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SPOT DIAGNOSIS OF FUNGICIDE (CARBENDAZIM) RESISTANCE IN RICE BY USING PCR WITH REFERENCE TO *Pyricularia Oryzae*

[1]Subramanian Gomathinayagam*, [2]Murugesan Rekha and [3]Lydia Ori
[1]Faculty of Agriculture and Forestry, University of Guyana, Berbice Campus, Tain, Guyana, South America
[2]School of Biotechnology, Kalasalingam University, Krishnana Kovil, Tamil Nadu, India
[3]Faculty of Technology, Anton de Kom, University van Suriname, Suriname
E-mails: [1]gomsrekha@uog.edu.gy , [2]rekhakarishma@gmail.com , [3]lydia50ori@hotmail.com
(**Corresponding Author*)

Abstract: A procedure was developed for detecting point mutations in the beta-tubulin gene of benomyl resistant laboratory mutant of *Pyricularia oryzae* using the Polymerase Chain Reaction (PCR) in combination with Allele Specific Oligonucletide (ASO) analysis. PCR was used to amplify a specific 1,919bp DNA sequence of the beta-tubulin gene in DNA extracts. The amplified DNA sequence was then probed with 18- mer end labelled oligonucleotides specific for the sensitive phenotype or for the benomyl-resistant phenotypes. The point mutations, converting codon 198 from glutamic acid in the sensitive strain to lysine or alanine, respectively, in high resistant and very high resistant strains were detected by ASO analysis. A point mutation, converting codon 200 for phenylalanine in sensitive strain to tyrosine, in moderately resistant strains was detected by ASO analysis. ASO analysis was a useful tool for detecting and characterizing benomyl-resistant strains *P. oryzae* and other pathogenic fungi in field and under laboratory conditions.
Keywords: *Pyricularia oryzae*, ASO, PCR, Carbendazim, Plant pathogen.

1. Introduction

Agricultural practices are often portrayed as significantly contributing to environmental problems. When organic pesticides were discovered they were hailed for their positive contributions to increased food production. The challenges we are facing is that the changing environmental ethics coincide with rapid population growth. It is expected that within the next 50 years there will be approximately 50% more people on earth to feed, house and cloth. The world is not only increasing in population, but also in affluence. Invasive pests are a significant threat to the statuesque of our natural ecosystems, our health and agriculture.

Many of the most serious plant diseases have already been spread throughout the world, but there is still good reason to be very diligent in monitoring and excluding those diseases that are still endemic to limited geographic areas. Emergence of new diseases and disease

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epidemics must be expected. One of the most important contributions of plant pathology research to science, is the recognition that pest evolve at a rapid rate and that single gene changes can turn an obscure microbe into the cause of an epidemic. The challenge for plant pathology is to be able to predict when such genetic changes will happen. The ability to genetically modified plants, animals and microbes in precise ways using molecular biology and biotechnology provides the present century best hope for meeting the food, fibre and nutritional needs of the growing population of the world without further compromising the quality of our environment. This technology is so important for the future that care has to be taken that no compromise is taken on long-term value by meeting short-term goals. With the modern biotechnology on hand, research area calls for more attention in basic and applied research programmes in product, mode of action, mechanism of action and application of fungicides for safety and economic use. Development of fungicide resistance is a threatening topic to farmers who practice with potential systemic fungicides. When a fungicide fails because of the development of resistance by the target organism, in practice it is very important to know whether the effectiveness of other fungicides has been affected as this will lead to many more problems.

The present research is case study on Carbendazim fungicide resistance in pathogen. *In vivo* and *in vitro* fungicide resistant mutants have been isolated and characterized for growth, sporulation and pathogenicity. The complete fitness and competition tests of the resistant strains we carefully carried out in the presence and absence of fungicide. Level of resistance, using Petri plate method was compared to spot diagnosis techniques using allele specific oligonucleotides. The test fungicide chosen was Carbendazim, a Benzimidazole compound whose mode of action is inhibition of microtubules assembly. Benzimidazole compounds are increasingly important and most widely known, owing to the excellent systemic control of much important plant diseases. Development of fungicide resistance is now one of the major problems in plant disease control, but could be easily delayed or prevented through careful practices. This requires stable information's on genetics of resistance, level of resistance and mechanisms of resistance. The Present research gives an insight on to the above factors to know more information's on fungicide resistance. New molecular techniques have been assessed for effective evaluation of the level of fungicide resistance at field level.

2. Materials and Methods

2.1 Chemicals

Chemicals and solvents used were of analar grade. Ethyl Methane Sulfate (EMS), calf thymus DNA, cesium chloride, agarose, β DNA, restriction enzymes and polyethylene glycol (PEG) were purchased from Sigma chemicals Co., St. Louis, USA. Novozym –234 was purchased from Novo Industry, Denmark.

2.2 Fungicides

BASF (India) Ltd., Chennai generously supplied Carbendazim. Other fungicide used in the investigation was purchased from various sources. Molecular Characterization of Carbendazim Resistance of Plant Pathogen (*P. oryzae*) 199

2.3 Preparation of stock solution of fungicide

Stock solutions of fungicide were prepared by dissolving the fungicide in 1 m L acetone or ethanol and made up to a known volume with distilled water. Required concentrations were prepared by diluting the stock solution. Fungicide solutions were prepared just before use and the final concentration of the solvent did not exceed 0.5% in the medium. Sterilization of stock solution was made by filtering through sterilized millipore filter system (0.22μ).

2.4. Test pathogen

The test pathogen *P. oryzae* causing brown leaf spot were chosen as test pathogen. The phytopathogenic strains of *P. oryzae* were collected from infected paddy leaves in paddy filed of Siddi Vinayaga farm, Bandikavnoor, Chennai, Tamil Nadu, India. Infected leaves were sterilized in mercuric chloride (0.01%) and placed onto PDA, to which streptomycin (50 μg / m L) was added to suppress bacterial growth and incubated at 20^{o}C for 3 days leaf bits were removed. Hundred agar blocks containing germlings of single conidia were picked up with a sterile needle under microscopic observation, transferred individually to PDA slants and incubated until they form sporulating colonies.

2.4.1 Culture conditions of the fungus

Fungus stock culture was maintained on PDA slants at 28^{o}C and transferred to new media at regular intervals. Petri plate cultures were maintained as follows. To 3 days old culture, 5 m L of sterile distilled water was added and scrapped with an inoculation needle. The conidial suspension was transferred to a 250 m L conical flask containing 100 m L molten PDA (40^{o}C), mixed thoroughly and poured into sterile Petri dishes. After 3 days of incubation, using a sterile cork borer, mycelial discs (8 mm dia) were cut at random of periphery region and were used for further experiments throughout the investigation.

2.4.2 Preparation of conidial suspension

Conidial suspension of phytopathogen of *P. oryzae* were prepared by washing the well sporulated slant cultures in sterile water containing one drop of Tween 20 and filtered through two layers of cheese cloth to remove hyphal fragments. The spore suspension was washed twice with sterile water and resuspended in sterile water. The concentration of conidia was determined using a Haemocytomer.

2.5.3 Pathogenicity test on rice plants (Chevalier et at., 1991)

Pathogenicity of rice pathogens was tested on rice plants under green house conditions. Forty five days old Ponni and IR 50 paddy plants were first sprayed with mycelial suspension of the sensitive strains of pathogen (*P. oryzae*) means of automizer. The plants were covered with individual's polythene bags to provide adequate humidity and kept at room temperature. The inoculated plants were observed after 7 days for characteristic symptoms.

2.5 Morphological and physiological characterization

2.5.1 Growth of test pathogen (sensitive strain) on solid media

Growth rate of pathogens was assessed on solid media in PDA. Mycelial disc (8 mm dia) was placed at the center of the Petri plate in an inverted position containing media and kept for incubation. At every 48 h, the diameter of the mycelial growth was measured. After incubation, 10 mycelial discs (8 mm dia) were cut at random from the periphery and transferred to 10 m L sterile water in 100 m L flasks. The flasks were kept on an orbital shaker for 30 min. The number of conidia /m L in media was counted using Haemocytometer.

2.5.2 Sensitivity of test pathogen (wild strain) to Carbendazim on PDA

Sensitivity of phytopathogen to Carbendazim was tested using poison food technique (Carpenter, 1942) by inoculating 8 mm mycelial discs at the centre of the Petri plates containing different concentration of the fungicide (i.e. 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000µM) amended with PDA. Four plates were maintained for each treatment. After incubation for 10 days, the diameter of mycelial growth was measured in all treatments and control. The percentange inhibition over control was calculated by plotting probit values of the percent inhibition of the growth against log concentration of the fungicide (Nageswara Rao, 1965) and ED_{50} dose was estimated.

2.6 Development of *in vitro* resistant mutant of test pathogen against Carbendazim

2.6.1 Collection of conidia

The fungus was grown in PD broth at 27°C on a reciprocal shaker for 5 days. The conidia were separated from mycelia by filtering through double layers of cheese cloth and the filtrate was centrifuged at 1000Xg for 10 min. The conidia were suspended in a known 10 volume of sterile water and the final concentration was adjusted to $1X10^6$ conidia /m L by using Haemocytometer.

2.6.2 Development of Carbendazim resistant mutants of test pathogen by UV – irradiation (Tanaka et al., 1988)

The *in vitro* mutants resistant to Carbendazim were developed by following the procedure of Tanaka et al., (1988). Aliquots of conidial suspension (1 X 10^3 conidia / m L) containing Tween 20 (0.02% V/V) was poured into a Petri plate and exposed to UV irradiation (254 nm Phillips TUV 51 W G15T8) at a distance of 30 cm for 15, 30, 45 and 60 min. After which, the conidia were incubated in the dark for 2 h to prevent photo reactivation. Conidia treated with mutagens as described above, were plated separately on PDA amended with Carbendazim 5 times ED_{50} concentration of the sensitive strain. Medium devoid of fungicide served as control. The Petri plates were incubated at 20°C for 8 days. Survival rates were calculated from the ratio of colonies which grew on the fungicide unamended medium and the number of conidia inoculated. Mutation rate was determined from the ratio of colonies which grew on fungicide amended media and number of conidia inoculated.

2.6.3 Induction and isolation of Carbendazim resistant mutants of phytopathogen by Ethyl Methyl Sulfate (EMS) treatment (Tanaka et al., 1988)

Conidia (1 X 106 /m L) of the sensitive strain of test pathogens were suspended in 100 m L conical flask containing 10 m L freshly prepared EMS solution at a concentration of 5, 10, 25, 50, 100, 250, 500, 1000,2000 and 5000µm in 0.1 M sterile phosphate buffer (pH 7.0). All the flasks were kept on a shaker for 6 h at 27°C. Treated conidia were washed twice with sterile distilled water by centrifugation at 1000 X g for 10 min to remove traces on EMS. Subsequently, the conidial concentration was adjusted to 1 X 10^6 m L and plated on PDA amended with fungicide (five times the concentration of ED_{50} value of sensitive strain). The seeded plates were incubated for 5 d at 20°C. The colonies (mutants) that survived were counted and isolated for further studies and percent survival was also calculated.

2.6.4 Collection of field resistant strains

Field resistant mutants of *P. oryzae were* collected from diseased parts of plants treated continuously with Carbendazim.

2.6.5 Stability test for fungicide resistance in the mutants of test pathogen

Carbendazim resistant mutants were subcultured on fungicide free PDA medium for 10 generations. After 10 generations, the mutants were transferred to the respective fungicide (5 X the ED_{50} concentration) amended PDA medium. Stability rate was determined from the ratio of colonies which grew on the fungicide amended medium and number of colonies inoculated. The Carbendazim resistant mutant strains of test pathogens obtained by various mutagenesis techniques were designated.

2.7 Level of fungicide resistance laboratory mutants and field mutants resistant to Carbendazim

2.7.1 Level of resistance based on the mycelial growth on PDA medium

Young mycelial discs (8 mm dia) were aseptically transferred to the centre of the Petri plates containing PDA medium amended with different concentrations (always above 5 times the concentration of ED_{50} value of sensitive strain) of Carbendazim (5µM to 1000µM) and incubated at 20^{o}C for 25days. Medium devoid of fungicide served as control. Four replicates were maintained for each treatment. Percent inhibition of growth was calculated and the ED_{50} values were derived by probit analysis.

2.7.2 Morphological characterization of the resistant mutants of test fungi

Colony morphology, pigmentation and conidial production of the resistant mutants in fungicide unamended and amended medium were compared with that of the sensitive strain. The conidia was measured using a calibrated microscope (Carl Zeiss, Germany) Molecular Characterization of Carbendazim Resistance of Plant Pathogen (*P. oryzae*).

2.8 Biochemical characterization of sensitive strain and resistant mutants

2.8.1 Measurement of oxygen uptake by sensitive strain and resistant mutants of test pathogen

Oxygen uptake was measured (Johnson, 1972) polar graphically in Clark type oxygen probe fitted to a YSI (Yellow Springs Instruments Ltd., Ohio., USA) model oxygen monitor. The probe was standardized with distilled water. The probe was inserted into the chamber and 100% saturation of air was set. For the measurements of oxygen, 1 g fresh mycelium suspended in 5 m L Potato Dextrose Yeast Extract (PDYE) broth was transferred into the chamber. The decrease in saturation percent was measured at 1 min interval for 10 min

(Arditti and Dunn, 1969). Percent saturation of air measured was converted to oxygen concentration using Rawson's monogram (Welch, 1948).

2.8.2 Determination of electrolytes

One gram fresh mycelium was washed with sterile double distilled water and incubated in 100 m L PD broth. One set of flasks was amended with ED_{50} concentration of the Carbendazim and incubated on an orbital shaker (150 rpm). Mycelium incubated in PD broth devoid of fungicide served as control. Four replicates were maintained for each treatment. Mycelium was harvested at every 4 h interval for 24 and washed with excess of distilled water. Washed mycelium was transferred to 25 m L of sterile double distilled water and incubated on an orbital shaker (150rpm) for 1 h. After incubation, myceliums were filtered and the conductance of the ambient water was measured using CM 82T Conductivity Bridge with a dip electrolytic cell. Dry weight of mycelium was determined.

2.8.3 Extraction of protein

Wet mycelium (500 mg) was ground with equal amount of acid washed sand and 0.1 M sodium phosphate buffer (pH 7.0) in a pre-chilled mortar and pestle for 20 min at 4^{o}C. The ground material was centrifuged at 15, 000 x g for 15 min and the supernatant was made up to a known volume with the same buffer and dialyzed overnight against large volume of glass distilled water. The dialyzed extract was used for protein estimation.

2.8.4 Protein estimation

Protein was estimated by the method of Lowry et al., (1951) using Bovin Serum Albumin (BSA) as standard and the amount of protein was expressed as mg protein/g dry wt. of mycelium.

2. 8.5 Analysis of protein by SDS –PAGE (Laemmli, 1970)

The discontinuous buffer system of Laemmli (1970), which is a modification of Ornstein (1964), was used in the present study for the separation of proteins.

2.9 Molecular characterization of sensitive strain and resistant mutants of test pathogen

2.9.1 Extraction of degraded DNA and RNA

For quantification, DNA and RNA were extracted by the modified method of Scheneider (Munro and Fleck, 1966). One gram fresh weight of the mycelium was treated with 5% trichloroacetic acid (TCA) for 60 min at 4^{o}C. The supernatant containing the cold acid soluble compounds was discarded after centrifugation at 15,000 X g for 15 min at 4^{o}C. The mycelium was their treated with 1 N perchloric acid (PCA) at 70^{o}C in water bath for 20 min to hydrolyse the nucleic acid. The suspension was again centrifuged at 15, 000 X g for 30

min at 4°C and the supernatant was collected. The pellet was re extracted with 1 N TCA under the same conditions and the supernatants were pooled and used for DNA and RNA estimation.

2.9.2 Determination of rate of uptake of Carbendazim

One gram fresh mycelium grown in PDYE broth was harvested by filtration on a Buchner filter and washed thrice with distilled water. The mycelium was washed twice with incubation medium (25mM KH_2PO_4, 12.5 mM $K_2HPO_4.3H_2O$ buffer, pH 7.0, with 0.1 mM $CaCl_2$. $2H_2O$ and 1% glucose) (De Waard and Nistelrooy, 1980) and resuspended in 50 m L of the same at 37°C for 30 min. After 30 min, ED_{50} of fungicide (25µm Carbendazim) was amended in the mycelial suspension. Uninoculated medium served as control and four replicates were maintained for each treatment. At 10 min interval, 5 m L sample was filtered over Whatman No.1 filter paper. The mycelial residues were washed thrice with the incubation medium without fungicide and finally the incubation media were pooled and made up to a known volume. Similarly, 5 m L incubation medium was drawn from the control flask (incubation medium without fungicide) and made up to same volume as that of the test sample. Percent uptake of fungicide by each strain was determined from the difference in the amount of residual fungicide in the test and control medium. Sodium azide was added to the mycelial suspension 15 min prior to the addition of fungicide.

2.10 Polymerase Chain Reaction (PCR)

The polymerize chain reaction and then sequence analysis of the genomic DNA were used to rapidly characterize the sequence of β–tubulin DNA from the pathogens. Genomic DNA was prepared from each strain of test pathogens and subjected to PCR by using two generic β–tubulin primers. Constraints on primer design were that the amplified DNA had to contain codons 167 and 241, in which mutations were associated with resistance to benomly and that the primers had to anneal to a conserved region with minimal variation in the sequence. The 22-mer oligonucleotide A (5'- CAAACCATCTCTGGCGAACACG) and 22- mer oligonucleotide B (5' – TGGAGGACATCTTAAGACCACG) were used as primers. Primer A was identical in sequence to codons 22-28, and primer B was complementary in sequence to codons 359-365 of β–tubulin genes of *V. inaequalis* With these primers, a 1,191- bp fragment of the beta-tubulin gene was amplified. The primer was synthesized in Bangalore Gennie Pvt. Ltd, Bangalore.

The reactions were performed in a thermal cycler (35 cycles) with the replication prime DNA amplification system (Du Pont) according to the manufactures procedures. Each PCR reaction was performed in 25 µl (final volume) of reaction mixture. It consisted of 1 µl of DNA, 4 µl (1.02 µl) 200 µM dNTPs, 5.5 µl (2.5 mM MgCl2), 5.5 µl (50 mM KCl), and 5.5 µl (10 mM Tris HCl), Triton –100 X 0.05 µl, 0.5 µM of each primer and 1 unit of Tag DNA polymerize (approximately 0.66 µl) 1.79 µl double distilled water. Negative controls were run in all the amplification reactions to detect contamination. In reactions involving primers A and B, 35 cycles were performed by pre –heating the sample for 5 min at 94oC for each reaction as follows. 94^{o}C, 1 min; 55^{o}C, 1min; and 72^{o}C, 2 min. Amplification products were analyzed for the expected 1,191-bp or 436-bp fragments by 1.0% agarose gel electrophoresis in 1X TBE buffer (0.1 M Tris-HCl, 0.1 M boric acid, 0.02 mM EDTA, pH 8.3. following electrophoresis, the DNA was visualized after staining with ethidium bromide.

2.11. Allele –Specific Oligonucleotide (ASO) analysis

The single spore isolate of test pathogen used this study was from a large collection of field strains and laboratory strains previously characterized in studies on the inheritance to benomly resistance negatively correlated cross-resistance to diethofencarb (Jones, et al., 1987). The PCR amplified β–tubluin DNA (25ng per sample) was denatured in 0.25 N NaOH for 10 min and then applied to a nylon membrane (Gen Screen-Plus, Du Pont, Boston, MA) in a dot blot manifold. The dot blots were incubated in prehybridization solution (1 M NaCl, 50 mM Tris –Hcl, pH 7.5, 10% dextran sulfate, 1% SDS, 0.2% Ficoll (MW 400,000), 0.2% polyvinylprrolidone (MW 40,000), 0.2% bovine serum albumin, 0.1% sodium pyrophosphate, and 0.25 mg/mL denatured salmon sperm DNA) for 2 h at 5oC according to the manufacture's producer. An end-labeled ASO probe was then added to the prehybridization solution and incubated at 37ºC for at least 4 h. The blots were washed three times for 15 min each in 2 X SSC buffer (Saline – Sodium Citrate) (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 6.8) at room temperature. A high –stringency wash, three times for 2 min each in 2X SSC buffer, was then used to remove ASO probe with a single base pair mismatch from the blots. The optimum temperature for the high-stringency wash with each probe was determined empirically. The dot blots were exposed to X-ray film for 0.5-2 days at –70^{o}C. Four ASO probes for detecting allelic mutations in the β-tubulin gene of *V. inaequalis* were synthesized in the macromolecular structure facility, Department of Biochemistry, Michigan State University, East Lansing, USA. The 18-mer oligo-nucleotides were

designated as ASOS-LR, ASOMR, ASOHR and ASOVHR probes according to the specificity of each probe.

3. Results

In recent years awareness of the importance of fungicide resistance in crop protection has been growing. Hence, in the present studies *P. oryzae* (Table 1) highly sensitive to benzimidazole of fungicide have been chosen to study the probability and level of resistance of Carbendazim in the pathogen and measured to overcome resistance by understanding the molecular mechanisms of resistance. The test pathogen was tested for their virulence through pathogenicity test. Growth kinetics of the pathogen of *P. oryzae* (Table 2) shows that 13-16 days for complete growth. Dry weight estimation in PD broth of test pathogen (Table 3) under static and shaken conditions always show that shaken condition the growth rate in fast with enhanced mycelial dry weight in test pathogen. Before understanding, the mechanism of resistance in different mutants the base line sensitivity of the parent strains to test fungicide Carbendazim is analyzed (Table 4). The ED_{50} value was calculated for pathogen of *P. oryzae* the most sensitive one to Carbendazim was ED_{50} value of *P. oryzae* 20μM.

To understand the mechanisms of resistance EMS, UV and adaptation methods have been used to produce number of laboratory mutants (Table 5, 6 and 7). In case test pathogen nearly 200 mutants were produced and screened on PDA amended with five times the ED_{50} concentration of respective sensitive strains (according to test pathogen) resistant colonies were picked up and stored in test tube slants amended with 5 times concentration of ED_{50} dose. The laboratory developed resistant mutants were subsequently tested for their stability for 10 generation in fungicide free medium (Table, 8). All the tested fungicides which retained resistance after 10 generation only were taken for further studies. In *P. oryzae* the UV mutants were more stable.

The stable resistant mutants were screened to evaluate the level of resistance and then to categorize them as LR, MR, HR and VHR based on to their level of resistance. To group the selected mutants, they were grown on 5-10 times (LR), 10-15 times (MR), 15-20 (HR), 20-25 times and above as VHR based on ED_{50} value of the sensitive strain of test pathogen. The ED_{50} value of *P. oryzae* is 20 μm, accordingly various concentrations were prepared and 60 mutants were screened (Table- 9) and categorized *P. oryzae* 20 as LR, 25 as MR 10, HR and 5 as VHR were screened.

The base composition of DNA of sensitive and resistant strain of *Pyricularia oryzae* results on the GC% (Table 10) and Tm show an increase in GC% and as a result increase in melting

temperature. Always the GC% was less in sensitive strains while all levels of mutants of test pathogens show an increase in GC%.

The spore germination and primary hyphal length were measured and presented in Table 11. Interestingly the results show a distinct reduction in spore germination and primary hyphal elongation as the level of resistant increase. The time taken for spore germination and hyphal elongation is more in resistant mutants over the control. Macromolecular changes were assessed in various mutants of test pathogen (Table.12). Total DNA content was increasingly more in all levels of mutants of all test pathogen. Similarly RNA and total protein content were also more in resistant mutants than sensitive strain.

Cross resistance studies was carried out and the results are presented in Table 13. The results clearly indicate that when the Q-value is 1 and less than 1 that compared is considered to be negatively correlated and by springing such chemical the resistant can broken and the disease can be controlled. Dithane M-45, Mancozeb appeared to be negatively correlated to carbendazim resistant in all levels of mutants. In *P.oryzae* also Dithane M-45 sowed negative correlation. Here in addition Kitazin and Fytolan showed negative correlation suggesting that these chemicals can be effectively used for controlling *P.oryzae* resistant to carbendazim at all levels of resistant.

Agrose gel electrophoresis (Plate 1) total DNA of various levels of mutants of all the test pathogen when compared to control did not show variation at all but the DNA was subjected to Hind III restriction enzyme and the restriction enzyme digested profile of various levels of mutants (Plate 2) showed distinct variation in the number of discrete bands. Those the profile pattern varied between the levels of mutants and test pathogen. The number of discrete bands developed varied among the levels of mutants.

4. Discussion

Agriculture crops are under continuous attack of serious and noxious organisms. They are powerful challengers of nature and manmade technologies. To safeguard world food production, crop protection measures especially choice based intelligent and ecofriendly chemicals are indispensable as they are instant and with known mode of action. However, control of pest and disease with chemicals also encounter several problems, when the pathogens resistance to the potential broad-spectrum chemical. The ability of a pathogen to develop stable resistance to any toxicant is the fundamental theory of survival of the fittest under unfavorable conditions.

In a fungal population that is originally sensitive to fungicide, forms may arise or already exist that are less sensitive to the fungicide. Such a decrease in sensitivity may be caused by genetic or non-genetic changes in the fungal cell. Decrease in sensitivity due to genetic changes in the pathogen is more serious and requires in-depth understanding to detect the resistance in the field and combat the resistance well in advance to avoid or delay build of resistance.

Progress in clarifying the biochemical mechanisms of resistance has been made with some systemic fungicides, viz. Carbendazim, Carboxamides and Organophosphorous compounds (Georgopoulos, 1977). However, it is usually better to act before the buildup of resistance starts. For this, one should collect information from experiments with test fungi *in vitro* about the chances of a resistance problem arising in practice. Laboratory mutants resistant to test fungicides can be developed through in vitro mutagenesis (mutagenic chemicals or Ultra violet irradiation). The wild or parent strains should be characterized throughout to compare the resistant mutants. Also, the wild strain should be thoroughly characterized for its sensitivity against the target fungicide. In the present studies paddy pathogen *P. oryzae* was taken as test pathogen.

Before proceeding to produce Carbendazim resistant strains the sensitivity of the test pathogen was carefully monitored and ED_{50} value of carbendazim for all test pathogen *P. oryzae*. Laboratory mutants were developed through UV irradiation, EMS mutagenesis and adaptation. There are several reports on the development of resistant mutants through EMS, UV irradiation and adaptation (Sanchez et al., 1975, Davidse, 1981, Leach et al., 1982; Gangawane et al., 1988; Rana and Sengupta, 1977, Hillderbrand et al., 1988). Many of the laboratory mutants were stable with high level of resistance.

In the present studies, though resistance to Carbendazim in test pathogen was suspected to be site modification, it was made very clear through results that site modifications is quiet rare and is not frequently observed in fields under practice with target fungicides. PCR amplification of β-tubulin and spot hybridization further confirmed that the resistance at various levels tested proved that there was no point mutation observed in all the test pathogen. Instead ample evidences are present to confirm that in majority of the test pathogen, the mechanism of resistance primarily and always observed in membrane modification which could easily be handled or controlled easily with negatively correlated chemicals. Hence, the present studies had given an authentic molecular proof that all benzimodazole resistance observed need not be site modifications which is very difficult to

manage but mostly membrane modification, clearly indicating the possibility that fungicide resistance at any level if predicted can easily be managed and it is recommended that selection of choice based chemical (alternate chemical) with careful monitoring will definitely give a stable and sustainable management of diseases successfully in the agricultural practices. This is an additional warranty for the management of uncontrollable diseases using biological control.

5. Summary

Many pathogens develop resistance under field conditions due to frequent application of fungicides. To evaluate the resistance risk, stable laboratory mutant of *P. oryzae* resistant to Carbendazim were developed under laboratory conditions. Important pathogen *P. oryzae* was selected for intensive studies on molecular mechanisms of the fungicide resistance to benzimidazole compound. For the precise understanding of resistant mutants the complete characterization of parent strains very carefully carried out. The growth kinetics of *P. oryzae* was estimated on PDA. Similarly the dry weights of the test pathogen were estimated under static and shaken conditions. The mycelial dry weight was more under shaken condition than the static condition. Test pathogen was screened for sensitivity against Carbendazim using different concentration. The ED_{50} value for *P. oryzae* was 40 µM. Induction of laboratory mutants using EMS, UV irradiation and adaptation technique were carried out. More than 200 resistant colonies were taken to select resistant strains 200 colonies were screened on PDA amended with 5 times the ED_{50} value of Carbendazim. The respective sensitive strains and resistant colonies were picked up and stored in test tube slants amended with test fungicides. The percent survival was assessed for all the test pathogen. Stability of resistance in all the mutants was checked. All the stable mutants of test pathogen when tested for pathogenicity proved to be pathogenic by causing respective disease through artificial inoculation. All the stable resistant mutants were categorized as LR, MR, HR and VHR based on their level of resistance over ED_{50} values of sensitive strain.

Selected mutants when grow on (5 – 10 times LR, 10 –15 times MR, 15-20 times HR and 20 – 25 times VHR). Sixty mutants in each pathogen were screened on the above concentration of Carbendazim and categorized as LR, MR, HR and VHR. Sporulation of the resistant mutants were evaluated for all test pathogens and compared. In sensitive strains more sporulation was observed than the sensitive strains. Agarose gel electrophoresis of genomic DNA of the all the levels of mutants of test pathogens did not show any variation. Cross resistance study showed that *P. oryzae* resistant mutants at all levels could be overcome by

alternative use of Dithane-45 or Mancozeb. The PCR amplification of β-tubulin in DNA extract was very poor and in most of the resistant mutants DNA, β–tubulin could not be amplified.

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Table 1: Test pathogen used for the present study

S. No	Organism	Place of collection	Disease caused
1	*Pyricularia oryzae*	Bandikkavanoor Agriculture field (Tamil Nadu, India)	Rice blast

Table 2: Radial growth of sensitive strain of *Pyricularia oryzae* on PDA

Day	Radial growth* (cm)
2	2.2
4	2.8
6	3.6
8	4.8
10	5.5
12	6.7
14	8.9
16	Plate fully covered

* Mean of 4 replicates

Table 3: Growth rate of sensitive strain of *Pyricularia oryzae* in PD broth

Incubation time (days)	Dry weight of the mycelium(mg)*	
	Static condition	Shaken condition
1	20.00	65.75
2	45.00	98.88
3	60.00	120.86
4	100.00	186.00
5	185.00	214.85

6	225.00	280.68
7	300.00	320.60
8	360.00	375.80
9	380.00	418.00
10	400.00	450.50
11	425.00	500.00
12	485.00	560.00

* Mean of 4 replicates

Table 4: Effect of Carbendazim on the growth of *Pyricularia oryzae*

Concentration (µM)	Mycelial dry weight (mg)	% inhibition
0	670	0
1	640	4.5
2.5	558	16.7
5	415	38.1
10	390	41.8
25	355	40.8
50	250	47.0
100	200	70.1
250	125	81.3
500	43	93.6

ED 50 value = 20 µM

Table 5: Induction of mutation of *Pyricularia oryzae using* EMS

Conc. (µM)	No. of colonies survival	% of survival
1	260	52
2.5	200	40
5	180	36
10	140	28
25	120	24

50	100	20
100	75	15
50	50	10
500	25	5

Spore concentration : 0.5 X 10^3
Incubation time : 8 h

Table 6: Induction of mutation *Pyricularia oryzae UV* irradiation

Exposure time (min)	No. of colonies survived	% of survival
15	250	50
30	210	42
45	160	32
60	80	16
75	50	1
90	-	-

Spore concentration: 0.5 X 10^3
Incubation time: 8 h

Table 7: Development of a resistant strain of *Pyricularia oryzae* by adaptation

Treatment	Concentration (µM)	Mycelial growth diameter (mm)
I	5	85
II	10	60
III	25	45
IV	50	25
V	100	12
VI	250	6
VIII	500	2
IX	1000	2

Incubation time: 8 d

Medium: PDA

Table 8: Stability of fungicide resistance in *Pyricularia oryzae* mutant

Mutant	No. of colonies examined	No. of stable colonies retained	Stability rate (%)
EMS	35	25	71.42
UV	40	35	87.50
AD	50	40	80.00

EMS : Carbendazim EMS resistant mutant
UV : Carbendazim UV resistant mutant
AD : Carbendazim Adapted resistant mutant

Table 9: Sporulation of resistant strains and mutants of test pathogen

Strain	*P. oryzae* No. of conida /m L	Mutants of *P. oryzae* No. of Colonies
SEN	$5.6 X10^5$	85
LR	$5.2 X 10^5$	20
MR	$4.9 X 10^5$	15
HR	$4.9 X 10^5$	10
VHR	$3.0 X 10^5$	5

Table 10: Base composition of DNA of sensitive and resistant strain of *Pyricularia oryzae*

S.No.	Name of the strains	GC % undegraded DNA		
		UV absorbance	Thermal Denaturation	Melting temperature (C^o)
1	Sensitive	59.10	60.96	86
2	MR (20)#	61.25 (3.64)	64.11 (5.17)	88
3	HR (15)	63.15 (6.85)	64.23 (5.36)	90
4	VHR (5)	65.18 (10.28)	65.73 (7.82)	91

Figure in parenthesis indicate per cent over sensitive strain
Mean of strain

Table 11: Rate of conidial germination and primary hyphal elongation under shaken conditions of *Pyricularia oryzae* (without fungicide)

Incubation	% of germination				Primary hyphae length (μM)			
Strain	SEN	MR	HR	VHR	SEN	MR	HR	VHR
3	0	0	0	0	0	0	0	0
6	25	0	0	0	55	0	0	0
12	35	34	32	33	73	69	67	65
15	48	44	42	45	89	80	83	86
18	65	64	62	61	94	91	90	89
21	70	68	66	65	99	98	91	90
24	79	70	69	68	158	147	155	150
27	90	89	88	87	175	168	158	155

Shaken conditions
Incubation : 10^3 conida/ m L
Temperature : 20^oC
Values are mean of 5 replicates.

Table 12: Macromolecular content of sensitive and resistant strain of test pathogens

Mg/g dry weight			
P. oryzae			
Strains	DNA	RNA	Protein
SEN	8.00	4.00	16.25
LR	8.90 (11.25)	4.25 (11.25)	18.12 (11.50)
HR	9.10 (13.75)	4.60 (15.00)	19.10 (17.35)
VHR	9.12 (14.00)	4.10 (2.5)	19.24 (18.46)

Table 13: Cross resistance in resistant mutant of *P. oryzae*

Fungicides	ED 50 value						Q – value				
	SEN	LR	MR	HR	VHR	FR	LR	MR	HR	VHR	FR
Benomyl	320	325	336	345	310	300	1.93	1.05	1.08	0.97*	0.94*
Biloxozol	360	368	345	412	440	312	1.02	0.96*	1.14	1.22	0.87*
Kitazin	410	425	435	460	480	440	1.04	1.06	1.12	1.17	1.07
Mancozeb	390	412	385	425	412	560	1.06	0.86*	1.09	1.07	1.44
Fytolan	420	425	400	418	475	510	1.01	0.95*	0.98*	1.13	1.21
Ziram	406	418	425	460	400	480	1.06	1.05	1.13	0.99*	1.18
Dithane M-45	425	435	400	456	467	476	1.02	0.94	1.07	1.09	1.12

* Negatively correlated

Plate 1

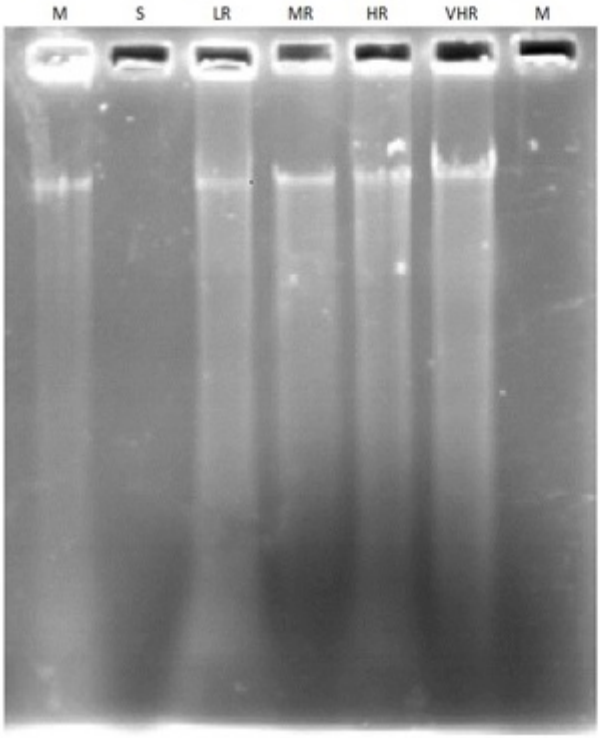

Plate 2