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Morphactin-Induced Changes in the Cytokinin Effect on Tissue and Organ Cultures of *Nicotiana tabacum*

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Summary

The influence of a morphactin, chlorflurenol-methylester (CFM), on the growth, the morphogenesis and the isoelectric peroxidase pattern was investigated in both callus cultures (two different tissue culture strains) and multiple bud cultures of *Nicotiana tabacum* var. *Wisconsin*. CFM (range of concentration between 10^{-6} g/ml and 10^{-4} g/ml) was applied singly, or in combination with a cytokinin, benzylaminopurine (BAP), or with an auxin, indoleacetic acid (IAA), or with IAA plus BAP.

In general, the callus growth was inhibited under the influence of CFM. In some of the experiments carried out in hormone-free media, growth stimulation was observed. Even minimal inhibition or stimulation of the callus growth was always accompanied by characteristic changes in the peroxidase patterns.

The following results show the influence of the morphactin CFM on cytokinin effects (endogenous cytokinin or equally the exogenously applied cytokinin, BAP). (1) In the multiple bud cultures, BAP and CFM (both substances combined with IAA) similarly caused inhibition of root formation and stimulation of bud formation. The bands in the peroxidase patterns, characteristic of cytokinin action, were accentuated also of those bud cultures which had been treated with BAP or with CFM. (2) In the callus cultures, the cytokinin characteristics appeared under CFM influence in the peroxidase patterns of one of the tissue culture strains only when CFM was applied in combination with BAP and not in combinations of CFM with IAA.

The observed morphactin-induced increase in the cytokinin effects could occur via changes in the hormone level of the tissue.

Keywords: Morphactin influence; Growth; Morphogenesis; Peroxidase pattern; Tissue culture; *Nicotiana tabacum*.

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1. Introduction

Morphactins, named according to their morphogenetical effects, are fluorene-carbonic acid derivatives. These substances cause changes in higher plants which indicate an interference in the complex effects of the phytohormones (SCHNEIDER 1969, 1970, ZIEGLER 1970, PARUPS 1980).

Tobacco tissue cultures, which respond actively to growth hormones, have shown close correlations between hormonal effects, changes in growth, and the isoelectrical peroxidase pattern in previous experiments; indoleacetic acid and benzylaminopurine cause characteristic changes of enzyme activity in the peroxidase patterns, which on the one hand are closely consistent with changes in the growth but, on the other hand are an expression of the hormones applied (RÜCKER and MARKOTAI 1978, 1980).

In an extention of these experiments the additional influence of the morphactin, chlorflurenol-methylester, on growth, morphogenesis, and peroxidase patterns of tobacco tissue and organ cultures was investigated in order to find further correlations caused by possible changes in the effects of the growth hormones.

2. Materials and Methods

2.1 Tissue and Organ Cultures and Culture Conditions

Two different tissue culture strains – strain b and strain c – and organ cultures – multiple bud cultures – of *Nicotiana tabacum* var. *Wisconsin*

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were examined. Both tissue strains were obtained from pith tissue of the same plant. The multiple bud cultures originated from buds induced in b-strain tissues.

As a result of cultivation under different growth hormonal treatments (strain b: IAA + BAP 10^{-7} g/ml each; strain c: dichlorphenoxyacetic acid (2,4 D) 10^{-6} g/ml), the two tissue culture strains vary in growth rate, morphology, and dependence on growth hormones. Their isoelectric peroxidase patterns differ also – corresponding bands exhibit different peroxidase activity. Strain c has higher growth rate then strain b and is independent of cytokinin; the tissue is strongly dissociated, thus of soft consistency. Tissue of strain b grows at slower rate, has a compact structure, and needs cytokinin for growth over several passages.

During the experiments, all cultures were grown in a day-night illumination rhythm of 12 hours each and at 22-24 °C. Each variant started with 12 to 24 cultures and was evaluated after 4-week periods (tissue cultures) or 8-week periods (organ cultures). Nutrient medium was chosen according to LINSMAIER and SKOOG (1965) with the addition of 3% sucrose in all experiments.

2.2. Growth

Growth was assessed at the end of the cultivation time by individual measurements of fresh and dry weight of the cultures.

2.3. Enzyme Analyses

The peroxidase systems were separated according to the method of thin-layer isoelectric focusing (RADOLA 1973). It was performed with carrier ampholite: Ampholine (LKB) pH 3.5-10 and with Sephadex 75 superfine on 20 × 20 glass plates. The enzyme detection was carried out with the print technique: Filter paper (Whatman 1, buffered with citrate-phosphate, pH 5.9) was impregnated with methanol-H_2O_2 solution containing 1% o-dianisidine as a secondary substrate (DELINCÉE and RADOLA 1972) before taking a print of the gel layer. The separated extracts of each sample were equivalent to 2 mg of dry tissue.

The isoelectric peroxidase patterns of tobacco tissue cultures show a series of bands forming 3 functional groups (MÄDER and BOPP 1976, NESSEL and MÄDER 1977, RÜCKER and MARKOTAI 1978). These are designated as groups A, B, C in our papers. The bands in the alkaline pH range – group C – proved to be very instable. Therefore, our observations are limited to the bands in the A and B groups, which are located close together in the acidic pH range.

2.4. Compounds Used

2-Chlorflurenol-Methylester (CFM), 99.6%, Celamerck, Ingelheim; 6-Benzylaminopurin (BAP), EGA-Chemie, Steinheim/Albuch; Indol-3-Essigsäure (IAA), Serva, Heidelberg.

2.5. Test Series

2.5.1. Tissue Cultures (see Figs. 1 and 2)

CFM $4 \cdot 10^{-6} - 10^{-5}$ g/ml (I).

CFM $4 \cdot 10^{-6} - 10^{-5}$ g/ml combined with IAA + BAP 10^{-7} g/ml each (II).

CFM $10^{-5} - 10^{-4}$ g/ml combined with IAA 10^{-7} g/ml (I a, II a).

CFM $10^{-5} - 10^{-4}$ g/ml combined with BAP 10^{-8} g/ml (III a).

The effects of tissue extracts were tested also; these extracts were prepared from cultures of strain c, cultivated a 6-week-passage under the influence of either IAA or BAP (I b, II b).

2.5.2. Multiple Bud Cultures (see Fig. 3)

IAA 10^{-7} g/ml (1, 1 a).

IAA 10^{-7} g/ml combined with BAP 10^{-7}, $5 \cdot 10^{-7}$, 10^{-6} g/ml (2, 3, 4).

IAA 10^{-7} g/ml combined with CFM 10^{-6}, $5 \cdot 10^{-6}$, 10^{-5} g/ml (2 a, 3 a, 4 a).

3. Results

3.1. Tissue Cultures

In most cases the morphactin, CFM, inhibits callus growth in concentrations applied. This is true for both tissue strains, b and c, and also for those tests combined with or without growth hormones: $4 \cdot 10^{-6}$ g/ml CFM causes extremely small enhancement of growth when applied alone. However, growth, enhanced under the influence of IAA and BAP markedly decreases when these substances are combined with CFM (see Fig. 1). As shown in the graphs the course of growth reduction with increasing CFM concentrations varies in the two tissue strains. Initially, concentrations up to $5 \cdot 10^{-5}$ g/ml CFM cause a stronger growth inhibition in b strain than in c strain (see Fig. 1; I a and II a); however, decrease of growth with increasing CFM concentrations progresses faster in c strain; finally, the concentration of 10^{-4} g/ml CFM inhibits growth of c strain stronger than of b strain. In all test series up to $5 \cdot 10^{-5}$ g/ml CFM, the final weight of the cultures follows the array strain c > strain b > strain c_0.

The results of enzyme separations (see Fig. 1) exhibit correlations between growth and certain changes in the peroxidase patterns: growth inhibition and stimulation are accompanied by an activity decrease or increase, respectively, in the A group bands. The intensity of these bands remains accentuated even in less inhibited tissues, becomes weaker in strongly inhibited tissues, in some of them the bands are not visible at all. Even the slight growth stimulation in two tests with $4 \cdot 10^{-6}$ g/ml CFM is visible as a small band intensification (Fig. 1: I). The intensities of B group bands are conforming with CFM influences but are independent of growth. However, in tests with strain c tissues, the B group bands are produced only in cultures treated simultaneously with CFM and BAP. Unlike the c strain tissues – cultured with 2,4 D – the b strain tissues – grown under the influence of IAA + BAP – don't need additional treatment with cytokinin for producing B group bands in the peroxidase patterns and for intensifying them in correspondence with CFM influence. The different reaction to CFM application, which can be observed on the peroxidase patterns, indicate variations in the endogenous growth hormone contents of the tissues of strain b and c.

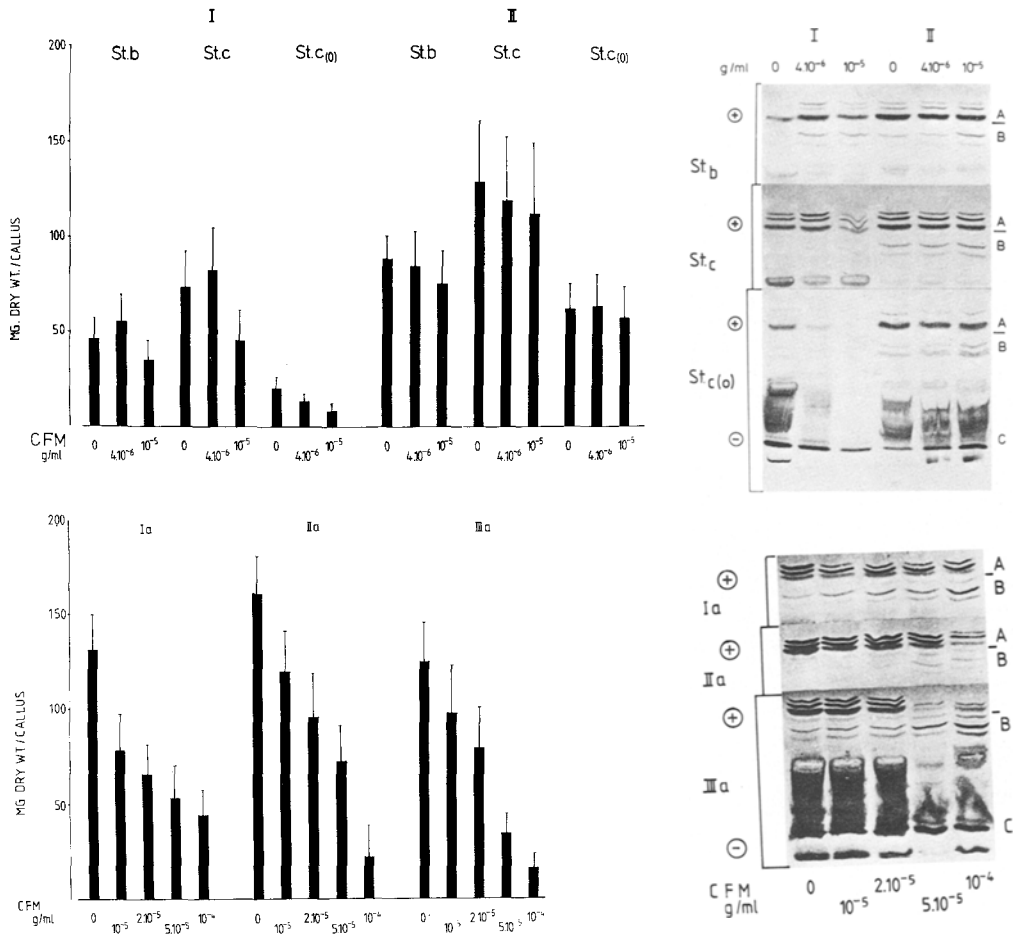

Fig. 1. The influence of CFM on growth and isoelectric peroxidase patterns of different strains of tobacco tissue cultures: strain b (St. b), strain c (St. c), and tissues of strain c grown one passage in nutrient medium without the addition of growth hormones, strain c_0 (St. c_0)
CFM applied singly (I) or combined with IAA + BAP 10^{-7} g/ml (II)
CFM with IAA 10^{-7} g/ml; tissue strain b (I a); tissue strain c (II a)
CFM with BAP 10^{-8} g/ml; tissue strain c (III a)
Stimulation and inhibition of tissue growth is accompanied by enzyme activity increase or-decrease, respectively, exhibited in the A group bands; they show very low activities in strongly inhibited cultures. The activity of the bands of group B corresponds to the CFM influence; however, in the test with strain c tissue, this effect occurs only when a small amount of BAP is applied simultaneously

In order to gain insight into the hormone level of the tissues treated with different growth substances, tissues were cultured in the presence of tissue extracts. The extracts were produced of c strain tissues grown in nutrient medium containing IAA or BAP for one cultivation passage. Fig. 2 shows growth and peroxidase patterns of c and b strain tissues cultured under the influence of IAA or BAP extracts. In strain c even the lowest extract concentration (2 g dry tissue) induces pronounced growth stimulation; increasing concentrations of IAA or BAP extracts cause increasing stimulation or a dose dependent inhibition of growth. On the contrary, in strain b tissues no growth differences after IAA or BAP extract treatment are noticable when compared with the control cultures grown in nutrient media without any growth substances.

In this experiment the intensity of A group bands in the peroxidase patterns corresponds to change in growth also under the influence of tissue extract: whereas almost no activity changes are observed in the peroxidase patterns of the b strain cultures – corresponding to insignificant growth differences. In strain c, however, stimulation and inhibition of growth due to the extract influence causes intensity increase or decrease in the A group bands. The activity of the B group bands hardly changes after addition of IAA extracts – that means, in strain b the intensity decreases

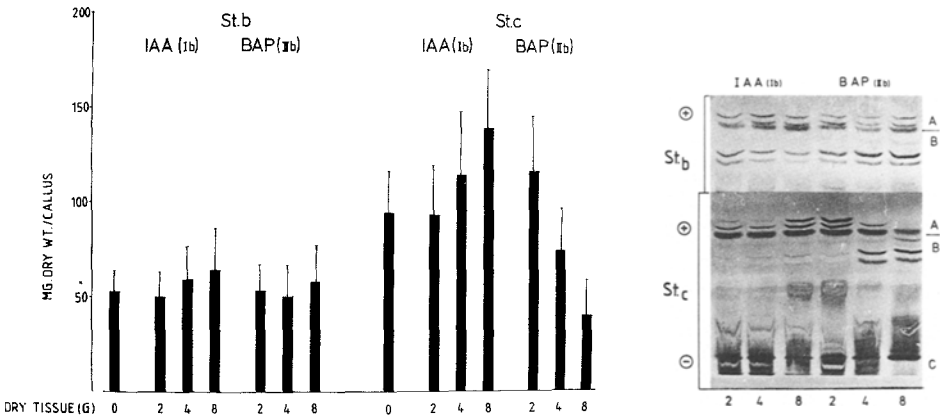

Fig. 2. Influence of different tissue extracts on growth and peroxidase patterns in tobacco tissue cultures: strain b (St. b) and strain c (St. c); the applied extracts were derived from c strain tissues, which had been grown one passage in either IAA (I b) or BAP-containing media. (II b) The applied extracts were equivalent to 2, 4 or 8 g dry tissue. Growth differences hardly appear in the cultures of strain b. In the cultures of strain c, the influence of IAA extracts cause growth stimulation depending on the concentration, whereas BAP extracts exert growth inhibition. In the isoelectric peroxidase patterns, stimulation and inhibition of growth are exhibited as an intensity increase or decrease, respectively, of the A group bands. The enzyme activities, exhibited by the B group bands, remain almost unchanged under the influence of IAA extracts, but are enhanced by BAP extracts depending on the concentration

slightly, and in strain c the bands were barely developed. Increasing BAP extract concentrations markedly pronounce the B group bands of both tissue strains.

3.2. Multiple Bud Cultures

Buds induced in b strain tissues under the influence of BAP can further be propagated in liquid medium with IAA. After cultivation for one month, they develop into plantlets with long-narrow leaves. When cultivated further, these plantlets partly evolve into fully developed small tobacco plants (see Fig. 3: 1 and 1 *a*). The addition of increasing BAP concentrations (10^{-7}–10^{-6} g/ml) to IAA containing medium causes an increase in bud formation and a decrease in root formation. The further development of the buds is likewise inhibited: the leaves remain small and show partly a long or round shape. They are deeper green than the leaves grown in control cultures under IAA influence alone (see Fig. 3: 2–4). CFM (10^{-6}–10^{-5} g/ml) causes morphogenetic effects similar to BAP (see Fig. 3: 2 a–4 a). The only difference is that all leaves have round shape and their colour is noticeably dark-green.

The isoelectric peroxidase patterns of all tests with bud cultures – control and original cultures included – exhibit remarkable pronounced A group bands. Their intensity increases further with increasing BAP-concentrations. The B group bands of the peroxidase patterns, not visible in cultures under IAA treatment alone, appear under the circumstance that IAA is combined with BAP or CFM. Their activity intensifies with increasing BAP or CFM dosage; at the highest concentration applied the intensity of these bands is markedly accentuated.

4. Discussion

In the experiments described above as well as in previous investigations (RÜCKER and MARKOTAI 1977, 1978, 1980) it has been demonstrated that correlations exist between the intensity of growth and the intensity of A group bands of the isoelectric peroxidase patterns. These correlations make it possible to draw conclusions about growth from the intensity of the A bands. Due to the slight intensification of these bands in the peroxidase patterns even the little growth stimulation of two tests with tissue cultures was ascertained. Moreover, the observed intensity increase of the A bands in the peroxidase patterns of the bud cultures under BAP influence allows to infer an enhancement of growth which could not be detected in the final weight. The different reaction of the two tissue strains to morphactin influence – observed in the peroxidase patterns – can be interpreted as a result of continuing influence of the growth hormones deriving from previous passage (2,4 D: strain c or IAA + BAP: strain b). This interpretation is based firstly on the fact that growth results of strain c become lower after inserting a culture passage in hormone free medium (strain c to

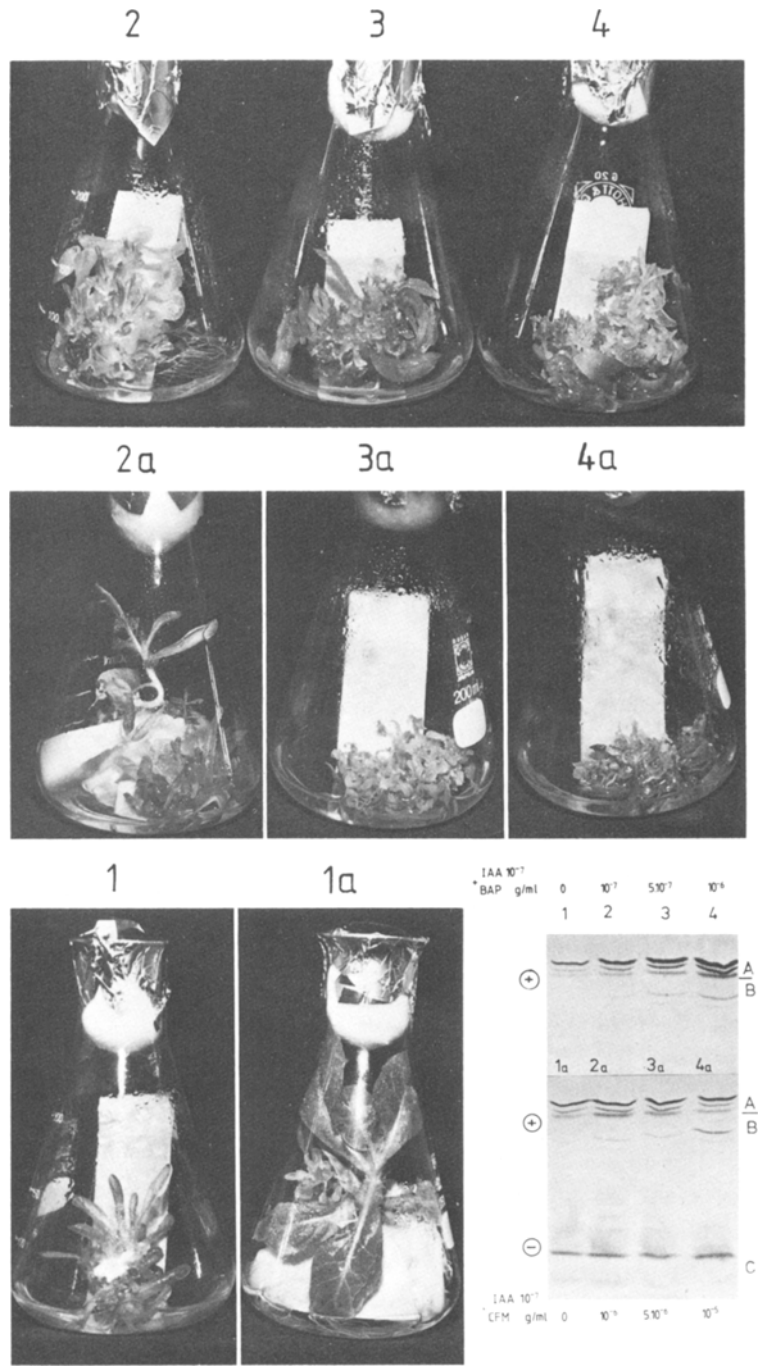

Fig. 3. Morphology and peroxidase patterns of multiple bud cultures of tobacco. (1): starting material; IAA 10^{-7} g/ml; cultivation period-four weeks; (1 a): control series; IAA 10^{-7} g/ml; cultivation period-two months; (2, 3, 4): the combined influence of IAA 10^{-7} g/ml + BAP 10^{-7}, $5 \cdot 10^{-7}$, 10^{-6} g/ml; (2 a, 3 a, 4 a): the combined influence of IAA 10^{-7} g/ml + CFM 10^{-6}, $5 \cdot 10^{-6}$, 10^{-5} g/ml. IAA combined with BAP or CFM stimulates bud differentiation and inhibits root development. In both instances, the morphology changes are similar. They are accompanied by changes in the isoelectric peroxidase patterns: Intensification of the bands of group A occurs only in the combined treatment of IAA + BAP. Accentuation of the B group bands appears in both experimental series corresponding to increasing BAP and CFM concentrations

strain c_0). Secondly, in tests with tissue extracts the same peroxidase patterns were observed in tissues serving as extract material and in tissues grown under the influence of these extracts. Similar results were obtained regarding the growth of c strain cultures; conforming with previous experience that IAA increasingly stimulates growth and BAP shows inhibiting effects even at low concentrations (RÜCKER and MARKOTAI 1980), IAA extract causes stimulation and BAP extract causes inhibition of growth. Plant cells are able to bind growth hormones to proteins, amino acids, or to other substances and to incorporate them in their metabolism afterwards. Such bindings have been proven for IAA, 2,4 D and also for cytokinin (FEUNG, HAMILTON, and MUMMA 1977, DAVIDONIS, HAMILTON, and MUMMA 1978, MOORE 1979, SEMBDNER, GROSS, LIEBISCH, and SCHNEIDER 1980).

Special attention should be paid to the findings that morphactin causes cytokinin-like effects; observed on the bud morphology as well as on the peroxidase patterns of the organ and of the tissue cultures. In the peroxidase patterns of c strain tissues, however, morphactin exhibits only an activity distribution similar to that of cytokinin when CFM is applied simultaneously with low cytokinin concentrations. Consequently this results show that morphactin does not imitate cytokinin but reinforces the cytokinin effects. The different reactions of the two tissue strains b and c could at least partly be explained by differences in cytokinin contents appearing as a result of different hormone treatments and their after effects. For this reason it is supposed that the cytokinin concentrations in the IAA + BAP treated tissue cultures (strain b) are higher than in the 2,4 D treated tissue cultures (strain c). The results of the tissue extract feeding tests confirm this hypothesis. The cytokinin-like effects of CFM in the multiple bud cultures probably are induced by combined action of morphactin and endogenous cytokinin. It has been demonstrated that also bud meristems produce cytokinins (KODA and OKAZAWA 1980). A combined action of morphactin and cytokinin has also been observed in other plants (HARADA 1969, CHAWAN and SENN 1971, OGURA 1975).

The results of this investigation obtained from observations of growth, morphogenesis and isoelectric peroxidase pattern suggest that the cytokinin-like morphactin influence is mediated by changes in the hormone level of the tissue; viz. an increase in the cytokinin concentration and a reduction in the auxin concentration (ZIEGLER, VOGT, TREICHEL, and STREITZ 1969).

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