Effect of Hormone Treatment on Growth Bud Formation and Free Amine and Hydroxycinnamoyl Putrescine Levels in Leaf Explant of *Nicotiana tabacum* Cultivated *in Vitro*

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Josette Martin-Tanguy*, Claude Martin, Michel Paynot, and Nadia Rossin
Laboratoire de Physiopathologie Végétale, Institut National de la Recherche Agronomique, BV 1540, 21034 Dijon Cédex, France

**ABSTRACT**

Foliar explants of *Nicotiana tabacum* cv Xanthi n.c. were cultured on four different media: a basal medium, basal medium plus benzyladenine, basal medium plus 2,4-dichlorophenoxyacetic acid (2,4-D), and the basal medium containing both hormones. No differentiation or cell division occurred in leaf explants cultured on the basal medium. Addition of benzyladenine caused the formation of buds on the explants, while 2,4-D caused callus formation and proliferation. Likewise, only callus was formed when explants were cultured on medium containing both hormones, but growth was significantly greater than that of callus grown on a medium containing 2,4-D alone. The levels of amines and hydroxycinnamoyl putrescines were determined in the four types of explants. In nongrowing explants, amines (except an aromatic amine, tyramine) and hydroxycinnamoyl putrescines were always at a low level and only small changes in their concentrations were observed. In callus cultures, amine (except an aromatic amine, phenethylamine) and hydroxycinnamoyl putrescine levels were higher than those found in bud cultures. In all the media, transitory accumulation of aromatic amines occurred after a few days of culture. Higher levels of hydroxycinnamoyl putrescines were attained in callus cultures with a slow growth rate (2,4-D alone) than in callus cultures with a fast growth rate (benzyladenine + 2,4-D). The formation of buds was accompanied by significant changes in putrescine and hydroxycinnamoyl putrescine levels. Increasing levels were found during the first 14 days in culture when cell multiplication was rapid, followed by a sharp decline after 20 days in culture as the rate of cell division decreased and differentiation took place. The relationship among amines, hydroxycinnamoyl putrescines, and cell division and bud formation is discussed.

Some functional roles for these compounds have been suggested. Thus, caffeoyl putrescine and other amides such as caffeoyl spermidine occur in large concentrations in the reproductive organs of tobacco, and it has been proposed that their formation is functionally linked with reproduction (6). In addition, since tobacco plants infected with tobacco mosaic virus react by increasing levels of PA conjugates and since viral multiplication is retarded in their presence, a role for these compounds in virus resistance has been suggested (15, 17). Recent results (16) indicate that hydroxycinnamoyl putrescines interfere *in vitro* with callus growth and bud formation of tobacco when included in the medium.

*In vitro* formation of buds or roots can be obtained directly without intermediate growth of callus from organ fragments of several tobacco varieties cultured on media solidified by agar and containing mineral nutrients, vitamins, carbohydrate, and growth substances (14). For this type of bud or root formation, the concentrations of auxin and cytokinin determine the nature of the organ formed (14, 25). Bud formation is obtained with a cytokinin-like BA. For callus induction without differentiation, 2,4-D is the most common and effective auxin (1). The growth of callus is activated by 2,4-D plus cytokinin (1). These cultures provide an excellent model system for studying the relations among amines, hydroxycinnamic amides, cell division, and bud formation.

In the present investigation, the accumulation of amines and hydroxycinnamic acid amides has been studied *in vitro* in tobacco leaf explants in relation to cell growth and hormone treatment and represents the first in a series of studies on free polyamines and conjugated PAs on cell proliferation and differentiation.

**MATERIALS AND METHODS**

Biological Material and Growth Conditions. *Nicotiana tabacum* cv Xanthi n.c. was utilized in all the experiments. Plants were grown from seeds in pots (diameter 25 cm) containing a mix of peat and gravel. Every day, 200 mL of a nutrient solution (6) was supplied to each plant. After 5 weeks, the plants were placed in controlled-environment chambers under the following conditions: 20 ± 0.5°C, 120 to 150 W m⁻² (Philips TLF 110 fluorescent tubes and 40 W incandescent lamps), photoperiod 16 h, RH 70 to 80%.

Leaf discs (diameter 8 mm) were cut from the youngest fully expanded leaves of tobacco plants after 65 d of growth. The explants were sterilized with 7% (v/v) calcium hypochlorite for 10 min and rinsed several times with sterile distilled water.

Culture Media. The basal medium consisted of macro- and microelements according to Murashige and Skoog (21) and of

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Polyamines and their biosynthetic enzymes may play an important role in many aspects of plant development including growth, differentiation, senescence, and response to stress (2, 11, 28). Increased polyamine synthesis appears necessary for cell proliferation (4). Amides formed between hydroxycinnamic acids and amines are widely distributed in the plant kingdom (17, 18). In several plants, accumulation of hydroxycinnamic acid amides correlates with leaf emergence and flowering (6, 18, 19, 22). Tobacco stem apices, callus tissues, and cell cultures often contain PAs conjugated with cinnamic acids (5, 6, 12, 20).

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1Abbreviations: PA, polyamine; BA, benzyladenine; Phe NH₂, phenethylamine; Put, putrescine; Spd, spermidine; Spm, spermine; Tyr NH₂, tyramine.
the following supplements: 5.5 × 10⁻⁴ M inositol, 6.8 × 10⁻⁴ M glutamine, 8.7 × 10⁻² M sucrose. Bud formation without intermediary callussing was promoted by the presence of 2 × 10⁻³ M BA. Callus formation without differentiation was promoted by the presence of 2 × 10⁻⁴ M BA and 10⁻³ M 2,4-D, or by 10⁻³ M 2,4-D only.

Solid medium was made by the addition of agar (7 g/L). The pH of the culture medium was adjusted to 5.8 with 1 M KOH, and the medium was then autoclaved for 30 min at 110°C. The cultures were grown in test tubes (25 × 150 mm) under controlled conditions: 25 ± 2°C, 25 to 30 W m⁻² (Philips TLF 110 fluorescent tubes), photoperiod 16 h (14).

Analysis of Amine Levels. Tissues were extracted in 5% (v/v) cold HCl O₃ at a ratio of about 100 mg/mL HCl O₃, according to the methods of Flores and Galston (10). After extraction for 1 h in an ice bath, samples were pelleted at 48,000g for 20 min, and the supernatant phase, containing the ‘free’ PA fraction, was stored frozen at −20°C. HCl O₃ extracts were stable for polyamine analysis by HPLC for more than 6 months under these conditions, provided excessive refreezing and thawing were avoided.

HPLC in combination with fluorescence spectrophotometry was used to separate and quantitate PAs (Put, Spd, and Spm) (29), prepared as their dansyl derivatives from plant tissue.

The PAs were derivatized according to the method of Flores and Galston (10). Fifty to 100 μL aliquots of the supernatant were added to 200 μL of saturated sodium carbonate and 400 μL of dansyl chloride in acetonitrile (7.5 mg/mL) in a 5-mm tapered reaction vial. After brief vortexing, the mixture was incubated in darkness at room temperature overnight. Excess dansyl reagent was removed by adding 10 μL of proline and incubating the mixture for 30 min. Dansylated polyamines were extracted in 0.5 mL benzene. The organic phase was collected and stored in glass vials at −20°C. Standards were processed in the same way, and 20 nmol were dansylated for each, alone or in combination.

HPLC was performed according to the method of Smith and Davies (29). The column used was reversed-phase Bondapak C18 (particle size 9 cm; 30 cm × 3.9 mm) (Waters Assoc.). Samples were eluted from the column with a programmed methanol:water (v/v) solvent gradient, changing from 60% to 95% in 23 min at a flow rate of 1 mL/min. Elution was completed by 27 min. The column was washed with 100% methanol for 5 min and reequilibrated at 60% methanol before the next sample was injected. Eluates from the column were detected with a fluorescence spectrophotometer equipped with an 8-μL flow through cell (model 650-10 LC, Perkin Elmer). For dansylated PAs, an excitation wavelength of 365 nm was used with an emission wavelength of 510 nm. Eluant peaks with their areas and retention times were recorded by an attached integrator (model 3390 A, Hewlett-Packard).

Analysis of Hydroxycinnamic Acid Amide Levels. The techniques which were used to extract, separate, and measure the quantity of phenolic amides have been described elsewhere (6, 18, 22). Plant tissue (1–5 g fresh weight) was extracted at 2°C in a homogenizer with chilled methanol (100 mL), filtered, and washed twice with some 80% (v/v) aqueous methanol. After centrifugation (10,000g for 15 min), the supernatant was evaporated in vacuo at 30°C down to ca. 10 mL, diluted with water (30 mL) and then treated with petroleum ether to remove Chl. The aqueous extract, containing basic hydroxycinnamic acid amides (p-coumaroyl-, caffeoyl-, and feruloyl putrescine) was passed through a column of Amberlite Serva CG-50 (H⁺ form) according to the method previously described (6). Elution with 3 M acetic acid furnished basic amides. The eluate was taken to dryness and dissolved in methanol (2 mL/g fresh weight). The basic amides were quantified by HPLC (23). The compounds were chromatographed on a Bondapak C18 reversed-phase column (particle size 9 μm) with different methanol-water gradients as the mobile phase. Hydroxycinnamoyl putrescines were a gift from Roussel-Uclaf, Industries, France.

Number of buds, fresh weight, levels of amines, and amides were measured at excision and at different stages of development between the 4th and 26th day of culture. The time of visible appearance of buds was noted. Fifty explants were cultured in each experimental medium. The dry weight was estimated by heating the explants at 60°C for 24 to 48 h to constant weight. Each experiment was performed in triplicate and was repeated on at least three separate occasions. Three sets of samples were used for dry weight determination.

RESULTS

Morphogenesis and Growth. Bud differentiation was initiated at d 14 of culture on medium containing BA (Table I). On d 26 there were about 200 buds per explant. Callus formation without differentiation was initiated by BA and 2,4-D or by 2,4-D alone (Table I). Both calli grown on (BA + 2,4-D) and calli grown on 2,4-D alone exhibited the same morphology; they lost Chl and became brown and amorphous after 20 d of culture. Complete absence of morphogenesis (the explants remain unchanged; there was no or only limited division) was found in leaf explants cultured in the absence of growth factors (hormone-free medium, Table I).

The growth (measured by an increase in dry weight) was strongly influenced by hormone treatment (Fig. 1). In both bud and callus cultures the growth appeared exponential throughout the 26-d period of development. Callus growth was higher on BA + 2,4-D than on 2,4-D alone.

Amine Levels. The main free amines on hormone-free medium and on media inducing callus formation were Put, Spd, Spm, and Tyr NH₂. In medium promoting bud formation, Phe NH₂ was also present in relatively large levels.

The effect of hormone treatment on the endogenous levels of aliphatic PAs are shown in Figure 2. On d 0, the levels of aliphatic amines were less than 1 μmol/g dry weight. In all the media, 28% remained at a low level throughout the 26-d period of culture. In hormone-free medium, Put and Spd were always low, and only small changes were observed. Considerable variations were observed in the concentrations of Put and Spd at different stages of callus growth and bud formation. Put and Spd were higher in callus cultures than in bud cultures. During early growth, higher levels of Put were attained in callus grown on (BA + 2,4-D) than on 2,4-D alone, whereas there was little difference on d 10 and later.

A rapid and substantial accumulation of Put was observed in both bud and callus cultures with the maximum level occurring at d 7. After d 7 and until d 14, a marked decrease was observed in all the media. In callus cultures, Put level seemed to show some increase during the later stages of development (Fig. 2).

Table 1. Morphogenesis in Vitro of Tobacco Leaf Explants in Relation to the Hormone Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response and Time of Response (days in culture)</th>
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<tbody>
<tr>
<td></td>
<td>Callus formation</td>
</tr>
<tr>
<td>No hormone</td>
<td>-</td>
</tr>
<tr>
<td>BA</td>
<td>-</td>
</tr>
<tr>
<td>BA + 2,4-D</td>
<td>+</td>
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<tr>
<td>2,4-D</td>
<td>+</td>
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* -, no response; +, response.
In bud cultures, Spd increased to a maximum at d 7. This was followed by a marked decrease during the later stages of culture, approximately down to the initial value. In callus cultures, the maximum level occurred at d 11. In all the cultures and at every stage of development, the levels of Put were higher than the levels of Spd (Fig. 2).

The effect of different culture conditions on endogenous levels of aromatic amines is shown in Figure 3. Initially, the level of Tyr NH₂ was low. Accumulation began after 4 d of culture and was transitory, with the maximum value on d 11. In hormone-free medium and in media inducing calli (especially in 2,4-D medium), a substantial accumulation of Tyr NH₂ took place, whereas lower values were obtained in bud-forming cultures.

Except for d 0, Phe NH₂ was observed only in the bud-forming cultures. It decreased during the first 4 d of culture and afterwards increased rapidly, reaching a maximum at d 14. After the first emergence of buds, this was followed by a marked decrease in Phe NH₂ level (Fig. 3).

**Hydroxycinnamoyl Putrescine Levels.** Hydroxycinnamoyl putrescines (p-coumaroyl putrescine, caffeoyl putrescine, feruloyl putrescine) were absent on d 0 but appeared in all the media after 4 d of culture (Fig. 4). The total levels were strongly influenced by hormone treatment. On hormone-free medium the concentrations were low, and only small changes were observed over the 26-d period of culture. In callus cultures the levels were higher than in bud cultures (Fig. 4). Considerable variations were observed in the levels of total hydroxycinnamoyl putrescines at different stages of callus growth and bud formation. In bud cultures, accumulation was transitory, and the maximum (12 μmol/g dry weight) occurred at d 14 of culture. After the first emergence of buds, the amides decreased to 6 μmol/g dry weight at d 20. In callus cultures, hydroxycinnamoyl putrescines sharply increased and reached a peak on d 7. A decrease was then observed from d 7 until d 19. This was followed by a marked increase during the last stage of culture. The hydroxycinnamoyl amides occurred in higher concentrations in callus grown on 2,4-D alone than in callus grown on (BA + 2,4-D).
Fig. 5. Changes in p-CP, CP and FP in relation to the hormone treatment of tobacco leaf explants cultivated in vitro. Symbols and statistics as in Figure 1.

\( \text{p-Coumaroyl putrescine was not at all detected on hormone-free medium (Fig. 5). In other cultures, p-coumaroyl putrescine appeared after 7 (callus-forming cultures) or 11 (bud cultures) days. It remained low throughout the 26-d period of culture especially in bud cultures.} \)

In bud cultures, caffeoyl and feruloyl putrescine changed more or less in parallel during the 26-d culture period, and on the whole seemed to follow the same pattern as total hydroxycinnamoyl putrescine (Fig. 5 as compared to Fig. 4). Caffeoyl putrescine reached a maximum at d 11, after which a constant decrease was observed until d 26. The maximum level of feruloyl putrescine in the budding cultures occurred on d 14. In callus cultures, caffeoyl putrescine showed an initial increase and reached a peak on d 7. After this a decrease was observed, followed by a marked increase. At d 26, caffeoyl putrescine constituted the major part of the hydroxycinnamoyl putrescine pool, namely 50 to 60% of the whole. The levels of caffeoyl putrescine were higher in callus than in bud cultures except at d 11. From d 14 and until d 26 of culture the levels of this amide were higher in callus grown on 2,4-D alone than in callus grown on (BA + 2,4-D).

In callus cultures feruloyl putrescine increased until d 7 and then decreased during the later stages of development (Fig. 5). Feruloyl putrescine was the predominant amide between d 4 and d 19. During the whole period of culture higher levels of feruloyl putrescine were attained in callus grown on 2,4-D than in callus grown on (BA + 2,4-D).

The same patterns as above were observed when the data were expressed on a fresh weight basis (data not shown).

From d 11 to d 26, Put was mainly present as cinnamic acid conjugates especially in callus cultures. On d 20 in callus cultures, the conjugated amides represented 80 to 90% of total Put.

**DISCUSSION**

Intracellular levels of both Put and hydroxycinnamoyl putrescines were markedly enhanced when the cultures were induced to proliferate (Figs. 2, 4, and 5), and higher levels of these metabolites were attained in callus cultures than in bud cultures. Higher levels of hydroxycinnamoyl putrescines (in particular caffeoyl putrescine) were attained in nondifferentiated cultures with a slow growth rate (2,4-D alone) that in those with a fast growth rate (BA + 2,4-D) (Figs. 4 and 5). The formation of buds was accompanied by significant changes in Put and hydroxy- cinnamoyl putrescine (Figs. 2 and 4). Increasing levels were found during the first 14 days in cultures, when cell multiplication was rapid. Then the levels declined as the rate of cell division decreased and differentiation occurred.

It seems that Figures 2, 4, and 5 are telling us something about the turnover of Put in the various cultures. The endogenous level of Put will be a function of the relative rates of synthesis and metabolism (conjugation and conversion to Spd and Spm). The low levels of Spd and Spm and the correspondingly higher levels of Put conjugates suggest that the major pathway of Put metabolism is via the conjugation pathway. If Put has an important role in the regulation of cell division, one would expect its levels to be tightly controlled, in an analogous fashion to plant hormones. The data in Figures 2, 4, and 5 are consistent with this notion: there is an accumulation of free Put that is correlated with cell proliferation, and when free Put levels decline, there is an increase in the levels of metabolites of Put.

PA biosynthesis is associated with cell division and changes in free PA levels during morphogenic processes are well known (3, 7, 26). Less is known about variations in conjugated PA levels and their significance during cell division and tissue organization.

The function of hydroxycinnamoyl putrescines during cell division and cellular differentiation is still under discussion. Are they a storage form of polyamines or are conjugates in themselves active? Sclocs and Galston (27) suggested that there is only a limited exchange between free and conjugated polyamines, at least in tobacco. However, Flores and Filner (8) suggested that the conjugation to cinnamic acids may be a way of regulating the free PA pool in the cell. In our system the molar proportion of total Put that is in the form of conjugates remained high even though free Put levels declined. This suggests that, in this system at least, the conjugates do not act as storage forms for PAs.

Recently, we have reported (16) that free Put did not promote in vitro cell multiplication but, when conjugated with a cinnamic acid, it promoted cell multiplication of leaf explants of a tobacco mutant RMB 7 cultivated in vitro on a medium promoting bud formation (BA medium as described above). Complete absence of morphogenesis (the explants remain unchanged, there was no or only limited division) and no accumulation of free Put and no accumulation of hydroxycinnamoyl putrescines occurred on leaf explants of this tobacco cultivated in vitro in this medium. When hydroxy cinnamoyl putrescines were added to the BA medium, a rapid proliferative growth of an undifferentiated callus took place. A significant growth promoting effect was obtained at 5 x 10^-5 M; the maximum growth being obtained at 2.5 x 10^-4 M. Above these concentrations the growth promoting effect diminished and tended towards zero at 5 x 10^-3 M. Even at this concentration no toxic effect, i.e. no necrosis, was observed. Also interesting are the results obtained with N. taba- cum Xanthi leaf explants cultivated on BA medium with hydroxy cinnamoyl putrescines. Put conjugates had no effects at 10^-5; leaf explants produced numerous buds like the control. Between 2.5 x 10^-3 M and 5 x 10^-3 M amides partially inhibited bud initiation but promoted callus formation. From 10^-4 M to 5 x 10^-3 M, these molecules strongly inhibited cell multiplication without toxic effect. Free Put had no effect (other than a toxic effect above 10^-3 M).

When foliar explants of N. tabacum cv Xanthi n.c. are cultivated in vitro in a medium promoting bud formation (BA medium as described above), growth (increase in fresh weight) and bud formation (time of emergence, number of buds) are strongly influenced by K-nutrition (13). In K-deficient medium and in high K medium growth and bud formation are markedly inhibited. No apparent relationship is found between free Put and growth or bud formation. In contrast, changes in hydroxy cinnamoyl putrescine levels are shown to correlate well with growth and bud formation. The greatest stimulation of budding and growth is correlated with the greatest accumulation of hy-
droxycinnamoyl putrescines. The highest amide levels are found during the first days in culture, when cell multiplication is rapid. Then, they decline sharply as the rate of cell division decreases and differentiation occurs.

Other results (24) also showed a relationship between the tobacco cell growth in suspension culture and the formation of cinnamoyl putrescines; and in a tobacco cell line selected for growth on Put as the sole nitrogen source, cinnamoyl putrescines may have a primary role in Put metabolism and serve as carrier for the oxidation of Put to hydroxy-Acyl putrescines (9).

These results report that a change in morphology or growth is accompanied by changes in Put levels and the largest changes occur in conjugated Put content specifically. A change in the metabolism of Put is clearly demonstrable as the cells undergo an alteration in their differentiative state. This result suggests that Put and conjugates may play an important role in the growth and development of higher plants, acting either as plant growth regulators themselves, or by acting synergistically with the other plant growth regulators.

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LITERATURE CITED

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