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THE INFLUENCE OF PLANT HORMONES AND GROWTH FACTORS ON GROWTH OF ERIOSTEMON AUSTRALASIUS PERS. IN TISSUE CULTURE

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Abstract. Following standard disinfestation treatments, cultures of apical and axillary buds of *Eriostemon australasius* Pers can be initiated on a simple minerals-sucrose-agar medium, i e , MZZ [ZM] and rapid multiplication can be induced in cultures transfered to medium — $[MH_{Fe}]Z$ $BAP_{31\ 6\mu M}$ $[H_{4+R}M]$ Apically-dominant growth can be induced on transfer of cultures to medium — $[MH_{Fe}]M$ KINETIN $_{10\mu M}$ $[H_{4+R}M]$, and roots can be induced to form on some cultures on medium — $[MH_{Fe}]$ $NAA_{31\ 6\mu M}$ $BAP_{0\ 0316\mu M}[M_{ALL\text{-}R}M]$

Only BAP and PBA were able to induce adventitious bud formation and these cytokinins had, in common, a benzyl ring as a substituent in the N^6 position

Interactions of auxins, cytokinins, riboflavin and other growth factors in producing various growth forms in culture are discussed

INTRODUCTION

The research done by Lilien-Kipnis and de Fossard (unpublished results) was primarily aimed at finding methods for the clonal propagation of *Eriostemon australasius* Pers. It succeeded in developing a high multiplication rate, but only one experiment was done on root induction (Lilien-Kipnis and de Fossard, unpublished results). Of great interest was the induc-

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tion of numerous adventitious buds on the leaves and stems of cultures and the interaction of cytokinins, auxin and riboflavin to produce strikingly different growth forms. This paper discusses the continuation of this work and includes not only the successful rooting and establishment of tissue-cultured *E. australasius* in soil but also additional research into the role of specific cytokinins in adventitious bud formation.

Much research has attempted to define the role of various cytokinins. For high cytokinin activity, the compound must have an intact adenine nucleus and a moderately sized N^6 substituent (11). The saturation level of the substituent also influences activity (10,11). The cellular pathway for cytokinin action is unknown. An association, described in this paper, between chemical structure of substituents and morphogenetic events might lead to new insights into the initial reactions of cytokinins in the plant.

Riboflavin, a vitamin which has been used only rarely in tissue culture, had striking effects with cultures of *Eucalyptus ficifolia* F. Muell (2,3,8,9) and also with cultures of *E. australasius*. It has been suggested that riboflavin acts by sensitizing the photo-oxidation of auxin (6,4) and enzymes (5). This paper describes experiments with riboflavin, auxins and other growth factors.

MATERIALS AND METHODS

Media (Table 1) were adjusted to a pH of 5.5 and were solidified with 9g Fluka agar per litre. Hot liquid media were dispensed in 10 ml aliquots into 8 × 2.5 cm polycarbonate tubes and then autoclaved for 20 minutes. Cultures were incubated in controlled-environment chambers with a 16 hr light/8 hr dark photoperiod at 25 ± 3°C for six weeks. Data were collected either by direct measurement or using a 1-10 scoring system and these were analysed using appropriate statistical methods. Controls were used in all experiments and there were 10 replicates of each treatment.

Initiation and Multiplication. Cultures, initiated 12 months earlier by Lilien Kinis and subcultured at approximately two-month intervals, were used. The multiplication medium developed at the end of this initial work was Medium A, i.e., $[MH_{Fe}]MH[HM]$ (Table 2) and this was subsequently modified to Medium B, i.e., $[MH_{Fe}]\ MH[H_{4+R}M]$ (Table 2), by the elimination of certain growth factors.

The experiments were based on the Broad Spectrum approach (1) and the chemical compositions of various categories of constituents used to prepare the different media cited in this paper are described in Table 1.

Table 1. Composition of minerals, auxins, cytokinins and growth factor categories used to make media described in Table 2.

Constituents of Medium	Chemicals (Concentration)	
Minerals — [MH_{Fe}]		
Macronutrients [mM]	NH_4NO_3(10),KNO_3(10), $MgSO_4$ (1 5)	NaH_2PO_4(1),$CaCl_2$(2),
Micronutrients (μM)	H_3BO_3(50),$MnSO_4$(50), Na_2MoO_4 (0 1);$CoCl_2$ Na_2EDTA (100),Na_2SO_4	$ZnSO_4$(20),$CuSO_4$(0 1), (0 5),KI (2 5),$FeSO_4$(100), (650)
Auxins — M (μM)	IAA (1), IBA(1), NAA(1), NOA(1), CPA(1), 2,4-D(1)	
Cytokinins -H (μM)	Kinetin (10), BAP (10)	
Sucrose -M (mM)	Sucrose (60)	

	Growth Factors Code		
Chemicals (μM)	H	H_{4+R}	M_{ALL-R}
Inositol	600	600	300
Nicotinic Acid	40	40	20
Pyridoxine HCl	6	6	3
Thiamine HCl	40	40	2
Biotin	1	—	0 2
D-Ca-Pantothenate	5	—	1
Riboflavin	10	10	—
Ascorbic Acid	10	—	1
Choline Chloride	10	—	1
L-Cysteine HCl	120	—	60
Glycine	50	—	5

Experiments with Cytokinins. Cytokinins were tested using Medium C, i.e., [MH_{Fe}]M*[H_{4+R}M] (Table 2) as a basal medium. The mineral, auxin, growth factor and carbon source constituents were held constant while the cytokinin constituents were varied. The effects of the following individual cytokinins (μM) were compared with the effects of 10 μM BAP and 10 μM Kinetin together with (Kinetin (10), BAP (10), IPA (10), Zeatin (10) and PBA (10). This was to determine the effect of these cytokinins on growth habit.

These cytokinins were also tested, using the same basal medium, over a range of concentrations. This was to determine the cytokinin concentration which produced the maximum number of adventitious buds. The concentrations used were (μM): 1.0, 3 16, 10, 31.6 and 100, i.e., 10^{-6}M, $10^{-5\,5}$M, 10^{-5} M, $10^{-4\,5}$M and 10^{-4}M. Controls of zero cytokinins were used in both experiments.

The effects of auxins, cytokinins and riboflavin were examined using the basal medium, [MH_{Fe}]* *[*M], Medium D (Table 2). Here the mineral constituents and main carbon source were held constant while the auxin, cytokinin and riboflavin constituents were varied. The cytokinins (μM) tested were: Kinetin (10), BAP (10), IPA (10), Zeatin (10), PBA (10) and a zero cytokinin control. Auxins (μM) were either present as:

IAA (1), IBA (1), NAA (1), NOA (1), pCPA (1), and 2,4-D (1) or absent. Riboflavin was either present at a concentration of 10 μM or absent.

Table 2. Combinations of Constituents Used to Make Different Media

	Medium Code							
Constituents of Medium	A	B	C	D	E	F	G	H
Minerals [MH^{Fe}]	✓	✓	✓	✓	✓	✓	✓	✓
Auxins M	✓	✓	✓	—	—	—	—	✓
Other	—	—	—	*	*	—	NAA 31 6μM	—
Cytokinins H	✓	✓	—	—	—	—	—	—
Other	—	—	*	*	—	BAP 31 6μM	BAP 0 0316μM	Kinetin 10μM
Growth Factors H_{ALL}	✓	—	—	—	—	—	—	—
H_{4+R}	—	✓	✓	—	—	✓	—	✓
M_{ALL-R}	—	—	—	—	✓	—	✓	—
Sucrose M	✓	✓	✓	✓	✓	✓	✓	✓
Agar 9g/l	✓	✓	✓	✓	✓	✓	✓	✓

Medium Code	Broad Spectrum Code
A	$[MH^{Fe}]$ MH [HM]
B	$[MH^{Fe}]$ MH $[H^{4+R}M]$
C	$[MH^{Fe}]$ M^{*} $[H^{4+R}M]$
D	$[MH^{Fe}]$ ** [*M]
E	$[MH^{Fe}]$ * $[M^{ALL-R}M]$
F	$[MH^{Fe}]$ $ZBAP^{31\ 6\mu M}$ $[H^{4+R}M]$
G	$[MH^{Fe}]$ $NAA^{31\ 6\mu M}$ $BAP^{0\ 016\mu M}$ $[M^{ALL-R}M]$
H	$[MH^{Fe}]$ M Kinetin $^{10\mu M}$ $[H^{4+R}M]$

Experiments to Develop a Rooting Medium. Attempts to induce root formation were made first with individual auxins using the basal medium $[MH_{Fe}]^{*}Z[M_{ALL-R}M]$, Medium E (Table 2) that is a cytokinin free medium containing the medium concentration of all growth factors except riboflavin. The following auxins were tested individually at 6μM and 10μM (Lilien-Kipnis and de Fossard, unpublished results). IAA, IBA, NAA, NOA, pCPA, 2,4-D.

Later a rooting experiment with Medium E, i.e., $[MH_{Fe}]^{*}Z$-$[M_{ALL-R}M]$ tested a range of concentrations of NAA in combination with a range of concentrations of BAP and Kinetin. NAA was tested at (μM). 0 1, 1.0, 3 16, 10, 31.6 and 100, i.e., 10^{-7}M, 10^{-6}M, $10^{-5\ 5}$M, 10^{-5}M, $10^{-4\ 5}$M and 10^{-4}M respectively. BAP and Kinetin were tested individually at (μM). 0.1, 0.0316 and 0.01, i.e., 10^{-7}M, $10^{-7\ 5}$M, 10^{-8}M.

RESULTS

Growth Habit. Adventitious buds formed in cultures with

See Appendix I for definition of abbreviations

either BAP or PBA present in the medium BAP, PBA and Kinetin stimulated the growth of axillary buds (Figure 1). The optimal concentration of BAP and PBA for adventitious bud formation was 31.6μM. The stimulation of axillary bud growth by BAP and PBA increased with increase in concentration however it decreased with increased kinetin concentration over the range tested. When no cytokinins were present the growth was apically dominant

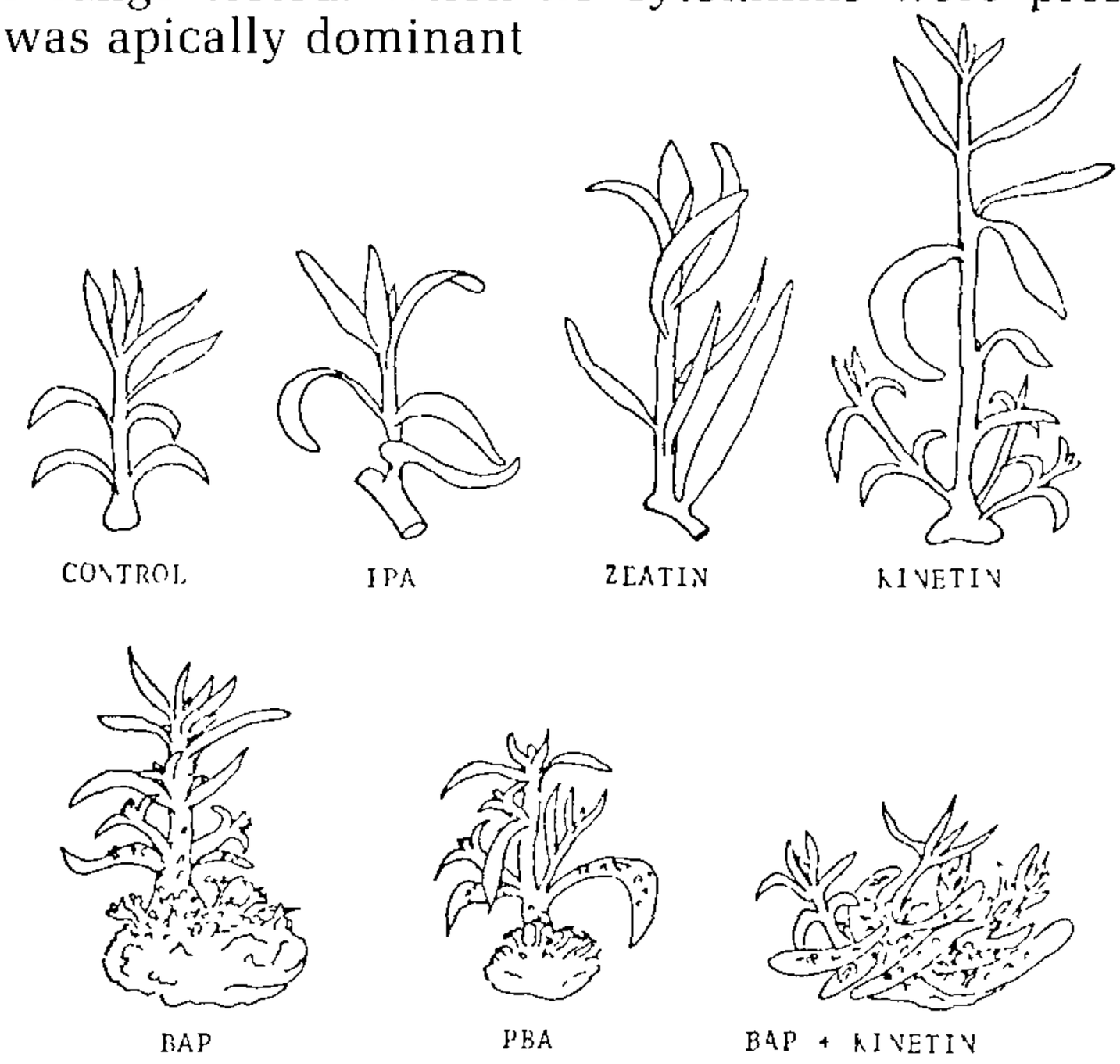


Figure 1. The response of cultures to the various cytokinins (all at 10μM) as shown by adventitious bud formation and growth habit

The presence of auxins in the media inhibited apically dominant growth in the case of cytokinin-free media and it inhibited adventitious and axillary bud development in the case of media containing either BAP or PBA. The addition of 10μM riboflavin to auxin-containing media appeared to nullify this inhibitory auxin-effect. These responses are shown on Table 3.

Table 3. Interactions of auxins, cytokinins and riboflavin on the growth habit of *E australasius* cultures, basal medium was [MH[MH^{Fe}]**[*M]

			Cytokinins
Auxins	Riboflavin	Zero	10μM BAP or PBA
Zero	Zero	Apically dominant growth	Adventitious and axillary bud development
	10μM	Apically dominant growth	Adventitious and axillary bud development
M	Zero	No growth	No growth
	10μM	Apically dominant growth	Adventitious and axillary bud development

Rooting Medium. In the experiment testing various auxins at two levels only one root formed where the culture medium contained 10μM NAA. In the following experiment the medium inducing most roots contained 31.6μM NAA and 0.0316μM BAP; this gave 40% (6/15) rooted cultures (Medium G, Table 2). The rooted cultures were successfully transferred to soil by first placing the culture tubes in a shaded glasshouse followed by the removal of their lids. Two days later rooted cultures were washed free of medium and placed in 5 cm diameter tubes containing a moist peat-sand (1.1) mixture. A plastic hood was placed over the plants and, when new roots had formed, the plants were gradually hardened by exposure to less humid air. This procedure was adapted from that used by Gorst et al. (7) with *Grevillea*.

DISCUSSION

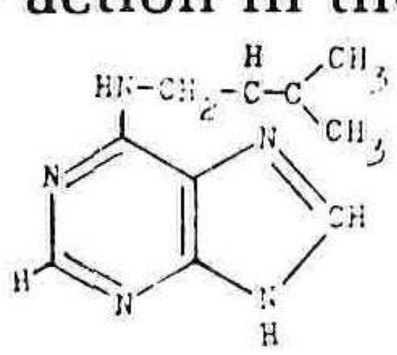
Propagation System. Medium F, i.e., $[MH_{Fe}]Z\ BAP_{31\ 6\mu M}$ $[H_{4+R}M]$ (Table 2) was found to be a good rapid multiplication medium with up to 200 adventitious and axillary buds forming over a 6-week incubation period. Usually 50 of these buds were sufficiently developed for transfer to the rooting medium G, i.e , $[MH_{Fe}]NAA_{31\ 6\mu M}BAP_{0\ 0316\mu M}$ $[M_{ALL-R}M]$ (Table 2). The remaining 150 may be intermediately transferred to Medium H, i.e., $[MH_{Fe}]$M Kinetin $_{10\mu M}[H_{4+R}M]$ (Table 2) to induce apically dominant development. However, due to the abundant supply of material formed by this system this extra step would not normally warrant its expense.

The rooting medium was successful in 40% of cultures. This figure is low compared to other tissue culture rooting systems; however it is greater than the 5% success rate using *in vivo* systems over the same period and plant material is abundant *in vitro*.

Cytokinins. As can be seen in Figure 2 the two cytokinins, BAP and PBA, that induced adventitious buds are structurally related. Skoog and Armstrong (10) found that high levels of unsaturation in the substituent, which is the benzyl ring of BAP and PBA, produce high activity. Therefore this greater unsaturation level compared with the other cytokinins tested may be responsible for their activity. However none of the other cytokinins induced adventitious bud formation, even in high concentrations.

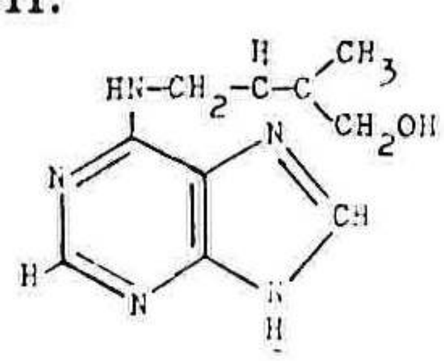
This suggests two other alternatives. One is that the other cytokinins did not enter the plant tissue or were rapidly broken down. Kinetin, however, did induce the development of axillary shoots showing that it was not completely broken down.

Another possibility is that a specific molecular structure is required at one of the steps that leads towards adventitious bud formation and that BAP and PBA satisfy this requirement. In this supposition, these compounds and others closely related to them may be suitable for experiments to determine their point of action in the cell.



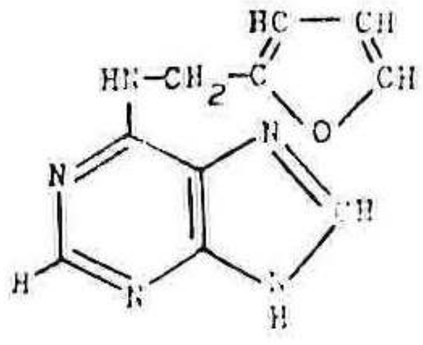
N^6 - isopentenyl amino purine

IPA



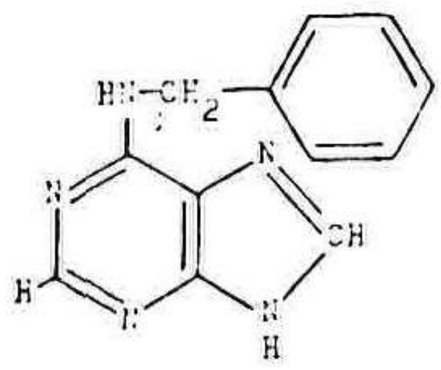
6-(4-Hydroxy-3-methylbut-2enyl)-amino purine

ZEATIN



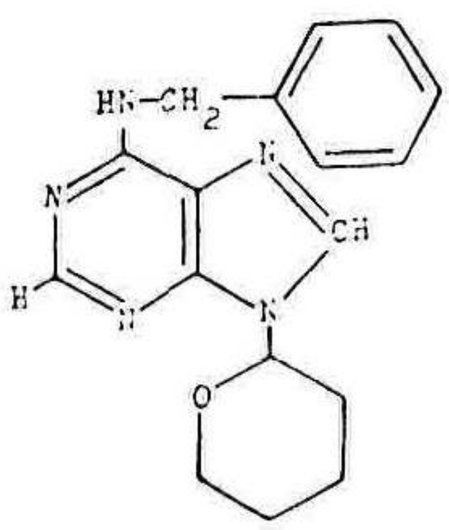
6-Furfuryl amino purine

KINETIN



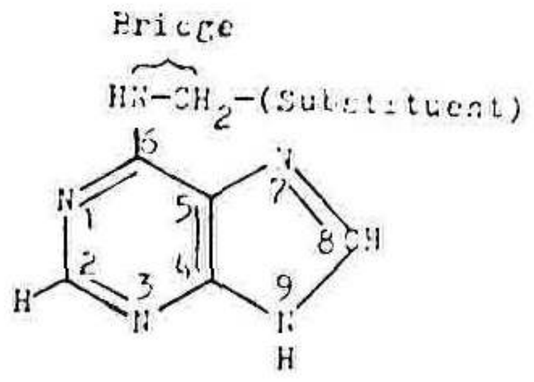
N^6-Benzyl amino purine

BAP



6-(benzylamino)-9-(2-tetra hydro-pyranyl)-9H-purine

PBA



Basic Arrangement and Numbering System for substituted adenines

Figure 2. The Structural Formulae For The Five Cytokinins Used.

Interaction Between Auxins, Cytokinins and Riboflavin on Growth Habit. In the absence of cytokinins, growth was apically dominant but in the presence of either BAP or PBA adventitious and axillary buds developed. Auxins inhibited both apically dominant growth in the cytokinin-free media and the development of adventitious and axillary buds in the case of media with either BAP or PBA. The addition of 10μM riboflavin to auxin-containing media appeared to nullify the inhibitory auxin effect.

Lilien-Kipnis and de Fossard (unpublished results) also tested the interaction between riboflavin and cytokinin but used different growth factors (Table 4).

Table 4. Interactions of riboflavin and cytokinins on growth of *E. australasius*; basal medium was [MH^{Fe}] M * [$H^{ALL-R}M$]; note that all four media had all growth factors (except riboflavin); these growth factors were at the high concentration.

	Cytokinins	
Riboflavin	Zero	High
Zero	Growth	No growth
10μM	No growth	Growth

In the presence of cytokinins on a medium containing auxins, growth is dependent on the addition of 10μM riboflavin to the medium — this result is depicted in both Tables 3 and 4. However, in the absence of cytokinins on a medium containing auxins, different responses to riboflavin were elicited in the separate experiments summarised in Tables 3 and 4. In Table 3, riboflavin induced growth of cultures on auxin-containing, cytokinin-free media, whereas in Table 4 riboflavin addition inhibited growth. Thus it would appear that one or more of the growth factors used in the Table 4 experiment induced growth in the absence of riboflavin but not in its presence. In contrast (Table 3) in the absence of these growth factors, riboflavin can induce growth These puzzling results indicate the need for further systematic work to unravel the active substances in these interactions.

APPENDIX 1. Full names of auxin and cytokinin compounds used in experiments and explanation of symbols

Auxins IAA (Indoleacetic acid), IBA (Indolebutyric acid), NAA (α-naphthaleneacetic acid), NOA (2-Naphthoxyacetic acid), pCPA (*para-chlorophenoxyacetic acid*), 2,4-D (2,4-dichlorophenoxyacetic acid)

Cytokinins BAP (N^6-benzyl amino purine), KINETIN (6-furfuryl amino purine), PBA (6(benzylamino)-9-(2 tetra hydropyranyl)-9H-purine), IPA (N^6 isopentenyl amino purine), ZEATIN (6-(4-hydroxy-3-methylbut-2-enyl) amino purine)

Symbols

Z Represents zero concentration, i e , this constituent is absent

* Represents the use of a number of compounds, i e , this constituent is changed for different treatments

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