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Effect of culture condition on the sporulation and virulence of *Magnaporthe oryzae* isolated from rice field of Hoogly, West Bengal

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The filamentous Ascomycetous fungi *Magnaporthe oryzae* causes rice blast disease which accounts for major loss in rice production world wide. We isolated this pathogenic fungus from diseased leaf sample, collected from rice field in Hoogly district, West Bengal in the 'Aman' season. We used three different media namely oat meal agar (OMA), Malt extract agar (MEA), and Potato dextrose agar (PDA) to find out if the media composition affects sporulation *in vitro* and the virulence of the pathogen. Maximum hyphal growth was observed on MEA followed by PDA and OMA, but maximum conidial development occurred on OMA ($33.9x10^2$ conidia/mm^2 of mycelia) followed by MEA ($5.19x10^2$ conidia/mm^2 of mycelia) and PDA ($1.17x10^2$ conidia/mm^2 of mycelia). Sizes of the spores developed on the three media did not show significant difference in size. Although hyphal growth was comparatively slower on OMA, maximum conidiophores development occurred on this media and least conidiophores developed on PDA. Virulence of the spores depended on the media type where they developed, e.g. about 72.89% spores from OMA developed appressoria on the hydrophobic parafilm surface within 8hrs of incubation where as only 31.78% and 27.42% spores from MEA and PDA respectively developed mature appressoria after the same period of incubation. Appressoria development from spores on OMA started within 4 hrs of incubation and within 6 hrs mature appressoria development occurred but in case of spores developed on MEA and PDA, appressorial development started after 6 hrs of incubation. Therefore OMA is the best media for conidiation and maintaining virulence of spores for M. *oryzae* strain isolated from hoogly district.

Key words : Virulence, *Magnaporthe oryzae*, sporulation

INTRODUCTION

The filamentous ascomycetous fungi *Magnaporthe oryzae*, previously called *M. grisea*, is the most devastating fungal pathogen of rice, accounting for more than 10 million tons of yield loss every year worldwide (Talbot 2003, Xu et al., 2007, Oh et al., 2007). The disease causes damage to rice crop at two main stages; during the seedling stage as leaf blast and also during seed setting stage as panicle blast (Ou, 1985).Not only rice, it also infects a large number of grasses and other cereals including wheat, barley etc. On the host surface, it first develops specialized dome shaped cells or 'appressoria' which develop enormous turgor pressure (de Jong et al., 1997). This turgor pressure is regulated by a number of genes like Mg*ATG1* (Liu et al., 2007), that helps to pierce the host cutin layer during infection process (Chen et al., 2004). Once inside the host cells, it rapidly develops invasive hyphae that become sealed in an extra-invasive hyphal membrane (EIHM) compartment and exhibit pseudohyphal growth as they fill the invaded cell for further infection by hemibiotrophic mechanism (Mosquera et al., 2009). But not always pathogens gain access to their hosts and establish themselves. A number of genes are involved in this host-pathogen interactions. Talbot et. al. (1996) identified *MPG1* of *M. oryzae* responsible for the pathogenicity of the organism. Viaud et al. (2002) identified *CYP1* which is a virulence determining factor of *M.oryzae*. On the other hand there are many *R-genes* e.g. *Pia*,

Pii, Pik, Pik-m, Pik-p, Piz, Pita, Pita2, Piz-t, Pib, and Pit (Kiyosawa et al., 1986) that are present in the host genome which can recognize the pathogen elicitors encoded by their *avr*-genes including *AVR-Pita1, PWL1, PWL2, ,AVR-CO39* (Mosquera et al.,2009) and restrict their spreading in the host tissues. Recently a comprehensive database called Genomic Resources of *Magnaporthe oryzae* (GROMO) has been developed for the convenience of research on *M. oryzae* (Thakur et al., 2009).

Developing novel means to control rice blast will require detailed understanding of both the infection process and the developmental biology of *M.oryzae* including stages of conidial development, the environmental factors controlling it (Lee et al., 2006) Therefore factors affecting appressorial development in vitro and in vivo is an important field of research. In the present study we have isolated rice blast fungus *M. oryzae* from Hoogly district of West Bengal. This is the first report of isolation of *M. oryzae* from the Baruipara locality of Hoogly. We used three different media to study their growth pattern and conidial development and examined whether there is any relationship between the pathogen virulence and source of nutrient used.

MATERIALS AND METHODS

Isolation of rice blast pathogen M. oryzae

Extensive occurrence of rice blast disease was observe in local rice cultivar 'Masuri' in rice field of Baruipara, Hoogly district of West Bengal during the 'Aman' season in the month of October, 2010. Leaf lesions of blast disease were collected in polythene bag to protect from over-drying. Lesions were cut into small sizes (approximately 5x5 mm^2) and surface sterilized in 30% liquid commercial bleach solution for 15-20 minutes. Lesions were then washed thoroughly with sterile distilled water for three times. Surface sterilized lesions were inoculated on Oat meal (HIMEDIA) (3%) – agar (1.5%) slant and incubated under continuous fluorescent light at 28°C temperature. After 3 days, fine thread like hyphae started to extend from the lesion. The growing tips of these hyphae were sub-cultured onto new slants and incubated under previous condition for 10 days to allow sporulation. After sporulation, spores or conidia were observed under compound microscope (Leica).Pure cultures were sub-cultured and stored in 4°C.

Study of growth pattern on different media

To find out suitable culture media for this fungus, three different media were used, namely oat-meal (3%) agar (OMA), malt extract (2%) agar (MEA), potato dextrose agar (PDA). Fungal inocula of about 3 mm diameter were placed onto three different media in 9cm diameter petriplates with 3 replicas for each medium. Petriplates were incubated under continuous fluorescent light at 28°C temperature. Diameters of the fungal colony were measured at 3 days intervals up to 12 days. To prepare the growth curve, 3 diameters were taken from a single colony on a single plate, and the average of the diameters was calculated. Finally the mean of the diameter of colony on a specific medium at specific time intervals was calculated from the average of three replicates. Development of vegetative hyphae and conidiophores was examined under the microscope.

Sporulation on different media

After 12 days of culture on different media, effect of the media on spore production was examined. Five fungal mats were scooped out by a 3mm diameter cork borer at 35 mm distance from the center, agar mass from each mat was discarded and mycelia was suspended in 500μl sterile distilled water in microfuge tubes. Number of spores was examined using a haemocytometer under compound microscope (Leica).Mean was calculated from three observations.

Measurement of spore sizes

Ocular divisions were standardized with stage micrometer. Spore sizes were taken by ocular divisions and it was converted into micrometer unit. Finally average size of the spores from different microscopic fields was calculated.

Preparation of spore suspension

Spores were scraped out from the upper surface of the media by a sterilized scalpel and suspended in sterile distilled water in a 50 ml beaker. The suspension was filtered through a triple folded cheese cloth to separate out the mycelia. Finally the spore concentration was checked under microscope using a haemocytometer to obtain spore concentration of approximately $2x10^5$ spores/ ml. 25ml water was used to suspend spores of three 12

days old colonies. This spore suspension was used for in vitro virulence assay.

In vitro virulence assay

Efficiency of appressorium development of the spores was studied after 12 days post inoculation to analyze the virulence of the spores developed on different media. 50μl of spore suspension (containing $2x10^5$ spores/ ml) was placed on hydrophobic parafilm surface and kept on moist tissue paper in petriplates at 25°C in dark for up to 8 hrs. Appressoria development was studied at 0, 2, 4, 6, 8 hrs post incubation (hpi).

Staining of the fungal spores, hyphae and appressoria

Fungal hyphae, spores and appressoria were stained with 0.01% trypan blue (dissolved in lactophenol) for 5 minutes. Excess stain was blotted dry and samples were observed under compound microscope.

Microscopic study

All the microscopic studies were performed using compound microscopes- (Olympus, LEICA DM*LS*). Magnifications used for this work were 10X, 20X and 40X with or without staining. Photographs were taken by SAMSUNG ES573 digital camera.

RESULTS AND DISCUSSION

Colony characteristics of M. oryzae on different media

In three different media, different growth patterns of this fungus were observed. Among these three media, hyphal growth was maximum on MEA followed by OMA and PDA. Figure 1 shows the comparative growth pattern of *M. oryzae* in three different media. Growth of the fungus started just after one day of inoculation. Hyphal growth was highest on MEA, followed by PDA and OMA (Figure 2). Awoderu et al., 1991 obtained maximum growth on PDA which was probably due to different strain of pathogen and environmental/experimental conditions. Initially growth rate was slightly slower in all of the three media upto 3 days; during this period the fungus acclimatized itself to the new environment. After this maximum growth was achieved. During the maximum growth phase, fungal colony increased about 6 mm in diameter per day on MEA. Ash colored aerial hyphae started to develop from the center of the colony after 4 days of inoculation on MEA, 5 days on OMA but completely absent in case of PDA. Dark color of the colony, which was due the development of dark green, thick, septate conidiophores, was also less in number in the colony on PDA medium. This was clearly observed when the mycelium was studied under compound microscope. But the conidiophores developed extensively forming a dark green mat in both OMA and MEA.

Table 1: Stages of appressoria development after incubation of spores from different media on hydrophobic surface at two hours interval.

Media used	2 hr post inoculation	4 hr post inoculation		6 hr. post inoculation	8 hr. post inoculation
	% of germi-nated spores	% of germi -nated spores appressoria	% of germinated spores with appressoria	% of germinated spores with appressoria	% of germinated spores with
OMA	89.54±3.4	98.87±3.1	19.02±4.2	64.39±8.36	72.89±2.11
MEA	68.05±5	97.33±3.8	x	11.01±3.4	31.78±4
PDA	62.22±3.1	96.42±6.2	*	22.14±2.1	27.42±6.6

*Appressoria development started but data statistically insignificant.

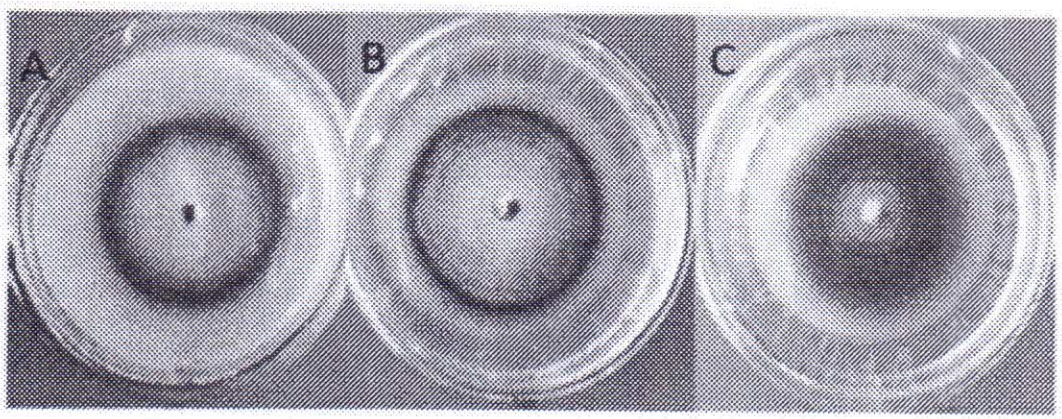

Fig.1: Colony morphology of *M. oryzae* on different media A. Oat Meal Agar (OMA), B. Malt Extract Agar (MEA) C. Potato Dextrose Agar (PDA).

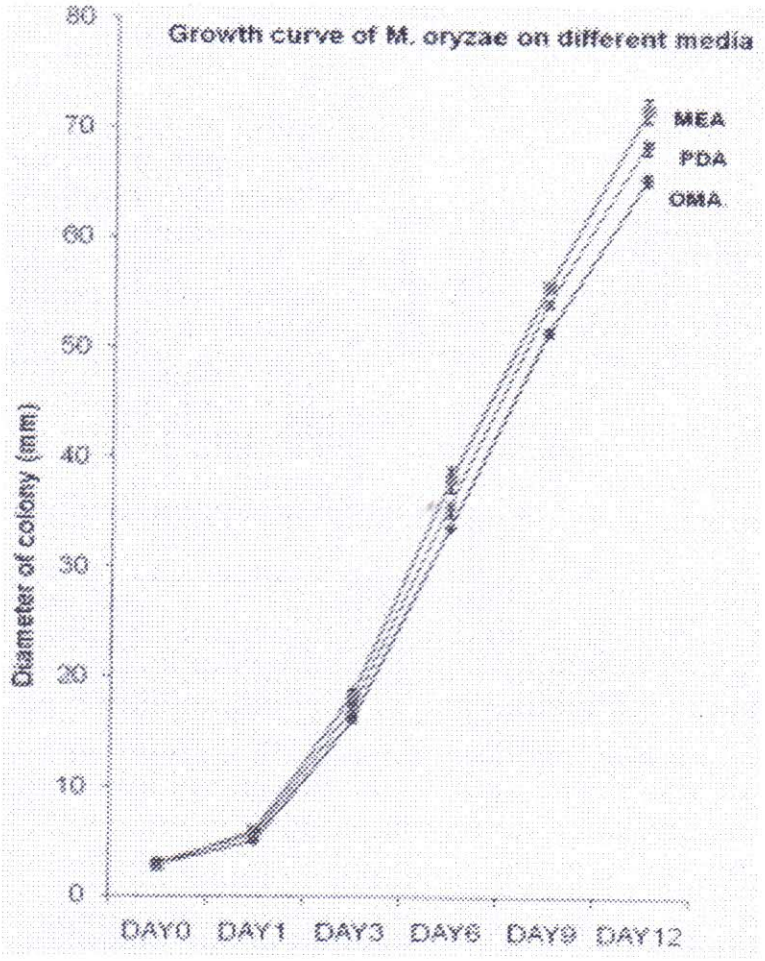

Fig. 2 : Growth curve of *M. oryzae* colony over 12 days post incubation on different media, showing greater colony size on MEA medium compared to OMA and PDA.

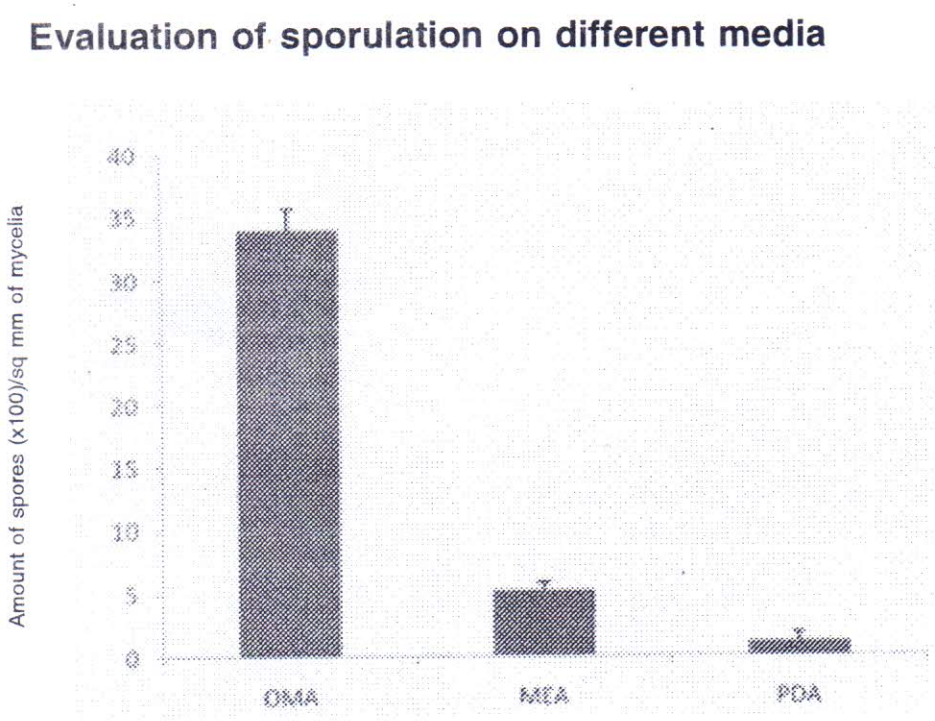

Fig. 3: Comparison of amount spore production on different media

Sporulation of M. oryzae depends on nutrient source

Remarkable difference in spore production of *M. oryzae* was observed in three different media. Highest number of spore production was found on OMA (approximately 33.9x 10^2 spores/mm^2 of the mycelia), followed by MEA (approximately 5.19x10^2 spores/mm^2 of mycelia) and PDA (approximately 1.17x10^2 spores/mm^2 of mycelia) which was consistent with the poor development of conidiophores on PDA (Figure 3).

Spores sizes remained unaffected on different media

Spore size was almost similar in three different media (approximately 2.5±0.2μm). This result indicates that the source of nutrient effect on the quantity of the spore production not the size of the spores.

Effect of media on the virulence of the spores

Virulence of the spores depends on the source of the nutrient. MEA is usually used for the culture of *Alternaria solani* to retain virulence of the fungus. Virulence was examined by their efficiency of appressoria formation on hydrophobic parafilm surface. In case of OMA, percentage of spores that developed appressoria was highest (72.89±2.1%). Most of the spores (89.54±3.4%) developed on this medium started to grow germ tubes within 2 hours of incubation on hydrophobic surface and appressoria development started within 4 hours. After 6hrs, most of the germinated spores (?64.4%) developed mature appressoria. But in case of spores developed on MEA and PDA, percentage of spore germination was lower in comparison to that of OMA. Appressoria development was also slower in spores developed on both of these media. Although germ tube development was started within 2 hpi, but appressoria development barely started at 4 to 6 hpi interval. Therefore at 4 hours most of the germinated spores on MEA and PDA have no

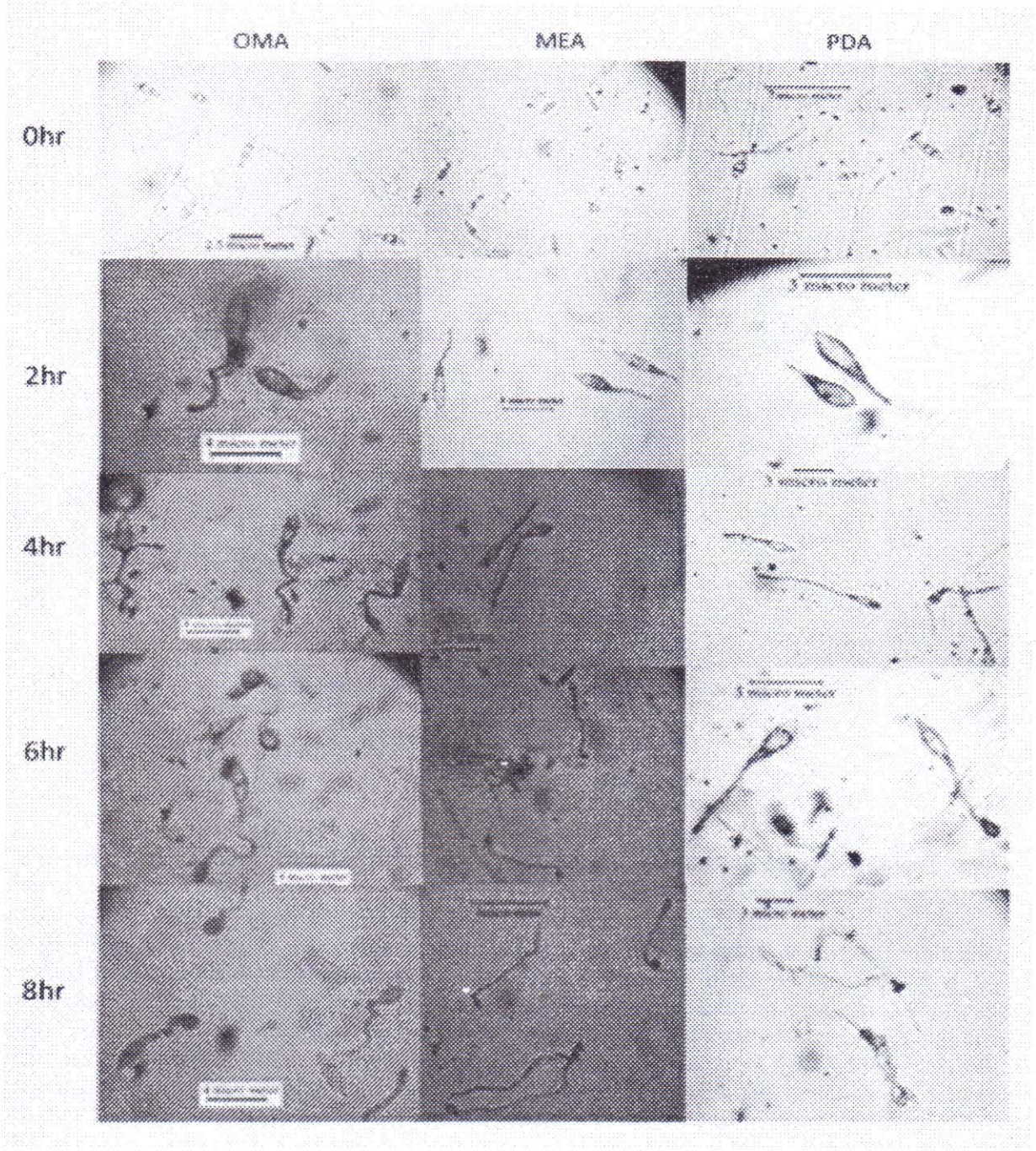

Fig. 4 : Development of germ tubes and appressoria from spores obtained from different media at two hours time intervals on hydrophobic surface

appressoria at the tip of germ tubes. Under developed appressoria and mature appressoria were found only at 6 and 8hpi. Some spores developed on PDA formed branched germ tubes without appressoria. These branched germ tube was not found in spores from OMA or MEA. These observations are shown in Table 1 and Figure 4.

On the whole the study indicates that media composition not only affects the hyphal growth of the pathogen, but the amount of spore production is inversely related to colony growth. OMA allows less colony growth and more spore production. OMA is also suitable for maintaining virulence of the spores. On the other side, the media composition was seen to affect the germ tube morphology, PDA showing brached germ tube formation. These observations were reproducibly seen in case of the strain of *M. oryzae* obtained from Baruipara locality of Hoogly district of West Bengal.

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