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Ethylene biosynthesis in sweet-potato root tissue induced by infection with black rot fungus (*Ceratocystis fimbriata*)

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Abstract. Ethylene production rapidly increased in sweet potato (*Ipomoea batatas* Lam.) root tissue in response to infection by black rot fungus (*Ceratocystis fimbriata* Ell. & Halst). Ethylene production increased in the surface layer (0-0.5 mm) of the root inoculated with endoconidia of *C. fimbriata*, reaching a maximum 1 day after inoculation and declining thereafter. The rise in the surface layer was followed by a subsequent increase in the inner, second (0.5-1.0 mm), and third (1.0-1.5 mm) layers as the fungus penetration progressed. Discs 10 mm in diameter and 0.5 mm thick excised from the surface of the infected root slices were used for the experiments. Our previous study demonstrated that ethylene synthesis in the infected tissue may occur via a pathway independent of the methionine-ACC (1-aminocyclopropane-1-carboxylic acid) pathway. Ethylene synthesis in the infected tissue was significantly suppressed by the inhibitors that interfere with NADPH oxidase, phospholipase A_2 and lipoxygenase, by metal ion chelators, and by scavengers of reactive oxygen species. The inhibition of metal ions was restored by the addition of cupric ion. These results suggest that in the infected sweet potato root tissue, polyunsaturated fatty acids are released from membrane phospholipids followed by peroxidation by lipoxygenase. The hydroperoxides could be decomposed in the presence of copper ion or copper enzyme and reactive oxygen species such as hydroxyl radical, leading to ethylene generation. De novo protein synthesis was required for ethylene synthesis to be induced in response to the fungal infection.

Keywords: *Ceratocystis fimbriata*; Copper ion; Ethylene biosynthesis; Infected tissue; *Ipomoea batatas*; Lipoxygenase; Membrane lipids; Reactive oxygen species; Unsaturated fatty acids.

Introduction

When sweet potato (*Ipomoea batatas* Lam.) roots are infected by black rot fungus (*Ceratocystis fimbriata* Ell. & Halst), a wide range of metabolic changes is induced in the host tissue (Uritani, 1971). These changes include an increase in respiration and stimulation of polyphenol synthesis, coumarin synthesis, furanoterpenoid synthesis, and ethylene synthesis (Uritani, 1967; 1971; Kojima, 1999). It has been reported that a large amount of ethylene is produced in response to infection with *C. fimbriata* in sweet potato roots (Stahmann et al., 1966; Imaseki et al., 1968a; Sakai et al., 1970; Kato and Uritani, 1972; Hyodo and Uritani, 1984; Hirano et al., 1991; Okumura et al., 1999).

The plant hormone ethylene plays an essential role in the growth, development, and senescence of higher plants and is synthesized normally by the pathway mediated by methionine and ACC (1-aminocyclopropane-1-carboxylic acid) (Adams and Yang, 1979; Yang and Hoffman, 1984). In the methione-ACC pathway, ACC synthase and ACC oxidase have a pivotal role in regulating ethylene synthesis. In lower plants, however, it is known that ACC fails to serve as a direct intermediate in ethylene biosynthesis (Osborne et al., 1996). Ethylene is also produced as a result of rapid response to abiotic and biotic stresses such as wounding and disease in plants (Yang and Hoffman, 1984; Boller, 1991; Hyodo, 1991; Abeles et al., 1992). In diseased tissue ethylene production is caused by host-parasite interactions.

In sweet potato roots it has been considered that most of the ethylene is generated in the living host cells adjacent to the necrotic region invaded by the fungus (Hirano et al., 1991; Okumura et al., 1999; Hyodo et al., 2003). A previous study has suggested that major sources of ethylene produced in sweet potato root tissue after infection by the fungus are derived from a pathway distinct from the methionine-ACC or glutamate pathway (Hyodo and Uritani, 1984; Hirano et al., 1991; Okumura et al., 1999; Hyodo et al., 2003).

The aim of the present study was to investigate the mechanism by which ethylene is biosynthesized in sweet potato root tissue infected by *C. fimbriata*. Our results suggest that phospholipase, lipoxygenase, reactive oxygen species, and copper ion are involved in ethylene formation.

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Materials and Methods

Plant Material and Fungal Inoculation

Sweet potato (*Ipomoea batatas* Lam. cv. Beniazuma) roots were obtained from a local market. The roots were surface-sterilized with 0.1% sodium hypochlorite solution, rinsed in running tap water, and sliced about 1 cm thick. One side of the cut surfaces of each slice was inoculated with spore (endoconidia) suspension (10^7 ml^{-1}) of sweet potato strain of *Ceratocystis fimbriata*. Ascospores of the fungus were subcultured on a potato dextrose (PD) agar medium at 28°C. A loopful of ascospores was taken from PD agar slant cultures and inoculated into a PD broth in a Sakaguchi flask, which was shaken cultured at 25°C for 2.5 days on a reciprocal shaker. Then, the broth was filtered through two layers of gauze, and the filtrate was centrifuged at 1,500 rpm for 10 min. The sediment comprised of endoconidia was suspended in sterilized water with the concentration adjusted to 10^7 ml^{-1}. The suspension was used to inoculate the cut surfaces with a soft brush. Non-inoculated slices were used for the cut control (wounding). The slices were incubated at 28°C in a moist chamber.

Assay of Ethylene Production

Discs 10 mm in diameter and 0.5 mm thick were excised from the inoculated slices using a cylinder microtome (Ikemoto Rika). Five discs were placed in a 63-ml Erlenmeyer flask, which was sealed with a rubber serum cap, and incubated at 25°C. The atmosphere in the flask was sampled at regular intervals with a 1-ml airtight plastic syringe. The sample gas was injected into a Hitachi 163 gas chromatograph equipped with a flame ionization detector and an activated alumina column at 70°C. The rate of ethylene production was expressed as nl per g fresh weight per h or nl per g fresh weight. Data were expressed as mean ± S.D. of two or three replicates.

Preparation and Purification of Polyclonal Antibodies for Lipoxygenase

Lipoxygenase antibodies were raised in rabbit against soybean lipoxygenase (Sigma-Aldrich Co., Type V). The antiserum was purified by the procedures of affinity chromatography fitted with lipoxygenase antigen. An aliquot of this purified fraction in PBS in 20 µl was administered to each disc excised from the inoculated slices.

Inhibitors of Ethylene Synthesis in the Infected Tissue

To study the inhibitory effect on ethylene synthesis, discs excised from inoculated slices were placed in an Erlenmeyer flask and fed with 20 µl of a solution consisting of 10 mM Tris-HCl buffer, pH 7.2, and the specified inhibitory substances of protein synthesis, NADPH oxidase, phospholipase A_2, and lipoxygenase, scavengers of reactive oxygen species, and chelators of metal ions.

Cycloheximide, dipenyleneiodonium chloride, aristolochic acid sodium salt, N-acetyl-L-cysteine, (S)-6-methoxy-α-methyl-2-naphthaleneacetic acid (naproxen) sodium salt, and 1,10-phenanthroline monohydrate were obtained from Sigma-Aldrich Co. Sodium N,N-diethyldithiocarbamate trihydrate was obtained from Wako Pure Chemical Industries, Ltd.

Results and Discussion

Ethylene Production Correlating with Fungal Infection

Ethylene production in sweet potato root tissue was initiated in the first 0.5 mm layer from the surface after inoculation with the endoconidia suspension of *C. fimbriata* (Figure 1). The ethylene production rate reached a peak one day after inoculation and then declined sharply. This was followed by a rise in the inner, second (0.5-1.0 mm) and third (1.0-1.5 mm) layers, respectively, correlating with the progress in fungal invasion (Figure 1). In the non-infected wounded root tissue only a tiny amount of ethylene was generated (Figure 1). The fungus itself was shown to make only a minor contribution to ethylene production in the infected tissue (Hirano et al., 1991). One day after inoculation mycelial penetration reached a few cell layers (approximately 0.2 mm) of the root tissue, causing the invaded host cells to be necrotic (Okumura et al., 1999). Most of ethylene was considered to originate from living

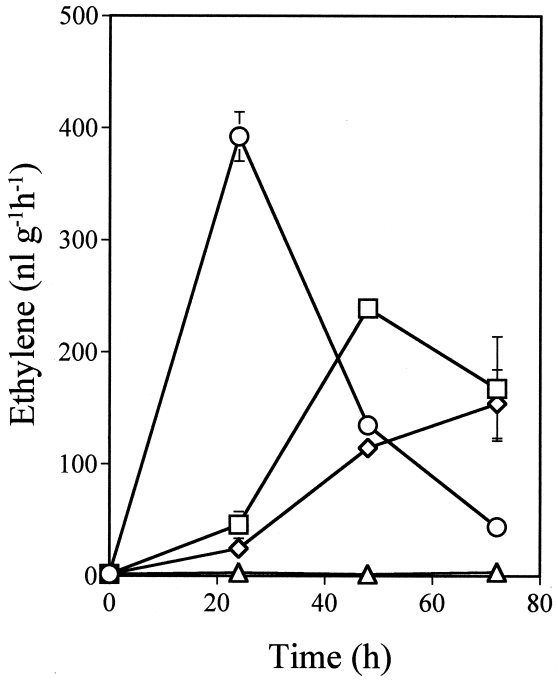

Figure 1. Time course of ethylene production rate in sweet potato root tissue infected by *C. fimbriata.* Bars represent S.D.s and are contained within the symbol when not shown. (○), first layer (0-0.5 mm); (□), second layer (0.5-1.0 mm); (◇), third layer (1.0-1.5 mm) of infected tissue, and (△), wounded tissue (0-0.5 mm), respectively.

root cells adjacent to the necrotic region. We used discs 10 mm in diameter and 0.5 mm thick excised from the first layer 1 day after inoculation for the experiments where ethylene production was observed.

We have previously shown that most of the ethylene formed in the infected root tissue may be derived from a pathway different from the ACC or glutamate pathway (Hyodo and Uritani, 1984; Hirano et al., 1991; Okumura et al., 1999; Hyodo et al., 2003). This conclusion was based on evidence that (1) the incorporation of radiolabeled methionine or glutamate into ethylene was insignificant, (2) ethylene synthesis was little affected by aminoethoxyvinylglycine (AVG), a potent inhibitor of ACC synthase, and (3) ACC oxidase activity extracted from the root tissue infected by the pathogen was extremely low compared to the levels of ethylene produced in the tissue. This mechanism of ethylene synthesis seemed different from what is predominantly operating in higher plants and is observed in plant tissues attacked by pathogens (Boller, 1991; Ohtsubo et al., 1999). Hence, we have further investigated some characteristics of this ethylene-synthesizing system.

Inhibition by Diphenyleneiodonium

Ethylene synthesis in infected tissue is strongly suppressed by diphenyleneiodonium (DPI), thought to be an inhibitor of membrane NADPH oxidase (O'Donnell et al., 1993; Lamb and Dixon, 1997) (Figure 2). In the present experiment DPI was used at 1 mM. At a concentration much lower than 1 mM, DPI was still effective in suppressing ethylene synthesis. The results suggest that superoxide anion (O_2^-) formed by the reaction of NADPH oxidase is involved in ethylene synthesis.

Inhibition by Aristolochic Acid

Ethylene synthesis was severely suppressed by the application of 100 mM aristolochic acid, which is known to arrest phospholipase A_2 at the plasma membrane (Viehweger et al., 2002) (Figure 3). At a concentration of 50 mM or 10 mM the inhibition by aristolochic acid was still noteworthy. Phospholipase A_2 cleaves phospholipids to free fatty acids and 2-lysophospholipids (Wang et al., 2002). That the activity of phospholipase A_2 was required for ethylene synthesis suggests that polyunsaturated fatty acids such as linoleic or linolenic acid that are liberated from membrane phospholipids play an important role in ethylene synthesis.

Inhibition by Naproxen and Lipoxygenase Antibodies

In a previous paper we reported that phenidone and ibuprofen, both known to inhibit lipoxygenase activity (Cucurou et al., 1991; Ellis et al., 1993), were highly effective in suppressing ethylene synthesis in infected tissue (Hyodo et al., 2003). In the present study we used naproxen as an alternative inhibitor of lipoxygenase (Creelman et al., 1992; Kolomiets et al., 2001) and also

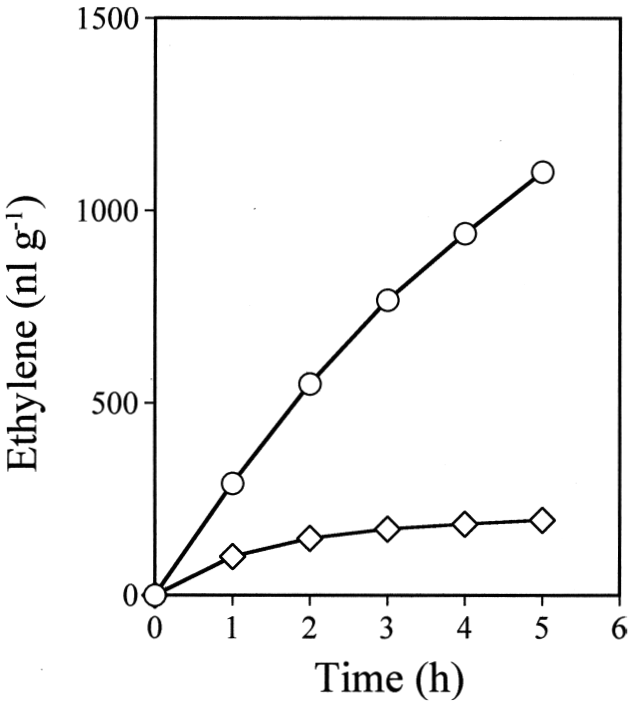

Figure 2. Effect of diphenyleneiodonium (DPI) (◇) on ethylene production in the first layer (0-0.5 mm) excised from sweet potato slices 1 day after inoculation. Twenty µl of 1 mM DPI in the buffer was applied to each disc. For control (○), the same volume of the buffer was given.

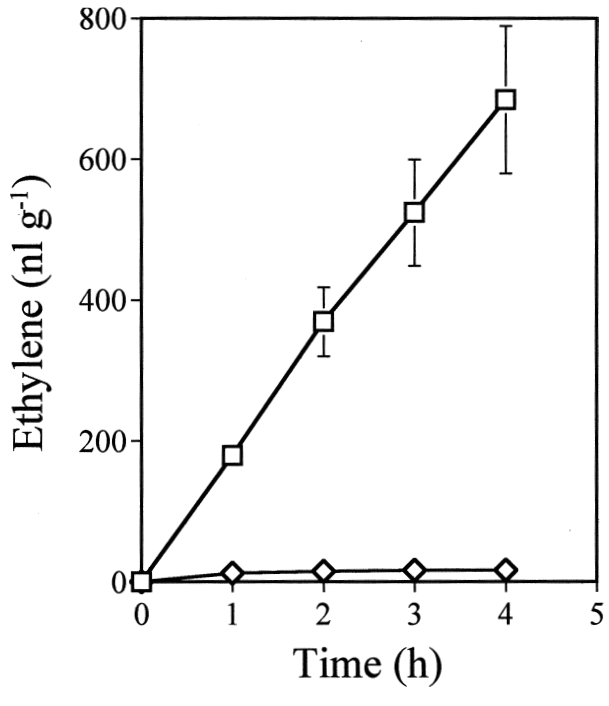

Figure 3. Effect of aristolochic acid (◇) on ethylene production in the first layer excised 1 day after inoculation. Twenty µl of 100 mM aristolochic acid in the buffer was added to each disc while the same volume of the buffer was added to control discs (□).

tested the inhibition by lipoxygenase antibodies to reconfirm whether lipoxygenase is involved in ethylene synthesis. The results shown in Figure 4 illustrate that naproxen at 100 mM totally suppressed ethylene production in discs removed from the first layer one day after inoculation. It also seems clear that polyclonal antibodies raised against soybean lipoxygenase rapidly reduced the rate of ethylene production in the discs when applied 1 h after the start of incubation (Figure 5). Here we tried to check the cross-reactivity by immunoglobulins other than the antibodies raised against soybean lipoxygenase. To this end we used polyclonal antibodies raised against broccoli ascorbate peroxidase prepared by Nishikawa et al. (2003). The results demonstrated that asorbate peroxidase antibodies exerted a substantial inhibition to ethylene synthesis in the infected tissue, although to a considerably smaller extent than lipoxygenase antibodies when compared at the same protein level (data not shown). This may indicate that immunoglobulins play a role in the inhibition at least partly in a non-specific manner such as free radical scavengers. These results taken together imply that lipoxygenase is closely involved in ethylene synthesis in infected tissue.

Lipoxygenase that contains non-heme iron catalyzes hydroperoxidation of polyunsaturated fatty acids with the addition of molecular oxygen to form hydroperoxy linoleic or linolenic acid (Hildebrand, 1989; Siedow, 1991; Porta and Rocha-Sosa, 2002). Lipoxygenase is committed to the initial step that leads to the synthesis of octadecanoid–derived signalling molecules (Schaller, 2001). We have examined the effect of lipoxygenase antibodies on wound-induced ethylene synthesis in mesocarp tissue of winter squash fruit (*Cucurbita maxima*), in which ethylene was synthesized through the methionine-ACC pathway (Hyodo et al., 1983; Kato et al., 2000). However, we failed to detect any rate of inhibition by the antibodies. This was consistent with the findings by Wang and Yang (1987) that the lipoxygenase system is not involved in ethylene production in vivo where ethylene synthesis is mediated by ACC.

Inhibition by N-Acetyl-L-Cysteine

Superoxide (O_2^-) generated by the reaction of NADPH oxidase is converted to H_2O_2 by superoxide dismutase, which is then converted to hydroxyl radical by the Harber-Weiss reaction or Fenton reaction in the presence of metal ions. We investigated the effect of N-acetyl-L-cysteine (NAC), which is reported to scavenge reactive oxygen species such as hydroxyl radicals (Aruoma et al., 1989; Green and Fluhr, 1995) on ethylene synthesis in the discs taken from the surface layer of slices infected by the fungus one day after inoculation. Results are illustrated in Figure 6 where ethylene production in the tissue was partially but significantly reduced by the addition of 100 mM NAC, suggesting that reactive oxygen species including hydroxyl radical (• OH) may be related to the mechanism of ethylene synthesis. Other chemicals thought to act as hydroxyl radial scavengers such as L-histidine (Schopfer, 2001) also partially inhibited ethylene synthesis (data not shown).

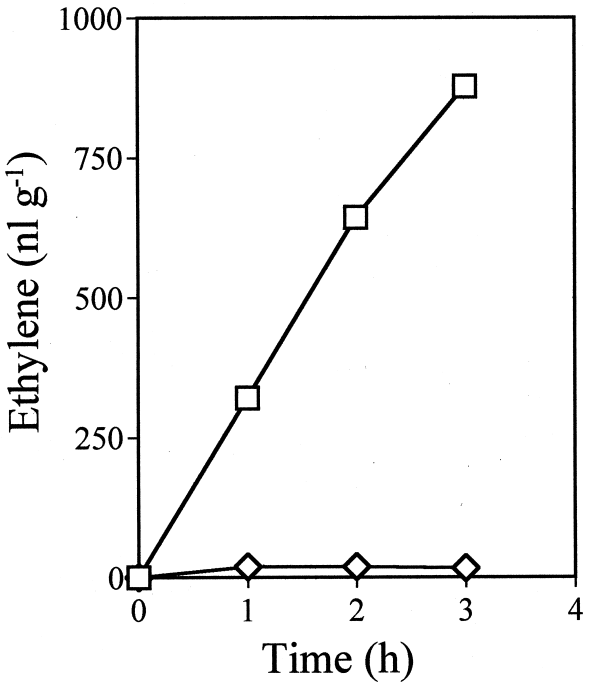

Figure 4. Effect of naproxen (◇) on ethylene production in the first layer excised 1 day after inoculation. Twenty µl of 100 mM naproxen was added to each disc. Control discs (□) were supplied with the same volume of the buffer.

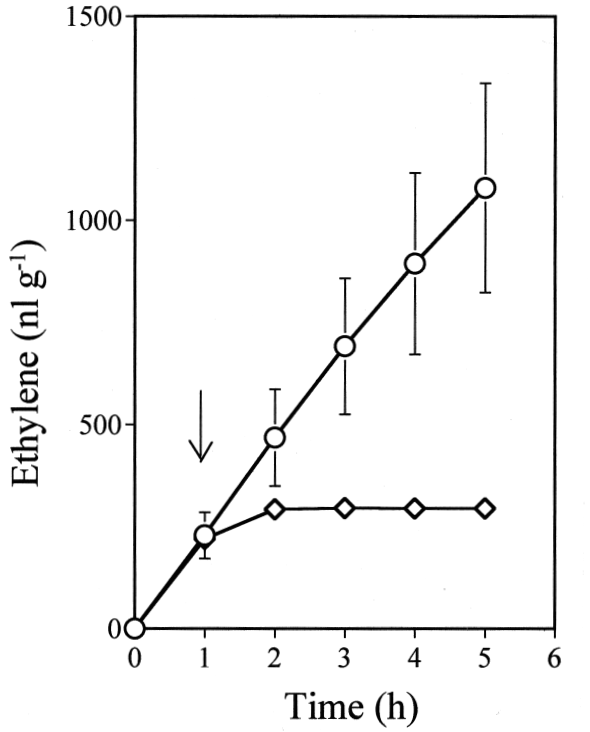

Figure 5. Effect of polyclonal antibodies raised against soybean lipoxygenase on ethylene production in discs of the first layer taken 1 day after inoculation. Antibodies in PBS were applied to each disc in 20 µl 1 h after the start of incubation as indicated by an arrow. (○), control discs; (◇), treated with antibodies (13.6 µg 20 µl^{-1}).

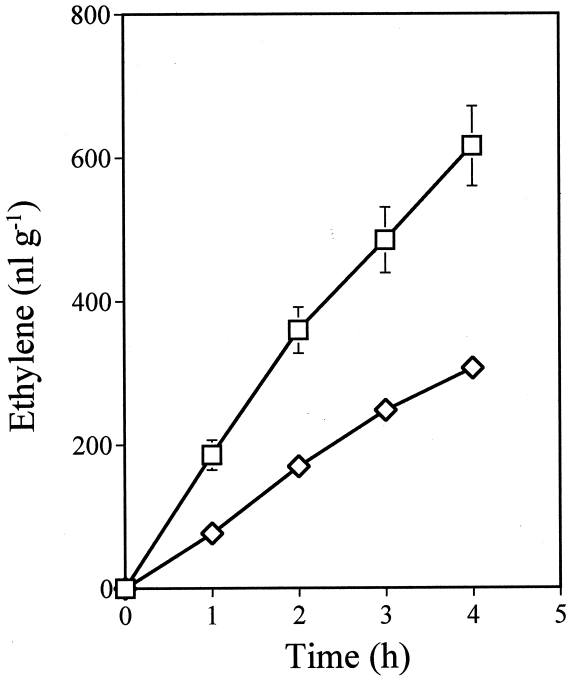

Figure 6. Effect of N-acetyl-L-cysteine (NAC) on ethylene production in the first layer excised 1 day after infection. Twenty µl of 100 mM NAC (◇) was added to each disc of the first layer, while control discs (□) were given the same volume of the buffer.

Inhibition by Metal Ion Chelators and its Restoration by Copper Ion

Ethylene synthesis in the infected tissue was suppressed in the presence of chelators of metal ions such as diethyldithiocarbamate (DIECA) and 1,10-phenanthroline (PA). As illustrated in Figures 7 and 8 ethylene evolution was inhibited by the addition of 10 mM DIECA or 5 mM PA, respectively, whose inhibition could be restored almost completely by the addition of 20 mM cupric ion. The ability of Cu^{2+} to reverse the inhibition could not be substituted by other metal cations such as Fe^{2+} or Zn^{2+} (data not shown). These results indicate that copper ion (or copper enzyme) may be closely associated with the mechanism.

Protein Synthesis is Required for Ethylene-Producing System

It was suggested that de novo protein synthesis was needed for maintaining ethylene synthesis in sweet potato root tissue infected by the pathogen. This was confirmed by the results of the present experiments, in which cycloheximide or puromycin was applied to the discs taken from the surface layer 1 day after inoculation (data not shown). These inhibitors were effective in suppressing ethylene biosynthesis in the infected tissue.

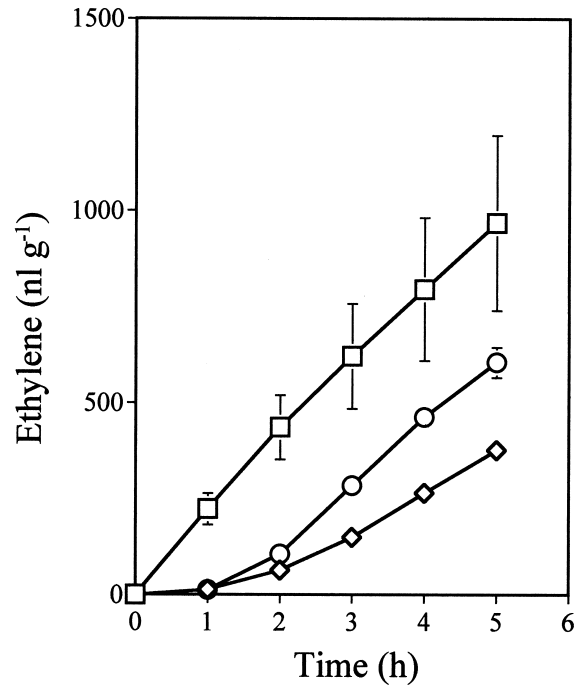

Figure 7. Inhibition of ethylene production by diethyldithiocarbamate (DIECA) and its recovery by cupric ion. Twenty µl of 10 mM DIECA in the buffer was applied to each disc excised from the first layer infected by the fungus. One h later another twenty µl of 20 mM $CuSO_4$ in the buffer was given to the discs, and measurement of ethylene production commenced. (□), Control; (◇), + DIECA; (○), + DIECA + $CuSO_4$.

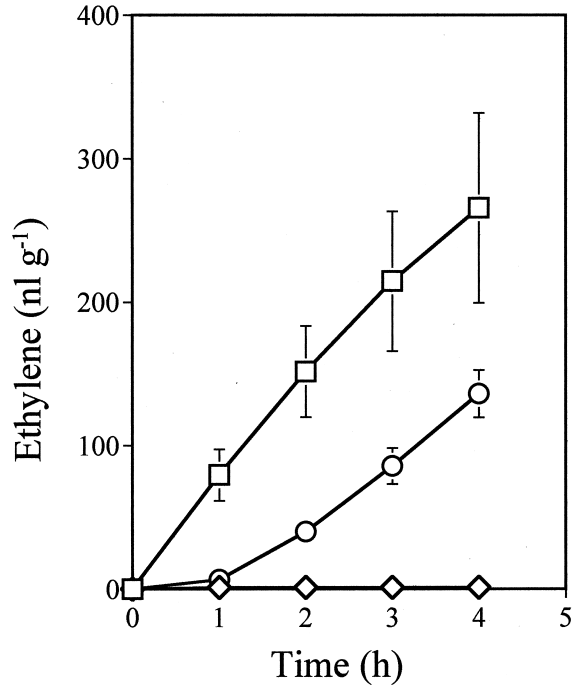

Figure 8. Inhibition of ethylene production by 1,10-phenanthroline (PA) and its restoration by cupric ion. Twenty µl of 5 mM PA in the buffer was applied to discs taken from the first layer 1 day after inoculation. One h later the same volume of 20 mM $CuSO_4$ in the buffer was provided to the discs, and measurement of ethylene production commenced. (□), control; (◇), + PA; (○), + PA + $CuSO_4$.

High rates of ethylene production are characteristic of events that occur in sweet potato roots in response to the infection by *C. fimbriata*, as well as increase in respiration, polyphenol synthesis, furanoterpenoid synthesis, and coumarin synthesis (Uritani, 1971). In sweet potato roots ethylene may enhance polyphenol synthesis (Imaseki et al., 1968b) through the activation of metabolic changes. The enhanced levels of phenolics and their oxidized products could be associated with forming necrotic lesions of sweet potato root cells challenged by the black rot fungus. Induction of the ethylene-synthesizing system with a rapid turnover rate appears to be an early event that underlies symptoms characteristic of host responses coping with the invading fungus.

Our study has suggested that ethylene produced in the diseased sweet potato root tissue can be formed from the peroxidation of polyunsaturated fatty acids and by the functions of copper ion (copper enzyme) and free radicals. As yet, the ethylene-synthesizing system has not been isolated from the diseased tissue for in vitro studies. Lieberman and Mapson (1964) demonstrated that activated linolenic acid was decomposed to give rise to ethylene by the catalytic activities of cupric ion and ascorbate in a chemical model system. Dumelin and Tappel (1977) described ethylene as being generated by the copper-catalyzed decomposition of hydroperoxides of methyl linolenate. The major hydrocarbon gas product that evolved from the ω-3 fatty acid by the decomposition of fatty acid hydroperoxides was ethylene or ethane (Dumelin and Tappel, 1977). The role of cupric ion (Cu^{2+}) was considered to facilitate the reaction of electron transfer oxidation of ethyl radicals as an electron acceptor to form ethylene and cuprous ion (Kochi, 1967). The mechanism of ethylene formation from decomposition of the 16-hydroperoxide of linolenic acid was discussed by Peiser and Yang (1978). The 16-alkoxy radical formed by the reaction with bisulfite would undergo cleavage to form ethylene mediated by the production of ethyl radical. In the present ethylene-forming system there arises a possibility that ethylene is generated by the cleavage of hydroperoxides of polyunsaturated fatty acids and that copper ion and superoxide or hydroxyl radical participate in this mechanism. As yet, the in vitro cell-free system remains to be studied to elucidate the pathway more precisely.

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黑腐菌（*Ceratoystis fimbriata*）感染甘藷塊根組織以誘導乙烯生合成

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甘藷（*Ipomoea batatas* Lam.）塊根組織以黑腐菌（*Ceratoystis fimbriata* Ell.& Halst）感染可以快速增加乙烯的產生。在甘藷塊根表皮層（0-0.5 mm）以 *C. fimbriata* 接種，在接種和斜面培養之後，乙烯的增加可以在一天達到最多。在塊根表皮層增加後隨後增加的是內層（0.5- 1.0 mm）和第三層（1.0-1.5 mm）如同真菌穿透生長。從被感染的塊根表皮切片切出直徑 10 mm 和 0.5 mm 厚的圓盤作為實驗所需。在我們的先前的研究中指出乙烯合成的主要來源是在感染的組織中，可能的來源是來自獨立的甲硫胺酸氨基環丙烷羧酸（1-aminocyclopropane-1-carboxylic acid）路徑。可以藉由抑制干擾 NADPH 氧化酶，磷酯酶 A_2，脂肪氧合酶，藉由金屬螯合和藉由清除有活性的氧化態自由基，使乙烯的合成在被感染的組織中有顯著的降低。金屬離子的抑制可藉由加入銅離子來恢復。這個結果指出在感染甘藷塊根組織中會從細胞膜的磷脂中釋放多不飽和脂肪酸，而後跟隨著是脂肪氧合酶的過氧化作用。氫過氧化物成分可以被分解，當有銅離子或含有銅的酵素和有活性的氧化態物質如羥基自由基時導致乙烯的合成。由真菌感染而誘導出乙烯合成的反應需要新的蛋白質合成。

關鍵詞：黑腐菌；銅離子；乙烯生合成；感染組織；甘藷；脂肪氧合酶；細胞膜磷脂；活性的氧化態自由基；不飽和脂肪酸。