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Influence of Polyamines on Growth of Hairy Root Cultures of Witloof Chicory (*Cichorium intybus* L. cv. Lucknow Local) and Formation of Coumarins

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Abstract. The effect of polyamines (putrescine, spermidine, and spermine) was examined for growth and production of two coumarins, esculetin and esculin, in the hairy roots of chicory (*Cichorium intybus* L. cv. Lucknow local). Of the polyamines administered, 1.5 mM putrescine alone resulted in a 2.3-fold higher increase in the growth of hairy roots as well as in the production of esculetin and esculin, which was 3.37 times more than that of the control on day 21. The endogenous level of conjugated putrescine was more than fivefold that of free putrescine levels in untreated samples. The production of esculetin and esculin in hairy root cultures strictly correlated with growth in all of the treatments. Putrescine at 1.5 mM resulted in a greater length of primary root (18.29 ± 1.37 cm) compared with the control (10.96 ± 0.82 cm) and more secondary and tertiary roots. This study also provides insight into the morphogenetic changes that occur in roots in response to the external supply of polyamines.

Key Words. *Cichorium intybus* L. cv. Lucknow local—Hairy root cultures—Polyamines—Coumarins

The endogenous levels of polyamines (PAs) such as putrescine (Put), spermidine (Spd), and spermine (Spm) are known to influence a variety of growth and developmental processes in higher plants (Evans and Malmburg 1989). They occur in both free as well as conjugated forms and titers depending on external conditions such as light and temperature (Galston and Kaur-Sawhney 1990). Increased polyamine titers have been noticed during sprouting in potato tubers (Kaur-Sawhney et al. 1982) and Jerusalem artichoke (Bagni and Fracassini 1985). It is also known that polyamine titers increase during seed germination (Gallardo et al. 1992, Huang and Villeneuva 1992) and root and shoot formation (Chriqui et al. 1986). The conjugated PAs are known to be associated with the physiology of flowering, metabolite synthesis, and response of the plant to viral infections (Slocum and Galston 1985).

The work reported here is aimed to study the influence of PAs on the growth of hairy roots of chicory (*Cichorium intybus* L. cv. Lucknow local) and formation of two coumarins, esculetin (6,7-dihydroxycoumarin) and esculin (6,7-dihydroxycoumarin 6-glucoside) in hairy roots. Esculetin is used widely as a UV filter in cosmetics (Shah 1945) and esculin, as a marker in microbiological media (Goodwin and Pollock 1954). Another objective was to check the morphological differences that occur in the hairy roots because of external feeding of PAs and accompanying changes associated with endogenous levels of PAs. This could probably help us to understand the nature and quantity of PAs for obtaining specific morphogenetic response in hairy root cultures.

Materials and Methods

Plants

Seeds of *C. intybus* L. cv. Lucknow local were washed by immersing them in water containing Tween 20 (5%) for 15 min; they were sterilized with 70% ethanol and 5% sodium hypochlorite for 10 min and then rinsed three times with sterile distilled water. Sterile seeds were sown on 40 mL of MS basal medium (Murashige and Skoog 1962) containing 3% sucrose and 0.8% agar (w/v) for germination. The seedlings obtained were grown at 25 ± 2°C under 16-h light (37.6 ± 10.1 μmol m^{-2} s^{-1}) and 8-h dark cycles.

Abbreviations: PAs, polyamines; Put, putrescine; Spd, spermidine; Spm, spermine; PCA, perchloric acid; HPLC, high performance liquid chromatography.

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Chemicals

Putrescine, spermidine, and spermine were obtained as their hydrochlorides from Sigma Chemical Co. They were incorporated into the culture medium to obtain final concentrations of 0.5 mM, 0.75 mM, 1.0 mM, and 1.5 mM. All other chemicals were of analytical grade, and solvents were of HPLC grade.

Induction of Hairy Roots

Transformed roots were initiated by inoculating the wounded stems of 4-week-old seedlings with *Agrobacterium rhizogenes* LMG 150 (mannopine type) obtained from Prof. P. J. Hooykaas Rijksuniversitat, Leiden, The Netherlands. Bacterial colonies were cultured for 3 days on solid YEB medium before inoculation (Verveliet et al. 1975) under the same conditions as for seedling growth. Transformed roots were found to appear within 10 ± 2 days of infection. Roots of at least 3 ± 0.225 cm were excised and transferred immediately to 40 mL of MS basal liquid medium containing 500 mg^{-1} carbenicillin in 150-mL Erlenmeyer flasks. Roots were periodically subcultured at three intervals of 3 days each in antibiotic containing MS basal liquid medium. The roots were subsequently transferred to MS basal liquid medium and incubated in the dark on a rotary shaker at 90 rpm and maintained at 25 ± 2°C to obtain axenic hairy root cultures.

Confirmation of Hairy Roots

Transformation in hairy roots was checked using the opine detection test (Trypsteen et al. 1991). Normal and hairy root samples (1 ± 0.1 g) were ground with an extraction buffer (0.1 M thiamine HCl, 0.5 M sucrose, 0.1% ascorbic acid, 0.1% cysteine HCl, pH 8.0) and centrifuged at 5,000 ×g, for 10 min. The supernatant (5–10 μL) was spotted onto Whatman No. 3 paper. This paper was mounted in an electrophoretic tank and run at 20 V. cm^{-1} for 50–55 min. Spots were visualised using silver nitrate staining method (Petit et al. 1983). The negative control was in vitro cultured normal roots of chicory.

Growth Measurement of Hairy Roots

The hairy root cultures were harvested at regular intervals, and the root mat was washed twice in sterile distilled water. After pressing between folds of filter paper, the fresh weight of the mat was recorded as g/culture. Each treatment had five replicates, and the average weight of the recordings was noted.

Estimation of Coumarins

Estimation of esculetin and esculin was carried out using spectrophotometry, HPLC, and later confirmed by ^{1}H NMR (Tamma and Miller 1985). Root samples (1 g) were extracted in 5 mL of solvent (ethanol: water, 70:30) and centrifuged at 5,000 ×*g* for 15 min. The supernatant was made up to a final volume of 5 mL. Aliquots of 20 μL were injected into HPLC after passing through a 0.45-μm filter. HPLC was performed by gradient elution maintained with a UV detector at 340 nm (Tamma and Miller 1985). The separation was performed on a 10-μm μ-Bondapak C_{18} (30 cm × 3.9-mm inner diameter) column at ambient temperature. A mixture of acetic acid and water (1:99 v/v) and pure acetonitrile was used as the solvent system. A flow rate of 1.5 mL · min^{-1} and column pressure of 1,200 p.s.i. were maintained. The retention time was recorded and compared with those of standards, and quantification was performed by calculating the peak area.

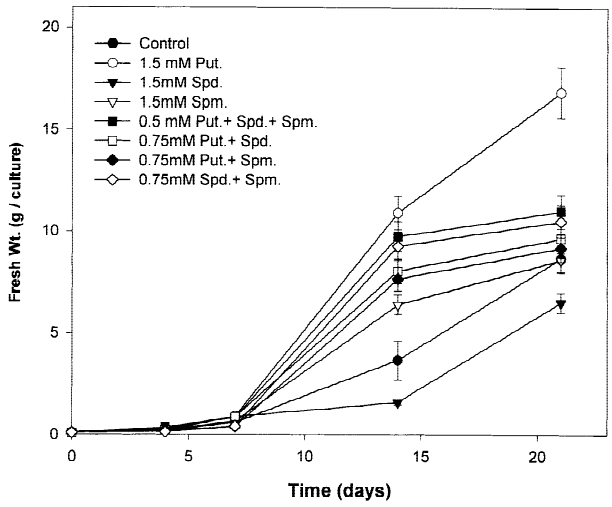

Fig. 1. Fresh weight (g/culture) of hairy root culture of *C. intybus* L. cv. Lucknow local on days 4, 7, 14, and 21 after treatment with polyamines, individually and in combination.

Extraction of Endogenous Polyamines

The extraction of endogenous PAs was carried out by acid hydrolysis of PCA-soluble and PCA-insoluble extracts, and analysis of PAs by benzoylation was performed by the method adopted by Flores and Galston (1982). Each sample was replicated three times for HPLC, and an average of the three values was expressed in μmol g^{-1} fresh weight of the tissue in untreated samples of hairy root cultures.

Results

An initial inoculum of 100 mg of hairy roots was used for all of the experiments. The hairy roots of chicory (*C. intybus* L. cv. Lucknow local) were analyzed periodically for growth and coumarin content during the culture period of 21 days. Of the three culture media tested, namely MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968), and White's (White 1963), MS was found to be superior for growth and production of coumarin in hairy root cultures. Among the three PAs administered, Put at 1.5 mM influenced maximum growth of hairy roots with a productivity of 16 ± 0.5 g/culture, which was 2.3-fold higher than the control (Fig. 1). Although 1.5 mM Spm enhanced the growth of hairy roots up to day 14, there was no difference in the fresh weight (g/culture) over control on day 21 of culture. Spd at 1.5 mM was found to reduce the root growth. A combination of Put with Spm or Spd at equimolar levels (0.75 mM) promoted growth higher than in the control on day 14; but compared with Put treatment at 1.5 mM, the growth was less

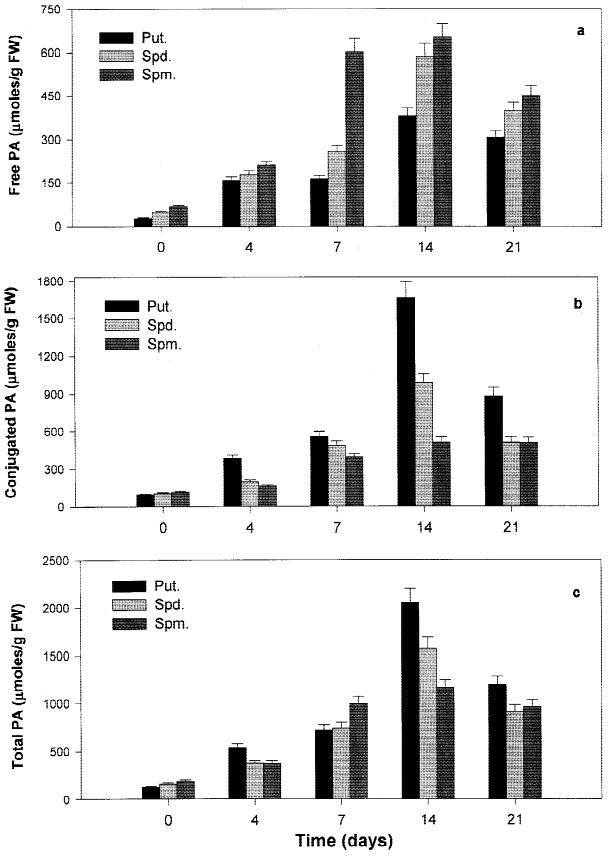

Fig. 2. Endogenous levels of free, conjugated, and total PAs (μmol/g FW) in hairy root cultures of *C. intybus* L. cv. Lucknow local on days 4, 7, 14, and 21. *Vertical bars* are ± S.D.

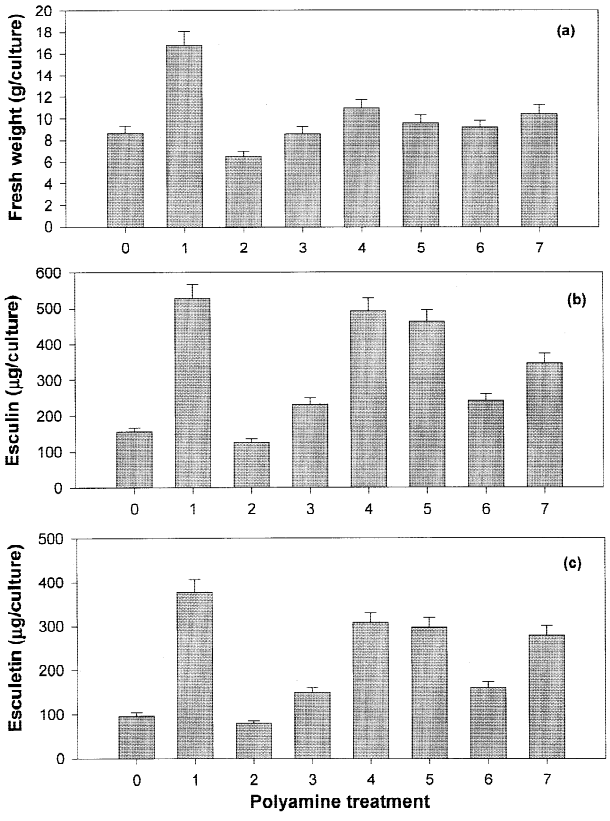

Fig. 3. Effect of polyamine treatment on (*a*) fresh weight (g/culture), (*b*) esculin, and (*c*) esculetin in (μg/culture) on day 21. *Vertical bars are* ± S.D. *0*, control; *1*, 1.5 mM Put; *2*, 1.5 mM Spd; *3*, 1.5 mM Spm; *4*, 0.5 mM Put + Spm + Spd; *5*, 0.75 mM Put + Spd; *6*, 0.75 mM Put + Spm; *7*, 0.75 mM spd + spm.

(Fig. 1). Similarly, a combination of all three PAs at 0.5 mM did not show a significant increase in the root fresh weight over the Put treatment at 1.5 mM. However, the combined effect of three PAs was found to exhibit enhanced growth over the control on day 14 (Fig. 1).

To determine their influence on growth, endogenous levels of free and conjugated PAs were assayed in hairy root cultures for growth in MS basal medium without PA treatment (Fig. 2). A significant increase in endogenous levels of conjugated Put was observed on the 14th day. The level of conjugated Put was more than fivefold that of free Put levels (Fig. 2). This phase coincided with the period of rapid increment of growth during days 7–21 (Fig. 1). Conjugated Put titers remained higher than other conjugated PAs throughout the growth period. A significant increase in the conjugated PA level on day 14 may also suggest their role in promoting growth and biomass accumulation in hairy root cultures (Fig. 2); however, the present experimental design does not rule out the possibility of an increment in endogenous levels of PAs as a result of hairy root growth. Therefore, external feeding of Put would have influenced the growth and biomass accumulation by enhancing endogenous Put titers. The production of esculetin and esculin in hairy root cultures correlated strictly with growth in all of the treatments (Fig. 3).

To understand the influence of PAs on the morphogenetic pattern of hairy root formation, parameters such as growth index, length of primary root, and the number of secondary roots and tertiary roots was investigated (Table 1). It was observed that in a sample treated with 1.5 mM Put, the maximum hairy root length of the primary root was achieved (18.29 ± 1.37 cm), which was 1.6-fold higher than the control (10.96 ± 0.82 cm). Furthermore, other PA treatments alone or in combination caused a significant increase in primary root length and the number of secondary and tertiary roots over the control (Table 1). The 1.5 mM Put-treated sample gave a maximum number of secondary roots (4.55 ± 0.34/25.4 mm of primary root), which was 1.7-fold more than the control (2.84 ± 0.28/25.4 mm of primary root). The maximum number of tertiary roots was observed in 1.5 mM Put-treated samples (31.92 ± 2.39), which was 3.5-fold higher than the control (9.1 ± 0.68).

Table 1. Morphogenetic changes in hairy root cultures in response to incorporation of polyamines in hairy roots of *C. intybus* L. cv. Lucknow local. Data were recorded after 21 days.

Polyamine treatment (mM)	Growth index[a]	Primary root length (cm)	No. of secondary roots (per 25.4 mm primary root)	No. of tertiary roots
Control	85.20 (±6.39)	10.96 (±0.82)	2.84 (±0.21)	9.10 (±0.68)
1.5 Put	162.60 (±12.19)	18.29 (±1.37)	4.55 (±0.34)	31.92 (±2.39)
1.5 Spd	63.20 (±4.74)	14.28 (±1.07)	3.71 (±0.28)	13.23 (±0.99)
1.5 Spm	87.20 (±6.54)	15.23 (±1.14)	4.28 (±0.30)	16.23 (±1.21)
0.75 Put + Spd	97.60 (±7.32)	13.22 (±0.99)	3.69 (±0.27)	23.00 (±1.72)
0.75 Put + Spm	97.32 (±7.29)	12.86 (±0.96)	3.86 (±0.29)	25.60 (±1.92)
0.75 Spm + Spd	109.60 (±8.22)	15.68 (±1.17)	4.42 (±0.33)	19.81 (±1.48)
0.5 Put + Spm + Spd	108.60 (±8.14)	15.92 (±1.19)	4.19 (±0.31)	24.86 (±1.86)

[a] G.I. = final wt. −initial wt./Initial wt.

Discussion

High levels of free PAs have been reported to influence growth by cell division and low levels with cell expansion (Egea-Cortines and Mizrahi 1991). Moreover, the endogenous concentration of PAs can be growth limiting as reported by Smith (1982). Exogenous incorporation of PAs, and Put in particular, have been shown to stimulate growth of several higher plants. In these experiments, PAs are believed to act merely as a source of nitrogen when stimulating growth, although this is unlikely in concentrations less than 100 μM in standard media.

A considerable increase in PA levels accompanying growth and induction of roots was observed in apple (Wang and Faust 1986). Chirqui et al. (1986) reported a synergistic effect between auxins and a PA precursor, ornithine, on rhizogenesis, a morphogenetic response, in *Datura innoxia* leaf explants. Furthermore, exogenous Put influenced root growth in auxin-containing medium (Evans and Malmburg 1989). Levels of PAs are reported to be highest in actively growing tissues and organs, such as root tips (Kulpa et al. 1985) and those that have undergone morphogenesis (Tanguy et al. 1988).

Based on the above study, it could be inferred that the choice of a particular type of PA would significantly enable correlation with morphogenetic pattern of hairy root and development, which in turn also influences the growth and formation of esculin and esculetin in hairy root cultures of chicory (Fig. 3). Although the addition of PAs was shown to induce changes in the type of phenolics in the culture as well as their quantity, neither one of these approaches has been followed for the production of coumarins (Matern et al. 1988). In our study, Put was found to result in increased esculin and esculetin productivity in hairy roots compared with other PAs (Fig. 3). Stress-induced phenylpropanoids, which are known to accumulate in the epidermal and hypodermal layers of stems and leaves, have been thought to play a role in protecting against UV irradiation by strongly absorbing light in UV-B wavelengths (Dixon and Pavia 1995). Coumarins, a class of phenylpropanoids, are also reported to accumulate in various diseased plants (Murray et al. 1982), and this protocol has been employed successfully for inducing coumarin synthesis in parsley (Tietjen et al. 1983). Esculetin formed from caffeic acid (catalyzed by a polyphenol oxidase) was shown to be incorporated into the cell walls of elicitor-treated parsley cells (Matern and Kneusel 1988); however, it was not detectable in cell extracts (Hahlbrock and Scheel 1989). An intracellular signaling system has been proposed for rapid induction of coumarin accumulation in cultured cells (Tietjen et al. 1983). Furthermore, it is believed that the phenylpropanoid pathway provides conjugation partners for PAs, and its activity contributes to the regulatory machinery of plant growth (Mader and Hanke 1997). It has been speculated that cellular levels of free PAs are regulated through reversible conjugate formation (Slocum et al. 1985).

Earlier, there have been attempts to scale-up hairy root cultures in appropriate bioreactors (Whitney 1992). Based on the results obtained from the present study, it may be inferred that PAs, especially Put, influenced growth of hairy root culture and the branching pattern. The latter would enable better root anchorage, desirable for scale-up of hairy roots in bioreactors.

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