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HORMONAL INDUCTION AND ANTIHORMONAL INHIBITION OF TRACHEARY ELEMENT DIFFERENTIATION IN *ZINNIA* CELL CULTURES*

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Zinnia elegans*; Asteraceae; cell suspension cultures; differentiation; tracheary element.

Abstract—Mechanically isolated mesophyll cells of *Zinnia elegans* L. cv Envy differentiate to tracheary elements when cultured in inductive medium containing sufficient auxin and cytokinin. Tracheary element differentiation was induced by the three auxins (α-naphthaleneacetic acid, indole-3-acetic acid, and 2,4-dichlorophenoxyacetic acid) and four cytokinins (6-benzyladenine, kinetin, 2-isopentenyladenine and zeatin) tested. Tracheary element formation is inhibited or delayed if the inductive medium is supplemented with an anticytokinin, antiauxin, or inhibitor of auxin transport.

INTRODUCTION

The hormonal control of tracheary element (TE) differentiation has been reported for many *in vitro* systems [1, 2]. In the *Zinnia* system, mechanically isolated leaf mesophyll cells are induced to differentiate by treatment with an auxin-cytokinin combination such as α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) [3] or 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kn) [4]. Neither mitosis nor a full round of DNA synthesis is necessary for such differentiation of isolated *Zinnia* mesophyll cells into TEs, and a high percentage of the cells differentiate without prior cell division [5–7]. Hormonal influences on TE differentiation in this system are therefore independent of influences on cell division. The purpose of the present study is to test the response of cultured *Zinnia* mesophyll cells to (a) various natural and synthetic auxins and cytokinins that induce TE differentiation in other systems, and (b) antiauxins and anticytokinins that act to inhibit TE differentiation.

RESULTS AND DISCUSSION

Dose/response studies with NAA and BA

Zinnia mesophyll cells were isolated and cultured as described below. NAA and BA were included in the culture medium at 0.005 to 5.0 μM. In the presence of adequate NAA and BA, TE differentiation is complete after three days of culture; few additional cells differentiate and nearly all of the TEs have gone through the final autolytic stages associated with TE formation. Maximum TE differentiation occurred when cells were cultured with 0.5 μM NAA + 0.5 μM BA (Table 1), in contrast with 0.5 μM NAA + 5.0 μM BA reported in ref. [3].

Table 1. % TE on day 3 of culture in media containing various concentrations of NAA + BA*

	0.005	0.15	0.5	1.5	5.0 μM NAA
0.005	0	—	0	—	—
0.05	—	—	8	—	13
0.15	—	0	22	18	20
0.5	0	0	**53**	34	17
1.5	—	0	31	**49**	10
5.0 μM BA	—	0	21	19	7

*Averaged % TE are given ($n=2$). For %TE > 0, the mean, median and maximum deviations from the values given are ±3%, ±2% and ±9%, respectively. Bold type indicates maxima.

Induction of TE differentiation by various auxins and cytokinins

Zinnia cells were cultured in media containing 0.5 μM BA and various auxins. The differential response of *Zinnia* cells to the different auxins is illustrated in Fig. 1. Two synthetic auxins, NAA and 2,4-D, and a natural auxin, indole-3-acetic acid (IAA), were tested; all three were effective in inducing the differentiation of *Zinnia* mesophyll cells to TEs. The oxidative breakdown of IAA in the culture medium may contribute to its relative inefficiency in the induction of TE differentiation [8]. Although differentiation is complete on the third day of culture in medium containing NAA or IAA, five days of culture are required for high levels of differentiation in medium containing 2,4-D (Fig. 1b).

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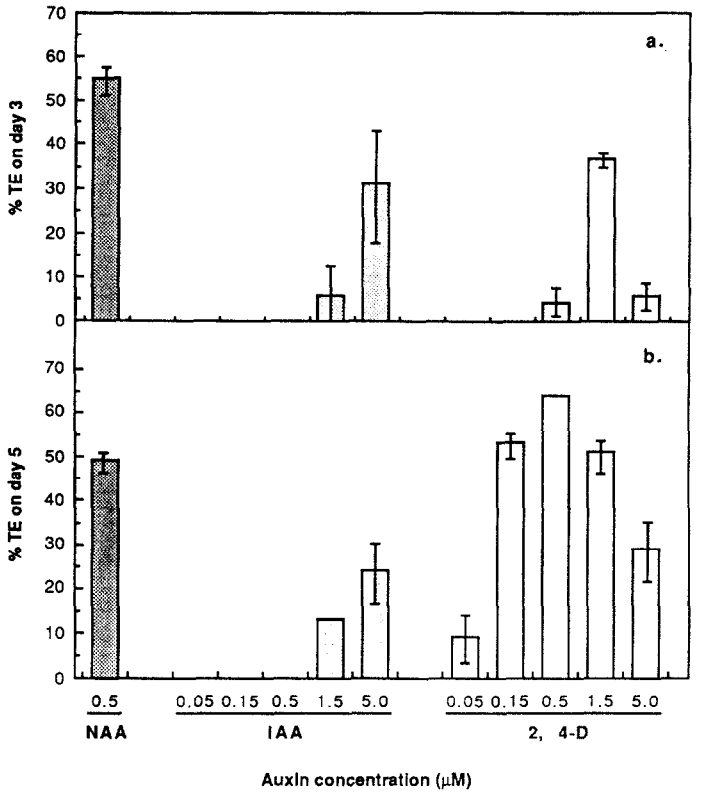


Fig. 1. Effects of auxins on TE differentiation. Cells were cultured in media containing optimal cytokinin (0.5 μM BA; see Table 1) $\pm$ NAA, IAA or 2,4-D. % TE was scored on days three and five. Averaged % TE ($n=2$) are shown from a single experiment. Error bars indicate the range of % TE at each auxin concentration.

The response of cultured *Zinnia* mesophyll cells to various cytokinins in combination with 0.5 μM NAA is shown in Fig. 2. Two natural cytokinins, zeatin (Z) and 2-isopentenyladenine (2-iP), and two synthetic cytokinins, BA and Kn, were tested. While each cytokinin induced differentiation, BA and 2-iP were especially effective at the concentrations used.

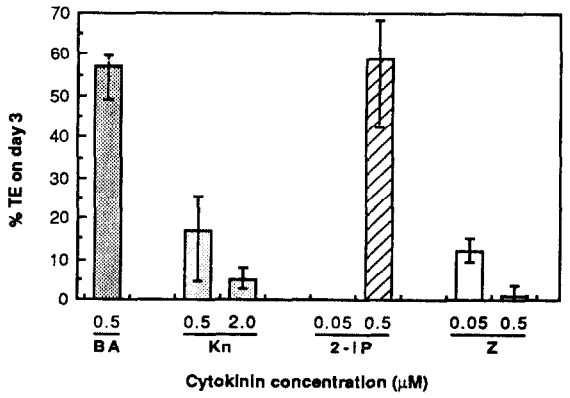


Fig. 2. Effects of cytokinins on TE differentiation. Cells were cultured in media containing optimal auxin (0.5 μM NAA; see Table 1) $\pm$ BA, kinetin (Kn), 2-isopentenyladenine (2-iP) or zeatin (Z). % TE was scored on day three. Averaged % TE ($n=4$) are shown from a single experiment. Error bars indicate the range of % TE at each cytokinin concentration.

Per cents 'viability' on day 3 {i.e., [no. live cells + no. TE/(no. dead cells + no. live cells + no. TE)] 100} were similar in all cultures, irrespective of the hormonal content of the culture medium.

Inhibition of differentiation by antiauxins and anticytokinins

The effect of antiauxins on hormone-induced TE differentiation was tested by supplementing media with two types of antiauxins: (i) structural analogues of active auxins, *p*-chlorophenoxyisobutyric acid (PCPIB) and β-naphthaleneacetic acid (β-NAA) and (ii) the auxin-transport inhibitor, 2,3,5-triiodobenzoic acid (TIBA). TIBA and PCPIB inhibit or delay differentiation when included in culture medium that normally induces TE differentiation on day three (Fig. 3a and 3b).

In the presence of 2 μM TIBA, less than 1% of the cells differentiate on day three and only moderate levels of differentiation occur by day six (Fig. 3a and 3b; see also [9]). Inhibition by 2 μM TIBA can be reversed by increasing the concentration of NAA from 0.5 to 5.0 μM (Fig. 3c); NAA concentrations between 0.5 and 5.0 μM were not tested. Although TIBA is generally known as an auxin-transport inhibitor that prevents auxin anion efflux from cells [10, 11, 12], our results suggest that it may act as a competitive inhibitor auxin binding in the *Zinnia* system. Inhibition of differentiation by TIBA could be overcome by increasing the level of NAA in the culture medium to a concentration that is supraoptimal for

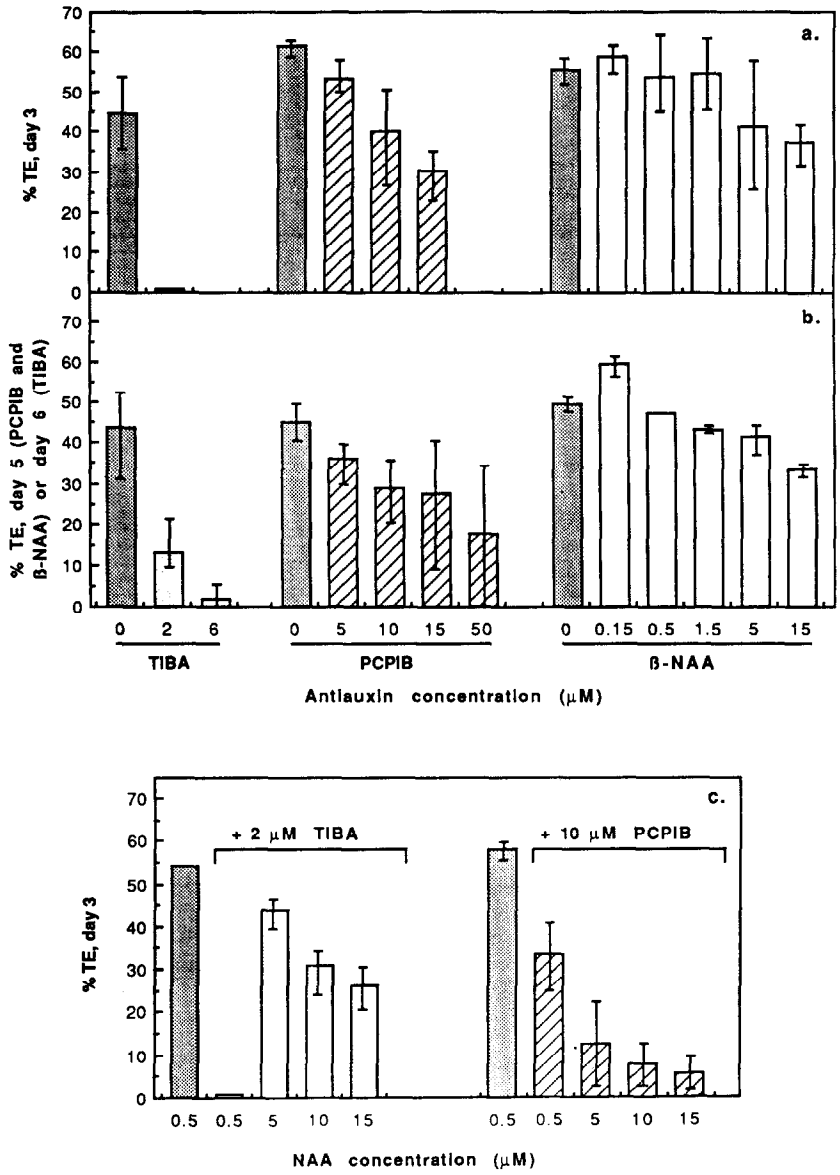
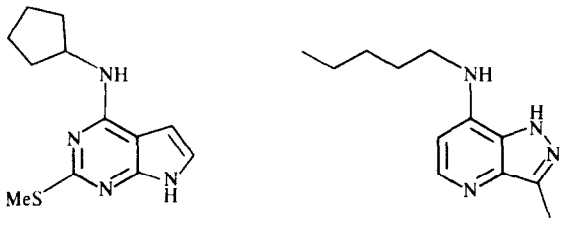


Fig. 3. Inhibition of differentiation by TIBA, PCPIB and β-NAA. Cells were cultured in media containing 0.5 μM NAA + 0.5 μM BA ± various concentrations of the antiauxins. % TE was scored on day three (Fig. 3a) and day five or six (Fig. 3b). Reversal of inhibition by TIBA using increased levels of NAA is shown in Fig. 3c. The averaged results for two, three or four independent experiments involving TIBA or PCPIB are graphed, while the averaged results of a single experiment using β-NAA are shown. Error bars indicate the range of %TE at each concentration.

differentiation in the absence of TIBA. TIBA was ineffective in inhibiting TE differentiation when added to cells previously exposed to the high levels of NAA present in the inductive medium (data not shown).

PCPIB is less effective than TIBA in inhibiting the differentiation of cultured *Zinnia* cells. Substantial TE differentiation is observed on day three if up to 15 μM PCPIB is included in medium containing 0.5 μM NAA + 0.5 μM BA. Although 50 μM PCPIB completely inhibits TE differentiation on day three (Fig. 3a), many of the inhibited cells go on to differentiate by day five (Fig. 3b). Higher NAA concentrations in culture medium containing PCPIB further inhibited TE differentiation (Fig. 3c).

β-NAA is relatively ineffective as an antiauxin (Fig. 3a) and, in fact, behaves as a weak auxin in the *Zinnia* system; 5.0 μM β-NAA can replace the 0.5 μM α-NAA required for maximum TE differentiation under standard conditions (data not shown). In the absence of α-NAA, 6 μM TIBA failed to induce TE differentiation, and higher TIBA concentrations were cytotoxic. Similarly, no TE differentiation was seen in auxin-free medium containing up to 50 μM PCPIB, although the cells remain healthy at this high antiauxin concentration.



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In addition to inhibiting TE differentiation, TIBA and PCPIB inhibit cell division (data not shown). Exogenous auxin is required for both TE differentiation and the division of cultured *Zinnia* mesophyll cells [3, 4, 13], but differentiation does not require prior mitosis [5–7]. Thus, antihormonal inhibition of TE differentiation is not dependent on the inhibition of cell division.

TE differentiation on day three was eliminated by 15 μM anticytokinin **1** (4-(cyclopentylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine) or anticytokinin **2** (3-methyl-7-*n*-pentylaminopyrazolo [4,3-*b*]pyridine) (Fig. 4a and 4b). Neither **1** nor **2** induced differentiation in the absence of BA. By day four, some cells initially inhibited by **1**, and most cells inhibited by **2**, had differentiated (Fig. 4c). Anticytokinin **1** acts as a specific competitive inhibitor of cytokinin-induced growth in the tobacco callus bioassay, based on reversal of inhibition by increased cytokinin concentrations [14, 15]. Due to the limited supply of **1** and **2** available, we did not attempt to reverse the anticytokinin inhibition of TE differentiaion by increasing the cytokinin concentration in the culture medium. Anticytokinins had no effect on the division of *Zinnia* mesophyll cells.

In conclusion, both natural and synthetic auxins and cytokinins induce TE differentiation in *Zinnia* mesophyll cell cultures. Antiauxins and anticytokinins inhibit differentiation, and might be useful in further studies of the hormonal induction of TE formation.

EXPERIMENTAL

Plant material. Seeds of *Zinnia elegans* L. cv Envy (W. Atlee Burpee Co., Warminster, PA, U.S.A) were germinated in vermiculite sub-irrigated twice daily with a 1.2 g/l solution of Hyponex (Hydroponics Chemicals Co., Copley, OH, U.S.A.). Plants were grown at 24° in a controlled environment growth room with a 16-hr photoperiod and light intensity of *ca* 20 W/m^2.

Isolation and culture of single cells. Leaf mesophyll cells were isolated from young *Zinnia* plants and cultured as previously described (Church and Galston, *Plant Physiology* in press). Briefly, first leaves from 10-day old plants were excised, surface-sterilized in NaOCl (*ca* 0.05%) for 10 min, rinsed in sterile distilled H_2O, and placed in a 0.2 M mannitol bath to reduce turgor. Cells were mechanically isolated by pressing the slightly wilted leaves against a 400 μm stainless steel mesh to release cells into hormone-free culture medium [3]. The cell suspension was poured through an 85 μm mesh, then washed in hormone-free medium. Four-ml aliquots of cell suspension (0.15×10^6 cells/ml; cells counted as described below) were cultured in 50 ml Erlenmeyer flasks. In each experiment, there were at least two culture flasks for each combination of hormones and inhibitors tested, and all experiments were repeated at least twice. Cells were shaken at 75 rpm on a 'gyrotory' shaker in the dark at 27°.

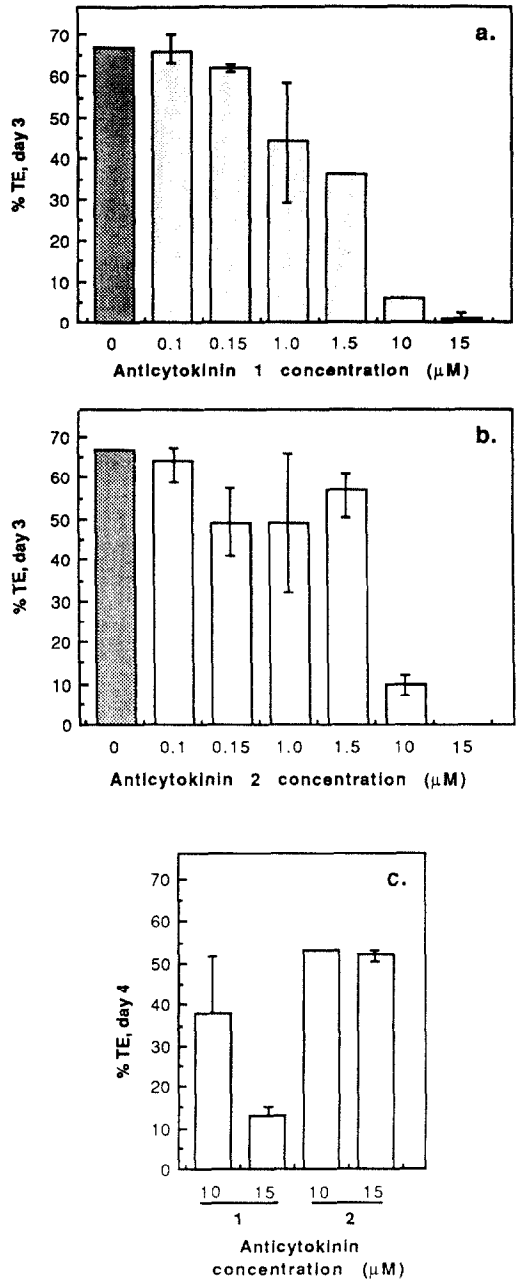

Fig. 4. Inhibition of differentiation by anticytokinins. Cells were cultured in media containing 0.5 μM NAA + 0.5 μM BA ± various concentrations of anticytokinins **1** or **2**. % TE was scored on day three (Fig. 4a and 4b) and day 4 (Fig. 4c). % TE on day four is shown only for those concentrations of anticytokinin that inhibited differentiation on day three. Error bars represent the range of % TE for the experiment illustrated.

The basal medium used was that of ref. [3]. Media were supplemented with auxins and cytokinins as indicated in the figure legends and Table 1. Inhibition of differentiation was tested freshly prepared stock solutions of antiauxins: TIBA (recrystallized twice from EtOH), PCPIB and β-NAA. All media were filter sterilized. Two anticytokinins, provided by Prof. Sidney M. Hecht (University of Virginia), were tested: 4-

cyclopentylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine (anticytokinin **1**) and 3-methyl-7-*n*-pentylaminopyrazolo[4,3-*b*]pyridine (anticytokinin **2**) [14, 15]. Anticytokinins were added directly to cell suspensions as 1 mg/ ml solns in DMSO. The final concentration of DMSO in all media used in anticytokinin experiments was 0.375%; this concentration does not inhibit differentiation.

To determine the percent differentiation at various times of culture, cells and TEs were stained with Evans Blue and counted on a hemacytometer; percent differentiation represents no. TE/no.(TE + no. live cells)] × 100.

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