Discussion

Under conditions of spectrophotometric assay the capacity of homogenized wheat embryos to oxidize DPNH or TPNH via an ascorbate sensitive pathway in the absence of peroxidase acceptor is 0.22 micromoles/mg N × 1 minute. This value is equivalent to the oxidation of 0.44 micromoles of cytochrome c. It is of interest, therefore, that under similar conditions of assay wheat embryo homogenates oxidize 2.0 micromoles of cytochrome c/mg N × 1 minute (5). The capacity of the oxidase is thus 20 % that of the cytochrome system. Allowing for the hazards of interpreting comparative in vitro activities, the capacity of this non-cytochromal oxidase system cannot be regarded as negligible. The fact that cytochrome c is present in limiting amounts in the ungerminated embryo (2) may add to the importance of this alternative channel of coenzyme oxidation during the early stages of embryonic growth.

The morphology of the structures which incorporate this oxidase system remains unrevealed. It is reasonably certain that the structures are not mitochondria. They may be discrete particles but there is no evidence compelling such a conclusion; it is equally possible that they are products of a mechanical breakdown of larger cytoplasmic structures. It would appear that the association between the oxidative moiety and the physical skeleton of the system is of a much looser kind than that existing between cytochrome oxidase and the mitochondrial framework. Sodium choleate solubilizes the pyridine nucleotide oxidases leaving the sedimentable components without oxidative activity, whereas under similar treatment cytochrome oxidase remains attached to the mitochondrial membrane (8).

Summary

In homogenates of viable wheat embryos 80 % of the capacity to oxidize DPNH and TPNH independently of the cytochrome system is localized in particulate structures. These particles are readily separated from the mitochondria by differential centrifugation.

The oxidase system has properties in common with those previously described for soluble oxidase preparations. It has a specific requirement for Mn^{++}, and is inhibited by catalase and a variety of reducing agents, most effectively by ascorbic acid. H_2O_2 is a reaction product, which in the presence of an appropriate acceptor (*p*-aminobenzoic and anthranilic acids) is reduced by the peroxidase component of the system.

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NICOTINE PRODUCTION AND GROWTH OF EXCISED TOBACCO ROOT CULTURES[1,2]

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Experiments with interspecific grafts have shown that, despite its lesser mass, the tobacco root produces much greater quantities of nicotine than does the aerial shoot (cf 4, 7, and 9 for review). Possible explanations for this inequality include (a) a gross difference in the intrinsic rates of nicotine synthesis of root and shoot, and (b) an unequal distribution between the major plant organs of the total mass of tissue responsible for synthesis. If, as has been suggested, the growing tips of root and stem were major loci of nicotine formation much of the difference could be explained by parallel differences in the numbers of such tips, the advantage obviously resting with the root system. Available evidence is insufficient to support either hypothesis.

The idea of concentration of alkaloid synthetic

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activity in the growing root and shoot tips is not new. Early experiments of Schmidt and Serrano (11) showed a direct relationship between the total nicotine content of the plant and the protein content of the root. Laschuk (8) observed a close parallelism between alkaloid production and the development of roots on intact tobacco plants. He concluded that the production of nicotine is dependent upon continued growth and development of the root tip and not upon the general metabolic activities of root tissues as such. Histochemical evidence demonstrates that nicotine appears first in the shoot tip as well as the root tip of germinating tobacco seeds which are initially alkaloid free (2, 6, 10). However, histochemical evidence is incapable of providing a distinction between loci of synthesis and loci of accumulation from a translocation stream.

A more detailed examination of nicotine production and growth was undertaken in the hope of learning whether or not there is a close correlation between the two. For study of the root, suitable experimental material was found in the excised root culture of *Nicotiana Tabacum* var. Turkish. This material possesses certain important characteristics: 1) There is no discernible secondary growth in root diameter (see below). Hence, it was possible to compare relative capacities for alkaloid synthesis of growing root tips and of matured regions of the primary root uncomplicated by the possibility that appreciable rates of such synthesis might occur in tissues of secondary origin (e.g., an active cambium). 2) The roots can be subcultured through an indefinite number of short-term passages. The clones thus established possess relative genetic stability. 3) The physical and chemical environment of the cultures can be controlled easily inasmuch as growth occurs in the dark and in a medium which is entirely synthetic except for a small amount of yeast extract. Physiological stability is thus also a possibility. 4) Nicotine is the only alkaloid that we have detected in properly prepared extracts of these roots.

As a result of this combination of circumstances, it has been possible to study the interrelations of growth[3] and nicotine production under essentially steady state conditions with respect to the individual root branch.

Materials and Methods

Root tips were isolated aseptically from two seedlings of *Nicotiana Tabacum* L. var. Turkish and cultured as separate clones in a medium modified from that described by White (13) by the addition of 0.01 ppm copper and 0.003 ppm molybdenum. In earlier experiments the pH was adjusted to 5.6 but this was found later to be unnecessary. The cultures were grown in 125-ml Erlenmeyer flasks containing 20 to 40 ml of autoclaved medium depending on the duration of the experiment. Except where otherwise stated each flask was inoculated with a single root tip approximately 10 mm long. Care was taken to use only secondary tips from rapidly growing cultures of uniform size. All cultures were grown in the dark at a temperature of 29 ± 0.5° C.

Nicotine was assayed in steam distillates by techniques (5) modified for increased sensitivity from those of Willits et al (14). Recovery of nicotine from twenty aliquots of a stock solution of the pure alkaloid by these methods averaged 99.4 ± 1.4 %. Since the root in culture has no aerial shoot to which the nicotine may be transported, the latter is excreted presumably through the cut end into the surrounding medium. This necessitates separate analyses of both root and medium. At harvest the root tissue was dried at 70° C and the pooled medium was concentrated in vacuo. Portions of each were separately steam-distilled for nicotine assay.

Results

Data on growth measurements and nicotine accumulation under standard conditions are found in table I. Cultures originally containing 30 ml of medium and 1 root tip were harvested at intervals to the 20th day. At the end of 20 days the surface of the medium was completely covered with a dense mat of intertwined branches which caused extreme difficulty in separation for purposes of length measurements. Consequently most experiments were terminated at this time.

Excised root cultures accumulate nicotine as they grow. When measurements are confined to the exponential phase of growth, plots of length, dry weight and nicotine against elapsed time show similar slopes.

Table I

Growth and Nicotine Production by a Clone of Excised Roots of Nicotiana Tabacum var. Turkish

Expt	Age of culture, days	No. of cultures	Mean dry wt mg	Mean length, mm	Mean no. of branches*	Mean nicotine yield, μgm
A	7	30	0.34	56	1.6	11
	11	27	0.67	99	4.6	23
	14	20	1.46	231	13.3	50
	17	10	2.86	488	29.7	91
	20	10	4.77	744	47.2	139
B	7	28	0.32	57	3.4	9
	11	22	0.89	163	7.5	26
	14	23	1.71	284	11.8	55
	17	12	3.27	564	33.2	110
	20	7	6.34	1098	73.5	182
C	7	18	0.36	...	...	10
	7	18	0.43	...	...	12
	14	14	2.12	...	...	70
	14	14	2.33	...	...	79
	20	15	9.38	...	...	294
	20	13	6.83	...	...	208

* Does not include main branch.

[3] Growth is defined as increase in root volume and is estimated in terms of one or more functions of volume, viz length, dry weight, etc. The relationship between length and dry weight appears in the discussion.

TABLE II

NICOTINE PRODUCTION, GROWTH AND RESPIRATORY ACTIVITY OF EXCISED ROOT SEGMENTS OF NICOTIANA TABACUM VAR. TURKISH DURING CULTURE FOR THE PERIODS INDICATED

SEGMENT TYPE AND TREATMENT	INITIAL			1 WEEK			2 WEEKS	
	NICOTINE	DRY WT	QO_2*	NICOTINE	DRY WT	QO_2*	NICOTINE	DRY WT
	μgm	*mg*		*μgm*	*mg*		*μgm*	*mg*
Mature segments	286	18.8	4.7	284	17.8	1.3	....	....
Tip segments	65	3.0	12.4	773	30.0	..	4437	130.0
Tip segments with 10^{-6} M IAA	...	...	...	134	15.4	5.9	147	29.0

* Microliter oxygen absorbed per mg dry tissue per hr.

This also means that a straight-line relationship exists between any pair of these dependent variables. Details of the statistical analysis of the data are presented in the discussion. Due to the more rapid growth obtained in experiment (b) the data from the 20-day harvest were eliminated from the statistical analysis. Under our conditions accurate measurement becomes almost impossible when total root length exceeds 800 mm. In addition, it appears from the few such cases so far analyzed that the effects of progressive diminution in nutrient supply make themselves felt at about this stage of culture development.

Differences in nicotine synthetic capacity between mature portions of the root and the root tips were demonstrated as follows. Two-hundred segments (5 per flask and 5 mm long of clone 1) of each were cultured separately in the usual medium. The mature sections were taken from the oldest part of the culture in order to eliminate the possibility of formation of new root branches. The averaged results of eight replications taken initially and after one week of culture are shown in table II. After one week the root tips had increased their alkaloid content as well as their dry weight approximately ten-fold. This increase could not be attributed to root branches since they did not appear until after the first week. On the other hand the older sections gained neither in dry weight nor in alkaloid content. Although growth did not occur in the segments there was still evidence of metabolic activity as shown by the respiratory gas exchange data contained in table II.

The observed connection between nicotine production and growth was examined under a different set of conditions. A series of concentrations of 3-indolylacetic acid (IAA) was provided in the standard culture medium. It was found that, at 10^{-6} M, linear growth was almost completely inhibited with the preservation of otherwise normal root appearance (i.e., absence of hypertrophy, etc.),

Four separate experiments were performed in which five root tips were placed in each of 50 culture flasks containing 20 ml complete nutrient solution 10^{-6} M with respect to IAA. Means for dry weight and nicotine production are contained in table II. During the first week, increase in dry weight was reduced 50 % by IAA although growth in length appeared to have been virtually halted by this compound. The corresponding yield of nicotine was reduced to 16 % of the control cultures. Changes during the second week were associated with the development of many branch roots in the control cultures. Dry weight of the latter increased 4.3-fold while the corresponding change in the treated cultures was 1.9-fold. IAA therefore decreased dry weight to 14 % of the control yields and nicotine production to 0.36 %. The respiratory gas exchange data are presented to give an index of metabolic activity in the treated roots.

It appears from these results that there is no simple relationship between dry weight and nicotine production when IAA is present. However this conclusion is complicated by the microscopic observation that large quantities of starch were stored in the treated

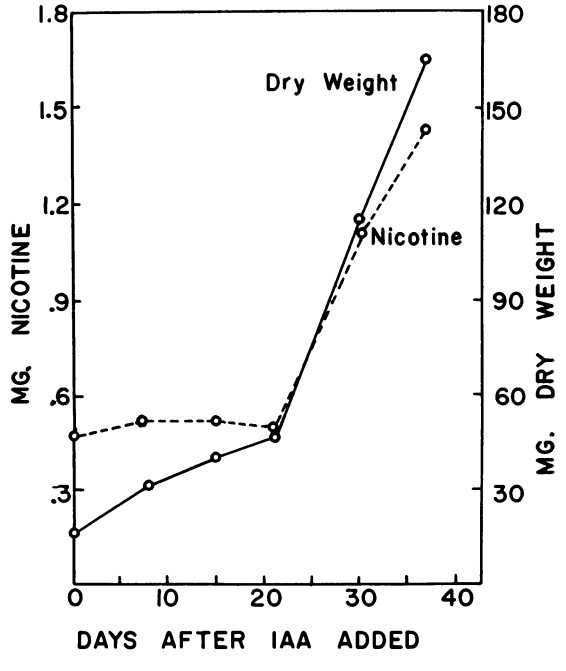

FIG. 1. Relationship between nicotine production and dry wt accumulation at various intervals after addition of IAA to sterile cultures of excised roots of *Nicotiana Tabacum* var. Turkish. Initial concentration of IAA in each case was 10^{-6} M.

roots. None was observed in the control roots. Further data were provided by the following experiment.

Six groups of 25 cultures, each flask containing 5 root tips and 20 ml of medium, were allowed to grow for 1 week before IAA was added to make a final concentration of 10^{-6} M. The groups were harvested consecutively. The results are given in figure 1. For the first three weeks dry weight increased at a slow rate while nicotine synthesis and elongation had apparently halted. This is in agreement with results in table II. Between the third and fourth weeks, these roots, which had shown little, if any, elongation up to that time, began to form many branch roots which elongated normally. Simultaneously, rapid increases in nicotine production and dry weight were observed. The cause of the suddenly renewed elongation was not completely investigated. There are two possibilities. First, there was some evidence that the roots might have become adapted to IAA, since on transferrence to medium without IAA they grew slowly. Second, the IAA may have been oxidized or destroyed by some other means. Whatever the cause, in the absence of elongation nicotine synthesis did not occur, while on resumption of elongation nicotine synthesis continued at a normal rate.

Discussion

A clone of excised tobacco roots can be subcultured through an indefinite number of passages provided secondary tips are used to inoculate the new cultures. These roots produce nicotine as they grow (3). When the duration of each passage is relatively short (less than 21 days), and when tips 10 mm in length taken from actively growing cultures are used for establishing each new passage, the following statements may be made which characterize the growth of the Turkish strain of *Nicotiana Tabacum* employed in our experiments:

(a) There was no discernible secondary increase in root diameter; hence, growth may be considered as essentially a matter of linear root extension. The line of least squares relating root length to dry weight during 20 days of growth possessed a slope of 5.95 μgm per mm with a standard error of estimate ($t = 0.05$) equal to $\pm$ 0.29 μgm per mm (table I). Assuming that appreciable secondary increase in diameter of older roots would have been associated with an axially asymmetric distribution of dry weight, it is clear that little or no secondary growth in diameter did occur.

(b) As the roots grew they also branched. Hence, the number of growing tips increased with age of culture. If the total root lengths of cultures harvested at different ages (table I) are related to numbers of root branches, there is obtained a second linear regression with slope 16.4 mm per branch (or per tip) with a standard error of estimate $\pm$ 1.12 ($t = 0.05$). There is thus no indication of gross inconstancy in developmental pattern during the growth periods employed.

(c) The total linear growth of the entire root with all its branches followed first order kinetics with respect to time, the rate constant being 0.2107 mm per day per mm. Although actually a discontinuous phenomenon, branch production also follows a first order trend of 0.227 branch per day per branch. Virtual equality of rate constants for branch production and for total linear extent implies constancy of growth rate of the individual branch. This justifies the claim for steady state conditions (see above).

The relationships of nicotine production in the same 20-day period to both length and dry weight can be expressed by simple proportionalities. The roots produced nicotine at a rate of 0.191 μgm per mm with a standard error of estimate of $\pm$ 0.010 ($t = 0.05$). Under the standardized conditions of our experiments nicotine production was also related proportionally to dry weight. The rate was 29.05 μgm per mg with a standard error of estimate of $\pm$ 2.14. Limited dry weight increase is possible without accompanying alkaloid formation (fig 1) providing the standard conditions of growth are varied. Hence, the above mentioned rates were dependent upon careful elimination of variations in conditions of culture. Some of these variations are the purity of distilled water, deterioration of the thiamine-glycine stock solution of White (13) during storage, and the presence of toxic contaminants in the salts and sucrose used in preparing the medium. The last-mentioned item is particularly troublesome and has been eliminated only by systematic pretesting of newly acquired stocks against acquisitions of known efficacy.

The close connection between growth in terms of increase in length (or dry weight) and nicotine yield suggests a relationship between the process of alkaloid production and one or more activties of the growing root tip. Dissection of the root into tip and mature region confirms that the alkaloid is synthesized only when tips are present. Therefore, it seems probable that nicotine production depends upon some activity in the root tip rather than upon the general metabolic activities of the entire excised root.

The use of inhibiting concentrations of IAA further shows that the root tip will not synthesize nicotine when it is not elongating. The results obtained from this treatment raise an interesting question. Burström (1) reports that "Supraoptimal concentrations of indoleacetic acid (IAA) inhibit root growth by reducing cell elongation with only small changes in cell multiplication." This has been confirmed by Street et al (12) for excised tomato roots. Microscopic observations of the treated roots in our experiments revealed an accumulation at the tips of many small cells. A comparative histological investigation is needed to ascertain quantitatively the effects of IAA upon rates of cell division and exact limits to the increase in cell volume. The question is whether there were enough cell divisions in the presence of IAA to permit analytical detection of the nicotine formed if division and synthesis are closely related. If there were, this approach can be used to decide which

principal cellular activity of the root tip, division or normal elongation and differentiation, is correlated more closely with nicotine production.

Summary

Excised roots of Turkish tobacco were grown in sterile culture, and a detailed study was made of the interrelations of their growth and nicotine production. During the exponential phase of growth the roots produced nicotine at a constant rate of 0.191 μgm per mm length or 29.05 μgm per mg dry weight. When the growing tips and mature regions of the root were cultured separately, nicotine increases resulted only in the case of the tips. By the use of indoleacetic acid in proper concentration, nicotine synthesis was observed only in those roots where elongation occurred.

Grateful appreciation is expressed for the counsel of Professor R. F. Dawson of Columbia University.

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NICOTINE PRODUCTION AND GROWTH OF TOBACCO SCIONS ON TOMATO ROOTSTOCKS[1,2]

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Although the primary role of the tobacco root in nicotine biosynthesis was established more than a decade ago there has been little investigation of the limited alkaloid productivity of the shoot (cf Mothes (8) for review).

Indications that the tobacco shoot may produce some steam-volatile alkaloid are evident in the earlier data of Dawson (2) where increases in this fraction took place slowly during growth of Turkish tobacco scions on tomato rootstocks. The magnitude of the change was 7.3 mg (calculated as nicotine) per scion in 84 days of growth or 0.017 mg/gm increase in fresh weight. However, the method of assay then employed was neither sensitive nor specific for pyridine alkaloids, and attempts to isolate the alkaloid were unsuccessful.

Again, working at or near the limits of sensitivity of available methods for nicotine assay, Mothes (7) reported that growing leaves, when excised and provided with a good nitrogen supply, accumulated small amounts of alkaloid. Later, Mothes and Romeike (9, 10) found traces of alkaloid in *Nicotiana rustica* scions grafted on tomato.

Similarly, Mashkovtsev and Sirotenko (6) grafted tobacco embryos almost completely devoid of nicotine on tomato rootstocks. By direct isolation as the crystalline dipicrate, a 100,000-fold increase in nicotine content was demonstrated during the growth and development of a single scion. From these and related data they concluded that the characteristic of nicotine synthesis is possessed by any cell of the above-ground part of *Nicotiana Tabacum*.

In this paper we wish to present further data in support of the hypothesis that limited alkaloid production occurs in the tobacco shoot and to describe the patterns of such alkaloid accumulation in relation to growth of the plant.

Materials and Methods

Grafts were made using three different tobaccos: *Nicotiana rustica*, var. *brasilia*, *N. Tabacum* var. Turkish, and *N. Tabacum* var. Connecticut 49. These scions were cleft-grafted with stocks of tomato, *Lycopersicum esculentum* var. Marglobe, and grown

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