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Molecular Characterization of Carbendazim Resistance of Plant Pathogen (*Bipolaris oryzae*)

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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 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, fiber 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 developing research on biocontrol practice needs supportive control of some chemicals in the control of diseases that cannot be controlled by biocontrol. It is high time to go further with in-depth research on fungicides, to combat and overcome fungicide resistance in practice. Systemic chemicals are best alternatives for any failure practices of plant protection, provided they are carefully monitored for their mode of action, method of application and understood more on the mechanism of development of fungicide resistance.

The present research is case study on Carbendazim fungicide resistance in many important pathogens. *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 give 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 General methods

Glassware was first soaked in chromic acid cleaning solutions (10% potassium dichromate solutions in 25% sulphuric acid) for few hours and washed thoroughly in tap water. After a second wash in detergent solution, they were again washed thoroughly in tap water, rinsed in distilled water and air dried. Glassware, media and buffers were sterilized at 120°C, 15 Ib pressure / inch^2 for 20 min.

2.2 Chemicals

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

2.3 Fungicides

BASF (India) Ltd., Chennai generously supplied Carbendazim. Other fungicide used in the investigation was purchased from various sources.

2.4 Preparation of stock solution of fungicide

Stock solutions of fungicide were prepared by dissolving the fungicide in 1 mL 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).

2.5 Isolation of fungi

2.5.1 Test pathogen

The test pathogen *Bipolaris oryzae* causing brown leaf spot were chosen as test pathogen. The phytopathogenic strains of *Bipolaris oryzae* were collected from infected paddy leaves in paddy filed of Siddi Vinayaga farm, Bandikavnoor, Chennai. Infected leaves were sterilized in mercuric chloride (0.01%) and placed onto PDA, to which streptomycin (50 µg / mL) 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.

2.5.2 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 mL of sterile distilled water was added and scrapped with an inoculation needle. The conidial suspension was transferred to a 250 mL conical flask containing 100 mL 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.

2.5.3 Preparation of conidial suspension

Conidial suspension of phytopathogen of *Bipolaris 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.

2.5.4 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 (*B. 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.

2.6 Morphological and physiological characterization

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

Before the assessment of growth on fungicide amended medium, the maximum growth and sporulation rate inducing medium was screened. Growth rate of pathogens was assessed on five different solid media viz. PDA, PDYEA, CDA, MEA and OMA. Mycelial disc (8 mm dia) was placed at the center of the Petri plate in an inverted position containing media and kept for incubation. Quadruplicates were maintained for each treatment. 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 mL sterile water in 100 mL flasks. The flasks were kept on an orbital shaker for 30 min. The number of conidia /mL in different media was counted using Haemocytometer.

2.6.2 Growth rate of test pathogen (sensitive strains) in PD broth

The growth rates of test pathogen were studied by inoculating 4 mycelial discs (8 mm dia) into 250 mL conical flask containing 100 mL PD broth. One set of flasks were incubated on an orbital shaker (120 rpm) while another's sets of flasks were incubated under static conditions for 8 days. Every day, 4 flasks were removed from each set, the mycelial mat was filtered and dried separately in preweighted Whatman No. 1 filter paper at 80^{o}C and the dry weights estimated.

2.6.3 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.

2.6.4 Sensitivity of test pathogen to Carbendazim in PD broth

In PD broth, the sensitivity of phytopathogens to Carbendazim was carried out by inoculating 4 mycelial discs (8mm dia) in 250 mL flasks containing 100 mL of PD broth amended with different concentrations of fungicides. The flasks were kept on an orbital shaker (120 rpm) for 4 days. Quadruplicates were maintained for each treatment. Then the mycelial mat was than collected and dried on a reweighed, Whatman No. 1 filter paper and the dry weight estimated. The ED_{50} dosage was calculated by plotting probit values of the percent inhibition of the growth against log concentration of the fungicide.

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

2.7.1 Collection of conidia

The fungus was grown in PD broth at 27^{o}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

volume of sterile water and the final concentration was adjusted to 1 X 10^6 conidia /mL by using Haemocytometer.

2.7.2 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 / mL) 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 was 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.

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

Conidia (1 X 10^6 /mL) of the sensitive strain of test pathogens were suspended in 100 mL conical flask containing 10 mL 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 mL 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.

2.7.4 Development of resistant strains of test pathogen by adaptation technique

Adaptability of phytopathogen to Carbendazim was carried out by the method of Rana and Sengupta (1977). Conidia (1 X 10^3 / mL) of the sensitive strain were placed on PDA medium amended with 5 times higher the ED_{50} value of fungicide and the plates were incubated for 5 d at 23°C. From the developed colonies, mycelial discs (8 mm dia) were cut from periphery using a sterile cork borer and then transferred to stepwise increasing concentration of the fungicide. All the laboratory mutants resistant to test fungicide Carbendazim were numbered and maintained.

2.7.5 Collection of field resistant strains

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

2.7.6 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.

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

2.8.1 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.

2.8.2 Level of resistance based on the mycelial growth and conidial production in PD broth

One mL conidial suspension (1 X 10^3 conidia / mL) of the resistant mutants was inoculated in 100 mL PD broth amended with different concentrations of Carbendazim (5 to 1000µM) and incubated orbital shaker (120 rpm) at 20^{o}C for 12 days. PD broth devoid of fungicide served as control. Four replicates were maintained for each treatment. After the incubation period, mycelia were filtered and dry weight was determined. Numbers of conidia were counted in each flask per replicate per treatment. Percent inhibition of mycelial dry weight and conidial production was determined and ED_{50} values derived by probit analysis.

2.8.3 Level of resistance based on germination and primary hyphal elongation in PD broth

One ml conidial suspension (1 X 10^3 conidia / mL) of the resistant mutants was inoculated in 25 mL PD broth amended with higher concentration of Carbendazim (100 to 1000µM) and incubated on an orbital shaker (120 rpm) at 20^{o}C. Flasks inoculated with Cassava Root meal (CRM) were incubated for 30 h. PD broth devoid of fungicide served as control. Four replicates were maintained for each treatment. Percent inhibition of germination and primary hyphal elongation were calculated and ED_{50} values of the fungicide were derived by probit analysis.

2.8.4 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)

2.9 Biochemical characterization of sensitive strain and resistant mutants

2.9.1 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 mL 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 Rawso's monogram (Welch, 1948).

2.9.2 Determination of electrolytes

One gram fresh mycelium was washed with sterile double distilled water and incubated in 100 mL PD broth. One set of flasks was amended with ED50 concentration of the carbendazim and incubated on an orbital shaker (150rpm). 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 mL 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.

2.9.3 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.

2.9.4 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.

2.10 Electrophoretic protein pattern of sensitive strain an resistant mutant

Electrophoretic pattern of total protein of sensitive and resistant mutants was analysed by the method of Ornstein (1964).

2.10.1 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.

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

2.11.1 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^{o}C and the supernatant was collected. The pellet was re extracted with 1 N PCA under the same conditions and the supernatants were pooled and used for DNA and RNA estimation.

2.11.2 Calorimetric determination of DNA

DNA was estimated by the improved method of Giles and Myers (1965) further modified by Lalithakumari et al (1975). The reaction mixture consisted of 2 mL of hydrolysate obtained after 1 N PCA treatment and 2 mL 1% freshly prepared diphenylamine in glacial acetic acid and was incubated at 30^{o}C for 16 h in dark. The blue colour developed was measured at 595 nm and 700 nm in a Hitachi 150 – 20 spectrophotometer and the difference in optical densities were calculated. The solution without hydrolysate mixture was used for blank. The quantity of DNA was estimated using the standard curve prepared with calf thymus DNA.

2.11.3 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 mL of the same at 37^{o}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 mL 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 mL 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.

2.12 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

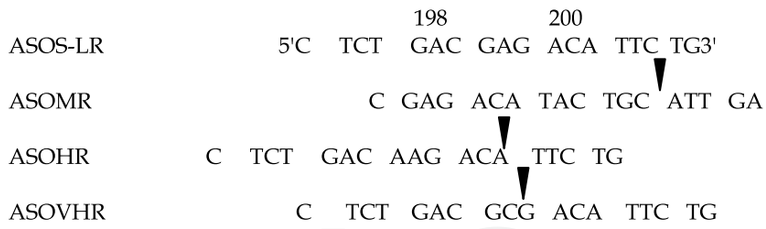
β–tubulin primers. Constraints on primer design were that the amplified DNA had to contain codons 167 and 241, in which mutations were associated with resistance to benomly and that the primers had to anneal to a conserved region with minimal variation in the sequence. The 22-mer oligonucleotide A (5'- 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 repliprime 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 (1.02µl) 200µM dNTPs, 5.5µl (2.5 mM MgCl2), 5.5 µl (50 mM KCl), 5.5 µl (10 mM Tris HCl), Triton –100 X 0.05µl, 0.5 µM of each primer and 1 unit of Tag DNA polymerize (approximately 0.66µl) 1.79µl 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 94^{o}C 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.

2.12.1 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 5^{o}C according to the manufacture's producer. An end-labeled ASO probe was then added to the prehybridization solution and incubated at 37^{o}C 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 *B. oryzae* (Table 1) highly sensitive to benzimidazole of fungicide have been chosen to study the probability and level of resistance of Carbendazim in these pathogens and measured to overcome resistance by understanding the molecular mechanisms of resistance. All the test pathogen was tested for their virulence through pathogenicity test.

S. No	Organism	Place of collection	Disease caused
1	*Bipolaris oryzae*	Bandikkavanoor, Tamil Nadu, India. Agriculture field	Brown spot disease

Table 1.Test pathogen used in the present study

3.1 Growth kinetics of sensitive strains

3.1.1 Radial growth of sensitive strains of test pathogens on PDA

Growth kinetics of the test pathogen *B. oryzae*, (Table 2) shows that *B. oryzae* takes 12 day for complete growth

Day	Radial growth* (cm)
2	2.7
4	3.3
6	4.6
8	6.9
10	8.8
12	Plate fully covered

* Mean of 4 replicates

Table 2. Radial growth of sensitive strain of *B. oryzae* on PDA

3.1.2 Growth rate of sensitive strains of test pathogens in PD broth

Dry weight estimation in PD broth of test pathogen (Table 3) under static and shaken conditions always showed that under shaken condition the growth rate was fast with enhanced mycelial dry weight in all the test pathogen.

3.1.3 Effect of Carbendazim on the growth of test pathogens

Before understanding, the mechanism of resistance in different mutants, the base line sensitivity of the parent strains to test fungicide, Carbendazim was analyzed (Table 4). The ED_{50} value was calculated for all the test pathogen. The test pathogen the most sensitive to Carbendazim *B. oryzae* with ED_{50} values of 40 μM.

Day	Dry weight of the mycelium(mg)*	
	Static condition	Shaken condition
1	65.00	95.00
2	96.00	120.00
3	125.00	168.60
4	165.00	285.00
5	195.00	345.00
6	260.00	370.00
7	300.00	400.00
8	310.00	420.00
9	315.00	410.00
10	400.00	690.00

* Mean of four replications

Table 3. Growth rate of sensitive strain of *B. oryzae* in PD broth

Concentration (μM)	Mycelial dry weight (mg)	% inhibition
0	650	0
1	545	16.2
2.5	468	28.0
5	425	34.6
10	400	38.5
25	385	40.8
50	300	53.8
100	268	58.8
250	225	65.4
500	143	78.0
1000	50	92.3

ED_{50} value = 40 μM

Table 4. Effect of Carbendazim on the growth of *B. oryzae*

3.1.4 Induction of mutation of test pathogen

To understand the mechanisms of resistance EMS, UV and adaptation methods have been used to produce number of laboratory mutants (Table 5, Table 6, and Table 7). In the case of

all the 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 results in tables 7-9 clearly show that the percent survival rate depends on the test pathogen and the mutagenesis.

Concentration of EMS (μM)	No. of colonies survived	% of survival
50	450	90
100	400	80
250	375	75
500	175	35
1000	75	15
2500	10	2

Spore concentration: 0.5 X 10^3
Incubation : 6 h

Table 5. Induction of mutation of *B. oryzae* by EMS

Exposure time	No. of colonies survived	% of survival
15	475	95
30	400	80
45	200	40
60	150	30
75	35	7

Spore concentration: 0.5 X 10^3
Incubation : 6

Table 6. Induction of mutation of *B. oryzae* by UV-irradiation

Treatment	Concentration of Carbendazim (μM)	Mycelial growth in diameter(mm)
I	5	75
II	10	60
III	25	58
IV	50	56
V	100	48
VI	250	45
VII	500	38
VIII	1000	20
IX	2,000	15
X	5,000	10

Incubation time : 6 d Medium : PDA

Table 7. Development of resistant strains of *B. oryzae* by adaptation

3.1.5 Stability of test pathogen

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 *B. oryzae* the EMS mutants were more stable.

Mutants	No. of colonies examined	No. of stable colonies retained	Stability rate%
EMS	50	35	70.00
UV	45	20	44.44
AD	40	25	62.50

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

Table 8. Stability of fungicide resistance in *B. oryzae*

3.1.6 Pathogenicity test

All the stable mutants of the test pathogen were subsequently tested for pathogenicity. From all the tested mutants only those that developed symptoms of the diseases were chosen for further studies.

3.1.7 Categorization of level of fungicide resistance

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 ED50 value of the sensitive strain of test pathogen. The ED50 value of *B. oryzae* was 40 µm. accordingly various concentrations were prepared and 60 mutants in pathogen were screened (Table 9) and categorized.

	Concentration of Carbendazim (µM)									
	10	**40**	**200**	**400**	**600**	**800**	**1000**	**1200**	**1400**	**1600**
S	300	275	-	-	-	-	-	-	-	-
1	300	289	285	-	-	-	-	-	-	-
2	302	292	290	-	-	-	-	-	-	-
3	303	295	292	-	-	-	-	-	-	-
4	305	298	295	-	-	-	-	-	-	-
5	306	299	295	-	-	-	-	-	-	-
6	307	299	292	-	-	-	-	-	-	-
7	310	298	295	-	-	-	-	-	-	-
8	313	302	300	-	-	-	-	-	-	-
9	315	308	302	-	-	-	-	-	-	-
10	317	306	305	-	-	-	-	-	-	-
11	318	310	308	-	-	-	-	-	-	-
12	319	310	307	-	-	-	-	-	-	-
13	320	312	310	-	-	-	-	-	-	-
14	321	316	313	-	-	-	-	-	-	-
15	323	321	320	-	-	-	-	-	-	-
16	324	320	318	313	310	-	-	-	-	-

	Concentration of Carbendazim (μM)									
	10	**40**	**200**	**400**	**600**	**800**	**1000**	**1200**	**1400**	**1600**
17	325	318	315	312	308	-	-	-	-	-
18	326	319	315	309	302	-	-	-	-	-
19	327	315	313	310	306	-	-	-	-	-
20	328	319	316	309	302	-	-	-	-	-
21	330	321	320	318	315	-	-	-	-	-
22	331	325	320	318	313	-	-	-	-	-
23	334	326	320	315	313	-	-	-	-	-
24	336	332	330	328	327	-	-	-	-	-
25	338	332	328	325	318	-	-	-	-	-
26	340	338	332	330	328	-	-	-	-	-
27	343	332	328	320	318	-	-	-	-	-
28	346	340	333	328	325	-	-	-	-	-
29	347	341	313	329	320	-	-	-	-	-
30	348	338	332	330	328	-	-	-	-	-
31	349	340	338	335	330	-	-	-	-	-
32	350	342	340	338	329	-	-	-	-	-
33	352	350	348	345	340	-	-	-	-	-
34	351	350	347	340	338	-	-	-	-	-
35	353	350	348	340	337	335	330	-	-	-
36	355	350	347	345	340	338	335	-	-	-
37	357	352	348	345	342	337	332	-	-	-
38	360	353	345	342	340	336	333	-	-	-
39	362	353	350	348	345	340	339	-	-	-
40	363	355	350	347	343	340	340	-	-	-
41	365	355	350	348	345	340	338	-	-	-
42	368	360	355	352	348	345	340	-	-	-
43	370	361	360	355	350	350	348	-	-	-
44	371	362	358	357	355	350	349	-	-	-
45	373	365	359	355	350	348	345	-	-	-
46	376	370	369	365	360	357	355	-	-	-
47	377	371	370	366	363	360	358	-	-	-
48	380	375	372	370	368	363	360	-	-	-
49	382	378	375	370	368	365	360	359	355	.350
50	383	370	370	368	365	360	359	355	354	352
51	385	380	375	370	360	360	358	355	350	348
52	387	382	380	378	370	368	365	360	630	358
53	390	385	380	378	375	370	368	362	360	374
54	393	388	385	380	378	375	370	368	362	360
55	397	390	388	385	380	365	350	358	358	348
56	398	393	392	390	385	382	380	375	370	365
57	402	400	395	392	390	387	380	375	370	365
58	405	400	399	397	390	388	380	375	370	365
59	407	405	400	396	394	391	390	387	385	370
60	410	407	404	395	391	387	385	380	379	374

ED50 of sensitive strain 12μM

1 –10 time	15 mutants	LR
10 - 15 time	20 mutants	MR
15 –20 time	10 mutants	HR

Table 9. Categorization of level resistance based on mycelial growth of mutants of *B. oryzae*

3.1.8 Grouping of mutants of test pathogen

In *B. oryzae* 15 mutants were classified under group of LR, 19 under MR, 14 under HR and 12 as VHR were obtained (Table 10).

Name of strains	LR	MR	HR	VHR
Bipolaris oryzae	15	19	14	12

Table 10. Grouping of mutants of test pathogen

3.1.9 Sporulation of resistant strains of test pathogen

After grouping the resistant mutants as LR, MR, HR, and VHR, morphological characterization studies were carried out. Sporulation of the resistant mutants were evaluated for all the test pathogen and compared with the sensitive strain (Table 11). The sporulation was always more in sensitive strain than the resistant mutants. The rate of sporulation decreased according to level of resistance.

Strain	*B. oryzae* No. of conidia / mL
SEN	5.4×10^4
LR	4.9×10^4
MR	2.8×10^2
HR	2.2×10^2
VHR	1.2×10^2

Table 11. Sporulation of resistant strains of test pathogen

3.1.10 Rate of conidial germination and primary hyphal elongation of test pathogen

Following sporulation, the spore germination and primary hyphal length were measured and presented in Table 12 for the test pathogen. 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 was more in resistant mutants over the control. The test pathogen, *B. oryzae* showed similar results.

3.1.11 Oxygen uptake by the sensitive and resistant mutant of test pathogen

Physiological characterization was initiated with oxygen uptake (Table 13). The oxygen uptake in n mole/min/mg dry wt of mycelium showed a distinct reduction in all the mutants irrespective of the test pathogen.

Incubation	% of germination*				Primary hyphal length (μM)			
Strain	SEN	MR	HR	VHR	SEN	MR	HR	VHR
3	0	0	0	0	0	0	0	0
6	20	0	0	0	35	0	0	0
12	40	30	38	45	50	45	48	47
15	45	38	46	59	69	55	50	49
18	78	49	67	69	68	60	55	51
21	89	53	71	79	80	70	69	70
24	90	69	65	60	150	145	140	125

Shaken conditions
Incubation : 10^3 conidia/ mL
Temperature : 20 ^oC
* Values are mean of 5 replicates.

Table 12. Rate of conidial germination and primary hyphal elongation of *B. oryzae* under shaken conditions (without fungicide)

Name of strains	*B. oryzae*
	Oxygen uptake n moles/min/mg/ dry wet. Mycelium
SEN	27.18
MR	22.12
HR	24.10
VHR	24.00

Table 13. Oxygen uptake by the sensitive and resistant mutant of test pathogen

3.1.12 Rate of efflux of electrolytes of sensitive and resistant mutants of test pathogen

Electrolytic leakage studies on various levels of mutants of test pathogens (Table 14) also showed decrease in efflux when compared to the sensitive strains. Though there was reduction in electrolytic leakage, it was increasing with incubation time.

3.1.13 Amino acid composition of sensitive and resistant mutants of test pathogens

Amino acid composition of sensitive and resistant mutants of test pathogen (Table 15) showed distinct variations in the quantity of amino acids. Trypsin, Cystine and Glycine were totally absent in sensitive and LR mutants of *B. oryzae*. In all the mutants of test pathogens, interestingly the Proline content was more and this was absent in sensitive strain.

Strains	Specific conductance+ μm hos / cm^2/ h/g/dry wt of (mycelium) Incubation time ($h_)$				
	2	**4**	**8**	**16**	**24**
Sensitive	2800	3680	3960	4850	4590
LR	2796 (0.14)	3560 (3.26)*	3920 (1.01)	4380 (9.69)	4580 (0.22)
MR	2786 (0.50)	3470 (5.70)	3890 (1.76)	4525 (6.70)	4420 (3.70)
HR	2775 (0.89)	3580 (2.71)	3770 (4.79)	4060 (16.23)	4360 (5.01)
VHR	2025 (27.67)	3660 (0.54)	3590 (9.34)	4050 (16.49)	4200 (8.49)
FR	2600 (7.14)	3000 (18.47)	3680 (7.07)	3960 (18.35)	4150 (9.59)

+ mean of 10 replication
* Figure in parenthesis indicate per cell (+) increase (or) (-) decrease over sensitive strain.

Table 14. Rate of efflux of electrolytes of sensitive and resistant mutants of *B. oryzae*

Amino acid	Composition (mg/g dry weight)					
	S	**LR**	**MR**	**HR**	**VHR**	**FR**
Arginine	0.48	0.49	0.55	0.65	0.60	0.55
Lysine	0.50	0.50	-	0.56	0.59	0.65
Aspartic acid	0.40	-	0.56	0.53	0.62	0.42
Glutamine	0.56	0.63	0.45	0.50	-	-
Asparagine	0.55	0.64	0.50	0.66	0.63	0.47
Serine	0.60	-	0.63	0.69	0.64	0.49
Valine	0.62	0.68	0.48	0.63	0.65	-
Phenylalanine	0.62	0.34	0.50	0.55	0.60	0.45
Proline	0.49	0.65	0.66	0.62	0.68	0.70
Histidine	0.48	Trace	-	0.60	0.59	0.75
Methionine	0.55	0.75	Trace	0.64	0.56	-
Leucine	Trace	Trace	0.35	0.65	0.57	0.78
Tryptophan	-	-	0.65	0.60	-	0.80
Cystine	-	-	0.66	0.61	0.60	0.82
Glycine	-	-	0.49	0.16	-	0.53
Ornithine	-	-	-	-	-	-
Threonine	-	-	-	-	-	-
Glutamic acid	0.49	0.60	0.62	0.63	0.65	0.54

*Mean of four replicates

Table 15. Amino acid composition of sensitive and resistant strains of *B. oryzae*

3.1.14 SDS gel electrophoresis of sensitive and resistant strains of test pathogen

Agarose gel electrophoresis of genomic DNA of all the levels of mutants of test pathogen did not show any variations among the levels of mutants and also between sensitive and resistant mutants.

3.1.15 GC% and Tm of resistant mutants of test pathogen

Results on the GC% (Table 16) and Tm showed 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 showed an increase in GC%.

S. No.	Name of the strains	GC% of undegraded DNA		
		UV absorbance	Thermal Denaturation	Melting temperature (°C)
1	Sensitive	60.12	60.18	80
2	MR (25)	65.14 (8.34)	64.18 (6.64)	88
3	HR (10)	63.80 (6.12)	64.19 (6.66)	90
4	VHR (5)	62.16 (3.40)	65.60 (9.00)	91

Figure in parenthesis indicate percent over sensitive strain
Mean of strain

Table 16. Base composition of DNA of sensitive and resistant strains of *B. oryzae*

S.No.	Strains	GC% of mt. DNA		
		UV absorbance	Thermal Denaturation	Melting temperature (°C)
1	Sensitive	50.00	60.60	89
2	MR (19)#	55.60 (11.20)	65.83 (8.63)	93
3	HR (14)	54.65 (9.30)	66.98 (10.52)	94
4	VHR (12)	56.80 (13.60)	68.80 (14.66)	95

Figure in parenthesis indicate percent over sensitive strain
Mean of strain

Table 17. Base composition of (GC%) of mitochondrial DNA of sensitive strain and resistant mutants of *B. oryzae*

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

In mitochondrial DNA GC% was assessed (Table 17) and always in all levels of mutants the GC% was more and consequently the melting temperature was always more than the control.

3.1.16 Cross resistance of resistant mutants of test pathogen

Fungicides	ED_{50} value						Q – value				
	SEN	LR	MR	HR	VHR	FR	LR	MR	HR	VHR	FR
Benomyl	360	390	475	510	575	340	1.10	1.32	1.42	1.59	0.94*
Biloxozol	450	490	400	515	525	540	1.08	0.88*	1.14	1.16	1.20
Kitazin	65	125	150	210	200	220	1.92	2.30	3.23	3.07	3.38
Mancozeb	250	320	375	300	385	340	1.28	1.5	1.2	1.54	1.36
Fytolan	200	250	380	395	200	360	1.25	1.90	1.97	1.00	1.80
Ziram	150	200	140	285	300	295	1.33	0.93*	1.90	2.00	1.69
Dithane M-45	335	256	260	270	340	285	0.79*	0.78	0.81	1.01	0.85*
Edifenphos	102	240	250	308	330	310	2.4	2.45	3.01	3.23	3.04

* Negatively correlated

Table 18. Cross resistance in resistant mutants of *B. oryzae*

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. The evolution of organisms would have been impossible without this property.

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 *B. oryzae* was taken as test pathogen.

Detailed chemical control work has recommended benzimidazoles as one of the best broad spectrum compound for the control of paddy pathogen, *B. oryzae*. Present study is an attempt to predict the disease control failure using modern molecular diagnostic method to detect under in vitro condition to combat the development of resistance.

Before proceeding to produce Carbendazim resistant strains the sensitivity of the test pathogens was carefully monitored and ED_{50} value of carbendazim for all test pathogens *B. 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 pathogens 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 pathogens. Instead ample evidences are present to confirm that in majority of the test pathogens, 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 *B. oryzae* resistant to Carbendazim were developed under laboratory conditions. Important pathogen *B. 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 *B. 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 pathogens were screened for sensitivity against Carbendazim using different concentration. The ED_{50} value for *B. 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 pathogens. Stability of resistance in all the mutants were checked. All the stable mutants of test pathogens 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 *B. 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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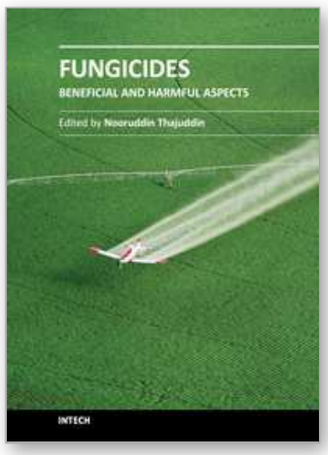

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Fungicides are a class of pesticides used for killing or inhibiting the growth of fungus. They are extensively used in pharmaceutical industry, agriculture, in protection of seed during storage and in preventing the growth of fungi that produce toxins. Hence, fungicides production is constantly increasing as a result of their great importance to agriculture. Some fungicides affect humans and beneficial microorganisms including insects, birds and fish thus public concern about their effects is increasing day by day. In order to enrich the knowledge on beneficial and adverse effects of fungicides this book encompasses various aspects of the fungicides including fungicide resistance, mode of action, management fungal pathogens and defense mechanisms, ill effects of fungicides interfering the endocrine system, combined application of various fungicides and the need of GRAS (generally recognized as safe) fungicides. This volume will be useful source of information on fungicides for post graduate students, researchers, agriculturists, environmentalists and decision makers.

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