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Inhibitory potential of fungi isolated from several weeds in Matsue city against *Colletotrichum orbiculare*, the causal agent of anthracnose disease in cucurbit crops

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Abstract Anthracnose disease, caused by *Colletotrichum orbiculare*, is a major disease of cucumber and other cucurbit crops worldwide. In this study, the culture and culture filtrates of symbiotic and parasitic fungi were isolated from several weeds in Matsue City (Shimane prefecture), and screened for their inhibitory potential against *C. orbiculare*. Symbiotic and parasitic fungi (20 isolates) were isolated from the stems of 7 weed samples (*Cerastium glomeratum, Equisetum arvense, Lamium purpureum, Rumex acetosa, Taraxacum albidum*, *Trifolium incarnatum*, and *Vicia sativa* subsp. *nigra*) collected from a field in 2020. Inhibition zone against *C. orbiculare* on the petri dish was observed for two isolates (301, and 504) using dual culture method. The direct effects of culture filtrate of the isolated symbiotic and parasitic fungi was determined by investigating the germination of *C. orbiculare* conidia. Sixteen of the 20 fungal culture filtrates inhibited *C. orbiculare* conidia germination by more than 50%, while germination in control was not inhibited. Nine of 16 culture filtrates inhibited more than 90% of *C. orbiculare* conidia germination. Additionally, three of the nine culture filtrates inhibited more than 100% of *C. orbiculare* conidia germination. These results strongly suggest that the inhibitory effects of symbiotic and parasitic fungi on plant pathogenic fungi may contribute to the development of new fungicides and new biological agent to control plant diseases caused by plant pathogens.

Keywords *Colletotrichum orbiculare*, Culture filtrate, Inhibitory activity, Symbiotic and parasitic fungi

Introduction

Cucurbits are consumed worldwide as popular food crops; however, cucurbit plants are vulnerable to damage, which can further increase their susceptibility to several diseases (Agrios, 2005). Anthracnose disease, a destructive disease caused by the hemibiotrophic fungal pathogen *Colletotrichum orbiculare* (Berk. & Mont.) Arx [syn. *C. lagenarium* (Pass.) Ellis & Halst.], engenders major losses to the production of cucumber and other cucurbit crops. The control strategies applied for the plant pathogenic fungi mainly involve the use of chemical fungicides, which over time, develops resistance to some of these chemicals. Therefore, it is essential to identify natural compounds to develop new agents to control fungal pathogens. Recently, the efficacy of biological control, to reduce the use of chemical fungicides, in controlling plant disease has been investigated (Li et al., 2007; You et al., 2019). Moreover, it is evident that the resistance developed towards biological control using antagonistic microorganisms has not yet been reported. In addition, microorganisms are recognized for their distinct physiological characteristics and production of different compounds, even within the same species. Therefore, it is necessary to examine a wide and diverse array of microorganisms. We recently discovered symbiotic and parasitic fungi in wild mushrooms and reported their potential in controlling plant diseases (Nguyen et al., 2017). Furthermore, we identified a new antifungal compound from the culture filtrate of symbiotic and parasitic fungi in wild mushrooms (Nguyen et al., 2018).

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The results provided compelling evidence in that the inhibitory compounds in the culture filtrate of symbiotic and parasitic fungi may contribute to the development of new fungicides. On the other hand, there exists several weed species worldwide. However, existence of symbiotic and parasitic fungi in several weeds and its effectiveness in controlling plant disease are not yet reported. Therefore, the objectives of this study were: (1) to isolate symbiotic and parasitic fungi from the weed; (2) to investigate the inhibitory activity of the isolated fungal against mycelial growth of *C. orbiculare*; and (3) to investigate inhibitory activity of the isolated fungal culture filtrate against conidia germination of *C. orbiculare*. In this study, symbiotic and parasitic fungi from several weeds were isolated and screened for their inhibitory potential against *C. orbiculare*.

Materials and Methods

Isolation of fungi from several weed samples

Fungi were isolated from the stems of 7 weed samples (*Cerastium glomeratum, Equisetum arvense, Lamium purpureum, Rumex acetosa, Taraxacum albidum*, *Trifolium incarnatum*, and *Vicia sativa* subsp. *nigra*) collected from a field in Shimane prefecture in 2020. Segments (approximately 1 cm) from each stem were cut with a blade, and surface-sterilized with 70% ethanol for 30 sec and 1% sodium hypochlorite solution for 2 min, and rinsed twice with sterile distilled water. Rinsed segments were placed on sterile filter paper in petri dishes to remove excess surface water. Each dried segment (approximately 2 mm) was subsequently placed on potato sucrose agar (PSA; 200 g/L potato extract, 2% w/v sucrose, 2% w/v agar) medium containing chloramphenicol (20 ppm) in petri dishes (9 cm diameter) and incubated for 4–5 days at 25 ± 2˚C. Fungal isolates that grew on the segments were transferred to fresh PSA medium, and fungal isolates obtained by a single spore or colony was maintained on PSA slants until further use.

Pathogens

Colletotrichum orbiculare was maintained on PSA slants until use. *C. orbiculare* was grown on rice bran agar (50 g/L rice bran, 20 g/L sucrose, 20 g/L agar, and H_2O) at 25 ±2°C for 7 days to induce abundant conidiation. Synchronously formed conidia were used to create the inoculum.

Dual culture technique

The inhibitory activity of the isolated fungi on the mycelial growth of *C. orbiculare* was investigated using the dual culture method with PSA medium. Mycelial plugs (8 mm diameter) of *C. orbiculare* and isolated fungi were placed on PSA plates, at a distance of 4 cm. Subsequently, PSA medium plug (8 mm) was treated on the PSA plates as control. All petri dishes were incubated at 25 ± 2°C for 17-18 days.

Preparation of culture filtrates from isolated fungi

Mycelial plugs (8 mm diameter) prepared from a single colony of each isolate grown on PSA medium were incubated at 25 ± 2°C in dark in a test tube containing PSB, for 10 days with constant shaking on a rotary shaker (110 rpm). The culture suspension filtered through 0.22-µm micropore membrane filters (Syringe Filter Nylon 0.22 µm / φ32 mm: AS ONE Corp., Osaka, Japan), was used as the culture filtrate.

Inhibitory activity of culture filtrates of isolated fungi against *C. orbiculare*

C. orbiculare conidia (5.0×10^4 conidia/ml) suspended in culture filtrates of isolated fungi were pipetted dropwise onto glass slides (30 µl / 1 drop, 3 drops) and maintained in a moist chamber at 25 ± 2°C. After 24 h, the germination of 150 conidia per experiment for each treatment were assessed using a light microscope, to calculate the percentage germination as: (number of conidia germinated/total number of conidia) × 100. The experiments were repeated thrice.

Statistical analysis

Data are reported as the mean(s) ± standard deviation (SD). Significant differences in the experimental values between groups were determined by a Tukey-Kramer test using SPSS Statistics ver. 22.0 for Windows (IBM, Armonk, NY, USA). The p-values<0.05 indicated a statistically significant difference.

Results and Discussion

The inhibitory potential of weed fungal isolates collected from 7 samples were assessed against *C. orbiculare* using dual culture methods. Fourteen of the 20 isolated fungi inhibited the mycelial growth of *C. orbiculare* (Fig. 2). Moreover, two isolates (301, and 504) showed inhibition zone against *C. orbiculare* on the petri dish (Fig. 2), indicating the production of inhibitory compound(s) against *C. orbiculare*. The direct effects of culture filtrate of isolated fungi were determined by investigating their involvement in the germination of *C. orbiculare* conidia. Among the 20 fungal culture filtrates, 16 inhibited *C. orbiculare* conidia germination by more than 50%, while germination in control was not inhibited (Fig. 3). Nine (301, 302, 401, 502, 503, 504, 601, 602, and 701) of the 16 culture filtrates inhibited more than 90% of *C. orbiculare* conidia germination (Fig. 3). Additionally, three (503, 504, and 701) of the nine culture filtrates inhibited more than 100% of *C. orbiculare* conidia germination (Fig. 3). The percentage of *C. orbiculare* conidia germination using the culture filtrates of isolates 101, 102, 103, 104, 201, 202, 203, 204, 205, 301, 302, 401, 501, 502, 503, 504, 601, 602, 603, and 701 was 31.6 ± 18.0%, 17.3 ± 26.1%, 79.6 ± 15.4%, 44.7 ± 25.1%, 55.8 ± 26.6%, 16.4 ± 11.9%, 27.1 ± 20.1%, 81.8 ± 8.0%, 34.9 ± 18.6%, 9.1 ± 9.3%, 7.6 ± 9.7%, 3.3 ± 4.5%, 34.9 ± 35.7%, 3.1 ± 5.1%, 0%, 0%, 4.6 ± 7.7%, 1.1 ± 2.7%, 58.0 ± 30.0%, and 0%, respectively. Germination in the PSB controls was 95.6 ± 0.9% (Fig. 3). These results indicated that the germination of *C. orbiculare* conidia was strongly inhibited by culture filtrates of fungi isolated from several weeds. On the other hand, the ability of the isolated fungi to suppress other pathogenic fungi and bacteria in greenhouse-grown plants has not yet been investigated. Therefore, further studies are required to investigate the control of multiple diseases by the isolated fungi in greenhouse-grown plants. In addition, the application of these cultures and culture filtrates in plant cultivation experiments for controlling anthracnose disease is now in progress.

In conclusion, this study on the inhibitory effects of symbiotic and parasitic fungi on plant pathogenic fungi may contribute to the development of new fungicides and new biological agent to assist in controlling plant diseases caused by plant pathogens.

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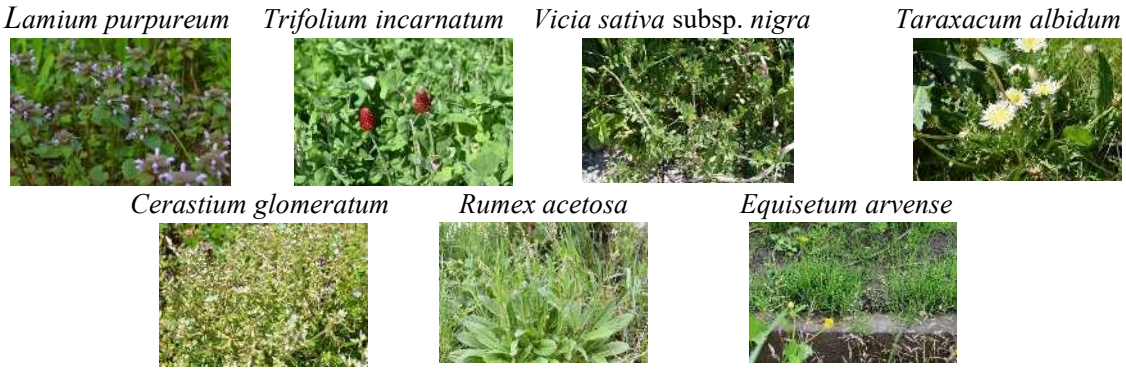

Fig. 1 Collected weed samples for isolation of fungi

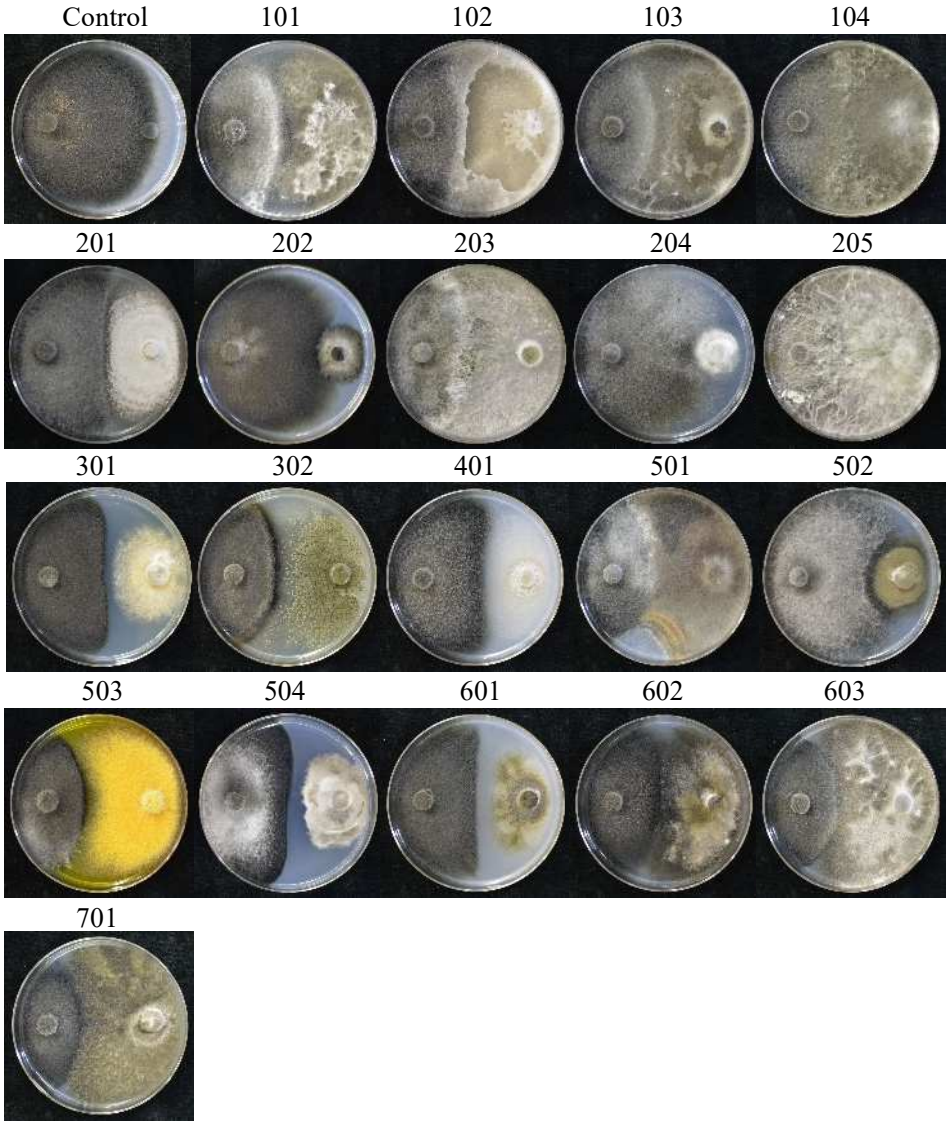

Fig. 2 Inhibitory potential of isolated fungi from several weeds in Matsue city (Shimane prefecture) against the growth of *Colletotrichum orbiculare* observed by dual culture on potato sucrose agar (PSA) plate. Mycelial plugs (8 mm) of *C. orbiculare* (left) and isolated fungi (right), were placed on PSA plates, at a distance of 4 cm. PSA medium plug (8 mm) was inoculated on the PSA plates as control. All petri dishes were incubated at 25 ± 2 °C for 17-18 days.

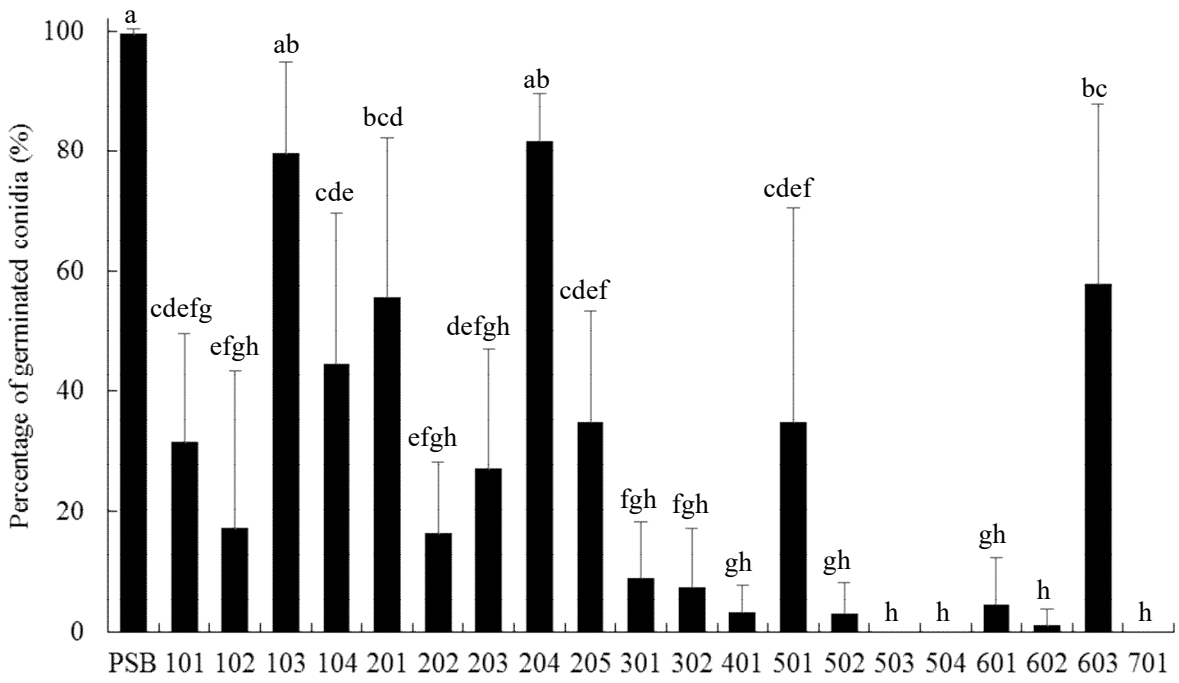

Fig. 3 Inhibitory activities of the culture filtrates of isolated fungi from several plant in Matsue city (Shimane prefecture) on the germination of *Colletotrichum orbiculare* conidia. *C. orbiculare* (5.0×10^4 conidia/ml) suspended in the culture filtrates of isolated fungi, were dropped onto glass slides and maintained in a moist chamber at 25 ± 2 °C. As a control, potato sucrose broth (PSB) was used. After 24 h, percentages of conidial germination were determined by assessing 150 conidia under a light microscope. Experiments were repeated three times. The bar at the top of each column represents the standard deviation of the mean. Mean values denoted by the same lowercase letter are not significantly different at the 5% level, as determined using Turkey's test.