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In vitro induction of haploid plants from unpollinated ovules and ovaries of the sugarbeet (*Beta vulgaris* L.)

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Summary. Haploid plantlets from male fertile and male sterile sugarbeet plants could be induced at frequencies up to 2.2% using ovule culture. Ovary culture on media without charcoal resulted in a similar induction frequency. Plant development was inhibited by callus development originating from the mother tissue. When the callus parts were removed and the ovule transferred to a new medium without 2,4 D, callus formation could be inhibited by adding 0.5% charcoal to the medium. Up to 6.1% haploids were induced. Chromosome counts in leaf tips, chloroplast counts and isozyme patterns revealed that all plants were haploid and originated from the haploid cells of the embryo sac. Root tips showed spontaneous polyploidisation.

Key words: Haploid induction – Ovary culture – Ovule culture – Gynogenesis – Sugarbeet – *Beta vulgaris*

Introduction

In sugarbeet many different approaches have been suggested for the induction of haploid or dihaploid plants.

Classical techniques such as natural polyembryony (Fisher 1956; Kruse 1980), pollen irradiation (Fisher 1956, Bosemark 1971), and crosses between diploid and tetraploid lines (De Jong and De Bock 1978) or with wild species (Bosemark 1971) yielded very low numbers of haploids.

The induction of haploids through androgenesis has proven unsuccessful until now. Although cell divisions, microcalli (Rogozinska et al. 1977; Van Geyt et al. 1985) and even pro- embryoids (Van Geyt et al. 1985) were reported, no haploid plants were regenerated.

By culturing mature ovules from male sterile plants, Hosemans and Bossoutrot (1983) induced 0.23% plantlets, and subsequently, the successful induction of plants from unpollinated ovules was reported (Bossoutrot and Hosemans 1985). The shoot meristems were haploid but root tips showed an important polyploidisation.

The present paper presents more generally applicable techniques for sugarbeet gynogenesis. The uses of mature ovules and mature as well as immature ovaries from male fertile and sterile plants are compared. The ploidy and origin of the regenerated plants were determined by chromosome counts on root and shoot tips, chloroplast counts of field or greenhouse grown plants, and isozyme techniques.

Material and methods

Plant material

Vegetative clones from individual seeds of different male fertile lines of the SES (Belgium), namely F3S52, Pr 3, MR 8/20, F1 were initiated and cultured as described previously (Van Geyt and Jacobs 1985). The plants rooted spontaneously on a PGo medium supplemented with 0.3 mg/l kinetin. Rooted plants were transferred to the soil and cultured for four months in a greenhouse.

Plants of Nickerson-Zwaanesse (Nick 1 and 2) were grown outdoors during the normal season. All plants were vernalised at 4 °C for at least 90 days. Flowering was induced in an open greenhouse during the normal flowering season or under standardized conditions (16 h/8 h light-dark; 18–20 °C/13–15 °C) throughout the year.

Sterilisation

Greenhouse grown inflorescences were sterilised by subsequent immersions in 70% alcohol (3 min), 0.5% $HgCl_2$ + 0.03% Tween 20 (1 min), washed with sterile 5 mM Na_2EDTA and rinsed several times with distilled water. Field grown inflorescences were sterilised for 20 min in 2% NaOCl + 0.03% Tween 20 and rinsed three times with distilled water.

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Culture conditions

Induction was performed on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose and hormones. The hormones BAP, NAA and 2,4 D were used at concentrations (0; 0.05; 0.1; 0.3 and 0.5 mg/l) either alone or in combinations of two or three. In certain experiments washed charcoal was added at a concentration of 0.5%. Cell culture conditions were 25 °C/2 °C and 16 h/8 h photoperiod.

Subculturing of the induced ovules was done on the same medium supplemented with BAP 0.1 mg/l and NAA 0.1 mg/l as soon as macroscopic development was observed. Ovaries were transferred after approximately 14 days on the same medium.

Determination of ploidy and origin

Chromosome staining was performed as described by De Jong (1980).

Chloroplast counts on the stomata guard cells of the lower epidermis were done by the method of Deuter (1970).

Electrophoretic and staining procedures have been described previously (Van Geyt and Smed 1984; Van Geyt 1986) for following enzyme systems: malate dehydrogenase (MDH), malic enzyme (ME), isocitrate dehydrogenase (ICD), glucose phosphate isomerase (GPI), phospho glucose mutase (PGM), leucine amino peptidase (LAP), glutamate dehydrogenase (GDH), glucose 6 phosphate dehydrogenase (G6PPH). Esterases, superoxide dismutase, 6-P-gluconate dehydrogenases were determined using a PAGE 1 system (Van Geyt and Smed 1984) and stained according to Vallejos (1983).

Results and discussion

Ovule culture

The state of development of the excised ovule was determining for the reaction. Only ovules with a typical comma form reacted positively. Young spherical ovules degenerated after a few days of culture. In contrast to Hosemans and Bossoutrot's technique (1983), the ovules were always dissected from closed flowers. This permitted us to work with male fertile as well as with male sterile flowers. Indeed, in sugarbeet flowers, the embryo sac is fully formed about six days before anther dehiscence and is even capable of being fertilised from five days before to twelve days after anthesis (Kharetchko-Savitskaya 1931).

During the first 14 days of culture, the excised ovules enlarged markedly in size. The ovule changed colour from opalescent white to brown. After three to eight weeks, small plantlets appeared at the micropylar end of the ovule. Similar observations have been reported by Hosemans and Bossoutrot (1983) and Bossoutrot and Hosemans (1985). The plantlets originated from embryogenic structures. Embryogenesis was also observed and cytologically investigated by Bossoutrot and Hosemans (1985).

Six to sixteen weeks after culture initiation, callus formation was observed. In a few cases, plants could be regenerated from the callus. The regeneration capacity, however, was lost after two subcultures.

The best results were obtained on media with BAP 0.3 mg/l, NAA 0.1 mg/l and 2,4 D 0.05 mg/l (Table 1). Slight differences in the cytokinin/auxin ratio did not influence to any great extent the induction frequencies. Depending on the genotype, 1% to 7.6% of the ovules formed callus. A maximum of 2.2% of the regenerants were obtained from the cultured ovules. Two genotypes did not give any regenerants. The wild relative *Beta procumbens* did not react at all. *Beta webbiana* formed callus in 2.2% of the cases and only one plantlet was regenerated out of 250 ovules. On media containing a higher ratio of cytokinin/auxin the induction percentage was approximately half the values indicated above. Media with only auxins induced mostly callus. In this case, regeneration was never higher than 0.5%.

Ovary culture

Ovaries of clone F3S52 at different stages of maturity were dissected and cultured with the cut in contact with the medium.

On media without charcoal, a massive induction of callus was observed at the cut side. The callus totally overgrew the explant and prevented further development of the proembryoids. Plants could only by regenerated if the calli were cut off and the original explant was transferred to a medium without 2,4 D (Table 1). If 0.5% washed charcoal was added to the medium lower or no callus induction was observed (Table 1). At the present time it is not known which substances are absorbed by the charcoal. The ovule enlarged until the ovary broke open and turned black. Transferring these ovules to a medium with BAP 0.1 mg/l and NAA 0.1 mg/l resulted in the development of plantlets. Two

Table 1. Percentage of callus formation and regeneration in ovule culture and ovary culture of different genotypes. The induction medium was supplemented with BAP 0.3 mg/l, NAA 0.1 mg/l and 2.4 D 0.05 mg/ml

Motherplant	No. of explants	% callus formation	% ovules forming at least one regenerant
Ovule culture			
F3SS2	500	7.6	2.2
MR 8/20	95	2.1	0
F_1	300	6.6	1
Pr3	83	2.4	0
Nick 1 male sterile	1,000	4.2	2
B. procumbens	128	0	0
B. webbiana	250	2.2	0.4
Ovary culture			
F3S52	1,000	35,3	2.2
F3S52[a]	1,000	7.5	6.1
F_1[a]	1,000	10.9	5.8
Nick 2 male fertile	2,000	1,0	0.5

[a] With addition of 0.5% charcoal

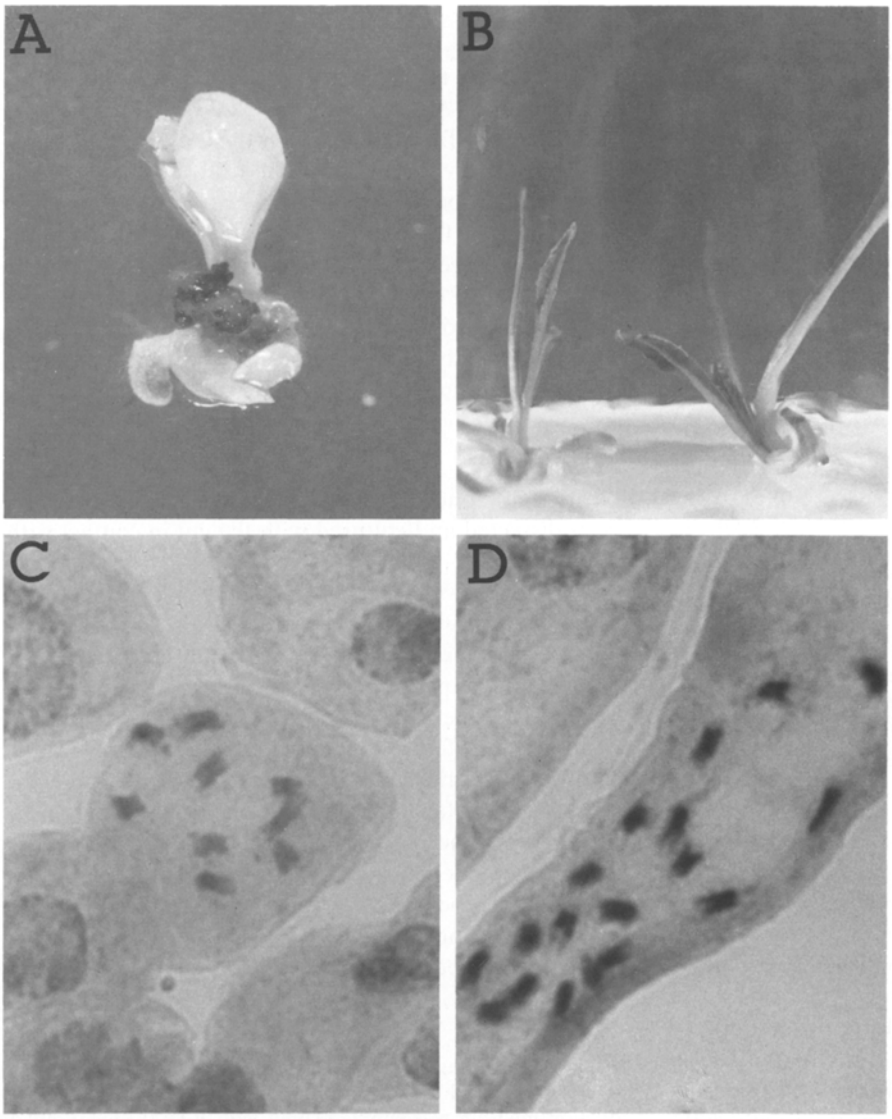

Fig. 1. **A** Plantlet development occurred directly after embryogenesis. The cotyledons can be seen. **B** Vegetative multiplication of the regenerants. **C** Chromosome staining in leaf tips revealed a haploid chromosome number. **D** In root tips, spontaneous polyploidisation occurred

clones were tested: 6.1% and 5.8% of the ovules, respectively, for F3S52 and F1 induced at least one plantlet.

In ovary culture, the state of development was less important. The ovules ripened in the excised ovary. Even ovaries with spherical ovules evolved to the comma form and were able to induce plantlets. In this case, however, plantlets were only formed after one or two months.

Regeneration

The small plantlets were transferred to media with halved cytokinin concentration and without 2,4 D. In all cases only the cotyledons and leaves developed (Fig. 1). The rootlike structure at the basis of the embryoid plantlets seldomly grew to maturity. In this case, when the plantlets were approximately two centimeters, they were transferred to a medium with BAP 0.3 mg/l and vegetatively propagated. Vegetative multiplication and rooting was performed as described before (Van Geyt and Jacobs 1985). In some genotypes more than 50% of the plantlets issued from ovule culture died in an early stage. Some clones degenerated totally. Most rooted plants grew poorly with regard to their corresponding motherplants. Fig. 1 shows some morphotypes regenerated from the same motherplant. No albino plants were detected.

Ploidy determination

The ploidy and origin of the different regenerants were checked using various methods. Chromosome counts in leaf and root tips and chloroplast counts in the guard cells of the stomata of the lower epidermis are presented in Table 2. All regenerated plants had a haploid chromosome number in the shoot tip cells. Chimaeric plants

Table 2. Comparison of the chromosome numbers of shoot tips, root tips and chloroplast counts in field or greenhouse grwon plants

Motherplant	Regenerated clone	Chromosome no.		Chloroplast no.
		Leaf tip	Root tip	
1.1	121	9	–	9 (8–10)
	128	9	–	–
	143	9	18, 18[a]	8 (7–9)
2.1	131	9 + < 1% 18	18	9 (8–12)
	134	9, 9, 9, 9, 9[a]	18, 18, 36, 18, 18[a]	8 (8)
	135	9	18	8 (8–9)
	136	9	18 + 1% 36	8 (8–9)
	137	9, 9, 9[a]	18, 18, 18[a]	9 (9)
	141	9	36	8 (8–9)
6.1	113	9		8.5 (7–10)
	114	9	–	8.5 (7–10)
	116, 117	9		
	118, 119	9		
	119	9	–	9.5 (8–11)
	123	9 + < 5% 18		
	124, 127	9		
	129, 130	9		
	133	9, 9, 9	9, 18, 18[a]	10 (9–11)
	138	9	50% 18 + 50% 36	8 (6–9)
	140	9	9	8 (6–9)
	142	9	19	9 (8–12)
6.4	125	9		–
7.1	120	9		
	126	9		

[a] Several individuals tested from the same clone

were observed in only two out of the 40 plants tested. The contamination with diploid cells was very low (less than 1% in plant 131; less than 5% in plant 123).

The corresponding root tips, however, were mostly diploid. Only two out of 25 plants showed the haploid chromosome number. Two root tips were tetraploid. The number of chimaeric plants was very low. Two plants were partly haploid, partly diploid. One plant was mixed diploid and tetraploid. No aneuploidy was observed.

The fact that in many cases several regenerants from the same clone had different chromosome numbers in their root tips indicated that endoploidization occurred after gynogenesis. In all cases the shoot tips remained haploid. A similar observation was described by Hosemans and Bossoutrot (1983) and Bossoutrot and Hosemans (1985).

Chloroplast counts of plants during the in vitro culture phase showed no correlation with the number of chromosomes. Regenerants growing in the greenhouse or under field conditions had an average chloroplast number between eight and ten, corresponding with a haploid nuclear genome.

Isozymes were used in order to determine the origin (mother tissue versus haploid tissue) of the regenerated plants at an early stage of development. The rationale for the test is that regenerants from gametophytic origin must exhibit homozygous isozyme patterns if they are derived from heterozygous mother plants. Working with the natural polymorphism of 12 isozyme systems (representing 30–40 loci), we generally found three to five isozyme systems showing the required heterozygous pattern among the mother plants. Fig. 2 shows a few examples of the comparison of the heterozygous pattern of a mother plant with patterns of regenerants. In all cases a homozygous type of pattern was detected. Where possible, multiple genes were screened in order to detect aneuploids or chimaera. Until now, no such forms were detected.

Peroxidase 1 is an example of a monomeric enzyme. The zymogram showed two bands in the mother plant. POD_2 is generally not polymorphic. The polymorphic zone of NAD-MDH is a dimeric enzyme showing a triple banded pattern; NADP-MDH (or ME) is characterized by three sets of dimeric enzymes. The regenerants showed phenotypes with the corresponding single band, characteristic for homozygotes. ICD is controlled by two genes forming inter- as well as intragenic dimers. The heterozygote mother plant showed 5 bands (only one of the genes shows polymorphism). The cor-

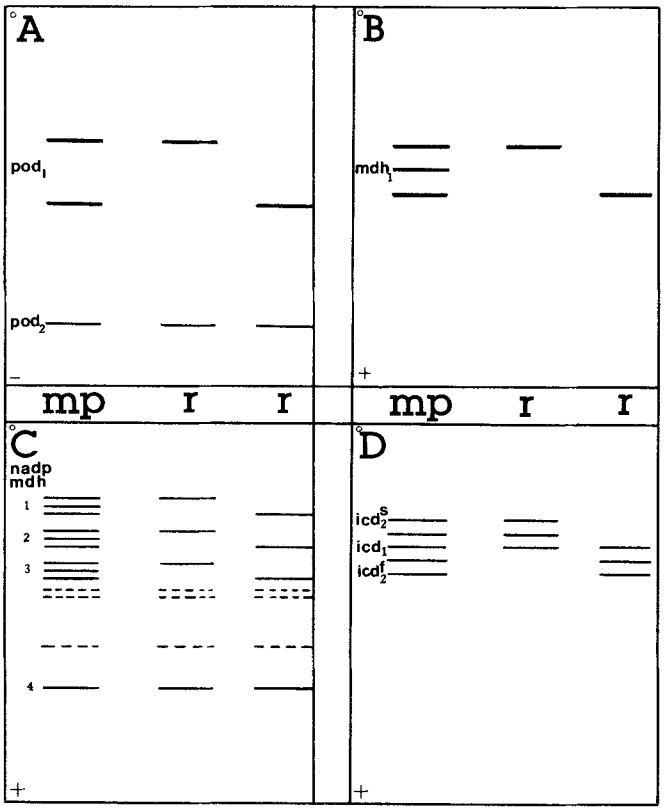

Fig. 2. Comparison of isozyme patterns of heterozygous mother plants (2n–MP) and several regenerants (n-R). All regenerants from ovule and ovary culture showed homozygous patterns. The enzyme systems shown are respectively: **A** Cathodal peroxidase; **B** NAD dependent malate dehydrogenase; **C** NADP dependent malate dehydrogenase-malic enzyme; **D** Isocitrate dehydrogenase

responding types with three bands (homozygous for both genes) were found in the regenerants (information on the genetical control of the different enzymes can be found in Van Geyt 1986).

The isozyme technique provided an elegant method for the determination of the origin of the regenerants at a very early stage. Less than 1 mg of tissue was found to be necessary. The technique should also be useful for the discrimination of dihaploid and diploid regenerants (originating from the mother tissue).

Conclusions

Haploids can be obtained using ovary as well as ovule culture. Ovary culture was more successful then ovule culture. Several reasons can be mentioned. With ovary culture there is less chance of injuring the ovule. As the ovule is not in direct contact with light and the environment, lower levels of phenolic substances are formed. A main advantage of ovary culture is that the state of development is less crucial than in the case of ovule culture. As the ripening of the flowers occurs gradually, one can only take a few ovules per inflorescence. The yield of reacting ovaries per inflorescence is much higher. When young ovaries are used, no fertilisation can occur so that one can work with male sterile as well as male fertile plants.

The dissection of ovaries appears to be much faster than that of ovules. A few weeks after induction the ovary breaks open and the swollen ovule can easily be transferred to a new medium without injuring it.

Doubled haploid breeding has proven to be useful in several plant species especially in self-pollinators (Baenziger and Schaeffer 1982). A computer simulation of the work of Choo and Kannenberg (1978) showed that in outbreeders doubled haploid mass selection can be as efficient as S_1 selection and more efficient than classical diploid mass selection.

The immediate use of doubled haploids in commercial sugarbeet lines is doubtful. As breeders generally work with heterogeneous populations, one should test several dihaploid clones derived from several individuals of the parental populations in order to find those combinations reaching a maximal heterosis effect and with as low as possible lethal genes. The production of the new parental lines should have to be coupled with vegetative multiplication and self-fertility in order to stabilise the selected genotypes. Practically, this means a reconstruction of completely new genotypes. In the long run, however, the doubled haploids could be included in breeding schemes for the homogenisation of parental populations. Haploids and doubled haploids could also be very useful at the basic genetical level. Mutants of single (recessive) genes could be selected for more easily (e.g. herbicide resistance, amino acid overproduction (fodderbeet)). Dihaploid lines are also well suited for the better understanding of the genetics of single and polygenic traits (disease resistances, the yield/sucrose content paradox, isozymes).

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