Phytochemistry, 1971, Vol. 10, pp. 1529 to 1532. Pergamon Press. Printed in England.

REGULATION OF NICOTINE BIOSYNTHESIS BY AUXINS IN TOBACCO CALLUS TISSUES*

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(Received 17 *July* 1970)

Abstract-Chemical constituents in 2,4-D callus and IAA callus of *Nicotiana tabacum* var. Bright Yellow grown on 2,4-D medium or IAA medium for about 5 years were compared. No alkaloids were found in 2,4-D callus, while nicotine, anatabine and anabasine were found in IAA callus. On transference of 2,4-D callus to IAA medium and IAA callus to 2,4-D medium, it was demonstrated that nicotine biosynthesis in the callus tissue is activated by the supply of IAA and suppressed by that of 2,4-D.

INTRODUCTION†

ALTHOUGH nicotine,[1,2] phytosterols,[3,4] triterpenoids,[3] scopoletin[4–6] and scopolin[5,6] have been demonstrated in tobacco callus tissues, studies on nicotine and other compounds, except scopoletin,[7] in tissue grown in media containing auxins such as 2,4-D or TAA have not been reported.

The callus tissue (named 2,4-D callus) derived from tobacco stem (*Nicotiana tabacum* var. Bright Yellow) was subcultured for about 5 years on the medium containing 2,4-D (2,4-D medium).[2,4] The other callus tissue (IAA callus) was transfered to the medium containing IAA (IAA medium) from the 2,4-D callus and subcultured for about 5 years. On transference of 2,4-D callus to IAA medium and IAA callus to 2,4-D medium, the difference of nicotine biosynthesis in the two callus strains was investigated.

RESULTS

Extracts of 2,4-D callus, IAA callus and reciprocally exchanged calluses were separated into fractions A, B, C and D (see Experimental).

Chemical Constituents of 2,4-D Callus

The presence of tobacco alkaloids could not be demonstrated in fraction C. On the other hand, the amount of neutral compounds in fraction A (260·6 mg) was much larger than that of IAA callus (72·5 mg). The occurrence of phytosterols such as β-sitosterol, stigmasterol, campesterol and cholesterol and triterpenoids such as cycloartenol and 24-

* Part VI in the series "Studies on Plant Tissue Cultures"; for Part V, see T. FURUYA, H. KOJIMA and K. SYONO, *Chem. Pharm. Bull.* **15,** 901 (1967).

† Abbreviations used: 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, 3-indolylacetic acid; YE, Yeast extract.

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methylenecycloartanol were detected by TLC and GLC. Scopoletin in fraction B, scopolin in fraction D, palmitic and stearic acids in fraction B were also detected by PPC, TLC and GLC. Scopoletin and scopolin were observed in smaller amounts than in IAA callus. The palmitic acid content was about five times higher than that of stearic acid.

Chemical Constituents of IAA Callus

A considerable amount of nicotine and a small amount of anatabine and anabasine were found, these being identical with authentic specimens by TLC[8] and GLC. Nicotine was confirmed by a mixed m.p. of its dipicrate and gas chromatography-mass spectrometry, the content being about 0·11 μg/mg dry weight. A small amount of nicotine was also found in the medium by TLC and GLC. The presence of phytosterols and triterpenoids were not detected. Scopoletin, scopolin, palmitic and stearic acids were demonstrated by PPC TLC and GLC. Palmitic acid content was shown to be about ten times higher than stearic acid.

Regulation of Nicotine Biosynthesis by Reciprocal Exchanges of Auxins

As indicated in Table 1 nicotine was detected in the callus tissue of the first, second and third generations obtained by the transference of 2,4-D callus to IAA medium. The nicotine increased gradually until it was about 60 % of that in IAA callus. If these exchanged callus tissues had grown normally, the amount might have reached the level of IAA callus.

TABLE 1. REGULATION OF NICOTINE BIOSYNTHESIS BY 2,4-D AND IAA

Culture* generation	FW† (g)	DW† (g)	DW/FW (%)	Nicotine (μg)	Nictoine/DW (%)	Ratio:
2,4-D	1257	16.4	1·30	0	0	0
(E)-1	770	7·9	1.03	340	0.0043	32.4
(E)-2	495	5.1	1.03	295	0·0058	43.6
(E)-3	436	5.2	1.19	439	0·0084	63.5
IAA	800	6.7	0·84	893	0.0133	100
E-l	1500	27.3	1.82	287	0·0011	8·2
E-2	1330	24.0	1·80	0	0	0
E-3	1100	22.5	2.04	0	0	0

* (E) or E-l, 2, 3; First, second and third generations, respectively after the transference of 2,4-D callus to IAA medium or IAA callus to 2,4-D medium.
† FW, fresh weight and DW, dry weight.
‡ Relative to 100 of nicotine amount in IAA callus.

On the other hand, the growth of callus tissue in the opposite case (IAA callus → 2,4-D medium) was far better than that of the converse mentioned above. The nicotine in the callus tissue after one generation was reduced rapidly, as expected. If nicotine in the inocula did not decompose, the *de novo* nicotine synthesis during this culture period might be completely suppressed, and it was shown that nicotine biosynthesis is considerably reduced in callus tissues of the second and third generations.

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Each callus tissue obtained by media exchange was regulated by exogenous auxins, 2,4-D or IAA. Therefore, it appears that the difference in alkaloid biosynthesis of the two callus strains has not resulted from genotypic mutation of callus tissue, but from phenotypic variation.

DISCUSSION

Speake et *al.*[1] showed that nicotine occurs in callus tissues derived from N. *tabacum* var. Virginica and Ourisson *et al.*[3] observed the absence of nicotine in habituated callus tissue from N. *tabacum* var. P-19. The presence of nicotine together with anatabine and anabasine has now been demonstrated in IAA callus from N. *tabacum* var. Bright Yellow. Ourisson *et al.*[3] isolated phytosterols from habituated tobacco callus tissues and we also found these in 2,4-D callus.

Recently, the regulation of anthocyanins,[9,10] coumarins[11] and chlorophyll[12] production by auxins was reported, but that of nicotine production by auxins using callus tissues has now been proved for the first time and shown to be clearer than in the entire tobacco plant.[13]

EXPERIMENTAL

Plant Tissue Culture

2,4-D Callus derived from stems of N. *tabacum* var. Bright Yellow in February, 1961, was subcultured every 4 weeks for 68 months on 2,4-D medium [White's basal medium, YE (Daigo Eiyō) 0·5%, sucrose 2%, agar (Wakō) 0·7% and 2,4-D 1 ppm]. IAA callus was obtained by transferring the 24-D callus onto IAA medium [White's basal medium, YE (Difco) 0·1%, sucrose 2 %, agar (Difco) 0·8% and IAA 1 ppm] after subculture for 4 months, and maintained for 64 months. These calluses were grown at about 25" in the dark in 100 ml conical flasks.

Reciprocal Exchanges of Media

To study the effect of the concentration and the kind of YE and agar on tobacco alkaloid biosynthesis, a part of 2,4-D callus was transferred onto the medium containing agar (Difco) 0·8%, YE (Difco) 0·1% and 2,4-D 0·1 ppm. Since it was shown that the biosynthesis of tobacco alkaloids was suppressed, we attempted to incubate each callus, weighing about 2 g per flask, on media exchanged reciprocally (IAA callus → 2,4-D medium and 2,4-D callus → IAA medium). After the third generation, each callus tissue (grown for 4 weeks) was subcultured or harvested. The growth was measured by fresh weight and the residual callus tissue after extraction with acetone was dried in a oven at 1 10° and estimated as dry weight.

Extraction and Separation Procedures

2,4-D Callus (1300 g) and IAA callus (1600 g) were homogenized in a Waring blender with cold acetone and allowed to stand for 7 days. The filtrates were adjusted to about pH 2·0 by citric acid and carefully concentrated below 40" under vacuum. The aqueous solution (I) was extracted with CH_2Cl_2, the acidic substances removed with 2N NaOH, and the CH_2Cl_2 evaporated to dryness (fraction A). The alkaline solution after acidification was extracted with CH_2Cl_2 and the CH_2Cl_2 evaporated to dryness (fraction B). (I) was made alkaline and re-extracted with CH_2Cl_2, which was evaporated to dryness (fraction C). The aqueous layer after neutralization, was extracted with H_2O-saturated BuOH and the BuOH layer was evaporated to dryness (fraction D). Extracts from the reciprocally exchanged calluses were separated by the same method.

Thin-Layer Chromatography of Each Fraction

Thin-layer chromatograms using silica gel G were developed with the following solvents; tobacco alkaloids (nicotine R_f 0.68, anatabine 0·57 and anabasine 0·53), $CHCl_3$–MeOH–conc. NH_4OH (60: 10: 1), reagent: modified König;[8] phytosterols (0·33), cycloartenol (0.42) and 24-methylenecycloartanol (0·42), benzene-ether (4: 1), reagent: 10% H_2SO_4 and 20% $SbCl_3$ in $CHCl_3$; scopoletin (0·47), $CHCl_3$–MeOH–CH_3COOH (60: 10: 1), Gibbs reagent.

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Gas-Liquid Chromatography

Using a flame-ionization detector the following were detected; tobacco alkaloids (nicotine t_R 4·7 min, anabasine 9·0 and anatabine 9·7), 1·5% SE-30 on Chromosorb W (60-80 mesh) (column temp. 140°, detector and injection temp. 200°, N_2 flow rate 57.9 ml/min); phytosterols (cholesterol 15·5, campesterol 20·8, stigmasterol 22.6 and β-sitosterol 26.1) and triterpenoids (cycloartenol 29·9, 24-methylenecycloartanol 34·1), 1% SE-30 on Gas-Chrom Q (SO-100 mesh), (240, 250, 300, 84.0); fatty acids (palmitic 7·8 and stearic acid methyl ester, 14·7), 2% SE-30 on Gas-Chrom P (80–100 mesh) (200°, 250°, 300°, 660). The glass column (2.6 m long, 4 mm i.d.) except phytosterols (1.8 m, 4 mm) was used. The relative amounts were determined by weighing the paper representing the peak area.

Isolation of Nicotine

Fraction C was subjected to preparative TLC and the nicotine band was extracted with acetone to afford a brown oil, which gave nicotine dipicrate (7·15 mg), identical with authentic derivatives by m.p. and mixed m.p., and gas chromatography-mass spectrometry.

Determination of Nicotine

The alkaloidal fraction dissolved in acetone (1·0–1·5 ml) was injected into the gas chromatograph under the conditions described. Quantitative analyses for nicotine were carried out using the peak half-width method. The nicotine amount of 0·9–4·0 μg range was proportional to its peak area.

Identification of Scopolin

On paper chromatogram of fraction D developed with BuOH–AcOH–H_2O (5: 1:2), the fluorescent band (R_f 0·5; cf. scopoletin R_f 0·81) was hydrolysed with N HCl (120°, 20 min) and shaken with CH_2Cl_2. The aqueous layer was neutralized and concentrated, re-shaken with CH_2Cl_2 and the scopoletin in this CH_2Cl_2 was detected by TLC. Glucose was identical with an authentic sample.

Acknowledgements-The authors are grateful to Dr. E. Tamaki and Dr. E. Masuda, Japan Monopoly Corporation for the kind gifts of authentic samples of tobacco alkaloids and tobacco plant, to Dr. H. Itokawa, Tokyo College of Pharmacy and to Dr. G. Ohta, Center Research Laboratory, Daiichi Seiyaku Co., Ltd. for the gifts of authentic samples of phytosterols and triterpenoids, respectively. Thanks are also due to Dr. H. Sato, the Naka Works of Hitachi, Ltd. for GC-MS measurements. This work was partly supported by a grant from the Ministry of Education of Japan.