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Hormonal Effects on Growth and Morphology of Normal and Hairy Roots of *Hyoscyamus muticus*

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Abstract. Treatment of normal and *Agrobacterium rhizogenes*-transformed root cultures of *Hyoscyamus muticus* with three different auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthaleneacetic acid (NAA), revealed that the response varied considerably among auxins, between transformed and normal roots, and depending on the parameter. In normal roots all three auxins provoked abundant branching, with IBA and NAA being the most effective at 2.5 and 0.5 μM, respectively, whereas IAA was most effective at low concentrations (0.05 and 0.1 μM). In transformed roots exogenously supplied auxins were generally inhibitory or, at best, without effect on growth and branching. Only 0.01 μM IAA significantly enhanced lateral root number, whereas at the higher concentrations IBA, although inhibitory, was the least effective auxin. In both root types IBA had little effect on primary root growth, but normal roots were more sensitive to IAA and NAA. These results suggest a different sensitivity to auxins of normal and transformed roots since there was no significant difference in endogenous free and conjugated IAA content nor in IAA uptake capacity. Ethylene production and biosynthesis were approximately threefold higher in hairy roots, but production could be stimulated up to tenfold that of control levels in normal roots by supplying NAA or 1-aminocyclopropane-1-carboxylic acid (ACC). Treatment with 2.5 μM NAA, but not IAA or IBA, also enhanced ethylene biosynthesis in normal roots but not in transformed ones. ACC and malonyl-1-aminocyclopropane-1-carboxylic acid accumulated to detectable levels only after treatment with an auxin (NAA).

Key Words. Hormonal effects—*Hyoscyamus muticus*—Roots

Hairy roots of *Hyoscyamus muticus*, obtained by transformation with the agropine-type *Agrobacterium rhizogenes* strain A4 and maintained as stable root cultures on solid hormone-free medium, have the same morphology as that already described for some phenotypic classes of hairy roots in other species. Compared with nontransformed roots, they are much thicker, elongate more slowly, are much more highly branched, and have many root hairs.

Characterization of the bacterial transferred DNA (tDNA) and studies on the expression of the genes located on this tDNA indicate that the *rolA, B,* and *C* genes of the T_L region of the root-inducing (Ri) plasmid are sufficient to induce adventitious roots (Spena et al. 1987, White et al. 1985), whereas the *aux* genes (bacterial genes encoding auxin biosynthesis) of the T_R region are responsible for the hairy root phenotype described above (Amselem and Tepfer 1992). Thus, auxin is considered the main factor controlling, if not root induction itself, at least hairy root growth and development.

To date, *rolB* is the tDNA gene that has attracted the most attention as it is the only one that, if inactivated, totally suppresses root formation (White et al. 1985) and the only one capable of inducing it, individually, on all host plants tested. The morphologic alterations of *rolB*-transgenic plants and the growth pattern of *rolB*-induced roots have led cells transformed by this oncogene to be

Abbreviations: tDNA, transferred DNA; Ri, root-inducing; IAA, indole-3-acetic acid; AVG, aminoethoxyvinylglycine; ACC, 1-aminocyclopropane-1-carboxylic acid; MACC, malonyl-1-aminocyclopropane-1-carboxylic acid; T, transformed; N, normal; IBA, indole-3-butyric acid; NAA, naphthaleneacetic acid; PR, primary root; LR, lateral root; IPR, initial primary root; NPR, new primary root; FW, fresh weight; HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry.

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regarded as in a ''hyperauxinic'' state possibly because of either an altered sensitivity to or an abnormally high intracellular concentration of the hormone (Costantino et al. 1994). Increased auxin content (resulting from the presence of *aux* genes and/or *rolB*) is in fact not the only possible cause for the different developmental pattern of hairy roots compared with nontransformed roots. Instead, transformed roots of tomato, *Catharanthus trichophyllus* (Shen et al. 1990), tobacco (Spanò et al. 1988), and *Lotus corniculatus* (Shen et al. 1988) exhibit a higher sensitivity to this hormone than their normal counterparts.

Transformed and nontransformed (normal) root cultures of *H. muticus* were used as a model system to study the morphologic effects of different natural and synthetic auxins supplied in a wide range of concentrations. Root development was evaluated on the basis of several parameters involving mainly primary and lateral root growth and lateral root number. These observations are discussed in the light of the indole-3-acetic acid (IAA) uptake capacity and endogenous IAA content in the two root types.

Given the well known interaction between auxin and ethylene, the possible role of the latter hormone in regulating hairy root development was analyzed by supplying aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor. Basal and auxin-enhanced ethylene production, 1-aminocyclopropane-1-carboxylic acid (ACC), and malonyl-1-aminocyclopropane-1-carboxylic acid (MACC) content (the ethylene precursor and its conjugated form, respectively) were also measured in both root types.

Materials and Methods

Establishment of Root Cultures

A transformed hairy root clone of *H. muticus* L., initiated by inoculating 3-week-old seedlings with *A. rhizogenes* (strain A4), was kindly supplied by Prof. Hector Flores, Pennsylvania State University. Untransformed root cultures were established from excised root tips of sterile seedlings germinated in vitro on basal White's (White 1938) medium.

Growth of Isolated Roots

Both transformed (T) and normal (N) root cultures were maintained in Petri dishes (internal diameter 9 cm), kept in darkness at 23°C, on the same hormone-free B5 medium (Gamborg et al. 1968) supplemented with 2% sucrose and solidified with 2.5 g/liter Gelrite (Schweizerhall, NJ). They were subcultured every 2–3 weeks by excising and transferring to fresh medium explants consisting of 1-cm apical segments (usually lacking lateral roots). Auxins were added to the culture medium prior to autoclaving, whereas AVG was filter sterilized. The following auxins were tested: IAA or IBA, 0.01, 0.05, 0.1, 0.5, 1, or 2.5 μM; NAA, 0.5, 1, or 2.5 μM.

Growth was monitored at intervals (for time course of lateral root appearance) or after 7–9 days after subculture. The following parameters were considered: primary root (PR) length, number of lateral roots (LRs) per PR segment (initial explant vs new portion of PR formed after subculture, called IPR and NPR, respectively), average length of LR, frequency classes of LR length, number of LRs per unit length of the PR (root density), and distance from apex to first LR.

Ethylene, ACC, and MACC Analysis by Gas Chromatography (GC)

After aerating the Petri dishes for about 30 min, approximately 1 g FW of roots were transferred to 11-mL test tubes containing 250 μL of distilled water. After sealing the tubes with rubber caps, 1-mL gas samples were withdrawn at 1-h intervals with a hypodermic syringe and injected into a gas chromatograph equipped with an activated alumina column and a flame ionization detector.

For ACC and MACC analysis, root samples were frozen in liquid nitrogen and ground in a mortar on ice. Extraction was carried out with 80% (v/v) ethanol. After centrifugation for 15 min at 1,500 × *g*, the supernatant was concentrated under vacuum at 45°C and then resuspended with 2 mL of water and 0.5 mL of chloroform. After a brief centrifugation, an aliquot of the aqueous phase was used to determine ACC using the method of Lizada and Yang (1979). MACC was assayed in the same way after hydrolyzing an aliquot of the aqueous phase with 7.2 N HCl at 100°C for 3 h.

[^{14}C]Methionine Incorporation into Ethylene

Ethylene biosynthesis was evaluated by measuring the incorporation of U-[^{14}C]methionine (specific activity 9.6 TBq mol^{-1}, purchased from DuPont de Nemours, Italia) according to the method described by Biondi et al. (1990). Approximately 0.5^{-1} g FW of intact roots was transferred 8 days after subculturing on medium supplemented or not with 2.5 μM IAA, IBA, or NAA to 50 mL flasks containing 5 mL of liquid medium having the same composition. To each of these 37 kBq in 10 μL of the labeled precursor was added. An ethylene trap, consisting of 0.5 mL of 0.25 M mercuric perchlorate, and a CO_2 trap, consisting of 0.5 mL of 1 N KOH, both in small hanging wells, were set in place and the flasks capped with rubber stoppers. After a 6-h incubation at room temperature, radioactivity in the mercuric perchlorate was measured using a liquid scintillation counter.

Auxin Analysis

Normal and transformed roots growing on hormone-free medium were harvested at 8 and 15 days after subculture, immediately frozen in liquid nitrogen, freeze-dried, and stored in vacuum until use. For auxin analysis, the equivalent of 0.5 g FW of freeze-dried samples was extracted in 65% isopropyl alcohol (v/v) with 0.2 M imidazole buffer at pH 7 to which were added [^{3}H]IAA as a radiotracer and [$^{13}C_6$]IAA (0.1–1 μg g^{-1} sample) as internal standard for quantitative mass spectral analysis (Chen et al. 1988). A Beckman System Gold coupled to a UV detector was used for HPLC purification of free and conjugated (esters plus amides) IAA. They were separated by chromatography on a 5-μm C_{18} Partisphere column (Whatman, 110- × 5-mm inner diameter) and eluted at a rate of 1 mL min^{-1} with 20% acetonitrile-water and 1% acetic acid. Quantitative analyses were carried out on a GC-MS (Hewlett Packard 5890-5970) equipped with a 12-m Chrompack CPSil 19 capillary column following the method described previously by

Baraldi et al. (1995). Ions monitored were *m/z* 130 and 136 for the base peak (quinolinium ion) and 189 and 195 for the molecular ion of the methyl ester of IAA and of [$^{13}C_6$]IAA, respectively. Ratios of 130:136 and 189:195 were used to calculate endogenous levels of IAA and to verify the analysis (Cohen and Schulze 1981).

[^{3}H]IAA Uptake

Normal and transformed roots were harvested 10 days after subculture, and 200–300 mg FW was transferred to flasks containing 2 mL of liquid B5 medium and 15 kBq [^{3}H]IAA (specific activity 999 TBq · mol^{-1}, pur-chased from Amersham, Italia). They were incubated for 60 min (when the system was surely at saturation), then collected, rinsed, and blotted dry. At the end of incubation, roots were chopped into small pieces and extracted overnight at 4°C with 80% methanol (2:1, v/w) containing 100 mg/liter ascorbic acid. One hundred-mL aliquots of methanol were counted in a scintillation counter (Beckman LS-1800).

Statistical Analysis

All data on root growth and LR formation are the mean values of two or three independent experiments with a minimum of 12 roots/treatment. Statistical analysis was carried out using Student's *t*-test. Assays on auxin content, IAA uptake, and ethylene formation were performed on a similar number of samples. Each treatment had three replicates, and experiments were repeated once.

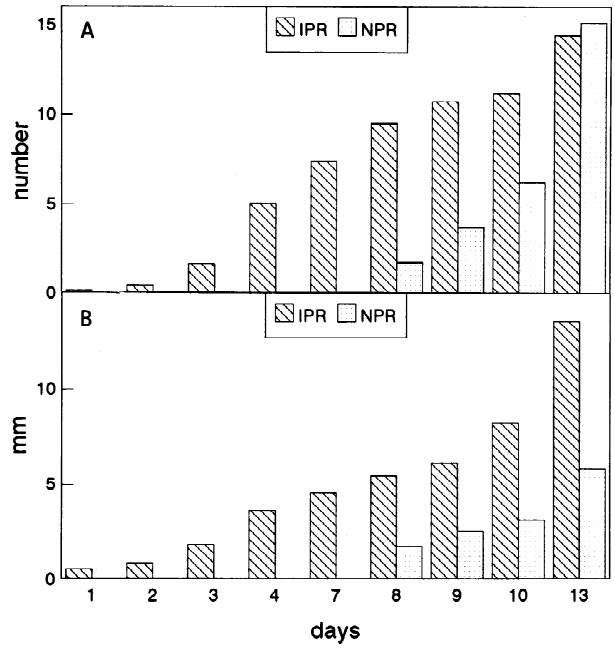

Fig. 1. Time course of LR development in *H. muticus* hairy roots cultured on hormone-free medium. *A*, mean LR number/PR; *B*, mean length of LRs.

Results

Number and Time of Appearance of LRs on Transformed Roots

LR number and mean length were evaluated at close intervals over a 13-day subculture period; those formed on the IPR segment were counted separately from those initiated on the NPR grown after subculturing (Fig. 1). Some of the former were already present as primordia at subculture time since 1 day later they had emerged and were on average about 0.5 mm long. LRs first appeared on the NPR on day 8 and rapidly increased in number; by day 13 their number equaled that on the IPR, but their mean length remained significantly shorter.

Auxin Effects on Growth

Normal roots. In N roots, which on basal medium display rapid elongation growth but no or sporadic branching, exogenous auxins, as expected, caused abundant LR formation. As shown in Fig. 2*B*, the effectiveness of the three auxins on this parameter was similar at 0.5 and 1 μM. At the highest concentration tested (2.5 μM), however, they behaved quite differently; compared with the other two auxins but also with the other concentrations, IBA gave the strongest stimulation; the NAA effect was intermediate, and IAA was inhibitory.

Elongation growth of the PR was strongly inhibited by IAA and NAA at 0.5, 1, and 2.5 μM, whereas IBA was stimulatory at 0.5 μM ($p < 0.05$), without effect at 1 μM, and inhibitory only at 2.5 μM (Fig. 2*A*).

In the lower concentration range (Table 1) IAA strongly enhanced LR formation and LR density at 0.05 and 0.1 μM, but IBA was without effect. IAA inhibition of PR growth was significant at and above 0.05 μM, whereas IBA was without effect at all three concentrations.

Although mean LR length was not significantly different, in the presence of auxin (IAA, IBA, or NAA) LRs tended to become shorter (Fig. 2*C*). IBA had the least effect also on this parameter.

Transformed Roots. Exogenous auxins were generally inhibitory or without effect depending on the parameter examined and on the concentration supplied. Only 0.01 μM IAA significantly enhanced LR number (but not LR density); however, this concentration was without effect on PR growth (Table 1). With 2.5 μM there was no growth at all of the PR and almost complete inhibition of LR formation (Fig. 3, *A* and *B*). As in N roots, IBA enhanced PR growth but in this case only at the lowest

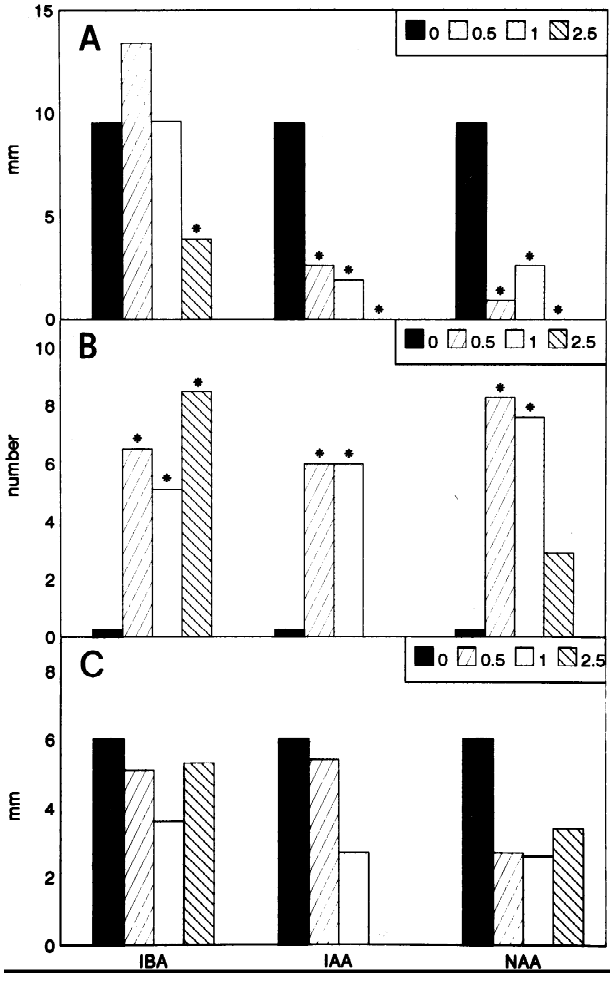

Fig. 2. Mean PR length (*A*), number of LR/PR (*B*), and mean LR length (*C*) in 7-day-old normal *H. muticus* roots cultured in the presence of 0, 0.5, 1, or 2.5 μM IBA, IAA, or NAA. *Bars* with * are significantly different ($p < 0.01$) from controls.

concentration (0.01 μM). In terms of growth reduction, IBA was, as in N roots, less effective than IAA, whereas the effect of NAA resembled that of IBA rather than IAA (Fig. 3*A*).

Mean LR length gave the most uniform response with respect to the three different auxins; in all cases the trend was toward a reduction in length, but only IBA and IAA caused significant growth inhibition (Fig. 3*C*).

Table 2 compares the effects of the three auxins given at the same concentration (1 μM) on different parameters in N and T roots. It shows clearly that the main difference between the response of N and T roots to applied auxin is in LR number, whereas their mean length is reduced to a similar extent (approximately 50%) in both root types. It also shows that in both N and T roots IBA has little or no effect on PR growth; IAA and NAA are strongly inhibitory.

Table 1. Auxin effects on PR growth (% increase from day 0) and on mean number, length, and density of LRs in normal and transformed roots of *H. muticus* 7 days after subculture.

Root type and treatment	PR growth	LR number	LR length (mm)	LR density
Normal				
Control	115	2.7	8.5	0.1
IAA				
0.01	78	5.2	5.2	0.2
0.05	28**	14.5**	6.1	0.7**
0.1	15**	14.2**	5.0	0.8**
IBA				
0.01	112	1.3	2.8	0.1
0.05	123	6.2	4.2	0.2
0.1	120	3.0	4.2	0.1
Transformed				
Control	26	10.0	4.4	0.5
IAA				
0.01	44	14.0*	6.7	0.5
0.05	33	7.5	3.2	0.4
0.1	10	6.5	3.6	0.4
IBA				
0.01	66**	8.5	7.0	0.3
0.05	48	9.5	7.5	0.4
0.1	56	12.7	5.1	0.5

Note. Concentrations are μM. Numbers followed by * and ** are significantly different from controls with $p < 0.05$ and $p < 0.01$, respectively.

AVG Effects on Transformed Roots

The ethylene biosynthesis inhibitor AVG (0.1μM) had no significant effect on the number of LRs on the IPR over a 12-day subculture period (data not shown), but it did stimulate their elongation dramatically (Fig. 4). Instead, LRs on the NPR portion were significantly more numerous (2.5-fold above untreated controls on day 12; data not shown), and they also emerged earlier, on day 6 instead of day 9 (Fig. 5). The PR also elongated more rapidly in the presence of AVG (Fig. 6). In addition, in the presence of AVG the distance from the primary root apex at which the first LR emerged increased more than fourfold (26.4 ± 5.7 mm compared with 6.3 ± 1.6 mm in controls).

Ethylene Production and Biosynthesis; ACC and MACC Content

When measured by GC, basal ethylene production was approximately threefold higher in hairy roots than in normal ones (1.1 ± 0.3 and 0.3 ± 0.1 pmol g FW^{-1} h^{-1}, respectively). In both cases 10 mM ACC stimulated ethylene production; while remaining lower in N roots, ACC enhanced ethylene production in N roots more than in T ones (eightfold and fivefold, respectively). Com-

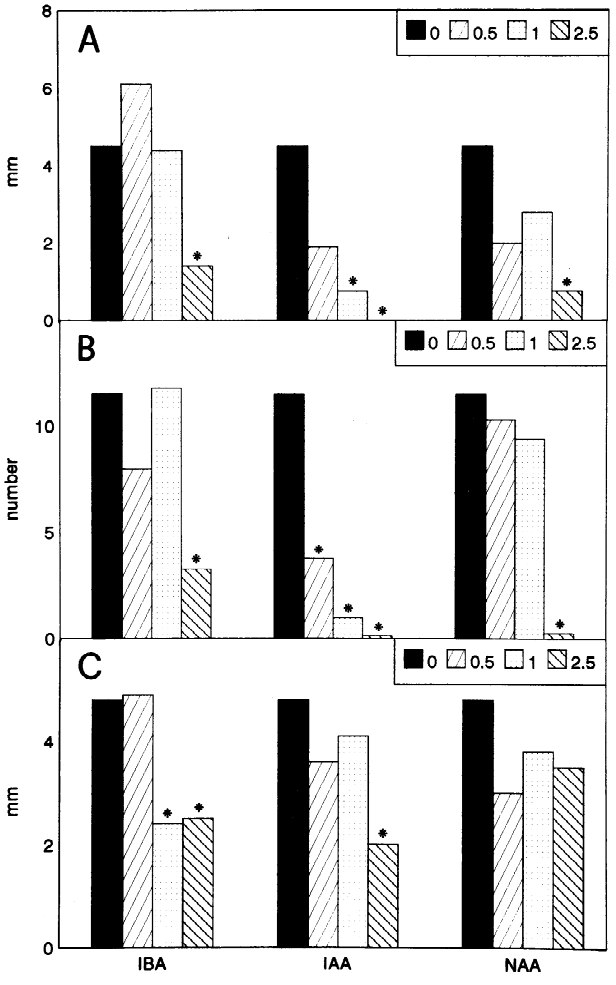

Fig. 3. Mean PR length (*A*), number of LRs/primary root (*B*), and mean LR length (*C*) in 7-day-old *H. muticus* hairy roots cultured in the presence of 0, 0.5, 1, or 2.5 μM IBA, IAA, or NAA. *Bars* with * are significantly different ($p < 0.01$) from controls

pared with untreated controls 1 μM NAA stimulated ethylene formation almost tenfold in N roots and only twofold in T roots (data not shown).

Labeled methionine incorporation into ethylene (Table 3) yielded similar results. There was a significant difference between N and T roots grown on basal medium. Furthermore, ethylene biosynthesis in roots treated with different auxins at a concentration (2.5 μM) shown to have strong effects on growth and development did not change except in NAA-treated N roots (2.5-fold increase).

ACC and its conjugated form MACC were not detectable unless roots were preincubated for 3 days with 10 μM NAA. In this case, ACC and MACC content in T roots was 5.4 and 19.3 nmol/g FW, respectively. On the other hand, treatment with 100 μM cobalt chloride or salicylic acid, both inhibitors of ACC oxidase activity,

Table 2. PR growth, LR number, and LR length in normal and transformed root cultures of *H. muticus* treated with different auxins at 1 μM concentration.

Root type and treatment	PR growth	LR number	LR length
Normal			
Control	1	1	1
IBA	1	41.7	0.58
IAA	0.20	46.7	0.35
NAA	0.27	58.3	0.55
Transformed			
Control	1	1	1
IBA	0.73	1	0.49
IAA	0.17	0.17	0.54
NAA	0.29	0.79	0.39

Note. Numbers are relative to controls (hormone-free medium) = 1.

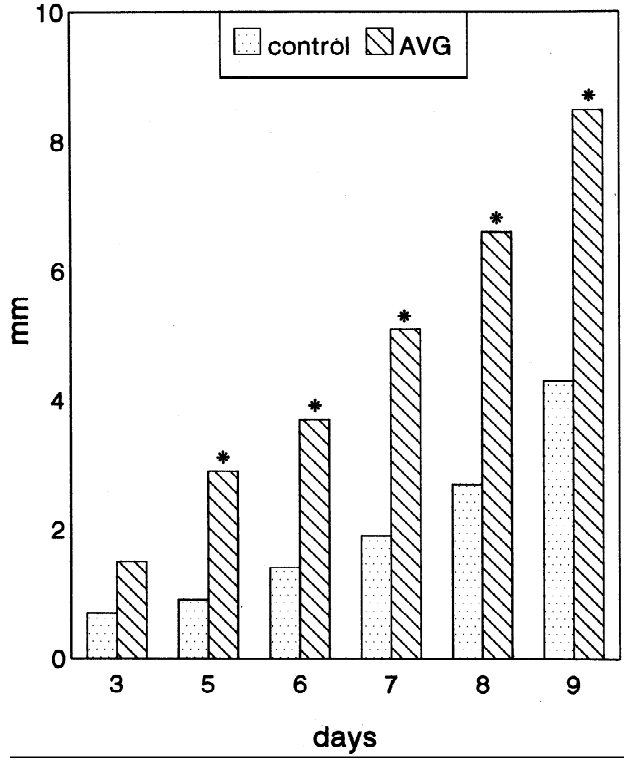

Fig. 4. Mean length of LRs on the IPR segment of *H. muticus* hairy roots cultured in the presence or absence of 0.1 μM AVG. *Bars* with * are significantly different ($p < 0.05$) from controls.

did not lead to ACC or MACC accumulation above detection levels (data not shown).

Endogenous IAA Levels and IAA Uptake

Levels of endogenous free and conjugated IAA measured 8 days after subculture were not markedly differ-

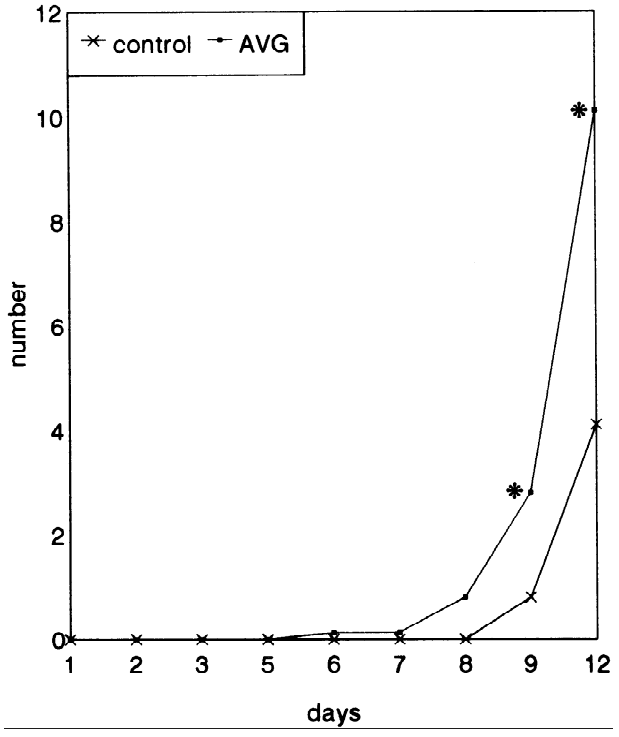

Fig. 5. Time course of the mean number of LRs on the NPR segment of *H. muticus* hairy roots cultured in the presence or absence of 0.1 μM ANG. *Asterisks* indicate values significantly different ($p < 0.05$ day 9, $p < 0.01$ day 12) from controls.

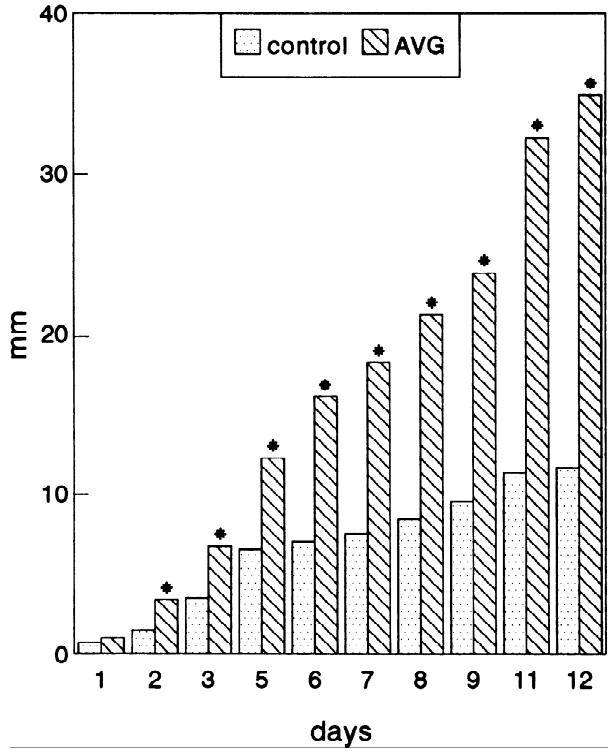

Fig. 6. Mean PR length of *H. muticus* hairy roots cultured in the presence or absence of 0.1 μM AVG. *Bars* with * are significantly different ($p < 0.05$) from controls.

ent: 7.5 (free), 22.9 (conjugated) ng/g FW and 5.6 (free), 16.8 (conjugated) ng/g FW for N and T roots, respectively. On day 15 these values did not change significantly except for conjugated IAA in N roots, which fell to 8 ng/g FW and free IAA in T roots, which rose to 9.2 ng/g FW. The latter values were significantly different from the respective ones measured on day 8.

When the younger portion (NPR and LRs) excised from 10-day-old T roots was incubated in [^{3}H]IAA-containing medium, total extractable radioactivity after 1 h amounted to 292,975 ± 28,500 dpm/g FW. This value was similar to the radioactivity extracted from N roots (226,762 ± 47,000 dpm/g FW).

Table 3. Ethylene biosynthesis (pmol g FW^{-1} 6 h^{-1}) in 8-day-old normal and transformed roots of *H. muticus* treated or not with 2.5 μM IAA, IBA, or NAA.

Treatment	Normal	Transformed
Control	5.0 ± 0.6	14.4 ± 3.1
IAA	5.9 ± 1.4	11.7 ± 2.9
IBA	6.1 ± 0.6	9.9 ± 0.9
NAA	12.4 ± 2.1	12.8 ± 3.1

Note. Values are means ± S.D.

Discussion

Roots are a useful system in which to study auxin action because of their morphologic simplicity and well characterized auxin responses. They generally respond to applied auxin by increased LR formation (branching) and reduced elongation. These are complex, long term responses based, on the one hand, on pericycle cell dedifferentiation and initiation of LR primordia, and on the other, by LR growth through repeated cell divisions and then cell elongation. Exogenous auxin promotes the formation of LRs apparently by stimulating cell division in the pericycle (Webster and Radin 1972). Instead, in the root apex meristematic activity is inhibited by exogenously supplied auxin (Zeadan and MacLeod 1984). In fact, root elongation is extremely sensitive to auxin, and thus screening for reduced inhibition of root elongation has been used to isolate auxin response mutants in *Arabidopsis*.

In N roots of *H. muticus* applied auxins strongly enhanced LR formation at concentrations that inhibited PR elongation. In T roots only a low concentration of IAA slightly stimulated LR number but was substantially

without effect on PR growth. That the response of N and T roots strongly differs mainly with respect to LR number but that it is similar with respect to PR growth and mean LR length emerges clearly when comparing the effects of the same concentration of auxin in the two root lines. Indeed, a recent study by Hobbie and Estelle (1995) using auxin response mutants led the authors to conclude that the complexity of the genetic interactions observed indicated that there may be differences in the mechanism of action during root elongation and the formation of LRs.

Most studies indicate no promotion of root elongation at any concentration of auxin (Mulkey et al. 1982), but stimulation, albeit at very low doses (about 10^{-11} M IAA), has been reported in a few cases (Evans et al. 1994). This trend is confirmed in N roots of *H. muticus* insofar as PR growth is significantly retarded by IAA even at rather low doses. Unexpectedly, however, transformed PR growth was not affected by IAA, except in the higher concentration range. Similarly, NAA also inhibited growth of N roots at concentrations lower than in T roots. It would seem as though T roots have a reduced responsiveness to exogenously supplied IAA and NAA in terms of inhibition of PR growth. Given that these results probably cannot be justified on the basis of a different auxin content or uptake capacity, this fact may be related to an altered apical meristem activity in T roots compared with N ones. Indeed, control T roots elongate more slowly than their nontransformed counterparts, and recent histologic evidence supports the notion of an altered cytologic pattern in the T root apex (Bitonti et al. 1996).

It is also noteworthy that IBA hardly ever affected PR elongation and in some cases even tended to enhance it (in T roots at 0.01 μM and in N roots at 0.5 μM). Few studies have compared the response to different auxins. Using horseradish hairy roots Nakashimada et al. (1994) studied the effect of IAA, 2.4-dichlorophenoxyacetic acid, and NAA on the kinetics of root growth. All three auxins increased the number of root apices with NAA being the most effective even at very high concentrations (approximately 5 μM). Elongation was of course strongly inhibited and almost totally blocked by 0.5 mg/liter (approximately 2.5 μM) NAA. Thus, compared with horseradish, *H. muticus* hairy roots respond similarly to NAA with regard to PR elongation, although IAA was more effective, but not with regard to LR formation, which was not enhanced by auxin except at very low concentration of IAA.

In *H. muticus,* IBA was the only auxin that continued to exert a stimulatory effect on LR number at the highest concentration (N roots) and the least effective auxin in suppressing it (T roots). Its effect on PR elongation was always the least inhibitory and in some cases even stimulatory. Instead, IAA was generally the most effective auxin in terms of LR suppression (T roots) or inhibition of PR growth (N and T roots). These data may suggest that, as reported for the induction of adventitious roots (Alvarez et al. 1989, Baraldi et al. 1995), the efficiency of exogenous IBA depends on its conversion to IAA.

Mannopine-type strains of *A. rhizogenes* confer an increased auxin sensitivity to the plant tissue (Shen et al. 1988, Spanò et al. 1988) but, since no auxin synthesis genes are transferred by these strains, transformation does not lead to elevated auxin levels (Cardarelli et al. 1985). According to Filippini et al. (1994) the increased sensitivity to auxin can be attributed to *rolB* alone, possibly in relation to an altered auxin reception/transduction mechanism. On the contrary, tissues transformed with the complete (T_L + T_R) DNA of agropine-type strains (such as strain A4), in addition to being hypersensitive to auxin, ought to have higher auxin levels. For example, the free IAA content in transformed (strain HRI) and nontransformed roots of *Duboisia* was not markedly different but nevertheless twofold higher in the former (Deno et al. 1987). On the other hand, Epstein et al. (1991) analyzed IAA content in hairy roots induced on carrot discs by strain A4 and reported that these contained significantly greater amounts of the hormone compared with control carrot discs. The levels of free IAA in N and T roots of *H. muticus* are comparatively low. They are, however, in line with data published by Schaerer and Pilet (1993) where long term root cultures of *Pisum sativum,* rather than newly initiated ones, were studied. In spite of their growth rate and prolific branching, all hairy root lines except one had lower IAA levels than intact primary roots. The line with auxin levels comparable to intact controls displayed a peculiar phenotype, namely abundant formation of calli. The authors concluded that extensive secondary root proliferation typical of hairy roots cannot be attributed merely to enhanced IAA content. This seems to be the case also with *H. muticus* root cultures.

Yet, as suggested by Arroo et al. (1995), in one way or another auxin appears to be the main factor controlling growth and morphology in hairy roots. Given that intracellular free and conjugated IAA levels as well as IAA uptake capacity are comparable in the two *H. muticus* root lines, the response of T roots to added auxin compared with that of N roots may be indicative of a greater sensitivity to the hormone as suggested by the following: (1) PR growth stimulated by IBA at 0.01 μM (T) and 0.5 μM (N); (2) LR number enhanced by IAA at 0.01 μM (T) and up to 1 μM (N). However, differences in compartmentation, metabolism, or conjugation of the hormone cannot be excluded, as suggested by the decline in conjugated IAA and the increase in free IAA observed in N and T roots, respectively, late in culture (day 15).

Auxin and ethylene are two closely related hormones, mainly because the first stimulates synthesis of the second. In addition, some auxin response mutants have altered responses to ethylene and abscisic acid (Wilson et

al. 1990). The higher ethylene production in T roots lends support to the idea that they are in a sort of ''hyperauxinic'' state, whereas in N roots ethylene synthesis, as well as overall growth and development, are optimized by supplying exogenous auxin. Despite considerable differences between the effects on growth of the different auxins even at relatively low concentrations, such as 1 and 2.5 μM, IAA and IBA at the latter concentration did not alter basal ethylene biosynthesis measured as labeled methionine incorporation; however, using either method, NAA-stimulated ethylene formation was observed in N roots. These results suggest that the auxin effects are generally not mediated by ethylene overproduction.

Primary root elongation is generally inhibited by ethylene, but LR elongation and root hair production can be increased (Crossett and Campbell 1975). In this study, the inhibition of endogenous ethylene production by AVG stimulated both PR and LR elongation, whereas the complete absence of root hairs in T roots of *H. muticus* treated with 100 μM silver thiosulfate, an ethylene antagonist, was reported previously (Biondi et al. 1995). Ethylene inhibits longitudinal auxin transport in the shoot and lateral auxin transport in roots (Lee et al. 1990); inhibition of root elongation by ethylene could thus derive from accumulation of auxin to high levels in certain cells, possibly the apical (meristematic?) ones. Conversely, exogenously supplied auxin may inhibit meristematic activity in the root apex by inducing supraoptimal ethylene production since it is reported to be already highest in that region (Finlayson et al. 1996). Thus, elevated ethylene levels correlate with high meristematic activity. This supports the notion that AVG acted by reducing the retarding effect of this hormone on elongation rather than by stimulating primordia initiation. In addition, since adequate cellular polyamine levels are necessary to support root growth and LR formation (Biondi et al. 1995), the well known relationship between these amines and ethylene may also play a role in the response to AVG.

Zeadan and MacLeod (1984) suggested that one or more inhibitors of LR emergence originate in the root apex and that laterals emerge when proliferation in that region is either depressed or at some distance from the apex. Thus, the faster the root lengthens, the greater the distance between the apex and the first LR (Zeadan 1982). In fact, in rapidly elongating AVG-treated *H. muticus* hairy roots, the distance between the tip and the point of LR emergence increased dramatically.

In conclusion, it seems likely that the control of growth and development in hairy roots, as opposed to *A. tumefaciens*-derived tumors, involves factors other than IAA overproduction. Hypersensitivity to auxin seems to be one of them. Nevertheless, many questions remain unanswered because of the complexity of the system. This complexity would appear to derive from the many interactions between tDNA genes and auxin. For instance, auxin is necessary for *aux* gene expression, and the level of expression is correlated with its concentration (Gaudin and Jouanin 1995). Thus even if initial endogenous content and uptake capacity are the same, treatment of T roots with a given concentration of auxin results in higher endogenous levels than in N roots because of biosynthetic activity. The *rolB* promoter also appears to be auxin regulated (Maurel et al. 1990). On the other hand, *rolA*, *B*, and *C* genes have been shown to depress the free IAA pool in T roots containing the *aux* genes (Gartland et al. 1991).

Finally, it has also been suggested that the different reactivity of transformed tissues may derive from an acceleration of the transduction chain following perception of the auxinic signal (Julliard et al. 1992). A more precise interpretation of ours and similar data will be possible when more detailed information is available on the *rol* gene products and on signal transduction mechanisms.

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