Deciphering β -tubulin gene of carbendazim resistant *Fusarium solani* isolate and its comparison with other *Fusarium* species

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Abstract

Carbendazim resistance in phytopathogenic *Fusarium* species is being an immense problem in agricultural practices. Carbendazim exerts fungicidal activity through preventing cell division of target fungi and by binding to β -tubulin, a major component of microtubule. Several point mutations in the β -*tubulin* gene contribute to carbendazim resistance. A full-length (1619 bp) β -*tubulin* gene has been cloned and sequenced from a carbendazim resistant (SF0301) and a sensitive (SF0104) isolates of *F. solani* isolated from agricultural fields of Murshidabad (24.23°N, 88.25°E), West Bengal, India. The phylogenetic position of the isolates was confirmed using ITS and β -*tubulin* gene sequences. The β -*tubulin* based phylogenetic tree revealed nine species complexes among *Fusarium* species with available data and separated members of both *F. solani* species complex and *F. fujikuroi* species complex into three clades. The β -*tubulin* gene of *F. solani* isolates was organized into five exons and four introns. The intron positions were conserved, but their lengths were shortest among the *Fusarium* species. The β -*tubulin* gene encoded a 446 amino acid protein with a calculated molecular mass 49.834 KD, extinction coefficient $41640 \text{ M}^{-1}\text{cm}^{-1}$ and an estimated isoelectric point of 4.64. Amino acid sequence comparison between the resistant and the sensitive isolates revealed that they were all identical, indicating that the mechanism of carbendazim resistance in *F. solani* SF0301 was not due to point mutation in β -*tubulin* gene. Homology model of β -tubulin of *F. solani* isolates was prepared and compared with that of other *Fusarium* species. The binding sites for GDP, carbendazim and α -tubulin were identified through molecular docking.

1. Introduction

β -tubulin is the key component of the microtubule wherein it is assembled with α -tubulin as heterodimer in head-to-tail manner and involved in many cellular activities, such as cell division, ciliar or flagellar motility, and intracellular transport in eukaryotic organisms (Zhao et al. 2014). This protein is also the target for benzimidazole fungicides such as carbendazim, that are used as foliar spray, seed treatment and post-harvest application to control many plant pathogenic fungi. Through binding at specific site(s) of β -tubulin they could inhibit cell division in target fungi. In most of the phytopathogenic fungi, resistance against the fungicide is associated with single point mutation at several target sites in the β -*tubulin* gene, which results in altered amino acid sequences and change in carbendazim-binding site (Ma and Michailides 2005). This causes a reduction in the affinity of the β -tubulin for the fungicide, without interfering with normal biological function of β -tubulin in the fungus. Sometimes a wide range of carbendazim-resistance could be found instead of carrying different mutations at similar amino acid residue and different substitutions at the same codon can exhibit distinct level of resistance (Albertini et al. 1999).

Tertiary structure of any protein provides an important insight into the molecular mechanism of its function, which forms the basis for intending various approaches for structure-based drug designing or altering its structure by site-directed mutagenesis. X-Ray crystallography and NMR are no doubt advance techniques to provide high definition structure of protein, but the disadvantages are that these techniques

are often prolonged, costly and require large quantity of purified protein. Homology based modeling of protein is an competent computational tool that can be used for predicting the structure of unknown protein based on previously resolved three-dimensional structure of other associates of the same relative having similar folds and/or function. To date, there are hardly any studies on structure-function relationship of β -tubulin of *Fusarium solani* exhibiting carbendazim resistance and in particular there is no study on structural properties of β -tubulin of *F. solani*. Hence, in order to understand their functionality and role in carbendazim resistance, it is imperative to understand molecular structure of the protein.

More than 300 phylospecies (i.e., phylogenetically distinct species) within the monophyletic genus *Fusarium* are distributed into 23 evolutionary lineages referred to as species complexes (Geiser et al. 2020; O'Donnell et al. 2020). *Fusarium solani* species complex (FSSC) is one of such lineage which evolved early and contains at least 60 phylogenetically distinct species (Schroers et al. 2016). On the basis of molecular phylogenetic relationships among isolates, O'Donnell (2000) identified three major clades of FSSC. The Clade 1 consisted of New Zealand isolates such as *F. illudens* which was originally isolated from *Metrosideros* species. The Clade 2 comprised the South American isolates representing soybean SDS (sudden death syndrome) and bean root rot pathogens, such as *F. brasiliense* etc. The Clade 3 encompassed all of the isolates formerly characterized as *Nectria haematococca* and some other species, now clustered as '*F. solani*' with different *formae speciales* based on host specificity. In addition, Asian isolates, such as *F. ambrosium*, African isolates *F. neocosmosporiellum* and a few South American isolates were included in Clade 3. The members of *F. solani* (Mart.) Sacc. are ubiquitous pathogens of many agriculturally important crops (Coleman 2016). At least 111 plant species from 87 genera are commonly infected by the pathogen (Kolattukudy and Gamble 1995). They cause rot in root and stem, wilt, and damping off in vegetables and legumes, and canker and dieback disease in tropical trees (Leslie and Summerell 2008). They are also responsible for post-harvest decay of many succulent fruits and vegetables. Some strains are associated with human infections. Over 70 *Fusarium* species have been reported to cause human infections, one-third of them are the members of FSSC which are approximately two-thirds of all the reported *Fusarium* infections (O'Donnell et al. 2020).

Previously, carbendazim resistant *F. solani* isolates (SF0204, SF0301 and SF1303) which could grow in 100 μ g/mL carbendazim and a sensitive isolate (SF0104) which failed to grow in 100 μ g/mL carbendazim have been identified from our laboratory (Tarafder et al. 2019). In this study, we have identified the phylogenetic position of the *F. solani* isolates SF0104 and SF0301 based on ITS sequence and reported for the first time, cloning and sequencing of the full-length of β -tubulin gene, that have been analyzed to detect any mutation responsible for the carbendazim resistance and to compare their nucleotide and amino acid sequences with other *Fusarium* species and the β -tubulin gene was used to construct a phylogenetic tree of *Fusarium* species. Furthermore, we have predicted the three-dimensional structure of the β -tubulin by homology modeling and the binding sites of GDP, carbendazim and α -tubulin were identified through molecular docking.

2. Materials And Methods

2.1 Fungal isolates

A number of *Fusarium solani* isolates were isolated from agricultural fields of Murshidabad (24.23°N, 88.25°E), West Bengal, India, identified based on morphological characterization and rRNA gene sequencing and their carbendazim sensitivity was studied (Tarafder et al., 2019). Among these, *F. solani* SF0104 was considered as carbendazim sensitive as it showed only 6.7% growth in 10 µg/mL carbendazim and failed to grow in 100 µg/mL carbendazim with ED₅₀ value 0.49 µg/mL. By contrast, *F. solani* SF0301 was carbendazim resistant and could grow in 100 µg/mL carbendazim with ED₅₀ value 0.98 µg/mL. The pure cultures of the isolates were maintained on potato dextrose agar (PDA) medium at the Mycology and Plant Pathology Research laboratory, Department of Botany, University of Kalyani, India.

2.2 Preparation of phylogenetic tree of FSSC based on ITS sequence

Genomic DNA was extracted from fresh mycelia using the DNeasy kit (Qiagen, Germany) according to the manufacturer's instruction. The ITS (internal transcribed spacer) of rDNA regions were amplified using ITS5 (F) [5'-GGAAGTAAAAGTCGTAACAAGG-3'] and ITS4 (R) 5'-TCCTCCGCTTATTGATATGC-3'] (Dutta et al. 2018). ITS sequences of the *Fusarium* species of FSSC were retrieved from GenBank (<https://www.ncbi.nlm.nih.gov/genbank>) and Al-Hatmi et al. (2018), and used for preparation of phylogenetic tree with MEGA X software (Kumar et al. 2018). The genetic relationships were investigated by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 44 nucleotide sequences with a total of 1189 positions in the final dataset. The confidence of the branches was determined with bootstrap analysis in 1000 samplings.

2.3 Cloning, sequencing and analysis of *β-tubulin* gene

Forward primers βOD F (5'-AACATGCGTGAGATTGTAAG-3') was designed from partial *β-tubulin* gene sequence of *F. solani* (Accession No. KF255996) and according to O'Donnell and Cigelnik (1997). The reverse primer βEx5 R (5'-TTACTCCTCGCCCTCAGGG-3') was designed from sequence alignment of a number of *Fusarium* species whose full sequences were available in database (<https://www.ncbi.nlm.nih.gov/genbank>). The start codon and termination codon in the primers are underlined. PCR amplification was performed using Perkin-Elmer PCR system in a 50 µL reaction mixture containing 5 µL of 10X PCR buffer, 25 mM MgCl₂, 10 mM dNTPs, 500 nM each primer, 1 U *Taq* polymerase (Thermo Scientific™), and 100 ng genomic DNA. The thermal programme used for PCR was as follows: a initial denaturation of 5 min at 95°C, followed by 35 cycle of denaturation for 30 sec at 94°C, annealing for 45 sec at 57°C, and extension at 72°C for 2 min, followed by a final extension for 10 min at 72°C. PCR products were purified with a gel purification kit (Qiagen, Germany), directly ligated into the pGEM-T Easy vector (Promega, USA) and sequenced with the vector primers and internal primers

using Automated DNA Sequencer (ABI 3500 Genetic Analyzer) (Perkin-Elmer, Applied Biosystem, Inc.) at the S. N. Bose Innovation Centre with Central Instrumentation Laboratory, University of Kalyani. The β -*tubulin* sequences of both the isolates were submitted to the GenBank (<https://www.ncbi.nlm.nih.gov/genbank>) and homology search was carried out using nucleotide BLAST and Fusarium MLST (<http://fusarium.mycobank.org>). Number, position and size of coding and non-coding region were identified through comparison with other sequences.

2.4 Comparison of β -tubulin nucleotide sequence and phylogenetic analysis

The β -*tubulin* sequences of both the isolates SF104 and SF301 were compared with almost full-length β -*tubulin* sequences of other *Fusarium* species which were available in GenBank database (<https://www.ncbi.nlm.nih.gov/genbank>). The alignment was made by CLUSTAL W (Thompson et al. 1994) using the Blosum matrix and standard default parameters and MEGA X software was used to construct a phylogenetic tree using 56 nucleotide sequences (Kumar et al. 2018).

2.5 Alignment of β -tubulin amino acid sequence

The DNA sequences from the putative coding region were translated into amino acid sequences with the standard code using ExPASy-Translate tool (<https://web.expasy.org/translate>). The G + C content of coding region, molecular mass, extinction coefficient and isoelectric point of the protein was calculated using web based tools (www.biologicscope.com; www.aatbio.com; www.isoelectric.org). To detect any mutation the deduced amino acid sequences of the resistant and sensitive isolates were aligned and compared. The deduced amino acid sequences of the *F. solani* isolates were compared with β -tubulin sequences of other *Fusarium* species through alignment by CLUSTAL W (Thompson et al. 1994) using MEGA X (Kumar et al. 2018).

2.6 Homology modeling of β -tubulin

Homology model of β -tubulin of *F. solani* isolates and other *Fusarium* species were carried out using the Swiss-Model programme (<https://swissmodel.expasy.org>) based on the crystal structure of *Bos taurus* (PDB code: 4O4I of chain D). There were no experimental three-dimensional structures available in PDB for β -tubulin of *F. solani*. The modeling data were then visualized and analyzed by BIOVIA Discovery Studio Visualizer. The secondary structure of the protein was developed in POLYVIEW-2D (<http://polyview.cchmc.org/>) (Porollo et al. 2004) and was compared with other *Fusarium* species.

2.7 Validation of the model

The backbone conformation of the modeled structure of β -tubulin of *F. solani* was intended by the analysis of phi (Φ) and psi (ψ) torsion angles using Ramachandran plot server (<https://zlab.umassmed.edu/bu/rama/> and <http://vadar.wishartlab.com/>), as resolved by Ramachandran plot statistics. The model was further scrutinized by ProSA (Wiederstein and Sippl 2007) and QMEAN (Benkert et al. 2009). ProSA was used to exhibit the Z-score and energy plots. VADAR

(<http://vadar.wishartlab.com/>) was used to calculate the volume area dihedral angle for fractional accessible surface area.

2.8 Identification of nucleotide binding site(s)

The nucleotide binding sites within the β -tubulin was identified using a topological approach. To recognize the nucleotide binding site of PDB file of the modeled β -tubulin we used software BIOVIA Discovery Studio v21.1.0 using ligand interaction menu.

2.9 Molecular docking

To recognize potential binding sites and the binding affinities of carbendazim (PubChem ID: 25429) for β -tubulin, web-based SwissDock program (www.swissdock.ch/docking) was used to perform automated molecular docking. The blind docking was performed using default parameters, with no region of interest defined. Docking results were visualized using UCSF Chimera v1.13.1 software and BIOVIA Discovery Studio v21.1.0.

To locate a consensus protein-protein binding conformation of α -tubulin and β -tubulin complex two different docking servers, viz., HADDOCK (High Ambiguity Driven protein-protein DOCKing) (Dominguez et al. 2003; de Vries et al. 2010) and ClusPro (Kozakov et al. 2017; Vajda et al. 2017) were used. The web-based docking tool ClusPro uses the rigid body docking method with the help of PIPER. In PIPER ligands were allowed to move while the conformation of the receptor was kept fixed. Top clusters were ranked with their representative centers on energy scoring functions. The best docked complex was chosen which would comply with the amino acid residues present in the binding interface. We also used HADDOCK web-based server for docking purpose. Rigid body docking server HADDOCK also followed the same steps as in ClusPro. The interfaces of all docked complexes were calculated using PISA server (www.ebi.ac.uk/pdbe/pisa/).

2.10 Superposition of three-dimensional protein

The constructed tertiary structure of *F. solani* was compared with that of other *Fusarium* species through superimposition in UCSF Chimera (<https://www.rbvi.ucsf.edu/chimera>) (Pettersen et al. 2004). Using web-based program SuperPose Version 1.0 (<http://superpose.wishartlab.com/>) the three-dimensional protein of *F. solani* SF0301 was superimposed with that of *F. oxysporum* f.sp. *cubense*. During the superimposition, the following parameters were deposited. To look for sub-domain matches and mismatches (e.g. hinge regions) for pair-wise sequence, the identities were considered above 80%. To identify as 'similar' aligned alpha-carbon atoms with RMSD (root-mean-square deviation) of atomic positions less than 2.0 Å were used as similarity cut off value. To identify as 'dissimilar' aligned alpha-carbon atoms with RMSDs greater than 3.0 Å were used as dissimilarity cutoff value. To set the dissimilar sub-domain, the minimum number of contiguous alpha-carbon atoms with RMSDs above the dissimilarity cutoff (above) required to be considered a 'dissimilar' sub-domain was 7 atoms.

3. Results

3.1 ITS sequence based phylogeny of FSSC members

The phylogenetic analysis based on ITS sequence separated three major clades of the FSSC members and the *F. solani* isolates SF104 and SF301 aggregated into the members belonging to Clade 3 (Fig. 1). *F. solani* SF104 was placed at the same branch with the Indian isolate *F. ambrosium* (FSSC19) and the Slovenian isolate *Fusarium* sp. (FSSC44), whereas *F. solani* SF301 was with the Italian isolate *F. solani* (FSSC5).

3.2 Molecular characterization of *β-tubulin* gene

Using the primer pair β OD F and β Ex5 R, an approximately 1.6 kb DNA fragment was amplified from both the *F. solani* isolates. Nucleotide BLAST of the DNA sequences revealed that 98 % identical with partial *β-tubulin* genes of different *F. solani* strains/isolates: WNQ3 (GenBank accession no. MK441724), FJBX18-1 (MN295050), FJBX18-2 (MN295051), and FJBX18-3 (MN295052); 97 % identical with CMFS007 (KU983876), MICMW-30.1 (KX912242), PaR-1 (MN692927), PaR-2 (MN692928), PaR-3 (MN692929), and *F. solani* strain causing root rot and stem canker on storage roots of sweet pea in China (KF255996); 96 % identical with FS-01403 (KJ572782). Based on *Fusarium* MLST identification, the nucleotide sequences showed highest similarity (97.12 %) with partial sequence (1333 bp) of *β-tubulin* gene of *F. solani* species complex NRRL 46706. The *β-tubulin* gene sequences of *F. solani* SF0104 and SF0301 were deposited in GenBank under accession numbers MZ409524 and MK900720, respectively.

By comparison with other fungi it was inferred that the full length (1619 bp) *β-tubulin* gene of both the *F. solani* isolates was organized into five exons and four introns (Fig. 3A). The intron positions were conserved and occurred after the codons for amino acids 5, 12, 54 and 316, but they varied in their lengths. All introns contained 5' and 3' consensus splice junctions similar to those in *β-tubulin* genes of other fungi that conformed to the GT-AG rule. The G+C content of the coding region was calculated as 58.61 %. Both the *β-tubulin* genes encoded a 446 amino acid protein, with a calculated molecular mass 49.834 KD, extinction coefficient 41640 M⁻¹cm⁻¹ and an estimated isoelectric point of 4.64.

Sequence comparison between the carbendazim resistant and sensitive isolates revealed that they were all identical, indicating that the mechanism of carbendazim resistance in *F. solani* SF0301 was different from other filamentous fungi, where point mutation at different sites on the *β-tubulin* gene is responsible for the resistance.

Protein BLAST of the deduced amino acid sequences revealed that 100 % identical with that of *F. euwallaceae* (AMD38824), a member *F. solani* species complex; 99.78 % identical with that of *F. heterosporum* (KAF5674510); 99.55 % with *F. oxysporum* (XP_018241948), *F. verticilloides* (XP018748359), *F. longipes* (RGP77670), *F. sacchari* (AMD38831), *F. denticulatum* (KAF5673514) and *F. pseudograminearum* (XP009254731); 99.33 % with *F. graminearum* (PCD17759), *F. fujikuroi* (AAB18275), *F. bulbicola* (KAF5979982) and *F. commune* (AMD38822); 99.1 % with *F. babinda* (AMD388320) and *F. poae* (AMD38828); and 98.88 % with *F. sporotrichioides* (RGP75200).

3.3 Phylogenetic analysis of *Fusarium* species based on β -*tubulin* nucleotide sequences

The phylogenetic analysis of the *Fusarium* species including *F. solani* based on β -*tubulin* gene sequence revealed their position in nine clusters considered as species complexes (Fig. 2). The species complexes are *F. sambucinum* species complex (FSaSC, nine species), *F. incarnatum-equiseti* species complex (FIESC, two species), *F. tricinctum* species complex (FTSC, two species), *F. heterosporum* species complex (FHSC, one species), *F. fujikuroi* species complex (FFSC, 25 species), *F. oxysporum* species complex (FOSC, four sequences), *F. redolens* species complex (FRSC, one species), *F. decemcellulare* species complex (FDSC, one species) and *F. solani* species complex (FSSC, 10 sequences). The phylogenetic tree showed a well-supported relationship (99 % MP bootstrap) among the *F. solani* isolates of FSSC under the Clade 3 including our Indian isolates, SF0104 and SF0301. The formation of three clades by FSSC isolates was also confirmed by the analyses based on β -*tubulin* sequences. The sister groups FSaSC and FIESC, FTSC and FHSC also showed well-supported lineage with 100 % bootstrap support. *F. decemcellulare* formed lineage from *F. solani* was in accordance with Aoki et al. (2014). The 25 species under the FFSC were phylogenetically placed in three different clades as American, Asian and African - the Asian clade was derived from the African clade with 82 % bootstrap support and American clade from the Asian clade with 100 % bootstrap support.

3.4 Comparison of β -*tubulin* gene of *F. solani* isolates with other *Fusarium* species

The β -*tubulin* gene of carbendazim resistant *F. solani* SF0301 was compared with complete β -*tubulin* gene of other *Fusarium* species available in GenBank (Fig. 3B). All *Fusarium* species including *F. solani* had β -*tubulin* gene with five exons except *F. graminearum* (AY303689), *F. praegraminearum* (KX260131) and *F. sambucinum* (AF484166) as they lack 4th intron; hence, their total β -*tubulin* length became shorter with 1631 bp, 1629 bp and 1643 bp, respectively. Other species also showed variation in total length due to variation of intron length and among these, 1st intron showed maximum variation. The β -*tubulin* gene of the *F. solani* isolate was found to be shortest with 1619 bp in length and their introns were shortest among the *Fusarium* species. Out of the total length of 1619 bp, coding region consisted of 1341 bp and non-coding region with only 278 bp was made up of 1st, 2nd, 3rd and 4th introns with 139 bp, 49 bp, 44 bp and 46 bp, respectively. All the introns in other *Fusarium* species were larger, thus their β -*tubulin* lengths were larger and in the range from 1668 bp in *F. redolens* (MT011043) to 1684 bp in *F. andiyazi* (MT011059). The nucleotide length of 2nd, 3rd and 4th introns were almost similar among the *Fusarium* species with an average of 58 bp, 48 bp and 50 bp, respectively. However, shorter 2nd intron was found in *F. sambucinum* (57 bp) and *F. solani* (49 bp), and larger in *F. verticillioides* (59 bp). The length of 3rd intron also varied in few species such as, 49 bp in *F. ramigenum* and *F. napiforme*, 47 bp in *F. redolens* and 44 bp in *F. solani*. The length of 4th intron was 49 bp in *F. brevicatenuatum*, *F. pseudoanthophilum*, *F.*

pseudonygamai, *F. verticillioides* and *F. redolens*, whereas in *F. solani* it was 46 bp. The variable 1st intron was largest in *F. sambucinum* (197 bp) and shortest in *F. solani* (139 bp). Among the total 1341 bp coding region, the length of exons of all *Fusarium* species was highly conserved and exon 1, 2, 3, 4 and 5 contained 12 bp, 24 bp, 123 bp, 791 bp and 391 bp, respectively. Thus, exon 4 was the largest. However, exon 4 of *F. graminearum* (AY303689), *F. praegraminearum* (KX260131) and *F. sambucinum* (AF484166) were larger than the average length (791 bp) due to lack of 4th intron and contained 1185 bp, 1182 bp and 1182 bp, respectively. The β -tubulin of all the *Fusarium* species encoded 446 amino acid protein except *F. graminearum* which encoded 447 amino acid protein. All these data were calculated only from β_1 -tubulin nucleotide sequence from those which had two β -tubulin genes.

3.5 Comparison of β -tubulin amino acid of *F. solani* isolates with other *Fusarium* species

The most frequent difference of β -tubulin amino acid sequence of *F. solani* isolates with other *Fusarium* species was found to be at 381st position with isoleucine (I) in *F. solani* isolates and valine (V) in other (Fig. 4). However, *F. avenaceum* and *F. heterosporum* also contained isoleucine at 381st position similar to the *F. solani* isolates. But they differed from *F. solani* isolates at 189th position where valine in *F. solani* was changed as isoleucine in these two species. Another difference was identified at 335th position where asparagine (N) in *F. solani* isolates was changed to serine (S) in other *Fusarium* species. However, this change was not detected in species such as *F. oxysporum*, *F. fujikuroi*, *F. verticillioides*, *F. decemcellulare*, *F. avenaceum* and *F. heterosporum*. The amino acid proline (P) was only found in *F. oxysporum* f.sp. *cubense* at 279th position, where as in others it was histidine (H). In addition, a few minor changes were found in different species (Fig. 4).

3.6 Homology modeling and structural analysis

To build a tertiary structural model of β -tubulin, an appropriate template has been searched using Swiss-Model (<https://swissmodel.expasy.org>). Of the yielded templates, the crystal structure of *Bos taurus* (PDB ID: 4O4I) was found to be the best template with the sequence identity of 83.15 % with the query coverage of 96.64 % and the QMEAN score of the built model was -1.85. A tertiary model structure of β -tubulin was again built up by BIOVIA Discovery Studio package using the crystal structure. The three-dimensional model of the protein was found to be composed of different secondary structural elements (Fig. 5).

3.6.1 Proposed secondary structural conformation

The secondary structure of β -tubulin of *F. solani* had proposed to contain 21 α -helices, 16 β -strands and several coils (Fig. 5A). The α -helices were formed with the amino acid position from 10-27, 41-44, 47-49,

71-78, 87-89, 101-105, 108-126, 143-158, 181-195, 204-213, 222-241, 250-257, 276-279, 286-293, 296-298, 305-307, 323-336, 338-340, 372-389, 396-399 and 405-427. The β -strands were found with the amino acid position from 4-9, 30, 36, 51-54, 58-61, 63-68, 90-92, 131-138, 163-170, 198-203, 265-271, 299, 310-319, 341, 249-354 and 363-371. When the constructed secondary structure of β -tubulin of *F. solani* was compared with that of other species using POLYVIEW-2D, it was observed that all the *Fusarium* species had identical secondary structural elements. However, *F. oxysporum* f.sp. *cubense* contains 20 α -helices due to presence of proline (instead of histidine in other) at 279th position and as proline is a helix breaker, the α -helix formed by ARG276, GLY277, ALA278 and HIS279 in other *Fusarium* species is absent in *F. oxysporum* f.sp. *cubense* (Fig. 5B).

3.6.2 Model reputation

The stereo-chemical behavior of the predicted model of β -tubulin protein of *F. solani* was analyzed through ProSA and QMEAN server's confirmation and evaluated by the Phi/Psi Ramachandran plot inspection. ProSA was used to confirm the three-dimensional model of β -tubulin protein for potential errors. The program displayed two attributes of the input structure: its Z-score and a plot of its residue energies. The ProSA Z-score of -10.11 indicates the overall model quality of β -tubulin protein (Fig. 6A). The deviation of total energy of the structure, measured by the Z-score, with respect to an energy distribution obtained from random conformations. The scores were well within the range of scores and indicated a highly reliable structure typically found for proteins of comparable size. The energy plot demonstrated the local model superiority by plotting the knowledge-based energies as function of the amino acid sequence position (Fig. 6B). QMEAN analysis was also employed to evaluate and validate the model. The QMEAN4 score of the model was 0.82 (Fig. 6C) and the Z-score was -1.85 which was close to zero and this confirmed the superior quality of the model. This is because the estimated consistency of the model was projected to be in between 0 and 1 (Table 1). Assessment between regularized QMEAN score (0.82) and protein size in non-redundant set of PDB structure in the plot revealed different set of Z-values for different parameters such as C-beta interactions (0.28), interactions between all atoms (0.24), solvation (0.85) and torsion (-2.31) (Table 1).

The constructed homology model was also assessed for structural and stereo-chemical competency. A Ramachandran Phi/Psi plot for β -tubulin (Fig. 6D) showed 97.436 % highly preferred observations, 1.865 % preferred observations and 0.699 % questionable observations. The analysis of the predicted structure provided strong evidence that the predicted three-dimensional structure of β -tubulin was of superior quality.

3.6.3 Determination of putative nucleotide binding sites

In order to explore the spatial relationships between nucleotide and β -tubulin we used a topological approach after homology modeling. The nucleotide-binding domain in β -tubulin was depicted in Fig. 7A and B. We assumed that the GDP-binding site of β -tubulin was composed of two components: a

guanine-binding component and a phosphoryl-binding component. The guanine-binding component was composed of CYS12, GLN15, SER176, GLU181, ASN204, TYR222 and ASN226 and the phosphoryl-binding component was composed of GLN11, SER138, GLY141, GLY142 and THR143. The binding pattern and the H-bond formation are according to the Fig. 7A and B and Table 2.

3.6.4 Molecular docking to identify carbendazim binding site(s)

Molecular docking was performed to map the interactions between carbendazim and β -tubulin and to find out putative binding site(s). The molecular docking of carbendazim and β -tubulin detected 48 clusters in eight different sites of the protein. The top-score cluster exhibited best “*full fitness*” and those parameters were calculated by averaging the 30 % most favourable effective energies of a cluster’s element and lower free energy than those obtained for other potential binding sites (Grosdidier et al., 2007; Vela-Corcía et al., 2018). The pocket atoms of β -tubulin for carbendazim binding site were VAL193, SER196, ARG251, VAL255, VAL258, PHE260, ARG262 and HSE(Half Sphere Exposure)264. Carbendazim bound with β -tubulin with different kind of interactive forces such as classical hydrogen bond, non-classical hydrogen bond, electrostatic interaction (charged) and alkyl hydrophobic interaction. There were a conventional hydrogen bond between MBC:H18 and β :PRO261:O; two non-conventional hydrogen bonds between MBC:H16 and β :GLU194:O, MBC:H17 and β :GLU194:O; seven charged-electrostatic interactions between β :ARG156:HZ2 and MBC:C1, β :ARG156:HZ2 and MBC:C3, β :GLU194:CA and MBC:C1, β :ASN195:CA and MBC:C1, β :ASP197:CA and MBC:C3, β :PRO261:CA and MBC:C1, β :PRO261:CA and MBC:C3; alkyl hydrophobic interaction between β :ALA254 and MBC (Fig. 7C and D). All the putative bonds with their bond distance were in accordance with Table 3.

3.6.5 Dimerization of α - and β -tubulins

Hetero-dimerization of α - and β -tubulin subunits was also checked by protein-protein docking. The two monomers were interacted to each other with H-bonds and electrostatic interactions. The H-bonds were formed through salt bridge formation, conventional H-bonds and non-conventional with carbon-hydrogen bond formation. Out of total eleven bonds/interactions, the monomer β -tubulin contributed six amino acids as H-donor and five amino acids as H-acceptor. The amino acid representatives of α - and β -tubulin monomers with their actual positions in the peptide chain, the bond distance and bond angles are represented in Table 4. The β -tubulin of *F. solani* isolates had sites for the putative beta/alpha domain interface at ASP128, CYS129, ARG162, LYS252, ASN256, ARG331, ASN347, GLN350 and GLY429 (Fig. 7E).

3.6 Comparison of β -tubulin tertiary structure

Homology models of β -tubulin of all the *Fusarium* species were constructed and to identify any discrimination with the *F. solani* protein, superimposition was performed. Though few regions in amino acid residues were dissimilar among the species, the tertiary structural superimpositions were nearly identical. This could be due to substitution of the amino acids with the same class of other amino acids. The three-dimensional model of β -tubulin of *F. oxysporum* f.sp. *cubense*, however, differed from that of *F. solani* SF0301 and other species (Fig. 8A and B). In β -tubulin of *F. oxysporum* f.sp. *cubense*, PRO279 (yellow) strongly refused to form an α -helix and acted as a helix-breaker (Fig. 8D); where as in all other *Fusarium* species including *F. solani*, HIS279 (yellow) formed an extra α -helix (Fig. 8C). In Fig. 8C, the schematic diagram of β -tubulin of *F. solani* SF0301 showed ARG213 (blue) as a component of an α -helix while in *F. oxysporum* f.sp. *cubense* (Fig. 8D) ARG213 was not integrated into the α -helix rather it formed loop with its C terminal amino acid residue.

4. Discussion

Geiser et al. (2020) used 19 protein coding genes to study phylogeny of *Fusarium* species. Among these, they considered rDNA and β -tubulin genes. In this study, we confirmed phylogenetic position of our *F. solani* isolates isolated from Indian subcontinent based on ITS sequence and β -tubulin gene sequence (Figs. 1 and 2). Although, β -tubulin gene sequences of both the isolates were identical, they differed slightly in ITS sequence and was placed apart in the ITS sequence based phylogenetic tree. However, both the ITS and β -tubulin gene sequence based phylogenetic trees separated the members of the *Fusarium solani* species complex into three clades and *F. solani* isolates, SF104 and SF301 were placed in Clade 3.

The β -tubulin gene has been widely used in fungal phylogenetic analysis because it possesses both variable and highly conserved regions, and it is supposed to have more stability and reliability in the demarcation of *Fusarium* species as compared with the rRNA gene (Myburg et al. 2002). The β -tubulin contains 3.5-fold more phylogenetic information than the SSU rRNA gene (Begerow et al. 2004). Unlike rRNA genes, it is non-repetitive and accumulates fewer mutations. Its intron positions are known to be conserved and in this respect an extensive analysis of the introns and their positions are important in phylogenetic studies. When β -tubulin gene was used as molecular marker for construction of phylogenetic tree, the positions of the *Fusarium* species within the nine species complexes was almost similar with the phylogenetic analysis of O'Donnell et al. (2013), inferred from a combined gene sequences of RPB1 and RPB2 datasets. Molecular phylogenetic analysis indicated that the genus *Fusarium* was originated in the middle Cretaceous period approximately 91.3 million years ago. The fragmentation of Gondwanaland during the upper Cretaceous through the Paleocene played a central role in the emergence of clades in several species complexes. The FSSC originated near New Zealand component of Gondwana because isolates collected from this region constituted the basal clade, Clade 1. Clade 2, included isolates of South America, and Clade 3, consisting of the isolates from Asia and Africa were most likely to have emerged later due to the reproductive barrier imposed by fragmentation of the ancient southern hemisphere super continent (Bogale et al. 2009). In FFSC, the emergence of the American clade, together with the Asian clade and African clade was initially recommended by O'Donnell

et al. (1998) due to the fragmentation of Gondwanaland. Afterward, the same authors reported that the complex emerged more recently (ca. 8.8 million year ago) and that the apparent biogeographic clustering was probably due to long distance dispersal from South America to Africa and then to Asia in the late Miocene (O'Donnell et al. 2013). Distribution of species in three clades of FFSC was in accordance with the Maximum likelihood phylogeny of the FFSC inferred from the combined translation elongation factor 1- α and β -tubulin gene regions sequence data of Herron et al. (2015). O'Donnell et al. (1998) also grouped the FFSC taxa into the American, African and Asian clades.

In the present study, we characterized organization of β -tubulin gene of *F. solani* isolates. The gene was organized into five exons of variable length (Fig. 3A). To the best of our knowledge, this was the first report of complete β -tubulin gene sequence of *F. solani*. The full-length nucleotide sequences were in agreement with partial sequences of various *F. solani* isolates available in database (<https://www.ncbi.nlm.nih.gov/genbank>). We also observed that β -tubulin genes of *Fusarium* species varied significantly in their length and the variation was due to difference of their intron lengths especially the 1st intron, which was largest among the four introns. However, the number and length of exon among *Fusarium* species were almost conserved (Fig. 3B). We also compared the deduced amino acid sequence of β -tubulin of different *Fusarium* species (Fig. 4) and constructed the secondary and tertiary structure of β -tubulin protein of *F. solani* through homology modeling and compared the structure with that of other *Fusarium* species (Fig. 5). The constructed β -tubulin was scrutinized by ProSA and QMEAN analysis and validated by the analysis of phi (Φ) and psi (Ψ) torsion angles using Ramachandran plot (Fig. 6).

During polymerization of microtubules, tubulin monomer shows GTPase activity and the GTP bound with β -tubulin is hydrolyzed shortly after being incorporated. Therefore, GDP is predominantly associated in the β -tubulin subunit near the newly formed plus end (Horio and Murata 2014). A highly conserved glycine-rich sequence, GGGTGAG, has been identified from residues 140 to 146 in the *F. solani* β -tubulin which formed part of the GDP-14 binding site as reported by Nogales et al. (1998). As similar to other fungi, the β -tubulin of *F. solani* isolates encoded an N terminal tetrapeptide MREI which was involved in the autoregulation of β -tubulin expression as detected in mammalian cells (Msiska and Morton 2009).

Carbendazim has been reported to bind at specific position of the β -tubulin in different fungi (Zhou et al. 2016; Vela-Corcía et al. 2018; Yang et al. 2018; Xu et al. 2019) while, to the best of our knowledge, no evidence has been raised for physical interaction between carbendazim and β -tubulin in *F. solani*. In this study, the interaction has been demonstrated through molecular docking. Zhou et al. (2016) showed that, in *F. graminearum* carbendazim inhibited the dimerization of tubulin by binding with the β_2 -tubulin; but unable to affect the polymerized tubulin. In our study, we investigated the mystery behind the binding of carbendazim to the monomeric form but not to the polymerized tubulin. The carbendazim binding pocket in β -tubulin in *F. solani*, consisting of amino acids at the position was close to the α/β -tubulin subunit interfaces (Fig. 7). Therefore, due to the presence of carbendazim binding pocket inside the β -tubulin, we can assume that the formation of carbendazim- β -tubulin complex resulted in conformation change in α/β -tubulin subunit interfaces followed by losing the ability of these subunits to accept other tubulin

molecules for further microtubule polymerization. In contrast, polymerized microtubules may have compacted organization that destroyed the accessibility of carbendazim to the β -tubulin.

Among the naturally occurring amino acids, proline is unique in that its side chain cyclically back to the backbone amide, leaving one of its dihedral angles (ϕ) fixed at -65° (Richardson and Richardson 1989; MacArthur and Thornton 1991). Proline residue is not often found at the center of secondary structures such as the α -helix and β -sheet in globular protein due to the structural consequence of this particular arrangement (Chou and Fasman 1978, 1974). When proline is located in the first turn, acting apparently as an N-capping residue, it does occur in an α -helix (Richardson and Richardson 1988). In our study, to compare the secondary structure formed in β -tubulin among different *Fusarium* species, homology modeling of the β -tubulin protein followed by superimposition of each β -tubulin with the β -tubulin of *F. solani* was carried out. Algorithms to superimpose protein's three-dimensional structures were applied to identify similarities of protein folds. The coordinates of a protein was superposed so that the backbone lies over the backbone of a reference protein. Distant homologues might not be recognized by their amino acid sequence because the sequences diverge more rapidly in evolution than the three-dimensional structure. The most significant difference was observed at the 279th position in *F. oxysporum* f.sp. *cubense* due to presence of proline instead of histidine (Fig. 5). In *F. oxysporum* f.sp. *cubense* proline was located in the last turn, acting apparently as a C-capping residue of α -helix and acted as a helix breaker whereas, in others including *F. solani* HIS279 was a part of α -helix (Fig. 8). Proline is unfavorable to the α -helical conformation for the several reasons. Firstly, due to the absence of an amide proton on an x-Pro (x = any amino acid residue) bond which participates in helix stabilization during intramolecular hydrogen bonding (Williams and Deber 1991). Secondly, its pyrrolidine ring is too bulky to place steric constraint on the conformation of the earlier residue in the α -helix (Hurley et al. 1992). Finally, proline, being a secondary amide, is comparatively a polar residue that exhibits an enhanced tendency to form strong hydrogen bonds in non-periodic structural motifs such as proline-induced β -turns (Smith et al. 1980) and γ -turns (Deber et al. 1990). Although, no significant differences in protein function were observed in *F. oxysporum* f.sp. *cubense* because PRO279 was not the interacting residue in both tubulin polymerization as well as carbendazim binding. The other amino acid substitution(s) in different location among the species was not significant for the changes of secondary structure probably due to the substitution by the amino acid of same chemical group.

The main objective of the study was to address the molecular basis of carbendazim resistance of a *F. solani* isolate which could grow in 100 $\mu\text{g/mL}$ carbendazim. Carbendazim resistance develops rapidly in many pathogenic fungi including *Fusarium* species (Zhang et al. 2016; Avenot et al. 2019; Liu et al. 2020). Resistant isolates can adapt to environmental conditions to become dominant in pathogen populations under selection pressure of the fungicides, and in turn decrease fungicide effectiveness. Similarly isolates typically arise as resistant with a very low natural rate of mutation in a specific gene (Ma and Michailides 2005). In several species of *Fusarium*, point mutation of nucleotides (underlined) and resulting change of amino acids at various positions of β -tubulin have been reported:

TYR(TAC)50ASP(AAC), TYR(TAC)50CYS(TGC), GLN(CAG)134LEU(CTG), PHE(TTT)167TYR(TAT), GLU(GAG)198LYS(AAG), GLU(GAG)198GLN(CAG), GLU(GAG)198ALA(GCG), GLU(GAG)198VAL(GTG),

PHE(TTC)200TYR(TAC), THR(ACA)351ILE(ATA) (Yan and Dickman 1996; Qiu et al. 2011; Suga et al. 2011; Chen et al. 2014; Petkar et al. 2017; Yang et al. 2018; Xu et al. 2019). In addition, substitutions have also been observed at the position of 6, 165, 235, 240 and 241 in other phytopathogenic fungi (Ma and Michailides 2005). Some fungi contained two β -tubulin genes and among these, only one was responsible of benzimidazole resistance. However, *F. solani* contained only one β -tubulin gene with four copies (Zhao et al., 2014). In this study, β -tubulin genes of the carbendazim resistant and the sensitive isolates were identical and no point mutations were found in *F. solani* SF0301 β -tubulin gene. This might be due to the resistance factor (the ration of ED₅₀ of resistant and sensitive isolates) of *F. solani* SF0301 was very low (2.0) than that of other plant pathogenic fungi (>1000) (Chen et al. 2007). However, this observation suggested that the mechanism of carbendazim resistance in *F. solani* SF0301 might be due to overproduction of the fungicide target, detoxification of the fungicide by some fungal metabolite, an active efflux or reduced uptake of the fungicide and some unknown mechanisms (Sevastos et al. 2017). In *Gibberella pulicaris* (= *F. sambucinum*) the amino acid sequences of the β -tubulin gene from several thiabendazole- resistant and -sensitive isolates were identical and β -tubulin gene was not linked to thiabendazole resistance (Kawchuk et al. 2002). In benomyl-resistant *Colletotrichum acutatum* isolates, putative leucine zipper protein CaBEN1 increased production of the target protein by enhancing β -tubulin gene (CaTUB1) expression (Nakaune and Nakano 2007). Recently, Vígla's and Olejníková (2021) stated that in filamentous fungi the ABC transporters play a key role in antifungal resistance by transporting various xenobiotics, including antifungal compounds. *F. graminearum* mutants with ABC4 transporters were significantly less tolerant to epoxyconazole and prothioconazole. Additionally, toxin-efflux ATP-binding cassette (ABC) transporters were involved in resistance to benzimidazole fungicides in *Aspergillus nidulans* (Andrade et al. 2000) and *Penicillium digitatum* (Nakaune et al. 1998). These reports demonstrated that the resistance to benzimidazole fungicides could be partially due to the membrane transport system for these compounds into the fungal cells. Some mutations in the α -tubulin gene of *Saccharomyces cerevisiae* increased benzimidazole sensitivity (Richards et al. 2000). Further investigations are warranted in understanding the existence of resistant mechanism of benzimidazole fungicides in *F. solani* SF301.

5. Conclusions

Carbendazim resistant *F. solani* SF0301 did not have any mutation in the β -tubulin gene. Sequence comparison of the ITS and β -tubulin gene confirmed the phylogentic placement of the Indian isolates of *F. solani* within Clade 3 of FSSC. The deduced amino acid sequence showed some variations with β -tubulin of other *Fusarium* species. Homology modeling and structural analysis of β -tubulin of *F. solani* resolved putative nucleotide (GDP) binding site, carbendazim binding site and binding pattern with α -tubulin.

Declarations

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Disclosure statement

We declare no conflict of interest

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Tables

Table 1

Z scores of individual component of QMEAN for β -tubulin protein model of *F. solani*

Scoring function term	Z-score
C-beta interactions	0.25
All-atom pairwise	0.24
Solvation	0.85
Torsion angle	-2.31
QMEAN4 score = 0.82	-1.85

Table 2

Nucleotide (GDP) binding sites with calculated bond distance and bond angle

Bond Type	H-Donor	H-Acceptor	Distance (Å)	Angle XDA	Angle DAY	Theta
Conventional Hydrogen Bond	β:GLN11:N	GDP14:O1B	2.88164	6.067	118.021	
Conventional Hydrogen Bond	β:CYS12:N	GDP14:O2A	2.81274	8.263	146.499	
Conventional Hydrogen Bond	β:GLN15:NE2	GDP14:O6	3.12362	51.361	135.514	
Conventional Hydrogen Bond	β:SER138:OG	GDP14:O2A	2.91155	58.906	93.549	
Conventional Hydrogen Bond	β:SER138:OG	GDP14:O5'	2.70812	111.374	99.188	
Conventional Hydrogen Bond	β:GLY142:N	GDP14:O3B	2.91525	43.167	127.701	
Conventional Hydrogen Bond	β:THR143:N	GDP14:O3B	2.51272	21.687	137.13	
Conventional Hydrogen Bond	β:GLY144:N	GDP14:O2B	2.79906	18.253	121.661	
Conventional Hydrogen Bond	β:ASN204:ND2	GDP14:O2'	3.12522	54.321	120.505	
Conventional Hydrogen Bond	β:TYR222:OH	GDP14:O2'	3.00163	42.302	72.907	
Conventional Hydrogen Bond	β:ASN226:ND2	GDP14:O6	2.55275	8.679	123.253	
Conventional Hydrogen Bond	GDP14:N2	β:ASN204:OD1	2.59664	15.55	131.126	
Carbon Hydrogen Bond	β:GLY10:CA	GDP14:O2B	3.46944	25.517	108.499	
Carbon Hydrogen Bond	β:GLY141:CA	GDP14:O3A	3.29923	21.382	104.781	
Carbon Hydrogen Bond	β:SER176:CB	GDP14:O2'	3.7051	10.179	13.992	
Carbon Hydrogen Bond	GDP14:C8	GDP14:O1A	3.44282	13.234	104.329	
Pi-Donor Hydrogen Bond	β:CYS12:SG	GDP14	3.14745			28.863
Pi-Donor Hydrogen Bond;Pi-Sulfur	β:CYS12:SG	GDP14	2.84006			14.331

Table 3

Hydrogen bonds formed in carbendazim (MBC) binding with β -tubulin with their calculated bond distance

Category	Bond Type	Bond Donor	Bond Acceptor	Bond Distance (Å)
Electrostatic	Attractive Charge	β :ARG156:HZ2	MBC:C1	4.12
Electrostatic	Attractive Charge	β :ARG156:HZ2	MBC:C3	3.95
Electrostatic	Attractive Charge	β :GLU194:CA	MBC:C1	4.78
Electrostatic	Attractive Charge	β :ASN195:CA	MBC:C1	5.27
Electrostatic	Attractive Charge	β :ASP197:CA	MBC:C3	4.46
Electrostatic	Attractive Charge	β :PRO261:CA	MBC:C1	5.24
Electrostatic	Attractive Charge	β :PRO261:CA	MBC:C3	4.43
Hydrogen Bond	Conventional Hydrogen Bond	MBC:H18	β :PRO261:O	2.23
Hydrogen Bond	Carbon Hydrogen Bond	MBC:H16	β :GLU194:O	2.62
Hydrogen Bond	Carbon Hydrogen Bond	MBC:H17	β :GLU194:O	2.65
Hydrophobic	Alkyl	β :ALA254	MBC	3.92

*MBC-the ligand and β - the receptor protein

Table 4

Hydrogen bond forming H-donor and H-acceptor amino acid residues with their positions in polypeptide chain and calculated bond-distance and bond-angle in protein-protein docking of α - and β -tubulin proteins.

Category	Bond Types	H-Donor	H-Acceptor	Distance (Å)	Angle XDA	Angle DAY
Hydrogen Bond; Electrostatic	Salt Bridge	β:ARG162:NH1	α:ASP98:OD2	2.58503	138.62	143.67
Hydrogen Bond; Electrostatic	Salt Bridge	β:LYS252:NZ	α:GLU71:OE1	3.75977	111.05	98.26
Hydrogen Bond; Electrostatic	Salt Bridge	β:ARG331:NH1	α:GLU207:OE2	3.81886	139.54	145.41
Hydrogen Bond; Electrostatic	Salt Bridge	α:LYS96:NZ	β:ASP128:OD1	3.05739	104.57	92.99
Hydrogen Bond	Conventional Hydrogen Bond	β:CYS129:SG	α:LYS96:O	3.75908	98.55	103.54
Hydrogen Bond	Conventional Hydrogen Bond	β:ASN347:ND2	α:PRO175:O	1.89782	124.85	173.65
Hydrogen Bond	Conventional Hydrogen Bond	β:GLN350:NE2	α:THR179:O	3.08042	93.34	140.43
Hydrogen Bond	Conventional Hydrogen Bond	α:LYS401:NZ	β:GLY429:O	3.09468	98.12	107.38
Hydrogen Bond	Conventional Hydrogen Bond	α:ARG402:NH2	β:GLY429:O	1.87444	119.31	138.60
Hydrogen Bond	Carbon Hydrogen Bond	α:SER178:CA	β:GLN350:OE1	2.69845	100.73	110.04
Hydrogen Bond	Carbon Hydrogen Bond	α:THR179:CA	β:ASN256:OD1	2.42814	96.09	118.38

Figures

Figure 1

Maximum-likelihood phylogenetic tree of *Fusarium solani* species complex (FSSC) inferred from ITS nucleotide dataset rooted on that of *F. staphyleae*. Accession number of each sequence is given in

parenthesis. Values in the nodes indicate bootstrap support. Our *F. solani* isolates are marked as red.

Figure 2

Maximum-likelihood phylogenetic tree of *Fusarium* species inferred from β -tubulin nucleotide dataset rooted on that of *Epichloe festucae*. Accession number of each sequence is given in parenthesis. Values in the nodes indicate bootstrap support. Different colours are represented by different species complexes which are abbreviated as FSaSC (*Fusarium sambucinum* species complex), FIESC (*Fusarium incarnatum-equiseti* species complex), FTSC (*Fusarium tricinctum* species complex), FHSC (*Fusarium heterosporum* species complex), FFSC (*Fusarium fujikuroi* species complex), FOSC (*Fusarium oxysporum* species complex), FSSC (*Fusarium solani* species complex) and FDSC (*Fusarium decemcellulare* species complex).

Figure 3

A. Organization of β -tubulin gene of *Fusarium solani*. The downward vertical arrow shows the initiator and terminator nucleotide number of each exon. The grey regions represent exon and the line drawings represent intron regions. The diagram is drawn to scale, with nucleotide length measured in base pair (bp). **B.** Comparison of complete β -tubulin gene of different *Fusarium* species. The species are arranged alphabetically in ascending order. The positions exon and intron are marked as dark and light, respectively; their lengths are given in bp. Total nucleotide length (bp) of each gene is shown at the end.

Figure 4

Alignment of translated amino acid sequence of β -tubulin of *Fusarium solani* with other *Fusarium* species. **A.** comparison of amino acid from 1-300. **B.** comparison of amino acid from 301-446. The matching residues are represented in dots. Abbreviation of species and the sequence accession numbers are given at the end.

Figure 5

Predicted secondary structures of *F. solani* (A) and *F. oxysporum* f.sp. *cubense* (B). The red, green and blue coloured regions are α -helix, β -strand and coil, respectively. The hydrophobicity levels of different amino acid residues like relative solvent accessibility (RSA) values are represented in 0 to 10 scales; where, 0 value represents completely buried i.e. 0-9 % RSA (Black) and the value 9 represents fully exposed i.e. 90-100 % RSA (White).

Figure 6

Structural justification of the β -tubulin model. **A.** ProSA overall Z score (-10.11) is indicated in the graph as black dot, marked with red circle. A negative value of overall energy profile confirmed the consistent structural conformation of β -tubulin. **B.** Energy profile of the β -tubulin homology model. **C.** QMEAN4 score 0.82 marked with red star. **D.** Ramachandran plot of the β -tubulin model showing 97.436 % of amino acid residues in the core region.

Figure 7

Homology model of tertiary structure of *F. solani* β -tubulin showing nucleotide binding, carbendazim binding, and α -tubulin binding domains. **A.** Space-filling model showing nucleotide binding domain with bound GDP-14. **B.** Enlarged view of A, the GDP-14 binding domain of *F. solani* SF0301 β -tubulin with amino acid residues hydrogen-bonded with GDP-14 (Blue). The conventional hydrogen bonds are marked as green and non-conventional carbon-hydrogen bonds as grey. **C.** Ribbon model showing carbendazim binding domains **D.** Enlarged view of C, showing putative carbendazim binding pocket of β -tubulin with conventional hydrogen bonds marked as green, charged electrostatic interactions marked as brown, non-conventional carbon hydrogen bonds marked as black, alkyl hydrophobic interaction marked as magenta. **E.** α / β -tubulin hetero-dimer with their interactions between them with calculated bond distance and interacting amino acid residue. α -tubulin is purple and β -tubulin is cyan in colour.

Figure 8

Homology model of tertiary structure of *F. solani* β -tubulin showing nucleotide binding, carbendazim binding, and α -tubulin binding domains. **A.** Space-filling model showing nucleotide binding domain with

bound GDP-14. **B.** Enlarged view of A, the GDP-14 binding domain of *F. solani* SF0301 β -tubulin with amino acid residues hydrogen-bonded with GDP-14 (Blue). The conventional hydrogen bonds are marked as green and non-conventional carbon-hydrogen bonds as grey. **C.** Ribbon model showing carbendazim binding domains **D.** Enlarged view of C, showing putative carbendazim binding pocket of β -tubulin with conventional hydrogen bonds marked as green, charged electrostatic interactions marked as brown, non-conventional carbon hydrogen bonds marked as black, alkyl hydrophobic interaction marked as magenta. **E.** α/β -tubulin hetero-dimer with their interactions between them with calculated bond distance and interacting amino acid residue. α -tubulin is purple and β -tubulin is cyan in colour.