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Chromosomal variation in immature embryo derived calluses of barley (*Hordeum vulgare* L.)

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Summary. Chromosome counts of ten morphogenic and seven non-morphogenic immature embryo derived calluses of barley, *Hordeum vulgare* L. cv. 'Himalaya', were determined. Morphogenic calluses carried the normal chromosome complement (2n = 2x = 14) in a majority of the cells. A low frequency of haploid (2n = x = 7), triploid (2n = 3x = 21), tetraploid (2n = 4x = 28) and octoploid (2n = 8x = 56) cells were also observed. In contrast, non-regenerability of a callus was attributed to the cells having numerical and structural chromosomal changes. In these calluses, aneuploid cells around diploid, triploid, and tetraploid chromosome numbers predominated. It has been demonstrated that chromosomal changes were induced during the culture and that they did not pre-exist in the cultured barley embryos. Based on this study, it is suggested that chromosome analysis of a non-regenerable callus should be conducted before altering the media composition.

Key words: Embryo culture – Chromosome aberrations – Plant regeneration – Tissue culture – *Hordeum vulgare*

Introduction

Why does callus not regenerate plants? This question is often asked among scientists engaged in cell and tissue culture programs. Frustratingly, alterations are often done in the media compositions, particularly for growth hormone ratios, without realizing their effect on the chromosomal constitution of the callus tissues. During the past two decades a voluminous amount of literature has been published on plant regeneration through cell and tissue cultures (see Ammirato et al. 1984). However, only a few reports describe chromosomal variation in the callus and its impact on plant regeneration (Evans and Reed 1981; Krikorian et al. 1983).

In general, the regenerability of a callus is impeded by numerical and structural changes in it chromosomes (Murashige and Nakano 1967). These chromosome aberrations have been attributed to media composition (Straus 1954; Torrey et al. 1962; Torrey 1967; Venketeswaran 1963; Demoise and Partanen 1969), growth hormones (Naylor et al. 1954; Nishiyama and Taira 1966; Van't Hof and McMillan 1969; Singh and Harvey 1975; Nuti Ronchi et al. 1976), age of the callus (Murashige and Nakano 1965, 1967; Shimada 1971; Novák 1974; Novák et al. 1978; Roy 1980), nature of the callus, compact versus friable, morphogenic versus nonmorphogenic (Partanen et al. 1955; Mitra et al. 1960; Murashige and Nakano 1965, 1967; Sacristán and Wendt-Gallitelli 1971) and genetic background (Browers and Orton 1982).

The present study was undertaken to investigate the chromosome constitution of morphogenic (regenerable) and non-morphogenic (non-regenerable) immature embryo derived calluses of barley (*H. vulgare*). This study will demonstrate that morphogenic potentiality of a callus is due to the presence of a high frequency of cells having a normal chromosome complement.

Materials and methods

Seeds of barley cv. 'Himalaya' were kindly supplied by Dr. T. Tsuchiya, Department of Agronomy, Colorado State University, Fort Collins, Colorado. The main reason in selecting barley for this particular study was that it is a basic diploid (2n = 2x = 14) and chromosomes are large and can be easily identified.

All plants were grown in clay pots in the greenhouse. Spikes were tagged at the time of anthesis and were harvested 19 days after pollination. Immature seeds were surface sterilized with 1.5% sodium hypochlorite (w/v) for 5 min and then rinsed twice with sterile distilled water. A total of 50 embryos were extracted and cultured on a callus induction medium

Table 1. Chromosome analysis in immature embryo derived calluses of barley cv. 'Himalaya'

Callus types	Callus nos.	2n chromosomes no.						Others[a]	Total cells	Diploid (%)
		7	14	15	21	28	56			
Morphogenic	1	–	190	–	–	–	–	–	190	100.0
	2	–	170	–	–	–	–	–	170	100.0
	3	2	198	–	–	–	–	–	200	99.0
	4	–	200	–	–	2	–	–	202	99.0
	5	–	100	–	–	1	–	–	101	99.0
	6	–	118	–	–	3	–	–	121	97.5
	7	–	147	–	–	17	–	–	164	89.6
	8	–	100	–	2	10	2	–	114	87.7
	9	–	60	–	–	16	2	–	78	76.9
	10	–	117	–	3	33	5	–	158	74.1
Non-morphogenic	1	–	49	2	2	49	2	44	148	33.1
	2	–	10	1	3	9	–	59	82	12.2
	3–7[b]									

[a] Others (no. of cells observed are in parenthesis): 13+2 telocentrics (5), 14+1 telocentrics (7), 14+2 metacentrics (2), 18 (2), 19 (5), 19+1 acrocentric (1), 21+1 telocentric (8), 21+2 telocentrics (5), 21+3 telocentrics (2), 21+1 ring (3), 21+1 dicentric (1), 22 (6), 22+1 ring (1), 22+2 dicentrics (4), 23 (1), 24 (6), 24+1 fragment (5), 24+1 acrocentric (1), 25 (2), 25+1 telocentric (3), 27 (4), 27+1 telocentric (1), 7+29 telocentrics (1), fragment chromosomes (3), clumped chromosomes (14), uneven cell division (5), high uncountable ploidy chromosome number (5)

[b] Lacked mitotic cell division

containing Murashige and Skoog (1962) salts, B-5 vitamins (Gamborg et al. 1968), 20 g/l sucrose, 2 mg/l 2,4-D (2,4-dichlorophenoxy-acetic acid) and 8 g/l purified bactoagar at pH 5.8. Five embryos were cultured per Petri dish and incubated for 4 weeks at 24 °C in the dark. Calluses were transferred to the same medium with 0.5 mg/l 2,4-D and were kept under a 9 h photoperiod of 5000 lux white fluorescent light for 3 weeks. Calluses were characterized as morphogenic (regenerable) and non-morphogenic (non-regenerable). Morphogenic calluses were compact, dark green, with green spots and small shoots while non-morphogenic calluses were yellow to brown color and were also friable.

For chromosome analysis, all calluses were pretreated with ice cold water for 16–18 h. They were then fixed for 48 h in a freshly prepared mixture of 3 : 1 absolute ethanol : propionic acid. Ferric chloride (1 g/100 ml fixtive) was added to the fixative to intensify the staining of chromosomes. Calluses were stained in 0.7% aceto-carmine and placed in a refrigerator for 2–6 days. A squash preparation was made in 45% acetic acid. Ten morphogenic and seven non-morphogenic calluses were studied cytologically. Attempts were made to analyze chromosomes from 5–15 preparations per callus.

Results

Morphogenic calluses

Ten morphogenic calluses were studied cytologically to determine their chromosome number (Table 1). The expected diploid (2n=2x=14) chromosome cells predominated in all morphogenic calluses (Fig. 1 a). The percentage of diploid cells ranged from 74.1% to 100.0% (Table 1), however, haploid (2n=x=7), tetraploid (2n=4x=28), octoploid (2n=8x=56) and triploid (2n=3x=21) cells were also recorded in low frequencies (Fig. 1 b–e; Table 1). The occurrence of these chromosomal types varied from callus to callus. The calluses with the higher number of tetraploid cells were not as morphogenic as those which showed largely diploid cells.

Non-morphogenic calluses

All seven non-morphogenic calluses analyzed cytologically were friable, rough in texture and yellow to brown in appearance. They did not show visible morphogenic potentialities. Low dividing mitotic cells were observed in only two calluses while cell division was absent in the remaining five calluses (Table 2). The frequency of diploid cells in both non-morphogenic calluses was comparatively lower than those observed in morphogenic calluses. The majority of aneuploid cells also carried chromosome structural changes such as ring (Fig. 2 a), acrocentric (Fig. 2 b), dicentric (Fig. 2 c) telocentric (Fig. 2 d) and fragment chromosome(s). The aneuploid cells were around haploid, diploid, triploid and tetraploid chromosome numbers. In addition, cells with uneven chromosome migration at telophase were seen occasionally (Fig. 2 e). The uneven chromosome separation during cell division may have contributed to the origin of cells with uncountable microchromosomes (Fig. 2 f). Thus, the loss of regenerability of a callus was attributed to an increased frequency of cells with aneuploid and polyploid chromosome numbers (Table 1).

Do chromosome aberrations occur during culture or do they pre-exist in the cultured tissues? To answer this

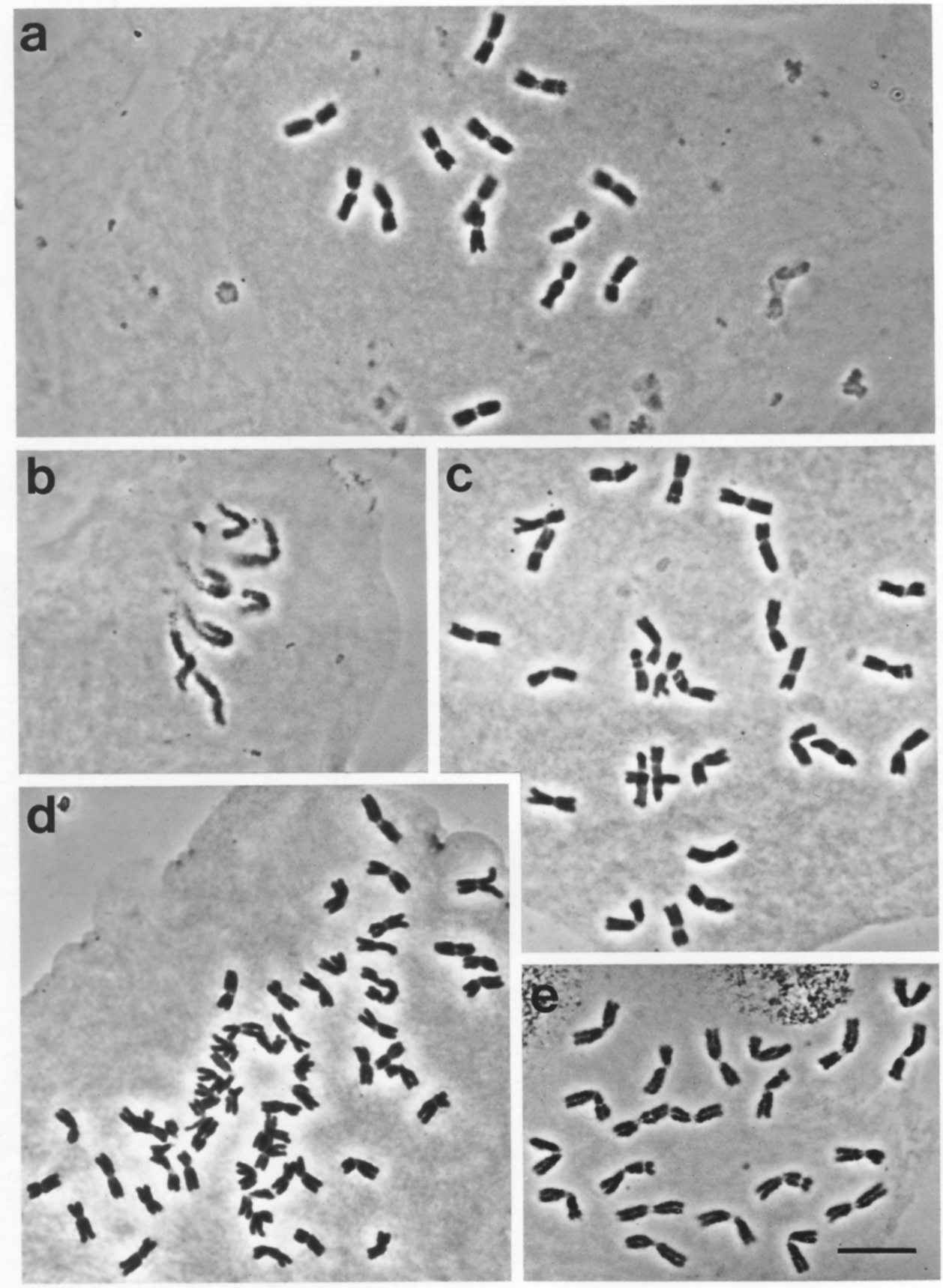

a
b
c
d
e

Table 2. Chromosome count in immature embryo derived callus of barley cv. 'Himalaya' after 1–15 days in culture

Days after embryo cultured	2n chromosome no.					Fragments	Total cells studied	% diploid
	14	14+1 telo	15	28	13+2 telo			
1	182	–	–	–	–	–	182	100.0
2	130	–	–	–	–	–	130	100.0
3	248	–	–	–	–	–	248	100.0
6	202	–	1	1	–	–	204	99.0
7	255	1	–	2	–	–	258	98.8
8	50	–	–	3	1	–	54[a]	92.6
10	372	–	–	9	2	2	385	96.6
15	149	–	–	11	–	–	160	93.1

[a] Low mitotic index, condensation of chromation in the interphase cells

question, chromosome counts of five calluses each were conducted after 1, 2, 3, 6, 7, 8, 10 and 15 days of embryo inoculation although immature embryos were not callused in the beginning. It is evident from Table 2 that chromosome aberrations, mainly tetraploid, initiated in culture after 6 days. After 8 days, all five cultured embryos showed low mitotic indexes. The interphase nuclei were enlarged with condensed chromatin. The tetraploid cells began to increase gradually after 10 days. These observations suggest that chromosome aberrations were induced during the culture.

Discussion

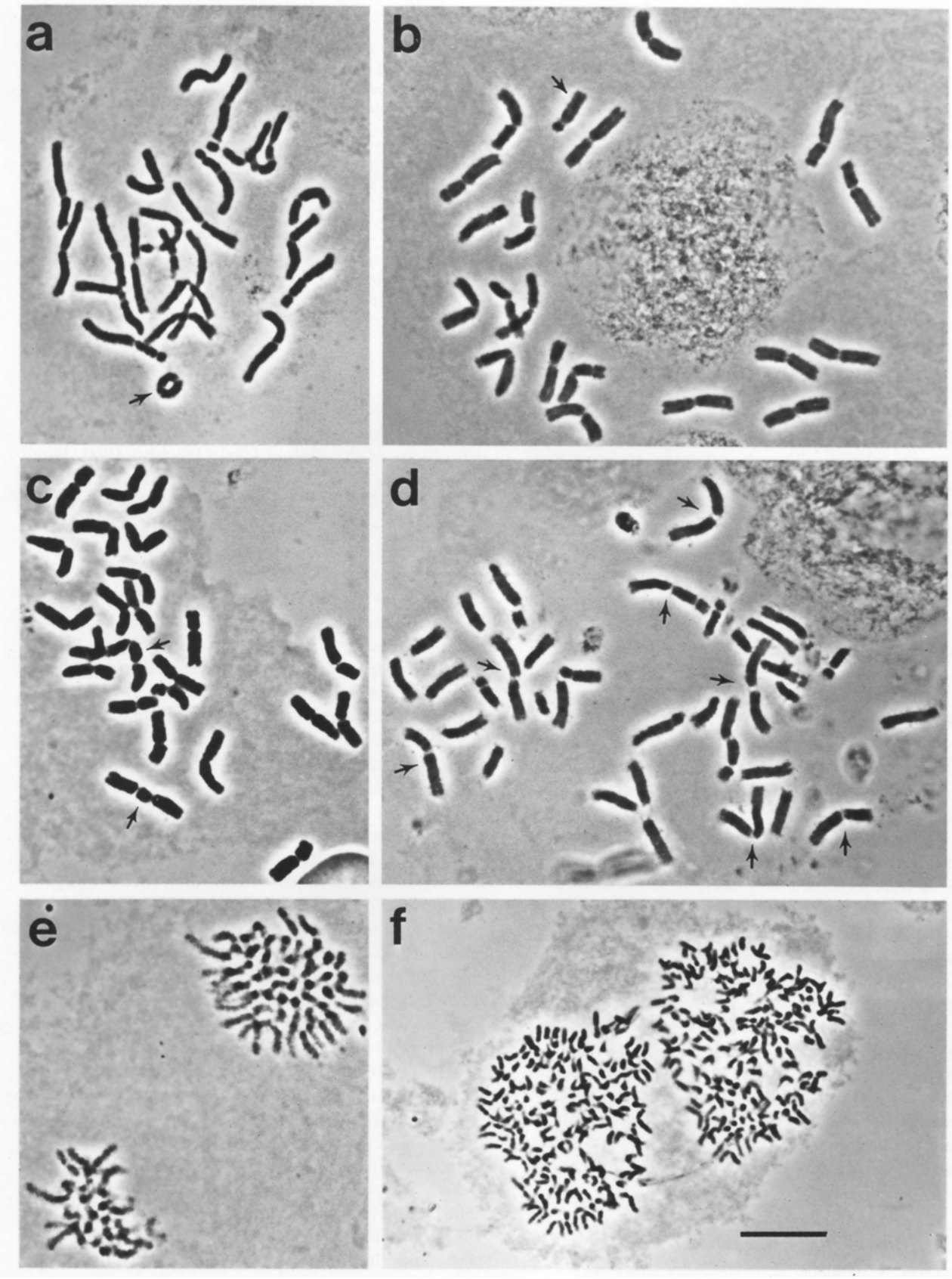
The above results have demonstrated that morphogenic ability of a callus depends upon the chromosome constitution of the cells. Expected diploid (2n=2x=14) cells predominated in morphogenic calluses of barley. In contrast, both numerical and structural chromosome changes were observed in a majority of cells of non-morphogenic calluses. This suggests that a balanced chromosome number in a callus is a prerequisite for regenerating plants. This is in agreement with the results reported in *Haworthia setata* Haw (Ogihara and Tsunewaki 1979), *Nicotiana tabaccum* L. (Murashige and Nakano 1965, 1967) and *Pisum sativum* L. (Torrey 1965, 1967).

The predominance of aneuploidy in non-morphogenic calluses may have contributed to the loss of plant regenerability. Barley being a basic diploid expresses diagnostic morphological changes when a complete chromosome (primary trisomic) or a telocentric chromosome (telotrisomic) is added to its normal chromosome complement of 2n=14 and cannot tolerate deficiencies at the diploid level (Tsuchiya 1960; Singh and Tsuchiya 1977). Thus, the mixture of polyploid and aneuploid cells disturbs the physiological and genetical balances of the cells causing the death of non-morphogenic calluses.

It is interesting to note that aneuploid cells carried chromosomes around haploid (2n=x=7), diploid (2n=2x=14), triploid (2n=3x=21) and tetraploid (2n=4x=28) chromosome number and, in addition to this, they possessed chromosome structural changes: for example, ring chromosome, acrocentric, telocentric, dicentric and fragment chromosomes. Analogous results have been recorded in several plant species (Torrey 1967; Sacristán 1971; Novák 1974; Orton 1980; Singh 1981; Murata and Orton 1984). Here, tissue culture may play an important role in restructuring the chromosome after interspecific and intergeneric hybridization. Once a desired chromosome combination is accomplished, efforts should be directed to bring the chromosome number to a genetic balance level by media modification which was demonstrated by Torrey (1959). He discovered by chromosome analysis of pea root callus that the multiplication of tetraploid cells was favored by the medium supplements, yeast extracts and kinetin, but by media modification he obtained uniformly diploid cells.

The results presented in this study clearly show that chromosomal breakage is a non-random phenomenon which occurred preferentially in the centromere or at the centromeric region and generated a high frequency of telocentric and acrocentric chromosomes, respectively. Since all of the barley chromosomes carry centromeric heterochromatin (see Singh and Tsuchiya 1982), it is likely that breaks occurred in the heterochromatin. In this aspect, the mode of chromosome breakage induced in culture is not different than those

Fig. 1. (a–e). Photomicrographs of cells observed in morphogenic calluses of barley. **a** Diploid (2n=2x=14); **b** Haploid (2n=x=7); **c** Tetraploid (2n=4x=28); **d** Octoploid (2n=8x=56); **e** Triploid (2n=3x=21). Bar represent 10 μm


a
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produced by X-ray treatment or any other mutagen; for example, Khush and Rick (1968) working with tomato found that about 60% of the breaks induced by X-ray treatment occurred in the heterochromatin.

The occurrence of tetraploid (2n=4x=28) and octoploid (2n=8x=56) cells in both morphogenic and non-morphogenic calluses suggests that polyploidization followed in a progression fashion (2x–>4x–>8x). This indicates that chromosome doubling occurred through endoreduplication or by c-mitosis (see D'Amato 1952; Torrey 1967). However, the observation of several anaphase cells revealed that chromosomes separated during mitotic anaphase but failed to reach to their respective poles. This may have been due to disturbed spindle formation (c-mitosis). Therefore, such cells will have a doubled chromosome number in the next mitotic division and the cycle will keep on repeating.

In the present study, a low frequency (2/200 cells) of haploid cells (2n=x=7) were observed in a morphogenic callus. Haploid cells in appreciably low frequency have been recorded in *Daucus carota* L. (2n=2x=18) suspension cultures (Mitra et al. 1960), callus cultures of *Allium sativum* L. (2n=2x=16) and *Triticum aestivum* L. (2n=6x=42) (Novák 1974; Novák et al. 1978) and protoplast derived calluses of *Solanum tuberosum* L. (2n=4x=48) (Sree Ramulu et al. 1984). However, such a low frequency of haploid cells may not compete with diploid or polyploid cells during cell division and they will be eliminated eventually.

It is evident from this study that chromosome aberrations are induced in the cultured tissues during the culture possibly by media components such as salts or growth hormones. The majority of reports agree that chromosome aberrations are generated during the culture and do not pre-exist in the cultured tissues (Dermen 1941; Huskins and Steinitz 1948; Naylor et al. 1954; Partanen et al. 1955; McMahon 1956; Van't Hof and McMillan 1969; Singh and Harvey 1975; Nuti Ronchi et al. 1976; Singh 1981). On the other hand, Shimada and Tabata (1967) observed variable chromosome numbers in pith tissues of *N. tabaccum* grown in vivo. If these tissues are grown in vitro, polyploid and aneuploid cells are expected to be generated more than those tissues lacking initial chromosome abnormalities.

In any case, the present study has demonstrated that morphogenic calluses have normal chromosome complements while numerical and structural chromosome changes predominate in non-morphogenic calluses. Thus, non-morphogenic callus becomes physiologically and genetically unbalanced, leading to the loss of the capacity to regenerate complete plants. It is suggested that chromosomes of a non-regenerating callus should be determined before altering the media composition.

Fig. 2 (a–f). Photomicrographs of cells observed in non-morphogenic calluses of barley. **a** 22+1 ring, *arrow* indicates a ring chromosome; **b** 19+1 acrocentric, *arrow* indicates an acrocentric chromosome; **c** 22+2 dicentric, *arrows* indicate dicentric chromosomes; **d** 7+29 telocentrics, *arrows* indicate complete chromosome; **e** telophase with unequal chromosome separation; **f** two daughter nuclei with numerous microchromosomes. Bar represents 10 μm

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