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Physiological and biochemical effects of morphactin IT 3233 on callus and tumour tissues of *Nicotiana tabacum* L. cultured in vitro

II. Protein synthesis and respiratory activity

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Abstract

It was found that the inhibition of callus tissue growth in *Nicotiana tabacum* L. cultured *in vitro* by the application of morphactin IT 3233 is associated with a rise of the protein level in spite of a 50 per cent depression of its synthesis. Respiratory activity of these tissues is also lowered. Tumour tissues, on the other hand, show only light changes in the parameters studied. It would seem that morphactin depresses a metabolic activity in the callus tissues, and probably causes deposition of a large quantity of metabolically inactive proteins.

INTRODUCTION

As it has been established earlier (Chirek — part I, this issue morphactin IT 3233 inhibits the growth of callus tissue, and, in a smaller degree, of tumor tissue in tobacco plants cultured *in vitro*. This reduction of growth was associated with an increase in dry mass and protein nitrogen fraction values. It seemed useful to verify with more precision the dynamics of proteins and their synthesis in different types of tissues treated with morphactin and in control tissues.

Earlier investigations of Rennert (1971) indicate a more active protein synthesis in tumour tissues of tobacco. It is not known, however, how morphactin affects protein synthesis. It was found that in wheat treated with morphactin changes occur in protein content

and in the proportions of the particular fractions (Krishchenko et al. 1969).

Growth inhibition and an increase in dry mass may be the result of changes in the respiratory activity of the tissues investigated. It seemed, therefore, purposeful to compare the oxygen uptake activity in callus and tumour tissues of tobacco exposed to the action of morphactin.

MATERIAL AND METHODS

The material for this study consisted of callus and tumour tissues of tobacco cultured under the conditions previously described (Chirek — part I, 1974). Usually 5-week-old colonies were used for analysis, and only for the experiments with isotopes 4-week-old ones.

Total protein content, respiratory activity of the tissues and the rate of ^{14}C-leucine incorporation into the ethanol and protein fractions of both types of control tissues and those treated with morphactin were determined.

Protein determination. Weighed 10-mg samples of dry, powdered tissues were ground in the cold with 5 cm^3 of 5% trichloroacetic acid (TCA) and centrifuged for 15 min at 18 000 g. The sediment was washed with 5% TCA portions and 80% ethanol. Protein was extracted with 5 cm^3 of 1 M NaOH for 10 min on a boiling water bath (Fletcher and Osborne, 1965). After cooling the liquid was passed through a filter paper and in the filtrate protein was determined by the method of Lowry et al. (1961). For the determinations 0.5 cm^3 of the extract were taken and diluted with water to 1 cm^3.

Extinction measurements in the solutions were taken in a "Spekol" photocolorimeter at the wavelength of 750 nm. Protein content was read from the standard curve plotted for bovine albumin dissolved in 0.5 M NaOH. The analyses were repeated 6 times.

^{14}C-leucine incorporation

1. Tissue incubation. Three weighed samples of each tissue (total mass ca. 600 mg for the callus tissue and ca. 300 mg for the tumour tissue) were placed in test tubes with 1 cm^3 of the liquid standard medium containing ^{14}C-DL-leucine with 2 μCi/cm^3 activity. The tissues were incubated for 4 h at continuous shaking. For each type of tissue four parallel tests were run and the experiment was replicated three times with different tissue series.

2. Extraction of radioactive compounds. The compounds soluble in 80% ethanol were extrated from the incubated tissues as well as proteins according to the procedure described by

Knypl (1971a). Protein was extracted with 1 M NaOH in the hot (as above described) and then precipitated with 4 N TCA in the cold and centrifuged at 18 000 g. The supernatant containing nonprotein compounds reacting positively with the Folin-Ciocalteau reagent was discarded (Knypl 1971b).

The sediment was dissolved in 5% NH_4OH and this solution was used for protein determination by the Lowry's method and for radiometric analysis.

For determinations by the Lowry method 0.1 cm^3 of the solution was taken and diluted with 0.5 M NaOH to a 1 cm^3 volume.

3. Radioactivity measurements. On two aluminium spot plates for each sample 0.2 cm^3 of the radioactive extracts (ethanol and protein) were placed dropwise, dried at 50°C and impulses were counted in a setup consisting of a scintillation probe SSU-4W and an automatic electronic scaler ZPA-1A. The counting effectiveness was 20 per cent.

Oxygen uptake. The measurements of oxygen consumption by tissues were performed in a Warburg apparatus according to Rennert (1968). In the Warburg vessels moistened with a drop of water 500-mg aliquots of tissues were placed (six more or less equal fragments). A roll of filter paper was placed in the central well and 0.2 cm^3 of 15 per cent KOH was pipetted into it for the absorption of atmospheric CO_2. Oxygen uptake measurements were carried on for 2 h at 30°C. Readings were noted at 15-min intervals. The results are given in cubic millimeters of O_2 consumed by 1 g fresh tissue mass in the course of 1 h. For each tissue three replications were run, and the measurements were repeated 3 - 4 times on various tissue series.

RESULTS

Protein level. The results of protein determination by the Lowry method in the particular tissues are shown in table 1 and confirm the earlier observed tendency to an increase in protein nitrogen in tissues treated with morphactin (particularly callus).

In callus tissues the protein level rises along with morphactin concentration by 19 - 34 per cent (in reference to the tissue dry mass). The differences are significant when compared with the control results at morphactin concentrations of 5 - 40 mg/dm^3.

As regards to the tumour tissues the changes are of the order of several per cent, only at the morphactin concentration of 20 mg/dm^3 the difference is significant (increase of protein content by 19%).

Table 1

Protein content in *Nicotiana tabacum* L. tissues subjected to the action of morphactin IT 3233 (mg/100 mg dry mass)

Kind of tissues \ Medium	C	M-1	M-5	M-10	M-20	M-40
Callus	6.33 100%	7.53 119%	7.62* 120%	8.02** 127%	7.64** 121%	8.80** 134%
Tumour	12.23 100%	12.19 100%	12.72 104%	12.89 105%	14.60* 119%	13.22 108%

Notations: C (control) without morphactin, M-1, ... M-40 morphactin concentrations 1, ... 40 mg/dm^3
* — difference significant at $\alpha = 0.05$
** — difference significant at $\alpha = 0.01$

^{14}C-leucine incorporation. In order to establish whether the rise in protein content noted previously is due to enhanced protein synthesis, the incorporation of labelled leucine into the tissues was tested. The results are listed in table 2.

It proved that the ability of ^{14}C-leucine uptake from the substrate (measured in terms of radioactivity of the ethanol fraction) by the callus and tumour tissues treated with morphactin is only slightly lowered (to 15%) as compared with the control results. On the other hand, if we compare the control callus and tumour tissues, a more than twofold higher ability of ^{14}C-leucine uptake by the latter is found.

Also the radioactivity of the protein fraction is higher in the tumour tissues, this being associated with the higher protein content in this tissue.

A small decrease of radioactivity of protein in tumour tissues and a greater one in the callus tissues are observed under the influence of morphactin. These differences are significant at a 40 mg/dm^3 morphactin concentration.

The metabolic activity of proteins is characterized by so-called specific protein activity. This value is higher by about 25 per cent for the control tumour tissues as compared with callus, which indicates a higher intensity of protein synthesis in the tumour tissue. Under the influence of morphactin the protein specific activity decreases to 17 per cent in tumour tissues (nonsignificant) and to 51 per cent in callus tissues (statistically significant). It may be concluded hence that in callus tissues subjected to the action of higher morphactin concentrations (10 - 40 mg/dm^3) a depression of protein synthesis occurs.

The diagrams present the specific activity of proteins and the protein level in the tissues examined.

Table 2

^{14}C-leucine incorporation into *Nicotiana tabacum* L. tissues subjected to the action of morphactin IT 3233

Medium	Compounds soluble in 80% ethanol imp./min/100 mg fr. wt.		Protein imp./min/100 mg fr. wt.		Protein mg/100 mg fr. wt.		Specific activity of protein imp./min/mg protein	
	callus	tumour	callus	tumour	callus	tumour	callus	tumour
C	21 660	50 590	3 860	8 220	0.22	0.39	17 440	21 620
M-1	20 830	48 360	3 770	7 780	0.23	0.43	16 530	18 560
M-10	19 100	43 210	3 120	8 080	0.27*	0.43	11 680	19 060
M-40	18 490	42 940	2 410*	7 090	0.28*	0.40	8 610**	17 870

Notations as in Table 1

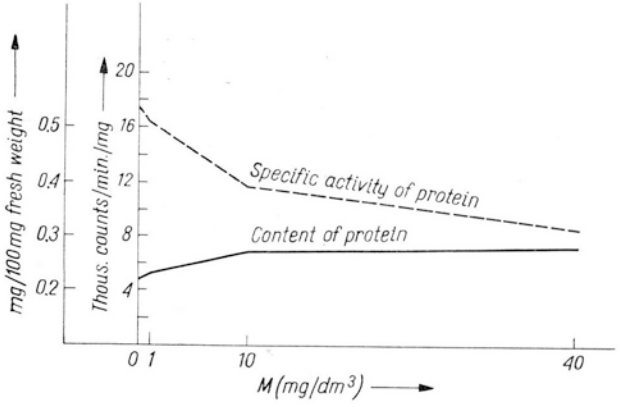

Diagram 1. Protein level and specific activity in callus tissue of tobacco treated with morphactin IT 3233 (M), cultured *in vitro*

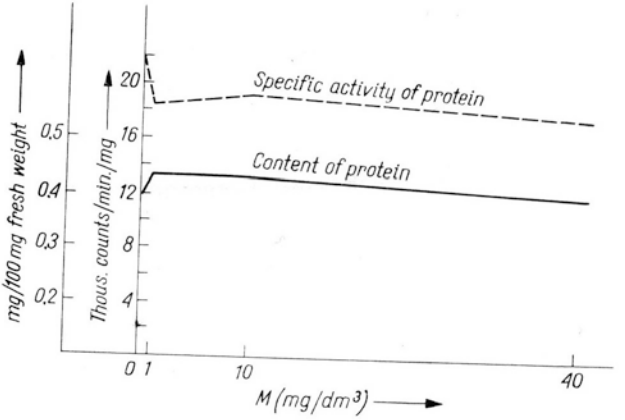

Diagram 2. Protein level and specific activity in tumour tissue of tobacco treated with morphactin IT 3233 (M), cultured *in vitro*

In callus tissues a greatly lowered specific protein activity is observed while the protein level is significantly higher. This proves that the metabolic processes, both protein synthesis and its breakdown, are limited.

Analogous results for tumour tissues do not show significant changes in their protein metabolism under the action of morphactin.

O x y g e n u p t a k e. The metabolic activity of tissues is connected with their respiratory activity.

Table 3 shows an oxygen uptake by callus and tumour tissues of tobacco cultured on a standard medium and with morphactin added.

Table 3

Oxygen uptake by *Nicotiana tabacum* L. tissues subjected to the action of morphactin IT 3233

Measurements	Substrate / tkanka	C	M-1	M-5	M-10	M-20	M-40
mm^3 O_2/l hr/1 g fr. wt. of tissues	callus	123 100%	147 119%	149 121%	136 110%	152 123%	157 128%
	tumour	145 100%	142 98%	173 119%	161 111%	173 119%	191 132%
mm^3 O_2/l hr/1 mg of protein	callus	33.4 100%	34,6 104%	33.0 99%	26.8 80%	29 87%	24 72%
	tumour	18.1 100%	16.5 91%	19.5 108%	18.2 100,5%	18.4 101%	21.9 121%

Notations as in table 1.

The results are given in reference to the fresh tissue mass and protein content in them.

Morphactin enhances oxygen uptake by callus tissues by 10 - 28 per cent as compared with the controls. In reference to protein, however, a respiratory activity of these tissues decreases under the influence of higher morphactin concentration by 13 - 28 per cent. This would confirm the supposition that metabolic activity is depressed by morphactin in callus tissues.

Under the action of morphactin the tumour tissues also take up oxygen more intensively as compared with the controls, but in conversion to protein these differences disappear.

If we refer oxygen uptake by callus and tumour tissues to the protein level, an almost twofold lower value is found in the latter tissue. This fact has already been established by Rennert (1968) and referred to the presence of a considerable amount of metabolically inactive proteins in tumour tissues.

DISCUSSION

The above presented results indicate that in callus tissues treated with morphactin a rise in the protein level accompanies growth inhibition, but at the same time the processes of protein synthesis are greatly limited and the respiratory activity of these tissues is depressed. This suggests, that the metabolic processes — both synthesis and degradation — are slowed down, and a large part of the protein produced is deposited and does not take part in the metabolism. The rise in the dry mass content may also be conditioned by the depression of the metabolic activity in these tissues.

The presence of metabolically inactive proteins was revealed in the fraction from the cell walls of plant tissues in culture *in vitro* (Steward et al., 1958; Dougall, Shimbayashi, 1960; Lamport, 1963).

This protein plays a structural role or serves as storage. The presence of a large quantity of hydroxyproline is characteristic for it (Pollard, Steward, 1959; Dougall, Shimbayashi, 1960; Olson, 1964; Lamport, 1963).

The presence of such protein in a tumour tissue of tobacco has been suggested by Rennert (1968) on the basis of the low respiratory activity of these tissues.

In view of the present results it may be supposed that morphactin increases the presence of this type of protein in tobacco callus tissue. Morphactin did not have a distinct effect on the protein content in

tumour tissues or on the intensity of protein synthesis, although a certain tendency was noted to its depression, and this could condition a certain limitation of growth.

The respiratory activity showed no significent changes in connection with the protein content. It would seem that the metabolism of tumour tissues does not change essentially under the influence of morphactin.

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Fizjologiczne i biochemiczne efekty działania morfaktyny IT 3233 na tkanki — kalusową i tumorową Nicotiana tabacum L. w hodowli in vitro

II. Synteza białek i aktywność oddechowa

Streszczenie

Morfaktyna IT 3233 wywołuje podwyższenie poziomu białek w tkankach kalusowych o 19—34% zależnie od stężenia. W tkankach tumorowych zmiany są nieistotne z jednym wyjątkiem — przy stężeniu 20 mg/dm^3 morfaktyny.

Zdolność pobierania leucyny —^{14}C z podłoża przez oba typy tkanek nie ulega istotnym zmianom pod wpływem morfaktyny, natomiast włączanie tego związku do frakcji białek jest obniżone do 50% w tkankach kalusowych traktowanych wyższymi stężeniami morfaktyny. W tkankach tumorowych — zmiany są nieistotne.

Aktywność oddechowa tkanek kalusowych również ulega obniżeniu pod wpływem morfaktyny, a tkanek tumorowych — w niektórych przypadkach — podwyższeniu.

Sądzi się, że morfaktyna powoduje obniżenie aktywności metabolicznej tkanek kalusowych, a nieznacznie działa na tkanki tumorowe.

Podwyższenie poziomu białek w tkankach kalusowych traktowanych morfaktyną tłumaczy się nagromadzaniem frakcji białek nieczynnych metabolicznie.