Precise Methods for Single Spore Isolation and Controlled Sporulation in *Magnaporthe oryzae* Isolates

Abstract:

The present investigation focuses on enhancing the understanding of *Magnaporthe oryzae*, the causal agent of rice blast disease, by developing simple and cost-efficient protocols for single spore isolation and controlled sporulation with minimal equipment. In pursuit of this objective, various natural hosts were evaluated for their sporulation potential at different time intervals. The results revealed the significant differences in sporulation of *M. oryzae* among different hosts at different time intervals, showing higher sporulation at 14 DAI compared to 10 DAI. Notably, rice leaves from TN-1 and HR-12 cultivars exhibited robust sporulation at 14 DAI under a 14-hr light + 8 hr dark conditions. Validating these findings, twelve isolates from various locations in Telangana State consistently confirmed that rice leaves from cultivar TN-1 supporting the highest mean sporulation rate, followed by the HR-12 cultivar. The implications of these findings extend to aiding researchers and rice breeders in comprehending disease dynamics, formulating effective control strategies, and developing rice cultivars resilient against rice blast.

Key Words: Rice blast, *Magnaporthe oryzae,* Single spore isolation**,** Sporulation Index.

Introduction

Rice is a global food grain crop for more than one-third of the human population and a source for food and nutritional security. Rice production is subjected to various biotic stresses (Asibi *et al.,* 2019). Among the various biotic stresses, rice blast caused by the fungal pathogen *Magnaporthe oryzae*, is one of the most devastating and economically significant diseases affecting rice crops worldwide (Reddy *et al.,* 2021). With the potential to cause yield losses of up to 70-80%, rice blast poses a significant threat to global food security and the livelihoods of millions of farmers (Vanaraj *et al.,* 2013). The pathogen's high genetic plasticity and adaptability to various environmental conditions exacerbate its impact, making it formidable challenge to rice production worldwide (Rao, 1994). Understanding the biology, behaviour, and mechanisms of *M. oryzae* infection is critical for effective disease management. Among the different methods available for managing the disease, resistance is a viable method of its control as long as it lasts. However, sometimes resistant varieties may become ineffective due to evolutionary changes in the pathogen population (Khadka, 2013).

The ability to isolate pure fungal cultures and assess sporulation characteristics is fundamental for conducting meaningful research in the field of plant pathology (Choi *et al.*, 1999). However, isolating pure cultures of *M. oryzae* has proven to be a complex task due to potential cross-contamination with other fungi and the lack of standardized protocols.

In recent years, research efforts have intensified to address these challenges, leading to the development of innovative techniques for single spore isolation and improved methods for assessing sporulation. These advancements are pivotal not only for enhancing our fundamental understanding of the pathogen but also for providing practical insights into disease management and resistance breeding strategies.

This research article provides insights of critical aspects of single spore isolation and the methodology of sporulation assessment in *Magnaporthe oryzae* that infects rice crop. By establishing a comprehensive and standardized approach to these techniques, this study aims to contribute significantly to the broader understanding of rice blast disease and its implications for agricultural productivity. Moreover, the findings of this research hold the potential to streamline future investigations, enabling researchers to effectively study the diversity of the pathogen population and evaluate the efficacy of control measures.

Material and methods

Single spore isolation of the pathogen:

- The rice plant (leaves, neck, collar, node, stem and panicle) showing typical symptoms of the blast disease were marked and washed with sterile double distilled water.
- Fine pieces of diseased tissue along with some healthy portion was cut with the help of a sterile scalpel blade. These fine sections were sterilized with 1% sodium hypochlorite solution for one min, then rinsed thrice in sterile double distilled water and air dried on sterilized filter paper.
- Later, sterilized infected plant pieces were transferred aseptically onto the glass slide positioned within sterilized petri dishes lined inside with 3 layers of sterilized moist filter paper (Plate.1).
- Ten ml of sterile distilled water is transferred to the lower half petri plate to create 100% relative humidity.
- Whole set up was incubated at 25 ± 1°C for 48 hrs to enhance sporulation. After incubation, these infected plant pieces were examined under stereo binocular

microscope to confirm the typical elliptical or spindle shaped *M. oryzae* conidia (Plate.1).

- Upon positive confirmation, the aforementioned plant fragments were aseptically transferred into sterilized test tubes containing 1 ml of sterile double distilled water, subsequently vertex for a duration of five minutes to prepare a conidial suspension.
- A volume of 100 microliters of appropriately diluted conidial suspension was uniformly spread across 0.8% water agar medium. The culture was then subjected to incubation at 25 ± 1°C for 10-12 hours.
- After incubation, single germinating conidia was marked with a glass marker on bottom surface of the Petri dish with the help of objective lens under a microscope.
- Each marked single conidium accompanied by a minute portion of agar medium, was aseptically cut with the help of sterilized scalpel blade. Subsequently, these excised elements were delicately transferred to fresh Petri dish containing oat meal agar medium.
- The petri dishes, hosting these transferred elements, were then subjected to an incubation period of 25 ± 1°C for a duration of seven days, during which the development of fungal cultures originating from single spores transpired. The identification of these cultures was based on meticulous assessment of spore morphology in alignment with established criteria by Ou, 1985.

Evaluation of natural hosts for sporulation of *M. oryzae*:

To assess the sporulation of *M. oryzae*, an investigation was conducted employing three distinct natural hosts, namely rice leaves (cv.TN-1 and cv.HR-12), rice grains (cv.TN-1 and cv.HR-12), and weed species (*Panicum repens*, *Echinochloa colonum*, and *Brachiaria mutica*). These hosts were collected from the Institute of Rice Research (IRR), PJTSAU, Rajendranagar.

- The collected leaves and grains were thoroughly cleaned using tap water, followed by meticulous drying utilizing sterile blotter paper.
- Subsequently, dried leaves were sectioned into small fragments measuring 1-5 cm in length. These fragments, comprising 20 grams of leaves and an equivalent amount of grains, were transferred into 150 ml Erlenmeyer flasks. To ensure optimal humidity, the flasks were tightly sealed with cotton plugs.
- These flasks were sterilized at 15 lbs (121.6^0C) for 15 min in an autoclave.

- After sterilization, each flask was inoculated with two 5 mm diameter mycelial discs of *M. oryzae* isolate (IRR) and incubated for 14 days at ambient room temperature.
- After 14 days of incubation, a conidial suspension prepared by crushing the leaf pieces in 50 ml sterile distilled water using sterile pestle and mortar. Conversely, conidial suspension from grains was harvested by inverting the flask for a duration of 5 minutes within 50 ml of sterile distilled water.
- The enumeration of spores was performed at both 10 and 14 Days after Incubation (DAI). This quantification was represented as the number of spores per microscopic field. To achieve this, a loopful of the spore suspension was carefully placed onto a clean slide, followed by the positioning of a cover slip. The sporulation rate was recorded across five distinct microscopic fields, thereby ensuring comprehensive assessment and accuracy in data acquisition.

Chart 1 : Rating scale for sporulation index (Aruna *et al.*, 2016)

Rate of sporulation	No. of spores / microscopic field	Sporulation index
Excellent	>30	4
Good	20- 30	3
Fair	10- 20	2
Poor	<10	1

Results and discussions

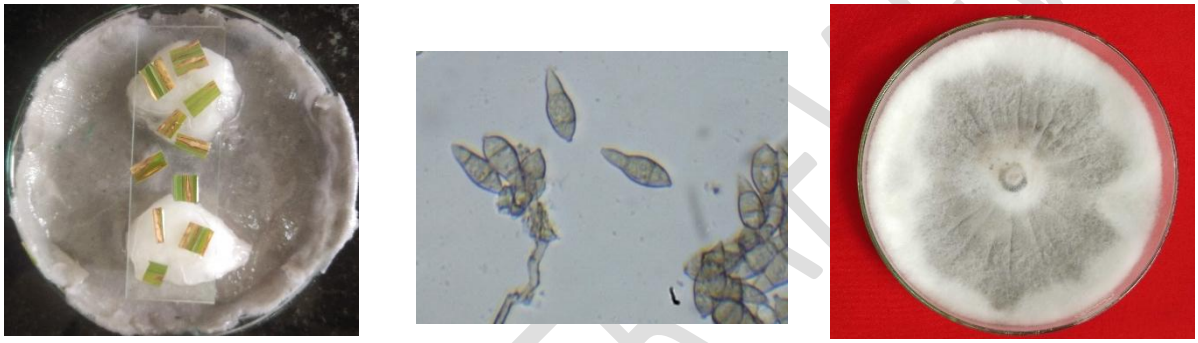
Single spore isolation:

Many of the researchers are facing the problem of single spore isolation of *Magnaporthe oryzae* pathogen as the isolation procedures are not given completely in most of the publications available for *M. oryzae*. In this study, we tried to isolate the single spore cultures of blast with less efforts by following moist chamber method. Our mono conidial isolation procedure is simple, inexpensive, can be easily carried out with minimum equipment. Therefore, even poorly funded laboratories can carry out the procedures that are outlined.

In many of the studies carried out on *M. oryzae* in order to understand the diversity of population, pathogen was isolated by using tissue segment method where the infected portion was cut into small pieces and surface sterilized by dipping in 1% sodium hypochlorite for 1 min, rinsing three times with sterile distilled water and transferred onto PDA plate

(Srivastava *et al.*, 2019; Gowrisri *et al.*, 2019). In such cases there are more chances of getting cross contamination with saprophytes as saprophytes dominates the pathogen growth.

In the present investigation sequential steps are explained so that any researcher is able to establish monoconidial culture with ease. In this method, when a diluted conidial suspension was spread on water agar, distance between the spores increased and it is easy to pick the separated individual germinated spore using transfer needle and transferred to fresh medium to obtain mono conidial cultures (Jagadeesh *et al.*, 2018). Hence this method has the advantage of being easy to locate well separated spores for mono conidial isolation.



Incubation under Moist chamber | **Spindle shaped conidia of *M. oryzae*** | **Pure culture of *M. oryzae***

Plate.1 Single spore isolation of *M. oryzae* under moist chamber method

Evaluation of different natural hosts for sporulation of *M. oryzae*

Significant differences were observed in sporulation of *M. oryzae* (IRR isolate) on three different natural hosts *viz.*, namely rice leaves (cv.TN-1 and cv.HR-12), rice grains (cv.TN-1 and cv.HR-12), and weed species (*Panicum repens*, *Echinochloa colonum*, and *Brachiaria mutica*) at 10 and 14 days after incubation. With respect to the incubation time, the mean amount of sporulation was higher at 14 DAI than at 10 DAI in all the tested natural hosts. Sporulation of the *M. oryzae* increased with increase in time but that increase from 14 DAI was found with no significant difference (Table.1). The present results are coincided with the findings of Bhaskar *et al.* (2018), who assessed the sporulation of *P. oryzae* on different natural hosts at 10 and 15 Days After Inoculation (DAI). Results revealed that the mean amount of sporulation was higher at 15 DAI (4.40×10^5 ml^{-1}) than at 10 DAI (4.26×10^5 ml^{-1}) in all the hosts, sporulation increased with increase in time but that increase from to 15 DAI (4.40×10^5 ml^{-1}) was found with no significant difference.

The present results also showed that the sporulation Index of *M. oryzae* on three different natural hosts *viz.*, namely rice leaves, rice grains and weed species was higher under 14 hr light + 8 hr dark conditions compared to constant light or dark conditions. This suggests that light conditions can influence the pathogen's sporulation ability, with alternating light and dark conditions potentially favouring sporulation. Similarly, a study conducted by Mahdieh *et al*. (2013) revealed that providing an alternation of 16 hr light + 8 hr darkness improved the fungal sporulation and increased production of conidia.

The present results also showed that among the three different types of natural hosts tested, rice leaves collected from the susceptible cultivars *viz.*, TN-1 and HR-12 exhibited excellent sporulation with sporulation index of 4 after 14 DAI at 14 hr light + 8 hr dark conditions. Among the rice grains tested, HR-12 exhibited good sporulation with sporulation index of 3 while the TN-1 grains exhibited fair sporulation (2.0) after 14 DAI at 14 hr light + 8 hr dark conditions. Among the weed sps. Tested, *Panicum* and *Brachiaria* found on par with each other by producing fair sporulation with sporulation index of 2 while *Echinochloa* exhibited poor sporulation (1.0) after 14 DAI at 14 hr light + 8 hr dark conditions (Table.1). Similar attempt was made by Bhaskar *et al.*, 2018 who reported that the sporulation on *Brachiaria mutica* leaf and stem segments was found as best method with the sporulation of 7.61×10^{5} ml^{-1}.

The present findings corroborate the research work carried out by Gaitonde *et al*. (2016), who revealed significant effects of different photoperiods on the mycelial colony growth of *M. oryzae*. The results also showed that the maximum colony growth and the highest number of conidia in *M. oryzae* was observed when the inoculated culture plates were subjected to a three-day period of darkness, followed by four days of exposure to light, and subsequently maintained a cycle of 8 hours of darkness and 16 hours of light. Similarly, Awoderu *et al*. (1991) reported that continuous light enhanced the conidial production of *M. oryzae*. In India, Padhi (1988) also showed that three races of *P. oryzae* (ID-5, 11-1 and IA-65) sporulated best under alternate light and dark regimes. However, Charkrabati and Wilcoxon (1976) indicated that light was not an essential condition for sporulation in *P. oryzae* but light enhanced its sporulation. Whereas, Kumar and Singh (1995) found that continuous darkness enhanced maximum growth of *M. oryzae*. These differences may be attributed to different isolates of *M. oryzae* at different culture media.

Interactions:

Natural Host × Time: There doesn't seem to be a strong interaction between "Natural Host" and "Time." The general trend of increased sporulation from 10 DAI to 14 DAI is consistent across hosts.

Natural Host × Light Condition: An interaction between "Natural Host" and "Light Condition" indicates that the effect of light conditions on sporulation index might vary depending on the host.

Time × Light Condition: This interaction indicates that the effect of light conditions on sporulation index might differ between 10 DAI and 14 DAI. The extent of sporulation increase from 10 DAI to 14 DAI might vary under different light conditions.

Table.1 Comparing Sporulation Index of *M. oryzae* Isolate (IRR) on Different Natural Hosts at Diverse Time Intervals

Natural host	Sporulation index					
	10 DAI			14 DAI		
	24hr Light	24hr dark	14 hr light + 8 hr dark	24hr light	24hr dark	14 hr light + 8 hr dark
1) Rice leaves:						
TN-1	1.0	1.0	3.0	2.0	2.0	4.0
HR-12	2.0	2.0	2.0	2.0	2.0	4.0
2) Rice grains:						
TN-1	1.0	1.0	1.0	1.0	2.0	2.0
HR-12	1.0	1.0	1.0	1.0	1.0	3.0
3) Weed species:						
Panicum	1.0	1.0	1.0	1.0	1.0	2.0
Echinochloa	1.0	1.0	1.0	1.0	1.0	1.0
Brachiaria	1.0	1.0	2.0	2.0	2.0	2.0

Based on the attained results, specifically concerning rice leaves collected from the susceptible cultivars *viz.*, TN-1 and HR-12, exhibited excellent sporulation with sporulation index of 4 after 14 days of incubation under the conditions of 14 hours of light followed by 8 hours of darkness.

To substantiate the sporulation capability of *M. oryzae* on these natural hosts, a total of twelve *M. oryzae* isolates were collected from diverse locations within Telangana State, including Mancherial, Jagtial, Karimnagar, Nizamabad, Peddapalli, Rangareddy, Nalgonda, Mahabubnagar, Medak, Warangal, Mahababad, Khammam, and Mahabubnagar districts (Table.2).

Table 2. Isolates of *Magnaporthe oryzae* collected from different locations of Telangana State.

S. No.	Location	Name of the District	Isolate Code
1.	Gangadhara	Karimnagar	Mo1
2.	Rajendranagar	Rangareddy	Mo2
3.	Makulapet	Mancherial	Mo3
4.	Polasa	Jagtial	Mo4
5.	Dharmaram	Nizamabad	Mo5
6.	Kanchanapalli	Nalgonda	Mo6
7.	Eligedu	Peddapalli	Mo7
8.	Garla	Mahabubabad	Mo8
9.	Marlapadu	Khammam	Mo9
10.	Makthal	Mahabubnagar	Mo10
11.	Peddashettipalli	Medak	Mo11
12.	Atmakur	Warangal	Mo12

Subsequently, these collected isolates were evaluated for their sporulating ability on rice leaves collected from two cv. TN-1 and HR-12 after 14 days of incubation under the illumination of 14 hours of light followed by 8 hours of darkness (Plate.2). The findings unveiled significant differences in sporulation both among the various isolates and between the two tested cultivars. Among the two rice cultivars tested, highest mean sporulation was supported by cv. TN-1 (3.1) followed by cv. HR-12 (2.2). Sporulation of twelve isolates on cv. TN-1 ranged from fair (2.0) to excellent sporulation (4.0) (Table.3). Whereas, on HR-12 sporulation of isolates ranged from poor (1.0) to excellent sporulation (4.0). High amount of sporulation on hosts leaf and stem bits was supported by the findings of Manjunath *et al*., 2013, who reported high sporulation (1983 conidia ml^{-1}) on maize stem bits. Maintenance of humidity in the flask by placing cotton swab and nutrient composition of host might have a major role in induction of sporulation in *M. oryzae*.

Mycelial growth of *M. oryzae* on rice leaves

Conidial suspension of *M. oryzae* isolates

Spores/ microscopic field

Plate. 2 Sporulation assessment of twelve isolates of *M. oryzae* on rice leaves at 14 DAS

Table.3 Comparative Sporulation Index of different isolates of *M. oryzae* on leaves of rice cultivars after 14 DAI under 14hr light + 8hr dark conditions.

Isolate	Sporulation Index 14 DAI (14hr light + 8hr dark)	
	Cv. TN-1	Cv. HR-12
Mo1	3.0	3.0
Mo2	4.0	4.0
Mo3	4.0	2.0
Mo4	2.0	2.0
Mo5	2.0	1.0
Mo6	4.0	3.0
Mo7	3.0	2.0
Mo8	3.0	3.0
Mo9	4.0	1.0
Mo10	2.0	1.0
Mo11	4.0	3.0
Mo12	3.0	2.0
Mean	3.1	2.2

Conclusion

During the current investigation, we have undertaken a systematic endeavor aimed at developing a meticulously detailed, step-by-step protocol for the precise isolation of monoconidial cultures, as well as the induction of sporulation in the *M. oryzae* pathogen. Moreover, through careful experimentation conducted under specific photoperiod conditions, we have discerned that the rice leaves of cultivars *viz.*, TN-1 and HR12 supported more sporulation at 14 DAI under the conditions of 14 hours of light followed by 8 hours of darkness.

To validate and ascertain the sporulation capacity of the aforementioned rice cultivars (TN-1 and HR12), we subjected them to testing using twelve distinct *M. oryzae* isolates. The outcomes of these rigorous tests unveiled a distinct pattern, with the cultivar TN-1 supporting the highest mean sporulation rate, followed by the HR-12 cultivar.

This study will become helpful for many researchers who are working in this line. Thus, the information on this method of isolation and sporulation will supplement the researchers and rice breeders to understand the disease epidemics, control and developing rice blast resistance cultivars.

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