Accumulation of 9β,19-Cyclopropylsterols in Cereals Treated with Fenpropimorph

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The effect of a systemic fungicide, fenpropimorph (Corbel/Mistal BASF), on the growth and sterol biosynthesis of wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) seedlings was studied to assess its phytotoxicity. Application of 250 μM fungicide retarded the growth of the shoot, primary leaf, and root of both the crops. It also inhibited the biosynthesis of major phytosterols, campesterol, stigmasterol, and sitosterol. These sterols were largely replaced by 9β,19-cyclopropyl- and Δ^8-sterols. These effects were probably due to the interaction of the fungicide with the sterol enzyme systems: (i) cycloeucalenol–obtusifoliol isomerase; (ii) Δ^8- → Δ^7-isomerase; and (iii) Δ^{22}-desaturase. At the lowest concentration (5 μM) tested, fenpropimorph was not phytotoxic.

INTRODUCTION

Fenpropimorph (Corbel/Mistal, BASF) (Figure 1) is a systemic fungicide used for the control of such phytopathogens as powdery mildews (*Erysiphales*), rusts (*Uredinales*), and smut (*Ustilaginales*) on cereals (Bohnen et al., 1979). Studies on the antifungal mode of action of this compound revealed that it inhibits ergosterol biosynthesis (Leroux and Credt, 1983; Baloch et al., 1984) by interacting with vital enzyme systems (Baloch and Mercer, 1987). Sterol intermediates that accumulate in the presence of the fungicide are incorporated into the fungal membrane instead of ergosterol. This affects normal membrane functions and is believed to be the ultimate cause of the observed fungistasis (Mercer, 1988).

Since ergosterol biosynthesis in fungi (Mercer, 1984) is identical in many respects with phytosterol biosynthesis (Goodwin, 1979), it is of interest to know the effect of fenpropimorph on crop plants that are treated with it. Adverse effects of this compound on the growth of potato seedlings (Elenwa et al., 1983) reflect its phytotoxicity, which could be due to its interaction with the enzyme systems of phytosterol biosynthesis. More recent studies indicated altered sterol composition of wheat (Costet-Corio and Benveniste, 1988) and barley (Mercer et al., 1989) seedlings treated with fenpropimorph. Insects reared on such plants showed a dramatic reduction in their cholesterol content and reproductive capability (Costet-Corio et al., 1987). The present work, initiated much earlier than the recent findings, further reveals the effect of low and high doses of fenpropimorph on the growth and sterol biosynthesis of maize grown in tropical conditions beside winter wheat.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L., cv. Aquila) and maize (*Zea mays* L., cv. Brutus) seeds were soaked in 5, 15, and 250 μM solutions of fenpropimorph (BASF, Limburgernof) and then sown in John Innes No. 2 compost. Germination occurred in a growth cabinet in temperate (wheat) or tropical (maize) conditions. The seedlings were watered daily with the control (water) or appropriate fungicide solution.

Morphological Measurements. Germination percentage of both crops was recorded soon after seedling emergence. Plants were randomly sampled at the 7th (maize) or 10th (wheat) day after germination for the measurement of the various growth parameters. Leaf area was measured by a portable area meter (Crump Scientific Products). In each case at least 10 seedlings were measured and the mean value was taken as a replicate. Four such replications were used for the statistical analysis of the data. Shoot samples were also taken for dry matter and lipid determination.

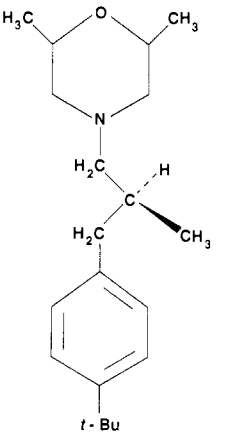

Figure 1. Structure of fenpropimorph (Corbel/Mistal).

Extraction of Lipids. About 10–15 g of fresh shoots was cut into small pieces (0.5 cm) and extracted with acetone in a homogenizer (Silverson Ltd). The homogenate was filtered through a sintered funnel and the residue twice re-extracted with acetone and refiltered. The acetone extracts were combined and mixed with water and peroxide-free diethyl ether (ether) in a separatory funnel. The ether phase containing lipids was separated, washed with water, and dried as described earlier (Khalil et al., 1990). The weight of the green lipid residue (acetone extracted) was recorded. The acetone extract (hereafter called lipids) was saponified with ethanolic KOH, and the unsaponified (unsap) lipid was separated in ether. It was then washed with water, dried, and weighed in the usual way.

The yellow unsap lipid was chromatographed on a column of acid-washed Brokmann grade III alumina (ICN Biomedicals GmbH) to remove the carotenoids from the sterols (Khalil and Mercer, 1990). The sterol-containing eluate was dried in vacuo and weighed.

Analysis of Phytosterols. Sterols were separated by TLC (Mercer et al., 1989) into three structural classes, 4-demethyl-, 4α-methyl-, and 4,4-dimethylsterols. These sterol fractions were located on the TLC plate by spraying lightly with a 0.25% (w/v) acetone solution of Rodamine 6G and then viewing under UV light. Each fraction was further analyzed by GLC and GC–MS

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0021-8561/91/1439-0404$02.50/0

Table I. Growth of Wheat and Maize As Influenced by Fenpropimorph

treatment[a]	G[b]	length, cm, of shoot[c]	leaf[c]	root[c]	leaf area,[c] cm^2	dry matter, %
wheat seedlings[d]						
control	78a	17.7a	12.2a	16.3a	5.3a	9.6a
5 μM F	76a	16.5a	11.6a	13.0a	5.0a	9.9ab
15 μM F	75a	15.2a	9.8b	11.8b	4.1b	10.2b
250 μM F	72a	11.0b	9.2b	11.2b	3.8b	10.5b
SEM±[e]	2.1	0.75	0.46	0.51	0.15	0.15
maize seedlings[d]						
control	85a	19.5a	8.6a	16.8a	7.8a	9.5a
5 μM F	84a	18.8a	8.1a	16.2a	7.4a	9.7a
15 μM F	81a	17.3a	7.8a	14.5b	7.2a	9.9ab
250 μM F	78a	11.5b	6.2b	13.8b	6.0b	10.2b
SEM±[e]	2.5	0.82	0.25	0.41	0.22	0.12

[a] F, fenpropimorph. In a given column means within each species that are followed by the same letter are not significantly different at a probability of 5% ($P = 0.05$). [b] C, germination percentage on fourth (maize) or fifth (wheat) day after sowing. [c] Primary. [d] Seedling age: 10 days (wheat) or 7 days (maize) after emergence. [e] Standard error of means.

Table II. Liquid Content of Wheat and Maize Seedlings As Affected by Fenpropimorph

treatment	mg/g dry weight of shoot lipids[b]	unsap[c] lipid	sterols
wheat seedlings[d]			
control	73.6a	24.2a	4.8a
5 μM F	75.2a	25.5a	5.2ab
15 μM F	80.5b	30.3b	5.8bc
250 μM F	95.1b	35.2c	6.4c
SEM±[c]	1.22	0.53	0.18
maize seedlings[d]			
control	69.1a	16.5a	3.2a
5 μM F	71.5a	17.2a	3.8ab
15 μM F	75.0ab	18.5b	4.2b
250 μM F	80.2b	20.1c	5.0c
SEM±[e]	1.51	0.28	0.16

[a] F, fenpropimorph. In a given column means within each species that are followed by the same letter are not significantly different at a probability of 5% ($P = 0.05$). [b] Acetone extracted. [c] Unsaponifiable. [d] Seedling age: Same as in Table I. [e] Standard error of means.

(Khalil and Mercer, 1990). GLC was carried out on a Pye-Unicam Model 204 chromatograph equipped with a flame ionization detector whose output was recorded by a computer integrator (Pye-Unicam PU4810). For GC–MS a Carlo Erba/Kratos MS-25 instrument coupled to a DS-55 data system was used. Identification of sterols was based on the coincidence of their mass spectral fragmentation patterns with published data (Audier et al., 1966; Berti et al., 1967) and those of authentic samples.

In fenpropimorph-treated plants some unusual 9β,19-cyclopropylsterols were present in the 4-demethylsterol fraction of TLC. They were identified from their characteristic fragments, ion a, b, and c (Audier et al., 1966). In 24-methylenepollinastanol ($M^+ = 412$) these ions appeared at m/e 300, 353, and 351, respectively. They were shifted to 302, 355, and 353, respectively, in the case of 24-methylpollinastanol ($M^+ = 414$). The mass spectra of both these sterols were in complete agreement with their established structures (Mercer et al., 1989). Two other unusual sterols containing a cyclopropyl ring appeared in the 4α-methylsterol fraction of TLC. Of these, 31-norcyclobranol ($M^+ = 426$) showed three distinctive peaks at m/e 300, 353, and 365. These ions were found at m/e 314, 367, and 379 in cyclofontuminol ($M^+ = 440$), also reported earlier (Mukam et al., 1973).

In the 4α-methylsterol fraction of TLC some Δ^8-sterols in the treated seedlings were also found in excess. These sterols were identified by the appearance of their fragments at $M^+ - 15$, $M^+ - 18$, $M^+ - 33$, and $M^+ -$ lateral chain. These fragments were in concert with their suggested structures reported elsewhere (Mercer et al., 1989). Authentic standards of these sterols also exhibited similar fragmentation patterns.

RESULTS AND DISCUSSION

The results showing the effect of low and high doses of fenpropimorph on the growth of wheat and maize seedlings are presented in Table I. The fungicide had no significant effect on seedling emergence. However, growth of both the crops was retarded by increasing concentration of fenpropimorph. The highest dose (250 μM) tested significantly ($P < 0.05$) reduced the length of shoot, primary leaf, and root of both species. Consequently, the primary leaf area was also decreased. However, low levels (5 and 15 μM) of the fungicide had little or moderate effect on seedling growth. In contrast to the inhibition of growth, the dry matter content of the shoot of both wheat and maize was increased with increasing concentration of fenpropimorph.

Since fenpropimorph prevents fungal growth by inhibiting ergosterol biosynthesis (Mercer, 1988), its plant growth retarding activity might also be due to altered phytosterol biosynthesis. In view of this hypothesis, a dramatic fall in the sterol content of the treated seedlings was expected. However, this was not found, and an increase in the sterol content in the treated seedlings of both wheat and maize (Table II) was observed. The lipids (acetone-extracted) and the unsap lipid contents were also increased with increasing levels of fenpropimorph. These increases were positively related to the dry matter content of the treated seedlings.

The sterol composition of the shoots of both wheat and maize seedlings treated with different levels of fenpropimorph (Table III) revealed that the synthesis of major phytosterols, campesterol, stigmasterol, and sitosterol, was diminished by the fungicide. These Δ^5-sterols, which constituted about 95–96% of the total sterols in untreated control shoots, were reduced to 13.6% in wheat and 10.3% in maize seedlings treated with 250 μM fenpropimorph. However, this reduction was not so pronounced (but still noticeable) in seedlings treated with low doses (5 and 15 μM) of the fungicide. Among the three major phytosterols, stigmasterol was the most sensitive to the presence of fenpropimorph since it totally disappeared in both crops even with the application of the lowest level (5 μM) of the fungicide.

These artifacts led to the accumulation of 9β,19-cyclopropyl- and Δ^8-sterols in the treated seedlings. Cycloeucalenol, a normal biosynthetic intermediate of phytosterols (Figure 2) containing a cyclopropyl ring, was found in greater proportion in the treated seedlings than in the untreated control seedlings. It rose from 1.5 to 45.4% of the total sterols in wheat seedlings treated with 250 μM fenpropimorph. Likewise, cycloeucalenol plus 24-dihydrocycloeucalenol content in the treated maize shoot increased from 0.8 to 18.8% of the total sterols. The amount of other such biosynthetic intermediates, cycloartenol and 24-methylene cycloartenol (Figure 2), also increased in the treated seedlings but to a small extent. These observations suggest that fenpropimorph inhibited the formation of obtusifoliol (Figure 2) and thus reduced the biosynthesis of major (Δ^5) phytosterols.

Some unusual 9β,19-cyclopropylsterols, absent in the control seedlings, were also detected in the treated shoots of both crops. Of these, 24-methylenepollinastanol was found in plants treated with 5 and 15 μM fenpropimorph, and 24-methylpollinastanol was observed in seedlings supplied with 250 μM fungicide. The latter was present in higher proportion in maize (42.8% of the total sterols) than in wheat (13.2% of the total sterols) seedlings, indicating variable response of the two species to fungicide. This was also evident by the formation of a small amount

Table III. Sterol Composition of Wheat and Maize As Influenced by Fenpropimorph[a]

	wheat seedlings[b]				maize seedlings[b]			
sterol (as % of total sterol)	control	5 μM F	15 μM F	250 μM F	control	5 μM F	15 μM F	250 μM F
campesterol	22.2	18.5	18.3	4.3	14.8	14.4	12.8	2.7
stigmasterol	10.4				21.1			
sitosterol	62.2	61.7	60.1	9.3	60.1	50.4	44.2	7.6
obtusifoliol + 24-dihydroobtusifoliol	0.4	1.0	0.8	1.4	0.5	0.3	1.2	1.1
cycloeucalenol	1.5	3.3	3.4	45.4				
cycloeucalenol + 24-dihydrocycloeucalenol					0.8	3.1	4.5	18.8
cycloartenol	0.3	0.4	0.6	2.1	0.4	0.7	0.6	3.3
24-methylenecycloartenol	1.4	1.8	1.7	5.8	1.5	0.4	0.7	1.7
amyrins[c]	0.8	0.8	0.9	1.3	0.6	0.7	1.0	1.9
24-methylenepollinastanol		9.9	10.2			5.1	8.0	
24-methylpollinastanol				13.2				42.8[f]
5α-ergosta-8,22-dien-3β-ol		*d*	*d*	6.7[e]				
5α-ergosta-8-en-3β-ol						22.3	20.2	8.5
5α-stigmasta-8-en-3β-ol		1.3	2.5	6.6		2.3	6.5	9.0
31-norcyclobranol		0.7	1.1	1.9				1.8
cyclofontumienol				1.3				
others	0.8	0.6	0.4	0.7	0.2	0.3	0.2	0.8
total Δ^5-sterols	94.8	80.2	78.4	13.6	96.0	64.8	57.0	10.3
total Δ^8-sterols	0.4	2.3	3.3	14.7	0.5	24.9	27.9	18.6
total 9β,19-cyclopropylsterols	3.2	16.1	17.0	69.7	2.7	9.3	13.9	68.4

[a] F, fenpropimorph. [b] Seedling age: See footnote in Table I. [c] These are not sterols, but peritacyclic triterpenes (M^+ = 426). [d] Trace amount of 5α-ergosta-8,22-dien-3β-ol cochromatographed with 24-methylenepollinastanol. [e] Also contained traces of 24-methylenepollinastanol and 5α-ergosta-8-en-3β-ol. [f] Contained trace amount of α-ergosta-8,22-dien-3β-ol.

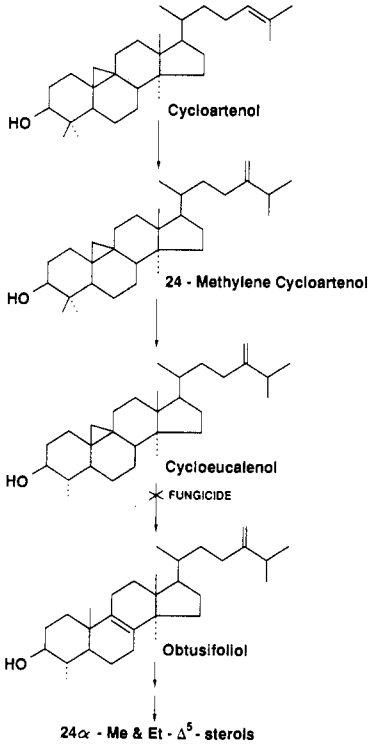

Figure 2. Phytosterol biosynthesis and the site of action of fenpropimorph.

of cyclofontumienol in wheat but not in maize seedlings. Again the latter contained 31-norcyclobranol only in the presence of 250 μM fenpropimorph. However, at lower levels (5 and 15 μM) some Δ^8-sterols accumulated in much higher proportion in maize than in wheat plants. These Δ^8-sterols, rarely found in excess in cereals, were 5α-ergost-8-en-3β-ol and 5α-stigmast-8-en-3β-ol. They were also found in barley and wheat crops in earlier studies (Costet-Corio et al., 1987; Mercer et al., 1989).

Fenpropimorph also caused accumulation of Δ^8-sterols in fungi (Leroux and Credt, 1983; Baloch et al., 1984) by inhibiting the enzyme Δ^{8}- → Δ^7-isomerase (Baloch and Mercer, 1987). Hence, it appears that the same enzyme was also susceptible to fenpropimorph in cereals. However, in contrast to its effect on the Δ^{14}-reductase in fungi (Baloch and Mercer, 1987), the plant Δ^{14}-reductase was not affected since there was no accumulation of $\Delta^{8,14}$-sterols in the treated plants. In the latter, the complete disappearance of stigmasterol revealed that fenpropimorph might have interacted with the sterol Δ^{22}-desaturase (Goodwin, 1979). More significant than these effects was the formation of excessive 9β,19-cyclopropylsterols in the treated seedlings. The same findings in other studies on barley (Mercer et al., 1989) and wheat (Costet-Corio and Benveniste, 1988) reflect that fenpropimorph blocks the opening of the cyclopropyl ring of cycloeucalenol (Figure 2), probably by interacting with the enzyme cycloeucalenol–obtusifoliol isomerase (COI) (Mercer et al., 1989) which catalyzes the above reaction. This led to the formation of unusual cyclopropylsterols, which probably arose from cycloeucalenol through irregular 4-demethylation, 26(28)-double bond saturation, and/or C-28 transmethylation (Mercer et al., 1989). Since no COI operates in ergosterol biosynthesis (Mercer, 1984), cyclopropylsterols were not detected in fungi treated with fenpropimorph (Baloch et al., 1984). It may also be noted that other ergosterol biosynthesis inhibiting (EBI) fungicides, also known as 14-demethylation inhibitors (DMIs), affect phytosterol composition differently and to a small extent (Khalil and Mercer, 1990; Khalil et al., 1990).

It appears from the preceding discussion that, unlike DMIs, fenpropimorph probably interacts with three enzymes in phytosterol biosynthesis: (i) COI; (ii) Δ^{8}- → Δ^7-isomerase; and (iii) Δ^{22}-desaturase. The latter effect, not predicted in any of the previous studies, may not be significant in terms of phytotoxicity. However, the other interactions might be responsible for the plant growth retarding activity of fenpropimorph. Hence, it is recommended to avoid excessive use of such compounds.

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Received for review May 7, 1990. Accepted August 7, 1990.

Registry No. Fenpropimorph, 67306-03-0; campesterol, 474-62-4; stigmasterol, 83-48-7; sitosterol, 83-46-5; obtusifoliol, 16910-32-0; 24(28)-dihydroobtusifoliol, 16910-33-1; cycloeucalenol, 469-39-6; 24-dihydrocycloeucalenol, 59780-40-4; cycloartenol, 469-38-5; 24-methylenecycloartenol, 124713-05-9; amyrin, 126236-47-3; 24-methylenepollinastanol, 34443-88-4; 24-methylpollinastanol, 34347-58-5; 5α-ergosta-8,22-dien-3β-ol, 6673-68-3; 5α-ergosta-8-en-3β-ol, 5259-28-9; 5α-stigmasta-8-en-3β-ol, 34350-84-0; 31-norcyclobranol, 64543-35-7; cyclofontumienol, 50906-50-8.