II. PLOIDY DEPENDENCE OF CHROMOSOMAL VARIATION IN CALLUS CULTURES OF HYOSCYAMUS MUTicus L.

Introduction

Although the regeneration of plants through callus cultures is essentially a consequence of an asexual process, interestingly enough the regenerants exhibit considerable phenotypic variability. Genetic factors like point mutations, changes in repetitive DNA and structural and numerical chromosome changes have been accounted for such variations (Karp and Bright 1985). Out of all these possibilities chromosomal instability is of most frequent occurrence in callus cultures (Bayliss 1980, D'Amato 1985) and may be considered a major contributor for somaclonal variation. Nevertheless, the extent of somaclonal variation in different systems is not a constant feature and differences exist depending upon genotypic differences, including the differences in the ploidy of the starting material (Karp et al. 1987a). The present study was performed to understand: (i) the effect of ploidy on chromosomal variation, (ii) sequence of chromosomal variation with respect to basic genome and time, and (iii) chromosome number in relation to morphogenetic capacity.

Materials and methods

Seeds of diploid \((2n = 28)\) and its autotetraploid strain, CIMAP/HMT-1 developed by Lavana (1986b) of the medicinally important solanaceous plant, Hyoscyamus muticus, were used to generate callus cultures. Surface sterilised seeds were germinated on MS basal medium (Murashige and Skoog 1962), and seedlings were allowed to grow up to the 4-5
leaf stage on MS basal medium supplemented with Nitsch's vitamins (Nitsch 1969). The stem explants from such seedlings were cultured for callusing on MS basal medium supplemented with NAA (1 mg/l) and kinetin (0.1 mg/l). Sufficient calli became available within one month in both diploid and tetraploid material. The calli were regularly sub-cultured on fresh medium at monthly intervals.

For cytological analysis the calli generated from both diploid and tetraploid explants were concurrently fixed overnight in Carnoy's fixative at regular intervals both directly, as well as following pre-treatment in saturated aqueous solution of para-dichlorobenzene for 3 hrs. Chromosomal data were recorded from approximately 100-150 well analysable cells in each fixation consecutively for 12 months. The peripheral soft callus tissue was squashed in 45% acetic acid following staining in 2% acetoorcein + 1N HCl (9:1), and microscopically examined.

Results

Since the chromosome size is very small in this species (1.28-2.57 μm), it is extremely difficult to work out structural changes in the chromosomes, so that observations have been confined to numerical variations. The data on chromosome counts are depicted in Figure 3, and the representative cytological features are illustrated in Figure 4. Remarkable variation of chromosome numbers was observed in both diploid and tetraploid cultures. Initially, the subcultures exhibited predominantly the genomic level of the starting material, but subsequently and gradually heterogenous population of euploid and aneuploid cells originated imparting the subcultures a aneusomatic status. With
Figure 3: Relative account of sequential chromosome flow in diploid and tetraploid callus cultures in *Hyoscyamus muticus*. 2X = 28±7, 3X = 42±7, 4X = 56±7, 4X+ = > 63.
Fig 4 Patterns of chromosomal instability and nuclear variation in callus cultures of *Hypecoum muticum*. Cells showing: a 28 chromosomes = 2x, b 53 chromosomes = 4x, c 42 chromosomes = 3x, d 84 chromosomes ≥ 6x, e > 12x, f lagging and extrusion of chromosomes at anaphase, g multinucleate condition with variable nuclear size.
time 2x calli gradually shifted to polyploidy, particularly tetraploidy, and the 4x calli maintained their 4x chromosome number state, but also attempted to return to the 2x level. Variations beyond pentaploidy were only occasionally observed in both cases. Some kind of mitotic cells synchrony was observed in the peripheral meristematic regions of the calli. Anaphase disjunctional anomalies like multipolar spindle, micronuclei formation, precocious separation and lagging of chromosome were occasionally encountered. Micronuclei formation was of relatively common occurrence, but the formation of multinucleate cells was a rare feature. The frequency of dividing cells decreased continuously in subsequent subcultures. Older callus cultures, i.e. subcultures grown for 20-30 days in the callus medium, allowed the emergence of adventitious roots. The examination of chromosomes from such roots revealed the euploid chromosome level (both 2x and 4x) as far as it could be examined.

Discussion

The survey of literature dealing with the chromosomal studies in callus cultures has established beyond doubt that disorganised callus growth is associated with chromosomal instability. Such chromosomal variation involves structural and/or numerical changes, the extent of which may be species-specific and genotype and organ dependent (Bayliss 1980, D'Amato 1985, Karp 1986, Orton 1987). Notwithstanding, polyploidy is the most frequently observed numerical variation encountered in the proliferating callus cultures. The specific question of the present study was: how does such chromosome change relate to genomic dosage? The callus cultures derived from the $2n = 2x = 28$ genotype of H. muticus
and its artificially induced stable tetraploid genotype (4x = 56) have been used for an attempt to answer this question. The callus cultures grown from both the genotypes (2x, 4x) exhibited significant amounts of chromosomal instability, increasing with time, but by deducing the variation to the unit genome dosage it was revealed that the 4x genotype was relatively more resistant than its 2x counterpart in terms of frequency and extent of chromosomal variability. Moreover, the callus of the 2x genotype attempted to attain the genomic dosage of 4x, possibly as a sequel of stress caused by callus growth under artificial conditions. The 4x genotype is probably lesser constrained and as such is able to retain its genomic level with minor fluctuations. Nagl (1978) has discussed extensively the role of endopolyploidy and polyteny in phylogeny and organogenesis and suggested polyploidy to be a genomic strategy to meet various biological situations. The occurrence of polyploid cells beyond the 4x level was meagre in the present case and was encountered only after long culture times. The observations suggest that a genomic threshold is attained, in the present material at the 4x status. This is consistent with the observations that, in experimentally induced polyploidy, a ploidy level beyond 4x is seldom successful (Gottschalk 1985).

The numerical chromosome variation during callus growth is evidently attained by some kind of endoreduplication and anaphase nondisjunction. Certain spindle anomalies do affect the exact genome multiplication and give rise to aneuploid cells, the frequency of which increases with time as a consequence of multiplication of divisional errors.
The spectrum of ploidy variation as observed in callus cultures in the present study could also be demonstrated at the level of regenerated plants derived from protoplast and explant cultures of diploid and tetraploid potato, *Solanum brevidens*. The diploid material passed through in vitro culture showed a tendency towards tetraploidy, whereas the tetraploid one exhibited relative stability of its genomic level (see Karp and Bright 1985). A similar tendency of genomic doubling following in vitro mediated regeneration has also been observed in monoploid cultures of potato which resort to diploid - tetraploidy (Karp et al. 1985), and haploid cultures of *Crepis capillaris* and *Nicotiana tabacum* resorting to diploidy (Sacristan 1971, Novak and Vyskot 1975).

In spite of aneusomaty and chromosomal instability also in organogenetic calli, only cells possessing balanced genomes (euploids) with minor variations are evidently potent to differentiate and give rise to regeneration. The aneuploid cells are selected against, and a sort of regeneration differential may operate on the heterogenous population of cells allowing mainly the euploid cells (possessing genomic balance) to proliferate and regenerate faster compared to heteroploid ones. For this phenomenon of regeneration differential the term 'morphogenetic sieve' is suggested. This has been clearly demonstrated by this study for the case of the initial adventitious roots. Nevertheless, improved culture conditions and changes in growth medium may impart the ability of differentiation to heteroploid cells as well, but still with a reduced potential as shown by Karp (1986) and Karp et al. (1987b).