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Full Length Research Paper

Antifungal activity of rice straw extract on some phytopathogenic fungi

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The antifungal activity of allelochemicals extracted from rice straw on the radial growth rate and the activity of some hydrolyzing enzymes of *Aspergillus flavus, Alternaria alternata and Botrytis cinerea* were studied *in vitro.* Five different concentrations (2, 4, 6, 8 and 10%, w/v) of water, methanol and acetone extracts of rice straw were tested. All extracts significantly (P<0.05) inhibited the radial growth rate and protease, carboxymethyl cellulase (CMCase) and amylase activities of the tested fungal species. The most potent solvent was methanol. The present study suggests that rice straw extract had antifungal properties, thus it can be used as a natural alternative approach to synthetic fungicide.

Key words: Rice straw, allelochemicals, antifungal, *Aspergillus flavus, Alternaria alternata, Botrytis cinerea*, amylase, protease, carboxymethyl cellulase.

INTRODUCTION

Plants are constantly exposed and threatened by a variety of pathogenic microorganisms present in their environments. Phytopathogenic fungi are the most problematic pests of agricultural crops worldwide (Savary et al., 2006). *Aspergillus flavus*, *Alternaria alternata* and *Botrytis cinerea* are among the prevalent pathogens causing diseases in many economic crop species in humid and semiarid areas worldwide (Timmer et al., 2003; Guimarães et al., 2004; Horn and Dorner, 2009). Although, there are fungicides for the control of phytopathogenic fungi, many classes of fungicides have failed due to their genetic plasticity. As the use of synthetic fungicides is being reduced globally (Waard et al., 1993), the use of agrochemicals to control plant diseases has recently attracted widespread interest (Gan et al., 2006; Kong, 2007; Gil et al., 2008).

Plants produce many kinds of low-molecular-mass secondary metabolites that are generally non-essential for the basic metabolic processes of the plant. Among these secondary plant metabolites, some are known as allelochemicals that improved defense against other plant competition, microbial attack or insect/animal predation. Allelochemicals can be applied in biological control of weeds and phytopathogens (Rice, 1995).

Rice (*Oryza sativa* L.) is one of the principal food crops in the world. Large amount of rice straw residues are produced after harves. A major portion of this residue is disposed by burning which cause environmental problems. Several allelochemicals belong to different classes of secondary metabolites detected in rice residues and root exudates. These include phenolics (Mattice et al., 1998; Seal et al., 2004), flavones, diterpenes, steroids (Kong et al., 2004; Macias et al., 2006) and momilactone B (Kato-Noguchi and Ino, 2005).

Previous studies on rice allelochemicals have mainly focused on weed suppression (Olofsdotter, 2001; Narwall, 2002; Xuan et al., 2005). Accordingly, the aim of the present work was to assess the effect of allelochemicals extracted from rice straw on the growth and enzyme activities of selected phytopathogenic fungi.

MATERIALS AND METHODS

Rice (*O. sativa* L., cv. Giza 101) straw was collected from a rice

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field (El Mansura, Egypt), air dried and ground to very fine chops. A virulent pure isolates of *A. flavus, A. alternata* and *B. cinerea* were obtained from the Department of Plant Pathology, Agricultural Research Center, Giza, Egypt.

Preparation of rice straw extracts

Extraction of allelochemicals from rice straw was carried out as described by Kagale et al. (2004), with some modifications. Briefly, 100 g of the chopped rice straw was added to 100 ml of sterile distilled water, ethanol or acetone (1:1 w/v) and incubated at room temperature for 24 h. Thereafter, the slurry was filtered through double-layered cheesecloth, followed by centrifugation at 5000 rpm for 10 min. The water extract was used directly for the antifungal assay while, the solvent extracts were evaporated completely under reduced pressure, and the residues were dissolved in 100 ml of sterile distilled water. Each of the prepared extracts was diluted to 4, 8, 12, 16 and 20% (w/v) with sterile distilled water. All extracts were filter sterilized using 0.22 mm filters.

Antifungal assay

The technique of Nene and Thapliyal (1979) was followed to study antifungal activity of rice straw extracts. Briefly, 50 ml of the 4, 8, 12, 16 and 20% (w/v) dilutions of each extract was mixed separately with 50 ml of 2X PDA (potato dextrose agar) medium to obtain final concentrations of 2, 4, 6, 8 and 10%, respectively, of each extract. 20 ml of this mixture was poured into the sterile Petri plates and a 5 mm mycelial disc of the test fungus was placed at the center of the plate, followed by incubation at room temperature (28°C). 1X PDA without leaf extract served as the control. The diameter of the radial growth of the fungi were measured daily and then used to determine the radial growth rate of the test fungi.

Assay of pathogenic enzymes

To study the effect of allelochemicals present in rice straw on the activity of some pathogenic enzymes (protease, carboxymethyl cellulase (CMCase) and amylase), the tested fungal species were grown in 250 ml flasks containing 50 ml of the 4, 8, 12, 16 and 20% dilutions of methanolic extracts and 50 ml of 2X Waksman's medium containing the appropriate substrate, to obtain final concentrations of 2, 4, 6, 8 and 10%, respectively. Thereafter, cultures were filtered through sterile Whatman filter paper No.1 and the filtrate was collected for assay of the pathogenic enzymes. The medium was prepared by replacing glucose content of the original medium with either carboxymethyl cellulose or solubilize starch (2% of each) to measure the production of CMCase and amylase, respectively. The peptone content of the original medium was also replaced with casein to measure the production of protease.

Protease activity assay

Protease activity was determined according to Kunitz (1947) using casein as a substrate. The reaction mixture containing 1 ml of the above prepared filtrate and 1 ml 1% (w/v) casein in 50 mM citrate phosphate buffer at pH 6.0 was incubated at 30°C for 20 min. The reaction was stopped with 3 ml 10% (w/v) TCA (trichloroacetic) acid and the mixture was centrifuged at 5,000 *g* for 10 min. The optical density of the supernatant was measured at 750 nm after Folin Reaction according to the method of Lowery et al. (1951). One unit protease activity was defined as the amount of enzyme liberating 1 µg tyrosine per minute under assay conditions. Enzyme units were measured using tyrosine (30 to 300 µg) as a standard.

CMCase activity assay

CMCase activity was assayed according to DNS method (Berlin et al., 2005) with little modifications. The reaction mixture containing 100 µl of filtrate, and 1.9 ml of 1% (w/v) CMC–Na (carboxymethyl cellulose) solution, was incubated in 50°C for 20 min. Thereafter, 600 µl of DNS (2,4-dinitrosalicylic acid) was added and thoroughly mixed. The mixture was heated in 100°C water bath for 10 min and then cooled. The mixture was colorimetrically determined (A_{520}). One unit of cellulase activity was defined as the amount of enzyme that produced l µg reducing sugar (glucose equivalents) per minute under the above assay condition. Glucose (Sigma, St Louise, Mo, USA) was used to prepare the standard curve under the same condition.

Amylase activity assay

Total amylase activity was assayed according to DNS method (Wu et al., 2008). The mixture contained 100 µl of the above prepared filtrate, 2 ml of 0.15 M citrate-phosphate buffer (pH 5.0) and 3 ml of 4% (w/v) soluble starch previously maintained at 40°C for 15 min. The reaction mixture was incubated at 40°C for 10 min. The enzymatic reaction was terminated by addition of 600 µl of DNS, and the released reducing sugar (glucose) was colorimetrically determined (A_{520}). One unit of amylase activity was defined as the amount of enzyme that releases 1 µg reducing sugar (glucose equivalents) per minute under the above assay conditions. Glucose was used to prepare standard curve.

Statistical analysis

Each *in vitro* experiment was performed in triplicate and repeated three times. Results were expressed as means ± standard error (SE) of three parallel measurements with one way ANOVA. Statistical analysis was performed by using SPSS version 15. Probability $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

In the current study, we evaluated the antifungal activity of allelochemicals extracted from rice straw on some phytopathogenic fungi including *A. flavus, A. alternata* and *B. cinerea.* The effect of different concentrations (2, 4, 6, 8 and 10%) of water, methanol and acetone extracts of rice straw on the radial growth rate of the tested fungal species is presented in Figure 1. All extracts significantly ($P<0.05$) inhibited the radial growth rate of the tested phytopathogens.

The inhibition in the radial growth rate was concentration and solvent dependent, where the most potent solvent was methanol followed by acetone. At concentration of 10%, methanolic extract caused about 76, 78 and 93% inhibition in the radial growth rate of *A. flavus, A. alternata* and *B. cinerea,* respectively.

The inhibition in the mycelial growth may be attributed to the cyto-morphological modifications induced by allelochemicals present in rice straw. In this context, in a previous work, Kocić-Tanackov et al. (2011) proved that basil (*Ocimum basilicum* L.) extract caused hyphae deformations of *Fusarium* sp. with a frequent occurrence of fragmentations, thickenings and diminished

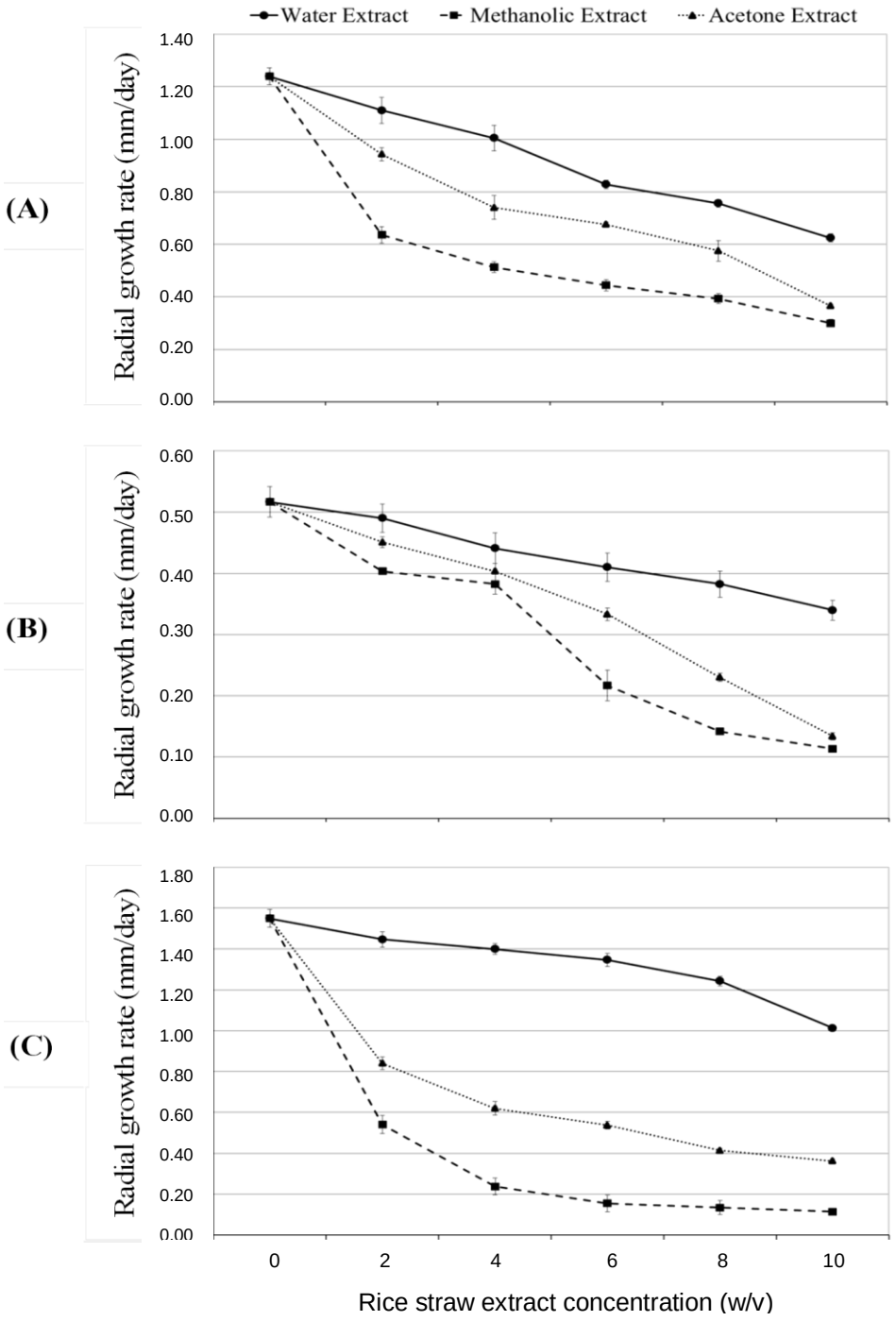

Figure 1. Effect of different rice straw extracts on the radial growth rate (mm/day) of *A. flavus* (A), *A. alternata* (B) and *B. cinerea* (C). Values are expressed as mean ± standard error of three replicates.

sporulation. In accordance with the present results, Nwachukwu and Umechuruba (2001) reported that leaf extracts of several plant species (basil, bitter, lemon grass, neem and paw-paw) significantly inhibited the radial growth of several phytopathogens including *A. flavus*. Also, the inhibitory effect of plant extracts on mycelial growth of *B. cinerea* was demonstrated (Bouchra et al., 2003; Ribera et al., 2008). Furthermore, Fawzi et al. (2009) reported that cinnamon, halfa barr, laurel, avocado and ginger extracts have strong antifungal activity with significant inhibition on the growth of *A. alternate*. In addition, Hao et al. (2010) showed that root exudates from rice significantly decreased spore germination and sporulation of *Fusarium oxysporum*.

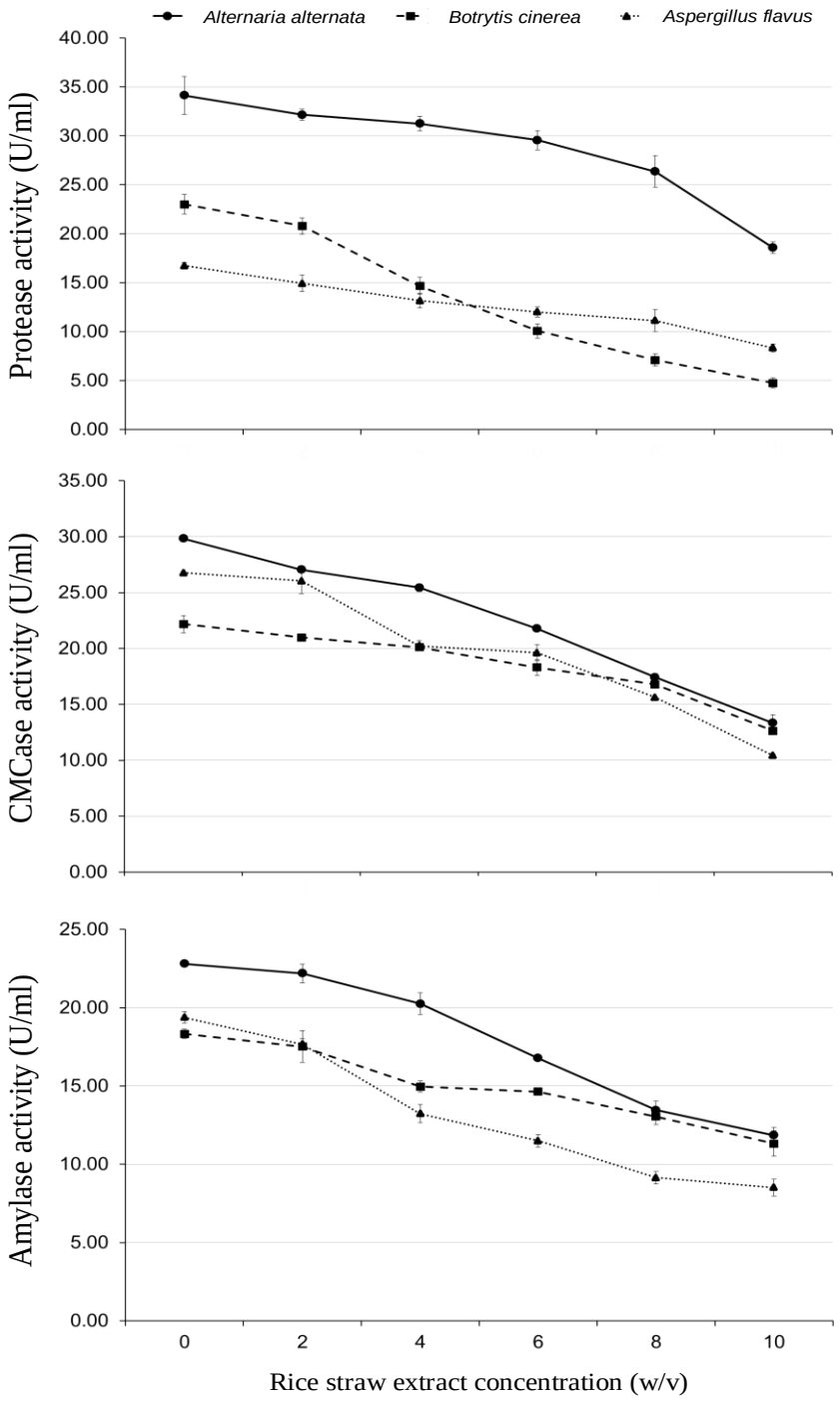

Figure 2. Effect of methanolic rice straw extract on protease (A), CMCase (B) and amylase (C) activities of *A. flavus, A. alternata* and *B. cinerea.* Values are expressed as mean ± standard error of three replicates.

Based on the results of the antifungal assay, the methanolic extract was selected to study its effect on the production of the hydrolyzing enzymes protease, CMCase and amylase by *A. flavus, A. alternata* and *B. cinerea*. Generally, enzyme activities of the tested fungal species in liquid cultures were significantly ($P<0.05$) suppressed by methanolic extract of rice straw (Figure 2). The degree of inhibition was dose dependent.

In this respect, Muhsin et al. (2001) reported high reductions in the activities of amylase, cellulase and

protease for *A. flavus*, *A. alternate* and other 16 fungal species subjected to garlic extract in the growth medium. Furthermore, Wu et al. (2008) demonstrated that the artificially applied coumarin inhibited the activity of protease, amylase and cellulase of *F. oxysporum*. In addition, Fawzi et al. (2009) reported that cinnamon, halfa barr, laurel, avocado and ginger extracts inhibited the production of glucosidase, pectin lyase and protease by *A. alternata* and *F. oxysporum*.

Finally, the significant inhibition in the radial growth rate and enzymes activity of *A. flavus*, *A. alternata* and *B. cinerea* by rice straw treatment suggests its efficiency as a natural environmental safe approach for controlling these phytopathogens.

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