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Inhibition of Gibberellin Production in the Fungi *Gibberella fujikuroi* and *Sphaceloma manihoticola* by Plant Growth Retardants

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ABSTRACT

The effect of different types of plant growth retardants on fungal gibberellin (GA) formation has been studied in cultures of *Gibberella fujikuroi* and *Sphaceloma manihoticola*. Quaternary ammonium compounds (chlormequat chloride, mepiquat chloride, Amo-1618), triazoles (uniconazole and several experimental compounds), and the norbornanodiazetine tetcyclacis inhibited GA biosynthesis in both fungal species. Concentrations between 2×10^{-4} and 10^{-9} M were required for a 50% inhibition of the production of gibberellin A_3 in *Gibberella fujikuroi* and of giberellin A_4 in *Sphaceloma manihoticola*. The formation of other prominent GAs was affected at a similar degree of intensity. Tetcyclacis was the most active compound in both fungi. Compared to the growth retardants mentioned above, the biological activity of chlorphonium chloride was low. The acylcyclohexanediones prohexadione and LAB 198 999 had virtually no activity. Most likely, this lack of activity is due to a rapid metabolism of the compounds in the cultures. For the triazole-type compounds and tetcyclacis, a relatively distinct correlation exists in their ability to inhibit GA formation in fungal cultures, to block *ent*-kaurene oxygenase in a cell-free system, and to reduce shoot growth of rice seedlings. Due to differences in their metabolic fate and species specificities, such conclusions cannot be made for the other compounds.

Plant growth retardants play an important role in agriculture and horticulture in reducing unwanted shoot elongation (21). At present, five major groups have been recognized. Ethylene-releasing compounds such as ethephon reduce shoot elongation mainly by inhibiting cell division. Daminozide most likely acts by causing a more rapid inactivation of GAs[1] and by inhibiting GA translocation (31). Quaternary ammonium and phosphonium compounds (e.g. CCC, mepiquat chloride, Amo-1618, and chlorphonium chloride), compounds with a nitrogen-containing heterocycle (e.g. ancymidol, tetcyclacis, paclobutrazol, uniconazole, and inabenfide), and acylcyclohexanediones (e.g. prohexadione, cimectacarb, and LAB 198 999) inhibit the biosynthesis of GAs. It is known that representatives of the latter three groups block, respectively, the cyclization of geranylgeranyl pyrophosphate via copalyl pyrophosphate into *ent*-kaurene, the oxidative reactions from *ent*-kaurene to *ent*-kaurenoic acid, or 3β-hydroxylation and related hydroxylations in the late steps of GA biosynthesis (for details see refs. 8, 10, 22).

GAs occur not only in higher plants, but also in fungi such as *Gibberella fujikuroi* and *Sphaceloma manihoticola* (25). The biosynthetic sequence and the type of enzymes involved in fungal GA formation are largely comparable to those in higher plants, although distinct differences must not be overlooked (1, 5, 8, 20). Contrary to the situation in the tissue of higher plants, far higher concentrations of GAs are present in the fermentation liquid of *G. fujikuroi* and *S. manihoticola* and can, thus, be analyzed much easier.

Due to the close relationship of GA metabolism in higher plants to that in fungi and to the technical advantages in the analysis of GAs from fungal cultures, it appears logical to study the effects of growth retardants on fungal GA biosynthesis. Such investigations should speed up the search for further inhibitors of GA biosynthesis and also help identify the mode of action of new plant growth retardants.

Older publications have shown that quaternary ammonium compounds such as CCC and Amo-1618 strongly inhibit GA biosynthesis in *G. fujikuroi* (12, 19, 32). It was demonstrated that ancymidol, flurprimidol (EL 509), and other pyrimidines also blocked GA formation in this fungus (5). Similar results were obtained with paclobutrazol (14). To complete the knowledge about influences of plant growth retardants on fungal GA biosynthesis, old and new compounds have been tested both in *G. fujikuroi* and *S. manihoticola*.

MATERIALS AND METHODS

Plant Growth Retardants Used

The following compounds were tested: CCC; 1,1-dimethylpiperidinium chloride (mepiquat chloride); (2-isopropyl-5-methyl-4-trimethylammonium chloride)-phenyl-1-piperidinium-carboxylate (Amo-1618); 2,4 dichlorobenzyl-tributyl-phosphonium chloride (chlorphonium chloride); 1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1, 2, 4-triazol-1-yl)-1-penten-3-ol (uniconazole); 1-(2,4-dichlorophenyl)-2-methoxy-1-methyl-2-(1H-1,2,4-triazol-1-yl)-ethanol (BAS 110 .. W); 1-phenoxy - 3 - (1H-1,2,4-triazol-1-yl)-4-hydroxy-5,5-dimethylhexane (BAS 111 ..W); 1-(4-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)-4,4-dimethyl-pentan-1-on (LAB 117 682); 1-(4-chlorphenyl) - 1 - hydroximino - 3 - (1H-1,2,4-triazol-1-yl)-4,4-dimethyl-pentane (LAB 129 409); 1-(4-chlorophenyl)-3-(1H-1,2,4-triazol-1-yl)-4,4-dimethyl-2-methyl-

[1] Abbreviations: GAs, gibberellins; GA_n, gibberellin A_n; CCC, chlormequat chloride.

pentan-1-on (LAB 130 827); 1-(4-trifluormethyl)-2-(1H-1,2,4-triazol-1-yl)-3-(5-methyl-1,3-dioxan-5-yl)-propen-3-ol (LAB 150 978); 5-(4-chlorophenyl)-3,4,5,9,10-pentaazatetracyclo [5,4,1,$0^{2,6}$,$0^{8,11}$]dodeca-3,9-diene (tetcyclacis); 4-(ethyl-α-hydroxymethy-lene)-3,5-dioxocyclohexanecarboxylic acid (prohexadione); 4-(*n* - propyl - α - hydroxymethylene)-3,5-dioxocyclohexanecar-boxylic acid ethyl ester (LAB 198 999). (For structures and further literature see refs. 22, 24, 27.)

Cultivation of Fungi

Gibberella fujikuroi (strain CBS 265.54 purchased from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) was grown on a nitrogen-limited nutrient solution (modified as in ref. 7) containing, per L, 80 g of sucrose, 5 g of KH_2PO_4/K_2HPO_4, 1 g of $MgSO_4 \cdot 7 H_2O$, 300 mg of NH_4NO_3 and 5 mg of $FeSO_4 \cdot 7H_2O$ complexed with Na-EDTA, Zn, Cu, Mn, and Mo in traces. The initial pH was adjusted to 6.0. *Sphaceloma manihoticola* (ATCC 442.92 [American Type Culture Collection, Rockville, MD], equivalent to strain No. 43 in ref. 25) was grown on an identical medium with the exception that maltose was used as a carbon source and the pH was adjusted to 4.5. Plant growth retardants were added to the sterile culture media as solutions in ethanol or acetone (1 mL/100 mL) giving final concentrations between 10^{-3} or 10^{-4} and 10^{-10} M. One milliliter of liquid precultures of the fungi growing in the exponential phase was used to inoculate 100 mL of medium. The cultures were incubated at 27°C in darkness on a gyratory shaker at 150 rotations/min for 5 (*G. fujikuroi*) or 12 d (*S. manihoticola*).

Extraction and Analysis of GAs

Mycelia were removed either by centrifugation or filtration. Dry matter of mycelia was determined after drying at 105°C. GAs were extracted and analyzed by GLC using methods previously described (26). Briefly, the clarified solution was acidified to pH 2.5 with HCl and then extracted four times with 35 mL of water-saturated ethyl acetate. The combined organic extract was partitioned three times against 70 mL of ethyl acetate-saturated 100 mM K_2HPO. After acidification to pH 2.5 with HCl, the combined aqueous phases were extracted four times with 70-mL portions of water-saturated ethyl acetate. The ethyl acetate extract was washed four times with 14-mL portions of water adjusted to pH 2.5 with HCl. An aliquot of an extract (usually 1%) was taken to dryness by evaporating the organic solvent with a stream of N_2. The sample was derivatized by methylation with etheral diazomethane followed by trimethylsilylation with *N*-methyl-*N*-trimethylsilyltrifluoracetamide at 90°C for 30 min in a sealed glass ampoule. In most cases, analyses were carried out on a Packard 430 gas chromatograph with flame ionization detection (glass column [1800 × 4 mm] packed with 2% [w/w] QF-1 on Chromosorb W/AW-DMCS [80–100 mesh]; 40 mL min^{-1} N_2 as carrier gas; column temperature maintained at 170°C for 3 min and then raised with 5°C min^{-1} to 240°C, where it was kept constant for 3 min). To verify the data obtained, analyses were occasionally repeated using a Hewlett-Packard 5890 gas chromatograph equipped with a 5971 A mass-selective detector. Here, samples of 1 μL were injected into a fused silica WCOT bonded HP 1 capillary column (25,000 × 0.2 mm with 0.33 μm film thickness) using the following temperature program: 1 min at 60°C followed by an increase at 20°C min^{-1} to 240°C and then at 4°C min^{-1} to 295°C. The He inlet pressure was 0.13 MPa and the temperatures of the injector, interface, and MS source were 220, 250, and 175°C, respectively. For identification, the full mass spectra or selected ions were compared with reference spectra.

Quantitations of GAs were corrected for extraction losses. An extraction efficiency of 85 ± 12% was estimated in separate experiments with spiked culture filtrates (cf. 26). All experiments were carried out at least twice and the resulting mean values were used for further calculations. Quantitative differences between parallels did not exceed 20%.

RESULTS AND DISCUSSION

Under the conditions chosen, both fungi produced several different GAs. Typically, control incubations of *G. fujikuroi* yielded GA_3 (approximately 30 mg L^{-1}), GA_4 (25 mg L^{-1}), GA_7 (17 mg L^{-1}), GA_{13} (10 mg L^{-1}), GA_9 (3 mg L^{-1}), GA_1 (3 mg L^{-1}), and other GAs. This observation agrees with previous results (2, 3, 12). In the absence of plant growth retardants, the main GAs found in the culture filtrate of *S. manihoticola* were GA_4 (15 mg L^{-1}), GA_{13} (4 mg L^{-1}), and GA_{14} (1 mg L^{-1}). GA_{24}, GA_9, GA_{15}, GA_{25}, GA_{36}, and GA_{37} were found to be present at lower concentrations (28).

In the experiments with growth retardants, analyses concentrated on GA_3 and GA_4 as the main GAs in *G. fujikuroi* and *S. manihoticola*, respectively. In most instances, the

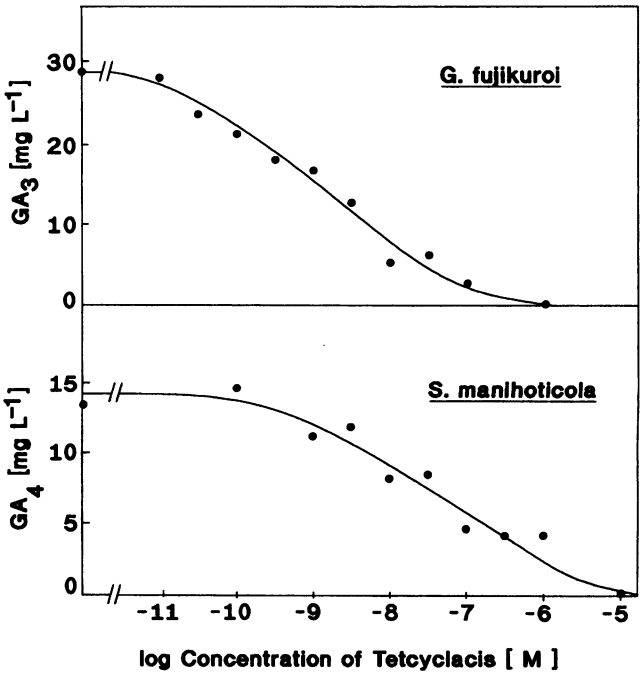

Figure 1. Dose-response curves for the inhibition by tetcyclacis of GA_3 and GA_4 formation in *G. fujikuroi* and *S. manihoticola*, respectively.

Table I. *Activities of Different Plant Growth Retardants on Fungal GA Formation*

Molar concentrations of different plant growth retardants required for a 50% inhibition of GA_3 production in *G. fujikuroi* and of GA_4 production in *S. manihoticola* (I_{50} values).

Retardant	*G. fujikuroi*	*S. manihoticola*
CCC	2×10^{-6}	5×10^{-7}
Mepiquat chloride	4×10^{-7}	7×10^{-7}
Amo-1618	3×10^{-6}	1×10^{-6}
Chlorphonium chloride	5×10^{-4}	$>10^{-3}$
Uniconazole	2×10^{-6}	6×10^{-7}
BAS 110 .. W	2×10^{-4}	2×10^{-7}
BAS 111 .. W	5×10^{-5}	5×10^{-7}
LAB 117 682	2×10^{-6}	8×10^{-7}
LAB 129 409	2×10^{-6}	not determined
LAB 130 827	3×10^{-7}	not determined
LAB 150 978	1×10^{-7}	8×10^{-8}
Tetcyclacis	1×10^{-9}	4×10^{-8}
Prohexadione	$>10^{-3}$	$>10^{-3}$
LAB 198 999	$>10^{-3}$	$>10^{-3}$

growth retardants inhibited GA production in both fungi. The results obtained with tetcyclacis are typical and are shown in Figure 1. Tetcyclacis is active over a wide range of concentrations and sigmoid dose-response curves are obtained in both fungi. From the dose-response curves, the I_{50} values for a 50% inhibition of GA production have been calculated for the different growth retardants and are shown in Table I.

The dose-response curves for the other GAs present in higher amounts yielded almost identical I_{50} values under the influence of all growth retardants found to be active. As a typical result, the I_{50} values for the formation of GA_1, GA_3, GA_4, GA_7, and GA_{13} in *G. fujikuroi* in the presence of LAB 117 682 are shown in Table II. These observations indicate that the biosynthesis of a precursor common to all of these GAs is inhibited. The same conclusion has been drawn for CCC and related compounds in *G. fujikuroi* (9) and fits also with the known mode of action of these compounds in higher plants (8, 10, 22).

The inhibitory effect of CCC on GA biosynthesis in *G. fujikuroi* matches relatively well with data published in older contributions. I_{50} values for CCC of approximately 6×10^{-7} M and 3×10^{-6} M, respectively, have been reported (9, 19). The present result (see Table I) is approximately the mean of these values. The differences observed between these investigations must be deemed relatively minor when taking into consideration that different fungal strains and different media were used. Also, bioassays used in the older studies could not yield accurate quantitations.

Other results from the literature are more difficult to interpret with regard to I_{50} values, but do not contradict the findings here. From these data, it can be concluded that the I_{50} value for Amo-1618 in *G. fujikuroi* lies markedly below 10^{-4} M (32). CCC at 1.1 mg L^{-1} (approximately 6×10^{-6} M) inhibited GA_3 production in the same fungal species by approximately 80% (6), and 10^{-4} M CCC inhibited more than 90% (30). CCC, Amo-1618, mepiquat chloride, and paclobutrazol at 10^{-4} M inhibited GA_3 production in *G. fujikuroi* by 79, 65, 67, and 62%, respectively (16). The I_{50} value for paclobutrazol in *G. fujikuroi* was found to be less than 10^{-5} M (14). The pyrimidines ancymidol and flurprimidol gave I_{50} values in this fungus of approximately 10^{-4} M (5). It must be noted, however, that paclobutrazol, as well as the pyrimidine compounds, is relatively fungitoxic, as determined by mycelial growth. Therefore, the inhibition of GA production might, at least in part, also be due to fungicidal activity. On the other hand, such an effect largely can be ruled out for the compounds used in this investigation because even the highest concentrations used did not significantly affect the formation of dry matter in either of the fungi (data not shown).

The quaternary ammonium compounds CCC, mepiquat chloride, and Amo-1618 exert a similar activity on GA formation in both fungi (Table I). In all cases, the I_{50} value lies in the order of magnitude of 10^{-6} M. The phosphonium compound chlorphonium chloride is known to be rapidly broken down in cultures of *G. fujikuroi* (9). This is also reflected by the results obtained in this investigation: no pronounced influence on GA formation could be detected in *S. manihoticola*. The I_{50} value of 5×10^{-4} M in *G. fujikuroi* is relatively high.

Triazoles appear to be generally more active in *S. manihoticola* than in *G. fujikuroi*. This is especially obvious with BAS 110 .. W, which had to be used at a rate 1000 times higher in *G. fujikuroi* than in *S. manihoticola* to obtain an equivalent effect. In addition to differences in reaching the target enzymes, one must take into consideration that the metabolic inactivation of the triazoles may be different in the two fungi. This may lead to pronounced variations in the activity of the particular compounds on GA biosynthesis (23).

Of all the compounds tested, the norbornanodiazetine tetcyclacis is the most active inhibitor of fungal GA production, with I_{50} values of 10^{-9} M and 4×10^{-8} M in *G. fujikuroi* and *S. manihoticola*, respectively. On the other hand, the acylcyclohexanediones prohexadione and LAB 198 999 are inactive. Even at 10^{-3} M, the highest concentration used, no inhibition of GA formation or mycelial growth could be observed in either fungus (data not shown). This result is in marked contrast to observations made with higher plants, where these and related compounds retard shoot growth very actively (13, 15, 18) due to their inhibition of the formation of growth-active GAs (17, 18, 29). This inconsistency probably is due to rapid metabolism of these acylcyclohexanediones in culture. For example, LAB 198 999 applied at 10^{-3} M could not be detected by physicochemical methods in the

Table II. *Activity of LAB 117 682 on the Formation of Major GAs in Fermentations of G. fujikuroi*

Molar concentrations required for a 50% reduction of GA production (I_{50} values).

Gibberellin	I_{50}
	[M]
GA_1	2.1×10^{-6}
GA_3	2.4×10^{-6}
GA_4	1.6×10^{-6}
GA_7	2.9×10^{-6}
GA_{13}	2.2×10^{-6}

Table III. *Activities of Various Triazole-Type Plant Growth Retardants and Tetcyclacis in Different Systems*

Concentration ranges required for a 50% inhibition[a].

Compound	*G. fujikuroi* GA_3 Production	*S. manihoticola* GA_4 Production	Rice Seedling Shoot Growth	*ent*-Kaurene Oxygenase
Uniconazole	+++	++++	++++	++++
BAS 110 .. W	+	++++	++	+++
BAS 111 .. W	++	++++	++	++++
LAB 117 682	+++	++++	+++	+++++
LAB 129 409	+++	n.d.	++	+++
LAB 130 827	++++	n.d.	+++	n.d.
LAB 150 978	++++	+++++	++++	++++
Tetcyclacis	++++++	+++++	++++	+++++

[a] 50% inhibition by concentrations of 10^{-3} to 10^{-4} M (+); 10^{-4} to 10^{-5} M (++); 10^{-5} to 10^{-6} M (+++); 10^{-6} to 10^{-7} M (++++); 10^{-7} to 10^{-8} M (+++++); 10^{-8} to 10^{-9} M (++++++).

medium of *G. fujikuroi* on the 2nd d of culture (data not shown). Similarly, experiments on the degradation of LAB 198 999 and related compounds in microbially active soils have revealed a biological half-life at 20°C of less than 2 d (W. Rademacher, unpublished data), and prohexadione calcium is also rapidly degraded by soil microorganisms (15).

It is very difficult to correlate the plant growth-retarding activity of the compounds investigated with their ability to inhibit fungal GA synthesis. As outlined above, metabolic inactivation in a fungal culture may be quite different from compound to compound. Retardants such as CCC, chlorphonium chloride, or Amo-1618 are of relevant biological activity only in selected plant species (4). Furthermore, uptake, translocation, and metabolic inactivation can vary between different compounds in a given higher plant species. With these restrictions in mind, a rough comparison has been made between the activities of the triazole-type growth retardants and tetcyclacis in the two fungal species and their activities on rice seedlings grown under standardized conditions (21, 27) and on in vitro *ent*-kaurene oxygenase prepared from pumpkin endosperm (refs. 11, 24; J.E. Graebe, personal communication).

As can be seen from Table III, the degree of activity in the fungal systems often parallels the results obtained with intact rice seedlings and the in vitro *ent*-kaurene oxygenase activity. This is especially the case with LAB 150 978 and uniconazole. However, results with other compounds, e.g. BAS 110 .. W, are more variable. A parallel between growth-retarding activity and inhibition of GA biosynthesis in *G. fujikuroi* has also been reported previously for CCC and related compounds (9); however, the scales employed in this investigation were less defined.

Although it can be concluded that inhibitors of GA biosynthesis in higher plants also inhibit GA formation in fungi, differences in the metabolism of the growth retardants, species specifities, and other factors prevent the occurrence of a general parallelism.

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