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Imidazole Fungicides and Paclobutrazol Enhance Cytokinin-Induced Adventitious Shoot Proliferation in Araceae

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Abstract. The imidazole fungicides imazalil, prochloraz, and triflumizole and the triazole growth retardant paclobutrazol strongly enhanced the shoot-inducing effect of 6-benzyladenine in *Spathiphyllum floribundum* 'Petite' Schott. Numerous small shoots and shoot meristems appeared at the basal part of the plant. This effect was confirmed when such widely different cytokinins as zeatin, *meta*-topolin, and thidiazuron were combined with imazalil. Neither these fungicides nor paclobutrazol showed cytokinin effects on cytokinin-free medium. The number of roots per explant could be augmented using particular concentrations, depending on the fungicide used. The combination prochloraz and 6-benzyladenine had a similar effect on *Anthurium andreanum,* which suggests that Araceae are especially sensitive to this interaction.

Key Words. Imazalil—Prochloraz—Triflumizole—In vitro—*Spathiphyllum*—*Anthurium*

When screening fungicides as potential tissue culture medium additives to control fungal development, we found that the imidazole fungicide imazalil (IMA) exuberantly enhanced the shoot (bud)-inducing effect of BA in *Spathiphyllum floribundum.* On cytokinin-free medium, IMA did not induce buds (Werbrouck and Debergh 1995). IMA, prochloraz (PRO), and triflumizole (TRI) are imidazole fungicides (Fig. 1). They share a structural feature with triazoles and pyrimidine-carbinoles: a heterocyclic ring containing a sp^2-hybridized nitrogen with a lone electron pair. Molecules with this feature can inhibit ergosterole biosynthesis in fungi and can block gibberellin (GA) in plant biosynthesis by inhibiting the oxidative reactions leading from *ent*-kaurene to *ent*-kaurenoic acid. Cytochrome P450-dependent mono-oxygenases (methylhydroxylases), which are not only involved in the biosynthesis of gibberellins but also of abscisic acid, cytokinins, and sterols, are also sensitive to molecules with this structural feature (Rademacher 1991, Grossman 1992).

We tested whether an interaction existed between BA and prochloraz or triflumizole and between BA and the triazole growth retardant paclobutrazol (PBZ) in *S. floribundum.* We also investigated whether these fungicides interacted with cytokinins other than BA: mT, a natural *meta*-hydroxylated BA analog recently discovered in poplar leaves by Strnad (Werbrouck et al. 1996); Z, naturally occurring in many plants (Letham 1978); and TDZ, a very active phenylurea compound which is not a true cytokinin, but has cytokinin-like effects due to the inhibition of cytokinin oxidase (Huetteman and Preece 1993, Hare and Van Staden 1994). In a previous experiment, we examined whether other Araceae such as *Anthurium* reacted in the same way as *Spathiphyllum.*

Materials and Methods

Plant Material, Medium, and Growth Conditions

S. floribundum Schott and *Anthurium andreanum* are important ornamentals, belonging to the Araceae family. They are usually micropropagated by axillary and adventitious shoots, both induced at the plant base (Fonnesbech and Fonnesbech 1979, Geier 1990). Unless stated otherwise, *S. floribundum* 'Petite' was micropropagated in 380-mL glass vessels (with a screw-on polycarbonate lid) on a basal medium (BMS) 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,

Abbreviations: BA, 6-benzyladenine; BMA, basal medium *Anthurium;* BMS, basal medium *Spathiphyllum;* f.m., fresh mass; GA, gibberellin; GA_3, gibberellic acid; IMA, imazalil; mT, 6-(3-hydroxybenzyl)adenine; NFT, Nutrient Film Technique; PAR, Photosynthetic Active Radiation; PBZ, paclobutrazol; PRO, prochloraz; TDZ, thidiazuron; TRI, triflumizole; Z, zeatin.

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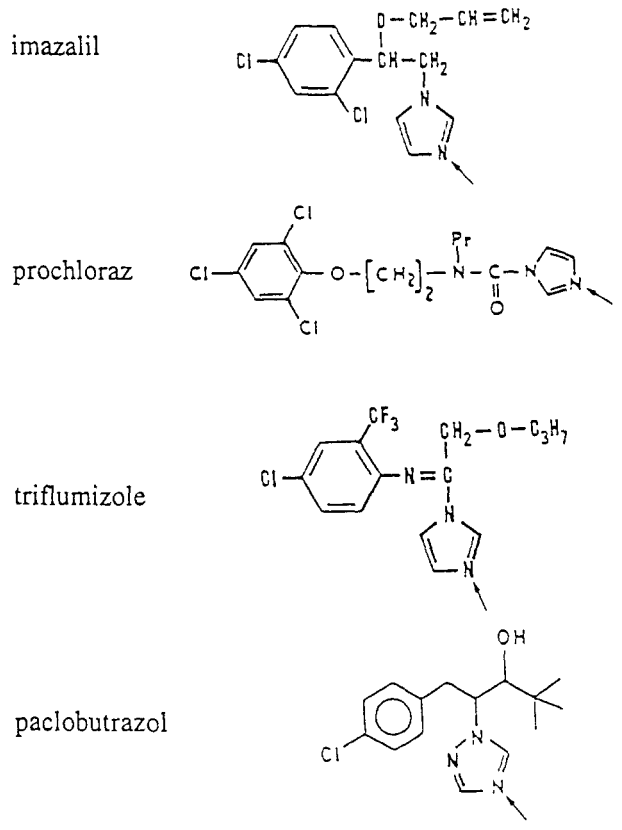

Fig. 1. Chemical structure of imazalil, prochloraz, triflumizole and paclobutrazol (Tomlin 1994). Arrows indicate the sp^2-hybridized nitrogen with its lone electron pair.

and 4 g/L BDH agar. Each culture vessel contained six shoots. *A. andreanum* 'Rother 2' was cultured in the same type of jars. Its basal medium (BMA) contained Murashige and Skoog (1962) macroelements at half strength (NH_4NO_3 concentration was reduced to 2.5 mM), Murashige and Skoog (1962) microelements, 95 μM NaFeEDTA, 555 μM *myo*-inositol, 0.89 μM thiamine HCl, 111 mM sucrose, and 7 g/L BDH agar. Each culture vessel was inoculated with six single shoots from which the leaf blades had been removed. The basal media were supplemented with cytokinins and/or fungicides 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} PAR. Stock plants were micropropagated on BMS and supplemented with 10 μM BA. Trade formulations of the fungicides were used: Fungaflor (Liro, Belgium) = 200 g/L imazalil (Janssen Pharmaceutica, Belgium); (molecular weight [m.w.] 297.2); Sporgon (Schering, Belgium) = 50% prochloraz (m.w., 376.7); Rocket (Nippon Soda, Japan; ProAgro, The Netherlands) = 150 g/L triflumizole (m.w., 345.8). Gas chromatographic (GC) analysis proved that these fungicides are autoclavable (results not shown).

Interaction of Prochloraz, Triflumizole, or Paclobutrazol with BA in S. floribundum

In three independent experiments, BMS was supplemented with 0 or 10 μM BA and combined with 0, 10, or 40 μM PRO, TRI, or PBZ. Each treatment consisted of six culture vessels. After a culture period of 10 weeks the number of induced shoots per explant was determined, as well as the root number and length. Furthermore, some explants which developed on BMS with 10 μM BA and 10 μM PRO were transferred to vessels with cytokinin-free BMS.

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

	BA					
	Shoot number		Root number		Root length (mm)	
	0 μM	10 μM	0 μM	10 μM	0 μM	10 μM
Prochloraz (μM)						
0	0 a	18 b	2.1 bc	1.2 a	74 d	15 a
10	0 a	79 c	3.4 d	1.4 ab	57 c	14 a
40	0 a	90 c	3.5 d	2.3 c	44 bc	36 b
Triflumizole (μM)						
0	0.03 a	28 b	1.5 bc	0.2 a	68 b	2.1 a
10	0.15 a	56 d	2.3 c	5.7 d	97.1 b	167 c
40	0 a	3 c	2.2 c	1.1 ab	80 b	27 a
Paclobutrazol (μM)						
0	0.1 a	13 b	3.1 c	1.0 b	109 c	6 ab
10	0.5 a	34 c	4.3 d	1.0 b	15 b	4 a
40	0 a	17 b	0.1 a	0.8 ab	0.7 a	4 a

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

Interaction of Imazalil with BA, mT, Z, or TDZ in S. floribundum

BMS was supplemented with 0 or 10 μM BA, 10 μM mT, 10 μM Z, or 1 μM TDZ, and combined with 0 or 34 μM IMA (10 mg/L). Each treatment consisted of five culture vessels. Only after a culture period of 15 weeks could the shoot (meristem) number of the treatment with TDZ be determined well; thus, the evaluation of the entire experiment was delayed. The number of induced shoots per explant was determined, as well as the root number and length.

Interaction of Prochloraz with BA in A. andreanum

BMA was supplemented with 0 or 2 μM BA, combined with 0, 10, or 40 μM PRO. Each treatment consisted of six culture vessels. After a culture period of 15 weeks, the number of induced shoots/explant was determined, as well as the root number and length. For histological studies, explants grown on 2 μM BA + 10 μM PRO were fixed in acetic acid and ethyl alcohol (1:3, v/v) for 48 h, dehydrated through a series of ethyl alcohol and tertiary butyl alcohol, and embedded in paraffin (58–60°C). Ten micrometer-thick sections were stained with a 2% safranin ethanol and a 1% fast green butanol solution.

Fig. 2. Interaction between PRO and BA in *S. floribundum* after 10 weeks. Left column, 0 μM BA; right column, 10 μM BA; 1st, 2nd and 3rd row, 0, 10, 40 μM PRO.

Results

Interaction of Prochloraz, Triflumizole or Paclobutrazol with BA in S. floribundum

The results are presented in Table 1 and illustrated in Figures 2 and 3. The lowest concentration of the two fungicides as well as PBZ synergistically enhanced the shoot-inducing effect of BA. A callus-like structure appeared, on which an expanding ring of numerous small shoots developed. After 10 weeks, this resulted in a globe-shaped cluster of adventitious shoots or shoot primordia. Combined with 10 μM BA, 10 μM PRO induced 79 shoots, 10 μM TRI induced 56 shoots, and 10 μM PBZ induced 34 shoots (Table 1). The leaves of the new shoots were narrow and pale green. Forty μM seemed to be supraoptimal for TRI and PBZ; the size of most of the shoots was reduced to a small meristematic dome, which made them very difficult to count. On BA-free medium, almost no shoots grew out. It is possible that the few shoots that were counted had been induced before the start of the experiment. Without BA, the lowest concentration of PRO, TRI, and PBZ augmented the number of

Fig. 3. Interaction between PBZ and BA in *S. floribundum* after 10 weeks. Left column, 0 μM BA; right column, 10 μM BA; 1st, 2nd and 3rd row, 0, 10, 40 μM PBZ. Magnified image, 10 μM BA + 10 μM PBZ.

roots, often at the expense of the total root length per explant. These roots emerged from the stem of the original explant. Forty μM PBZ proved to be highly supraoptimal for root number as well as total root length per explant. As expected, BA inhibited root formation, which was indicated by a reduced root number and a reduced total root length per explant. Forty μM PRO and 10 μM TRI could counteract this effect. In contrast with PRO and TRI, PBZ caused a significant growth reduction of the main shoot of the explant (Fig. 3). In combination with BA, TRI did not reduce growth of the new shoots, in contrast with PRO and PBZ. Some globe-shaped clusters of adventitious shoots which had developed on BMS with 10 μM BA and 10 μM PRO were each transferred to a vessel with cytokinin-free BMS. After 10 weeks they had produced long roots and completely filled the vessels with shoots (Fig. 4).

Interaction of IMA with BA, mT, Z, or TDZ in S. floribundum

The results are presented in Table 2. The effect of all tested cytokinins was enhanced by IMA. 1 μM TDZ induced more and smaller shoot meristems (50) on IMA free-medium than 10 μM BA, mT, or Z. In combination with IMA, TDZ also produced significantly more shoots (77) than did the other cytokinins. Without cytokinins in the medium, there was no shoot multiplication, only root formation (Table 2). In contrast with mT and Z, BA and TDZ did not allow root formation, irrespective of the presence of IMA. The number of roots and the total root length per explant were significantly diminished by adding IMA to the medium with mT or Z.

Fig. 4. A globe-shaped cluster of adventitious shoots, which developed on BMS with 10 μM BA and 10 μM PRO, was transferred to a vessel with cytokinin-free BMS. After 10 weeks it produced long roots and completely filled the vessel with shoots.

Table 2. Interaction of imazalil with mT, Z and TDZ regarding the number of new shoots, root number, and total root length (mm) per *S. floribundum* explant after a culture period of 10 weeks.[a]

	Imazalil					
	Shoot number		Root number		Root length (mm)	
Added cytokinin	0 μM	34 μM	0 μM	34 μM	0 μM	34 μM
0	0 a	0 a	4.3 d	4.7 d	177 c	183 c
Ba (10 μM)	8 b	44 d	0 a	0 a	0 a	0 a
mT (10 μM)	18 c	43 d	2.7 c	1.0 b	80 b	17 a
Z (10 μM)	21 c	43 d	3.5 cd	0.4 ab	72 b	4 a
TDZ (1 μM)	50 d	77 e	0 a	0 a	0 a	0 a

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

Interaction of Prochloraz with BA in A. andreanum

In *A. andreanum* 10 μM PRO enhanced the shoot-inducing effect of BA: an average of 60 small shoots (meristems) was calculated (Table 3, Fig. 5). Without BA, 10 μM PRO considerably augmented the number of roots. 40 μM was supraoptimal: the plants remained small and did not develop roots. The meristems which developed on medium with 2 μM BA + 40 μM PRO were too small to be counted. The sections in Figure 6 show that the formed structures were shoot meristems.

Discussion

IMA (Werbrouck and Debergh 1995) is not the only imidazole fungicide which can considerably enhance the shoot-inducing effect of BA; related fungicides such as

Table 3. Interaction of prochloraz with BA regarding the number of new shoots, root number, and total root length (mm) per *A. andreanum* explant after a culture period of 15 weeks.[a]

	Ba					
	Shoot number		Root number		Root length (mm)	
	0 μM	2 μM	0 μM	2 μM	0 μM	2 μM
Prochloraz (μM)						
0	0 a	13 b	4.4 b	7.7 c	128 c	237 d
10	0 a	60 c	11.1 d	1.8 a	202 d	34 b
40	0 a	n.c.	0 a	0 a	0 a	0 a

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

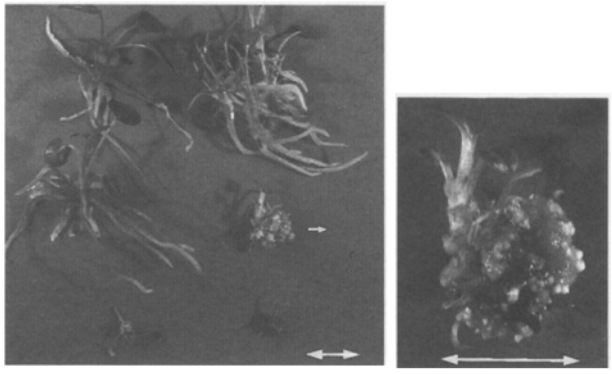

Fig. 5. Interaction between PRO and BA in *A. andreanum* after 10 weeks. Left column, 0 μM BA; right column, 2 μM BA; 1st, 2nd and 3rd row, 0, 10, 40 μM PRO. Magnified image, 2 μM BA + 10 μM PRO. Double arrow = 1 cm.

PRO and TRI as well as the growth retardant PBZ, have comparable effects. This synergistic effect was confirmed for such widely different cytokinins as zeatin, *meta*-topolin, and thidiazuron in combination with imazalil. The interaction with this wide selection of cytokinins indicates that IMA probably affects a general mechanism of cytokinin action.

A heterocyclic ring containing a sp^2-hybridized nitrogen with a lone electron pair is a structural feature shared by IMA, PRO, TRI, and PBZ. In PBZ, it is partially responsible for growth reduction caused by an inhibition of the GA biosynthesis (Rademacher 1991, Grossman 1992). Although PRO, TRI, and IMA (Werbrouck and Debergh 1995) were not definite growth retardants (the growth of the main shoot was not reduced in our experiments), they were strong enhancers of the shoot induction effect of BA. PBZ, which severely reduced growth of the main shoots, seemed to enhance the shoot-inducing effect of BA at a lower extent. However, growth reduction of the induced meristems by PBZ could mask the actual number of induced meristems. Although

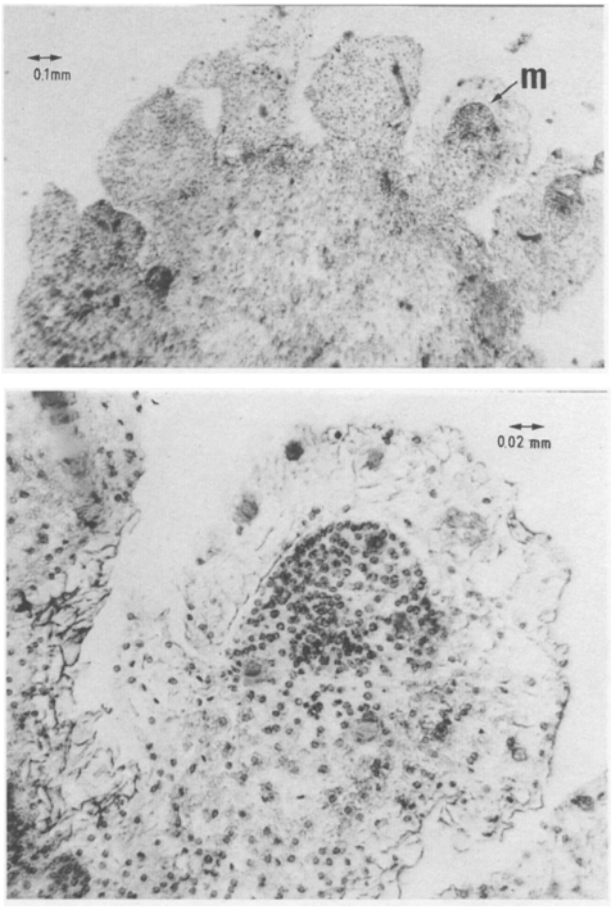

Fig. 6. Adventitious meristems (m) appear at the base of *A. andreanum* explants cultured for 10 weeks on a basal medium supplemented with 10 μM PRO + 2 μM BA.

a possible role of gibberellins in these interactions cannot be excluded, other mechanisms remain possible. Our results suggest that molecules with this structural feature can interact with cytokinins. The metabolism of exogenous cytokinins can change in favor of biologically active derivatives, or cytokinin receptors can be affected. The investigated imidazoles and triazoles had no caulogenic effect on cytokinin-free medium. This suggests that whether or not the metabolism of endogenous cytokinins is influenced, this had no significant effect on bud induction. Endogenous auxins or their receptors could be affected as well, because the number of roots and the total root length per explant were greatly increased at particular concentrations of PRO, TRI, or PBZ, depending on the presence of BA.

A. andreanum reacted in the same way as its relative *S. floribundum*. We were not able to observe an effect of IMA on the shoot-inducing effect of BA in species from other genera, i.e., *Ficus, Rosa, N. tabacum,* and *Cordyline* (results not shown). Only Araceae seems to be sensitive to this interaction. It took quite some time for the symptoms to become visible; the first meristems appeared only after 6 weeks. Final observation of the experiments with *S. floribundum* and *A. andreanum* were made after 10 and 15 weeks, respectively, so that the shoots could be counted.

Imidazole fungicides may serve as an additional means to induce adventitious shoots in Araceae. For genetic transformation and mutagenesis, where a large number of adventitious shoots are wanted, this may be especially useful. Regarding *Agrobacterium*-mediated transformation, we observed that a bacterial contamination of the explants often completely inhibited the shoot-boosting effect.

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