

Influence of the Percentage of Polar and Non-polar Amino-acids in the β -tubulin gene of the Nematophagous fungus: *Arthrobotrys musiformis* in conferring Benzimidazole Resistance

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Abstract. *Arthrobotrys musiformis* is a biocontrol agent 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 our earlier study improved strains of *Arthrobotrys musiformis* were obtained using random mutagenesis technique. Reports suggest benzimidazole resistance to be caused by point mutations in β -tubulin gene. The current study therefore focuses on analyzing the β -tubulin genes of the selected mutants in comparison with the wild type fungus to understand its role in benzimidazole resistance. All the mutants were analyzed for their LD50 values followed by sequencing of the β -tubulin genes. A cladogram was generated of the sequenced β -tubulin genes followed by a detailed study of the sequences in terms of their amino acid composition and mutations. The results suggest increased percentage of non-polar amino acids in the β -tubulin gene to confer higher benzimidazole resistance to this fungus.

Keywords: β -tubulin genes, *Arthrobotrys musiformis*, benzimidazole, non-polar amino acids.

1. 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. The use of recombination in commercial strain improvement can be significant especially for the alteration of qualitative traits (2). 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 (3) or protoplast fusion (4) Improved strains of *Arthrobotrys musiformis* were obtained using random mutagenesis technique.(5) Earlier reports suggest Benzimidazole resistance to be caused by point mutations in β -tubulin gene (6, 7). In an attempt to understand the molecular basis of resistance to benzimidazole the current study focuses on a detailed analysis of the β -tubulin genes of the selected mutants in comparison with the wild type fungus.

2. Materials and Methods

2.1. Fungal Cultures

Wild type strains of *A. musiformis* along with mutants showing high benzimidazole resistance obtained from random mutagenesis studies of B. M. S. Jarullah et. al. (5) were used. Mutant M1, M2, M3, M4 and M5 were chosen based on their growth parameters and nematode capturing ability. The cultures were maintained on 2% CMA (Hi Media, Mumbai), containing 35mg/ml Tetracycline and 100mg/ml Ampicillin. 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 (Hi Media, Mumbai), containing 35mg/ml Tetracycline and 100mg/ml Ampicillin, was inoculated in Potato Dextrose Broth (Hi Media, Mumbai) containing 35mg/ml Tetracycline and 100mg/ml Ampicillin and incubated in static conditions for seven days. These cultures were further used for DNA isolation.

2.2. 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 Ampicillin 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.

2.3. Isolation of DNA

Isolation of DNA was done using liquid nitrogen as per the standard protocol described by B. M. S. Jarullah et. al. (8)

2.4. Study of β -tubulin sequences

With the aim to understand the molecular basis of resistance to benzimidazole the β -tubulin genes of the selected mutants and the wild type fungus were sequenced and compared.

2.4.1 Sequencing of β -tubulin gene

2.4.1.1 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 µl 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 µM of each dNTP and 2 unit of Taq DNA polymerase in final reaction volume of 50 µl. 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.

2.4.1.2 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 (9). Sequencing of purified gene was carried out using fluorescent dye terminator method in an ABI 377 PRISM sequencer (Applied Biosystems, California) at Genei, Bangalore.

2.4.2 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 benzimidazole resistance. A detailed study of the sequences of these β -tubulin genes in terms of their amino acid composition and mutations was also done using Genedoc.

3. Results and Discussion

The study of the LD50 values 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. (Table 1)

The cladogram generated using the β -tubulin gene sequences of the mutants M1, M5, and M4 along with *Arthrobotrys musiformis*, showed M5 to be closest to *Arthrobotrys musiformis*, whereas M1 and M4 were found in two separate nodes. (Figure 1)

Although with many advantages, a major drawback of random mutagenesis technique is its non-specificity. 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. 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. (Table 2) 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 overall 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. (10)

4. References

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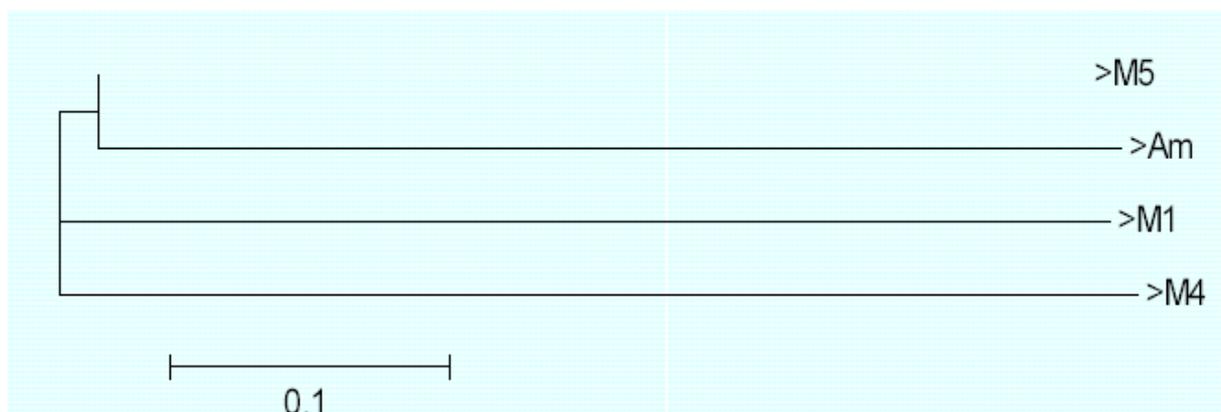


Fig. 1: Cladogram generated using the β -tubulin sequences of three mutant strains along with the wild culture using Clustal W

Table 1: LD50 values of the five mutant strains in the presence of benzimidazole

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

Table 2: Comparison of the β -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 \pm 0.56	D	Polar	C	Polar uncharged
M-1	41.8 \pm 0.23	A	Non Polar	F	Non Polar
M-4	39.2 \pm 0.53	G	Polar uncharged	A	Non Polar
M-5	38.9 \pm 0.43	L	Non Polar	A	Non Polar