in the southeast coast. The deep turbid layers observed near the coast are attributed to resuspension of the bottom sediments due to the tsunami. The present study however calls for a long-term monitoring of the ecosystem changes caused by the tsunami near the affected coast.

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Molecular characterization of Trichoderma taxa causing green mould disease in edible mushrooms

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Trichoderma taxa associated with green mould disorder of edible mushrooms were isolated from both fruit bodies and substrates of Agaricus bisporus, A. bitorquis, Calocybe indica, Ganoderma lucidum, Lentinula edodes, Pleurotus sajor-caju, and Volvariella volvacea. Emerged fruiting bodies in the affected portion of the substrate were badly spotted, brownish in colour and reduced in both growth rate and yield performance. Microscopic examination of cultures revealed significant differences in fungal morphology, but were difficult to designate as Trichoderma species. The nucleotide sequence comparisons of 5.8S rRNA gene using BLAST network facilitated molecular identification and genetic cataloguing of 18 Trichoderma isolates into two taxa, namely T. harzianum and T. virens. RAPD primers exhibited both inter- and intra-specific variations among the test isolates and separated them into eight distinct phylogenetic sub-clades. The present study underlines the potential threat of green mould disorders in cultivated mushrooms and validates existence of significant intraspecific diversity in isolates of both T. harzianum and T. virens.

Keywords: Edible mushrooms, green mould disease, molecular identification, Trichoderma.

MUSHROOM cultivation is a controlled biological system, which depends upon many biotic and abiotic factors, and contamination by several microorganisms during cultivation is manifested. Among the biotic factors, fungi both parasitic and competitor, are the most important group which are generally encountered during cultivation of mushrooms. Trichoderma species causing green mould is one of the most serious problems in the mushroom industry globally. Sinden and Houser1 were the first to recognize Trichoderma species as a potentially important pathogen and/or competitor that may effect the production of white button mushroom. Trichoderma outbreaks have led to a total loss of mushroom production in many commercial mushroom farms2. Different levels of crop losses ranging from 10 to 80% have been reported from various parts of the world2–6. Serious crop losses due to Trichoderma species have not only been reported in Agaricus bisporus but also in other commercially cultivated edible mushrooms.7–10

Trichoderma species grow rapidly in versatile conditions and utilize various kinds of substrates11. Different species

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of Trichoderma, viz. T. harzianum, T. Viride, T. viresns, T. longibrachiatum, T. Koningii and T. polysporum have been found frequently associated with mushrooms. Among these, T. harzianum is recognized as the most important species and cause of potential losses. In the last two decades, isolates that have caused the greatest losses in mushroom production in England, Canada, USA and Australia have been molecularly classified as T. harzianum.

Identification of Trichoderma species has had a chequered history and the literature contains conflicting identification. Rafal suggested that more than one species of Hypocrea produces similar Trichoderma states and most species, such as T. harzianum are defined as aggregates. This implies that a number of species may exist with one species name. Therefore, additional parameters beyond morphological criteria are required to designate the species/isolates.

The present study enumerates morphological and molecular characterization of Trichoderma taxa associated with cultivation of edible mushrooms by direct sequencing of 5.8S rRNA gene and RAPD analysis.

Green mould samples were collected from different edible mushrooms and their substrates (Table 1). Isolations were made and pure cultures raised in pre-sterilized petri plates using standard laboratory techniques on malt extract agar culture medium (malt extract, 10 g l⁻¹; glucose, 5 g l⁻¹; agar, 20 g l⁻¹) amended with 50 ppm streptomycine. Cultures were incubated at 25°C in a BOD incubator for four days. Morphological characterization of Trichoderma species was done following the classification of Rafal.

For DNA extraction, mycelial cultures were raised individually in 150 ml conical flasks containing 50 ml liquid culture medium (malt extract, 10 g l⁻¹; glucose, 5 g l⁻¹) for seven days at 25 ± 1°C under darkness and stationary culture conditions. Total DNA was extracted from approximately 100 mg of Trichoderma mycelium, which was dried and crushed with micro-peslet in a conical micro-centrifuge tube under liquid nitrogen. The protocol suggested by the manufacturer (Qiagen) of DNeasy plant mini kit was followed. DNA was eluted in 100 μl of elution buffer (Qiagen) and quantified using calf thymus DNA as standard in a DNA fluorometer (DyNA Quant 200, Amersham Biosciences).

The polymerase chain reaction (PCR) primers ITS-1 and ITS-4, developed by White et al., were used to amplify the internal transcribed spacer regions of ribosomal DNA, which encompass the 5.8S rRNA gene and both ITS-1 and ITS-2 regions. The reaction mixture contained 50 μl of 10 Taq DNA polymerase, 5 μl of 10X PCR buffer (10 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂), 160 μM each of dATP, dCTP, dGTP and dTTP (MBI Fermentas), 50 μM of each ITS-1 and ITS-4 primers, 2 μl of 5% glycerol and 50 ng of genomic DNA in sterile distilled H₂O. The reaction was performed in a thermal cycler (MJ. Research, USA) with standard PCR conditions consisting of 34 cycles of 1 min denaturation at 95°C, 30 s annealing at 50°C, 1 min 20 s elongation at 72°C, and ending by a 10 min final elongation step at 72°C.

Random amplified polymorphic DNA (RAPD) analysis was performed using four decamer arbitrary primers supplied by Operon Technologies, namely OPA-1, OPA-4, OPP-16 and OPP-12. Each amplification was performed in a total reaction mixture of 25 μl containing decamer primer, 2 μl (50 pmol μl⁻¹); dNTP mix, 2 μl (2 mM each); MgCl₂, 1 μl (25 mM); Taq DNA polymerase, 1 μl (6 U μl⁻¹); 10X PCR buffer, 2.5 μl (100 mM), Tris-HCl, pH 8.3, 15 mM MgCl₂, 250 mM KCl and 16.5 μl of distilled H₂O. To this, 4 μl of genomic DNA (approx. 40-60 ng) was added. RAPD-PCR amplification was performed with an initial denaturation step of 94°C for 3 min followed by 36 amplification cycles of 94°C for 40 s, 50°C for 40 s and 72°C for 2 min and final elongation at 72°C for 10 min.

PCR amplification products were electrophoretically separated on 1.6% agarose gel prepared in 1X TAE. The gel was run for 3 h at 45 V. Staining was done with ethidium bromide and gels visualized at 300 nm UV and photographed. Gel photographs were scored for presence and absence of scorable bands with the assumption of positional homology. To establish the genetic relationship among isolates, similarity coefficients were calculated between isolates and dendrogram drawn using UPGMA employing the NTSYS-pc, ver. 2.02h program.

PCR product of ITS-amplified region containing ITS-1, 5.8S rDNA and ITS-2 was directly sequenced using ITS-1 (forward primer) and ITS-4 (reverse primer) by Big dy terminator method on an ABI prism DNA sequencer. The sequence data obtained from ITS-4 reverse primer were inverted using Gene doc software and clubbed with sequence data of ITS-1 to obtain complete sequences of amplified ITS product. Nucleotide sequence comparisons were performed using BLAST network. Trichoderma species were designated to the sequenced cultures and analysed based on similarity with the best-aligned sequence of the BLAST search. The 5.8S rRNA gene sequence alignments were performed using Clustal x 1.83 software.

The fungus initially produced a dense pure white mycelium, which was difficult to differentiate from the mushroom mycelium. The mycelial mat gradually turned green in colour due to heavy sporulation, which is a characteris-

Table 1. Trichoderma isolates along with host isolated from various substrates and fruit bodies.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Isolate</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-1</td>
<td>Agaricus bisporus</td>
<td>T-16</td>
<td>Pleurotus sajor-caju</td>
</tr>
<tr>
<td>T-1-1</td>
<td>A. bisporus</td>
<td>T-18</td>
<td>P. sajor-caju</td>
</tr>
<tr>
<td>T-2</td>
<td>Lentinula edodes</td>
<td>T-19</td>
<td>P. sajor-caju</td>
</tr>
<tr>
<td>T-5</td>
<td>Ganoderma lucidum</td>
<td>T-21</td>
<td>A. bisporus</td>
</tr>
<tr>
<td>T-6</td>
<td>Lentinula edodes</td>
<td>T-22</td>
<td>A. bisporus</td>
</tr>
<tr>
<td>T-7</td>
<td>A. bisporus</td>
<td>T-23</td>
<td>A. bisporus</td>
</tr>
<tr>
<td>T-9</td>
<td>A. bisporus</td>
<td>T-24</td>
<td>Volvariella volvacea</td>
</tr>
<tr>
<td>T-11</td>
<td>A. bisporus</td>
<td>T-25</td>
<td>A. bisporus</td>
</tr>
<tr>
<td>T-12</td>
<td>L. edodes</td>
<td>T-26</td>
<td>Calocybe indica</td>
</tr>
</tbody>
</table>
tic symptom of green mould disease. Subsequently, the mould crepted in and infected the developing newly born primordia. This produced brownish lesions and spots on the developing fruit bodies, which later joined together and completely covered them. Emerged fruiting bodies in the affected portion of the substrate were badly spotted, brownish in colour, and reduced in both growth and yield performance.

Growth patterns at 25°C showed significant differences in the nature of mycelial growth and sporulation patterns of *Trichoderma* isolates (Figure 1). There were significant differences in fungal morphology after four days of incubation. The phialides were short to very short and close type, conidia were subglobose in *T. harzianum* isolates and ovoid in *T. virens*. The culture sporulation pattern varied considerably within and between the two species. Although conidial shape and arrangement and hyphal branching pattern helped in distinguishing species from each other, they failed to designate *Trichoderma* species. Seaby\(^15\) also reported that differentiation of *Trichoderma* spp. using classical microscopic features alone was difficult since cultural morphology varied widely on different media and spore size varied significantly with incubation temperature. Moreover, variation among the isolates based on size of the phialides, and their arrangements was small. However, sporulation pattern and size of the spores within the species were highly variable.

PCR amplification of ITS region of 5.8S rRNA gene yielded an ITS fragment of 600–650 bp length in all the eighteen isolates of *Trichoderma*. No inter- or intra-species ITS length diversity was detected (Figure 2). This is due to the fact that 5.8S rRNA gene is known to be highly conserved at genus level, and this only confirmed that all the isolates belong to a single genus. Functionally and evolutionary conserved rRNA gene blocks contain both highly non conserved sequences which have been used in various studies to determine phylogenetic relationships\(^23\)–\(^25\).

Further investigations of nucleotide sequence comparisons using BLAST network services against NCBI, USA databases facilitated molecular identification and genetic cataloguing of 18 *Trichoderma* isolates into two taxa, namely *T. harzianum* and *T. virens*. Nucleotide sequence comparisons confirmed that *T. harzianum* was associated with green mould disorders in all substrates and edible mushroom samples examined, whereas *T. virens* was associated with *A. bisporus* disorders. The sequences of the isolates studied were compared with that of NCBI world databases. The
test isolates exhibited 98–100% identities with NCBI Gen accessions AF 414329, AY 266646, AY 154948, AJ 507137 and AF 483586. Serious crop losses due to Trichoderma species have been reported in A. bisporus [12,13,27]. Lentinula edodes [28-30], Pleurotus ostreatus and P. pulmonarius [29], P. sajor-caju [10], Volvariella volvacea [31] and A. bitorquis [32]. In addition to the above edible mushrooms, we have recorded green mould disorders in Ganoderma lucidum and Calocybe indica by T. harzianum.

All the four arbitrary decamer RAPD primers amplified the genomic scorable DNA fragments and exhibited both inter and intra-specific variations among the test isolates and separated them into eight distinct phylogenetic sub-clades (Figure 3). Group-I included isolate T-1, Group-2 (T-2; T-5; T-9; T-11; T-16; T-19; T-23; T-26), Group-3 (T-6; T-7; T-24), Group-4 (T-22; T-25), Group-5 (T-12), Group-6 (T-18), Group-7 (T-1-1) and Group-8 (T-21). Joint phylogenetic analysis of all the four primer RAPD profiles exhibited significant phylogenetic distances of about 63% among the isolates of T. harzianum and T. virens. More than 96% similarity was observed among the isolates of Groups 2–4 of T. harzianum. Isolates T-1, T-12 and T-18 were identified as T. harzianum and they were significantly different not only from other isolates of the same species but were also different from each other. Intra-species variations amongst the isolates of T. harzianum causing green mould disease in mushrooms has been reported on the basis of virulence pattern and morphological variation [15], ITS sequencing of conserved genes [27-33], and RAPD profiles [12,34]. The nucleotide sequence comparisons of Trichoderma lines studied with NCBI Gene Bank exhibited 98–100% sequence identities with a number of Trichoderma strains from other parts of the world conforming worldwide distribution of both T. harzianum and T. virens.

Four strains of T. harzianum have been recognized globally using molecular techniques like RAPD, RFLP and ITS sequencing and have been designated as Th-1, Th-2, Th-3 (refs 16, 32) and Th-4. In America, T. harzianum biotype-4 (identified by RAPD) is responsible for almost 90% of the epidemic-related episodes which is more closely allied to T. harzianum biotype-2, the predominant and most frequently associated pathogenic genotype in Europe [29]. Ospino-Giraldo et al. [36] separated T. harzianum in two distinct groups on the basis of nucleotide sequences of rDNA repeat. One group contained biotype Th-1, Th-2 and Th-4, while the other contained biotype Th-3 and other isolates of T. harzianum/latrovride from worldwide sources.

The results of our studies underline the potential threat of green mould disorders in cultivated mushrooms and validate the existence of significant intraspecific diversity in isolates of both T. harzianum and T. virens.

Standardization of environmental conditions for induction and retention of post-transcriptional gene silencing using tobacco rattle virus vector

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Post-transcriptional gene silencing technique (PTGS) using virus-induced gene silencing system (VIGS) is a highly useful gene knockout approach for functional analysis of endogenous genes in plants. Tobacco rattle virus based VIGS vector is a suitable vector for gene silencing in model plants like Nicotiana benthamiana and Lycopersicum esculentum (tomato). The effectiveness of Agroinfection and TRV–VIGS is influenced by environmental conditions. The vector having plant endogenous Phytoene desaturase (PDS) gene cloned either from N. benthamiana or tomato was used for targeted PTGS by Agro-infiltration approach. The extent of PDS RNA silencing was found to be highly temperature-sensitive and silencing was enhanced under low ambient temperature (less than 24°C), low light intensity (250 μmol m$^{-2}$ s$^{-1}$) and high humidity (85–90%). The efficiency of silencing was better in N. benthamiana than tomato. For examining the functional relevance of genes associated with specific physiological processes or abiotic stress, it is essential to retain the phenotype for longer period under greenhouse condition. N. benthamiana plants that were induced PTGS of PDS in growth room retained the phenotype for 30–35 days in the greenhouse maintained at high temperature 28–30°C and light intensity (1000 μmol m$^{-2}$ s$^{-1}$). The silenced plants maintained low PSII quantum yield and did not show any photosynthesis.

**Keywords:** Agro-infiltration, post-transcriptional gene silencing, Nicotiana benthamiana, tobacco rattle virus, VIGS.

The genomes of the important crop plant Oryza sativa, and the model plant Arabidopsis thaliana, have been completed and the analysis predicts that there can be approximately 55,000 and 25,000 protein-coding sequences in the former and latter respectively$^{1-3}$. The major focus is to evaluate the function of these genes, which can be further used for genetic manipulation of crops for better productivity. The most recent reverse-genetic approach of post-transcriptional gene silencing (PTGS) using Virus-Induced Gene Silencing (VIGS) system is considered as an attractive tool for gene function analysis$^{4-6}$. VIGS can be effectively used to silence known gene sequences in

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