J Plant Growth Regul (1999) 18:159–165

Journal of
Plant
Growth
Regulation

Putrescine Influences Growth and Production of Coumarins in Hairy Root Cultures of Witloof Chicory (*Cichorium intybus* L. cv. Lucknow Local)

H. P. Bais, G. Sudha, and G. A. Ravishankar*

Department of Plant Cell Biotechnology, Central Food Technological Research Institute, Mysore 570 013, India

Received July 14, 1999; accepted October 5, 1999

Abstract. The effect of putrescine (Put) on the growth and production of two coumarins, esculin and esculetin, in hairy roots of chicory (*Cichorium intybus* L. cv. Lucknow local) was examined. To study the role of Put on growth and production of coumarins, polyamine inhibitors, namely α-DL-difluromethylornithine and α-DL-difluromethylarginine were used at 1 mM concentration. Put treatment at 1.5 mM produced a 1.9-fold increase in the growth of hairy roots, as well as the production of esculin and esculetin. The treatments with polyamine (PA) inhibitors resulted in much lower growth and production of coumarins compared with both 1.5-mM Put treatment and the control. Both free and conjugated PAs were studied over the whole culture period, and conjugates of all three PAs, namely Put, spermidine, and spermine, were higher than free PAs throughout the culture period. The treatments with PA inhibitors showed lower levels of endogenous PAs compared with Put-treated samples. The treatment with 1.5 mM Put showed maximum accumulation of endogenous conjugated Put (2,098 ± 157 nmoles gm^{-1} fresh weight). The production of esculin and esculetin was strictly correlated with growth in all treatments. Put at 1.5 mM resulted in greater length of primary root (18.3 ± 1.4 cm) as compared with the control (11 ± 0.9 cm) and larger numbers of secondary and tertiary roots.

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

Abbreviations: ADC, arginine decarboxylase; DFMA, α-DL-difluromethylarginine; DFMO, α-DL-difluromethylornithine; HPLC, high-performance liquid chromatography; ODC, ornithine decarboxylase; PA(s), polyamine(s); PCA, perchloric acid; Put, putrescine; Spd, spermidine; Spm, spermine.

*Author for correspondence: E-mail: pcbt@cscftri.ren.nic.in

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 Malmberg 1989). PAs occur both in free and conjugated forms, and titers depend on external conditions of light and temperature (Galston and Kaur-Sawhney 1990). Increased PA titers have been noted during sprouting in potato tubers (Kaur-Sawhney et al. 1982) and Jerusalem artichokes (Bagni and Fracassini 1985). It is also known that PA titers increase during seed germination (Gallardo et al. 1992, Huang and Villeneuva 1992) and root and shoot formation (Chriqui et al. 1986). 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).

Many of the studies demonstrating PA function have been made possible through the use of inhibitors of PA biosynthesis, such as α-DL-difluromethylornithine (DFMO) and α-DL-difluromethylarginine (DFMA) that selectively inhibit ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively (Kallio and McCann 1981). Inhibition of PA biosynthesis blocks differentiation in plants and the effect can be reversed by addition of PAs (Fierer et al. 1984). Unlike animals, plants synthesize Put through ODC and arginine through an alternative pathway involving ADC (Fierer et al. 1984). The two pathways to Put have different tissue distributions and differential regulation (Hiatt and Malmberg 1988) because ADC has been linked to stress responses and ODC to cell cycle and division (Cohen et al. 1982).

In previous work (Bais et al. 1999) we demonstrated the influence of PAs on growth of hairy root cultures of chicory (*Cichorium intybus* L. cv. Lucknow local) and formation of two coumarins—esculetin (6,7-dihydroxycoumarin) and esculin (6,7-dihydroxycoumarin 6-glucoside). Put at the 1.5 mM level influenced maximum growth and production of the metabolites.

This study deals with the role of Put for growth and coumarin formation by use of PA inhibitors, namely DFMO and DFMA. Another objective was to check the morphological differences that occur in the hairy roots caused by external feeding of Put and PA inhibitors and accompanying changes associated with endogenous levels of free and conjugated PAs. This may help to understand the nature and quantity of PAs required for obtaining specific morphogenetic responses in hairy root cultures.

Materials and Methods

Plant Material

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

Chemicals

Put was obtained from Sigma Chemical Co. (St. Louis, MO); DFMA and DFMO were obtained from Marion Merrell Research Co. (Cincinnati, OH). They were incorporated into the culture medium to obtain final concentrations of 1.5 mM, 1.0 mM, and 1.0 mM, respectively. All other chemicals were of analytical grade and solvents were of high-performance liquid chromatography (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 P. I. J Hooykaas Clusius Laboratorium, Rijksuniversiteit, 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 ± 0.2 cm were excised and immediately transferred to 40 mL MS basal liquid media containing 500 mg L^{-1} carbenicillin in 150 mL Erlenmeyer flasks. Roots were periodically subcultured three times at intervals of 3 days each in carbenicillin (500 mg L^{-1}) containing MS basal liquid media. 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 with 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. The negative control was in vitro cultured normal roots of chicory. The paper was mounted in an electrophoretic tank and run at 20 V. cm^{-1} for 50–55 min. Spots were visualized with silver nitrate staining method (Petit et al. 1983).

Growth Measurement of Hairy Roots

Hairy root cultures were harvested at regular intervals and the root mat 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 calculated.

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 solvent (ethanol/water 70:30) and centrifuged at 5,000 × *g* for 15 min. The supernatant was made up to final volumes of 5 mL. Aliquots of 20 μL were injected into HPLC after filtering through a 0.45 μm filter. HPLC was performed by gradient elution maintained with an ultraviolet 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 ID) column at ambient temperature. A solvent 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 psi were maintained. The retention time was recorded and compared with those of standards, and quantification was performed by calculating peak area.

Extraction of Endogenous PAs

The extraction of endogenous PAs was carried out by acid hydrolysis of perchloric acid (PCA)-soluble and PCA-insoluble extracts. PAs were analyzed by benzoylation performed by the method adopted from Flores and Galston (1982). Each sample was replicated three times for HPLC, and an average of the three values was expressed in nmole g^{-1} FW of the tissue.

Results

An initial inoculum of 100 mg of hairy roots was used for all the experiments. The hairy roots of *C. intybus* were analyzed periodically for growth and coumarin content during the culture period of 21 days. Among the various treatments tested, it was found that a 1.5 mM concentration of Put caused maximum growth of hairy roots (16.92 ± 0.5 g/culture), 1.93-fold greater than that of controls (Fig. 1). Treatments with DFMO and DFMA showed minimum growth of untransformed roots, or was 6-fold less than that obtained with 1.5 mM Put (Fig. 1). The treatment combining Put along with 1 mM DFMA/

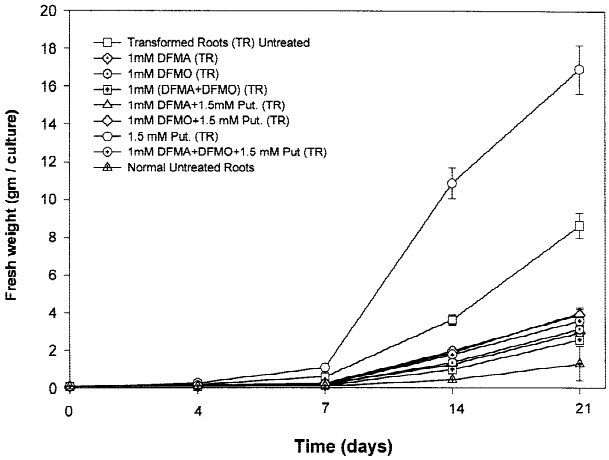

Fig. 1. Influence of Put and PA inhibitors on growth in transformed roots of *C. intybus.*

DFMO showed a slight increase in root productivity, similar to the PA inhibitor treatments (Fig. 1).

To determine the influence of exogenous feeding of Put on growth, parameters such as endogenous levels of free and conjugated PAs were assayed in hairy root cultures in MS basal medium in all treatments (Figs. 2 and 3). A significant increase in titers of endogenous free PAs (Put/Spd/Spm) was observed on day 14 in Put-treated samples (Fig. 2). Endogenous free PA titers of all three PAs were 3.5-fold greater than those of controls on day 14 (Fig. 2). This increase coincided with a period of rapid growth during days 7–21 (Fig. 1). The DFMO and DFMA treatments at the 1.0 mM level resulted in hairy roots with lower levels of free endogenous PAs (Fig. 2). However, when Put was fed along with the inhibitors, there was a slight increase in free endogenous PA levels compared with hairy roots subjected to DFMO and DFMA treatments alone (Fig. 2). Untransformed untreated samples showed minimal growth and the lowest accumulation of free endogenous PAs (Figs. 1 and 2).

Conjugated PA titers remained higher in the case of 1.5 mM Put-treated samples compared with all the other treatments (Fig. 3) and attained a peak on day 14. Treatment with DFMA and DFMO at the 1.0-mM level resulted in lower levels of conjugated PAs compared with 1.5-mM Put-treated samples (Fig. 3). Hairy roots, when treated with Put and DFMA/DFMO showed an increase in titers of endogenous PAs compared with PA inhibitor treatments (Fig. 3). Of the three conjugated PAs, Put conjugates were the greatest on day 14 (2,098 ± 157 nmoles gm^{-1} FW) under 1.5 mM Put treatment, 3-fold greater than control levels (Fig. 3). The production of esculetin and esculin in hairy root cultures was strictly correlated with growth in all the treatments (Fig. 4).

To understand the influence of PAs on the morphogenetic pattern of hairy root formation, parameters such as growth index, length of primary root, number of secondary roots, and tertiary roots were recorded (Table 1). Samples treated with 1.5 mM Put resulted in maximum lengths of primary hairy roots (18.3 ± 1.4 cm), 1.6-fold greater than in controls (11 ± 0.9 cm). Treatments with DFMO and DFMA showed a lower growth index, shorter primary roots, and fewer secondary/tertiary roots compared with controls and 1.5 mM Put treated samples (Table 1). Put-treated roots produced the maximum number of secondary roots (18.2 ± 1.3), 1.7-fold more than the control (11.3 ± 0.9) (Table 1). The maximum number of tertiary roots was observed in 1.5-mM Put- treated samples (32 ± 2.4), 3.5-fold greater than controls (9.1 ± 0.68) (Table 1).

Discussion

On the basis of the data, it can be inferred that Put plays an important role in root growth and development and in turn also influences the growth and productivity of esculin and esculetin in hairy root cultures of chicory (Fig. 4). A significant increase in the titers of free and conjugated PAs on day 14 may also suggest a role for Put in promoting root growth and biomass accumulation in hairy root cultures (Figs. 2 and 3). Therefore exogenous feeding of Put would have influenced growth and productivity by enhancing endogenous PA titers (Figs. 1–4). The promotive role of Put was quite evident in the case of inhibitor-treated hairy roots, wherein the addition of Put restored root growth, biomass, and endogenous PA pools (Figs. 1–4). The role of Put in root differentiation and morphogenesis is supported by results from combined treatments of 1.5 mM Put with DFMO and DFMA (1.0 mM) that showed marginal increases in the growth index and morphogenesis over PA inhibitor treatments (Table 1).

These results may also imply the possibility of involvement of both ODC and ADC in PA synthesis as has been reported by Tiburcio et al. (1987). Similarly, Berta et al. (1997) found that thinning of cell walls of tobacco thin cell layer explants after DFMA and DFMO administration could be reversed by the supply of PAs.

Put levels were found to be high in rapidly growing tumors of *Nicotiana glauca*. Martin Tanguy et al. (1990) reported that alterations in PA levels are associated with morphological changes of plant tissue. Tiburcio et al. (1987) reported that addition of DFMA or D-arginine decreased the total endogenous Put content and alkaloid titers and also inhibited rooting in tobacco root cultures. Furthermore, exogenous Put administration influenced root growth in auxin-containing medium in hairy root cultures of *Datura inoxia* and increased titers of endogenous PAs (Evans and Malmberg 1989). PA levels are reported to be greatest in actively growing tissues and

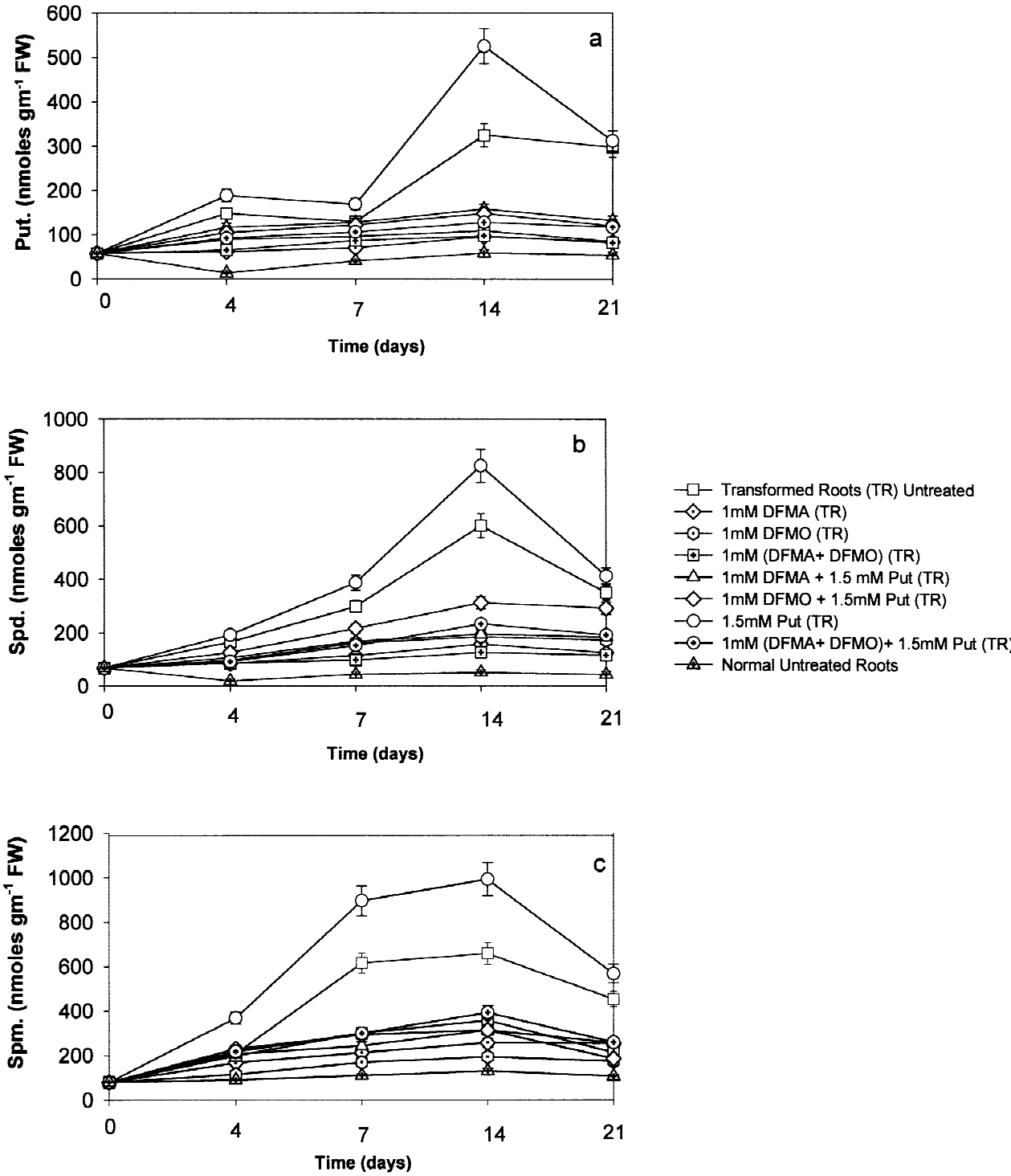

Fig. 2. Influence of Put and PA inhibitors on titers of free endogenous PAs in transformed roots of *C. intybus.*

organs, such as root tips (Kulpa et al. 1985) and those that have undergone morphogenesis (Martin Tanguy et al. 1988).

On the basis of the results obtained from this study, it may be inferred that PAs, especially Put, influence growth and branching patterns of hairy root cultures. The latter enabling better root anchorage desirable for scale-up of hairy roots in bioreactors.

Acknowledgments. H. P. B. and G. S. acknowledge the C. S. I. R, New Delhi for the award of Senior Research Fellowships. This research work was supported by a grant from Department of Biotechnology, DBT, New Delhi, India, BT/18/08/PR0320/96-PID.

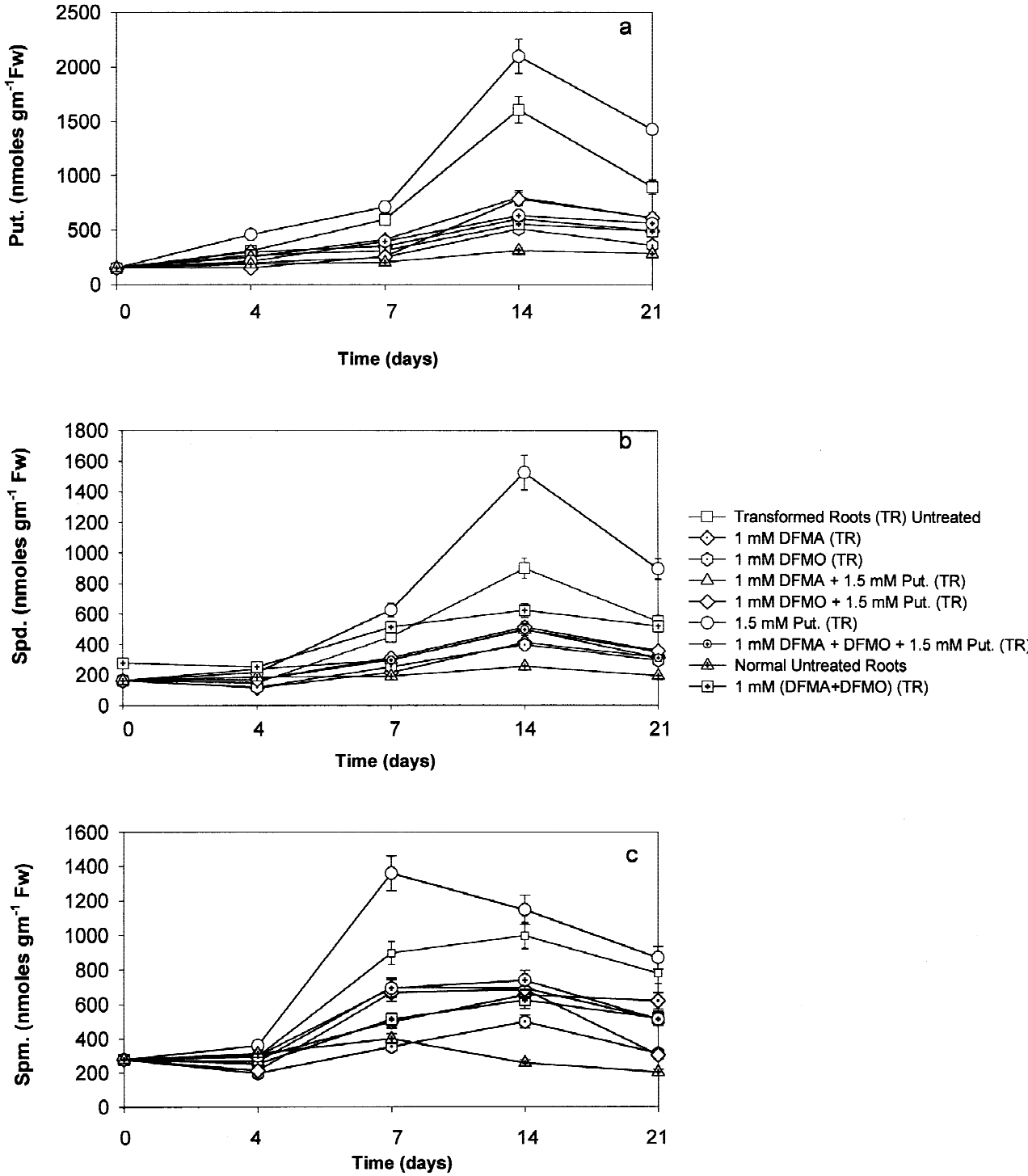

Fig. 3. Influence of Put and PA inhibitors on titers of endogenous conjugated PAs in transformed roots of *C. intybus.*

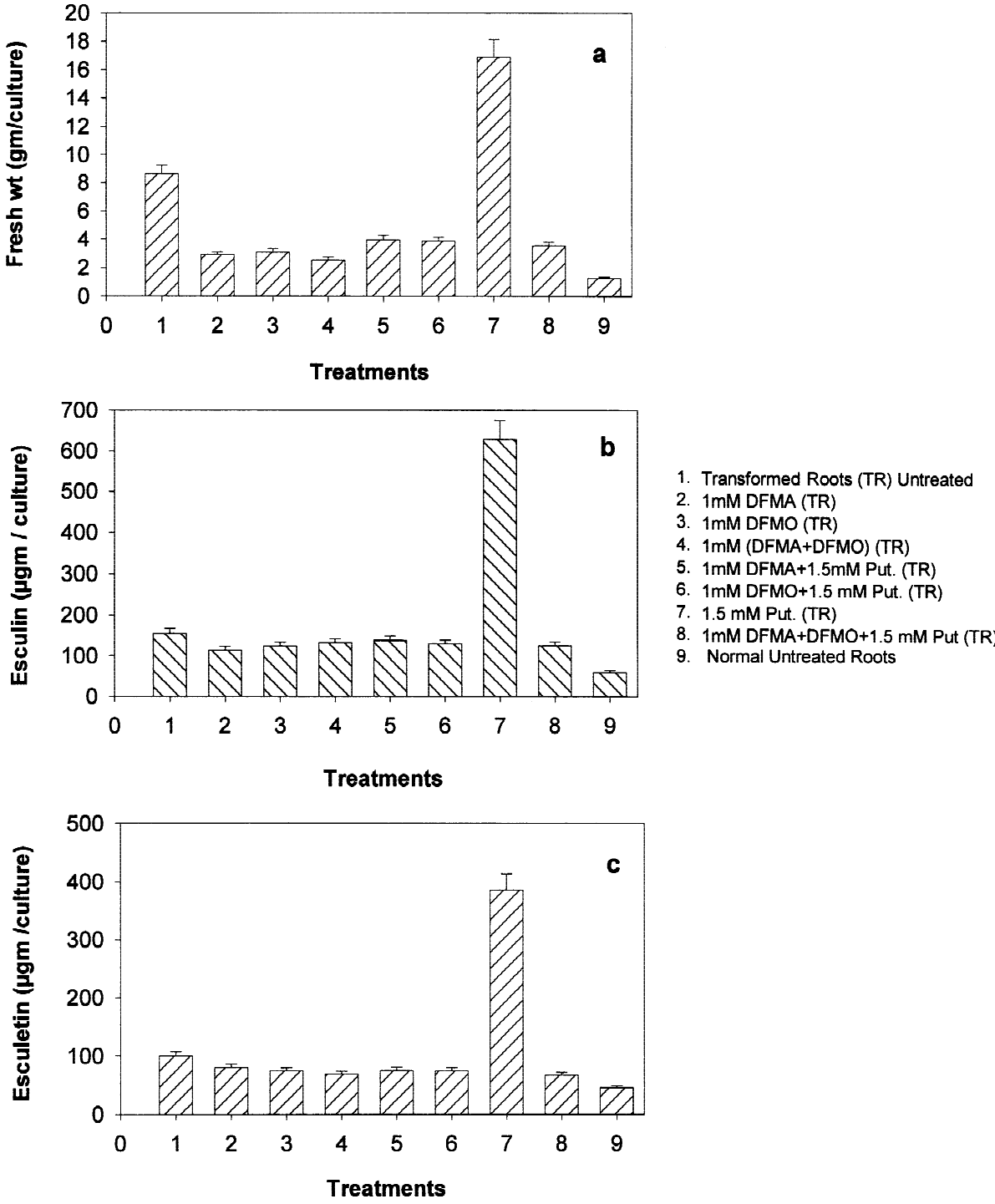

Fig. 4. Influence of Put and PA inhibitors on growth and production of coumarins in transformed roots of *C. intybus* on day 21.

Table 1. Influence of putrescine and polyamine inhibitors on morphogenetic response in transformed roots of *Cichorium intybus* L.cv. Lucknow local.

S1. no.	Treatments[a]	Growth index[b]	Primary root length (cm)	Number of secondary roots	Number of tertiary roots
1.	Transformed roots untreated	85.20 ± 6.39	11.00 ± 0.9	11.3 ± 0.9	9.1 ± 0.7
2.	1 mM DFMA	28.3 ± 6.39	4.96 ± 0.36	6.96 ± 0.52	7.21 ± 0.54
3.	1 mM DFMO	30.2 ± 2.2	4.28 ± 0.36	5.89 ± 0.44	7.31 ± 0.54
4.	1 mM (DFMA + DFMO)	24.6 ± 1.8	3.62 ± 0.27	5.28 ± 0.39	6.36 ± 0.47
5.	1 mM DFMA + 1.5 mM putrescine	30.8 ± 2.9	3.86 ± 0.28	4.98 ± 0.37	5.96 ± 0.44
6.	1 mM DFMO + 1.5 mM putrescine	37.9 ± 2.0	3.92 ± 0.29	4.86 ± 0.36	5.78 ± 0.43
7.	1.5 mM putrescine	162.8 ± 12.1	18.3 ± 1.4	18.2 ± 1.4	32 ± 2.4
8.	1 mM (DFMA + DFMO) + 1.5 mM putrescine	34.6 ± 2.59	3.58 ± 0.26	4.56 ± 0.34	5.36 ± 0.4
9.	Normal untreated roots	11.9 ± 0.89	1.99 ± 0.14	2.36 ± 0.17	3.98 ± 0.29

S1. No. 1–8 experiments with transformed roots.
[a] Data recorded after 21 days of culture period.
[b] Growth index = (Final weight − Initial weight)/Initial weight.

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