Environmental Parameters Influencing Phenolics Production by Batch Cultures of *Nicotiana tabacum*

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The influence of temperature, illumination, hormonal levels (2,4-D and kinetin), carbon to nitrogen ratios, antibiotics, and precursor feeding on phenolics production by *Nicotiana tabacum* (tobacco) was studied. This plant cell system was chosen as a model system to learn more about secondary product formation in plant cell tissue cultures. This is the first study to manipulate all of these environmental parameters with a single plant cell system. The most striking results were with 2,4-D manipulation. The removal of 2,4-D resulted in significant phenolics production during the stationary phase, while normal levels strongly suppressed phenolics production during the stationary phase. The addition of phenylalanine stimulated phenolics production per gram of cells but strongly inhibited growth.

INTRODUCTION

The industrial potential for the production of medicinals, fragrances, food ingredients, and other high-value biologicals from plant cell tissue culture has been well recognized.[1-3] The realization of the potential has been hindered by a lack of understanding of how environmental parameters influence the formation of secondary metabolites in plant cell tissue cultures.

The purpose of this article is to systematically explore the batch kinetics of plant cell growth and product formation under different environmental and nutritional conditions. The product criterion in this study is the formation of *total* (ethanol soluble) *phenolics* from cell suspensions of *Nicotiana tabacum* (tobacco). Phenolic compounds occupy an important place among secondary substances produced by higher plants, and their metabolic regulation provides a useful model system to test schemes to improve product yields of secondary plant metabolites. Zaprometov[4] states that the number of known natural phenolic compounds exceeds 3000, second only to alkaloids in their variety. As distinct from alkaloids the simplest phenolic compounds are synthesized by all plants and perhaps by every plant cell. Phenolics are classified as secondary metabolites since no role for them has been found in the basic metabolism of cells. Lignin, a structural component of plants, is made by polymerization of phenolic compounds.

Phenolics are also commercially important because of the role they play in plant disease resistance as well as the role in imparting certain desirable textural and flavor characteristics to various food products (e.g., tea, coffee, wine, tobacco, etc.).

Phenylalanine ammonia lyase (PAL) is one of the key enzymes in the regulation of phenolic metabolism, and much of the research on the regulation of secondary products has centered on the metabolic control of PAL.[5,6] The main phenolic constituents identified in tobacco tissue culture include free acids and putrescinal derivaties of *p*-coumaric, ferulic, and caffeic acids.[7-9]

Based on reports in plant cell culture literature, the following parameters were selected for further experimentation with tobacco cell-phenolics system: 1) temperature, 2) glucose and nitrate levels of the feed, 3) precursor addition, 4) hormonal additions (e.g., 2,4-D and kinetin), 5) illumination, and 6) antibiotics. Many of the above parameters have been shown to influence cell growth of product yields in plant cells but no general trends are evident. In no case have all of these parameters been tested with a single cell line and product combination. The purpose of this article is to report data on the influence of each of the above parameters on tobacco cell growth and phenolics formation. Such data is a prerequisite to the rational design of multistage continuous culture systems as well as other reactor configurations.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Suspension cultures of tobacco cells (*Nicotiana tabacum*) were provided by Dr. Roy Chaleef, Department of Plant Breeding, Cornell University (Ithaca, NY) in September, 1979. The tobacco cells were grown in a modified Linsmaier and Skoog medium (LS)[10] at 26 ± 1°C and 150 rpm in a New Brunswick psychrotherm

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shaker (New Brunswick Scientific Co., Inc.). Details of the medium are given in Table I. Cells were subcultured every 20–25 days using approximately 20% inoculum (v/v) of a dense, early stationary phase culture in a way to exclude large lumps or aggregates. The cultures were maintained in the dark for routine transfers. Prior to autoclaving, the pH of the LS medium was adjusted to 6.0 using 1*N* KOH. Deionized distilled water was used in preparing the media.

The batch experiments to study the effect of different environmental and nutritional variables were initiated in 500-mL Erlenmeyer flasks with ca. 200 mL medium in each flask. The inocula used were early stationary phase cells grown in dark at 26°C and 150 rpm for all the experiments. For all tobacco cell experiments, normal LS medium was used for developing the inoculum except for the 2,4-D, kinetin experiments, where cells grown on non-2,4-D, kinetin medium for one generation were used as inoculum.

Assays

The standard procedure for sampling and assays for all experiments was as follows: after collection of 10–12 mL of a sample in a graduated centrifuge tube (15 mL capacity), the volume was noted, and the sample centrifuged for five minutes at setting 50 in an Adams Dynac Centrifuge (Clay Adams, Inc.). About 5 mL of the supernatant was withdrawn and frozen in a 20-mL polyethylene scintillation vial (Fisher Scientific Co.) for later analysis of glucose, soluble phenolics, nitrate, etc. The dry weight was determined by filtering the rest of the sample through a prewashed, dried, and preweighed 0.45-μm filter (Millipore Corp., Bedford, MA) under vacuum and washing the cells with three volumes of distilled water. The filter and cells were dried at 70°C for 24 h and reweighed. All weighing measurements were done with a balance (Mettler H10, VWR Scientific Co., Rochester, NY) sensitive to the nearest 0.1 mg.

Table I. Medium composition.

Substance	Amount (g/L)
NH_4NO_3	1.65
KNO_3	1.9
$CaCl_2 \cdot 2H_2O$	4.4
$MgSO_4 \cdot 7H_2O$	3.7
KH_2PO_4	1.7
Na_2EDTA	3.73
$FeSO_4 \cdot 7H_2O$	2.78
2,4-D	1×10^{-3}
Kinetin	0.3×10^{-3}
Glucose	0.30
Micronutrients	
H_3BO_3	6.2 mg/L
$MnSO_4 \cdot H_2O$	16.8 mg/L
$ZnSO_4 \cdot 7H_2O$	10.6 mg/L
KI	0.83 mg/L
$Ma_2MoO_4 \cdot 2H_2O$	0.25 mg/L
$CuSO_4 \cdot 5H_2O$	0.025 mg/L
$CoCl_2 \cdot 6H_2O$	0.025 mg/L
Vitamins	
Nicotinic acid	1 mg/L
Thiamine HCl	10 mg/L
Pyridoxine HCl	1 mg/L
Myo-Inositol	100 mg/L

To a second sample (ca. 10–12 mL), 10 mL of a 70% ethanol solution was added, and the sample stored in the refrigerator at 4°C for total phenolics determination.

To prepare the stored samples for further analysis the cells were first homogenized in a tissue homogeniser (15 mL capacity and 150 μm clearance) to break large clumps, if any, and then sonicated in a 40-mL beaker at a setting of 50 for 10 min in a sonic dismembrator (model 300, Fisher Scientific, Rochester, NY). Extraction was carried out two more times with 8 mL ethanol each time, the total extracts were pooled together in a centrifuge tube, and centrifuged for 10 min at 10,000 rpm in a model B-20A centrifuge (International Equipment Co.). The supernatant was collected in plastic vials for phenolics measurement.

The ethanolic extracts and filtrates were assayed for phenolics using the technique described by Swain and Hillis[11] with the modification suggested by Singleton and Rossi.[12] The absorptivity was determined in 1 cm cells at 725 nm in an ACTA MVI spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) using as blank water and reagents only.

Gallic acid (Aldrich Chemical Co., Milwaukee, WI) was used as the standard. Since glucose in the medium contributed to the final optical density at 725 nm, appropriate corrections were made for all the samples. The glucose concentration was measured with the "Calbiochem" enzymatic assay kit (Calibiochem, Glucose-S.V.R., La-Jolla, CA). Nitrate was measured using the Nitrate-Nitrite Assay Kit, model NI-12 (Hach Chemical Co., Loveland, CA).

Sterility testing for batch suspension cultures was done routinely using Trypticase Soy Broth Agar (BBL Microbiology Systems, Cockeysville, MD). A few drops of the culture were added to the TSB plate, and the plate incubated at 30°C for 48 h. No microbial contaminants were ever observed in any of the plates. In addition, the cells were examined under the microscope (WILD, M20-35214, Hearbrugg, Switzerland) at magnifications of ($\times$100) and ($\times$1000) for presence of contaminants and for cytological examination of the plant cells.

RESULTS AND DISCUSSION

Influence of Temperature

Figure 1 shows the influence of temperature on cell growth and total phenolics formation by tobacco cells. The doubling time at 15°C was 210 h compared to 61 h at

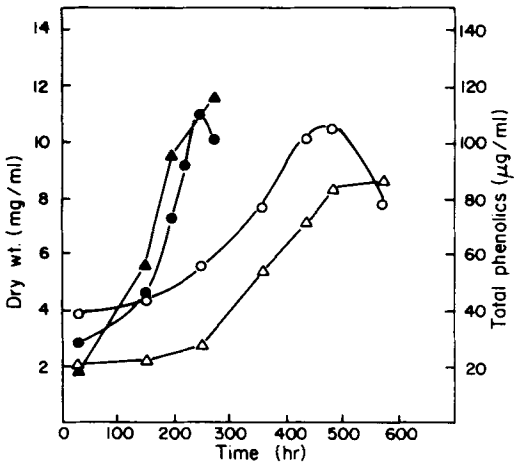

Figure 1. The effect of temperature on cell growth and phenolics formation for dark grown tobacco cells. Symbols are: (○) dry weight (mg/mL) at 15°C; (●) dry weight (mg/mL) at 27°C; (△) total phenolics (μg/mL) at 15°C; (▲) total phenolics (μg/mL) at 27°C.

27°C. The maximum observed biomass levels were comparable (10.5–11.0 mg/mL dry wt). The maximum total phenolics accumulation at 15°C of 86.8 μg/mL was roughly 75% of that obtained at 27°C (i.e. 116.0 μg/mL). At higher temperatures, there is a higher metabolic activity of tobacco cells with increased growth rates accompanied by an increase in total phenolics accumulation. Similar effects have been observed with ubiquinone biosynthesis by tobacco cells in the range 27–32°C.[13] Courtois and Guern[14] have observed a sixfold decrease in growth rate with temperature decreases from 27 to 16°C, but the amount of alkaloid per cell *increased* 12-fold. No other investigations on the effect of temperature on phenolics accumulation in any plant species have been reported, to the best of our knowledge.

Influence of Phenylalanine Addition

Phenylalanine functions as a precursor to phenolic acids on the shikimic acid pathway. The conversion from phenylalanine to cinnamic acids is catalyzed by the enzyme PAL. It was decided to test the response of exogeneous supply of the product precursor on growth and total phenolics formation in tobacco cells.

The effect of different levels of phenylalanine on cell growth at a large and a small initial inoculum level are given in Table II. As is apparent from the data, phenylalanine was inhibitory to cell growth at 0.01% and 0.1% concentrations.

The maximum phenolics level observed were 118.0 μg/mL for controls as compared to 13.1 μg/mL for medium supplemented with 0.01% phenyalanine and 7.0 μg/mL for medium with 0.1% phenylalanine. The total phenolics yield for the control was very stable throughout the growth curve (i.e. between 9.1 and 9.8 μg phenolics /mg dry wt). With 0.01% phenylalanine supplementation, however, the apparent phenylalanine incorporation into phenolics was low during the first 36 h of inoculation; but

Table II. Effect on phenylalanine on growth and total phenolics accumulation in tobacco cells.

	Time (h)	*X* Dry wt. (mg/mL)	*P*, total phenolics (μg/mL)	*Y*(*P*/*X*) μg/mg
Low inoculum level				
Control	36	0.50	4.9	9.8
	299	3.13	30.2	9.7
	377	12.13	118.0	9.7
	511	9.74	88.5	9.1
0.01% Phenylalanine	36	0.72	7.8	10.8
	299	0.76	13.1	17.4
	377	0.75	13.0	17.5
	511	0.75	12.0	16.1
0.1% Phenylalanine	36	0.37	7.0	19.0
	299	0.39	4.4	11.4
	377	0.35	2.6	7.3
	511	0.43	4.4	10.2
High inoculum level				
Control	36	2.20	19.6	8.9
	177	4.95	43.8	8.8
	230	9.37	81.6	8.7
	299	13.71	134.0	9.8
	377	8.94	90.0	10.1
	511	10.34	85.6	8.3
0.1% Phenylalanine	36	1.40	17.5	12.5
	177	1.70	16.0	9.4
	230	1.64	16.5	10.1
	299	1.44	17.6	12.2
	377	1.20	15.2	12.7
	511	1.08	15.2	14.1

within the next 260 h, the phenolics level increased almost 1.7 times. The cell mass, however, remained the same. This improved the yield factor from an initial 10.8 μg/mg to a final 17.35 μg/mg. This would amount to a percent incorporation of phenylalanine to total phenolics of roughly 5.3%.

These results compare well with the results of phenylalanine feeding to wild and resistant strains of tobacco cells by Berlin and Witte.[9] In this study wild cells of tobacco incorporated between 2.9 and 3.3 mg phenylalanine into cinnamoyl putrescines if the medium was supplemented with 20 mg/flask (approximately 0.03% phenylalanine). The total incorporation was only slightly higher (3.4–3.7 mg) at 50 mg/flask phenylalanine. Cells resistant to *p*-fluorophenylalanine, however, incorporated three times as much into cinnamoyl putrescines.

In presence of 0.1% phenylalanine and a low inoculum level the major incorporation occurred in the first 36 h, with a yield of 19.0 μg/mg. Subsequently, it declined to 11.4 μg/mg in the next 260 h. Since the total phenolics level immediately after inoculation was not measured, it is difficult to make value comparisons in terms of percent of phenylalanine incorporated into total phenolics. The

effect of 0.1% phenylalanine with a high initial inoculum level displays similar behavior.

The flasks with 0.1% and 0.01% phenylalanine show consistently higher $Y_{P/X}$ values when compared to controls. This enhancement, however comes at the expense of a diminished dry weight; and the net phenolics obtained from the batch system in presence of phenyalanine are much lower than controls. These results may be compared to the variable and contradictory results reported in the literature for feeding amino acids as precursors. Sairam and Khanna[15] found that the inclusion of 0.1% phenylanaline and tyrosine increased alkaloid formation in *Datura tatula* tissue manifold but decreased cell growth rates drastically. The addition of phenylalanine did[16] or did not[17] influence the amount of rosmarinic acid formed by Coleus blumei. Feeding the precursor L-tryptophan to *Catharantheus roseus* has been reported to positively[16] or negatively[18] affect the formation of alkaloids.

The results of our experiments indicate that the separation of growth and product formation stages may be necessary if precursor incorporation experiments to enhance overall yields are to be successful.

Influence of Hormones

Two sets of shake flask experiments were conducted to investigate the effects of 2,4-D and kinetin on growth and total phenolics formation. In set A, the inoculum used was grown for a generation devoid of both 2,4-D and kinetin, while in set B the normal complement of kinetin and 2,4-D were used in preparing the inoculum.

The procedure in set A was used to reduce the residual levels of intracellular kinetin and 2,4-D concentrations in the cells. The cells were then inoculated into flasks with 2% glucose and the following hormonal concentrations: a) normal kinetin, normal 2,4-D; b) normal kinetin (i.e. 0.3 mg/L), no 2,4-D; c) normal 2,4-D, no kinetin, d) no kinetin, no 2,4-D; e) 4 mg/L, 2,4-D, normal kinetin; and f) 0.06 mg/L kinetin, normal 2,4-D (i.e. 1 mg/L). Duplicate flasks were run for each case. The results are displayed in Figures 2(a)-(f). It is apparent that in the absence of both 2,4-D and kinetin the cell growth rates, the maximum attainable cell mass, and total phenolics accumulation are absolutely diminished when compared to controls with the normal supplement of 2,4-D and kinetin. Table III lists the cellular doubling times at different hormonal compositions of the growth medium; the normal amount of 2,4-D appears optimal for growth rate, but a reduced level of kinetin appears to improve the growth rate. It is interesting to compare kinetics of total phenolics formation at different levels of 2,4-D but in presence of normal kinetin [i.e. Figs. 2(a), 2(b), and 2(e)]. Without 2,4-D, the culture shows a mixed growth associated or a non-growth-associated behavior.[19] Thus, the total phenolics concentration continues to increase beyond the point of attainment of maximum cell mass. In contrast, in presence of 1 or 4 mg/L 2,4-D, the product concentration reaches a high value at the point the maximum dry weight is obtained and then subsequently declines or increases slightly (i.e. ca. 5-6%) within the next 100 h, exhibiting an apparent growth-associated product formation pattern. The presence of 2,4-D suppress some aspects of secondary metabolism in tobacco suspension cultures, giving the false impression that phenolics formation is growth associated or is a product of primary metabolism of the cells.

If total phenolics accumulation at the maximum dry weight (i.e. $t = 255$ h) is compared for the three cases, it seems a normal 2,4-D (i.e. 1 mg/L 2,4-D) provides the highest phenolics concentration, i.e. 86.6 μg/mL when compared to 47.2 μg/mL for 2,4-D free medium and 76.1 μg/mL for medium with 4 mg/L 2,4-D. However, within the next 100 h, the cells devoid of 2,4-D accumulate 41.5% more phenolics (but still less than the control) as compared to + 5-6% change in product concentration observed for cells growing in presence of 2,4-D.

Complete absence of kinetin, but with the normal complement of 2,4-D showed a product kinetic pattern akin to the controls (i.e. normal LS medium with 0.3 mg/L kinetin). However, in presence of 0.06 mg/L kinetin, even on achieving $X = X_{max}$, the quantity of phenolics kept on increasing. Also, under this condition, the doubling time was slightly lower (i.e. an average of 53 vs. 60 h for controls). The recorded X_{max} of 9.5 mg/mL dry wt under this condition is slightly higher than 9.0 mg/mL dry wt for control and 8.43 mg/mL dry wt for nonkinetin conditions. The presence of kinetin significantly enhances phenolics production; P_{max} for nonkinetin conditions is approximately 56.5% of that with 0.06 mg/L kinetin and 65% that with 0.3 mg/L kinetin.

The effect of kinetin on total phenolics formation can be compared with the work of other authors looking at different polyphenols in plant cells. Thus, Bergmann[20] observed increasing polyphenol formation with kinetin in calluses of *Nicotiana tabacum*. Kinetin stimulated the accumulation of lignin in the calluses, though it did not exert a significant effect on the formation of soluble polyphenols.[4] Bagratishirli et al.,[21] however, observed increased polyphenol formation with kinetin in tea plant cultures.

While assessing the effect of kinetin on cellular metabolism, it must be realized that cell cultures preserve the ability to synthesize cytokinins to a greater extent than to synthesize auxins. The lack of information on intracellular level of hormones foils an attempt at making a reliable interpretation of the observed behavior. Kinetin has been found to stimulate the rate of protein synthesis in soybean and tobacco[2]; and according to Hagen and Harcus,[22] an important function of kinetin is the inhibition of cell enlargement, thereby facilitating a balance between cell division and cell expansion.

In set B experiments, the inoculum was grown in normal LS medium with the usual supplement of 2,4-D and kinetin. It was then inoculated into flasks with 3% glucose and six varying concentrations of 2,4-D in the range 0-4

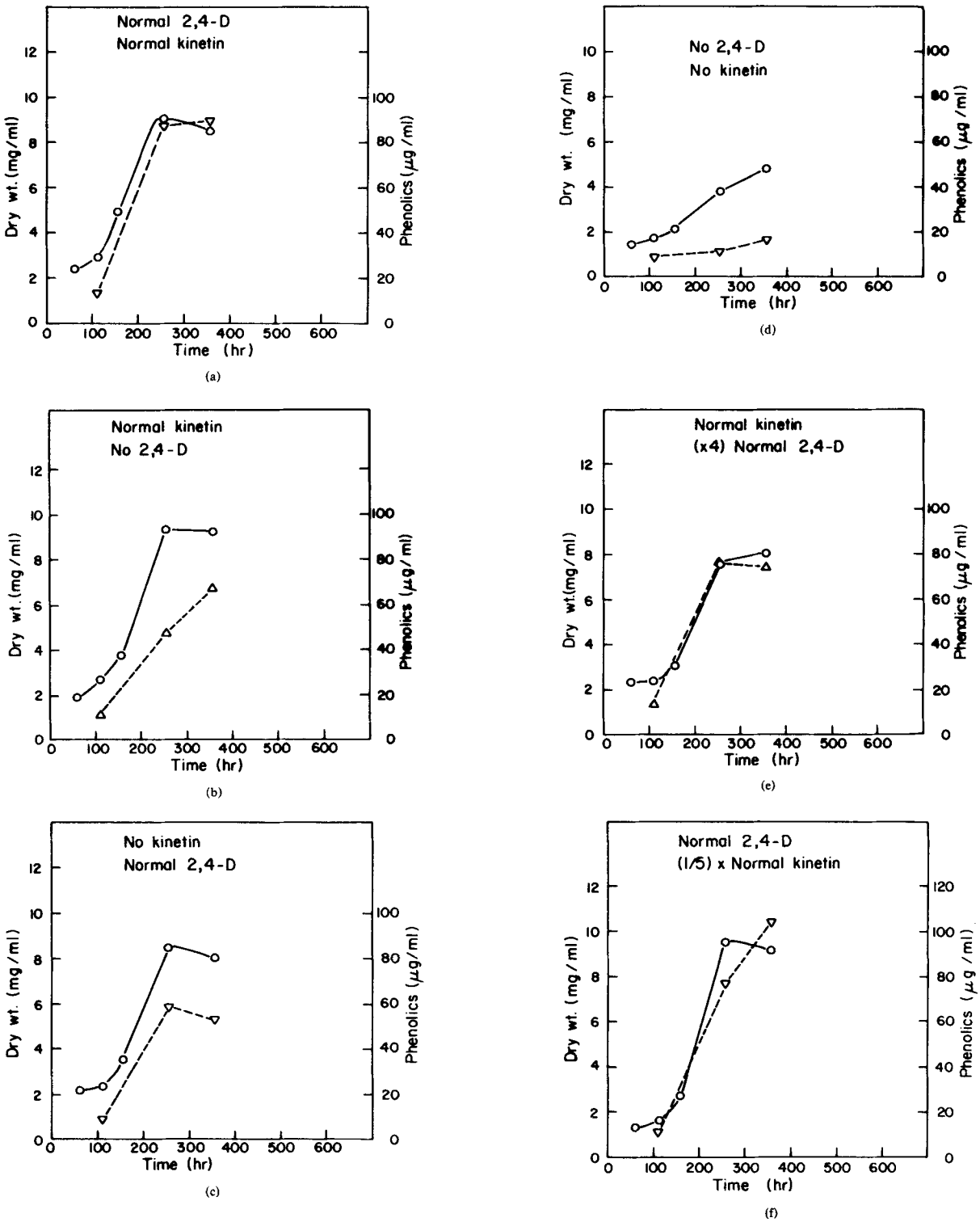

Figure 2. The effect of hormone treatments on growth and phenolics formation in dark grown tobacco cells (set A). The temperature was 27 ± 1°C and the glucose concentration was 20.0 g/L. The inoculum consisted of 26-day-old stationary-phase cells grown for one-generation in non-2,4-D, non-kinetin medium. In all cases, the symbols (○) and (△) were used for dry weight (mg/mL) and total phenolics (µg/mL), respectively: (a) normal kinetin and no 2,4-D; (b) normal kinetin and no 2,4-D; (c) no kinetin and normal 2,4-D; (d) no kinetin and no 2,4-D; (e) normal kinetin and four times normal 2,4-D; and (f) one-fifth normal kinetin and normal 2,4-D.

Table III. Effect of hormones on cell growth.

Hormonal composition of medium	Doubling time (h)
No 2,4-D, no kinetin	130
No 2,4-D, normal kinetin	75
No kinetin, normal 2,4-D	62
Normal 2,4-D, normal kinetin	61
2,4-D (×4), normal kinetin	75
Kinetin (×0.2), normal 2,4-D	53

mg/L 2,4-D. Such a sequence represents an analog of the response of a multistage continuous system. Duplicate flasks were used for each condition.

The results of these different levels of 2,4-D on cell dry weight and total phenolics production are displayed in Figures 3(a)-3(f). The difference in kinetic behavior of product formation with cultures at low and high levels of 2,4-D, alluded to in the discussion of results from Set (A), was markedly pronounced in this study. In the culture devoid of 2,4-D, a high concentration of total phenolics is reached as X reaches X_{max} (i.e. 107.1 μg/mL), and this increased further to 159.6 μg/mL in the next 106 h. In contrast, those with 2,4-D (0.05, 0.5, 1, 2, and 4 mg/L) show an almost growth associated behavior. The maximum phenolics content obtained in the presence of 2,4-D was very close to the time when $X = X_{max}$.

It is difficult to discern why the suspension cultures in set A in absence of 2,4-D failed to accumulate high levels of total phenolics when compared to the controls. It is likely that the nature of the inoculum (i.e. grown in presence or absence of 2,4-D and kinetin) may have contributed to the difference. However, the substantially different kinetics of product formation in presence and in complete absence of 2,4-D are consistent with both sets. In both cases, phenolics concentration increased substantially after reaching $t_{X=X_{max}}$, when the initial level of 2,4-D in the medium was zero. In set A, this increment was roughly 41.5% during a period of 100 h, whereas in set B there was a 49% increase over 106 h.

The enhancement in phenolics formation at very low levels of 2,4-D has been shown in turbidostat experiments with suspensions of *Acer pseudoplatanus*.[23] Using a turbidostat to maintain a constant cell density, prolonged steady states were established at several different low 2,4-D concentrations. In contrast to the cell composition obtained at the highest input 2,4-D concentration $4.5 \times 10^{-6}M$, at the lowest 2,4-D concentration ($0.5 \times 10^{-6}M$), the total soluble phenolics increased roughly 62%, the coumaric acid increased 225%, and the lignin content increased 376%. Our results in set B are consistent with these observations.

If a continuous flow immobilized cell reactor were to be constructed, the formation of phenolics might be enhanced by using a 2,4-D-free nutrient solution either continuously or alternating with normal 2,4-D medium in a cyclic manner.

Influence of Illumination

Since synthesis and accumulation of many cinnamic acids and flavanoid compounds have been shown to be light dependent,[6,24] it was decided to test the effect of continuous illumination from white cool fluoresence lamps on cell growth and total phenolics accumulation. Within 48 h of illumination with six white fluoresence lamps the suspensions turned green, indicative of substantial accumulation of chlorophyll. The controls (i.e. suspensions cultivated in the dark) remained brown. This development of protoplastids into typical chloroplasts and increase in chlorophyll content in presence of illumination is a sign of some level of cellular differentiation. Similar observations have been reported with calluses of carrot, tobacco, and *Atropa*.[25] Many factors other than light are known to affect the chlorophyll content of tissue cultures, including iron availability, auxins, and cytokinins, and sugars, especially sucrose. Some strains of tobacco calluses have been shown to have an obligate requirement for exogenous cytokinins (i.e. kinetin) for chlorophyll development which was shown to be distinct from its requirement for cell division.[26]

Batch cultures grown with and without illumination gave nearly equal growth rates, maximum cell masses, and phenolics production. Since PAL is under photocontrol and is a branch point enzyme which provides and controls precursor flow to lignin biosynthesis, a more dramatic shift in metabolism was expected.

The PAL activity of many plant tissues has been shown to be stimulated by light, so that it would be expected that the absence of light would reduce PAL activity to a low level and restrict the synthesis of phenolic compounds. However, as concluded by Everett and Street[27] after testing the combined effect of light and hormones on total phenolics accumulation in cells of *A. pseudoplatanus*, further metabolism of soluble phenolics into alcohol-insoluble compounds may be light stimulated. This could explain in part the increased levels of soluble phenolics they obtained for *A. pseudoplatanus* cells in the absence of 2,4-D in dark as compared to light controls. They have also suggested that either PAL activity is not significantly reduced in the absence of light or that PAL activity and phenol polymerization may be closely linked.

Since only ethanol-soluble total phenolics were measured for the tobacco cells, it is possible that illumination may have caused substantial polymerization into lignins in lighted cultures or conversion of phenols into alcohol-insoluble compounds. This would explain the lower phenolics yields obtained in the presence of light.

Influence of C/N Ratio

The purpose of this set of experiments was to study the combined effect of illumination and different initial levels of glucose and NO_3^- source in the medium on growth and total phenolics accumulation. Three different levels of glucose (i.e. 2, 3, and 6%, with the normal complement of

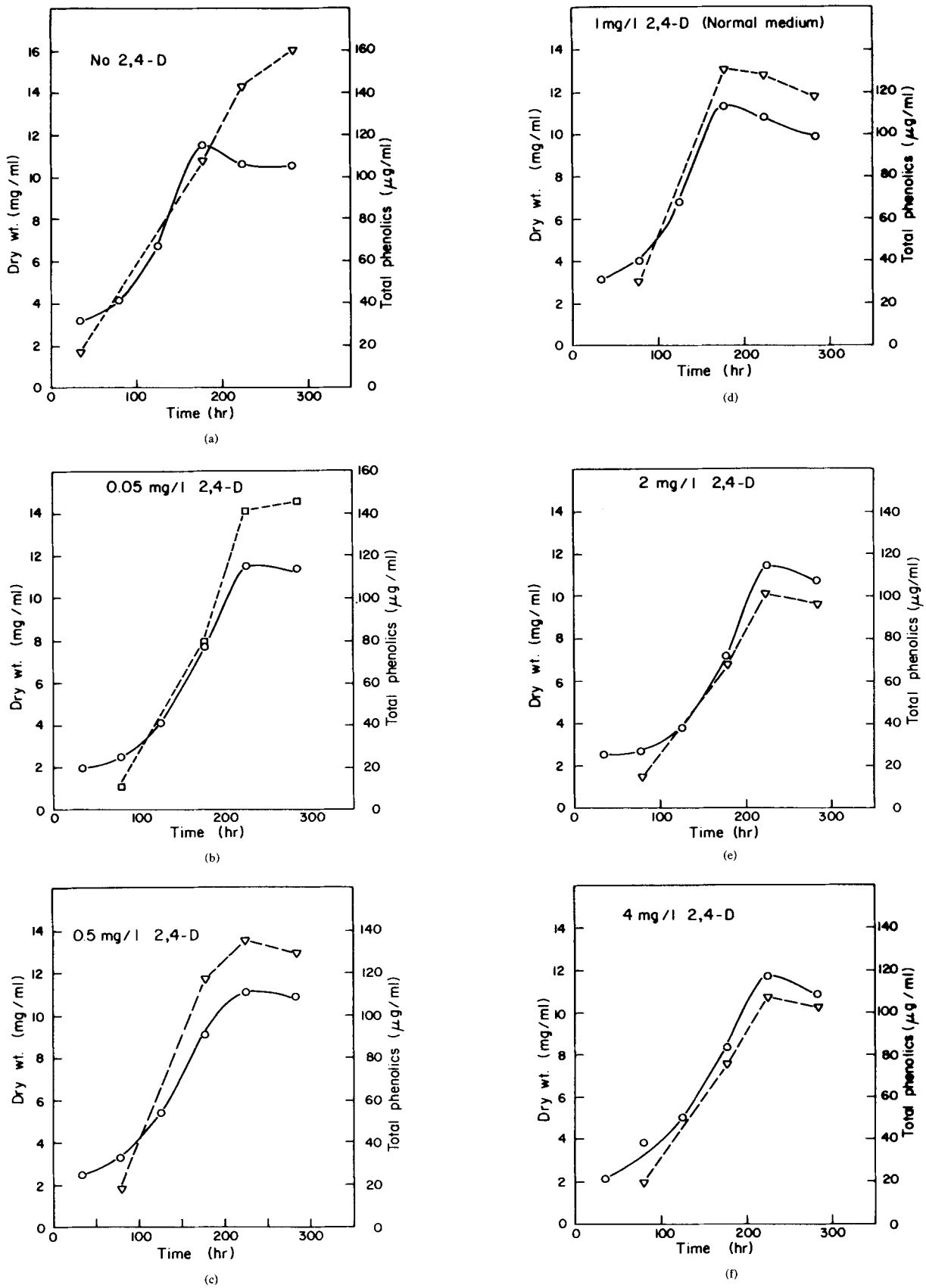

Figure 3. The effect of hormone treatments on growth and phenolics formation in dark grown tobacco cells (set B). The temperature was 27 ± 1°C and the glucose concentration was 30.0 g/L. The inoculum consisted of 20-day-old stationary-phase cells grown on a normal LS medium. In all cases, symbols (○) and (△) were used for dry weight (mg/mL) and total phenolics (μg/mL), respectively: (a) normal LS medium with no 2,4-D; (b) 2,4-D increased to 0.05 mg/L; (c) 2,4-D increased to 0.5 mg/L; (d) 2,4-D increased to 1.0 mg/L; (e) 2,4-D increased to 2.0 mg/L; and (f) 2,4-D increased to 4.0 mg/L.

KNO_3 and NH_4NO_3 (designated as 1N) as also three different levels of NO_3^- source (i.e. 1/8N, 1/4N, and 1/2N) in the presence of 2% glucose were chosen for experimentation.

Growth and total phenolics formation curves under these conditions are displayed in Figures 4(A)-4(F). In all cases the flask contents turned green within 48 h of illumination, and microscopic examination of cells revealed the presence of chloroplasts. Though the chlorophyll content of the cells was not measured, it was observed that at the highest level of glucose (i.e. 6% C), the intensity of color of the flask contents was much lower than the others (i.e. greenish-brown rather than green). This would indicate that significantly less chlorophyll was formed in presence of high glucose concentrations. Similar observations have been reported for other plant tissues with sucrose as the carbon source.[28]

However, the effect of NO_3^- levels on chlorophyll formation has not been reported. It was observed that at t = 185 h the contents of the flasks supplemented with 1/8N turned brown and the cells were highly agregated. The effect was probably due to the depletion of NO_3^-. The same phenomenon was repeated in flasks supplemented with 1/4N at 208 h the precise time at which all the nitrate was exhausted from the medium. A minimum level of NO_3^- may be associated with chlorophyll formation.

On comparing the maximum phenolics obtained in flasks with different initial levels of NO_3^-, it was found that the flasks with normal complement of nitrate (i.e. 1N) produced equivalent levels of phenolics as those with 1/2N. However, as the nitrate levels were further reduced P_{max} decreased slightly to 73 μg/mL at 1/4N and then fell sharply to 36 μg/mL at 1/8N. However, if the product yield (i.e. $Y_{P/X}$ = yield of total phenolics/mg initial NO_3^-) is compared for the four cases, a behavior as indicated in Figure 5 is obtained. Thus, with increasing nitrate concentrations from 1/4N to 1N a progressive dimunition of specific phenolic yield is obtained.

Antagonism between nitrogen and polyphenol metabolism has been reported in many cell cultures.[4] A decrease in nitrate concentration in the media has been found to be accompanied by a significant increase in the production of phenolic compounds. In interpretating the observed results it should be noted that ethanol soluble total phenolics constitute only one part of the net phenolics formed by the cell culture. In the presence of illumination, increased synthesis of complex phenols (e.g. leucoanthocyanins) have been observed.[29] Thus, at nitrate concentrations of 1/8N [Fig. 4(f)] the optimal rate of simple phenol synthesis appears to be in the logarithmic phase of growth, and there is a marked decline as the cells enter the stationary phase. It is possible that increased conversion from simple to complex phenols occur at these low levels of nitrogen, as the cells move from the logarithmic to the stationary phase.

The diminution of the specific phenolic yield could possibly be due to nitrite toxicity. Since nitrite was not measured in these experiments, we can not eliminate nitrite toxicity as a factor in the reduction of phenolic yields.

The decrease in P_{max} with decreasing levels of nitrate may also be explained on the basis of complex phenol synthesis. For example, one might contend that the synthesis of complex phenols depends on the "acetate pool" and the availability of cinnamic acid precursors. Increasing nitrate concentration in the culture medium is ineffective in the control of cinnamic acid precursor availability, but results in the shunting of acetyl CoA to the TCA cycle which results in an inadequate pool of acetyl CoA for the synthesis of complex phenols.

Increasing glucose concentrations in the medium, however, appear to have a significant effect on the promotion of total phenol synthesis in tobacco cell cultures. In the presence of illumination, P_{max} levels obtained at 2, 3, and 6% were 85.6, 131.6, and 219.0 μg/mL, respectively. Similar results have been obtained with rose suspension cultures[29,30] and with *Acer pseudoplatanus* suspensions.[31] It should be noted that the ratio of C/N is important,[30] and the response to glucose and NO_3^- changes can involve synergetic effects. Additionally, the data on cultures with high glucose concentrations suggests a shift from an apparent primary association of phenolics with cell growth to a mixed growth-associated pattern.

The kinetics of growth and total phenolics accumulation in dark-grown tobacco cells were measured for flasks with 0.5 and 1% glucose. Values of 17.7 and 29.3 μg/mL, respectively, were obtained. Figure 6 exhibits the variation in P_{max} with change in glucose concentrations.

Influence of Antibiotics

Since plant cells are easily susceptible to microbial contamination, addition of antibiotics would be very valuable if they controlled the growth of the contaminants without any adverse effect on plant cell growth and metabolism. The influence of addition of antifungal agent Nystatin (25 units/mL) and antibacterial agent Tylosin (20 μg/mL) and penicillin (G 100 units/mL) on tobacco cell growth was investigated. The specific growth rate and maximum attainable biomass were identical to those of the controls for all three antibiotics.

SUMMARY

These studies then suggest some strategies for increasing the output of ethanol soluble phenolics from tobacco cell cultures. Dark-grown cultures at 27°C appear adequate for phenolics production. If the cell growth phase can be separated from a productive stage (e.g., immobilized cell cultures or multistaged continuous units), then phenolics production might be enhanced by phenylalanine feeding to the production phase or the removal of 2,4-D in the product formation stage. Glucose feed to the production phase should improve phenolics production *if* another nutrient is limiting growth and the glucose addition does not cause renewed cellular proliferation. Finally, the addition of antibiotics to prevent significant contamination of continuous culture systems may be possible.

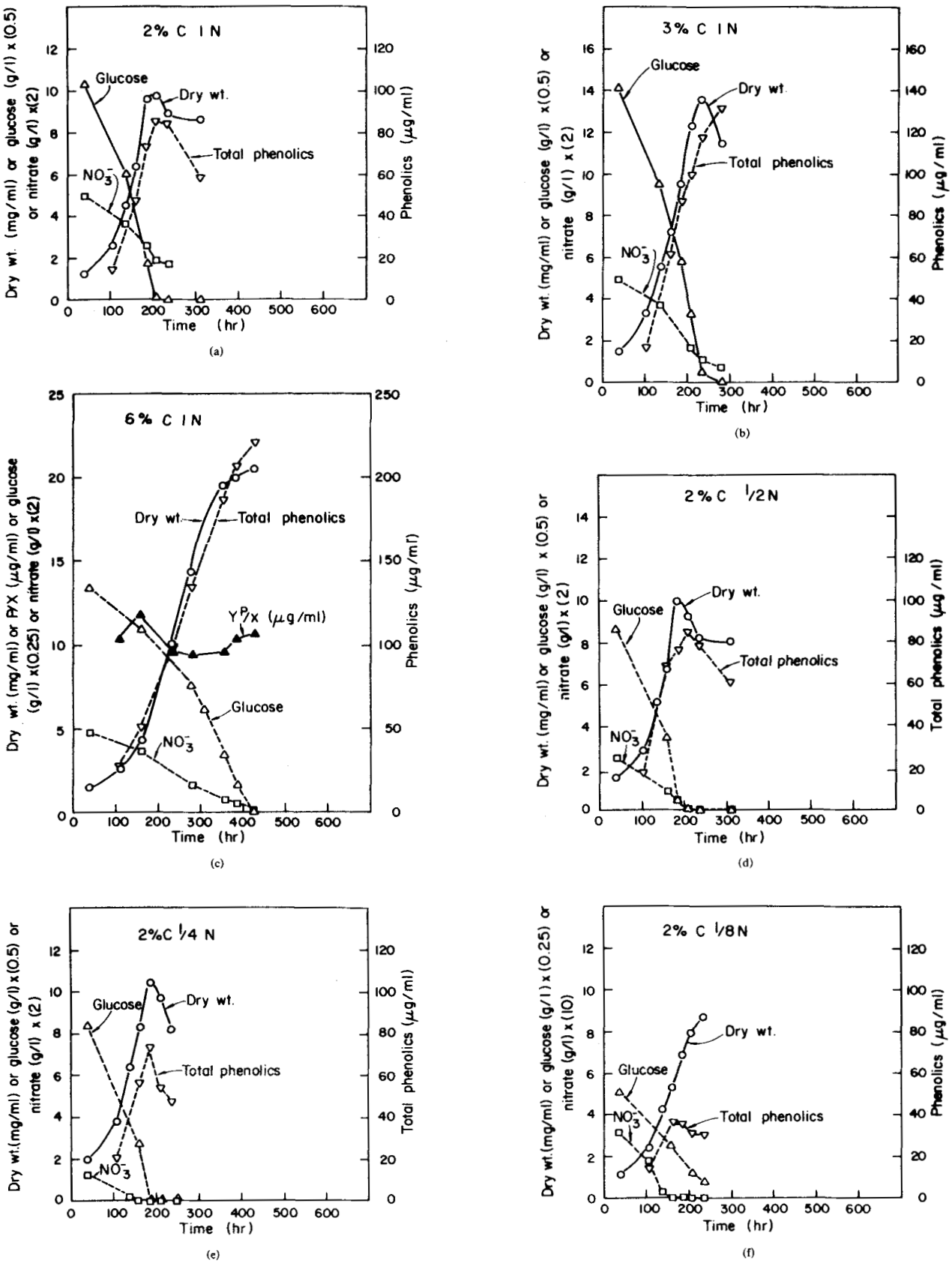

Figure 4. Variation in cellular dry weight (○), total phenolics (▽), NO_3^- (□), and glucose (△) for various levels of carbon and nitrogen sources. Cells were grown in the presence of six cool, white fluorescent lamps at 27 ± 1°C. The inoculum contained stationary-phase cells (21 days postinoculation) grown on normal LS medium: (a) 20 g/L glucose, 1.65 g/L NH_4NO_3 and 1.9 g/L KNO_3; (b) as in (a) except 30 g/L glucose; (c) as in (a) except 60 g/L glucose ($Y_{P/X}$ is yield of phenolics per unit cell wall); (d) 20 g/L glucose, 0.825 g/L NH_4NO_3, and 0.95 g/L KNO_3; (e) 20 g/L glucose, 0.413 g/L NH_4NO_3, and 0.475 g/L KNO_3; and (f) 20 g/L glucose, 0.206 g/L NH_4NO_3, and 0.238 g/L KNO_3.

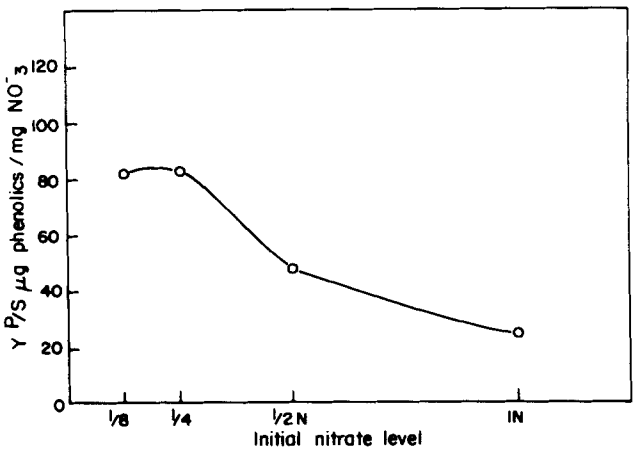

Figure 5. The quantity of phenolics consumed per unit mass of NO_3^- consumed is a function of the initial nitrate concentration in a batch culture. The "1N" refers to a medium with 1.65 g/L of NH_4NO_3 and 1.9 g/L of KNO_3. The "1/2N" refers to one-half of the concentration of NH_4NO_3 and KNO_3, while "1/4N" and "1/8N" refer to concentrations of nitrogen sources at one-fourth and one-eighth the level of "1N", respectively.

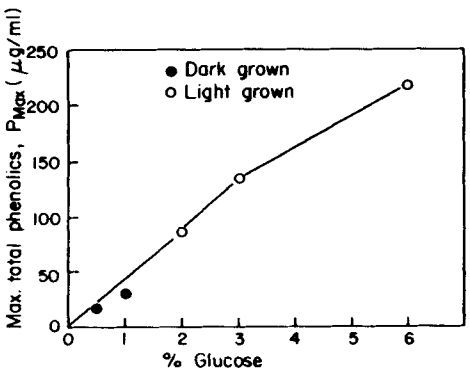

Figure 6. The concentration of phenolics formed by tobacco depends on the initial concentration of glucose.

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References

1. S. L. Lee and A. I. Scott, *Dev. Ind. Microbiol.*, **20,** 381 (1979).
2. D. K. Dougall, in *Cell Substrates*, J. C. Petricciani, H. E. Hopps, and P. J. Chapple, Eds. (Plenum, New York, 1979), p. 135.
3. M. H. Zenk, in *Frontiers of Plant Tissue Culture*, T. A. Thorpe, Ed. (International Association of Plant Tissue Culture, Calgary, Alberta, Canada, 1978), p. 1.
4. M. N. Zaprometov, in *Frontiers of Plant Tissue Culture*, T. A. Thorpe, Ed. (International Association of Plant Tissue Culture, Calgary, Alberta, Canada, 1978), p. 335.
5. L. L. Creasy and N. Zucker, in *Metabolism and Regulation of Secondary Plant Products— Recent Advances in Phytochemistry. Vol. 8*, V. C. Runeckles and E. E. Conn, Eds. (Academic, New York, 1974), p. 1.
6. H. Grisebach and K. Hahlbrock, in *Metabolism and Regulation of Secondary Plant Products—Recent Advances in Phytochemistry, Vol. 8*, V. C. Runeckles and E. E. Conn, Eds. (Academic, New York, 1974), p. 21.
7. S. A. Brown and M. Tenniswood, *Can. J. Bot.*, **52,** 1091 (1974).
8. S. Mizusaki, Y. Tanabe, M. Noguchi, and E. Tamaki, *Phytochemistry*, **10,** 1347 (1971).
9. J. Berlin and L. Witte, *J. Nat. Prod. (Lloydia)*, **45,** 88 (1982).
10. E. M. Linsmaier and F. Skoog, *Physiol. Plant.*, **18,** 100 (1965).
11. T. Swain and W. E. Hillis, *J. Sci. Food Agric.*, **10,** 63 (1959).
12. V. L. Singleton and J. A. Rossi, *Am. J. Enol. Vitic.*, **163,** 144 (1965).
13. T. Ikeda, T. Matsumato, and M. Noguchi, *Agric. Biol. Chem.*, **41,** 1197 (1977).
14. D. Courtois and J. Guern, *Plant Sci. Lett.*, **17,** 473 (1980).
15. T. V. Sairam and P. Khanna, *Lloydia*, **34,** 170 (1971).
16. M. H. Zenk, H. El Shagi, H. Areus, J. Stockigt, E. W. Weiler, and B. Deus, in *Plant Tissue Culture and Its Biotechnological Application*, W. Barz, E. Reinhard, and M. H. Zenk, Eds. (Springer-Verlag, New York, 1977), p. 27.
17. A. Razzague and B. E. Ellis, *Planta*, **137,** 287 (1977).
18. G. Doller, A. W. Alfermann, and E. Reinhard, *Planta Med.*, **30,** 14 (1976).
19. E. L. Gaden, Jr., *J. Biochem. Microbial. Technol. Eng. (Biotechnol. Bioeng.)*, **1,** 413 (1959).
20. L. Bergmann, *Planta*, **62,** 221 (1964).
21. D. G. Bagratishvili, M. N. Zaprometov, and R. G. Butenko, *Fizol. Rast.*, **27,** 404 (1980) (as translated by Plant Physiology from Russian).
22. G. L. Hagen and A. Marcus, *Plant Physiol.*, **55,** 90 (1975).
23. P. J. King, *J. Exp. Bot.*, **27,** 1053 (1976).
24. K. Hahlbrock and E. Wellmann, *Biochim. Biophys. Acta*, **304,** 702 (1973).
25. P. A. Aitchison, A. J. Macleod, and M. M. Yeomen, in *Plant Tissue Culture*, H. E. Street, Ed. (University of California Press, Berkeley, CA, 1977), p. 267.
26. K. Kaul and P. S. Sabharwal, *Plant Physiol.*, **47,** 691 (1971).
27. N. P. Everett and H. E. Street, *J. Exp. Bot.*, **30,** 409 (1979).
28. J. Edelman and A. D. Hanson, *J. Exp. Bot.*, **23,** 469 (1972).
29. H. V. Amorin, D. K. Dougall, and W. R. Sharp, *Physiol. Plant.*, **39,** 91 (1977).
30. D. J. Kubek and M. L. Shuler, *J. Nat. Prod. (Lloydia)*, **43,** 87 (1980).
31. R. Phillips and G. G. Henshaw, *J. Exp. Bot.*, **28,** 785 (1977).