

Ethylene production by *Alternaria alternata* and its association with virulence on inoculated grape berries

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Abstract Ethylene has been shown to promote spore germination and hyphal growth in the phytopathogenic fungus *Alternaria alternata*. However, little is known about the ethylene biosynthetic pathway in this fungus. In the present study, the ethylene biosynthetic pathway in *A. alternata* was investigated to explore ethylene-associated virulence of this fungus. The strain A0 of *A. alternata* did not produce ethylene on basal medium with different possible precursors or intermediates for ethylene biosynthesis (glutamate, aspartate, 2-oxoglutarate and 1-aminocyclopropano-1-carboxylic acid). However, ethylene production was observed when methionine was added as a precursor to the medium and was further promoted by continuous light illumination. Furthermore, addition of 2-keto-4-methylthiobutyric acid (KMBA) promoted ethylene production in the absence of methionine, indicating that the KMBA pathway was mainly responsible for ethylene biosynthesis in this fungus. The strain A0 was inoculated into grape berries to examine the effect of ethylene production on its virulence (as assessed by lesion formation at the inoculation site). The results indicated that higher ethylene production caused larger lesion formation. Similar results were also obtained when isolates of *A. alternata*, obtained from infected grapes, were inoculated. Thus, the present study thus demonstrated that *A. alternata* produces ethylene via the

KMBA pathway and utilizes it for enhanced virulence expression during infection.

Keywords Ethylene biosynthetic pathway · KMBA pathway · *Alternaria*

Introduction

Alternaria alternata is a ubiquitous filamentous pathogen that causes black rot in wide varieties of fruits and vegetables at postharvest level (Ma et al. 2009). More importantly, this species is not only causing serious losses to plant crops, but also posing threat to human health as airborne allergens (Nguyen et al. 2016; Thomma 2003). The major aim of our research was to elucidate various biotic and abiotic factors affecting virulence of *A. alternata* in order to establish an efficient postharvest pathogen control for major agricultural crops.

Many microbial plant pathogens can produce ethylene as a biotic signaling factor (Iiag and Curtis 1968), which has been shown to play an important role in microbe-plant interactions (Fukuda et al. 1993). Ethylene production in *Pseudomonas syringae* is associated with its pathogenicity (Weingart et al. 2001). In higher plants, ethylene is produced from methionine via the 1-aminocyclopropane-1-carboxylic acid (ACC) pathway (Adams and Yang 1981; Johnson and Ecker 1998). However, in microorganisms the ACC pathway is not commonly found, and two other pathways, the 2-keto-4-methylbutyric acid (KMBA) and 2-oxoglutarate, seem to be mainly responsible for ethylene biosynthesis

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(Chague et al. 2006). Ethylene plays a dual role in fungus-plant interactions, both as a virulence factor in pathogens and as a signaling compound in disease resistance in higher plants (Chague et al. 2006).

Ortuno et al. (2008) demonstrated that application of ACC or ethephon to citrus fruit tissues inoculated with *A. alternata* results in accelerated disease development. Furthermore, Kepczynska (1994) reported that endogenous ethylene promotes spore germination and hyphal growth in *A. alternata*. Despite these interesting findings, the mechanism of ethylene biosynthesis by *A. alternata* has not yet been determined, and therefore we focused on the ability of this fungus to produce ethylene. In the present study, we examined the ability of *A. alternata* to produce ethylene, using a verified strain of this fungal species and elucidate the main ethylene biosynthetic pathway in this fungus. In addition, we analyzed the crucial role of ethylene production in virulence by this fungus upon inoculation into host grape berries. On the basis of the results obtained in present study using natural isolates of *A. alternata* from infected grapes, we propose that ethylene-mediated expression of virulence is important in disease development.

Materials and methods

Fungal cultures and growth conditions

The strain A0 (ACCC310001) of *A. alternata* was purchased from the China General Microbiological Culture Collection Center. Four isolates (A4, A5, A8, and A10) of *A. alternata* were isolated from infected grapes and cultured on potato dextrose agar (PDA) medium containing 200 mg/l streptomycin at 25 °C. To prepare conidial suspensions, the mycelia were removed from agar plates by gently brushing the plate surfaces with a sterile platinum loop and were then suspended in distilled water. The mycelia were removed from the suspension culture by filtration with four layers of gauze to separate the conidia. Density of the conidia obtained was determined using a hemocytometer.

Determination of *in vitro* ethylene production by *A. alternata*

To determine the ethylene producing ability of *A. alternata*, 10 day-old single spore-derived mycelial cultures of the strain A0 were used. The cultures were

grown in a 25-ml flask containing 15 ml of potato dextrose broth (PDB) medium supplemented with either one of the possible precursors (10 mM L-methionine, L-aspartate, L-glutamate and L-arginine) or intermediates (10 mM 2-oxoglutarate, ACC and KMBA) for ethylene production. Specifically, each flask was inoculated with A0 conidia (at a final density of 10^5 spores/ml) and shaken at 150 rpm at 25 °C for definite periods and was either illuminated with white fluorescent light ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) or incubated in the dark. For ethylene measurement, flasks were sealed with a rubber stopper and left for 4 h. To determine the levels of ethylene produced, gas sample (1 ml) of each flask was aspirated with a syringe and injected into a Varian GC9800 gas chromatograph. Data were expressed as nanoliters of ethylene produced per hour.

Detection of KMBA and assay for conversion to ethylene

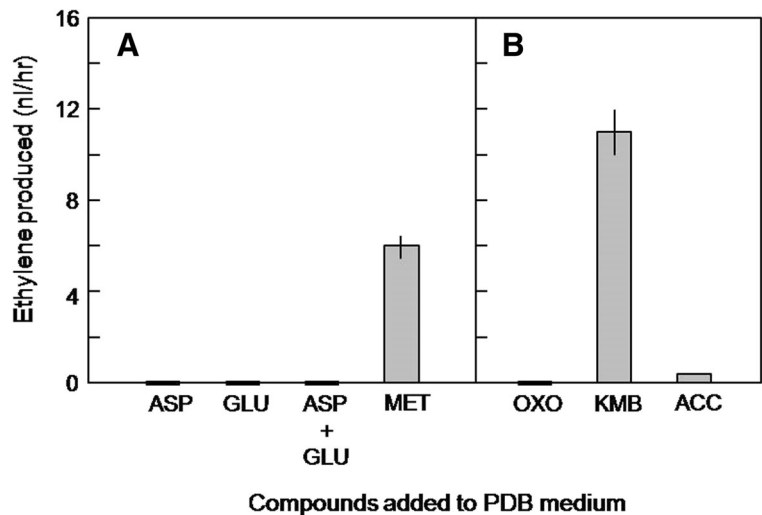
The presence of KMBA was determined by precipitating it with 4-dinitrophenylhydrazine (Primrose 1977). Briefly, the mycelia were eliminated by filtration and the culture filtrate (10 ml) was mixed with 1 ml of 0.1% 2, 4-dinitrophenylhydrazine in 2 M HCl. The mixture was stirred at room temperature in the dark. After 30 min, appearance of yellow precipitate confirmed the presence of KMBA.

The conversion of KMBA to ethylene was examined as described previously (Chague et al. 2002). Mycelia were grown in the dark in PDB supplemented with 10 mM methionine for 24 h. The mycelia were separated from the culture solution by filtration, washed with fresh PDB (without methionine) and put into a flask containing fresh PDB. The washed mycelia and original culture filtrate were incubated separately for an hour under light illumination. Production of KMBA and ethylene was examined by the above-mentioned method.

Inoculation assay

Agar discs about 5 mm in diameter containing the mycelia of strain A0 and four isolates were excised from seven day-old cultures on PDA and placed at the needle-wounded site of grape berries (*Vitis vinifera* cv. 'Thompson Seedless'). The inoculated grapes were placed in a closed sterilized box and kept at 25 °C under light or dark conditions as mentioned above. The disease symptoms and ethylene emission (microliters of ethylene

Fig. 1 Effect of various compounds on ethylene formation in *A. alternata* strain A0. The strain was cultured for three days in dark in PDB medium supplemented with various compounds including possible precursors (a) or intermediates (b) for ethylene production; 10 mM of L-Asparatate (ASP), L-Glutamate (GLU), L-Methionine (MET), 2-Oxoglutarate (OXO), 2-keto-4-methylbutyric acid (KMBA), and 1-aminocyclopropane-1-carboxylic acid (ACC)



produced per gram of grape per hour) were scored at seven days post inoculation.

Statistical analysis

For statistical analyses, the Student's *t*-test was used to generate *p*-values. The alpha level was 0.05, and the tests were one-tailed. The data were normalized and all samples were normally distributed with homogeneity of variance.

Results and discussion

In the initial experiment, we examined whether the strain A0 can produce ethylene when cultured in PDB media supplemented with one or more of the possible precursors or intermediates for ethylene production. In a preliminary experiment, we confirmed that the fungus did not produce ethylene in the medium without any test compound. Figure 1a shows that ethylene production was stimulated only when methionine was added to the medium, indicating that methionine can serve as a precursor of ethylene in *A. alternata*.

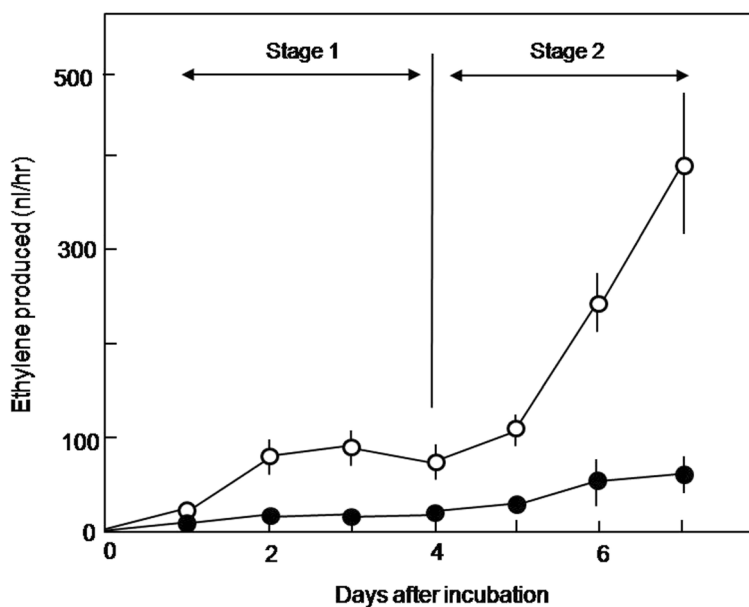
Among microorganisms, the ACC pathway has only been described in the slime mold *Dictyostelium mucoroides* (Amagai and Maeda 1992) and *Penicillium citrinum* (Jia et al. 1999). On the other hand, the KMBA-mediated pathway was reported in *Escherichia coli* (Ince and Knowles 1986), *Cryptococcus albidus* (Fukuda et al. 1989) and *Botrytis cinerea* (Chague et al. 2002; Cristescu et al. 2002), while another

pathway via 2-oxoglutarate was reported in *Penicillium cyclopium* (Pazout and Pazoutova 1989), *Penicillium digitatum* (Fukuda et al. 1989), *Fusarium oxysporum* (Hottiger and Boller 1991) and *Pseudomonas syringae* (Nagahama et al. 1991). In the present study, we investigated the ethylene biosynthetic pathway in *A. alternata* by supplementing the conidial suspension of A0 (PDB without methionine) with the main intermediates of three ethylene biosynthetic pathways (ACC, KMBA and 2-oxoglutarate). As shown in Fig. 1b, ACC and 2-oxoglutarate did not stimulate ethylene production, while KMBA significantly promoted ethylene emission. These results suggested that ethylene is mainly produced by the KMBA-mediated pathway in *A. alternata*.



Fig. 2 Demonstration of the formation of typical yellow precipitate of KMBA by addition of 2, 4-dinitrophenylhydrazine. The precipitate was detected in fresh PDB medium supplemented with KMBA (a) and culture filtrate (c). The filtrate was obtained after *A. alternata* strain A0 was cultured in methionine-supplemented PDB medium. No yellow precipitate was formed in the fresh methionine-supplemented medium (b) treated with 2, 4-dinitrophenylhydrazine

Fig. 3 Change in ethylene production over the course of time by *A. alternata* strain A0. The fungus was cultured 10 ml PDB supplemented with 10 mM methionine under continuous light illumination (*open circle*) or in the dark (*closed circle*) during the entire experimental period. Note that the strain A0 produced ethylene at two distinct stages during the incubation period

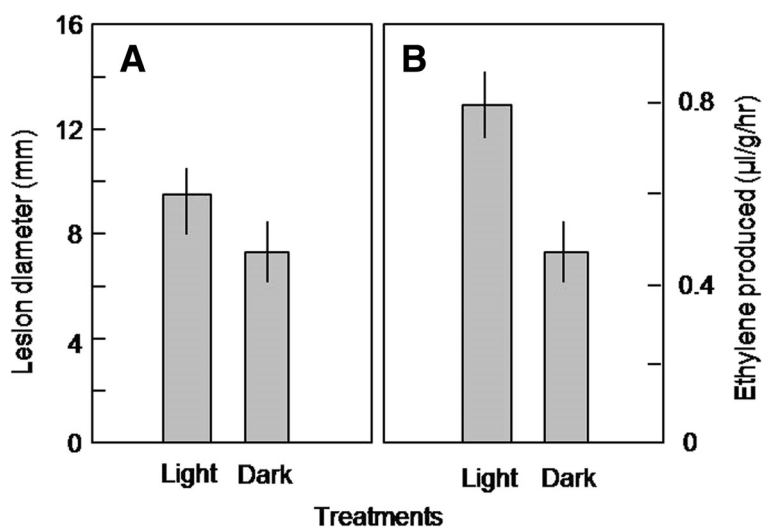


KMBA is a transaminated derivative of methionine, and its presence can be detected by the appearance of yellow precipitate when mixed with 2,4-dinitrophenylhydrazine (Primrose 1977). Figure 2 demonstrates KMBA formation in the strain A0. A typical yellow precipitate of KMBA was detected in the control sample (fungus-free, KMBA-added PDB) (left tube) and the culture filtrate of strain A0 (methionine-enriched PDB) (right tube). On the other hand, the yellow precipitate was not observed in the methionine-enriched medium without the fungus

(middle tube) or methionine-free PDB with the fungus (data not shown). These results indicated that *A. alternata* produced KMBA in the presence of methionine, and KMBA can be secreted into the medium and then converted to ethylene.

Billington et al. (1979) reported that ethylene was produced from KMBA by photochemical oxidation, suggesting that light plays an important role in ethylene production. Also in the present study, we examined the effect of light exposure on ethylene production in *A. alternata*. Figure 3 shows the change in

Fig. 4 Effect of light exposure and darkness on lesion formation (a) and ethylene production (b) by *A. alternata* strain A0. Grape berries were inoculated with A0 and lesion development and ethylene emission were measured seven days after inoculation



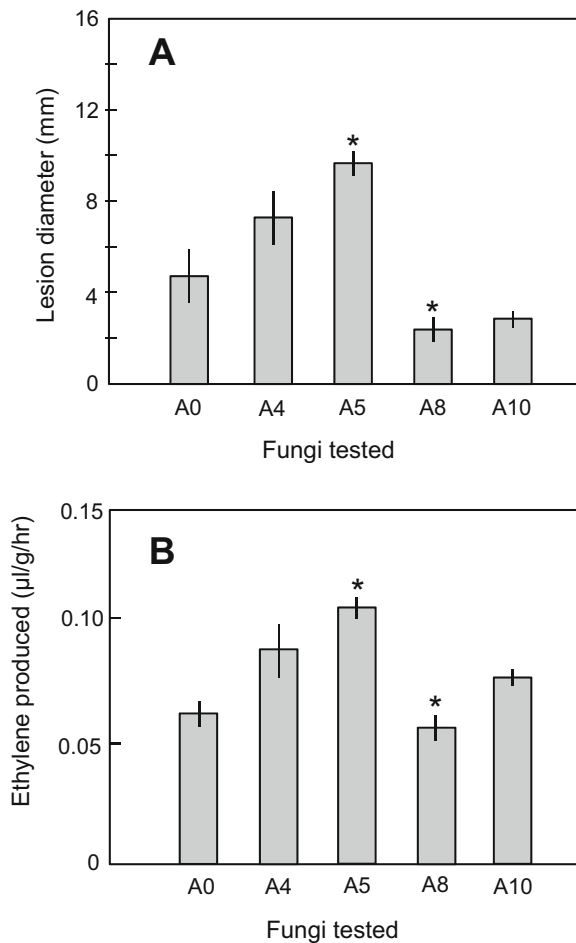


Fig. 5 Lesion development (a) and ethylene emission (b) from grape berries inoculated with *A. alternata* strain A0 and four isolates (A4, 5, 8 and 10). Grape berries were inoculated with the test fungi, and lesion development and ethylene emission were measured seven days post inoculation. Values are means $\pm$ SEM for at least three replicate experiments. Asterisks indicate significant difference ($p < 0.05$)

ethylene production over the course of time by the strain A0 under light and dark conditions. High ethylene emission was detected when the fungus was cultured under continuous light illumination. In fact, the initial intense production of ethylene was detected within three days of incubation (Stage 1), in contrast to lower ethylene production by A0 cultured in dark. During this stage, we confirmed that KMBA accumulated in the culture filtrate of the fungus grown in the dark, which could be converted to ethylene following exposure to light (data not shown). These results suggest that light exposure can accelerate the extracellular reaction that converts

KMBA into ethylene, especially during the initial stage of incubation (Stage 1).

A drastic increase in the ethylene production was observed in the present study during the later stage of incubation (Stage 2). A similar increase was also detected when the fungus was cultured in the dark, although the levels were much lower. These results suggested that an additional mechanism or another pathway was involved in ethylene production at Stage 2. It was likely that there was a change in the composition of the culture solution or depletion of the supplemented methionine owing to increased fungal growth during the long period of cultivation. These possibilities are currently being explored in our laboratory.

Light can regulate growth, pigment formation, asexual and sexual reproduction of various fungi (Idnurm and Heitman 2005), and can also influence interactions between host plants and fungal pathogens (Zeier et al. 2004). To analyze the role of light in ethylene-mediated virulence in *A. alternata*, table grapes were inoculated with the strain A0 and cultured under continuous light illumination or in dark. Figure 4a shows the effect of light exposure and darkness on the development of lesions at the site of inoculation. Grapes placed in dark after inoculation showed reduced lesion formation, in contrast to much larger lesions formed under light. Figure 4b indicated that ethylene production in the inoculated grapes exposed to continuous light illumination was about two-fold higher than that of the samples grown in dark. These results suggest a positive role for light-mediated ethylene production in the virulence of *A. alternata*. However, in spite of the close relationship between the ethylene production and fungal virulence, the present study failed to identify the exact sources of ethylene production in inoculated grape berries. This problem may be crucial, especially in terms of virulence determination as a result of host and pathogen interactions, and therefore this problem will be the next target in our research.

In the final experiment, we examined four isolates (A4, A5, A8, and A10) of *A. alternata* for ethylene production and lesion development after inoculation into grape berries in comparison to strain A0 (Fig. 5). Upon preliminary examination, all isolates were confirmed to produce ethylene under light and dark conditions, although there were considerable differences in ethylene production among these isolates (data not shown). Figure 5a shows the results obtained from the

inoculation of strain A0 and the four isolates into grape berries. All test fungi produced similar lesions at the inoculation site, but two isolates (A4 and A5) were highly virulent and produced lesions that were larger than those of A0; in fact, A5 produced the lesions that were twice in diameter of A0. Figure 5b shows ethylene production after inoculation. The levels of ethylene produced by the test fungi correlated well with the strength of their virulence (as assessed by the lesion formation) in the inoculated host grapes. These results suggested that the differences in virulence of these isolates are partly attributed to variability in their ethylene production.

Various stress factors, including pathogen infection, can induce ethylene biosynthesis in many plants. The relative contribution of pathogen-produced ethylene versus host-produced ethylene in the host-pathogen relationship is still not clear. The present work demonstrated the ability of *A. alternata* to produce ethylene both in an artificial culture as well as in the host plant upon infection. This ability of the pathogen has a crucial role in disease development. Further work will be carried out to clarify the role of ethylene produced by *A. alternata* on its virulence.

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