

Comparative In-Silico Study of β -Tubulin Proteins from *Arthrobotrys musiformis* for Resistance to Benzimidazole

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Abstract—*Arthrobotrys musiformis* is a biocontrol agents which can be used in the integrated pest management strategies. Although this isolate was found to show high benzimidazole resistance as compared to the other biocontrol agents, considering the dose of administration of anthelmintics, a need for developing an improved strain of this fungus resistant to chemical anthelmintics was observed. In the present study improved strains of *Arthrobotrys musiformis* were obtained using random mutagenesis technique carried out both by chemical mutagenic agent, Ethyl methane sulfonate (EMS) and physical mutagenic agent, UV radiation. The obtained mutants were screened using their ability to survive in high benzimidazole concentration, isolated, selected based on their biocontrol activity and characterized for their suitability as biocontrol agents. The β -tubulin genes of the selected mutants were finally studied.

Index Terms—*Arthrobotrys musiformis*, benzimidazole resistance, beta-tubulin genes, biocontrol agents, mutagenesis.

I. INTRODUCTION

Biological control is an important component of an integrated pest management strategy. It is defined as the reduction of pest populations by natural enemies and typically involves an active human role. For the biological control of nematodes the most common approach is the use of soil borne fungi. In an attempt to obtain improved strains of nematode trapping fungi from different agro-climatic regions of India we have already reported a new isolate of *Arthrobotrys musiformis* as a potential biocontrol candidate against *Haemonchus contortus* [1]. Although this isolate showed high benzimidazole resistance as compared to the other biocontrol agents, considering the dose of administration of anthelmintics, a need for developing an improved strain of this fungus resistant to chemical anthelmintics was observed. (Table I)

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TABLE I: DOSAGE OF ANTHELMINTIC (BENZIMIDAZOLE) IN VARIOUS RUMINANTS.

Species	Thiabendazole Safety Ratio	
	First Sign of Reaction	Maximum Recommended Dose (mg/kg)
Goat	66	>1600
Sheep	66	>600
Cattle	100	>1600
Horse	44	>1200

Earlier reports suggest Benzimidazole resistance to be caused by point mutations in β -tubulin gene [2], [3]. The use of recombination in commercial strain improvement can be significant especially for the alteration of qualitative traits [4]. However, in this group of fungi; i.e. fungi imperfecti, which lack the sexual mode of reproduction recombination becomes difficult. Therefore, in such cases strain improvement is normally achieved by mutagenesis [5] or protoplast fusion [6].

In the present study improved strains of *Arthrobotrys musiformis* were obtained using random mutagenesis technique carried out both by chemical mutagenic agent, Ethyl methane sulfonate (EMS) and physical mutagenic agent, UV radiation.

The obtained mutants were screened using their ability to survive in high benzimidazole concentration, isolated, selected based on their biocontrol activity and characterized for their suitability as biocontrol agents. The β -tubulin genes of the selected mutants were finally studied.

II. MATERIALS AND METHODS

A. Fungal Culture

A nine mm diameter plug from the edge of the colony, of a one week old pure culture of *A. musiformis* maintained on 2% CMA (Corn Meal Agar) (Hi Media, Mumbai), containing 35 μ g/ml Tetracycline and 100 μ g/ml Ampicillin, was inoculated in Potato Dextrose Broth (Hi Media, Mumbai) containing 35 μ g/ml Tetracycline and 100 μ g/ml Ampicillin and incubated in static conditions for seven days.

B. Harvesting of Spores

The culture obtained above was shaken vigorously and filtered through sterile muslin cloth, followed by centrifugation of the filtrate at 5000 rpm for 10 minutes. The supernatant was discarded and the pellets washed twice with sterile double distilled water. Spores were counted using Neubeurg's chamber and diluted to a concentration of 10^6 - 10^7 .

C. Development of Mutants

1) Treatment with physical mutagen

One ml of spore suspension was taken and exposed to UV irradiation for ten minutes. [7] Of this 200µl was taken and spread on 2% CMA plates without benzimidazole to serve as control. The remaining spore suspension was spread in the batches of 200µl on four plates of 2% CMA containing 100µl/ml benzimidazole (Selection Media) and allowed to grow at 27°C.

2) Treatment with chemical mutagen

100µl of EMS was mixed with 8.9 ml of 0.05M phosphate buffer [8]. To this 1ml of spore suspension was added and allowed to incubate for one hour (10 times dilution). After one hour the above suspension was further diluted ten times. Of this 200µl was spread on 2% CMA plates without benzimidazole and incubated at room temperature. The remaining spore suspension was spread in batches of 200µl on four plates of 2% CMA containing 100µl /ml benzimidazole (Selection Media) and allowed to grow at 27°C.

D. Isolation of Mutants

On incubation the mutated spores of interest tend to survive on media containing Benzimidazole. The colonies obtained were plugged out and placed inverted on to fresh plates containing selection media and allowed to grow at 27°C.

E. Selection of Mutants

In order to ascertain the presence of biocontrol activity of the mutants, they were subjected to a bioassay for capturing. Plates containing pure seven days old colonies of mutants were inoculated with 50 nematodes per plate and incubated at room temperature. After 24 hours, capturing of nematodes was checked by observing the plates under a compound microscope. [9] The assay was carried out twice.

F. Characterization of Mutants

In order to select the most suitable mutant for field application, growth parameters such as optimum pH, optimum temperature, nematode capturing efficiency and LD50 for benzimidazole were studied.

G. Morphological Analysis

Five day old pure fungal cultures were used. Slides were prepared by spreading small inoculum of the fungi using sterile forceps taken from the periphery of the cultures. These were mounted in 25% Lactophenol Blue stain and covered slowly with clean cover slips avoiding bubbles. These were then observed in a compound microscope and images of the fields of interest were taken using a Carl-Zeiss MPM 400 image analyzer.

H. Optimization of Parameters

Nine mm diameter mycelial plugs, taken from the edge of the colonies of seven days old culture plates of each mutant, were inoculated individually in the center of four different plates containing 2% CMA, 5.4% PDA, 4.9% CDA (Czapek Dox Agar) and 4.83% WA (Wort Agar), each amended with 50 µg/ml Benzimidazole, 100µg/ml Ampicilin and 35µg/ml Tetracycline. The plates were incubated at 27°C and the

radial growth (colony diameter) of each isolate was measured, with the original mycelial plug subtracted from each measurement, at the interval of 24 hours for 5 days. Two measurements, each perpendicular to the other, were taken for each plate and the mean calculated. From the above data the rate of growth for each fungus was calculated. The experiment was performed in duplicate and repeated twice in order to optimize the media of growth. Similar experiments were performed to optimize the pH and temperature of growth.

I. Determination of LD50 for Benzimidazole

Nine mm diameter mycelial plugs, taken from the edge of the colonies of seven days old pure cultures of each mutant, were inoculated individually in the center of 9 plates containing 2% CMA (Hi Media, Mumbai) or PDA (Hi Media, Mumbai), depending on the culture requirements, amended with 0, 50, 100, 150, 200, 250, 300, 350, 400 µg/ml Benzimidazole, 100µg/ml Ampicilin and 35µg/ml Tetracycline. The plates were incubated at 27°C and the radial growth (colony diameter) of each isolate was measured, with the original mycelial plug subtracted from each measurement, at the interval of 24 hours for 5 days. Two measurements, each perpendicular to the other, were taken for each plate and the mean calculated. From the above data the rate of growth for each fungus along with its LD50 was calculated. The experiment was performed in duplicate and repeated twice.

J. Nematode capturing Efficiency

A set of five petri dishes containing 2% CMA (Hi Media, Mumbai) or PDA (Hi Media, Mumbai), depending on the culture requirements, amended with 50 µg/ml Benzimidazole, 35mg/ml Tetracycline and 100µg/ml Ampicilin, were inoculated with nine millimeter diameter mycelial plugs taken from the edge of a one week old pure culture of each mutant in the center of the plates. These plates were incubated at 27 °C in the dark for 1 week. Hundred nematodes were added to these petriplates. Petri plates inoculated with 100µl of water instead of nematodes served as control.

The dishes were kept in dark at 27°C. After 24 hours one of the plates was flushed with water and the drained water collected. The nematodes in the collected water were counted using optical microscope (10X), taking three 10µl aliquots, counting the average number of nematodes and extrapolating the total volume.

This procedure was repeated at 36 and 48 hours using the remaining plates. From this data the percentage capturing at various time intervals was calculated. The experiment was performed in duplicate and repeated twice.

K. RAPD Analysis

To identify which of the obtained mutants was closest to *Arthrobotrys musiformis* in terms of the genetic constitution, a molecular characterization by RAPD was done as described by B. M. S. Jarullah et. al. [10]

1) Study of β-tubulin sequences

With the aim to verify the increase in resistance to be linked to the mutations in the β-tubulin gene, the β-tubulin genes of the mutants genetically closer to *Arthrobotrys*

musiformis to were sequenced and analyzed

2) Sequencing of β -tubulin gene

a) Synthesis of β -tubulin primer and PCR amplification

β -tubulin gene specific primers were obtained from Genei, Bangalore. These were used to amplify β -tubulin gene from the genomic DNA of each mutant along with the wild strain. PCR reaction was set up under sterile conditions in 200 μ l capacity PCR tubes. The PCR mixture contained 200 ng of template DNA, 33 ng of oligonucleotide primer (5' aac atg cgt gag att gta agt 3', 5' tct gga tgt tgt tgg gaa tcc 3'), 1X PCR reaction buffer with 1.5 mM MgCl₂, 250 μ M of each dNTP and 2 unit of Taq DNA polymerase in final reaction volume of 50 μ l. PCR reaction was conducted using Eppendorf thermal cycler. The thermal profile used was as follows: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds, primer annealing at 51°C for 1 minute, extension at 72°C for 1.5 minutes and final extension at 72°C for 10 minutes. After completion of the PCR reaction, amplification product was electrophoresed at 100 volts in 2% Agarose gel, stained with ethidium bromide, viewed under UV light and photographed. DNA ladder (100 bp) was used as the molecular size marker.

b) Sequencing of β -Tubulin gene

The β -tubulin gene of each mutant was eluted and purified. Purity of the eluted DNA was checked on 2% agarose gels containing ethidium bromide. Electrophoresis was performed at 100V for approx. 2 h [11]. Sequencing of purified gene was carried out using fluorescent dye terminator method in an ABI 377 PRISM sequencer (Applied Biosystems, California) at Genei, Bangalore

3) Analysis of β -tubulin sequences

The sequenced β -tubulin genes were aligned and a cladogram was generated using ClustalW program (random seed number, 111; bootstrap value, 1000). This data was then compared with the results obtained from the assay for the carbendazim resistance. A detailed study of the sequences of these β -tubulin genes in terms of their amino acid composition and mutations was also done.

III. RESULTS

A total of 15 mutants resistant to Benzimidazole, one from U.V. treatment and the others from EMS treatment were obtained. Of these, only five mutants, all obtained from EMS treatment, showing capturing ability were chosen for further characterization.

The study of the morphological changes of the mutants as compared to the wild culture showed dark pigmented colonies in all mutants. Contrary to the wild culture, which showed a thread like appearance with aerial mycelia growing vertically on the surface of the media, these mutants had a mat like appearance.

All the mutants exhibited smaller spores in comparison to the spores of the wild culture. This was accompanied by loss of the candle-like pattern in all mutants except in M4. The conidia were bottle shaped and small chlamydospores were observed in mutants M1, M2 and M5. (Fig 1)

Although all the mutants showed good growth in both CDA as well as CMA, however mutants M2 and M4 were

similar to *Arthrobotrys musiformis*, which showed higher growth rate in CMA as compared to CDA. All the mutants were found to have reduced rate of growth in Wort agar.

The pH 7 was found to be the optimum pH for all the mutants except M4, which showed higher rate of growth at pH 9.

The range of temperature between 20-30°C provided a good environment for rapid growth of all mutants. The optimal growth temperature for mutants M1, M3 and M5 was $27 \pm 2^\circ\text{C}$, similar to that of *Arthrobotrys musiformis*, with mutants M2 and M4 showing higher rate of growth at 30°C.

The growth rates of the mutants at the various benzimidazole concentrations showed a very interesting pattern. Not only did all the mutants survive at a high concentration around 400 μg of benzimidazole per ml of media, but mutants M2, M3, M5 and M4 showed a better rate of growth in the presence of 50 μg of benzimidazole per ml of media. For the mutant M1 highest rate of growth was observed in media amended with 100 μg of benzimidazole per ml. Based on the rate of growth at various concentrations of benzimidazole the LD50 values of the five mutants were also obtained. (Table II)

In the biocontrol efficiency studies highest activity was observed for mutant M2 with 100 % capturing within 24 hours of baiting of nematodes. Mutants M1, M3 and M4 showed 100% capturing only after 48 hours where as mutant M5 showed total capturing within 36 hours of baiting.

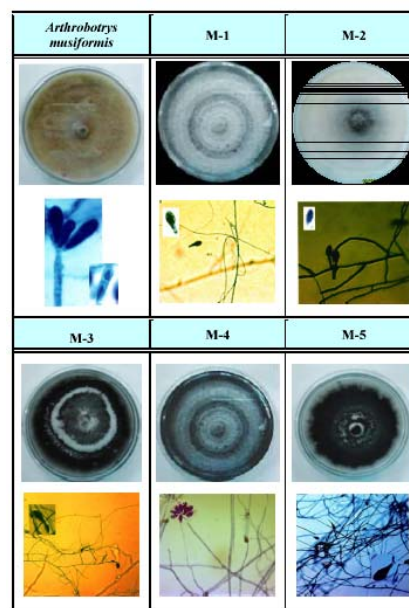


Fig. 1. Colonial and spore morphology of the mutants and wild culture

TABLE II: LD50 VALUES OF THE FIVE MUTANT STRAINS IN THE PRESENCE OF BENZIMIDAZOLE.

Sr. No.	Mutant	LD50 ($\mu\text{g/ml}$)
1	M1	231 \pm 0.35
2	M2	228 \pm 0.56
3	M3	230 \pm 0.17
4	M4	238 \pm 0.67
5	M5	225 \pm 0.35
6	Wild	0.5 \pm 0.12

A lot of variations in the RAPD profiles were observed based on the combination of the primer and the mutant used.

The Jaccard's similarity coefficient based on the RAPD data ranged from 0.08 to 0.5.

The final dendrogram generated showed two main clades; one in which *Arthrobotrys musiformis* and mutant M5, formed a sub-node in close proximity to the mutant M1 and the other in which mutants M4, M3 and M2 were grouped together. (Figure II)

Mutant M5 and the wild strain showed maximum

similarity in the parameters studied. The cladogram generated using the β -tubulin gene sequences of the mutants M1, M5, and M4 along with *Arthrobotrys musiformis*, although with some variation, showed pattern similar to that of the dendrogram generated in the RAPD analysis. M5 was found closest to *Arthrobotrys musiformis*, where as M1 and M4 were found in two separate nodes. (Figure III)

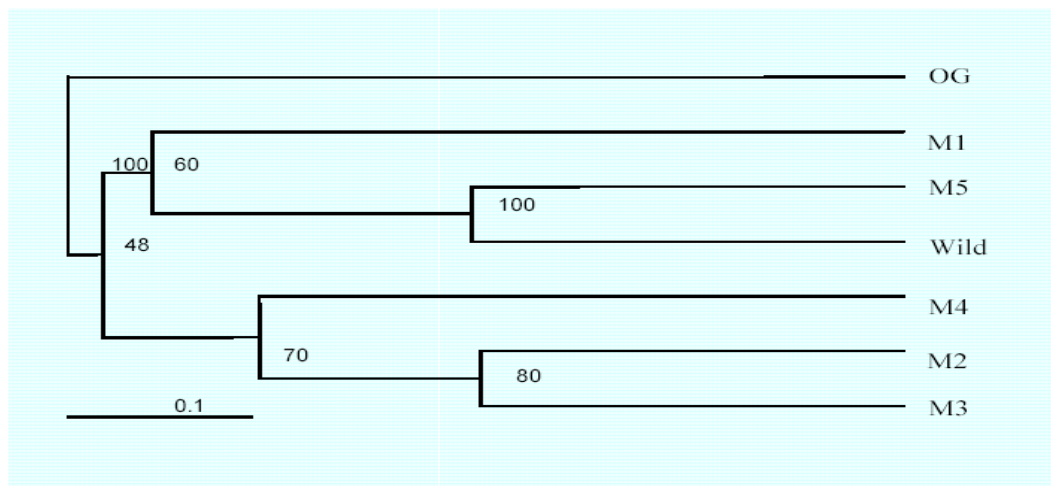


Fig. 2. Phylogenetic relationship of the wild culture, mutants and the out group based on genetic distance estimated by Jaccard's similarity coefficient

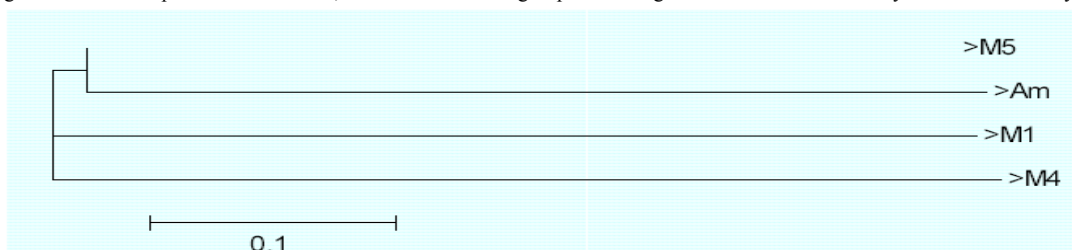


Fig. 3. Cladogram generated using the β -tubulin sequences of three mutant strains along with the wild culture using Clustal W.

TABLE III: COMPARISON OF THE B-TUBULIN GENE OF *ARTHROBOTRYS MUSIFORMIS* AND MUTANTS.

Variants	Percentage of non-polar amino acids	Amino acid substitution			
		At position 198		At position 200	
Wild	38.8 ± 0.56	D	Polar	C	Polar uncharged
M-1	41.8 ± 0.23	A	Non Polar	F	Non Polar
M-4	39.2 ± 0.53	G	Polar uncharged	A	Non Polar
M-5	38.9 ± 0.43	L	Non Polar	A	Non Polar

The analysis of the amino acid sequences of the β -tubulin genes of the mutants, showed multiple mutations in all the sequences with no specific pattern. The total percentage of non polar amino acids was found to be increased in all the mutant sequences with point mutations at codon number 198 and 200. A detailed comparison of the sequences of the β -tubulin genes from the mutants and *Arthrobotrys musiformis* is shown in Table III.

IV. DISCUSSION

Based on the above results, it is clear that EMS mutagenesis is more reliable as compared to physical mutation using Ultra Violet radiations, to obtain

benzimidazole resistant mutants. Probably, due to the photoreactivation of the fungi on exposure to visible light, mutation by UV irradiation is less effective.

Of the total of fifteen mutants obtained from the mutation experiments, only 5 showed positive results in the nematode capturing bioassay. It was therefore assumed that in the other 10 mutants the gene responsible for the biocontrol activity might have been mutated. The five mutant showing nematophagous activity were thus selected for further studies.

An attempt was then made, to analyze these mutants for their proximity with the original wild fungus, in terms of their growth parameters. This was necessary, as the original culture of *Arthrobotrys musiformis* had already been characterized for its compatibility in field application and had showed very promising results [1] Similar growth parameters would ascertain that the obtained mutants would also be as compatible to field conditions. The results of the above study showed that all the mutants except M4 were close to the wild culture in their growth characteristics. M4, however, not only showed 30°C as an optimum temperature of growth, but was also found to have an optimal pH of 9. Although it could grow well at other temperatures also, its inability to survive well at pH 7 made it unfit for field application. However, the over all rate of growth of all the mutants was found to be

lower to that of the wild culture.

Since, the mutant fungi would have to resist high concentrations of the benzimidazoles present in the gut of the ruminants supplied in the form of anthelmintics, a higher LD50 value would therefore enable a better survivability of the fungi in field conditions. The study of the LD50 values was thus another important criteria that would lead to an understanding of most suitable mutant.

This study showed very promising results. The mutant strains of *A. musiformis* had LD50 values ranging between 225 and 240 µg of benzimidazole per ml of media, with M5 having the highest value of 238 µg.

When the rate of growth of the mutants at various benzimidazole concentrations was studied, a very interesting pattern was observed. Not only did all the mutants survive at a high concentration around 400 µg of benzimidazole per ml of media, but mutants M2, M3, M5 and M4 showed a better rate of growth in the presence of 50 µg of benzimidazole per ml of media. For the mutant M1 highest rate of growth was observed in media amended with 100 µg of benzimidazole per ml. Probably these mutants had some mutation, which showed preferential growth in the presence of benzimidazole.

Each of the mutants also showed high biocontrol efficiency with 100 % capturing within 48 hours of baiting of nematodes. All these parameters made these mutants equally suitable for field application.

Although with many advantages, a major drawback of random mutagenesis technique is its non- specificity. Apart from the expected mutations in the beta tubulin gene conferring resistance to Benzimidazole, various other mutations in many other genes might also have occurred. Therefore, a characterization of these mutants at the genetic level is absolutely necessary. Therefore, to complete the search for the right mutant, a molecular characterization of these mutants to assess the genetic variability with respect to the wild type using Randomly Amplified Polymorphic DNA (RAPD) was carried out.

The dendrogram generated based on the RAPD profiles obtained using eight different primers, distinctly indicated M5 to be genetically closest to *Arthrobotrys musiformis*. Of all the mutants obtained M5 was closest to the wild culture with a Jaccard's similarity coefficient of 0.531 followed by M1 having 0.189 similarity coefficient. M4, M2 and M3 had very low similarity coefficients and fell into entirely separate nodes. All the above data therefore indicated that the mutant M5 was highly suited for field applications as a new strain of biocontrol agent against nematodes.

To study the mutations caused in the beta tubulin genes of these mutants a final study to analyze the β-tubulin sequences was carried out.

Owing to very high levels of mutations with no specific

pattern, not much analysis of the sequences could be done. It was however observed that there was a definite increase in the percentage of non polar amino acids in the mutant β-tubulin sequences. Furthermore, all the sequences from the mutants had point mutations at codon 198 as well as 200, which lead to an amino acid change from polar to non polar. This study further supports our earlier view that not only the presence of specific mutations in the β-tubulin gene correlated to the variation in resistance to benzimidazole, but the over all sequence of the gene with the three dimensional structure of its protein and the percentage of polar and non-polar amino-acids also play a vital role in conferring benzimidazole resistance to these fungi. [12]

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