Regulation of nicotine biogenesis 2: Increased production of nicotine by urea in tobacco tissue cultures

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Summary. The floral bud callus of *Nicotiana tabacum* L. was successfully grown on 10 mM urea as sole nitrogen source. Nicotine content in the urea-treated tissues was 3.5 times higher than in the control on the basis of dry weight, and 2.5-fold higher per culture. Contents of ornithine, citrulline and arginine, urea cycle intermediates, and known precursors of nicotine, were higher in urea-treated callus. The implications of the results are discussed.

Cell and tissue cultures of various varieties of *Nicotiana tabacum* have exhibited their potential for producing alkaloids[2-7]. Earlier studies[2] have demonstrated that tobacco tissues lose their biogenetic potentiality after sequential subcultures. However, a few others have reported stabilized nicotine production[8, 9]. Precursor feeding experiments have shown that L-glutamic acid, L-ornithine and L-putrescine are intermediates of nicotine biogenesis.

In the present study, we examined the effect of urea on growth and nicotine production in callus cultures of *Nicotiana tabacum* L. var. Anand-2. Earlier work in our laboratory[10] revealed that urea-fed callus tissues of *Datura metel* L. yield more tropane alkaloids than nitrate-fed ones. Since the initial steps in biogenesis of tropane and nicotine alkaloids are the same it was of interest to examine how tobacco callus would behave.

Material and methods. Initiation and establishment of callus cultures. Young floral buds of *Nicotiana tabacum* var. Anand-2 obtained from Beedi Tobacco Research Station of the Gujarat Agricultural University, Anand, were thoroughly washed with water and treated with 70% EtOH for 1-2 min and then with 0.1% (w/v) mercuric chloride for 5 min. Subsequently the explants were copiously washed with sterile distilled water and inoculated on Murashige and Skoog's (MS)[11] medium containing 2% sucrose and fortified with 2.0 mg/l each of indole-3-acetic acid (IAA), naphthalene acetic acid (NAA) and kinetin (K). Callus formation was noticed on day 15 of culture and almost completely covered the explant by day 30. Such callus masses have been maintained on MS medium with 2% sucrose, and reduced K level (0.4 mg/l) by regular transfers every 30 days.

Analysis of alkaloid. Quantitative determination of nicotine was carried out by steam distillation method[12]. A powdered dry sample of callus weighing 100 mg was mixed with 100 mg of MgO and the mixture was steam distilled. The distillate (90 ml) was collected in a volumetric cylinder containing 10 ml of 0.5 N HCl. The chromatographic analyses of distillates, which were found to contain nicotine as the only alkaloid, were carried out as described by Tabata and Hiraoka[8]. The UV-absorbance of distilled nicotine was measured at 236, 259 and 282 nm. The recovery of nicotine by the above method ranged between 95 and 100%. An average value of nicotine from 5 replicates was taken as the absolute amount of nicotine in a particular sample.

Amino acid analysis. Dry callus tissue weighing 100 mg was extracted in 80% (v/v) EtOH and a suitable aliquot was spotted on Whatman No. 1 chromatographic paper. The chromatograms were run in 2 solvent systems[13]: chloroform/methanol/17% ammonia (2:2:1, v/v/v); n-butanol/glacial acetic acid/water (4:1:1, v/v/v).

Table 1. Effect of urea on growth and nicotine biogenesis in *N. tabacum* callus cultures[a]

Treatment[b]	Fresh weight (mg/culture)	Dry weight (mg/culture)	Percentage of nicotine	Nicotine (µg/culture)
Standard nitrogen supply (control)	6526.67 (±496.8)	225.50 (±16.2)	0.220 (±0.006)	497.00 (±35.7)
10 mM urea	2073.90 (±181.1)	162.36 (±10.2)	0.783 (±0.018)	1271.28 (±79.8)
25 mM urea	1518.00 (±100.7)	85.88 (±5.7)	0.754 (±0.022)	647.53 (±42.9)
50 mM urea	398.36 (±29.5)	17.15 (±2.1)	0.725 (±0.023)	124.34 (±14.92)
100 mM urea	402.70 (±26.8)	19.78 (±3.3)	0.551 (±0.012)	108.98 (±18.09)
150 mM urea	381.00 (±30.0)	19.71 (±2.3)	0.290 (±0.012)	57.16 (±6.66)
200 mM urea	316.90 (±25.5)	18.14 (±2.4)	0.104 (±0.003)	18.94 (±2.50)
250 mM urea	376.05 (±29.7)	23.04 (±2.8)	0.087 (±0.004)	20.04 (±2.43)

[a] Data represents an average of 5 replicates. [b] All media contained 2 mg/l IAA, 2 mg/l NAA, 0.4 mg/l K and 2% sucrose. All values are (± SE).

Table 2. Amino acid content in urea treated callus tissues of *N. tabacum*[a]

Treatment[b]	Amino acid µg/100 mg dry weight of callus					
	L-Glutamic acid	L-Phenylalanine	L-Tyrosine	L-Ornithine	L-Citrulline	L-Arginine
Standard nitrogen supply	55.00	162.50	51.27	77.50	92.50	67.50
10 mM urea	50.00	75.00	37.50	145.50	102.25	82.00
25 mM urea	55.50	37.50	25.00	132.50	98.00	86.35
50 mM urea	35.00	–	16.00	92.21	30.73	78.20
100 mM urea	08.00	–	–	89.20	40.60	83.00
150 mM urea	12.60	12.50	15.55	75.25	38.00	42.00
200 mM urea	14.00	–	–	16.00	42.63	15.00
300 mM urea	15.00	–	–	05.00	61.25	12.50

[a] Data represent an average of 5 replicates. [b] All media contained 2 mg/l IAA, 2 mg/l NAA, 0.4 mg/l K and 2% sucrose.

The chromatograms were sprayed with 0.2% ninhydrin reagent and subjected to 80 °C for 15 min. The spots of amino acids were marked and were identified by comparing R_f with that of authentic samples. Later they were eluted in 5 ml of 75% EtOH containing 0.2 mg of $CuSO_4$ 5 H_2O. An Uncoloured area was utilized for the blank. Absorbance was measured at 540 nm using a colorimeter. Quantitative measurement was done by comparing the absorption of an identified spot with the standard curve obtained for the corresponding authentic amino acid treated as above.

Results. Studies on growth of callus under the influence of urea: Agar media containing various concentrations of urea (filter sterilized) ranging from 10 mM to 300 mM were prepared. Nitrates were completely omitted. However, controls received a standard nitrogen supply. Callus pieces weighing 300±30 mg were inoculated in 150 ml Erlenmeyer flasks containing 40 ml of the medium and cultures were incubated at 25±2 °C in continuous light (3000 lx). At the end of 30 days growth of the tissue was measured in terms of fresh and dry weight.

Effect of urea on growth and nicotine biogenesis: It was interesting to find that the callus tissues of *N. tabacum* could be successfully grown on 10 mM urea as sole nitrogen source, but at a slower rate than on MS medium with the standard nitrogen supply of 18.8 mM KNO_3 and 20.6 mM NH_4NO_3. Such a phenomenon has been demonstrated earlier in cell cultures of soybean[14] and tobacco[15]. Of the various levels of urea administered, 10 mM supported maximum growth of the callus and highest yield of nicotine (table 1). Increasing levels of urea reduced growth and nicotine production. The nicotine content of urea-treated tissues was found to be 3.5- and 2.5-fold higher than the control on percent dry weight and per culture basis respectively. Similar increase in nicotine content by foliar application of urea to tobacco plants has been reported[16] from the Beedi Tobacco Research Centre at Anand in Gujarat, India.

Discussion. Possible role of urea in enhancement of nicotine biogenesis: Ornithine is an established precursor of nicotine biogenesis[17] and a key compound in the ornithine-urea cycle. It seemed that feeding of urea increased the endogenous urea level of the tissues, thereby suppressing the activities of enzymes of the urea cycle. Such a situation would result in an increased ornithine level, since its turnover via the urea cycle is restricted. This hypothesis is substantiated by the fact that urea-treated tissues accumulated higher levels of ornithine, citrulline and arginine than the control (table 2). Thus urea seemed to increase nicotine production by suppressing the entry of ornithine into the urea cycle, thereby increasing the availability of ornithine for nicotine biogenesis. The enzymatic evidence for the above statements has been obtained and will be reported elsewhere.

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Apparent inhibition of erythrocyte glutamic oxaloacetic transaminase in subjects on large dosages of B-6[1]

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Summary. An apparent inhibition of the erythrocyte glutamic oxaloacetic transaminase E-GOT system of Kishi and Folkers has been observed with erythrocytes from individuals on large amounts of B-6. Evidence is presented that this inhibition is an artefact due to excess pyridoxal phosphate reacting with 2,4,dinitrophenylhydrazine. Excess pyridoxal phosphate competes with pyruvic acid for the available 2,4,dinitrophenylhydrazine which results in a product that is not extractable into toluene, therefore, less chromophore is present as more pyridoxal phosphate is available.

The erythrocyte glutamic oxaloacetic transaminase system (E-GOT) procedure of Kishi and Folkers[2] was used to evaluate the B-6 status of a psychiatric out-patient population who were taking various levels of B-6. In those subjects taking B-6 in amounts greater than 10 mg per diem Sohler and Pfeiffer[3] noted an apparent inhibition of the overall in vitro reaction instead of the maximum reaction rate expected when there is complete saturation of the apoenzyme with cofactor.

Kishi and Folkers have also observed this inhibition with erythrocytes from subjects who were receiving i.v. hyperalimentation with preparations containing about 100 mg B-6 per day and Folkers has reported instances of this inhibition in subjects taking 50–100 mg of oral B-6 daily. We