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Isolation and Characterization of a Phytotoxin from *Rhizoctonia solani*: The Causal Agent of Rice Sheath Blight

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To isolate and characterize the bioactive components of the Rs-toxin produced by the rice sheath blight pathogen *R. solani*, various extraction and purification methods, several chemical reactions, ultraviolet- and visible-wavelength light scanning, gas chromatography/mass spectrometry (GC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS) analyses, were used. The leaf floating soakage method was established for evaluating the phytotoxicity of Rs-toxin. The results of Molish's reaction, ninhydrin test and Fehling's reaction suggested that the bioactive components of Rs-toxin were carbohydrates other than amino acids or proteins. By using GC/MS technique, the partially purified Rs-toxin was found to contain six saccharides. By means of peak area normalization, the relative amounts of these six saccharides were calculated. The result of LC/MS/MS indicated that neither phenylacetic acid nor its derivatives was detected in Rs-toxin. Taken together, it was concluded that Rs-toxin consists of six saccharides and has obvious maceration effects on rice leaf tissues.

Key Words: Rice sheath blight, *Rhizoctonia solani*, Phytotoxin, Gas chromatography/mass spectrometry, Liquid chromatography-tandem mass spectrometry, Saccharide.

INTRODUCTION

Sheath blight is one of the most serious and economically important diseases of rice (*Oriza sativa* L.) in most of rice-growing countries[1,2]. This disease is caused by a soil-borne fungus *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk], one of the most important fungal pathogens, which has a broad range of plant hosts and causes diverse necrotic symptoms such as sheath blight and root rot in mono- and dicotyledonous plants, respectively[3,4]. It is well-known that *R. solani* is recognized as a complex species consisting of at least 14 anastomosis groups (AGs) differing in morphology, pathogenicity, host range, molecular characteristics and distribution in nature[5-8]. In addition, 5 AGs of this fungus were further divided into intraspecific groups[6]. One important intraspecific group within the *R. solani* complex is AG-1 IA (*R. solani* AG-1 IA), the causal agent of rice sheath blight[2]. The taxonomy, biology, ecology and population genetics of *R. solani* have been well documented by many researchers[3,8-14].

Phytotoxins and cell wall-degrading enzymes are thought to be the two important pathogenicity determinants of *R. solani*[15-25]. Previous studies revealed that phytotoxins (Rs-toxin) produced by *R. solani* are involved in lesion development and thus play a significant role in pathogenesis[18,20-22,25,26]. Several investigations have been carried out to isolate and characterize the chemical constitution of Rs-toxin, but the results vary by study[17,20,27-33]. So far, several chemical substances, including phenyl acetic acid (PAA) and its derivatives[17,28,30-33], phenolic and glycosidic substances[27,29] and carbohydrates[20,26], have been reported to be the main components of Rs-toxin. Phenyl acetic acid, a well-known plant growth regulator, however, did not cause necrosis (*e.g.*, root rot and sheath blight), which was a characteristic symptom of diseases caused by *R. solani*, thus it seemed unlikely that phenyl acetic acid was the principal phytotoxin produced by this fungus[28]. Therefore, a further investigation on the isolation, purification and characterization of the phytotoxin (hereafter, Rs-toxin) produced by *R. solani*, in particular the rice sheath blight pathogen *R. solani* AG-1 IA and its maceration (pathogenic) effects on rice leaf tissues is necessary.

The objectives of this research were to: (1) establish a simple and reliable bioassay system for evaluating the phytotoxicity of Rs-toxin on rice leaves; (2) characterize the bioactive components of Rs-toxin; and (3) evaluate the maceration (pathogenic) effects of Rs-toxin on rice leaf tissues.

EXPERIMENTAL

Fungal isolate and pathogenicity test: A highly virulent isolate[23] of *R. solani*, GD118, originally isolated from a diseased leaf of a rice plant with typical symptom of sheath blight[34], was selected from the culture collections of the Laboratory of tropical and subtropical fungi, Department of Plant Pathology, South China Agricultural University, Guangzhou, China. The isolate GD118 was incubated on potato dextrose agar (PDA) in dark at 26 ºC for preparation of inocula. To further confirm the pathogenicity of isolate GD118 to rice before phytotoxin production, the detached leaf inoculation method[34] was adopted in present research. In brief, each of 10 segments of 12-cm long rice leaves was inoculated with a 5-mm diameter mycelial plug and incubated at 26 ºC in an illuminated plant growth chamber (ZPX-300ESW, Hangzhou, China) with 12 h photoperiod and the inoculated leaves were scored 2 days post-inoculation using the 5-grade standard followed the method described by Zhou *et al*.[34].

Phytotoxin production: For Rs-toxin production, fungal cultures were grown in 1 L Erlenmeyer flasks, each containing 200 mL of Richard 's liquid medium (KNO_3 10 g; KH_2O_4 5 g; $MgSO_4 \cdot 7H_2O$ 2.5 g; $FeCl_3$ 0.02 g; sucrose 50 g; distilled water 1000 mL; pH 7.0; autoclaved at 121 ºC for 20 min). The Richard's liquid medium in each flask was inoculated with 10 mycelial plugs (5-mm diameter) cut from the edge of a 36 h old colony grown on PDA medium. Inoculated flasks were then incubated in the dark at 26 ºC with manual shaking once per 12 h for 15 days as described previously[19].

After 15 days of incubation at 26 ºC in Richard's liquid medium, the fungal cultures was first filtered through three layers of gauze to remove mycelia and followed by vacuum filtration through two layers of Whatman No. 1 filter paper *in vacuo*. The resulting culture filtrate was used for bioassay as described below or stored at 4 ºC for the purification of bioactive components of Rs-toxin in the next step.

Bioassays: Bioassays of culture filtrate and various purified fractions of Rs-toxin were carried out on detached healthy leaf-segments of rice (susceptible cultivar Huahang Simiao). For bioassays, rice leaves were detached and cut into 8 cm long segments so as to fit into a 9 cm diameter Petri dish.

In all bioassays with culture filtrate, various purified fractions and partially purified Rs-toxin as indicated bellow, non-inoculated Richard's liquid medium or distilled water served as a control. Six replications were maintained for each treatment, all assays were repeated at least once. In order to establish a simple and reliable bioassay method suitable for evaluating the phytotoxicity of culture filtrate or partially purified Rs-toxin, the following four bioassay methods were used and their effectiveness were compared.

Floating soakage method: Leaf segments of rice were placed into 9 cm diameter Petri dishes containing culture filtrate and ensuring that every leaf segment touched culture filtrate completely, the Petri dishes together with the treated leaf segments were then incubated at 28 ºC (12 h photoperiod) for 2 days or until symptoms were clearly visible.

Puncture method: Two punctures were made in the middle of each leaf segment with the tip of a #4 insect-needle. The leaf segments with punctures were then placed onto the surface of moistened filter paper dipped in culture filtrate in 9 cm diameter Petri dishes and ensuring that every puncture wound closely touched the wet filter paper. The incubation conditions are the same as those described in the floating soakage method.

Puncture and floating soakage method: Two punctures were made as above described. The leaf segments with punctures were placed into 9-cm diameter Petri dishes containing culture filtrate and ensuring that every leaf segment touched culture filtrate completely. The incubation conditions were the same as those described in the floating soakage method.

Seed germination suppression method: Rice seeds were sterilized in 70 % ethanol for 30 s and further sterilized in 0.1 % mercuric chloride for 3-5 min, followed by rinsing three times with sterile distilled water. Two layers of filter paper dipped in culture filtrate or partially purified Rs-toxin solution were placed into 9 cm diameter Petri dishes, with the disinfected seeds laid on the surface of moistened filter paper. The incubation conditions were the same as those described in the floating soakage method.

Isolation and purification of Rs-toxin: To isolate and purify the phytotoxic compound(s), the culture filtrate was extracted in various ways and the phytotoxicity of various extracted fractions of Rs-toxin was measured by the floating soakage method.

Organic solvent extraction: To explore the extraction of Rs-toxin effectively, six organic solvents, *i.e.*, benzene, petroleum ether, ethyl acetate, cyclohexane, epichlorohydrin and chloroform, were used to extract the bioactive components from the culture filtrate of *R. solani* AG-1 IA in this research. Equal volumes of the above mentioned six organic solvents and culture filtrate were combined and mixed overnight to extract the bioactive compounds from the aqueous phase into an organic solvent phase. Organic and aqueous fractions were evaporated to dryness *in vacuo* at 60 ºC and the residue was dissolved in 30 mL sterile distilled water for phytotoxicity assay using the floating soakage method.

Activated charcoal adsorbent method: For the isolation and purification of Rs-toxin from the culture filtrate of *R. solani* AG-1 IA, an activated charcoal adsorbent method, which was based on that of Kang *et al*.[19] and Vidhyasekaran *et al*.[20] with some minor alterations, was used. In brief, activated charcoal was mixed with fungal culture filtrate at a ratio of 7:10 (w/v) in a 1 L Erlenmeyer flask. The mixture was then placed at 4 ºC for 24 h for complete adsorption of components in culture filtrate by activated charcoal, after which the mixture was transferred to a 80 mm × 500 mm chromatography column filled with activated charcoal. The piston of the column was opened and the remnant filtrate was collected. 100 mL of sterile distilled water was added on the top of column for elution and this step was repeated twice. The water elution solution was collected separately, followed by keeping the piston open for 2 h to allow the liquid to flow out completely. Both the remnant filtrate and water elution solution were evaporated to dryness *in vacuo* at 60 ºC and the residues were then dissolved in sterile distilled water for later bioassay. After no liquid dropped, the column was eluted with 100 mL of absolute methanol for 5 times, the methanol elution solution was collected, combined

and evaporated to dryness *in vacuo* at 60 ºC. The residue resulting from the evaporated methanol fraction was a yellowish substance, hereafter called crude Rs-toxin. For further purification, the crude Rs-toxin was dissolved in methanol and loaded into chromatography column containing silica gel. The column was eluted with absolute methanol until no colour remained in the sample. The methanol elution solution was collected and evaporated to dryness *in vacuo* at 60 ºC and the resulting substance, which was called "partially purified Rs-toxin" was used for bioassays, GC/MC and LC/MS/MS analyses for the presence of the bioactive components. For activity detection, "partially purified Rs-toxin" at the concentration of 30 mg/mL was used in the bioassay and the result revealed that it was still active (data not shown). Therefore, the GC/MC and LC/MS/MS analyses were conducted for detecting the presence of bioactive components in Rs-toxin. The detailed procedures for isolation, purification and characterization of Rs-toxin from culture filtrate of *R. solani* AG-1 IA isolate GD118 were summarized in Fig. 1.

Characterization of Rs-toxin

Heat treatment: Culture filtrate obtained from 15-day-old culture of isolate GD118 grown in Richard's liquid medium was autoclaved at 121 ºC for 20 min. Phytotoxicity of autoclaved culture filtrate was assessed by detached rice leaf assay as described in the floating soakage method.

Molish's reaction: The method described by Cai *et al.*[35] was followed with some minor modifications. Briefly, 1 mL of partially purified Rs-toxin solution at concentration of 10 mg/mL, together with 10 µL of α-naphthol, was added into a test tube. The tube was shaken gently to mix the reaction solution well, followed by the addition of 1 mL of conc. sulfuric acid from the tube wall. Dextrose solution at 10 mg/mL served as a positive control and distilled water served as a negative control. The appearance of purple red ring in the reaction mixture suggested that it was a positive reaction and indicated that the reaction solution contained saccharides. In the absence of immediate positive reaction, tubes were further incubated at a 50 ºC hot water bath for 5 min and re-observed.

Ninhydrin test: The method described by Cai *et al.*[35] was used with some minor modifications. Briefly, 1 mL of partially purified Rs-toxin solution at a concentration of 10 mg/mL, together with 0.5 mL of ninhydrin at concentration of 50 mg/mL, was added into a test tube. The tube was shaken gently to mix the reaction solution well and kept at boiling water bath for 5 min. Glycine solution at 50 mg/mL with pH 5-7 served as a positive control and distilled water served as a negative control. After cooling down, if the colour in the reaction solution became dark blue from light pink, it was a positive reaction and indicated that the reaction solution contained amino acids or proteins.

Fehling's reaction: Followed the method as described previously[36] with minor modifications. Briefly, 1 mL of partially purified Rs-toxin solution at a concentration of 10 mg/mL, together with 1 mL of HCl solution at concentration of 5 mol/L, was added into a test tube and kept in 100 ºC water bath for 6 h. The solution mixture was adjusted to pH 7.0 with 6 mol/L NaOH solution. 1 mL of solution mixture was added to a new tube, followed by the addition of 20 µL of Fehling reagent, keeping the tube in a boiling water bath for 5 min. Dextrose solution at 10 mg/mL served as a positive control and distilled water served as a negative control. The appearance of brown red colour in the reaction solution revealed that it was a positive reaction and indicated that reducing sugar existed in the toxin solution.

Ultraviolet (UV) absorption peak: The partially purified Rs-toxin was dissolved in methanol or distilled water. The toxin solution was then scanned with different ultraviolet (UV)- and visible-wavelength light ranging from 200-500 nm in a spectrophotometer (Type U-2910, Hitachi, Japan) and its absorption spectrograms were obtained.

Detection of saccharides: With eight authentic saccharides (ribose, arabinose, xylose, mannose, glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine) as standards, the partially purified Rs-toxin was tested for the presence of saccharides by using the method of GC/MS (GC/MS Model: Agilent 6890 GC/5973i MS) described previously[37].

Detection of phenylacetic acid and its derivatives: With three authentic phenylacetic acid (PAA) and its derivatives (*p*-hydroxy-PAA and α-methoxy-PAA) as standards, the partially purified Rs-toxin was tested for the presence of PAA and its derivatives by using the method of LC/MS/MS (LC/MS/MS Model: Agilent 1200 LC /6410 Triple Quad MS) described by Ma *et al.*[38].

Maceration effects of Rs-toxin on rice leaf tissues

Measurement of reducing sugar from rice leaves treated with Rs-toxin: Five grams of rice leaves at 5-leaf-stage (susceptible cultivar Huahang Simiao) were cut into small pieces approximately 0.5-0.8 cm long, rinsed with sterile distilled water, dried with sterile blotting paper and placed into Rs-toxin solutions in test tubes with 2 mL of serially diluted Rs-toxin solution at concentrations of 1, 5, 10, 15 and 20 mg/mL, respectively. Air in the tube was pumped *in vacuo* for 10 min, making leaves sink into the toxin solutions. After incubation in the dark for 24 h, the leaves were taken out and rinsed with sterile double distilled water 5 times, dried up with blotting paper and placed into new test tubes with 5 mL of double distilled water, followed by air-pumping *in vacuo* for 10 min. Finally, after stationary incubation for 20 h at 28 ºC, the amounts of leaky reducing sugar were measured using 3,5-dinitrosalicylic acid[36]. Double distilled water replacing of toxin solution served as a control and at least three replications were used for each assay.

Mensuration of rice cell membrane damage by Rs-toxin: Damage of cell membranes of rice leaf tissues caused by Rs-toxin was measured by an electrolyte leakage assay. The treatments of rice leaf samples were the same as those mentioned above. After stationary incubation for 20 h at 28 ºC, the conductivity of leaf leachate was measured using a conductivity meter (DDS-Type IIA Conductivity Meter, Shanghai, China). Then the treated tissues were boiled for 15 min allowing rice cell membranes to lyse and after cooling down of the samples, the maximum conductivity of leaf leachate was determined. Double distilled water replacing of toxin solution served as a control. Finally, the damage ratio was calculated by means of the following equation:

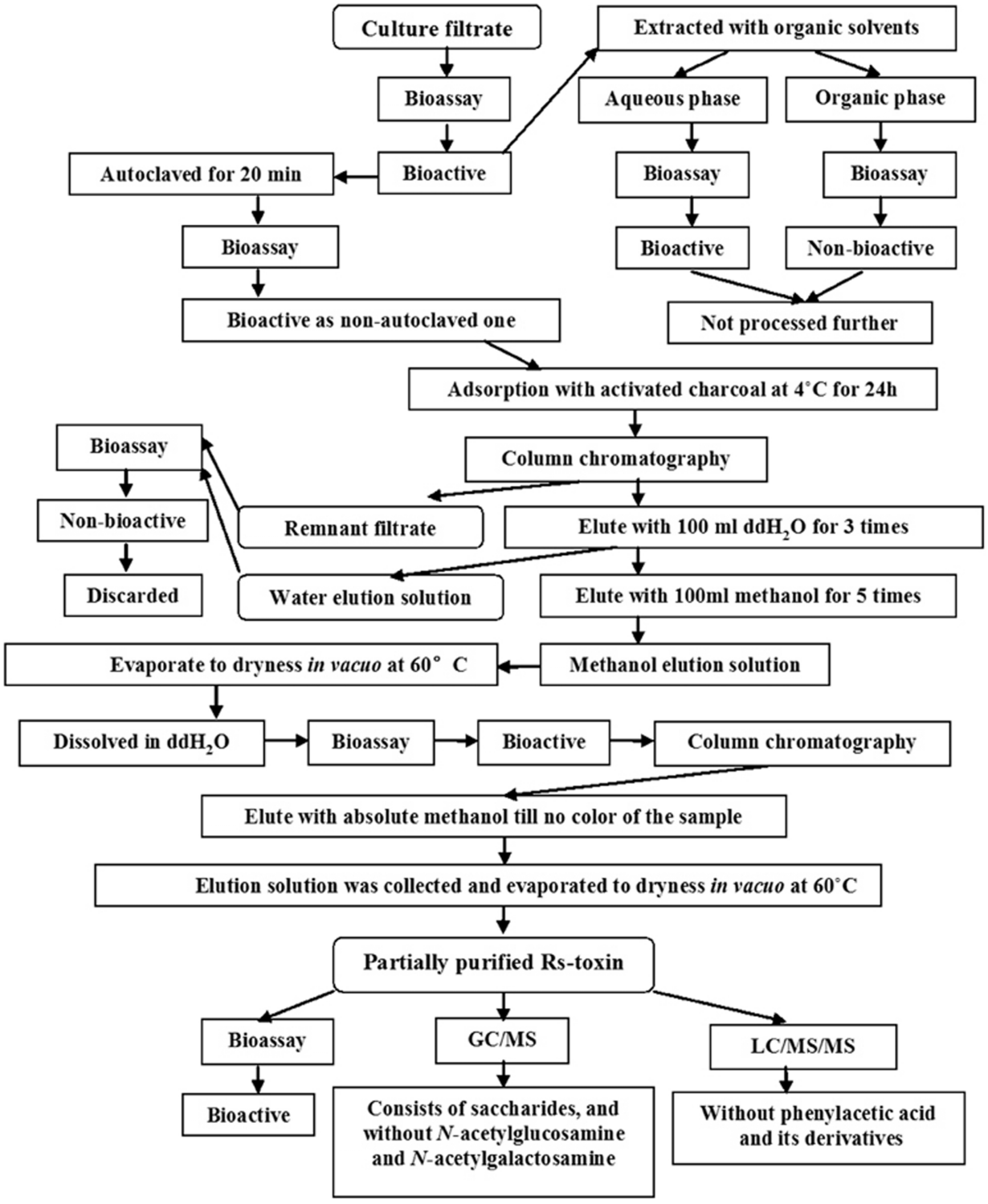


Fig. 1. Schematic diagram of isolation, purification and characterization of Rs-toxin from culture filtrate of *R. solani* AG-1 IA isolate GD118

Damage ratio (%) = (conductivity of toxin treatment - conductivity of water control)/(maximum conductivity of toxin treatment after boiling - maximum conductivity of water control after boiling) × 100.

RESULTS AND DISCUSSION

Pathogenicity test: The result of pathogenicity test revealed that 2 of 10 segments of rice leaves were identified as disease grade 4 and other 8 segments of rice leaves were identified as disease grade 5 and the average disease grade of isolate GD118 was 4.8. The disease grade was between 4 and 5, for which isolate GD118 was categorized as high virulent isolate according to the 5-grade standard as described by Zhou *et al.*[34]. Moreover, it was reported that the toxin production was positive correlation with pathogenicity of *Rhizoctonia solani*[21]. Therefore, the isolate GD118 was suitable for use as a good toxin-producing isolate in the present study.

To further confirm the pathogenicity of isolate GD118 on rice, detached leaf inoculation method[34,39] was adopted and the result of pathogenicity test showed that isolate GD118 was a highly virulent isolate as described previously[34]. Therefore, the isolate GD118 is suitable for use as a toxin-producing isolate in the present study.

Bioassays: Among the four bioassay methods, the floating soakage method, which could cause obvious lesions (Fig. 2a), was found to be the best one and thus was chosen as a standard bioassay method for all phytotoxicity tests in the present study for its simplicity, sensitivity, convenience and reproducibility (data not shown). Although the needle puncture method could also cause obvious lesions on leaves (Fig. 2b), its operability was somewhat complicated. The needle puncture and floating method often caused too many lesions on a leaf to be observed and distinguished easily (Fig. 2c). Seed germination suppression method relies on the quality of rice seeds very much and different degrees of disinfection of seeds would affect the germinating ability of seeds, therefore, it is also not a suitable assay method for evaluating the phytotoxicity of Rs-toxin (Fig. 2d).

Isolation and purification of Rs-toxin

Organic solvent extraction: The results of bioassay revealed that the six organic solvent phases of the culture filtrate did not cause visible symptoms on rice leaves (data not shown), indicating that the solvent extracts did not contain any phytotoxic component and thus no organic solvent was effective for extracting the bioactive components from the culture filtrate of *R. solani* AG-1 IA. The aqueous phase of the culture filtrate after extraction with organic solvents, however, did cause typical lesions on rice leaves (data not shown), indicating that the phytotoxic components were present in the aqueous fraction after extraction with organic solvents (benzene, petroleum ether, ethyl acetate, cyclohexane, epichlorohydrin and chloroform). Therefore, the activated charcoal

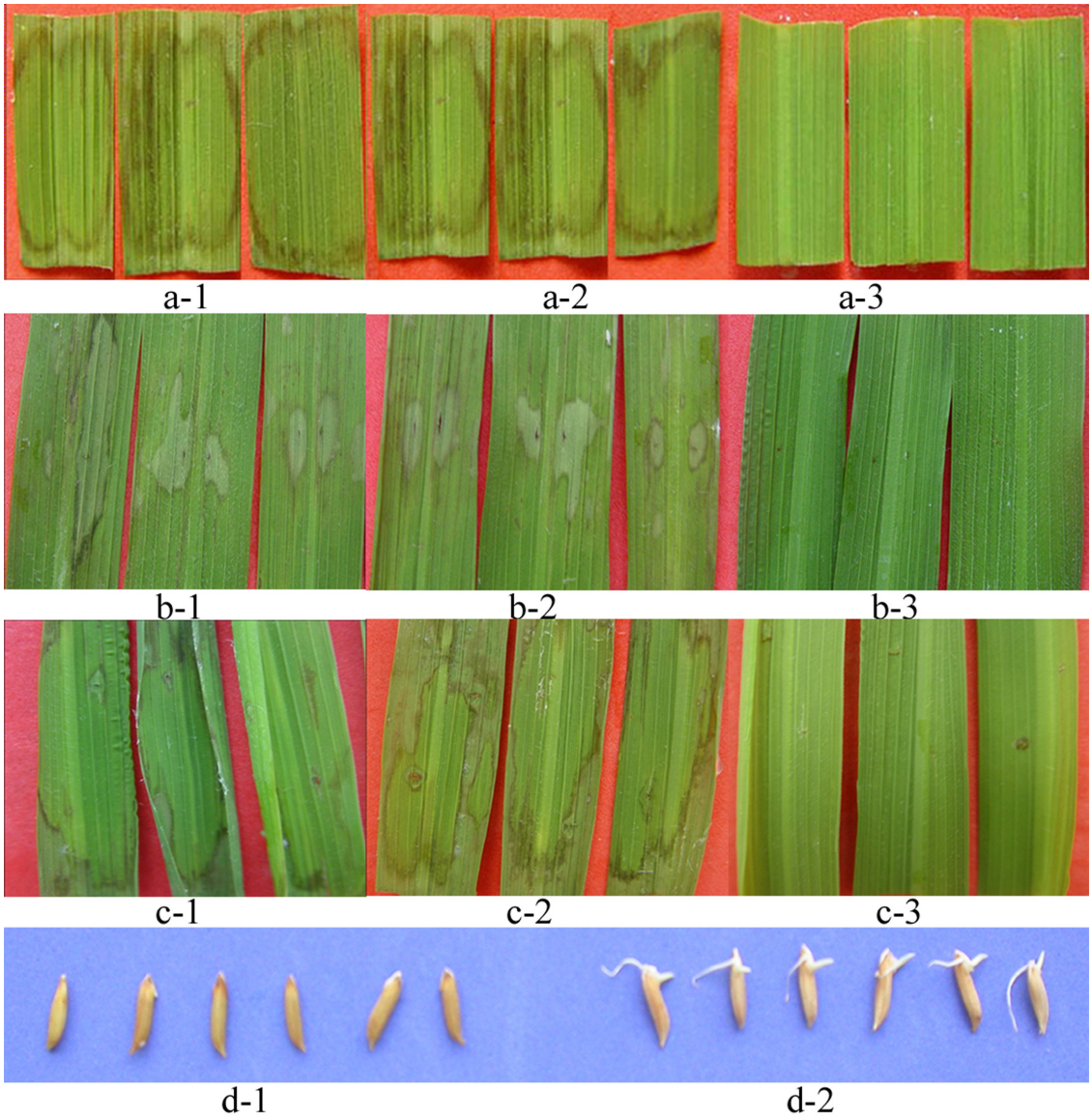


Fig. 2. Bioassays. a. Floating soakage method. a-1. Culture filtrate; a-2. Autoclaved culture filtrate; a-3. Non-inoculated Richard's liquid medium. b. Puncture method. b-1. Culture filtrate; b-2. Autoclaved culture filtrate; b-3. Non-inoculated Richard's liquid medium. c. Puncture and floating soakage method. c-1. Culture filtrate; c-2. Autoclaved culture filtrate; c-3. Non-inoculated Richard's liquid medium. d. Seed germination suppression method. d-1. Partially purified Rs-toxin; d-2. Sterile distilled water.

adsorbent method was chosen and used for further isolation and purification of the bioactive components from the culture filtrate of rice pathogen *R. solani* AG-1 IA.

Activated charcoal adsorbent method: The activated charcoal adsorbent method was then used for further isolation and purification of the bioactive components from culture filtrate because no organic solvent was effective for extracting the bioactive components from the culture filtrate of *R. solani* AG-1 IA in the above mentioned experiments.

In the activated charcoal adsorbent method, the phytotoxicity of the remnant filtrate, water elution fraction and methanol elution fraction from the chromatography column of activated charcoal were assessed by using the floating soakage method (all these parts were evaporated to dryness and dissolved in sterile distilled water before bioassay) and the bioassay results revealed that both the remnant filtrate and water elution fraction were not phytotoxic, whereas the methanol elution fraction was found to be phytotoxic to rice leaf (data not shown), indicating that the bioactive components were present in the methanol elution fraction. Therefore, the methanol elution solution was collected and evaporated to dryness *in vacuo* at 60 ºC and finally the "partially purified Rs-toxin" was obtained in present studies (Fig. 1).

Characterization of RS-toxin

Heat treatment: The culture filtrates before and after autoclaving at 121 ºC for 20 min were evaluated for their phytotoxicity to rice leaves using the floating soakage method. The results showed that the autoclaved culture filtrate still caused typical lesions on rice leaves, as did the non-autoclaved filtrate, indicating that the heat treatment of culture filtrate did not reduce its phytotoxicity to rice leaves in the bioassays (Fig. 2a-c). The results suggested that the bioactive components of Rs-toxin were thermostable. Negative controls were autoclaved and non-autoclaved non-inoculated Richard's liquid medium, both them could not cause any lesion on rice leaves.

Molish's reaction: The result of the Molish's reaction showed a purple red ring appeared in the reaction tubes of the Rs-toxin solution and the positive control, respectively, nor was the negative control (Fig. 3a), indicating the presence of saccharides in Rs-toxin solution.

Ninhydrin test: The result of the ninhydrin test revealed that the colour of the Rs-toxin and the negative control reaction mixture did not change from light pink to dark blue (Fig. 3b), while the colour of the positive control reaction mixture changed from light pink to dark blue (Fig. 3b), indicating that there were no amino acids or proteins in Rs-toxin solution.

Fehling's reaction: The result of Fehling's reaction suggested the appearance of orange red precipitation took place in Rs-toxin reaction mixture and the positive control, nor did in the negative control (Fig. 3c), which proved the existence of saccharides in Rs-toxin. From the results of above three separate experiments of chemical reactions, it is evident that the Rs-toxin contains saccharides and does not contain amino acid or proteins. Therefore, it is concluded that saccharides should be the main bioactive components of Rs-toxin.

UV- and visible-wavelength light absorption peak of Rs-toxin solution: To obtain the absorption peak, the partially purified Rs-toxin was dissolved in distilled water or methanol and scanned with UV- and visible-wavelength light ranging from 200-500 nm. The results indicated that in methanol, the maximum absorption peak was at 240 nm (Fig. 4a) and in water, the maximum was at 235 nm (Fig. 4b). As is known to all, when the wavelength is shorter than 210 nm, disruptors in the sample own strong absorption, which prevents the MS detection. Therefore, the partially purified Rs-toxin, with 240 nm as the maximum absorption peak in methanol and 235 nm as the maximum absorption peak in water, is suitable for GC/MS and LC/MS/MS analyses.

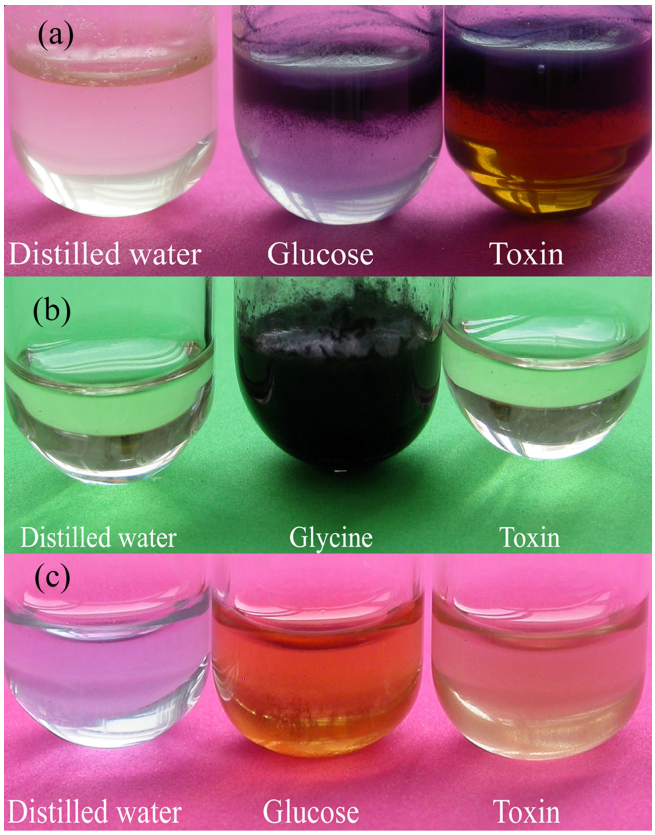
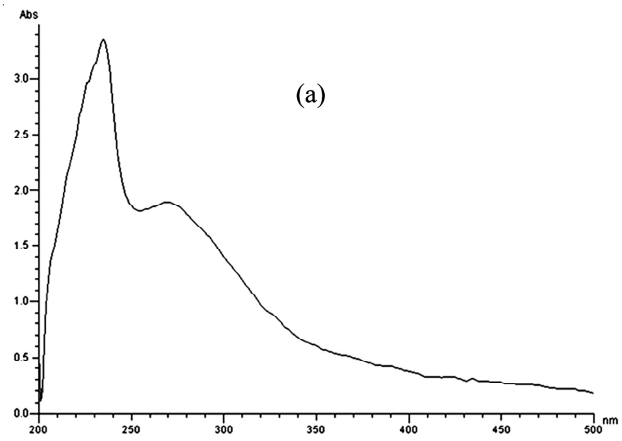


Fig. 3. Chemical reactions. (a) Molish's reaction. Left: Distilled water (negative control); Center: Glucose solution at 10 mg/mL (positive control); Right: Partially purified Rs-toxin solution at 10 mg/mL. (b) Ninhydrin test. Left: Distilled water (negative control); Center: Glycine solution at 50 mg/mL (positive control); Right: Partially purified Rs-toxin solution at 10 mg/mL. (c) Fehling's reaction. Left: Distilled water (negative control); Center: Glucose solution at 10 mg/mL (positive control); Right: Partially purified Rs-toxin solution at 10 mg/mL



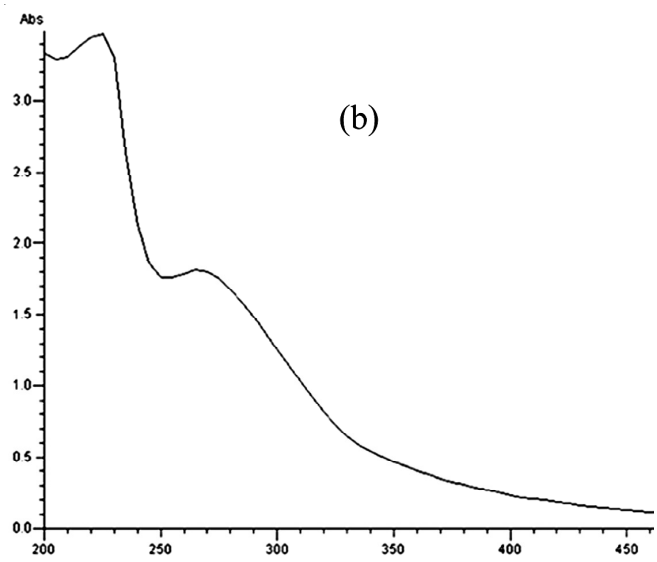
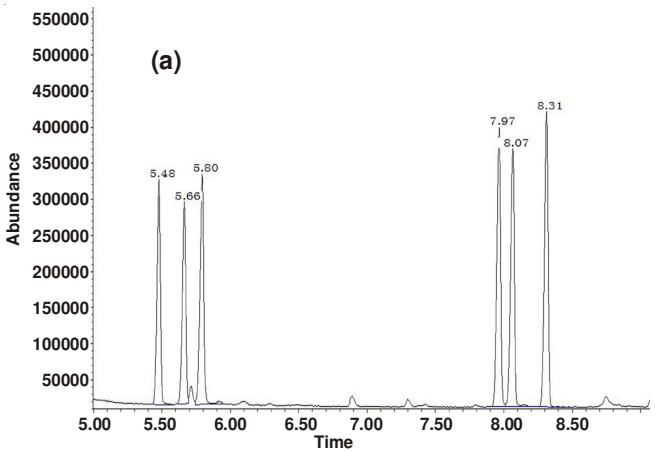


Fig. 4. Ultraviolet and visible absorption spectrograms of partially purified Rs-toxin. (a) In methanol, with a 240 nm point as its maximal absorption peak. (b) In double distilled water, with a 235 nm point as its maximal absorption peak

Detection of saccharides: Through a comparison with eight authentic standard monosaccharides (ribose, arabinose, xylose, mannose, glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine) by using GC/MS method, the results showed that the bioactive components of Rs-toxin consisted of six monosaccharides, *i.e.*, ribose, arabinose, xylose, mannose, glucose and galactose and without N-acetylglucosamine and N-acetylgalactosamine in this toxin. The relative amounts of these six monosaccharides calculated by means of peak area normalization were as follows: 0.20 % for ribose, 0.83 % for arabinose, 0.83 % for xylose, 14.3 % for mannose, 83.63 % for glucose and 0.07 % for galactose, among them glucose and mannose occupied the biggest proportions (Fig. 5a-c).

Detection of phenylacetic acid and its derivatives: To identify whether the phenylacetic acid (PAA) and its derivatives presented in Rs-toxin or not, the LC/MS/MS method was taken. The TIC MRM figure of three authentic standard PAA and its derivatives, *i.e.*, PAA, hydroxy-phenylacetic acid and methoxy-phenylacetic acid, was included in this research (Fig. 6a). The result of TIC MRM revealed that only interferential signals



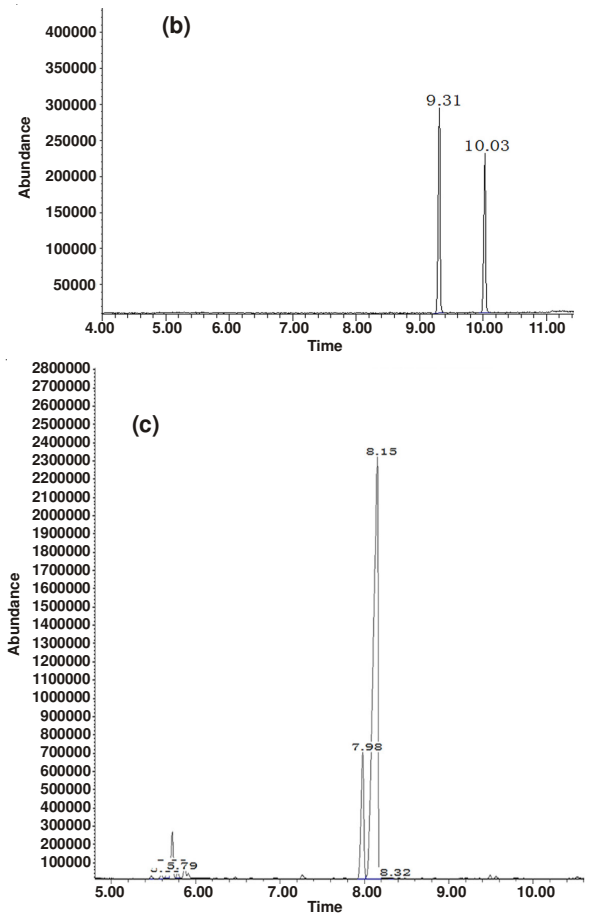


Fig. 5. Total ion current chromatograms of eight authentic standard monosaccharides and Rs-toxin sample. (a) The absorption peaks with retention times of six authentic standard monosaccharides. From left to right: ribose, arabinose, xylose, mannose, glucose and galactose at the retention time of 5.48, 5.66, 5.80, 7.97, 8.07 and 8.31 min, respectively. (b) The absorption peaks with retention times of two authentic standard monosaccharides. From left to right: N-acetylglucosamine and N-acetylgalactosamine at the retention time of 9.31 and 10.03 min, respectively. (c) The absorption peaks with retention times of Rs-toxin sample. From left to right: ribose, arabinose, xylose, mannose, glucose and galactose were detected at the retention time of 5.48, 5.66, 5.79, 7.98, 8.15 and 8.32 min, respectively which corresponding to those of six authentic standard monosaccharides in (a) although the peak signals were faint at the retention time of 5.48, 5.66, 5.79 and 8.32 min. Neither N-acetylglucosamine nor N-acetylgalactosamine was detected in Rs-toxin sample at the retention time of 9.31 and 10.03 min, respectively

and no targeted mass spectra were detected in Rs-toxin sample (Fig. 6b), indicating that targeted PAA and its derivatives were absent in Rs-toxin sample.

Maceration effects of Rs-toxin on rice leaf tissues: To further confirm the maceration (pathogenic) effects of partially purified Rs-toxin on rice leaf tissues, the leaky reducing sugar and relative conductivity of leaf leachate from rice leaf tissues treated with Rs-toxin were determined. The results indicated that the Rs-toxin was pathogenic and the damage degrees of Rs-toxin to the rice leaf tissues, indicated by reducing sugar and relative conductivity, increased with the increase of concentrations of Rs-toxin (Fig. 7a,b).

Biological active substances have been isolated and characterized on fractionation of hexane and methanol extracts of leaves of *Vitex negurndo* Linn, which expresses antimicrobial

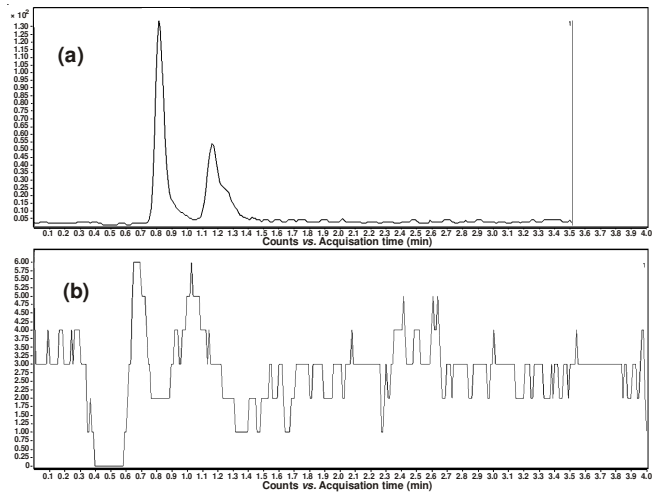


Fig. 6. TIC MRM of three authentic standard PAA and its derivatives and Rs-toxin sample. (a) TIC MRM of three authentic standard PAA and its derivatives. From left to right: hydroxy-phenylacetic acid, PAA and methoxy-phenylacetic acid. (b) TIC MRM of Rs-toxin sample. The scale on Y-axis of Fig. 6a was $\times 10^2$, while the scale on y axis of Fig. 6b was $\times 1$ (10^0), which is 100 times difference. Only a straight line could be seen if Fig. 6b was $\times 10^2$ because the values were too small. Therefore, only interferential signals were found and no targeted mass spectra were detected in Rs-toxin sample

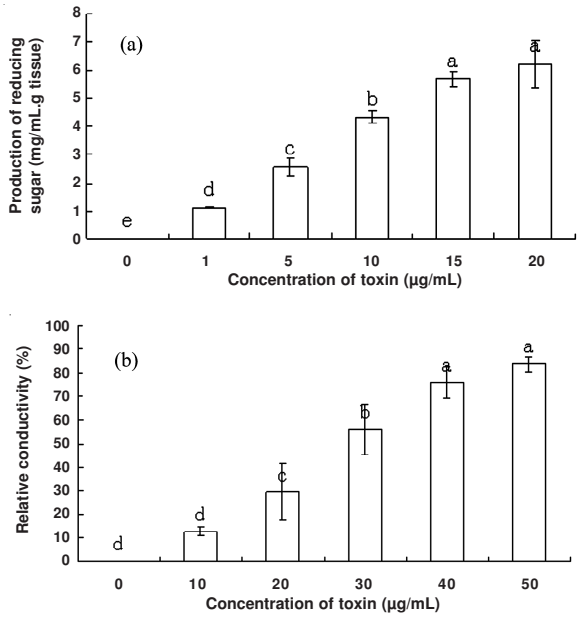


Fig. 7. Maceration effects of Rs-toxin on rice leaf tissues. (a) Production of leaky reducing sugar from rice leaf tissues treated with Rs-toxin. X-axis stands for toxin concentration. Y-axis stands for production of reducing sugar. (b) Leaf cell membrane damage by toxin treatment. X-axis stands for toxin concentration. Y-axis stands for cell membrane damage by toxin treatment expressed by relative conductivity. Data shown are the mean ± SE of six replicates; data (column) denoted with different letters are significantly difference at 5 % level (P = 0.05) analyzed by using Duncan's Multiple Range Test (DMRT)

activities against *R. solani*[40], phytoxin of *R. solani* which do damage and cause necrosis on rice tissues has been investigated in present study. Different nutrient media were used in different studies on phytotoxins. The potato-sucrose agar plates were utilized for the phtotoxin production of bipolaris bicolour[41], while the Richard solution was used in the present study for the production of Rs-toxin by *R. solani*, corresponding with other reports in *R. solani*[19,22]. A suitable bioassay is crucial in the study of phytotoxins[25,42]. Through a comparison of four bioassays used, it is established that an effective bioassay system-the floating soakage method, which is sensitive, simple, convenient and reliable for the evaluation of phytotoxicity of culture filtrate and Rs-toxin. Compared with other bioassays[17,20,25], the advantage of the present bioassay is that it is relatively fast and does not need a specific device such as a pump for the infiltration of toxin solution into plant tissues. With the present bioassay, it is easy and accurate to evaluate the phytotoxicity of culture filtrate, various extracted fractions and partially purified Rs-toxin on rice detached leaves at each step during the isolation and purification of Rs-toxin.

To isolate and characterize the bioactive components of Rs-toxin from the culture filtrate of rice isolate GD118 of *R. solani*, several organic solvents were explored for their effectiveness in Rs-toxin extraction in this research. The results showed that the phytotoxic activity was observed only in the aqueous fraction but not in the organic fractions after extraction with six organic solvents (benzene, petroleum ether, ethyl acetate, cyclohexane, epichlorohydrin and chloroform), suggesting that no organic solvent was effective for extracting the bioactive components from the culture filtrate of *R. solani*. In the isolation of the bioactive components of Rs-toxin, the activated charcoal adsorbent method was proved to be effective as verified by bioassay at each step by using the floating soakage method.

The results of three separate experiments of chemical reactions, *i.e.*, Molish's reaction, Ninhydrin test and Fehling's reaction, have clearly demonstrated the saccharide nature of the Rs-toxin. Saccharides were further confirmed and characterized to be the main bioactive components of Rs-toxin in the later GC/MS and LC/MS/MS detection and finally six kinds of saccharides were detected, but no N-acetylgalactosamine, N-acetylglucosamine, phenylacetic acid or its derivatives were found in Rs-toxin in the present study. The present results are somewhat similar to that of Vidhyasekaran *et al.*[20]. Present findings, however, are quite different from those reported by Sherwood and Lindberg[27], Aoki *et al.*[28], Sherwood[29], Mandava *et al.*[30], Iacobellis *et al.*[17] and Siddiqui *et al.*[32] who found that the Rs-toxins belong to phenolic and glycosidic compounds or phenylacetic acid and its derivatives. Vidhyasekaran *et al.*[20] reported that the Rs-toxin is a kind of carbohydrate containing glucose, mannose, N-acetylgalactosamine and N-acetylglucosamine. Among the six saccharides detected in present study, glucose and mannose were the same as those of Vidhyasekaran *et al.*[20]. However, the other four kinds of saccharides (ribose, arabinose, xylose and galactose) detected in the present study were different from those reported by Vidhyasekaran *et al.*[20]. Though phenylacetic acid and its derivatives had also been reported to be the principal components of Rs-toxins by several researchers[17,28,30,32], we could not detect any one of them from the present study with present rice isolate GD-118 even in the analysis of LC/MS/MS. We also tried to prove the existence of N-acetylgalactosamine and N-acetylglucosamine[20] in the Rs-toxin by using GC/MS technique, but they could also not be detected in the present study. The main reason for the differences in chemical constitutions of Rs-toxins in different investigations might be the use of different isolates with different AGs and host origins. From the results of Rs-toxin

characterization in several laboratories, it is also suggested that *R. solani* is indeed a diverse species which is capable of producing several different non-enzymatic phytotoxic metabolites in culture filtrates[17,20,27-33].

Several authors have evaluated Rs-toxins as virulence factors[17,19-22,25,26,43] and also determined whether the Rs-toxin is host-specific or not[20,25] in the pathogenesis of *R. solani*. Present results of maceration of Rs-toxin on the rice leaf tissues further confirmed the pathogenic actions of Rs-toxin and also verified the effectiveness of the activated charcoal adsorbent method in isolating Rs-toxin from culture filtrate of *R. solani*. Saccharides were confirmed to be the bioactive components of Rs-toxin in the present study and in the report of Vidhyasekaran *et al*.[20]. It was reported that polysaccharides served as a virulence factor in several pathogens including bacteria and fungi[44-50]. However, little is known about how saccharides serve as a pathogenicity factor in the pathogenesis of rice sheath blight and other *Rhizoctonia* diseases. We postulate that the Rs-toxin might be a kind of glycoside or polysaccharide consisting of a group of monosaccharides and it was broken during the GC/MS analysis. For elucidating the pathogenic mechanism of saccharides in the pathogenesis of *R. solani*, more intensive studies will need to be conducted.

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