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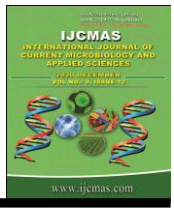

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An Innovative Technique for Artificial Inoculation of *Rhizoctonia solani* Kuhn for Field Experiments

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A B S T R A C T

Rhizoctonia solani is one of the most important soil borne plant pathogenic fungus of many agricultural and horticultural crops worldwide. The detailed analysis of sheath blight resistance at genetic, molecular, biochemical and functional levels is facilitated by development of effective and uniform inoculation techniques. The efficiency of *R. solani* infection plays a pivotal role in accurate evaluation of disease and screening of resistant cultivars. In the present study, an innovative technique for artificial inoculation of *R. solani* in rice was evaluated under field conditions using maize sand meal as inoculum type. Development of sheath blight disease symptoms was found to be effective and uniform in inoculated plots.

Introduction

Rhizoctonia solani Kuhn (Basidiomycota: Cantharallales: Ceratobasidiaceae) having teleomorphic stage as *Thanatephorus cucumeris* Frank, is an important soil borne plant pathogenic fungus of many agricultural and horticultural crops worldwide (Sneh *et al.,* 1996). *Rhizoctonia* is widely known for causing collar rot of cowpea and tomato (Dutta and Das, 2002) root rot of wheat and sugar beet, damping off of vegetables, wire stem of cabbage, web blight of cowpea, black scurf of potato, sheath blight of rice (Das, *et al.,* 1997), stem rot of soybean (Dutta and Das, 1999; 2000) etc. The genus *Rhizoctonia* is broadly classified into 14 anastomosis (AGs) groups based on their hyphal fusion, morphology, pathogenicity and physiology of which, AG1-1A is the most destructive one causing sheath blight of rice (Wang *et al.,* 2015).

The fungus is identified as septate, multinucleate mycelium branching at right angles from dolipore septum having constriction at the branching point. The hypha is initially hyaline and later turns brown with maturity producing numerous, small, light to dark brown sclerotia scattered irregularly

from the center to periphery (Oyetunde and Bradley, 2018). The characteristic symptoms caused by *R. solani* appear as water soaked chlorotic patches on leaf sheaths initially at culms just above water level. Patches later develop into oval or elliptical greenish grey spots of about 10 mm long with greyish white centre surrounded by irregular blackish or brown margin.

The detailed analysis of sheath blight resistance at genetic, molecular, biochemical and functional levels is important to devise accurate disease assay as well as to screen resistant cultivars. Quantification of sheath blight disease resistance are facilitated by development of effective inoculation techniques, where the type and amount of inoculum plays a key role in determining infection efficiency. Several inoculation techniques *viz.* placement of sclerotia, mycelia or colonized rice straw or grain on detached leaf, sheaths, at the base of plants or among tillers either in greenhouse or field condition have been used (Yoshimura and Nishizawa, 1954; Gangopadhyay and Chakraborthy, 1982), yet uniform infection and high degree of resistance is not easily achieved.

Further, in actively growing field plants in water logging conditions, the above methods showed variation in pathogenicity and resulting in confusion among the workers. Moreover, if proper conditions (moisture or humidity, temperatures, age of the plants, etc.) is not maintained the pathogen fails to cause infection in the target site. The present study has been conducted to develop an innovative technique for effective and uniform infection development of *R. solani* in rice plants, that can be used to differentiate minor differences in susceptibility of rice cultivars and for accurate evaluation of sheath blight disease.

Materials and Methods

Isolation and maintenance of *Rhizoctonia solani*

Freshly infected leaf sheath samples showing peculiar sheath blight symptoms, were collected from lowland rice field located in Umeit, Meghalaya (25º40’55.3’’ N, 91º56’55.4’’ E). Samples were collected in ice box and taken to the Plant Pathology laboratory of School of Crop Protection, CPGSAS, CAU (Imphal), Umiam, Meghalaya. Collected samples were washed carefully in running tap water to remove dirt and preserved in polypropylene bags at 4ºC in refrigerator for further use.

Samples including borderline of healthy and diseased tissues were cut into small sections (1 cm^2), disinfected with 5.0% sodium hypochlorite (NaOCl) solution followed by passing through double distilled water (DDW) for thrice and blot dried with sterile filter paper (Whatmann no. 1, Sigma-aldrich). Sections were inoculated into PDA plates (90 mm) amended with 0.1% streptomycin sulphate solution and incubated at 28±1ºC in BOD incubator (Equitron). Constant observation was made till radially grown hypha emerged out from inoculated sections. The isolated putative incitant was purified by hyphal tip culture method (Dhingra and Sinclair, 1995). The pure culture was maintained by period subculturing on fresh PDA slants and stored in refrigerator at 4ºC.

Pathogenicity test of *R. solani*

The pathogenicity test was conducted on healthy rice plants (var.: CAUS107) grown in 25 cm diameter plastic pots containing 5 kg sterilized soil. We tested two different methods viz., mycelial disc inoculation methods and application of mass culture inoculum in water logged conditions. For the

first method, mycelial disc (5.0 mm dia.) of actively growing 5 days old culture of *R. solani* were inoculated between sheaths and stem with the help of sterile forceps and wrapped with aluminum foil to maintain optimum moisture (Plate 1). Observations on sign, symptom and disease development were made.

Preparation of MSM media

Maize sand meal (MSM) media was prepared by grinding 40 g of pre-soaked maize kernels into fine powder and mix uniformly with 960 g of white sand (Plate 2). Optimum moisture was maintained by adding 400 ml of double distilled water for 1000 g of MSM media. MSM media was then filled into polypropylene bags (8 x 10 inch) upto 1/3rd of the capacity and and were plugged with non-absorbent cotton. MSM media filled bags were moist sterilized at 121ºC (15 lbs) for 15-20 minutes in autoclave (Equitron) twice at 24-hour interval.

Multiplication of *R. solani*

Actively grown mycelial disc (9 mm dia.) of *R. solani* from PDA plates (4 days old) were cut from the periphery of the plates with the help of cork borer (nichrome; 9 mm diam.). Three mycelial discs were inoculated into the MSM media axenically in laminar air flow cabinet (Biosafety) near Bunsen flame and tied back by pressure releasing of air. Inoculated bags were kept at room temperature till white coloured mycelial run completely covers the media. Any contamination observed in bags was immediately discarded.

Experimental site

The present study was conducted in low land rice field (400 m^2 area) CPGS-AS, CAU(Imphal), Umiam, which is situated in Ri bhoi district of Meghalaya . The experimental farm lies between 90˚55’ 15 to 91˚16’ latitude and 25˚40’ to 25˚21’ longitude at an altitude of 1010 m above mean sea level (msl). Ri bhoi district is classified under agro ecological sub region (ICAR) as North-Eastern Hills (Purvanchal) with warm pre-humid eco-region with an average annual rainfall of 1907.6 mm (129 normal rainy days). The maximum rainfall of 1239.3 mm (100 normal rainy days) occurs during kharif season (June-September). Rice variety (CAUS107), 150 days duration crop was selected for the present study.

The experimental farm was divided into 64 plots of 1.5 X 1.5 m^2 area having bunds of 30 cm width and surrounded by water channels (Plate 3). Plots were prepared by ploughing thoroughly for thrice at 15 days interval followed by pulverisation and application of farm yard manure (FYM) @ 12 tonne/ha. Paddy (var: CAUS107) was sown in nursery during last fortnight of May and transplanted @ 3 seedlings/hill at first fortnight of June at 25 days after sowing (DAS). Intercultural operations were done at 30 days interval from transplanting till heading stage.

Pathogen inoculation

Inoculation of *R. solani* was done at active tillering stage i.e. 60 days after transplanting (DAT). Fifteen (15) days old MSM media with mycelium and were mixed well by shaking and breaking sand clogs. About 100 g of the media with sufficient inoculum of the pathogen were uniformly distributed by fist-sprinkling near waterline in experimental plots. Control plots were inoculated with MSM media without any inoculum of *R. solani*. Regular observations on development of initial symptoms as elliptical to oval greenish grey spots in sheaths near waterline were made at every 24 hours interval.

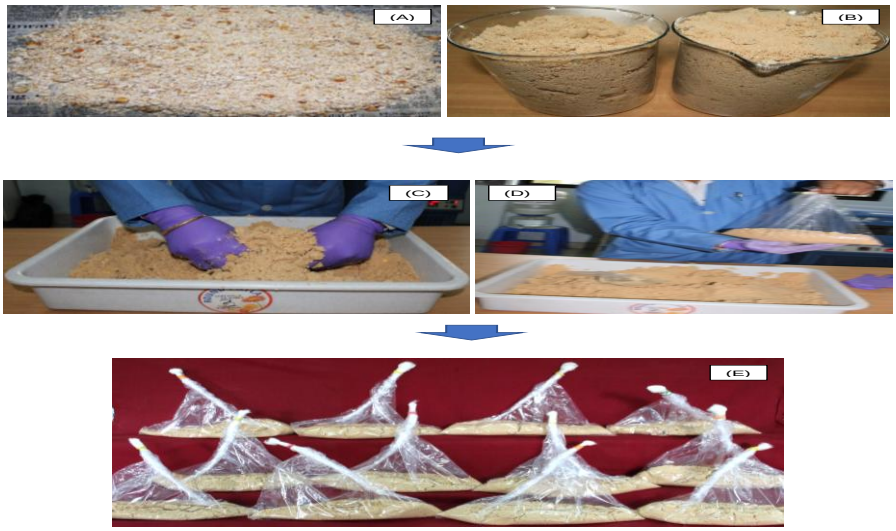
Results and Discussion

Identification of *Rhizoctonia solani*

The cultural characteristics of *R. solani* on PDA plate was identified as growth of whitish, fluffy, uprising mycelia at young vegetative stage that attains full growth (90 mm) after 4 days post incubation (Plate 4a). After 5th days of incubation, mycelia turned light brown producing numerous, circular, thick-walled, brown sclerotia initiated from centre of petriplate and developing until periphery that darkened with prolonged incubation (Plate 4b). Under light microscopy (Leica), *R. solani* showed septate, multinucleate, hyaline hyphae having right-angled branching pattern with typical constriction at the point of branching junction (Plate 4c). Earlier, Debbarma and Dutta (2015) also reported the similar identification characters of *R. solani* of different crops including rice. Similar morphological characters were also reported by Basbagchi *et al.*, (2019), Oyetunde and Bradley (2018), Desvani *et al.*, (2018).

Plate.1 Pathogenicity test for *R. solani;* Inoculation of *R. solani* mycelial disc in rice sheaths (A) and incubation for disease development (B)

Plate.2 Preparation of MSM media; Grinding of maize kernels into fine powder (A), white sand (B), uniform mixing of powdered maize with white sand with double distilled water (C), filling of polypropylene bags with MSM (D) and MSM media in polypropylene bags (E)



a) Plate 2: Preparation of MSM media; Grinding of maize kernels into fine powder (A), white sand (B), uniform mixing of powdered maize with white sand with double distilled water (C), filling of polypropylene bags with MSM (D) and MSM media in polypropylene bags (E)

Plate.3 General view of experimental field

Plate 3: General view of experimental field

Plate.4 Front (A) and rear view (B) of *Rhizoctonia solani;* (C) Micro-image of *R. solani* at 100x magnification under compound microscope (Leica)

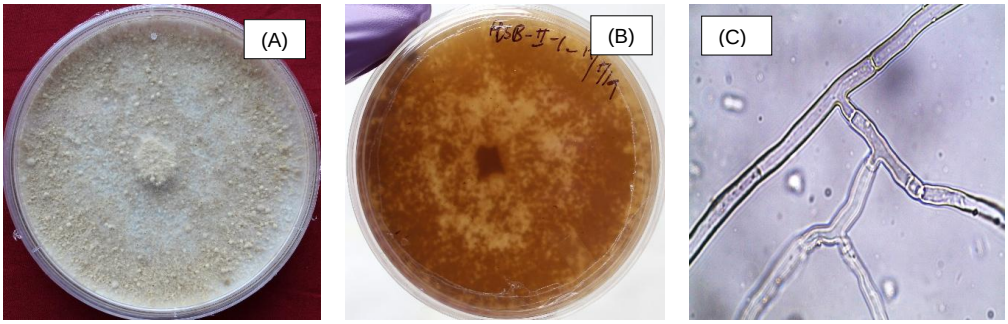


Plate 4: Front (A) and rear view (B) of *Rhizoctonia solani;* (C) Micro-image of *R. solani* at 100x magnification under compound microscope (Leica)

Plate.5 Development of sheath blight symptoms

Plate.6 Mycelial run (A, B) and development of brown coloured sclerotia of *R. solani* in MSM media (C)

Plate 6: Mycelial run (A, B) and development of brown coloured sclerotia of *R. solani* in MSM media (C)

Plate.7 Development of sheath blight symptoms under field conditions; at 10 days post inoculation dpi (A), oval to elliptical grey spots with brown margin at 25 dpi (B), drying of entire plant (C) and leaf blight symptoms (D)

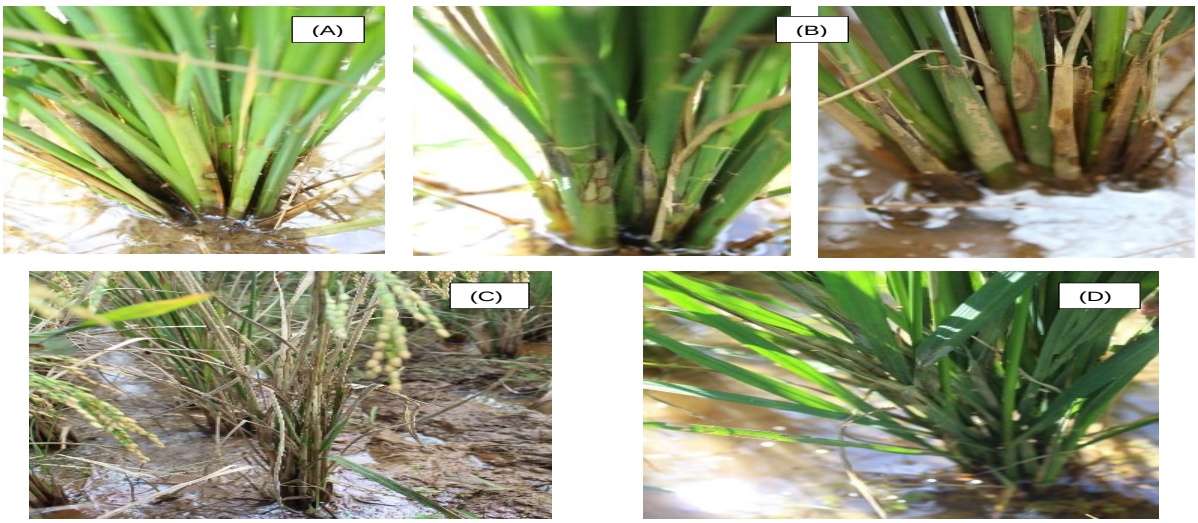


Plate 7: Development of sheath blight symptoms under field conditions; at 10 days post inoculation dpi (A), oval to elliptical grey spots with brown margin at 25 dpi (B), drying of entire plant (C) and leaf blight symptoms (D)

Pathogenicity test

The pathogenicity test was characterized by appearance of symptoms like development of irregular, water soaked, chlorotic patches initially, that further developed into oval or elliptical spots with grey centre and brown margin (Plate 5). In later stage, spots coalesce covering entire sheath and spread towards other parts of plants ultimately killing whole plant. Similar results were also reported by Das *et al.*, (2013), Botha *et al.*, (2003), Alghuthaymi *et al.*, (2015), Basbagci *et al.*, (2019), Yildirim and Erper (2016) in potato, strawberries, cotton, rice and maize, chickpea and vegetable crops respectively.

Multiplication of *R. solani* in MSM media

Initiation of fungal mycelia of *R. solani* in MSM media was observed from 3-4 days post incubation (dpi) and complete mycelial run in

the form of white mycelia was observed 7 dpi (Plate 6). Formation of numerous, initially white later brown coloured, thick-celled, small, round sclerotia were observed scattered in the media and maturity of sclerotia was observed within 15 dpi. Developed mycelial strands were re-isolated into PDA Petri plates and showed similar morphological and microscopic characteristics with the inoculated test pathogen. Silva *et al.*, (2017) and Buddemeyer *et al.*, (2004) have also used MSM media for inoculum multiplication of *R. solani* and reported similar results.

Development of sheath blight symptoms in rice under field condition

Under field conditions, initial symptoms of *R. solani* was observed at 10^{th} day post inoculation as small, irregular chlorotic patches on leaf sheath near waterline (Plate 7a). The patches were further developed uniformly in entire plots as irregular, oval to elliptical spots with greyish centre and brown margin that coalesce and gradually covering entire sheaths at 25^{th} days post inoculation (Plate 7 b, c). The symptoms were further aggravated by spreading of infection to other parts of plants covering entire plant leading to the development of leaf blight symptoms, ultimately whole plants get killed (Plate d, e). Similar symptoms were also reported in Maize by Dutta *et al.*, (2013) and rice (Das *et al.*, 1997).

It can be concluded that The present study showed that inoculam of *R. solani* produced in maize sand meal (MSM) media can be inoculated artificially in water logged field inoculation. This technique will help the researcher to overcome the problem faced by the earlier artificial methods of inoculation of *R. solani* in water logged conditions. The present technique is an innovative and effective technique for artificial inoculation of the pathogen and its uniform invasion and development of diseases in targeted host plant in water logged condition. This novel method will play an effective role in detailed analysis of sheath blight at physiological, biochemical and molecular level for development of effective management strategies.

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