Research Square

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Identification of antifungal compound isolated from Cercospora sp. ME202 and evaluation of the inhibitory activity against Colletotrichum orbiculare

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Research Article

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Abstract

Cucumber anthracnose, an important disease caused by *Colletotrichum orbiculare*, is controlled using fungicides. However, this can lead to the development of fungicide resistance. We have previously shown that an ethyl acetate extract of the culture filtrate of *Cercospora* sp. ME202 (ME202-ECF) exhibits fungicidal activity and inhibits the formation of anthracnose lesions caused by *C. orbiculare*. However, studies on the control of plant diseases using the antimicrobial compounds produced by *Cercospora* have not been conducted. Here, we aimed to isolate and identify antifungal compounds produced by *Cercospora* sp. ME202 and evaluate their antifungal activity against *C. orbiculare.* We identified traversianal, a tricyclic diterpenoid aldehyde with a molecular weight of 316.4 from ME202-ECF. The traversianal structure was determined by liquid chromatography-mass spectrometry using electrospray ionization and nuclear magnetic resonance spectroscopy after purification by column chromatography and high-performance liquid chromatography. The inhibitory activity of isolated traversianal was detected as a mycelial growth inhibition zone at Rf 0.92 by thin layer chromatography bio-assay. Traversianal also inhibited the conidial germination of *C. orbiculare* at 2–10 ppm and exhibited fungicidal activity at 5–10 ppm. Furthermore, pretreatment of cucumber leaves with 10, 20, and 30 ppm traversianal significantly suppressed lesion formation in *C. orbiculare* without phytotoxicity. These results indicate that the traversianal produced from *Cercospora* sp. ME202 is a potential candidate as a new fungicide against *C. orbiculare*.

Introduction

Anthracnose, a major plant disease in many crops, including grains, vegetables, and fruit trees, is caused by fungi belonging to the genus *Colletotrichum*. *C. orbiculare* (Berkeley. & Mont) Arx infects members of the family Cucurbitaceae, including cucumber (*Cucumis sativus* L.), squash (*Cucurbita maxima*), watermelon (*Citrullus lanatus*), and melon (*Cucumis melo* L.), causing significant yield losses by harming aboveground parts, such as leaves, stems, and fruit (Farr and Rossman 2021; Joshi et al. 2022; Tamilmalar et al. 2022). The disease is generally controlled using fungicides (Palenchar et al. 2009). However, resistance to benzimidazoles, quinone external inhibitors (QoIs), and sterol demethylation inhibitors (DMIs) has been reported in the genus *Colletotrichum* (Chung et al. 2006; Inada et al. 2008; Wong and Midland 2007). Therefore, it is important to overcome the acquisition of pesticide resistance in plant pathogens, and continued development of novel and effective pesticides is necessary for stable agricultural production.

Natural products, such as secondary metabolites produced by microorganisms, have recently attracted attention because of the high level of safety required for living organisms and the environment in agricultural production. The presence of antibacterial and antifungal compounds of microbial origin has been reported by many researchers (Deshmukh et al. 2022; Singh et al. 2017), and we have previously reported that endophytic fungi isolated from weeds produce secondary metabolites that inhibit *C. orbiculare* (Ino et al. 2021). Pesticides derived from secondary metabolites of micro-organisms often degrade easily and quickly in the natural environment and have a low effect on other environmental

organisms (Kim and Hwang 2007). Therefore, secondary metabolites of microbial origin are potential chemical control agents for various plant diseases.

The genus *Cercospora* comprises several species, most of which are plant pathogens. It causes leaf spot disease, sometimes stem blight, and fruit and ear rot, resulting in significant crop production losses worldwide (Gouda et al. 2022; Skaracis et al. 2010). Therefore, *Cercospora* is considered an important control subject. In contrast, some *Cercospora* species have been found to produce secondary metabolites with antibacterial and antifungal activities (Felisbino et al. 2021; Mookherjee et al. 2020). We previously reported that *Cercospora* sp. ME202 isolated from crimson clover (*Trifolium incarnatum*) produced antifungal compounds (Ino et al. 2022). These results suggest that secondary metabolites produced by *Cercospora* spp. are potential candidates for chemical control agents. However, studies on the control of plant diseases using the antimicrobial compounds produced by *Cercospora* have not been conducted. This study aimed to isolate and identify antifungal compounds produced by *Cercospora* sp. ME202 and evaluate their antifungal activity against *C. orbiculare*.

Materials and methods

Microorganisms

Cercospora sp. ME202 was isolated from crimson clover (*Trifolium incarnatum*), an important forage plant from the legume family, collected in the Shimane Prefecture per the previously reported procedure (Ino et al. 2021).

Colletotrichum orbiculare (strain CO-01) was used as the cucumber pathogen. *C. orbiculare* was inoculated onto rice bran ager (50 g/1 rice bran, 20 g/1 sucrose, 20 g/1 agar, and water) and incubated at 25 ± 2 ℃ for 7 days to produce abundant conidia for inoculum.

Purification of active compound

Mycelial disks of ME202 grown on potato sucrose agar (PSA) were incubated in potato sucrose broth (PSB) at 25 ± 2 ℃ in the dark for 10 days with shaking (150 rpm). The culture liquid (6.0 L) was filtered through No.101 filter paper (Advantech Toyo, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and extracted twice with double the quantity of ethyl acetate. The ethyl acetate soluble fraction was used as ethyl acetate extract of the ME202 culture filtrate (ME202-ECF) in thin layer chromatography (TLC) bioassay and subjected to silica gel column chromatography (Wako Gel C-200; 30 mm i.d. × 10 cm, Wako Pure Chemical, Osaka, Japan) with ethyl acetate/hexane solution (0, 20, 50, and 100%). A fraction of 20% ethyl acetate with antifungal activity was collected, and antifungal compounds were detected by TLC bioassay with hexane: ethyl acetate (4:1, v/v), in accordance with a previously reported method (Ino et al. 2022). The silica gel containing the antifungal compound was scraped from the TLC plates and extracted using chloroform: methanol (2:1, v/v). The crude extract was filtrated using a PTFE filter (Syringe Filter PTFE 0.22 μm/φ32 mm: AS ONE Corp., Osaka, Japan) and subjected to high-performance liquid chromatography (HPLC) using a COSMOSIL 5C_{18}-AR-II column (10×250 mm; Nacalai Tesque, Kyoto,

Japan) with 75% acetonitrile (3 ml/min). The main peak (Rt = 8.0 min) was collected and used as the purified compound to investigate the inhibitory activity against *C. orbiculare*.

LC/MS and NMR analysis of the antifungal compound

The purified compound was dissolved in methanol and subjected to liquid chromatography-mass spectrometry (LC-MS) analysis under electrospray ionization (ESI) conditions using a Acquity UPLC BEH C18 1.7 µm (2.1×50 mm; Waters, Milford, MA, USA) to determine its molecular weight. The mobile phases were Milli-Q 0.1% formic acid (solvent A) and acetonitrile 0.1% formic acid (solvent B). The linear gradient was 5–100% B for 0–10 min at a flow rate of 0.2 ml/min (detection: 230 nm).

To identify the antifungal compound, the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra and two-dimensional NMR data (^{1}H–^{1}H COSY, HMQC, and HMBC) of the compound in $CDCl_3$ were recorded using a JNM-ECZ600R/S1 spectrometer (JEOL, Tokyo, Japan).

Detection of the antifungal compound on thin-layer chromatography (TLC)

The purified compound was detected using a TLC bioassay. Silica Gel TLC plate (Silica Gel 60; Merck KGaA, Darmstadt, Germany) was spotted with ME202-ECF (50-fold, 10µl) and 200 µl of purified compound (10 ppm) adjusted with ethyl acetate, and then developed with chloroform: ethanol: water (40:10:1, v/v/v) solvent. After the solvent had completely evaporated, the plate was sprayed with potato sucrose agar (PSA) containing the antibiotic chloramphenicol (20 ppm) suspended in the conidia of *C. orbiculare* (> 1×10^7 conidia/ml) to detect the antifungal compound. After 4 days of incubation in moist camber at 25 ± 2 ℃, the mycelial growth inhibition zone was observed.

Inhibitory activity of the purified compound on conidial germination of C. orbiculare

The purified compound was prepared at different concentrations (1, 2, 5, and 10 ppm) with a maximum concentration of 0.1% dimethyl sulfoxide (DMSO). Conidia of *C. orbiculare* (1.0×10^5 conidia/ml) were suspended in different concentrations of the purified compound (1, 2, 5, and 10 ppm), while 0.1% DMSO was used as a control. The conidial suspensions were dropped onto glass slides and incubated in moist conditions at 25 ± 2 ℃. After 24 h, the percentage of germinating conidia was determined by counting the number of germinating conidia among 300 conidia in each treatment. The experiments were repeated thrice.

Fungicidal activity of the purified compound on C. orbiculare

C. orbiculare was treated with the purified compound from ME202-ECF to determine its fungicidal activity, following a previously described method (Ino et al. 2022). Conidia of *C. orbiculare* (1.0×10^5 conidia/ml) were suspended with purified compound (5 and 10 ppm) and incubated at 25 ± 2 ℃ for 24 h; 0.1% DMSO was used as a control. After incubation, the liquid phase was replaced with sterile distilled water by centrifugation and applied to the PSA containing chloramphenicol (20 ppm). All plates were incubated at

25 ± 2 ℃ for 3 days to count the number of colonies formed. The fungicidal activity of the purified compound was evaluated by viability staining using fluorescein diacetate (FDA) and propidium iodide (PI) under a fluorescence microscope (BZ-X700 all-in-one; KEYENCE Corp., Osaka, Japan) (Tang et al. 2020).

Pretreatment with the antifungal compound on the cucumber leaves

The suppressive effect of the compound purified by pretreatment was evaluated according to a method described by a previous report (Ino et al. 2022). In brief, cucumber seedlings (*Cucumis sativus* L. "Tsuyataro") were grown to first true-leaf development in an incubator at 25 ± 2 ℃ under a 12 h/12 h light/dark cycle. Conidia of *C. orbiculare* (1.0×10^5 conidia/ml) were inoculated on the true leaves pretreated with purified compound (5, 10, 20, and 30 ppm) and the lesion area was measured 7 days after inoculation of the pathogen. We used 0.3% DMSO as a control and the experiments were repeated thrice. Furthermore, the infectious behavior of *C. orbiculare* on inoculated leaves has been observed (Ino et al. 2022).

Statistical analyses

Data are presented as mean ± standard deviation (SD). Significant differences in the means between treatment groups were determined using the Tukey–Kramer test with SPSS Statistics ver. 22.0 for Windows (IBM, Armonk, NY, USA).

Results

Purification and structural analysis of the antifungal compound

The purified antifungal compound (4 mg) was obtained as crystals from the culture filtrate of *Cercospora* sp. ME202 (6 L) and detected at Rt 8.04 min by HPLC (Fig. S1a).

The antifungal compound was identified by liquid chromatography-mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy. The molecular weight of the purified compound was determined to be 316 by ESI-LC-MS based on the molecular-related ions at *m/z* 317.2 $[M + H]^+$ in the positive mode (Fig. S1b), and *m/z* 315.2 $[M-H]^-$ in the negative mode (Fig. S1c). The peak data of NMR were obtained as follows: ^{1}H-NMR (600 MHz, $CDCl_3$) δ_H 9.12 (s, 1H), 7.16 (t, *J* = 9.0 Hz, 1H), 4.81 (q, *J* = 1.2 Hz, 1H), 4.72 (d, *J* = 2.4 Hz, 1H), 3.34 (td, *J* = 11.4, 8.4 Hz, 1H), 3.02 (dd, *J* = 20.7, 9.2 Hz, 1H), 2.85 (s, 1H), 2.66–2.58 (m, 4H), 2.33–2.26 (m, 2H), 1.89–1.84 (m, 1H), 1.81 (t, *J* = 11.4 Hz, 1H), 1.72–1.70 (m, 4H), 1.50–1.47 (m, 2H), 1.34 (dd, *J* = 14.4, 12.0 Hz, 1H), 1.17 (s, 3H), 0.81 (d, *J* = 7.8 Hz, 3H). ^{13}C-NMR (600 MHz, $CDCl_3$) spectrum showed twenty carbon signals: δ_C 220.69, 193.16, 166.70, 147.60, 145.94, 113.87, 78.76, 54.65, 50.84, 49.39, 44.13, 43.67, 43.55, 42.51, 30.55, 28.12, 24.85, 22.40, 18.68, and 16.90. These results indicate that the

molecular formula of the antifungal compound is $C_{20}H_{28}O_3$. Stoessl et al. (1988) reported the isolation and structure of traversianal, a tricyclic diterpenoid aldehyde, from *Cercospora traversiana*, a pathogen of fenugreek. Finally, by comparing the molecular weight and spectral data obtained in this study with those reported by Streel et al. (1988), the antifungal compound isolated from ME202-ECF was identified as traversianal (Fig. 1).

Inhibition of mycelial growth and conidial germination of C. orbiculare by traversianal

To evaluate the antifungal activity of traversianal against *C. orbiculare*, TLC bioassays and conidial germination tests were performed. Purified traversianal from the isolate ME202 was detected as a mycelial growth inhibition zone at Rf 0.92 on a TLC plate (Fig. 2). A decline in conidial germination rate of *C. orbiculare* due to treatment with traversianal was observed at 2, 5, and 10 ppm compared with 0.1% DMSO in the control, and conidial germination was completely inhibited at 5 and 10 ppm (Fig. 3).

Fungicidal activity of traversianal against C. orbiculare

The fungicidal activity was investigated by treating the conidia of *C. orbiculare* with traversianal for 24 h (Fig. 4a). The number of colonies formed on PSA was significantly reduced when treated with traversianal at 5 and 10 ppm (3.5 ± 2.7 and 0.5 ± 0.5 cfu/plate) compared to 325.1 ± 40.3 cfu/plate in the control (Fig. 4b). Double fluorescence staining with FDA and PI was used to observe the viability of *C. orbiculare*. The conidia of *C. orbiculare* showed green fluorescence (live cells) in the control, whereas red fluorescence (dead cells) was observed in conidia treated with traversianal at 5 and 10 ppm (Fig. 4c).

Suppressive effect of traversianal on cucumber anthracnose

The preventive effect of traversianal on cucumber anthracnose was evaluated by pretreatment on cucumber leaves. As shown in Fig. 5, the lesion area was 31.8 ± 7.3 mm^2 in the control treated with 0.3% DMSO. Whereas, the pretreatment with traversianal resulted in a concentration-dependent reduction in lesion area, and the lesions formed by *C. orbiculare* were significantly suppressed at 10, 20, and 30 ppm (Fig. 5). Furthermore, no phytotoxicity was observed in cucumber leaves after treatment with 30 ppm triversianal (data not shown).

Discussion

We have previously reported that the ethyl acetate extract of the culture filtrates from *Cercospora* sp. ME202 (ME202-ECF) exhibited inhibitory activity against *C. orbiculare* (Ino et al. 2022). However, the substance responsible for the antifungal activity of ME202-ECF remained to be identified. Hence, this study aimed to identify antifungal compounds in ME202-ECF and evaluate their potential use as fungicides by determining their inhibitory activity against cucumber anthracnose.

The genus *Cercospora* is composed of numerous species, most of which are phytopathogens. Some species of the genus *Cercospora* produce antifungal compounds and cercosporin, a photoactivated polyketide toxin involved in pathogenicity (Felisbino et al. 2021; Souza et al. 2019). Cercosporin exerts broad toxicity on plants, bacteria, and fungi (Daub and Briggs 1983; Daub 1987; Yamazaki et al 1975). However, the antifungal compound in ME202-ECF is different from cercosporin (Ino et al. 2022).

The antifungal compound in ME202-ECF was identified using liquid chromatography/mass spectrometry and NMR analyses. The results indicated that the active compound was traversianal, a diterpenoid aldehyde with a molecular weight of 316.4. Traversianal has been isolated from *Cercospora traversiana*, a pathogen of fenugreek, one of the oldest medicinal plants originating from India and North Africa, and has a 5-8-5 tricyclic carbon skeleton (Stoessl et al. 1988). Stoessl et al. (1989) examined the biological properties of traversianal and reported that it is highly toxic at low concentrations to brine shrimp and snails and lyses human red blood cells at 5×10^{-7} M. Additionally, the study reported that traversianal has no harmful effects on oral crop intubation (100 mg/kg) or any other level tested on chicks and is not toxic to various bacteria and fungi in standard growth and germination inhibition tests. In contrast, in the present study, traversianal isolated from ME202-ECF inhibited mycelial growth of *C. orbiculare* in the TLC bioassay (Fig. 2). In addition, the inhibition of conidial germination caused by traversianal was observed at > 2 ppm in the conidial germination test (Fig. 3), and its fungicidal activity was demonstrated at 5 and 10 ppm (Fig. 4). This is the first report on the antifungal and fungicidal activity of traversianal. However, the toxicity of traversianal in humans and animals has not been reported in detail. Therefore, the toxicity of triversianal in humans and animals must be investigated in detail.

We also investigated the inhibitory activity of traversianal against *C. orbiculare* in cucumber leaves. The pretreatment of cucumber leaves with traversianal suppressed lesion formation in *C. orbiculare* in a concentration-dependent manner above 5 ppm. These results indicate that traversianal can inhibit the infection behavior of *C. orbiculare* on cucumber leaves. The same tricyclic carbon framework is present in fusicoccins, cotylenins, and ophiobolins, some of which are phytotoxic (Camusso et al. 2007; Kim et al. 1999). The phytotoxicity of fusicoccins, cotylenins, and ophiobolins strongly depends on both the substituents and the tricyclic stereochemistry (Stoessl et al. 1989). Therefore, the substituents and the tricyclic carbon framework may also be involved in the antifungal activity of traversianal against *C. orbiculare*. However, traversianal was negative in phytotoxicity tests for young wheat leaves and seedlings of tomatoes, peas, peanuts, and fenugreek (Stoessl et al. 1989). In the present study, no phytotoxicity was observed in cucumber leaves treated with 30 ppm triversianal (data not shown). These results indicate that traversianal is a potential fungicide against cucumber diseases. The efficacy of anthracnose control in the field and the antifungal mechanism of traversal should be investigated in the future.

In summary, traversianal, a tricyclic diterpenoid aldehyde isolated from *Cercospora* spp. ME202 inhibits mycelial growth, conidial germination, and lesion formation in *C. orbiculare* without causing phytotoxicity in cucumber. These results indicate that traversianal is a potential fungicidal candidate for controlling cucumber anthracnose.

Declarations

Acknowledgments

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

Not applicable

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Figures

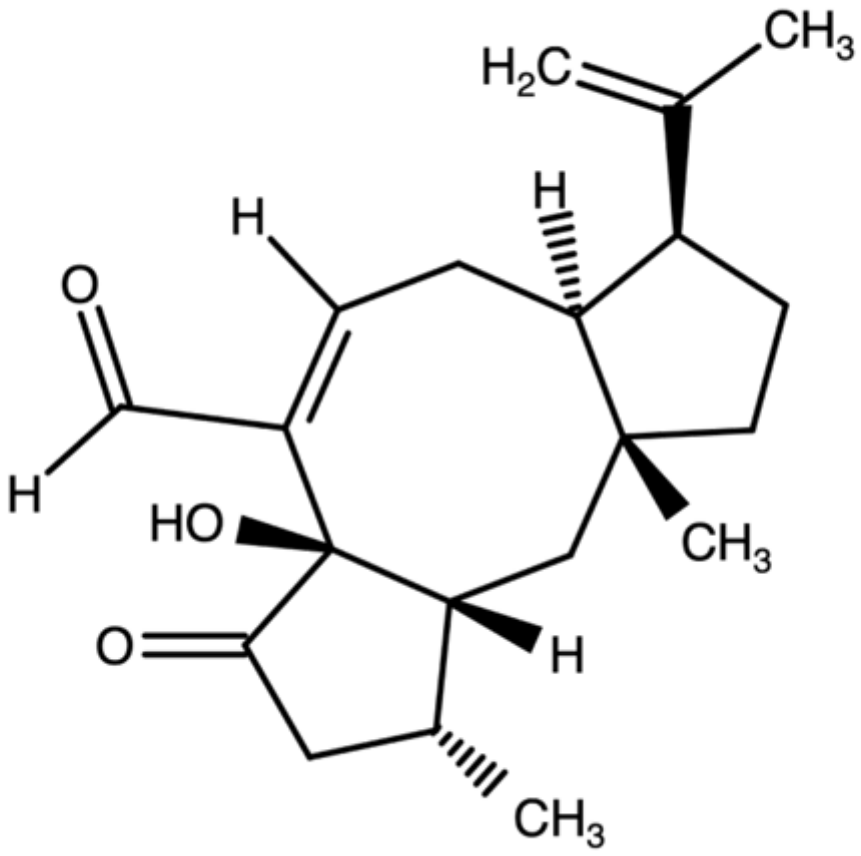


Figure 1

Structure of traversianal from ethyl acetate extract of the culture filtrate of *Cercospora* sp. ME202

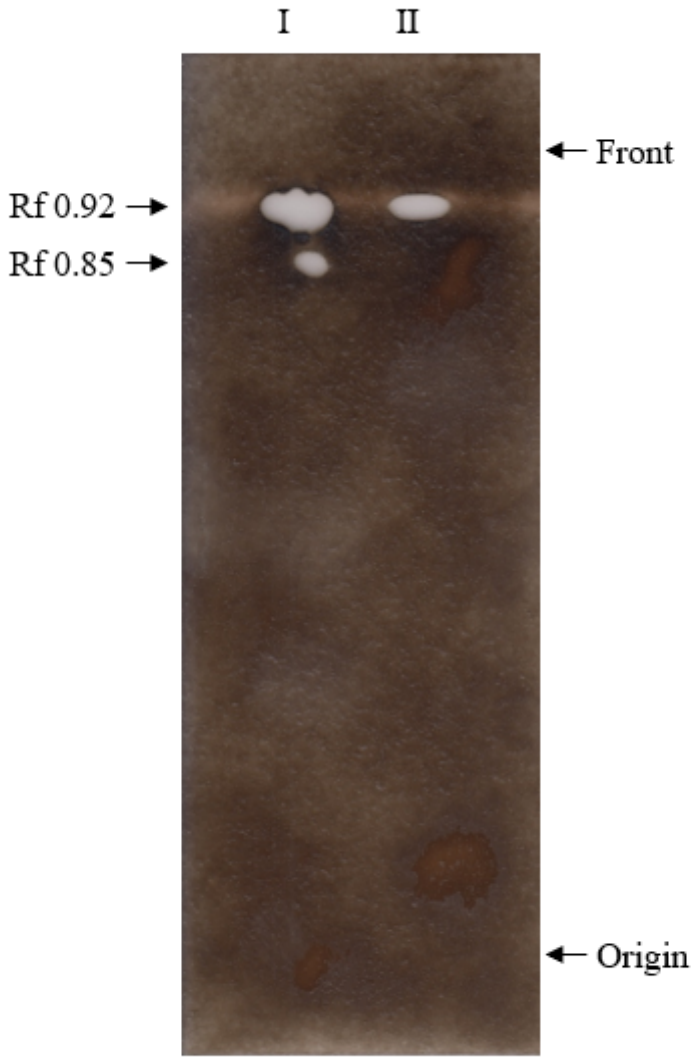


Figure 2

Thin layer chromatography (TLC) bioassay to detect the inhibitory compound from the ethyl acetate extract of the culture filtrate of *Cercospora* sp. ME202 (ME202-ECF). Silica gel TLC plate was spotted with ( ) ME202-ECF and ( ) Traversianal from ME202-ECF and developed with chloroform:ethanol:water (40:10:1, v/v/v). The plate was sprayed with molten potato sucrose agar containing *Colletotrichum orbiculare* (> 1 × 10^7 conidia/ml) and incubated at 25 ± 2 ℃ for 4 days

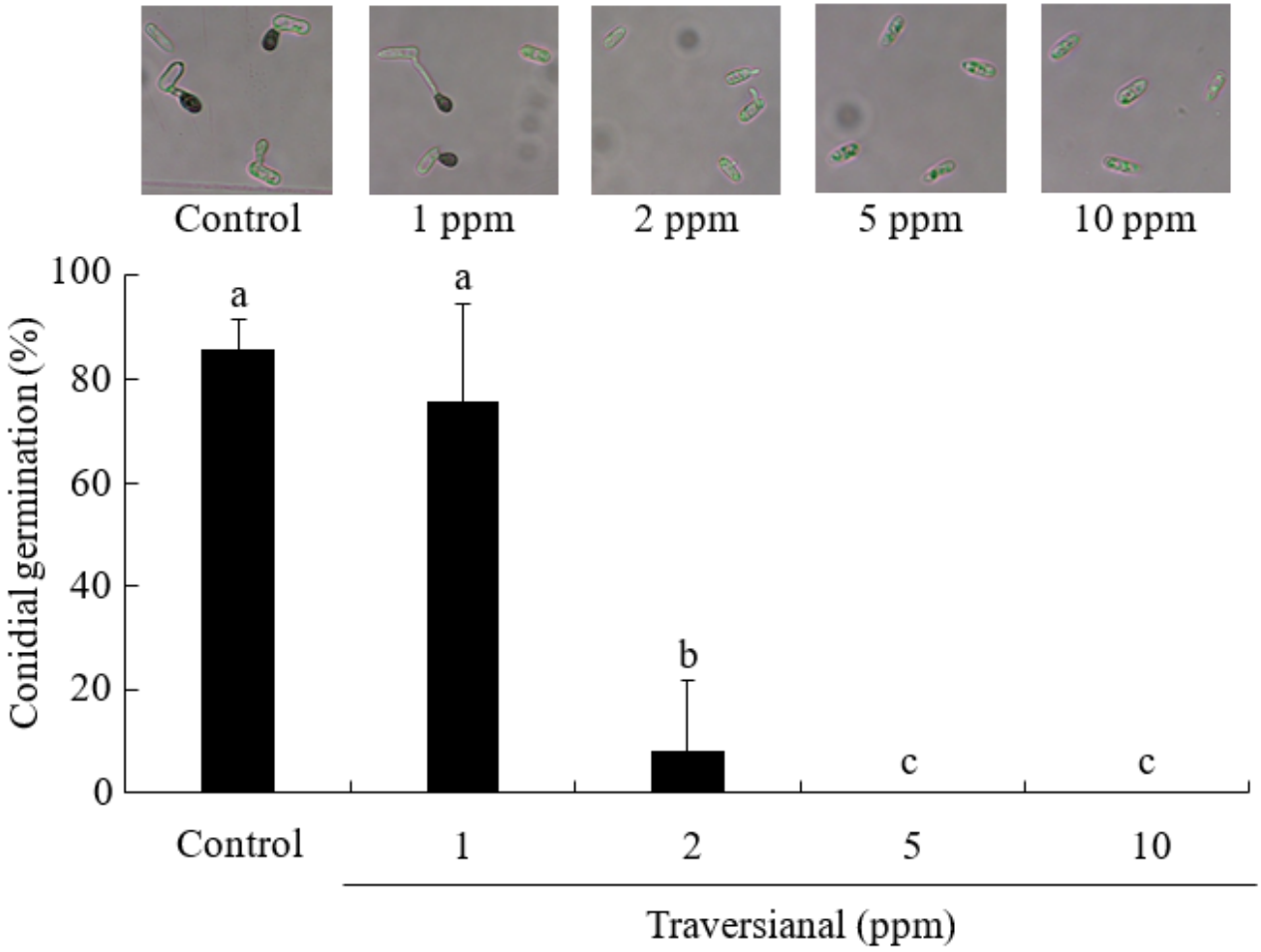


Figure 3

Inhibitory activity of traversianal from ethyl acetate extract of the culture filtrate of *Cercospora*sp. ME202 on conidial germination of *Colletotrichum orbiculare*. Conidia of *C. orbiculare*were mixed with traversianal (1–10 ppm) with 0.1% dimethyl sulfoxide (DMSO). As a control, conidia of *C. orbiculare*were mixed with 0.1% DMSO (Control). After 24 h, conidial germination (%) was determined by counting the number of germinating conidia in 300 conidia in each treatment. The bar at the top of each column represents the standard deviation of the mean. Means followed by different letters indicate a significant difference (Tukey–Kramer test, $P < 0.05$)

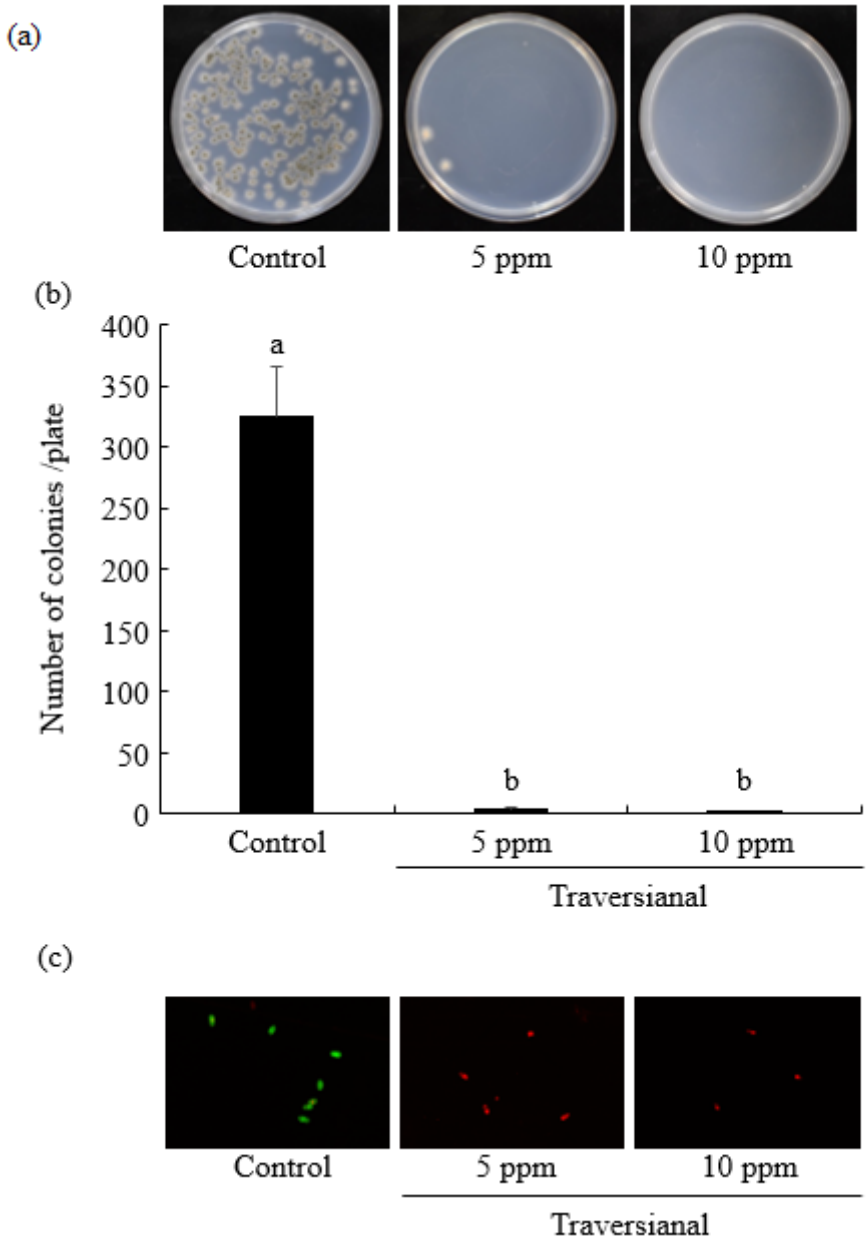


Figure 4

Fungicidal activity of traversianal from ethyl acetate extract of the culture filtrate of *Cercospora*sp. ME202 on *Colletotrichum orbiculare*. (a) *C. orbiculare* was mixed with traversianal (5 and 10 ppm) with 0.1% dimethyl sulfoxide (DMSO) and incubated on PSA containing chloramphenicol. As a control, conidia of *C. orbiculare* were mixed with 0.1% DMSO (Control). (b) After 3 days, the colonies formed were counted. (c) Conidia of *C. orbiculare* treated with traversianal were stained by fluorescein diacetate (FDA) and propidium iodide (PI) and observed with an all-in-one fluorescence microscope

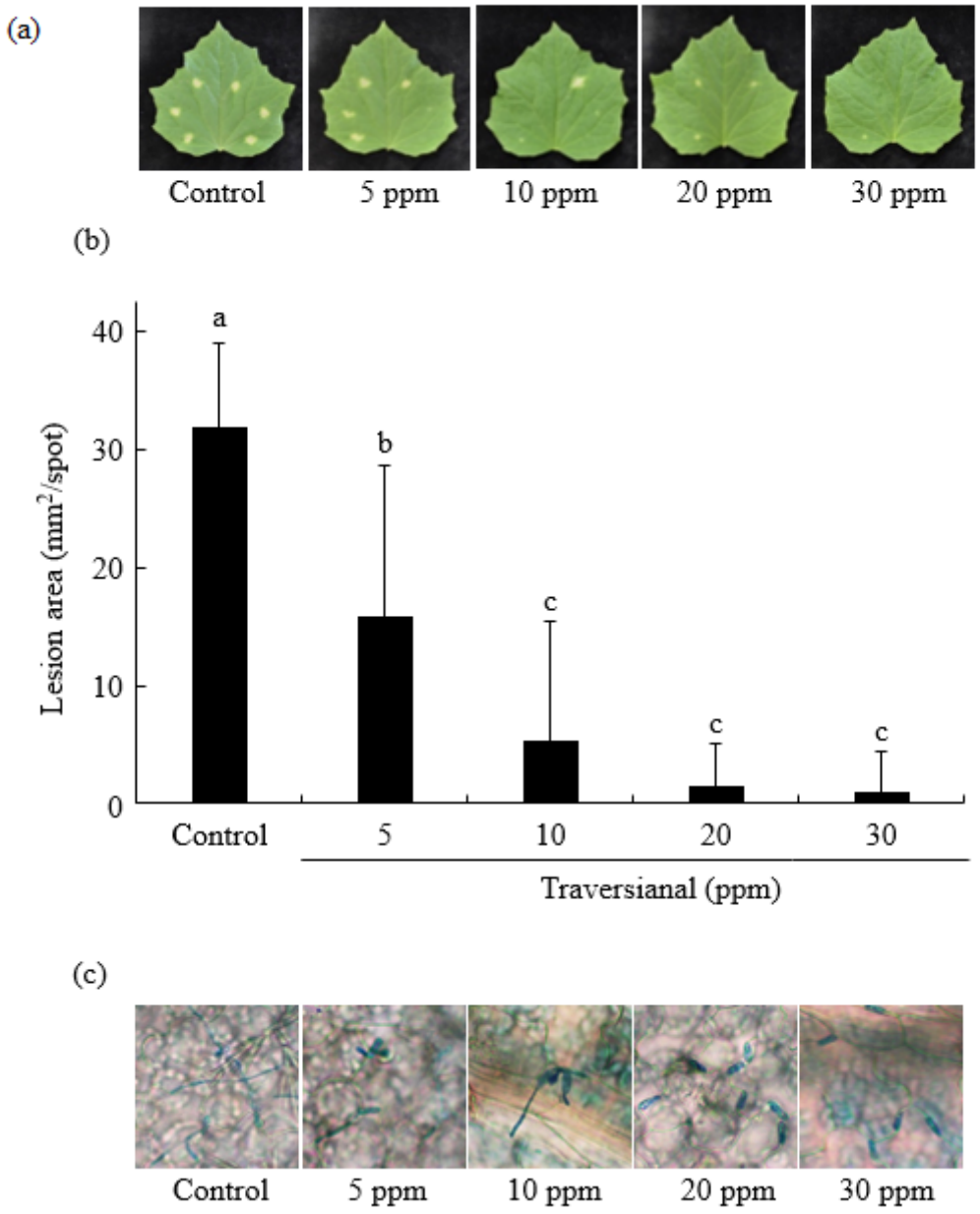


Figure 5

Suppressive activity of pretreatment with traversianal from ethyl acetate extract of the culture filtrate of *Cercospora* sp. ME202 on lesion formation by *Colletotrichum orbiculare*. (a) Cucumber leaves were pretreated with traversianal (5, 10, 20, and 30 ppm) with 0.3% dimethyl sulfoxide (DMSO). As a control, 0.3% DMSO was pretreated. After 24 h, pretreated cucumber leaves were inoculated with a conidial suspension of *C. orbiculare* (1.0×10^5 conidia/ml). After 7 days, anthracnose lesions were formed (a), and the lesion areas (b) were recorded. The bar at the top of each column represents the standard deviation of the mean. Means followed by different letters indicate a significant difference (Tukey–Kramer test, $P < 0.05$). (c) Light micrographs of representative decolorized leaf samples are shown in (a) and stained with 0.01% w/v lactophenol cotton blue

Supplementary Files

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