J Plant Growth Regul (1990) 9:221–225

Journal of
Plant
Growth
Regulation

Inhibition of Abscisic Acid Biosynthesis in *Cercospora rosicola* by Triarimol

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Received November 14, 1989; accepted February 9, 1990

Abstract: The fungicide triarimol was tested for its effect on abscisic acid (ABA) accumulation in growing cultures of *Cercospora rosicola*. ABA accumulation was reduced by approximately 50% with 10^{-8} M triarimol. Growth of *C. rosicola*, as measured by dry weight accumulation, was inhibited by triarimol concentrations at or greater than 10^{-7} M. These results are compared with those obtained with clomazone, ancymidol, and paclobutrazol, which inhibit ABA accumulation by 50% at concentrations of 5×10^{-5}, 5×10^{-6}, and 5×10^{-7} M, respectively. Triarimol, therefore, is among the most potent inhibitors of ABA biosynthesis reported to date. Feeding studies with [^{14}C]mevalonic acid confirmed the inhibition of ABA biosynthesis by 5×10^{-8} M triarimol. These results support previous suggestions that one or more of the steps in the ABA biosynthetic pathway from mevalonic acid is catalyzed by cytochrome P-450. Feeding studies with 1′-deoxy-[^{2}H]-ABA in resuspended cultures of *C. rosicola* show that the conversion of this substrate is not inhibited by triarimol.

Abscisic acid (ABA) is a sesquiterpenoid plant hormone. As an isoprenoid compound, ABA is biosynthetically related to gibberellins (GAs) and cytokinins, and these hormones are in some instances antagonistic to each other.

Most of the initial work on ABA biosynthesis was done with *Cercospora rosicola* (Assante et al. 1977) and *C. cruenta* (Oritani et al. 1982), because these fungi produce high yields of ABA in a short time. This work indicated that ABA is synthesized from farnesyl pyrophosphate or another derivative of farnesol (Bennett et al. 1984) with the intermediates α-ionylideneethanol, α-ionylideneacetic acid, and 1′-deoxy-ABA (Neill and Horgan 1983, Neill et al. 1982, Neill et al. 1984). The normal terpenoid precursors, such as acetate and mevalonic acid (MVA), are converted by *C. rosicola* to ABA. Bennett et al. (1981) showed that 1,2-[^{13}C]sodium acetate was incorporated into ABA through MVA via the isoprenoid pathway in resuspension cultures of *C. rosicola*. Robinson and Ryback (1969) demonstrated that one molecule of ABA could be formed from three molecules of MVA. Neill et al. (1981, 1982) found that [^{3}H]MVA was incorporated into 1′-deoxy-[^{3}H]-ABA and [^{3}H]ABA by *C. rosicola*.

ABA biosynthesis in *C. rosicola* is inhibited by a number of sterol synthesis inhibitors, including fenarimol and nuarimol (Norman et al. 1988), and plant growth regulators, including ancymidol, paclobutrazol (Norman et al. 1983, 1986), and cytokinins (Norman et al. 1982/83). Ancymidol and some cytokinins also inhibit GA biosynthesis in cell-free preparations from wild cucumber liquid endosperm (Coolbaugh 1984, Coolbaugh et al. 1978). In the wild cucumber system, ancymidol and cytokinins yield cytochrome P-450-binding spectra and block specific oxidative reactions catalyzed by cytochrome P-450. These observations suggest that at least one of the reactions in the ABA biosynthetic pathway involves a mixed function oxidase.

Triarimol is a chemical analog of ancymidol, fenarimol, and nuarimol. It is considerably more active than ancymidol in inhibiting GA biosynthesis in the fungus *Gibberella fujikuroi* (Coolbaugh et al. 1982). The present study was undertaken to study the effects of triarimol on ABA biosynthesis in *C. rosicola*. These effects are compared with those of ancymidol, paclobutrazol, and the herbicide clomazone, which is reported to act at a site within this pathway (Sandmann and Boger 1987).

Materials and Methods

Fungus Cultures

Cercospora rosicola Passerini (strain no. 138.35) was obtained

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from R. Bennett (USDA-ARS, Pasadena, CA, USA) and cultured on potato dextrose agar supplemented with 1.25 mg/L thiamine (Neill and Horgan 1983). Liquid cultures were grown in a chemically defined liquid medium (Norman et al. 1981a). Potential inhibitors (ancymidol, paclobutrazol, clomazone, and triarimol) were added to the media prior to autoclaving.

Fifty milliliters of sterilized liquid medium in 250-ml Erlenmeyer flasks were inoculated with 1 ml mycelial suspension prepared from two agar slants blended in 25 ml sterile deionized water for two 30-s bursts at full speed in a blender. The suspension was passed through two layers of sterile cheesecloth before inoculation. The inoculated cultures were incubated on a linear shaker at 100 rpm at room temperature (~24°C) under continuous fluorescent light. Treatments were given to two or three replicate cultures, and all experiments were repeated three or more times with similar results.

[^{14}C]MVA Metabolism

Experiments were conducted to test the effect of triarimol on the incorporation of [^{14}C]MVA into [^{14}C]ABA and other metabolites. Nine micromolar 4,5-[^{14}C]-MVA (specific activity, 0.72 μCi/μmol) were added to growing 4-day-old cultures, containing 0, 10^{-8}, and 5×10^{-8} M triarimol. These cultures were maintained an additional 48 h prior to harvesting.

1′-Deoxy-[^{2}H]-ABA Metabolism

Experiments were conducted to test the effect of triarimol on the incorporation of 1′-deoxy-[^{2}H]-ABA into [^{2}H]ABA. In these experiments, treated cultures contained 5×10^{-8} M triarimol. The cultures were filtered, rinsed with sterile H_2O, and resuspended in an equal volume of triarimol-containing medium on the fifth day of growth. Ten milliliters of the resuspension were dispensed into 50-ml Erlenmeyer flasks. The resuspended cultures were incubated for 12 h, fed 1′-deoxy-[^{2}H]-ABA, and then incubated an additional 36 h prior to harvesting.

Isolation of ABA

Cultures were filtered through Whatman no. 1 paper using a Buchner funnel. The dry weight of the mycelia was recorded after drying at 70°C for 24 h. The filtrate was adjusted to pH 3.0 with 1 N HCl and extracted three times with equal volumes of EtoAc. The acidic EtoAc fractions were combined, dried over 5–10 g sodium sulfate, and evaporated to dryness on a rotary evaporator at 30–40°C. The residue was taken up in MeOH and passed through a C_{18} Sep-Pak (Baker), which was rinsed with 2 × 1 ml MeOH. The MeOH was evaporated, the residue was redissolved in 1 ml MeOH and 1.5 ml 0.01 M H_3PO_4 (pH 3.0) for high-performance liquid chromatography (HPLC). In experiments using 1′-deoxy-[^{2}H]-ABA, the whole sample was dissolved in 20 μl MeOH.

HPLC and Gas Chromatography–Mass Spectrometry (GC-MS)

In experiments with [^{14}C]MVA, 1 ml of the final extract was injected into a 1 × 25 cm HPLC column (Beckman ODS) and eluted with a gradient of 40–100% MeOH in 0.01 M H_3PO_4, pH 3.0, over 28.3 min at 2.5 ml/min. The gradient program used was isocratic from 0–7.5 min at 40% MeOH and linear from 7.5–16.25, 16.25–22.5, and 22.5–25 min at 40–75%, 75–80%, and 80–100% MeOH, respectively. The effluent was monitored at 205 nm with a Gilson variable wavelength detector, and ^{14}C-metabolites were detected with a Ramona-D detector. Samples containing 1′-deoxy-[^{2}H]-ABA metabolites were separated on a 0.46 × 25 cm column (Beckman ODS) with a flow rate of 1 ml/min. The effluent was monitored at 254 nm.

HPLC fractions containing ABA were collected and prepared for analysis by GC-MS. The MeOH was evaporated under reduced pressure and the aqueous residue was adjusted to pH 3.0, and extracted (three times) with equal volumes of EtoAc. The acidic EtoAc fraction was evaporated to dryness and methylated with diazomethane. The samples were dried and redissolved in 50 μl MeOH, and 2 μl were injected into the Hewlett Packard model 5890 gas chromatograph with a model 5970 mass selective detector. Helium was the carrier gas at 26 ml min^{-1}. The split ratio ranged from 38–42:1. The column used was a 30 m × 0.25 mm i.d. DB-1 WCOT fused silica capillary column (J & W Scientific). The identity of ABA was confirmed by comparison of mass spectra with that of an authentic standard.

Source and Purity of Reagents

Ancymidol [α-cyclopropyl-α-(p-methoxyphenyl)-5-pyrimidine methyl alcohol] and triarimol [α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methyl alcohol] were of technical grade and provided by Eli Lilly and Co. Clomazone (also known as FMC 57020 and Command) was of technical grade and obtained from FMC. 1′-Deoxy-[^{2}H]-ABA was obtained from D. Walton, SUNY Syracuse, NY, USA. 4,5-[^{14}C]-R,S MVA (specific activity, 110 μCi/μmol) was from Amersham. Paclobutrazol [(2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol] was of technical grade and obtained from ICI.

Results

Cercospora rosicola entered a linear phase of growth in liquid cultures 4 days after inoculation (Fig. 1). ABA accumulated rapidly in these cultures from day 4–6. Typically, more than 7.0 μg/ml accumulated by day 6. While both the (*2Z,4E*)-ABA and (*2E,4E*)-ABA were isolated from culture filtrates starting on day 3, (*2Z,4E*)-ABA was always more abundant (>90%). The mass spectrum of Me-(*2Z,4E*)-ABA included the following ions: m/z 278 [M^+] (1%), 190 (100%), 162 (49%), 134 (52%), and 125 (41%). Quantification of ABA was based on standard curves prepared with authentic ABA using full spectra.

The effects of triarimol on the growth and ABA accumulation in *C. rosicola* are shown in Fig. 2D. Triarimol had a significant inhibitory effect on both growth and ABA production. There was 93% inhibition of growth and 96% inhibition of ABA production with 10^{-6} M triarimol. At 10^{-7} M, the inhibi-

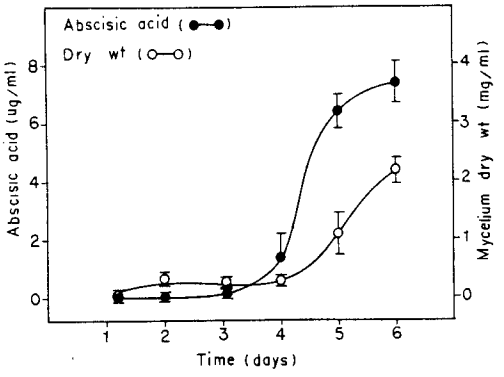

Fig. 1. Accumulation of dry weight and abscisic acid over 6-day period following inoculation with *C. rosicola*.

tion of ABA production was 97%, but that of growth was only 32%. Lower concentrations of triarimol seemed to only slightly stimulate growth compared to the controls, whereas ABA accumulation was substantially inhibited. Based on these results, we estimate that the effective dose for triarimol (ED_{50}, concentration causing 50% inhibition of ABA accumulation) is approximately 10^{-8} M. Identical experiments with clomazone, ancymidol, and paclobutrazol (Fig. 2A–C) were considerably less effective than triarimol. The ED_{50} of these compounds under the same conditions was approximately 5×10^{-4}, 5×10^{-6}, and 5×10^{-7}, respectively.

Incubation of [^{14}C]MVA in actively growing cultures routinely led to accumulation of substantial quantities of [^{14}C]ABA (Fig. 3A, retention time at 21.2 min) as previously reported by Neill et al. (1981, 1982). A second [^{14}C] peak was observed at a retention time of 22.7 min (Fig. 3A and B). The level of this compound was low in the control, but greater in the presence of triarimol. A third [^{14}C] peak at a retention time of 9.9 min was observed only in the elution profile of the control. This compound was absent with 5×10^{-8} M triarimol.

1′-Deoxy-[^{2}H]-ABA was incorporated into [^{2}H]ABA by actively growing cultures of *C. rosicola* as previously reported by Horgan et al. (1983) and Neill et al. (1981, 1982, 1987). GC-MS evidence of the incorporation of 1′-deoxy-[^{2}H]-ABA into [^{2}H]ABA was obtained by monitoring m/z 193, 165, 137, and 128, the four major ions of [^{2}H]ABA. These ions were also monitored in cultures that were not fed with 1′-deoxy-[^{2}H]-ABA. The ion chromatograms of extracts from control cultures and those containing 5×10^{-8} M triarimol, are shown in Fig. 4A–D. Fig. 4A and B indicates that there is incorporation of 1′-deoxy-[^{2}H]-ABA into [^{2}H]ABA in both control cultures and those containing 5×10^{-8} M triarimol. These data demonstrate that triarimol has no inhibitory effect on the incorporation of 1′-deoxy-[^{2}H]-ABA into [^{2}H]ABA. The presence of significant peaks at m/z 165, 137, and 128 (but not 193) in the control cultures without

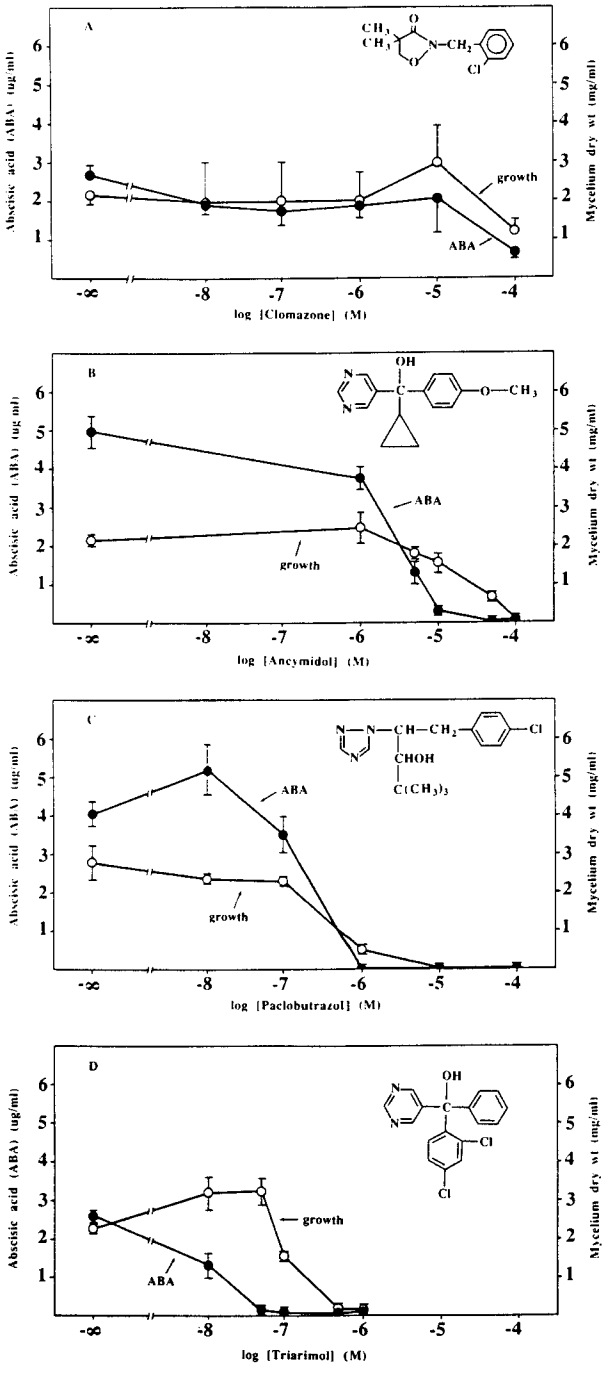

Fig. 2. Effects of clomazone (A), ancymidol (B), paclobutrazol (C), and triarimol (D) on growth and ABA accumulation in 5-day-old cultures of *C. rosicola*.

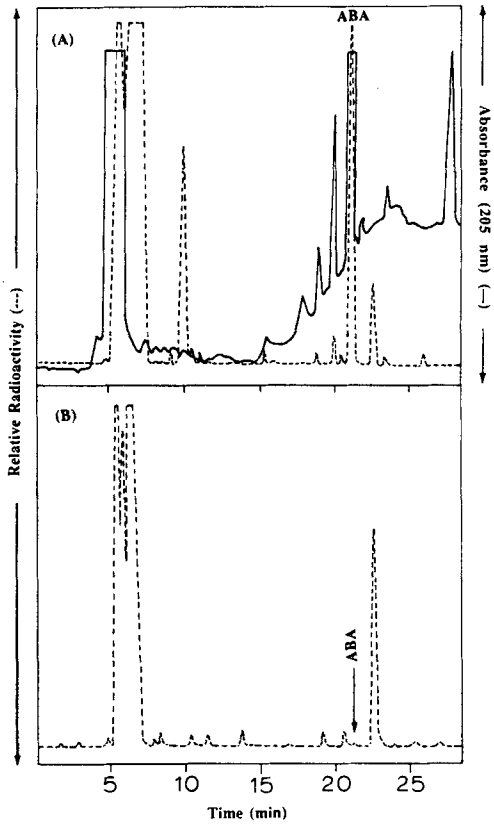

Fig. 3. HPLC profile of products of incorporation of [^{14}C]MVA metabolism in a 50-ml liquid culture of *C. rosicola* in the absence (A) and presence of 5×10^{-8} M triarimol (B).

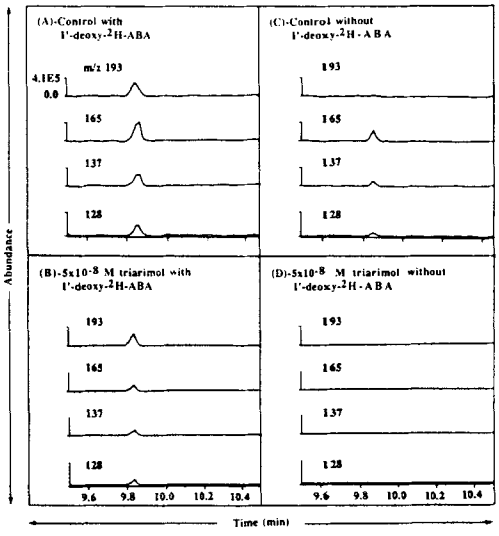

Fig. 4. Ion chromatogram profiles of m/z 193, 165, 137, and 128 obtained by GC-MS of extracts from control cultures (A,C) and those containing 5×10^{-8} M triarimol (B,D). A complete description of these results is provided in the Results section. Profiles A and B represent cultures fed with 1'-deoxy-[^{2}H]-ABA, whereas C and D were cultured without feeding.

feeding (Fig. 4C) was due to the production of unlabeled ABA by the fungus under these conditions. Boiled fungal cultures obviously do not contain any ABA (data not shown).

Discussion

The time course for growth and ABA accumulation in liquid-shake cultures of *Cercospora rosicola* over a 20-day period was described by Norman et al. (1981b). Others have reported shorter time courses (Neill and Horgan 1983). We selected conditions leading to the accumulation of up to 7.4 μg/ml ABA by day 6. At this time, growth and ABA accumulation began to level off.

The effects of ancymidol, nuarimol, fenarimol, and triarimol on growth and GA biosynthesis in the fungus *Gibberella fujikuroi* have been reported previously (Coolbaugh et al. 1982). Triarimol is a more effective inhibitor of GA biosynthesis than ancymidol in that fungus. The data presented in Fig. 2D demonstrate that triarimol also inhibits ABA biosynthesis in *C. rosicola* with an apparent ED_{50} of 10^{-8} M. At the relatively low concentrations of 5×10^{-8} and 1×10^{-8} M triarimol, ABA production was inhibited but growth was not affected. Thus, inhibition of ABA synthesis is not due to injury, but rather to a more direct effect on the ABA biosynthetic pathway. These data are consistent with those of Norman et al. (1988). They compared the effects of ancymidol, paclobutrazol, and fenarimol on ABA synthesis in *C. rosicola* and demonstrated that fenarimol is relatively more effective than the others. In that study, fenarimol, which is a close analog of triarimol, was shown to have an ED_{50} of approximately 10^{-6} M.

Like ancymidol and paclobutrazol, triarimol inhibits ABA biosynthesis at a concentration one order of magnitude lower than that which inhibits growth. It is, however, much more effective than either of these. Clomazone has comparatively little effect on growth and ABA synthesis in *C. rosicola*. The inhibition at concentrations greater than 10^{-5} M is consistent with the report by Sandmann and Boger (1987), which showed this to affect the isoprenoid biosynthetic pathway in several organisms with I_{50} values ranging from 10–60 μM. However, this seems to be a relatively high effective concentration. As such, it seems unlikely that this is the most sensitive site of action of this herbicide.

The inhibitory effect of triarimol on the incorporation of [^{14}C]MVA into [^{14}C]ABA was clear at a

concentration of 5×10^{-8} M. At this concentration, the enhanced radioactive peak was commonly present at a retention time of 22.7 min. Based upon comparative HPLC analysis, we are confident that this compound is not 1′-deoxy-ABA (retention time of 23.67 min) or α-ionylidineacetic acid (retention time of 31.81 min). At the present time the identity of this metabolite is unknown.

1′-Deoxy-[^{2}H]-ABA was incorporated into [^{2}H]ABA in resuspended cultures of *C. rosicola*. As shown in Fig. 4, a concentration of 5×10^{-8} M triarimol had no apparent inhibitory effect on the incorporation of 1′-deoxy-[^{2}H]-ABA into [^{2}H]ABA. This indicates that the site of triarimol inhibition is between MVA and 1′-deoxy-ABA in the ABA biosynthetic pathway. Triarimol may prove to be a useful tool in studying ABA biosynthesis in *C. rosicola*, and its inhibitory action might lead to new information on intermediates in this pathway.

Acknowledgments. We wish to thank Dr. Rodney Bennett (USDA-ARS, Pasadena, CA, USA) for supplying a culture of *C. rosicola*, Dr. Roger Couture (ICI) for a sample of paclobutrazol, Dr. Ed Tschabold (Eli Lilly and Co.) for samples of ancymidol and triarimol, Dr. David Simcox (FMC Corp.) for a sample of clomazone, and Dr. Daniel Walton (SUNY, Syracuse, NY, USA) for a generous sample of 1′-deoxy-[^{2}H]-ABA. Appreciation is expressed to Dr. Jan Hazebroek, Hanna Targowski, and Chris McCoy for their technical assistance and to Joanne Nystrom for preparing the manuscript.

This research was supported in part by NSF grant PCM 8415924 and USDA grant no. 87-CRCR-1-2589.

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