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Laila Freih Eid Al-Nimri
Iowa State University

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Al-Nimri, Laila Freih Eid, Ph.D.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

Abscisic acid biosynthesis in *Cercospora rosicola* and related fungi

by

Laila Freih Eid Al-Nimri

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TABLE OF CONTENTS

ABSTRACT

Abscisic acid (ABA) is an important plant hormone involved in controlling normal growth and development in higher plants. The fungus *Cercospora rosicola* synthesizes large quantities of ABA (Assante et al., 1977), which has made it a useful organism for studies of ABA biosynthesis.

The purpose of this study is to examine the ABA biosynthetic pathway in *C. rosicola*. Feeding experiments with labeled mevalonic acid (MVA), farnesyl pyrophosphate (FPP), and 1'-deoxy-ABA have confirmed the conversion of these intermediates into ABA. This conversion was inhibited in the fungicide triarimol-treated cultures fed with MVA and FPP, but not 1'-deoxy-ABA.

The development of a cell-free enzyme system from fungal extracts capable of converting any of these intermediates into ABA has not been previously reported. A cell-free enzyme system has been developed in our laboratory to convert 1'-deoxy-^{2}H-ABA into ^{2}H-ABA. The reaction products were chromatographed by reverse phase HPLC. The presumptive ABA fractions were collected and ^{2}H-ABA was quantified by GC-MS using a ^{2}H-ABA standard curve. 1'-deoxy-^{2}H-ABA was converted to an average of 1.47 pmol ^{2}H-ABA mg^{-1} protein min^{-1}. Most of the enzymatic activity was found in the microsomal fraction. The reaction required NADPH and was enhanced by FAD; it was not inhibited by triarimol. These data, taken together, suggest that cytochrome P-450 may be involved in some oxidative reactions prior to 1'-deoxy-ABA in the ABA biosynthetic pathway.

The phytopathogenic fungi *Gnomonia veneta* and *Cercospora mori* infect sycamore and mulberry leaves, respectively. These fungi do not appear to produce ABA in liquid cultures. However, infected leaves with these fungi contain elevated levels of ABA and abscise prematurely.

LIST OF TABLES

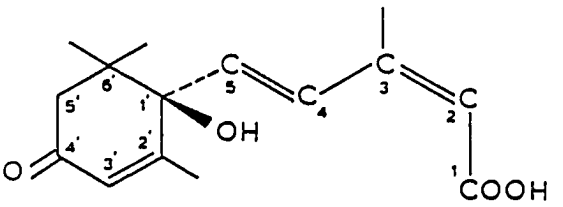
LIST OF FIGURES

INTRODUCTION

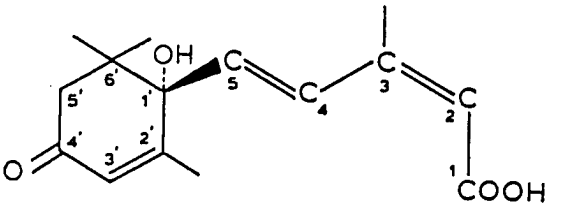
Abscisic acid [3-methyl-5-(1'-hydroxy-4'-oxo-2',6',6'-trimethyl cyclohex-2'-en-1'-yl)-2,4-pentadienoic acid] is a plant growth regulator and one of the five major groups of plant hormones: abscisic acid, auxins, cytokinins, ethylene, and gibberellins (GAs). It was known as inhibitor β (Bennet-Clark and Kefford, 1953), dormin (Wareing et al., 1964 cited in Hirai, 1986), and abscisin II (Ohkuma et al., 1963). Addicott et al. (1968) proposed the name abscisic acid with the abbreviation ABA. The chemical structure was proposed by Ohkuma et al. (1965), and confirmed by synthesis by Cornforth et al. (1965a).

ABA is a sesquiterpene compound. Chemically, it is related by its biosynthesis to monoterpenes, diterpenes (including GAs), triterpenes, and carotenoids. It is especially interesting that ABA and GAs, two classes of hormones that are antagonistic to each other in some responses, are synthesized by branches of the same isoprenoid pathway. Synthetic abscisic acid is a racemic mixture of (S)- and (R)-ABA as shown in Figures 1a and b, respectively. Naturally occurring ABA is in the (S) form (Addicott et al., 1968). The more biologically active form of ABA is the *cis,trans*-isomer [*(2Z,4E)*-ABA] (Fig. 1c). This form is photoisomerized to its *trans,trans*-isomer [*(2E,4E)*-ABA] (Fig. 1d) by natural sunlight, possibly in a UV control mechanism (Brabham and Biggs 1981). The photoisomerization is partially quenched by oxygen. Unless otherwise indicated, the term ABA will be used throughout this dissertation to refer to naturally occurring abscisic acid in its active form.

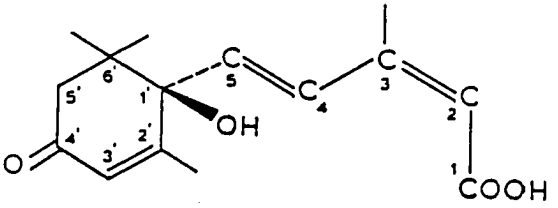
The structural requirements for ABA activity were deduced from bioassay studies conducted by several researchers to test the activity of ABA and related



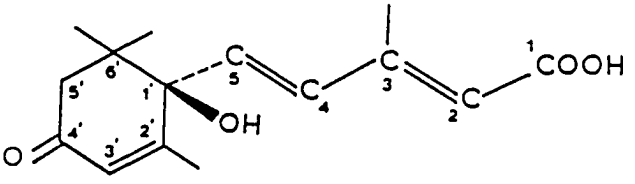
a- (S)-ABA



b- (R)-ABA



c- *(2Z,4E)*-ABA



d- *(2E,4E)*-ABA

Figure 1. Structures of different forms of abscisic acid

compounds on various kinds of plant tissues. It was found that the presence of the C-2 double bond in the *cis*-form (Sondheimer and Walton, 1970), the 4-*trans* double bond (Milborrow, 1974a), the conjugation of the 2-*cis*-double bond to a carboxyl group (Sondheimer and Walton, 1970; Milborrow, 1974a), and the ring double bond (McWha et al., 1973) are essential for ABA activity.

The physiological roles of ABA in plant growth and development have been reviewed extensively by Milborrow (1974a) and Walton (1980). Some of these roles involve the regulation of stomatal opening, seed development and germination, and bud and seed dormancy.

ABA has been identified in a variety of angiosperms and gymnosperms (Milborrow, 1969). Among the bryophytes, it has been detected in mosses (Pryce, 1972), but not in any liverwort (Pryce, 1971). The brown alga *Ascophyllum nodosum* (Boyer and Dougherty, 1988) and seven species of green algae (Tietz et al., 1989) have been shown to produce ABA. ABA has been found in the brains of pigs and rats (Le Page-Degivry et al., 1986), as well as in those of rodents and ruminants (Chen et al., 1988). Goodwin and Mercer (1983) indicated that ABA can be found in roots, stems, buds, leaves, fruits, and seeds, and in xylem, phloem, and nectar sap of higher plants. They also state that ABA levels differ from one tissue to another, and even within a given tissue depending on the developmental stage and the environment. Most plant tissues may contain 4×10^{-8} to 2×10^{-7} M ABA. This level may increase to 10^{-6} M in water-stressed leaves, developing seeds, and dormant buds and seeds (Walton, 1988).

ABA has also been detected in fungi. Assante et al. (1977) reported the isolation of ABA from the fungus *Cercospora rosicola*. They found that ABA was produced in a yield of 6 mg/100 ml medium when the fungus was grown on potato

agar under suitable conditions of temperature, pH, and light. Since then, eight other species of fungi have been shown to produce ABA; these are *Cercospora cruenta* (Oritani et al., 1982), *Botrytis cinerea* (Marumo et al., 1982), *Ceratocystis coerulescens*, *Ceratocystis fimbriata*, *Rhizoctonia solani*, and *Fusarium oxysporum* (Dorffling et al., 1984), *Cercospora pini-densiflorae* (Okamoto et al., 1988a), and *Alternaria brassicae* (Dahiya et al., 1988).

All known ABA-producing fungi are phytopathogenic. The fungus *Cercospora rosicola* infects the leaves of roses, causing a disease called *Cercospora* leaf spot of rose (Davis, 1938). In general, it is not known whether ABA produced by these fungi is involved in the disease process. As a plant growth regulator, ABA might cause disease symptoms by disrupting host cellular functions (Dunkle, 1984). Another possibility is that a nonspecific toxin called cercosporin produced by *Cercospora* species might play an important role in causing pathogenesis (Daub, 1987).

Cercospora rosicola and *Cercospora cruenta* are among the ABA-producing fungi in which the ABA biosynthetic pathway has been studied extensively. *Cercospora* is a genus of hyphomycetous fungi growing generally on dead or discolored spots on living leaves (Ellis and Everhart, 1885). *C. rosicola* has been classified as a deuteromycete fungus in the family Dematiaceae. Davis (1938) described the characteristics of this fungus as follows:

> "stromata inconspicuous, scattered over the necrotic area or grouped together, brown; conidiophores in loose fascicles, brown, strongly geniculate, typically continuous but sometimes one- to two-septate, 45-120 X 4.6-6 u, usually 50-60 x 5 u; conidia obclavate, with a beveled base, olivaceous, 1-6 septate, straight or slightly curved, 30-75 x 3.5-5.5 u, usually 40-60 x 4-5 u."

Pathways of ABA biosynthesis

Although it has been 25 years since ABA was described, the details of its biosynthetic pathway are still somewhat obscure. Studies of ABA biosynthesis in plant tissues have been difficult because of the low concentration of ABA and its metabolites in plant tissues, the inadequate knowledge of the exact time of ABA synthesis in plant tissues except for water-stressed leaves and developing seeds, and the low incorporation of radioactive presumed intermediates into ABA (Neill et al., 1984; Walton, 1988).

Two pathways of ABA biosynthesis have been proposed: the direct C-15 pathway involving direct synthesis from a C-15 precursor derived from farnesyl pyrophosphate (FPP), and the indirect pathway involving ABA synthesis from a C-15 compound, such as xanthoxin, resulting from the cleavage of a C-40 carotenoid intermediate (Figure 2). The possibility that xanthoxin might be formed via a pathway other than that of carotenoid degradation cannot be ruled out (Zeevaart and Creelman, 1988; Sindhu and Walton, 1987). Neither of these biosynthetic pathways has been conclusively demonstrated either in higher plants or in the fungus *C. rosicola.*

The direct biosynthetic pathway

In *Cercospora rosicola* The normal terpenoid precursors, such as acetate and MVA, are incorporated into ABA by *C. rosicola.* Bennett et al. (1981) found that 1,2-^{13}C-sodium acetate was incorporated into ABA in resuspended cultures of *C. rosicola.* They found that six acetate units were incorporated into ABA through MVA via the isoprenoid pathway. Similarly, Robinson and Ryback (1969) found that a molecule of ABA could be formed from three molecules of MVA. Neill et

al. (1982a, b) have shown that MVA can be incorporated into 1'-deoxy-ABA and ABA by *C. rosicola*. Also, ^{3}H-labeled 1'-deoxy-ABA was incorporated into ABA in 11% yield by the fungus. Neill et al. (1981) suggested that 1'-deoxy-ABA is the immediate precursor of ABA and that ABA is synthesized in *C. rosicola* by a route involving successive oxidations of a 3-methyl-5-(2',6',6'-trimethylcyclohex-2'-en-1'-yl)-2,4-pentadienyl intermediate (α-ionylidene) which arises from farnesol or a farnesyl derivative. Neill and Horgan (1983) proposed some possible biosynthetic routes to ABA. These routes involve the conversion of *(2E,4E)*-FPP to α-ionylidene which would be oxidized successively to α-ionylidene derivatives with the 1'-hydroxylation occurring at the last step as shown in Figure 3. Norman et al. (1985c) suggested that FPP acts as both a precursor and an inducer of ABA. These researchers also found that farnesol appears to induce ABA biosynthesis.

Feeding experiments using *C. rosicola* resuspended cultures have demonstrated incorporation of α-ionylideneethanol and α-ionylideneacetic acid into 4'-hydroxy-α-ionylideneacetic acid, 1'-deoxy-ABA, and ABA (Norman et al., 1985b; Neill et al., 1982a, b, 1984, 1987; Neill and Horgan, 1983; Horgan et al., 1983) and that 1'-deoxy-ABA is metabolized to ABA (Neill et al., 1981, 1982a, b, 1984, 1987; Neill and Horgan, 1983; Horgan et al., 1983; Norman et al., 1985b). ^{2}H-labeled *(2Z,4E)*-α-ionylideneethanol and *(2Z,4E)*-α-ionylideneacetic acid were also converted to 1'-deoxy-ABA and ABA by *C. rosicola,* while the *(2E,4E)*-isomers of these compounds were converted to *(2E,4E)*-1'-deoxy-ABA but not to *(2E,4E)*-ABA. ^{3}H-labeled β-ionylideneethanol and β-ionylideneacetic acid were not converted into 1'-deoxy-ABA or into ABA (Bennett et al., 1984; Neill et al., 1982a, b, 1984; Neill and Horgan, 1983; Horgan et al., 1983). Thus, isomerization of the 2,3-double bond occurs early in the biosynthetic pathway, before or at the same

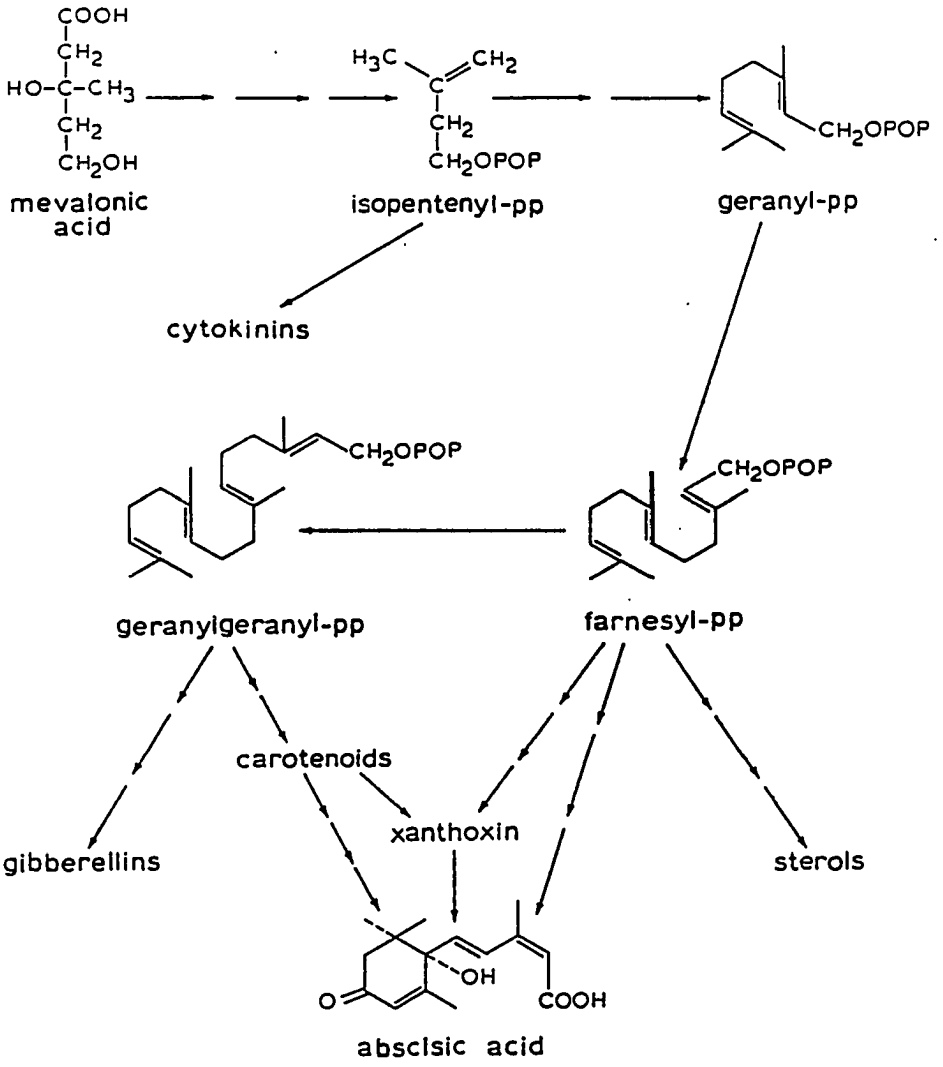


Figure 2. Proposed biosynthetic pathways of ABA

time as formation of the first desaturated cyclic intermediate. These results also suggest that the geometry of the side chain of 1'-deoxy-ABA has a role in its recognition by the final hydroxylase enzyme, which would not use the *(2E,4E)*-isomer of 1'-deoxy-ABA as a substrate. Conversion of *(2E,4E)*-forms of α-ionylideneethanol and α-ionylideneacetic acid to *(2E,4E)*-1'-deoxy-ABA suggests the non-specificity of the early oxidizing enzymes in the ABA biosynthetic pathway. The failure of incorporation of β-ionylideneethanol and β-ionylideneacetic acid into 1'-deoxy-ABA and ABA suggests the formation of a 2'-double bond in the intermediate resulting from cyclization and desaturation of farnesol derivatives.

Norman et al. (1985b) suggested the presence of another biosynthetic pathway through a 1'-hydroxy-α-ionylidene type intermediate in addition to the proposed pathway through 1'-deoxy-ABA. The conversion of the ABA precursor α-ionylideneacetic acid to 1'-hydroxy-α-ionylideneacetic acid and the ethyl derivatives of the *cis* and *trans* forms of that precursor into the respective ethyl esters of ABA led Norman et al. (1985a, b) to conclude that hydroxylation at the 1'-position can precede oxidation at the 4'-position and that ABA might have a precursor in its biosynthetic pathway in which 1'-hydroxylation of the ring occurs first. Norman et al. (1985b) found that the conversion of α-ionylideneacetic acid to 1'-deoxy-ABA is more rapid than that of 1'-deoxy-ABA to ABA. Because of the rapid oxidation at the 4'-position, the detection of the 1'-hydroxy precursor in the ABA biosynthetic pathway would be difficult. On the other hand, Neill et al. (1987) found that a low yield of ABA was produced from the oxidation of 1'-hydroxy-α-ionylidene derivatives applied to resuspended cultures of *C. rosicola*.

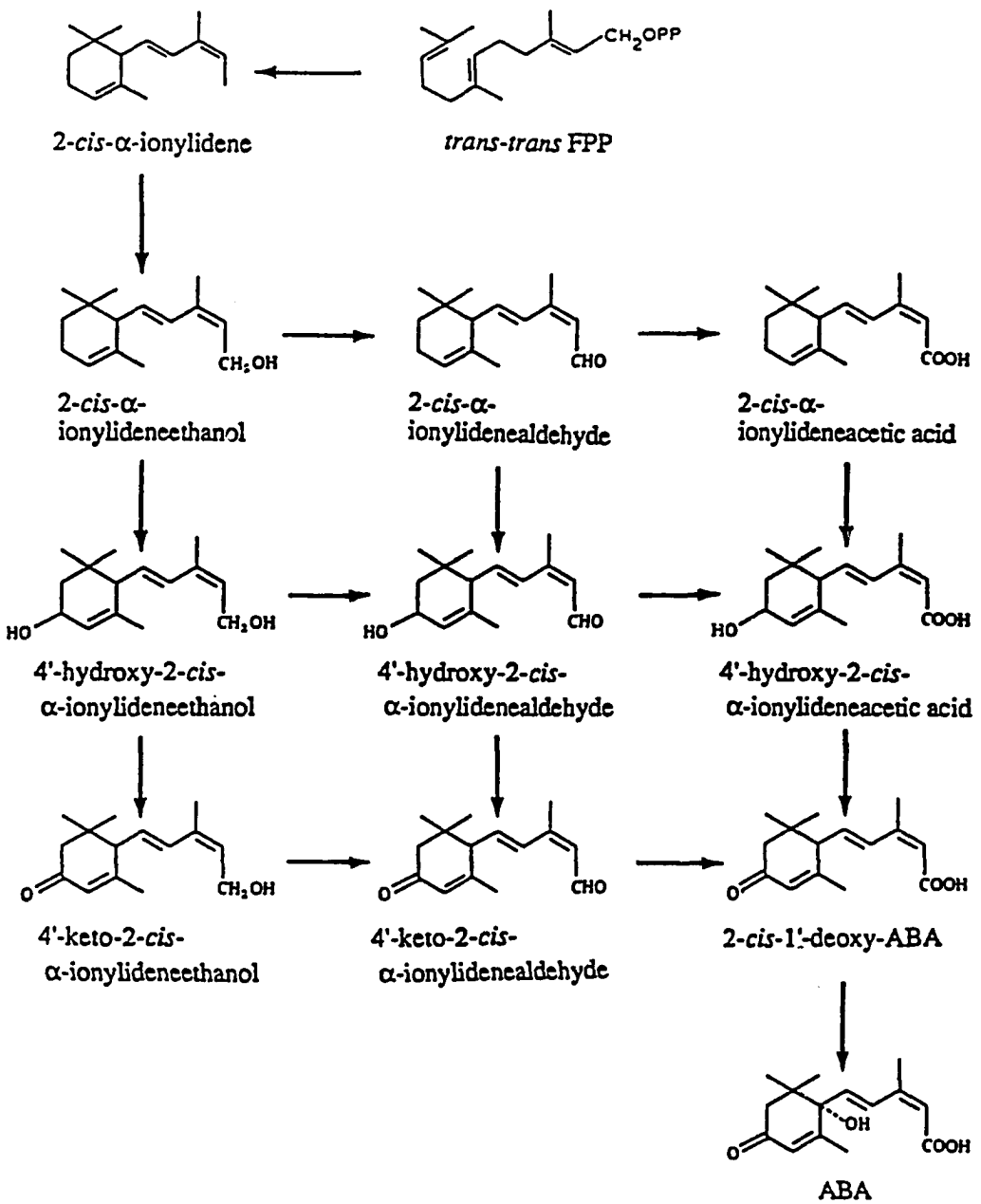


Figure 3. Possible biosynthetic pathways for ABA in *C. rosicola*. From Neill and Horgan (1983)

In plants ^{3}H- and ^{14}C-labeled MVA have been incorporated into ABA in several plant tissues (Robinson and Ryback, 1969; Noddle and Robinson, 1969; Milborrow 1975). The yield of ABA produced was always low and no radioactive intermediate has been identified (Hirai, 1986; Zeevaart and Creelman, 1988). This low incorporation of MVA into ABA might be because MVA is a precursor in other terpenoid pathways, so that the radioactive MVA might be diluted extensively by a large precursor pool prior to ABA formation (Zeevaart and Creelman, 1988). The inability to identify any radioactive intermediates might be due to the instability and rapid conversion of these intermediates into ABA (Hirai, 1986).

Feeding experiments using ^{2}H-labeled intermediates in the ABA biosynthetic pathway in *C. rosicola* were conducted to test their incorporation into ^{2}H-ABA in various plant tissues. These intermediates included α-ionylideneacetic acid, and 1'-deoxy-ABA. In avocado fruits and *Phaseolus vulgaris* cuttings, these ABA precursors were found to be metabolized to compounds other than ABA. However, low yields of 1'-deoxy-ABA and ABA were synthesized from α-ionylideneacetic acid and 1'-deoxy-ABA in *Vicia faba* cuttings (Neill et al., 1982b, 1984; Horgan et al., 1983). The above results may be due to the absence of the necessary enzymes involved in the ABA biosynthetic pathway in avocado or *Phaseolus vulgaris* during the feeding period. Alternatively, the high activity of conjugating enzymes may prevent a sufficient amount of precursor from reaching the subcellular sites for ABA synthesis for incorporation at detectable levels. Lehmann and Schutte (1976) suggested that neither (*2Z,4E*)- nor (*2E,4E*)-[2-^{14}C]-α-ionylideneacetic acid is a precursor for ABA in barley shoots, since they were converted into 1'-deoxy-ABA but not into ABA. Similar results were obtained by

Oritani and Yamashita (1979, cited in Hirai, 1986) who found that [2-^{14}C]-α-ionylideneacetic acid is metabolized to 1'-deoxy-ABA and 3'-keto-β-ionylideneacetic acid, but not to ABA in tomato shoots and seedlings of rice and soybean. The same authors found that 1'-hydroxy-α-ionylideneacetic acid was metabolized to ABA in the same plant tissues. On the basis of investigations by a number of researchers, Neill et al. (1984) suggested that the *cis*-1'-4'-ABA-diol might be a natural precursor of ABA in the plant tissues.

More work needs to be done to determine whether these compounds are truly ABA intermediates in plants.

The indirect (carotenoid) pathway

Structurally, there is similarity between ABA and the end portion of certain carotenoids. This led Taylor and Smith (1967) to suggest that the degradation of a carotenoid such as violaxanthin could give rise to ABA (Figure 4). Violaxanthin can be oxidized by light *in vitro* or by neutral zinc permanganate in aqueous acetone to give xanthoxin, a potent plant growth inhibitor (Burden and Taylor, 1970). Firn and Friend (1972) found that xanthoxin could also be formed by the incubation of violaxanthin with soybean lipoxygenase and linoleate. Subsequent feeding experiments with 2-^{14}C-(*2Z,4E*)-xanthoxin applied to shoots of tomato and dwarf bean led Taylor and Burden (1973) to conclude that xanthoxin could be converted into ABA *in vivo*. However, an experiment suggesting that ABA is not synthesized via the carotenoid pathway was conducted by Robinson (unpublished results cited in Milborrow, 1974a). He found that both ^{14}C-phytoene, an uncyclized carotenoid, and ^{3}H-MVA when fed to an avocado fruit were incorporated into carotenoids, which shows that both compounds had entered the

sites of carotenoid synthesis within the cells. Although ABA was heavily labeled with ^{3}H, it contained no ^{14}C; this suggests that carotenoids are not involved in ABA biosynthesis in this tissue.

Zeevaart and Creelman (1988) introduced several lines of evidence in favor of the indirect pathway:

1. The synthesis of *(2Z,4E)*- and *(2E,4E)*-ABA from *(2Z,4E)*- and *(2E,4E)*-xanthoxin in tomato and bean shoots.
2. ^{18}O-labeling studies.
3. The conversion of xanthoxin to ABA in cell-free extracts from leaves of various plant tissues.
4. Results with inhibitors of carotenoid biosynthesis.
5. Results with mutants deficient in ABA.

Labeling experiments with $^{18}O_2$ in maize embryos led Gage et al. (1989) to suggest that an indirect pathway via an oxygenated carotenoid (xanthophyll) operates under non-stress conditions. Their results agree with those obtained by Creelman and Zeevaart (1984), in which $^{18}O_2$ labeling experiments provided evidence for the indirect biosynthetic pathway via an oxygenated precursor in water-stressed leaves of *Xanthium strumarium*. The ^{18}O in ABA was in the carboxyl group. If a pathway similar to that in *C. rosicola* operates in water-stressed leaves (with 1'-deoxy-ABA as the immediate precursor of ABA), one would suspect that the ^{18}O would appear in the 1'-hydroxyl group.

The results of Li and Walton (1987, 1990) indicate that ABA is derived from preformed xanthophyll(s). They found that in water-stressed leaves of *Phaseolus vulgaris*, the labeling of ABA and the major xanthophylls by $^{14}CO_2$ was inhibited

by the carotenoid inhibitor fluridone. They also suggested that an ^{18}O-labeled violaxanthin is a precursor of a portion of stress-induced ABA.

Incubation of water-stressed leaves of different species in an atmosphere of 20% $^{18}O_2$ resulted in incorporation of ^{18}O into the carboxyl group of ABA. These studies led Zeevaart et al. (1989) to suggest the presence of a universal biosynthetic pathway in higher plants involving ABA production from a larger oxygenated carotenoid.

Cell free extracts from the leaves of several plants capable of converting xanthoxin to ABA by NAD/NADP-dependent reactions were developed by Sindhu and Walton (1987, 1988). Sindhu and Walton (1988) used cell-free extracts from wild-type and wilty mutants of tomato (*sitiens, notabilis,* and *flacca*) to convert xanthoxin to ABA. These data supported the assumption that xanthoxin is an intermediate in the ABA biosynthetic pathway. ABA aldehyde was suggested as a final precursor of ABA in the plant tissues tested.

To convert xanthoxin to ABA, two oxidations and an opening of the epoxide ring must take place. This led Sindhu and Walton (1987) to suggest that more than one enzyme is involved in that conversion. From their results on the incorporation of xanthoxin into ABA by cell-free extracts, they were unable to show the existence of specific enzymes for xanthoxin oxidation. They suggested that enzymes whose main functions are oxidations of other substrates might have oxidized xanthoxin to ABA in these extracts.

Sindhu and Walton (1987) and Zeevaart and Creelman (1988) suggested that xanthoxin might be an intermediate in both a direct and an indirect ABA biosynthetic pathways in plants. Its role as an ABA precursor is still uncertain.

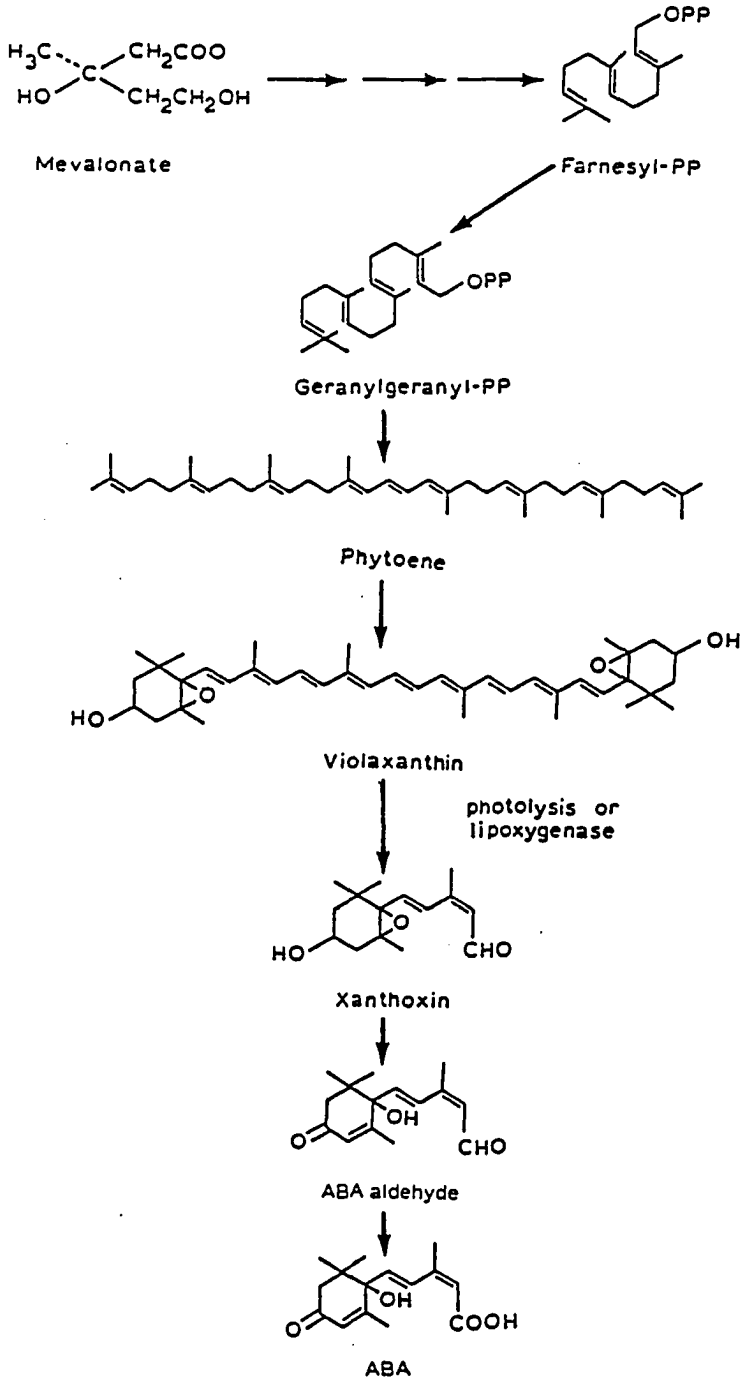


Figure 4. The indirect (carotenoid) pathway of ABA biosynthesis

Effect of triarimol on ABA biosynthesis in *C. rosicola*

ABA biosynthesis in *C. rosicola* is inhibited by a number of plant growth regulators, including cytokinins, ancymidol, and paclobutrazol (Norman et al., 1982/83, 1983, 1986). Ancymidol and some cytokinins also inhibit gibberellin biosynthesis in cell-free preparations from wild cucumber liquid endosperm and other similar systems (Coolbaugh et al., 1982a; Coolbaugh, 1984).

Norman et al. (1986, 1988) compared the effects of ancymidol, paclobutrazol, and fenarimol on ABA biosynthesis in *C. rosicola*. Fenarimol, which is a close analog of triarimol, was shown to have an ED_{50} (concentration at which ABA accumulation was inhibited by 50%; ED = effective dose) of approximately 5×10^{-6} M. Triarimol was tested for its effects on ABA accumulation in growing cultures of *C. rosicola*. ABA accumulation was reduced by approximately 50% with 10^{-8} M triarimol (Al-Nimri and Coolbaugh, 1990). Triarimol is the most potent inhibitor of ABA biosynthesis reported to date.

Ancymidol and cytokinins yield cytochrome P-450 binding spectra and block specific oxidative reactions in gibberellin biosynthesis catalyzed by Cyt P-450 mixed function oxidases (Coolbaugh et al., 1982b; Coolbaugh, 1984). Walton and Griffin (unpublished results cited in Neill et al., 1984) found that ABA production as well as incorporation of ^{14}C-acetate into ABA in *C. rosicola* were markedly reduced by the mixed function oxidase inhibitor piperonyl butoxide at a concentration of 4×10^{-4} M. These data, taken together, suggest that at least one of the reactions in the ABA biosynthetic pathway is catalyzed by a mixed function oxidase.

ABA production in fungi other than *C. rosicola*

ABA biosynthetic pathways have been studied in a number of fungi. In *Botrytis cinerea*, 1',4'-*trans*-diol-ABA has been found to be a possible precursor but not a metabolite of ABA (Hirai et al., 1986). Feeding ^{2}H-labeled α-ionylideneacetic acid to *Cercospora pini-densiflorae* led Okamoto et al. (1988b) to suggest that ABA was synthesized from α-ionylideneethanol via (1'R)-(4'S)-hydroxy-α-ionylideneacetic acid and the 1',4'-*trans*-diol of ABA, which in turn was converted to ABA. The conversion of the 1',4'-trans-diol to ABA was easier than that of 1'-deoxy-ABA in this fungus. Okamoto et al. (1988b) suggested that the ABA biosynthetic pathway in *C. pini-densiflorae* is different from that in *C. rosicola* and *C. cruenta*, but similar to that in *Botrytis cinerea*. *C. cruenta* has been found to produce relatively high levels of ABA and 1'-deoxy-ABA (Oritani et al., 1982). 1',4'-dihydroxy-γ-ionylideneacetic acid was, however, converted by this fungus to ABA with a higher incorporation ratio than that of 1'-deoxy-ABA (Oritani and Yamashita, 1985). On the basis of this information, the authors suggested an ABA biosynthetic pathway in *C. cruenta* via 1'-4'-dihydroxy-γ-ionylideneacetic acid rather than 1'-deoxy-ABA. Using carotenoid biosynthesis inhibitors, Oritani and Yamashita (1985) concluded that the ABA biosynthetic pathway in *C. cruenta* may not pass through the C-40 carotenoid route.

The previous information related to ABA biosynthesis in different species of fungi suggests that the ABA-producing fungi might not have a common pathway of ABA biosynthesis.

Purpose of this study

Despite the intensive work of many researchers to elucidate the ABA biosynthetic pathway, there are still no definite answers to a number of questions related to its biosynthesis. It seems highly likely that the ABA biosynthetic pathway differs between higher plants and fungi, and also among different fungi. The present studies were undertaken to gain a better understanding of ABA biosynthesis in *C. rosicola*. Specific objectives were:

1. Optimize ABA production in *C. rosicola* liquid cultures.
2. Study specific steps in the ABA biosynthetic pathway in *C. rosicola* through feeding experiments with labeled precursors of ABA.
3. Isolate and characterize a cell-free enzyme system from mycelia of *C. rosicola* capable of converting 1'-deoxy-ABA, the probable immediate precursor of ABA, into ABA.
4. Use our previous results concerning the inhibition of ABA biosynthesis by triarimol to characterize specific steps in the ABA biosynthetic pathway.
5. Determine if ABA is produced by the phytopathogenic fungi *Gnomonia veneta* and *Cercospora mori*, which infect sycamore and mulberry leaves, respectively.

MATERIALS AND METHODS

Culture of fungi

Cercospora rosicola Passerini (strain No. 138.35) was obtained from R. Bennett, USDA-ARS, Pasadena, CA and cultured on potato dextrose agar (PDA) supplemented with 1.25 mg/l thiamine (Neill and Horgan, 1983). A chemically defined liquid medium described by Norman et al. (1981a) was used to grow liquid cultures of the fungus. The liquid culture medium contained glucose (20 g), $MgSO_4$ (0.2 g), KCl (0.5 g), $CaCl_2.2H_2O$ (0.1 g), KH_2PO_4 (0.8 g), thiamine (0.001 g), monosodium glutamate (3.0 g), and trace elements solution (1 ml) in purified water to a total volume of one liter. The trace elements solution contained $FeSO_4.7H_2O$ (0.05 g), $MnCl_2$ (0.033 g), $ZnSO_4$ (0.25 g), $CuSO_4.4H_2O$ (0.4 g) and H_3BO_4 (0.00005 g) in 100 ml purified water.

Agar plates were inoculated by streaking with a colony of *C. rosicola*, which results in fungal growth all over the agar surface. The inoculated agar plates were incubated under continuous fluorescent light at 23° C for 10 to 14 days. They were refrigerated until used as a source for starting *C. rosicola* liquid cultures.

Inoculum of liquid cultures was prepared by scraping half the surface of a PDA plate into 25 ml sterile liquid medium and homogenizing in a teflon to glass hand-held homogenizer. Two ml mycelial suspension was used to inoculate 50 ml liquid medium in a 250 ml cotton-plugged Erlenmeyer flask. The inoculated cultures were incubated on a reciprocatry shaker at 90 rpm at room temperature (approximately 23° C) under continuous fluorescent light (1.8×10^{13} quanta s^{-1} cm^{-2}). After 7 days, two ml mycelial suspension was used as an inoculum for 50 ml

culture medium. Incubation was continued under the same conditions for a period depending on the type of experiments to be done.

Preparation of *C. rosicola* medium The effect of the order of autoclaving and mixing the components of the liquid medium on growth and ABA production by *C. rosicola* was studied. In one preparation the glucose, salts, thiamine, monosodium glutamate, and trace elements were mixed in a total volume of one liter before autoclaving. Alternatively, the ingredients were autoclaved separately, cooled, and mixed. Fifty ml of each liquid medium preparations was inoculated with 2 ml mycelial suspension from a six-day-old *C. rosicola* liquid culture. Mycelial cultures were incubated under the growth conditions given previously for 7 days before harvesting.

Addition of citric acid and orange peels to *C. rosicola* liquid medium Experiments were conducted to study the effects of citric acid and orange peels on growth and ABA production by *C. rosicola* liquid cultures. Citric acid (1 mM) or oven dried orange peels (50 mg) were added to 50 ml liquid medium before inoculation. Fungal cultures were incubated under the growth conditions described above. Duplicate cultures were harvested 5 and 7 days after incubation.

Time course for growth and ABA production

Growth and ABA production over a 9 day period were studied. Five ml suspensions of *C. rosicola* liquid cultures were pipetted from duplicate 50 ml cultures at 24 h intervals, unless otherwise indicated. Mycelial dry weights were measured, and the filtrates were analyzed by gas chromatography-mass spectrometry (GC-MS) for ABA.

Isolation of ABA

Fifty ml fungal cultures were vacuum filtered through Whatman #1 filter paper using a Buchner funnel. Small volumes (5-15 ml) of mycelial suspensions were filtered through Whatman #1 in a Millipore SWINNEX attached to a syringe. The mycelia were dried at 70° C for 24 h. The filtrates were stored at -20° C if not extracted immediately after filtration.

Solvent partitioning The filtrates were acidified to pH 3.0 with 1M HCl to protonate ABA and thus make it less soluble in the aqueous phase. The acidic aqueous filtrates were extracted three times with an equal volume of ethyl acetate (EtoAc). The acidic EtoAc fractions were dried over 5-10 g of sodium sulfate and evaporated *in vacuo* at 30-40° C. The dried residue was redissolved in methanol (MeOH) and then concentrated *in vacuo* to 0.5 ml. The methanolic fraction was passed through a MeOH-treated C-18 Sep-Pak followed by two equal volumes of MeOH to elute the ABA. The MeOH in the eluate was evaporated *in vacuo* to dryness. The dried sample was redissolved in MeOH and subjected to high performance liquid chromatography (HPLC) analysis.

HPLC HPLC was used as an efficient means of ABA purification, but not for quantification unless otherwise indicated. A Beckman System Gold^{R} HPLC was used. The samples were injected onto an Ultrasphere column (Beckman ODS 5 um C-18, 4.6 mm x 25 cm) pre-equilibrated with 40% MeOH in 0.01 M H_3PO_4, pH 3.0, and eluted with a gradient of 40-100% MeOH in 0.01 M H_3PO_4 for 40 min at a flow rate of 1 ml/min. The gradient program used was isocratic at 40% MeOH for the first 7.5 min, linear from 7.5-16.25, 16.25-22.5, 22.5-30 min at 40-75%, 75-80%, and 80-100% MeOH, respectively, and isocratic at 100% MeOH from 30 to 40 min. Effluent was monitored at 254 nm with a programmable

detector module 166. The gradient was controlled and peaks were integrated by an IBM personal system/2 model 50 computer connected to the pumps by an analog interface module 406. Radioactive isotopes were monitored by a Beckman 171 radioisotope detector containing a solid scintillator flow cell with a 125 ul volume.

An ABA standard was chromatographed prior to each set of samples to monitor the stability of its retention time.

A twenty ul injection volume, which represented the whole sample, was chromatographed each time unless otherwise indicated. A standard curve was prepared with authentic ABA (Sigma) when needed for quantification.

GC-MS HPLC fractions presumably containing ABA were collected and prepared for analysis by GC-MS. The presumptive ABA-containing fractions were dried *in vacuo*. The dried residue was redissolved in 250 ul MeOH, transferred to 100 ul conical vials, where it was dried again, and methylated with excess ethereal diazomethane. The samples were then dried under a stream of N_2. In analyses where more than 5 ml filtrates were extracted, the dried samples were redissolved in 500 ul MeOH and 2 ul aliquots were injected into GC-MS. In cell-free experiments where ABA was produced in very small quantities, the samples were redissolved in 10 ul MeOH and reduced under a stream of N_2 to 2-3 ul for injection into the GC.

A Hewlett-Packard model 5890 gas chromatograph coupled with an HP model 5970 mass selective detector (MSD) was used. An HP model 9133 computer controlled these instruments and data collection. He was used as the carrier gas at a linear flow rate of 30 cm^3/min. A splitless glass liner was used. The chromatography column used was a J & W Scientific DB-1 W.C.O.T. fused

silica capillary column (30 m long and 0.25 mm inner diameter). Both full scan and selected ion monitoring (SIM) methods were used in monitoring ABA methyl ester (Me-ABA) content, depending on the expected quantity of ABA in the analyzed sample. In the SIM method used in ^{2}H-ABA detection, the mass/charge (m/z) values monitored were 193 (base peak), 165, 137, 128, and 94, which are the tri-deuterated forms of the m/z values 190, 162, 134, 125, and 91 characteristic of ^{1}H-ABA. In both full scan and SIM methods, data were aquired for 18.0 min. The temperature program started at 100° C, increased at 20° C per min for 9 min, and was maintained at 280° C until the end of the run. The injector and detector (MSD) temperatures were 280° C. All spectra were measured at 70 eV.

In vivo incorporation of ABA precursors into ABA

Selected ABA precursors were tested for their incorporation into ABA in *C. rosicola* cultures. ^{14}C-MVA; ^{2}H,^{3}H,^{13}C-FPP; and ^{2}H-labeled 1'-deoxy-ABA were fed to resuspended cultures of *C. rosicola*. The effect of triarimol on the incorporation of these compounds into ABA was also tested.

^{14}C-MVA metabolism Previous work has shown that ^{14}C-MVA can be incorporated into ^{14}C-ABA in growing fungus cultures without resuspension (Al-Nimri, 1986). In an attempt to extend this work, experiments were conducted to test the effect of triarimol on the incorporation of ^{14}C-MVA into ^{14}C-ABA and other metabolites. ^{14}C-MVA (0.65 umol; specific activity 10 uCi/umol) was added to two- and three-day-old cultures in the absence or presence of 5.0×10^{-8} M triarimol. These cultures were maintained an additional 24, 36, 48, and 72 h prior to harvesting.

The effect of triarimol on this incorporation was also tested in resuspended cultures of *C. rosicola*. In these experiments, five-day-old cultures grown in the absence or presence of 5.0×10^{-8} M triarimol were vacuum filtered under sterile conditions. The mycelia were washed with an equal volume of sterile purified H_2O and resuspended in the same volume of corresponding liquid medium. Ten ml of this suspension was dispensed into a 50 ml cotton-plugged Erlenmeyer flask. The resuspended cultures were re-incubated for an additional 12 h, at which time they were fed 0.2 umol ^{14}C-MVA (specific activity 10 uCi/umol). The cultures were filtered 34 to 48 h after feeding. The filtrates were extracted as previously described for HPLC. The incorporation of ^{14}C-MVA into ^{14}C-ABA and other radioactive products was monitored with the Beckman 171 radioactive detector.

^{2}H,^{3}H,^{13}C-FPP metabolism In a preliminary feeding experiment, ten ml *C. rosicola* resuspended cultures were fed with 1 uM and 10 uM ^{2}H,^{3}H,^{13}C-FPP 12 h after resuspension. Sodium pyrophosphate was added to each resuspended culture to a concentration of 0.3 mM before feeding FPP to reduce the effect of endogenous phosphatase (Bennett et al., 1984). The cultures were harvested 24 h later. The mycelia were oven dried and the dry weights were measured. The filtrates were extracted and purified by HPLC. Presumptive ABA-containing fractions were collected and prepared for GC-MS. One replicate of these fractions was counted on a Beckman LS 5000 CE liquid scintillation counter. Another feeding experiment was done in which fungus cultures were fed with 15 uM ^{2}H,^{3}H,^{13}C-FPP in the absence or presence of 5.0×10^{-8} M triarimol. The acidic EtoAc extracts of the filtrates of these cultures were purified on HPLC. One ml HPLC fractions were collected and counted on the scintillation counter.

^{14}C-FPP prepared in our laboratory was incorporated into ^{14}C-ABA and ^{14}C-GA_3 by resuspended cultures of *C. rosicola* and *Gibberella fujikuroi*, respectively. Other unidentified ^{14}C-labeled metabolites were observed in HPLC chromatograms.

1'-Deoxy-^{2}H-ABA metabolism The HPLC gradient previously described was used for the purification of ^{2}H-labeled 1'-deoxy-ABA. Presumptive 1'-deoxy-^{2}H-ABA-containing fractions were collected, combined, and evaporated to dryness *in vacuo*. The dried residue was redissolved in ethanol (EtOH) and stored at -20° C for use as a substrate. The purity and identity of the collected 1'-deoxy-^{2}H-ABA were confirmed by GC-MS.

The conversion of 1'-deoxy-^{2}H-ABA into ^{2}H-ABA by *C. rosicola* liquid cultures was studied in resuspended mycelial cultures as in the ^{2}H,^{3}H,^{13}C-FPP experiments. The 10 ml resuspended cultures were fed with 1.5 uM 1'-deoxy-^{2}H-ABA 12 h after resuspension. The cultures were filtered 24 h after the feeding. The filtrates were extracted and purified by HPLC. Presumptive ABA-containing fractions were collected, concentrated, methylated with excess ethereal diazomethane, and analyzed by GC-MS. The ion range m/z 80-350 was scanned. The percent conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA was calculated as:
[A/(A + B)] x 100% where:
A = Area of m/z 193 (Me-sample) - Area of m/z 193 (Me-ABA standard).
B = Area of m/z 190 (Me-sample) - Area of m/z 190 (Me-^{2}H-ABA standard).

The integrated areas of both ABA and ^{2}H-ABA standards in the total ion chromatograms were close to those of the corresponding sample.

In vitro conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA

Experiments using enzyme preparations from *C. rosicola* were conducted to study the conversion of the immediate probable precursor of ABA, 1'-deoxy-ABA, to ABA. 1'-deoxy-^{2}H-ABA was used in these experiments as a labeled substrate. *C. rosicola* enzyme extracts were prepared from mycelia collected after filtration of 4 to 5-day-old liquid cultures. The mycelia were rinsed with an equal volume of water followed by another equal volume of 50 mM tricine, pH 8.0. The mycelia were then frozen in liquid N_2, crushed in a Sager press at 15,000 psi, and suspended in 50 mM tricine, pH 7.8, containing 0.25 M sucrose and 10 mM β-mercaptoethanol. The ratio of the volume of tricine buffer to mycelial fresh weight was 3:1. The suspension was centrifuged 10 min at 10,000 g_{av}. The supernatant (S_{10}) was used as an enzyme source in some of the experiments. In other experiments, the S_{10} was recentrifuged for 60 min at 100,000 g_{av}. Microsomes in the 100,000 g_{av} pellet were resuspended in a volume of buffer equal to the original mycelial fresh weight. The high speed supernatant (S_{100}) and the resuspended microsomal fraction (P_{100}) were frozen in liquid N_2 to be used later as an enzyme source.

In an attempt to characterize the enzyme catalyzing the conversion of 1'-deoxy-ABA to ABA, a wide range of concentrations of substrate and enzyme preparations, and of incubation times were tested for their effects on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA. The effects of NADPH, FAD, and 10^{-7} M triarimol on this conversion were also tested.

Unless otherwise indicated, reaction mixtures contained 300 ul enzyme extract, 10 uM 1'-deoxy-^{2}H-ABA, 1 uM $MgCl_2.6H_2O$, and 1 mM NADPH. The volume of the reaction mixture was adjusted to 1 ml with 50 mM tricine containing

0.25 M sucrose at pH of 7.8. Reactions were incubated for 7 to 20 min in a shaking water bath at 30° C and 200 rpm. Boiled enzyme or zero time reactions were included in each experiment as controls. Reactions were stopped by addition of 1 ml acetone. Mixtures were extracted with 1 ml benzene:acetone, 3:1. The aqueous residue was acidified to pH 3.0 with 1M HCl and extracted three times with 1 ml aliquots of EtoAc. The organic fraction was dried *in vacuo*. The dried residue was dissolved in 20 ul MeOH and injected into the HPLC. The presumptive ABA-containing fractions were collected and prepared for GC-MS analysis as mentioned previously. The methyl ester of ^{2}H-ABA was identified and quantified by GC-MS. The SIM method was used in most of the analyses.

ABA production in plant tissues

Diseased and healthy sycamore and mulberry leaves were tested for their ABA contents. Plant tissues were collected in the summers of 1987, 1988, and 1989, weighed, and kept at -70° C until extraction.

Extraction of ABA from plant tissues Plant tissues were homogenized in 80% MeOH using a Waring blendor with three 30 s bursts at full speed interrupted by three 30 s rests. The homogenate was stirred overnight at 4° C. Grinding the plant tissues with a mortar and pestle in liquid N_2 and homogenizing with a Polytron (Brinkmann Instruments) were sometimes used as substitutes for blending and overnight stirring. The homogenate was vacuum filtered through Whatman #1 filter paper. The MeOH was removed under vacuum. The pH of the aqueous residue was adjusted to 8.0 with 1M KOH and extracted three or more times with chloroform to remove lipophilic basic and neutral compounds (Dorffling and Tietz, 1983). The chloroform phase was discarded. The pH of the

aqueous residue was changed to 3.0 with 1M HCl and partitioned three times against one half volume of EtoAc. The organic phase was dried over sodium sulfate and evaporated *in vacuo*. The dried residue was dissolved in about 7 ml MeOH and concentrated to 1 ml *in vacuo*. The sample was then passed through a C-18 Sep-Pak followed by 2 ml aliquots of MeOH. The MeOH was evaporated *in vacuo*. The dried residue was redissolved with 0.2 to 1 ml MeOH and purified further by HPLC as previously described. Quantification of ABA was done by GC-MS. ^{2}H-ABA was used as an internal standard in some experiments.

ABA production in fungi other than *C. rosicola*

Two phytopathogenic fungi were studied for their production of ABA. *Gnomonia veneta* and *Cercospora mori* were isolated by N. Vakili (USDA-ARS, Iowa State University) from infected sycamore and mulberry leaves, respectively. The isolated fungi were grown on PDA plates prior to being used as an inoculum source for liquid cultures. The medium defined for *C. rosicola* was used as a liquid medium for both fungi. Fungus harvesting and ABA extractions from fungal filtrates were as described previously for *C. rosicola*. ^{2}H-ABA was used as an internal standard in some of these studies.

Source and purity of reagents

Tri-^{2}H-ABA was a gift from R. Horgan, The University College of Wales, England, to C. LaMotte, Iowa State University, Ames, IA. 1'-Deoxy-^{2}H-ABA was obtained from D. C. Walton, SUNY, Syracuse, NY. The ^{2}H,^{3}H,^{13}C-FPP sample was obtained from R. Horgan, the University College of Wales, England. Ethereal diazomethane was prepared as described by De Boer and Backer (1963). 2-^{14}C-

MVA-lactone (1 mCi in benzene, specific activity 53 uCi/umol) was purchased from Amersham; benzene was removed under a stream of N_2 and the lactone was hydrolyzed by treating overnight with 10 ml 5 mM NaOH. Two hundred ul of final solution was diluted with 93 ul cold MVA and 297 ul H_2O to bring the specific activity to 200 uCi/20 umol/5 ml. Triarimol [α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methyl alcohol] was provided in technical grade by Eli Lilly and Co., Indianapolis, IN. All other chemicals were reagent grade except for MeOH, EtoAc, and acetone, which were HPLC grade.

RESULTS

Culture of fungi

The report of ABA production as a secondary metabolite by the fungus *Cercospora rosicola* Passerini by Assante et al. (1977) introduced this fungus as a valuable organism for studies on the ABA biosynthetic pathway. Extensive studies on ABA biosynthesis in *C. rosicola* have taken place since then.

A chemically defined liquid medium was developed by Norman et al. (1981a) to support growth and ABA production by *C. rosicola* liquid shake cultures. This liquid medium was used in the present studies to grow *C. rosicola*. Autoclaving the glucose, salts, thiamine, monosodium glutamate, and trace elements in a total volume of 1 l of liquid medium rather than autoclaving them separately yielded no significant difference in ABA production.

In an attempt to stimulate ABA production in liquid cultures of *C. rosicola*, the effect of adding orange peels and citric acid was studied. The addition of 50 mg oven-dried orange peels to 50 ml liquid cultures of *C. rosicola* showed an inhibitory effect on both growth and ABA production compared to control cultures. In contrast, adding citric acid at a concentration of 1 mM in 50 ml liquid cultures enhanced both growth and ABA production, as shown in Figure 5.

Purification of ABA

HPLC HPLC was used to purify ABA in the acidic EtoAc extracts. A standard of ABA was chromatographed prior to each set of samples to monitor the stability of ABA retention time, which was approximately 19.5 min under the chromatographic conditions used. HPLC was not used for identification or

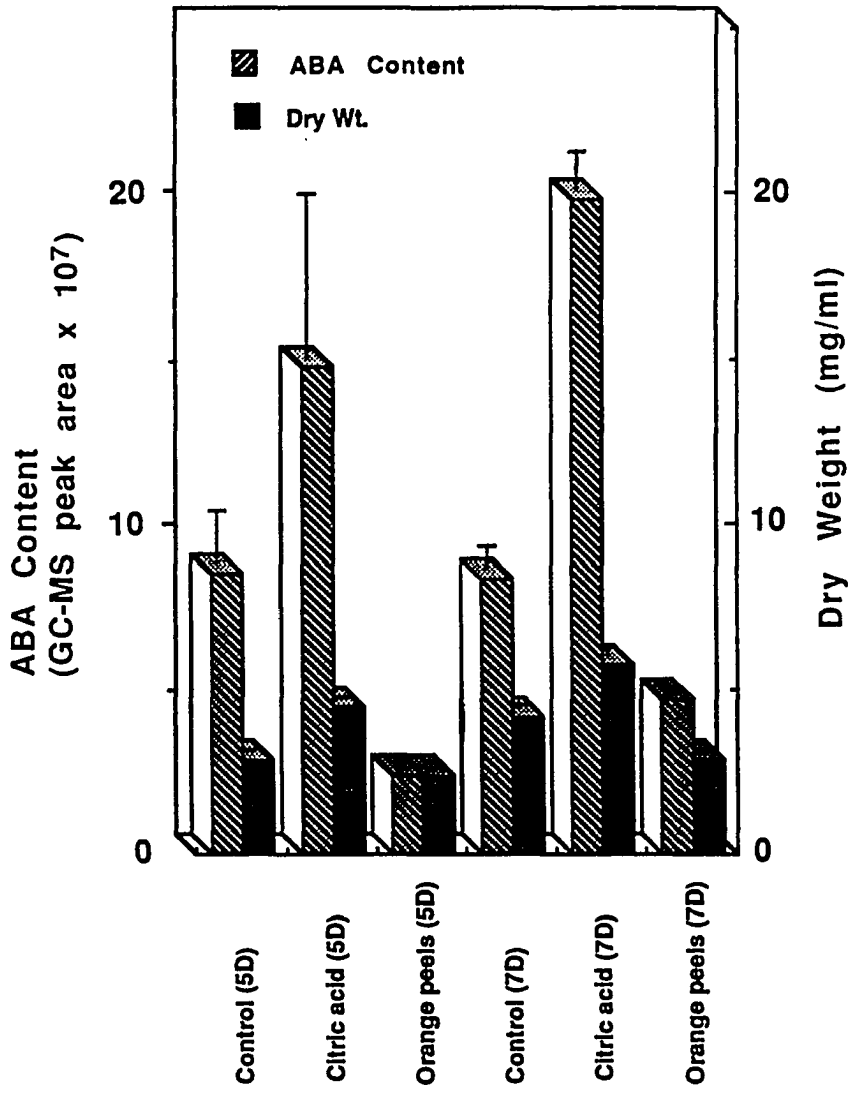


Figure 5. Effects of citric acid (1 mM) and orange peels (50 mg) on growth and ABA production in 50 ml liquid cultures of *C. rosicola*. 5D and 7D represent 5 and 7 days, respectively. Bars represent SE

quantification unless otherwise indicated. The 50 ng low detection limit of ABA at A_{254} and the possibility of other compounds having the same retention time as that of ABA restricted our use of HPLC to purification. In cell-free experiments, ABA production was less than 10 ng/reaction mixture. The use of the radioactive detector, Beckman 171, made it possible to monitor small amounts of radioactivity.

GC-MS GC-MS proved to be a powerful method for identifying and quantifying ABA in fungal and plant extracts after purification by HPLC. Ethereal diazomethane was used to methylate ABA in order to increase its volatility for chromatography by GC. Me-ABA is fragmented by the mass spectrometer to a number of characteristic ions, which facilitates its easy identification regardless of the weak molecular ion, m/z 278 (1%). These ions are m/z 190 (100%), 162 (49%), 134 (52%), and 125 (41%). Gray et al. (1974) have illustrated the scheme of the major fragmentation mechanism of Me-ABA. Quantification of Me-ABA was based on standard curves prepared with authentic Me-ABA. Methylated deuterated ABA (Me-^{2}H-ABA) produced from the conversion of 1'-deoxy-^{2}H-ABA in cultures and cell-free extracts from *C. rosicola* displayed major ions at m/z 193, 165, 137, and 128. Quantification of Me-^{2}H-ABA produced in cell-free experiments was based on the Me-^{2}H-ABA standard curve.

Time course for growth and ABA production

The results of time course experiments are shown in Figure 6, which shows growth and ABA production as determined from 5 ml aliquots pipetted from duplicate 50 ml cultures of *C. rosicola* at 24-36 h intervals. The first three days of growth yielded no significant amounts of ABA. ABA accumulation began near the end of the log growth phase, after 4 days, and continued to increase. The rapid

growth phase started from the fourth day and continued till the seventh day, when growth leveled off. ABA production increased from the fourth day through the eighth day, the last day of monitoring in these experiments. The amount of ABA detected at the end of the experiment was 29 ug/ml liquid medium.

In vivo incorporation of ABA precursors into ABA

From the time course experiments, ABA production started increasing at the fourth day of growth. Therefore, the fifth day was selected to resuspend *C. rosicola* cultures for experiments on the incorporation of ^{14}C-MVA, ^{2}H,^{3}H,^{13}C-FPP, and 1'-deoxy-^{2}H-ABA into the corresponding labeled forms of ABA. The resuspension of triarimol-treated cultures along with the untreated ones provided some information about the ABA biosynthetic pathway in *C. rosicola.*

^{14}C-MVA metabolism [2-^{14}C]- and [(4R)-4-^{3}H]-MVA are incorporated into ABA by higher plants (Noddle and Robinson, 1969; Robinson and Ryback, 1969). Neill et al. (1981) provided evidence that [2-^{3}H]-MVA was incorporated into ABA and 1'-deoxy-ABA in *C. rosicola* grown on modified Miller's medium. In our laboratory, *Cercospora rosicola* cultures grown in a defined liquid medium were also capable of incorporating ^{14}C-MVA into ^{14}C-ABA. Two sets of experiments were conducted to test the effect of triarimol on the incorporation of ^{14}C-MVA into ^{14}C-ABA and other metabolites or intermediates in the ABA biosynthetic pathway. In one set, ^{14}C-MVA (0.65 umol; specific activity 10 uCi/umol) was added to 50 ml growing 2 to 3-day-old cultures in the absence or presence of 5.0 x 10^{-8} M triarimol. These cultures were incubated an additional 24 to 72 h before harvesting. Upon purification of the concentrated samples by HPLC, a ^{14}C-peak at the retention time 22.0 min was observed on the elution profile of both

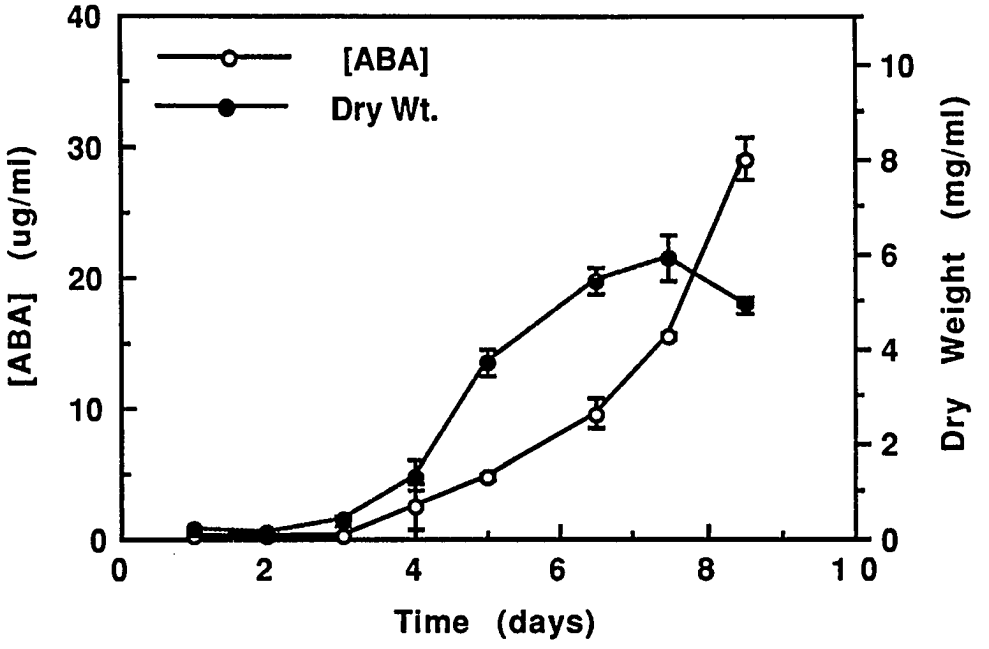


Figure 6. Time course for growth and ABA production in liquid cultures of *C. rosicola*. Bars represent SE

triarimol-treated and untreated samples. The expression "unknown A" is used to identify this peak.

In recent experiments, ten ml resuspended cultures were used. Two tenths umol ^{14}C-MVA (specific activity 10 uCi/umol) was fed on the fifth day after inoculation, when the cultures are within the rapid phase of growth and ABA production. Under these conditions, in the absence of triarimol, ^{14}C-MVA was incorporated into ^{14}C-ABA without any incorporation into ^{14}C-unknown A. However, triarimol-treated cultures incorporated ^{14}C-MVA into ^{14}C-unknown A without any ^{14}C-ABA production (Fig. 7).

^{2}H,^{3}H,^{13}C-FPP metabolism In a preliminary experiment, one or ten uM ^{2}H,^{3}H,^{13}C-FPP was fed to resuspended cultures of *C. rosicola* in order to test its incorporation into labeled ABA. HPLC fractions containing the presumptive ABA contained 185 and 441 DPM for cultures fed with 1 and 10 uM ^{2}H,^{3}H,^{13}C-FPP, respectively. The distribution of radioactivity in HPLC fractions from boiled, triarimol-treated and untreated cultures was determined by liquid scintillation counting (Fig. 8). ^{2}H,^{3}H,^{13}C-FPP was metabolized in growing cultures in the absence or presence of 1.5×10^{-7} M triarimol. However, triarimol inhibited the formation of presumptive ABA and 1'-deoxy-ABA by 66% and 43%, respectively. In addition, a significant amount of label eluted later in the run (at 25 min). These results were reproduced in another experiment. No further purification was done with these samples.

1'-Deoxy-^{2}H-ABA metabolism Figure 9 shows a GC-MS total ion chromatogram of purified 1'-deoxy-^{2}H-ABA methyl ester. The mass spectrum of the peak at 9.26 min (A) included the following ions and their relative abundances:

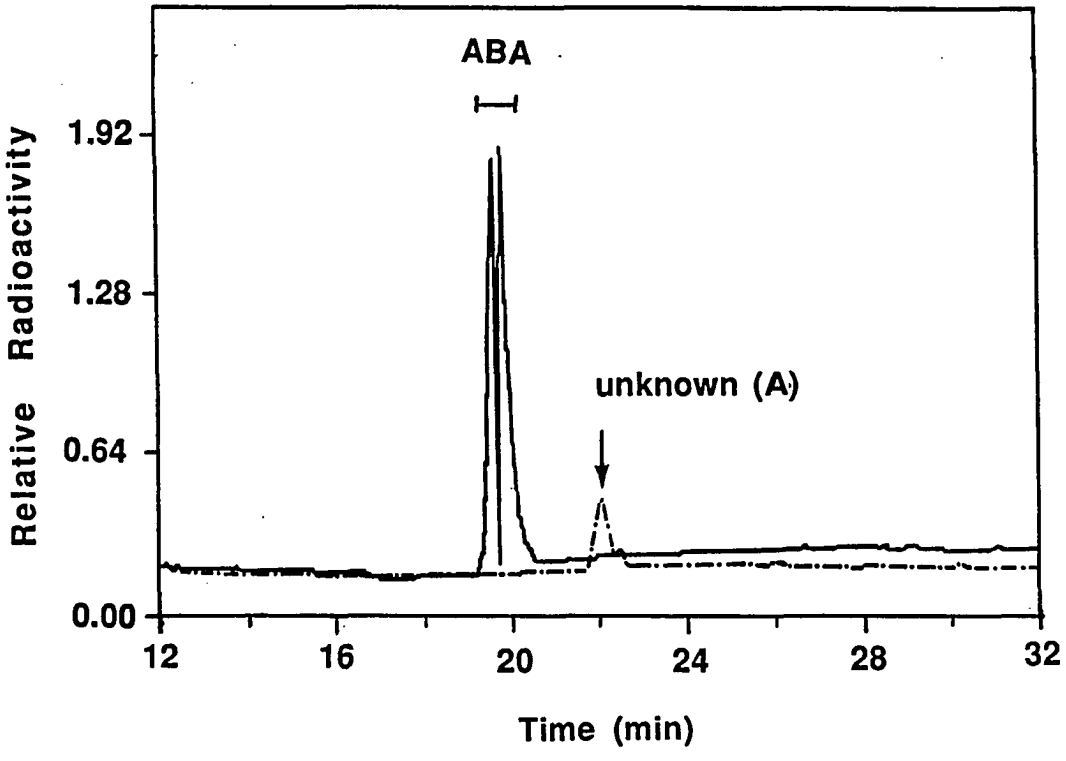


Figure 7. HPLC elution profile of metabolites following the feeding of ^{14}C-MVA to resuspended cultures of *C. rosicola* in the absence (—) or presence of 5 x 10^{-8} M (-·-) triarimol

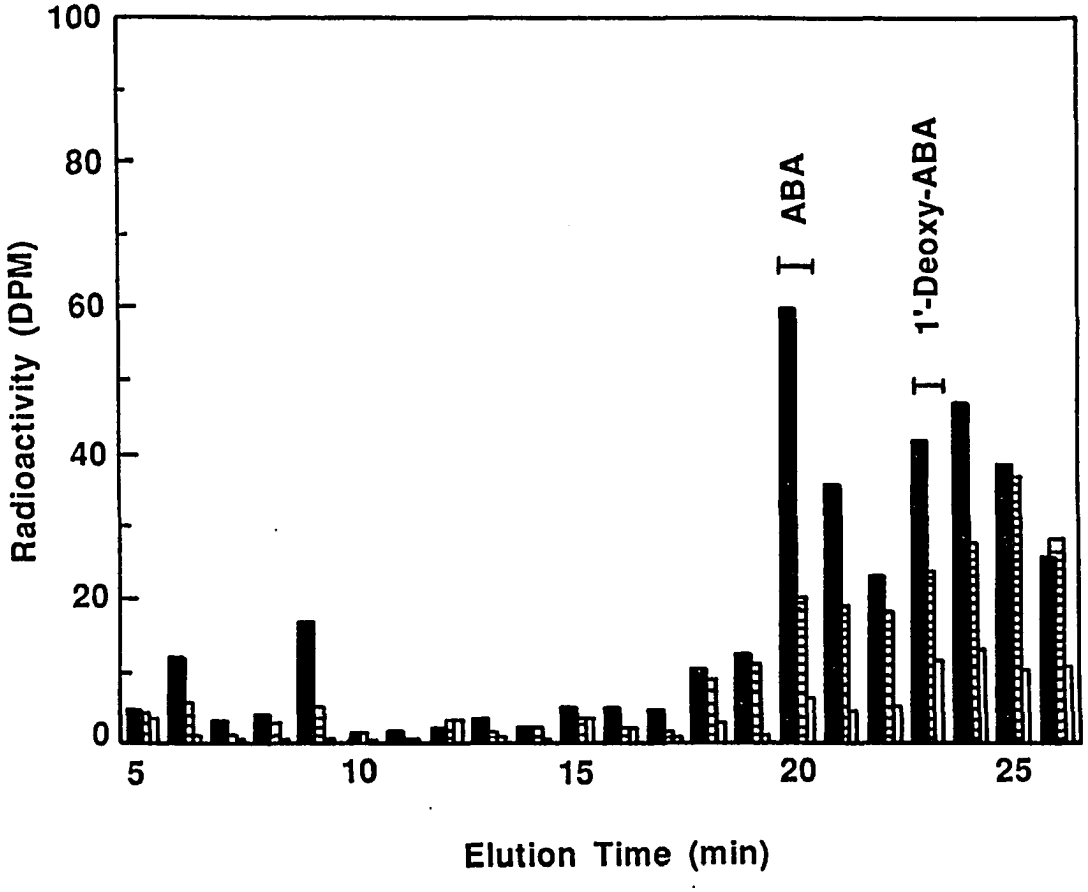


Figure 8. Distribution of radioactivity following the feeding of $^{2}H,^{3}H,^{13}C$-FPP to resuspended cultures of *C. rosicola* in the absence (■) or presence of 1.5×10^{-7} M (▤) triarimol. Boiled fungus (□) without triarimol is included as a control

m/z 265 (0), 192 (2), 190 (2), 177 (16), 150 (26), 149 (61), 148 (35), 128 (100), 122 (10), 115 (6), 108 (4), 94 (5) (Fig. 10A). The analogous ions of 1'-deoxy-[^{1}H]-ABA methyl ester are m/z 262 (5.3), 189 (3.8), 187 (3.3), 174 (22.1), 147 (20.9), 146 (44.8), 145 (13.6), 125 (100), 119 (14.7), 112 (9.2), 105 (6.8), 91 (9.5) (Neill et al., 1982a). The other peak (B) at 9.59 min was present in every purified sample of 1'-deoxy-^{2}H-ABA and its mass spectrum had a base peak of m/z 149 (Fig. 10B). The concentration of the purified Me-1'-deoxy-^{2}H-ABA was quantified based on a standard curve of authentic Me-ABA.

1'-Deoxy-^{2}H-ABA was converted to ^{2}H-ABA by actively growing cultures of *C. rosicola*, as previously reported by Horgan et al. (1983) and Neill et al. (1981, 1982a, b, 1987). GC-MS evidence of the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA was obtained by monitoring m/z 193, 165, 137, and 128, the four major ion fragments of Me-^{2}H-ABA. The same ions were monitored in cultures that were not fed with 1'-deoxy-^{2}H-ABA.

The ion chromatograms of extracts from liquid cultures grown and resuspended in the absence and presence of 5.0 x 10^{-8} M triarimol are shown in Figure 11 (A-D). Figures 11A and 11C show that there is conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in the fungal cultures in the absence and presence of 5.0 x 10^{-8} M triarimol. These data demonstrate that triarimol has no inhibitory effect on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA. The presence of significant peaks at m/z 165, 137, and 128 (but not 193) in the triarimol-free cultures without feeding (Fig. 11B) was due to the production of unlabeled ABA, the mass spectrum of which contains significant amounts of these three ions. Boiled cultures without triarimol do not contain any ABA (Figs. 11E and F).

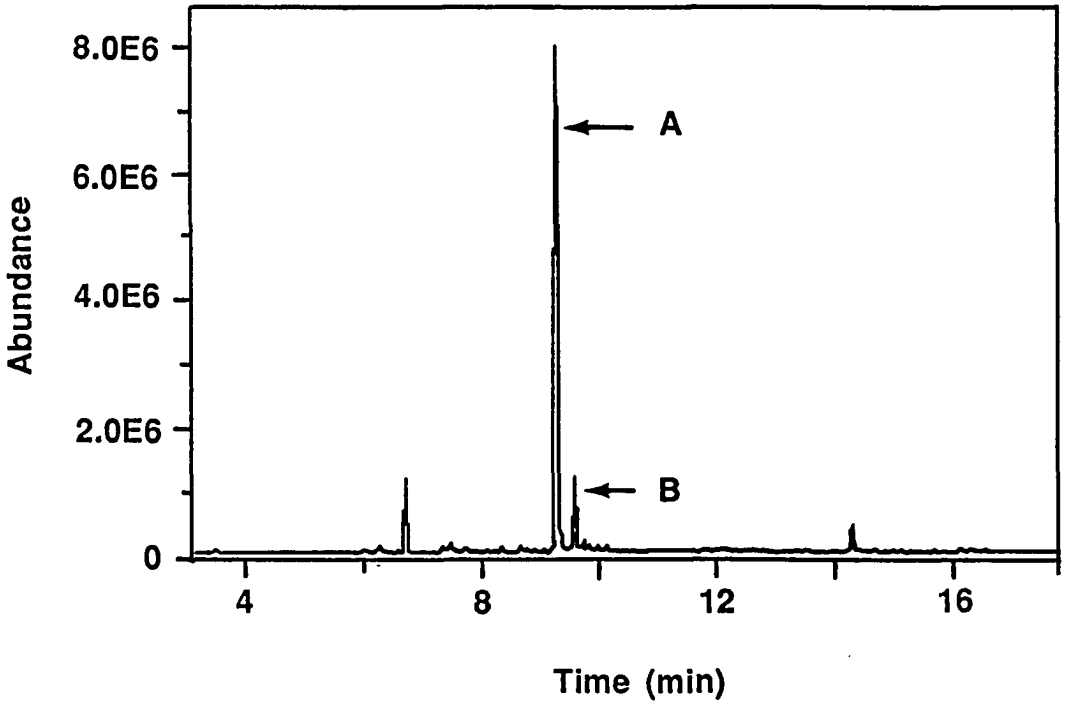


Figure 9. Total ion chromatogram of the methyl ester of purified 1'-deoxy-^{2}H-ABA

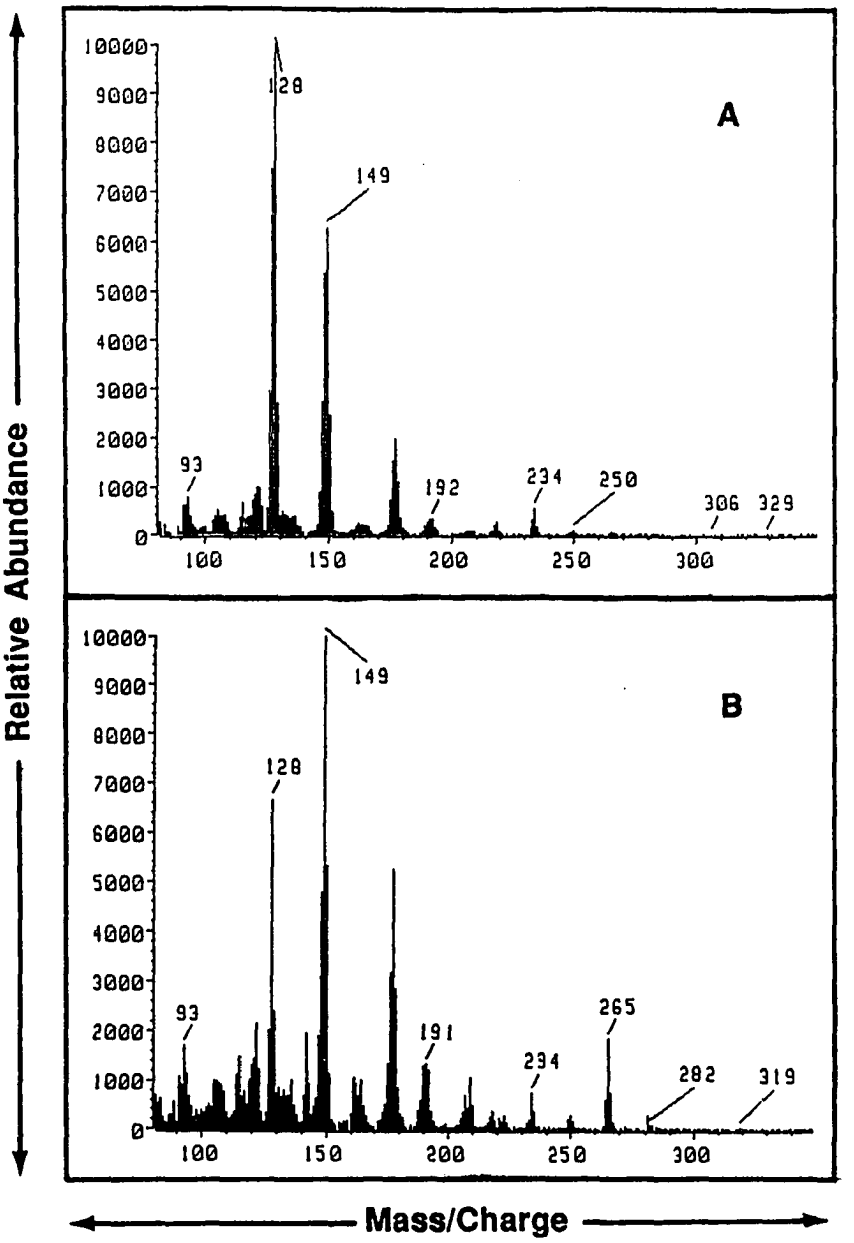


Figure 10. Mass spectra of 1'-deoxy-^{2}H-ABA methyl ester eluted at a retention time of 9.26 min (A) and peak at a retention time of 9.59 min (B) shown in the purified 1'-deoxy-^{2}H-ABA samples

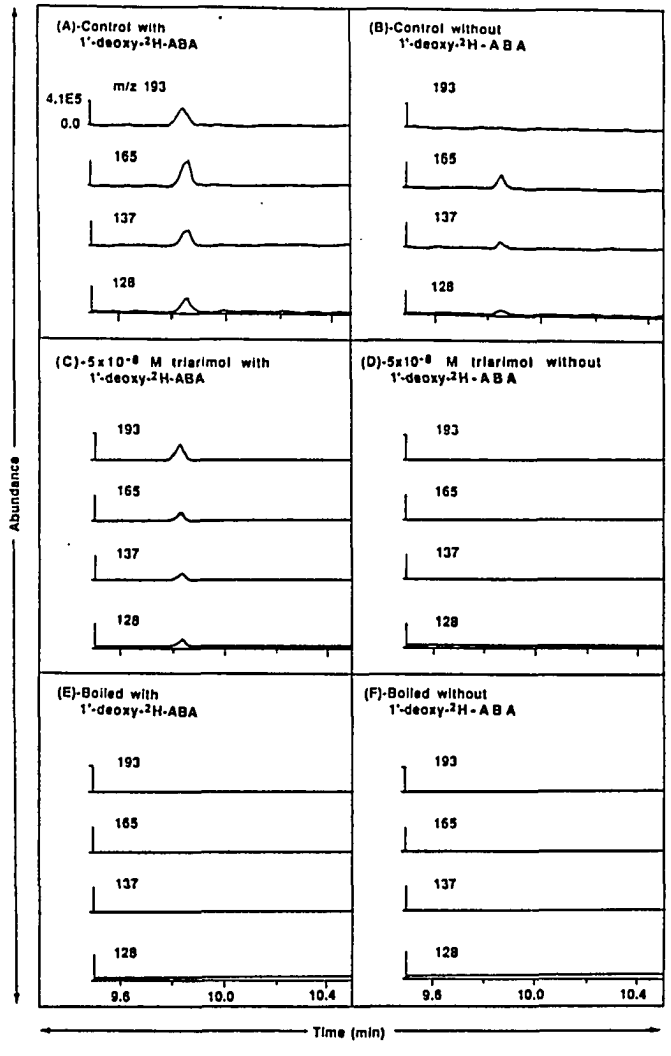


Figure 11. Ion chromatograms of m/z 193, 165, 137, and 128 obtained by GC-MS of extracts from cultures grown and resuspended in the absence (A, B) or presence (C, D) of 5.0×10^{-8} M triarimol, and boiled cultures without triarimol (E, F). Profiles A, C, and E represent cultures fed with 1'-deoxy-^{2}H-ABA, while B, D, and F are from cultures without feeding

The percent conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in cultures grown with and without triarimol is shown in Table 1. In experiments 1 and 2, the concentration of triarimol was 1.5 x 10^{-7} M, and in experiment 3 it was 5.0 x 10^{-8} M. Based upon GC-MS analysis, triarimol-treated and untreated cultures of *C. rosicola* were capable of converting 1'-deoxy-^{2}H-ABA to ^{2}H-ABA. In experiments 1 and 3 the percentage of conversion was higher in triarimol-treated than in untreated cultures; in experiment 2 conversion was the same.

Figure 11 and Table 1 strongly suggest that the presence of triarimol at a concentration that inhibits ABA production in liquid cultures of *C. rosicola* has no inhibitory effect on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA. There was no conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in boiled fungus cultures.

In vitro incorporation of ABA precursors into ABA

Using fungal cell-free extracts to incorporate labeled precursors into ABA is an important step to be taken for a better understanding of the ABA biosynthetic pathway in *C. rosicola*. So far, there has been no published report of using a cell-free system in *C. rosicola* or any of the other ABA-producing fungi for ABA biosynthesis.

^{14}C-MVA and ^{2}H,^{3}H,^{13}C-FPP metabolism Experiments using cell-free extracts of *C. rosicola* with ^{14}C-MVA or ^{2}H,^{3}H,^{13}C-FPP as a substrate were conducted. No identifiable products were observed.

1'-Deoxy-^{2}H-ABA metabolism The first active enzyme preparation was obtained from a 10,000 g_{av} supernatant (S_{10}) of a 5-day-old culture. One hundred ul of this fungal preparation was used in a cell-free reaction that contained 30 uM 1'-deoxy-^{2}H-ABA, 1 mM NADPH, 1 uM $MgCl_2.6H_2O$, and 50 mM tricine buffer

Table 1. Apparent percent conversion[a] of 1'-Deoxy-^{2}H-ABA to ^{2}H-ABA and ABA production (ug/ml) in resuspended cultures of *C. rosicola*

Treatment	Exp. 1		Exp. 2		Exp. 3	
	%	[ABA]	%	[ABA]	%	[ABA]
With triarimol						
+1'-Deoxy-^{2}H-ABA	79.63	0.28	38.82	0.30	97.05	0.18
-1'-Deoxy-^{2}H-ABA	1.22	0.13	0.72	0.20	8.94	0.01
Without triarimol						
+1'-Deoxy-^{2}H-ABA	12.92	1.62	38.94	0.35	2.80	2.97
-1'-Deoxy-^{2}H-ABA	0.09	1.50	0.47	0.24	0.15	2.47

[a]Apparent percent conversion of 1'-deoxy-^{2}H-ABA into ^{2}H-ABA was calculated as follows:
[A/(A + B)] x 100% where:
A = Area of m/z 193 (Me-sample) - Area of m/z 193 (Me-ABA standard).
B = Area of m/z 190 (Me-sample) - Area of m/z 190 (Me-^{2}H-ABA standard).

containing 0.25 M sucrose, pH 7.8, in a total reaction volume of 1 ml. Another reaction containing boiled enzyme was included as a control. Reactions were incubated in a water bath at 30° C, 225 rpm for 3 h. GC-MS analysis of ABA-containing fractions collected from HPLC showed conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in the active enzyme reaction, but not in the boiled one.

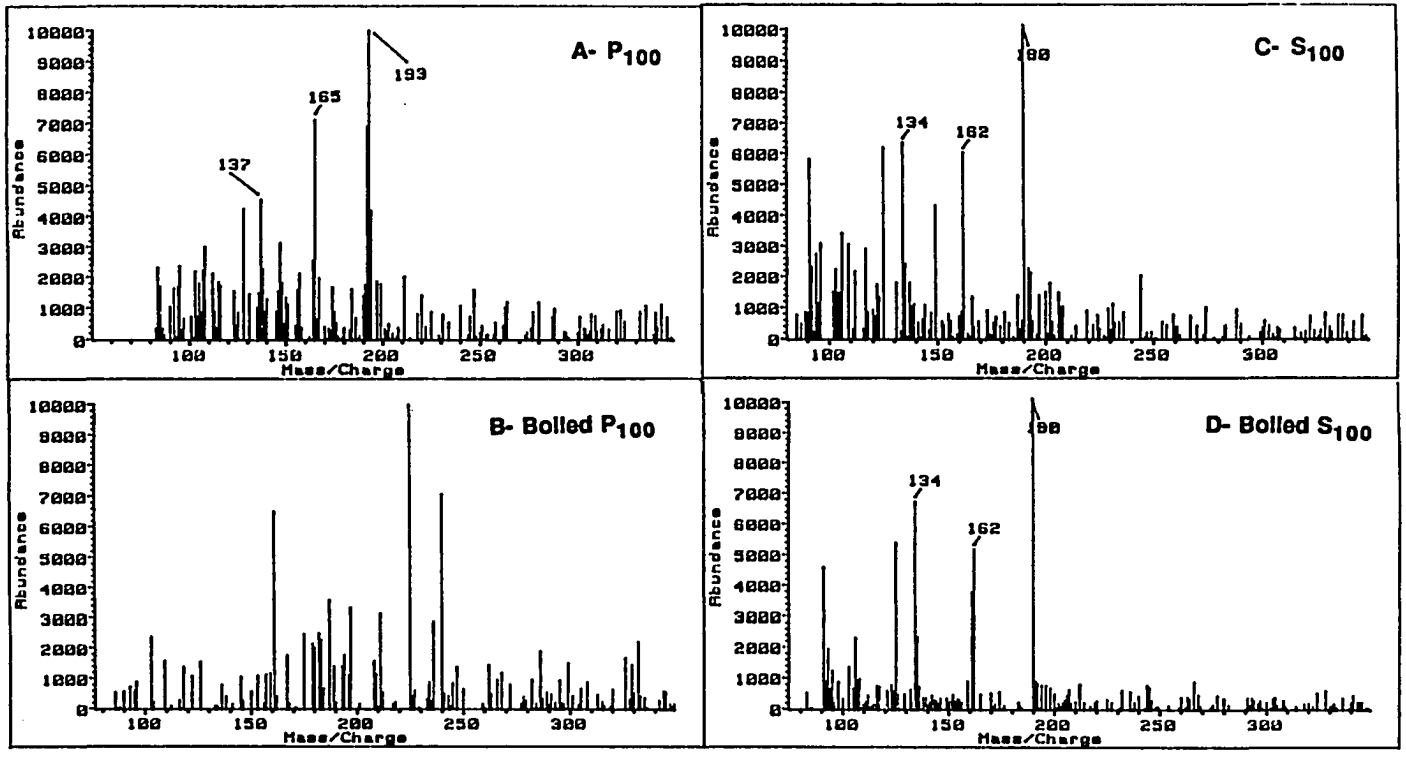
In an attempt to localize the site of the enzymatic (hydroxylase) activity, further fractionation of S_{10} into a 100,000 g_{av} supernatant (S_{100}) and a microsomal fraction (P_{100}) was done. Both these fractions were used as an enzyme source in separate reactions. GC-MS analysis of the reaction products revealed that most of the activity was in the resuspended microsomal pellet rather than in the corresponding supernatant (Fig. 12). The average mass spectra of Me-^{2}H-ABA resulting from the conversion of 1'-deoxy-^{2}H-ABA in active and boiled P_{100} enzyme preparations are shown in Figures 12A, B, respectively, while those of active and boiled S_{100} enzyme preparations are given in Figures 12C, D, respectively. There is clear conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA by the active P_{100} enzyme preparation. This conversion is clearly enzymatic since there is no conversion with the boiled enzyme preparation. There were no detectable differences between the mass spectra obtained using the active and boiled S_{100} fractions (Figs. 12C and D). The high abundance of m/z 190 in both S_{100} reactions was due to the original presence of endogenous ABA in S_{100} extracts of *C. rosicola* mycelia.

The effect of 1'-deoxy-^{2}H-ABA concentration on its conversion to ^{2}H-ABA is shown in Figure 13. Activity peaked at 15 uM and declined at higher concentrations. Ten uM was used as a substrate concentration in further experiments.

Figure 12. Mass spectra of methylated ABA fractions from cell-free experiments using (A) P_{100}, (B) boiled P_{100}, (C) S_{100}, and (D) boiled S_{100} as an enzyme source from *C. rosicola* enzyme preparations.

Prominent native Me-ABA ions: m/z 190, 162, 134.

Prominent deuterated Me-ABA ions: m/z 193, 165, 137


A- P100
B- Boiled P100
C- S100
D- Boiled S100
Abundance
Mass/Charge
137
165
193
134
162
198

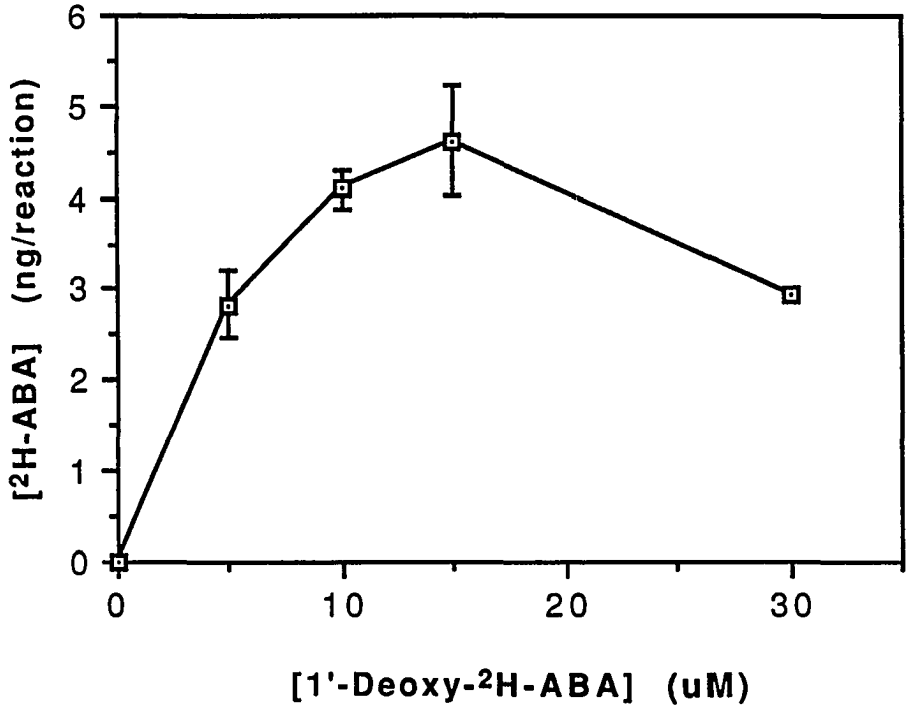


Figure 13. Effect of 1'-deoxy-^{2}H-ABA concentration on ^{2}H-ABA production in a cell-free enzyme extract from *C. rosicola*. ^{2}H-ABA value at 30 uM 1'-deoxy-^{2}H-ABA represents one reaction without replication. Bars represent SE

An increase in ^{2}H-ABA production was also observed with increasing P_{100} concentration. As shown in Figure 14, the relation between ^{2}H-ABA production and enzyme concentration was linear up to at least 400 ul P_{100} per reaction. The protein concentration in the P_{100} enzyme preparation was 1.50 mg/ml.

The time course for the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA is shown in Figure 15. Under the conditions of this assay, ^{2}H-ABA production was quite rapid during incubation for 10 min.

The effects of NADPH, Mg^{++}, FAD, and 10^{-7} M triarimol on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA are shown in Table 2. The presence of NADPH and Mg^{++} in a reaction using S_{10} as an enzyme source enhanced this conversion. Using P_{100} as an enzyme source in the presence or absence of NADPH showed that NADPH was necessary for the reaction to take place. 10^{-7} M triarimol had no inhibitory effect on this conversion using S_{10} as an enzyme source. Table 2 also shows that FAD was not necessary for the reaction to take place, but enzymatic activity was enhanced in its presence.

ABA production in plant tissues

ABA has been detected in various plant tissues. ABA levels change in response to environmental factors such as drought and photoperiod. The purpose of the experiments on plant tissues described here was to determine if fungal infection of leaves is accompanied by an increase in ABA levels. ABA was quantified in diseased and healthy leaves of sycamore and mulberry. These species were chosen for this study because of the observation that diseased leaves fall off earlier in the season than healthy ones. Using the purification and quantification procedure for ABA described earlier, it was found that ABA levels in the diseased

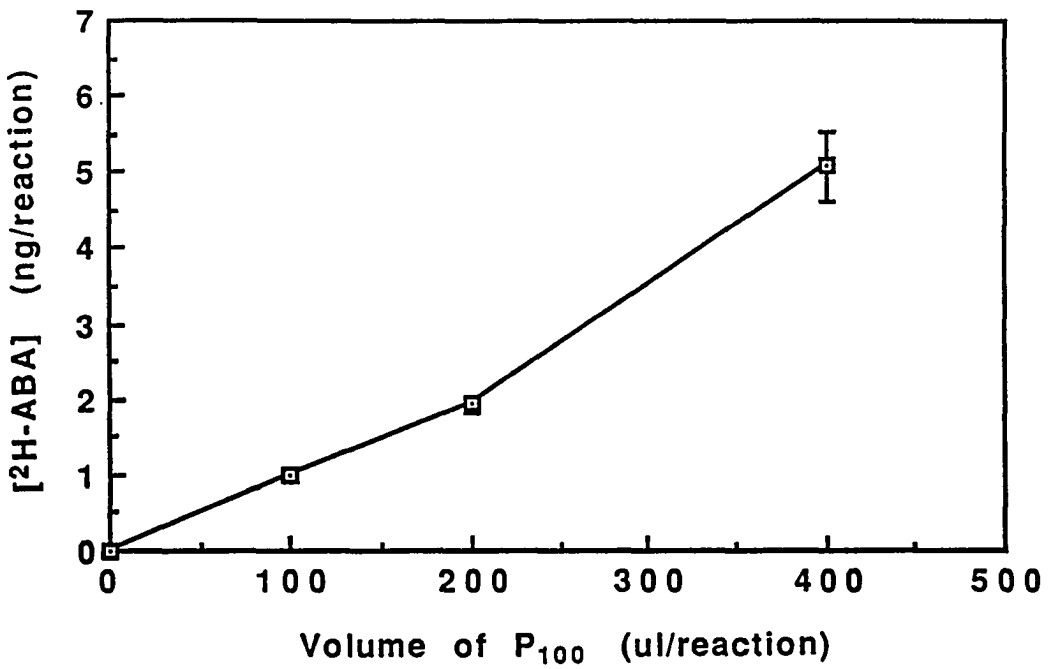


Figure 14. Effect of the microsomal (P_{100}) enzyme concentration on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in cell-free extracts from *C. rosicola*. Bars represent SE

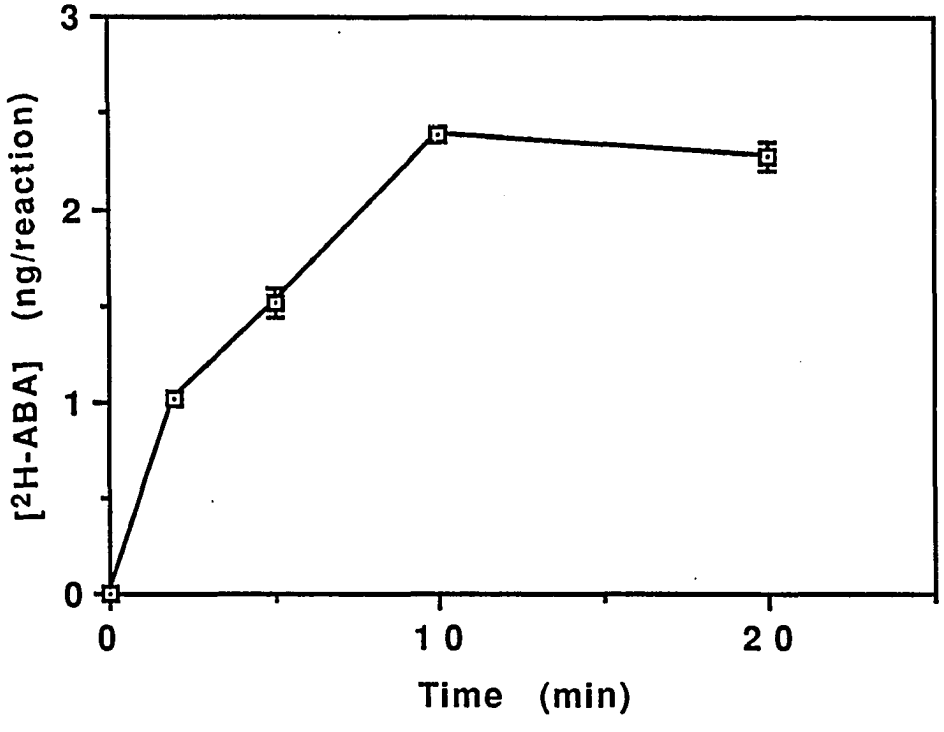


Figure 15. Time course for the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in cell-free extracts from *C. rosicola*. Bars represent SE

Table 2. Effects of NADPH, Mg^{++}, FAD, and 10^{-7} M triarimol on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in cell-free extracts from *C. rosicola*

Enzyme source	[^{2}H-ABA] (ng/reaction) (±SE)				
	Complete	- NADPH	- Mg^{++}	- FAD	+ Triarimol
S_{10}	2.66±0.03[a]	1.08±0.32	1.72±0.29	--	2.16±0.67
S_{10}	2.99±0.24[a]	--	--	--	3.63±1.00
P_{100}	0.51±0.19[b]	0.00	--	0.25±0.05	--
P_{100}	0.80±0.20[c]	0.00	--	0.58±0.03	--

[a]Reaction mixture contained 30 uM 1'-deoxy-^{2}H-ABA, 50 mM tricine buffer containing 0.25 M sucrose at pH 7.8, 1 mM NADPH, 1uM $MgCl_2.6H_2O$, and 100 ul S_{10} enzyme extract in a total volume of 1 ml. Reactions incubated for 3 h at 30°C.

[b]Reaction mixture contained 10 uM 1'-deoxy-^{2}H-ABA; buffer, NADPH, and Mg^{++} as in (a) above; 300 ul P_{100} enzyme extract, and 4.7 uM FAD in a total volume of 1 ml. Reactions incubated for 7 min at 30°C.

[c]Reaction mixture contained the same as (b) except for [NADPH] which was 50 uM.

leaves were higher than in the comparable healthy ones (Table 3).

ABA production in fungi other than *C. rosicola*

Gnomonia veneta and *Cercospora mori* are pathogens that cause the abscission of diseased leaves of sycamore and mulberry, respectively. These fungi have been tested in our laboratory for their production of ABA. Both fungi were grown in the liquid medium used to culture *C. rosicola*. ^{2}H-ABA was used as an internal standard in selected samples during ABA extraction from liquid media. Purification of ABA was done by HPLC, while identification and quantification were performed by GC-MS. Analysis of the data from a number of experiments using the relative intensities of prominent ions in cultures ranging in ages from 4 to 8 days did not lead to unequivocal evidence for the presence of ABA in these cultures.

Table 3. ABA accumulation in healthy and diseased sycamore and mulberry leaves

Species	Exp	[ABA] (ug/g f wt.)	
		Healthy	Diseased
Sycamore:	1	0.59	1.11
	2	1.17	2.78
		ABA (GC-MS peak Area)[a]	
Mullberry:	1	30,021	87,553
	2	17,631	46,524

[a]Peak areas were not within the range used for the standard curve.

DISCUSSION

The discovery of high yields of ABA in *C. rosicola* by Assante et al. (1977) has created an interest among a few groups of researchers in using this fungus to elucidate the ABA biosynthetic pathway. The average ABA production by *C. rosicola* as detected by Assante's group was 60 ug/ml growth medium compared to 10 to 50 ng/g fresh weight in most plant tissues. Realizing the importance of this fungus in ABA studies, we have chosen it for study of ABA production. In previous research, using inoculum from resuspension of agar-grown mycelia gave us a maximum ABA yield of 7.4 ug/ml by the 6th day. In our current work, the inoculum source used was from a 5 to 7-day-old liquid culture which in turn was started from an agar-grown mycelial resuspension. These fungal cultures produced up to 29 ug/ml by the 8th day.

Citric acid enhanced ABA production significantly in 5 to 7-day-old *C. rosicola* cultures. A possible role of citric acid is blocking the enzyme phosphofructokinase in glycolysis. This leads to the accumulation of glucose-6-phosphate, which can be used to generate NADPH by the pentose phosphate pathway (Stryer, 1988). The enhancement of ABA production by citric acid might be due to that of NADPH production, which is required for some oxidative reactions involved in ABA biosynthesis.

Growth and ABA production *in C. rosicola* occurred over an extended period in the defined liquid medium. Over a 20-day period, Norman et al. (1981b) found that growth and ABA production increased with time until the 17th and 13th day, respectively. A considerable variation in *C. rosicola* growth and ABA production observed in our laboratory seems to depend upon culture medium, age and size of

the inoculum culture, and many other factors (Al-Nimri, 1986). For growing *C. rosicola* cultures, we used the conditions described in Materials and Methods, which yield reasonably consistent results.

The effects of ancymidol and triarimol on growth of and GA biosynthesis by the fungus *G. fujikuroi* were reported by Coolbaugh et al. (1982b). In that report, triarimol was found to be a more effective inhibitor of GA biosynthesis than ancymidol. Effects of ancymidol and triarimol on both growth and ABA production in *C. rosicola* liquid cultures have been compared more recently. These results (Al-Nimri and Coolbaugh, 1990) support the earlier work with another fungus and another phytohormone biosynthetic pathway (Coolbaugh et al., 1982b), in that these plant growth regulators exerted an inhibitory effect on both growth and ABA production and that triarimol has a greater inhibitory effect than ancymidol. The ED_{50} of ancymidol and triarimol in *C. rosicola* liquid cultures were 5×10^{-6} and 10^{-8} M, respectively. Norman et al. (1988) studied the effect of many sterol inhibitors on the ABA biosynthetic pathway and found that 10^{-4} M fenarimol, a triarimol analog, caused 92% inhibition of ABA production. An ED_{50} of fenarimol was found to be approximately 5×10^{-6} M.

Comparison of the effects of many inhibitors of ABA biosynthesis tested in our laboratory (Al-Nimri and Coolbaugh, 1990) as well as those tested by Norman et al. (1983, 1986, 1988) suggests that triarimol is the most effective inhibitor of ABA biosynthesis reported to date.

Feeding *C. rosicola* resuspended cultures with labeled precursors of ABA in the presence or absence of triarimol was an approach that shed some light on the ABA biosynthetic pathway in this fungus. ^{14}C-MVA, ^{2}H,^{3}H,^{13}C-FPP, and

1'-deoxy-^{2}H-ABA were incorporated into the corresponding labeled ABA in resuspended cultures.

Previous work in our laboratory had shown that ^{14}C-MVA could be incorporated into ^{14}C-ABA with a yield of 0.64%, and that ^{14}C-MVA in *C. rosicola* cultures treated with 5.0 x 10^{-8} M triarimol could be incorporated into an unknown compound, "unknown A", at an HPLC retention time later than that of ABA (Al-Nimri, 1986). The possibility that ^{14}C-unknown A might represent an intermediate in the ABA biosynthetic pathway has not been ruled out. Indeed, it seems likely that unknown A is a substrate for a mixed function oxidase because it accumulates in the presence of a mixed function oxidase inhibitor, triarimol. The HPLC retention times of ABA, unknown A, and 1'-deoxy-ABA have been observed to be approximately 19.5, 22, and 23 min, respectively. In an attempt to determine the role of unknown A in ABA biosynthesis, a number of experiments have been conducted to accumulate it in significant quantities. The first attempts to reproduce the previous results were unsuccessful in getting consistent incorporation of ^{14}C-MVA into ^{14}C-unknown A in triarimol-treated cultures. This problem was overcome by using resuspended cultures of *C. rosicola* and feeding them with ^{14}C-MVA at a higher substrate concentration and at a time within the rapid phase of ABA production. Experiments involving feeding ^{14}C-unknown A to resuspended cultures of *C. rosicola* are planned. It would be a good indication that this compound is an intermediate in the ABA biosynthetic pathway if it is incorporated into ^{14}C-ABA in these cultures. No further work has been done to identify this compound.

Feeding ^{2}H,^{3}H,^{13}C-FPP to *C. rosicola* resuspension cultures resulted in its incorporation into ABA and several other metabolites (Fig. 8). The incorporation

of FPP into ABA was also reported by Bennett et al., 1984. Our results show that FPP can be incorporated into metabolites other than ABA and 1'-deoxy-ABA. This is expected, since FPP is a common precursor of many isoprenoid compounds in addition to ABA and GAs. The inhibition of FPP incorporation into ABA and 1'-deoxy-ABA by triarimol is an indication of triarimol acts prior to 1'-deoxy-ABA. FPP feed results do not support the ^{14}C-MVA results concerning unknown A. If this compound comes after FPP in the ABA biosynthetic pathway and accumulates in the presence of triarimol, triarimol treatment should cause unknown A accumulation in both ^{14}C-MVA- and ^{2}H,^{3}H,^{13}C-FPP-fed cultures. This inconsistency between the two feeding results is hard to explain since none of the reactions between MVA and FPP is oxidative, so that none would be an expected site of triarimol action. However, our previous difficulty in observing consistent accumulation of unknown A in triarimol-treated cultures labeled with ^{14}C-MVA suggests that if these experiments were done with variable substrate concentrations of FPP, unknown A might accumulate.

A third precursor of ABA used in our laboratory was ^{2}H-labeled 1'-deoxy-ABA, the likely immediate precursor of ABA in *C. rosicola*. Feeding 1'-deoxy-^{2}H-ABA to resuspended cultures of *C. rosicola* resulted in its conversion to ^{2}H-ABA in both triarimol-treated and untreated (control) cultures. The percent conversion in triarimol-treated cultures was higher than in the control cultures. This might be due to the competition between 1'-deoxy-^{2}H-ABA and endogenous 1'-deoxy-ABA for the binding site(s) of the hydroxylase enzyme. In triarimol-treated cultures, triarimol might have blocked 1'-deoxy-ABA synthesis by the fungus, and therefore less competition for the active site(s) would have occurred than in the controls. In experiment 2 (Table 1), the percent conversion was the same in both triarimol-

treated and control cultures of *C. rosicola*. In this experiment the fungus produced less ABA, which is probably associated with less 1'-deoxy-ABA and consequently less competition with 1'-deoxy-^{2}H-ABA. Although 1'-deoxy-ABA was shown by Mallaby (1974, cited in Hirai, 1986) to hydroxylate automatically at C-1' in air, its hydroxylation in *C. rosicola* cultures to ABA seems to be enzymatic, since boiled fungus cultures did not convert 1'-deoxy-^{2}H-ABA to ^{2}H-ABA (Fig. 11C).

The conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA by *C. rosicola* is in agreement with previous results obtained by other groups. Neill et al. (1981; 1982a, b; 1984) and Horgan et al. (1983) found that 1'-deoxy-ABA is converted to ABA by *C. rosicola* and Neill et al. (1982a) suggested it is the immediate precursor of ABA.

Cell-free studies can provide significant information about biosynthetic pathways and metabolism of compounds of interest. Using cell-free systems to study the GA biosynthetic pathway in immature pea seeds has shown that the enzymes catalyzing (-)-kaurene biosynthesis occur in the soluble fraction of the cotyledons of these seeds and in pea shoot tips (Coolbaugh and Moore, 1971; Coolbaugh et al., 1973).

The first cell-free system related to ABA biosynthesis was developed by Milborrow (1974b) using ^{14}C-MVA with a preparation of lysed chloroplasts isolated from ripening avocado fruit in reactions that contained ATP, FAD, FMN, NAD, NADH, NADP, NADPH. Cell-free systems for the study of ABA biosynthesis in plants were not used again until Sindhu and Walton (1987) tried to incorporate xanthoxin into ABA in cell-free extracts from *Phaseolus vulgaris* leaves. In these extracts, xanthoxin was incorporated into ABA in an NAD/NADP-dependent reaction. The enzymatic activity appeared to be cytosolic. They were

unable to detect any intermediates between xanthoxin and ABA. Using wild type and wilty mutants of tomato, Sindhu and Walton (1988) showed that xanthoxin is a normal intermediate in the ABA biosynthetic pathway, and ABA aldehyde was suggested to be the final precursor of ABA.

Our few attempts to incorporate ^{14}C-MVA and ^{2}H,^{3}H,^{13}C-FPP into ABA using *C. rosicola* cell-free extracts were unsuccessful. This might be because the biosynthetic pathway from MVA and/or FPP to ABA involves many reactions.

The development of a cell-free system to convert 1'-deoxy-ABA to ABA was an approach taken to advance our understanding of ABA biosynthetic pathway in *C. rosicola*. The first active enzyme preparation was obtained from an S_{10} enzyme extract of a five-day-old culture. A reaction containing boiled enzyme was used as a control. GC-MS results confirmed the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in the active enzyme reaction, but not in the boiled one. From this point, a number of experiments were conducted to characterize the system. Most of the enzymatic activity was found in the microsomal fraction, indicating that the hydroxylase enzyme is membrane bound. In subsequent experiments using the microsomal fraction as an enzyme source, 10 uM 1'-deoxy-^{2}H-ABA was converted to ^{2}H-ABA in an average of 1.47 pmol ^{2}H-ABA mg^{-1} protein min^{-1}.

The results of testing the effect of NADPH on the rate of the reaction indicate that NADPH is necessary for the reaction to occur. Using S_{10} for these studies showed that there was production of ^{2}H-ABA in reactions with and without NADPH, but to a lesser extent in reactions with no NADPH. Using the microsomal fraction as an enzyme source for the same kind of studies showed no production of ^{2}H-ABA in the absence of NADPH. Thus, it seems there is some endogenous NADPH in S_{10}, but not in the microsomal fraction.

The effect of triarimol on the oxidation of 1'-deoxy-^{2}H-ABA was tested in S_{10} fungal extracts. Results using *C. rosicola* liquid cultures showed that triarimol inhibited endogenous ABA production, but had no inhibitory effect on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA. The cell-free system results with triarimol confirm our previous conclusion that the site(s) of triarimol inhibition is(are) prior to 1'-deoxy-^{2}H-ABA in the ABA biosynthetic pathway.

The proposed ABA biosynthtic pathway in *C. rosicola* involves several oxidative reactions of the precursor α-ionylidene as suggested by Neill and Horgan (1983). A mixed function oxidase enzyme is expected to catalyze these oxidative reactions with the involvement of Cyt P-450. Walton and Griffin (unpublished results cited in Neill et al., 1984) found that ABA biosynthesis in *C. rosicola* was inhibited by the Cyt P-450 inhibitor piperonyl butoxide at a concentration of 4×10^{-4} M. An indication that Cyt P-450 is involved in the ABA biosynthetic pathway in *C. rosicola* came from our previous results (Al-Nimri and Coolbaugh, 1990), which showed that ABA production was adversely affected by ancymidol, triarimol, and paclobutrazol (cytochrome P-450 inhibitors). Norman et al. (1986) also found that ancymidol and paclobutrazol inhibited ABA production in *C. rosicola*. Testing the effects of fenarimol, a close triarimol analog, and several sterol inhibitors on the ABA biosynthetic pathway in *C. rosicola* led Norman et al. (1988) to conclude that a mixed function oxidase is probably involved in one or more steps of the pathway. These data, taken together with those presented here, suggest that triarimol's site of action is on one or more of the oxidative reactions prior to 1'-deoxy-ABA, and that the last step in ABA biosynthetic pathway, the hydroxylation of 1'-deoxy-ABA into ABA, may not be a Cyt P-450 catalyzed reaction, as it is not inhibited significantly by triarimol.

The discovery of ABA production by *C. rosicola* in 1977 by Assante et al. initiated a number of investigations to detect ABA in other phytopathogenic fungi. As noted earlier, eight other species of fungi have been found to produce ABA. We have also searched for ABA production in phytopathogenic fungi. The observation that the diseased sycamore and mulberry leaves abscise early in the season led us to isolate the fungus that causes each disease. *Gnomonia veneta* and *Cercospora mori* were isolated from infected sycamore and mulberry leaves, respectively. Growing both of these fungi in liquid medium gave variable results regarding ABA production. In some experiments, one replicate had an indication of the presence of ABA, while the other replicate of the same treatment did not. This kind of results, obtained on many occasions, casts doubt on ABA production by both fungi.

ABA, under the name of dormin, was detected in sycamore and birch leaves when the conditions changed from long-day to short-day (Cornforth et al., 1965b). Our identification of ABA in sycamore and mulberry leaves confirm earlier reports of the occurrence of ABA in almost every vascular plant investigated. We also were able to detect ABA in higher amounts in infected sycamore and mulberry leaves than in healthy ones. It is still not known whether the elevated ABA levels in infected leaves are due to the production of ABA by the pathogenic fungi in the leaf tissues or to the induction of ABA production in plants by the infection process. The absence of ABA production by *G. veneta* and *C. mori* is not a conclusive evidence that these fungi are unable to synthesize ABA, since nonsupplied nutrients and/or different growth conditions might be necessary for ABA production by them. On the other hand, the infection process might explain the elevated levels of ABA in infected leaves as well as the premature abscission of

these leaves. Both ABA and ethylene tend to initiate and accelerate abscission (Addicott, 1982). Yang and Pratt (1978) found that fungal infection of the plants often leads to the formation of large amounts of ethylene. Ethylene was found by Goldschmidt et al. (1973) to increase ABA levels in the outer, colored peel layer of Shamouti orange (*Citrus sinensis*) fruit. On the contrary, Hyodo (1978) found that ABA increased ethylene production in segments of Satsuma mandarin (*Citrus unshiu*) fruit. If a similar interrelation between ABA and ethylene operates in sycamore and mulberry leaves, and *G. veneta* and *C. mori* induce ethylene formation, this could explain the elevated levels of ABA and the premature abscission of the infected leaves. The possibility of ABA production by the pathogenic fungi in the plant tissues cannot be ruled out.

CONCLUSIONS

1. A good yield of ABA is produced by *C. rosicola* in shaking liquid cultures incubated for less than ten days.

2. *C. rosicola* resuspended cultures are able to incorporate labeled ABA precursors (MVA, FPP, 1'-deoxy-ABA) into labeled ABA.

3. A cell-free enzyme system has been developed to oxidize 1'-deoxy-^{2}H-ABA into ^{2}H-ABA with a yield of 1.47 pmol mg^{-1} protein min^{-1}.

4. The enzyme responsible for the oxidation of 1'-deoxy-ABA requires NADPH and is stimulated by FAD.

5. The site(s) of action of triarimol is prior to 1'-deoxy-ABA in the ABA biosynthetic pathway in *C. rosicola*. This conclusion is based on the results that:
   a- Conversion of ^{14}C-MVA into ^{14}C-ABA was inhibited completely in the presence of triarimol in *C. rosicola* resuspended cultures;
   b- Conversion of ^{2}H,^{3}H,^{13}C-FPP into ^{2}H,^{3}H,^{13}C-ABA was inhibited 66-80% in resuspended triarimol-treated compared to untreated cultures;
   c- Conversion of 1'-deoxy-^{2}H-ABA into ^{2}H-ABA is not affected by triarimol in *C. rosicola* resuspended cultures and in the cell-free enzyme system from the same fungal cultures.

6. The phytopathogenic fungi *Gnomonia veneta* and *Cercospora mori* do not appear to produce ABA in liquid cultures. However, leaves infected with these fungi contain elevated levels of ABA and abscise prematurely. Additional work is required to determine whether the additional ABA is derived from the fungus or from the infected leaf tissue.

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