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An investigation on the effects of photoperiod, aging and culture media on vegetative growth and sporulation of rice blast pathogen *Pyricularia oryzae*

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ABSTRACT

Pyricularia oryzae (Tel. *Magnaporthe grisea*) is currently used as a fungal model for plant-microbe interaction studies as well as an indicative model for anticancer drug discovery. The present study introduces the optimal condition in which *P. oryzae* grows and sporulates best on common culture media. We have considered three fungal culture media, i.e. PDA, PCA and WA, based on which *P. oryzae* sporulation inducers like rice polish, rice extract or rice leaf segments could be added, and evaluated both for vegetative growth and sporulation. Three light regimens, i.e. continuous light, 16/8 hr light/darkness, and continuous darkness were applied in combination with nine synthetic culture media. Mycelial growth was measured after 11 days, but sporulation was tracked on the 10th, 20th, and 30th day after incubation at 26ºC. The findings indicate that PDA culture medium could provide the best medium for *P. oryzae* vegetative growth, regardless of light condition. However, *P. oryzae* could sporulate when light was provided either continuously or at intervals. A combination of 16/8 hr light/darkness intervals and adding rice materials to culture media could induce *P. oryzae* for a better sporulation. RPCA can be used as the best culture medium for *P. oryzae* in order to obtain a high number of conidia under light alterations. Moreover, aging increases the total number of conidia.

Key Words: conidiation, fungal development, light regimen, *Pyricularia oryzae*.

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Introduction

Rice is one of the most important cereal crops worldwide, particularly in Asia where almost half of the population relies on it as the main food. Rice blast disease is the most important and the most destructive disease of rice, which is caused by the ascomycetous fungus *Pyricularia oryzae* (Teleomorph *Magnaporthe oryzae* Couch) formally known as *Pyricularia grisea* (Cooke) Sacc.] (4), [Teleomorph *Magnaporthe grisea* (Herbert)]. The disease is spread worldwide, but its occurrence and severity vary by year, location and environmental conditions. Rice blast symptoms can occur on all above-ground parts of the plant and is observed at earlier growing stages until the final grain production. In addition to rice blast disease (12), *P. oryzae* can also cause gray leaf spot disease in monocotyledonous plants *Stenotaphrum secundatum* (Waltz.) Kuntze and *Lolium perenne* (L.) (10).

The anamorph *P. oryzae* produces pyriform shaped gray or hyaline spores, known as conidia, with one to two transversal septa. Conidiophores are slightly browned, septated, rarely branched, suggesting sympodial growth. The teleomorph *M. grisea* has only been observed under laboratorial crosses between compatible isolates.

Virulent *Pyricularia* species employ a hemibiotrophic strategy to invade host, in which biotrophic and necrotrophic stages of infection are sequentially established (7). Upon germination of conidia of these fungi, the hyphae penetrate the host cell lumen through host cuticle and cell. These intracellular hyphae are biotrophic (7, 9). Afterwards, necrotrophic hyphae are formed, extensively spread and kill the host tissue. Each lesion from a susceptible host can produce more than 2×10^4 conidia over several days (1).

Understanding the development of the fungus, most notably its vegetative growth and sporulation, is crucial for large scale studies, especially for production of fungal spores when disease screening studies are concerned. Moreover, *P. oryzae* is currently being used as a model organism for anticancer metabolite screenings (6,8). For such studies, obtaining over 10^4 conidia is the first prerequisite. It is reported that cultivation of the fungus on crushed rice leaves (12) or including rice polish, or rice plant materials in Potato Dextrose Agar medium (PDA) favors fungus sporulation (3,2,5). We tried several of those media without sufficient success in obtaining adequate conidia. Therefore, in this research we aimed at quantifying and comparing vegetative mycelial growth and sporulation of *P. oryzae* on nine novel combinations of simple cheap culture media based on PDA, PCA, and WA, and three light regimens.

Materials and methods

Fungal strain

Pyricularia oryzae wildtype strain HS-1390 was isolated from leaf lesions of *Oryza sativa* in Iran (Provided by Salar Jamali, Guilan University, Iran). The isolated fungus was purified by the hyphae tip method, and identified by a set of its morphological characters. That isolate was used as the model in our experiments. Fungus strain was grown on Potato Dextrose Agar (PDA) medium (Merck, Darmstadt, Germany) at 26ºC. For long term usage, the fungus was maintained under liquid paraffin at +4ºC.

Culture media and light regimens

Nine fungal solid culture media, i.e. PDA (Potato Dextrose Agar), PDA-W (PDA+ Whatman filter paper), PDA-R1 (PDA+Rice leaf extract), PDA-R2 (PDA+Rice Polish), PDA-R3 (PDA+segments of Rice Leaf), WA-R1 (Water Agar+segments of Riceleaf), WA-R2 (WA+Rice Polish), PCA (Potato Carrots Agar), and RPCA (Rice Potato Carrots Agar) were employed for development studies (Merck, Darmstadt, Germany). The effects of three light conditions, i.e. 24 hrs fluorescent light, 8 hr dark/16 hr fluorescent light and 24 hr darkness on fungal development were evaluated in combination with the culture media. All experiments contained three replicates and were repeated twice.

Assessing fungal development

Mycelial discs of 7mm diameter cut from the growing margins of the fresh fungal culture were placed at the center of each 9 cm Petri plate containing culture media. Radial zone of mycelial growth (mm) was measured on the 11^{th} day, when the fungal colonies first covered the whole Petri plate area. Sporulation of the fungus (×10) under different regimens was measured on a daily (10, 20, 30) interval. The conidia were harvested by adding a 10 mL aliquot of sterile deionized water (pH 7.5) per 9 cm Petri plate and gently scraping the surface. A conidial suspension from each media was prepared and conidial densities were counted using a haemacytometer.

Statistical analyses

ANOVA and SAS procedures and programs were used for statistical analyses. In cases where the F-test showed significant differences between means, the differences among treatments were compared using least significant differences (LSD) test at 1% significance level (11). In cases where there was zero number, like absence of sporulation, the non-parametric statistics and Wilcokson's test were also applied.

Results

Mycelia growth and sporulation of *P. oryzae* under 24 hr fluorescent light at 26ºC on nine different culture media

After eleven-day incubation of *P.oryzae wildtype* strain HS-1390 under continuous fluorescent light on nine solid culture media, fungal vegetative growth was measured as the radial zone of the colony that covered the Petri plate. Data analyses indicated significant differences among the treatments ($P \leq 0.01$; not shown). The interaction of culture media and light was not statistically significant ($P \leq 0.01$).

Under 24 hr fluorescent light (Table 1), PDA-based culture media improved mycelial growth much more than PCA-based and WA-based culture media did. Of the PDA-based culture media, the best growth of *P. oryzae* was observed on the PDA medium, without adding any rice plant material. However, among the mixed media of PDA/Rice materials, PDA containing segments of rice leaf, better favored the growth of fungus. The least zone of growth was obtained on WA-based media.

However, under 24 hr fluorescent light, the fungus could only sporulate on PCA culture medium after 20 days, up to 7.5×10^4 conidia per mL, which was raised to ~7.9×10^4 conidia per mL after 30 days of incubation. Under this condition, the other culture media did not show any effect on fungal sporulation, nor could added rice material favor this phenomenon (Table 1).

Table 1. Mycelial growth of *P. oryzae* wild type strain HS-1390 incubated on nine culture media for 24 hours at 26ºC with light.

Culture Media	Radius Zone of Mycelia growth (mm)[&,*]
PDA	37.8 (A)
PDA-R3	33.49 (B)
PDA-R2	29.98 (C)
PDA-R1	27.8 (CD)
RPCA	27.8 (CD)
PDA-W	27.16 (CD)
PCA	25.99 (D)
WA-R1	17.66 (E)
WA-R2	16.99 (E)

[&]Averages of six replicates. *Media whose corresponding growth indices are not significantly different ($P \leq 0.01$) are indicated with same letter (e.g. E) or combination of letters (e.g. CD) in parenthesis.

Mycelia growth and sporulation of *P. oryzae* under 16/8 hr fluorescent light/ darkness at 26ºC on nine different culture media

After eleven-day incubation of *P. oryzae* wildtype strain HS-1390 under 16/8 hr fluorescent light/darkness on nine solid culture media, fungal vegetative growth was measured as the radial zone of the colony covered the Petri plate. Data analyses indicated significant differences between the treatments ($P \leq 0.01$; not shown). The interaction of culture media and light was not statistically significant ($P \leq 0.01$).

Under 16/8 hr fluorescent light/darkness (Table 2), PDA-based culture media improved mycelia growth much more than PCA-based and WA-based culture media did. This almost resembles the growth of fungus under continuous 24 hr light on the same media. Among the PDA-based culture media, the best growth of *P. oryzae* was again observed on PDA itself, without adding any rice plant material. Once again, among the mixed media of PDA/Rice materials, PDA containing segments of rice leaf, better favored the growth of fungus. The least zone of growth was obtained on WA-based media.

As appeared in Table 2, fungal sporulation on the same nine culture media was improved under 16/8 hr fluorescent light/ darkness, compared with 24 hr fluorescent light. Here, the fungus could sporulate not only on both PCA-based culture media (PCA and RPCA) after 20 days, but on other rice material containing culture media like PDA-R2 (PDA + Rice Polish) and WA-R1 (Water Agar +segments of Rice leaf), all in the range of $\times 10^5$ conidia per mL which was 10-fold higher than that under continuous light. Although under this condition the PCA medium generated the least number of conidia, compared with other media which favored sporulation, the maximal sporulation was still obtained on the rice extract containing PCA (i.e., RPCA), in the range of 3.6×10^5 (at 20^{th} day) to $\sim 4.4 \times 10^5$ (at 30^{th} day) conidia per mL. This was followed by PDA-R2 in which sporulation increased between $\sim 3.4 \times 10^5$ (at 20^{th} day) and 3.6×10^5 (at 30^{th} day) conidia per mL; and WA-R1 which induced sporulation from 2.7×10^5 to $\sim 3.2 \times 10^5$ on 20^{th} and 30^{th} days, respectively. Under this condition, the other culture media did not show any effect on fungal sporulation (Table 2).

Table 2 . Mycelial growth and sporulation of *P. oryzae* wild type strain HS-1390 incubated on nine culture media under 16/8 hr fluorescent light/darkness cycle at 26ºC.

Culture Media	Radius Zone of Mycelia growth (mm)[&,*]	Sporulation on 20th day (Conidia/mL)[&,*]	Sporulation on 30th day (Conidia/mL)[&,*]
PDA	44.33 (A)	0	0
PDA-R3	38.98 (B)	0	0
PDA-R2	33.33 (C)	3.38×10^5 (B)	4.23×10^5 (B)
PDA-W	33.33 (C)	0	0
RPCA	33 (C)	3.6×10^5 (A)	4.36×10^5 (A)
PDA-R1	32.33 (CD)	0	0
PCA	31.33 (DE)	1.68×10^5 (D)	2.3×10^5 (D)
WA-R1	21.6 (I)	2.71×10^5 (C)	3.16×10^5 (C)
WA-R2	21.46 (I)	0	0

[&]Averages of six replicates. *Media whose corresponding growth indices are not significantly different ($P \leq 0.01$) are indicated with same letter (e.g. I) or combination of letters (e.g. CD) in parenthesis.

Mycelia growth and sporulation of *P. oryzae* under 24 hr darkness at 26ºC on nine different culture media

After the eleven-day incubation of *P. oryzae* wildtype strain HS-1390 under continuously darkness on nine solid culture media, fungal vegetative growth was measured as the radial zone of the colony covered the Petri plate. Data analyses indicated significant differences between the treatments ($P \leq 0.01$; not shown).

Under 24 hr darkness (Table 3), mycelial growth and fungal sporulation was almost identical to that of treatment under 24 hr fluorescent light. Indeed, PDA-based culture media improved mycelial growth much more than PCA-based and WA-based culture media did. Among the PDA-based culture media, the best growth of *P. oryzae* was observed on the PDA itself, without adding any rice plant

Table 3 . Mycelial growth of *P. oryzae* wild type strain HS-1390 incubated at 26ºC on nine culture media under 24 hr darkness.

Culture Media	Radius Zone of Mycelia growth (mm)[&,*]
PDA	39.99 (A)
PDA-R3	34.65 (B)
PDA-R2	30.45 (C)
PDA-W	29.8 (CD)
PDA-R1	29.49 (CD)
RPCA	29.33(CD)
PCA	28.48 (DE)
WA-R1	18.48 (F)
WA-R2	17.99 (F)

[&]Averages of six replicates. *Media whose corresponding growth indices are not significantly different ($P \leq 0.01$) are indicated with same letter (e.g. F) or combination of letters (e.g. CD) in parenthesis.

material, as it was under continuous light. Once again, among the mixed media of PDA/ Rice materials, PDA containing segments of rice leaf, better favored the growth of fungus. The least zone of growth was obtained on WA-based media.

However, in contrast to 24 hr fluorescent light and 16/8 hr fluorescent light/darkness, fungus could not sporulate under 24 hr darkness at all, even after 30 days of incubation (Table 3).

Discussion

Pyricularia oryzae is currently used as a fungal model for plant-microbe interaction studies as well as an indicative model for anticancer drug discovery. At fungal natural niches, light and nutrients are among the most influencing environmental factors affecting the success and fitness of the fungus. However, for a better handling of the fungus under laboratory conditions there is a need for understanding its development i.e. vegetative growth and sporulation. Several researches have introduced a number of mixed solid media for gaining the maximal colonization of the fungus and obtaining enough conidia for further experiments. We initially aimed at using *P. oryzae* as a model target fungus for drug discovery in our laboratory. Yet, by using the formerly introduced culture media, we could not obtain sufficient conidia. Hence, it was tried to find the optimized condition in which *P. oryzae* sporulates best. We considered three fungal culture media namely PDA, PCA and WA, based on which *P. oryzae* sporulation inducers like rice polish, rice extract or rice leaf segments could be added and evaluated both for vegetative growth and sporulation. Three light regimens, i.e. continuous light, 16/8 hr light/darkness, and continuous darkness were applied in combination with nine synthetic culture media. Mycelial growth was measured after 11 days, but sporulation was tracked on 10th, 20th, and 30th day after incubation at 26 ºC.

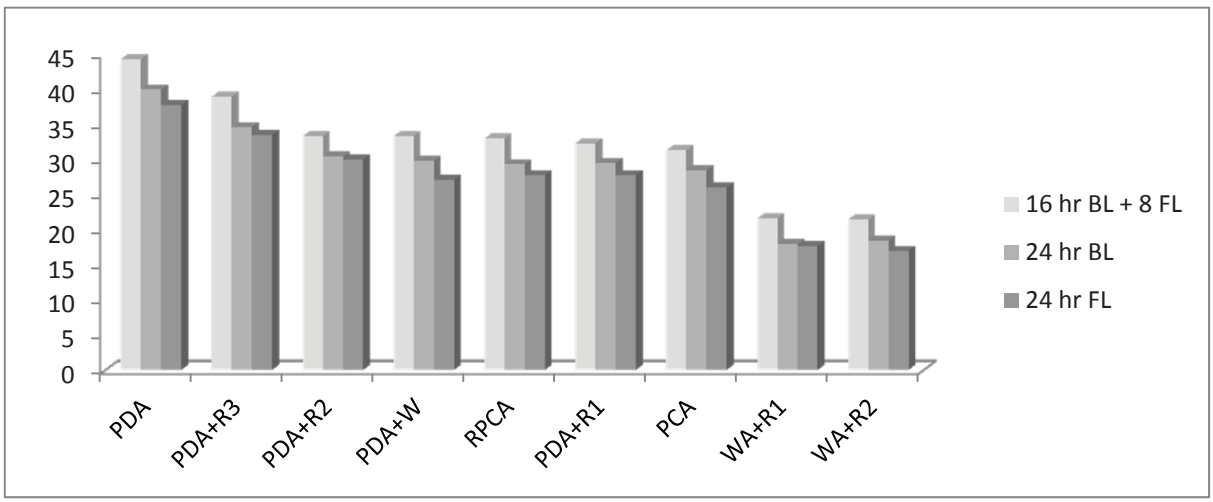

Figure 1. Comparison of vegetative growth of *P. oryzae* HS-1390 on PDA-, PCA-, and WA-based culture media, under three different light regimens. Growth was assessed after eleven days. For a full description of culture media abbreviations, refer to Materials and methods section.

Fungal vegetative growth, as observed in Figure 1, was almost identical on nine different culture media under three different light regimens. Indeed, the fungus could grow

on all the culture media tested, regardless of light conditions.

This means that mycelial growth was independent of light, although an alternation of 16/8 hr light/darkness improved the fungal vegetative growth, compared with either continuous light or continuous darkness. Broadly put, the PDA culture medium could provide the best condition for *P. oryzae* vegetative growth, regardless of light regimen.

Fungal sporulation, as seen in Figure 2, on nine different culture media differed greatly under three different light regimens. Indeed, the fungus could not sporulate on any culture medium under continuous darkness, at all. However, it could sporulate when light was provided either continuously or at intervals.

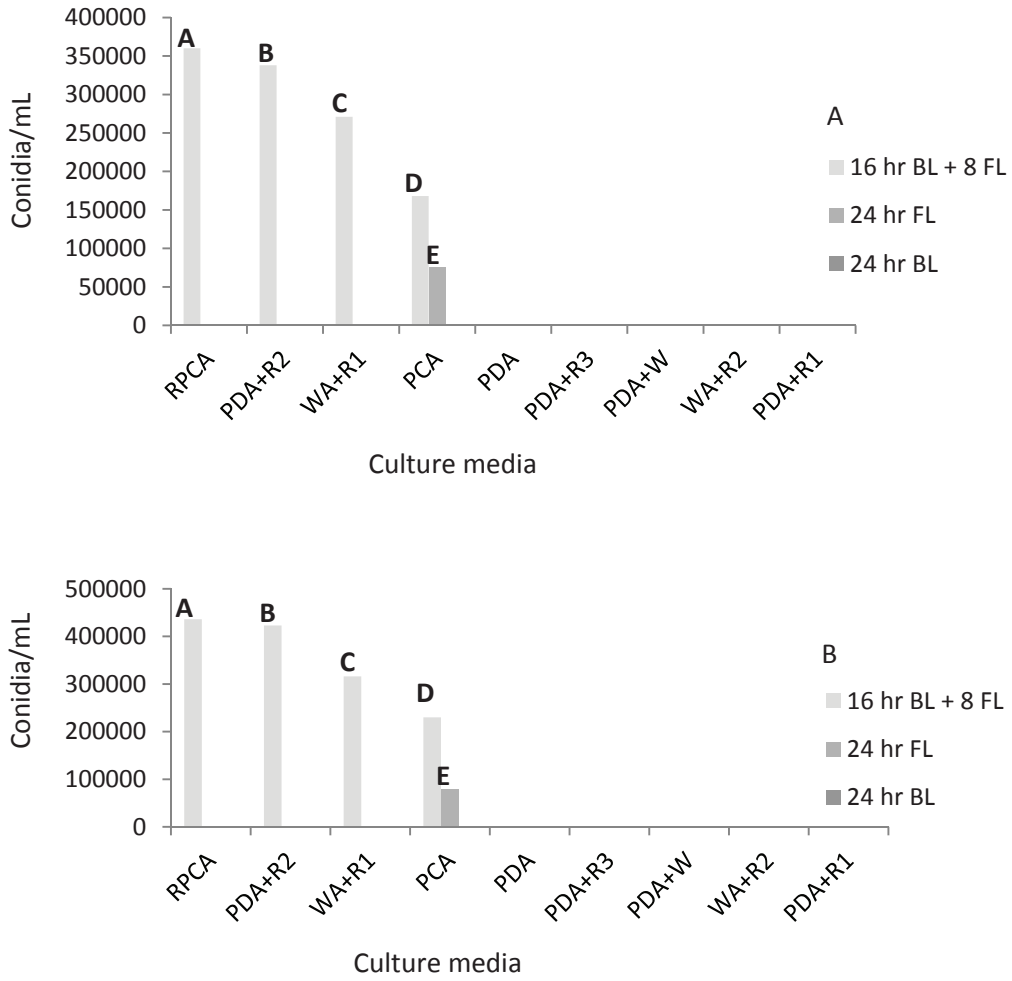

Figure 2 . Comparison of sporulation of *P. oryzae* HS-1390 on PDA-, PCA-, and WA-based culture media, under three different light regimens. A- After twenty days. B- After thirty days. For a full description of culture media abbreviations refer to Materials and methods section.

This indicates that *P. oryzae* sporulation is light-dependent. Moreover, under continuous light, the fungus could sporulate only on PCA culture medium at a concentration of $\times 10^4$. Under this condition, adding rice materials or extracts to the culture media did not lead

to any improvement in fungal sporulation. Furthermore, compared with the 20th day, by aging the number of conidia was increased, as on the 30th day the total number of conidia was raised.

Providing an alternation of 16/8 hr light/ darkness improved the fungal sporulation and increased production of conidia on four culture media, i.e. PCA.RPCA, PDA-R2 and WA-R1 at concentrations of $\times 10^5$. Under this condition, the rice materials (extract, polish or leaf segments) added to culture media as RPCA, PDA+R2 and WA+R1 could also improve the sporulation of *P. oryzae*, compared with PCA. Thus, these data indicate that a combination of 16/8 hr light/darkness intervals and adding rice materials to culture media could induce *P. oryzae* for a better sporulation. Generally, PCA was the only culture medium on which the fungus could sporulate under continuous or discontinuous light, and also with or without rice material inducers. However, in order to obtain the highest number of conidia, RPCA can be used as a culture medium of choice for *P. oryzae* under 16/8 hr light/darkness. Aging also plays a role in increasing the number of conidia at this condition, as the total number of conidia on the 30th day was raised, compared with the 20th day.

Taken all together, our findings could also indicate that in nature, at its niches, *P. oryzae* does not depend on light for its vegetative growth as a biotrophic or necrotrophic phytopathogen. However, the fungus needs both rice inducers and light intervals for its sporulation, as this phenomenon resembles the life of the pathogen on plant tissue lesions.

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References

1. Barksdale, T. and Asai, G.N. (1961) Diurnal spore release of *Pyricularia oryzae* from rice leaves. *Phytopathology*,51,313-317.
2. Bhattacharyya D.,and Bose S.K.(1981) Studies on standardizing the condition of sporulation in *Pyricularia oryzae*. *Ind.Phytopath*.,34, 382-383
3. Chen, Y. X. (1983) Methods for inducing sporulation of rice blast fungus (*Pyricularia oryzae* Cav.). *J.Nanjing Agri*. Col.,2, 39.
4. Couch B.C., and Kohn L.M.(2002) A multilocus gene genalogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae* from *M. grisea*. *Mycolgia*,94, 683-693
5. Dhingra O.D., and Sinclair J.B. (1995) Basic Plant Pathology Methods.2nd ed. Boca Raton FL. CRC Press, 434 pp.
6. Dong, Q.F.,Wang,J.L.,Zhang,S.F.,Wang,Z.,Zhang,C.X., Gao, .,Zhang,H.M., and Zhang,L. (2008) Antifungal activity of crudeextracts and fat-of crude extracts and fat soluble conctituens of *Holotrichia diomphalia* larvae. *Bioresource Technol*.,99, 8521–8523.
7. Kankanala, P.; Czymmek, K. and Valent, B.(2007) Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell*,19, 706-724.
8. Kobayashi, H., Namikoshi, M., Yoshimoto, T., and Yokochi, T. (1996) A screening method for antimitotic and antifungal substances usingconidia of *P. oryzae*, modification and application to tropical marine fungi. *J. Antibiot*.,49, 873–879.
9. Marcel, S., Sawers, R., Oakeley, E., Angliker, H. and Paszkowski, U.(2010) Tissue-adaptedinvasion strategies of the rice blast fungus *Magnaporthe oryzae. Plant Cell*,22,3177-3187.
10. Smiley, R.W., P.H. Dernoden, and B.B. Clarke. (2005) Compendium of Turf grass Diseases. 3rd. ed. St. Paul, MN: *The American Phytopathological Society.*
11. Steel, R.G.D., Torrie, J.H., and Dicky, D.A.(1997) Principles and Procedures of Statistics. A Biometrical Approach, 3rd ed. *McGraw Hill Book International Co*.,Singapore,pp. 204–227.
12. YorionoriJ.T.,and Thurston H.D.(1974) Sporulation of *Pyricularia oryzae* on crushed rice leaves. *Fitopathologia*,9, 24-27.