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The Effect of Initial Phosphate and Sucrose Levels on Nicotine Accumulation in Batch Suspension Cultures of *Nicotiana tabacum* L.

S. H. Mantell, D. W. Pearson, L. P. Hazell and H. Smith

Department of Botany, University of Leicester, University Road, Leicester LE1 7RH, UK

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Abstract

A system of liquid batch culture of tobacco (*Nicotiana tabacum* cv NC2512) is described in which up to 2% nicotine accumulates on a dry weight basis. Nicotine accumulation is first detected in cultures when medium phosphate is completely depleted. By reducing initial medium phosphate to 1/10th levels normally employed, alkaloid accumulation is accelerated while the raising of initial medium sucrose (30-50 g l^{-1}) results in a five-fold increase in peak accumulation of nicotine. The roles of medium phosphate and sucrose levels in culture growth and nicotine production are discussed.

Introduction

Cultural factors which influence accumulation of secondary metabolites in plant cell and tissue cultures include ingredients of media, the degree of aeration and culture macromixing and culture media pH (Mantell and Smith, 1982). Of these medium components, growth regulators (notably auxins) have been established to exert the most significant effect on secondary metabolite productivity of cell cultures (Staba, 1980). Nicotine production by tobacco cell and tissue cultures is no exception, (Furuya *et al.*, 1971; Shiio and Ohta, 1973). With establishment of a two stage auxin/cytokinin stepdown protocol, batch suspension cultures of tobacco can be obtained which produce 1-4% nicotine on a dry weight basis (Pearson, 1978). Maintenance of these levels of productivity can be achieved through use of productive cell strains obtained from screening of established cell lines through appropriate techniques of single cell selection and cloning (Tabata *et al.*, 1978; Ogino *et al.*, 1978). Cell suspension culture systems provide excellent opportunities for studying the influence of culture factors on the biosynthesis, accumulation and biodegradation of secondary plant metabolites. Furthermore, the use of culture systems larger than sub-litre rotary shake cultures facilitates time course studies on more uniform batches of cells.

The importance of sucrose and phosphate regimes in triggering the production of various secondary metabolites including antibiotics and alkaloids in microbial cultures is well recognized (Floss *et al.*, 1974; Drew and Demain, 1977). For example, during ergot alkaloid production by cultures of *Claviceps purpurea*, a high concentration of phosphate in the medium favours abundant synthesis of cells and phosphorylated compounds such that alkaloid is only formed when there is not sufficient phosphate for rapid growth. Surprisingly little attention has been paid to the effect of phosphate on secondary metabolite biosynthesis in plant cell cultures. In view of this and because preliminary observations suggested that initial sucrose levels may also be important in determining the nicotine productivity in our tobacco cell culture system, studies on the effect of these two medium constituents on alkaloid production were carried out.

This paper reports the effect of varying initial phosphate and sucrose levels of media on the pattern of accumulation of nicotine in liquid batch suspension cultures of tobacco.

Materials and Methods

Initiation and maintenance of the flue-cured tobacco, *Nicotiana tabacum* cv NC2512, as callus and suspension cultures were carried out using conventional methods (Street, 1977). Calluses were derived from commercial seed, obtained from the Agricultural Experimental Station of the University of Kentucky, U.S.A., germinated in the dark at 25°C on 0.8% agar-solidified MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg l^{-1} naphthaleneacetic acid (NAA) and 0.2 mg l^{-1} kinetin. The pH of media was adjusted to 5.8 before autoclaving. At 3-5 weeks, 5-10 g callus was transferred to 70 ml liquid MS medium (stock culture medium i.e. SCM) and cell suspensions initiated in aluminium foil-capped 250 ml conical flasks held on orbital shakers (120 $r.min^{-1}$) at 25°C in continuous diffuse light provided by 25W cool-white fluorescent lamps (5.3 W m^{-2}).

Routine subculturing of stock cell suspensions was carried out every 14-21 days using a dilution (c. 1:7) of one 70 ml stock culture, previously passed through wire sieves of 500 µm mesh to remove large cell aggregates, to 500 ml fresh medium. Since variation in coloration and aggregation of mother stocks was sometimes observed during the course of a few culture generations (Pearson, unpublished data), cell stocks for seedling were raised in 4 litre batches. This enabled a series of fermentors to be seeded from a single stock of cells.

To induce NC2512 cells to produce nicotine, stocks were transferred to nicotine production medium (NPM), consisting of MS medium (which contains 150 mg l^{-1} orthophosphate) minus casein hydrolysate and supplemented with 0.2 g l^{-1} NAA and 0.02 g l^{-1} kinetin, at

a dilution of 1:10 (to give an initial density of 10^5 cells ml^{-1}) in either 250 ml rotary shake flasks or in 5-litre batch fermentors previously used by Wilson *et al.* (1971) for *Acer pseudoplatanus* cell cultures though specially modified for tobacco cell cultures. Internal heating coils were omitted and instead of magnetic stirrers, which produced deleterious sheer, air inlet tubes were extended to within 1 cm of the base of each vessel to provide aeration (3l min^{-1}) and macromixing.

Nicotine was determined using either GLC or UV spectrophotometric methods. GLC separations were carried out in 180 cm glass analytical columns packed with 10% carbowax 20M with 5% KOH supported on 80-100% mesh chromosorb W. Column temperature was 160°, injection port 200° and detectors 250° on a Pye Unicam 204 machine. Gas flow rates were N_2 at 30 ml min^{-1}, H_2 at 30 ml min^{-1} and air at 300 ml min^{-1}. Nicotine was measured in a 5 µl diethyl ether sample and quantified using quinaldine as an internal standard. UV spectrophotometry was performed using a modification of the method of Willits *et al.* (1950). Cell and medium samples were placed in 2x volume 0.1M HCl and homogenized in a 'Polytron' for 1-2 min. A 20 ml aliquot was spun at 1,800g for 10 min to deposit cell debris and triplicate 4 ml supernatant samples and nicotine standard (160 µg ml^{-1} 0.1M HCl) each placed in 4 ml 0.1M NHCl. After mixing, 2 drops of 1N NaOH were added to raise the pH above 7 and nicotine extracted by addition and mixing (at least 2 min for each sample) with 4 ml diethyl ether. The ether layer (1 ml) was transferred to 4 ml 0.1M HCl and after removal of ether at 70°C, cooled extracts were examined in a scanning Unicam spectrophotometer (λmax for nicotine = 259 nm in 0.1M HCl).

Cell fresh weight and nicotine values presented are means of at least four replicates taken at each sampling time. Cells were separated from 100 ml culture by vacuum filtration through 'Miracloth' and both cells and filtrate used for determinations of cell and medium nicotine, respectively. Total accumulations of nicotine (w/v) were calculated by combining values of 'cell' nicotine and 'medium' nicotine. For other analyses, 10 ml aliquots of media were filtered through Whatman's No. 1 paper and stored at -20°C. Medium sucrose and phosphate levels were determined colorimetrically using described methods (see Gould *et al.*, 1981).

Results

Growth and nicotine production by *N. tabacum* cv NC2512 cells grown in 5-litre batch fermentors is shown in Fig. 1. Nicotine accumulation was first detected in cultures between 5 and 10 days after inoculation in NPM. It reached a maximum level between 20 and 25 days. Substantial increases in fresh weight occurred subsequent to complete phosphate depletion although once sucrose depletion had occurred increases in cell fresh weight ceased. The effects of placing NC2512 cells in media with different levels of these nutrients were therefore examined. Contrasting levels of sucrose (Fig. 2) produced markedly different patterns of growth and nicotine accumulation. At 20 g l^{-1} initial sucrose, cell fresh weight increase was of the typical pattern with a depletion of sucrose in the medium occuring by 14 days. In contrast, detectable levels of sucrose were maintained throughout the 32-day culture period in media containing raised initial sucrose (50 g l^{-1}). Under these conditions, cell fresh weight increases occurred after 14 days, although cell fresh weight never reached the levels obtained with 20 g l^{-1} sucrose. Nicotine accumulation followed a similar pattern in both cultures up to 10 days. Thereafter, there was a marked increase in the level of nicotine accumulating in the culture with 50 g l^{-1} initial sucrose.

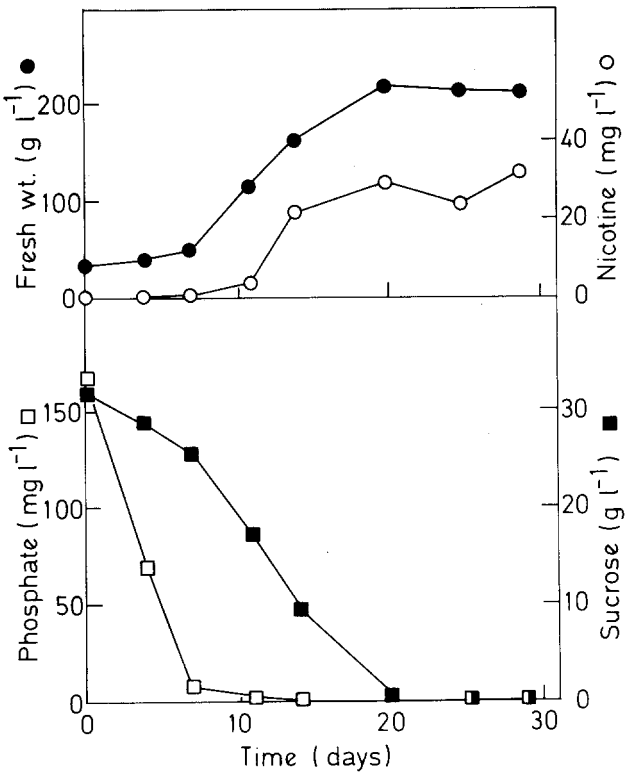

Fig.1. Time course of cell fresh weight (g l^{-1}) and nicotine content (mg l^{-1}) in a 5-litre batch culture of *N. tabacum* cv NC2512 and of the depletion of sucrose and phosphate in the medium.

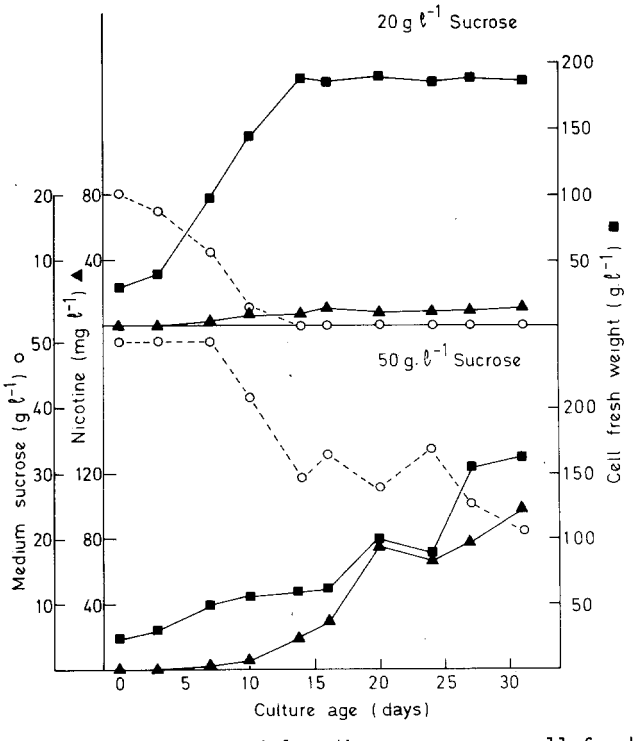

Fig.2. Effect of initial medium sucrose on cell fresh weight and nicotine accumulation in 4-litre batch cultures of *N. tabacum* cv NC2512.

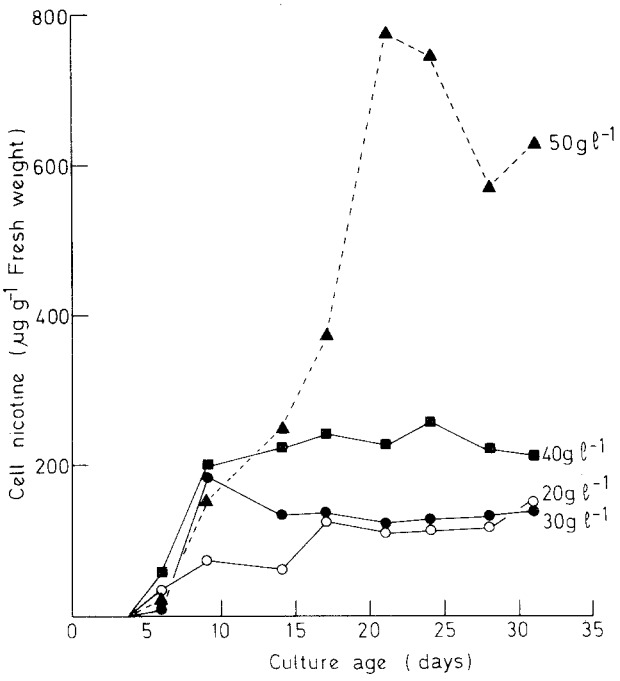

Fig.3. Effect of initial medium sucrose on nicotine accumulation in 40 ml cultures of *N. tabacum* cv NC2512.

To test whether the presence of high initial levels of sucrose stimulated nicotine accumulation in our NC2512 lines, cells were grown in NPM containing a range of initial sucrose (20-50 g l^{-1}) in 40 ml rotary shake cultures. A time course of nicotine accumulation in these cultures (Fig. 3) confirmed this effect. The patterns of accumulation were similar during the initial stages of culture development. However, after 10 days highest levels of accumulation were observed in cultures grown in media containing highest levels of initial sucrose. The maximum peak accumulation in 50 g l^{-1} initial sucrose at 21 days was 780 μg g^{-1} fresh weight which represented an equivalent dry weight accumulation of 1.3% (based on a mean dry to fresh cell weight ratio of 0.06 at 21 days).

Since the depletion of phosphate in NPM appeared to be closely correlated with onset of nicotine accumulation in NC2512 cultures (Fig. 1), the following experiment was carried out to assess the effect of depleted levels of initial phosphate on alkaloid accumulation. Three 4-litre batch cultures were inoculated with a single stock of NC2512 cells grown for 21 days in a 5-litre fermentor. The initial sucrose level in all three cultures was 30 g l^{-1}. Time course of cell fresh weight, medium phosphate and nicotine accumulation in cells and media (Fig. 4) showed that phosphate depletion had marked effects on the timing of nicotine accumulation. Phosphate was rapidly removed from NPM containing 15 mg l^{-1} (by 5 days) whereas at higher initial levels (75 and 150 mg^{-1}), complete phosphate removal did not occur until 11-15 days. At normal initial phosphate levels (150 mg l^{-1}), cell nicotine showed a steady increase over culture time whereas at the lower initial phosphate levels tested, there was a peak of accumulation at between 12 and 14 days. These patterns of accumulation were also reflected in total nicotine levels. Reduced initial phosphate therefore appeared to accelerate the accumulation of nicotine and it also induced contrasting patterns of alkaloid accumulation in the tobacco cells (maximum levels of accumulation were equivalent to 0.3% cell dry

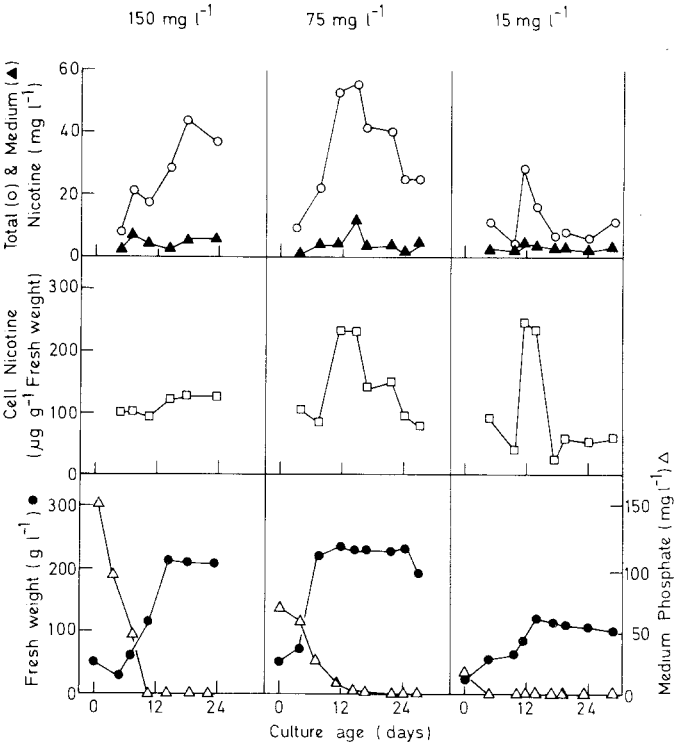

Fig.4. Effect of initial medium phosphate (150, 75 and 15 mg l^{-1}) on cell fresh weight and nicotine accumulation in 4-litre batch cultures of *N. tabacum* cv NC2512.

weight). The combined effect of raising sucrose and reducing phosphate in single cultures was not assessed. Cells were grown on NPM supplemented with 15 mg l^{-1} initial phosphate and two contrasting levels of sucrose (30 and 50 g l^{-1}). Results (Fig. 5) showed that nicotine accumulation peaked in both cultures at 10-12 days and that raised initial sucrose induced at least a five-fold increase in nicotine accumulation (from c. 0.4 to 2.2% cell dry weight). Data indicated, therefore, the contrasting and apparently independent roles of intial sucrose and phosphate on growth and nicotine accumulation in NC2512 cell cultures.

Discussion

Efforts to raise the levels of secondary metabolites produced by batch cultures of plant cells have tended to concentrate on the manipulation of growth regulators in media. The approach adopted has generally been one of reducing auxin and cytokinin to levels which allow expression of secondary metabolism but which also support reasonable levels of biomass for the production of acceptable yields of metabolites. A two-stage strategy has proved satisfactory for doing this on the laboratory scale (Alfermann and Reinhard, 1978). In the tobacco system described, stock cultures (first stage) were propagated in a rapid growth medium (SCM) until stationary phase was reached (21 days) when cells were transferred to a second stage production medium (NPM). This contained 1/10th level of growth regulators and as a result nicotine accumulation was stimulated. The net accumulation of nicotine during both the log and stationary phases of cell growth in NPM (Fig. 1) is probably due to the heterogenous nature of batch cultures brought about by asynchronous division in their cell populations (King, 1980). In NC2512 batch cultures, nicotine appears to be synthesized in cells which are not undergoing cytokinesis.

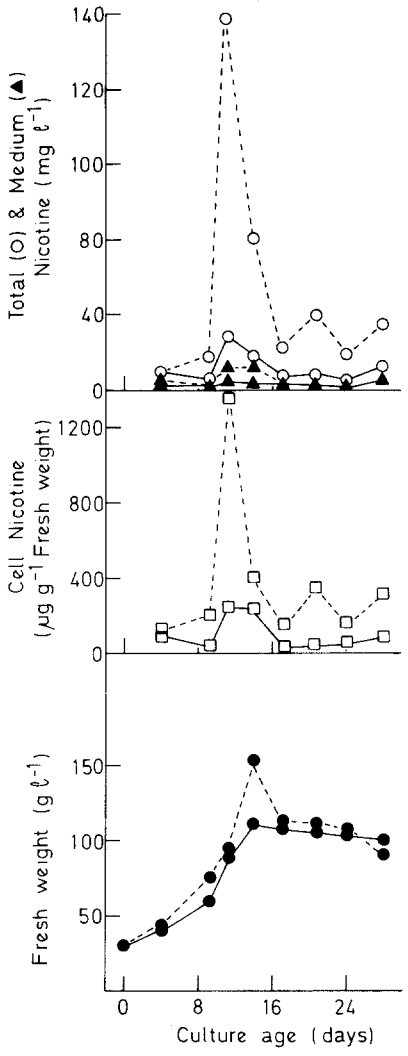

Fig.5. Cell fresh weight and nicotine accumulation in low initial medium phosphate (15 mg l^{-1}) cultures of *N. tabacum* cv NC2512 containing initial medium sucrose levels of 30 g l^{-1} (solid lines) and 50 g l^{-1} (broken lines).

Cells of this type are characteristically elongated and highly vacuolated and have been found to increase significantly in number as the level of nicotine accumulating in cultures increases (Mantell and El-Tigani, unpublished data).

Apart from studies by Nettleship and Slaytor (1974) on the effect of reduced medium phosphate levels on alkaloid production by *Peganum* callus cultures, very little attention has been paid to the effect of phosphate on secondary metabolite biosynthesis in plant cell cultures. Levels of orthophosphate used in the MS medium are more than adequate for rapid growth of tobacco cells (Murashige and Skoog, 1962) and no advantage is gained in raising phosphate levels in media since these have resulted in no significant increases in fresh weight or nicotine accumulation (Pearson, 1978).

In batch cultures of *Catharanthus roseus* (MacCarthy *et al.*, 1980) and open chemostats of *Acer* (Gould *et al.*, 1981), sucrose and phosphate starvations cause cells to arrest in G_1 and G_2 stages of the cell cycle in an approximate ratio of 4 to 1. Whereas sucrose-starved cells begin to die soon after entry into stationary phase, a phosphate-limited cell population shows high viability late into stationary phase. Observations on the viability of NC2512 cells under phosphate- and sucrose-depleted conditions have substantiated this (Mantell, unpublished data). Data presented in Figs. 1 and 4 indicate that phosphate depletion in NPM coincided with substantial accumulations of nicotine. Sucrose depletion on the other hand (Figs. 1 and 2) had a significant effect on delimiting growth (fresh weight increase). Data shown in Figs 4 and 5, confirmed that raised levels of sucrose amplified nicotine productivity of cultures. Data in Fig. 3 confirms the latter

The beneficial effect of raising initial sucrose levels on metabolite productivity has been a commonly observed phenomenon in plant cell cultures. For example, cells of Paul's Scarlet Rose grown in media containing raised levels of sucrose (4%), accumulated 150% more polyphenols than did cells grown in an identical medium containing 2% sucrose (Davies, 1972). The effect of phosphate depletion on secondary metabolite production in plant cell cultures is not so widely reported. Some secondary metabolites do not accumulate in cultures containing low phosphate. This is true of anthraquinone production in cultures of *Morinda citrifolia*, which is enhanced in media containing high levels of phosphate (Zenk *et al.*, 1975), and in those of *Gallium mollugo* where phosphate-limiting chemostat cultures do not produce these metabolites (Wilson, 1978). Nevertheless, medium phosphate depletion appears to be particularly effective in triggering the production of other secondary metabolites in plant cell cultures. For instance, recent data from Berlin's group (Knobloch and Berlin, 1981) have shown that phosphate depletion of culture media leads to marked increases in phenylalanine ammonia lyase activity followed by increases in the biosynthesis of cinnamoyl putrescines.

One major effect of reducing initial phosphate levels in NPM was the rapidity with which accumulated alkaloid was apparently degraded (see fig. 4). It is likely that once phosphate became limiting in media, the nicotine which accumulated was rapidly degraded possibly by substances released into the medium as a result of the death of cells (caused by the onset of sucrose delimitation) because the rapid degradation of alkaloid took place shortly after the time sucrose limitation would be expected to occur in the cultures (i.e. when increases in fresh weight ceased). This might explain the continued accumulation of nicotine in NC2512 cultures grown in media containing raised levels of sucrose and normal levels of phosphate (Fig. 3) since nicotine might be expected to continue accumulating in the living cells sustained under conditions of ample sucrose. Clearly, phosphate limitations influence the degree to which nicotine is biodegraded in the NC2512 culture system and more detailed investigations on this aspect are required. In the meantime, we reiterate the value of phosphate-limited batch cultures as useful experimental systems for the study of biosynthesis and biodegradation of alkaloids.

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Abbreviations: NAA, naphthaleneacetic acid; NPM, nicotine production medium

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