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EFFECT OF NORLEUCINE ON THE METABOLISM OF TOBACCO CELLS IN SUSPENSION CULTURES

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Key Word Index—*Nicotiana tabacum*; Solanaceae; cell suspension culture; free amino acids; phenolics; protein composition; norleucine; ethylene.

Abstract—The phenolic content of tobacco cells in suspension cultures rose during the initial stages of growth and then fell again. Norleucine treatment prevented this initial rise in production of phenolics, but a steady level of the compounds was maintained. No significant changes in the type of phenolics produced was observed. Norleucine had a slight effect on the amino acid composition of proteins probably by changing the relative amounts of soluble proteins. The free amino acid pool was changed drastically and the amount of free glutamine especially was reduced. A slight increase of ethylene production was noted, perhaps due to a sparing action of norleucine on methionine. The results are discussed in terms of the possible use of an amino acid antagonist such as norleucine to change cell metabolism and cell composition without changing cell growth.

INTRODUCTION

Plant tissue cultures are able to adapt to amino acid analogues and as a result resistant cell lines develop. This process involves many generations of cells. The number of reports is extensive [1–4]. As a result of resistance various metabolites may accumulate. For example resistance to fluorophenylalanine leads to the accumulation of amides and amines [5].

We have reported that norleucine can affect catechol oxidase activity in tobacco cells without materially changing their growth [6, 7], in a process not involving selection for a resistant cell line. It seemed that the system using tobacco cell suspension cultures and the methionine antagonist norleucine might be useful to study how an amino acid analogue can change metabolic pathways in conditions under which there is no selection and apparently no adaptation.

Three effects of the amino acid analogue were studied. The first is its effect on the free amino-acid pool of the cells. There have been very few reports on the free amino acid composition of cell tissue cultures [8]. It was therefore of interest to determine whether the free amino acid pool changes due to norleucine treatment. The second point which we wished to investigate was whether the incorporation of norleucine into the cell proteins changes with time, since the amino acid analogue does appear in the soluble protein of tobacco suspension cells [7]. There seem to be no other data on norleucine in the protein of plants although its incorporation into protein of bacterial cells has been reported [9]. Lastly, since norleucine represses catechol oxidase activity in tobacco suspension cultures [6], we wished to determine whether it also affected the phenolics composition of the cells. There is very little data to show whether the level of catechol oxidase activity and the level and variety of phenolics are in any way related. The control of total tissue phenolics content has been studied in continuous cultures [10, 11]. The use of norleucine as a repressor of enzyme activity might provide an answer on the question of regulation of phenolic contents, especially as data on the phenolics composition and content of tobacco cell suspension or callus is scant [12–17]. In this report we present some of the results of the studies carried out on the effect of norleucine on amino acid and phenolics composition and content of tobacco cells.

RESULTS AND DISCUSSION

Phenolics

Cultures grown in the absence of norleucine were subcultured in medium, with or without addition of 5 mM norleucine and allowed to grow for various periods, and then sampled. Cell density sufficient for extraction was reached after about 5 days. Growth rates have been previously described [7]. As can be seen from the results there was no dramatic change in the phenolic content (total and *ortho*-diphenols) due to the norleucine treatment (Fig. 1). The treated cells appeared to contain less phenols than the controls between five and ten days of culture and their level did not change during growth, while in the controls there is a clear initial rise in phenolic content, at the onset of growth followed by a drop to the initial value of the old cultures, before subculturing.

TLC of the phenolics after hydrolysis showed the presence of caffeic, ferulic, chlorogenic, gallic, 3,4-dihydroxy benzoic and *p*-coumaric acids. HPLC confirmed the presence of *p*-coumaric acid and chlorogenic acid and also showed clearly the presence of syringic and gallic acids as well as the presence of catechin. The failure of HPLC to detect ferulic acid and 3,4-dihydroxy benzoic acid is probably due to the presence of a very large peak of an unidentified substance on the elution pattern, which seems to be due to a fluorescent substance also observed but not identified on TLC. The phenolics identified correspond in general to those described previously [13–17] for tobacco,

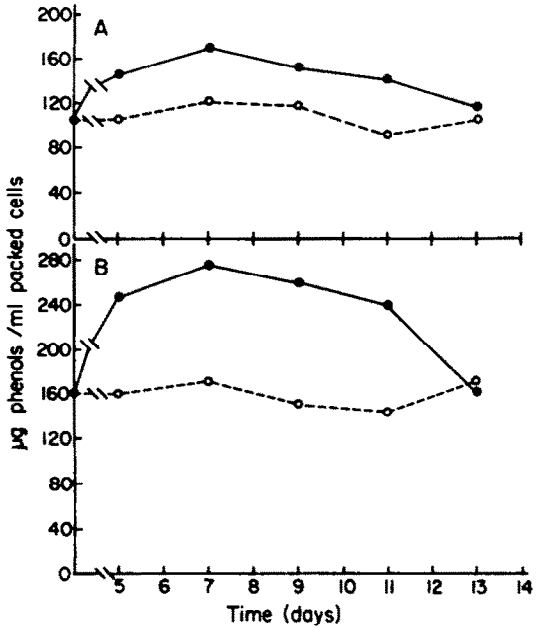

Fig. 1. Changes in phenolics content of control and norleucine treated tobacco suspension cultures during growth. A: *Ortho*-diphenols; B: total phenols. ●—●, controls; ○—○, norleucine.

Table 1. Amino acid composition of soluble protein fraction from control and norleucine treated tobacco cell suspension cultures (results as amino acid relative to leucine)

Amino acid	B_5	N_5	B_{10}	N_{10}
Asp	1.48	1.39	1.39	1.48
Thr	0.67	1.1	0.87	0.80
Ser	1.0	1.0	1.0	0.97
Glu	1.1	1.1	1.18	1.10
Pro	2.1	1.0	1.75	1.37
Gly	1.5	1.5	1.42	1.37
Ala	1.4	1.9	1.39	1.34
Cys	0.2	—	0.27	0.28
Val	0.58	0.87	0.60	0.62
Met	0.29	0.57	0.20	0.31
Ile	0.35	0.8	0.45	0.45
Leu	1.0	1.0	1.0	1.0
Norleu	—	trace	—	0.02
Tyr	0.77	1.38	0.9	0.8
Phe	0.25	0.27	0.39	0.42
His	0.63	0.53	0.54	0.62
Lys	0.32	0.26	0.34	0.37
Arg	0.32	0.22	0.26	0.27

Cells grown for 5 or 10 days in absence (B) or presence (N) of 5 mM norleucine.

although not all the compounds have been previously reported as being present together in a given callus or suspension culture. This is probably caused by differences in culture conditions and lack of sensitivity in detection. There was no basic difference in the compounds detected in norleucine treated and control cells. Our analysis did not permit a quantitative evaluation of the individual phenolic compounds detected.

The results on the phenolics show that in tobacco, as in other tissues, the level tends to rise and then drop during culture as has been described by others [10, 11, 18]. Norleucine treatment apparently prevented the changes in level of phenolics during growth and ageing of the culture. It may therefore be possible to prevent fluctuations in the level of a secondary metabolite by chemical manipulation of the cells.

The results do not point to any major interference of the antimetabolite in the biosynthesis of phenolics but the phenolic content was smaller. There was no indication that the methionine antagonist changed the methylation pattern of the phenolics formed.

Protein amino acids

We have previously shown that treatment of tobacco cell suspensions with norleucine resulted in the appearance of this amino acid in soluble cell protein [7]. At the time only one cell age was studied. In order to investigate this point further we repeated the analysis of control and treated cells at the beginning of growth, after five days, and towards the onset of ageing, after ten days (Table 1).

In general norleucine did not cause any major changes in the amino acid composition of the soluble protein. Initially norleucine appeared to cause a drop in the proline content of protein which rose again during cell ageing, while the reverse was the case for the controls. Changes were also detected in methionine content, which surprisingly increased in the protein of treated cells despite the fact that norleucine also appeared in the protein. Minor changes also occurred in a number of other amino acids. It seems likely that the changes observed are due to alterations in the amino acid composition of some proteins due to treatment, while others probably were not affected at all. It should be recalled that norleucine incorporation into purified catechol oxidase was five times as great as into soluble protein [7]. It is also possible, although we have no evidence that treatment changes the relative amounts of certain proteins, differing in amino acid composition, so that the composition of the soluble fraction changes.

The results support the idea that it is possible to manipulate cell protein using an amino acid analogue. Further studies on well defined protein fractions are needed to ascertain which of the two alternative explanations is correct or whether both mechanisms are operating.

Free amino acids

Cells were grown in the absence or presence of norleucine and then sampled and free amino acid content determined (Table 2). A very striking effect of norleucine on the free amino acid pool of the cells can be seen. Amino acids related to glutamic acid (glutamic acid, glutamine, proline, γ-amino butyric acid and ornithine) account for the greater part of the pool, about 80%. In this group a big change was observed. Glutamine content dropped due to norleucine treatment, indicating perhaps less protein breakdown. Minor changes were also observed in other components of this group. The other major change was in the tyrosine + norleucine content. In this group norleucine + tyrosine rose very sharply due to treatment,

Table 2. Composition of free amino acid pool of tobacco cell suspension culture grown in absence or presence of norleucine (results given as amino acid % of total at three cell ages)

	B_5	N_5	B_7	N_7	B_{10}	N_{10}
Asp	0.70	0.74	1.47	0.56	0.84	1.34
Thr	0.61	0.74	1.13	0.73	0.59	0.94
Ser	0.94	3.54	1.78	4.3	1.05	4.60
As	1.25	0.68	1.94	1.04	6.28	1.56
Gl	61.45	33.7	48.6	26.3	67.1	40.0
Glu	3.9	4.57	5.79	2.78	3.6	5.1
Pro	5.4	3.42	1.76	5.35	5.62	1.63
Gly	2.27	2.91	2.26	2.95	1.31	2.24
Ala	4.95	11.2	5.64	5.35	4.44	5.1
Val	0.5	1.14	0.58	0.62	0.28	1.0
Met	—	0.2	0.21	0.12	0.059	0.06
Ile	0.16	0.52	0.58	0.62	0.22	0.35
Leu	0.1	1.52	0.42	0.32	0.28	0.96
Tyr + Norleu	0.325	15.6	6.12	35.4	0.73	16.0
Phe	0.095	0.75	0.48	0.39	0.31	0.17
GABA	14.1	14.6	15.9	10.0	5.39	14.9
Orn	0.19	0.1	—	—	—	0.53
Lys	0.34	0.65	0.43	0.51	0.21	0.28
His	0.445	0.55	0.55	0.41	0.30	0.62
Arg	0.115	0.38	0.32	0.35	0.10	0.34
Cys	0.90	0.4	0.29	0.36	0.26	—
Ethanolamine	0.61	0.74	1.17	0.28	0.30	0.96
β-Ala	0.695	1.22	2.28	1.14	0.59	1.84
Total	100	99.9	99.7	99.9	99.8	100

Cells grown for 5, 7 or 10 days in absence (B) or presence (N) of 5 mM norleucine.

presumably because the treated cells took up a considerable amount of norleucine from the medium. After 10 days norleucine + tyrosine fell again apparently due to depletion of norleucine in the medium. After 10 days growth the cultures were extremely dense (3.1 ml packed cells/10 ml medium). The columns used for analysis did not separate norleucine adequately from tyrosine, due to the fact that they ran very closely together. This makes it difficult to be certain that the tyrosine content did not also change, although this is not very likely.

There are no comparable data on the free amino acid pool of tobacco cell suspension cultures, although there are some data on callus [8]. The effect, particularly on the glutamine content of the cells, is extremely striking. How exactly this effect is brought about is still unclear.

Clearly the effect of norleucine on free amino acid composition of the cells deserves much more detailed study. The results so far obtained are consistent with the idea that the composition of cell suspension cultures can be changed by chemical treatment of the cell, which did not change their growth rate (as previously shown [7]).

Ethylene

Since norleucine is a methionine antagonist and the latter is the immediate precursor of ethylene, ethylene production by the cells was examined. The cells were grown in Gamborg's medium as in the previous experiments but in this case in tightly stoppered flasks with a side arm closed with latex rubber, to allow sampling. After suitable periods gas samples were withdrawn and ethylene determined using gas chromatography. No major change in the ethylene content was observed, but the rate of ethylene production did rise slightly due to treatment, from 14.7 nl/100 mg dry wt/hr to 18.8 nl/100 mg dry wt/hr after 5 days. After 10 days of culture the ethylene content of the culture flasks did not show any further rise, indicating that ethylene production had virtually ceased. The changes are not very large and the absolute amounts are of the same order of magnitude as those reported for example by others [19, 20], for apple and pear cell suspension cultures respectively. Lieberman *et al.* [19] also showed that exogenous methionine was converted to ethylene, as had previously been shown for apple slices [21]. The slightly elevated ethylene production, observed by us could be due to a greater availability of methionine which results from the norleucine treatment. Norleucine might be considered as having a sparing action on methionine.

EXPERIMENTAL

Suspension cultures. The cultures of *Nicotiana tabacum* L. cv Xanthi were grown as previously described [6, 7] on the medium of Gamborg *et al.* [22], in 250 ml Erlenmeyer flasks at 26° on a rotary shaker, operated at 120 cycles/min. Packed cell volume was determined as described in [6]. Norleucine was added to the

cultures after subculturing at a concentration of 5 mM. During subculturing, a culture grown in the absence of norleucine was divided, and inoculated into fresh medium, with or without norleucine (5 mM). Standard methods of sterilization were used and all transfers carried out in a laminar hood.

Extraction of phenols. Cells were collected by centrifugation at 1500 *g* for 10 min. Usually 5 ml packed cells (equivalent to 116 mg dry wt) were extracted with 20 ml 80% EtOH.

Total and ortho *diphenols analysis.* Total phenols were determined according to [23] with the Folin Denis reagent.

Ortho diphenols were measured according to Mapson [24].

TLC. Analysis of phenolic compounds in tobacco cultures by TLC chromatography was performed according to Jende-Strind [25], after hydrolysis in 2 M HCl at 100° for 20 min. TLC was on Merck precoated cellulose F254 plates (0.1 mm), using 15% acetic acid as solvent. The phenolic compounds were visualized under UV light before and after treatment with NH_3 fumes, or by spraying with 0.3% ethanolic $FeCl_3$.

HPLC of phenolic acids was carried out on acid hydrolysed samples on a Lichrosorb RP 18 column which was eluted with two solvents: solvent A (MeOH–HOAc–H_2O, 4:1:15) and solvent B (MeOH–HOAc–H_2O, 4:1:11). Solvent A was used isocratically for 10 min followed by a linear gradient of B for a further 10 min, followed by solvent B isocratically for a further 20 min. Fifty μl samples of extract were injected into the column. Elution was at 30 or 40°.

Free amino acids. Usually 5 ml of tobacco packed cells were extracted with 20 ml of 80% EtOH for 20 min. The sample was centrifuged for 15 min at 20000 *g*. The supernatant was retained and the tissue re-extracted with 40% EtOH (× 3) and once with H_2O. All the extracts were combined and reduced to dryness in a dessicator. Free amino acids were determined on a Biotronik automatic amino acid analyser using a physiological fluids standard programme and lithium buffer.

Soluble protein bound amino acids. The protein in the supernatant fraction of the cell homogenate after centrifugation at 20000 *g* for 20 min was precipitated by adding 10% TCA, the suspension was centrifuged for 15 min at 20000 *g*, and the pellet was resuspended in 10% TCA and heated for 20 min at 90° and then centrifuged.

The protein in the pellet was hydrolysed at 110° overnight with 6 M HCl. The amino acid composition was determined with a L. K. B. Biochem model 4400 amino acid analyser. Protein was determined according to Lowry *et al.* [26].

Ethylene was determined in samples of gas withdrawn from the flasks, by GC. The samples were injected directly into the columns.

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