IN VITRO EVALUATION *OF* FUNGICIDAL RESISTANCE OF RICE BLAST PATHOGEN (*Magnaporthe oryzae* L) STRAINS

[1]Aremu, M. B. and [2]Adebola, M. O.

[1] Rice Research Program, National Cereal Research Institute, Badeggi, Niger State.
[2.] Biological Science Department, Federal University of Technology, Minna, Niger State.

ABSTRACT

Rice blast disease caused by the fungus, *Magnaporthe oryzae,* is one of the major fungal diseases affecting rice (*Oryza sativa*) cultivation. The resistance of this fungus to fungicides is imperative as the presence of fungicide resistant strains (biotypes) has been associated with control failure, therefore managing resistance is critical to disease control. This study was carried out to evaluate the *in vitro* fungicidal resistance among the strains of *M. oryzae* found in rice fields in Niger State. The blast infested leaves, stems and panicles of rice plants were collected in November, 2015 from five farmers' field located in Gbako, Katcha and Lavun Local Government Areas in Niger state, Nigeria. Isolation of the pathogen was carried out on Potato Dextrose Agar (PDA) and the fungicidal sensitivity test of the isolates was conducted in PDA amended with Mancozeb and Benomyl fungicides. Ten *M. oryzae* strains designated as MOR001- 0010 were isolated from all the samples collected and exposed to both fungicides (Mancozeb and Benomyl). Out of these, five strains (MOR001, MOR002, MOR004, MOR005 and MOR008) were resistant to mancozeb with varying percentage inhibitions (91.67, 45.45, 84.13, 86.67 and 83.88% respectively) while three isolates (MOR004, MOR005 and MOR010) were resistant to benomyl with varying percentage inhibition (85.25, 91.67 and 75.00% respectively). However, isolates MOR004 and MOR005 were resistant to both fungicides (84.13% and 86.67% in mancozeb while 85.25% and 91.67% in benomyl respectively). Molecular evaluation is recommended to actualise the relationship between the ten strains because species within the same fungal genus may respond differently to certain fungicides.

Keys: *M. oryzae*, Rice, Fungicides, *In vitro,* Blast, Pathogens

INTRODUCTION

In the world's major rice growing areas, reduction in production, is caused by rice blast disease caused by *Magnaporthe oryzae.* However, *M. oryzae* remains a particular threat because of its unpredictable outbreaks as well as resistance to fungicidal treatment. Rice blast pathogen (*Magnaporthe oryzae)* remain*s* the most devastating agent causing serious damage to upland rice in Nigeria. It is the most important disease of rice worldwide and amajor threat to food security. *Magnaporthe oryzae* (syn *Pyricularia oryzae*) the rice blast pathogen, is a filamentous, haploid heterothallic fungus belonging to the phylum Ascomycota (Gilbert *et al.,* 2004). Rice blast is an infectious fungal disease that prevails in more than 85 countries of the world causing more than 14-18% yield losses (Scardaci *et al.,* 1997; Mew and Gonzales, 2002; Jamal-U-deen *et al.,* 2012). Heavy yield losses have been reported in many rice growing countries like India (75%), Phillipines(50%), Nigeria(40%) and also in other West African contries (Ghazanfar *et al.,* 2009).. The fungus attacks all stages of the crop and symptoms appears on leaves, nodes, neck and panicle (Seebold *et al.,* 2004; Ghazanfar *et al*., 2009). Blast occurs in upland and rainfed rice ecologies because water deficiency predisposes the rice field to severe infection in all environments. In the past, several fungicides have been employed in the control of fungal disease of rice and other crops. Agrochemical like Benlate, bavistin, apron plus 50 DS, femasan-D and Dithane M-45 were used to control a number of diseases caused by fungal pathogens of rice. However, new and potentially virulent species of the fungal pathogens are being discovered every day (Ibiam *et al.,* 2008).

Newer melanin biosynthesis inhibitors such as carpropamid (Motoyama *et al.,* 1999; Thieron *et al.,* 1999) or broad spectrum fungicides like azoxystrobulin (strobilunin) (Lee and Beaty, 1999) have gained much patronage as a result of their mode of action and effectiveness particularly for resistance management.

Fungicide resistance is a stable, inheritable adjustment by fungus to a fungicide, resulting in reduced effectiveness of the fungicides. The presences of different strains of this pathogen leading to frequent resistant to fungicides, has called for a great concern on the biology of *M. oryzae*. Understanding biology of fungicide resistance is critical for insuring sustainable disease control with fungicide. However, the information available about the different strains of the pathogen in the study area is not adequate.

Thus the aim and objectives of this study are to isolate different strains of *M. oryzae* from the infested leaves, stem and panicle of rice plants and evaluate the *in vitro* resistance patterns of strains in different concentrations of mancozeb and benomyl.

MATERIALS AND METHODS

Collection of Materials

Blast infested leaves of rice were collected into labelled polythene bags from five farmer's field in Edozhigi, Chanchaga, Kataeregi, Busu and Agaie across Gbako, Katcha and Lavun Local Governments in Niger State, Nigeria (N9^{o}.06'26.79 E5^{o}.51'43.49; N9^{o}21'40.82 E6^{o}.17'18.89 and N9^{o}02'28.11 E6^{o}01'30.31 respectively). The samples were for further studies. Mancozeb (Dithane M45) and Benomyl (Benlate WP 50) were purchased from Agrochemical shops in Minna, Niger State.

Isolation of fungal pathogen

The blast infected leaves were sterilized in mercuric chloride (0.01%) and 5 discs taken from the periphery of necrotic regions were placed on Potato Dextrose Agar (PDA), to which streptomycin (50 µg /m L) has been added to suppress bacterial growth and incubated at 28$\pm$2^{O}C for 3 days. Pure culture of the isolate was obtained by picking a single conidium with a sterile needle under microscopic observation, transferred individually to PDA plates and incubated at ambient temperature (Gomathinayagam *et al.,* 2011). The fungal isolates were identified using the fungal family of the world mycological monograph and stored on PDA slants at 4^{o}C. Subculturing was made at regular intervals unto fresh culture media.

Pathogenicity test

The rice seed variety FARO 52 that was used for pathogenicity test was collected from National Cereal Research Institute (NCRI) Badeggi, Niger State, Nigeria. Pathogenicity of *M. oryzae* was tested on healthy rice plants grown in the greenhouse. Forty five days old FARO 52 rice plants were sprayed with mycelial suspension (10^{5}spore/ml) of the strains of pathogen (*M. oryzae*) (10ml/pot) by means of automizer. The plants were individually covered with polythene bags to provide adequate humidity. The inoculated plants were observed after 7 days for characteristic symptoms of blast (Subramanian *et al.,* 2013).

Sensitivity of *M. oryzae* in PDA amended with fungicides

Sensitivity of the pathogen to fungicides was tested using poison food technique (Subramania *et al.,*

2013). Two (2) mm mycelia discs were inoculated at the centre of the Petri dish containing PDA amended with different concentrations of the fungicides (i.e. 1.0, 1.5, 2.0, 2.5, 3.0mg/ml) and unamended as control. Three replicates were maintained for each treatment, and incubated for 3 days. The diameter of mycelial growth was measured and percentage inhibition was calculated using:

$$\frac{R1-R2}{R1} \text{ X } 100\ \%$$

(R1=radial growth in control, R2=radial growth in amended media) (Adebola and Amadi 2012) The ED_{75} was determined by

plotting percentage inhibition of the growth against log concentration of the fungicides (Wei-Jen *et al.,* 2007; Gomathinayagam *et al.,* 2011)

RESULTS

Isolated Pathogens

A total of ten *M. oryzae* strains (Table 1) were isolated from all the samples collected from the five sites (Edozhigi, Badeggi, Kataeregi, Bussu and Agaie farmer's field). The conidium of the fungi was typically pyriform with rounded base, narrowed apex and 2-3 septate. The mycelia were highly branched, septate, superficial, bearing conidia at the tip and bunch at the side of the conidiophores, The conidiophores of the isolates were slender, straight grayish white to grayish black, smooth bearing clusters of conidia which are typically of pyriform and 2-3 septate. The result of the pathogenicity test revealed that all the strains isolated are causative organisms of rice blast.

Sensitivity of fungal pathogen to fungicides on PDA

The results on the sensitivity test (Table 2 and 3) on solid medium revealed that out of the ten *M. oryzae* subjected to different concentration of Mancozeb (Table 2), Five ("MOR003" "MOR006", "MOR007", "MOR009" and "MOR010") were completely susceptible to the fungicide with 100% inhibition of radial growth and were significantly different from the remaining five ($P<0.05$). The most resistant isolates was MOR002 whose percentage inhibition of the radial growth varied between 36.36% and 45.45% in all the concentrations it was exposed. The percentage inhibition of all the resistant strains (MOR001, MOR002, MOR004, MOR005 and MOR008) were significantly different at ($P<0.05$). The ED_{75} (Effective dose required to achieve 75% mortality of the pathogen) for these five isolates was greater 3.0mg/ml. However, at 2.0mg/ml; the manufacturer's specification, only MOR002 was less susceptible and significantly different ($P<0.05$) with 45.45% inhibition. Other

isolates were between 79.36-100% inhibitions.

The results of susceptibility of the ten isolates in Benomyl on solid medium (Table 3) revealed that three of the isolates (MOR004, MOR005 and MOR010) were resistant to fungicides at various concentrations. The most resistant isolates was MOR004 with percentage radial growth inhibition between 52.28 and 85.25% which was significantly different ($P<0.05$) from other isolates. The ED_{75} (Effective dose required to achieve 75% mortality of the pathogen) for these three isolates were greater than 3.0mg/ml. At manufacturer's specified concentration (2.0mg/ml), the percentage inhibition was generally high.

DISCUSSION

Fungicide treatments are essential for maintaining healthy crops and reliable, high-quality yields. They form a key component of integrated crop management, and their effectiveness must be sustained as long as possible. Pathogen resistance to fungicides is wide spread and the performance has been affected to some degree. Therefore, monitoring of resistance is very important

The results on the sensitivity of *M. oryzae* to both fungicides showed that the percentage inhibition of mycelia growth of the resistant isolates ranges from 52.38% to 93.75% across the different concentration of benomyl. Seventy percent (70%) of the isolates were completely sensitive while 30%

Table 1 Number of *M. oryzae* strains isolated

S/N	Strains code*	Locality/collection site
1	MOR 001	Edozhigi
2	MOR 002	Edozhigi
3	MOR 003	Chanchaga
4	MOR 004	Chanchaga
5	MOR 005	Chanchaga
6	MOR 006	Kataeregi
7	MOR 007	Busu
8	MOR 008	Busu
9	MOR 009	Busu
10	MOR 010	Agaie

Key; MOR- *Magnoporthe oryzae* strains from rice

Table 2: Growth inhibition *M. oryzae* at different concentrations of Mancozeb (Dithane M45)

Isolates	Percentage inhibition					
	1.0mg/ ml	1.5mg/ ml	2.0mg/ ml	2.5mg/ ml	3.0mg/ ml	ED 75
MOR001	58.33^{a}	66.66^{b}	83.33^{b}	86.67^{b}	91.67^{c}	<2.0mg/ml*
MOR002	36.36^{a}	36.36^{a}	45.45^{a}	45.45^{a}	45.45^{a}	<1.0mg/ml*
MOR003	100.00^{d}	100.00^{d}	100.00^{d}	100.00^{c}	100.00^{d}	>3. 0mg/ml
MOR004	76.19^{c}	76.19^{c}	79.36^{b}	84.13^{bb}	84.13^{b}	<2.0mg/ml*
MOR005	75.00^{c}	80.00^{c}	85.00^{b}	86.67^{bb}	86.67^{b}	=1.0mg/ml*
MOR006	100.00^{d}	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{d}	>3.0mg/ml
MOR007	100.00^{d}	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{d}	>3.0mg/ml
MOR008	66.67^{bc}	75.00^{c}	83.33^{b}	83.33^{b}	83.33^{b}	=1.5mg/ml*
MOR009	100.00^{d}	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{d}	>3.0mg/ml
MOR010	100.00^{d}	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{d}	>3.0mg/ml

Values followed by the same alphabet are not significantly different at P<0.05. Duncan multiple range test was use for mean separation

Table 3; Growth inhibition *M. oryzae* at different concentrations of Benomyl (Benlate 50WP)

Isolates	%inhibition					
	1.0mg/ ml	**1.5mg/ ml**	**2.0mg/ ml**	**2.5mg/ ml**	**3.0mg/ ml**	**ED75**
MOR001	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{b}	100.00^{c}	>3.0mg/ml
MOR002	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{b}	100.00^{c}	>3.0mg/ml
MOR003	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{b}	100.00^{b}	>3.0mg/ml
MOR004	52.38^{a}	63.33^{a}	66.67^{a}	76.19^{a}	85.25^{ab}	<2.5mg/ml
MOR005	66.67^{b}	66.67^{a}	75.00^{a}	83.33^{a}	91.67^{b}	=2.0mg/ml
MOR006	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{b}	100.00^{c}	>3.0mg/ml
MOR007	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{b}	100.00^{c}	>3.0mg/ml
MOR008	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{b}	100.00^{c}	>3.0mg/ml
MOR009	100.00^{d}	100.00^{c}	100.00^{c}	100.00^{b}	100.00^{c}	>3.0mg/ml
MOR010	93.75^{c}	87.5^{b}	81.25^{b}	77.5^{a}	75.00^{a}	=3.0mg/ml

Values followed by the same alphabet are not significantly different at P<0.05. Duncan multiple range test was use for mean separation

were resistance at ED75 of greater than 3.0mg a.i/ml. In different concentrations of mancozeb, the percentage inhibition of mycelia growth ranges from 36.36% to 91.67%. Fifty percent (50%) of the isolates were totally sensitive while 50% were resistance. The results was in agreement with Nithyameenakshi *et al.* (2006); Gholve *et al.* (2012) and Bhojyanai and Jamadar (2014); who separately reported that systemic fungicides (Tricyclazole, Benomyl, Carbendazim) were very efficient in inhibiting the mycelial growth of *P. grisea in vitro* as compared to non-systemic fungicide (Mancozeb, Captan), and at higher concentration of mancozeb (Dithane M45), about 52% of the fungi germinate with 48% inhibition. However, among the five fungicide used against *M. Oryzae* only Mancozeb appears as highly effective which completely inhibit mycelia growth of the organism (Jamal –u-deen *et al.*, 2012).

The results also showed that only two isolates "MOR004 and MOR005" completely resist both fungicides, probably as a result of change in the genetic makeup over time. When fungicide resistance results from modification of several interacting genes, pathogen isolates exhibit a range in sensitivity to the fungicide depending on the number of gene changes. It may also be as a result of continuous application of these fungicides or cross resistance as reported earlier by Igeleke and Ayanru (2007). From this research, the resistance frequency of *M. oryzae* to Mancozeb, which has been in used for many years, was as high as 50%. This might be attributed to development of cross-resistance to Mancozeb and corroborated the report of Kumar *et al.* (2013). Therefore in other to ascertain the reason for their resistance, molecular evaluation is recommended to actualise the relationship between the ten strains because a change in base pair of DNA can render the fungicide less effective or ineffective and species within the same genus of fungi may respond differently to certain fungicides.

CONCLUSION

From this study, ten strains of rice blast pathogen (*M. oryzae)* were isolated from blast infested leaves, stem and panicle of rice. The results of pathogenicity confirmed that all isolated *M. oryzae* are causative organisms of rice blast disease

Evaluation of the sensitivity or resistance of the *M. oryzae* in media amended with mancozeb and benomyl showed that isolates MOR004 and MOR005 were completely resistant to both fungicides.

REFERENCES

Adebola, M. O. and Amadi, J. E. (2012). The Efficacy of *paecilomyces* specie and *Penicillium digitatum* on Black pod Disease pathogen on the field. *European Journal of Applied Science,* 4(3):101-104

Anon, (1992). Nohyaku-Yoran (Statistics of agro-chemicals in Japan). *Japan Plant Protection Association*, Tokyo.

Anwar, A., Bhat., G.N and. Singhara. G.N. (2002). Management of sheath blight and blast in rice through seed treatment. *Association Plant Protection Scientist*, 10, 285-287.

Akator, k., Awande, S., Adjata, D., Séré, Y. and Silue, D. (2013). Rice common disease in West Africa and integrated management of rice diseases: *Rice pathology consulting firm* (ccpr) 260

Bhojyanai V. K and Jamadar, M. M. (2014). *In- vitro* bioassay of different fungicides against blast of pearl millet caused by *Pyricularia grisea* (Cooke.) Sacc. *Karnataka journal of Agriculture science* 27 *(1):88-90*

Canno P. F. and Kirk P. M. (2007). *Fungal Family of the world*. Wallingford UK, CABI:Singapore. 456

Filippi, M.C. and Prabhu, A.S. (1997). Integrated effect of host plant resistance and fungicidal seed treatment on rice blast control. *Brazil Plant Diseases,* 81: 351-355

Ghazanfar, M.U., Waqas Wakil, S.T. and Saleem-il-yasin (2009). Influence of various fungicides on the management of rice blast disease. *Mycopathology,* 7(1), 29-34

Gholve, V. M., Jogdand, S., M., Jagtap, G., P. and Dey V. (2012). Invitro Evaluation of fungicides, bioagents and aqeous leaf extracts against Alternaria leaf blight of cotton. *Scientific Journal of Veterinary Advance.* 1(1): 12-21

Gilbert, M.J., Soanes, D.M. and Talbot, N.J., (2004). Functional Genomic Analysis of the Rice Blast Fungus *Magnaporthe grisea*. *Applied Mycology and Biotechnology*, **4**: 331-352

Gohel N.M., Chauhan H.L., and Mehta, A.N. (2008). Bio-efficacy of fungicides against *Pyricularia oryzae* the incitant of rice blast. *Journal of Plant Diseases Science*, **3**(2): 189-192.

Gomathinayagam, S., Balasubramanian, N., Shanmugaiah, V., Rekha, M., Manoharan, P.T. and Lalithakumari, D. (2011). Molecular characterization of

carbendazim resistance of plant pathogen (*Bipolaris oryzae*), Fungicides -Beneficial and Harmful Aspects, *InTech* Available from: http://www.intechopen.com/books/fungicides-beneficial-and harmfulaspects/molecular-characterization-of-carbendazim-resistance-of-plant-pathogen-bipolaris-oryzae.1,307-451, ISBN: 978-953

Ibiam, O.F.A., Umechuruba, C. I. and Arinze, A. E. (2008). In-vitro seed dressing Techniques for the control of seedborne fungi of rice variety Faro 29. *Journal of Applied Science Environment Management*, **12**(3): 39-43

Igeleke, C. L and Ayanru D. K.G (2007). Evaluation of fungicides on growth and conidial germination of *Verticillium theobromae* isolated from plantain. *Journal of Applied science,* 7: 531-535

Jamal-U-deen Hajano, A.M., Muntaz, A.P., Alikhanzada, A. and Serwarshah, G. (2012). Invitro evaluation of fungicides, plant extracts and bio-control agents against rice blast pathogen *Magnaporthe oryzae* couch. *Pathology Journal of Botanist* 44 (5):1775-1778

Kumar prasanna, M.K., Sidde Gowda, D.K., Rishikant Moudgal, Kumar Kiran N, Pandurange Gowda, K.T and Vishwanath, K. (2013). Impact of fungicides of rice production in India.. Fungicides- showcase of integrated plant disease management from around the world. *Intech open science*, 4: 77-98

Lee, F.N. and Beaty, T., (1999). Evaluation of foliar fungicides for control of neck blast of rice, *FandN Tests*, 55: 329

Leroux,P. Gredt, M., Remuson, F., Micoud, A., Walker, A.S. (2013). Fungicide resistance status in French populations of the wheat eyespot fungi *Oculimacula acuformis* and *Oculimacula yallundae*. Pest. Manag. Sci .69:15-26.

Mew, T.W. and Gonzales, P. (2002). *A Handbook of Rice Seed borne Fungi*. International Rice Research Institute, Los Banós, Philippines. 83

Motoyama, T., Nakasako, M., Yamaguchi, I., Lyr, H., Russell, P.E., Dehne, H.W. and Sisler, H.D., (1999). Molecular action mechanism of a new melanin biosynthesis inhibitor. In: *Modern fungicides and antifungal compounds II.* 12th International Reinhardsbrunn Symposium, Friedrichroda, Thuringia, Germany, 111-119.

Nithyameenakshi S., Jeyaramraja, P.R. and Manian S.(2006) Investigation

on phytotoxicity of two new fungicides. Azoxystrobin and Difenoconazole. *American Journal of Plant Physiology,* **1**: 89-98

Scardaci, S.C., Webster, R.K., Greer, C.A., Hill, J.E., William, J. F., Mutters, R.G., Brandon, D.M., McKenzie, K.S and Oster, J.J. (1997). *Rice blast*: *A new disease in California*. Agronomy Fact Sheet Series. Department of Agronomy and Range Science, University of California, Davis. http://agronomy.ucdavis.edu/uccerice/AFS/agfs0297.htm. Retrieved; November 2015

Seebold, K.W., Datnof, J.L.E., Correa-Victoria, F.J., Kucharek, T.A. and Snyder, G.H. (2004). Effects of Silicon and fungicides on the control of leaf and neck blast in upland rice. *Plant Diseases,* **88**: 253-258.

Srivastava, D., Shamim, M. D., Kumar, D., Pandey, P., Khan, N. A. and Singh, S. N. (2014). Morphological and molecular characterization of Pyricualria oryzae causing blast disaease in rice(O. sativa) from North India. *Internaational journal of scientific and research publication* **4**(7): 2250-3153

Subramania, G., Murugesan, R. and Lydia, O. (2013). Spot diagnosis of fungicide (Carbendazim) resistance in rice using PCR with reference to *Pyricularia oryzae*; International journal of Science, Environment and Technology, **2**(5): 1039-1059

Taware, M. R., Golve, V. M. and Dey U. (2014). Bio-efficacy of fungicides, bioagents and plant extracts/botanical agents *Alternaria carthiami*, the causal agent of Alternaria blight of safflower (*Carthamus trinctorius* L). *African Journal of Microbiology Research* **8**(13):1401-1412

Thieron, M., Pontzen, R., Kurahash,i Y., Lyr, H., Russell, P.E., Dehne, H.W., and Sisler, H.D. (1999). Molecular action mechanism of a new melanin biosynthesis inhibitor. In: Modern fungicides and antifungal compounds II. *12th International Reinhardsbrunn Symposium*, Friedrichroda, Thuringia, Germany, 24th-29th May 1998, 101-109

Wei-Jen, C., Francois, D., Sylvie, R-C., Lisette, D., Charles, G and Marie-france C-C.(2007).At least two origins of fungicides resistance in Grapevine Downey mildew population. *Journal of Applied and Environmental Microbiology* **73(**16): 5162-5172

Zhou M. G. (1998). Chemical Control of Plant Disease in China. Beijing: Chinese *Agricultural Science and Technology Press*,1: 50-62.