Biological Control xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Isolation of indigenous antagonistic microorganism to inhibit *Rigidoporus microporus* and other plant pathogens and analysis of the bioactive compounds

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ARTICLE INFO

Keywords:
Bioactive compounds
Plant pathogens
Rigidoporus microporus
Trichoderma asperellum

ABSTRACT

The objectives of this study were to screen chitinase producing microorganisms to inhibit *Rigidoporus microporus* (white root rot) and to analyze the bioactive compounds produced by the selected strain using gas chromatography mass spectrometry (GC–MS). A total of 148 microbial isolates were screened from different sources such as composts and soils for chitinolytic activity by colloidal chitin hydrolysis, and then tested for inhibition by dual culture technique. The result showed that 78 isolates exhibited inhibitory activity against *R. microporus*. Among them, the fungal strain F14 showed the most chitinase production. It was identified as *Trichoderma asperellum* based on 18S rDNA. *T. asperellum* F14 was then cultured in 1/5 potato dextrose broth for 28 days to obtain the bioactive compounds using various type of solvents including methanol, n-butanol, ethyl acetate and hexane. Crude ethyl acetate extract displayed the highest inhibitory activity against many plant pathogens such as *Agrobacterium tumefaciens, Erwinia carotovora* subsp. *carotovora*, *Xanthomonas oryzae*, *Aspergillus parasiticus*, *Fusarium culmorum*, *F. merismoides*, *F. oxysporum f.* sp. *gladioli*, *R. microporus*, *Verticillium albo-atrum* and *V. dahliae*. The GC–MS analysis of the bioactive compounds produced by *T. asperellum* F14 showed that they were composed of many known bioactivities. This study indicates that *T. asperellum* F14 can be used to inhibit white root rot disease and other plant diseases.

1. Introduction

Rubber, rice, cassava and tropical fruit are the main agricultural export products of Thailand (Phairuang et al., 2017). In 2017, Thailand's total export value of agricultural export products was 7,365,792 million baht (OAE, 2018). However, it was reported that the yield losses caused by plant pathogenic microorganisms such as bacteria, actinomycetes and fungi. Especially *Rigidoporus microporus* is the main causes of white root rot disease of rubber trees in rubber plantations (Kaewchai et al., 2010; Jayasuriya and Thennakoon, 2007). Moreover, *Erwinia carotovora* subsp. *carotovora* and *Xanthomonas oryzae* are the bacterial pathogen of soft rot disease on cassava tubers and stems and leaf blight of rice disease, respectively (Shalini et al., 2017; McCallum et al., 2017). In some countries, it is the dominant cause of economic losses to rubber, cassava and rice farmers, among diseases and pests which yield losses of up half in old rubber plantations (Ogbebor et al., 2015) and rice yield losses up to 80% (Perumalsamy et al., 2010).

These diseases are commonly controlled by the chemical bactericides or fungicides, which can negatively impact on human health, pollute to the environment, and leave residues in agricultural soil (Haggag and Mohamed, 2007). Moreover, several plant pathogenic microorganisms have developed resistance to chemical fungicides (Barickman et al., 2017). To avoid the negative or harmful effects of chemicals, the biological control is a preferred alternative for disease control by reducing exposure to inoculums and inhibiting spread of the disease. Several studies were conducted to evaluate the ability of selected microorganisms against *R. microporus* and other plant pathogens. Among those antagonistic microorganisms, *Trichoderma* sp. showed the highest potential that can be used as biocontrol agents. According to

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https://doi.org/10.1016/j.biocontrol.2018.01.007
Received 18 July 2017; Received in revised form 15 January 2018; Accepted 16 January 2018

report of Kaewchai and Soytong (2010) that tested ten species of antagonistic fungi against *R. microporus* and found that *A. niger*, *Ch. bostrychodes*, *Ch. cupreum*, *T. hamatum* and *T. harzianum* could inhibit the growth of *R. microporus* over 50% by rapid growth over the colony within a few days. Similar to Sharma et al. (2017) also reported that *T. harzianum* was highly antagonistic against four fungal pathogens *Colletotrichum capsici*, *C. truncatum*, *F. oxysporum* and *Gloesercospora sorghi*. Moreover, Singh et al. (2016) also reported that *T. harzianum* was highly antagonistic against mycelium of *Rhizoctonia solani* which was cause of sheath blight in rice. A side from that, Aleandri et al. (2015) isolated the *Trichoderma* species from the rhizosphere of holm oak, lavender and olive in nursery pots, using the soil dilution plate method, and tested them for the control of *Rhizoctonia solani*, *Phytophthora cinnamomic*, *Sclerotinia sclerotiorum*, and *Verticillium dahliae* using a dual culture technique. *T. asperellum* showed the highest mycelial growth inhibition of *R. solani*, *S. sclerotiorum*, and *V. dahlia*, while *T. hamatum* showed the largest inhibition zone of *P. cinnamomic*.

In this study, we screened the antagonistic microorganisms from composts and soils that showed the ability to control *R. microporus*. Subsequently, the selected microorganisms were identified and evaluated the production of bioactive compounds. In addition, the GC–MS analysis of the bioactive compounds produced by the selected strain was studied. Overall, our result suggested that selected antagonistic strain is potential biocontrol agents of *R. microporus* and other plant pathogens.

2. Materials and methods

2.1. Pathogens

The reference isolate of *R. microporus* was obtained from the Office of Agricultural Research and Development Region 8, Songkhla, Thailand. Three pathogenic fungi, *Aspergillus flavus, Fusarium moniliforme* and *Penicillium citrinum* were obtained from the Enzyme Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Thailand. Three pathogenic bacteria, *Agrobacterium tumefaciens, Erwinia carotovora* subsp. *carotovora* and *Xanthomonas oryzae* and seven pathogenic fungi, *Aspergillus parasiticus*, *Fusarium culmorum*, *F. merismoides, F. oxysporum f.* sp. *gladioli*, *R. microporus*, *Verticillium albo-atrum* and *V. dahliae* were obtained from the stock culture of Toyama Prefectural University, Toyama, Japan.

2.2. Isolation of bacteria, actinomycetes and fungi showing chitinolytic activity

The microorganisms were isolated from various sources such as composting pile of decomposed rubber bark, lutoid, activated sludge, fats from grease and shrimp shell; liquid inoculum of composted molasses and shrimp shell fermented by effective microorganisms; pile of shrimp shell from the frozen shrimp factory and soil from naturally grown bamboo, in Hat Yai, Thailand. Fifty grams of sample were added to 450 mL of sterile 0.85% NaCl. The solution was mixed and then allowed to settle. One milliliter of the solution was serially diluted and 0.1 mL was spread evenly on an agar plate of the minimal medium (K_2HPO_4 1.5 g/L, $NaNO_3$ 2 g/L, $MgSO_4$ 1.4 g/L, agar 15 g/L) supplemented with 1.0% colloidal chitin. The pH of the medium was adjusted to 7.0 for bacteria and actinomycetes and 5.6 for fungi (Malathi and Viswanathan, 2013). The plates were incubated at room temperature (30 °C) until colonies developed for 3–7 days. The various colonies were selected based on their morphology, size and color appearance. These isolated microorganisms were purified by repeated streaking and were kept at 4 °C for further identification and testing of growth inhibition of *R. microporus*.

2.3. Inhibition of R. microporus by dual culture technique

Inhibition of *R. microporus* growth by the selected microorganisms was determined by dual culture technique (Jayasuriya and Thennakoon, 2007). Isolated microorganisms were prepared in cultures on appropriate culture media: nutrient agar (NA) for bacteria; starch casein agar (SCA) for actinomycetes; and potato dextrose agar (PDA) for fungi. The plates were incubated at 30 °C for 3–5 days. *R. microporus* was cultured on PDA plate and incubated at room temperature for 3 days. An agar plug of a 3-day old culture of *R. microporus* was placed approximately 3 cm from the edge of the dual culture PDA plates (90 mm diameter). A loopful of isolated bacteria was streaked opposite to the *R. microporus* agar plug. Alternatively, an agar plug of isolated actinomycetes or fungi from 3 to 5 days old culture was placed on the plate opposite to the *R. microporus* agar plug. The dual culture plates were incubated at room temperature and monitored daily for microbial growth over 7 days (Kumar et al., 2012). In the control experiment, only an agar plug of *R. microporus* was placed on the PDA plate. The percentage inhibition of radial growth was calculated according to Adebola and Amadi (2010):

$$\text{Percentage inhibition}(\%) = 100 \times ((R1-R2)/R1) \quad (1)$$

where R1 = radius of radial growth of *R. microporus* towards the opposite side on the control plate; and R2 = radius of radial growth of *R. microporus* towards the antagonist on the test plate. All treatments were performed in triplicate.

The mycelial portion in the interaction area with a prominent antagonist was viewed under the scanning electron microscope.

2.4. Determination of chitinase and β-1,3 glucanase activities

Colloidal chitin was prepared from chitin powder (Hi Media) by the method of Saima et al. (2013). The antagonistic isolates were cultured in 100 mL of the minimal medium without agar, amended with 1.0 g colloidal chitin, in 250 mL Erlenmeyer flasks at room temperature under 150 rpm shaking. The bacterial isolates were cultivated for 3 days, and actinomycetes and fungal isolates for 7 days (Malathi and Viswanathan, 2013). The supernatant was collected by centrifugation at 6060*g* and 4 °C for 15 min to determine chitinase activity by the method of Ahmadi et al. (2008) and β-1,3 glucanase activity by the method of Marcello et al. (2010). One unit of chitinase activity was defined as the amount of enzyme that released 1 µmol of N-acetyl-D-glucosamine (GlcNAc) per min under the assay conditions. One unit of β-1,3 glucanase activity was defined as the amount of enzyme that released 1 µmol of glucose per min under the assay conditions. All treatments were performed in triplicate.

2.5. Identification of the isolated microorganisms

Those microbial isolates that showed the highest chitinase activity were identified by the morphology and DNA sequencing. First, genomic DNA was extracted by using the E.Z.N.A. ® Tissue DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's recommendations. The 16S rDNA was PCR amplified using the 8F and 1492R primers for bacteria and actinomycetes. Amplification by PCR was performed according to Hayes and Lovley (2002). For the fungal isolates, the 18S rDNA was amplified from the genomic DNA with NS1 and EF3 primers. Amplification by PCR was performed according to Hoshino and Morimoto (2008). The PCR products were confirmed by agarose gel electrophoresis of 5 µL of the reaction product in a 1% agarose gel. The PCR products were purified with the HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Biotech Co., Taipei County, Taiwan). The purified PCR products were sequenced by BioDesign. Co., Ltd., Thailand. The similarity of the resulting DNA sequences was analyzed with NCBI blast tool available online at www.ncbi.nlm.nih.gov/blast.