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HABITUATION OF PLANT CELLS DOES NOT MEAN INSENSITIVITY TO PLANT GROWTH REGULATORS

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SUMMARY

Fully habituated organogenic and nonorganogenic sugarbeet calluses reacted to application of the synthetic auxin [3-benzo(b) selenienyl] acetic acid by changes in growth and ethylene production. Treatment of fully habituated cells of periwinkle with 2,4-dichlorophenoxyacetic acid led to the decrease of free cytokinin contents (isopentenyl adenine, zeatin riboside, and zeatin) during the late exponential phase of growth. The polyamine contents were also modified and the capacity to biotransform secologanin into ajmalicine was decreased. Treatment of the habituated periwinkle cells with zeatin greatly increased the amount of a polypeptide of 16 kDa; this response was more marked than that displayed by the auxin-dependent line. These data show that hormone-independent calluses and cell suspensions can retain some sensitivity to growth hormones. However, differences of responses were observed between the auxin-dependent lines and the habituated lines.

Key words: Beta vulgaris; *Catharanthus roseus*; ethylene; habituation; periwinkle; plant growth regulators; polyamines; proteins; sugarbeet; 2D-PAGE.

INTRODUCTION

In 1942, Gautheret reported that some strains of carrot gradually lost their requirement for exogenous auxin. He called the phenomenon "accoutumance à l'auxine," and later "anergie à l'auxine," which was further translated as "auxin habituation" (Braun and Morel, 1950; White, 1951; Gautheret, 1955). Requirements for exogenous cytokinins and vitamins can be lost as well (Gautheret, 1955; Street, 1969; Ikeda et al., 1979). Today, habituation is defined as a stable heritable loss in the requirement of cultured plant cells for growth hormones (Butcher, 1977; Meins et al., 1982, 1989). The cause of such an autonomy for growth substances remains unknown. It might be due to either: (a) increased biosynthesis of the growth substances; (b) decrease in their rate of degradation; (c) altered sensitivity of the cells to the growth substances; or (d) interaction of some or all of these (Christou, 1987; Jackson and Lyndon, 1990). Although early studies have reported that habituated tissues accumulate higher amounts of auxins (Kulescha and Gautheret, 1948; Kulescha, 1952; Smith, 1972), this was never fully confirmed (Nakajima et al., 1979; Everett, 1981; Kevers et al., 1981; Kutacek et al., 1981; Pengelly and Meins, 1982; Mousdale et al., 1985; Chen, 1987; Campell and Town, 1991; Szabò et al., 1994). Bishop (1987) proposed that the loss of growth control in plant or animal neoplasia is due either to the increased endogenous accumulation of growth factors, or to the activation and expression of genes bypassing the requirement for specific growth factors. Campell and Town (1991) found an altered hormone balance in some autonomous phenotypes only. A similar debate has concerned the reversibility of the phenomenon of habituation and the loss of organogenic totipotency (Nanney, 1958; Meins, 1982; Hervagault et al., 1991; Syono and Fujita, 1994). Habituation should be reversible if due to epigenetic changes that leave the heritable altered cells totipotent (Meins, 1982). It should be irreversible if it results from mutations affecting proto-oncogenes (Bishop, 1987; Hagège, 1993). Much of the discrepancies can be explained when considering that the autonomous lines were habituated at different levels and/or degrees, and that habituation takes part of neoplastic progression leading to a complete loss of totipotency (Gaspar et al., 1991, 1995). Within this context, the present work contributes to give some responses of partially and fully habituated plant cell lines to exogenously applied growth hormones. We show that hormone-independent sugarbeet calluses, as well as periwinkle cell suspensions, retain some sensitivity to applied plant growth hormones.

MATERIALS AND METHODS

Plant material. Experimental conditions for obtaining and maintaining normal (N), habituated organogenic (HO), and habituated nonorganogenic (HNO) callus cultures of sugarbeet (*Beta vulgaris* L., subsp. *altissima*) have been reported elsewhere (Kevers et al., 1981). The calluses were subcultured every 2 wk on hormone-free medium (habituated lines) or on medium containing 0.1 mg l^{-1} (0.45 μM) 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg l^{-1} (0.44 μM) benzylaminopurine (normal lines). Some morphological and biochemical characteristics of these lines have previously been described (Kev-

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TABLE 1

EFFECT OF THE AUXIN BSSA (0.1 MG l^{-1}) ON GROWTH AND ETHYLENE PRODUCTION OF N, HO, AND HNO SUGARBEET TISSUES (GROWN WITH OR WITHOUT ACC). THE AUXIN 2,4-D USED IN THE MAINTENANCE MEDIUM OF N CALLUS WAS REPLACED BY BSAA IN THIS EXPERIMENT. RELATIVE GROWTH IS EXPRESSED BY THE RATIO : (FINAL FW-INITIAL FW)/INITIAL FW. ETHYLENE PRODUCTION IS EXPRESSED IN nL (g FW)$^{-1}$ DAY^{-1}. DATA ARE THE MEAN OF FIVE REPLICATES ± STANDARD DEVIATION [a]

	Days	N		HO		HNO	
		2,4D	BSAA	–	BSAA	–	BSAA
Growth	14	1.67 ± 0.13	1.75 ± 0.15	1.46 ± 0.11	1.48 ± 0.09	1.62 ± 0.11	2.38 ± 0.21
	21	2.37 ± 0.16	3.46 ± 0.31	2.68 ± 0.24	3.81 ± 0.27	2.65 ± 0.15	1.22 ± 0.08
Ethylene emission (without ACC)	14	189 ± 4	21 ± 1	1.25 ± 0.11	1.21 ± 0.14	4.95 ± 0.21	7.22 ± 0.45
	21	84 ± 2	14 ± 1	4.60 ± 0.24	5.01 ± 0.43	4.44 ± 0.39	3.96 ± 0.44
Ethylene emission (with ACC)	14	527 ± 49	295 ± 15	317 ± 23	168 ± 12	37.3 ± 2.9	50.7 ± 2.1
	21	345 ± 21	117 ± 10	83 ± 10	84 ± 12	43.6 ± 4.4	43.5 ± 3.9

[a] BSAA = [3-benzo (b) selenienyl] acetic acid; N = normal; HO = habituated organogenic; HNO = habituated nonorganogenic; ACC = amins aminocyclopropane-1-carboxylic acid; 2,4-D = 2,4-dicholorophenoxyacetic acid; FW = fresh weight.

ers et al., 1981, 1982; Gaspar et al., 1988, 1991; Hagège, 1990; Hagège et al., 1990a, 1992a; Crèvecoeur et al., 1992; Le Dily et al., 1993; Gaspar, 1995).

The 2,4-D-dependent line (C20) and the fully habituated line (C20A) of periwinkle [*Catharanthus roseus* (L.) G. Don] were maintained as suspension cell cultures on a 7-d growth cycle regime, in 250 ml Erlenmeyer flasks containing Gamborg et al.'s (1968) medium with 1 mg l^{-1} (4.5 μ*M*) 2,4-D (C20 line), or without any hormone (C20A line). Data concerning some biochemical characteristics of these lines have been presented in Mérillon et al. (1989, 1993, 1995a, 1995b).

Methodology of growth experiments. The effect of [3-benzo(b) selenienyl] acetic acid BSAA on growth of *Beta vulgaris* calluses was studied by introducing this new powerful synthetic auxin (Gaspar, 1995) in the culture media (HO and HNO calluses) or by replacing 2,4-D by it (N callus) at the same concentration (0.1 mg l^{-1}, 0.42 μ*M*). For experiments continuing beyond Day 14, the calluses were left on the same medium without subculture. Callus growth was expressed by relative fresh weight (FW) increase: (final FW − initial FW)/initial FW. For fresh weight measurements, callus from petri dish was rapidly blotted, placed on aluminum foil, and weighed. Initial FW was calculated by the difference between full and empty petri dishes at the time of subcultures. The measurements were repeated at least three times on different series.

C. roseus cells were grown in Gamborg et al.'s (1968) medium containing or not 2,4-D for one passage. Zeatin 1.2 mg l^{-1} (5 μ*M*) and/or various concentrations of secologanin were added on the third day of culture. The 2,4-D, zeatin, and secologanin solutions were sterilized by filtration (0.22 μm, Millipore Corp., Bedford, MA 01730, U.S.A.). The cells were harvested on the indicated days (*see* below) by filtration under reduced vacuum. Weighed cells were immediately frozen in liquid nitrogen, then stored at −80° C until use (for hormone measurements and protein electrophoreses), or freeze-dried (for polyamine measurements and indole alkaloid quantitation). Three experimental flasks containing 50 ml of medium were used per condition.

Measurement of auxin and cytokinin contents. Indole-3-acetic acid, zeatin, zeatin riboside, and isopentenyladenosine were quantitatively analyzed according to Jemmali et al. (1995).

Measurement of ethylene and polyamine contents. The capacity of sugarbeet calluses to produce ethylene without added aminocyclopropane-1-carboxylic acid (ACC) or to transform ACC into ethylene was investigated in calluses grown in the presence or absence of BSAA. Fourteen- or 21-d-old calluses were placed on disc filters (20 mm diameter) imbibed with 1 ml MOPS (3-[N-Morpholino] propanesulfonic acid) buffer (25 m*M*, pH 6.1) containing or not 1 m*M* ACC and/or 0.1 mg l^{-1} (0.42 μ*M*) BSAA. The filters were put into 25-ml glass vials sealed with a serum cap. After 24-h incubation at 25° C, the ethylene content was determined by gas chromatography in 1-ml gas samples of internal atmosphere (Hagège et al., 1991).

The accumulation of polyamines was measured in periwinkle cells grown in medium containing or not 1 mg l^{-1} 2,4-D. The periwinkle cells were harvested on the 3rd, 6th, and 10th days. The procedure of Walters and Geuns (1987) was used with some modifications to quantitate the polyamines. Ten mg of freeze-dried material was homogenized in 1 ml 4% perchloric acid containing 1,7-diamino heptane-2 HCl (2 mg l^{-1}) as internal standard. After 1 h at 4° C, the homogenate was centrifuged and 50 μl of a solution of dansyl chloride (10 mg per ml of acetone) was added. After vortexing, the mixture was heated at 60° C (dark) during 15 min. The dansylated polyamines were extracted in 0.6 ml toluol to which 0.6 ml hexane was added to lower the polarity in order to get a quantitative absorption on silicagel purification columns. They were made of 250-ml toluol/triethylamine (10/0.3 vol/vol). They were sucked dry and the dansylated polyamines were eluted with 2 × 0.3 ml ethylacetate. The solvent was evaporated in a vacuum centrifuge and the residue was dissolved in 250 μl MeOH of which 10 μl were injected in a fully automated HPLC system. Columns: 10 cm long × 3 mm int. diam., 5 μm ODS; solvent: 1.5 ml·min^{-1}; gradient: 0–0.5 min: 58% Ac-CN; 0.5–4CN. Analysis time: 7.5 min. Detection: fluorescence detector (excit: 340 nm; em.: 510 nm).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Frozen periwinkle cells (400 mg) were homogenized in the so-called 2D-MH extraction buffer of Mayer et al. (1987), using a motor-driven homogenizer. Homogenates transferred to microtubes were treated with protamine (1.5 mg ml^{-1}), agitated on gyratory shaker for 10 min at room temperature, and microfuged at 15 000 ×g for 15 min. The supernatants were adjusted to 9 *M* urea. Aliquots were stored at −80° C until use. Protein contents were determined according to the Bradford's (1976) procedure modified by Ramagli and Rodriguez (1985). Bovine serum albumin dissolved in extraction buffer adjusted to 9 *M* urea was used as standard. 2D-PAGE was carried out with a minielectrophoresis system (Miniprotean II, Bio-Rad Laboratories) as described in Ouelhazi et al. (1993). Aliquots of 10–20 μg of proteins were fractionated using 12% polyacrylamide mini-slab gels. After electrophoresis, the gels were fixed in methanol-acetic acid-water (40/10/5, vol/vol/vol), then silver stained (Oakley et al., 1980). The whole experiment (treatment of the cells, protein extraction, and separation) was repeated three times, and each 2D-PAGE was performed with at least two replicates of each single sample.

Quantitation of indole alkaloids. The ability of the fully habituated cells to biotransform secologanin into indole alkaloids was studied in the presence or absence of 1 mg l^{-1} 2,4-D and/or 1.2 mg l^{-1} zeatin. The alkaloids were extracted by methanol from aliquots of 100 mg freeze-dried cells, and separated by thin layer chromatography as previously described (Mérillon et al., 1989). Contents in ajmalicine (taken as a marker of alkaloid accumulation) were determined by spectrofluorometry.

Results

Effect of the auxin BSAA on normal and habituated tissues of sugarbeet. The effect of BSAA on the growth of normal (N), habituated organogenic (HO), and habituated nonorganogenic (HNO) sugarbeet calluses is given in Table 1. BSAA is an indole 3-acetic acid analog in which a selenium atom has replaced the nitrogen (Gaspar, 1995). This powerful synthetic auxin had no effect on N and HO lines at Day 14 but enhanced the growth of these two calluses at Day 21.

TABLE 2

CYTOKININ AND AUXIN CONTENTS IN THE 2,4-D-DEPENDENT LINE (C20) AND FULLY HABITUATED LINE (C20A) OF PERIWINKLE, AND EFFECT OF 2,4-D ADDITION ON THEIR HORMONAL CONTENTS. THE CELLS WERE SUBCULTURED IN GAMBORG ET AL.'S (1968) MEDIUM CONTAINING OR NOT 4.5 μ*M* 2,4-D AND HARVESTED ON THE 3RD AND 5TH DAY (HORMONE CONTENTS) OR ON THE 7TH DAY (CELL GROWTH). DATA ARE GIVEN AS THE MEAN OF THREE REPLICATES ± STANDARD DEVIATION[a]

Cell Line	2,4-D (μ*M*)	Day of Harvest	Cytokinin Contents (pM. g^{-1} FW)				IAA Contents (nM g^{-1} FW)	Growth (mg DW $flask^{-1}$)
			ipA	ZR	Z	Total		
C20A	0	3	5.30 ± 0.61	1.12 ± 0.19	1.38 ± 0.38	7.8	283 ± 14	495 ± 13
	0	5	19.70 ± 2.19	7.54 ± 1.35	6.95 ± 1.59	34.19	228 ± 36	
	4.5	3	5.93 ± 0.30	1.41 ± 0.21	1.35 ± 0.18	8.69	153 ± 19	442 ± 12
	4.5	5	4.10 ± 0.85	0.87 ± 0.20	0.98 ± 0.28	5.95	292 ± 32	
C20	0	3	8.94 ± 1.29	4.46 ± 0.82	4.27 ± 0.81	17.67	118 ± 23	506 ± 18
	0	5	24.12 ± 3.12	12.40 ± 1.17	4.64 ± 0.98	41.16	142 ± 22	
	4.5	3	16.49 ± 2.44	3.72 ± 0.83	6.56 ± 1.04	26.77	ND	518 ± 15
	4.5	5	21.34 ± 1.98	5.09 ± 1.17	9.34 ± 1.08	35.78	189 ± 42	

[a]2,4,D = 2,4-dichlorophenoxyacetic acid; DW = dry weight; FW = fresh weight; IAA = indole-3-acetic acid; ipA = isopentenyladenosine; ND = not determined (broken flask); Z = zeatin; ZR = zeatin riboside.

BSAA enhanced the growth of HNO calluses at Day 14, but was inhibitory at Day 21. Early appearance of necrosis zones on these BSAA-treated HNO calluses indicated the toxicity of such auxin treatments.

Table 1 also shows the effect of BSAA on the production of ethylene in the different sugarbeet cell lines and on their capacity to biotransform ACC into ethylene. The calluses were harvested on the 14th and 21st d of culture and the ethylene production was studied after 24 h incubation in the presence or absence of ACC. The auxin reduced the conversion of ACC in N calluses, and had practically no effect on the ethylene production by HO calluses (although some reduction of the capacity to convert ACC into ethylene was noticed at Day 14). By contrast, the auxin greatly increased the capacity of HNO calluses to convert ACC into ethylene and increased their ethylene production without ACC at Day 14.

Effect of the auxin 2,4-D and/or the cytokinin zeatin on the 2,4-D-dependent line (C20) and fully habituated line (C20A) of periwinkle. We have previously observed that subculturing the 2,4-D-dependent line in a 2,4-D-free medium leads to cell necrosis after several passages. However, growing these cells without 2,4-D for only one passage, or growing the fully habituated cells C20A in the presence of 2,4-D does not affect the cell growth (Mérillon et al., 1989). Each line was, therefore, grown in both media in order to distinguish changes related to habituation per se from those related to auxin treatment.

As shown in Table 2, isopentenyladenine is the main endogenous cytokinin in both lines under all culture conditions. Comparison of cytokinin amounts in cells grown in their respective maintenance medium shows a higher level at Day 5 (late exponential phase of growth) than at Day 3 (mid-exponential phase of growth). At the beginning of the culture, the habituated line accumulated lower amounts of cytokinins than did the 2,4-D-dependent line. Treating the habituated cells with 2,4-D, deeply decreased cytokinin contents at the 5th day of culture. By contrast, 2,4-D treatment of the C20 cells did not affect their auxin content at Day 5. Concerning auxin, Table 2 shows that the indole-3-acetic acid (IAA) levels were about 1.5-fold higher in the habituated line than in the 2,4-D dependent line. Treatment with 2,4-D did not affect auxin content at Day 5 in both lines.

The effect of 2,4-D on the polyamine level in both lines is shown in Fig. 1. Adding the auxin to the culture medium of the C20A cell line increased the putrescine and spermidine levels from Day 3 onwards, and the spermine levels on the 10th day of culture. On the other hand, deleting the auxin from the culture medium of the C20 cells decreased the spermidine levels, but increased the putrescine levels at Day 10, and the spermine levels at Day 3.

We also investigated by 2D-PAGE the effect of zeatin on the polypeptide profiles of C20 and C20A cells. The cytokinin was added to 2,4-D-free culture medium at Day 3 and the cells were harvested 48 h later. A few changes in the polypeptide profile were observed. As an example, we present changes in the accumulation pattern of one polypeptide (kDa = 16; pHi = 5.35) referred to as m_1 (Fig. 2). This polypeptide was scarcely detectable in the C20 cells grown in hormone-free medium, but adding 5 μ*M* zeatin to this medium slightly enhanced the polypeptide content. On the other hand, m_1 was easily noticeable in the C20A cells grown in their maintenance (hormone-free) medium; addition of zeatin greatly enhanced the m_1 level.

Lastly, we examined the effect of zeatin on the capacity of the C20 and C20A cells to biotransform the *seco*-iridoid secologanin into ajmalicine. Secologanin is not produced in the C20 cells grown in the presence of 2,4-D, but feeding this terpenoid to the cultures restores the alkaloid production (Mérillon et al., 1986). In the same way, secologanin is not produced in the C20A cells (which therefore do not accumulate alkaloids); adding the terpenoid to the C20A cultures also led to ajmalicine production (Fig. 3). In both lines, addition of 2,4-D to the medium decreased the transformation of secologanin into ajmalicine. Co-addition of zeatin and 2,4-D led to a slight (but reproducible) enhancement of ajmalicine production, compared to that of cells grown without zeatin.

Discussion

Present data show that habituated sugarbeet callus tissues responded to external application of the synthetic auxin BSAA. We found that the N callus as well as the HO callus (the latter retains organogenic capacities) responded to BSAA quite similarly in their growth behavior and biochemical changes. As previously observed (Hagège et al., 1990b; Carrié et al., 1992), a growth stimulation was

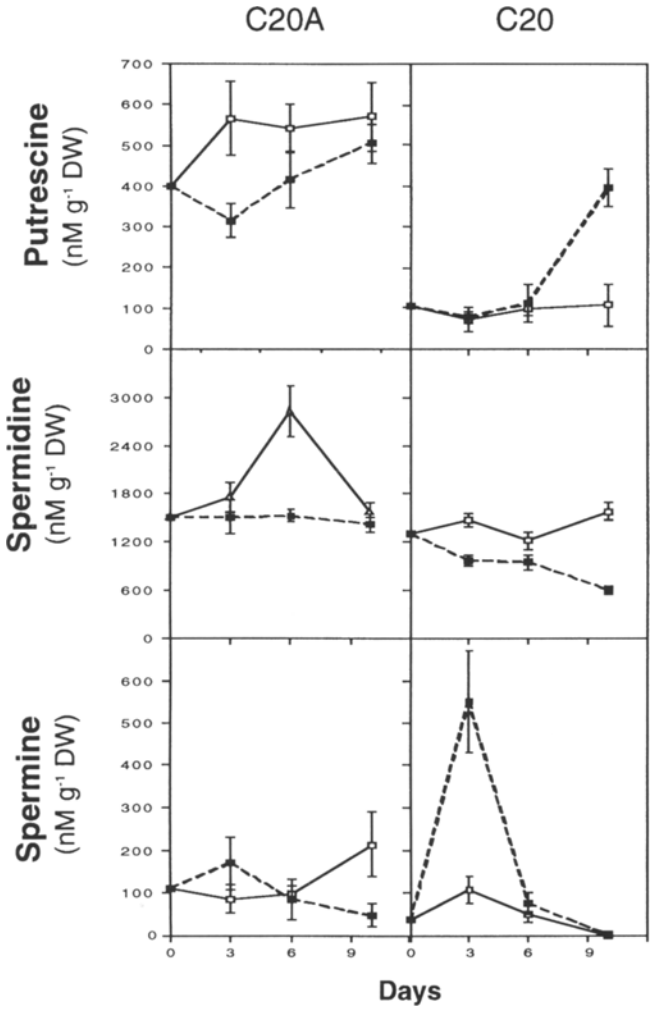

FIG. 1. Effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on polyamine levels in the 2,4-D-dependent line (C20) and the fully habituated line (C20A) of periwinkle. The auxin 2,4-D was added to the culture medium at Day 0 (final concentration: 1 mg l^{1}). The cells were harvested at Days 3, 6, and 10. Results are the mean (± standard deviation) of three replicates. —□— = with 2,4-D; --■-- = without 2,4-D; DW = dry weight.

accompanied by a lower ethylene production in both calluses. On the contrary, the HNO callus, which had lost organogenic capacities and showed altered metabolism of ethylene (Hagège et al., 1991, and Table 1), nitrogen (Le Dily et al., 1993), and tetrapyrrole-containing compounds (Kevers et al., 1981; Hagège et al., 1992b), responded differently to BSAA application. The different behavior of the HNO callus has already been shown, for example through response to NaCl treatment (Hagège et al., 1990b). The calcium-uncontrolled peroxidase secretion by HNO cells in suspension cultures was shown to be unaffected by the presence of auxins, while it was enhanced in the case of N cells. However, subculturing the HNO callus for 5 wk on auxin-containing medium led to responsiveness of the cells to auxins after transferring them in liquid medium (Gaspar et al., 1983), an effect later related to a long-term effect of auxins on ethylene production in HNO cells (Kevers et al., 1985). Lastly, it was shown that applying 2,4-D to HNO cells modulated the level of auxin protectors (Kevers, 1981). All these data support the hypothesis that HNO callus has acquired a peculiar homeostatic behavior that renders it uncomparable to N and HO calluses.

We also compared the effect of auxin or cytokinin on two periwinkle cell lines, a 2,4-D-dependent line (C20) and a fully habituated line (C20A). As shown in Table 2, free cytokinin contents were about threefold higher in the C20 line, whereas free IAA contents were about twofold higher in the C20A line. It seems unlikely that this low increase in IAA level may explain the autonomous phenotype of C20A cells and other hypotheses should be examined in the future, for example, an altered sensitivity of the cells to endogenous growth hormones (Jackson and Lyndon, 1990), or the activation of dehy-

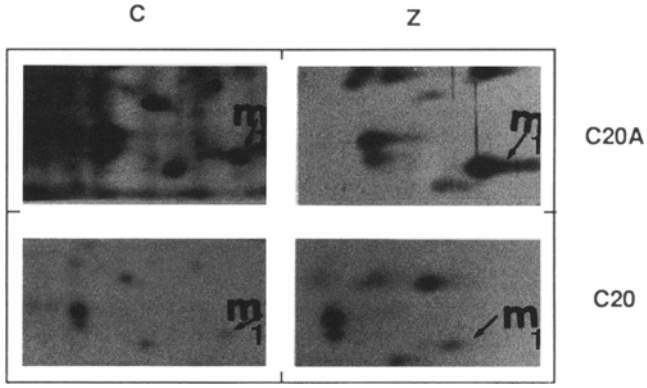

FIG. 2. Effect of zeatin on the accumulation of the polypeptide m1 (kDa = 16; pHi = 5.35) in C20 and C20A cell lines of periwinkle. The cytokinin was added (1.2 mg l^{-1}) at Day 3. The cells were harvested at Day 5. Ten µg of total proteins were loaded on IEF rods. Second dimension used 12% acrylamide mini-slab gel (1.0 mm thick). The polypeptides were silver-stained. C = cells without zeatin in a 2,4-dichlorophenoxyacetic acid (2,4-D) free medium; Z = cells grown with zeatin.

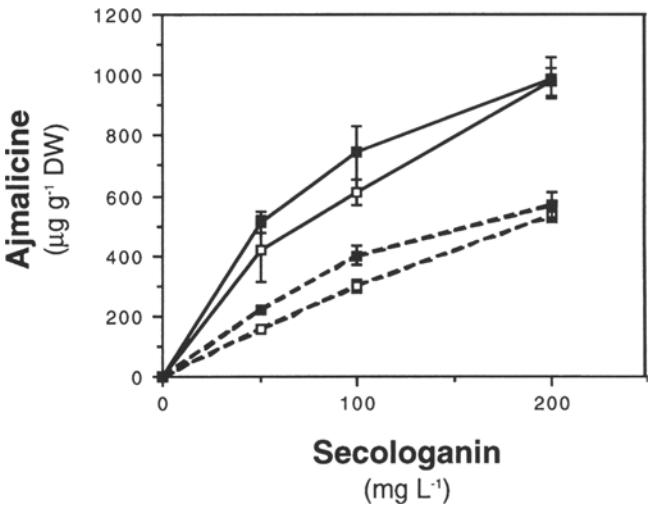

FIG. 3. Effect of 1.2 mg l^{-1} zeatin and/or 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) on the capacities of C20A cells of periwinkle to transform secologanin into ajmalicine. 2,4-D was added at Day 0; zeatin and secologanin were added at Day 3. The cells were harvested at Day 7. All data are the mean (± standard deviation) of three replicates and give the amounts in ajmalicine in µg per g of dry weight (DW). Cells grown without 2,4-D (——) or with 2,4-D (------) in presence (■) or absence (□) of zeatin.

drodiconiferylalcohol glucosides and/or other signaling molecules stimulating cell divisions (Binns et al., 1992). Adding 2,4-D to the medium of C20A cells deeply decreased the amounts of all endogenous-free cytokinins (including isopentenyl adenosine, the first one of the pathway) during the late exponential phase of growth. In contrast, this auxin did not change the cytokinin contents of C20A cells. The promotive or inhibitory effect of exogenously applied auxins on the metabolism of endogenous cytokinins has been reported (Hansen et al., 1987; Vankova et al., 1992). Our data probably reflect a high sensitivity of C20A cells to 2,4-D; a senescencing effect of this auxin may also be hypothesized because C20A cell growth was slightly decreased on Day 7 (Table 2). 2,4-D also affected the polyamine contents of both lines (even though differences were noticeable between the lines; Fig. 1), as well as the ability of C20A cells to transform secologanin into ajmalicine (Fig. 3). Concerning cytokinin, we previously reported that treatment of C20 cells by zeatin greatly enhanced their alkaloid production (Décendit et al., 1992), whereas treatment of C20A cells did not induce any alkaloid synthesis (Ouelhazi et al., 1993). As shown in Fig. 2, zeatin increased the amount of a 16 kDa polypeptide in both lines. All these results show that fully habituated cells of periwinkle can retain some sensitivity to applied growth hormones in spite of some differences in the responses of the lines.

That habituated tissues may respond to external application of plant growth regulators has already been noticed by a few authors. Syono and Furuya (1974) reported that an auxin-independent culture of tobacco could apparently be reverted into auxin-dependent culture by a treatment with auxin. In the same way, shoot regeneration was obtained by treating sugarbeet autonomous callus with cytokinin (Saunders and Daub, 1984). Everett (1981) was unable to reverse 2,4-D-independent cells by 2,4-D treatment, but it was also found that sensitivity to 2,4-D increased in some of the independent clones. Similar data were obtained by Kevers et al. (1985) with sugarbeet tissues and by Ramawat et al. (1987) with *Ruta graveolens* tissues. An interesting result has been reported by Campell and Town (1991): the authors produced several autonomous tissue lines from γ-irradiated *Arabidopsis thaliana* seeds: each habituated tissue displayed a unique set of responses to exogenously applied growth regulators, suggesting that a different event led to the formation of each habituated line (Persinger and Town, 1991) but at present such a hypothesis has not been proven.

Above results show that both HNO sugarbeet line and fully habituated periwinkle C20A cells have acquired peculiar physiological and biochemical characteristics that may change their response to growth hormones. Some modification in hormone signal transduction pathways can be hypothesized. There are evidences that cell membranes are one of the targets for biochemical changes connected to habituation (Hervagault et al., 1991), and marked changes in both lipid and protein composition as those recently reported in C20A cells (Mérillon et al., 1995a), may accompany changes in the physiological properties of several receptors, including those for growth hormones. In this context, we recently found that cell habituation in periwinkle led to a decrease of both the dissociation constants and the number of binding sites to verapamil, a calcium channel blocker belonging to the phenylalkylamine type (Mérillon et al., 1995b).

In conclusion, our data shows that habituated tissues in spite of their autonomous growth may conserve some sensitivity to exogenously applied growth regulators. Investigations on the degree of saturation of auxin- or cytokinin-binding sites for natural or synthetic hormones, as well as studies on the regulation of growth regulator-binding sites by external applied hormones should be of interest for a better knowledge of habituation processes.

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