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The Specificity of the Effect of 2,4-D and NAA of the Growth, Micromorphology, and Occurrence of Starch in Long-Term *Nicotiana tabacum* L. Cell Strains

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Abstract. The characteristic micromorphology of the tobacco cell strains, or its cyclic changes in the course of the subcultivation interval can be affected by auxin composition of the medium, *i.e.* by the application of either 2,4-D alone, or NAA, or their combination. On omitting one of the auxins, the over-all growth of the cultures is not substantially affected; however, the participation of various cell types, as well as the occurrence of starch grains are altered. The presence of 2,4-D alone results in an inhibition of starch occurrence, NAA alone causes a stimulation. There is no causal dependence of the occurrence or absence of starch grains on the stimulation of elongation (volume) growth, or, on the contrary, on cell division.

One of the factors influencing the utilization of plant cell cultures in hybridization and mutation experiments, is knowledge of the stability of phenotype characters, specifying the given culture. Therefore the previous paper (OPATRNÝ 1971) dealt with a detailed study of the micromorphology of two highly friable *Nicotiana tabacum* cell strains. The types of cells and cell aggregates were defined which form the population of these cultures, and the dynamics of their occurrence in the course of a subcultivation interval (SBI) was investigated. The variations observed proved to be specific for the given cell strain under standard cultivation conditions.

The aim of the present paper was to verify to what extent the growth and micromorphological characterization of the two strains can be affected by a change in auxin composition of the medium, *i.e.* by omission of one or both auxins (NAA and 2,4-D) employed in the standard cultivation. At the same time we followed the specificity of the effect of these auxins on the occurrence of starch grains in the cells of both cultures, as has already been observed (OPATRNÝ 1971, OPATRNÝ and OPATRNÁ 1972).

Material and Methods

The cell strains, denoted as VBI-T and VBI-O, were derived eight years ago from primary cultures of stem pith cuttings of *Nicotiana tabacum* L.,

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cv. 'Virginia Bright Italia'. The stock cultures of both strains were cultivated on the medium of mineral composition according to Heller (GAUTHERET 1959), containing further per 1 litre: sucrose 30 g, inositol 100 mg, vitamins according to WHITE (1943), Difco Bacto Casamino Acids 1 g, agar 7 g, and growth substances NAA 1 mg plus 2,4-D 1 mg. The usual length of the subcultivation interval (SBI) is 4 weeks; the cultures were kept in the dark at 25 ± 1 °C.

Prior to setting up experimental subcultures the tissue of the stock culture was shaken by hand in a standard volume of 3 per cent sucrose; by filtering through a stainless sieve cell aggregates larger than 2 mm in diameter were removed and the suspension was spread onto agar media (in Drigalski dishes) of the following composition: a) complete stock medium with both NAA and 2,4-D; b) the same medium without NAA; c) the same medium without 2,4-D; d) the same medium without auxins. The spreadings were cultivated in the dark for 5 weeks. The samples of tissue for the micromorphological analysis and for starch detection were taken on the 3rd, 7th, 13th, 20th, 27th and 34th days after spreading.

The cultures based on subculturing aliquot heaps of tissue were grown under the same conditions as the spreadings (for detailed technique see OPATRNÝ 1971). The weight of individual colonies (means from 24 samples per the sampling and variant given) served as the criterion of growth of the cultures.

Micromorphological Characterization

The basic micromorphological characterization of both cultures was obtained by direct counting of the frequency of the individual cell types (see further). In one determination 1000—1200 cell units were evaluated. The results of three independent repetitions were very similar, therefore their mean values are presented.

The changes in the micromorphological composition of the cell populations cultivated on auxin-deficient media were so great that we did not consider it necessary to express them numerically.

Evaluation of the Occurrence of Starch Grains

Starch was proved histochemically in non-fixed material using a 0.3 per cent solution of iodine in a 1.5 per cent solution of potassium iodide (IIK). The intensity of the blue stain, as well as the quantity and size of starch grains, were evaluated microscopically according to the scale: 0 — starch was not proved, 0.5 — isolated minute starch grains giving a red-violet colouration, 1 — medium-sized, simple starch grains in most cells, giving a blue colouration, 2 — large composed dark-blue starch grains in most cells.

Each of the values presented in Fig. 2, represents a mean from ten tissue samples evaluated in one of the three repetitions of the experiment. The differences between the repetitions were negligible.

Results

Growth

Cell strains VBI-T and VBI-O exhibit approximately the same growth rate when cultured on the medium containing simultaneously 2,4-D and

NAA. The omission of one of the two auxins did not substantially affect the growth of the cultures. The simultaneous absence of both auxins did not influence the growth of culture VBI-O; however, that of culture VBI-T was inhibited in the second half of SBI (Fig. 1).

Micromorphology of the Cultures

Both cell strains were characterized in detail as concerned the type and frequency of cells and multicellular formations. The study revealed that in the two strains six basic types of cell units could be distinguished:

A. small free cells, spherical or slightly elongated, of a meristemic character (smaller than 100 μm),
B. cells elongated to a varying degree (100 × 200—500 μm),
C. large spherical cells (100—200 μm),
D. filaments formed by cells of type A to B,
E. spherical or irregular cell clusters,
F. other types.

The frequency of the participation of the individual types in the cell population of both strains investigated is presented in Table 1. Culture VBI-T is formed mostly of spherical cells associated in spherical clusters (Fig. 3a), culture VBI-O of elongated cells which form filaments (Fig. 3b). The value presented in the table are relatively stable in the course of the exponential growth phase. Towards the end of SBI (4th week), the number

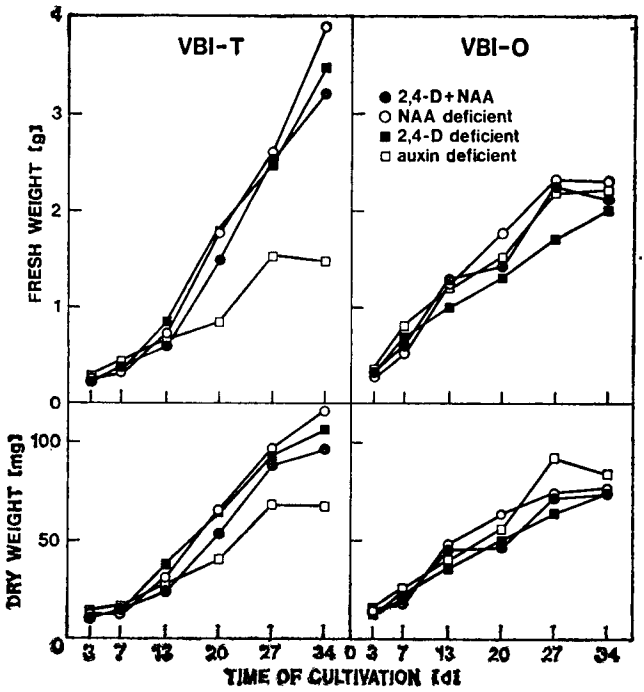

Fig. 1. The growth of tissue cultures VBI-T and VBI-O on the media of varying auxin composition: ● — 2,4-D and NAA, ○ — NAA deficient, ■ — 2,4-D deficient, □ — auxin deficient.

of cell clusters increased with culture VBI-T, in culture VBI-O that of free long cells increased, whereas in both strains the number of small cells of the meristemic character and that of filaments decreased.

The changes in auxin composition of the medium characteristically affected the micromorphological composition of the strains examined.

The omission of NAA (the presence of 2,4-D alone) resulted in a polarization of volume growth of small spherical cells of culture VBI-T. The cell wall protruded at one site and gradually the whole cell elongated. In the second half of SBI the frequency of long cells increased to such a degree, that the overall pattern resembled that of culture VBI-O.

TABLE 1

The frequency of the participation (in per centages) of the individual cell types in the cell population of stock cultures of strains VBI-O and VBI-T

	A	B	C	D	E	F
VBI-T	48	5	35	8	10	4
VBI-O	34	33	3	19	9	2

13th day of SBI, exponential growth phase of the subculture.

NAA omission had quite a different effect on the micromorphology of culture VBI-O. As early as from the third day of SBI a striking increase occurred in the number of simple and branched filaments, formed by meristemic cells of type A. The high proportion of these filaments persisted in the course of the entire SBI (Fig. 4b).

The omission of 2,4-D (the presence of NAA alone) had little influence on the micromorphology of culture VBI-T. Only towards the end of SBI fewer clusters were formed and the amount of long cells increased insignificantly.

However, the volume growth of cells of culture VBI-O was markedly stimulated. In the second half of SBI, cells of all types were twice to three times larger than the cells of the culture growing on the complete medium.

The simultaneous omission of 2,4-D and NAA did not result in any change of the micromorphology of culture VBI-T; in culture VBI-O, as in the previous case, an increased volume growth was observed with all cell types.

Occurrence of Starch

In both cell strains, cultivated on the standard medium with 2,4-D and NAA, the occurrence of starch grains was observed in the cytoplasm of cells of all types. Their size, stainability and number fluctuated irregularly in the course of SBI (Fig. 2).

The omission of one or both auxins substantially and conformly affected the occurrence of starch in the cells of both cultures. When omitting NAA, starch was either not proved, or only small simple grains occurred in the cytoplasm of the cells of both cultures, which gave a red-violet stain (Fig. 2, Fig. 4a, b).

When omitting 2,4-D, a considerable amount of dark-blue stained, simple and composed grains appeared in the cells of both cultures (Fig. 2, Fig. 5a, b), often concentrated around the cell nucleus (Fig. 5c). Starch occurred in cells of all basic types.

The simultaneous absence of both auxins had a similar effect as the omission of 2,4-D (Fig. 2).

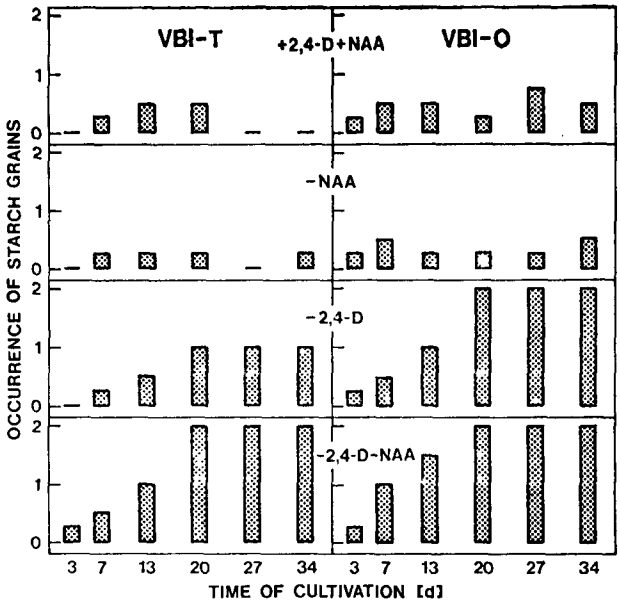

Fig. 2. The results of the histochemical evaluation of the occurrence of starch grains in the cells of cultures VBI-T and VBI-O, cultivated on the media of varying auxin composition (for explanation of the classification scale see the text).

Discussion

The comparison of the present and previous (OPATRNÝ 1971) results of the evaluation of the growth and micromorphology of both strains confirmed the fact that in the course of an eight-year cultivation on the standard medium, no change took place in the phenotype of the cultures. The characters investigated may thus be considered highly stable, and, under the standard cultivation conditions for the strain given, specific.

However, qualitative changes in auxin composition of the medium affected, to a varying degree, both the growth rate of cultures and their micromorphological characterization in a short time period (during one single SBI). The overall growth was influenced least: strain VBI-O proved to be fully auxin-autotrophic, strain VBI-T exhibited a drop in the increase of fresh and dry weights only when there was a simultaneous absence of both auxins.

The micromorphology of cultures was affected substantially more, and often specifically for the given strain and the hormonal composition of the medium. Previous observations were confirmed (WITHAM 1968, MILLER 1969, OPATRNÝ 1971, DAVEY *et al.* 1971) concerning not only a quantitatively, but

also a qualitatively different effect of NAA and 2,4-D on tissue cultures. In our experiments the absence of one of the auxins resulted in a specific morphogenic response of each of the strains. The application of 2,4-D alone, for example, resulted in a stimulation of VBI-T cell elongation; on the contrary, in strain VBI-O the division of cells was enhanced, the subculture becoming meristemized. These differences may be due to an interaction of the given exogenous factor with a different, strain-specific endogenous level of growth substances. A different sensitivity to exogenous auxins thus represents a further character of a series of characters (ability of organogenesis, chlorophyll formation, sensitivity to cytokinins — see OPATRNÝ 1971, OPATRNÝ and OPATRNÁ 1972) in which the two strains differ from each other and whose connection with the metabolism of growth substances is indisputable.

In this respect the finding concerning an almost identical effect of a given change in auxin composition of the medium on the occurrence of starch in cells of both strains was rather surprising. A connection has already been observed between the occurrence of starch and morphogenic processes in growth apices (USCIATTI *et al.* 1972), or in tissue culture (THORPE and MURASHIGE 1968, 1970, BROSSARD 1970). Starch grains were mostly localized at the site of future cell division or in parenchymatous cells adjacent to the meristemic tissue. From these observations a hypothesis was formed on the connection between the occurrence of starch (or differentiation of amyloplasts) and a certain subsequent cell fate, *i.e.* division or volume growth of cells. LUŠTINEC *et al.* (1974) found a negative correlation between the volume growth of cells and starch formation in pith explants of tobacco, cultivated in media with various auxin concentrations. However, the behaviour of pith explants of kale was somewhat different, and the authors concluded, that: . . . "the relationship between both processes is determined by their respective sensitivity to the auxin present in the cell and depends on the type of the tissue . . .".

The creation of physiological gradients in compact tissues of primary explants or callus cultures evidently results not only in a localized starch formation, formation of meristemoids, or in the change of respiration (see THORPE and MEIER 1972, ROSS and THORPE 1973, ROSS *et al.* 1973), but also in a certain type of "non-organized" growth in certain callus zones. A simultaneous occurrence of starch formation and differentiation of a certain type of cells at the same site and time may lead to an evidently erroneous conception on the causality of the two processes.

In our *a priori* highly friable cultures which were cultivated as thin spreadings on agar plates, the possibility of the rise of gradients of growth or nutritive substances was greatly reduced. Under these conditions the occurrence of starch grains was not linked to the formation of certain cell types, *e.g.* by the application of 2,4-D alone, the formation of starch in cells of both strains was conformly inhibited, the morphogenic effect being different, *i.e.* in one strain meristemic growth, in the other volume growth of the cells occurred. Both processes thus took place under a similar hormonal control, but independently of each other.

References

BROSSARD, D.: Évolution de la synthèse d'amidon durant l'organisation des nodules puis des méristèmes primaires obtenus *in vitro*, à partir d'explants de moelle de tabac. Comparaison avec le point végétatif de la plante entière. — Compt. rend. Acad. Sci. (Paris) Sér. D **271** : 56 to 59, 1970.

DAVEY, M. R., FOWLER, M. W., STREET, H. E.: Cell clones controled in growth, morphology and pigmentation isolated from a callus culture of *Atropa belladonna* var. *lutea*. — Phytochemistry **10** : 2559—2575, 1971.

GAUTHERET, R. J.: La culture des tissus végétaux; techniques et réalisations. — Masson, Paris 1959.

LUŠTINEC, J., KAMÍNEK, M., HADAČOVÁ, V.: Hormonal control of starch accumulation and cell expansion in tobacco stem pith. — Proc. IV Symp. Plant Growth Regulators, Toruń 1974.

MILLER, C. A.: Control of deoxyisoflavone synthesis in soybean tissue. — Planta **87** : 26—35, 1969.

OPATRNÝ, Z.: [Plant tissue cultures in genetics.] In Czech. — Diss. Thesis, Czech. Acad. Sci. Praha 1971.

OPATRNÝ, Z., OPATRNÁ, J.: The influence of growth substances (auxins and cytokinins) on the starch synthesis and morphogenesis of two cell strains of *Nicotiana tabacum* L. — Proc. Conf. Natural Growth Substances, Liblice 1972.

ROSS, M. K., THORPE, T. A.: Physiological gradients and shoot initiation in tobacco callus cultures. — Plant Cell Physiol. **14** : 473—480, 1973.

ROSS, M. K., THORPE, T. A., COSTERTON, J. W.: Ultrastructural aspects of shoot initiation in tobacco callus cultures. — Amer. J. Bot. **60** : 788—796, 1973.

THORPE, T. A., MEIER, D. D.: Starch metabolism, respiration and shoot formation in tobacco callus cultures. — Physiol. Plant. **27** : 365—369, 1972.

THORPE, T. A., MURASHIGE, T.: Starch accumulation in shoot-forming tobacco callus cultures. — Science **160** : 421—422, 1968.

THORPE, T. A., MURASHIGE, T.: Some histochemical changes underlying shoot initiation in tobacco callus cultures. — Can. J. Bot. **48** : 277—285, 1970.

USCIATTI, M., CODACCIONI, M., GUERN, J.: Early cytological and biochemical events induced by a 6-benzylaminopurine application on inhibited axillary buds of *Cicer arietinum* plants. — J. exp. Bot. **23** : 1009—1020, 1972.

WHITE, P. R.: A Handbook of Plant Tissue Culture. — Jacques Cattell Press, Lancaster, Pensylvania 1943.

WITHAM, F. H.: Effect of 2,4-dichlorphenoxyacetic acid on the cytokinin requirement of soybean cotyledon and tobacco stem pith callus tissue. — Plant Physiol. **43** : 1455—1457, 1968.

Figures at the end of the issue

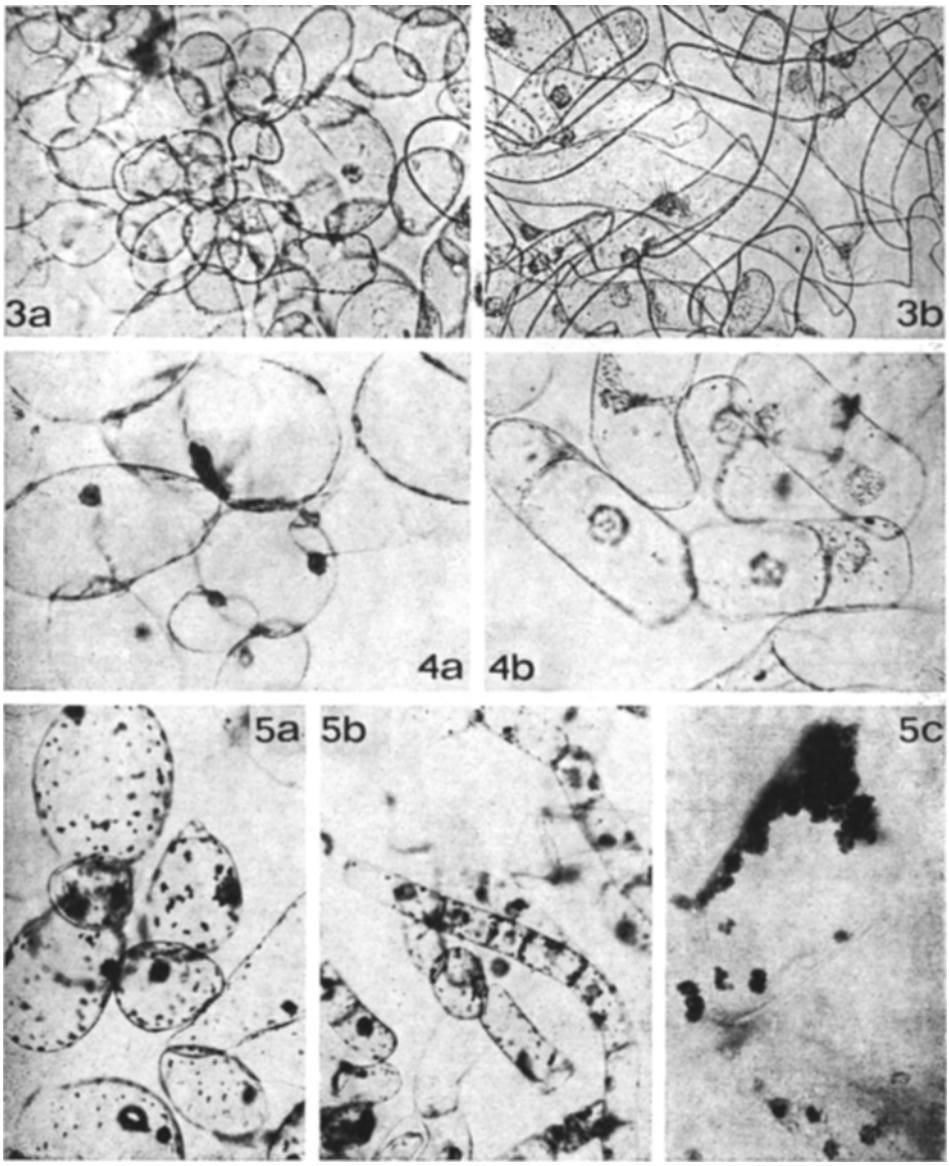

Fig. 3. The micromorphology of cultures VBI-T and VBI-O, cultivated on the standard medium with 2,4-D and NAA: a) spherical cells of types A and C, predominating in the cell population of strain VBI-T, b) elongated cells of type B, predominating in culture VBI-O. Stational growth phase. Magn. 250×.

Fig. 4. The absence of starch grains in the cells of cultures cultivated on the medium containing 2,4-D as the only auxin: a) strain VBI-T, b) strain VBI-O. Exponential growth phase. Magn. 500×.

Fig. 5. The occurrence of starch grains in the cells of cultures, cultivated on the medium with NAA alone: a) strain VBI-T. Magn. 500×.b) strain VBI-O. Magn. 200×. c) a detail of composed starch grains, strain VBI-O. Magn. 1000×. Exponential growth phase.