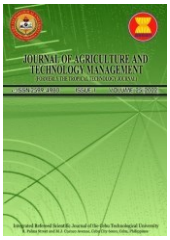
Journal of Agriculture and Technology Management (JATM)
25(1): 5-10 (January-June 2022)



Original article

Host-specificity and bioassay of toxin from *rhizoctonia solani* kuhn causing sheath blight disease of rice

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ABSTRACT

Sheath blight is one of the major diseases of rice worldwide which is caused by *Rhizoctonia solani* and known to produce phytotoxin. In this study, the pathogenic ability of pure culture isolate was assessed in standing susceptible check rice plants and host specificity *R. solani* toxin was evaluated through detached leaf bioassay. The effect of high temperature (by boiling) and different concentrations to toxin activity were also assessed through seed germination and seedling growth of susceptible (TN1 variety) and moderately resistant (IR72) rice varieties. Extracted toxin from the pure culture of *R. solani* isolate from Los Baños, Laguna, Philippines induced sheath blight disease in rice but not in corn (IPBVar2) and tomato (Red Cherry) which implied host specificity of the toxin. Percent germination and seedling growth of susceptible (TN1) and moderately resistant (IR72) rice varieties were significantly affected when applied with crude and/or boiled toxin indicating stability of RS toxin at high temperature. Low concentration of the RS toxin, however, reduced its infectivity in both rice varieties and was comparable with the control in terms of percent germination and seedling growth. Findings of the present study further confirmed (1) the pathogenic activity of RS toxin to rice (TN1 variety) but not pathogenic to tomato (Red Cherry) and corn (IPBVar2), (2) the high temperature did not affect *R. solani* toxin pathogenic activity while (3) level of concentration of the *R. solani* toxin affects its effectiveness on the diseases.

KEYWORDS: bioassay, host specificity, pathogenicity, rice, *Rhizoctonia solani*, sheath blight disease

1 INTRODUCTION

Sheath blight disease caused by *Rhizoctonia solani* Kuhn is one of the most serious and economically important diseases of cultivated rice worldwide. The disease is widely distributed in most rice-producing countries (Webster and Gunnell, 1992) like China, Japan and in Southeast Asian countries including the Philippines (Lee & Rush, 1983; Singh, Sunder and Kumar, 2016; Sandoval and Cumagun, 2019). In Asia, sheath blight disease is reported to affect approximately 15-20 million ha of irrigated rice fields and causes a yield loss of 6 million tons of rice grains per year (Bernardes *et al*., 2009). Yield losses vary on the factors that contribute to disease development, losses due to this disease may reach up to 50% when condition is favorable for pathogen and disease development (Groth, 2008; Bernardes de Assis *et al*., 2009) and 40% losses were recorded in rice crop found with highest inoculum density (Tan et al., 2007). Annual yield losses of 10% and 20% due to sheath blight are observed in India and Thailand, respectively (Gianessi, 2019). In the Philippines, around 5-80% rice grain yield reduction and about 0.27 to 1.29 tons per hectare yield losses was recorded in the dry season while 0.23 to 1.27 tons/ ha in the wet season (as cited by Cumagun et al., 2019).

R. solani 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. In rice, initial infections start at the water line as water-soaked lesions on leaf sheaths and move upward causing lesions on the upper leaf sheaths and leaf blade. Infected plants produce symptoms that include greenish, elliptical or oval-shaped spots with yellow margins mostly found on leaf sheaths. The disease usually infects the plant at late tillering or early internode elongation growth stages and can be spread through leaf-to-leaf or leaf-to-sheath contacts, by irrigation water and by the movement of soil and infected crop residues during land preparation. The pathogen survives in the soil as sclerotia (asexual stage of the fungus) in the absence of a host for two years and can accumulate over time (Webster *et al*., 1992; as cited by Turaidar et al., 2018).

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p-ISSN: 2599-4875 e-ISSN: 2599-4980

R. solani is known to produce phytotoxins and cell-wall degrading enzymes that play an important role in the pathogenicity or virulence of the fungus. The toxin isolated from the rice sheath blight pathogen was designated as RS toxin and its production was consistently associated with the necrotrophic nature of the pathogen (Danson *et al*., 1999; Mandava *et al*., 1980; Aoki *et al*., 1963). Several studies revealed that RS toxins are involved in lesion development playing a major role in pathogenesis (Pooja and Babu, 2017, Yang *et al*., 2011). Previous reports have indicated that RS toxins are carbohydrates that contain glucose, mannose, N-acetylgalactosamine (Vidhyasekaran *et al*., 1997). Other studies have also revealed some chemical substances such as phenyl acetic acid (PAA), derivatives of PAA, and a phenolic compound that acted as phytotoxins of *R. solani* (Laksman *et al*., 2006; Mandava *et al*., 1980). These various phytotoxins have been attributed to the broad host range and diversity within the *R. solani* species (Carling *et al*., 2002). Phytotoxins can increase the virulence of pathogens or are often ("pathogenicity determinants") required for a pathogen to incite disease. Brooks (2007) reported that sensitivity to a phytotoxin from *R. solani* correlated with sheath blight susceptibility in rice.

The virulence factor of any pathogen must be known in order to formulate effective management strategies. Since *R. solani* is known to produce toxins, assays such as host-specificity must be performed in order to get information about its host-range. The information of host-range is important in order for the researchers and scientists to study the possible yield losses in various crops at different conditions involved in disease development. Further, information on host range will help the farmer decide what to crop to cut the life cycle of *R. solani*. On the other hand, exploration on the sensitivity of these toxins to abiotic factors such as temperature is necessary in order to have basic data for the formulation of effective management strategies. Hence, this study aims to evaluate the host-specificity of the *R. solani* toxin (RS toxin) isolated from rice, determine the effect of dilution on the infectivity of the RS toxin, and determine the effect of heat temperature treatment on the toxin activity.

2 MATERIALS AND METHODS

Isolation of rice sheath blight pathogen

The sheath blight infected leaves of TN1 rice plants grown in pots at the screenhouse of Crop Protection Cluster (CPC), University of the Philippines Los Baños (UPLB), Philippines, were collected and brought to the Wing B, Plant Pathology Laboratory, CPC, UPLB for microbial isolation (Figure 1A) on 2016. Plant samples were washed with distilled water and blot dried on sterile tissue paper. Thin cut tissues (2-4 mm) were obtained from the advanced lesions and surface sterilized with 10 % sodium hypochlorite solution for 3 minutes, rinsed thrice with sterile distilled water, and blot dried in sterile tissue paper. Surface sterilized thin tissues sections were planted on Potato Dextrose Agar (PDA) in Petri dishes for 7 days at room temperature. The isolate of *R. solani* was further purified and maintained in the same culture medium.

Pathogenicity assay

The Taichung Native 1 (TN1) rice variety from the Department of Agriculture - Philippine Rice Research Institute (DA-PhilRice), UPLB, College, Los Baños, Laguna was used as a susceptible check for the pathogenicity assay of the isolated pathogen. The 4 mm mycelial discs were obtained from a 2-day old *R. solani* pure culture and used to inoculate the ligule and basal leaf sheath of a tiller of 45 days-old plants grown in pots at about 3 to 4 cm above the waterline. The inoculated plants were incubated with plastic cellophane until the first appearance of symptoms and were observed for further disease symptom development (Figure 1B). The disease plant part was cut and brought to the Wing B, Plant Pathology Laboratory, CPC, UPLB for isolation, and isolate characteristics were compared to the original pure culture.

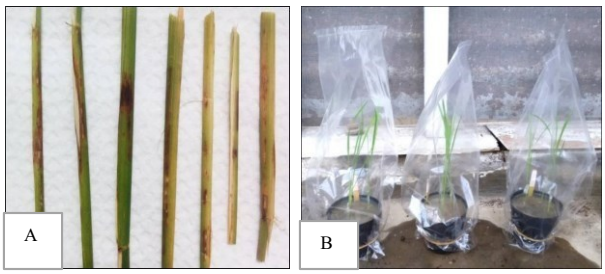

Figure 1. Pathogenicity test. A, rice leaves infected with sheath blight disease; and B, pathogenicity test set-up.

Production of *R. solani* crude toxins

The method on the study of Vidhyasekaran et al. (1997) was followed in the production of RS toxin. Fungal cultures were grown in 250 mL Erlenmeyer flasks, each containing 50 mL of Richard's liquid medium (50 g sucrose; 10 g KNO_3; 5 g KH_20_4; 2.5 g $MgSO_4$; 0.02 g $FeCl_3$; 1000 mL distilled water; pH 7.0; autoclaved at 121^0C for 20 min). Each flask was inoculated with 4 to 6 mycelial disc (4 mm diameter) taken from the edge of a 36 hr old colony of *R. solani* grown in PDA medium. Inoculated flasks were then incubated in the dark for 14 days at room temperature with manual shaking once per 12 hr as described previously.

After two weeks of incubation, the fungal cultures were first filtered through three layers of gauze to remove mycelia and followed by filtration through two of sterile

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Whatman No. 1 filter paper in a Seitz filter under vacuum (Figure 2B). The harvested culture filtrate was used for bioassay.

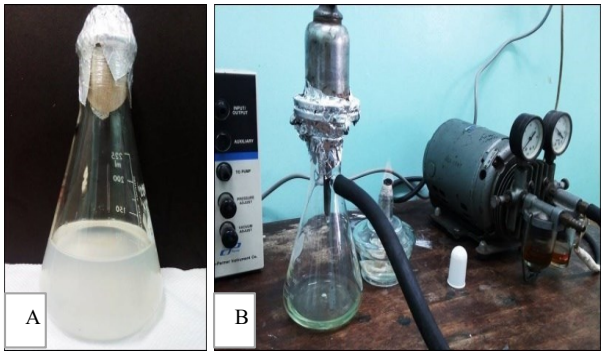

Figure 2. Culture and filtration of crude toxin produced by *R. solani*. A, Richard's medium; B, set-up of toxin filtration using Seitz filter under vacuum.

Host specificity of the *R. solani* toxin

The detached leaf bioassay method on the study of Vidhyasekaran et al. (1997) was followed in the host-specificity assay of RS toxin. Leaf sheaths of rice (TN1 variety), corn (IPBVar2), and tomato (Red Cherry) were detached from the healthy mother plant and used for the bioassay. Whole leaves of tomato and 4 cm pieces of rice and corn leaves were surface sterilized in 10% sodium hypochlorite for 5 min and were rinsed thrice with sterile distilled water. Each leaf sample was placed on a glass slide and the inoculation point was wounded with the tip of the sterile syringe needle. The set-up was kept inside a sterile petri dish lined with wet sterile filter paper. The 4 mm-diameter filter paper discs were soaked to *R. solani* crude toxin for 1 hr for complete absorption of toxin. One paper disc was then placed on the wounded leaf section. The petri dishes were incubated at room temperature. After 5 days of incubation, symptom development was assessed. The intensity of the symptoms was graded into four categories based on the leaf area affected: 1 = 1 to 10%; 2 = 11 to 25%; 3 = 26 to 50% and 4 = more than 50% affected leaf sheath area. Ten leaf samples were scored for symptom development.

Bioassay of R. solani toxin on rice cultivars

The infectivity of different concentrations of *R. solani* crude toxins on percent seed germination and seedling growth were assessed in the moderately resistant rice cultivar and susceptible variety. The IR72 rice variety (moderately resistant) from International Rice Research Institute, UPLB, Los Baños, College, Laguna and TN1 (susceptible check) from DA-PhilRice were used to assess the activity of *R. solani* toxin at different dilutions and the effect of boiling through seed germination and seedling growth. The treatments consisted of: T_1, distilled water (control); T_2, crude toxin; T_3, boiled crude toxin; T_4, 1:10; T_5, 1:100; and T_6, 1:200 crude toxin dilutions. Three replications were maintained for each treatment, all assays were repeated at least once. Rice seeds were disinfected with 10% sodium hypochlorite for 1 min and rinsed thrice with sterile distilled water. Ten seeds were placed into each petri dish containing 3 ml of each treatment, including the control. Percent germination, root, and shoot length were assessed after five days.

Data analysis

The host specificity data was analyzed descriptively while the bioassay data such as percent germination and the seedling growth which were determined by root and shoot length of rice seeds applied with *R. solani* toxin were subjected to Analysis of Variance (Anova) to test the significant difference between the treatments. The mean comparison was carried out using Tukey Pairwise Mean Comparison test using Statistical tool IBM SPSS Statistics Software version 22.

3 RESULTS AND DISCUSSION

Characteristic and pathogenic ability of isolated *R. solani*

Isolated culture of R. solani grown on the PDA medium showed the distinct characteristics of the hyphae and sclerotia of R. solani (Figure 3A). In this study, fungal mycelia were whitish during the early growth and became buff-colored to dark brown as culture aged while sclerotia formed were irregular in shape and light to dark brown in color. These characteristics of mycelial and sclerotial growth on PDA medium conformed to the previous report of Gnanamanickam (2009) and Banniza et al. (2007). At an early stage, *R. solani* was observed to produce hyaline mycelium which eventually turned yellow to brown when matured (Gnanamanickam, 2009). Sclerotial bodies of *R. solani* isolates in this study were irregular in shape while in the study of Banniza et al. (2007), its shape was relatively spherical, however, both studies showed similar color of the sclerotia which is brown to dark-brown color.

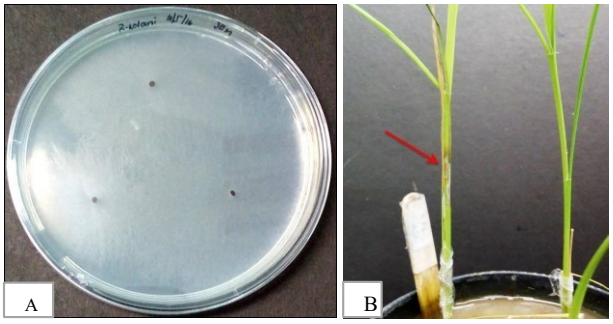

Figure 3.A. Fungal growth in PDA medium and B. Symptoms (red arrow) produced three days after inoculation of *R. solani*.

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The ability of *R. solani* isolate to infect rice plants was confirmed by a pathogenicity test. Infected plants were observed to produce oblong, gray-green, water-soaked spots on leaf sheaths three days after fungus inoculation (Figure 3B). The observed symptoms were characteristics of sheath blight infection confirming pathogenicity or ability of *R. solani* isolate

Host specificity of the *R. solani* toxin

R. solani is known to produce host selective toxins that can increase pathogen virulence and are often required in disease development (Danson *et al*., 1999). In this study, rice (TN1 variety), corn (IPBVar2), and tomato (Red Cherry) were tested for sensitivity to *R. solani* toxin by leaf bioassay. Results showed that only rice was sensitive to the *R. solani* toxin that was indicated by more than 50% disease leaf area, while the other plant leaves did not show the symptom (Table 1, Figure 4). This indicated that corn and tomato were not sensitive to the *R. solani* toxin. Thus, the toxin produced by *R. solani* isolate in this study showed host specificity which has been demonstrated already in several studies using non-hosts and host plants. In addition, the symptoms showed in the bioassay test were similar to the symptoms observed in the pathogenicity test. Vidhyasekaran *et al*. (1997) tested several rice cultivars along with other non-hosts crops and reported that all rice cultivars susceptible to the fungus were also susceptible to the fungal toxin while Sapota and coconut leaves were less susceptible to the fungus and the toxin.

Table 1. Degree of symptom development of different host plants applied with toxin.

Treatments	Disease intensity [a,b]
T_1 (Rice + dH_2O)	0
T_2 (Rice + crude toxin)	4
T_3 (Corn + dH_2O)	0
T_4 (Corn + crude toxin)	0
T_5 (Tomato + dH_2O)	0
T_6 (Tomato + crude toxin)	0

[a] Scored from 10 sample leaves per treatment.
[b] 0 = no symptoms; 1= 1 to 10%; 2 = 11 to 25%; 3 = 26 to 50%; and
4 = more than 50% affected leaf area.

***R. solani* toxin infectivity to rice cultivars**

Seed germination of both susceptible and moderately resistant rice varieties was significantly reduced by crude RS toxin application (Figure 5). Percent germination of susceptible TN1 rice seeds was significantly lower as compared to the control and to those applied with different RS toxin dilutions. The same observation was noted with moderately resistant rice seeds (IR72) wherein crude RS toxin application resulted in no germination of seeds and was significantly different from the control and those applied with different RS toxin concentrations. Percent germination of both rice seed varieties applied with boiled crude toxin was significantly lower than the control. This showed that crude RS toxin significantly affected the germination of rice seeds regardless of its variety. On the other hand, subjecting the toxin to high temperature (by boiling) did not affect the phytotoxicity or infectivity of the RS toxin. This result conformed with previous reports that heat treatment of culture filtrates of *R. solani* did not reduce its phytotoxicity to rice leaves in the bioassay (Yang *et al*., 2011). They noted that the autoclaved culture filtrate still caused typical lesions on rice leaves, as did the non-autoclaved filtrate. Yang *et al.* (2011) established that the bioactive components of RS toxin were thermostable

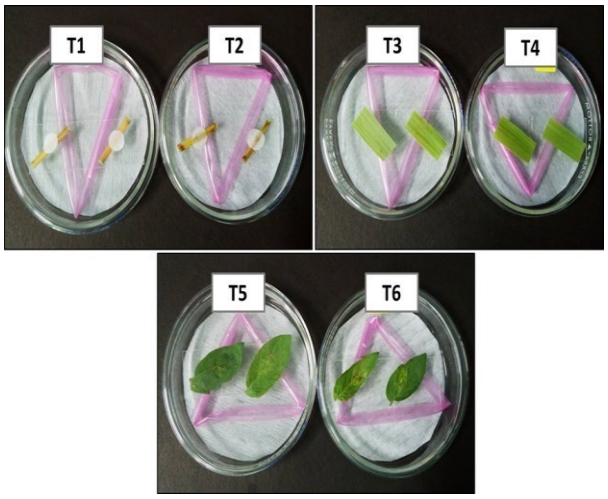

Figure 4. Leaf bioassay of Rhizoctonia solani toxin (RS toxin) on different plant species. T_1, rice leaf + dH2O; T_2, rice leaf + crude RS toxin; T_3, corn leaf + dH_2O; T_4, corn leaf + crude RS toxin; T_5, tomato leaf + dH_2O; and T_6, tomato leaf + crude RS toxin.

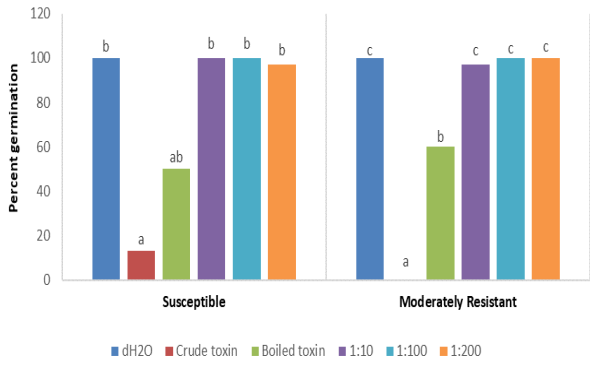

Figure 5. Percent germination of rice seeds applied with R. solani toxin. Bars/treatments with different letters are significantly different at P<0.05 using Tukey's post hoc test which was also manifested in the present study. Dilution of toxin to 1:10 (T_4), 1:100 (T_5) and 1:200 (T_6)

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resulted in a significantly higher percent germination of both TN1 and IR72 rice seeds which was comparable to the control (T_1). This result implied that lower concentration of the toxin reduced its phytotoxicity against rice seeds.

Effects of RS toxin on seedling growth of two rice cultivars were evaluated by shoot and root length. Crude (T_2) and boiled (T_3) toxin completely inhibited shoot growth of both susceptible & moderately resistant rice cultivars (Figure 6). Dilutions of toxin to 1:10 (T_4) resulted in a significantly lower shoot length of 1.8 cm in the susceptible variety but not in the moderately resistant variety. Toxin dilutions of 1:100 (T_4) and 1:200 (T_5) did not affect the shoot length and were comparable to those of the control (T_1). In terms of root length, similar observations with that of shoot length were noted. Root development in both cultivars was significantly hindered by crude (T_1) and boiled (T_2) crude toxin treatment. Lower dilution of toxin (1:10) significantly reduced root length of susceptible varieties but higher dilutions (1:100 and 1:200) had comparable effects with the control (T_1). While in moderately resistant cultivar, the three dilutions (1:10, 1:100 and 1:200) had no adverse effects on root development and were comparable to the control. Thus, seedling growth of two rice varieties was affected by the RS toxin applied as crude and as boiled. This indicated that temperature did not affect infectivity of the toxin to inhibit seed growth. As regards to concentration of RS toxin, high concentration (1:10) affected seed growth but not with low concentration (1:100 and 1:200) of RS toxin. In general, the reactions of two cultivars to RS toxin did not vary much implying the ability of the toxin to induce disease in host plants.

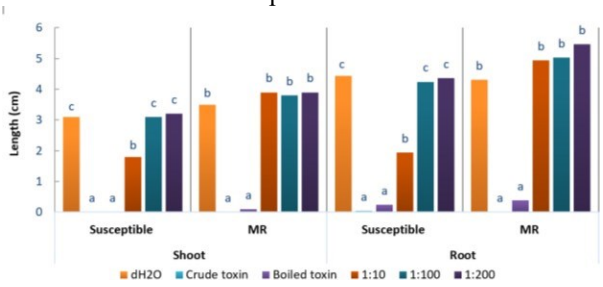

Figure 6. Seedling growth of two rice cultivars applied with R. solani toxin. Bars/treatments with different letters are significantly different at P<0.05 using Tukey's post hoc test.

4 CONCLUSIONS

RS toxin produced by *Rhizoctonia solani* infecting rice is pathogenic to its host plant (rice) but not to non-host plants (tomato and corn). Crude and boiled RS toxin affect seed germination and seedling growth of both susceptible and moderately resistant rice varieties. High temperature does not affect the infectivity of RS toxin to its host plant. Low concentration of RS toxin reduces its infectivity to both susceptible and moderately resistant rice cultivar.

ACKNOWLEDGMENT

We extend our thanks to IRRI for providing rice seeds and the Crop Protection Cluster Faculty and Staff for the support. This work was partly supported by the Department of Science and Technology through the Accelerated S&T Human Resource Program.

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