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Investigations on some metabolites of *Tecoma stans* Juss. callus tissue

Part I. Tissue culture

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Abstract

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Abstract

Callus tissue satisfactorily growing was established from *Tecoma stans* Juss. seedlings in static and suspension cultures on Murashige medium modified by Mei-Lie-Lin (M-L) and on Murashige-Skoog Revised Tobacco Medium supplemented with 0.3 ppm kinetin (RT-k). Faster growth, better growth efficiency and higher anatomical organization of the cultured tissue were observed on RT-k medium.

INTRODUCTION

The exotic species *Tecoma stans* Juss. (*Bignoniaceae*) (Fig. 1) arouses the interest of phytochemists regarding the occurence of physiologically active alkaloids and the posibility of their application in therapy. *T. stans* is a woody plant of tropical climates originating from Central America and the Western part of South America. In other tropical regions it is cultivated as an ornamental plant (Brenan, and Greenway, 1949; Thonner, 1915. repr. 1962; White and Angus, 1962; Engler, 1964).

The first report on the presence of alkaloids in this plant appeared in the end of the 19th century (Boorsma, 1897), and detailed investigations in recent years led to the identification of 9 compounds of monoterpene alkaloid character (Dickinson and Jones, 1969; Gross, Berg and Schütte, 1972; Hammouda, Plat and Le Men, 1963; Hammouda and Le Men, 1963).

In Mexico the leaves of many *Tecoma* species are used as an antidiabetic. The main alkaloid of *T. stans* — tecomanine and also tecostanine —

show in animal tests a strong hypoglycemic activity with potency equal to 180 per cent of that of *Tolbutamide* (Hammouda, Rashid and Amer Samir, 1964; Hammouda and Amer Samir, 1966).

Lapachol, a compound of the naphthoquinone group possesing an antimalarial and cytostatic action (Dudley and Chiang, 1969; Marini-Bettolo, 1971; Pettit and Houghton, 1968; Venditti and Abbot, 1967) is common in the family *Bignoniaceae* (Hegnauer, 1964). The wood of plants from the *Bignoniaceae* family containing lapachol is used in Brazil in treatment of neoplastic diseases (Jewers, Manchanda and Rose, 1972).

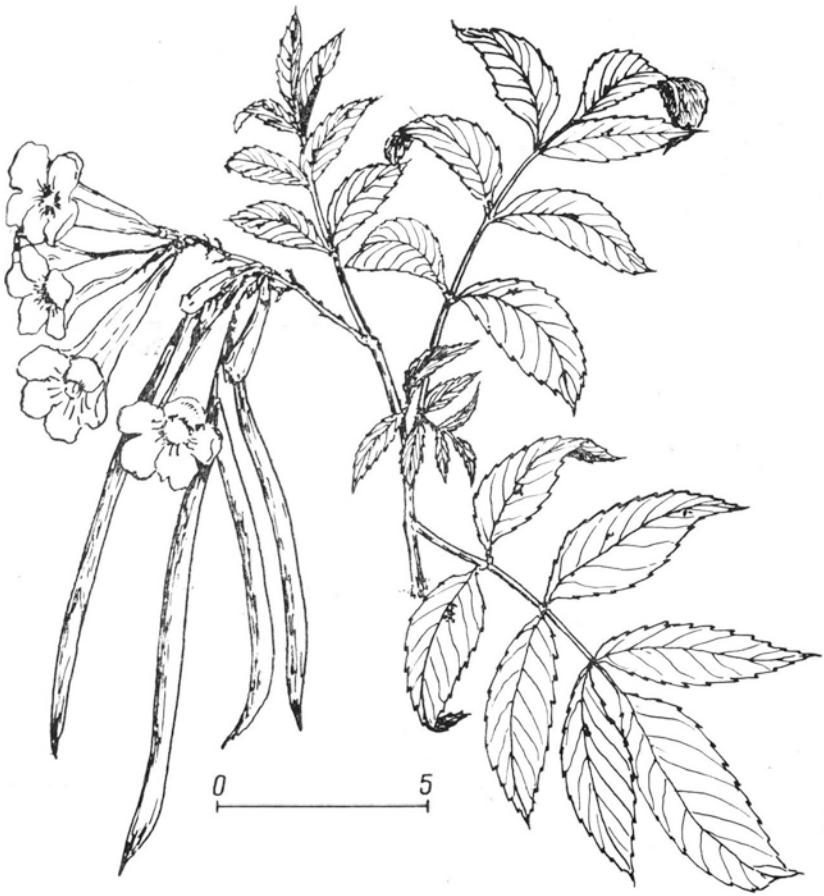
Culture of callus tissue from *T. stans* was undertaken in view of its content of biologically active alkaloids, and also of the possibility of the presence of lapachol. Investigation on these compounds in the cultured tissue will be the object of the second part of the present study.

MATERIAL AND METHODS

As outset material for tissue culture served seeds received from the Instituto de Investigacao de Agronomica de Angola, Nova Lisboa, Angola. Part of the seeds was sown into soil in greenhouse to obtain plant material for comparison. The seeds for tissue culture were sterilized with a 1.5 per cent aqueous solution of calcium hypochlorite for 30 min and placed in Petri dishes with filter paper moistened with distilled water and put in a thermostat (30°C). The seeds germinated after 3-6 days and were then placed on filter paper discs in test tubes with Knop medium (Gautheret, 1959). After about 7 weeks the seedlings reached a height of 6-8 cm and developed 3 pairs of leaves about 1-1.5 cm long. Stem thickness was 1-2 mm (Fig. 2). Pieces of the stem about 1 cm long and leaf blades were taken and inoculated on agar medium after incising them in several places to induce callus formation.

Sterile plants were cultivated on Knop medium diluted with water 1 : 1 and enriched with growth substances (kinetin 0.1 ppm, β-indol-acetic acid 0.01 ppm and glucose 2 per cent). For callus tissue culture the following media were used: Murashige medium modified by Mei-Lie-Lin (M-L), (Lin and Staba, 1961) and modified Murashige-Skoog Revised Tobacco Medium (Khanna and Staba, 1968) enriched with kinetin (RT-k) (Table 1). Suspension culture was run on both the above mentioned media with reduction of auxin concentration (2,4-dichlorophenoxyacetic acid 10^{-7} g/cm^3) and addition of kinetin and β-indolacetic acid (Table 1). The medium was adjusted to pH 5.6-5.8 with 1 per cent potassium hydroxide solution or hydrochloric acid. For solidification 0.9 per cent of agar was added. The media were sterilized in a steam autoclave and the vitamin solutions were passed through a Seitz bacterial filter.

Plate I



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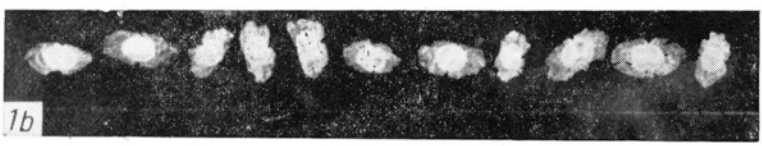

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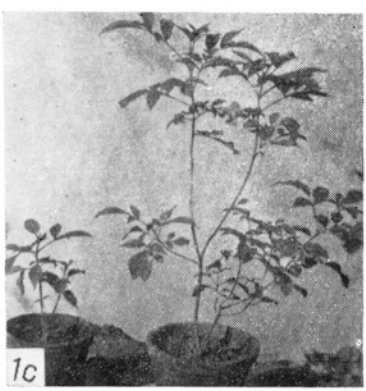

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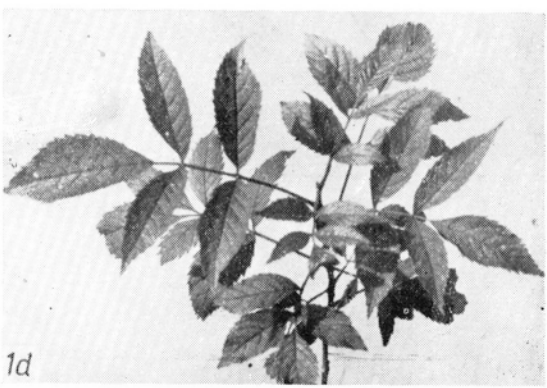


1d

1. *Tecoma stans* Juss. *a* — Shoot top with fruiting inflorescence (after El Hadid N. N., Boulos L., 1968); *b* — Seeds; *c*, *d* — Plants growing in greenhouse: *c* — one- and two-year-old plants, *d* — shoot top

Table 1
Tecoma stans tissue culture media

	M - L Murashige - Mei-Lie-Lin, mg/l		RT - k modified Murashige-Skoog, mg/l	
Basal medium	Inorganic salts			
	KNO_3	950.00	$MgSO_4.7H_2O$	370.00
	NH_4NO_3	720.00	KNO_3	1900.00
	$MgSO_4.7H_2O$	185.00	NH_4NO_3	1650.00
	$CaCl_2$	166.00	$CaCl_2.2H_2O$	440.00
	KH_2PO_4	68.00	KH_2PO_4	170.00
	$MnSO_4.H_2O$	7.00	$MnSO_4.H_2O$	23.30
	$ZnSO_4.7H_2O$	4.05	$ZnSO_4.7H_2O$	8.60
	H_3BO_3	2.40	H_3BO_3	6.20
	KJ	0.375	KJ	0.83
	$/NH_4/_6Mo_7O_{24}.4H_2O$	0.0925	$CuSO_4.5H_2O$	0.025
	$CuSO_4.5H_2O$	0.01	$Na_2MoO_4.2H_2O$	0.25
			$CoCl_2.6H_2O$	0.025
			$FeSO_4.7H_2O$	27.85
	FeEDTA	39.75	Na_2EDTA	37.25
	Growth substances and vitamins			
	Biotin	1.00	Cyanocobalamine	0.0015
	Choline	1.00	Folic acid	0.50
	Folic acid	1.00	Riboflavin	0.50
	Nicotinamide	1.00	Biotin	1.00
	Pantothenic acid	1.00	Choline	1.00
	Pyridoxine HCl	1.00	Ca Pantothenate	1.00
	Thiamine HCl	1.00	Pyridoxine HCl	1.00
	Riboflavin	0.10	Thiamine HCl	1.00
	2.4-D /static culture/	6.00	Nicotinamide	2.00
	2.4-D /suspension cul./	0.10	2.4-D /static culture/	1.00
			2.4-D /suspension cul./	0.10
	Myo-inositol	5000.00	Myo-inositol	5000.00
	Sucrose	20000.00	Sucrose	30000.00
Supplements	Static culture			
			Kinetin	0.30
	Suspension culture			
	Kinetin	0.03	Kinetin	0.03
	IAA	0.10	IAA	0.10

The culture was kept in a photothermostat at 26-28°C under constant illumination with fluorescent 40 W tubes "White" 5-61, with a light intensity of 800-1000 lux.

Callus tissue forming on the inoculated pieces was passaged to fresh medium every 4-5 weeks. After several months of growth on agar medium the static and suspension cultures were continued on liquid medium.

For static culture Petri dishes were used. The tissue was grown on glass U-tubes placed inside dishes and wrapped in filter paper to keep the culture above the liquid level and ensure supply of the medium (Fig. 4). In these conditions the tissue was passaged after 4-7 weeks of growth, that is when the medium was exhausted. The Erlenmayer flaks of 250 and 1000 cm^3 capacity contained 250 and 1000 cm^3 of medium, respectively were used for the suspension culture. They were aerated by shaking (100 swings/min) in rotating shakers of Vibroterm 1381 LE-204 type (Labor

Müszeripari Müvek, Hungary) with the water bath (28-30°C). Callus tissues from suspension cultures were filtered off after about 5 weeks of growth.

The increment of fresh callus tissue weight and cell suspensions was measured in growth efficiency units (S t a b a, 1969) expressing the mass increment in time and medium volume units.

$$\text{Growth efficiency} = \frac{W_1 - W_0}{M \cdot t}$$

where W_1 — end weight of tissue in grams, W_0 — initial tissue weight in grams, M — amount of medium in liters, t — duration of culture in days.

Callus tissue samples for microscopic observation were taken in the first phase of growth (7—10th day after inoculation) and from older, 3-4--week old cultures and prepared as smears. For preparing older tissues with a compact structure the standard paraffin method was used. The preparations were stained with 1 per cent gentian blue, floroglucin with hydrochloric acid, zinc chloride with iodine and Sudan III (F i l u t o w i c z, K u ż d o w i c z, 1951; J o h a n s e n, 1940).

RESULTS

Static culture of callus tissue

a) on Murashige medium, Mei-Lie-Lin (M-L) modification

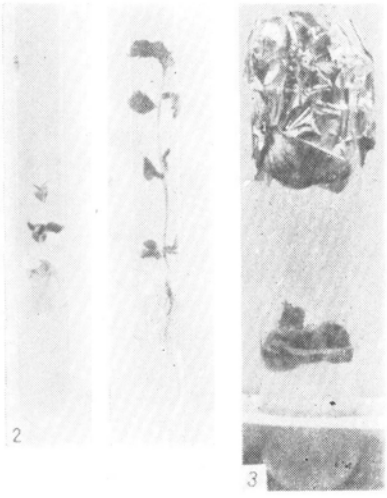
On the segments taken from the seedlings there formed after 2-4 weeks of culture a translucent light-grey soft callus tissue which expanded into a thick layer of rather loose consistence. This tissue after 6 months of culture on agar medium when transfered to liquid medium continued to develop in characteristic meander-like forms (Fig. 4).

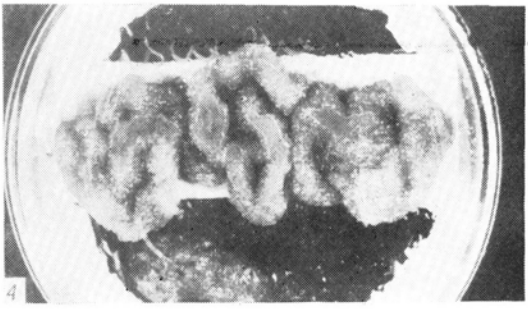
This tissue consists of a loose mass of long chains of thinwalled prosenchymatic cells varying in dimensions from 30-60 × 40-400 μ. Cells containing protoplast with a distinct nucleus and cytoplasmic threads as well as cells almost completely filled with plate-like granules of plastide character are noted (Fig. 5a, b). In older tissues vacuolized and dead cells prevail. This tissue resembles, mycelial structure and shows no trace of organization (Fig. 5).

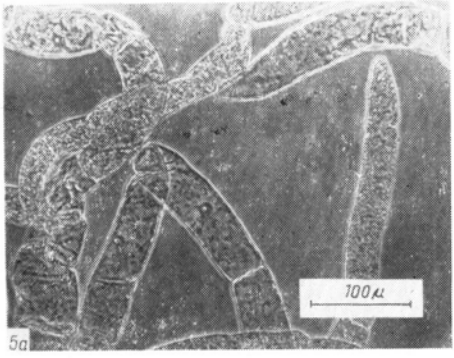
b) on Murashige-Skoog modified medium (RT-k)

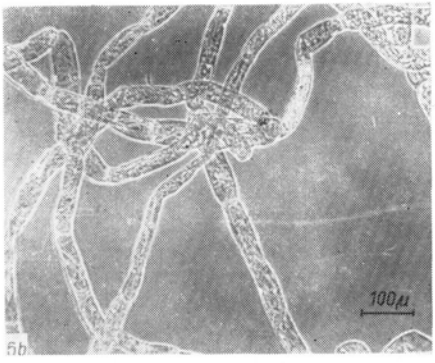
RT-k liquid medium was applied to the culture after 3 years growth of callus tissue on M-L medium. The change of medium, the remaining culture parameters remaining the same, caused marked differences in the

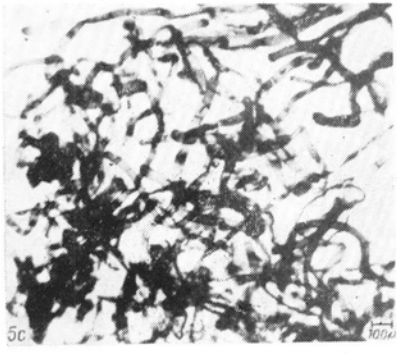
Plate II

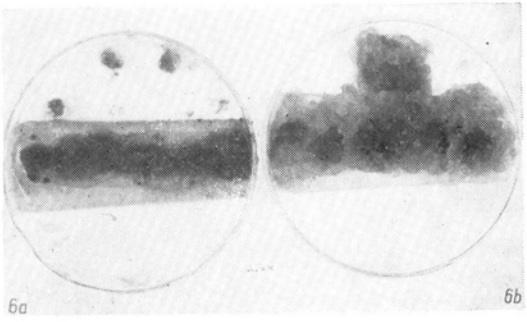




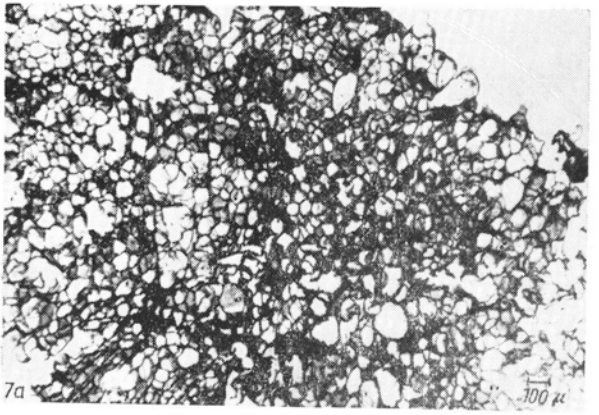


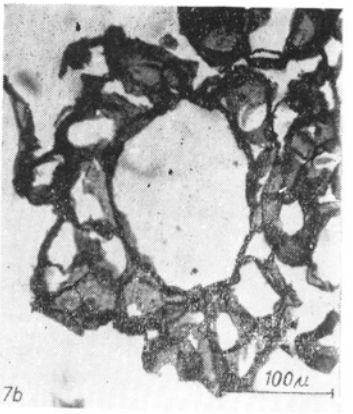


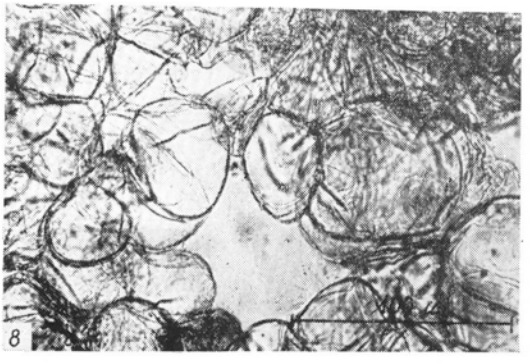


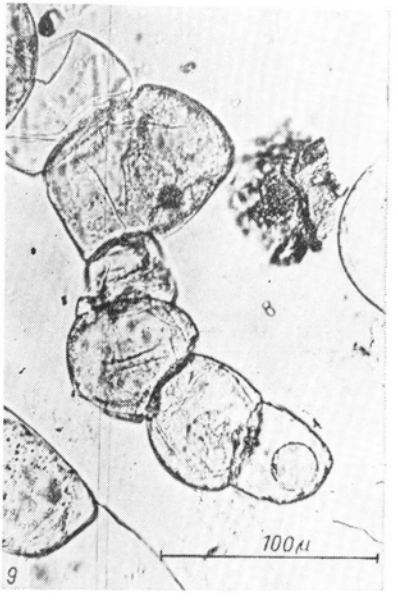


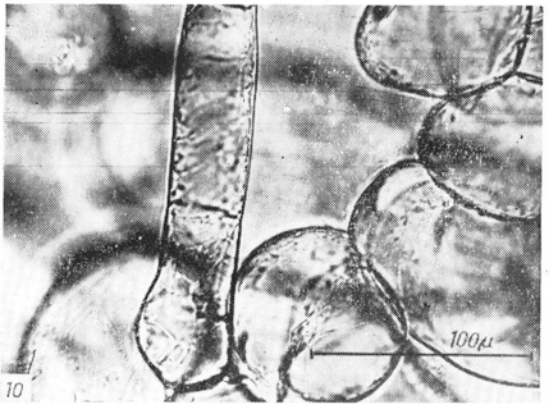
2. Two- and six-week-old seedlings on Knop medium; 3. Callus tissue arising from leaf fragment on M-L solid medium; 4. Static culture of callus tissue on liquid M-L medium; 5. Callus tissue from static culture on liquid M-L medium *a*, *b* — fixed material, phase-contrast photographs, *c* — smear technique, staining with gentian violet; 6. Callus tissue on medium: *a* — M-L, *b* — RT-k











7. a, b — Sections of callus tissue from static culture on RT-k medium, fixed older layer stained with gentian violet; 8. Intercellular spaces in fixed callus tissue from static culture on RT-k medium; 9. Cell aggregate from suspension culture; 10 Older partially vacuolized cells from suspension culture

tissue structure (Fig. 6). Instead of the soft loose mass, more compact tissue growth appeared consisting of nodular friable fragments which in the course of transplantation separated into smaller clumps.

In the course of 2-year culture on RT-k medium the tissue developed very well and in time began to produce chlorophyll strains. However, no trace of organogenesis was ever noted in the form of bud or root formation.

The young tissue consisted of small thinwalled almost isodiametric cells of 40-100 μ diameter, joined into short chains or small spherical aggregates.

Older parts of the callus tissue show a compact parenchymatous stucture (Fig. 7a). Cells with wide lumen (100-200 μ) and thicker cell walls occur in it, filled with protoplasmic content as well as empty dead cells. Such single larger cells or groups of them are surrounded by thinwalled cells of much smaller dimensions with diameter not exeeeding 50 μ (Fig. 7b). These cells of probably cambial character surround also the intercellular spaces (Fig. 8).

Groups of larger and smaller cells are scattered randomly in the tissue mass. Formation of an organized cambial tissue producing vascular bundles was never observed. Neither were the presence of starch and traces of lignification or suberization of cell membranes noted.

Suspension culture

In the suspension culture several days after inoculation occurred a phase of rapid cell division leading after 3-5 weeks to the formation of a more or less dense grey or brown suspension. Further culture did not give any mass increment and the inhibition of cell division was associated with a darkening of the culture (stationary phase).

In the cultures on both media (M-L and RT-k) single cells may be seen with thin membranes beside the small chains or spherical aggregates of them (Fig. 9). In the initial phase of growth (8-10 days after inoculation) young cells prevail filled with granular protoplasmic contents with a usually centrally situated distinct nucleus. Beside them there occur in small numbers more or less vacuolized cells and single dead ones (Fig. 10). After 3-4 weeks of growth the number of autolysed cells increases until they become dominating in 5-6-week suspensions.

Tissues mass increment

Mean data of growth efficiency for the fresh callus tissue mass and cell suspensions as well as water content in these tissues were obtained in several replications and are listed in Table 2.

Table 2
Water content and growth rate of Tecoma stans tissues cultivated on liquid media

Culture	Medium	Growth Efficiency tissue wet weight g/liter/day	Water content %	Growth Efficiency calculated for tissue dry weight g/liter/day
Static	M-L	17.0	97.9	0.36
	RT-k	22.0	95.1	1.00
Suspension	M-L	9.0	98.2	0.16
	RT-k	13.0	98.1	0.25

DISCUSSION AND CONCLUSIONS

Good results have been obtained with the use of M-L and RT-k media for tissue culture of *T. stans*. The growth yields were higher in static cultures, reaching on the average values of 17-22 g/l/day, whereas in the suspension cultures they did not exceed 13 g/l./day. According to some authors (S t a b a, 1969), the mean growth yields are higher in suspension cell cultures than in static cultures of callus and reached to 111 g/l./day in dependence on the technique applied and the kind of tissue.

In tissue cultures of *T. stans* better growth yields were obtained on lqiuid RT-k medium in static culture. Moreover, from this tissue with lowest moisture per cent the dry mass amount exceeded 3-6 times that obtained in the remaining combinations.

In the supension culture, beside the lower growth yield than in static culture, difficulties occurred owing to a higher per cent of infections or culture failure manifested in a lack of mass increment and darkening of the implanted inoculum without any noticeable cause. Therefore the material obtained from static cultures was mainly used for elaboration.

In these tissue cultured on liquid M-L and RT-k media wide differences were noted in the morphological and anatomical structure. The high auxin content (2,4-D 6 ppm) in M-L obviates the tendency to development of proper cambial tissue forming the vascular elements, but it only stimulates random cell (division (G a u t h e r e t, 1959). In the case of such division giant cells arise forming a loose homogeneous tissue without any organization, resembling thallus.

Callus tissue of *T. stans* preserved on M-L medium in the course of 3 years of culture (more than 30 passages) the character of such primary unorganized succulent tissue formed of a loose cells mass.

On the other hand, callus tissue obtained on RT-k medium is organized to some extent and characteristic for the morphotype of complex colonies which arise as the result of degraded cambium division (G a u t h e r e t,

1959). Cambial cells of such tissue are not capable of producing a proper layer with vascular bundles, but they aggregate into islets forming a homogeneous parenchyma. Chlorophyll strains frequently occur in such tissues.

The changes in the character of cell division and anatomical structure of the callus tissue of *T. stans* after transfer from M-L to RT-k medium seems to be caused by significant changes in the proportions of the main medium components, namely: an increased mineral salts and sugar content, a 6-fold decrease in auxin concentration and addition of kinetin in a favourable ratio to auxin (1 : 3).

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Badania niektórych metabolitów tkanki kalusowej Tecoma stans Juss. Hodowla tkankowa

Streszczenie

Tecoma stans Juss., roślina drzewiasta klimatu tropikalnego, zawiera alkaloidy monoterpenowe o działaniu hipoglikemicznym.

Z nasion pochodzących z Angoli wyhodowano na pożywce Knopa sterylne siewki, z których pobrano wycinki łodyg i liści w celu uzyskania tkanek kalusowych. Hodowlę prowadzono na pożywkach: Murashige modyf. Mei-Lie-Lina (M-L) i zmodyfikowanej Murashige-Skooga, Revised Tobacco Medium (RT-k), agarowych i płynnych: zawiesinową w kolbach Erlenmayera i stabilną w szalkach Petriego. Najwyższe wydajności wzrostu tkanki kalusowej (22 g świeżej masy/l/dzień) uzyskano na płynnej pożywce RT-k.

Stwierdzono różnice w budowie anatomicznej tkanek rosnących na pożywkach o odmiennym składzie, głównie w zakresie stężeń substancji wzrostowych, soli mineralnych (związki azotowe) i cukru. Miękka, rozlewająca się tkanka kalusowa hodowana na pożywce M-L, przedstawia luźne łańcuchy komórek prozenchymatycznych, bez śladów organizacji tkankowej. Kalus ten przepasażowany na pożywkę RT-k staje się bardziej zbity i twardy, dzieląc się na mniejsze, brodawkowate fragmenty. Jest zbudowany z komórek izodiametralnych, formujących zwartą tkankę o charakterze homogennej parenchymy, czyli wykazuje już pewną organizację tkankową.