Original Research Article

Unveiling of refined isolation and inoculation assay for *Ephelis japonica* inciting Udbatta disease in paddy

Abstract

Single spore isolation method from diseased samples is a crucial in obtaining pure cultures of fungal pathogens. Isolation of *Ephelis oryzae*, the causative agent of black choke or Udbatta disease in rice, is challenging due to its hindered growth and the prevalence of other rapidly proliferating fungi or bacteria. In this study, a streamlined spore-drop technique was standardized for rapid isolation of *E. japonica* from infected rice panicles. This method achieved pure cultures with minimal contamination (7.50%) compared to the spore dilution method (32.50%) and the standard tissue isolation method (50.25%). Additionally, the spore-drop technique produced single-spore isolates in the shortest time (25 days) with a growth rate of 1.7 mm/day, outperforming the tissue isolation (70 days, 0.45 mm/day) and spore dilution methods (65 days, 0.83 mm/day). Optimal conditions for fungal growth in axenic culture were also standardized. *E. japonica* exhibited the fastest growth on potato dextrose agar (PDA) medium at an incubation temperature of 27°C under dark conditions, which were more conducive to growth than light incubation. The fungus recorded a maximum radial growth of 35.60 mm in 25 days on PDA supplemented with 4% dextrose as compared to 30.27 mm in 2% dextrose. Furthermore, a standardized inoculation method for inducing Udbatta disease involved treating rice seeds with a fungal suspension for 1–2 hours, drying them overnight, and then sowing them. Diagnostic symptoms, characterized by black, erect, cylindrical spike-like structures, appeared at the panicle initiation stage. This refined isolation technique, with optimized culture conditions, provides a robust framework for advancing research on *E. japonica* and Udbatta disease in India.

Keywords: Udbatta, Rice, Spore-drop, Isolation, *Ephelis japonica*

Introduction

Rice (*Oryza sativa* L.) is a staple food crop for over one-third of the global population. Udbatta disease, a sporadic rice disease caused by *Ephelis oryzae* Syd., was first reported in India by Sydow in 1914. In recent years, Udbatta disease has been observed not only in rice but also in several other host plants, including *Cynodondactylon*, *Leptochloa chinensis*, *Panicum*spp., *Pennisetum* spp., *Eragrostistenufolia*, and Kodo millet (*Paspalum scrobiculatum*), as documented by Govindu and Thirumalachar (1961). The collateral host for Udbatta disease is *Echinochloacrusgalli*, as it exhibits symptoms identical to those found in rice. During the

Kharif season, disease severity reached up to 50% in the Tunga variety in the Sakleshpura region of the Hassan district. In other varieties such as BR2655 and MTU 1001, the severity ranged from 10% to 30% in certain fields of the Mandya district.

Infected rice plants suffer from complete panicle damage, and even a single infected panicle can result in total yield loss, as noted by Sanengowda and Pandurangegowda (1986).

Plants infected with Udbatta disease remain indistinguishable from healthy ones until they reach the boot leaf stage, as the disease does not affect the plant's growth or overall appearance (Ranganathaiah, 1985). The causal pathogen, *Ephelis oryzae*, produces characteristic symptoms in the form of erect, greyish-white, cylindrical to rod-shaped structures resembling incense sticks (commonly known as Agarbatti), giving the disease its name. Instead of normal panicle emergence, these Agarbatti-like structures emerge from the boot leaf sheath. Infected panicles have florets glued together to the main rachis by a spore mass, which hardens into a crust, preventing grain formation and causing 100% yield loss in affected panicles. In some cases, the size of the infected panicles and flag leaves is reduced. The flag leaf, along with the second and third leaves and their respective sheaths, displays a lustrous, greyish-white appearance. While the disease is typically noticeable after the ear head emerges, it can sometimes be observed as early as the boot leaf stage. In high-humidity regions like Malnad, infected leaves exhibit a silvery shine visible from a distance.

Isolation of pathogen is a fundamental step for studying pathogenicity, virulence, host plant resistance, and fungicide resistance. Purified mono-conidial isolates collected from fields are essential for these analyses. Several methods, such as spore dilution, leaf press, tissue isolation, and micromanipulator-based isolation, are used to obtain monoconidial cultures.However obtaining pure culture with these method was cumbersome as the pathogen is weakand slow growing and difficult to overcome the competitive contaminants. In the present study different methods of isolation was tested finally a refined method standardized with which pure culture devoid of contaminants can be obtained. These involve isolating and sub-culturing a single spore under a microscope (Fei *et al*., 2019). Understanding the pathogenicity of the Udbatta pathogen is critical for developing control strategies, breeding resistant varieties, preventing crop losses, and conducting resistance screening. Artificial inoculation of the pathogen into rice plants is necessary to confirm its identity, satisfy Koch’s postulates, and facilitate further characterization studies. Various inoculation methods include mixing dry seeds with spore dust, combining soaked seeds with spore dust, nodal inoculation, inoculating rice heads before flowering, inoculating germinated seeds, and treating healthy seeds with fungal suspensions. These techniques are pivotal for studying the disease and advancing control measures.

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Ephelis japonica is a slow-growing fungus, making it challenging to isolate in pure form. During isolation and incubation, it is frequently overrun by fast-growing saprophytes, complicating the process. A pure culture of the pathogen is crucial for advancing research on its disease cycle, host-pathogen interactions, and management strategies. However, in India, scientific knowledge on the successful isolation and cultural conditions for *E. japonica* remains limited (Kumar *et al*., 2016).

Existing methods for isolation are often labor-intensive and prone to contamination. Thus, there is a pressing need to develop a simple, efficient method for single-spore isolation of the rice Udbatta pathogen from diseased samples (Jia, 2009; Qiu *et al*., 2011; Yang *et al*., 2010). To address this, an effort was made to standardize an isolation technique with minimal contamination by evaluating various methods and optimizing the culture conditions and inoculation process for *E. japonica.*

Material and methods

Sample collection

The panicles showing erect, greyish white, cylindrically shaped agarbatti or karikaddi or udbatta-like and silvery or lustrous greyish white fungal growth symptoms appeared on leaves suspected to be infected by *Ephelis japonica* were collected from the different rice growing ecosystems of Karnataka. The collected panicles and leaves were packed adequately in polythene packets. Upon bringing to the laboratory, specimens were preserved in the refrigerator at 4 ± 1 °C and were utilized to isolate fungus under in vitro conditions.

Media preparation

The spore-drop technique for isolating *Ephelis japonica* requires two types of media: water agar (WA) and paddy grain extract agar (PDA). Water Agar (WA) was prepared by dissolving 20 g (2%) of agar powder in 1000 ml of double-distilled water. After autoclaving, streptomycin sulfate (40 mg/L) was added to prevent bacterial contamination. For preparing Potato Dextrose Agar (PDA), two hundred grams of cleaned, washed, and peeled potato tubers were chopped and boiled in distilled water. The potato extract was filtered through muslin cloth. Twenty grams each of dextrose and agar were dissolved in the potato extract, and the final volume was adjusted to 1000 ml with distilled water. The prepared medium was distributed into several conical flasks, which were plugged with non-absorbent cotton, wrapped in brown paper, and sterilized at 1.1 kg/cm² pressure for 20 minutes.

Rice leaf extract agar was also utilized for sub-culturing and rapid culture revival alongside PDA. Healthy green paddy leaves were collected, rinsed thoroughly under running water to remove dust and contaminants, and weighed to 200 g. The leaves were chopped into small

pieces and boiled in 1 liter of water until softened. The softened leaves were squeezed to release nutrients, and the extract was filtered using clean muslin cloth. To the filtrate, 20 g of agar was added and boiled until fully dissolved. Subsequently, 20 g of dextrose was added and stirred until completely dissolved. The final volume was adjusted to 1000 ml with distilled water, and the prepared medium was sterilized as previously described.

Isolation of *E. japonica* by spore-drop technique

To isolate *E. japonica*, rice panicles displaying typical Udbatta disease symptoms were collected in brown paper bags. The black stromata from the panicles were placed in a sterilized moist cotton setup within separate Petri plates and incubated for 24–48 hours to promote sporulation. After incubation, the samples were transferred to a Lesion or Black Stromata Print (LP) setup. The infected tissues were affixed to sterilized moist cotton on the inner surface of the upper lid of a Petri plate. The bottom portion of the plate was filled with either paddy grain extract agar or water agar medium, and the setup was sealed with parafilm tape(Fig 1).

The LP setup Petri plates were incubated at 25 ± 1 °C for three or more days until tiny fungal colonies became visible on the medium from the diseased samples. A mycelial disc from a single colony was then transferred to a sterile Petri plate containing 1 mL of paddy grain extract broth, which was macerated with a sterile glass rod. Lukewarm paddy grain extract agar medium was added and thoroughly mixed. After three to four days of incubation at 25 ± 1 °C, individual fungal colonies from *E. japonica* spores developed on the paddy grain extract agar medium. This method is a modified approach for isolating rice blast fungus. (Rajashekara*et al.*, 2017).

After fungal growth, a small loop of the culture was taken from the colonies and placed on a clean glass slide with a drop of lactophenol. The slide was then examined under both low and high magnification to check for the presence of fungal conidia.

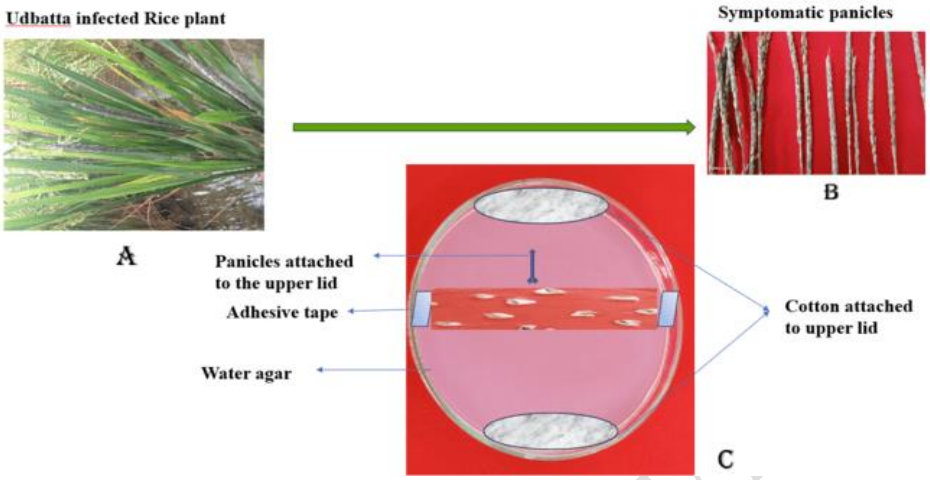

Fig 1. Pictorial representation of the isolation set-up used in the isolation of *E. japonica* by the spore-drop method. a) Udbatta infected rice plant b) Cut panicles of rice with udbatta black stromata c) Petri plate with upper lid attached with cut panicle bits and moistened cotton and lower lid containing paddy grain extract agar or water agar (WA).

Isolation of *E. japonica* by spore dilution method

For the spore-dilution technique, a diseased panicle was washed under tap water and cut into small pieces containing the ephelidial fructifications. These pieces were then surface sterilized with 1% sodium hypochlorite for 90 seconds, followed by three rinses with sterile distilled water. The sterilized pieces were placed on the upper lid of a Petri dish containing moist filter paper and positioned over a lower dish with 10 ml of sterile distilled water to maintain 100% relative humidity inside the chamber (Fig. 2). The setup was incubated at 28°C for 48 hours to facilitate sporulation. After incubation, the black ephelidial fructifications were examined under a stereo binocular microscope for sporulation. Sporulating leaf tissues were then transferred to an Eppendorf tube containing 1 ml of sterile water and gently mixed to release the spores. About 100 µl of this suspension was spread onto 2% WA medium using a sterilized spreader and incubated overnight at 25±1°C (Fig. 2). The incubated Petri plates were examined under a microscope, and germinating conidia were identified. Finally, a single spore was transferred to fresh PDA plates containing streptomycin sulfate (40 mg/L) and incubated at 25±1°C for 7-10 days (Fig. 2).

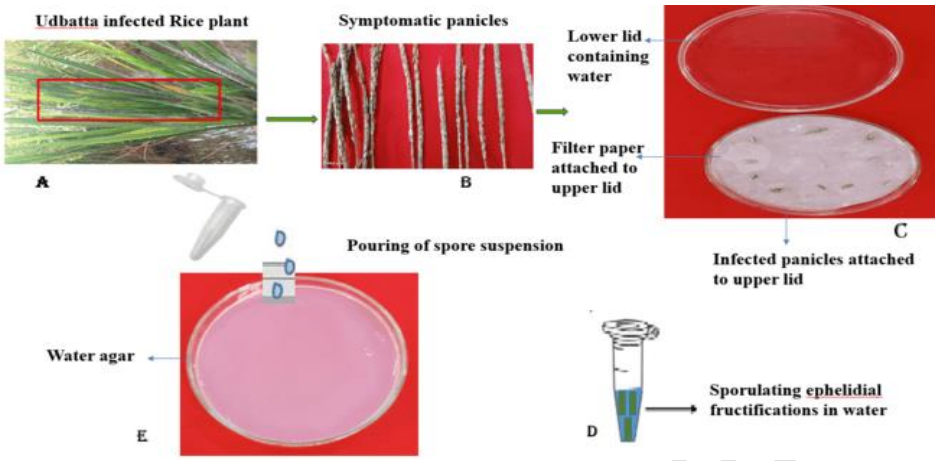

Fig 2. Pictorial representation of the isolation of *E. japonica* by the spore-dilution method. a) Udbatta infected rice plant; b) Rice panicles with udbatta symptoms; c) Petri plate with upper lid attached with cut panicle bits on filter paper and lower lid containing water; d) Panicle bits in Eppendorf tube containing sterile water; e) Spore suspension spread on water agar.

Isolation of *E. japonica* by the conventional tissue isolation method

In another method, infected host tissue isolation, the infected panicles and leaves were cut into small pieces of up to 1 cm in size and surface sterilized with a 0.1% mercuric chloride solution for 1 minute to eliminate external contamination. After sterilization, the pieces were washed several times with sterile distilled water. These small infected tissue samples were then placed on Potato Dextrose Agar (PDA) in Petri dishes and incubated at 26°C for 15 days. The Petri dishes exhibited full growth of white mycelia from the pathogen.

In all three methods, observations were made to record the percentage of contamination and the time taken to obtain a pure culture, measured from inoculation until a colony with a 5 cm radius was formed.

Culture conditions

Two different media, Potato Dextrose Agar (PDA) and Rice Leaf Extract Agar, were used for isolation, with PDA modified to include varying dextrose concentrations of 2%, 4%, 6%, 8%, 10%, and 12% for in-vitro culturing of the fungus. The cultures were incubated at temperatures of 25°C, 27°C, and 29°C.

Effect of light on growth of fungus

Petri plates containing fungal cultures were placed in darkness by turning off the light in the BOD incubator. The plates were periodically observed and their growth compared with that of

cultures incubated under light conditions.

Pathogenicity or study on suitable inoculation assay of *E. japonica*

Inoculum preparation

Randomly selected *E. japonica* isolates were used in this experiment. Spore suspensions of the respective fungal isolates were prepared to a concentration of approximately 1×10^6 conidia/ml. Prior to use, Tween 80 was added to the spore suspension for inoculation. For spraying inoculation, 0.5% gelatin was added to the suspension to prevent spore desiccation.

Artificial inoculation of rice

A spore suspension of *E. japonica* (1×10^6 spores/ml) was used to inoculate the Jyothi variety of rice. Three artificial inoculation methods were employed: soaking the seeds in spore suspension, nodal inoculation, and inoculating rice heads before flowering. Healthy Jyothi rice seeds were soaked in the spore suspension (1×10^6 spores/ml) for 24 hours, after which the inoculated seeds were air-dried overnight and then sown in plastic pots filled with sterile soil. For conidia spraying, the spore suspension was sprayed onto the leaf surfaces until runoff using an air compressor. For conidia injection, 1 ml of the suspension was injected into the upper part of the leaf sheath covering the developing panicle using a syringe. In a separate set, only sterile distilled water was sprayed or injected as a negative control. The inoculated rice plants were maintained under moist conditions, and the pots with seedlings were transferred from the humid chamber to a glasshouse. Observations were made to detect the development of typical Udbatta disease symptoms.

To confirm the pathogen's identity, the fungal pathogen was re-isolated from the infected plant parts of the artificially inoculated rice plants. The resulting culture on Potato Dextrose Agar was compared with the original culture to verify the pathogen's identity.

Statistical analysis

The results were statistically analyzed using a completely randomized design and interpreted through analysis of variance, performed with SPSS software (Sundarraju*et al*., 1972).

Results and Discussion

Among the various methods, the spore-drop technique exhibited the lowest contamination rate at 7.5%, whereas the spore-dilution and tissue isolation methods showed significantly higher contamination rates of 32.50% and 50.25%, respectively (Table 1). Additionally, the spore-drop technique significantly reduced the time required to obtain a pure culture of the pathogen, achieving this in just 25 days, compared to 70 days for tissue isolation and 60-65 days for the spore-dilution method.

Although the spore-dilution method shows a higher contamination rate compared to the spore-drop technique, the latter is more effective for routine isolation of the pathogen in rice-udbatta

studies. While earlier researchers (Jagadeesh *et al.,* 2018; Rajashekara*et al.,* 2017) have employed the spore-dilution method for similar slow-growing fungi like *Ustilaginoidea virens* and the blast pathogen, this approach requires additional surface sterilization with 1% sodium hypochlorite and 95% ethyl alcohol, followed by serial dilution. In contrast, the spore-drop method simplifies sample preparation, eliminating the need for toxic chemicals or surface sterilization. Pathogen growth rates on PDA plates were observed to be significantly higher with the spore-drop technique (1.7 mm/day) compared to the spore-dilution (0.83 mm/day) and tissue isolation methods (0.45 mm/day).Though the tissue isolation method seems to be easy, but involvement of lab contaminanats was higher which masked the growth of required pathogen and the sample needs to be processed first for incubation and then transformed to plate itself; therefore, we observed a higher contamination percentage.

For the isolation of *E. japonica*, regardless of the method used, it is essential to use fresh rice panicle samples since the pathogen is a weak saprophytic competitor (Ou, 1985). During isolation, other pathogens and saprophytes can outcompete *E. japonica*. Previous studies have documented the isolation of *E. oryzae*by creating a humid chamber in Petri dishes containing moistened filter paper (Narayan, 2014; Ranganathaiah, 1985). In this study, we adopted a similar approach with a slight modification where, cotton was attached along the inner margins of the upper lid, moistened with sterile water using a micropipette, and placed over a lower dish containing 2% WA.While the spore-dilution method has been widely used to obtain monoconidial cultures of slow-growing fungi like *U. virens*(a member of the Clavicipitaceae family) (Jagadeesh *et al*., 2018; Rajashekara*et al*., 2017), the spore-drop method described in this study proved to be more efficient and robust, with significantly lower contamination rates. This is largely because the entire process is conducted within a laminar airflow cabinet. The spore-dilution method, on the other hand, reported higher contamination rates (32.50%) due to the longer processing time, which allows saprophytes to proliferate.The spore-drop method, as standardized in this study, is a reliable and efficient technique for obtaining pure cultures of *E. japonica* from blast-infected rice panicles. It offers a significant advantage by eliminating the need for toxic surface sterilizing chemicals during the isolation process.

Methods	**Contamination (%) #***	**Days to obtain a pure culture**	**Growth per day**
Spore-drop	7.50 (15.89)	25	1.7 mm
Spore dilution	32.50 (34.76)	70	0.83 mm

Conventional tissue isolation	50.25 (45.14)	65	0.45 mm
S.Em.±	0.33		
CD @ 1%	1.18		

Table 1. Contamination recorded in different methods

Total number of contaminated plates/total number of plates inoculated

*Values in the parenthesis are angular transformed

Effect of light and darkness

The growth of *E. japonica* was found to be influenced by light conditions during incubation. The fungus exhibited optimal and faster growth when incubated in darkness compared to exposure to light at an intensity of 200 lux.

Cultural characteristics

After 9–10 days of inoculation, *E. japonica* began to grow on PDA culture medium, forming creamy-white colonies that were flat or slightly raised with gentle undulations. The mycelium appeared fluffy, compact, and leathery (Fig. 3). Initially, the colony color on potato dextrose agar was dull white, gradually turning brown with a shiny, waxy appearance. The colonies became thick, flat, and radiating with brown stripes. On paddy leaf extract agar, the colonies appeared nut-brown to dull brown with a shiny, leathery, and waxy texture. They were thick, flat, and exhibited profuse mycelial growth, accompanied by hyaline conidia (Fig. 4).A total of ten pure isolates were successfully obtained. The conidia were observed to be needle-shaped, aseptate, and hyaline, while the mycelium was initially aseptate and hyaline, transitioning to a brown color as it aged.The results were in accordance with Shivanandappa and Govindu (1977), Nagaraja *et al.* (2021) and Tai and Siang (1948).

Fig 3- Culture plate of *Ephelis japonica* on PDA after 28 days of isolation

Fig 4- Culture plate of *Ephelis japonica* on Paddy leaf extract agar after 28 days of isolation

Identification of the isolated fungus

Morphological identification of fungal pathogen was done based on cultural characteristics and description of *E. japonica* given by Tsukiboshi, *et al.* (2008) and Nagaraja *et al*. (2021), Kumar (1998) and Srivastava (2020).

Culture media for isolation

Between the two media tested, potato dextrose agar (PDA) and rice leaf extract agar, *E. japonica* exhibited superior growth on PDA for most isolates, with colony diameters ranging from 25 to 42 mm. The maximum growth (42 mm) was observed in isolate UEO9, 30–35 days after incubation, while the minimum growth (25 mm) was recorded for isolate UEO6. However, isolates UEO7 and UEO2 achieved the highest growth (40 mm) on rice leaf extract agar, while the other eight isolates showed maximum growth on PDA after 30 days of incubation. All isolates displayed their maximum radial growth at 35 days post-inoculation, consistent with the pathogen's slow-growing nature, as growth typically begins 9–10 days after inoculation. Radial growth steadily increased with incubation time (15, 25, and 35 days) across all isolates, indicating consistent and sustained fungal proliferation under the tested conditions. Growth at 35 days was significantly higher than at earlier time points for all isolates. Notably, isolates UEO7 and UEO9 demonstrated robust radial growth, making them potential candidates for applications requiring high fungal proliferation. Conversely, the limited growth observed in isolate UEO6 suggests a need for optimization or alternative growth conditions. Statistical analysis confirmed the reliability and significance of the observed differences in radial growth, with minimal error margins.These findings align with earlier studies by Kumar (1998), Mohanty (1976), and Manmohan et al. (2000), which reported *Ephelis*colonies on PDA achieving growth of approximately 35 mm

Isolate	(PDA) Radial growth (mm)		
	15#	**25#**	**35#**
UEO1	16.35	30	36
UEO2	15.65	36	40
UEO3	14.20	22.5	28
UEO4	15.5	25.5	29.5
UEO5	23.50	34.55	37.5
UEO6	11.58	19.00	25
UEO7	25.10	35.80	40
UEO8	18	29.55	34
UEO9	23	38	42
UEO10	14	21.50	25
S.Em ±	0.47	0.45	0.57
CD @ 1%	1.42	1.33	1.70

 # = Days after inoculation

Table 2. Growth of different isolates of *E. japonica* at 27°C and pH 6.0 in Potato dextrose agar at different days after inoculation

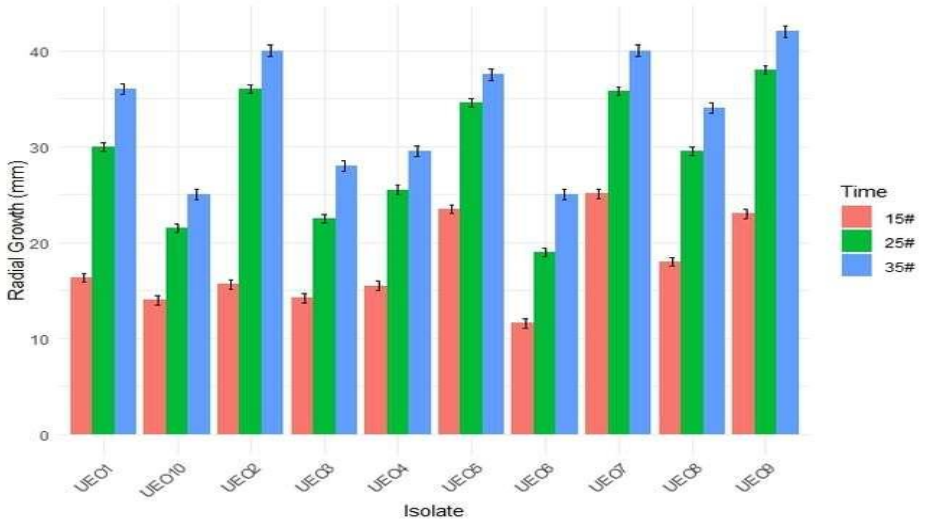

Fig 5. Growth of different isolates of *E. japonica* at 27°C and pH 6.0 in Potato dextrose agar at different days after inoculation

Temperature set up for isolation and growth

To determine the optimal temperature for the isolation and growth of *E. japonica*, cultures were incubated on PDA medium at different temperatures (25°C, 27°C, and 29°C). The highest growth rates for most isolates were observed at 27°C, with colony diameters ranging from 25 to 42 mm. However, isolates UEO5 and UEO7 showed the best growth at 29°C (Table 3, Fig. 6). These findings indicate that 27°C is the optimal temperature for the isolation and growth of *E. japonica* in most cases.Similar findings were reported by Harsha *et al.* (2016) and Das *et al.* (2000).

Isolate	**Temperature (Growth in mm at pH 6)**		
	25°C	**27°C**	**29°C**
UEO1	30	35.5	33
UEO2	38	42	40
UEO3	25	28	26.55

UEO4	26	29.5	28
UEO5	32	34	37.5
UEO6	25	27	23
UEO7	34	37	40
UEO8	30	34	31.45
UEO9	34.5	42	32
UEO10	21	25	20
S.Em ±	0.44	0.49	0.51
CD @ 1%	1.33	1.54	1.58

Table 3. Effect of temperature range on isolation and growth of *E. japonica* in PDA medium

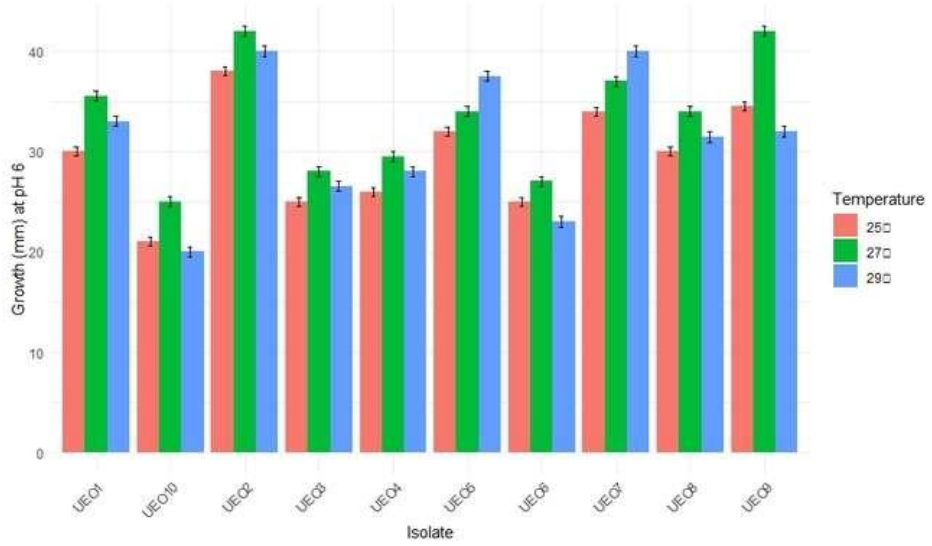

Fig 6. Effect of temperature range on isolation and growth of *E. japonica* in PDA medium

Effect of different dextrose concentration on isolation and growth of *Ephelis japonica*

Fungi rely on various natural substrates to obtain nutrients and energy, and no single artificial medium supports the growth of all fungi equally. To identify an optimal medium for

isolating and growing *Ephelis japonica*, an experiment was conducted using potato dextrose agar (PDA) with modified dextrose concentrations of 2%, 4%, 6%, 8%, 10%, and 12%. The methods used are detailed in the Materials and Methods section, and the results are shown in Table 4 and Figures 7 and 8. The radial growth and sporulation of the fungus were recorded at the point of maximum growth. The results demonstrated significant differences in how dextrose concentration affected the isolation and growth of *E. japonica*. The highest mean radial growth occurred on PDA with 4% dextrose (35.60 mm), which was significantly superior to all other concentrations. This was followed by PDA with 2% dextrose (30.27 mm), 6% (23.39 mm), 8% (19.98 mm), and 10% (11.40 mm). The lowest growth was observed on PDA with 12% dextrose (8.60 mm). Sporulation also varied across treatments. Excellent sporulation occurred on PDA with 4% and 2% dextrose, while good sporulation was noted on PDA with 6%, 8%, and 10% dextrose. In contrast, moderate to very low sporulation was recorded on PDA with 12% dextrose. These results align with studies by Lewis and White (2006), Indrasen *et al*. (1981), and Rajashekara*et al.* (2017).

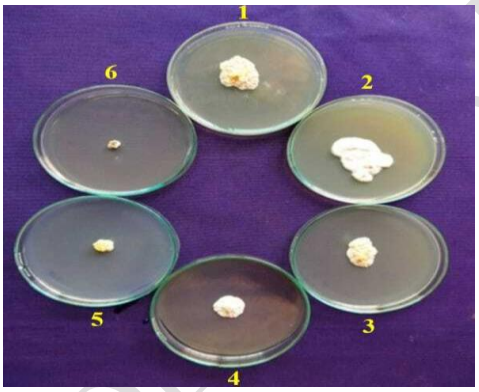

Fig 7. Effect of different dextrose concentration on isolation and growth of *Ephelis japonica*

Treatment No.	Dextrose concentrations	Mean radial growth (mm)	Sporulation
T1	2 %	30.27	++++
T2	4 %	35.6	++++
T3	6 %	23.39	+++
T4	8 %	19.98	+++

T5	10 %	11.4	+++
T6	12 %	8.6	++
	S.Em. ±	0.18	
	CD @ 1%	0.62	

Table 4. Effect of different dextrose concentration on isolation and growth of *Ephelis japonica*

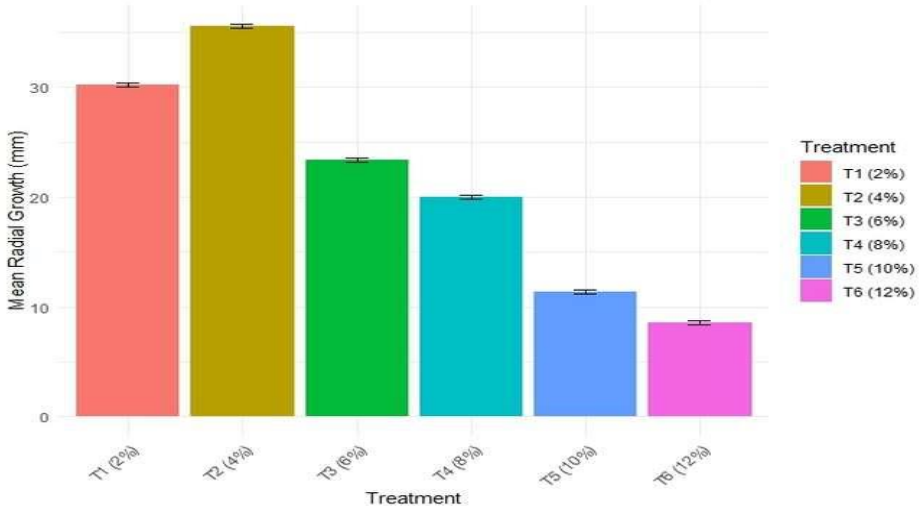

Fig 8. Effect of different dextrose concentration on isolation and growth of *Ephelis japonica*

Pathogenicity

To fulfill Koch's postulates, various methods were employed, including wet soaking of seeds in spore suspension, nodal inoculation, and inoculation of rice heads before flowering. Among these, the seed inoculation method using spore suspension proved most effective, producing typical udbatta symptoms. Seeds of the susceptible paddy variety Jyothi were artificially inoculated by soaking in the spore suspension. Following inoculation, the emerging panicles displayed characteristic symptoms: a straight, single, dirty-colored cylindrical appearance resembling an

incense stick (agarbatti), covered with white mycelial growth. Infected plants exhibited stunted growth, and the flag leaf showed a shiny, greyish-white fungal growth. In contrast, control plants that were not inoculated with the pathogen showed no symptoms, maintaining normal growth and appearance.

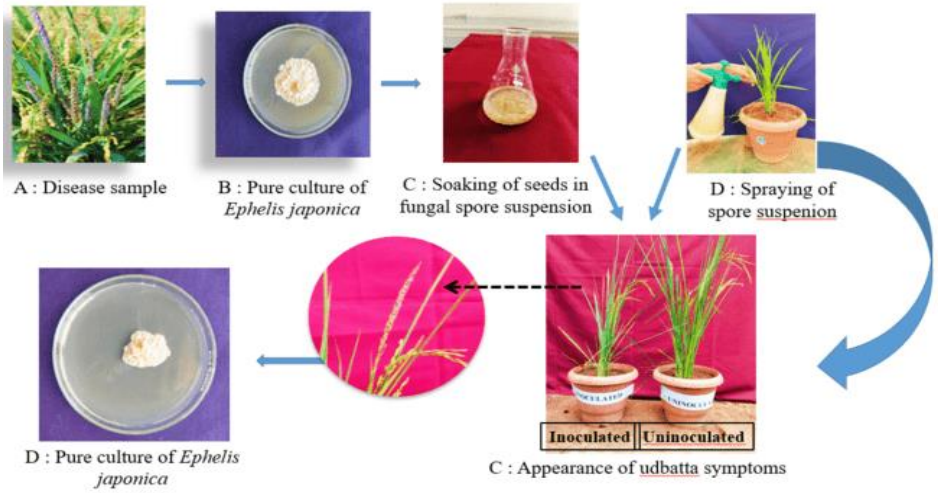

Fig 9.Inoculation assay standardization and proving the pathogenicity of *E. japonica*

The symptoms of Udbatta disease caused by the pathogen *Ephelis japonica*on artificially inoculated plants closely resembled those observed on naturally infected paddy plants (Fig. 9). The pathogen was re-isolated from the artificially infected paddy plants using Potato Dextrose Agar. The resulting culture matched the one initially used for inoculation, confirming the fungus as pathogenic to the paddy plant(Fig 10).

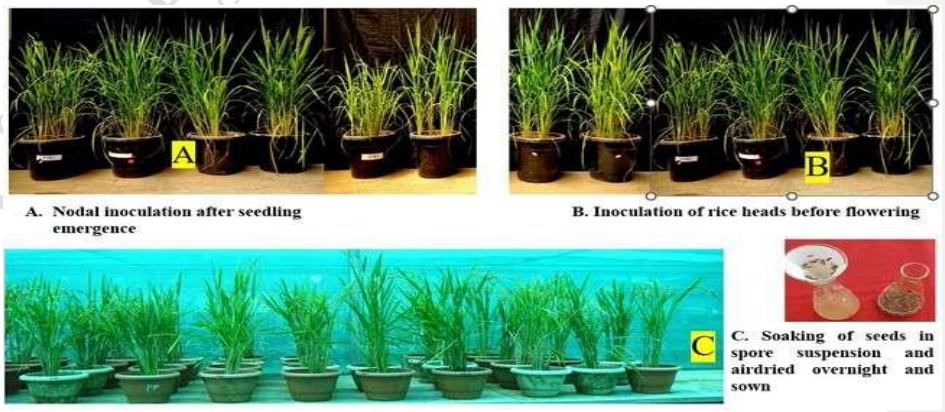

Fig 10. Different methods of inoculation of *E. japonica* used for rice plants to incite udbatta

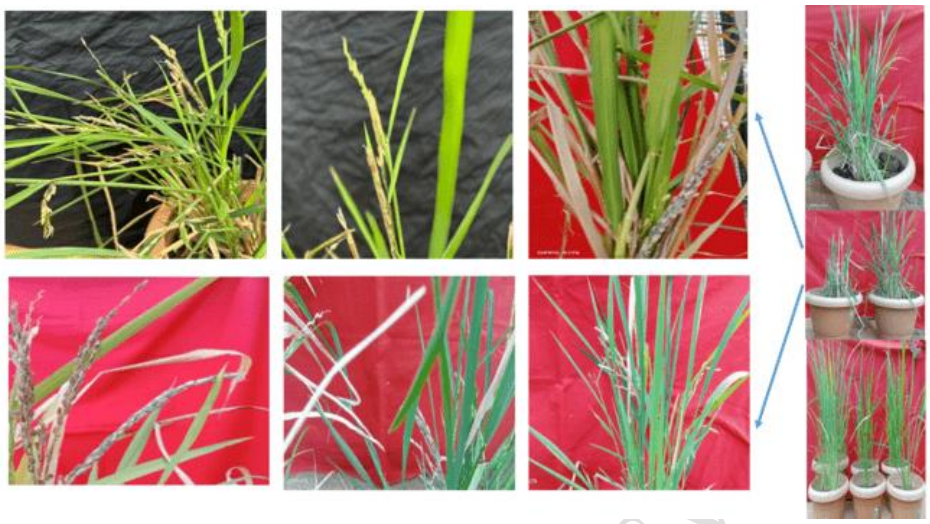

Fig 11. Symptoms of udbatta on rice plants due to artificial inoculation of *Ephelis japonica*

The disease was observed when rice plants were inoculated through seed inoculation with spore suspension, rather than by conidia injection (Figure 11), spraying during the tillering stage, or nodal inoculation, all of which showed no symptoms of Udbatta at later stages (Table 5). The results from the combined analysis clearly indicated that seed inoculation was the most suitable inoculation technique for this experiment (Table 5), and it should be performed before sowing.

Among the three inoculation methods applied at different rice growth stages, seed inoculation before sowing proved to be the most effective in causing Udbatta incidence. In contrast, conidia spraying or inoculating rice heads before flowering and nodal inoculation did not result in disease. This suggests that the pathogen is seed-borne and that seed infection plays a critical role in the pathogen's persistence. The growth stage is also a key factor in the success or failure of inoculation. Infected seeds are believed to be the primary source of inoculum. Similar findings have been reported by Nagaraja *et al.* (2010), Sannegowda and Panduranga (1986), Narayan (2014), Tanaka *et al*. (2001), and Mohanty (1979), who observed higher Udbatta incidence when rice seeds were inoculated with conidia suspension, with panicle initiation being the critical stage for disease development.

The consistent response of the Jyothi cultivar to *E. japonica* observed in both field conditions and this experiment suggests that its resistance may be polygenic. The higher disease scores seen in the experiment could be due to the high concentration of inoculum applied to the rice seed and the artificial nature of the inoculation process. Given these limitations of artificial inoculation, it is essential to repeat the screening under field conditions.

SI. no	Method of inoculation	Reaction
1	Artificial Seed inoculation by fungal spore suspension	+++
2	Nodal inoculation- during tillering stage	-
3	Inoculation of rice heads before flowering- around late booting stage	-
	Control	-

Table 5. Udbatta disease on rice inoculated by three different methods at different growth stages of rice

Rice variety	Major locality of growing in Karnataka	Reaction to udbatta
Jyothi	Mandya	Susceptible
Sahyadri megha	Shivamogga	Susceptible
Tunga	Talakaadu	Susceptible
Sona masuri, IR 64, Jaya etc	North Karnatka	Resistant

Table 6. Rice varieties and their observed reaction to Ephelis japonica employed in the study

Isolating *E. japonica*from Udbatta-infected rice plants is challenging due to its slow growth, which often results in contamination by faster-growing saprophytes during isolation and incubation. The surface sterilization methods previously described (Atia, 2004) did not consistently yield successful isolations. However, the newly developed technique proved to be more effective than the ones reported by Atia (2004). It is simpler, and the fungus can be isolated with ease. Surface sterilization of ephelidial fructifications using a 0.1% mercuric chloride solution was found to inhibit spore germination, likely by affecting both the spores and other surface contaminants or saprobes on the spore balls.

Fresh samples from mature Udbatta-infected panicles exhibited better spore germination when cultured, but prolonged storage (over two months) led to a rapid decline in germination percentage. The fungus was found to be sensitive to light conditions during the incubation period,

with growth of *E. japonica*being better in darkness than under light. These findings are consistent with those of Hong-ping (2001) and Ranganathaiah (1985).

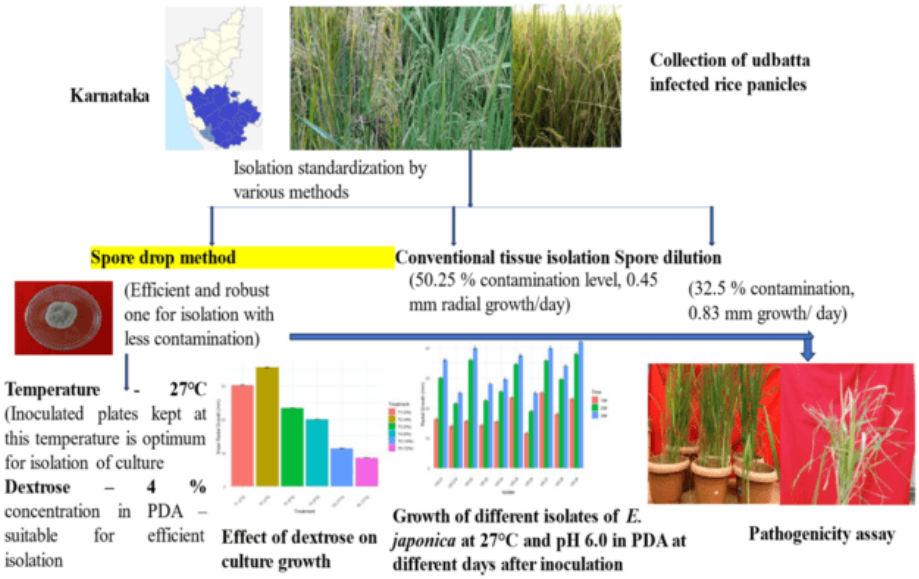

Fig -12 Schematic representation of collection of udbatta infected panicles, isolation of *E. japonica* and by various methods and standardization and inoculation assay for the pathogen

Conclusion

The fungus grew well on both media, but the best growth was observed on Potato Dextrose Agar (PDA). The alternative medium, Host Leaf Extract Agar, supported comparatively slower growth. PDA facilitated the fastest mycelial growth, with a rate of 1.70 mm/day. Of the three incubation temperatures tested, 27°C proved to be the most optimal, as the fungus displayed the highest growth rate at this temperature compared to 25°C and 29°C. 4 % dextrose in PDA revealed higher radial growth after isolation. Seed inoculation was proven to be best standardized method for artificial induction of disease.

In our study, *E. japonica* was successfully isolated using artificial media. The challenge of isolating the udbatta pathogen has been overcome with the successful isolation of *E. japonica*, along with the establishment of its optimal growth conditions, including the ideal culture media, temperature, and light. This study also provides, for the first time in India, detailed cultural images of *E. japonica*, which will assist in the precise identification of the fungus.

The standardized isolation technique is anticipated to be a valuable resource for researchers investigating this pathogen in the near future. The data documented in this study will serve as a helpful reference for future identification of the fungus. The deposited cultures at ITCC, New Delhi, will offer easily accessible material for ongoing research. This work opens new opportunities for further studies on this rapidly emerging rice disease in India.

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