Theor Appl Genet (1986) 72:244–251

Cytogenetics of protoplast cultures of *Brachycome dichromosomatica* and *Crepis capillaris* and regeneration of plants

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Received September 10, 1985; Accepted December 5, 1985
Communicated by G. Wenzel

Summary. Callus derived protoplasts of *Brachycome dichromosomatica* (2n=2x=4) and *Crepis capillaris* (2n=2x=6) have been regenerated into karyologically normal plants, i.e. plants without visible alterations of the diploid chromosome set. However, metaphase analysis of protoplast cultures derived from both callus as well as mesophyll cells showed karyological changes in the overwhelming majority of cells in both species leading to multinucleated, polyploid and aneuploid cells. Furthermore, callus derived protoplasts sometimes exhibited changes at the chromosome level as indicated by translocations. The vast majority of aberrant karyotypes arose from failures during mitosis and cytokinesis, pointing to inadequate microtubules as a possible underlying cause. Karyological events of the kind described herein greatly affect the plating efficiency of isolated protoplasts and the viability of protoplast derived calli. Plant regeneration, although demonstrated in this study for the first time in both species, seems to be limited to rarely occurring, protoplast-derived colonies with a relatively stable genome. Our experiments, performed with chromosomal model species, emphasize the need for controlled, non-mutagenic culture conditions.

Key words: Protoplast culture – Ploidy mutations – Plant regeneration – *Brachycome dichromosomatica* – *Crepis capillaris*

Introduction

Plant regeneration from protoplasts has been successful in a number of plant species and families (e.g. review by Evans and Bravo 1983), and this number is constantly increasing. However, the number of species or varieties which have not been regenerated, despite extensive efforts, may be even longer. There are only a few indications why regenerations are successful in some species and not in others (Hahne and Hoffmann 1984; Hoffmann et al. 1984), and why regeneration of certain species takes place only rarely while other species regenerate reproducibly from protoplasts.

Karyological aberrations could be one cause for a low viability and regeneration ability in protoplast cultures, in analogy to callus cultures where chromosomal abnormalities are common (see review by Sunderland 1977). Accordingly, we analyzed early karyological events in cultured protoplasts of two selected representatives of the Asterales. Protoplast regeneration to plantlets has only been reported occasionally in this family (Binding and Nehls 1980; Binding et al. 1981; Crepy et al. 1982).

For our studies we have chosen two species with few but large and characteristic chromosomes, *Brachycome chromosomatica* and *Crepis capillaris*. Both species can be considered karyological models with 2n=2x=4 and 2n=2x=6 chromosomes, respectively (inserts Figs. 1a and 4a). Such distinctive genomes allow detailed observations of the chromosomes during culture, which might reveal karyological events that lead to the inability of protoplasts to divide and to regenerate calli and plants. These two species should also be well suited for certain protoplast fusion studies, in which they could increase our knowledge about interactions between individual chromosomes in somatic hybrids, such as interspecific chromosome recombination (Kao 1977; Hoffmann and Adachi 1981).

Tissue-derived callus cultures of both species have been extensively studied. Gould (1978, 1979) found that some cytogenetically stable calli of *B. dichromosomatica* regenerated plants for over a year but later, frequent translocations were reported (1983). In *C. capillaris* cultures translocations as well as polyploidizations are also often observed (Sacristán 1971).

We succeeded in the regeneration of diploid plantlets from callus protoplasts of both species. Regenerants of *B. dichromosomatica* could be transferred to soil, and flowering plants were obtained. Cytological analysis

revealed massive changes in chromosome number as the main reason for a decline in viability of protoplast cultures after a few days or weeks in culture.

Materials and methods

Plant material and callus induction

Seeds of *Brachycome dichromosomatica* C. R. Carter, var. 'dichromosomatica' (Carter 1978) were kindly supplied by A. R. Gould in 1980. All our plants were derived from one heterozygous seedling by self-fertilization. Seeds of *Crepis capillaris* (L.) Wallr. were provided by G. Melchers. *B. dichromosomatica* plants were grown aseptically. Seeds were surface-sterilized by immersing them for 30 min in 2.5% sodium hypochlorite to which about 1% of detergent had been added. After thorough washing in sterile water the seeds were placed onto 50 percent concentrated B5 medium (Gamborg et al. 1968) with 5 g/l sucrose and without hormones. Seed germination took place within 6 to 12 weeks at 23 °C on a 16 h light/8 h dark cycle. Entire leaves of young non-flowering plants were used for callus induction and protoplast isolation. *C. capillaris* was grown in the greenhouse. Leaves were surface sterilized by immersing them in 0.05% sodium hypochlorite with about 1% of detergent for 20 min and subsequent washings in sterile water. Calli of both species were induced and maintained on MS-MG medium (standard medium of Müller and Grafe 1978) in the dark at 23 °C. When used for our studies, the callus cultures were about 10 months old and, unless otherwise stated, exhibited no chromosomal abnormalities.

Protoplast isolation and callus regeneration

All protoplasts were isolated in a mixture of 0.2% cellulase CELF (Worthington Diagnostics), 0.4% Macerase (Calbiochem), and 0.5 M mannitol at pH 5.6 and 30° for 2 to 3 h. Protoplast preparations from callus were constantly and gently agitated. The protoplast suspensions were passed through a cotton layer, centrifuged at 50×g for 10 min and washed twice. Wash solutions were 0.5 M mannitol for mesophyll protoplasts and a solution of 70 mM KCl, 60 mM $CaCl_2$, and 60 mM $MgCl_2$ for callus protoplasts. Protoplasts were cultured in 8p medium (Kao and Michayluk 1975) at 23 °C in the dark and subcultured every 4 to 8 days. Developing calli were transferred to solid MS-MG.

Plant regeneration

Morphogenesis was achieved on MS inorganic salts (Murashige and Skoog 1962) supplemented per liter with 100 mg m-inositol, 10 mg thiamin×HCl, 1 mg nicotinic acid, 1 mg pyridoxin×HCl, 30 g sucrose, and either 4 mg indole-4-acetic acid and 2.5 mg 6-benzylaminopurine ("IAA-MS") or 1 mg gibberellic acid and 1 mg 6-benzylaminopurine ("GA3-MS"). Regenerants were transferred to rooting media, alternating between hormone-free 50 percent concentrated B5, containing per liter 5 g sucrose, and MSO (MS inorganic salts, containing per liter 100 mg m-inositol, 1 mg thiamin×HCl, 30 g sucrose, and 3 g activated charcoal). Cultures were kept at 23 °C and a cycle of 16 h of light ad 8 h of darkness.

Chromosome staining

For chromosome studies of protoplast cultures and developing calli that were still in liquid culture, samples were taken aseptically, left at 4 °C for 6 h, centrifuged, and incubated in Kao's fixative (Kao 1975) for 4 to 48 h at 4 °C. Calli were either cold-treated as above or incubated in 2 mM 8-hydroxyquinoline for 5 h at room temperature, fixed in acetic acid/ethanol (1:3, v/v) overnight, and macerated in 1 N HCl for 0 to 2 h depending on the presence of tracheids in the callus. Aliquots were nearly completely dried onto slides, stained with orcein (saturated in 45% acetic acid) for 5 min and squashed. Root tips and young leaves were treated in a manner similar to that for calli except that the tips and leaves were macerated for only 5 min and stained for 10 to 15 min.

Results

A Protoplast regeneration

1 Plant regeneration from callus protoplasts of Brachycome dichromosomatica. When karyologically normal diploid callus protoplasts of *B. dichromosomatica* were cultured they usually developed into calli consisting of spherical cells, the latter having few cytoplasmic strands. The calli started to degenerate from the center and, after two to eight weeks of culture, became necrotic. In four out of 25 independent protoplast isolations, however, most calli developed, having non-spherical elongated cells with many cytoplasmic strands. In one of the four cases, the calli grew large enough to be transferred to solid medium (MS-MG) 1.5 months after protoplast isolation and to regeneration medium another 2.5 (clone 91) and 4.5 (clone 230) months later. The first shoot-like structures appeared 1 (clone 91) or 3 (clone 230) months afterwards. Clone 91 regenerated shoots in only two petri dishes. These two dishes contained GA3-MS, an atypical shoot induction medium. Prolific growth and further shoot induction ensued upon transfer to IAA-MS. Shoot induction, however, was never successful when IAA-MS was used as the first shoot-inducing medium. In contrast, clone 230 developed shoots only on IAA-MS. The anatomy of the shoots was at first quite variable, exhibiting teratomous growth, but after transfer to hormone-free medium they eventually resembled *B. dichromosomatica* plants grown in vitro from seeds. Regenerates could be propagated by cuttings. Flowers emerged about 6 months after shoot induction. Root development was poor for the first year after shoot induction, but then became prolific. Plants were potted 4 months later. Survival upon transfer to soil was approximately 40%, which is about the same rate we observed for *B. dichromosomatica* seedlings grown in vitro. The morphology of the regenerates was very much alike in both clones; no somaclonal variations were observed. All plants examined (3) were diploid and without visible metaphase chromosome alterations. Only clones 91 and 230, derived from the same protoplast isolate, yielded plants. None of the other 50 clones from this

protoplast culture were morphogenetically active, nor was morphogenesis inducible in the three other *B. dichromosomatica* callus protoplast cultures that regenerated viable calli. (Curiously, three of the callus regenerations, including clones 91 and 230, were achieved in cultures that had undergone a fusion treatment).

Mesophyll protoplasts of *B. dichromosomatica* divided readily in contrast to callus protoplasts, and frequently developed into calli when cultured at a density of 10^4 protoplasts/ml and diluted with culture medium every 4 to 8 days. However, most regenerated mini-calli died before they could be transferred to regeneration medium. Morphogenetic activity, restricted to poor root development, was observed in only one regenerated callus.

2 Plant regeneration from callus protoplasts of Crepis capillaris. Only one out of 50 callus protoplast cultures of *C. capillaris* proliferated sufficiently to give rise to transferable colonies, and only one of the resulting clones (out of 10 tested) regenerated plants. Calli of this clone were transferred to GA3-MS 8 months after the initiation of the culture. No morphogenesis was achieved on IAA-MS. It took two more months for the first leaves to appear. Another two months later, after transfer to MSO, shoot development was prolific. These shoots were morphologically perfect. Roots developed, but they always originated from little pieces of callus at the base of the plantlets and could not sustain the plants upon transfer to soil. The chromosome set of the regenerated plants was diploid and not notably aberrant, as determined in cells of young leaves.

C. capillaris mesophyll protoplast cultures seldom divided, and the rarely developing calli did not survive transfer to solid medium.

B Cytogenetics of protoplast cultures

The recalcitrance of *B. dichromosomatica* and *C. capillaris* protoplasts to regenerate calli, even though the protoplasts looked healthy and stayed alive for over 10 days under the culture conditions applied, could be caused by early macromutations. With this possibility in mind, we studied the nuclear characteristics of mesophyll and callus protoplast preparations. Cytogenetic analyses were performed by evaluation of metaphases.

1 Mesophyll protoplasts of Brachycome dichromosomatica. Mesophyll protoplasts of *B. dichromosomatica* divided readily in culture; usually up to 90% underwent division within the first 4 days. Karyological analysis showed that the very first mitoses, detectable after about 2 days, exhibited one diploid metaphase per cell. This finding indicates that the mitotically competent

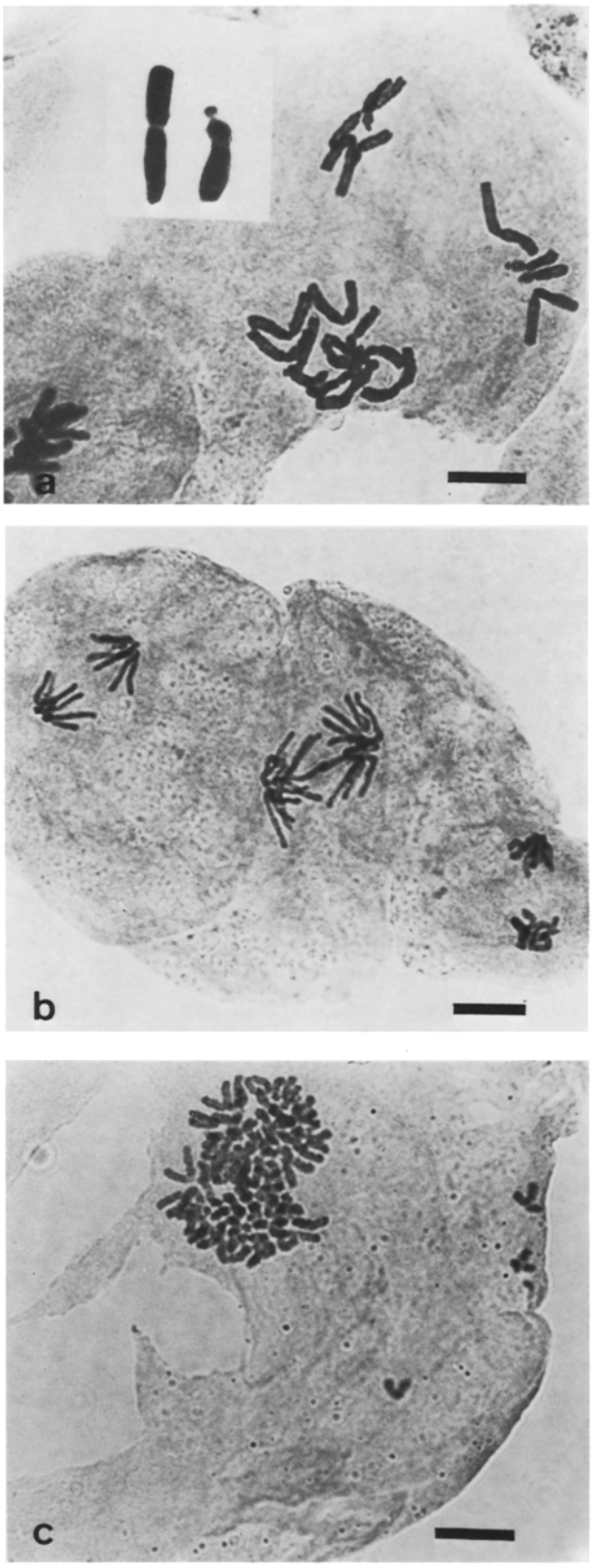

Fig. 1a–c. Examples of karyologically aberrant cells in protoplast cultures of *B. dichromosomatica* mesophyll. Bar = 10 μm. **a** A cell in an 8-day-old culture having two diploid and one tetraploid nuclei. Insert: the two chromosomes of *B. dichromosomatica;* **b** a small callus on the 5th day of culture. Two cells are diploid and one is tetraploid. **c** a highly polyploid cell with several chromosomes outside the metaphase plate, leading to an aneuploid cell (5 day old culture)

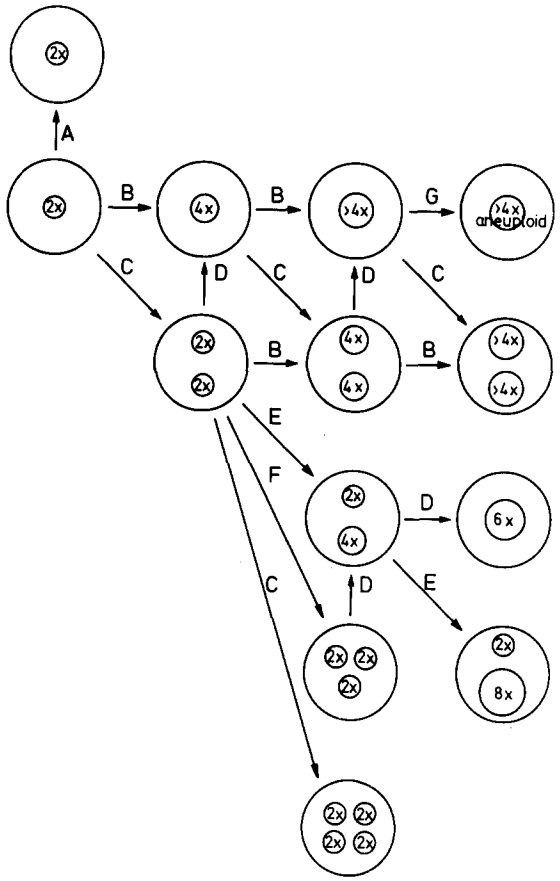

Fig. 2. Metaphase plates observed in *B. dichromosomatica* mesophyll protoplast cultures and possible explanations of their mode of formation. Other configurations found but not included in this scheme were e.g. 4x, 4x, 8x, 8x and 2x, 4x, 8x and 2x, 2x, 4x. *A*: normal mitosis with cytokinesis (e.g. 2x → 2x); *B*: endomitosis or endoreduplication (e.g. 2x → 4x); *C*: normal mitosis without cytokinesis (e.g. 2x → 2x, 2x); *D*: nuclear fusion (e.g. 2x, 4x → 6x); *E*: endomitosis or endoreduplication of only one nucleus in a binucleated cell (e.g. 2x, 2x → 2x, 4x); *F*: normal mitosis of a multinucleated cell with unequal distribution of the daughter nuclei in cytokinesis or mitosis of only one nucleus (e.g. 2x, 2x → 2x, 2x, 2x); *G*: chromosome loss during mitosis (e.g. > 4x → aneuploid > 4x)

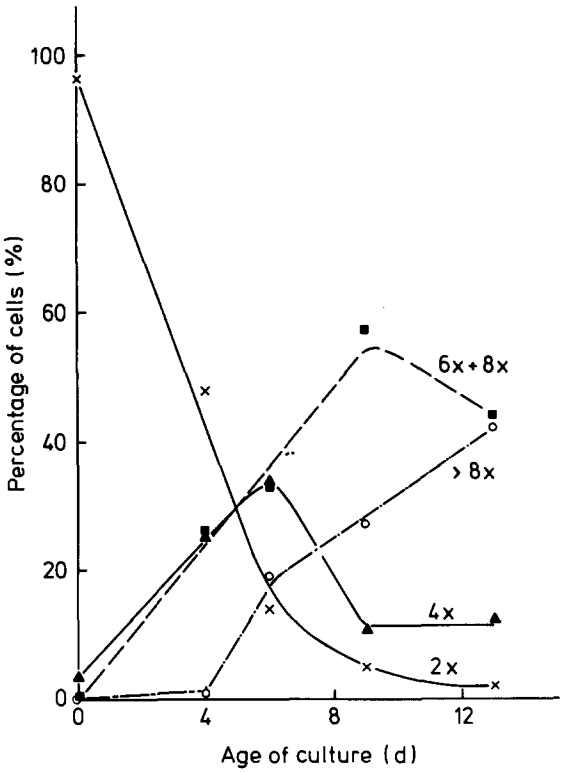

Fig. 3. Distribution of ploidy in a protoplast culture of *B. dichromosomatica* mesophyll as a function of the age of the culture. Ploidies of single nuclei in multinucleate cells were added up. There is a constant decrease in the percentage of diploid cells, whereas the percentage of highly polyploid cells is constantly increasing. Tetraploid cells are relatively most abundant around the 6th day of culture, and hexa- and octoploid cells have a maximum around the 9th day. Soon after the 13th day of culture, most single cells and calli became necrotic, correlated with their high ploidies. On average, 50 cells in metaphase were evaluated per sampling day. ×—×=2x, ▲—▲=4x, ■--■=6x and 8x, ○···○= > 8x ("highly polyploid")

cells of *B. dichromosomatica* mesophyll are mainly diploid. With increasing time of culture, however, most cells – either single or within small calli – became multinucleate and/or possessed polyploid nuclei (Fig. 1 a, b). Figure 2 shows some representative karyotypes, all of which were observed in cultures 2 to 13 days old, together with possible pathways leading to them. Some karyotypes could only have arisen from a combination of different mitotic defects in consecutive cell cycles. For example, in some cells two metaphases of different ploidies were seen, which suggests an early division without cytokinesis followed by endomitosis of one of the nuclei. Cells containing three metaphases, e.g. 2x/2x/2x, can be explained by an unequal distribution of the four daughter nuclei of an already binucleate cell or by mitosis without cytokinesis of one of the two nuclei. If several mitoses occurred in a cell they were almost always synchronous. The maximum number of metaphases per cell was four. Multinucleate cells constituted about 40% of all metaphase cells after a few days in culture. Metaphases were mostly euploid except those of some highly polyploid cells where chromosome loss was evident (Fig. 1 c). Also chromosome numbers other than 2^nx occasionally occurred, e.g. 6x or 12x. Chromosome breakage was observed only once and can therefore be regarded as an extremely rare event.

Figure 3 illustrates the increase in ploidy per cell. The percentage of highly polyploid cells increased with the duration of culture. The number of tetraploid and hexaploid plus octoploid cells reached a maximum

around days 4 and 9, respectively. Few cell were still diploid after the 9th day of culture. Later data were unobtainable due to a rapid decline in viability of the culture(s). The behavior of other cultures of *B. dichromosomatica* mesophyll protoplasts was very similar with respect to polyploidization, though with slight time shifts. The number of calli that could be established on solid medium varied greatly with each culture. Those examined were tetraploid or hexaploid and the cells were uninucleate.

2 Mesophyll protoplasts of Crepis capillaris. *C. capillaris* mesophyll protoplast cultures showed essentially the same development as the corresponding *B. dichromosomatica* cultures, viz. rapid polyploidization, multinucleate cells and concomitantly a decline in viability. However, the frequency of cell division was much lower in *C. capillaris,* and, interestingly, the rate of polyploidization was higher. For example, in a culture in which 0.1% of the cells had undergone division on the 6th day of culture, at least 2% of the cells were in metaphase. This culture survived for slightly longer than 20 days. On the 20th day, all cells, the majority still undivided, were polyploid, mostly highly polyploid, up to 52x (Fig. 4a). In other cultures even fewer cells divided, and the rate of polyploidization was much faster resulting in an earlier decrease in viability. As in *B. dichromosomatica* mesophyll protoplasts, karyotypes were found (e.g. 4x, 4x, 8x or 2x, 2x, 4x, 8x), that can only be explained by different mitotic abnormalities in two or more previous cell cycles (compare Fig. 2). Multinucleate cells could frequently be seen (Fig. 4b). They contained up to 6 nuclei per cell. Figure 4c shows an endoreduplicating polyploid cell in metaphase. The chromosomes are still attached to each other at the centromere ("diplochromosomes"), resulting in a further increase in ploidy. This configuration was only observed twice.

3 Callus protoplasts of Brachycome dichromosomatica. The diploid callus protoplast cultures of *B. dichromosomatica,* one of which gave rise to shoot regenerants, also occasionally underwent polyploidization. The calli that regenerated from these cultures were initially predominantly diploid. Later they became increasingly aneuploid, polyploid, or otherwise aberrant, including clone 91 which showed chromosomal translocations and polyploidization on several independent sites on the callus. The ability to regenerate shoots ceased with the appearance of chromosome abnormalities.

Tissue derived callus cultures of *B. dichromosomatica* also often proved to be karyologically aberrant after several months in culture. These aberrations, e.g. additional and/or translocated chromosomes, often resulted in more vigorous callus growth compared to

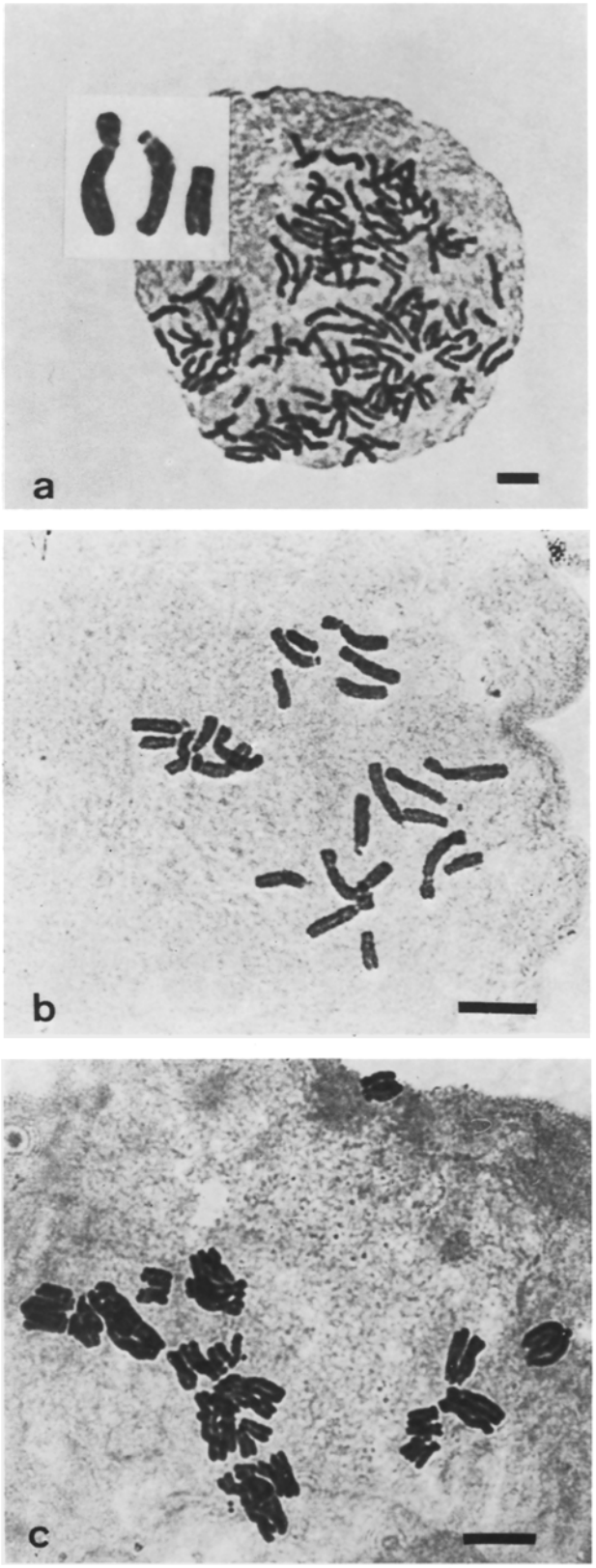

Fig. 4a–c. Examples of karyologically aberrant cells in protoplast cultures of *C. capillaris* mesophyll. Bar = 10 μm. **a** A 52x undivided cell on the 20th day of culture. Insert: the three chromosomes of *C. capillaris;* **b** a 6-day-old cell with four 2x metaphases; **c** a 6-day-old cell having diplochromosomes showing that endoreduplication took place

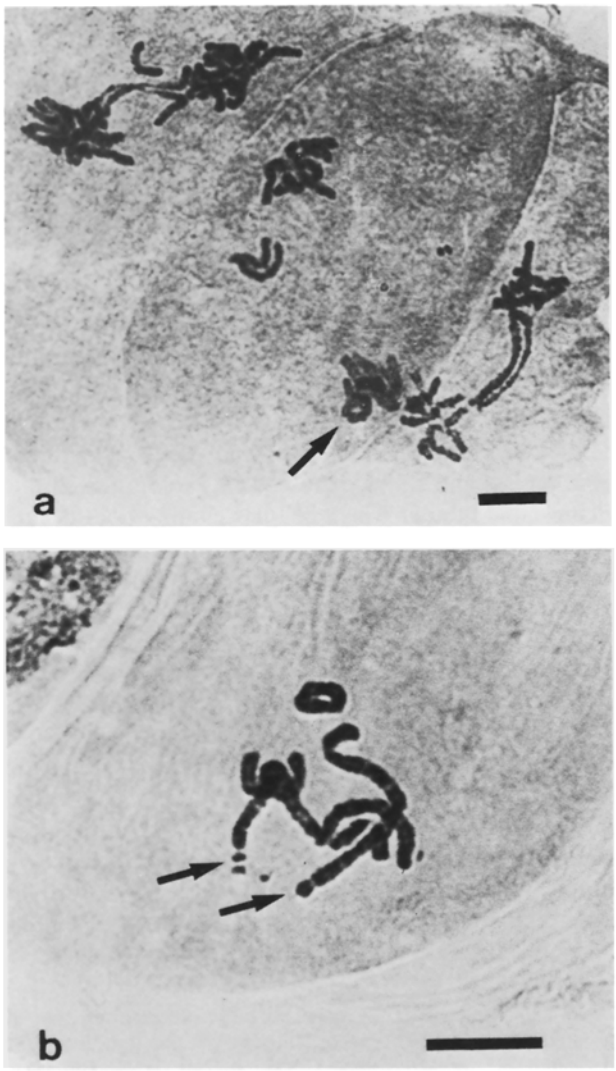

Fig. 5 a, b. Aberrant karyotypes in callus protoplast cultures of *B. dichromosomatica* and *C. capillaris*. Bar = 10 μm. **a** A ring chromosome (*arrow*) and chromosome bridges in a 5-day-old protoplast culture of *B. dichromosomatica* callus. The callus already exhibited several karyotypes; **b** a ring chromosome and translocated chromosomes (*arrows*) in a 6-week-old callus derived from non-aberrant diploid protoplasts of *C. capillaris*

wildtype cultures. Obviously, certain aberrations could impart selective advantage to some cells. Protoplasts derived from these aberrant callus cultures seemed to "inherit" the higher viability and, in most cases, readily regenerated calli. These calli frequently showed further alteration in ploidy, especially polyploidization. Dicentric or ring chromosomes (Fig. 5 a) were often found in these protoplast derived calli. Some of these aberrations, however, might already have been present in the callus used as a source of protoplasts, and therefore might not be a result of the protoplasting itself.

4 Callus protoplasts of Crepis capillaris. Only one of 50 protoplast cultures of callus from *C. capillaris* regenerated calli, and this also produced diploid shoots (see above). If mitoses occurred at all in the other 49 cultures, extremely high frequencies of polyploid cells were observed. After only 4 days of culture about two-thirds of the metaphase cells were usually octoploid or of a higher ploidy. The one regenerating culture, however, once stabilized, consisted of diploid and tetraploid cells after several months of culture. Before the genomic stabilization had been completed, some higher polyploids, and several aneuploids, e.g. 4x-1 or 4x-2 were observed. Several chromosome breakages and translocations were also visible (Fig. 5 b).

Discussion

Regeneration of callus from protoplasts as well as induction of complete plants seem to be difficult to achieve for the two species of Asterales studied here. In the first step, we found that a high and largely lethal rate of polyploidization hinders the formation of calli from protoplasts in both species. The second step of regeneration was also impaired. Morphogenesis rarely occurred in either species, and only regenerants of *B. dichromosomatica* could be transferred to soil, whereas in *C. capillaris* root development was insufficient to support the plants under greenhouse conditions. The low frequency of plant formation implies that this problem of plant regeneration must involve unknown factors other than obvious alterations of the genome.

Our study examined primarily the first step of protoplast regeneration, the formation of callus. We found much polyploidy, up to 52x, and multinucleate cells, which sometimes contained nuclei of different ploidy (Figs. 1 and 4). Multinucleate cells probably resulted from karyokinesis without cytokinesis, because no indication of nuclear fragmentation, as reported for soybean by Miller et al. (1971; see also D'Amato et al. 1980) was observed. We did not see nuclear fusion in interphase, as reported for multinucleate soybean protoplasts (Miller et al. 1971). Our findings relate all polyploid or multinucleate cells to defects during mitosis and cytokinesis: 1) DNA is duplicated in S-phase, but in M-phase the chromosomes merely condense, and the chromatids are not separated leading to diplochromosomes (endoreduplication). 2) One further step occurs, namely the chromatids are separated, but not pulled apart in an anaphase (endomitosis). In contrast to endoreduplication, the process of endomitosis cannot be directly observed. But as endoreduplication was only seen twice in our cultures, we believe endomitosis to be the most frequent cause of polyploidization. 3)

Anaphase proceeds, but in multinucleate cells with synchronous nuclei a common metaphase plate and common spindle poles are formed which results in a fusion of nuclei. (This mechanism increases the ploidy of the nuclei, which decrease in number). 4), finally, the nuclear division proceeds correctly, but the cells did not undergo cytokinesis, leading to multinucleate cells.

Another aberration during mitosis was chromosome loss which sometimes occurred in polyploid metaphases. This points to a failure of the spindle apparatus to distribute high numbers of chromosomes correctly. Chromosome translocations were only observed in callus protoplast cultures. These protoplasts might be predisposed to translocations as a result of prolonged in vitro culture of the callus (Gould 1983; Sacristán 1971).

Our findings are in good agreement with previous studies on the DNA-content of cultured mesophyll protoplasts. In the first days of protoplast culture, rapid polyploidization was observed to occur in tobacco (Galbraith et al. 1981) and potato (Sree Ramulu et al. 1984), and multinucleate cells were found in potato (Carlberg et al. 1984). Galbraith et al. (1981) propose that these abnormal genomes can be corrected by subdivision of polyploid cells into diploid cells, resulting in a decreased frequency of polyploid cells. In callus protoplast cultures of *C. capillaris,* we also found a decrease in highly polyploid cells after a few weeks of culture, but we feel this as owing to selection against highly polyploid cells. Our interpretation is supported by our data for mesophyll protoplasts of *B. dichromosomatica* and *C. capillaris.* The cultures became necrotic when they were highly polyploid, and small calli (all of low ploidy) developed only in those cases where at least a few cells of low ploidy had always been present. The three publications cited above also report the frequent occurrence of aneuploids. We rarely found aneuploids, and only in highly polyploid cells. *B. dichromosomatica* and *C. capillaris* possess few chromosomes compared to tobacco and potato (both $2n=4x=48$). Loss of a chromosome in a diploid species with only 4 or 6 chromosomes would probably be lethal immediately because of genomic imbalance. We believe that chromosome loss does not often occur in the diploid or tetraploid state in our species, because we would have observed this occasionally as a lagging chromosome in anaphase.

Polyploidization and multinucleate cells seem to be occurring regularly in the initial phases of callus (Cionini et al. 1978; Doležel and Novák 1984) and protoplast cultures. Mitotic abnormalities are probably influenced by general culture conditions (including preculture of the plant material and protoplast isolation), because the frequency of polyploidization varied with the history of our cultures. Investigations with other members of the Asterales, in which the composition of the culture medium and the plating density were varied, showed that both parameters are crucial for division and survival rates of cultured protoplasts (Binding et al. 1981; Crepy et al. 1982). Presently, we do not know the molecular basis of the correlation between culture conditions and chromosome variations. Protoplast cultures of *B. dichromosomatica* and *C. capillaris* should be an excellent system for studies on this correlation: they exhibit massive karyological changes soon after being cultured, and most changes are easily identified because of their few, distinctive chromosomes. On the other hand, regeneration of karyologically normal diploid shoots was achieved, showing that the genomes can be stable over many cell generations in protoplast and callus culture. This exploration of the circumstances under which cellular cloning does occur, i.e., the avoidance of any culture-induced variation, is urgently needed for future, fully controlled genetic manipulations of single cells.

There is only one example so far in which the recalcitrance of a protoplast species to divide and regenerate calli could be traced to the molecular level, namely the absence of a network of cortical microtubules (Hahne and Hoffmann 1984). It is not known, however, which parameter in protoplast isolation and/or culture caused this absence of microtubules. Microtubules are also involved in all the mechanisms of mitotic aberration observed in this study. A further search for non-mutagenic culture conditions for protoplast regeneration should focus on this coincidence.

Acknowledgement. Part of this work was supported by a stipend of the Deutscher Akademischer Austauschdienst to B.H.

References

Binding H, Nehls R (1980) Protoplast regeneration to plants in *Senecio vulgaris* L. Z Pflanzenphysiol 99: 183–185

Binding H, Nehls R, Koch R, Finger J, Mordhorst G (1981) Comparative studies on protoplast regeneration in herbaceous species of the dicotyledoneae class. Z Pflanzenphysiol 101: 119–130

Carlberg, I, Glimelius K, Eriksson T (1984) Nuclear DNA-content during the initiation of callus formation from isolated protoplasts of *Solanum tuberosum* L. Plant Sci Lett 35: 225–230

Carter CR (1978) Taxonomy of the *Brachycome lineariloba* complex (Asteraceae). Telopea 1: 387–393

Cionini PG, Bennici A, D'Amato F (1978) Nuclear cytology of callus induction and development in vitro. 1. Callus from *Vicia faba* cotyledons. Protoplasma 96: 101–112

Crepy L, Chupeau M-C, Chupeau Y (1982) The isolation and culture of leaf protoplasts of *Cichorium intybus* and their regeneration into plants. Z Pflanzenphysiol 107: 123–131

D'Amato F, Bennici A, Cionini PG, Baroncelli S, Lupi MC (1980) Nuclear fragmentation followed by mitosis as mechanism for wide chromosome number variation in tissue cul-

tures: its implications for plant regeneration. In: Sala F, Parisi B, Cella R, Ciferri O (eds) Plant cell cultures: results and perspectives. Elsevier, Amsterdam, pp 21–25

Doležel J, Novák FJ (1985) Karyological and cytophotometric study of callus induction in *Allium sativum* L. J Plant Physiol 118:421–429

Evans DA, Bravo JE (1983) Protoplast isolation and culture. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture, vol I. Macmillan, New York, pp 124–176

Galbraith DW, Mauch TJ, Shields BA (1981) Analysis of the initial stages of plant protoplast development using 33258 Hoechst: reactivation of the cell cycle. Physiol Plant 51: 380–386

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gould AR (1978) Diverse pathways of morphogenesis in tissue cultures of the composite *Brachycome lineariloba* (2n=4). Protoplasma 97:125–135

Gould AR (1979) Chromosomal and phenotypic stability during regeneration of whole plants from tissue cultures of *Brachycome dichromosomatica* (2n=4). Aust J Bot 27: 117–121

Gould AR (1983) Chromosome and heterochromatin rearrangement in morphogenic tissue cultures. J Cell Biochem (Suppl) 7B:254

Hahne G, Hoffmann F (1984) Dimethylsulfoxide can initiate division of arrested callus protoplasts by promoting microtubule assembly. Proc Natl Acad Sci USA 81:5449–5453

Hoffmann F, Adachi T (1981) "Arabidobrassica": chromosomal recombination and morphogenesis in asymmetric intergeneric hybrid cells. Planta 153:586–593

Hoffmann F, Hahne B, Hahne G (1984) Are cortical microtubules essential for cell formation and cell division by isolated callus protoplasts? In: Dugger WM, Bartnicki-Garcia S (eds) Structure, function and biosynthesis of plant cell walls. Waverly Press, Baltimore, pp 444–448

Kao KN (1975) A nuclear staining method for plant protoplasts. In: Gamborg OL, Wetter LR (eds) Plant tissue culture methods. National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, pp 15–18

Kao KN (1977) Chromosomal behaviour in somatic hybrids of soybean – *Nicotiana glauca*. Mol Gen Genet 150:225–230

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126:105–110

Miller RA, Gamborg OL, Keller WA, Kao KN (1971) Fusion and division of nuclei in multinucleated soybean protoplasts. Can J Genet Cytol 13:347–353

Müller AJ, Grafe R (1978) Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol Gen Genet 161:67–76

Murashige T, Skoog F (1962) A revised medium for rapid growth and assays with tobacco tissue cultures. Physiol Plant 15:473–497

Sacristán MD (1971) Karyotypic changes in callus cultures from haploid and diploid plants of *Crepis capillaris* (L.) Wallr. Chromosoma 33:273–283

Sree Ramulu K, Dijkhuis P, Roest S, Bokelmann GS, De Groot B (1984) Early observance of genetic instability in protoplast cultures of potato. Plant Sci Lett 36:79–86

Sunderland N (1977) Nuclear cytology. In: Street HE (ed) Plant tissue and cell culture. University of California Press, Berkeley, pp 16–20