Phytochemistry, 1971, Vol. 10, pp. 723 to 729. Pergamon Press. Printed in England.

REGULATION OF NICOTINE PRODUCTION IN TOBACCO TISSUE CULTURE BY PLANT GROWTH REGULATORS

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(*Received* 6 *May* 1970)

Abstract—Effects of plant growth regulators on nicotine production in the callus tissues derived from the stem of *Nicotiana tabacum* L. var. "Bright Yellow" were examined. Experiments clearly demonstrated that kinetin promotes nicotine production in the absence of auxin (IAA or 2,4-D) in the culture medium, whereas auxin strongly inhibits nicotine formation even in the presence of kinetin, without affecting tissue growth. When cultures were grown on an auxin-free medium containing kinetin, the amount of nicotine increased strikingly after a lag phase. It was shown further that 2,4-D brings about marked changes in the composition of free amino acids in the cultured tissues, particularly regarding the quantities of glutamic and aspartic acids known as initial precursors of nicotine.

INTRODUCTION

IT IS well known that nicotine is synthesized mainly in the roots of *Nicotiana* species.[1] The biosynthesis of nicotine by excised roots in sterile culture was demonstrated by Dawson *et al.*[2] Recently, however, Speake *et al.*[3] isolated nicotine from callus cultures derived from root, stem or leaf of *Nicotiana tabacum* var. "Virginia" and suggested that there is no inherent difference between the cells of different parts of the plant with respect to the ability to produce nicotine. Benveniste *et al.*,[4] on the other hand, were unable to detect nicotine in the stem callus of *N. tabacum* var. "P-19". Furuya *et al.*[5] identified small amounts of nicotine and anatabine in the stem callus (*N. tabacum* var. "Bright Yellow") maintained on a medium containing indole-3-acetic acid (IAA), but detected no alkaloid in the callus maintained with 2,4-dichlorophenoxyacetic acid (2,4-D). We have shown that only a small amount of nicotine is present also in the stem callus of the tobacco variety "Wisconsin No. 38", but found that the intact plants regenerated from the callus tissue produce normal amounts of nicotine.[6]

The various results mentioned above suggest that cultured cells of tobacco usually possess the genetic potentiality to form nicotine, but its quantitative expression must be largely dependent upon some physiological factors or events peculiar to organization of the plant. We report here that nicotine production in the callus cultures of "Bright Yellow" tobacco can be regulated by growth regulators and that it is intimately associated with bud formation induced in the cultures.

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RESULTS AND DISCUSSION

Effects of Kinetin

The culture stock BY-12, which had been derived originally from stem pith of "Bright Yellow" tobacco and maintained on the nutrient agar medium containing 2·0 ppm IAA and 0·2 ppm kinetin, usually grew as yellowish, compact callus without showing organogenesis. Chromatographic analyses of the tissue extracts showed that only nicotine was detectable as the alkaloidal component. Nicotine contents of these undifferentiated tissues were found to be 0·002–0·006% on dry weight basis in marked contrast to 1·6% for leaf of the intact plant grown in the greenhouse. However, when small pieces of the callus were transfered from the stock medium mentioned above to a new medium containing no IAA, a remarkable increase in nicotine content was observed accompanied with the formation of pale green buds. As shown in Table 1, fresh weight, number of buds, and nicotine content of cultures increased as the kinetin concentration was increased from 0 to 2·0 ppm. At the highest concentration (10 ppm), however, both nicotine production and growth of tissue were rather reduced, although the number of buds per piece of tissue was greatest. The TLC of the tissue extracts revealed no alkaloids other than nicotine regardless of the kinetin concentration in the medium.

TABLE 1. RESPONSE OF TOBACCO CALLUS CULTURES (BY-12) TO KINETIN. TISSUE PIECES WERE GROWN ON BASAL MEDIA CONTAINING NO IAA BUT VARIOUS CONCENTRATIONS OF KINETIN IN LIGHT FOR 8 weeks AT 25°

Kinetin concentration (ppm)	Fresh weight (g/piece)	Dry weight (mg/piece)	Number of buds per piece	Nicotine content (% of dry wt.)
0	0·97	55	0·4	0·057
0·02	1·54	80	1·2	0·112
0·2	2·84	132	4·1	0·149
2·0	4·59	212	25·3	0·255
10·0	3·24	150	31·0	0·090

It should be stressed here that no roots were formed in the cultured tissues; the buds formed in the callus continued to elongate to the length of 1–2 cm without forming roots. Thus nicotine production can be stimulated by kinetin without induction of root which is the main site of nicotine synthesis in the intact plant. The morphogenetic response of this culture strain to kinetin is similar to that observed by Skoog and Miller[7] in the stem callus of "Wisconsin No. 38" tobacco, in which 0·2 ppm kinetin permitted sufficient growth to produce a bud or two per piece of tissue in the absence of IAA.

The time course of nicotine production in relation to bud formation during a culture period of 8 weeks is shown in Fig. 1. In this experiment small pieces of callus from the stock culture BY-12, which had been grown with 2·0 ppm IAA and 0·2 ppm kinetin, were inoculated on an IAA-free medium supplemented with 2·0 ppm kinetin. After a lag phase of about 2 weeks the amount of nicotine per dry weight of tissue increased remarkably; it reached a maximum after 6 weeks and then tended to decrease.

[7] F. SKOOG and C. O. MILLER, *Symp. Soc. Exptl. Biol.* **11**, 118 (1957).

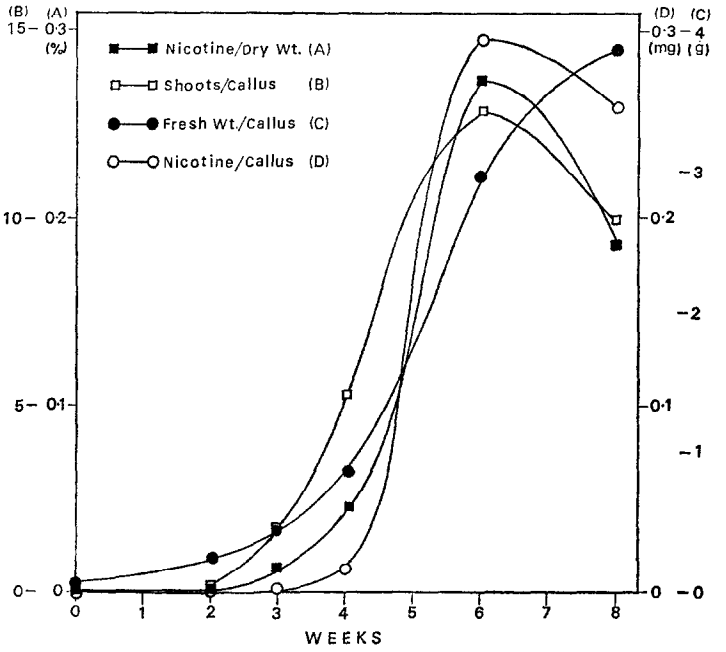

FIG. 1. TIME COURSE OF NICOTINE PRODUCTION IN THE TOBACCO TISSUE CULTURES (STRAIN BY-12) GROWN ON THE IAA-FREE MEDIUM CONTAINING 2·0 ppm KINETIN IN LIGHT AT 25°. CURVES FOR TISSUE GROWTH AND BUD FORMATION ARE ALSO SHOWN.

For unknown reasons, organogenesis was not induced in some of the tissue pieces tested in this experiment and assay for nicotine of the samples harvested after 8 weeks of culturing showed that the average nicotine content of the pieces without buds were 0·119% in comparison with 0·257% for those with buds. Nevertheless, it is significant that nicotine production was greatly enhanced, even without organogenesis, by removing IAA from culture medium. This seems to suggest therefore that the promotion of nicotine production is not directly coupled to bud formation in spite of the apparent parallelism observed between them. Since the cultures were neither examined microscopically for organization when no bud formation was apparent nor ascertained for occurrence of root primordia when bud formation did occur, any relationship between nicotine synthesis and the development of organized structures in the cultures is a subject for further and more detailed study.

As to the time course of nicotine production, essentially similar results were obtained from an experiment with a variant strain derived from BY-12 by heat treatment (Fig. 2). This substrain, BY-12-H, is characterized by its ability to produce a higher amount (nearly 0·1%) of nicotine in the presence of both IAA (2·0 ppm) and kinetin (0·2 ppm). Interestingly, its nicotine content declined to a very low level during the lag phase of growth after the transfer of callus pieces to the test medium containing no IAA but kinetin. Since no release of nicotine from these tissues to the culture medium was detectable, most of the nicotine present in the inocula would have been metabolized during the lag phase. The callus of BY-12-H formed more buds than that of BY-12, but its maximum nicotine content reached after 6 weeks was lower than that of BY-12. Besides, the nicotine content per tissue piece ceased to increase after 6 weeks, while the number of buds per piece was still increasing.

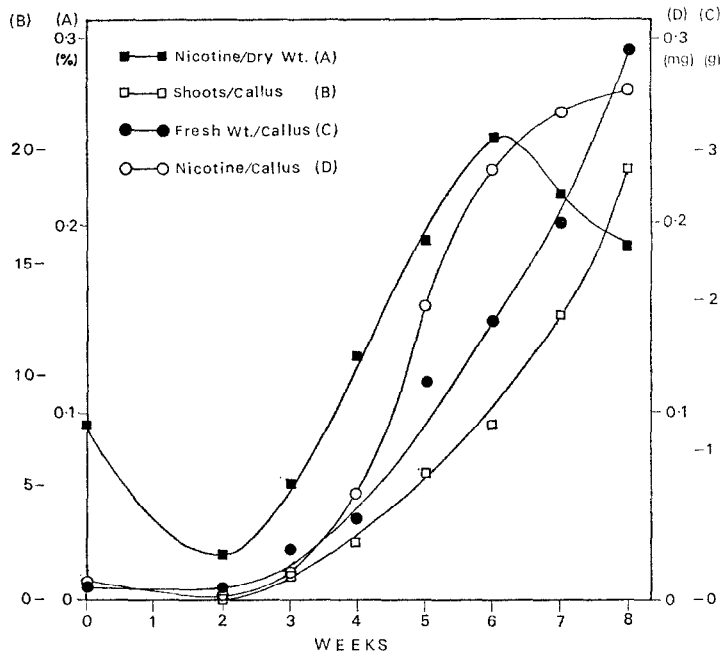

FIG. 2. TIME COURSE OF NICOTINE PRODUCTION IN THE TOBACCO TISSUE CULTURES (SUBSTRAIN BY-12-H) GROWN ON THE IAA-FREE MEDIUM CONTAINING 2·0 ppm KINETIN IN LIGHT AT 25°. CURVES FOR TISSUE GROWTH AND BUD FORMATION ARE ALSO SHOWN.

The relatively long period of lag phase observed again in the experiment might have been caused partly by possible carry-over of IAA in the inocula transferred from the previous medium. Detailed investigation on biochemical modifications expected to occur during the lag phase is needed in order to elucidate the mechanism of induction of nicotine synthesis in the cultured tissues.

Effects of 2,4-D

Effects of 2,4-D, a synthetic auxin, on alkaloid production were examined by transfering callus pieces of BY-12 from the stock medium to test media containing various concentrations of 2,4-D in addition to 2·0 ppm kinetin. The results obtained after a culture period of 8 weeks are presented in Table 2. In the absence of 2,4-D many buds and large amounts of nicotine were produced in cultures, as already observed in the previous experiments. For the nicotine assay of these cultures, pieces with buds were separated into bud and the remaining undifferentiated parts, and the result disclosed that the former contained 1·5 times as much nicotine as the latter. In the presence of 2,4-D, on the other hand, both bud and nicotine formations were strongly inhibited. At higher concentrations of 2,4-D neither bud nor measurable amount of nicotine was present and the analyses by TLC of the basic fractions of tissue extracts failed to detect any tobacco alkaloids except for a small amount of an unidentified Dragendorff-positive compound (R_f 0·0). In spite of these striking effects, the growth of cultures in terms of total fresh or dry weight was little affected by 2,4-D except at the highest concentration. It appears therefore that bud and nicotine formations are inhibited by 2,4-D independently of cellular proliferation.

TABLE 2. RESPONSE OF TOBACCO CALLUS CULTURES (BY-12) TO 2,4-D. TISSUE PIECES WERE GROWN ON BASAL MEDIA CONTAINING 2·0 ppm KINETIN AND VARIOUS CONCENTRATIONS OF 2,4-D IN LIGHT FOR 8 weeks AT 25°

2,4-D concentration (ppm)		Fresh weight (g/piece)	Dry weight (mg/piece)	Bud formation	Nicotine content (% of dry wt)
0	*	1·15	51	abundant	0·222
	†	2·78	111		0·158
0·022		4·03	169	scarce	0·015
0·22		4·08	143	absent	trace
2·2		3·18	92	absent	trace

* Buds isolated from the cultures.
† Undifferentiated callus portions of the cultures.

Although the experiment has demonstrated an almost complete inhibition of nicotine production by 2,4-D, the mechanism of action is not yet known. As auxins are known to influence many physiological processes[8] including nitrogen metabolism[9] of plant tissues, it is possible that the effect of 2,4-D may be mediated through its effects on amino acid metabolism that is related to alkaloid biosynthesis. Actually the results of comparative analyses of free amino acids extracted from cultures revealed striking differences in the overall quantity and the relative composition among cultures grown with varied concentrations of 2,4-D (Table 3). It is particularly noted that the tissues grown with higher concentrations

TABLE 3. FREE AMINO ACIDS IN THE TOBACCO TISSUE CULTURES (BY-12) GROWN ON BASAL MEDIA CONTAINING 2·0 ppm KINETIN AND VARIOUS CONCENTRATIONS OF 2,4-D IN LIGHT FOR 8 weeks AT 25°

Amino acid	Concentration of 2,4-D (ppm) 0	0·022	0·22	2·2
Aspartic acid	57·4	101·4	3·9	12·3
Threonine	—	31·9	17·9	13·1
Serine†	159·2	181·9	25·8	30·0
Glutamic acid	189·9	1050·0	33·3	47·4
Proline	—	—	9·8	7·1
Glycine	0·9	12·0	13·9	19·2
Alanine	1·0	13·1	69·2	73·4
Valine	0·8	8·9	13·5	9·9
Methionine	0·5	1·0	0·8	0·7
Isoleucine	1·2	4·4	9·8	5·6
Leucine	2·2	11·9	19·0	10·9
Tyrosine	6·7	10·2	7·6	4·0
Phenylalanine	12·3	15·1	8·4	6·8
Lysine	0·4	1·7	1·3	1·3
Histidine	3·9	6·2	1·5	1·1
Total	436·4	1449·7	235·7	242·8

* Unit of amino acid = μmole/100 g fr. wt.
† This fraction probably contains also asparagine and glutamine in high proportions, which were found by PPC.

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(0·22, 2·20 ppm) of 2,4-D showed much smaller pools of glutamic and aspartic acids in contrast to larger pools of alanine, glycine, and proline as compared with the control. On the contrary, the accumulation of glutamic and aspartic acids was apparently increased when 2,4-D had been supplied to medium at the lowest concentration (0·022 ppm). Unexpectedly ornithine[10–12] and arginine,[13] known precursors of the pyrrolidine ring of nicotine, could not be detected in all the cultures irrespective of the 2,4-D concentration.

It is difficult to interpret the 2,4-D-induced changes in amino acid metabolism in relation to nicotine production, but the apparent reductions in the pool sizes of both glutamic and aspartic acids, probably the initial intermediates of the biosynthetic sequence leading to the pyrrolidine ring of nicotine,[14–16] might be of special significance for the inhibition of nicotine production by 2,4-D. Since these amino acids are closely related to the Krebs tricarboxylic acid cycle, the primary effect of 2,4-D might be exerted through the operation of this cycle. However, it remains to be determined whether the nicotine production in tobacco tissue cultures is hormonally regulated through certain aspects of amino acid metabolism or it is subjected to other biochemical processes which are controlled also by plant growth regulators.

EXPERIMENTAL

Culture Stock and Culture Method

The stock culture of callus (BY-12) originally derived from stem pith of *Nicotiana tabacum* L. "Bright Yellow" had been maintained on the Linsmaier–Skoog basal medium[17] containing 2·0 ppm indole-3-acetic acid (IAA) and 0·2 ppm kinetin for about 30 months through successive transfers carried out at 1-month intervals. For culture experiments four pieces of callus (averaging 150 mg each) were aseptically planted in each 100 ml Erlenmeyer flask containing 50 ml of the Linsmaier–Skoog medium supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. For each lot of an experiment about 50 pieces of tissue were cultured. The tissues were grown at 25° and exposed to the light from fluorescent lamps at approximately 7000 lx for 12 hr per day.

Extraction and Analysis of Alkaloid from the Tissue Cultures

The cultured tissues were assayed for nicotine by the following procedure. Fresh tissues harvested from culture flasks were dried at 50° for 2 days and then kept in a desiccator. The dried sample (2–5 g each) was extracted with MeOH for 6 hr in a Soxhlet-extractor and the extract was evaporated to dryness. The residue was dissolved in 0·5 *N* HCl (20 ml) and extracted with ether (50 ml). The aqueous layer was made alkaline (pH 9·5) with 2 *N* NaOH and extracted with ether thrice (30 ml each). After drying (K_2CO_3) the ether was added with 0·5 *N* HCl (10 ml), and then the mixed solution was evaporated to dryness. Nicotine was isolated from the residude by means of multi-buffered paper chromatography i.e. the residue dissolved in a mixture of MeOH–ammonia water (9:1) was spotted with a microsyringe on Toyo filter paper No. 51 (2 × 40 cm) with three buffer zones (pH 6·0, 5·8, and 5·6,) and developed with *n*-BuOH saturated with acetate buffer (pH 5·6). The nicotine spot located at the buffered zone of pH 5·8 was extracted with 0·5 *N* HCl for 2 hr, and its concentration was estimated by the spectrophotometric method of Willits *et al.*[18]

The nicotine isolated from callus was compared with authentic nicotine by ultraviolet absorption spectrum in acidic solution (λ_{max} 259 mμ), infrared absorption spectrum in $CHCl_3$, and chromatographic behaviors on paper with buffer saturated *n*-BuOH as the solvent and on Silica gel G (Merck) with $CHCl_3$–EtOH (10:3) as the solvent. Nicotine spots on chromatograms were located by Dragendorff's reagent. In all tests, isolated alkaloid behaved identically with authentic nicotine.

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Analysis of Free Amino Acids from the Tissue Cultures

Each sample of fresh cultured tissues (100 g) was homogenized in a mortar, extracted twice with 75% EtOH, and filtered off by a Büchner funnel. The filtrate was concentrated under reduced pressure, passed through a column of Amberlite IR-120 (H^+ form, 100–200 mesh), and amino acids were eluted with 2 *N* ammonia water. The eluate was concentrated to dryness and the residue dissolved in water was passed through a column of Amberlite IRA-400 (OH^- form, 100–200 mesh). The amino acid fraction eluted with 2 *N* HCl was evaporated to dryness and the residue was dissolved in 2·0 ml of water to provide a sample for amino acid analysis, which was done by Hitachi amino acid analyzer.

Acknowledgement—We should like to thank Mr. Ebata of Shionogi Research Laboratory, Osaka, for amino acid analysis of the samples.