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Callus induction, plant regeneration and chromosomal variations in barley

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SUMMARY

Callus cultures were induced on mature embryo mesocotyl explants in *Hordeum vulgare* cv. Zafer-160. The callus induction ratio was 54% in Murashige–Skoog (MS) medium supplemented with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). After transfer at 22, 45, 360, 540 days of culture to MS medium containing lower concentrations of 2,4-D (0·5, 0·1, 0·01 mg l^{-1}) or MS medium lacking 2,4-D, 45-day-old callus cultures showed somatic embryogenesis. The optimum 2,4-D concentration for somatic embryogenesis was 0·01 mg l^{-1}. Callus cultures of 360 days showed regeneration via both somatic embryogenesis and organogenesis under the same conditions. Abnormalities in both number and structure of chromosomes increased with the age of calli. This phenomenon might be related to the loss of regeneration ability in 540-day-old calli. *In-vitro* regenerated plantlets gave rise to normal-looking plants after their transfer to soil. Regenerated plants had the normal diploid chromosome number ($2n = 14$) in their root tips.

Key-words: chromosomal variation, *Hordeum vulgare*, organogenesis, somatic embryogenesis.

INTRODUCTION

In crop plants, and graminaceous crops in particular, due to their economic importance in both food and brewing industries, cell and tissue culture systems have a potential importance for plant improvement through genetic manipulation techniques such as mutation, somaclonal variation and gene transfer. At present, further studies are needed for the initiation and manipulation of such cultures for cereals. In barley, different explant sources have been used for the induction of regenerable callus cultures (Cheng & Smith 1975; Saalbach & Koblitz 1978; Orton 1979; Lupotto 1984; Weigel & Hughes 1985; Hanzel *et al.* 1985; Köhler & Wenzel 1985; Thomas & Scott 1985; Wei & Harada 1986; Lührs & Lörz 1987; Ukai & Nishimura 1987; Gaponenko *et al.* 1988; Rotem-Abarbanell & Breiman 1989). Regeneration appeared to be strongly dependent on the genotype (Hanzel *et al.* 1985; Ahloowalia 1987; Lührs & Lörz 1987).

Our overall goal is to develop a tissue culture system with high regeneration capacity for the native barley variety *Hordeum vulgare* L. cv. Zafer-160. In this paper, the establishment of callus cultures from mature embryo mesocotyl explants of Zafer-160 barley and

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plant regeneration at high frequency from these calli is reported. In addition, chromosome analyses were carried out. The possible relationship between increasing chromosomal abnormalities and the loss of morphogenesis is discussed.

MATERIALS AND METHODS

Mature seeds of six-rowed winter barley (*Hordeum vulgare* L.cv. Zafer-160 ($2n = 2x = 14$)) were obtained from Halkali Agricultural Research Institute in Istanbul. Segments of embryo shoot axis, mesocotyl, root apex and root and leaf explants from seedlings were used for callus induction. Approximately 50 segments were used in each set of experiments.

Dry seeds were surface-sterilized by rapid immersion first in 95% ethanol and then in 20% sodium hypochlorite for 15 min and rinsed three times with sterile water. Embryos were isolated from the seeds, divided into three parts (shoot axis, mesocotyl, root portions) and placed on Murashige–Skoog medium (MS) (Murashige & Skoog 1962) supplemented with 100 mg l^{-1} myo-inositol, 0·5 mg l^{-1} nicotinic acid, 0·5 mg l^{-1} pyridoxine–HCl, 0·1 mg l^{-1} thiamine–HCl, 2 mg l^{-1} glycine, 0·5–2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose, and 0·8% agar. These cultures were incubated in the dark at 25°C. Explants originating from leaves and roots of seedlings were cultured on the same media. These cultures, however, were kept in a growth chamber illuminated with fluorescent light (*c.* 7000 lux) for 16 h per day at 25°C.

Once induced, the callus cultures were maintained in the growth chamber and subcultured at monthly intervals. After 22, 45, 360 or 540 days of culture, a part of each callus line was transferred to MS media containing 0, 0·01, 0·1 or 0·5 mg l^{-1} 2,4-D.

For cytogenetic analyses, calli were fixed at the days mentioned above for 24 h in 1:3 (v/v) acetic acid–ethanol and stained using the Feulgen method. Ten-day-old calli were used as controls and were processed accordingly. For each callus line, five preparations were analysed.

Thirty regenerated plantlets were potted in soil and grown to mature plants in the greenhouse. Roots of regenerated plants stained with Feulgen were used for chromosome countings. Statistical analyses were carried out by chi-square test.

RESULTS

Under the conditions tested, the various embryo segments gave different responses to culture conditions: shoot axis explants produced leafy shoots, root portion explants gave rise to roots, mesocotyl-originated explants produced white, soft and watery calli in MS medium containing different concentrations of 2,4-D. MS media supplemented with 1 mg l^{-1} 2,4-D gave the highest callus induction ratio (54%, see Table 1). Therefore this medium was used in further experiments on callus induction with mature embryo mesocotyl explants. On the 22nd day of culture, calli showed slight regeneration signs such as leafy spots and root-like structures but never developed shoot or whole root systems (Fig. 1a). The 45-day-old callus cultures regenerated, with a frequency of 33 calli out of 173, into plantlets via somatic embryogenesis after transfer to MS medium containing lower concentrations of 2,4-D (0·5, 0·1 or 0·01 mg l^{-1}) and MS medium lacking 2,4-D (Fig. 1b, d and e). The plantlets that regenerated from globular somatic embryos in callus material were characterized by the presence of the globular base of the somatic embryos (Fig. 1d). The optimum 2,4-D concentration for regeneration was 0·01 mg l^{-1} (Table 2).

Table 1. The influence of 2,4-D on the induction of callus from mature embryo mesocotyl explants of *Hordeum vulgare* L. cv. Zafer-160

Culture medium and 2,4-D concentrations		No. of explants	No. of calli	Callus induction ratio (%)
1	MS + 0·5 mg l^{-1} 2,4-D	144	52	36·1
2	MS + 1 mg l^{-1} 2,4-D	140	76	54·2*
3	MS + 2 mg l^{-1} 2,4-D	127	44	34·6

*Differences between media (1–2 and 2–3) for callusing response were significant ($P < 0·01$).

Callus cultures of 360 days showed regeneration via both somatic embryogenesis and organogenesis in a 45-day period when transferred to the MS medium containing 0·01 mg l^{-1} 2,4-D (Fig. 1c). In this material, leaf formation mostly occurred in the absence of recognizable globular embryoids, which is an indication of organogenesis rather than embryogenesis. From 540-day-old callus, embryogenesis or organogenesis was never observed.

On the 10th day of callus culture all metaphases were diploid. On the 22nd day and 45th day of culture both diploid and tetraploid cells were observed, the frequency of tetraploids being 0–4% and 0–9·5%, respectively (Table 3). On the 360th and especially on the 540th day, diploid, tetraploid, pentaploid, endomitotic hyperploid and aneuploid cells were observed (Fig. 2). In addition to abnormalities in the number of chromosomes, structural abnormalities such as bridges, disorganized anaphases, rings, etc. were also observed (Fig. 3).

Chromosome countings of regenerated plants cultured under greenhouse conditions showed the normal diploid chromosome number ($2n = 14$).

DISCUSSION

The results indicate that in *H. vulgare* L. cv. Zafer-160 callus can be induced on mature embryo mesocotyl explants. From this callus, dependent on its culture age, somatic embryos and/or shoots were regenerated. While callus material at 45 days of culture showed regeneration via somatic embryogenesis, at 360 days of cultivation regeneration occurred via both somatic embryogenesis and organogenesis. Finally, at 540 days of culture the calli no longer showed any morphogenesis. Weigel & Hughes (1985) reported that leaf formation in the apical meristem cultures of barley took place by both embryogenesis and organogenesis. The occurrence of both phenomena in the same species was reviewed by Roest & Gilissen (1989) in *Triticum aestivum, Brassica juncea, Eruca sativa, Cucumis melo*, etc. Similar to our study, in immature embryo-derived calli Gaponenko *et al.* (1988) observed a time-dependent decrease of the regeneration capacity which was already substantial for calli of immature embryos cultured for 4 months.

The use of mature embryos rather than immature ones or other explants has several advantages, such as season independence, easy handling and relative sterility of tissue. Rengel (1987) obtained a non-embryogenic (NE) callus from whole *H. vulgare* mature embryos cultured on modified MS medium supplemented with different concentrations of 2,4-D. Following transfer of NE callus tissue to fresh medium supplemented with the

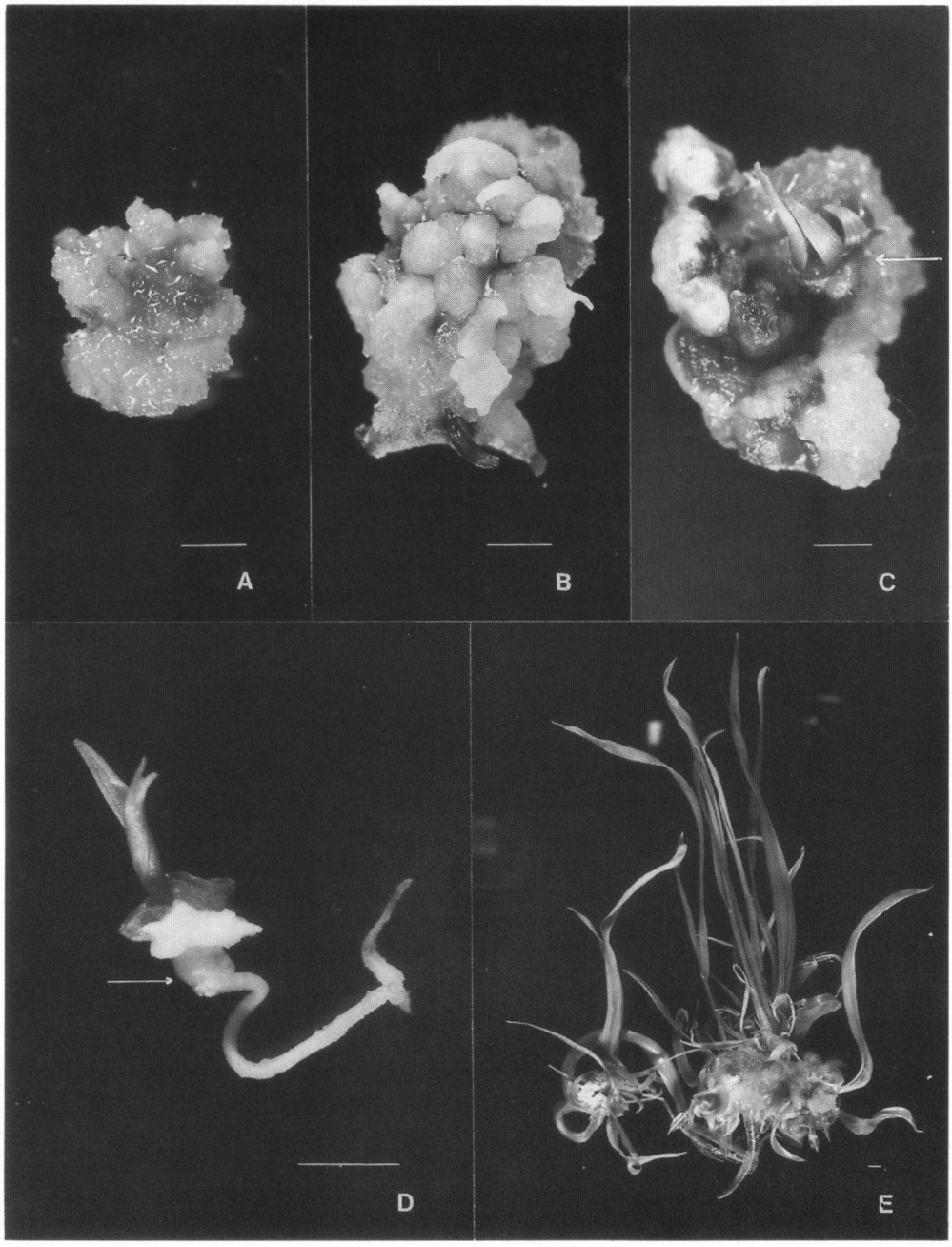

Fig. 1. Callus culture from mature embryo mesocotyls of barley (*Hordeum vulgare* L. cv. Zafer-160). Growth and developmental stage after 45 days of culture on MS medium containing 0·01 mg l^{-1} 2,4-D of callus, induced and maintained for 22 days (A), 45 days (B), and 12 months (C), respectively on MS medium containing 1 mg l^{-1} 2,4-D. Regeneration of a plantlet from somatic embryo (D) and numerous plantlets (E) on embryogenic callus represented in (B). Arrow indicates globular base of the somatic embryo. Bars represent 2 mm.

Table 2. Regeneration response of *Hordeum vulgare* L. cv. Zafer-160

Culture medium	No. of calli producing somatic embryos/ no. of calli transferred
MS + 1 mg l^{-1} 2,4-D	0/39
MS + 0·5 mg l^{-1} 2,4-D	6/40
MS + 0·1 mg l^{-1} 2,4-D	8/33
MS + 0·01 mg l^{-1} 2,4-D	15/34
MS	4/27

Callus initiated and maintained for 45 days in MS medium containing 1 mg l^{-1} 2,4-D, then transferred and maintained in MS medium containing various concentrations of 2,4-D for 45 days.

same concentration of 2,4-D, a hard opaque and nodular embryogenic callus tissue was produced. Attempts to induce somatic embryo formation in the culture of isolated parts of barley embryos, however, have failed. In our study no selection of regenerative parts was carried out. We obtained callus formation and somatic embryo formation from mesocotyl parts of mature embryos at high frequency. Rotem-Abarbanel & Breiman (1989) compared the plant regeneration of calli from immature and fully mature embryos of *Hordeum marinum*. They indicated the importance of the physiological state of explants because in their study mature embryos gave rise to morphogenic calli, each producing only one plantlet except in a few cases. They discarded germinating embryos before transfer to regeneration media. In our study, we did not observe mature embryo germination as we discarded the root and shoot meristems before starting the culture, and in the genotype multiple plantlet formation on mesocotyl-originated calli was noticed.

In cereals, the synthetic herbicide 2,4-D is the most important auxin required for the induction and maintenance of embryonic calli (Vasil & Vasil 1986). Organization of somatic embryos generally takes place by transfer to media with low concentrations of 2,4-D. However, quantitative data on the concentrations of 2,4-D for callus induction and plant regeneration are not easily comparable because cultural conditions, such as frequency of subculture and type of explant employed for regeneration, are dissimilar. In our study, the optimum 2,4-D concentrations for callus induction and regeneration are 1 and 0·01 mg l^{-1}, respectively.

Our results showed that abnormalities, both in number and structure of chromosomes, increase with the age of calli from mature embryo mesocotyl. However, Ruiz & Vazquez (1981) have reported that calli obtained from embryos show a high stability of diploidy after a period of 3 years, although at the beginning of their culture some polyploid or aneuploid cells were also present. Singh (1986), studying the chromosome constitution of morphogenic and non-morphogenic calli from immature embryos of barley, has shown that non-morphogenic calli had a large quantity of cells carrying numerical and structural changes. Gaponenko *et al.* (1988) observed high heterogeneity and significant differences in cytogenetic processes between different callus lines from immature embryos. Although these authors recorded a high percentage of polyploid metaphases in some callus lines, and did indicate chromosome stickiness, chromatin hypercondensation and chromosome supercoiling, they did not observe bridges, fragments and rings. In our aged (540 days old) callus material in one of the polyploid cells, occurrence of a pair of sister rings indicated the stability of such structures during mitotis. Differences in findings by different authors

Table 3. Chromosome analysis of calli derived from mature embryo mesocotyls of barley (*Hordeum vulgare* L. cv. Zafer-160, $2n = 2x = 14$)

Days after mature embryo cultured	Callus line no.	Metaphase + anaphase no.	Number of cells with abnormalities in the number of chromosomes				Number of cells with abnormalities in the structure of chromosomes			Total abnormality (%)
			4*n*	5*n*	Hyperploid	Aneuploid	Ring	Disorganised anaphase	Anaphase bridge	
	1	15	—	—	—	—	—	—	—	0
10	2	17	—	—	—	—	—	—	—	0
	3	20	—	—	—	—	—	—	—	0
	4	15	—	—	—	—	—	—	—	0
22	5	25	1	—	—	—	—	—	—	4
	6	18	—	—	—	—	—	—	—	0
	7	17	—	—	—	—	—	1	—	5
45	8	21	2	—	—	—	—	1	—	9·5
	9	25	—	—	—	—	—	—	—	—
	10	25	2	—	—	—	—	1	1	16
360	11	27	4	—	—	—	—	1	—	18·5
	12	15	5	—	—	—	—	—	—	33·3
	13	25	15	1	1(56)* Diplochromosome	1(36)*	—	2	—	88
540	14	25	18	—	—	—	—	—	—	72
	15	25	12	1	—	—	2	—	—	60

*Number of chromosomes given in parentheses.

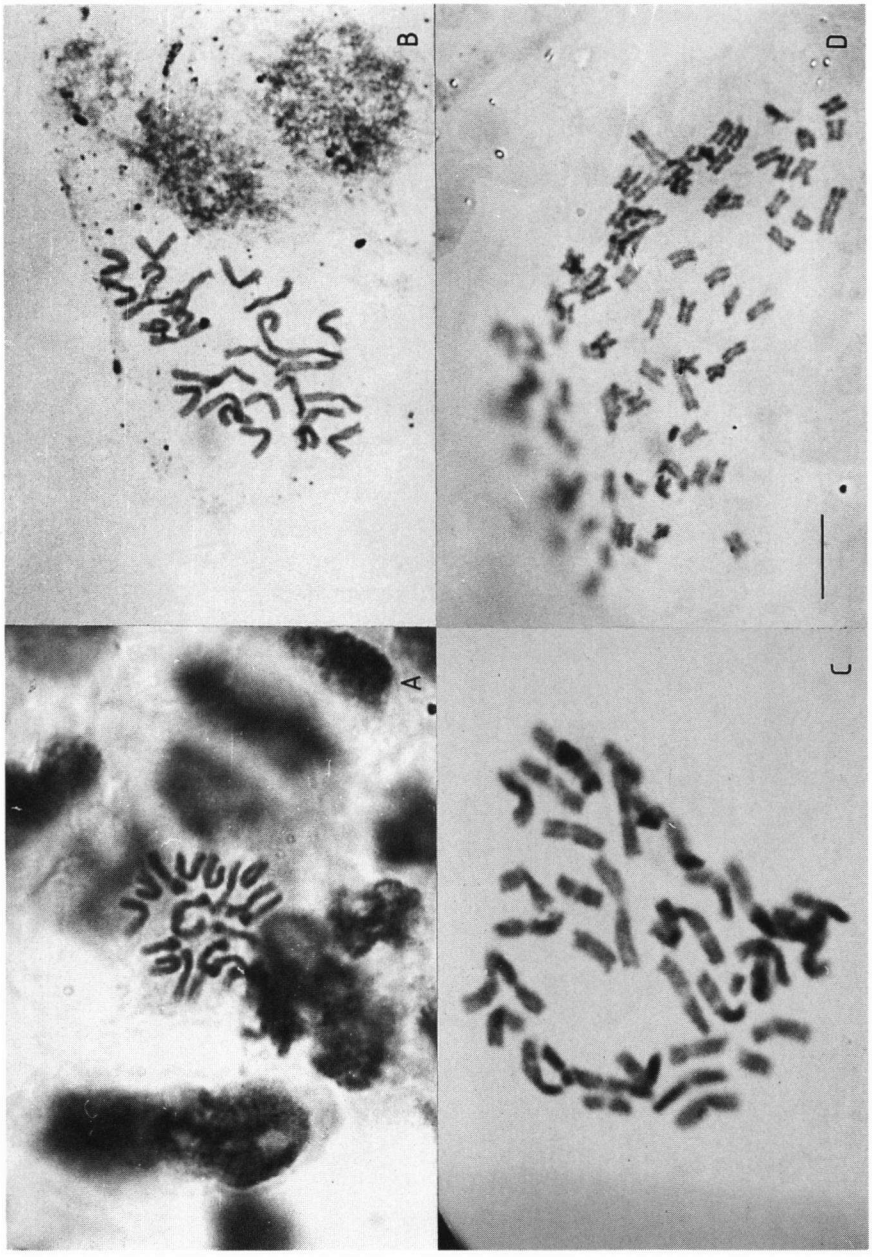

Fig. 2. Photomicrographs of cells observed in mature embryo-derived calli in a 540-day-old culture. (A) Diploid $2n = 2x = 14$; (B) tetraploid $2n = 4x = 28$; (C) pentaploid $2n = 5x = 35$; (D) endomitotic hyperploid (more than 56 diplochromosomes; the autonomously separated chromatids remain in a typical parallel arrangement). Bar represents 10 μm.

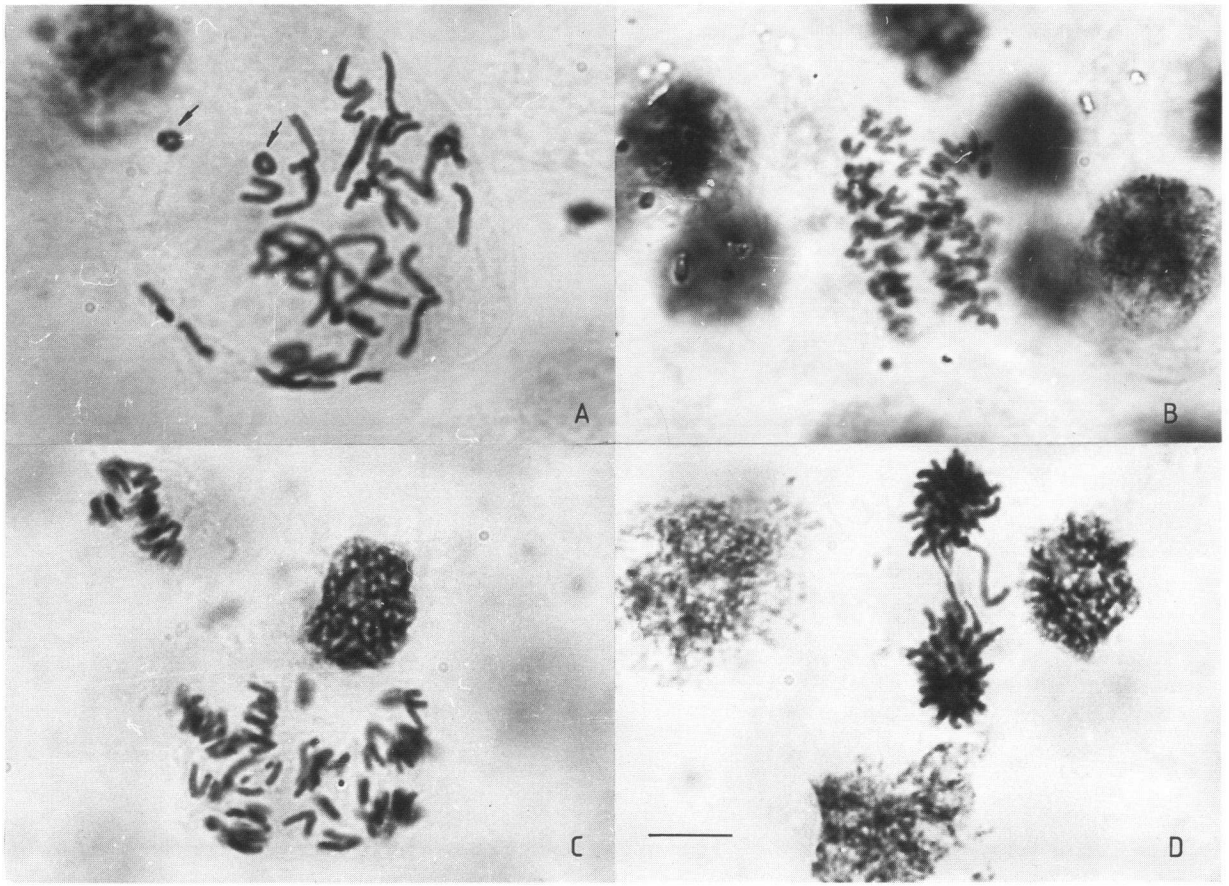

Fig. 3. Photomicrographs of cells observed in mature embryo-derived calli in a 540-day-old culture. (A) Polyploid cell with chromosome rings (arrows indicate the similar pair of sister rings); (B) disorganized anaphase in a tetraploid cells; (C) disorganized anaphase; (D) telophase with bridges. Bar represents 10 μm.

on chromosome behaviours could be due to genotype-dependent differences of the chromosomal behaviour in response to different medium (hormone) compositions and/or to different culture conditions (temperature, light intensity, etc.).

Finally, we may consider the possible correlation between the increasing age and decreasing ability to regenerate callus culture. Accumulation of gross chromosomal changes with subsequent mitosis and the occurrence of endomitosis markedly diminished the number of the normal diploid cells in aged callus material. As we did not observe polyploid plants among the regenerants from young callus cultures we can conclude that there is a close relationship between the loss of regenerative potential and age-dependent chromosomal abnormality accumulation.

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