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Antifungal Properties of *Rhizopus oligosporus* Against Apple Anthracnose Fungi

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This study was carried out to assess the antifungal potential of *R. oligosporus* and its ethyl acetate (EtOAc) extract against the fungal pathogens causing anthracnose disease in apple fruits using disc diffusion, antagonistic effect and morphological abnormalities in fungal mycelia. The percentage of inhibition of antifungal effect of the ethyl acetate extract (5 μl disc^{-1}) of the *R. oligosporus* against *C. acutatum* KACC 40848, *C. gloeosporioides* KACC 40897, *C. higginsianum* KACC 40806, *C. orbiculare* KACC 40808, *C. coccodes* KACC 40008, *C. musae* KACC 40947, *C. boninense* KACC 40893, *C. liliacearum* KACC 40981, *C. caudatum* KACC 41028 and *Colletotrichum* sp. KACC 40811 was found to be 44.4, 35.5, 40, 31.1, 33.3, 37.7, 40, 51.1, 28.8 and 28.8%, respectively. Also the fungus *R. oligosporus* showed potential antagonistic effect of antifungal activity against the tested pathogens of *Colletotrichum* spp. Further, *R. oligosporus* had a potential detrimental effect on the morphology of the tested fungi of *Colletotrichum* spp. such as wrinkle abnormalities, abnormal cell formation, lysis of mycelium, empty cell formation, distorted cell formation and breakage of the mycelium. These findings strongly support the role of *R. oligosporus* to serve as a potential antifungal agent to control plant pathogenic fungi causing anthracnose disease in apple fruits.

Key Words: Antagonism, Antifungal activity, Anthracnose fungi, *Colletotrichum* spp., *Rhizopus oligosporus*

INTRODUCTION

The apple is the pomaceous fruit of the apple tree, species *Malus domestica* in the rose family Rosaceae which has potential nutritive and therapeutic values. Research suggests that apples may reduce the risk of colon cancer, prostate cancer and lung cancer(NRCR, 2008). Compared to many other fruits and vegetables, apples contain relatively low amounts of Vitamin C as well as several other antioxidant compounds(Boyer and Liu, 2004). The fiber content, while less than in most other fruits, helps regulate bowel movements and may thus reduce the risk of colon cancer. They may also help with heart disease, weight loss, and controlling cholesterol, as they do not have any cholesterol, have fiber, which reduces cholesterol by preventing re-absorption, and are bulky for their caloric content like most fruits and vegetables (Sharma, 2005; AKYFH, 2008). There is evidence that *in vitro* apples possess phenolic compounds which may be cancer-protective and demonstrate antioxidant activity (Lee et al., 2004). The predominant phenolic phytochemicals in apples are quercetin, epicatechin, and procyanidin B2(Lee et al., 2003).

Fungi are major causes of plant disease, accounting for perhaps 70% of all the major crop diseases (IAPSC, 1985). Some of these fungal plant pathogens are termed biotrophic because they establish an intricate feeding relationship with living host cells. Others are termed necrotrophic,because they invade the plant tissues aggressively, killing the host cells to obtain nutrients(Deacon, 1997). Apple anthracnose is an important disease hampering the quality and

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texture of apple fruits worldwide. This disease is usually caused by *Colletotrichum* spp. The disease occurs as quiescent infections on immature fruit and the damage it incites is more important in the post-harvest period(Muirhead and Gratitude, 1986); Dodd et al., 1997).

Fungicides, either as pre-harvest or post-harvest treatments, from the main approach to reduce losses from anthracnose. However, their use is increasingly restricted due to public concerns over toxic residues. Moreover, fungicides are unaffordable for many apple growers in developing countries.

R. oligosporus is one of the common microbes in East Asia region, and usually is used for the production of several fermented food products. Previously it has been reported that *R. oligosporus* can serve as a beneficial agent to produce fermented food with a higher amount of functional properties(Handoyo and Morita, 2006). Also solid- state bioprocessing of cranberry pomace using the food grade fungus *R. oligosporus* has improved phenolic and antioxidant profiles, thereby showing potential antibacterial effect against foodborne pathogenic bacteria(Vatten et al., 2004).

In the present study, we tested the antifungal efficacy of a food grade fungus *Rhizopus oligosporus* and its ethyl acetate extract against a panel of fungi causing anthracnose disease in apple fruits in Korea.

MATERIALS AND METHODS

Fungal strain and culture media

The fungal pathogen *Rhizopus oligosporus* used in this study was provided by B & L Agro Co. Ltd, Andong, Gyeongbuk, Republic of Korea. The germination and the growth of isolated fungus was maintained on potato dextrose agar (PDA) medium containing per liter 17 g enzymatic digest of casein, 3 g enzymatic digest of soybean meal, 5 g NaCl, 2.5 g dipotassium phosphate and 2.5 g dextrose.

Test pathogens

Ten isolates of apple anthracnose fungi *Colletotrichum acutatum* KACC 40848, *C. gloeosporioides* KACC 40897, *C. higginsianum* KACC 40806, *C. orbiculare* KACC 40808, *C. coccodes* KACC 40008, *C. musae* KACC 40947, *C. boninense* KACC 40893, *C. liliacearum* KACC 40981, *C. caudatum* KACC 41028 and *C.* sp. KACC 40811 used in this study were maintained on potato-dextrose agar, which were obtained from Korean Agriculture Culture Collection, Suwon, Republic of Korea.

Preparation of the ethyl acetate extract of *Rhizopus oligosporus*

The fresh mycelium plugs of *Rhizopus oligosporus* grown on PDA medium at 24 ± 1℃ for 5-6 days were transferred separately into 3000 ml Erlenmeyer flasks containing 1500 ml potato dextrose broth (PDB) medium. After 12 days of incubation at 24 ± 1℃, 12 liter culture liquid was extracted with ethyl acetate (EtOAc) followed by incubation for 2 - 3 days at 24 ± 1℃ and 150 rev min^{-1} on a rotary shaker. Evaporation of the solvent from the extract *in vacuo* gave a residue, which was subjected to the antifungal potential.

Disc diffusion assay

Petri dishes (9 cm diameter) containing 20 ml of PDA medium were used for the antifungal activity assay, performed in solid media by the disc diffusion method(Duru et al., 2003). Sterile Whatman paper discs of 6 mm diameter were impregnated with 5 μl $disc^{-1}$ EtOAc extract of *R. oligosporus* and placed in the agar plates equidistantly. A disc of fungal inoculum 6 mm in diameter was removed from a previous culture of all the fungal strains tested and placed upside down in the centre of the petri dishes. The plates were incubated at 28℃ for 5 - 7 days. The inhibition of the growth of each fungal strain was calculated as the percentage of inhibition of radial growth relative to the control. The plates were used in three replicates for each treatment.

The growth inhibition of treatment against control was calculated by percentage, using the following formula:

Inhibition ratio (%) = {1- mycelium growth of treatment (mm) / mycelium growth of control (mm)} × 100

Antagonistic effect and morphological observations

Antagonistic activity of *Rhizopus oligosporus* was evaluated against 5 days old culture isolates of anthracnose fungi of *Colletotrichum* spp. by dual culture technique at 28℃. Zones of inhibition were observed after 4 - 5 days incubation. Plates inoculated with only

fungus served as control.

Fungal growth inhibition was recorded and calculated by the following formula:

Inhibition (%) = Radial growth in control (C) − Radial growth in dual culture (T) / Radial growth in control (C) × 100

For microscopic observations, fungal mycelia growing towards the zone of inhibition were processed. Specimens were examined under a compound light microscope (Nikon, Alphaphot-2/YS2, Shanghai, China) for morphological abnormalities in mycelia that occurred due to antagonism mediated by the *Rhizopus oligosporus*. Images of fungal deformities were captured using Image analyzer (Paxcam 2).

RESULTS

Antifungal activity of the ethyl acetate extract of *R. oligosporus*

The results of the antifungal activity of the ethyl acetate extract of *R. oligosporus* are shown in Table 1. The ethyl acetate extract of *R. oligosporus* at the concentration of 5 μl $disc^{-1}$ had potential antifungal effect against the tested pathogens of *Colletotrichum* spp. The extract exhibited low to moderate antifungal effect against the tested pathogens. The percentage of inhibition of antifungal effect of the ethyl acetate extract of the *R. oligosporus* against *C. acutatum* KACC 40848, *C. gloeosporioides* KACC 40897, *C. higginsianum* KACC 40806, *C. orbiculare* KACC 40808, *C. coccodes* KACC 40008, *C. musae* KACC 40947, *C. boninense* KACC 40893, *C. liliacearum* KACC 40981, *C. caudatum* KACC 41028 and *C.* sp. KACC 40811 was found to be 44.4, 35.5, 40, 31.1, 33.3, 37.7, 40, 51.1, 28.8 and 28.8%, respectively. *C. acutatum* KACC 40848, *C. higginsianum* KACC 40806, *C. boninense* KACC 40893 and *C. liliacearum* KACC 40981 were found to be the most susceptible fungal pathogens to the ethyl acetate extract of the *R. oligosporus*.

Antagonistic effect of *R. oligosporus* in dual culture

The *Rhizopus oligosporus* showed potential antagonistic effect of antifungal activity against the tested pathogens of *Colletotrichum* spp. As shown in Table 2, the *R. oligosporus* in dual culture showed remarkable inhibitory effect against *C. acutatum* KACC 40848 (62.2%), *C. gloeosporioides* KACC 0897 (64.4%), *C. higginsianum* KACC 40806 (64.4%), *C. orbiculare* KACC 40808 (66.6%), *C. coccodes* KACC 40008 (71.1%), *C. musae* KACC 40947 (77.7%), *C. boninense* KACC 40893 (64.4%), *C. liliacearum* KACC 40981 (60%), *C. caudatum* KACC 41028 (68.8%) and *C.* sp. KACC 40811 (66.6%). *R. oligosporus* displayed higher antifungal effect in dual culture against *C. coccodes* KACC 40008, *C. musae* KACC 40947 and *C. caudatum* KACC 41028. However, other fungi were also inhibited moderately (Table 2).

Effect of *R. oligosporus* on the morphology of tested fungi

As shown in the Fig. 1-4, *R. oligosporus* had a potential detrimental effect on the morphology of the tested fungi of *Colletotrichum* spp. such as *C. orbiculare* KACC 40808, *C. musae* KACC 40947, *C. higgnsianum* KACC 40806 and *C.* sp. KACC 40811. *R. oligosporus* in

Table 1. Antagonistic effect of *Rhizopus oligosporus* against anthracnose fungi of *Colletotrichum* spp. in dual culture

Test pathogens	Antagonistic effect in dual culture	
	Radial growth (mm)	Radial growth inhibition (%)
Colletotrichum acutatum KACC 40848	17	62.2
Colletotrichum gloeosporioides KACC 40897	16	64.4
Colletotrichum higginsianum KACC 40806	16	64.4
Colletotrichum orbiculare KACC 40808	15	66.6
Colletotrichum coccodes KACC 40008	13	71.1
Colletotrichum musae KACC 40947	10	77.7
Colletotrichum boninense KACC 40893	16	64.4
Colletotrichum liliacearum KACC 40981	18	60.0
Colletotrichum caudatum KACC 41028	14	68.8
Colletotrichum sp. KACC 40811	15	66.6

Table 2. Antifungal effect of ethyl acetate extract of *Rhizopus oligosporus* against anthracnose fungi of *Colletotrichum* spp.

Test pathogens	Antifungal effect of ethyl acetate extract (5 μl $disc^{-1}$)	
	Radial growth (mm)	Radial growth inhibition (%)
Colletotrichum acutatum KACC 40848	25	44.4
Colletotrichum gloeosporioides KACC 40897	29	35.5
Colletotrichum higginsianum KACC 40806	27	40.0
Colletotrichum orbiculare KACC 40808	31	31.1
Colletotrichum coccodes KACC 40008	30	33.3
Colletotrichum musae KACC 40947	28	37.7
Colletotrichum boninense KACC 40893	27	40.0
Colletotrichum liliacearum KACC 40981	22	51.1
Colletotrichum caudatum KACC 41028	32	28.8
Colletotrichum sp. KACC 40811	32	28.8

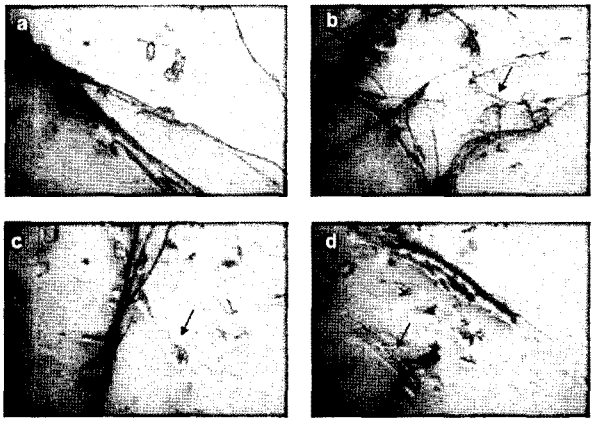

Fig. 1. Effect of *R. oligosporus* on the morphology of the tested pathogen of *Colletotrichum orbiculare* KACC 40808 in dual culture. a) control; b) empty cell formation; c) lysed cell; d) distorted cells.

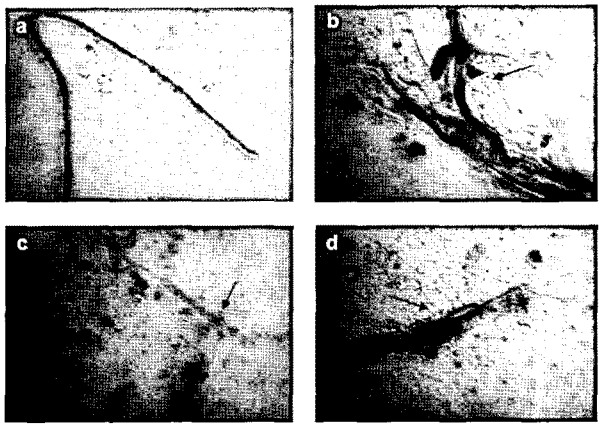

Fig. 2. Effect of *R. oligosporus* on the morphology of the tested pathogen of *Colletotrichum musae* KACC 40947 in dual culture. a) control; b) lysed and distorted cells; c) empty cell formation; d) distorted cells.

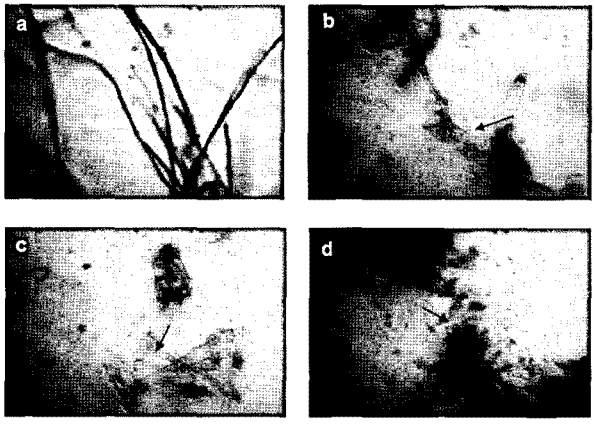

Fig. 3. Effect of *R. oligosporus* on the morphology of the tested pathogen of *Colletotrichum higgnsianum* KACC 40806 in dual culture. a) control; b) lysed and distorted cells; c) swelling and empty cell formation; d) distorted cells.

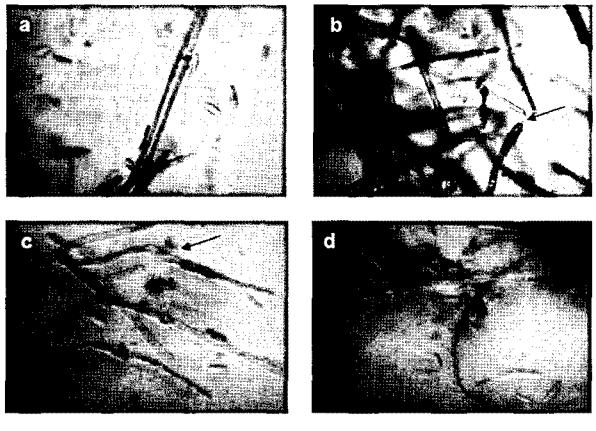

Fig. 4. Effect of *R. oligosporus* on the morphology of the tested pathogen of *Colletotrichum sp.* KACC 40811 in dual culture. a) control; b) swelling and deformed cells; c) lysed and distorted cells; d) swelling and empty cell formation.

dual culture showed remarkable deteriorative effect on the mycelium of the tested fungi such as wrinkle abnormalities, abnormal cell formation, lysis of mycelium, empty cell formation, distorted cell formation and breakage of the mycelium (Fig. 1-4).

DISCUSSION

The use of biological control methods to reduce disease incidence caused by plant pathogens is continually being developed and is being used in a variety of crops, fruits and vegetable plants(Soytong et al., 2001). Several researchers have reported the biocontrol efficacy of fungal pathogens to inhibit the growth of several plant pathogens including *Colletotrichum* spp. In this study, we tested the efficacy of *R. oligosporus* and its ethyl acetate extract against a panel of the plant pathogenic fungi of *Colletotrichum* spp. which cause severe disease of anthracnose in apple fruits growing in Korea using a dual culture antagonistic technique and disc diffusion assay to prove it as a potential biocontrol agent. In the present study, the ethyl acetate extract of *R. oligosporus* considerably inhibited the growth of the tested pathogens of *Colletotrichum* spp. Recently, it has been reported that *Colombian propolis* extracts of microbial origin exerted antifungal activity against some anthracnose fungi (Meneses et al., 2009). In further, the *R. oligosporus*also revealed strong antagonistic effect as well as showed potential detrimental effect on the morphology of the tested pathogens of *Colletotrichum* spp. showing wrinkle abnormalities, abnormal cell formation, lysis of mycelium and empty cell formation. Similar findings on the morphological abnormalities were observed using the extract of microbial origin against *Colletotrichum muase*(Taechowisan et al., 2009).

Secondary metabolites produced by microorganisms can be used potentially for controlling of many plant fungal diseases. Earlier studies on the analysis of antifungal effect of various fungal pathogens showed that they hadvarying degree of antifungal effect against different anthracnose fungi(Jeon et al., 2009). Our study revealed comparatively better results of the antifungal effect of *R. oligosporus* and its ethyl acetate extract against the tested pathogens of *Colletotrichum* spp. It has been reported that biocontrol products have significantly reduced the incidence of fungal disease when compared with chemical treatment(Soytong et al., 2005). These results suggest the availability of various crude extracts from the different origins for trials in controlling such anthracnose fungal pathogens. These pathogens are responsible for serious fungal disorders in apple fruits in various parts of the world and, although control measures are available, they are of limited effectiveness(Kefialew and Ayalew, 2008). As a result, work on alternative approaches to control such pathogens is important. Therefore, it can be estimated that *R. oligosporus* and its ethyl acetate extract have strong antifungal effect and can be used as in the biocontrol of the fungal pathogens of *Colletotrichum* spp. causing anthracnose disease in apple fruits in Korea.

In conclusion, the primary significance of this study is the observation that *R. oligosporus* and its ethyl acetate extract could effectively inhibit the growth of anthracnose fungi that have caused apple growers to seek various biocontrol strategies. To our knowledge, this is the first study to apply *R. oligosporus* and its ethyl acetate extract successfully to apple anthracnose fungal pathogens. Thus we anticipate developing and identifying novel bioactive molecules present in the ethyl acetate extract of *R. oligosporus* which might be responsible for this antifungal effect. We also believe that such antifungal effects of *R. olisosporus*, a food grade fungus and its ethyl acetate extract on apple anthracnose fungi will significantly contribute as a potential supplement to control anthracnose fungi. Thus from the above findings, it can be concluded that development of new biocontrol agents from microbial origin could be considered as an affective addition to plant pathology as the fast and reliable alternative to control pre-harves and post-harvest fungal pathogens.

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