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Cultural studies on *Ustilaginoidea virens*, the incitant of false smut of rice (*Oryza sativa*)

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ABSTRACT

A study was carried out to find out the favourable culture media for mass multiplication and the effect of carbon sources and duration on chlamydospore germination of *Ustilaginoidea virens* (Cook.) Tak. Different synthetic and semi synthetic solid, liquid media, substrate media were evaluated for mass multiplication of *U. virens*. Potato sucrose broth was the best medium for fast mycelia growth (104.80 mg) and sporulation (6.78×10^5 spores/ml), potato dextrose broth also favored mycelial growth, whereas corn meal dextrose broth was not suitable. Out of four solid media tested, maximum colony diameter (68.74 mm) and sporulation (6.86×10^5 spores/ml) was produced by potato sucrose agar followed by potato dextrose agar. Maximum sporulation was produced on substrate media based on barley seeds (5.54×10^5 spores/ml) followed by rice seeds (3.72×10^5 spores/ml) whereas rice husk based media produced minimum sporulation. Higher concentration of sucrose and dextrose at 2 per cent supported maximum germination of spores of *U. virens* and sucrose was found superior in inducing germination than dextrose. The results of this study help to understand the physiological and biochemical requirements for the growth and development of the pathogen, which could serve as an input in disease management to minimize the effect of false smut disease on rice.

Key words: Colony, Growth, Germination, Media, Substrate, Sporulation, *U. virens*

False smut of rice caused by *Ustilaginoidea virens* (Cooke.) Tak. is a serious disease worldwide as well as important constraint affecting rice yield and quality of rice throughout major rice-growing countries in Asia (Deng 1989, Yaegashi *et al.* 1989, Sugha *et al.* 1992, Zhou *et al.* 2008, Ladhalakshmi *et al.* 2012). False smut infection in the field is known to occur at rather short period just before heading (Ikegami 1960) and primarily affects quality since the fungus produces orange brown smut balls that contaminate rice grain at harvest.

Bagga and Kaur (2006) reported that false smut had become an important problem in the Punjab state and caused widespread concern in commercial cultivar PR 116 when environmental conditions were very favourable for the disease development. *U. virens* is a ubiquitous and one of the most destructive pathogens of rice (Hu *et al.* 2014). Quite a few attempts have been made to multiply the fungus using semi-synthetic media and solid substrates. The development of a suitable medium for growth and sporulation would facilitate studies of factors affecting the formation of metabolites in pure culture, from a practical stand point, a medium capable of stimulating sporulation of the fungus would aid in the preparation of spore suspensions required for inoculation of large populations in disease resistance breeding programs (Sharma and Joshi 1975). An understanding of the environmental conditions required for mycelial growth and spore germination is needed to identify the conditions required for spikelet infection and to develop appropriate control measures for the disease (Fu *et al.* 2013). A culture medium that can well support the conidial production is therefore essential as it is the primary step for evaluation or resistance against the pathogen. Therefore, the aims of the study were to determine the effect of different synthetic/semi synthetic and substrates medium on mycelial growth and sporulation & the effect of carbon sources and duration on chlamydospore germination of *U. virens*.

MATERIALS AND METHODS

The studies were carried out in the Department of Plant Pathology, Punjab Agricultural University, Ludhiana during 2014 to find out the favourable culture media for mass multiplication and the effect of carbon sources and duration on chlamydospore germination of *Ustilaginoidea virens*.

Isolation, purification and maintenance of causal pathogen: Initially the smutted balls taken from infected panicle were thoroughly washed with running tap water and surface sterilized with 0.1 per cent mercuric chloride

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solution for 1 minute and subsequently washed three times with sterile distilled water. Using a sterilized needle, the mass of chlamydospores was streaked onto Petri dishes containing potato sucrose agar (PSA) medium (peeled potato 200g, sucrose 20 g and agar 20 g in 1000 ml distilled water) under complete sterile and aseptic conditions. To check the bacterial contamination, the medium was incorporated with streptomycin (100 ppm). The Petri dishes were incubated in BOD incubator at 25 ± 2^0C for 2 weeks for obtaining fungal growth. A single, well isolated colony of the fungus (arising from a single spore) was picked up using sterilised needle and transferred to the fresh PSA slants and maintained as a pure culture.

Liquid nutrient media: The pathogen, *U. virens* was grown on potato sucrose agar (PSA) for 2 weeks at 25 ± 2°C. From the margin of actively growing fungus, 5 mm discs were plugged out. Sterile Petri dishes containing potato dextrose broth (PDB), potato sucrose broth (PSB), rice meal-dextrose (RMD) and corn meal dextrose (CMD) were inoculated each with a single 5 mm disc of the fungus and incubated at 25 ± 2°C for 2 weeks for determining the growth on different media. Three replications were maintained for each medium. At the end of incubation period, the mycelium containing conidia and conidiophores was filtered through two layers of tissue paper and observations on dry weight of mycelium were recorded. To count the number of spores, a mycelial bit of 5 mm diameter was taken from each flask and added to 5 ml of sterilized water in the test tube and was shaken well. The number of conidia/ml present in each suspension was calculated with the help of haemocytometer.

Solid nutrient media: Sterile Petri dishes containing Richard's medium (RM), Czapek's medium (CM), potato dextrose agar (PDA) and potato sucrose agar (PSA) were inoculated each with a single 5 mm disc of the fungus and incubated at 25 ± 2°C for 2 weeks for determining the growth on different media. Three replications were maintained for each medium. At the end of incubation period, observations for mycelial growth (diameter) were recorded and to count the number of conidia, a mycelial bit of 5mm diameter was taken from each Petri dish and was added to 5ml of sterilized water in the test tube and was shaken well. The number of conidia/ml present in each suspension was calculated with the help of haemocytometer.

Substrate nutrient media: Three natural media, i.e. barley seeds, rice seeds and rice husk were pre-soaked in 2% sucrose solution for 12 hr. The flasks containing the substrate were autoclaved twice at 121.6°C for 20-25 min. The flasks containing media were inoculated with a three mycelial bits of 5 mm diameter from the 2 weeks old culture of isolate of *U. virens* under aseptic conditions. Then the inoculated flasks were incubated at 25 ± 2°C for 2 weeks for determining the growth on different media. Three replications were maintained for each medium. Flasks were regularly shaken after every 2-3 days for the uniform spread of the fungus. At the end of incubation period, 1 g of sample was taken from each flask and was added to 5 ml of sterilized water in the test tube and was shaken well. The number of conidia/ml present in each suspension were calculated with the help of haemocytometer.

Effect of carbon sources on germination of chlamydospores of U. virens: The viability of the spores from spore balls of *U. virens* was assessed. Different substrates, viz. distilled water, dextrose and sucrose at concentration 1 and 2% were tested to find out the suitable substrate for inducing maximum spore. The spore suspension was prepared in sterile tubes by placing spores of *U. virens* in distilled water, 1 and 2% dextrose and sucrose solution with concentration of 2 × 10^5 spores per ml and incubated at 25 ± 2^0C. After incubation for 2, 4, 8, 12, and 24 hr, 10 µl spores suspension from incubated tubes were placed on a glass slide. The number of spores that produced visible germ tubes was calculated under a microscope at 40X magnification. The spores were considered to have germinated if germ tube was visible as half the length of the spore.

RESULTS AND DISCUSSION

Evaluation of nutrient/substrate media for mass multiplication of U. virens

Liquid nutrient media: The results presented in Table 1 reveal that the maximum mycelial growth (average dry weight = 104.80 mg) was produced by potato sucrose broth followed by potato dextrose broth (78.50 mg). The rice meal dextrose broth produced mycelial growth of 60.17 mg and the corn meal dextrose broth produced the least dry weight, i.e. 41.23 mg. The number of conidia/ml produced in all the media ranged between 2.60 × 10^4 conidia/ml to 6.78 × 10^5 conidia/ml. The maximum number of conidia was produced on potato sucrose broth (6.78 ×10^5 conidia/ml), whereas minimum was produced on corn meal dextrose (2.60 × 10^4 conidia/ml). The potato dextrose broth and rice meal dextrose broth produced 5.18 × 10^5 and 3.82 × 10^4 conidia/ml, respectively. Similarly, Haiyong (2012) tested seven media of different composition to obtain optimal liquid culture condition for sporulation and indicated that PSB was the best medium for conidia production. It has been reported by many workers that sucrose is the best carbon source for conidial production of *U. virens* (Wang 1992, Ji 2002). A culture medium which supported the maximum conidial production is therefore essential for undertaking further studies on different aspects of *U. virens*.

Table 1 Evaluation of liquid nutrient media for mass multiplication of *U. virens*

Liquid medium	Dry weight (mg)	Conidial concentration (/ml)
Potato dextrose broth	78.50	5.18 × 10^5
Potato sucrose broth	104.80	6.78 × 10^5
Rice meal dextrose	60.17	3.82 × 10^4
Corn meal dextrose	41.23	2.60 × 10^4
CD (P=0.05)	3.58	3.00

Table 2 Evaluation of solid nutrient media for mass multiplication of *U. virens*

Solid medium	Colony diameter (mm) after 14 days	Conidial concentration (/ml)
Potato sucrose agar	68.74	6.86×10^5
Potato dextrose agar	59.26	5.12×10^5
Czapek's medium	32.54	3.72×10^4
Richard's medium	16.76	2.18×10^4
CD (P=0.05)	2.78	1.17

Solid nutrient media: Colony diameter and spore production in all the solid media were observed after 2 weeks of inoculations (Table 2). Maximum colony diameter was produced by potato sucrose agar (68.74 mm) followed by potato dextrose agar (59.26 mm) and minimum was produced by Richard's medium (16.76 mm). The colony diameter on Czapek's medium was 32.54 mm. Similar trend was observed in spore production. However, maximum sporulation was observed in potato sucrose agar medium (6.86×10^5 conidia/ml) followed by potato dextrose broth (5.12×10^5 conidia/ml), whereas minimum sporulation occurred in Richard's medium (2.18×10^4conidia/ml) followed by Czapek's medium (3.72×10^4 conidia/ml). Similarly, Fu *et al.* (2013) also that out of eight media tested, potato sucrose agar (PSA) was the best medium for fast mycelial growth of *Villosiclava virens* (teleomorph of *U. virens*). The present findings are also in agreement with the observations as reported by Li *et al.* (2008) who also observed the fast growth of fungus on potato sucrose agar (PSA).

Substrate nutrient media: Data given in Table 3 show that maximum sporulation was produced on substrate media based on barley seeds (5.54×10^5 conidia/ml) followed by rice seeds (3.72×10^5 conidia/ml), whereas rice husk based medium produced minimum sporulation (2.18×10^4 conidia/ml). In case of barley based substrate medium, the colour of mycelium changed from white to yellow and finally green whereas in case of rice seeds and rice husk based media the colour remains white throughout. Similarly, Mohan *et al.* (2013) evaluated different natural and semi-synthetic media for growth and sporulation of *Pyricularia oryzae* and reported that substrate/media based on makra grass (*Dactyloctenium aegyptium*) and acrachne grass (*Eleusine racemosa)* yielded higher number of spores, i.e. 5.6×10^6/ml and 5.5×10^6/ml, respectively. Extracts of carrot, cereal grains and grassy host also enhanced the colony growth of the pathogen but they did not had much effect on sporulation. They reported that natural media can be used for mass multiplication of the pathogen.

Table 3 Evaluation of substrate nutrient media for multiplication of *U. virens*

Natural medium	Conidial concentration (/ml)	Colour of mycelium
Barley seeds	5.54×10^5	Green
Rice seeds	3.72×10^5	White
Rice husk	2.18×10^4	White
CD (P=0.05)	3.33	

Table 4 Effect of carbon sources on germination of chlamydospores of *U. virens*

Nutrient solution	Chlamydospores germination at different intervals (%)				
	2 hr	4 hr	8 hr	12 hr	24 hr
2% sucrose	34.03 (35.66)	47.00 (43.26)	57.80 (49.47)	68.97 (56.12)	70.30 (56.99)
2% dextrose	36.07 (36.09)	46.13 (42.76)	53.07 (46.74)	64.87 (53.63)	65.90 (54.27)
1% sucrose	34.23 (35.78)	45.20 (42.22)	56.00 (48.44)	66.32 (54.51)	67.20 (55.04)
1% Dextrose	38.17 (38.13)	43.93 (41.50)	52.23 (46.26)	62.40 (52.19)	64.00 (53.12)
Distilled water	33.77 (35.51)	41.25 (39.94)	49.20 (44.52)	49.33 (44.60)	46.90 (43.22)
CD (P=0.05)					
Nutrient media = 0.68					
Germination hours = 0.52					
Nutrient media Germination hours = 1.62					

*Figures in parentheses are angular transformed values

Effect of carbon sources on germination of chlamydospores of U. virens

The data on effect of carbon sources on germination of chlamydospore of *U. virens* are given in Table 4. In the beginning, the dextrose at 1 and 2% concentration slightly stimulated the germination of spores of *U. virens* (38.17 and 36.07%) than sucrose solution (34.23 and 34.03%) after 2 hr of incubation. However, later on with further delay in incubation from 4 to 24 hr, the higher concentration of sucrose and dextrose (2%) supported maximum germination of chlamyspores of *U. virens.* Among the two carbon sources tested, sucrose was found superior in inducing germination than dextrose. Maximum germination was induced by 2% sucrose (70.30%) followed by 1% sucrose (67.20%), whereas dextrose at 2% concentration induced a germination of 65.90% and lower dose of 1 per cent induced germination of 64.00% after 24 hr of incubation. In distilled water only 46.90% germination was observed. Schroud and Tebeest (2005) also studied viability of *U. viren*s and found that spores germinated very rapidly in sucrose, and nutrient broth solutions. Therefore, these findings in agreement to the results obtained in the present investigation that indicated that carbon sources stimulated the spore germination of *U. virens.*

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