The Effect of Methionine on Ethylene and 1-Aminocyclopropane-1-Carboxylic Acid Production by *Bipolaris sorokiniana*

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ABSTRACT

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Research was initiated to determine the effect of exogenous methionine on the ability of the fungal pathogen *Bipolaris sorokiniana* to produce ethylene and intermediates of the ethylene biosynthetic pathway of higher plants. When grown on leaf-blade infusion media of the host (*Poa pratensis*) supplemented with methionine, the pathogen produced ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) was secreted into culture filtrates, and the protein fraction of the pathogen had low ACC synthase activity. On the leaf-blade infusion media without methionine, ethylene production was negligible to nondetectable, the initial level of ACC in the leaf-blade infusion media was decreased, and ACC synthase activity of the protein fraction of the pathogen was high. Addition of ACC to the leaf-blade infusion media resulted in low ethylene production by the pathogen compared with that produced with the addition of methionine. Differences in the use of ACC and methionine for ethylene production by the pathogen suggests that the pathogen does not efficiently convert ACC to ethylene and that ethylene may be produced via more than one pathway. The low ACC synthase activity of the protein fraction of the pathogen after 10 days' growth on leaf-blade infusion media with methionine suggests that methionine or a metabolite of methionine may decrease the production of ACC synthase.

Additional key words: *Drechslera sorokiniana*, *Helminthosporium sativum*, Kentucky bluegrass, leaf spot.

Bipolaris sorokiniana (Sacc. & Sorok.) Shoem. is a serious pathogen of numerous grass species. Leaves of *Poa pratensis* L. infected by this pathogen often produce a leaf spot that is characterized by a necrotic lesion surrounded by a chlorotic halo (9). As the disease progresses, symptoms are characterized by midvein chlorotic streaking interconnecting lesions and, eventually, complete chlorosis of all leaf tissue not directly infected. The midvein and complete chlorosis is suggestive of premature senescence and the involvement of ethylene. Recent studies have established an increase in endogenous ethylene in leaves of *P. pratensis* infected by *B. sorokiniana* (9). The maximum increase in endogenous ethylene precedes the appearance of midvein and general chlorosis of infected leaves and then decreases. The order of the ethylene and chlorosis responses suggests that ethylene may be responsible for the chlorosis. This potential relationship has been further established by sharply decreasing the chlorosis when the disease is permitted to develop on leaves of intact plants grown under hypobaric conditions in a controlled atmospheric-environmental system (9,14).

Ethylene has long been known to be a factor in pathogenesis (15) and the evidence accumulated to date clearly involves ethylene in the chlorosis of *P. pratensis* leaves infected by *B. sorokiniana*. The source of the increase in endogenous ethylene in *P. pratensis* infected by *B. sorokiniana* is unknown. It seems to be the general consensus that in most host-pathogen interactions where ethylene is produced, the ethylene is primarily of host origin (8,15). Production of ethylene by some fungi is well documented (10) and the fungal plant pathogens *Drechslera graminea* and *Cylindrocladium floridanum* Sobers & Seymour produce ethylene when grown in the presence of methionine (2,17).

The biosynthesis of ethylene in higher plants requires methionine as a precursor (1,13,19) and occurs via the following pathway: methionine → S-adenosylmethionine (SAM) → ACC → ethylene (1). This pathway is not believed to occur in fungal organisms and several alternative pathways for ethylene in microorganisms have been proposed (7,11,12,19). Some fungal pathogens, however, produce ethylene in the presence of methionine (2,3,5,17,18). Methionine metabolites also seem to be associated with enzymatic and nonenzymatic production of ethylene by culture filtrates (3,5). Our studies were initiated to determine the effect of methionine on ethylene production by *B. sorokiniana* on a substrate derived from host tissue, and to assay the mycelium and culture filtrates for intermediates (ACC synthase and ACC) common to the biosynthetic pathway of ethylene in higher plants in an effort to determine whether this pathogen might contribute ethylene directly or indirectly to the host-pathogen interaction during pathogenesis.

MATERIALS AND METHODS

Effect of methionine on ethylene production by *B. sorokiniana*. The pathogen was grown in static culture at 23 C in glass culture vials (18 ml total volume). Treatments consisted of all combinations of leaf tissue (75 mg fresh weight of leaf disks of *P. pratensis* cut with a #2 cork borer), 5 ml of 1 mM methionine (distilled H_2O control), and *B. sorokiniana* (on 2 disks of 3% bacto-agar cut with a #2 cork borer from stock cultures in 100- × 15-mm plastic petri dishes). The leaf disks and methionine or water were placed in the vials, stoppered with Styrofoam plugs, autoclaved for 30 min, and cooled before aseptic introduction of the fungus (Day 0).

Culture vials were alternately stoppered with Styrofoam plugs during the 12-hr day cycle and with serum caps for gas accumulation during the 12-hr night cycle. Ethylene production

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was determined every 24 hr for 9 days by injecting a 1-ml sample of the headspace gas from the culture vials into a gas chromatograph [injector 150 C, column 155 C, flame ionization detector (FID 250 C)]. The signal of the FID was fed to a Carey 401 electrometer coupled to a Spectra-Physics 4100 computing integrator. Subsequent to calibration, sample data were plotted and quantified by the computing integrator. Ethylene was expressed in picomoles. Each treatment was repeated three times with two analytical replications each. Controls consisted of sampling the internal atmosphere of empty vials and the atmosphere of vials containing only the individual components of the culturing system (i.e., methionine solutions, leaf disks, and pathogen). Standard error of the mean was determined.

Effects of varying methionine and ACC concentrations on ethylene production by *B. sorokiniana*. The pathogen was grown on 0.1, 1.0, and 10.0 mM solutions of methionine or ACC (Sigma Chemical Co.) with 75 mg of grass leaf disks in vials as previously described. Ethylene production was measured each day and the study was replicated six times.

ACC synthase determination. Fifty cultures of *B. sorokiniana* were grown 10 days in 125-ml Erlenmeyer flasks on 100 ml of *P. pratensis* leaf-blade infusion media with and without 1 mM methionine. The leaf-blade infusion was prepared by boiling 150 g of fresh weight of shredded grass leaves in 2 L of distilled water for 1 hr, after which the leaves were removed with cheesecloth and the remaining infusion was diluted to 10 L. The infusion with and without methionine was autoclaved for 30 min before introduction of the fungus. Styrofoam stoppers were placed in the flasks for the first 9.5 days of growth to provide aeration. The Styrofoam stoppers of six flasks were replaced with septa for the last 12 hr of the study to trap ethylene. The experiment was replicated three times and each treatment (± methionine) had two analytical replications. Different batches of the fungus showed different levels of enzyme activity, but the overall trends due to the presence or absence of methionine in the medium were the same. A representative determination is presented.

Fungal tissue collected from each flask after 10 days' growth was homogenized in 50 ml of ice-cold grinding buffer (50 mM potassium phosphate, pH 7.2, 5% $(NH_4)_2SO_4$ (w/v), 4 mM dithiothreitol, 5 μM pyridoxal phosphate) with 5 g of grinding sand (sea sand:Carborundum 80 grit, 2:1, w/w) for 30 min. The suspension was centrifuged at 10,000 *g* for 10 min and the pellet discarded. The supernatant was brought to 90% saturation with $(NH_4)_2SO_4$ and allowed to stand for 1 hr. The protein suspension was centrifuged at 10,000 *g* for 10 min and the pellet was redissolved in dialysis buffer (10 mM potassium phosphate, pH 7, 0.1 mM dithiothreitol, 2 μM pyridoxal phosphate) and dialyzed against five changes of dialysis buffer. After dialysis, the $(NH_4)_2SO_4$ precipitated protein fraction was centrifuged at 10,000 *g* for 10 min to clarify the supernatant.

The supernatant was immediately assayed for ACC synthase activity by incubating 500 μl of the protein fraction with 100 μl of a reaction mixture containing buffered SAM (Sigma Chemical Co.) for 4 hr at 30 C. The final amounts of the reaction mixture components were 600 nmol of SAM and 40 μmol of 4-(2-hydroxyethyl)-piperazine-propanesulfonic acid (EPPS), pH 8.5. ACC produced during incubation of fungal protein from cultures with and without methionine and in the presence and absence of the substrate was determined by a previously described method (20). The assay also was conducted with denatured (boiled) protein.

Determination of ACC content of mycelium and culture filtrates by alkaline degradation. Cultures of *B. sorokiniana* were grown in 125-ml Erlenmeyer flasks on 100 ml of *P. pratensis* leaf-blade infusion with and without 1 mM methionine as previously described. Each treatment (± methionine) consisted of a flask from which mycelium was collected on a 60-μm sieve after 6, 7, 8, 9, and 10 days' growth. Each treatment was replicated six times. The mycelium (1–2 g) was homogenized with 2 ml of distilled water and centrifuged at 10,000 *g* for 10 min. The supernatant was adjusted to 3 ml with distilled H_2O and assayed for ACC as previously described. ACC content of mycelium was expressed as picomoles of ACC per gram of fresh weight.

Culture filtrate (90–100 ml) was concentrated to 2 ml under reduced pressure at 35 C, centrifuged at 10,000 *g* for 10 min, and then diluted to 5 ml with distilled water. The ACC content of the filtrate was assayed for ACC (20) and expressed as picomoles of ACC per milliliter.

Determination of ACC content of mycelium and culture filtrates by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). Mycelium (12 g) of *B. sorokiniana* from 10-day-old cultures grown on 100 ml of leaf-blade infusion was homogenized in water. The homogenate was extracted three times with 20 ml of water. Extracts were concentrated to 1 ml under reduced pressure at 50 C and 100 μl of the concentrate was applied directly to TLC plates. Culture filtrate of *B. sorokiniana* (600 ml) also was concentrated to 1 ml under reduced pressure. Extracts of the mycelium or culture filtrate were treated with 100 μl of 10% trichloracetic acid and centrifuged at 30,000 *g* for 30 min. The supernatant was collected and the components separated by TLC following procedures of Boller et al (4), except that each plate was developed twice to improve resolution. Samples were applied to cellulose plates as either spots or streaks, and sample components were separated after two developments using 1-butanol, acetone, diethylamine, and water (30:30:6:15) (4). The run time was about 2 hr for each development. The plates were air-dried and sprayed with 0.1% ninhydrin in 95% ethanol. Samples to be harvested for HPLC analysis were streaked between ACC standards placed at the edge of the cellulose plates. These plates were sprayed with ninhydrin only along the margin that contained the ACC standards. The sample region between the ACC standards was cut out with a razor blade and extracted three times with water, concentrated to 1 ml under decreased pressure, and stored frozen for HPLC analysis. Samples were derivatized according to the PICO-TAG procedure (Waters Associates, Milford, MA) and analyzed using a reverse-phase micro-Bondapak column and a Waters HPLC. Standard amino acids and standard ACC were analyzed with the samples. The retention times for standard and sample components were stable.

RESULTS

Effect of methionine on ethylene production by *B. sorokiniana*. Incubation of various combinations of 1.0 mM methionine, leaf disks of *P. pratensis*, and the pathogen *B. sorokiniana* suggest that the pathogen can use methionine to produce ethylene. Ethylene production by *B. sorokiniana* grown on leaf disks in the absence of exogenous methionine was negligible to nondetectable over the 9-day observation period (Table 1). When the pathogen was grown on leaf disks in the presence of 1.0 mM methionine, ethylene production was substantially increased (Table 1); peak ethylene production occurred 4 days after the pathogen was placed on the methionine-supplemented leaf disk medium. The pathogen was unable to use methionine as its sole source of carbon in the absence of leaf disks; ethylene production was negligible or nondetectable.

TABLE 1. Ethylene produced by *Bipolaris sorokiniana* on *Poa pratensis* leaf-blade disks in the presence or absence of 1 mM methionine (Met)

Treatment combinations			Ethylene (pmol)[b] at day								
Met (+) or (−)	Leaf disks[a] (+) or (−)	*B. sorokiniana* (+) or (−)	1	2	3	4	5	6	7	8	9
−	−	−	0	0	0	5	0	0	7	0	1
−	−	+	0	0	0	5	0	0	2	0	0
−	+	−	0	0	0	6	3	5	7	8	1
−	+	+	0	2	3	5	2	0	1	0	0
+	−	−	0	0	0	4	0	0	0	0	0
+	−	+	0	0	0	2	0	0	9	0	0
+	+	−	3	2	4	9	8	12	15	15	17
+	+	+	1	14	206	251	84	39	34	20	16

[a]Tissue contained about 17 nmol/mg of methionine.
[b]Zeros indicate ethylene not detected; detection limit = 1 pmol of ethylene. Standard error of the mean = 11 pmol ethylene.

Ethylene production from the individual components of the culturing system (i.e., methionine, leaf disks, or pathogen alone in the vials) was negligible or nondetectable (Table 1).

Effect of varying concentrations of methionine and ACC on ethylene production by *B. sorokiniana*. The addition of methionine or ACC in the range of 0.1, 1.0, and 10.0 mM to vial cultures of *B. sorokiniana* growing on leaf disks of *P. pratensis* resulted in different levels of ethylene production by the pathogen (Fig. 1A and B). Methionine at 0.1 mM stimulated a low and brief period of ethylene production. Greater levels of methionine (1 and 10 mM) resulted in greater ethylene production, which peaked on day 4 and gradually declined over the observation period (Fig. 1A). In contrast, 0.1 and 1.0 mM ACC had very little effect on ethylene production and 10 mM ACC stimulated ethylene production to about 20% of the level observed with methionine. Peak ACC-induced ethylene production by *B. sorokiniana* occurred 2 days later than that observed with methionine (6 days of fungal growth) and did not diminish to the extent methionine-induced ethylene production did during the experiment (Fig. 1B).

TABLE 2. ACC synthase activity of *Bipolaris sorokiniana* mycelium cultured for 10 days on *Poa pratensis* leaf-blade infusion with or without the addition of 1 mM methionine (Met)

Culture media[a] (+) or (−) Met	Protein	C_2H_4[b] (pmol)	ACC[c] (pmol)	Corrected ACC[d] (pmol)	Specific activity[e] (pmol $4h^{-1}$ mg $protein^{-1}$)
−	Fungal	6	568	455	1,311
−	Denatured fungal		113	0	0
+	Fungal	1,160	186	107	267
+	Denatured fungal		79	0	0

[a] Cultures of *B. sorokiniana* grown for 10 days on leaf-blade infusion with or without the addition of 1 mM methionine. On day 10, cultures were assayed for ethylene production and harvested, and the protein fraction extracted and assayed for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity.

[b] Ethylene determined in the headspace of the 125-ml culture flask by trapping during the last 12 hr of the study. The standard deviation for cultures with and without methionine was 223 and 5 pmol of ethylene, respectively.

[c] pmol of ACC detected after 4 hr of incubation of the protein fraction with buffered substrate (600 nmol of S-adenosylmethionine and 40 μmol of 4-(2-hydroxyethyl)-1-piperazine-propanesulfonic acid, pH 8.5) at 30 C.

[d] ACC produced by the active protein fraction minus ACC detected in the denatured protein fraction. The standard deviation for cultures without methionine was 28 pmol of ACC in the active fraction and 41 pmol of ACC in the denatured protein fraction. The standard deviation for cultures with methionine was 34 pmol of ACC in the active fraction and 46 pmol of ACC in the denatured protein fraction.

[e] Protein amount was 0.347 mg for mycelium grown without methionine and 0.401 mg for mycelium grown with 1 mM methionine.

ACC synthase activity of *B. sorokiniana*. SAM was converted to ACC by a fungal protein fraction from cultures of *B. sorokiniana* grown with or without 1 mM methionine (Table 2). ACC synthase activity was five times higher in 10-day-old fungal cultures grown without methionine than those cultured in the presence of methionine. The enzyme activity was unstable to heat and was sharply decreased by boiling. Control blanks containing only SAM were not degraded to ethylene by alkaline degradation.

ACC content of mycelium and culture filtrates of *B. sorokiniana*. Mycelium of *B. sorokiniana* and its culture filtrates assayed by alkaline degradation contained ACC. The level of ACC in mycelium varied during the growth of the fungus and was not consistently affected by the presence or absence of 1 mM methionine in the medium (Fig. 2A).

Culture filtrate assayed by alkaline degradation for ACC content over the 6- to 10-day growing period showed an increase in

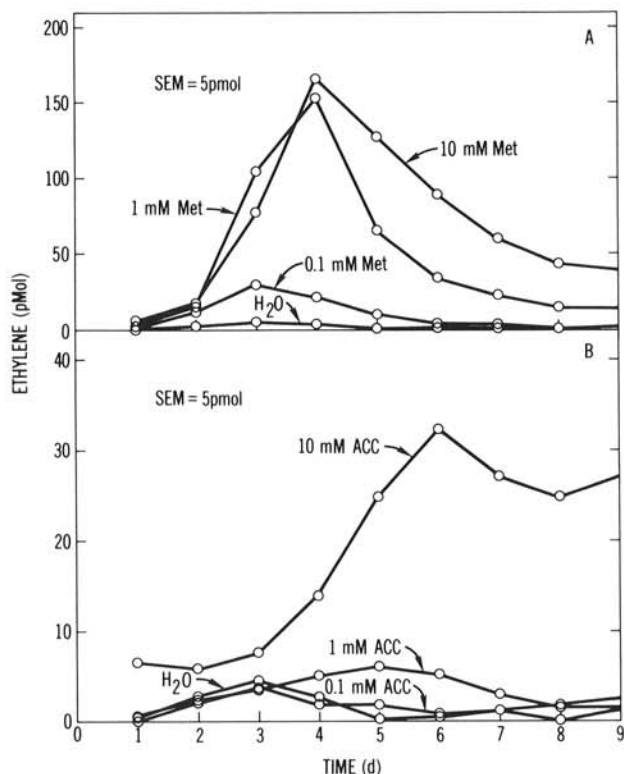

Fig. 1. The effect of various concentrations of methionine (Met) **(A)** and 1-aminocyclopropane-1-carboxylic acid (ACC) **(B)** on ethylene production by *Bipolaris sorokiniana*. SEM = standard error of the mean.

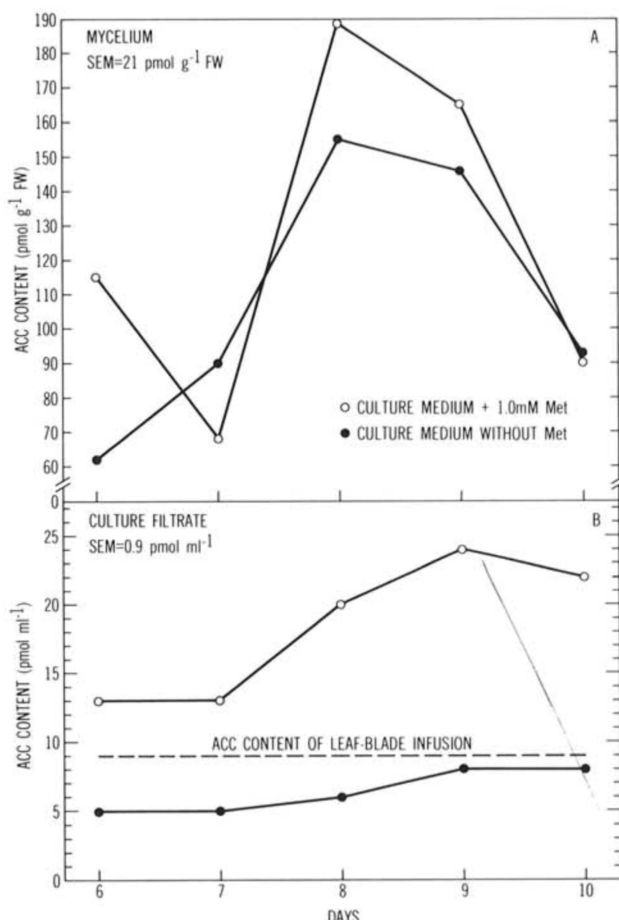

Fig. 2. The effect of methionine (Met) on 1-aminocyclopropane-1-carboxylic acid (ACC) production by mycelium **(A)** of *Bipolaris sorokiniana* and on secretion of 1-aminocyclopropane-1-carboxylic acid (ACC) into the culture filtrate **(B)**. SEM = standard error of the mean. FW = fresh weight.

ACC content over the initial ACC level (9 pmol ACC ml^{-1}) in the leaf-blade infusion medium when methionine was included in the medium (Fig. 2B). When the fungus was cultured on the medium without methionine over the same period the ACC content was 10–45% lower than the initial ACC content of the grass leaf-blade infusion.

Pure ACC analyzed by HPLC had the same retention time as threonine (4.9 min) and was close to alanine (5.06 min) and arginine (5.13 min). TLC analysis using one development was not sufficient to resolve ACC from threonine or alanine. TLC chromatograms subsequently developed twice provided adequate separation of ACC from threonine and arginine (Table 3). A small amount of alanine contamination in the ACC band probably occurred, but conditions of the HPLC analysis were adequate to ensure complete resolution of ACC from alanine (Table 3). Samples from *B. sorokiniana* mycelium and its culture filtrate contained a ninhydrin positive component that comigrated with ACC and yielded ethylene upon alkaline degradation. Subsequent HPLC analysis of this region demonstrated the presence of ACC and alanine from mycelium and culture filtrates of *B. sorokiniana*.

TABLE 3. Separation of 1-aminocyclopropane-1-carboxylic acid (ACC), alanine (Ala), threonine (Thr), arginine (Arg), and methionine (Met) by TLC and HPLC

Standard amino acid	TLC[a] Rf	HPLC[b] Rf
Thr	0.80	0.416
Met	0.75–0.80	0.598
ACC	0.62–0.64	0.416
Ala	0.52	0.429
Arg	0.09	0.403

[a] TLC done according to Boller et al (4), except that the chromatograms were developed twice.

[b] HPLC analysis done using the PICO-TAG procedure (Waters Associates, Milford, MA).

DISCUSSION

It is well established that some fungal organisms need or can use methionine for the production of ethylene, but not via the ACC synthase, ACC, ethylene pathway (2,3,5,16). Several pathways have been proposed for the use of methionine in the biosynthesis of ethylene by fungi, but none of the pathways has been conclusively established (16,19), and species differences seem probable.

The observation in this study that *B. sorokiniana* can produce ACC is of special interest because ACC is known only to be an intermediate of ethylene biosynthesis in higher plants. ACC is present in *B. sorokiniana* mycelium (Fig. 2A), but is not efficiently converted to ethylene (Fig. 1B). If uptake of exogenous ACC is not restricted compared with methionine, then its subsequent metabolism to ethylene seems limited. Ethylene production from ACC exhibits several differences when compared with methionine-dependent ethylene production (lower total conversion, delayed and relatively undiminished production) (Fig. 1A and B). These differences suggest that this fungal pathogen may produce ethylene by more than one pathway.

Grass-leaf infusion media prepared from *P. pratensis* had an initial ACC content of 9 pmol ml^{-1}. When the pathogen was grown on the media supplemented with 1 mM methionine, assays indicated a secretion of ACC by the fungus into the culture filtrate (Fig. 2B). ACC content of mycelium, however, was not influenced by methionine. In contrast, when the fungus was cultured on grass-leaf infusion without supplemental methionine, assays of the culture filtrate showed a decrease in the initial ACC content of the grass-leaf infusion media (Fig. 2B). This observation suggests that the pathogen may metabolize ACC on low-nutrient media without production of detectable levels of ethylene.

ACC synthase activity was detected in the protein fraction of *B. sorokiniana* after 10 days' growth on grass leaf-blade infusion with or without 1 mM methionine (Table 2). Enzyme activity was highest in those cultures grown without supplemental methionine and that produced little ethylene. Addition of methionine to the medium resulted in increased ethylene production, but at the time of the assay, after 10 days' growth, ACC synthase activity was low. Grass-leaf tissue contains methionine (about 17 nmol/mg dry weight) and if available to the fungus may be sufficient to induce the production of ACC synthase. When methionine is present at much higher levels (1 mM supplemented to the medium) it seems possible that either methionine or some metabolite of methionine may decrease the production of ACC synthase. Future analysis of the time-course of enzyme activity should help to clarify this point.

Observations that methionine can be used by *B. sorokiniana* to produce ethylene and ACC and that ACC synthase activity is present provide a basis for future research. The detection of ACC and ACC synthase activity suggests that elements of the biosynthetic pathway of ethylene in higher plants may exist in this fungal pathogen. The ability of the pathogen to produce ethylene and ACC from methionine, combined with its inability to efficiently convert ACC to ethylene, could have significant implications in pathogenesis of the host-pathogen interaction. Ethylene produced in most host-pathogen interactions is generally believed to be of host origin (8,15) and there is evidence that ethylene is, in part, responsible for the midvein and general chlorosis of *P. pratensis* leaves infected by *B. sorokiniana* (9). On the basis of the present observations, it is possible that *B. sorokiniana* may directly or indirectly contribute to the increase in endogenous ethylene of this host-pathogen interaction (9). Such phenomena closely approximate a recently formulated model (6) whereby the attack by a fungal pathogen may result in formation of ACC (perhaps of host and/or pathogen origin in the case of the present observations) that is metabolized to ethylene by the host.

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