

Original Research Article

Techniques for Isolation, Conidiation and Pathogenicity of *Ustilagoidea virens* Isolates (Uv3, Uv4, and Uv15) Causing False Smut Disease in Rice

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Abstract

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Rice false smut, caused by *Ustilagoidea virens* (Uv3, Uv4 and Uv15) showed small mycelial growth during isolation from chlamydospores and mycelial discs of smut balls, and exhibited varied in density of growth at 7 days of incubation. Twenty-seven days after incubation, isolate Uv3 exhibited the highest growth at 77.00 mm, characterized by raised elevation and white-yellow surface color with yellow-green pigmentation. Isolate Uv4 showed circular growth with a mycelial growth of 64.00 mm, while isolate Uv15 displayed the lowest growth at 55.66 mm, with flat elevation and white-yellow-green appearance. In BPT 5204 rice leaf extract (BRLE), conidia counts increased significantly with higher concentrations: Uv3 reaches 31.38×10^6 conidia/ml, Uv4 27.71×10^6 conidia/ml, and U15 20.11×10^6 conidia/ml. TN1 rice leaf extract (TRLE) media yields lower counts but shows similar concentration-dependent trends. PSB yields notably fewer conidia. Pathogenicity assessments on TN1 and BPT 5204 rice varieties revealed Uv3 as the most aggressive isolate, causing higher numbers of smut balls per panicle and greater grain infection percentages compared to Uv4 and Uv15.

Keywords: False smut of rice; *Ustilagoidea virens* isolation; Rice leaf extract; Pathogenicity test; Microscopic observation

1. INTRODUCTION

Rice is the world's second most vital cereal crop, serving as a primary food source for 60% of the global population. Annually, the global rice production stands at 503 million metric tons [14]. In India, during the 2021-22 season, rice cultivation spanned 46.38 million hectares, yielding 130.29 million tons with an average productivity of 2.809 tons per hectare [1]. Nonetheless, rice production is hindered by biotic stresses (such as pests and diseases) and abiotic stresses (such as environmental factors), leading to yield losses between 10% and 30% [10].

False smut, caused by the fungus *Ustilagoideia virens*, is among the most prevalent and damaging diseases affecting rice fields globally. The first recorded instance of this disease was in the Thirunelveli district of Tamil Nadu, India [4]. Symptoms of false smut manifest on the spikelets of mature rice crops, appearing as green smut balls covered in powdery dark green chlamydospores. Historically, false smut was known as 'Lakshmi disease' and was considered a sign of a good harvest [5]. However, it has recently emerged as one of the most destructive diseases impacting rice grains. Since 2001, the severity of false smut has intensified in major rice-growing states in India, causing notable yield reductions and deterioration in rice grain quality.

Add something about the strains and isolates previously studied and the type of media used or optimized for the *Ustilagoideia virens* to support the study or to provide base to the study. The study should be similar to the current study.

If the isolation techniques is novel, then discuss the previously sued isolation techniques with appropriated references.

Furthermore, pathogen produces large amount/number of mycotoxins (ustiloxin and ustilaginoidins), which have carcinogenic properties and pose a significant risk to both human and animal health when contaminated rice grains and straw are consumed [6, 15]. False smut balls are associated with many saprophytes, making the isolation of the pathogen very difficult. This experiment details the methods for isolating the false smut pathogen, conidia production using rice leaf extracts, and conducting pathogenicity tests for *Ustilagoideia virens* isolates (Uv3, Uv4 and Uv5).

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2. MATERIALS AND METHODS

2.1 Collection of smut balls

In this study, three smut ball samples collected from Telangana State were used: Uv3 from Morthad Village, Nizamabad District; Uv4 from RS & RRS, Rudrur, Nizamabad; and Uv15 from the Institute of Rice Research, ARI, Rajendranagar, Hyderabad. The experiment was conducted from April to December 2022. Laboratory experiments were performed in the Department of Plant Pathology, College of Agriculture, Rajendranagar. The pathogenicity test was conducted in a

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polyhouse at the Institute of Rice Research (IRR), Agricultural Research Institute (ARI), Rajendranagar, Hyderabad.

2.2 Isolation of rice false smut pathogen (*U. virens*)

Two methods were used for the isolation of rice false smut pathogen, in first method pathogen were isolated ~~from using~~ chlamydospores of smut balls (Fig 1A). Initially, the collected smut balls were washed with tap water and then surface-sterilized ~~using with a~~ 4% sodium hypochlorite (NaOCl) solution for 30 seconds, followed by thorough rinsing with sterilized distilled water three times. Chlamydospore suspension was prepared from the sterilized smut balls and used for pathogen isolation. Potato Sucrose Agar (PSA) was used as the growth medium; ~~and added with~~ streptomycin sulphate (100 ppm) was added to prevent bacterial contamination. The spore suspension was carefully streaked onto Petri dishes containing PSA, and these plates were placed in BOD incubator at a controlled temperature of $26 \pm 1^\circ\text{C}$ for seven days. In the second method, pathogen were isolated from the central mycelial portion of the smut ball (Fig 1B); ~~for that~~ ~~The~~ inner part of smut ball was cut into small pieces and subjected to surface sterilization ~~using with~~ 4% ~~sodium hypochlorite~~ (NaOCl) solution for 30 seconds, followed by thorough rinsing with sterilized distilled water ~~for~~ three times. The smut ball hard mycelial pieces were placed on sterilized blotter papers to remove excess water from the surface, then placed onto PSA plates and incubated similarly as described in the first method. After 7 days of incubation, mycelial growth was measured using digital colony meter (mention model, company name, country of origin of instrument used). The pure culture was prepared using the hyphal tip method for subsequent analysis [2, 3]. Mycelium, ~~ehlamydospores~~ chlamydospores, and conidia ~~was~~ were observed under the compound microscope at 10 and 40x. SEM imaging was done for the chlamydospores (5000 and 10000x). (mention model, company name, country of origin of instrument used)

2.3 Preparation of rice leaf extract media for the *U. virens* conidiation

Rice leaves collected at the early booting stage were used for media preparation. Specifically, the media included BPT 5204 rice leaf extract (BRLE) and TN1 rice leaf extract (TRLE) at various concentrations (0.02 g/ml, 0.04 g/ml, 0.06 g/ml, 0.08 g/ml, and 0.10 g/ml), along with a control medium of potato sucrose broth (2% sucrose in potato extract). For media

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preparation, the respective quantity of fresh leaves was taken and washed with tap water, followed by rinsing with distilled water. Subsequently, the leaves were chopped into small pieces, which were then blended in a blender with an appropriate amount of distilled water added. The resulting leaf extract was strained and sterilized in an autoclave at 121 °C, 15 psi (121°C) for 20 minutes at 15 psi. Mycelial discs (10 to 15 in number) from actively growing pathogens (Uv3, Uv4, and Uv15) were added to the autoclaved leaf extract media under laminar air flow, and the inoculated flasks were placed in a rotary incubator at $26 \pm 1^\circ\text{C}$ for 7 days at 120 rpm. The number of Conidia were counted using a hemocytometer (reference);.....After counting how the concentration was determined? You must have used some formulas to calculate the formulas, mention them.

2.4 Pathogenicity test for the U3, Uv4 and Uv15 on rice plants

To confirm the pathogenicity of three isolates (Uv3, Uv4 and Uv15) and to prove the Koch's postulates, polyhouse experiment was conducted from June to October 2022 at Institute of Rice Research, Agriculture Research Station, Rajendranagar, Hyderabad.

In this studies, The false smut susceptible rice cultivars TN 1 and BPT 5204 were used for the experiment. The seeds were surface-sterilized by soaking them in 4% sodium hypochlorite NaOCl for 30 seconds and then washed thoroughly with sterilized distilled water three times to remove chemical residues. Pots with a diameter of 12 inches and a height of 10 inches were filled with a mixture of sterilized farmyard manure and soil in a 1:3 ratio. The nursery was established and transplantation was performed after 30 days in pots under polyhouse conditions. Inoculation was done performed with a surgical syringe loaded with 2 ml of conidial suspension of *U. virens* (2×10^5 conidia ml^{-1}) was by injecting into leaf sheaths covering the developing panicle of BPT 5204 and TN 1 plants s at late booting stage (2 to 3 days before heading). After injection of inoculum, the plants were kept in polyhouse at a temperature of $26 \pm 1^\circ\text{C}$ for 10 days and 95% relative humidity was maintained using a fogging system. Subsequently, they were maintained under at normal room temperature until the appearance of false smut balls from the emerging spikelets. [7]. Data on the number of smut balls per panicle (NSBPP) and percent infected grains (PIG) was were calculated as given below [13, 9].

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$$\text{Number of smut balls per panicle (NSBPP)} = \frac{\text{Total number of observed smut balls/hill}}{\text{Total number of infected panicles/hill}}$$

$$\text{Per cent infected grains (PIG)} = \frac{\text{Number smut balls/panicle}}{\text{Total number of grains/panicle}} \times 100$$

2.5 Statistical Analysis

The experiments were carried out using a completely randomized design (CRD), and the data were analyzed through a one-way analysis of variance (ANOVA) at a 5% significance level ($P \leq 0.05$) in OPSTAT. Each experiment included three replications.

3. RESULTS

3.1 Growth of *Ustilagoideia virens* from smut balls

After 7 days of incubation, small colonies with white or green-colored mycelia formed from germinating chlamydospores and central mycelial pieces of smut balls... [on which media?](#) (Fig. 1). The maximum mycelial growth was observed in growth from the mycelial discs of smut balls: 7.20 mm (Uv3), 5.31 mm (Uv4), and 4.43 mm (Uv15), compared to growth from chlamydospore germination: 5.56 mm (Uv3), 3.94 mm (Uv4), and 3.31 mm (Uv15). The density of the mycelia was higher in growth from chlamydospore germination and lower in mycelial growth from mycelial discs of smut balls (Table 1).

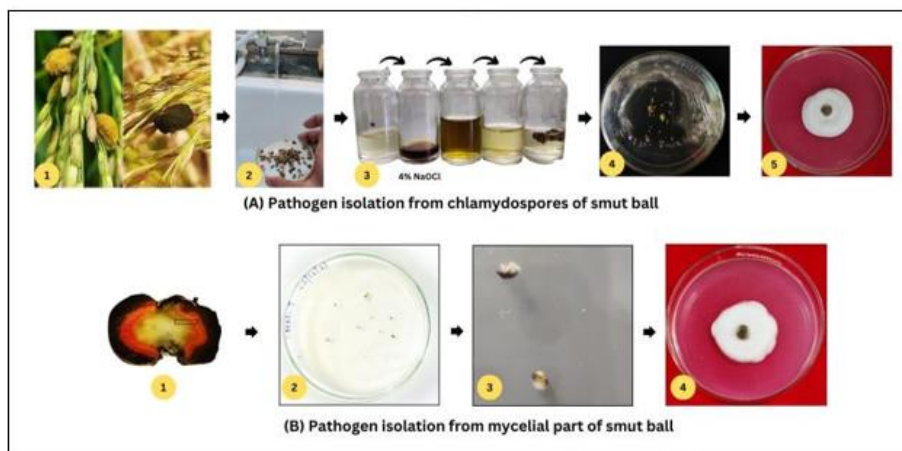
After obtaining the pure culture, growth was normalized for the pathogen isolated from both methods. At 27 days after incubation, Isolate Uv3 displays raised elevation with irregular growth forms and entire mycelial margins. It shows zonation and has a white-yellow surface color with yellow-green reverse pigmentation. Chlamydospores ~~are~~ were produced, and it had the highest radial mycelial growth among the three isolates, measuring 77.00 mm. Isolate Uv4 ~~was~~ characterized by a flat elevation, circular growth form, and filiform mycelial margins. It ~~lacked~~ zonation and sectoring and exhibited a white-green surface color with yellow-green reverse pigmentation. It ~~produce~~ produced chlamydospores and shows a mycelial growth of 64.00 mm. Isolate Uv15 ~~had~~ a flat elevation, circular growth form, and filiform mycelial margins. It ~~does~~ did not exhibit zonation or sectoring. The surface color ~~was~~ white-yellow-green, and the reverse pigmentation ~~was~~ yellow-green. Chlamydospores ~~were~~ produced, and the radial mycelial growth ~~was~~ 55.66 mm, the lowest among the three isolates.

Table 1. Mycelial growth from germinating chlamydo spores, smut ball mycelial discs and pure cultures

Isolate	Mycelial growth (mm) at 7 days after incubation		Mycelial growth (mm) from pure cultures at 27 days after incubation
	Chlamydo spores of smut balls	Mycelial disc of smut balls	
Uv3	5.56±1.03*	7.20±1.52	77.00 ±3.33
Uv4	3.94±0.52	5.31±0.57	64.00 ±2.65
Uv15	3.31±0.51	4.43±0.37	55.66 ±2.46
C.D. (<i>p</i> <0.05)	0.374	0.407	3.265
SEm±	0.106	0.115	0.925

*Mean standard error

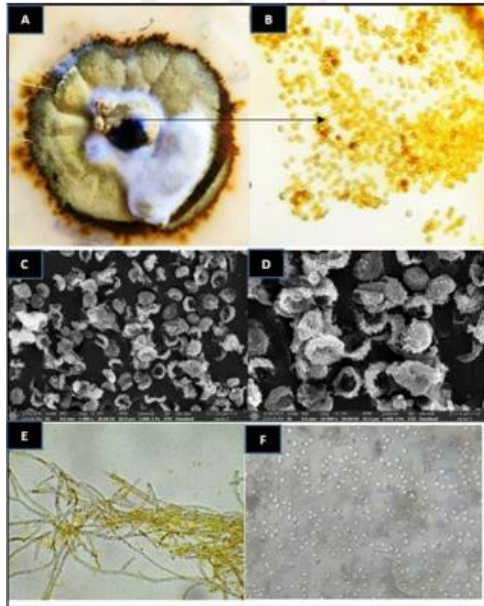
**** for better comparison among the different isolates, Tukey HSD or Duncan test should be done.



A1- collection of smutted rice grains from field, **A2-** washing of smut balls under running tap water, **A3-** surface sterilization of smut balls at 4% NaOCl, thoroughly washing of smut balls using sterilized water for three times and spore suspension preparation, **A4-** small mycelial colonies developed from chlamydo spore suspension streaked on PSA media at one week after incubation, **A5-** 15 days old pure culture of *U. virens*. **B1-** small mycelial pieces were cut from inner portion of smut balls, **B2-** surface sterilized mycelial pieces of smut balls placed on PSA media, **B3-** mycelial growth from mycelial pieces of smut balls at one week after incubation, **B4-** pure culture of 15 days old *U. virens*.

Fig 1. Isolation of rice false smut pathogen (*U. virens*) from smutted grain

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A– Chlamydospores (Uv3) formed on PSA media, **B**– Microscopic observation of chlamydospores (40x), **C–D**– SEM images of Chlamydospores (5000x and 10000x), **E**– Geniculate mycelium of *U. virens* (40x), **F**– Oval-shaped conidia formation in rice leaf extract media (40x).

3.2 Conidiation in rice leaf extract media (RLEM)

The Table 2 provided, the number of conidia produced by three strains of *Ustilagoidea virens* (Uv3, Uv4, and U15) at 7 days after incubation in media includes BPT 5204 rice leaf extract (BRLE) and TN1 rice leaf extract (TRLE) at various concentrations (0.02 g/ml, 0.04 g/ml, 0.06 g/ml, 0.08 g/ml, and 0.10 g/ml), along with a control medium (PSB) have been provided in Table 2. In the BRLE medium, the number of conidia for Uv3 starts at 5.32×10^6 conidia/ml at a concentration of 0.02 g/ml and increases significantly with higher concentrations, reaching 31.38×10^6 conidia/ml at 0.10 g/ml. Similarly, Uv4 produces 4.11×10^6 conidia/ml at the lowest concentration, increasing to 27.71×10^6 conidia/ml at the highest concentration. U15 shows the lowest conidia counts among the three strains, starting at 1.64×10^6 conidia/ml at 0.02 g/ml and rising to 20.11×10^6 conidia/ml at 0.10 g/ml. These results indicate a positive correlation between the concentration of BRLE and the number of conidia produced for all three strains. In the TRLE

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medium, the conidia counts ~~are were~~ generally lower compared to the BRLE medium. For Uv3, the conidia count is 3.67×10^6 at 0.02 g/ml and increases to 25.56×10^6 at 0.10 g/ml. Uv4 follows a similar trend, with conidia counts starting at 1.45×10^6 at the lowest concentration and reaching 21.48×10^6 at the highest concentration. U15 again shows the lowest numbers, with 0.57 ± 0.01 conidia at 0.02 g/ml and increasing to 22.16×10^6 at 0.10 g/ml. Despite the lower overall conidia counts in TRLE, [a noticeable increase in conidia was still observed at higher concentrations, the increase in conidia with higher concentrations is still evident.](#)

The control medium, PSB, showed significantly lower conidia counts for all strains. Uv3 produces 0.84×10^6 conidia/ml, Uv4 produces 0.57×10^6 conidia/ml, and U15 produces only 0.15×10^6 conidia/ml. This stark contrast underscores the influence of the rice leaf extracts on conidia production, highlighting the effectiveness of BRLE and TRLE in promoting conidia formation in *Ustilagoidea virens*.

Table 2. Effect of different concentrations of rice leaf extract on conidia formation

Media	Conidiation ($\times 10^6$ Conidia/ml) at 7 days after incubation		
	Uv3	Uv4	U15
0.02 g/ml BRLE	5.32±0.05	4.11±0.04	1.64±0.02
0.04 g/ml BRLE	9.91±0.13	8.63±0.04	4.37±0.05
0.06 g/ml BRLE	15.67±1.21	13.87±0.26	8.72±0.16
0.08 g/ml BRLE	23.28±1.16	20.16±0.83	14.49±1.13
0.10 g/ml BRLE	31.38±1.91	27.71±1.24	20.11±0.71
0.02 g/ml TRLE	3.67±0.07	1.45±0.02	0.57±0.01
0.04 g/ml TRLE	7.14±0.06	3.16±0.04	2.14±0.04
0.06 g/ml TRLE	12.94±0.62	7.22±0.01	5.31±0.03
0.08 g/ml TRLE	19.48±0.01	13.93±0.86	11.75±0.61
0.10 g/ml TRLE	25.56±1.17	21.48±0.66	22.16±0.77
PSB	0.84±0.02	0.57±0.01	0.15±0.00
C.D. ($p < 0.05$)	2.56	1.66	1.48
SEM±	0.87	0.56	0.50

*Mean standard error, BRLE- BPT 5204 rice leaf extract, TRLE- TN1 rice leaf extract

3.3 Microscopic observations of *U. virens*

Microscopic observation (Fig. 2) revealed that the mycelium of the fungi was septate and hyaline in color. On PSA media, chlamydospores formed from pseudomorphs started as smooth spheres with an orange-yellow hue, maturing into dark brown echinulate structures with a rough surface. On leaf extract media, oval-shaped conidia formed for all three isolates.

3.4 Pathogenicity test for *U. virens*

After 20 days of inoculation, the inoculated plants ~~have shown~~ exhibited the symptoms of smut balls (Fig. 3e). Again, the *U. virens* ~~have been was~~ successfully isolated from ~~these those~~ smut balls on PSA medium and Koch's postulates was proved for *U. virens*. The fungus infected the young ovary of the individual kernels and transformed them into large, velvety green balls. Smut balls were initially white in colour, visible between glumes, grew gradually and covered with a mycelial membrane which bursted in the later stage and released the ~~ehlamydospores~~ chlamydospores which gave the yellow to orange colour appearance to the smut balls, later turned to olive green to black (Fig 4a). When smut balls were young, they were fleshy inside ~~and which~~ hardened over time. The smut ball consisted of a central hard mycelial tissue consisting of thin, hyaline septate hyphae (Fig 4b).

The results of the study on the number of smut balls per panicle (NSBPP) and the percent infected grains (PIG) by *Ustilagoideia virens* isolates Uv3, Uv4, and Uv15 on two rice varieties, TN1 and BPT 5204, demonstrated significant differences in the pathogenicity (Table 3). For the TN1 variety, isolate Uv3 showed the highest NSBPP at 15.55% and PIG at 23.32%, indicating its aggressive nature in causing false smut disease. This was followed by Uv4, which had an NSBPP of 8.41 and a PIG of 12.62%, and Uv15, with an NSBPP of 5.02 and a PIG of 7.53%. Similarly, for the BPT 5204 variety, Uv3 again exhibited the highest levels of infection, with an NSBPP of 9.56 and a PIG of 21.03%. Uv4 and Uv15 followed, with NSBPPs of 3.71 and 2.75, and PIGs of 8.16% and 6.05%, respectively.

Table 3. False smut disease caused by Uv3, Uv4 and Uv15 in TN1 and BPT 5204

Isolate	TN1		BPT 5204	
	NSBPP	PIG	NSBPP	PIG
Uv3	15.55±1.89*	23.32±1.28	9.56±1.01	21.03±1.32

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Uv4	8.41±1.48	12.62±1.22	3.71±0.68	8.16±0.57
Uv15	5.02±0.51	7.53±1.14	2.75±0.50	6.05±0.43
C.D. ($p<0.05$)	3.972	3.773	2.423	2.873
SEm±	1.126	1.07	0.687	0.815

*Mean standard error, NSBPP- number of smut balls per panicle, PIG- percent infected grains

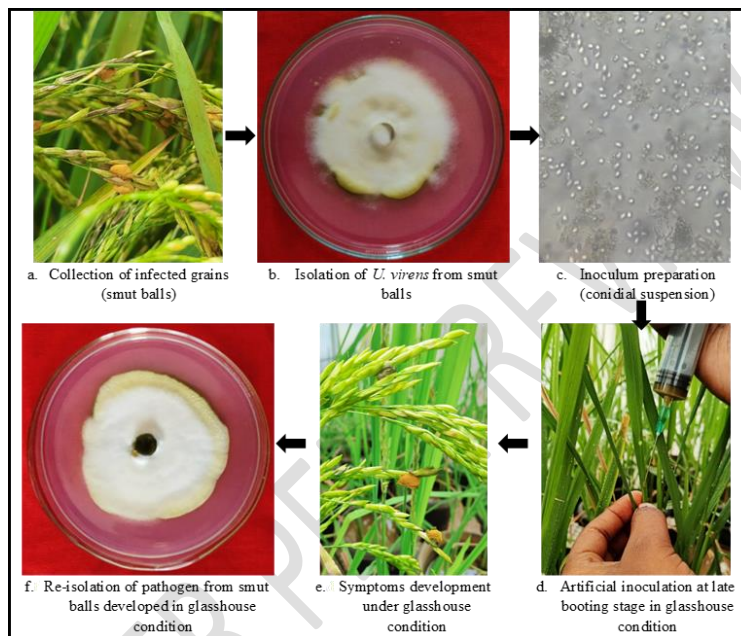


Fig 3. Pathogenicity test for false smut of rice caused by *Ustilagoideae virens*

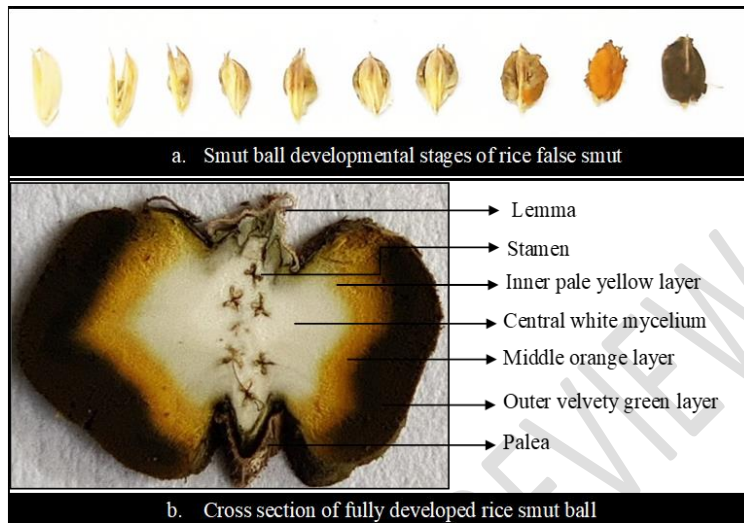


Fig 4. Developmental stages and cross section of rice false smut ball

4. DISCUSSION

Rice false smut pathogen, *Ustilagoidea virens* (Uv3, Uv4 and Uv15) showed varied mycelial growth from chlamydospores and mycelial discs of smut balls at 7 days after incubation. Maximum growth was observed from smut ball discs: 7.20 mm (Uv3), 5.31 mm (Uv4), and 4.43 mm (Uv15), compared to chlamydospore germination: 5.56 mm (Uv3), 3.94 mm (Uv4), and 3.31 mm (Uv15). Mycelial density was higher from chlamydospores and lower from smut ball discs. Prior studies used chlamydospores for isolating the false smut pathogen [3], noting its slow growth over one to two weeks to form large colonies [8]. After obtaining pure cultures, the growth characteristics of *Ustilagoidea virens* isolates Uv3, Uv4, and Uv15 were standardized and analyzed. Uv3 exhibited the highest mycelial growth at 77.00 mm, followed by Uv4 at 64.00 mm and Uv15 at 55.66 mm. Additionally, these isolates displayed distinct cultural characteristics. Uv3 showed raised elevation with irregular growth form, a white-yellow surface color, and yellow-green reverse pigmentation, while Uv4 and Uv15 exhibited flat elevation with circular growth forms and varying surface and reverse pigmentation patterns. These findings are consistent with previous studies ([give references of previous studies here](#)). Savitha et al. [11] reported colony diameters ranging from 42 mm to 71.75 mm in *U. virens*, reflecting variability similar to what we

observed. Similarly, Sekhar et al. [12] documented colony diameters spanning from 10.14 mm to 85.68 mm, with growth rates ranging from 0.33 mm to 2.85 mm per day, indicating significant variation in growth patterns across different isolates. Baite et al. [2] also reported mean colony diameters ranging from 25 mm to 40 mm, further supporting the variability in growth characteristics observed in *U. virens* isolates.

Ustilagoidea virens produces more conidia in BPT 5204 rice leaf extract (BRLE) compared to TN1 rice leaf extract (TRLE), with higher concentrations of these extracts leading to increased conidia production. Among the strains, Uv3 is the most prolific in conidia production, followed by Uv4 and U15. The control medium, PSB, results in significantly lower conidia counts, highlighting the positive impact of rice leaf extracts on the conidia production of *Ustilagoidea virens*. These findings are consistent with those of Wang et al. [16] who reported that among the tested media, a 0.10 g/ml panicle medium was most efficient for conidiation. Furthermore, certain rice leaf media, except for the 0.10 g/ml panicle medium, were more effective in increasing conidiation than panicle media. This underscores the potential of rice leaf extracts in enhancing the conidiation of *U. virens*, providing a valuable insight into optimizing media conditions for the study and management of this pathogen.

After 20 days, plants showed smut balls, and *Ustilagoidea virens* was isolated, confirming Koch's postulates. The fungus infected the young ovary, turning kernels into large, green balls that started white, grew, burst to release spores, and turned from yellow to black over time. The study revealed differences in pathogenicity among isolates Uv3, Uv4, and Uv15 on TN1 and BPT 5204 rice varieties. For TN1, Uv3 had the highest smut balls per panicle (15.55) and the highest infected grains percentage (23.32%), followed by Uv4 and Uv15. For BPT 5204, Uv3 also had the highest infection levels, followed by Uv4 and Uv15. These findings align with previous studies by Fu et al. [17], Yong et al. [19], Ladhakshmi et al. [20], and Ashizawa et al. [18], who examined the pathogenicity of false smut fungi in rice cultivars.

5. CONCLUSION

The study demonstrated that *Ustilagoidea virens* isolates Uv3, Uv4, and Uv15 ~~showed~~ exhibiting varying growth patterns and conidiation rates under ?. Uv3 exhibited the highest mycelial growth and conidia count, indicating its higher virulence. Pathogenicity tests confirmed

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Uv3 as the most aggressive isolate, causing the most severe infections on TN1 and BPT 5204 rice varieties. These findings highlight the importance of identifying and managing the most virulent strains to control rice false smut effectively.

6. REFERENCES

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