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In vitro studies on *Ustilaginoidea virens*, a rice false smut pathogen

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Abstract

False smut of rice is caused by *Ustilaginoidea virens* (Cke.) Tak. [teleomorph: *Villosiclava virens* (Nakata) Tanaka & Tanaka] has emerged an important grain disease causing significant yield losses in major rice producing states of India. In the present study, false smut pathogen was successfully isolated from the infected smut balls and pathogenicity of *U. virens* was proved in BPT-5204 by artificial inoculation of conidial suspension (2 x 10^5 conidia) during booting stage. Growth of *Ustilaginoidea virens* (CG-1 isolate) was evaluated in different media, wherein Potato Sucrose Agar (PSA) medium recorded the maximum growth (with a growth rate of around 2.12 mm/day) and minimal growth was recorded in Czapek's Dox Agar medium. Among the natural substrates tested for mass multiplication, rice and barley grains supported maximum growth of the pathogen. The temperature *viz*., 55^{0}C, 60^{0}C and 65^{0}C affected the germination of chlamydospores.

Keywords: *Ustilaginoidea virens*, pathogenicity, chlamydospore, mass multiplication

Introduction

Plant diseases reduces yield and affects the quality and stability of production in addition to their affect on agricultural sustainability. Earlier, false smut disease of rice was considered as farmer's friendly disease and locally known as 'lakshmi disease' as it was found associated with bumper yield. The occurrence of the disease was irregular/ sporadic and the symptoms were mostly restricted to one or two grains per panicle. Use of high fertilizer-responsive varieties and hybrids, heavy application of nitrogenous fertilizer and changes in climatic conditions had paved the way for the outbreak of rice diseases. Thus, false smut disease emerged as one of the important grain diseases of rice in India as well as in the world. Presently, the false smut pathogen can affect huge number of grains in a panicle which can lead to disease epidemic and heavy yield loss. The pathogen affects the young ovary of the individual spikelet and transforms it into large, yellow colour smut balls and symptoms are visible from milky stage onwards. Initially, the smut balls are small in size and remain confined between glumes, white in colour, gradually change into yellow and covered with white colour membrane. Later, the membrane bursts and the colour changes to yellowish orange, olive green and finally greenish black. In India, the disease has been reported to occur in moderate to severe intensity from 2000 onwards (Guo *et al.,* 2012; Singh and Pophaly, 2010; Ladhalakshmi *et al.,* 2012; Laha *et al.,* 2016). The yield losses in different states of the country have been estimated to vary between 0.2% to 49% depending on the disease intensity and rice varieties grown in those areas (Dodan and Singh, 1996). Apart from direct loss, *U. virens* also produces a toxins known as ustiloxins and ustilaginodins (Koiso *et al.,* 1994; Sun *et al.,* 2017). Due to the increased importance of this disease the present work focussed on basic studies on false smut which including isolation and pathogenicity of *U. virens*, identification of suitable media for mass multiplication and also temperature influence on germination of chlamydospores.

Materials and Methods

Isolation, purification of *U. virens*

Isolation was done from the false smut diseased samples collected from different rice growing regions of India. The smut balls collected were thoroughly washed with running tap water and surface sterilized with 1% sodium hypochlorite for one minute and subsequently washed three times with sterile distilled water. Smear of chlamydospores

was streaked onto petri dishes containing Potato Sucrose Agar (PSA) medium using a sterilized inoculation loop. under complete aseptic conditions. To check the bacterial contamination, the medium was added with streptomycin (100 ppm). The petri dishes were incubated in BOD incubator at 25 ± 2° C for one week for obtaining fungal growth (Ladhalakshmi *et al.*, 2012).

Pathogenicity of *U. virens*

Multiplication of *U. virens* isolates

Pure culture of *U. virens* was multiplied by inoculating with an 8 mm mycelial disc of 2 weeks old culture in 100 ml Potato Sucrose Broth (PSB) in 250 ml flask and incubated in a shaking incubator at 120 rpm at 25 ± 2°C for a week.

Preparation of inoculums

The conidia of *U. virens* was harvested 6-7 days after inoculation from inoculated PSB. The culture was filtered using a muslin cloth and centrifuged at 4500 rpm for 20 minutes. The supernatant is discarded and 2-3 ml of sterile distilled water was added to the pellet and the concentration of conidial suspension (2×10^5 conidia ml^{-1}) was adjusted with the help of haemocytometer.

The susceptible rice cultivar *viz.*, BPT-5204 was used and the plants with booting stage were selected and injected with conidial suspension of 2 ml (2×10^5 conidia/ml) using a syringe. The plants were incubated at 24-26°C for 6 days at 95% R.H. in a humidity chamber and then kept under normal room temperature, until appearance of symptoms. The rice panicles injected with sterile distilled water served as control (Ladhalakshmi *et al.*, 2012).

Effect of different media on the radial growth of *U. virens*

Different media were evaluated against the growth of *U. virens.* Studies were made on eight different culture media *i.e.* Potato Sucrose Agar (PSA), Potato Dextrose agar (PDA), Oat meal agar, Corn meal agar, Rice polish agar, Rice polish yeast extract, Malt extract with dextrose and Czapeck-Dox agar.

Evaluation of natural substrate for mass multiplication of *U. virens*

Five natural substrates *i.e.* Rice, Barley, Ragi, Maize and Sorghum grains were selected for the study. Except rice, all the grains were pre-soaked in 2 per cent sucrose solution for 12 hrs. To the rice grain, double the quantity of water with 2 per cent sucrose was added before autoclaving. The flasks containing the substrates were autoclaved twice at 121°C for 15-20 minutes. Three mycelial discs of 8 mm diameter from the 2 week old culture of *U. virens* (C.G-1) were used for inoculation under aseptic conditions. Then the inoculated flasks were incubated at 25 ± 2°C for 30 days. Three replications were maintained for each medium. Flasks were regularly shaken after every 2-3 days for the uniform growth of the fungus (Rani, 2014).

Effect of temperature on the germination of chlamydospore

Different temperatures were evaluated to find out the optimum temperature for the germination of the chlamydospore. Black coloured smut ball (1 No.) from the diseased sample was sterilized with 0.1 % mercuric chloride and washed with sterile distilled water for 2-3 times repeatedly and finally about 3 ml of sterile distilled water was added and mixed thoroughly. From the glass vial about 200 µl was transferred into sterilized eppendorf tubes. Then individual eppendorf tube was subjected to heat at different temperature starting from 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C for ten minutes and the control treatment without heat. Potato Sucrose Agar (PSA) medium was melted and poured @ 20ml into the sterilized petri plates under aseptic conditions. Using a sterilized inoculation loop, a smear of solution was taken from each heat-treated eppendorf tubes and was streaked into the pre-poured petri plates with PSA medium. A control plate was also maintained. Three replications of each were maintained. Observations were recorded after 7 days of inoculation.

Results and Discussion

U. virens culture

Small tiny white colour colony was observed after seven days of incubation. Mycelium is septate in nature, produces primary conidia. The white colour of the fungus gradually changed into yellow and then changed into green colour. Sometimes, pathogen produced small ball like structures or clumps either on the middle or end of mycelia. Later, these balls changed into mass of chlamydospores and bursting of the clump was observed.

Pathogenicity

After 10-15 days of inoculation, the inoculated plants expressed the symptoms of 5-6 smut balls per panicle and the *U. virens* was isolated from these smut balls on PSA medium and Koch's postulate was proved for *U. virens.*

Effect of different solid media on radial growth of *U. virens*

Eight different media were tested and *U. virens* isolate CG-1 used for the study. Colony diameter was recorded at 10 days interval. From the eight-media used, PSA medium supported the maximum growth with a colony diameter of 63.16 mm and Czapeck's-Dox Agar medium supported the minimal growth with a diameter of 19.83 mm. (Table 1). Two of eight media, Rice Polish Agar (RPA) and Rice Polish Yeast Extract Agar (RPYA) media exhibited colony diameter of 54.83 mm and 55.17 mm, respectively and they were on par (Fig 1).

Table 1: Effect of different media on the growth of *U.virens*

Name of the Media	Media Composition	Radial Growth (mm)			Average growth rate per day(mm)	Observation on mycelial growth
		10 DAI	20 DAI	30 DAI		
Potato Sucrose Agar	Potato (from 200 g) extract; Sucrose – 10 g; Agar – 20 g; Water – 1 lt	25.00	55.30	63.16	2.12	White, yellow coloured mycelium
Potato Dextrose Agar	Potato (from 200 g) extract ; dextrose – 10 g; Agar – 20 g; Water – 1 lt	21.33	34.5	49.5	1.65	White, yellow coloured mycelium
Oat meal Agar	Oat meal – 60g; Agar – 12.5 g ; Water – 1 lt	24.33	35.33	45.5	1.50	White coloured mycelium
Corn Meal Agar	Corn meal – 40g; Agar- 15g; Water – 1 lt	18.33	39.16	59	1.96	White, light yellowish mycelium
Rice Polish Agar	Rice polish – 10g; Sucrose – 10g; Agar – 10g	11.33	31.33	54.83	1.81	White coloured mycelium
Rice Polish Yeast Extract Agar	Rice polish – 10g; Yeast extract – 0.5 g; Peptone – 0.5 g; Sucrose – 10g; Agar – 10g	12	29.50	55.17	1.84	White coloured mycelium
Malt Extract with Dextrose Agar	Malt extract – 20g; Peptone – 1 g; Sucrose – 20 g; Agar – 15 g; Water – 1 lt	8.67	15.17	22.5	0.77	Yellowish white coloured mycelium
Czapeck's-Dox Agar	$NaNO_3$- 2g ; K_2HPO_4 – 1 g; $MgSO_4$. 7 H_2O - 0.5 g; KCl – 0.5 g; $FeSO_4$ – 0.01 g; Sucrose – 30 g; Agar – 20g; Water – 1 lt	8.5	15.17	19.83	0.66	Very poor mycelial growth
CD at 0.05%		1.33	2.04	1.808		
CV (%)		4.71	3.67	2.24		

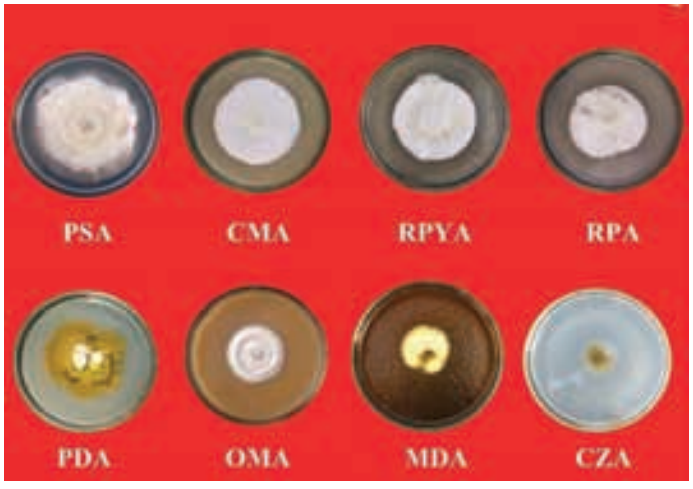

Figure 1. Evaluation of different media for the growth of *U. virens*

PSA - Potato Sucrose Agar; CMA- Corn meal agar; RPYA - Rice polish yeast extract; RPA - Rice polish agar; PDA- Potato Dextrose agar; OMA- Oat meal agar; MDA - Malt extract with dextrose; CZA - Czapeck-Dox agar

The above experimental findings were in accordance with the reports of Rani (2014), Baite *et al.*, (2014) and Fu *et al.,* (2013), who evaluated different carbon sources and different media. Results have shown that sucrose and starch were the best carbon sources and Potato Sucrose Agar medium was the best suitable medium for the growth of *U. virens.*

U. virens growth on different natural substrates

Different natural substrates like rice, barley, ragi, sorghum and maize grains were tested for mass multiplication of *U. virens.* Results indicated that rice and barley grains were shown maximum growth and the minimum growth was observed in maize grains. Colour of the culture was changed from white to yellow and green was observed in rice, barley, ragi; white to yellow in sorghum grains and only white colour in maize grains. In maize grains,

the growth of pathogen was minimum. Change in the colour of the mycelium indicates the formation of the chlamydospores. These results were supported by Rani (2014) who evaluated the different natural substrates for mass multiplication of *U. virens* and reported that the barley grains have shown the maximum growth compared to rice seeds and rice husk.

Effect of different temperatures on the germination of chlamydospore

The results revealed that the germination of the chlamydospores were not affected at temperature *viz*., 30°C, 35°C, 40°C, 45°C and 50°C, and hence colony growth was observed, Whereas temperatures of *viz*., 55°C, 60°C and 65°C affect the germination of the spore and thereby colony growth was not observed. The control treatment has shown the good germination and colony growth. Similar results were reported by Muraleedharan (2004), wherein the germination of chlamydospores was observed up to 40°C. Results of this study would help to have an idea on the survival of chalmydospores during extreme weather conditions.

False smut disease of rice is gaining importance because of its effect on both quantity and quality yield loss under favourable conditions. In the recent years, basic studies on the false smut disease *viz*., pathogen isolation, artificial culturing of the pathogen, pathogenicity and infection process were studied in detail. The results of the present study will assist in the development of the artificial inoculation screening technique for the identification of resistant sources..

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