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.

1. Banse, K., On upwelling and bottom-trawling off the south west coast of India. *J. Mar. Biol. Assoc. India*, 1959, **1**, 33–49.
2. Brock, J. C., McClaim, C. R. and Hay, W. W., A south-west monsoon hydrographic climatology for the northern Arabian Sea. *J. Geophys. Res.*, 1992, **97**, 9455–9465.
3. Darbyshire, M., The surface waters off the coast of Kerala, south-west India. *Deep Sea Res.*, 1967, **14**, 295–320.
4. Jayaraman, R., Ramamirtham, C. P., Sundaram, K. V. and Aravindakshan Nair, C. P., Hydrography of the Laccadive offshore waters. *J. Mar. Biol. Assoc. India*, 1960, **2**, 24–34.
5. La Fond, E. C., Oceanographic studies in the Bay of Bengal. *Proc. Indian Acad. Sci. (Earth Planet. Sci.)*, 1957, **46**, 1–46.
6. Muraleedharan, P. M. and Kumar, S. P., Arabian Sea upwelling – A comparison between coastal and open ocean regions. *Curr. Sci.*, 1996, **71**, 824–846.
7. Patil, M. R. and Ramamirtham, C. P., Hydrography of the Laccadive offshore waters – A study of winter conditions. *J. Mar. Biol. Assoc. India*, 1963, **5**, 159–169.
8. Sastry, A. A. and Myrland, P., Distribution of temperature, salinity and density in the Arabian Sea along the south Malabar coast (south India) during the post-monsoon season. *Indian J. Fish.*, 1959, **6**, 223–255.
9. Shetye, S. R., Gouveia, A. D., Shenoi, S. S. C., Sundar, D., Michael, G. S., Almedia, A. M. and Santanam, K., The coastal current off western India during the northeast monsoon. *Deep Sea Res.*, 1991, **38**, 1517–1529.
10. Sharma, G. S., Upwelling off the southwest coast of India. *Indian J. Mar. Sci.*, 1978, **7**, 209–218.
11. Vardachari, V. V. R., Murty, C. S. and Sankaranarayanan, V. N., Physical characteristics of waters and water masses off the west coast of India during late spring. *Indian J. Mar. Sci.*, 1974, **3**, 1–3.
12. Prasanna Kumar, S. and Prasad, T. G., Winter cooling in the northern Arabian sea. *Curr. Sci.*, 1996, **71**, 834–841.
13. Prasanna Kumar, S. *et al.*, Intrusion of the Bay of Bengal water into the Arabian Sea during winter monsoon and associated chemical and biological response. *Geophys. Res. Lett.*, 2004, **31**, L15304, doi:10.1029/2004GL020247.
14. Thompson, R. E. and Fine, I. V., Estimating mixed layer depth from oceanic profile data. *J. Atmos. Oceanogr. Technol.*, 2003, **20**, 319–329.
15. Luis, A. J. and Kawamura, H., Seasonal SST patterns along the west India shelf inferred from AVHRR. *Remote Sensing Environ.*, 2003, **86**, 2006–2015.
16. Bhattathiri, P. M. A., Aditi Pant, Sureka Sawant, Gauns. M., Mathondkar, S. G. P. and Mohanraju, M., Phytoplankton production and chlorophyll distribution in the eastern and central Arabian Sea. *Curr. Sci.*, 1996, **71**, 857–862.

ACKNOWLEDGEMENTS. We thank the Secretary, Department of Ocean Development, New Delhi for successful planning and execution of this post-tsunami expedition, and the help provided for making the research ship available for this study to acquire relevant datasets.

Received 6 April 2005; revised accepted 28 December 2005

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

S. K. Singh*, V. P. Sharma, S. R. Sharma, Satish Kumar and Mugdha Tiwari

National Research Centre for Mushroom (ICAR), Chambaghat, Solan 173 213, India

***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 Houser[1] 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 farms[2]. Different levels of crop losses ranging from 10 to 80% have been reported from various parts of the world[3–6]. 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 substrates[11]. Different species

*For correspondence. (e-mail: sksingh1111@hotmail.com)

of *Trichoderma*, viz. *T. harzianum*, *T. Viride*, *T. virens*, *T. longibrachiatum*, *T. koningii* and *T. polysporum* have been found frequently associated with mushrooms[2,12,13]. Among these, *T. harzianum* is recognized as the most important species and cause of potential losses[14,15]. 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*[4,7,16].

Identification of *Trichoderma* species has had a chequered history and the literature contains conflicting identification[17,18]. Rafai[17] 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 r RNA 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 gl^{-1}; glucose, 5 gl^{-1}; agar, 20 gl^{-1}) amended with 50 ppm streptocycline. Cultures were incubated at 25°C in a BOD incubator for four days. Morphological characterization of *Trichoderma* species was done following the classification of Rafai[17].

For DNA extraction, mycelial cultures were raised individually in 150 ml conical flasks containing 50 ml liquid culture medium (malt extract, 10 gl^{-1}; glucose, 5 gl^{-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-pestle 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 flurometer (DyNA Quant 200, Amersham Biosciences).

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

Isolate	Host	Isolate	Host
T-1	*Agaricus bisporus*	T-16	*Pleurotus sajor-caju*
T-1-1	*A. bisporus*	T-18	*P. sajor-caju*
T-2	*Lentinula edodes*	T-19	*P. sajor-caju*
T-5	*Ganoderma lucidum*	T-21	*A. bisporus*
T-6	*Lentinula edodes*	T-22	*A. bisporus*
T-7	*A. bisporus*	T-23	*A. bisporus*
T-9	*A. bisporus*	T-24	*Volvariella volvacea*
T-11	*A. bisporus*	T-25	*A. bisporus*
T-12	*L. edodes*	T-26	*Calocybe indica*

The polymerase chain reaction (PCR) primers ITS-1 and ITS-4, developed by White *et al.*[19] 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 1U Taq DNA polymerase, 5 µl of 10X PCR buffer (10 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 160 µM each of dATP, dCTP, dGTP and dTTP (MBI Fermentas), 50 pM of each ITS-1 and ITS-4 primers, 2 µl of 5% glycerol and 50 ng of genomic DNA in sterile distilled H_2O. The reaction was performed in a themal cycler (M.J. 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); $MgCl_2$, 1 µl (25 mM); Taq DNA polymerase, 1 µl (6U μl^{-1}); 10X PCR buffer, 2.5 µl (100 mM, Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl) and 16.5 µl of dH_2O. 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[20,21].

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 dye terminator method on an ABI prism DNA sequencer. The sequence data obtained from ITS-4 reverse primer were inversed 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 × 1.83 software[22].

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-

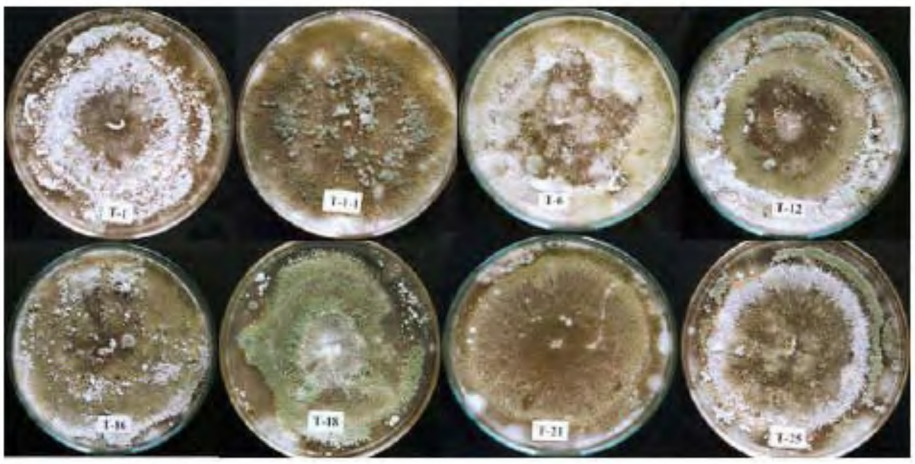

Figure 1. *Trichoderma* isolates showing variation in colony morphology.

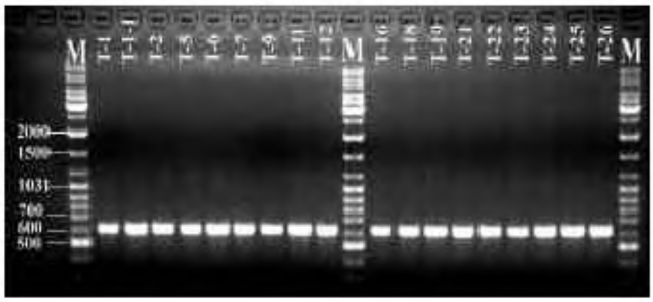

Figure 2. ITS lengths of 5.8S gene regions of *Trichoderma* isolates.

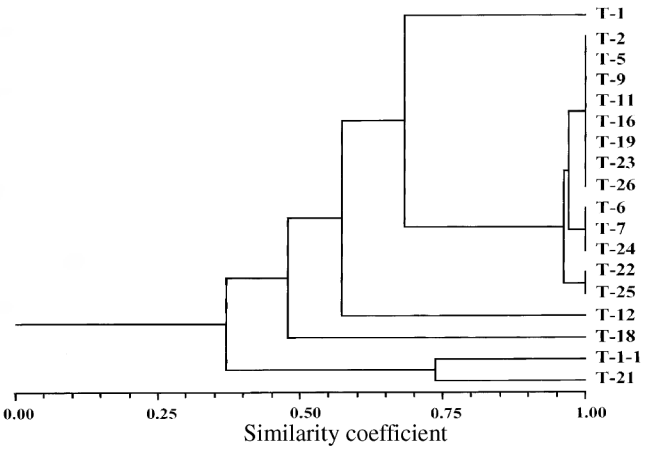

Figure 3. Dendrogram of *Trichoderma* isolates based on UPGMA analysis.

tic symptom of green mould disease. Subsequently, the mould creeped 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 obvoid 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 r RNA gene is known to be highly conserved at genus level, and this only confirmed that all the isolates belongs 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 phylogenic relationships[23–26].

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*[12,15,27]. *Lentinula edodes*[7,28–30], *Pleurotus osrteatus* and *P. pulmonarius*[31], *P. sajor-caju*[10], *Volvariella volvacea*[9] 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 variation 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[35]. Ospino-Giraldo *et al.*[7] 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/atroviride* 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*.

1. Sinden, J. W. and Houser, E., Nature and control of three mildew diseases of mushrooms in America. *Mushroom Sci.*, 1953, **1**, 52–56.
2. Morris, E., Doyle, O., Clancy, K. J. and Elliott, T. J., A profile of *Trichoderma* species II – mushroom growing units. *Science and Cultivation of Edible Fungi*, Proceedings of the 14th International Congress, Oxford, 17–22 September 1995, pp. 619–625.
3. Rinker, D. L. and Alm, G., Effect of supplementation at spawning or casing on the expression of *Trichoderma harzianum* biotype Th-4 in commercial mushroom production. *Mushroom News*, 6–11 Nov. 1977.
4. Seaby, D., Infection of mushroom compost by *Trichoderma* species. *Mushroom J.*, 1987, **179**, 355–361.
5. Sharma, S. R. and Vijay, B., Prevalence and interaction of competitor mould in *Agaricus bisporus*. *Mushroom Res.*, 1996, **5**, 13–18.
6. Staunton, L., *Trichoderm*a green mould in mushroom compost. *Mushroom J.*, 1987, **17**, 62–63.
7. Ospino-Giraldo, M. D., Royse, D. J., Thon, M. R., Chen, X. and Romaine, C. P., Phylogenetic relationships of *Trichoderma harzianum* causing mushroom green mold in Europe and North America to other species of *Trichoderma* from world-wide sources. *Mycologia*, 1998, **90**, 76–81.
8. Park, W. W., Kim, D. S., Park, Y. H. and Chung, H. S., Factors affecting growth of *Trichoderma* spp. with special reference to control of green mildew in *Agaricus bisporus*. *Kor. J. Plant Prot.* 1977, **10**, 91–95.
9. Pitakpaivan, P., Choobambroomg, W., Sontirat, P., Tontyaporn, S. and Maher, M. J., Studies on species of fungi associated with the cultivation of straw mushroom, *Volvariella volvacea* (Bull. Ex.Fr.) sing. in Thailand. *Mushroom Sci.*, 1991. **131**, 385–388.
10. Sharma, A. D. and Jandaik, C. L., Studies on competitors of two cultivated edible fungi. *Indian J. Mushroom*, 1980, **6**, 30–35.
11. Eveleigh, D. E., *Biology of Industrial Microorganisms* (eds Demainand, A. L. and Soloman, N. A.), Benjamin Cummings Publishing Co, Menlo Park, California, 1985.
12. Choi, I.-Y., Hong, S.-B. and Yadav, M. C., Molecular and morphological characterization of green mold, *Trichoderma* spp. isolated from oyster mushrooms. *Mycobiology*, 2003, **31**, 74–80.
13. Goltapeh, E. M. and Danesh, Y. R., Studies on interaction between *Trichoderma* species and *Agaricus bisporus* mycelium. *Sci. Cultivation Edible Fungi*, 2000, 661–666.
14. Morris, E., Doyle, O. and Clancy, K. J., The occurrence and diversity of *Trichoderma* spp. in commercial mushroom production. *Soc. Irish Plant Pathol. Newsl.*, 1991, 21 (abstr.).
15. Seaby, D., Differentiation of *Trichoderma* taxa associated with mushroom production. *Plant Pathol.*, 1996, **45**, 905–912.
16. Muthumeenakshi, S., Mills, P. R., Brown, A. E. and Seaby, D. A., Intraspecific molecular variation among *Trichoderma harzianum* isolates colonizing mushroom compost in the British Isles. *Microbiol. Read.*, 1994, **140**, 769–777.
17. Rafai, M. A., A revision of the genus *Trichoderma*. *Mycol. Pap.*, 1969, **116**, 56.
18. Webster, J., Culture studies on *Hypocrea* and *Trichoderma*. 1. Comparison of the perfect and imperfect states of *H. gelatinosa*, *H. rufa* and *Hypocrea* sp. 1. *Trans. Br. Mycol. Soc.*, 1964, **47**, 75–96.
19. White, T. J., Bruns, T., Lee, S. and Taylor, J., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols, a Guide to Methods and Applications* (eds Innis, M. A. *et al.*), Academic Press, New York, 1990, pp. 315–322.
20. Rohlf F. J., NTSYS-pc: Numerical taxonomy and multivariate analysis system ver. 2.02h, Exeter software, New York, 1997.
21. Sneath, P. H. A. and Sokal, R. R., *Numerical Taxonomy*, W. H. Freeman, San Francisco, 1973.
22. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G., The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 1997, **25**, 4876–4882.
23. Forster, H., Coffey, M. D., Ellwood, H. and Sogin, M. L., Sequence analysis of small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. *Mycologia*, 1990, **82**, 306–312.

24. Gaudet, J., Julien, J., Lafay, J. F. and Brygoo, Y., Phylogeny of some *Fusarium* species, as determined by large subunit rRNA sequence comparison. *Mol. Biol. Evol.*, 1989, **6**, 227–242.
25. Olsen, G. L., Lane, D. J., Giovannoni, S. J., Pace, N. R. and Stahl, D. A., Microbial ecology and evolution: A ribosomal RNA approach, *Annu. Rev. Microbiol.*, 1986, **40**, 337–365.
26. Sreenivasaprasad, S., Mills, P. R. and Brown, A. E., Nucleotide sequence of the rDNA spacer 1 enables identification of isolates of *Colletotrichum* as *C. aculatum*. *Mycol. Res.*, 1994, **98**, 186–188.
27. Samuels, G. L., Dodd, S. L., Gams, W., Castlebury, L. A. and Petrini, O., *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia*, 2002, **94**, 146–170.
28. Badham, E. R., Growth and competition between *Lentinus edodes* and *Trichoderma harzianum* on saw dust substrate. *Mycologia*, 1991, **83**, 455–463.
29. Hashioka, A. Y. and Komatsu, M. Y., *Trichoderma viride* as an antagonist of the wood inhabiting Hymenomycetesa III. Species of *Hypocrea* occuring on logwoods of *Lentinus edodes*. IV. Physiological properties of different forms of *Trichoderma* derived from the different *Hypocrea* species and soil. V. Lethal effect of different *Trichoderma* forms of *Lentinus edodes* in side logwoods. *Rep. Tottori Mycol. Inst.* 1965, **4**, 1–20.
30. Pandey, M. and Tewari, R. P., Antagonism of *Pleurotus sajor-caju* by some weed fungi. *Mushroom J. Trop.*, 1990, **10**, 101–104.
31. Mignucci, J. S., Hernandez-Baco, C., Rivera-Vargas, L., Betancourt, C. and Alameda, M., Diseases and pests research on oyster mushrooms (*Pleurotus* spp.) in Puerto Rico. *Int. J. Mushroom Sci.*, 2000, **3**, 21–26.
32. Steane, R. G., *Trichoderma* and mushrooms. *Mushroom J.*, 1978, **62**, 46–48.
33. Muthumeenakshi, S., Brown, A. E. and Mills, P. R., Genetic comparison of the aggressive weed mould strains of *Trichoderma harzianum* from mushroom compost in North America and the British Isles. *Mycol. Res.*, 1998, **102**, 385–390.
34. Muthumeenakshi, S. and Mills, P. R., Detection and differentiation of fungal pathogens of *Agaricus bisporus*. In *Science and Cultivation of Edible Fungi* (ed. Elliott, T. J.), Balkema, Rotterdam, 1995, pp. 603–610.
35. Chen, X., Romaine, C. P., Tan, Q., Schlagnhaufer, B., Ospina-Giraldo, M. D., Royse, D. J. and Huff, D. R., PCR-based genotyping of epidemic and pre-epidemic *Trichoderma* isolates associated with green mold of *Agaricus bisporus*. *Appl. Environ. Microbiol.*, 1999, **65**, 2674–2678.

ACKNOWLEDGEMENTS. We thank the Director, National Research Centre for Mushroom, Solan for providing necessary laboratory facilities.

Received 4 May 2005; revised accepted 14 October 2005

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

P. Nethra, K. N. Nataraja*, N. Rama and M. Udayakumar

Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore 560 065, India

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

*For correspondence. (e-mail: nataraja_karaba@yahoo.com)