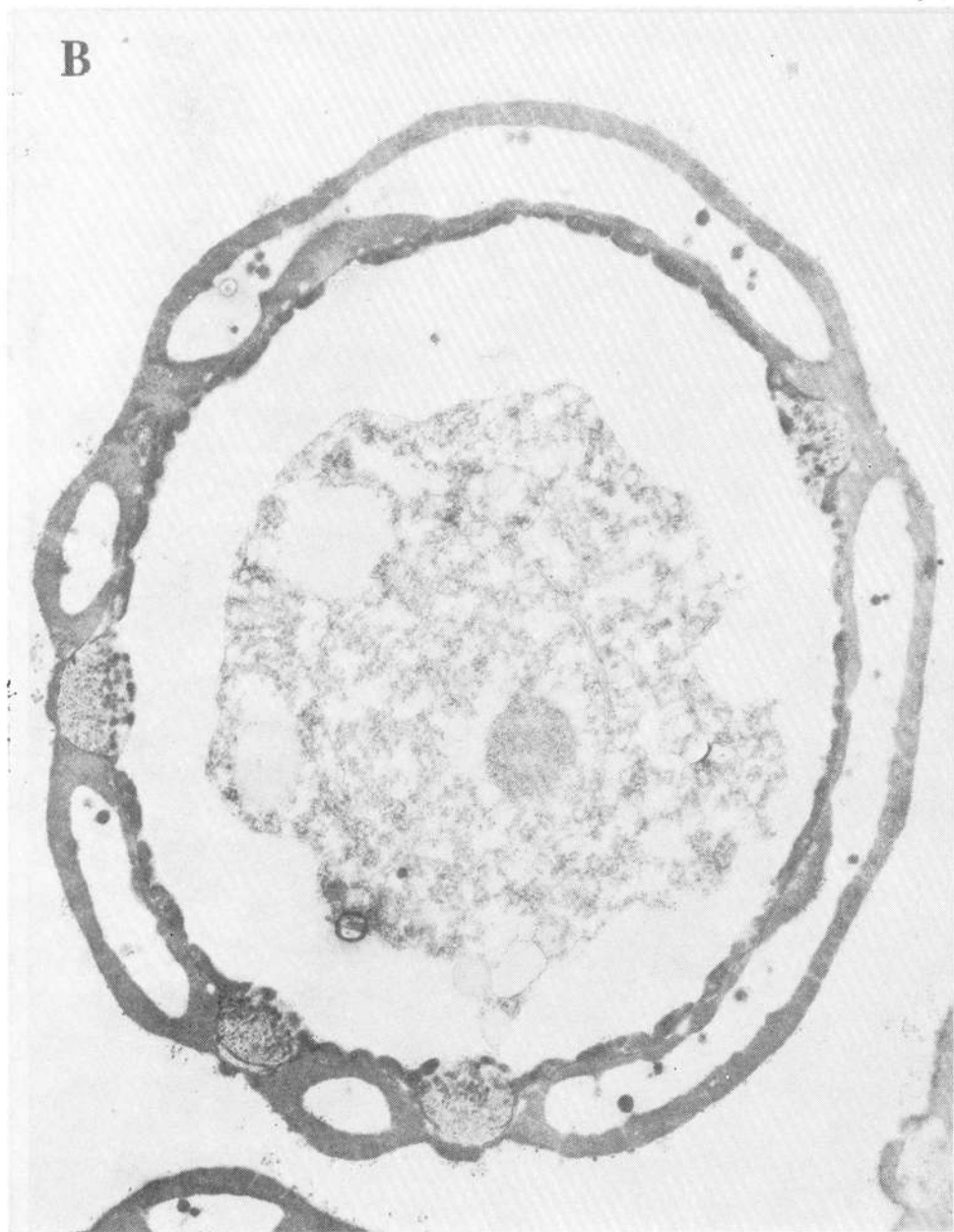


Fig. 11B. Shrunken and dried up pollen grain. Anthers were fixed after 15 day culture on LS medium with 20 μM BAP + 5 μM NAA. 13 000 \times

*Próby uzyskania haploidów w kulturach pylnikowych
buraków cukrowych, pastewnych i gatunków dzikich*

Streszczenie

W pracach nad uzyskaniem haploidów buraka z pylników przebadano wpływ pożywek mineralnych (Linsmaier i Skooga, Nitscha i Nitsch oraz White'a), ekstraktu ziemniaczanego i buraczanego oraz p-fluorofenyloalaniny (PFP) w kombinacji z substancjami wzrostowymi. Ponadto badano wpływ głodzenia roślin i pylników, niskiej temperatury oraz fotoperiodu. Na wszystkich pożywkach mineralnych pylniki tworzyły kalus i korzenie, jednak ich procent był zależny od zastosowanej kombinacji substancji wzrostowych. Optymalną pożywką dla różnicowania była pożywka Linsmaier i Skooga z 25 μ M zeatyny lub 6-(3-metylo-2-butenyloamino)puryny w kombinacji z 5 μ M kwasu naftylo-1-octowego (25,5%). Dodanie PFP zwiększało procent różnicowania pylników (41,6) jednak nie wywołało powstania haploidów. Oprócz kalusa i korzeni na jednym z pylników (na ok. 140 000) tworzyły się pączki wegetatywne z których uzyskano liczne roślinki (2n). Głodzenie roślin i pylników nie zwiększyło procentu różnicowania ani nie wywołało rozwoju haploidów, podobnie jak traktowanie kwiatostanów i wyizolowanych pylników niską temperaturą. Pylniki gatunków dzikich buraka wykazały mniejszą zdolność do tworzenia kalusa i korzeni niż pylniki buraków cukrowych i pastewnych. Badania cytologiczne hodowanych pylników wykazały bardzo małą liczbę pylników ze zmianami wzrostowymi. Tworzenie struktur wielokomórkowych obserwowano do 12 dnia hodowli pylników, poczym zachodziła ich degeneracja.