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Correlation between Polyamines and Pyrrolidine Alkaloids in Developing Tobacco Callus[1]

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ABSTRACT

Since the diamine putrescine can be metabolized into the pyrrolidine ring of tobacco alkaloids as well as into the higher polyamines, we have investigated the quantitative relationship between putrescine and these metabolites in tobacco callus cultured *in vitro*. We measured levels of free and conjugated putrescine and spermidine, and pyrrolidine alkaloids, as well as activities of the putrescine-biosynthetic enzymes arginine and ornithine decarboxylase. In callus grown on high (11.5 micromolar) α-naphthalene acetic acid, suboptimal for alkaloid biosynthesis, putrescine and spermidine conjugates were the main putrescine derivatives, while in callus grown on low (1.5 micromolar) α-naphthalene acetic acid, optimal for alkaloid formation, nornicotine and nicotine were the main putrescine derivatives. During callus development, a significant negative correlation was found between levels of perchloric acid-soluble putrescine conjugates and pyrrolidine alkaloids. The results suggest that bound putrescine can act as a pool for pyrrolidine alkaloid formation in systems where alkaloid biosynthesis is active. In addition, changes in arginine decarboxylase activity corresponding to increased alkaloid levels suggest a role for this enzyme in the overall biosynthesis of pyrrolidine alkaloids.

Polyamines and their biosynthetic enzymes ADC[3] (EC 4.1.1.19) and ODC (EC 4.1.1.17) may play an important role in many aspects of plant development including growth, differentiation, senescence, and response to stress (7). However, relatively little is known about their role in secondary plant metabolism. Putrescine is a precursor not only of PAs (7), but also of parts of important alkaloids such as the pyrrolidine rings of tobacco alkaloids (nicotine and nornicotine), tropane alkaloids (hyoscyamine, hyoscine, and meteloidine), pyrrolizidine alkaloids (retronecine), and possibly phenanthroindolizidines (tylophorine) (10). The putrescine-derived PAs (spermidine, homospermidine, and spermine) may be conjugated to cinnamic acids or to fatty acids, giving rise to other more complex alkaloids (18).

PAs are present in the plant cell in both free and conjugated forms (19, 20). In tobacco, where PAs are often conjugated with cinnamic acids and their derivatives (3, 14), their formation has been correlated with the onset of reproduction (4) and virus resistance (12), but not with alkaloid biosynthesis.

Several publications support the view that auxin levels are involved in the regulation of nicotine production by tobacco callus cultures (22) and root cultures (21) as well as in the intact tobacco plant (25). Recently, we found (23) that the alkaloid level increased dramatically when tobacco callus grown on 11.5 μM NAA (suboptimal for alkaloid biosynthesis) was transferred to a medium containing 1.5 μM NAA (optimal for alkaloid biosynthesis). In the present study, we have used this experimental approach to investigate the relation between PAs (free and conjugated) and pyrrolidine alkaloids in tissues where alkaloid biosynthesis is active. The activities of ODC and ADC in relation to pyrrolidine alkaloid formation were also investigated.

MATERIALS AND METHODS

Plant Material and Callus Cultures. *Nicotiana tabacum* L. cv Wisconsin-38 plants were grown in plastic pots containing vermiculite, subirrigated twice daily with 1.2 g/L solution of Hyponex (Hydroponics Chemical Co., Copley, OH) and maintained in a controlled growth room under a 16-h light/8-h dark photoperiod (9:1 energy mixture of fluorescent and incandescent light at 1.96 $w \cdot m^{-2}$) at 24°C.

Explants were obtained from petioles of plants grown to the 17 to 20 leaf stage, surface sterilized in 70% (v/v) ethanol for 30 s and 0.5% (v/v) NaOCl for 10 min, then rinsed several times in sterile distilled H_2O. Petiolar explants of 4 to 5 mm length were cultured on modified MS (16) medium containing 11.5 μM NAA, 1 μM kinetin, and Kao-Michayluk organic acids (8), and 1% purified BBL agar (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) in sterilized Petri dishes (100 × 20 mm) at 25°C in the dark. The agar was purified as described elsewhere (23). After callus induction on high (11.5 μM) NAA for 4 weeks, the calli were transferred to a medium containing lower (1.5 μM) NAA, and cultured at 25°C in the dark. During the subsequent 6 weeks of callus growth on low NAA, triplicate samples were taken for measurement of growth parameters as well as PA and alkaloid levels. These were compared with callus grown on higher NAA for 4 weeks (referred to as '0 week' as this is the time of their transfer to low NAA media). ADC and ODC activities were determined at weeks 0, 3, and 6 of callus growth and compared with the corresponding pyrrolidine alkaloid titers.

Determination of Dry Weight. The dry weight was estimated by heating the callus at 60°C for 24 to 48 h to constant weight.

Polyamine Analysis. Samples were extracted by homogenizing in 5% (v/v) cold PCA at 600 mg fresh weight/ml PCA. The homogenates were kept at 2°C for 2 h, then centrifuged at 27,000*g* for 20 min. The supernatant was set aside and the pellet was resuspended in the original volume with 1 N NaOH by vortexing. Aliquots (200 μl) each of the pellet suspension and the original supernatant were mixed 1:1 (v/v) with 12 N HCl and hydrolyzed

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[3] Abbreviations: ADC, arginine decarboxylase; PA, polyamine; Put, putrescine; Spd, spermidine; Spm, spermine; Cad, cadaverine; ODC, ornithine decarboxylase; NAA, α-naphthalene acetic acid; PCA, perchloric acid.

for 16 to 18 h at 110°C in flame-sealed ampoules. The hydrolysates were filtered through glass wool to eliminate carbonized material, dried at 70°C under a stream of air, and then resuspended in 200 μl PCA. The nonhydrolyzed PCA supernatant containing the free PAs (S), the hydrolyzed PCA supernatant (SH), and the hydrolyzed pellet (PH) containing PAs liberated from conjugates were dansylated and chromatographed as previously described (5). Dansyl-polyamines were separated on high-resolution silica gel TLC plates (Whatman LK6D) and developed with either of two solvents, *i.e.* chloroform:triethylamine (4:1, v/v) or cyclohexane:ethylacetate (5:4, v/v). The PA standards Put, Cad, Spd, and Spm were run each time PA levels were analyzed. After development of the plates, bands were scraped into 2 ml of ethyl acetate and the fluorescence at 495 nm determined in an Aminco spectrophotofluorimeter with an activating wavelength of 350 nm.

Protein Analysis. Protein was determined according to the method of Bradford (2) in the insoluble PCA-pellet resuspended in 1 N NaOH. Bovine γ-globulin (Sigma) was used as a standard.

Alkaloid Analysis. Determination of alkaloids was performed according to the method of Saunders and Blume (17). Samples dried to constant weight at 60°C for 24 h were extracted in 20 volumes (w/v) of 25 mM sodium phosphate buffer (pH 7.8) at 30°C for 24 h with constant agitation. The aqueous extract was filtered under reduced pressure through a Whatman No. 2 filter paper. Each extract was filtered through a 0.45-μm Millipore filter, diluted 10-fold with water and injected into a HPLC system as a 20-μl aliquot. HPLC analysis was carried out with a programmable Altex-Beckman model 322 liquid chromatograph. Alkaloids were separated at room temperature on a 4.6 × 250 mm 5-μm particle size reverse-phase (C_{18}) column (Altex-octadecylsilane) and eluted with an isocratic mobile phase of 40% (v/v) methanol containing 0.2% (v/v) phosphoric acid buffered to pH 7.5 with triethylamine, at a flow rate of 1 ml/min and detected at 254 nm. Alkaloids were quantified with a 3370 A Hewlett-Packard integrator using nicotine (K & K Laboratories, Inc., Plainview, NY) and nornicotine (Roth Atomergic Chemetals Corp., Plainview, NY) as standards. The chromatographic solvents used were HPLC grade from J.T. Baker Chemical Co. (Phillipsburg, NJ).

Determination of ADC and ODC Activities. Samples were ground in chilled mortars at a ratio of 600 mg fresh weight/ml of 100 mM K-phosphate (pH 7.5) containing 10 mM DTT, 20 mM sodium ascorbate, 5 mM EDTA, and 1 mM pyridoxal phosphate. Insoluble polyvinylpyrrolidone ('Polyclar AT'; GAF Corp., New York, NY), purified according to the method of Loomis (11), and pre-equilibrated in the appropriate buffer, was added to the crude extract at 0.5 g wet weight/g tissue to absorb phenolics. The extract was sonicated (Branson S-75 sonicator; Branson Ultrasonic Corp., Stamford, CT) twice for 1 min in an ice bath and then pelleted for 20 min at 27,000*g*. The supernatant fraction was made 60% saturated with $(NH_4)_2SO_4$, allowed to stand for 30 min, then centrifuged for 20 min at 27,000*g*. The resulting pellet was resuspended in half the original volume of 100 mM K-phosphate (pH 8.0) containing 1 mM DTT, 0.1 mM EDTA, and 0.05 mM pyridoxal phosphate, and dialyzed against the same buffer for 24 h in the dark. All the procedures were carried out between 0 and 4°C.

The activities of ODC and ADC were determined according to methods already published (6, 29). The reaction mixture consisted of 160 μl of extract, 20 μl of dialysis buffer (pH 8.0), and 20 μl of the radiolabeled compound. For assay of ODC activity, the labeled compound consisted of 20 μCi/ml DL-[1-^{14}C]ornithine (58 mCi/mmol; New England Nuclear) diluted with unlabeled ornithine to give a final concentration of 50 mM. For assay of ADC activity, the labeled compound was 20 μCi/ml L-[U-^{14}C]arginine (276 mCi/mmol; ICN) diluted with unlabeled arginine to give a final concentration of 10 mM. Reaction mixtures were incubated for 45 min with gentle shaking at 37°C, at which time the reaction was stopped by adding 0.2 ml of 10% (v/v) PCA. Trapping of the labeled CO_2 onto the KOH-impregnated collection disc continued for 45 min at 37°C. The discs were then removed and immersed in 2 ml of Biofluor (NEN). The radioactivity liberated was determined by counting for 10 min in a Beckman LS 7000 scintillation counter. Enzyme activity is expressed as μmol $^{14}CO_2$ released/h·g dry weight.

RESULTS

Callus Growth. Calli grown on 11.5 μM NAA (suboptimal for alkaloid biosynthesis) appeared undifferentiated, and reached, after 4 weeks, a mean fresh weight of 1520 mg (estimated from six calli). In contrast, calli grown under optimal conditions for

Table I. *Parameters of Growth during Callus Development on 1.5 μM NAA*

Numbers represent means ± SE of at least three replicates.

Week	Fresh Wt	Dry Wt	Protein
	mg	*mg/g fresh wt*	*mg/g fresh wt*
0[a]	113 ± 6.6	36.3 ± 1.7	4.1 ± 0.1
1	210 ± 10.0	38.1 ± 4.7	5.7 ± 0.05
2	384 ± 19.0	44.3 ± 6.0	3.3 ± 0.3
3	596 ± 32.0	43.6 ± 1.7	3.7 ± 0.5
4	1007 ± 57.0[b]	55.6 ± 2.9[c]	5.8 ± 0.9
6	1860 ± 87.0	82.2 ± 2.7[d]	6.6 ± 0.1

[a] Initial piece of callus grown on 11.5 μM NAA for 4 weeks and then transferred to medium containing 1.5 μM NAA. [b] The comparable fresh weight on 11.5 μM NAA was 1520 mg. [c] Significantly different from 0 week at the 5% level. [d] Significantly different from 0 week at the 1% level.

Table II. *Polyamine Titers during Callus Development on 1.5 μM NAA*

Week	PCA Fraction	Polyamine Titer	
		Put	Spd
		μmol/g dry wt	
0	S[a]	3.3 ± 0.5 (100)[b]	0.4 ± 0.04 (100)
	SH	30.7 ± 1.5 (100)	2.6 ± 1.0 (100)
	PH	17.9 ± 1.5 (100)	1.2 ± 0.2 (100)
1	S	2.2 ± 0.2 (66)	0.8 ± 0.04 (217)
	SH	29.2 ± 2.2 (95)	4.4 ± 0.8 (164)
	PH	10.4 ± 1.4 (58)	2.6 ± 0.2 (220)
2	S	2.7 ± 0.2 (85)	1.1 ± 0.1 (275)
	SH	25.6 ± 3.8 (83)	5.1 ± 1.1 (190)
	PH	10.9 ± 1.2 (61)	1.9 ± 0.1 (163)
3	S	0.9 ± 0.2 (30)	0.9 ± 0.2 (254)
	SH	15.9 ± 1.7 (52)	0.5 ± 0.04 (17)
	PH	6.7 ± 0.7 (38)	1.3 ± 0.04 (113)
4	S	3.9 ± 0.9 (121)	1.2 ± 0.04 (30)
	SH	15.3 ± 2.0 (50)	2.5 ± 0.2 (93)
	PH	8.7 ± 1.1 (49)	2.1 ± 0.2 (177)
6	S	0.4 ± 0.1 (11)	0.1 ± 0.04 (30)
	SH	6.4 ± 0.2 (21)	0.5 ± 0.04 (18)
	PH	3.2 ± 0.5 (18)	0.1 ± 0.04 (10)

[a] (S), Free, soluble PAs; (SH), PCA-soluble conjugated PAs released by hydrolysis; (PH), PCA-insoluble conjugated PAs. [b] Numbers in parentheses represent percentage of PA titers relative to '0 week.'

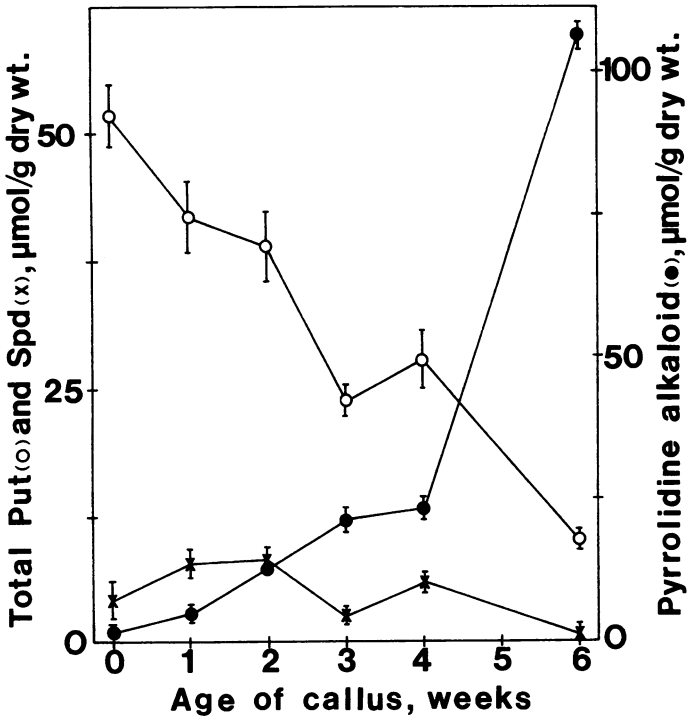

FIG. 1. Total Put and Spd (free and conjugated) and pyrrolidine alkaloids (nornicotine and nicotine) levels during tobacco callus development. Callus developed on 1.5 μM NAA (weeks 1–6) was compared with callus grown on 11.5 μM NAA for 4 weeks (0 week). Bars represent ± SE.

Table III. *Decarboxylase Activity and Pyrrolidine Alkaloids in Tobacco Callus*

Week	ODC Activity	ADC Activity	Nicotine	Nornicotine
	μmol CO_2/h·g dry wt		*μmol/g dry wt*	
0	0.170 ± 0.03	0.750 ± 0.02	0.9 ± 0.3	0.4 ± 0.1
3	0.230 ± 0.06	0.960 ± 0.07	8.0 ± 1.9	13.8 ± 1.2[a]
6	0.160 ± 0.04	1.300 ± 0.08[b]	36.2 ± 2.0[a]	71.8 ± 4.8[a]

[a] Significantly different from 0 week at the 1% level. [b] Significantly different from 0 week at the 5% level.

alkaloid biosynthesis (1.5 μM NAA) showed profuse root initiation reaching, after 4 weeks, a mean fresh weight of 1007 mg (Table I). In addition, a progressive increase of dry weight and protein content (on a fresh weight basis) was observed (Table I).

Polyamine Titers. Put and Spd are the main PAs present in callus grown on suboptimal (0 week) or optimal conditions for alkaloid biosynthesis (1–6 weeks; Table II). Cadaverine was not detected, and Spm was present only in trace amounts. A band with R_F similar to Spm was detected when samples were chromatographed with chloroform:triethylamine (4:1, v/v) as a solvent system. This band was identified as tyramine when TLC plates were developed with cyclohexane:ethyl acetate (5:4, v/v).

PAs were present mainly in the conjugated form; Put conjugates (SH + PH) represented 64 to 86% and Spd conjugates 8 to 20% of total PAs. Total Put is high in callus grown on suboptimal medium (week 0 of Fig. 1), but declines 5-fold during the succeeding 6 weeks on optimal medium. This decrease in Put is due mainly to changes in the SH fraction (Table II). Levels of the different Spd fractions, unlike Put, increase during the first 2 weeks when callus is transferred from suboptimal to optimal conditions for alkaloid biosynthesis (Table II).

Alkaloid Titers. Nicotine, nornicotine, anabasine, and traces of anatabine were detected in the callus (23), but in these investigations only the pyrrolidine alkaloids (nicotine and nornicotine) were considered. Pyrrolidine alkaloid levels were negligible (1.3 μmol/g dry weight) in callus grown on suboptimal medium, but increased progressively until the 4th week and then dramatically in the 6th week on optimal medium for alkaloid biosynthesis (Fig. 1), reaching a final concentration of 108 μmol/g dry weight. The increased alkaloid accumulation rate observed between weeks 4 and 6 may be related to the decreased hydration state of the tissue, since an increase in dry weight of more than 2-fold occurred from week 0 to week 6 (Table I). During callus development, an activation of nicotine demethylation occurs (23); thus, nornicotine is the main alkaloid present (Table III). We have also observed that less than 3% of the total alkaloids in the callus were excreted into the medium.

Correlation between PA and Alkaloid Levels. Since PA titers decreased during callus development while pyrrolidine alkaloid levels increased (Fig. 1), we tried to establish a correlation between levels of the different fractions. From 0 week to the 4th week, a significant negative correlation ($r = -0.98$; $P < 0.01$) was found between titers of PCA-soluble Put conjugate (SH fraction) and pyrrolidine alkaloid. No significant correlation was found between levels of the other Put fractions or total Spd with alkaloid titers.

Activity of Biosynthetic Enzymes. The data in Table III show that both ADC and ODC activities are present in callus grown on media optimal and suboptimal for alkaloid biosynthesis. However, ADC activity was significantly higher than ODC and increased with age of callus, corresponding with increased alkaloid levels. In contrast, a temporary increase of ODC activity was observed at the 3rd week of callus growth, but this had disappeared by the time of maximum alkaloid accumulation (6th week).

DISCUSSION

Tobacco stem apices (3), callus tissues (14), and cell cultures (1, 9) often contain PAs conjugated with cinnamic acids to form hydroxycinnamic acid amides. Some functional roles of these compounds have been suggested. Thus caffeoyl putrescine and other amides have been found in large concentrations in tobacco reproductive organs and it has been proposed that their formation is functionally linked with reproduction (4). In addition, since tobacco plants infected with tobacco mosaic virus react by increasing their levels of PA conjugates, and viral multiplication is retarded in their presence, a role for these compounds in virus resistance has been suggested (12). Mizusaki *et al.* (14) observed several Put conjugates in tobacco callus and suggested that these compounds might be related to nicotine biosynthesis, but made no further investigations.

This report describes a correlation between PA-conjugates and pyrrolidine alkaloid formation in tobacco callus. We have found that in callus grown on high NAA levels, suboptimal for alkaloid biosynthesis, the main Put derivatives are the PA conjugates; this has also been observed in tobacco ovary tissues (Slocum and Galston, personal communication). In contrast, in callus grown on low NAA, optimal for alkaloid biosynthesis, the main Put derivatives are pyrrolidine alkaloids, such as probably occur in the roots, the main site of nicotine formation in the tobacco intact plant. During callus development (from 0 week to the 4th week), we have found that there is a negative correlation between levels of PCA-soluble Put conjugates and pyrrolidine alkaloid.

The above results suggest that NAA regulates not only nicotine biosynthesis but also PA metabolism in tobacco tissue cultures. Furthermore, the present data suggest that bound Put, particularly in the PCA-soluble fraction, can act as a pool for pyrrolidine alkaloid formation in systems where alkaloid biosynthesis is operative. This is further supported by independent tracer studies with [1,4-^{14}C]Put in which has been observed that a considerable

amount of radioactivity is incorporated into several Put conjugates (14) as well as into nicotine (13) in tobacco cultures. In addition, Yang *et al.* (24) have found that the content of Put, Spd, and Spm decreased, while nicotine levels in the roots increased after decapitation of tobacco stems. The decrease in PA titers was due to Put, which decreased by 50 and 80% at 2 and 12 d, respectively, after decapitation. They suggested that the increased Put formed after decapitation was converted into nicotine, but indicated nothing about PA conjugates.

The comparative roles of ADC and ODC in the formation of pyrrolidine alkaloids via putrescine is not clear. In the past, ODC rather than ADC activity has been related to the biosynthesis of nicotine (15) and, in fact, pyrrolidine alkaloids have been classified as ornithine-derived compounds (10). We have found that ADC activity is much higher than ODC in callus grown on all media and that changes in ADC activity, but not ODC, correspond to increased alkaloid levels in the callus. Yoshida (27, 28) demonstrated that Put can be formed from both arginine and ornithine in tobacco, and feeding experiments with labeled arginine indicated that this amino acid can also participate in nicotine formation (26). The hypothesis which maintains that ODC is the main enzyme involved in nicotine biosynthesis is based on experiments in which tobacco plants were decapitated (15, 24). Increase of both ODC activity and nicotine levels were observed in the roots after decapitation of the stems, and this effect was related to the decrease of auxin content in the roots (15). However, in the roots of nondecapitated plants, ADC was 4.5-fold higher than ODC activity (24), and the roots of these plants also produced alkaloids. Thus, the role of ODC activity in nicotine biosynthesis remains questionable, since effects other than decreased auxin levels in the roots can result from decapitation of the plant.

To confirm possible main and alternative pathways for pyrrolidine alkaloid formation in tobacco, we are now using specific inhibitors of ADC and ODC activity. Preliminary experiments show that addition to the cultures of 1 mM D-arginine (competitive inhibitor) and 1 mM DL-α-difluoromethylarginine, an irreversible inhibitor of ADC, strongly inhibited pyrrolidine alkaloid levels. In contrast, 1 mM DL-α-difluoromethylornithine, an irreversible inhibitor of ODC, was less effective. This suggests that ADC is the main enzyme involved in furnishing Put for pyrrolidine alkaloid formation in cultured tobacco callus.

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