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# Hormonal Autonomy of *Arabidopsis thaliana* Calli

M. ONDŘEJ, K. PEŠINA and DANIELA PAVINGEROVÁ

Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha*

**Abstract.** The growth of *Arabidopsis thaliana* calli on media without growth regulators was studied. Calli under study showed autonomy for cytokinins independently whether cultivated on the light or in the dark. When cultivated in intense light, they were able to grow, either transiently or permanently, on the medium without any growth regulators. In the dark, they were strictly dependent on 2,4-D in the medium. Both the intensity of growth and the duration of the transient growth on the medium without growth regulators in the light decreased with the duration of the previous cultivation on the medium with growth regulators. The intensity of growth on the medium without growth regulators was best and the growth was permanent in the callus clone of spontaneous origin which was never treated by growth regulators. The degree of chromosomal variability (assessed as the number of chromocentres) in this callus line was lower than that in calli induced on media with growth regulators and then transferred onto medium without growth regulators.

One of the criteria of crown gall tumor transformation of plant cells by *Agrobacterium tumefaciens* is their unlimited *in vitro* growth without external growth regulators (WHITE and BROWN 1942). In our experiments with *Arabidopsis thaliana* (ONDŘEJ *et al.* 1984, PAVINGEROVÁ *et al.* 1984) we have found that the hormonally autonomous *in vitro* growth of calli in the light is not limited to crown gall tumors but it occurs also in untransformed calli. As hormonal autonomy of calli of *Arabidopsis thaliana* has never been described, we studied this phenomenon in detail and we also tried to look for a possible relationship between hormonal autonomy and the degree of chromosomal variability.

Undifferentiated *in vitro* growth is usually accompanied by increased chromosomal variability (TORREY 1967, NOVÁK 1981). The relationship between hormonal autonomy and the chromosomal variability was studied in tobacco and either no correlation was found at all (BINNS and MEINS 1980), or the relationship was not clear-cut (SACRISTAN and MELCHERS 1977, MOURAS and LUTZ 1979). On the other hand, SHERIDAN (1974) described hormonally autonomous calli of *Lilium longiflorum*, which were able to grow only in the light; they were diploid and chromosomally stable.

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* *Address:* 370 05 České Budějovice, Na sádkách 702, Czechoslovakia.

Of course, the degree of chromosomal variability in tissue cultures is conditioned, first of all, by the genotype of the plant, secondly by the tissue of origin of the explant and, last but not least, by exogenous growth regulators; when no growth regulators were added to the medium, chromosomal numbers of some objects might be relatively constant.

In this study we tried first to define the range of the variability of the intensity of the hormonally autonomous growth of *Arabidopsis thaliana* calli in dependence on the interval of previous *in vitro* cultivation with exogenous supply of growth regulators. Secondly, we tried to estimate the contribution of the exogenous growth regulators to the degree of chromosomal variability.

## MATERIAL AND METHODS

Seeds of *Arabidopsis thaliana* (L.) HEYN., cv. Dijon, obtained from Dr. T. Gichner (Institute of Experimental Botany, Praha) were used in most of the experiments. One of the calli under study appeared spontaneously on *A. thaliana* cv. Landsberg carrying mutations erecta (er) and chlorate resistance 1 (chl-1), obtained from Dr. F. Braaksma (Biological Centrum, University of Groningen).

The PG media of NEGRUTIU *et al.* (1975) were used for the cultivation of calli. Calli were derived from plants cultivated *in vitro* on agar $PG_0$ medium. The $PG_1$ medium was used for the callogenesis and $PG_2$ for the maintenance of calli. $PG_0$ is a medium without growth regulators; $PG_2$ has a similar composition as $PG_0$, but in addition it contains 1 mg 2,4-D and 0.05 mg kinetin per litre. $PG_1$ has half the concentration of inorganic compounds but otherwise a similar composition as $PG_2$. If not stated otherwise, calli on $PG_1$ and $PG_2$ were cultivated in the dark and calli and plants on $PG_0$ in the light (5 000 lx, 16 h photoperiod). Temperature was kept on 25 °C. The explants were subcultivated at four week intervals.

For the evaluation of the growth intensity, calli of the initial mass of 10 mg were cultivated in scintillation vials with diameter 2 cm containing 5 ml of the appropriate medium. After four weeks of cultivation, calli were weighed with the accuracy $\pm$ 1 mg. The growth of 200 calli was evaluated in each experimental variant and all experiments were replicated at least twice. Slight modifications are described in Results. The lysopine dehydrogenase or nopaline dehydrogenase activities were tested by the method of OTTEN and SCHILPEROORT (1978).

For anatomical and cytological studies, calli were fixed in modified Carnoy (ethanol : acetic acid : chloroform 6 : 3 : 1) or in Taylor's fixative, if the material was embedded in paraffin and cut slices of the thickness 9 μm were made. Either objects *in toto* (without embedding in paraffin) or slides were stained by Feulgen's method.

As the mitotic index in *A. thaliana* calli was very low (under 1%) and *A. thaliana* chromosomes are extremely small, the numbers of interphase nuclear chromocentres (prochromosomes) were scored as a substitute of the numbers of metaphase chromosomes, as introduced by RÖBBELEN (1957). Diploid chromosome number in *A. thaliana* is 10. The homogeneity of distribution

*Abbreviation used:* 2,4-D = 2,4-dichlorphenoxyacetic acid.

of the number of chromocentres was tested by Brand-Snedocor's formula of the $\chi^2$ test (WEBER 1964) before pooling the results obtained in different calli.

## RESULTS

In the preliminary experiments we found that *A. thaliana* calli can grow on $PG_0$ in the light, but when cultivated on this medium in the dark, they turn brown and die within four weeks of cultivation. When cultivated on $PG_2$ medium, calli can grow equally well in the light and in the dark. Intense illumination is a necessary condition of autonomous *in vitro* growth also in *Arabidopsis thaliana* crown galls (ONDŘEJ *et al.* 1984).

### Degree of Hormonal Autonomy of Calli Cultivated for Two Years on Medium with Growth Regulators

The growth of calli, which have been cultivated on $PG_2$ medium for about two years either with or without kinetin was compared and was found to be similar. A callus line, cultivated on $PG_2$ (with kinetin) for four weeks showed fresh mass increase from 10 mg to $120.8 \pm 3 \times 2.5$ mg. When kinetin was omitted, the fresh mass increase was to $120.4 \pm 3.4$ mg.

The calli can survive and increase their mass on $PG_0$ in the light (Fig. 1). As kinetin does not cause any difference in growth, the observed retardation of the growth should be attributed to the absence of external auxin in the medium.

In further succeeding subcultivations, some calli survived, but just a negligible growth was observed.

### Hormonal Autonomy of Calli which Have Been Cultivated for a Short Period with Growth Regulators

Calli were derived from the leaves and shoots of *A. thaliana* cv. Dijon on $PG_1$. After four weeks they were transferred on $PG_0$ in the light and $PG_2$ in the dark. Individual callus clones of independent origin were further subcultivated at four week intervals in two ways, which established two variants of experiment. Each callus was divided either into pieces weighing about 10 mg each (first variant) or into pieces weighing 50 mg each (second variant) and a single piece of the callus tissue was put into each vial. About

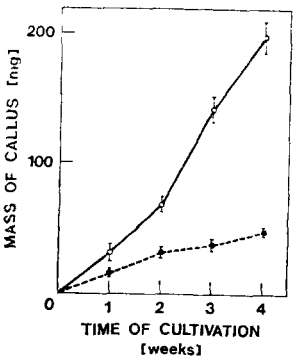


Fig. 1. The growth intensity of *Arabidopsis thaliana* callus clone which has been cultivated for two years on $PG_2$ medium. ○ — the growth on $PG_2$ medium; ● — the growth on $PG_0$ medium. Means and their 5% confidence intervals are given.

100 independent callus clones were cultivated in each of these variants. The growth of calli was, by visual inspection, equally intense as on $PG_2$ for at least two subcultivations, but the appearance of calli changed gradually. The original calli, derived on $PG_1$ and maintained on $PG_2$ were of a soft, globular consistence with globulae 1—2 mm and of creamy colour. During subcultivation of 10 mg pieces on $PG_0$ the colour of the calli changed to yellowish and finally different tints of green appeared in different clones and the consistence of the calli became hard. The same change of appearance of calli on $PG_0$ was found in crown galls (ONDŘEJ *et al.* 1984, PAVINGEROVÁ *et al.* 1983). In the third subcultivation, shoots or leaves differentiated occasionally.

In the 50 mg variant, roots often appeared on the whole surface of the calli. Roots never appeared together with leaves of floral axes. After the roots reached the length of 5—10 mm, the growth of the calli with roots ceased and the calli died.

Generally, leaf calli grew better and they were able to survive longer than these derived from shoots. After one year, 11 callus clones out of 182 initial ones are still propagated. Their growth is very slow and when the calli reach the size of about 5 mm, they get brown and die, if not subcultured before reaching this stage.

Hormonally autonomous tissues were derived, by a similar way and to a similar extent, also from the calli derived on $PG_1$ and subcultured twice on $PG_2$. In the third subcultivation, they were transferred and further subcultivated on $PG_0$. In this experiment, all the calli changed their appearance much more quickly than in the previous one; the differences were already clear-cut in the first subculture. Either greening and change of the consistence of calli or intense root formation appeared. The calli with roots died in the next subculture. Part of the calli without roots was further subcultivated, but it was impossible to maintain them as long as in the previous experiment; they died sequentially during succeeding subcultivations and no clone out of the original 200 was propagated longer than six months.

Despite the broad scale of these series of experiments no callus intensively growing without external supply of growth regulators has been selected.

#### Hormonal Autonomy of Callus of Spontaneous Origin

During cultivation of intact plants of *A. thaliana*, cv. Landsberg on $PG_0$, a single callus appeared spontaneously on the hypocotyl of a plant. After detachment from the plant, the tissue proliferated rapidly on $PG_0$ in the light as a green, soft callus, which showed a low degree of differentiation of buds, leaves and even fertile shoots. The growth intensity corresponds approximately to that of calli on $PG_2$. This callus is capable of unlimited growth on $PG_0$ in the light and the growth intensity does not decrease during succeeding subcultivations. The original plant and the resulting callus clone were never treated by growth regulators or by *Agrobacterium tumefaciens*. The detection of lysopine dehydrogenase or nopaline dehydrogenase activities was negative, so accidental infection by *Agrobacterium tumefaciens* and tumor transformation can be excluded.

#### Anatomical and Caryological Studies of Calli

The hormonally autonomous leaf calli, derived on $PG_1$, cultivated for 2 subcultivations on $PG_2$ and then on $PG_0$ were anatomically differentiated.

Except large parenchymatic cells and groups of small meristematic cells they consisted of a considerable proportion of tracheal cells which, in some calli, formed the main proportion of cells. The calli regenerated roots. It was possible to distinguish anatomically the epidermis, the cortex and vascular tissues on the differentiated roots.

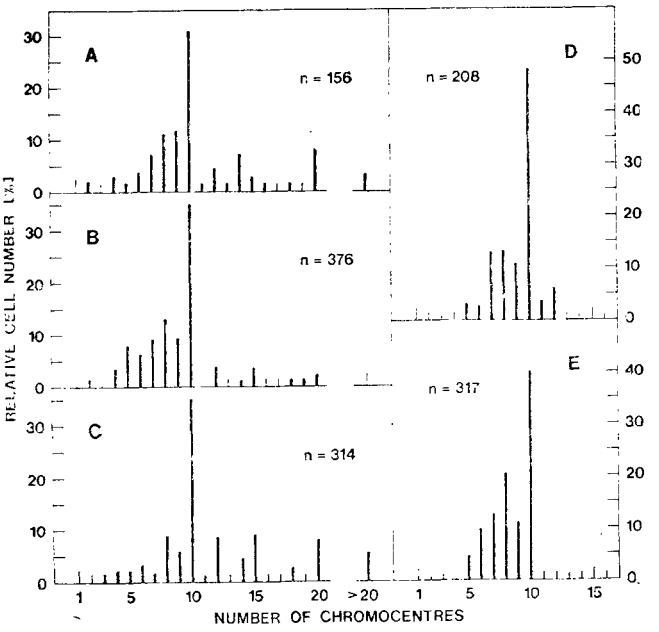


Fig. 2. Distribution of chromocentre numbers in the cell nuclei of the habituated callus clones HB 11 (A), HB 18 (B), HB 34 (C), of the callus of spontaneous origin (D) and of leaf cells (E). $n$ = cell number.

Three callus clones, HB 11, HB 18 and HB 34 were randomly chosen for the evaluation of the number of chromocentres in their interphase nuclei. Results are seen in Fig. 2 A—C. Except diploid and supposedly aneuploid cells, also cells with higher numbers of chromocentres were observed, which corresponded to 4 n, 5 n, and 6 n, mainly in parenchymatic tissues. A cell with 10 chromocentres is shown in Fig. 3 A and cells with higher numbers of chromocentres in Figs. 3 B and 4. Diploid and polyploid (Fig. 5) metaphases were also occasionally found. Binucleate and trinucleate cells occurred and also nuclei with extraordinarily large chromocentres appeared. The last are a probable manifestation of endopolyploidy.

The tissues of the growth-regulators autonomous callus derived from the ecotype Landsberg consist of large parenchymatic and small meristematic cells. Tracheal cells occur only sporadically. Most of the cells show numbers of chromocentres, corresponding to diploidy and hypodiploidy. Only a small part of the cells showed chromocentre numbers above ten (Fig. 2 D).

For comparison, the chromocentre numbers in the cell nuclei of leaf tissues

of plants growing from seeds were evaluated (Fig. 2 E). It shows approximatelly the same variation scale, as the hormonally autonomous culture.

The distribution of chromocentre numbers in all samples showed significant differences ($\chi^2_{exp.} > \chi^2_{th.}$, $\alpha < 1\%$).

## DISCUSSION

We have found that *A. thaliana* calli cultivated in the light are capable of transient and in some cases even permanent hormonally autonomous growth. The degree of the ability to grow without external supply of growth regulators was dependent on the previous cultivation of the calli. The growth was best when the callus was of spontaneous origin and provided that it was never cultivated on media with growth regulators. External supply of 2,4-D in the medium even during single subculture caused a long-term decrease of the ability of further autonomous growth, probably by induction of an irreversible decrease of the synthesis of auxins by the callus cells themselves. If the calli are propagated as large pieces (50 mg), they show less hormonal autonomy than when they are propagated as smaller pieces (10 mg). The decrease of the autonomous growth seems to be the more pronounced the longer was the cultivation on medium with growth regulators. This situation is the reverse of habituation. Habituation is the lost of requirement for exogenous growth regulators after a variable number of transfers in culture (MEINS and LUTZ 1980). There is either habituation for cytokinins or for both auxins and cytokinins. These two degrees arise either as succeeding events or as a single event. Habituation is either spontaneous or it can be induced by different factors, for instance by heat or by growth regulators (MEINS and LUTZ 1980).

In *A. thaliana*, freshly derived calli are relatively autonomous for growth regulators. If subcultivated on media with 2,4-D, the autonomy is gradually, but irreversibly lost and the tissue became more and more dependent on supply of 2,4-D in the medium. When cultivated without growth regulators, *A. thaliana* calli are capable of transient, but long-term or even permanent very slow autonomous growth. This autonomy is strictly dependent on intense illumination and it is probable that some intermediate products of photosynthesis are involved.

The general occurrence of hormonal autonomy, observed in *A. thaliana* calli is a rare phenomenon. Although both auxin and cytokinin habituated tissues were derived occasionally in calli of several plant species, their general occurrence is limited mainly to genetic tumors of *Nicotiana* and other genera (BAYER 1982) and to crown gall tumors (WHITE and BROWN 1942).

The degree of hormonal autonomy observed in our experiments with *A. thaliana* is very near to that described in *Lilium longiflorum* (SHERIDAN 1974). Calli of this species also grow generally as independent of the external growth regulators, but dependent on illumination. They are green and they grow very slowly. It is very interesting that they are always diploid and chromosomally stable. On the contrary, our *A. thaliana* hormonally autonomous calli showed always some degree of chromocentre number variability, which was broader if the calli were, previously in their history, treated by growth regulators.

In our experiments, the number of chromocentres in interphase instead of the number of chromosomes in metaphase was evaluated, as generally

recommended for *Arabidopsis* (RÖBBELEN 1957). Chromocentres in interphase actually present partly spiralized centromere heterochromatin. The equality of the number of chromocentres and the number of chromosomes probably is not quite reliable in all tissues; in our experiments a considerable proportion of leaf interphase nuclei showed numbers of chromocentres considerably lower than ten.

The number of chromocentres certainly cannot exceed the number of chromosomes and therefore the numbers of chromocentres, indicating polyploidy can be taken as proven polyploidy. On the other hand, two homologous and even nonhomologous chromocentres can fuse. In consequence, the number of chromocentres might sometimes be lower than the actual number of chromosomes. This is one of the possible explanations of most of the numbers of chromocentres under ten in the leaf tissues (Fig. 2 E) and probably also in the callus tissues (Fig. 2 A—D).

Although the externally supplied growth regulators certainly are not the only cause of the chromosomal variability observed in callus tissues, we have found that the chromosomal variability of the callus tissue of spontaneous origin was lower than in calli induced by medium with growth regulators. On the other hand, hormonally autonomous *A. thaliana* calli cannot be considered as chromosomally stable, in contrast to hormonally autonomous calli of *Lilium longiflorum* (SHERIDAN 1974).

The experiments described here show that the growth of *A. thaliana* calli without growth regulators cannot be used as a criterion for their tumor transformation by *A. tumefaciens*. Actually, differences between untransformed *A. thaliana* calli and *A. thaliana* crown gall tumors in the *in vitro* growth are only slight, as comes out from the comparison of the results presented here and those of ONDŘEJ *et al.* (1984). The main difference lies in the kinetics of the growth. While *Arabidopsis teratomas* and undifferentiated crown gall calli proliferate much better on $PG_0$ in the light than on $PG_2$ in the dark, in *A. thaliana* untransformed calli it is the other way round. Unfortunately, this variability is so broad that this relationship cannot be taken as a criterion for distinguishing between transformed and untransformed states.

## REFERENCES

BAYER, M. H.: Genetic tumors: physiological aspects of tumor formation in interspecific hybrids. — In: KAHL, G., SCHELL, J. (ed.): Molecular Biology of Plant Tumors. Pp. 33—66. Academic Press, New York—London—Toronto—Sydney—San Francisco 1982.

BINNS, A. N., MEINS, J. R.: Chromosome number and the degree of cytokinin habituation of cultured tobacco pith cells. — Protoplasma **103** : 179—187, 1980.

MEINS, F., LUTZ, J.: The induction of cytokinin habituation in primary pith explants of tobacco. — Planta **149** : 402—407, 1980.

MOURAS, A., LUTZ, A.: Dénobrement et analyse morphologique des chromosomes chez quelques clones issus d'une souche anergiée de tabac. Relation avec l'anergie, le pouvoir tumoral et l'organogenèse. — Genetica **50** : 195—199, 1979.

NEGRUTIU, I., BEEFTINK, F., JACOBS, M.: *Arabidopsis thaliana* as a new model system in somatic cell genetics I. Cell and tissue culture. — Plant Sci. Lett. **5** : 293—304, 1975.

NOVÁK, F. J.: Chromosomal characteristics of long-term callus cultures of *Allium sativum* L. — Cytobios **49** : 371—379, 1981.

ONDŘEJ, M., PAVINGEROVÁ, D., NAŠINEC, V., HROUDA, M.: Growth requirements of *Arabidopsis thaliana* crown galls. — Biol. Plant. **26** : 5—10, 1984.

OTTEN, L., SCHILPEROORT, R. A.: A rapid micro scale method for the detection of lysopine and nopaline dehydrogenase activities. — Biochem. biophys. Acta **527** : 497—500, 1978.

PAVINGEROVÁ, D., ONDŘEJ, M., MATOUŠEK, J.: Analysis of progeny of *Arabidopsis thaliana* plants regenerated from crown gall tumors. — Z. Pflanzenphysiol. (In press) 1984.

RÖBBELEN, G.: Untersuchungen an strahleninduzierten Blattfarbmutanten von *Arabidopsis thaliana* (L.). — Z. indukt. Abstammungs. Vererbungsl. **88** : 189—252, 1957.

SACRISTAN, M. D., MELCHERS, G.: Regeneration of plants from "habituated" and "*Agrobacterium*-transformed" single-cell clones of tobacco. — Mol. gen. Genet. **152** : 111—117, 1977.

SHERIDAN, W. F.: Long-term lilly callus cultures. — In: LEDOUX, L. (ed.): Genetic Manipulation with Plant Material. Pp. 263—295. Plenum Press, New York—London 1974.

TORREY, J. G.: Morphogenesis in relation to chromosomal constitution in long-term plant tissue cultures. — Physiol. Plant. **20** : 265—275, 1967.

WEBER, E.: Grundriss der biologischen Statistik. — VEB Gustav Fischer, Jena 1964.

WHITE, H. P., BRAUN, A. C.: A cancerous neoplasm in plants. Autonomous bacteria-free crown gall tissues. — Canc. Res. **2** : 597—617, 1942.

*Figs. 3—5 are at the end of the issue.*

## BOOK REVIEW

DANOVICH, K. N., SOBOLEV, A. M., ZHDANOVA, L. P., ILLI, I. E., NIKOLAEVA, M. G., ASKOCHENSKAYA, H. A., OBRUCHEVA, N. V., KHAVKIN, E. E.: FIZIOLOGIYA SEMYAN. [PHYSIOLOGY OF SEEDS.] — Nauka, Moskva 1982. 318 pp. Cloth Rbl. 2.50.

Seeds are interesting objects from both the theoretical and practical point of view: as objects for developmental, evolutional or genetic studies and for their nutritional value. So there is no wonder that short after the monograph by Bewley and Black (1978, 1980) appears a Soviet book, where 8 specialists present their own experimental results together with literature data concerning the main aspects of seed physiology, as may be seen from the titles of individual chapters: I. Structure and formation of seed (Danovich) II. Storage of reserve compounds (Sobolev, Zhdanova) III. Seed vitality (Illi) IV. Seed dormancy (Nikolaeva) V. Water relations of seeds (Askochenskaya) VI. Seed germination (Obrucheva) VII. Metabolism of germinating seeds (Khavkin). There is a rich experimental material concerning e.g. plant behaviour under extreme conditions (Siberia, arid zone in Central Asia and Kazakhstan); in some chapters evolutional aspects are discussed, and some problems, like the function of phytohormons in the vitality of seeds and in germinating are treated from different theoretical aspects. Special interest is given to the differences between the structure and germination of seeds in Monocots and Dicots, to different types of cell growth and metabolic processes connected with them *etc.* Nomogramme of seed vitality and vitality tests, *etc.* are interesting for practice.

The text is accompanied by numerous figures and tables. World and Soviet literature is followed up to 1980.

The book brings interesting information to plant physiologists, ecologists, biochemists as well as to agronomists.

VĚRA HADAČOVÁ (*Praha*)

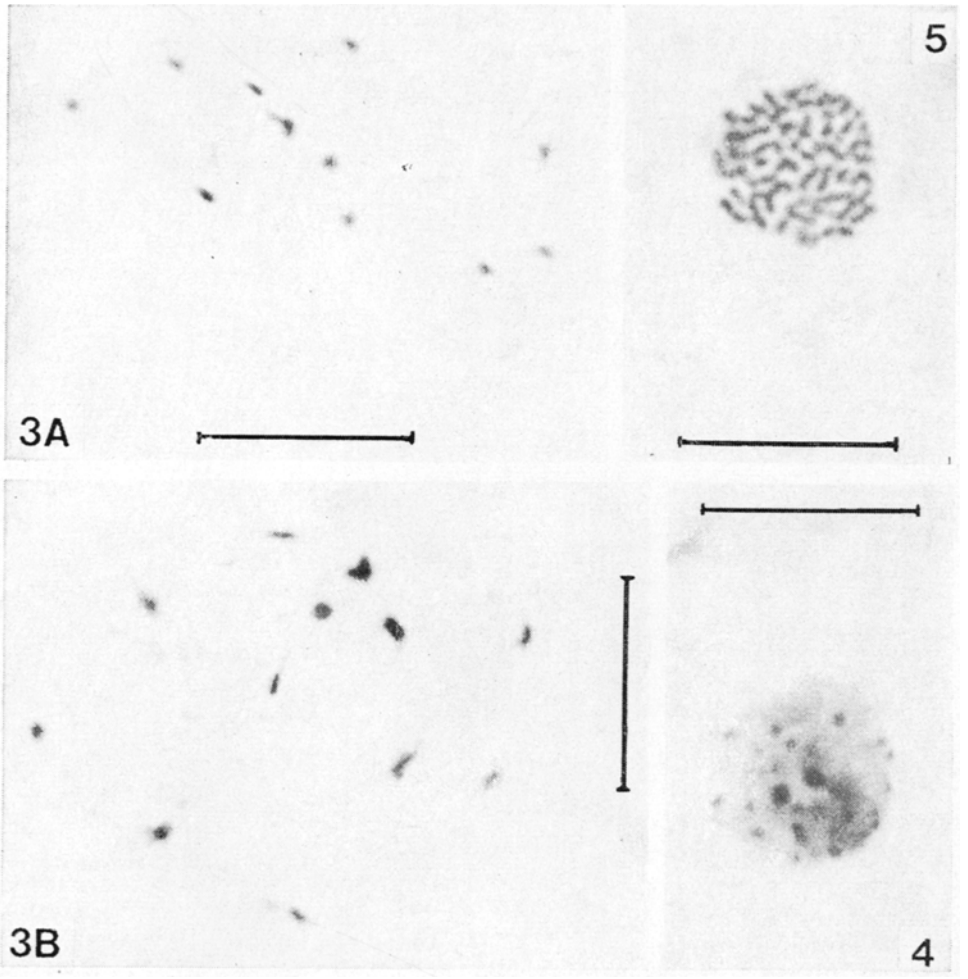


Fig. 3. Cell nucleus of the callus of spontaneous origin, showing 10 chromocentres (A) and 12 chromocentres (B); bar = 10 μm.
Fig. 4. Cell nucleus of the hormonally autonomous callus HB 34 containing 30 chromocentres. 18 chromocentres are seen at the level of focussing.
Fig. 5. The metaphase plate of the hormonally autonomous callus line HB 11, showing about 50 chromosomes.