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# Isolation of *Microdochium oryzae* and *Pinatubo oryzae* from rice seeds and their survival on stored seeds

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**Abstract**

The aim of these studies was to develop a semi-selective medium to differentiate *Microdochium oryzae* and *Pinatubo oryzae*, determine the frequency of seed infection of *M. oryzae*, study survival of the pathogen in stored seeds, and determine the frequency of infection of seed components. To simulate epidemics of differing intensities, panicles of rice cultivars that are susceptible (IR36) and resistant (IR42 and IR46) to *M. oryzae* were either non-inoculated, inoculated once, twice, or three times with a conidial suspension of *M. oryzae*. Both *M. oryzae* and *P. oryzae* colonies were recovered from seeds and were similar in culture. A semi-selective medium developed to detect *M. oryzae* seed infection rates aided in differentiating *M. oryzae* and *P. oryzae* by stimulating aerial conidiogenesis of *P. oryzae*. The conclusions taken from these results were: (a) seeds of IR36 had higher infection of *M. oryzae* than of IR42 and IR46 from plants grown in the dry season, but had lower infection of *M. oryzae* than of IR42 and IR46 from plants grown in the wet season; (b) *M. oryzae* infected seeds increased with an increase in the epidemic intensity with the highest occurring after three inoculations, the least occurring with non-inoculation, and intermediate with one, or two inoculations; (c) survival of *M. oryzae* decreased over time in seed lots stored at 10 °C and 40% relative humidity and (d) all components of the rice seeds of IR36, IR42 and IR46 lots were infected with *M. oryzae* with the highest frequency in the endosperm and lemma, intermediate in the basal glumes and palea, and the least in the embryo.

## Introduction

Leaf scald of rice (*Oryza sativa* L.), caused by *Microdochium oryzae* (Hashioka & Yokogi) Samuels & Hallett [teleomorph *Monographella albescens* (Thümen) Parkinson, Sivanesan & Booth] is widespread throughout the rice growing regions of Africa (Thomas, 1983, 1984), Asia (Anonymous, 1979, 1982; Mia et al., 1985; Sanchez et al., 1979) and the Americas (De Gutierrez, 1960; Faiad et al., 1993), causing up to 30% yield losses (Thomas, 1984). Although *M. oryzae* commonly infects leaves and sheaths, seed infection is also important because it reduces seed germination and seedling vigor (Singh and Gupta, 1985). Seed rot and seedling blight of rice, caused by *Pinatubo oryzae* Manandhar & Mew, is an important seedborne pathogen in the Philippines, reducing seed germination and seedling vigor (Manandhar and Mew, 1996). Both *M. oryzae* and *P. oryzae* are frequently recorded as *M. oryzae* since the growth characteristics of the both pathogens are similar on blotters (De Tempe and Binnerts, 1979). If aerial conidiophores of *P. oryzae* are absent (Manandhar and Mew, 1996) then *M. oryzae* may be overestimated. Since there is no difference in detecting *M. oryzae* in six methods, viz. blotter test, 2,4-D, Deep-freezing blotter,

potato-dextrose agar (PDA), guaiacol agar and rice extract agar (Shetty and Shetty, 1988), a semi-selective medium seems necessary to differentiate *M. oryzae* and *P. oryzae*.

*Microdochium oryzae* survives three to 18 months in infected rice seeds under ambient conditions (Mia et al., 1987; Sanchez et al., 1979; Singh and Gupta, 1986), but it can survive up to 11 years at 5 °C (Mia et al., 1985). All rice seed components are potential infection sites for *M. oryzae* (Kim et al., 1984; Mia et al., 1986). The precise location of the pathogen in the seed can help determine the efficacy of the seed treatments (Faiad et al., 1993). The objectives of this work were to develop a semi-selective medium to differentiate *M. oryzae* and *P. oryzae* for accurate estimate of seed infection, determine the frequency of seed infection of *M. oryzae* in plots differing in epidemic intensities, study survival of the pathogen by estimating the rate of decline in seeds stored at 10 °C and 40% relative humidity (RH), and locate the pathogen in seeds by estimating the frequency of infection in specific seed components.

## Materials and methods

*Inhibition of hyphal growth of fungi in a semi-selective medium.* A *M. oryzae* semi-selective medium (MOSM) was developed from a basal medium containing 15 g KCl, 1 g sucrose, 10 g granulated agar (Difco Laboratories, Detroit, MI) in 1 l distilled water and autoclaved at 121 °C and 100 kPa for 15 min. After cooling to 45 °C, 5 mg chloramphenicol (Sigma Chemical Co., St. Louis, MO), 5 mg erythromycin sulfate (Sigma Chemical Co., St. Louis, MO), 25 mg tetracycline hydrochloride (Sigma Chemical Co., St. Louis, MO), 20 mg neomycin sulfate (Sigma Chemical Co., St. Louis, MO), 1 mg chlorothalonil (tetrachloroisophthalonitrile, Daconil 2787® 75WP, ISK Biosciences Corp., Marietta, GA) and 20 mg mancozeb (manganese ethylenebisdithiocarbamate polymeric complex with zinc salt, Dithane M45® 80WP, Rohm & Haas, Philadelphia, PA) were added. Approximately 25 ml of prepared basal medium (all ingredients except chlorothalonil and mancozeb, pH 6.5) or MOSM (all ingredients including chlorothalonil and mancozeb, pH 6.5) was poured into each of 9-cm diam Petri dishes. Single conidial-isolates of *Alternaria padwickii* (Ganguly) M.B. Ellis, *Curvularia lunata* (Walker) Boedijn, *Drechslera oryzae* (Breda de Haan) Subram. & Jain, *Fusarium moniliforme* Sheld., *F. semitectum* Berk. & Cav., *F. solani* (Mart.) App. & Wr., *M. oryzae* and *P. oryzae* were isolated from rice seeds grown in the 1993 wet season at the Central Research Farm, International Rice Research Institute (IRRI) at Los Baños. Identities of conidial isolates were based on comparison with valid descriptions. The Mo-1 isolate of *M. oryzae* was matched to Type D cultural characteristics (Parkinson, 1980). Five-day-old colonies of all fungal species grown on PDA in dark were cut off from the margin using a 5-mm diam screw borer and plated at the center of 9-cm diam dishes containing basal medium or MOSM. There were three plates of either basal medium without fungicides or MOSM for each pathogen. The inhibition of the hyphal growth of the pathogen on the MOSM was calculated from the colony diam in 5 days after incubation at 28 °C as (colony diam in basal medium – colony diam in MOSM)/colony diam in basal medium.

*Assessment of seed infection using MOSM.* Low-land irrigated rice cultivars, susceptible IR36 and resistant IR42 and IR46 to *M. oryzae* (Anonymous, 1979, 1982) were sown in a dry seed bed nursery on 23 December 1993 and transplanted on 13 January 1994 for dry season production. The same cultivars were sown again on 6 June 1994 and transplanted on 28 June for wet season production. Experimental design was a split-plot with 4 replications. The main plot was cultivar and the sub-plot was differing epidemic intensities generated by non-inoculating or inoculating rice panicles with *M. oryzae* conidia either once, twice, or three times. Isolate Mo-1 of *M. oryzae* was used for all tests. Individual plots were 3 m × 5 m with hills at 20 cm within and between rows, and seedlings were transplanted 3 per hill. Fertilizers (N : P : K :: 30 : 30 : 30 kg per ha) were applied pre-planting, and 30 kg per ha N was applied at both maximum tillering and grain filling stages. Herbicides and insecticides were applied as recommended by the pesticide monitoring unit of IRRI. To produce conidial inoculum, three 3-mm diam hyphal plugs were cut from the edge of 5–7 day-old colonies grown on potato-dextrose agar (PDA) and placed 4 cm apart in 9-cm diam Petri dishes containing 25 ml of potato-sucrose salt agar (PSSA). PSSA was prepared by adding 20 g sucrose, 12 g KCl, and 15 g granulated agar to 770 ml of distilled water plus 230 ml of potato decoction. Potato decoction was prepared by simmering 300 g potato in 1 l of distilled water for 1 h (Naito, 1982). Plates were flooded with 10 ml sterile 0.01% Tween-20 in 7–10

days after incubation in the dark at 25–28 °C, conidia were dislodged with a sterilized brush and the resulting conidial suspension was filtered through two layers of cheese cloth. A conidial suspension was adjusted to $1 \times 10^6$ conidia per ml in a total volume of 500 ml using a hemacytometer and sprayed on rice panicles (300 ml per plot) during anthesis between 10:00 and 11:00 AM with a low volume sprayer. The first inoculation was applied on 17 March 1994 for IR36, 29 March 1994 for IR46 and 4 April 1994 for IR42 during the dry season; and on 25 August 1994 for IR36, 10 September 1994 for IR46 and 17 September 1994 for IR42 during the wet season. The second and third inoculations were at 3 and 6 days respectively following the first inoculation. Control plots were not spray-inoculated.

Harvested seed lots were dried for 96 h at 45 °C to 10% moisture content. The effect of epidemic intensity on seed infection rates was determined by examining seeds on MOSM. One hundred seeds per lot were plated on MOSM to differentiate *M. oryzae* and *P. oryzae*. Twenty five seeds per dish were incubated in the dark for 7 days at 30 °C. The number of *M. oryzae* or *P. oryzae* infected seeds was recorded using a binocular microscope.

*Survival of* M. oryzae *in stored seeds.* Seeds of IR36 were harvested from plots inoculated with *M. oryzae* during the dry season in 1994. Infection by *M. oryzae* determined by blotter (De Tempe and Binnerts, 1979) was 11%. Seeds were stored in a cold room adjusted to 10 °C and 40% RH, and 100 seeds from the same cold room were plated on MOSM for detection of *M. oryzae* at 105, 145, 210, 245, 294, 326, 355, 372, and 403 days after harvest (DAH). Similarly, 100 seeds of IR42 from a seed lot having 18% seed infection of *M. oryzae* were assayed after cold room storage at 85, 120, 155, 190, and 224 DAH for comparison. To determine survival trends, the same seed lots were assayed by blotter test using 400 seeds per assay at 44, 96, 131, 159, 217, 224, 251, 281, 312, 341, 369, 391, 414, 498, 530, 568, 594, 622, 660, 688 and 733 DAH for IR36 lot grown in the dry season and at 44, 102, 133, 162, 190, 212, 235, 319, 351, 389, 415, 443, 481, 509 and 654 DAH for IR42 lot grown in wet season.

*Seed components infected by* M. oryzae. Seed lots of IR36, IR42 and IR46 that were field inoculated with *M. oryzae* in the wet season had 11, 18 and 9% of *M. oryzae* infection respectively based on the blotter method. Three hundred seeds of each cultivar were dissected into basal glumes (referred to as the rachila with two sterile lemmas and with or without two vestigial lodicules), embryo, endosperm (with two vestigial stigmas), lemma, and palea (with or without two vestigial lodicules). Each of the components (25 per plate) was plated on MOSM (four plates per replication). Plates were sealed with a plastic bag and incubated for 7 days in the dark at 30 °C. There were three replications. Incidence of *M. oryzae* was determined using a binocular microscope.

*Statistical analysis.* Percent inhibition of hyphal growth of eight fungi was analyzed by analysis of variance (ANOVA) using completely randomized design and means were separated by Duncan's multiple range test (DMRT) at $P = 0.01$. Seed lots obtained from inoculated plots in the dry season and wet season experiments were analyzed separately and means separated by least significant difference (LSD) at $P = 0.05$ (Gomez and Gomez, 1984). Data from survival of *M. oryzae* in stored seeds were analyzed using linear correlation and plotted percent seeds with *M. oryzae* against DAH. Data from seed components infected by *M. oryzae* were analyzed using randomized complete block design with cultivar and seed component as factorial combinations.

## Results

*Inhibition of hyphal growth of fungi in a semi-selective medium.* Percent inhibition of hyphal growth of *M. oryzae* on MOSM was significantly ($P = 0.01$) lower than *C. lunata* and *F. semitectum* but higher than *A. padwickii*, *D. oryzae*, *P. oryzae* and *F. solani*, and not different from *F. moniliforme* (Table 1). Inhibition of *P. oryzae* was lower than 6 test fungi but higher than *D. oryzae* (Table 1).

*Assessment of seed infection using a semi-selective medium.* Percent seed infection due to *M. oryzae* was significantly ($P = 0.05$) higher in IR36 (5.6%), then followed by IR46 (4.9%) and IR42 (2.1%) for the dry season means, whereas IR42 (4.0%) or IR46 (4.4%) had higher seed infection than IR36 (1.6%) for the wet season (Table 2). There was significant ($P = 0.05$) interaction between cultivar and inoculation for the dry season but not for the wet season (Table 2). However, percent seed infection due to *P. oryzae* was not different among IR36 (1.1%), IR42 (0.4%) and IR46 (0.8%)

*Table 1.* Percent inhibition of hyphal growth on a semi-selective medium (MOSM) of eight different fungi at 28 °C in dark

| Fungus | Hyphal inhibition (%) |
|---|---|
| *Alternaria padwickii* | 72 d[1] |
| *Curvularia lunata* | 85 b |
| *Drechslera oryzae* | 33 f |
| *Fusarium moniliforme* | 76 c |
| *F. semitectum* | 100 a |
| *F. solani* | 70 d |
| *Microdochium oryzae* | 79 c |
| *Pinatubo oryzae* | 62 e |

[1]Numbers followed by the same letter are not significantly different using Duncan's multiple range test ($P = 0.01$).

*Table 2.* Percent rice seeds infected by *Microdochium oryzae* in lots harvested from plots differing epidemic intensities generated by either non-inoculating (0) or inoculating (1, 2 or 3) panicles with *M. oryzae* conidia as determined by examining 100 seeds per plot on a semi-selective medium (MOSM)

| Cultivar | Dry season[1] | | | | | Wet season[2] |
|---|---|---|---|---|---|---|
| | *Number of Inoculations* | | | | | |
| | 0 | 1 | 2 | 3 | Mean[2] | |
| IR36 | 1.5 | 4.3 | 7.5 | 9.3 | 5.6 a[3] | 1.6 b |
| IR42 | 0.8 | 1.5 | 2.5 | 3.8 | 2.1 c | 4.0 a |
| IR46 | 1.8 | 4.8 | 5.8 | 7.3 | 4.9 b | 4.4 a |
| Mean[4] | 1.3 d | 3.5 c | 5.3 b | 6.8 a | | |

[1]Means over four replications. Least significant difference (LSD, $P = 0.05$) between the number of inoculations within the same cultivar = 1.5 and in different cultivars = 1.4.
[2]Means over four replications and four epidemic intensities (0, 1, 2, and 3).
[3]Numbers followed by the same letters are not significantly different with least significant difference (LSD, $P = 0.05$).
[4]Means over four replications and three cultivars.

for the dry season, whereas significantly ($P = 0.05$) higher seed infection was found in IR36 (9.8%), then followed by IR46 (6.5%) and IR42 (4.0%) for the wet season (Table 3).

Percent seeds infected due to *M. oryzae* was significantly ($P = 0.05$) increased as the number of inoculations increased, with the lowest in non-inoculated lots (1.3%), then inoculated lots with once (3.5%), twice (5.3%), and three times (6.8%) for the dry season (Figure 1). Similarly, percent seed infection due to *M. oryzae* were not significantly ($P = 0.05$) different between lots of non-inoculated (1.7%) and once inoculated (2.5%), or between once and twice (3.6%) inoculated lots, but was found highest in lots inoculated three times (5.6%), intermediate in lots inoculated once or twice and lowest in lots non-inoculated or inoculated once for the wet season (Figure 1).

*Table 3. Pinatubo oryzae* seed infection rates in plots differing epidemic intensities by either non-inoculating (0) or inoculating (1, 2 or 3) panicles with *Microdochium oryzae* conidia as determined examining 100 seeds per plot on a semi-selective medium (MOSM)

| Cultivar | Dry season[1] | Wet season[1] |
|---|---|---|
| IR36 | 1.1 | 9.8 a[2] |
| IR42 | 0.4 | 4.0 c |
| IR46 | 0.8 | 6.5 b |
| | NS[3] | |

[1]Means over four (0, 1, 2, and 3) inoculations and four replications.
[2]Numbers followed by the same letters are not significantly different using least significant difference (LSD, $P = 0.05$).
[3]Nonsignificant.

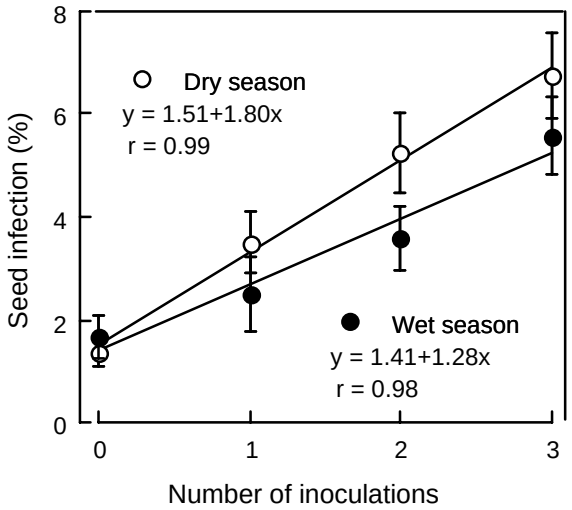


*Figure 1.* Percent of seeds infected with *Microdochium oryzae* in plots of differing epidemic intensities generated by either non-inoculating or inoculating rice panicles either once, twice, or three times (means of three cultivars and four replications). Vertical bars represent standard error of the means.

*Survival of* M. oryzae *in stored seeds.* Seed infection due to *M. oryzae* decreased significantly ($P = 0.05$) with increased storage for both the IR36 seed lot harvested in the dry season and the IR42 seed lot harvested in the wet season with negative coefficient

of correlation, $r = -0.99$ (Figure 2). The result of the blotter test showed a decline in survival of *M. oryzae*-like fungi (*M. oryzae*, *P. oryzae*, and possibly other fungi) over time for both dry and wet seasons (Figure 3).

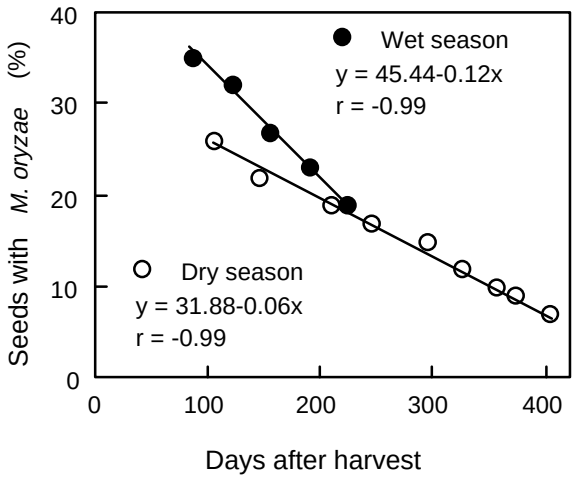


*Figure 2.* Longevity of *Microdochium oryzae* in IR36 seed lot harvested in the dry season and IR42 seed lot harvested in the wet season and stored at 10 °C and 40% relative humidity using 100 seeds per assay on a semi-selective medium (MOSM).

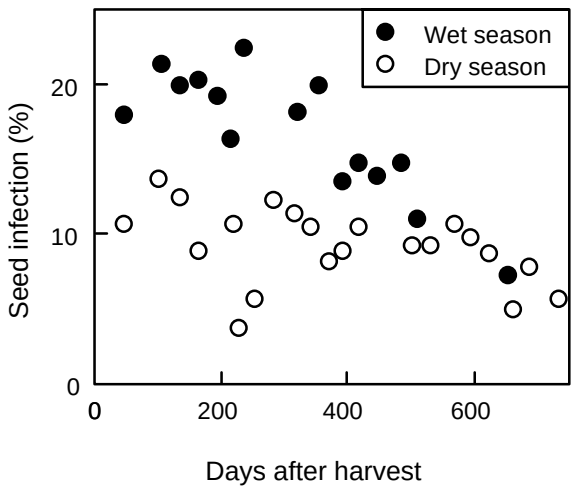


*Figure 3.* Longevity of *Microdochium oryzae*-like fungi (*M. oryzae* and *Pinatubo oryzae* and possibly other fungi having similar colony characteristics) on seeds of IR36 lot harvested in the dry season and IR42 lot harvested in the wet season and stored at 10 °C and 40% relative humidity using 400 seeds per assay on the blotters.

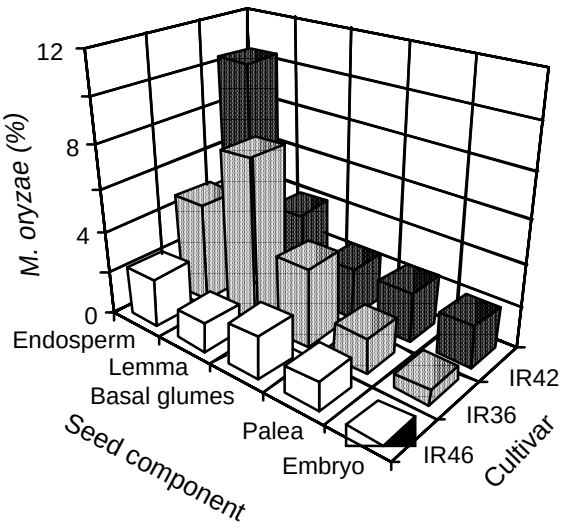


*Figure 4.* Infection of seed components with *Microdochium oryzae* on a semi-selective medium (MOSM) for cultivars IR36, IR42, and IR46 seed lots harvested from the wet season 1994. (Means of 3 replications, 100 components per replication, standard error = 0.5)

*Seed components infected by* M. oryzae. All five seed components of three test cultivars were infected or infested with *M. oryzae* (Figure 4). Cultivar, seed component, and their interaction were found significant by ANOVA test with standard error, $\sigma = 0.5$. Mean *M. oryzae* infection in the endosperm (5.8%) was significantly ($P = 0.05$) highest over three cultivars and three replications, then followed by the lemma (4.3%), basal glumes (2.7%), palea (1.8%) and embryo (0.9%). In IR42, highest *M. oryzae* infection was found in the endosperm (10.3%) followed by the lemma (4.0%), the basal glumes (2.3%), the palea (2.3%), and the embryo (2.0%); but in IR36, infection was the highest in the lemma (7.7%), followed by the endosperm (4.7%), the basal glumes (3.7%), the palea (1.7%), and the embryo (0.7%) (Figure 4). Similarly, highest *M. oryzae* infection was found in the endosperm (2.3%) and the basal glumes (2.0%) followed by the lemma (1.3%), the palea (1.3%), and the embryo (0%) among the seed components of IR46 (Figure 4).

## Discussion

Although *M. oryzae* is taxonomically different from *P. oryzae* (Manandhar and Mew, 1996), their cottony

hyphal colonies and salmon-colored conidial masses (sporodochia) are similar on seeds plated on blotters or other media. Sparse aerial conidiogenesis of *P. oryzae* (Manandhar and Mew, 1996) is often overlooked or absent in assays using the blotter test. Since *M. oryzae* and *P. oryzae* colonies appear similar, colonies of both fungi are frequently recorded as *M. oryzae* overestimating *M. oryzae* and underestimating *P. oryzae*. Both *M. oryzae* and *P. oryzae* can also occur on the same seed making the estimate of *M. oryzae* seed infection difficult. A selective medium for *Microdochium* spp. has not been previously reported. Many fungi can grow slowly on MOSM. However, MOSM was effective in differentiating *M. oryzae* from *P. oryzae* by stimulating the latter to form abundant aerial conidiogenesis. A binocular microscope is necessary to confirm the presence of the aerial conidiogenesis.

Leaf clipping and then spraying a conidial suspension of *M. oryzae* resulted in leaf and sheath scald (Anonymous, 1979, 1982). Grain abortion and spotted seeds due to leaf scald were reported (De Gutierrez, 1996; Naito, 1982; Soave et al., 1997). Leaf and sheath scald, grain abortion, and discolored seeds were not observed in these studies. Although seed infection is related to leaf and sheath scald (Mia et al., 1986; Naito, 1982), conidial inoculation of *M. oryzae* was targeted to rice panicles during anthesis to obtain high rate of seed infection without causing leaf and sheath scald. Leaves were neither clipped nor targeted during inoculation. In these studies epidemics of differing intensities were generated by inoculating rice panicles with *M. oryzae* either once, twice, or three times during anthesis. Seed infection by *P. oryzae* was all due to the natural infection. The effect of epidemic intensity on seed infection rates was then determined by examining seeds on MOSM.

Seed infection due to *M. oryzae* or *P. oryzae* may be attributed to varietal susceptibility to one or both pathogens or could be influenced by dry or wet season. *Microdochium oryzae* seed infection was expected to be higher in cultivar IR36 (susceptible to leaf scald) than cultivars IR42 and IR46 (resistant to leaf scald). However, percent seed infection due to *M. oryzae* was high in IR36, low in IR42, and intermediate in IR46 lots grown in the dry season. For wet season lots, seed infection was higher in both IR42 and IR46 than IR36. This discrepancy in *M. oryzae* seed infection might have influenced by the natural infection of *P. oryzae*, which was higher during the wet season and lower during the dry season. The highest epidemic level generated by inoculating rice panicles three times during anthesis yielded a high percent *M. oryzae* seed infection for both dry and wet seasons except that IR42 did not show difference between lots of non-inoculated and inoculated once, inoculated once and twice, or inoculated twice and three times. Similarly, IR46 was not different between lots inoculated once and twice in the dry season. IR42 may be more resistant to *M. oryzae* seed infection than IR46 or may be influenced by the natural infection of *P. oryzae*. Mean percent *M. oryzae* seed infection was low in lots grown in the wet season, which may be due to increased natural infection of *P. oryzae*. It is not known whether *M. oryzae* and *P. oryzae* compete for the same site in the seed, since there was indication that *M. oryzae* seed infection decreased as *P. oryzae* seed infection increased or vice versa.

Plots inoculated three times were expected to show one-hundred percent *M. oryzae* seed infection but resulted in low. Conidia of *M. oryzae* are discharged at mid-night and germinate to form appressoria-like structures that infect healthy rice tissues (Naito, 1982). Conidial inoculation commenced between 10:00 and 11:00 AM which may not favor development of appressoria-like structure. The pathogen may also be sensitive to desiccation that occurred in drying process of harvested seed lots at 45 °C for 96 h. Although the addition of polypeptone to conidial suspension seems to encourage the formation of appressoria-like structures and stomatal penetration on the leaves (Yamaguchi and Ito, 1975), polypeptone was not used in these studies.

Survival of *M. oryzae* in naturally infected rice seeds declines depending upon storage conditions (Mia et al., 1987; Sanchez et al., 1979; Singh and Gupta, 1986). The rate of decline of *M. oryzae* seed infection may be important from a non-chemical approach to disease management. One report (Mia et al., 1985) indicated survival of *M. oryzae* up to 11 years at 5 °C. In this study with the blotter tests only a small decrease in percentage of seeds with *M. oryzae*-like fungi was observed over two years supporting an earlier report (Sanchez et al., 1979). *Microdochium oryzae* is probably recorded for *M. oryzae*, *P. oryzae*, and possibly other fungi that form similar colony characteristics in the blotter assay. Rice seed lots grown in the Philippines usually harbor *P. oryzae* (Manandhar and Mew, 1996). Blotter test shows rapid decline of *M. oryzae* in countries where *P. oryzae* is not reported in seed lots (Mia et al., 1987; Shetty and Shetty, 1988; Singh and Gupta, 1986). In this study, survival of *M. oryzae* in seeds

declined rapidly only when seeds were assessed on MOSM.

Rice seed consists of the embryo, endosperm and hulls, with hulls reported to have the highest frequency of *M. oryzae* infection (Kim et al., 1984; Mia et al., 1986). This report is the first to show the highest frequency of *M. oryzae* infection in the endosperm. When hulls were separated into basal glumes, lemma and palea, the second highest frequency of *M. oryzae* infection was found in the lemma. *Microdochium oryzae* appears to reside in between endosperm and lemma. Location of the pathogen in rice seeds influences the potential efficacy of biological or chemical seed treatments (Faiad et al., 1993) and the formulation (Suzuki et al., 1994) for effective control. Unlike other rice pathogens, literature on *M. oryzae* or *P. oryzae* is sparse, and the results presented here may contribute to the knowledge of *M. oryzae* and *P. oryzae* seed infections and aid in rice seed rot and seedling disease control strategies.

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