Theor Appl Genet (1996) 93:414–420

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The morphology, cytology, and C-banded karyotypes of *Brassica campestris*, *B. oleracea*, and *B. napus* plants regenerated from protoplasts

Received: 10 November 1995 / Accepted: 24 November 1995

Abstract The behaviour of *Brassica campestris* (2n = 20, AA), *B. oleracea* (2n = 18, CC), and *B. napus* (2n = 38, AACC) were studied during a tissue-culturing process. Hypocotyl-protoplasts were cultivated into calli from which new plants were regenerated. The regenerated plants were compared, and mitotic root-tip cells were C-banded and karyotyped. A majority of the plants were tetraploid. The meioses were studied in the PMCs. A number of abberations were observed, mainly due to faulty spindle function. There was a difference between the three species in that *B. campestris* performed the most poorly with many fewer regenerated plants. These plants were more morphologically disturbed and had more problems during pollen production than *B. oleracea* and *B. napus* plants.

Key words *Brassica* sp. · Regeneration process · In vitro protoplast culture

Introduction

Protoplasts, as the basic material for in vitro culturing of regenerated plants, have been used as a tool in different methodological approaches to yield large numbers of specially designed plants. There are three main areas in which they have been used: (1) for protoplast fusion to produce somatic hybrids; (2) for the production of a large number of cells which through the lack of cell walls can be used for micromanipulation; and (3) for release of the potential for somaclonal variation to produce variant plants. When the method is used in the first two applications the ideal situation is a population of uniform protoplasts which will, if not manipulated, give rise to identical regenerated plants. If the third application is of interest, a maximum variation between the regenerated plants is desired. At present, it is very difficult, if possible, to determine the outcome of the regeneration process. The variation between regenerated plants is still only partly understood, although several attempts to elucidate the complicated process that results in plant regeneration have been made (for review see D'Amato 1985; Geier 1991).

Species in Brassicaceae have been very popular for use in producing different types of regenerated plants, especially somatic hybrids (e.g. Sundberg 1991). Flow cytometric determination of DNA content has been a convenient tool to determine the ploidy level (Fahlesson 1988). A more detailed chromosome analysis of regenerated plants has so far not been a commonly used method of assessment. In the investigation described here, three *Brassica* species, namely *B. campestris* L, *B. oleracea* L., and *B. napus* L., were analysed after plant regeneration. These species have a special relationship in that *B. napus* is the amphidiploid offspring of *B. campestris and B. oleracea* (U 1935). Because of this close relationship they were chosen as a model for a study of the regeneration behaviour in diploids and polyploids (in this case amphidiploids) of a plant family where the diploids are also supposedly of polyploid origin (Quiros et al. 1987; Chen et al. 1989, 1990; Slocum et al. 1990; McGrath et al. 1990; Song et al. 1991). Morphology and seed set after selfing in the regenerated plants were studied. Chromosome numbers were determined, differentially stained karyotypes were made, and meiotic behaviour was monitored in order to compare the behaviour of the three species. The aim was to study how the regulation of the regeneration process manifested itself. This may give new options to a continued search for an understanding of the possibility to control the outcome of regenerated plants, which seems to be limited at this point.

Communicated by P. M. A. Tigerstedt

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Materials and methods

Protoplasts were harvested from hypocotyls of spring turnip rape *B. campestris* var 'oleifera' L. breeding line Svalöf 03242 (diploid 2n = 20,

AA), winter kale *B. oleracea* var 'acephala' L. cv 'Hammenhögs Extra Mosskrusig' (diploid 2n = 18, CC), and oilseed rape *B. napus* L. cv 'Weibulls Olga' (amphidiploid between *B. campestris* and *B. oleracea*, 2n = 38, AACC).

The protoplasts were cultivated according to Glimelius (1984) using 8p media (Kao and Michalyk 1975). The resulting regenerated plants were maintained as shoot cultures on a Murashige-Skoog medium containing 1% sucrose and 0.3% Gelrite, without hormones.

Root tips for chromosomal analysis were harvested 3–4 weeks after transfer to new media. Metaphases were collected and stained for C-bands as described by Olin-Fatih and Heneen (1992). Karyotypes from all three species were made in agreement with the guidelines used by Olin-Fatih and Heneen (1992), i.e., with the chromosomes organized in m(median), sm(submedian), st(subterminal), and t(terminal) groups according to the position of the centromere. Meiosis and flowering were studied by transferring some randomly selected plants to the glasshouse, the exception being *B. campestris*: due to the low number of regenerated plants, all were kept. *B. oleracea* was vernalized for 10 weeks at 8 °C to induce flowering. For the cytological studies of the meiotic divisions in the PMCs, inflorescences were fixed in ethanol:acetic acid (3:1) with ferric chloride as mordant, stained in Snow's carmine, and mounted in Hoyer's medium. The plants were left for open pollination and were shaken daily to facilitate pollen spread.

Results

Regeneration, morphology, and meiosis of regenerated plants

The regeneration of plants from protoplast cultures could be performed in all three species. However, the species behaved differently and showed various degrees of plant regeneration from calli (Table 1). The species differed also in their morphological response to in vitro cultivation with respect to the number of normally looking plants and common abnormalities.

In turnip rape, a total of 348 calli regenerated 7 plants, all from different calli (2%). One plant died at an early stage. The remaining 6 plants had a bushy growth without a main shoot; 3 of them were dwarfs. The flowers lacked petals, and the siliqua were malformed. Only 2 plants produced a few seeds. Chromosome numbers were determined in 4 plants. All were tetraploid (2n = 40), with some hypotetraploid cells (35 or 36 chromosomes). Two of the dwarf plants, with the same abnormal morphology, behaved completely different during the meiotic division. One plant had a well-organized metaphase II and a normal tetrad development (Fig. 1a); the other plant had a highly irregular metaphase II, usually with the spindle asymmetrically organized (Fig. 1b). The 'tetrads' had multiple nuclei with varying amounts of nuclear material (Fig. 1c).

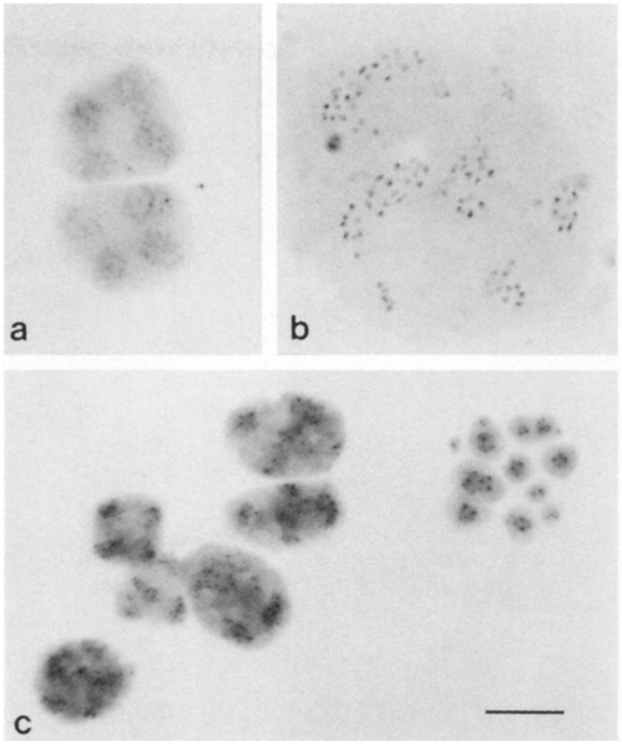


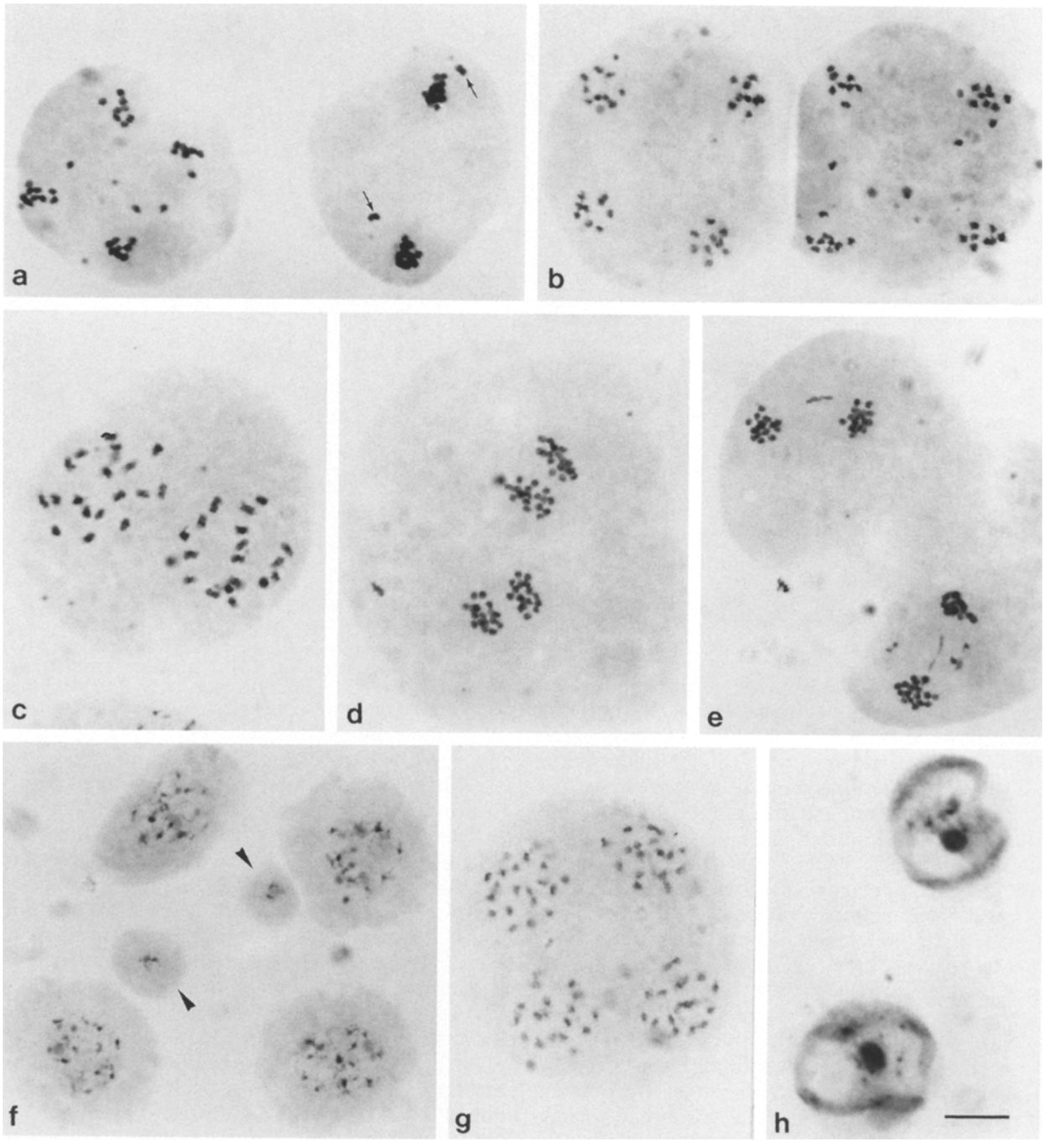
Fig. 1a–c *Brassica campestris* male meiosis in regenerated plants. **a** Normal tetrad in a tetraploid plant, **b** anaphase II with the chromosomes distributed in random groups, **c** 'tetrads' and 'disintegrating tetrads' from a plant with an anaphase as shown in **b** *Bar.* 10 µm

Winter kale tolerated the tissue culture better than turnip rape. Out of 138 calli as many as 85 calli regenerated plants (61.5%). Thirteen plants were kept for cultivation, and these were vernalized to induce flowering. Eleven plants showed normal growth. In 2 plants only the lateral buds developed, and these plants had a somewhat more bushy appearance than normal and no siliqua developed. These 2 plants together with 5 others had no seedset. The remaining 6 plants had normal siliqua but a low seedset. One plant had around 30 seeds, while the others had fewer than 10 seeds per plant. One plant had yellowish-brown seeds instead of the parental black seed colour. The chromosome number was determined in 10 plants. Seven plants were tetraploid (2n = 36). In 1 of these plants cells with 35 chromosomes were found as well as 1 cell with 71 chromosomes. One plant was diploid (2n = 18, Fig. 2a, b). The remaining 2 plants had a mixture of diploid (2n = 18) and tetraploid (2n = 36) cell. It was not possible to predict the meiotic

Table 1 Results of in vitro regeneration from callus in *Brassica*

Species	Number of calli	Number of plants	%Regenerating calli	2n[a]	Morphology	Seed set	Meiosis
B. campestris	348	7	2	40	Abnormal	No-very low	Normal-abnormal
B. oleracea	138	85	62	18, 36	Normal-abnormal	No-low	Normal-irregular
B. napus	132	56	42	(38), 76	±Normal	low-high	Normal-irregular

[a] Main chromosome number



behaviour from the general morphology and mitotic behaviour of the plant. One tetraploid plant had mostly normal tetrads with few lagging chromosomes (Fig. 2c, d). Another plant, with the same morphological and cytological characteristics, showed a number of different misdivisions, such as non-synchronous anaphase II, univalents, bridges, laggards (Fig. 2e), and tetrad formations with micronuclei (Fig. 2f). A third plant, with a bushy growth habit and no developed silique, had seemingly normal tetrads but shrivelled pollen (Fig. 2g, h). The diploid plant had usually one pair of univalents and in anaphase II cells often showed

Fig. 2a–h *Brassica oleracea* male meiosis in regenerated plants. **a** Diploid plant: *left* anaphase II, non-disjunction with 8 chromosomes at two poles and 10 chromosomes at the other two poles; *right* telophase I, *arrows* indicate lagging univalents. **b** Anaphase II, diploid plant: left cell contains chromosome groups with 9, 9, 8, and 10 chromosomes, respectively, due to non-disjunction. **c** Anaphase I, tetraploid plant, 18 chromosomes at each pole. **d** Anaphase II, the same tetraploid plant as in **c**, no lagging chromosomes or bridges. **e** Anaphase II, tetraploid plant, with bridges and lagging chromosomes. **f** Late-tetrad configuration with two micronuclei containing material. **g** Normal tetrad, tetraploid plant. **h** Shrivelled pollen from the same plant as in **g**. *Bar:* 10 μm

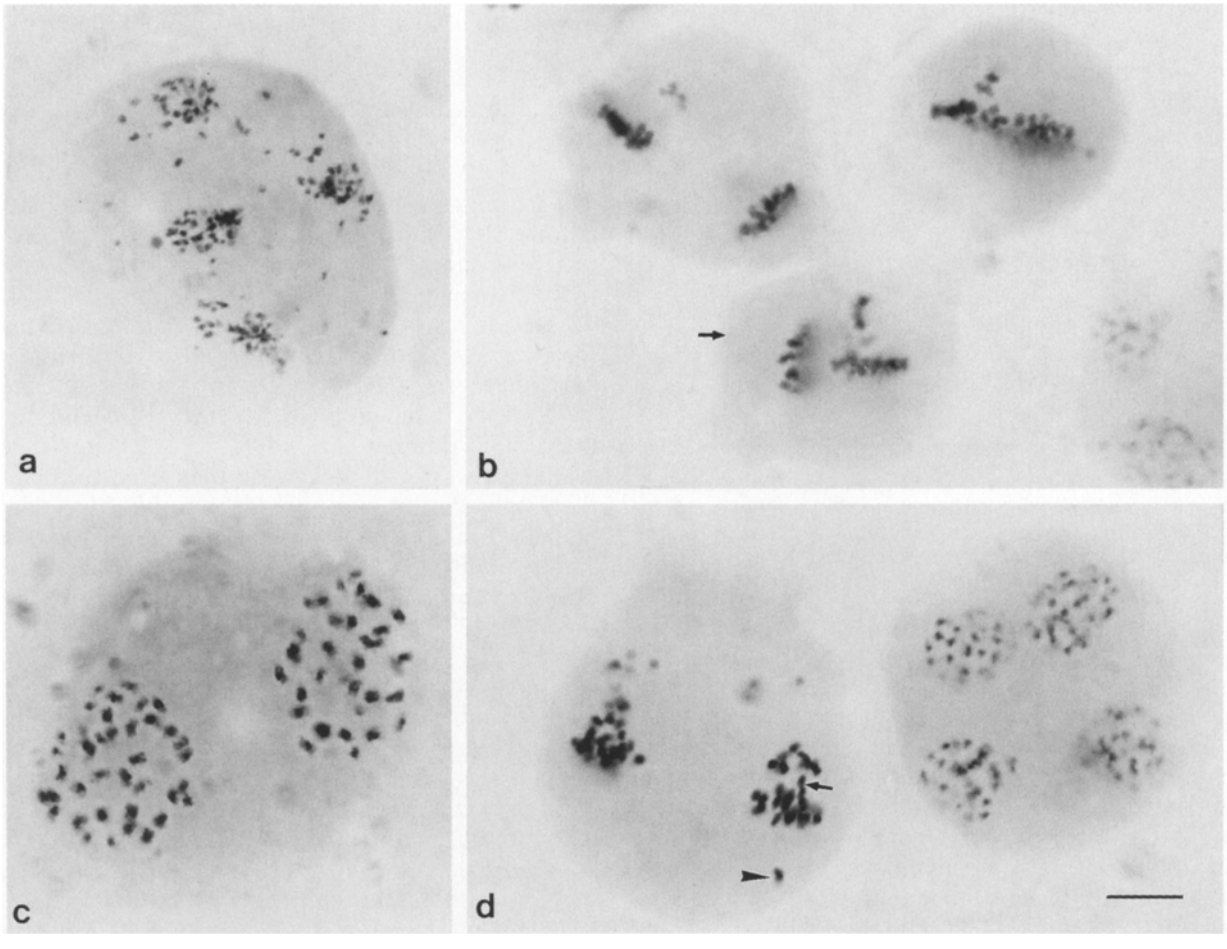
non-disjunction with 8 and 10 chromosomes at the respective poles (Fig 2a, b).

Oilseed rape had a regeneration percentage lower than that of kale. Out of 132 calli 56 regenerated plants (42%). Twenty-two plants were transferred to the glasshouse. Fourteen of these had a normal appearance; another 4 also looked normal but developed many lateral green shoots. Two plants grew in a stunted manner. One plant had two main shoots, and 1 was bushy. The shape of the silqua as well as the seedset was normal in 7 of the plants with normal appearance. Mitotic chromosomes were counted in 7 plants. Most cells were amphitetraploid $2n = 76$ or derivatives of amphitetraploid cells. A few cells in 2 different plants had $2n = 43$. Meiotic analyses were performed on 12 plants. One of the plants with normal growth, siliqua, and seedset was amphidiploid ($2n = 38$) with normal meiotic divisions; the other 11 plants were amphitetraploid and had a number of different meiotic aberrations, a frequent one being multipolar division (Fig. 3a, b). Despite this, 6 of the plants had normal seedset, although bridges and fragments were common during the meiosis of 5 of them (Fig 3d). In the 6th plant unbalanced chromosome segregation at anaphase was common (Fig. 3c). In general, the plants with a low seedset had the same type and frequency of abnormalities (non-disjunctions, laggards, and bridges) as plants with normal seedset. In 1 plant, which had a normal appearance but with many secondary green shoots, not one cell had a normal balanced segregation of the chromosomes during meiosis.

Karyotypes of regenerated plants

It was possible to identify a majority of the differentially stained chromosomes and to designate them to their respective pairs in the karyotypes of all three species (Fig. 4, 5, and 6). There were only a few examples of chromosomes with a new morphology, and it was not possible to determine the reason for this changed morphology. Turnip rape is represented by a C-banded cell (Fig. 4) with 36 chromosomes. Two chromosomes, A_1 and A_2, differ from the rest, being similar and seemingly more condensed. It is likely that they are the 2 missing

Fig. 3a–d *Brassica napus* male meiosis in regenerated plants. **a** Anaphase II with many chromosomes randomly distributed in the cell, **b** metaphase II with a tripolar spindle organization (*arrow*), **c** late anaphase I, segregation with 36 and 30 chromosomes at each pole, respectively, **d** anaphase II (*left* cell) with univalents (*arrowhead*) and bridge (*arrows*). *Bar* 10 μm



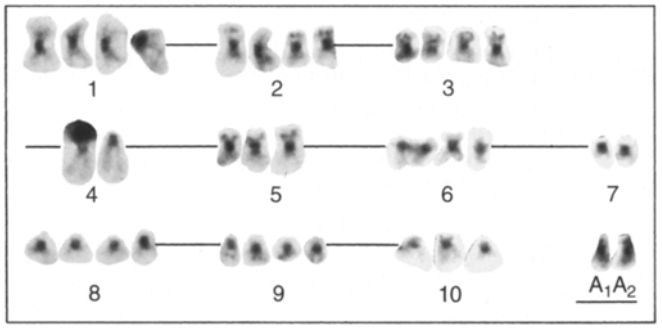


Fig. 4 *Brassica campestris.* Near-tetraploid plant (2n = 36). C-banding. *Pairs 1–3* M-group, *pairs 4–7* sm-group, *pairs 8–9* st-group, Pair 10 *t-group,* Chromosomes A_1 and A_2 are more condensed than the rest of the chromosomes and not identified as belonging to a certain pair. *Bar:* 5 μm

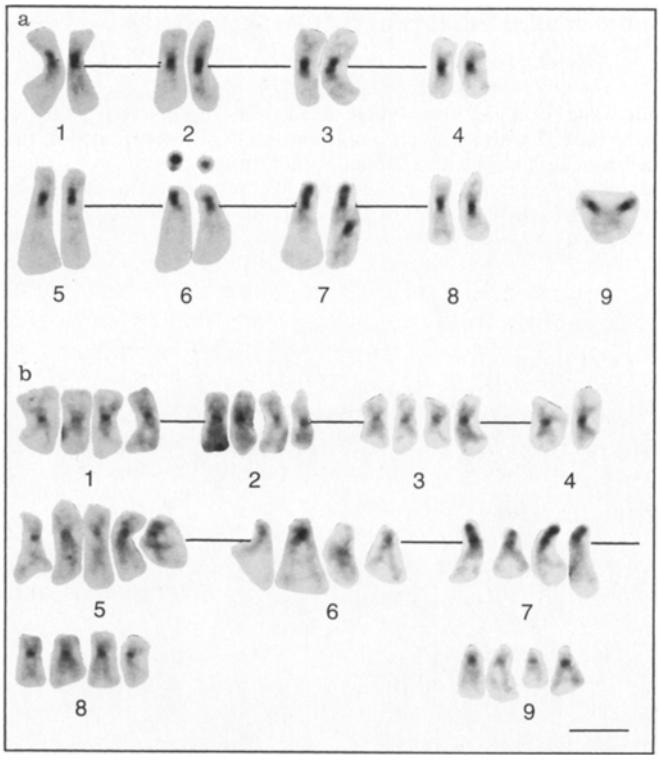


Fig. 5a–b *Brassica oleracea.* **a** Diploid plant (2n = 18). C-banding. *Pairs 1–4* M-group, *pairs 5–8* sm-group, *pair 9 st-group.* (In *pair 7,* a chromosome is overlapping the long arm in one of the chromosomes; in pair 9, the long arms are overlapping each other). **b** Near-tetraploid plant (2n = 34). Groups as above. *Bar:* 5 μm

chromosomes no. 4 which are carrying the NOR (nucleolar organizing region) and that the more condensed state reflects an inactivation of the nucleolar region. In the karyotypes of winter kale (Fig. 5a,b) the individual chromosome pairs are easily identified. The C-banded diploid cell (Fig. 5a) has a normal karyotype pattern. In the tetraploid cell, with 35 chromosomes (Fig. 5b), there are only 2 chromosomes in pair 4 (m group), but in pair 5 (group sm) there are 5. The amphitetraploid oilseed rape

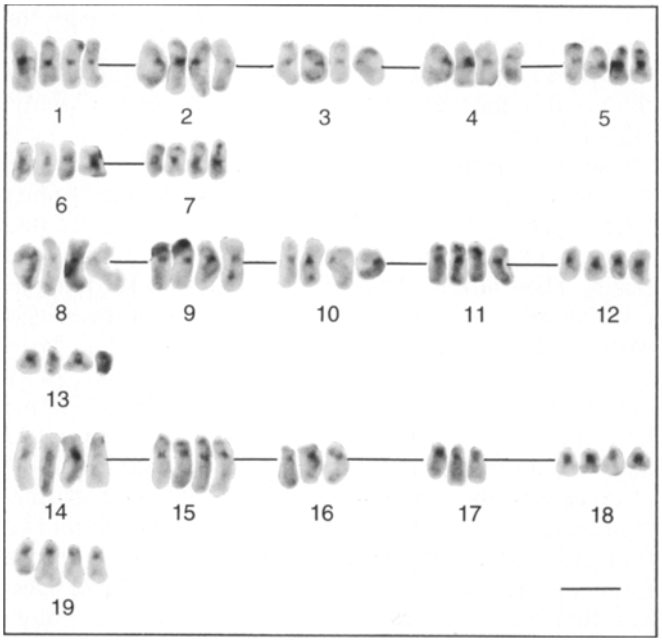


Fig. 6 *Brassica napus* (2n = 74). Near-amphitetraploid plant. C-banding. *Pairs* 1–7 M-group, *pairs 8–13* sm-group, *pairs 14–18* st-group, *pair 19* t-group. *Bar:* 5 μm

cell with 74 C-banded chromosomes (Fig. 6) has also the most intact pairs; only in pair 16 and 17 is 1 chromosome is missing.

Discussion

The main problem in regenerating plants via protoplast culture is the difficulty in controlling the process and thus obtaining the desired plants. An important parameter to evaluate is which type of plant tissue should be used for producing the protoplasts. Embryonic or meristematic tissues are presumed to give rise to less variable regenerated plants than mature tissues (Geier 1991). If the tissue used already has cells deviating from the diploid number this will be reflected in the regenerated plants (Geier 1991). Another important factor is that regeneration from protoplasts will give rise to a higher variability in chromosome number of the regenerated plants than regeneration from tissue explants. This has been demonstrated in oilseed rape by Newell et al. (1984). In the present study hypocotyls were used. It is a tissue that is in a phase of active division, and the protoplasts from hypocotyls of the varieties used here readily continue to divide during culturing. The culturing procedure and media used here are routinely used for *Brassica* species (Glimelius 1984). A better evaluation was achieved by standardizing both the plant tissue and the culturing method. Observed differences between species are then due to inherited differences in the response to the culturing procedure and not to environmental factors. The reaction of the genotypes of

each species used here can, however, not be generalized and taken to be valid for the entire respective species.

There were marked differences between the regeneration capacity displayed by the studied species. While winter kale and oilseed rape easily regenerated plants, with 61.5% and 42%, respectively, of the calli giving rise to plants, turnip rape proved to be difficult and only 2% of calli gave rise to plants. Turnip rape is known to be recalcitrant relative to kale and oilseed rape, and there have been few reports of the successful regeneration of plants from protoplasts. Of these, Glimelius (1984) had plant regeneration in 1% of the calli and Jourdan and Earle (1989) in less than 1%. However, Zhao et al. (1994) recently reported that in the variety 'Bunyip' as many as 20% of the calli, originating from cotyledonary protoplasts, regenerated shoots. All of the regenerated turnip rape plants from the present study were malformed. This was not the case in either oilseed rape or winter kale which had a normal morphology in a majority of the plants.

In all of the species the regenerated plants were most commonly tetraploid. The appearance of multiploid cells in the callus is due either to the presence of such cells in the originally used tissue (D'Amato 1964) or to misdivisions in the first divisions after protoplast production (Simmonds 1992). One explanation for the fact that the majority of the regenerated plants showed increased polyploidy could be that the culturing procedure favoured polyploid cells. In this case a modification of the procedure to favour dipoloids is necessary. The diploid *Brassica* species are considered to be of polyploid origin (Quiros et al. 1987; Chen et al. 1989, 1990; Slocum et al. 1990; McGrath et al. 1990; Song et al. 1991), and as polyploids they seem to be more tolerent to chromosome variation (D'Amato 1985). The eventual advantage of this tolerance in the somatic tissue is, however, lessened by the problems most new tetraploids encounter during meiotic divisions, a situation also seen in these species. The *B. campestris* line used here had a higher number of cells with malformed spindle organization and more multinucleated cells at this early stage of culturing (unpublished results), resulting in a more aberrant cell population than the other species. A possible explanation for the poor performance of the turnip rape could be that although it has a polyploid origin it behaves like a primary diploid species and does not tolerate further polyploidization. This process of diploidization may have gone further in turnip rape than in winter kale. A culturing procedure that favours a production of diploid cells will then possibly enhance the regeneration potential of *B. campestris*. Such a modified procedure can include keeping the cells in a frequently transferred suspension culture to produce somatic embryos instead of regenerating plants from a growing callus on solid medium, which seems to favour polyploid cells (Geier 1991). In a hybrid *Triticum aestivum* × *Thinopyrum ponticum*, Bai and Knott (1992) showed that when calli were maintained during a prolonged period the concentration of auxins had to be reduced to sustain the organogenesis. The range in chromosome number in the regenerated plants initially increased but seemed to stabilize when the regenerating calli were cultured for 18 months or more (Bai and Knott 1992). Also, in a *Brassica oleracea* recalcitrant inbred line, improved protoplast regeneration occurred after the proliferation time of the calli was reduced (Fransz et al. 1994). Thus, a shortening of the period for callus proliferation can be favourable for reducing the number of tetraploids seen in this study.

As a majority of the differentially stained chromosomes were recognized in the karyotype, the individual chromosomes showed a stable morphology and only a few chromosomes deviated from the normal pattern. The limitations of the staining method must, however, be considered. The resolution is restricted since the *Brassica* chromosomes are small with only few bands and a simple banding pattern (Olin-Fatih and Heneen 1992; Olin-Fatih 1994). Rearrangements like translocations, deletions, or duplications of chromosome arms or parts of arms which can cause an altered chromosome morphology can be hidden in the uniformly stained part of the chromosome. A change in the heterochromatic regions or in the chromatin condensation pattern is more likely to be noticed (Fig. 4). In species like *Crepis capillaris* (Ashmore and Gould 1981) and *Vicia faba* (Papes et al. 1983), with more differentiated C-banding patterns, chromosomal rearrangements are more easily recognized and have been reported to occur in cultured cell populations. The absence of visible rearrangements in the *Brassica* plants may be due to methodological shortcomings. The aneuploidy observed in some of the polyploid cells is probably due to nondisjunction. Analysis of events like non-disjunction is facilitated if differential staining is applied, especially when the chromosome number is unaltered and both gains and losses of chromosomes have occurred.

The meiotic division is sensitive to the changes brought about by the polyploidization of the regenerated plants that occurred during the culturing process. Gene regulation of meiosis obviously can be affected since there is a difference in behaviour between different polyploid plants. Some plants do not produce viable pollen although they show the same meiotic characteristics as plants with normal pollen production. A multipolar spindle resulting in a disturbed tetrad configuration is common. This results in cells with only a few chromosomes, which can not develop into pollen. A similar phenomenon described as 'budding' is reported from the male meiosis in a hybrid between *Hordeum lecheri* × *H. vulgare* (Linde-Laursen and Bothmer 1993). There is a marked difference in meiotic behaviour between the three *Brassica* species studied here. Turnip rape already has a much higher load of cell aberrations at an early stage of the tissue culturing process (unpublished results). This results in a poor performance both in regard to the number of plants regenerated and the performance of these plants. If turnip rape is compared to winter kale, which also is an outbreeding, diploid species, the

winter kale has a significantly higher number of regenerated plants, and these plants also perform better. This difference is probably a result of an inherited ability to deal with changing environments at the cell level since turnip rape has more aberrant cells at all levels from protoplast production up to pollen production. That oilseed rape tolerates tissue culturing well could be a result of such an ability already enhanced by the amphidiploid character of that species. Only plants which could overcome the disorder brought about by the polyploidization in the hybrid became ancestors of the new *B. napus* species. The best method for enhancing the performance of the regenerated plants within the genotypes used here seems to be to reduce the number of polyploids. However, the results of this investigation indicate that it is probably more difficult to reach that goal with turnip rape than with kale and oilseed rape.

Acknowledgements Financial support from The Scandinavian Contact Agency for Agricultural Research (The Swedish Council for Forestry and Agricultural Research), Hilleshög AB, and The Royal Physiographic Society is gratefully acknowledged.

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