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Gibberellins Play a Role in the Interaction Between Imidazole Fungicides and Cytokinins in Araceae

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Abstract. Imidazole fungicides such as imazalil, prochloraz, and triflumizole and the triazole growth retardant paclobutrazol promote the shoot-inducing effect of exogenous cytokinins in Araceae, such as *Spathiphyllum floribundum* Schott and *Anthurium andreanum* Schott. The mechanism of their action could partially be based on the inhibition of gibberellic acid (GA) biosynthesis, because administration of GA_3 inhibits the phenomenon completely in *S. floribundum.* Not only is the suppression of GA biosynthesis involved, but also the metabolism of endogenous cytokinins is significantly altered. Although the balance between isopentenyladenine, zeatin, dihydrozeatin, and their derivatives was shifted to distinguished directions by administration of BA and/or imazalil and/or GA_3, no correlation between these changes in metabolic pathways and the number of shoots could be found. The metabolism of BA was not significantly altered by adding imazalil to the micropropagation medium of *S. floribundum.*

Key Words. Imidazole—Paclobutrazol—Cytokinin—GA—Araceae

When screening fungicides as potential tissue culture medium additives to control fungal development, we found that the imidazole fungicide imazalil exuberantly enhanced the shoot(bud)-inducing effect of 6-benzyladenine (BA) in *Spathiphyllum floribundum.* On cytokinin-free medium imazalil (IMA) did not induce buds (Werbrouck and Debergh 1995). Later on we observed that IMA also enhanced the effect of other cytokinins as Zeatin (Z), 6-(3-hydroxybenzyl)adenine (mT), and even thidiazuron (TDZ) in *S. floribundum.* Prochloraz (PRO) and triflumizole (TRI), which are also imidazole fungicides, had the same effect. PRO and BA interacted in the same way in *Anthurium andreanum,* also a representative of the Araceae. At the same time we were confronted with problems encountered by an *Anthurium* grower. Six-month-old plants of an *A. andreanum* cv., grown on a Nutrient Film Technique (NFT)-system, accidently received PRO with the irrigation solution for several weeks so that the fungicide accumulated in the substrate. These plants developed a very high number of basal shoots (Fig. 1) and had a normal, well-developed root system. In this report we attempted to find a common explanation for the in vitro effects of imidazole fungicides and the extreme bud proliferation in the greenhouse (Fig. 1).

IMA and PRO are imidazole fungicides. The fungicidal action of imidazoles, triazoles, and pyrimidine-carbinoles is based on the inhibition of the ergosterol biosynthesis. Several triazoles, such as paclobutrazol

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; [9R-5′P]DHZ, 9-β-D-ribofuranosyl-dihydrozeatin-monophosphate; [9R-5′P]iP, 6-isopentenyl-9-β-D-ribofuranosyladenine-monophosphate; [9R-5′P]Z, 9-β-D-ribofuranosyl-zeatin-monophosphate; [9G]BA, 6-benzyl-9-β-D-glucopyranosyladenine; [9G]DHZ, 9-β-D-glucopyranosyl-dihydrozeatin; [9G]iP, 6-isopentenyl-9-β-D-glucopyranosyladenine; [9G]Z, 9-β-D-glucopyranosyl-zeatin; [9R]BA, 6-benzyl-9-β-D-ribofuranosyladenine; [9R]DHZ, 9-β-D-ribofuranosyl-dihydrozeatin; [9R]iP, 6-isopentenyl-9-β-D-ribofuranosyladenine; [9R]Z, 9-β-D-ribofuranosyl-zeatin; BA, 6-benzyladenine; DHZ, dihydrozeatin; ES^+ LC-MS/MS, HPLC coupled Electrospray Tandem Mass Spectrometry; f.m., fresh mass; mT, 6-(3-hydroxybenzyl)adenine; IMA, imazalil; iP, isopentenyladenine; NAA, 1-naphthalene acetic acid; NFT, Nutrient Film Technique; (OG)[9R]DHZ, O-β-glucopyranosyl-9-β-D-ribofuranosyl-dihydrozeatin; (OG)[9R]Z, O-β-D-glucopyranosyl-9-β-D-ribofuranosyl-zeatin; (OG)DHZ, O-β-D-glucopyranosyl-dihydrozeatin; (OG)Z, O-β-D-glucopyranosyl-zeatin; PAR, Photosynthetic Active Radiation; PBZ, paclobutrazol; PRO, prochloraz; TDZ, thidiazuron; TRI, triflumizole; Z, zeatin.

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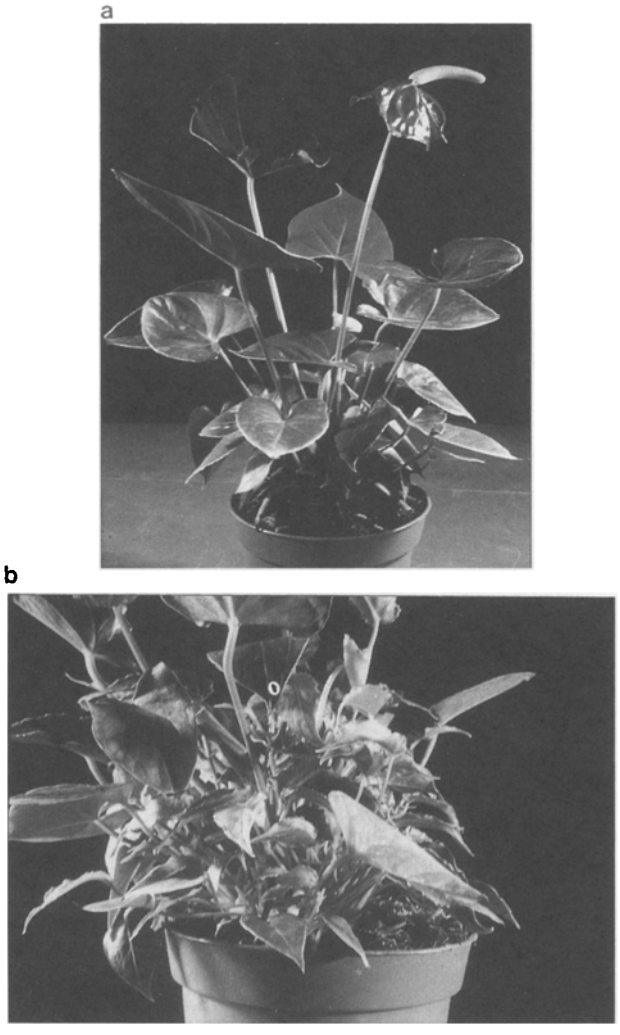


Fig. 1. *A. andreanum:* a, normal plant; b, bushy plant.

(PBZ), are known as growth retardants for plants. They inhibit the oxidative reactions leading from *ent*-kaurene to *ent*-kaurenoic acid and thus inhibit the gibberellic acid (GA) biosynthesis (Graebe and Ropers 1978, Rademacher 1991). Although IMA and PRO do not belong to the triazoles, they share a structural feature common to all these compounds: a heterocyclic ring containing a sp^2-hybridized nitrogen with a lone electron pair. The target enzymes of molecules with this structural feature are cytochrome P450-dependent mono-oxygenases, such as methylhydroxylases. These enzymes are particularly important in the terpenoid pathway. This includes the biosynthesis of gibberellins, but also of abscisic acid, cytokinins, and sterols (Rademacher 1991, Grossman 1992).

In vitro, the effect of imidazole fungicides on shoot proliferation could only be observed in the presence of exogenous cytokinins, such as BA. This suggests an interaction between imidazoles and applied cytokinins. To test this hypothesis we first examined the effect of IMA on the metabolism of BA in *S. floribundum.* Without IMA, *S. floribundum* converts BA mainly to [9G]BA (most likely physiologically inactive) or [9R]BA (physiologically active) (Werbrouck et al. 1995). A shift in balance in favor of [9R]BA could explain the abundant shoot proliferation.

The case of the bushy *A. andreanum* plants, grown in vivo, contradicts the in vitro observations made on *S. floribundum.* It indicates that PRO might interact with endogenous cytokinins. It is likely that 6 months after micropropagation, no remains of exogenously applied cytokinins would be present in these plants. The sensitive cultivar that showed extreme bushiness belongs to a particular breeding line which requires a low cytokinin level for micropropagation. Presumably, this line produces higher amounts of endogenous cytokinins compared with other cultivars.

Palni et al. (1984) established the following sequence of activity in the soybean callus bioassay: [9R]Z > Z > (OG)Z = (OG)[9R]Z > [7G]Z = [9G]Z. Dihydrozeatin (DHZ) and its conjugates are as active as their Z analogs (Letham et al. 1983). *N*-glucosides are extremely stable, in contrast with *O*-glucosides, which are storage forms rather than inactivation products (Palni et al. 1984). Cytokinin oxidases have a definite preference for iP and [9R]iP as substrate, and recognize Z and [9R]Z to a lesser extent. *O*-glycosylation prevents enzymatic oxidation in contrast with *N*-glycosylation which has little effect on the susceptibility to cytokinin oxidase (McGaw and Horgan 1983). [9R-5′P]iP (Laloue and Fox 1989) and DHZ and its derivatives are resistant to attacks by cytokinin oxidases (Hare and Van Staden 1994). To test the hypothesis that imidazole fungicides change the metabolism of endogenous cytokinins, we analyzed the pool of endogenous cytokinins *S. floribundum,* at the moment that the first meristems appeared at the plant base (after 6 weeks in vitro). We also investigated the possibility that imidazoles might inhibit the GA biosynthesis, as did the triazole paclobutrazol. A reduced level of endogenous gibberellins might be responsible for the exuberant shoot induction. To test this hypothesis we combined IMA or PBZ with BA and/or GA_3 and observed their effects on shoot induction.

Materials and Methods

Plant Material, Medium, and Growth Conditions

S. floribundum Schott, an important micropropagated ornamental plant, belongs to the Araceae family and is usually micropropagated by axillary and adventitious shoots, both induced at the plant base (Fonnesbech and Fonnesbech 1979). Unless otherwise indicated, *S. floribundum* 'Petite' was micropropagated in 380-mL glass vessels, with a screw-on polycarbonate lid, on a basal medium containing Murashige and Skoog (1962) macroelements, Nitsch and Nitsch (1969) microelements, 95 μM NaFeEDTA, 555 μM *myo*-inositol, 0.89 μM thiamine

HCl, 167 mM sucrose, 3 g/L Roth agar, and 4 g/L BDH agar. Each culture vessel contained 6 single shoots with leaves removed. The basal medium was supplemented with BA, IMA and/or GA_3, according to the experiment. Each culture vessel contained 100 mL autoclaved medium (120°C, 20 min). The cultures were maintained at 23 ± 2°C under a 16-h photoperiod at 40 μmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR). A trade formulation of the fungicide was used: Fungaflor (Liro, Belgium) = 200 g/L IMA (Janssen Pharmaceutica, Belgium), molecular weight (m.w.), 297.2.

Effect of Imazalil on BA Metabolism in S. floribundum

S. floribundum was cultured in test tubes filled with 20 mL basal medium, supplemented with [8-^{14}C]BA (2.00 GBq $mmol^{-1}$) (Amersham, UK). BA (0 or 10 μM) was combined with 0 or 35 μM IMA, added before autoclaving. Each treatment consisted of five test tubes, each containing one shoot. After 6 weeks, the basal part of the plants were analyzed separately by means of high-performance liquid chromatography (HPLC).

Effect of GA_3 on the Interaction Between IMA or PBZ and BA in S. floribundum

The basal medium for *S. floribundum* was supplemented with 0 or 10 μM BA, combined with 0 or 35 μM IMA or 10 μM PBZ. These six treatments were combined with 0 or 10 μM GA_3 (filter sterilized). Each treatment consisted of six culture vessels. After a culture period of 10 weeks, the number of induced shoots was counted, and root number and length were determined.

Effect of GA_3, IMA, and BA on the Pool of Endogenous Cytokinins in S. floribundum

The basal medium for *S. floribundum* was supplemented with 0 or 10 μM BA, combined with 0 or 35 μM IMA and 0 or 10 μM GA_3. Each treatment consisted of six test tubes, each containing 20 mL medium and one shoot. After a culture period of 5 weeks the endogenous cytokinins in the basal part of the plant were analyzed.

Harvest, Extraction, and Purification of BA and Its Derivatives

The plant fractions were rinsed, dried between paper, and homogenized with mortar and pestle in liquid nitrogen. [2-^{3}H]BA, [2-^{3}H][9R]BA, and [2-^{3}H][9G]BA (1.0 TBq · $mmol^{-1}$) (Olchemim, Czech Republic) were added as internal standards. ([2-^{3}H][9R-5′P)]BA was not added because it was not commercially available.) Extraction with 10 mL methanol/chloroform/water/formic acid (60:25:10:5, v/v/v/v) (20 h, −20°C) (Bieleski 1964) was followed by centrifugation (24000*g*, 4°C, 20 min). The supernatant was dried by rotary film evaporation and redissolved in 10 mL H_2O. The cytokinins were bound on a RP-C18 column (Varian Bond-Elut). They were eluted with 7 mL 80% MeOH and concentrated by rotary film evaporation. The samples were stored at −20°C until HPLC analysis.

Harvest, Extraction, and Purification of Endogenous Cytokinins

The plant fractions were rinsed, dried between paper, and homogenized with mortar and pestle in liquid nitrogen. Bieleski (1964) solvent and deuterated standards were added (free bases, nucleosides, and nucleotides of Z, iP, and DHZ) (Olchemim, Czech Republic). Extraction and purification were performed according to Redig et al. (1996) by means of immune affinity columns, RP-C18 columns, and DEAE (HCO_3^- form). The samples were stored at −20°C until LCMS analysis.

High-performance Liquid Chromatography and Analysis of Radioactive Fractions

Cytokinins and their derivatives were separated on a Microsorb C18 5-μm column (Rainin, USA) with (80% MeOH + 0.6% HOAc, pH 3.5) and (10% MeOH + 0.56% HOAc, pH 3.35) (both sparged with helium) using a partially linear gradient system (M. Strnad, Antwerp, pers. comm.). The UV-absorbance (240–350 nm) of relevant nonradioactive standards (Apex, U.K.) was monitored on-line with a diode array detector (Waters PDA991) for 40 min. One hundred microliters of each sample was injected, and 40 one-minute fractions per sample were collected and used for liquid scintillation counting (Packard 3-CARB 1500). By measuring ^{14}C and ^{3}H in each fraction, recovery calculations were possible. [2-^{3}H][9R]BA was also used for approximate recovery calculation of [9R-5′P]BA.

Liquid Chromatography—Mass Spectrometry (Electrospray Tandem Mass Spectrometry)

The fractions were analyzed by HPLC linked to a Quatro II mass spectrometer with an electrospray interface (ES^+ LC-MS/MS) (Prinsen et al. 1995). 10 μL samples were injected on a C-8 reversed-phase column (Merck; LiChrosphere 60 RP Select B; 5 μm; 125 × 4 mm) and eluted with methanol + ammonium acetate 0.01 M (70/30, v/v) at 800 μL/min. Using a post-column split of 1/20 the effluent was introduced into the electrospray source (source temperature 80°C, capillary voltage +3.5 kV, cone voltage 20 V). Under these conditions, full scan spectra of the molecule could be recorded (scan array 50–550 Da, scan speed 300 Da/s). Collision-activated dissociation spectra of $[MH]^+$ were obtained at a collision energy of 20 eV (P_{Ar}:1.10^{-3} mbar). Quantization was performed by multiple-reaction monitoring of $[MH]^+$ (dwell time 0.1 s) and the appropriate product ion. Data were processed by Masslynx software.

Results

Effect of IMA on BA Metabolism in S. floribundum

Adenine and adenosine were not separated. Four relevant derivatives could be identified by means of their UV-spectrum and co-eluting standards: [9R-5′P]BA (24.3 min), [9G]BA (26.9 min), BA (35.2 min) and [9R]BA (37.2 min). Adding imazalil had no significant effect on the concentration of [9R-5′P]BA, [9G]BA, BA or [9R]BA (Fig. 2). Only adenine + adenosine was halved.

Effect of GA on the Interaction Between IMA or PBZ and BA in S. floribundum

Results are illustrated in Figure 3. On BA-free medium, no shoots were induced (Table 1). Adding BA to the medium resulted in a moderate multiplication rate (8.7

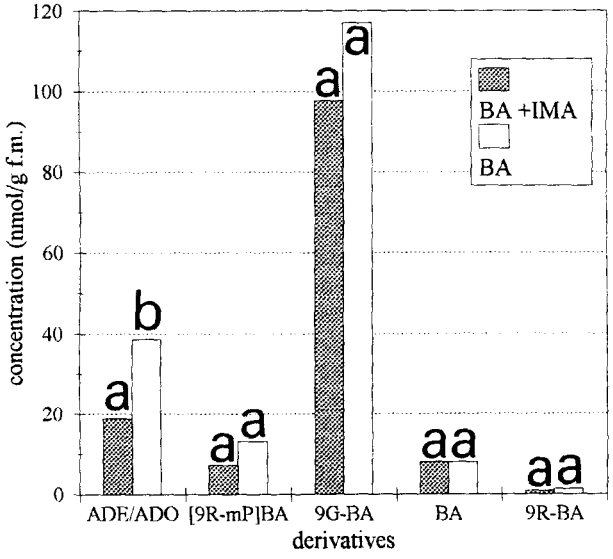


Fig. 2. Effect of combinations of BA and IMA on the metabolism of BA in the basal part of *S. floribundum* after a culture period of 6 weeks. Means of the same derivative followed by the same letter are not significantly different (LSD, 95%).

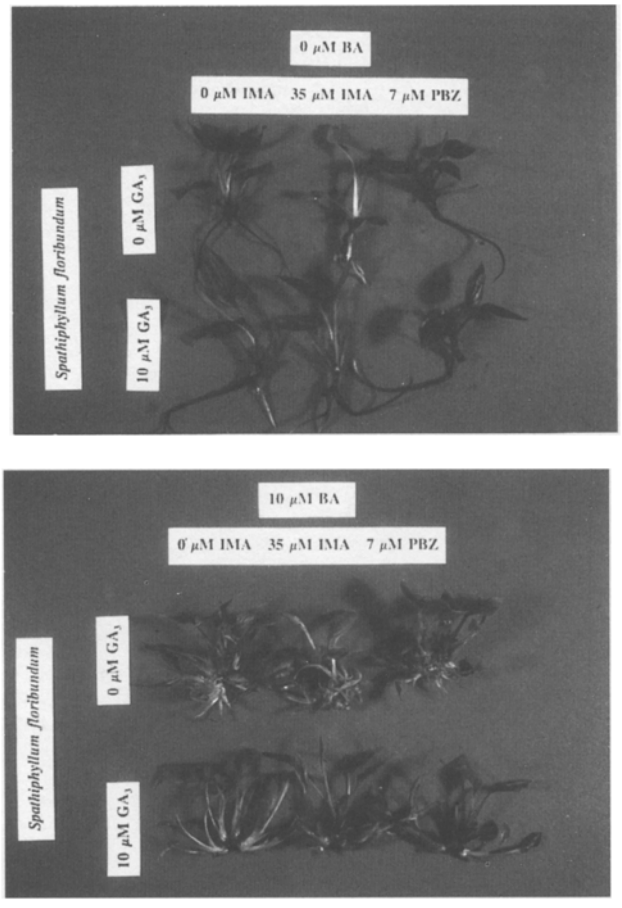


Fig. 3. Interactions between BA, IMA, and GA_3 in *S. floribundum* after a culture period of 10 weeks.

new shoots). Shoot induction by BA was significantly enhanced by IMA (51 new shoots), and to a lesser extent, but still significantly, by PBZ (33.7 new shoots). The shoots were often reduced to the size of a meristem. When GA_3 was added to these three treatments, shoot induction was almost completely abolished (Table 1): a few axillary shoots developed, but no adventitious shoots.

Roots were not observed in any of the treatments with BA; however, roots did develop on BA-free media. Adding IMA increased the number of roots per explant, but at the expense of total root length per explant. A higher total root length did not compensate for the negative effect of PBZ on root number. Adding GA_3 to the BA-free media had a slight negative effect on root number and total root length per explant. In all treatments involving IMA or PBZ, the roots were thicker (Table 1).

Effect of GA_3, IMA, and BA on the Pool of Endogenous Cytokinins in S. floribundum

Results are presented in Table 2. A simplified scheme of the biosynthesis and metabolism of endogenous cytokinins is added to this table. Only main changes will be described here. In the shoot base of plants which developed for 6 weeks on basal medium *Spathiphyllum* (BMS) without BA, IMA, or GA_3, the most important derivatives were (OG)Z, (OG)DHZ, and (OG)[9R]Z. When 10 μM BA was added to BMS, DHZ (430 pmol/g fresh mass [f.m.]) and [9G]iP (240 pmol/g f.m.) became predominant. *O*-glycosides fell under the detection limit because of a bad recovery of this fraction in this sample. When IMA was added to BMS, *O*-glucosides remained important derivatives. Combining BA and IMA favored the formation of (OG)DHZ (107 pmol/g f.m.). The addition of GA_3 to these treatments changed this picture: the peaks of DHZ and [9G]iP, which arose when BA was added to BMS, disappeared.

Discussion and Conclusion

The hypothesis that IMA alters the metabolism of BA in *S. floribundum* can be rejected. None of the concentrations of [9R]BA, [9R-5′P]BA, or BA, all biologically active cytokinins, underwent significant changes. The abundant shoot proliferation in the combination BA + IMA could be explained by great differences in cytokinin concentrations, but this was not the case. Although the existence of a BA-oxidase has not yet been reported (Hare and Van Staden 1994), BA is broken down to Ade + Ado. Halving the concentration of Ade + Ado when

Table 1. Interaction of GA_3 and BA and imazalil or paclobutrazol regarding the number of new shoots, root number and total root length (cm) per *S. floribundum* explant after a culture period of 10 weeks (results from three independent experiments).[a]

	BA					
	Shoot number		Root number		Root length (cm)	
	0 μM	10 μM	0 μM	10 μM	0 μM	10 μM
0						
0 μM GA_3	0 a	8.7 c	9.5 d	0 a	2.1 c	0 a
10 μM GA_3	0 a	1.9 ab	7.0 c	0 a	1.7 bc	0 a
IMA (35 μM)						
0 μM GA_3	0 a	51.0 e	7.0 c	0 a	3.2 d	0 a
10 μM GA_3	0 a	2.7 b	5.4 bc	0 a	2.2 c	0 a
PBZ (7 μM)						
0 μM GA_3	0 a	33.7 d	5.1 bc	0 a	1.3 b	0 a
10 μM GA_3	0 a	1.5 ab	4.5 b	0 a	1.5 bc	0 a

[a] Within a frame, means followed by the same letter are not significantly different (LSD, 95%).

IMA is added (Fig. 2) could indicate a partial inhibition of the breakdown process of BA. However, halving the amount of breakdown products cannot explain the abundant shoot proliferation.

On the other hand, it seems that GA is involved in the process. Indeed, GA_3 inhibits the normal induction of new shoots by BA (Table 1, Fig. 3). This phenomenon has often been reported for other crops and is reviewed by George (1993). It could be interpreted that in *S. floribundum,* the endogenous gibberellins block the full expression of the shoot-inducing potential of exogenous cytokinins, like BA. Adding IMA or PBZ removes the block by inhibiting GA biosynthesis and, as a consequence, (adventitious) shoot proliferation is enhanced. When GA_3 is added to the aforementioned media, the GA balance is restored and the full caulogenic potential of the cytokinins is again suppressed (Table 1).

The mechanism by which GA affects cytokinin expression remains open for speculations. It is possible that gibberellins affect cytokinin receptors. IMA is a much weaker growth retardant then PBZ; nevertheless, its promotion of the shoot-inducing effect of cytokinins is at least as powerful as that of PBZ. Inhibition of GA biosynthesis might not be the only possible explanation for the abundant shoot proliferation.

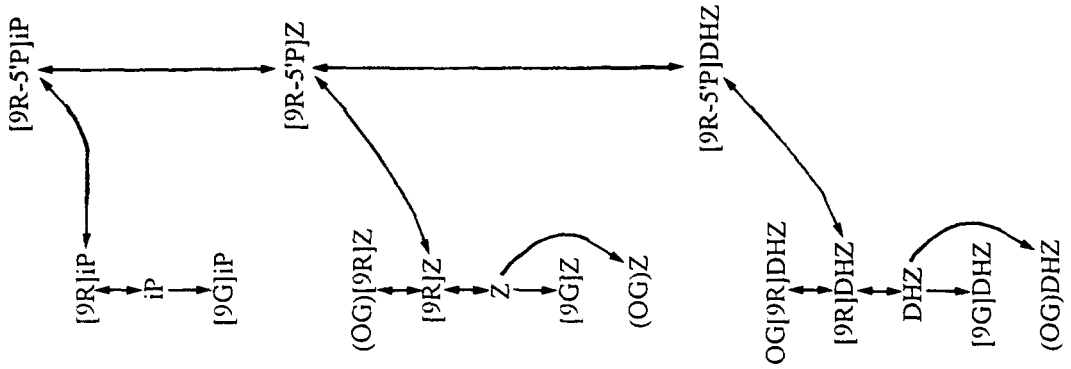
GA_3 has weak negative effect on root number and total root length (Table 1). Growth reduction of the roots by PBZ was not restored by adding GA_3. The inhibitory effect of cytokinins on the rooting process was not alleviated by GA_3, therefore, not all the effects of cytokinin are affected to the same extent by gibberellins.

The analysis of the endogenous cytokinin pool shows that the balance between biosynthesis of iP, Z, and DHZ, formation of their conjugates, and catabolic reactions, can be shifted in particular directions 6 weeks after administration of BA and/or IMA and/or GA_3. The shoot proliferation observed in the combination IMA + BA cannot be explained by the presence of a particular endogenous cytokinin derivative. The supplementation by GA_3 changed the existing equilibrium somewhat, but here too, no correlation between any derivative and the observed morphological effects could be detected. Although biosynthetic and metabolic pathways are altered by BA, IMA, and/or GA_3, they cannot explain the presence or absence of new shoots or shoot meristems. The absence of shoots when IMA is added to a BA-free medium indicates that the level of endogenous cytokinins is not adequate to induce shoots, even when the blocking effect of endogenous GA is alleviated. After all, *S. floribundum,* endogenous cytokinin concentrations are 100 to 1000 times smaller than the concentrations of BA and its derivatives (Werbrouck et al. 1995).

For other compounds with a N-containing heterocycle analog, in vitro effects have also been found. Chin (1982) combined ancymidol with NAA and kinetin in the medium of *Asparagus* and observed an increased in vitro shoot and root formation. Ancymidol belongs to the pyrimidines; it possesses a N-containing heterocycle, and inhibits the GA biosynthesis in the same way as do imidazoles and triazoles (Rademacher 1991). Sankhla et al. (1993) applied various levels of PBZ, uniconazole, and prohexadione calcium in the medium of *Albizzia julibrissin.* Significantly more green nodule-like structure per hypocotyl explant were induced in contrast with the control treatment. Administration of 2.9 μM GA_3 to the control medium decreased the number of shoot by 50%. The shoot-inducing effect of PBZ, uniconazole, and prohexadione calcium was severely inhibited by 2.9 μM GA_3. Although the authors do not mention which cytokinins were applied in their media, these results support our hypothesis. It is interesting that prohexadione calcium belongs to the cyclohexanetriones, another group of GA biosynthesis inhibitors, which possess no N-containing heterocycle. It blocks a different step in the GA biosynthesis, namely the conversion of GA_{20} to GA_1. Some authors have reported on the effect of growth retardants in liquid cultures. PBZ, ancymidol, daminozide and uniconazole inhibited leaf development and induced the formation of bud clusters in liquid-cultured *Gladiolus* (Ziv 1990). PBZ, and to a lesser extent ancymidol, had the same effect in liquid cultures of the Araceae *Philodendron* (Ziv and Ariel 1991). The growth retardants were always combined with BA. This reported effect of PBZ is similar to that of imidazole fungicides in combination with cytokinin.

In greenhouse-grown *Aechmea fasciata,* Ziv et al. (1986) reported that spraying of 6-month-old *A. fasciata* with 5 mg/L PBZ resulted in an abundant bud proliferation at the plant base. This observation, and the similar case of the bushy *A. andreanum,* can be combined in our hypothesis: GA biosynthesis is inhibited by PRO (or

Table 2. Effect of combinations of BA, IMA and GA_3 on the pool of endogenous cytokinins in *S. floribundum* after a culture period of 6 weeks (a simplified scheme of the biosynthesis and metabolism of the measured derivatives is added).



Added hormones (μM)			Detected cytokinins (nM)														
BA	IMA	GA	[9R]iP	iP	[9G]iP	[9R-5'P]Z	(OG)[9R]Z	[9R]Z	Z	[9G]Z	(OG)Z	[9R-5'P]DHZ	(OH)[9R]DHZ	[9R]DHZ	DHZ	[9G]DHZ	(OG)DHZ
0	0	0	6	45	11	8	<50	7	38	20	194	5	160	8	<10	<1	170
10	0	0	<25	<25	240	16	—	32	90	<18	—	9	—	16	430	<18	—
0	37	0	7	7	12	21	84	13	92	30	107	5	117	4	<17	2	130
10	37	0	<2	<14	19	2	44	4	23	5	59	0.5	<30	<1	<10	<2	107
0	0	10	2	15	6	5	63	4	17	11	130	1	50	3	20	<0.5	80
10	0	10	7	20	13	4	36	3	66	12	78	<0.8	<18	<1.5	40	6	130
0	37	10	3	11	3	<5	63	4	45	11	78	<5	<18	2	18	2	77
10	37	10	5	<15	22	6	56	6	30	17	72	1	<15	<1.4	<14	<1.5	107

PBZ) and this removes barriers that reduce the shoot-induction capacity of endogenous cytokinins in these greenhouse-grown plants.

The results allow us to formulate the following hypothesis: in micropropagated *S. floribundum,* endogenous gibberellins alleviate the shoot-induction capacity of exogenous cytokinin by means of an unknown mechanism. Imidazoles such as IMA and triazoles such as PBZ inhibit the biosynthesis of gibberellins, and as a consequence cytokinins can manifest their full shoot-induction potential. The changes in the pool of endogenous cytokinin induced by adding imidazoles and/or GA_3 have no effect because in *S. floribundum,* their concentrations are too low for shoot induction.

Only a part of the complex interactions between cytokinins, auxins, gibberellins, and ethylene is fully understood at this moment. By adding a few micromoles of IMA to the medium, the interrelations between growth substances are changed, with large developmental results as a consequence. Perhaps the role of gibberellins in the shoot-induction process has been underestimated. In the future, more chemicals will be discovered which might be of practical use for tissue culture, but which also might serve physiologists who want to unravel the mode of action of growth substances and growth regulators.

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References

Bieleski RL (1964) The problem of halting enzyme action when extracting plant tissues. Anal Biochem 9:431–442

Chin C (1982) Promotion of shoot and root formation in Asparagus in vitro by ancymidol. Hortscience 17:590–591

Fonnesbech M, Fonnesbech A (1979) In vitro propagation of *Spathiphyllum.* Sci Hortic 10:21–25

George EF (1993) Plant propagation by tissue culture. Part 1. The Technology, 2nd edition, Exegetics Ltd., England, pp 446–453

Graebe JE, Ropers HJ (1978) Gibberellins. In: Letham DS, Goodwin PB, Higgins TJV (eds) Phytohormones and related compounds—a comprehensive treatise. Vol I: The biochemistry of phytohormones and related compounds. Elsevier/North Holland Biomedical Press, Amsterdam, 147 pp

Grossman K (1992) Plant growth retardants: their mode of action and benefit for physiological research. In: Karssen CM, van Loon

LC, Vreugdenhil D (eds) Progress in Plant Growth Regulation, pp 788–797

Hare PD, Van Staden J (1994) Cytokinin oxidase: biochemical features and physiological significance. Physiol Plant 91:128–136

Laloue M, Fox JE (1989) Cytokinin oxidase from wheat: partial purification and general properties. Plant Physiol 34:163–197

Letham DS, Palni LMS, Tao GQ, Gollnow BI, Bates CM (1983) Regulators of cell division in plant tissues. XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassay. J Plant Growth Regul 2:103–115

McGaw BA, Horgan R (1983) Cytokinin oxidase from *Zea mays* kernels and *Vinca rosea* crown gall tissue. Planta 159:30–37

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Palni LMS, Palmer MV, Letham DS (1984) The stability and biological activity of cytokinin metabolites in soybean callus tissue. Planta 160:242–249

Prinsen E, Redig P, Van Dongen W, Esmans EL, Van Onckelen HA (1995) Quantitative analysis of cytokinins by Electrospray Tandem Mass Spectrometry. Rapid Commun Mass Spectrom 9: 948–953

Rademacher W (1991) Biochemical effects of plant growth retardants. In: Gausman HW (ed) Plant Biochemical Regulators Marcel Dekker, NY, pp 169–199

Redig P, Schmülling T, Van Onckelen H (1996) Cytokinin metabolism in *ipt* transgenic tobacco. Plant Physiol (in press)

Sankhla D, Davis TD, Sankhla N (1993) Effect of gibberellin biosynthesis inhibitors on shoot regeneration from hypocotyl explants of *Albizzia julibrissin*. Plant Cell Rep 13:115–118

Werbrouck SPO, van der Jeugt B, Dewitte W, Prinsen E, Van Onckelen HA, Debergh PC (1995) The metabolism of benzyladenine in *Spathiphyllum floribundum* Schott 'Petite' in relation to acclimatisation problems. Plant Cell Rep 14:662–665

Werbrouck SPO, Debergh PC (1995) Imazalil enhances the shoot inducing effect of benzyladenine in *Spathiphyllum floribundum* Schott. J Plant Growth Regul 14:105–107

Ziv M (1990) The effect of growth retardants on shoot proliferation and morphogenesis in liquid cultured *Gladiolus* plants. Acta Hortic 280:207–214

Ziv M, Ariel T (1991) Bud proliferation and plant regeneration in liquid-cultured *Philodendron* treated with ancymidol and paclobutrazol. J Plant Growth Regul 10:53–57

Ziv M, Yogev T, Krebs O (1986) Effect of paclobutrazol and chlormequat on growth pattern and shoot proliferation of normal and variant *Aechmea Fasciata* Baker plant regenerated in vitro. Isr J Bot 35:175–182