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

Part II. Chromatographical analysis of alkaloid and quinone compounds

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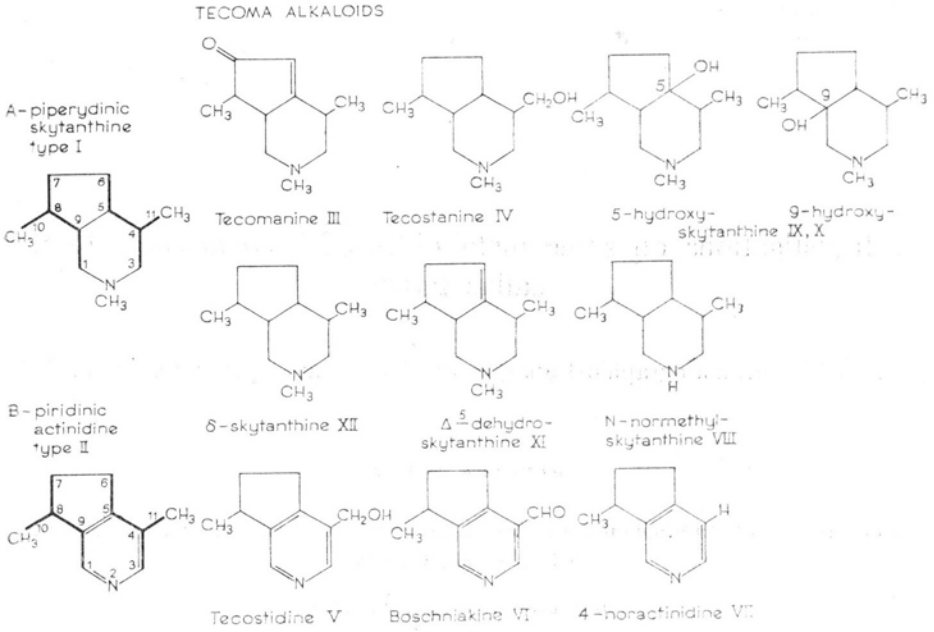
Abstract

Tecoma stans Juss. callus tissue grown on Murashige-Mei Lie Lin (M-L) and Murashige-Skoog (RT-k) medium supplemented with *Tecoma* alkaloid precursors like lysine, mevalonic acid lactone and quinolinic acid, were investigated for their alkaloid content by thin-layer (TLC) and paper (PC) chromatography methods. The results were compared with those obtained by parallel analysis of greenhouse plant leaves. Seven alkaloid spots were detected in the leaf extracts, namely: actinidine, 4-noractinidine, boschniakine, tecomanine, two spots of skytanthine derivatives and one unidentified spot. Only 1 spot corresponding to skytanthine derivatives appeard in the extracts of callus tissues cultured on basal M-L medium or supplemented with lysine or mevalonic acid lactone. However, 3 alkaloid spots were revealed in extracts of homogeneous parenchyma-like callus tissues obtained on RT-k medium. Beside the skytanthine derivatives mentioned above, actinidine and tecomanine were identified, and moreover, in callus tissues growing on the same medium (RT-k) supplemented with quinolinic acid, boschniakine was found. It was found that *T. stans* leaves as well as callus tissues cultured on RT-k medium contained traces of lapachol and another quinone-type compound.

INTRODUCTION

In the species *Tecoma stans* Juss. monoterpene alkaloids containing a piperidine ring (skytanthine type — I) or a pyridine ring (actinidine type — II) and a conjugated cyclopentane ring are present. The particular compounds differ in the degree of oxidation level of both rings and the number of methyl groups.

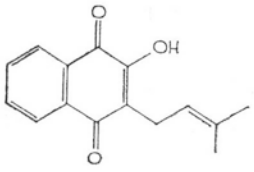


Nine alkaloids have been isolated from this plant and their structure was established: tecomanine (III), tecostanine (IV), tecostidine (V) (Hammouda, Plat and Le Men, 1963a, b; Hammouda and Le Men 1963), boschniakine (VI), 4-noractinidine (VII), N-normethylskytanthine (VIII), 5- and 9-hydroxyskytanthine (IX and X) Dickinson and Jones, 1969), Δ^5-dehydroskytanthine (XI) and δ-skytanthine (XII) (Gross, Berg and Schütte, 1972a, b and 1973).

The last named authors (1972a) by using labeled precursors confirmed the monoterpene origin of these alkaloids and their biosynthesis via mevalonic acid as intermediate product. They also stressed the significance of 7-deoxyloganinic acid as key metabolite on the way from nitrogenless iridoid compounds to alkaloids of this type.

Qualititative and quantitative differences concerning these compounds were found in the alkaloid spectrum of material originating from various geographical regions (Gross, Berg and Schütte, 1973; Dickinson, and Jones, 1969).

In the family *Bignoniaceae* lapachol, 2-hydroxy-3-(3-methyl-2-butenyl)1,4-naphthoquinone (XIII) is common. So far, however, this compound has not been detected in *T. stans*. In the leaves of this plant, on the other hand, a group of enzymes was found converting anthranilic acid to catechol (Nair and Vaidyanathan, 1964; Ellis, and Towers, 1969), and further catechol to diphenylene-dioxy-2,3-quinone (DDQ) (Kandaswami and Vaidyanathan, 1973).



Lapachol (XIII)

The presence of quinone compounds and among them of lapachol, chemotaxonomically characteristic for the *Bignoniaceae* family, is therefore, to be expected.

The first part of this paper concerned tissue culture of *Tecoma stans* Juss. (D o h n a l, 1976) for investigation of the biosynthetic properties of this tissue, particularly as regards compounds of the alkaloid group.

The present study comprises chromatographic analysis of extracts from callus tissue and leaves of glasshouse plants to establish whether under *in vitro* culture conditions they are capable to synthesis the secondary metabolites of alkaloid type or quinone type (lapachol), which are characteristic for the intact plant.

MATERIAL AND METHODS

Callus tissue, medium after tissue culture and leaves of the glasshouse plants were used as the starting material for the chromatographical examinations.

Callus tissue of *T. stans* was collected successively from static cultures on liquid Murashige-Mei Lie Lin (M-L) medium and modified Murashige-Skoog (RT-k) medium supplemented with compounds of the type of *Tecoma* alkaloid precursors. Five kinds of tissues cultured on several modifications of the basal media were examined (denoted by the symbols given in Table 1).

Table 1

Tecoma stans callus tissue material, applied media and supplements

Callus tissue	Basal medium*	Precursor added	Concentration
K	M - L	—	
K - l	M - L	lysine	10^{-5} g/cm^3
K - m	M - L	mevalonic acid lactone	10^{-4} g/cm^3
R	RT - k	—	
R - q	RT - k	quinolinic acid	10^{-4} M

* Medium composition see: Dohnal B., 1976.

The medium was filtered off from the tissue mass which was then washed repeatedly with water, dried at 50—60°C and ground to powder. The medium from tissue culture in the amount of 1 l. was concentrated under reduced pressure at 50—60°C to a volume of 100 ml. Leaves collected from 2-3-year old plants reared in soil were dried at the same temperature and ground to powder.

Lapachol content was determined in callus tissues R and in the leaves of glasshouse plants.

The extracts for alkaloid determination were prepared according to Dickinson and Jones (1969) (Fig. 1).

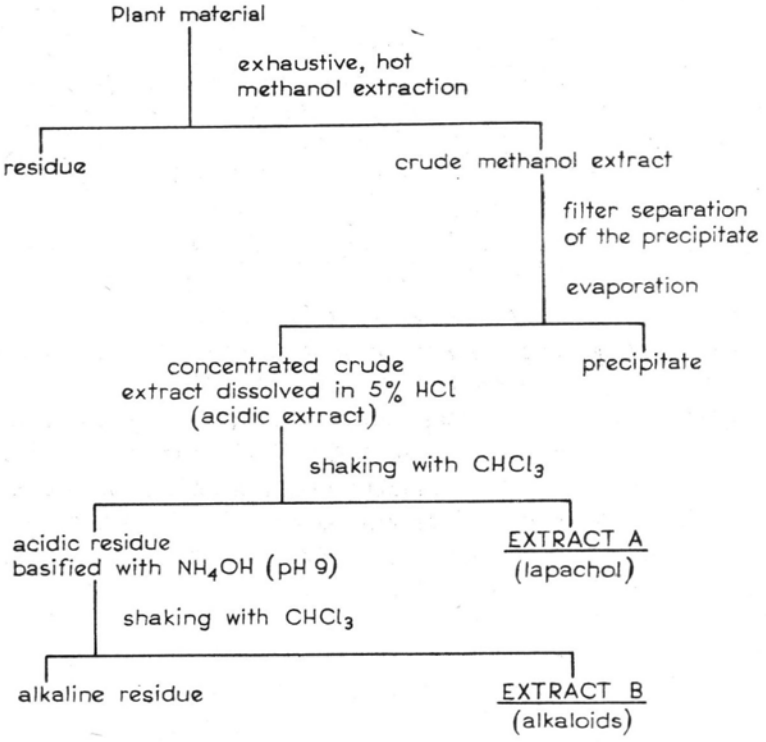


Fig. 1. Extraction procedure

The dried material (callus tissues and leaves) in the amount of 50 g were refluxed three times with hot methanol on a water bath. The combined crude extracts were concentrated under reduced pressure to a thick oily residue which was shaken with 5 per cent hydrochloric acid. The acid extract (ca. 20 ml) was thrice shaken with 50 ml of chloroform (extract A). The aqueous acidic extract was alkalized with ammonia to pH 9 and thrice shaken with 50 ml of chloroform (extract B).

The concentrated medium culture was acidified with hydrochloric acid to pH ca 4 and shaken with chloroform. The further procedure was the same as with tissue extract.

The chloroform extracts A and B were dried with anhydrous sodium sulphate and after filtration concentrated under reduced pressure. For chromatographic determinations the oily residue was dissolved in several milliliters of chloroform.

The acid extracts (Fig. 1) were first examined for the presence of alkaloids by the precipitation method using the following reagents: [1] Meyer's reagent (Turowska, Kohlmünzer and Molik-Węgiel, 1970), [2] Dragendorff's the modification of Thies-Reuter (Stahl, 1969) and [3] 12 per cent silicic-tungstic acid (Tylor, Stuntz, 1962).

B extracts were analysed by paper (PC) and thin-layer chromatography (TLC): [a] Whatmann No. 1 paper, descending technique, path length 45 cm, solvent system n-butanol:glacial acetic acid:water (4:1:5 v/v), organic layer, developing time 18-20 h (Partridge, 1948); [b] silica gel H (E. Merck), solvent system chloroform:benzene:methanol (10:2:1 v/v), development time 1 h (Gross, Berg and Schütte, 1972b); [c] silica gel G (E. Merck) in 0.5 N NaOH, solvent system chloroform:methanol (4:1 v/v), development time 1.5 h (Hammouda, Plat and Le Men, 1963); [d] silica gel G (E. Merck) in 0.5 per cent KOH, solvent system n-butanol:glacial acetic acid:water (4:1:1 v/v), development time 4 h (Jerzmanowska, 1967); path length in TLC was 17 cm.

The extract amounts corresponding to 500 mg-1g dry weight of callus tissue, 50-100 mg of leaf dry weight and 50-100 ml medium were spotted on the starting line. Standard alkaloid substances: tecomanine, actinidine, 4-noractinidine and boschniakine, were dissolved in chloroform and spotted on the plates parallely with the extract. Developed chromatograms dried at room temperature were visualized with Dragendorff's reagent in the Thies-Reuter modification.

Extracts A (Fig. 1) were analysed for the content of quinone compounds by chromatographic methods (PC and TLC) in the following solvent systems: [a] Partridge — as for alkaloids; [e] silica gel H (E. Merck), solvent system benzene : ethyl acetate : glacial acetic acid (90:10:1 v/v), development time 1 h (Dudley and Chiang, 1969); [f] silica gel H (E. Merck), solvent system chloroform:benzene (3:7 v/v) development time 40 min (Gavindachari, Patankar and Viswanathan, 1971).

The extract amounts corresponding to 500-700 mg of dry tissue weight were placed on the chromatograms and 20-30 μ lapachol parallely for comparison. After development the plates were inspected under UV light and the spots were visualized in ammonia vapour and after spraying with 2 per cent ferric chloride solution in ethyl alcohol.

RESULTS AND DISCUSSION

Alkaloid compounds

Preliminary investigation of acid extracts by the precipitation method demonstrated the presence of considerable amounts of alkaloids in leaves and minimal quantities in callus tissues cultured on M-L medium. The restricted ability of alkaloid synthesis of the callus tissue suggested the idea of running cultures on medium of another composition in order to stimulate biosynthesis of these compounds. The presumable piperidine, pyridine and monoterpene alkaloid precursors, such as lysine, quinolinic acid and mevalonic acid lactone were also added to the medium for this aim. In media from tissue cultures alkaloids were not found, with the exception of the RT-k medium with quinolinic acid added in which trace amounts were revealed. The results of chromatographic analysis of leaf extracts from 5 kinds of callus tissues cultured on variously modified media are listed in Table 2 and Fig. 2.

In leaf extract, in dependence on the solvent system applied 4-7 alkaloid spots appear, three of which are noted in all systems with identical R_f value to those of the standards — actinidine, boschniakine and tecomanine. The characteristic of the remaining alkaloid spots was based on literature data (B e r g, 1972). In some cases slight differences (lower R_f values) were found owing to the lower purity of the extracts. In the [b] system a spot with R_f 0.14 was found which may be considered as corresponding to δ-skytanthine (R_f 0.18) and 5-hydroxy-skytanthine (R_f 0.13) which develop next to each other. The lowest situated spot with R_f 0.06 is attributed to one of the most strongly polar *Tecoma* alkaloids of skytanthine type with similar R_f values (tecostanine 0.08 and N-normethylskytanthine 0.04) identified by B e r g (1972). In the quoted paper no counterpart was found either to the spot with R_f 0.35 (system b) occurring between tecomaine and actinidine, or to actinidine (R_f 0.45) appearing so distinctly in the leaf extract in the present investigations.

In callus tissues in all analyses a much lower amount and number of alkaloids were found than in the leaves.

One elongated alkaloid spot appears in tissue extracts K, K-1 and K-m (Table 1) only in the [f] system, another unidentified weak spot of alkaloid character was noted with a counterpart in the leaf extract. The R_f value 0.05 [System b] of the elongated spot common to all the above mentioned tissues was compared with the data for this system in the quoted paper by B e r g (1972). This spot seems to correspond to the mixture of highly polar skytanthine derivatives identified by Berg in root, leaf and seed extracts from *Tecoma stans* and also found by the present author in leaves.

In callus tissue extracts R and R-q (Table 1) there appeared, moreo-

Table 2

Paper and thin-layer chromatography of alkaloid extracts (B) from *Tecoma stans* callus tissues and leaves

Solvent system	R_f values									
	Extracts B						Standard alkaloids**			
	Leaves	Callus tissues*								
		K	K-l	K-m	R-q	R	Tec	Act	nAct	Bosch
PC a)	0.57				0.57	0.57	0.57			
	0.68									
n-butanol:glac.	0.75				0.75	0.75		0.76	0.73	
acetic acid:	0.80	0.81	0.81	0.81						
water	0.87				0.87					0.87
4:1:5										
TLC b)	0.06	0.05	0.05	0.05	0.05	0.05				
	0.14									
chloroform:ben-	0.22				0.20	0.19	0.20			
zene:methanol	0.33									
	0.45							0.45		
10:2:1	0.50				0.48	0.49			0.50	
	0.62				0.61					0.62
TLC c)	0.17	0.18								
	0.27	0.27	0.27	0.27	0.27	0.27				
chloroform:me-	0.35									
thanol	0.57									
	0.62				0.62	0.62	0.62			
4:1	0.73				0.74	0.73		0.75	0.75	
	0.86				0.86					0.87
TLC d)	0.04	0.04	0.03	0.03	0.03	0.03				
	0.18				0.18	0.18	0.18			
n-butanol:glac.	0.29									
acetic acid:	0.40				0.40	0.40		0.41	0.39	
water	0.48									
4:1:5	0.56				0.57					0.57

Abbreviations for: * callus tissues see table 1, ** alkaloids: Tec — tecomanine, Act — actinidine, nAct — 4-nor-actinidine, Bosch — boschniakine.

ver, spots corresponding to tecomanine and actinidine or its 4-nor derivative and in the extract from R-q tissue also boschniakine.

The scant alkaloid spectrum of callus tissues cultured on M-L medium points to the lack of the enzymes indispensable for biosynthesis of this group of compounds. This probably may result from the creative character of this tissue without any organization. This tissue growing on medium with high auxin content (2,4-D 6.10^{-6}g/cm^3) is mainly active in cell division and basic metabolites synthesis with considerable limitation of secondary metabolites biosynthesis.

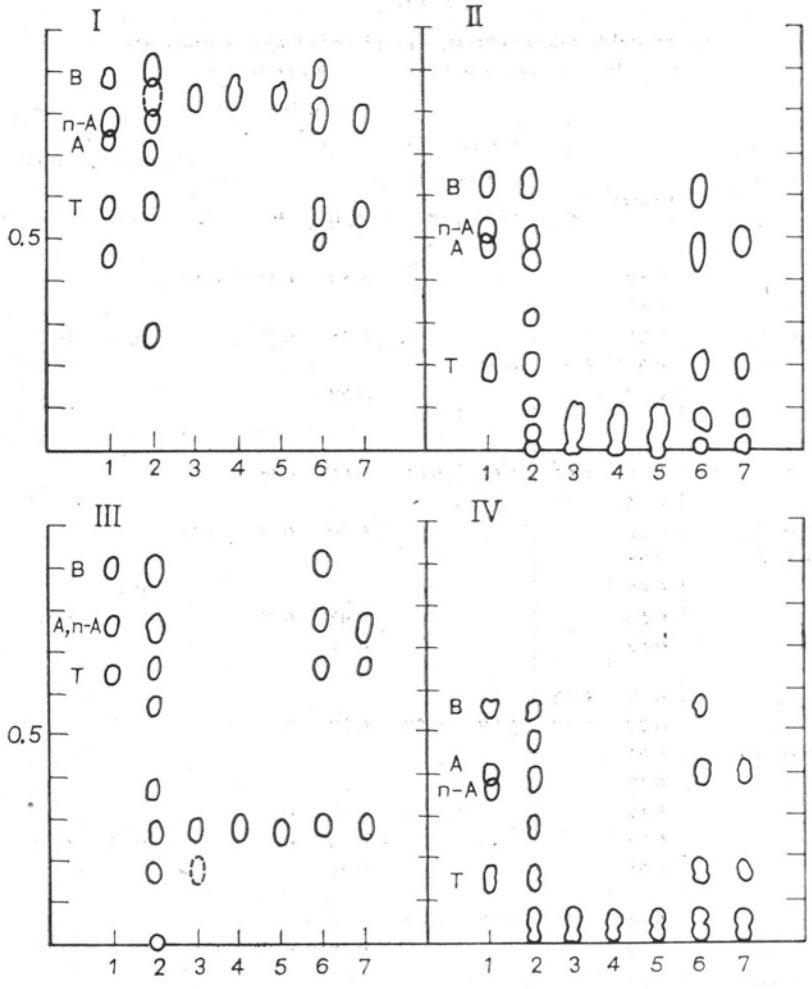


Fig. 2. Chromatograms of alkaloid extracts (B)

I — paper chromatography, solvent system [a]; II, III, IV — thin-layer chromatography, solvent systems [b, c, d] respectively; 1 — standard alkaloids: A — actinidine, n-A — 4-nor-actinidine, B — boschniakine, T — tecomanine; 2 — leaf extracts; 3 — 7 extracts of callus tissues: 3 — K, 4 — K-1, 5 — K-m, 6 — R-q, 7 — R, for explanation see Table 1. Spots encircled with broken lines — in trace amount

The failure of lysine addition to stimulate these processes seems to confirm the opinion that this compound should be excluded as monoterpene alkaloid precursor (G r o s s, 1970). On the other hand, the unexpectedly negative result for mevalonic acid lactone, the participation of which in biosynthesis of *Tecoma* alkaloids was convincingly demonstrated by G r o s s, B e r g and S c h ü t t e (1972a), might be interpreted as a too low dose of the compound used or a strong inhibition of enzymes catalyzing cyclization of two isoprene units in this undifferentiated tissue.

The alkaloid biosynthesis stimulation (3 compounds) observed in R callus tissue may be connected with its higher degree of organization. Such tissue, cultured on RT-k medium richer in nutrient substances but with a lower auxin concentration, developed a kind of homogeneous parenchyma, when compared with K tissue it achieved a certain anatomical organization. In culture *in vitro*, the higher tissue organization is usually associated with activation of biosynthesis of secondary metabolites (Patterson and Carew, 1969; Raj Bhandary, Collin, Thomas and Street, 1969; Reinhard, 1967; Teuscher, 1973).

Certain analogies may also be noticed with the results of Berg (1972) who identified in young 7-day-old seedlings of *T. stans*, that is in tissues undergoing rapid division, only one alkaloid — tecomanine, in the seeds two — tecomanine and 5-hydroxyskytanthine, in 10-day plants four — tecomanine, tecostanine, δ-skytanthine and Δ^5-dehydroksytanthine and in the mature plant as many as 7 compounds of this group, and beside the above mentioned ones N-normethylskytanthine and boschniakine.

In callus tissue cultured on RT-k medium a further stimulation of alkaloid biosynthesis occurs after quinolinic acid addition. Although the role of this compound as *Tecoma* alkaloids precursor is questionable, this fact may be explained according to the Leete's (1965) suggestions that a compound which does not fit into the given biosynthesis scheme, may be incorporated into its end product after conversion to one of the intermediate metabolites proper.

For a clearer illustration of the differences found in the alkaloid spectrum of callus tissue and leaves and for comparison of the observed relations between the number of alkaloids and the morphological structure of the tissue and composition of the medium, the results are listed in Tables 3 and 4.

Table 3

Alkaloids revealed by chromatography (PC,TLC) in *Tecoma stans* leaves and callus tissues

Alkaloids	Leaves	Callus tissues*				
		K	K-l	K-m	R-q	R
Actinidine	+	—	—	—	+	+
4-noractinidine	+	—	—	—	—	—
Boschniakine	++	—	—	—	+	—
Tecomanine	++	—	—	—	+	+
Skytanthine derivat.	++	++	++	++	+	+
Unidentified	+	—	—	—	—	—

* Abbreviations for callus tissues see table 1; ++ spots very pronounced, + spots well marked, — no spots.

Table 4

Correlation between nutritional requirements of *Tecoma stans* callus tissue with its morphology and ability to alkaloid biosynthesis

Medium	Essential differences	Callus tissue			
		Morphology		Growth efficiency* g/l/day	Number of alkaloids
		External	Internal		
Murashige modified (M-L)	2,4-D 6 mg/l Kinetin — Sucrose 2 %	White-gray, loose, soft, dividing into threads	Matted chains of elongated cells 30-60 μ × 40-400 μ "Thallus" type	0.36	1
Murashige-Skoog modified (RT-k)	2,4-D 1 mg/l Kinetin 0,3 Sucrose 3 % Inorganic salts concentration — 2-2,5 fold higher than in M-L medium	Light green, fine, more compact, hard, consisting of granular agglomerates	Homogeneous parenchyma Isodiametric cells groups of cambium character (⌀ 50 μ) and older ones (⌀ 100 μ), disorderly scattered in tissue mass "Complex colony" type	1.00	3

* calculated for tissue dry weight.

Quinone compounds

In extract A from callus tissues and leaves the occurrence of spots corresponding to quinone compounds was demonstrated chromatographically (PC and TLC). These spots exhibited a deep violet fluorescence in UV light, salmon-orange in visible light, salmon-red in ammonia vapour and rusty-red in ferric chloride.

In the solvent system [a] there occurred one elongated spot with R_f 0.91-0.93, that is somewhat lower as compared with that of the lapachol standard (R_f 0.98). In the systems [e] and [f] two characteristic spots were detected one of which with R_f 0.56 [system e] and 0.34 [system f], corresponds to the lapachol standard, whereas the other with lower values, R_f 0.28 [system e] and 0.19 [system f] indicates the presence of another quinone compound which has not been identified.

When observing the intensity of colour and the size of the spots it was remarked that in light coloured, quickly extending callus tissues the lapachol spot is visible as a trace only, while in older tissues turning brown it is somewhat more pronounced. This would suggest that the intensive metabolism of young tissue inhibits enzymatic oxidizing processes leading to naphthoquinone formation.

In extracts from media after callus tissue culture no quinone derivatives were detected by the same chromatographic methods.

The author is greatly indebted for the gift of standard substances to: professor dr H. R. Schüte of the Institute of Plant Biochemistry, Halle, Saale (East Germany) for actinidine and diethylacetal-boschniakine picrates, dr G. Jones of the Keele University (Great Britain) for tecomanine and 3-noractinidine picrates and dr K. C. Joshi of the Rajasthan University, Jaipur (India) for the lapachol standard.

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Badania niektórych metabolitów tkanki kalusowej Tecoma stans Juss. Cz. II. Analiza chromatograficzna związków alkaloidowych i chinonowych.

Streszczenie

Tkanki kalusowe *Tecoma stans* Juss. przebadano na zawartość alkaloidów monoterpenowych, które były identyfikowane w tym gatunku (9 związków tego typu, pochodne skytantyny i aktynidyny). Przeprowadzono porównawcze oznaczenia tych związków w tkankach kalusowych, uzyskanych z hodowli na dwóch pożywkach podstawowych: Murashige-Mei Lie Lina (M-L) i Murashige-Skooga (RT-k), które uzupełniono przewidywanymi prekursorami alkaloidów Tecoma: lizyną, laktonem kwasu mewalonowego i kwasem chinolinowym. Wyniki odniesiono do analogicznych oznaczeń w liściach roślin szklarniowych, uzyskanych z tego samego materiału siewnego.

Do badań używano tkanek suchych, ekstrahując je metanolem. Związki alkaloidowe w wyciągach chloroformowych z frakcji alkalicznej oznaczano chromatograficznie (PC i TLC) w 4 układach rozwijających, używając do identyfikacji plam wzorców i porównując wyniki z danymi z literatury.

W wyciągach z liści stwierdzono siedem plam alkaloidowych, odpowiadających aktynidynie, 4-noraktynidynie, boschniakinie, tekomaninie, dwu pochodnym skytantyny i jedną niezidentyfikowaną.

W wyciągu z plechowatej tkanki kalusowej, wyhodowanej na pożywce M-L występowała jedna plama, odpowiadająca pochodnym skytantyny, a dodanie do pożywki lizyny i laktonu kwasu mewalonowego nie dało wzbogacenia spektrum alkaloidowego w tkankach hodowanych na tych pożywkach.

W wyciągu z tkanki kalusowej o charakterze homogennej parenchymy, otrzymanej na pożywce RT-k, wystąpiły trzy plamy alkaloidowe. Poza wykrytymi w wymienionych tkankach pochodnymi skytantyny, stwierdzono aktynidynę i tekomaninę, a po dodaniu kwasu chinolinowego do pożywki jeszcze i boschniakinę.

Zaobserwowano zależność między wzrostem organizacji tkankowej a zawartością związków alkaloidowych oraz stymulujący wpływ kwasu chinolinowego podanego w pożywce, na biosyntezę alkaloidów monoterpenowych *Tecoma*.

W wyciągach chloroformowych frakcji kwaśnej z liści i tkanek kalusowych hodowanych na pożywce RT-k, stwierdzono występowanie nieznacznych ilości lapacholu oraz związku bliżej nie zidentyfikowanego, o charakterze chinonu. Z tkanek kalusowych jasnych, intensywnie rosnących, otrzymano plamy śladowe tych związków, natomiast w starszych, brunatniejących, zaznaczają się one nieco wyraźniej.