Isolation and molecular characterization of β-tubulin gene from *Arthrobotrys musiformis*—A nematode trapping fungus

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A new isolate of *Arthrobotrys musiformis*, a naturally occurring predacious hyphomycete, has been found to be effective against *Haemonchus contortus*, a roundworm, which seriously affects milk and meat production in ruminants. However, further studies on the suitability of this fungus for use in integrated pest management (IPM) showed that it is highly susceptible to benzimidazole derivatives, which are the most common anthelmintics currently used for deworming cattle. Injudicious and indiscriminate use of heavy metals, pesticides, fungicides and various other agrochemicals are now known environmental pollutants, adversely affecting even the beneficial micro flora and fauna. A recent study on *A. musiformis* has indicated that the fungus varies in its sensitivity to different heavy metals and fungicides.

Benzimidazoles are a group of broad-spectrum systemic fungicides, which interact with tubulin and especially with β-tubulin protein. Resistance to benzimidazole and its derivatives in filamentous fungi has been reported to be associated with specific mutations in the β-tubulin gene. In the present study, the authors report the isolation, cloning and partial sequencing of the β-tubulin gene of *A. musiformis*.

Genomic DNA was isolated from 10 d-old culture of *A. musiformis* by homogenizing the fungal mat using liquid nitrogen. DNA was extracted with extraction buffer containing 0.1 M Tris (pH 7.1-7.4), 0.5 M EDTA, 3% SDS followed by phenol-chloroform step. DNA was precipitated by ethanol and the purity was checked both quantitatively and qualitatively.

β-tubulin gene specific forward (5′ GGTAACC-AAATCGGTGCTGCTTTC 3′) and reverse (5′ ACCCTCAGTGATGACCCCTTGCC 3′) primers were obtained from Genei, Bangalore. They were used to amplify β-tubulin gene of *A. musiformis* through PCR. The 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 each of forward and reverse primer (24 mer), 1X PCR reaction buffer with 1.5 mM MgCl\(_2\), 250 µM each of dNTPs and 2 units of *Taq* DNA polymerase in a final reaction volume of 50 µL. PCR was carried out using an Eppendorf thermal cycler. All amplifications were carried out with an annealing temperature of 27°C for 1 min and an extension time of 5 min, followed by 40 cycles. After completion of the PCR reaction, the amplified product was electrophoresed at 100 volts in 2% agarose gel, stained with ethidium bromide and viewed in UV light.

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Nematophagous fungi are mainly soil living fungi that are parasitic on nematodes. Their predacious activity suggests that they are the determinants of the population density of nematodes in nature and may be of value for the biological control of nematode pests. A strain of *Arthrobotrys musiformis* was isolated from soil samples in Gujarat and the laboratory studies indicated that it fulfills all the necessary criterion to be a potential biocontrol agent against *Haemonchus contortus*, a roundworm, which seriously affects milk and meat production in ruminants. However, further studies on the suitability of this fungus for use in integrated pest management (IPM) showed that this fungus is highly susceptible to benzimidazole derivatives, which are the most common anthelmintics currently used for deworming cattle.
Amplified genomic DNA of *A. musiformis* (900 bp) purified from agarose gel using Geneclean (Bio 101) was cloned into a pGEM T-Easy vector (Promega). Ligation reaction was set-up in a final volume of 10 μL containing 1:1 ratio of vector (50 ng pGEM-T-Easy) insert, 1X ligation buffer and 1 unit T4 DNA ligase. The resultant reaction mixture was incubated at 14-16°C for 16 h.

50 μL of *Escherichia coli* competent cells DH5α (Lac Z repressor) were mixed with two micro litres 0.5M β-mercaptoethanol by gentle stirring as per methods of Sambrook *et al*. To this, 2 μL of thoroughly mixed ligation product was added, mixed and incubated on ice for 30 min. Heat shock treatment was given at 42°C in a water bath exactly for 2 min followed by incubation on ice for 5 min. To this, 250 μL of SOC medium was added and incubated at 37°C for 1 h at 225 rpm.

Fifty and 200 μL of the transformation product obtained were spread on LB/X-Gal/IPTG plates containing 100 μg/mL of ampicillin followed by incubation at 37°C for 18 h. These plates were kept at 4°C for 2-3 h in order to allow proper development of colour. Insert containing white colonies were used for plasmid DNA isolation and restriction enzyme digestion. Plasmid DNA was prepared from one of the positive clones by alkali lysis method. The recombinant plasmid DNA was subjected to restriction digestion using EcoRI. 5 μL of recombinant plasmid DNA, 2 μL of EcoRI assay buffer (10X), 1 μL of enzyme and 12 μL of sterile distilled water was mixed thoroughly and incubated at 37°C for 1 h. The mixture was then analyzed by agarose gel electrophoresis.

Sequencing of purified recombinant plasmid DNA was carried out by automated DNA sequencer with fluorescent dye terminator using ABI 377 PRISM™ sequencer (Applied Biosystems, California) at Genei, Bangalore using SP6, T7 promoters for primer annealing in the cloning vector. Sequence alignments were carried out employing CLUSTAL W program. Similarity search against the databases was done using the basic local alignment search tool in the BLAST (National Center for Biotechnology Information, Bethesda).

Agarose gel electrophoretic analysis of PCR product revealed a single amplicon of approximately 900 bp when *A. musiformis* genomic DNA was amplified by using β-tubulin gene specific forward and reverse primers. PCR product obtained was successfully cloned into pGEM-T-Easy vector. Eighty per cent of the transformed *E. coli* colonies were white in colour when plated on X-Gal/IPTG plates. Plasmid DNA was isolated and insert was released after digestion with *Eco*RI. Two fragments of approximately 450 bp were obtained, which indicated the presence of an internal *Eco*RI site in the insert (Fig. 1).

The PCR product (900 bp) obtained from β-tubulin PCR was ligated into the pGEM-T-Easy vector, generating the pAMTub construct. The insert was sequenced and a readable sequence of 650 bp was obtained. Out of this, a sequence of 321 bp was submitted to NCBI after removal of primer and vector contamination and was assigned a GenBank accession number AY: 963560. The homology search of GenBank and EMBL databases using BLAST program revealed 95% homology with β-tubulin gene of *A. oligospora* followed by other filamentous fungi.

Benzimidazoles are important antitubulin agents used in the veterinary medicine as well as plant disease control. Resistance is a practical problem correlated with single amino acid change in β-tubulin and is often linked to greater sensitivity to phenylcarbamates. What is particularly interesting about benzimidazole resistance is its link with increased sensitivity to phenylcarbamates, which do...
not affect wild type strains. This negative cross-resistance creates opportunities for durable anti-resistance strategies. Attempts to understand the molecular basis of benzimidazole resistance have been hampered by the inability to purify tubulin from filamentous fungi.

β-Tubulin plays a major role in conferring resistance to fungicides, where the property is very important in field application of biocontrol agents. Resistance is a major practical problem in the use of the fungicides; it is caused by point mutations in β-tubulin, which replaces Glu_{198} with Ala, Val or Gly, or Phe_{200}, or with Tyr or His to Tyr. The last mutation mentioned is particularly said to be very significant since it confers a high degree of resistance to fungicides. The same mutations are also encountered in veterinary medicine where parasitic nematodes have become resistant to treatment with benzimidazoles. At least one of these mutational sites within β-tubulin confers resistance to benzimidazoles in laboratory strains.

The β-tubulin gene sequence, which was analyzed from our study lacks mutations that confer resistance against fungicides. The bioassay using the fungus with carbendazim, a benzimidazole derivative, showed that the fungi is susceptible to this compound. In the present investigation, the mutation at position number 6 on Histidine in the β-tubulin gene sequence of A. musiformis was not encountered. Our study, therefore, confirms earlier reports, which linked mutation in β-tubulin gene sequence to resistance against benzimidazole and its derivatives.

The results may prove to be useful for future programmes to improve the fungal strain, A. musiformis, for commercial application.

References