OLEIC ACID: A MYCOHERBICIDE FROM Cochliobolus lunatus FOR MANAGEMENT OF Echinochloa crusgalli IN PADDY

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ABSTRACT

Cochliobolus lunatus a fungal pathogen of Echinochloa crusgalli (major weed in paddy) was in vitro isolated and proved its pathogenicity against E. crusgalli (a major weed of paddy). Furthermore, phytotoxic compounds responsible for pathogenicity were also isolated from fungus and tested it against E. crusgalli under laboratory conditions. The effective fraction showing 100% mortality of weed was purified through reverse phase HPLC and characterized through NMR, IR and LC-MS spectroscopy. Based on the spectral analysis, the compound was identified as Oleic acid with molecular weight 283 and molecular formula C_{18}H_{34}O_{2}.

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1 Introduction

Barnyard grass (E. crusgalli) is the most dominant weed in both transplanted and direct seeded rice throughout the world (Holm et al., 1977). It reduces rice yield up to 40% annually in direct seeded condition in Malaysia (Azmi, 1988), about 21% in Indonesia (Tjitorsemoto, 1994), 25 to 47% in India (Manandhar et al., 2007), 90% in Philippines (IRRI, 1990) and 85% in China (Chang, 1970). With an increase in direct seeding rice practices, barnyard grass is becoming a major problem in rice fields (Loux & Barry, 1991; Azmi & Baki, 2002). This particular weed is capable of accumulating significant amounts of macronutrients and negatively impacting crop plants, especially when these nutrients are under scarcity (Maun & Barrett 1986). Many post emergence chemical herbicides are available for the control of weeds but with continuous usages of herbicide has lead to resistant in weeds and also accumulation of chemical residues leading to environmental pollutions and health hazards. Thus many alternative ways are being in search to overcome the problem.

Biological control of weeds by using plant pathogens has gained acceptance as a practically safe and environmentally beneficial weed management practices of agro ecosystems (Charudattan, 1986; Flint & Thomson, 2000; Pemberton & Strong, 2000; Bouda et al., 2001). An alternative approach of weed control is the use of toxic metabolites produced by weed pathogens for integrated weed control programmes. The replacement or the integration of traditional chemical control methods of plant disease by the use of microorganisms and or their bioactive metabolites reduces the environmental impact of agricultural productions and gives effort to the agricultural biological production which is more and more present in the national and international markets. In this context these bioactive secondary metabolites could play an interesting role in the induction of disease symptoms (phytotoxins, antibiotics and phytohormones) or of defense response (elicitors). They have built-in species-selectivity as they have isolated from host-specific plant pathogens and weed hosts (Pearce et al., 1997). This is a highly desirable property in avoiding injury to crop plants which is a goal of synthetic herbicide development programs (Heap, 2005).

Use of phytotoxins from weed pathogens is also a potential approach to control the noxious weeds (Strobel & Sugawara, 1986; Saxena & Pandey, 2001; Vurro et al., 2001; Singh & Angrias 2003). Phytotoxins are alternative weed control methods different in chemical structure and size. These bioactive components invade into the host plant, cause pathogenicity, destroy their structure and lead them to produce necrotic lesions or chlorosis (Li et al., 2003). They are less toxic to mammalian systems, easily degraded and so far result in no biological disaster compared to chemical herbicides (Charudattan, 1991). Leakage of cell constituents frequently occurs after the application of host-specific phytotoxins and non-host specific phytotoxins. Few examples like Curvularia sps. are reported to produce secondary metabolites belonging to different classes of chemicals and possessing different toxic activities such as phytotoxic, antifungal and cytotoxic activities (Coombe et al., 1968; Hartwing & Stossel, 1979; Wells et al., 1988; Kobayashi et al., 1988; Abraham et al., 1995; Alam et al., 1997; Jadulco et al., 2002). The aim of this study was to investigate the effect of phytotoxins isolated from C. lunatus for the control of E. crusgalli in paddy.

2 Materials and methods

2.1 Isolation, Identification and maintenance of C. lunatus

The C. lunatus strain was isolated from infected leaf of E. crusgalli and tested its pathogenicity on same weed as method described by Jyothi et al.(2013). To identify secondary metabolites from isolated strain, fungus was cultured on rice grain media. 250g of rice grain was soaked in double distilled water for overnight and next day the excess water was removed by using muslin cloth. The moistened substrate were then supplemented with yeast extract (3g) and molasses (10ml) mixed well and transferred into polypropylene (PP) bags (65x40cm) and then covered with sponges at both sides. Then the bags were autoclaved at 121°C, 15lb pressure for 30 min. After sterilization, the bags were transferred to laminar air flow bench and inoculated with fungal pathogens of 4 days old seed culture containing 1 X 10⁶ conidia/ml. Then the bags were tightly closed with the sponges to avoid cross contamination and incubated in growth chamber for 14 days.

2.2 Extraction of phytotoxins from fermented rice substrate

For extraction of phytotoxins from fermented rice substrate, the substrate was ground to make powder, and to this added ethyl acetate (1:2 ratio) and kept on shaker for overnight at room temperature. Then, the substrate was filtered through Whatman No: 41 filter paper and collected organic phase, concentrated using rotavaporator (Buchi R - 210®) to evaporate the solvent completely. The concentrated crude phytotoxins were collected and subjected to column chromatography for purification.

2.3 Purification of phytotoxins by open silica column chromatography

The active EtOAc crude extract was dissolved in methanol followed by mixing with silica gel and the solvent was removed using vacuum under heating condition. Column was packed with silica gel (100 - 200 mesh) and the slurry was applied at the top of the column and eluted with Hexane: Ethyl acetate (EtOAc; commercial grade). Initially the column was run with 300 ml of 100% hexane (Low polar), followed by gradual increase of polarity with increasing concentrations of EtOAc and final elution with 300ml of 100% methanol. The compounds were eluted depending on the polarity of the mobile phase and the purity of the fractions was monitored by TLC and pooled the identical fractions followed by concentration using Rotovapor.

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2.4 Leaf bioassay of purified fractions on target weed

The phytotoxin fractions collected from column were tested for their phytotoxicity on target weed through leaf bioassay under in vitro conditions and also on host crop. The weed and rice leaves were collected from one month old seedlings, washed under running tap water followed by surface sterilization with 0.5% NaOCl and washed three to four times with sterile distilled water. The each phytotoxin fraction (1000 ppm) were taken in sterilized test tubes and labeled and then weed and rice leaves were dipped in test tubes. Control was kept with sterile distilled water and also with solvent. Test tubes were incubated in plant growth room at 25°C and 80% RH at different intervals of 24, 48, and 72 hrs. The disease incidence were recorded at different intervals (24, 48 and 72 hours after incubation) using the standard scale as per Horsfall and Barrat, 1945.

2.5 Purification of phytotoxin compound by RP-HPLC

The active phytoxin fraction (fraction no -5) which showed 100% mortality on weed were further purified through RP-HPLC (Simazu) using a reverse phase C\textsubscript{18} column with two different solvents viz solvent A: water: 0.2% acetic acid and solvent B: methanol (Merck): 0.2% Acetic acid, Flow rate: 8ml/min, UV detection: 280nm. The peak eluted at RT 16.5min was collected and concentrated using lyophilisation. Then the purified phytotoxins fraction was subjected to NMR, IR and Mass spectroscopy for characterization of active compound.

2.6 Chemical characterization of active compound by NMR, IR and Mass spectroscopy

Infrared (IR, KBr) spectra were recorded on JASCO FT-IR spectrophotometer (Model 5300) with polystyrene as reference. \textsuperscript{1}\text{H}-NMR (400 MHz) and \textsuperscript{13}\text{C}-NMR (100 MHz) spectra were recorded on Bruker-AC-200 and Bruker Avance-400 Spectrophotometer with chloroform-d as solvent and TMS as reference (δ = 0 ppm). The chemical shifts are expressed in downfield from the signal of internal TMS. The purified active metabolite was vacuum dried under heating followed and purified metabolite was weighed separately and dissolved in CDCl\textsubscript{3} followed by recorded both \textsuperscript{1}\text{H} (proton) and \textsuperscript{13}\text{C} (carbon) NMR separately. Mass spectra were recorded using LC-MS-2010 (Shimadzu). The active metabolite was dissolved in minimum volume of methanol, filtered with 0.2μm syringe filter and injected in to LC-MS/MS (Agilent technologies). The mobile phase was used water and methanol (50: 50), flow rate 0.2 ml/min and the eluted peak was subjected to MS/MS analysis.

2.7 Comparison of active compound with standard through analytical HPLC:

The active metabolite was compared with standard Oleic acid procured from sigma using analytical HPLC method. The mobile phase was: methanol: water, detection at 280nm, flow rate 1ml/min, column C\textsubscript{18} with particle size 0.5μm.

2.8 Statistical analysis

Data obtained from all the experiments were analyzed by analysis of variance ANOVA using SPSS, version 20.0. Least significance difference (LSD) at 5 % level of significance (P = 0.05) was used to compare the mean values of different treatments in the experiment.

3 Results

3.1 Leaf bioassay of purified phytotoxin fractions

The phytotoxins extracted from C. lunatus were purified through column chromatography and collected eight different fractions and each fraction was tested for its activity on target weed and host crop (rice) through leaf bioassay. The bioassay results revealed that fraction five showed maximum phytotoxicity on weeds leaves (100%), followed by fraction four (90%) after 72hrs of incubation without any effect on host crop (Table 1). Least phytotoxicity was observed with fraction six followed by fraction one and four. No phytotoxic effect was observed with fraction eight on weed.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>24h Weed</th>
<th>48h Weed</th>
<th>72h Weed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 01</td>
<td>25.0±d</td>
<td>45.0±d</td>
<td>50.0±e</td>
</tr>
<tr>
<td>Fraction 02</td>
<td>31.6±c</td>
<td>53.3±c</td>
<td>71.7±c</td>
</tr>
<tr>
<td>Fraction 03</td>
<td>41.7±b</td>
<td>70.0±b</td>
<td>85.0±b</td>
</tr>
<tr>
<td>Fraction 04</td>
<td>50.0±b</td>
<td>81.6±a</td>
<td>90.0±a</td>
</tr>
<tr>
<td>Fraction 05</td>
<td>61.7±a</td>
<td>84.0±a</td>
<td>100±b</td>
</tr>
<tr>
<td>Fraction 06</td>
<td>15.0±f</td>
<td>30.00±e</td>
<td>40.0±e</td>
</tr>
<tr>
<td>Fraction 07</td>
<td>21.7±a</td>
<td>35.00d±a</td>
<td>28.3±c</td>
</tr>
<tr>
<td>Fraction 08</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>0.00±f</td>
<td>0.00±f</td>
<td>0.00±f</td>
</tr>
</tbody>
</table>

Values with different letters show significant difference (P<0.05) as determined by DMR Test. Mean of triplicate determination
Figure 1 IR spectrum of purified phytotoxin fraction of *C. lunatus*.

Figure 2 $^1$H NMR spectrum of purified phytotoxin fraction of *C. lunatus*.

Figure 3 $^{13}$C NMR spectrum of purified phytotoxin fraction of *C. lunatus*. 
3.2 Characterization of active phytotoxins

The active phytotoxins were further purified through RP-HPLC and characterized through NMR, IR and Mass spectroscopy. The IR spectra shown different functional groups at IR (KBr) cm⁻¹: 3000.00 (=C-H), 2849.32 (C-H), 1742.47 (C=O), 1726.03 (C=O), 1468.49 (= -CH₃), 1369.86 (C-H), 1271.23 (O-H), 1243.84, 1161.64, 1117.81, 969.86, 728.77 (Figure 1). The ¹HNMR spectrum recorded at (400 MHz, CDCl₃) δ ppm: 5.35 M06 (m12, 11), 2.36, 2.34 M01 (m 1, 4), 2.05 M03 (m 13, 10), 2.03, 2.01, 1.65, 1.60, 1.33, 1.28 M03 (m 5), 0.92, 0.89 M05 (1, 20), 0.83 (Figure 2) and ¹³CNMR spectrum showed peaks at (200 MHz, CDCl₃) δ ppm: 173.31, 129.99, 34.17, 31.89, 29.09, 22.66, and 14.10 (Figure 3). The LC-MS spectrum shown major peak of the compound at 283 molecular weight (Figure 4). Based on the spectral analysis, the active compound was identified as Oleic acid with molecular weight 283 and molucual formula C₁₈H₃₄O₂. The active compound Oleic acid was compared with that standard procured from Sigma through analytical HPLC observed similar peak for both the compounds at RT 6.72.

4 Discussions

Fraction 5 showing maximum phytotoxicity on weed and no effect on rice was selected and further purified characterized and found Oleic acid as a major compound which is having weedicidal properties. Small necrotic spots were observed after 24hrs of inoculation with phytotoxin and the phytotoxicity was increased with increase of incubation period. After 72hrs of incubation the weed leaves were completely wilted and dried. Zhan & Watson (2000) reported that phytotoxins extracted from weed pathogen C. lunatus showed significant reduction in the population of Echinochloa species. Juraimi et al., 2005 reported that the phytotoxins isolated from aqueous extracts of Exserohilum longirostratum are effectively suppressed the growth of different ecotypes of barnyard grass Jiang et al. (2008) isolated α,β- dehydrocurvularin from Curvularia eragrostidis and reported it as potential toxin for control of Digitaria sanguinalis weed.

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References


