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INCORPORATION OF α-IONYLIDENE ETHANOL AND α-IONYLIDENE ACETIC ACID INTO ABSCISIC ACID BY *CERCOSPORA ROSICOLA**

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Key Word Index—*Cercospora rosicola*; Hyphomycetes; biosynthesis of abscisic acid; abscisic acid; α-ionylidene ethanol; α-ionylidene acetic acid; β-ionylidene ethanol; β-ionylidene acetic acid; farnesol.

Abstract—^{2}H-Labelled α-ionylidene ethanol and α-ionylidene acetic acid are converted in high yield to 1′-deoxy-abscisic acid (1′-deoxy-ABA) and abscisic acid (ABA) by *Cercospora rosicola*. Incorporation of label was confirmed by mass spectrometry. The 2-*trans* isomers of α-ionylidene ethanol and α-ionylidene acetic acid were converted to 2-*trans*-1′-deoxy-ABA but not to 2-*trans*-ABA. ^{3}H-Labelled β-ionylidene ethanol and β-ionylidene acetic acid are not incorporated into ABA by this fungus.

INTRODUCTION

We have previously presented evidence that 1′-deoxy-ABA (**3**) is the immediate precursor to ABA (**4**) in the fungus *Cercospora rosicola* [1–3]. Our results led us to suggest that the pathway of ABA biosynthesis in this fungus may proceed via the successive oxidations of a 3-methyl-5-(2′,6′,6′-trimethylcyclohex-2′-en-1′-yl)-2,4-pentadienyl intermediate. These oxidations could take place via several routes, as shown in the metabolic grid outlined in Fig. 1. This paper describes the incorporation of α-ionylidene ethanol (**1**) and α-ionylidene acetic acid (**2**) into 1′-deoxy-ABA (**3**) and ABA (**4**). Additional evidence is also presented that cyclization of farnesol or a farnesyl derivative proceeds via the formation of 2′-ene as opposed to 1′-ene intermediates (numbered as per the ABA skeleton).

RESULTS AND DISCUSSION

The time course of ABA production in liquid medium is shown in Fig. 2. ABA synthesis started after 4–5 days and lasted 3–4 days, by which time the ABA concentration had usually reached 15–20 μg/ml of culture filtrate. Mycelial growth was essentially complete after 5 days. Labelled compounds were administered at day 5 or 6 so that usually 60–80 % of the total ABA was synthesized during the time the precursors were present.

Both ^{2}H- and ^{3}H-labelled compounds were administered to the fungus. After feeding, ABA and 1′-deoxy-ABA were purified from the acid ether fractions by HPLC (system A). After methylation, ABA and 1′-deoxy-ABA were further purified as their methyl esters by HPLC (systems B and C, respectively). Methyl-ABA and methyl-1′-deoxy-ABA were then analysed by GC/MS. Actual incorporation (%) was calculated from incorporation of radioactivity (corrected for recovery losses in the case of ABA). Specific incorporation, i.e. the percentage of biosynthesized ABA synthesized from the labelled precursor, was calculated from the expression $DT/B \times 100$, where $D = \%$ ^{2}H content (determined by multiple-ion monitoring), T = total amount of ABA and B = amount of ABA synthesized during the feeding period.

As previously observed, 1′-deoxy-ABA (**3**) was converted in high yield to ABA by *C. rosicola*. α-Ionylidene ethanol (**1**) and α-ionylidene acetic acid (**2**) were both metabolized to 1′-deoxy-ABA and also to ABA in high yield. The results of these feeding experiments are shown in Table 1. The observed higher incorporation of α-ionylidene acetic acid (**2**) compared to that of 1′-deoxy-ABA (**3**) probably reflects differences in the ABA contents of the fed flasks.

Incorporation of label was detected by GC/MS of the extracted product (after methylation) as shown in Fig. 3. The mass spectrum of non-deuterated methyl-ABA displayed major ions at m/z 278 $[M]^+$, 190 (base peak), 162 and 125. The mass spectrum of methyl-ABA extracted from *C. rosicola* culture filtrate after application of ^{2}H$_3$-labelled α-ionylidene ethanol (**1**), α-ionylidene acetic acid (**2**) or 1′-deoxy-ABA (**3**) in addition displayed ions 2 and 3 amu heavier than those above, i.e. at m/z 281, 193, 165, 128, etc. The presence of these ions demonstrated unequivocally that the ^{2}H$_3$-labelled precursors had been incorporated into ABA. That the label was present in the side-chain methyl group was demonstrated by the presence of an ion at m/z 128 [4]. As the ^{2}H-labelled precursors were ^{2}H$_3$-labelled in the side-chain methyl group, this is good evidence that the compounds are incorporated *per se* without any prior rearrangement. Quantitative assessment of ^{2}H incorporation was determined by monitoring the ions at m/z 190 and 193.

Incorporation of label from α-[^{2}H$_3$]ionylidene ethanol (**1**) and α-[^{2}H$_3$]ionylidene acetic acid (**2**) into 1′-deoxy-ABA (**3**) was confirmed by GC/MS of the methylated, extracted 1′-deoxy-ABA (**3**) in an analogous manner to that described above (see Fig. 3). Quantitative assessment of incorporation was determined by monitoring the ions at m/z 125 and 128. The [3-methyl-^{3}H]-*trans* isomers of **1**,

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*Part 2 in the series "Biosynthesis of ABA in *C. rosicola*". For Part 1 see ref. [2].

†To whom correspondence should be addressed.

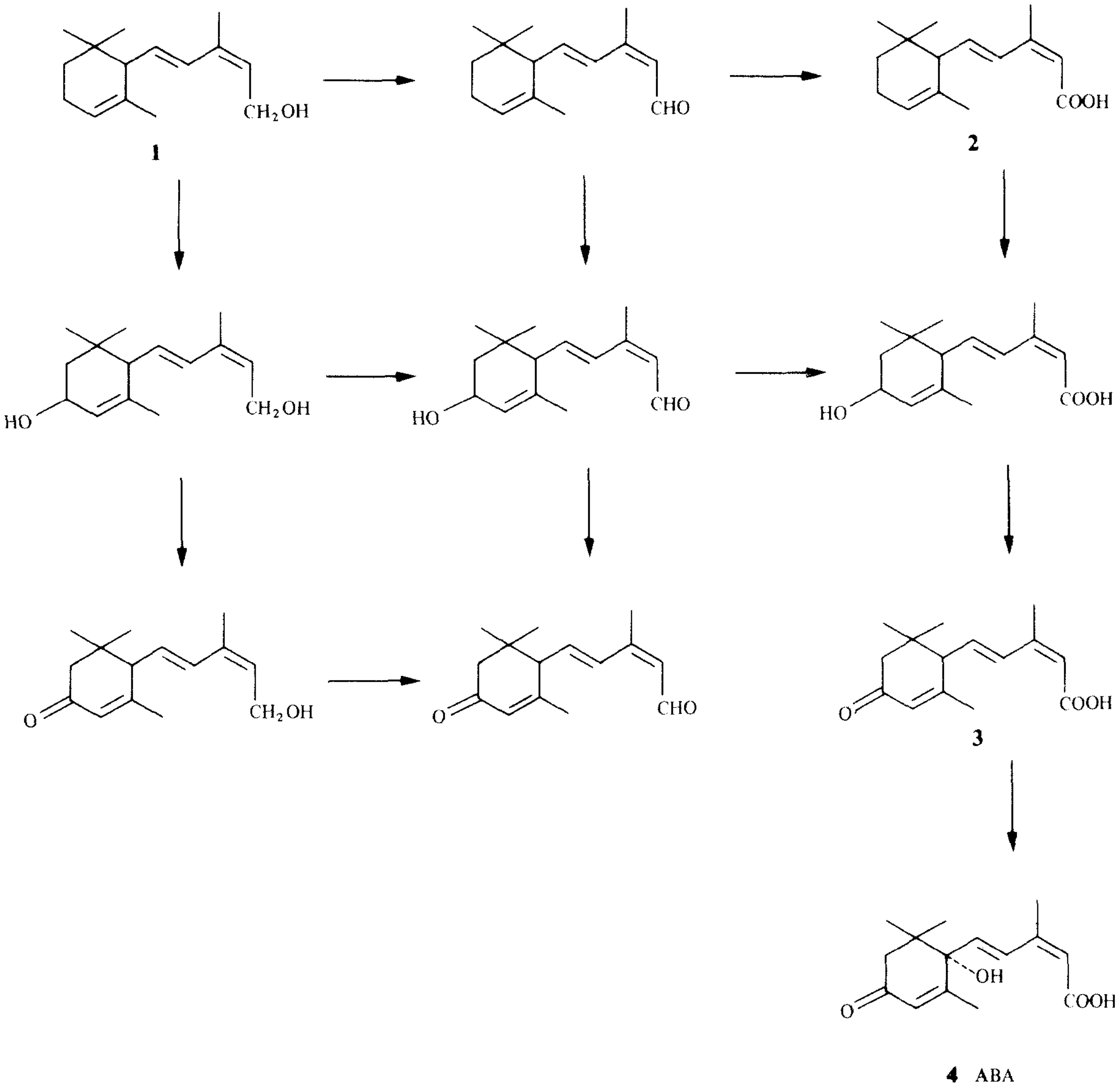


Fig. 1. Possible biosynthetic routes to ABA.

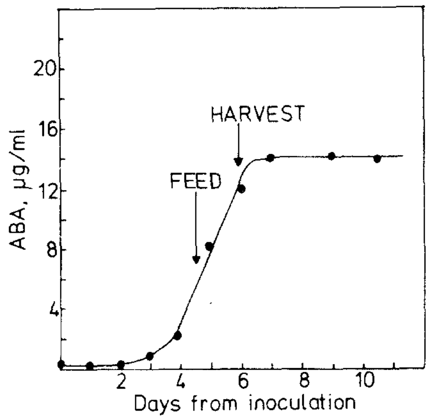


Fig. 2. ABA production by *C. rosicola* in defined liquid medium.

2 and **3** were administered to *C. rosicola*. 2-*Trans*-1′-deoxy-ABA (**7**) was not converted to ABA or to 2-*trans* ABA. However, 2-*trans*-α-ionylidene ethanol (**5**) and 2-*trans*-α-ionylidene acetic acid (**6**) were converted to 2-*trans*-1′-deoxy-ABA (**7**) (see Table 1). The results imply that the early oxidizing enzymes are rather non-specific; on the other hand, the final hydroxylase enzyme is sensitive to the geometry of the side-chain and will not utilize 2-*trans*-1′-deoxy-ABA as a substrate. No compound was transformed by a boiled control.

To determine whether or not β-cyclization of a farnesyl derivative can give rise to ABA, ^{3}H-labelled 1′-ene derivatives were applied to *C. rosicola*. Both β-ionylidene ethanol (**8**) and β-ionylidene acetic acid (**9**) were not converted to ABA. Neither was there any incorporation of the 2-*trans* isomers of β-ionylidene ethanol (**10**) or β-ionylidene acetic acid (**11**). This lack of incorporation of the β-cyclized compounds suggests: (a) that cyclization

Table 1. Utilization of α-ionylidene ethanol (**1**), α-ionylidene acetic (**2**) and 1'-deoxy-ABA (**3**) by *C. rosicola*

	2-Trans-1'-deoxy-ABA	1'-Deoxy ABA		ABA	
Compound fed	Incorporation (% dose)	Incorporation (% dose)	(Specific)	Incorporation (% dose)	(Specific)
α-Ionylidene ethanol	—	1*	53†	20	15
2-Trans-α-ionylidene ethanol	11	0	0	0	0
α-Ionylidene acetic acid	—	4*	71†	30	39
2-Trans-α-ionylidene acetic acid	13	0	0	0	0
1'-Deoxy-ABA	—	—	—	28	24
2-Trans-1'-deoxy-ABA	—	—	—	0	0

*Not corrected for extraction losses.

†Not corrected for initial endogenous 1'-deoxy-ABA.

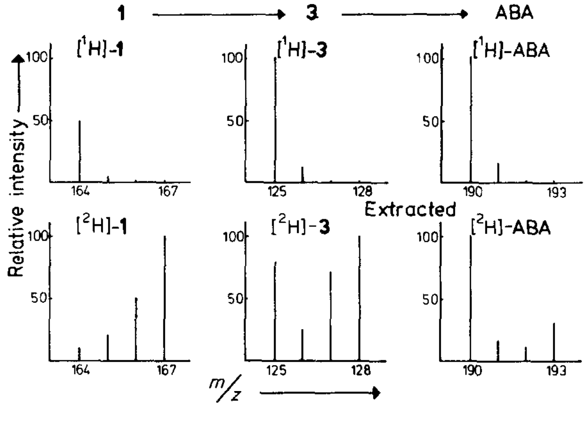
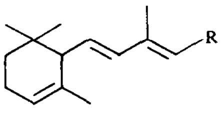
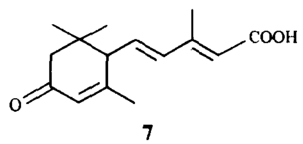


Fig. 3. Conversion of ^{2}H-labelled α-ionylidene ethanol (**1**) to 1'-deoxy-ABA (**3**) and ABA. Partial MS of methylated [^{1}H]-**1**, **3** and ABA, [^{2}H]-**1** and **3** and ABA extracted from culture filtrate following application of [^{2}H]-**1**.



5 R = CH_2OH

6 R = COOH



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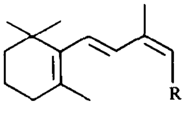

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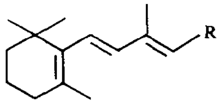
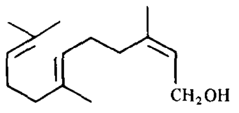
9 R = COOH



10 R = CH_2OH

11 R = COOH



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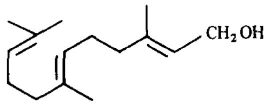

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occurs in a manner analogous to ε-ring formation during carotenoid biosynthesis, i.e. the ring is formed ε- and not β-, followed by subsequent isomerization; and (b) that β-cyclized compounds such as xanthoxin are excluded as intermediates.

The lack of incorporation of the 2-*trans* isomer of α-ionylidene ethanol (**5**) into ABA suggests that isomerization precedes or is concomitant with the formation of the first desaturated cyclic intermediate. Preliminary results indicate that neither the *cis,trans* (**12**) nor the 2-*trans* (**13**) isomers of farnesol are incorporated into ABA. It may be that farnesol pyrophosphate is the actual intermediate involved, the pyrophosphate group being necessary for desaturation and/or cyclization. Milborrow [5] has suggested that dehydrofarnesol may be an intermediate in ABA biosynthesis; experiments are underway to test this.

The incorporation of α-ionylidene ethanol (**1**) and α-ionylidene acetic acid (**2**) into ABA by *C. rosicola* demonstrates only that they can be utilized as biosynthetic precursors. Recently, however, Oritani *et al.* [6] have reported that another species of *Cercospora*, *C. cruenta*, can also incorporate α-ionylidene acetic acid into ABA. Nevertheless, the presence of these compounds as endogenous metabolites has still to be demonstrated.

EXPERIMENTAL

Materials. Cercospora rosicola Passerini (strain No. 138.35 from C.B.S., The Netherlands) was maintained on potato dextran agar supplemented with 1.25 mg/l thiamine. For feeding studies fungus was grown in 250 ml conical flasks containing 50 ml of a defined liquid medium [7], modified to contain 55 mM glucose, 8.6 mM K^+ glutamate and 1 mM NaH_2PO_4. Phosphate and thiamine were autoclaved separately. Mycelium was stripped from the agar and ground to a brei in distilled H_2O using a VirTis homogenizer (*ca* 30 sec, ½ full speed). Aliquots of this suspension were then dispensed into the liquid medium. The cultures were incubated on an orbital shaker (*ca* 150 rpm) at 26° under dim light.

Chromatography. HPLC was performed as described previously. HPLC systems: A: ODS-Hypersil (150 × 10 mm i.d.), eluted with a linear gradient of 20–100 % MeOH in 0.1 M HOAc over 40 min, 5 ml/min; B: Spherisorb (250 × 4.5 mm i.d.), eluted with hexane–*iso*-PrOH (19:1), 2 ml/min; C: Partisil-PAC (250 × 4.5 mm i.d.) eluted with hexane–EtOH (95:0.5), 2 ml/min; D: ODS-Spherisorb (150 × 4.5 mm i.d.), eluted with 45 % MeOH–0.1 M HOAc, 2 ml/min.

Measurement of ABA levels. 0.5 ml of medium plus mycelium was removed from duplicate flasks at increasing times after inoculation. A known amount of 2-*trans*-ABA was added to an aliquot of the medium, which was acidified and extracted with EtOAc. The organic phase was dried and analysed by HPLC using system D. ABA content was calculated by comparison of the heights due to the peaks of *trans*-ABA and ABA.

MS. GC/MS: Kratos MS/DS 55C linked to a Carlo Erba 4200 GC. GC: 3% OV-1 (1 m × 4 mm i.d.), 219° (methyl-ABA), 208° (methyl-1′-deoxy-ABA), He 20 ml/min. MS: source 200°, jet separator 250°, 70 eV. ^{2}H incorporation was initially assessed by full MS taken at 1 sec/decade across the GC peak. Quantitative measurement of ^{2}H incorporation was carried out by computer-controlled multiple-ion monitoring of the appropriate ions of the relevant compounds. Monitoring was carried out at a rate of 50 μsec/sample with a dwell time of 140 msec on each ion.

Administration of labelled compounds. Labelled compounds (*ca* 0.5 mg of [^{2}H] compound + *ca* 3×10^5–6×10^6 dpm [^{3}H] compound) were administered in 200 μl EtOH at or near the onset of the phase of rapid ABA synthesis. ABA levels were determined at the time of both feeding and harvest. Thus the exact quantity of ABA produced during the feeding period could be calculated. After harvest, acid Et_2O fractions of the culture filtrates were prepared.

Synthesis of α-[3-Me-^{2}H] *and* α-[3-Me-^{3}H]*ionylidene acetic acids* (**2** and **6**). ^{2}H- and ^{3}H-labelled *cis,trans*- and *trans,trans*-methyl-α-ionylidene acetates were prepared as described previously [2]. 10 mg of each ester was hydrolysed with 0.25 ml 5 N KOH and 0.5 ml EtOH for 6 hr. The hydrolysis mixture was acidified to pH 2.5 and the acids were extracted into 3 × 2 ml Et_2O. The acids were purified by HPLC in system A prior to feeding to the fungus. Portions of ^{1}H- and ^{2}H-labelled α-ionylidene acetic acid were methylated and examined by GC/MS.

[^{1}H]-**2**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 248 (7), 192 (13), 160 (9), 159 (9), 133 (37), 125 (100), 112 (19), 107 (26), 105 (11), 91 (7); [^{1}H]-**6**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 248 (19), 192 (67), 177 (22), 160 (28), 159 (17), 145 (26), 133 (100), 125 (75), 112 (59), 107 (33), 105 (16), 91 (8); [^{2}H]-**2**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 252 (0.6), 251 (1.2), 250 (0.6), 195 (15), 194 (8), 193 (3), 192 (1); 163 (14), 162 (14), 161 (6.5), 160 (3), 136 (48), 135 (40), 134 (17), 133 (9); 128 (100), 127 (62), 126 (30), 125 (4), 115 (28), 114 (20), 113 (7), 112 (2), 108 (8), 107 (24), 106 (7), 105 (11), 94 (6), 93 (13), 92 (11), 91 (14).

Synthesis of ^{2}H- *and* ^{3}H-*labelled α-ionylidene ethanols* (**1** and **5**). ^{2}H- and ^{3}H-labelled *cis,trans*- and 2-*trans*-methyl-α-ionylidene acetates were reduced separately with $LiAlH_4$ in Et_2O at 0° for 30 min. After decomposition of excess $LiAlH_4$ with EtOAc and saturated NH_4Cl the alcohols were extracted into Et_2O. The purity of the products was checked by GLC on 3% OV-1 at 210° and by HPLC in system A. The alchols were fed to the fungus without further purification. Portions of the ^{1}H- and ^{2}H- labelled alcohols were analysed by GC/MS.

[^{1}H]-**1**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 220 (2), 202 (15), 164 (36), 146 (79), 145 (18), 133 (62), 131 (100), 123 (16), 122 (26), 121 (21), 119 (33), 117 (33), 107 (40), 105 (35), 91 (47); [^{1}H]-**5**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 220 (8), 202 (15), 164 (44), 146 (71), 133 (60), 131 (100), 123 (11), 122 (21), 121 (18), 119 (28), 117 (34), 107 (42), 105 (38), 91 (51); [^{2}H]-**1**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 223 (5), 222 (3), 205 (10), 204 (9), 167 (100), 164 (47), 165 (16); 149 (42), 148 (43), 147 (23), 146 (8), 137 (93), 135 (80), 134 (60), 133 (45), 132 (35), 131 (31), 123 (90), 122 (71), 121 (71), 120 (15), 119 (13), 117 (13), 107 (70), 105 (34), 91 (52).

The ^{3}H-labelled acids and alcohols had a specific activity of 8.6 mCi/mmol. The ^{2}H-labelled compounds showed an isotopic composition, $3 \times [^2H]:2 \times [^2H]:1 \times [^2H]:0 \times [^2H]$, of 56:30:12:2.

Synthesis of β-[3-Me-^{3}H]*ionylidene acetic acids* (**9** *and* **11**). The *cis,trans* and *trans,trans* isomers of β-ionylidene acetic acid were prepared from β-ionone by methods analogous to those used for the α-compounds [2]. Tritiation of β-ionone was performed as for α-ionone. Portions of non-labelled β-acids were methylated and examined by GC/MS.

9: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 248 (55), 233 (18), 217 (15), 201 (22), 192 (16), 177 (18), 173 (38), 163 (16), 159 (47), 145 (26), 133 (56), 125 (26), 119 (100), 112 (30), 107 (20), 105 (36), 91 (33); **11**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 248 (45), 233 (15), 217 (14), 201 (20), 177 (14), 173 (38), 163 (16), 159 (30), 145 (17), 133 (30), 131 (23), 125 (18), 119 (100), 112 (10), 107 (13), 105 (27), 91 (18).

Synthesis of ^{3}H-labelled *β-ionylidene ethanols* (**8** *and* **10**). *Cis,trans*- and 2-*trans*-methyl-β-ionylidene acetates were reduced with $LiAlH_4$ as described above. Portions of the non-labelled β-alcohols were examined by GC/MS.

8: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 220 (21), 202 (74), 187 (50), 159 (40), 146 (76), 145 (46), 131 (100), 129 (21), 119 (70), 117 (38), 107 (41), 105 (58), 91 (69); **10**: EIMS (GC/MS), 24 eV, *m/z* (rel. int.): 220 (25), 202 (96), 187 (65), 159 (52), 146 (77), 145 (58), 131 (100), 129 (19), 119 (81), 117 (49), 107 (54), 105 (63), 91 (60).

The specific activity of the ^{3}H-labelled β-compounds was estimated at 1.7 mCi/mmol.

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