rhythms. In view of these previous studies and the present investigations, the *in situ* conservation of the tropical rhododendron population assumes greater significance.

The higher levels of genetic variation found in ssp. *arboreum* make the Himalayan populations an important reservoir of potentially useful genes which could be used for breeding them with their reproductively compatible counterparts in Nilgiri Hills showing low levels of genetic variability. In crop plants, hybrids showing heterosis are usually developed from parental lines which are diverse in genetic relatedness, geographic origin and ecotypes[11,25]. This is important from conservation and management perspective, as breeding of temperate and tropical rhododendron populations would widen their genetic base and possibly extend the ecological niche of the hybrid rhododendrons. The populations of tropical rhododendron should also be conserved *in situ* for the preservation of the species genetic resources by allowing periodic mixing of alleles with adaptive significance.

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Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermo-hyalospora* MTCC 1384

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Endosulfan, a chlorinated cyclodiene insecticide, is of environmental concern because of its apparent persistence and toxicity to many non-target organisms. The fungus, *Mucor thermo-hyalospora* MTCC 1384 was found to bring about transformation of endosulfan molecules. The identification of endosulfan metabolites by thin layer chromatography, gas liquid chromatography using electron capture detector, ^{1}H nuclear magnetic resonance, mass spectrometry and infrared spectra revealed the formation of a major non-toxic metabolite, endosulfan diol and also production of insignificant amount of endosulfan sulfate. This indicates that the fungus is involved in both oxidative and hydrolytic pathways for degradation of this compound.

ENDOSULFAN (1,2,3,4,7,7-hexachlorobicyclo-2,2,1-heptene-2,3-bis-hydroxy methane-5,6 sulfite) is a broad spectrum cyclodiene insecticide. It is used extensively throughout the world to control the insect pests of a wide range of crops including cereals, tea, coffee, cotton, fruits, oil seeds and vegetables[1]. The technical endosulfan is a mixture of two stereoisomers[2], i.e. alpha- and beta-endosulfan (Figure 1 *a* and *b*) in the ratio of 7 : 3. Endosulfan is of great concern because of its persistence and extreme

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toxicity to fish and aquatic invertebrates[3,4]. Endosulfan has been classified as a moderately hazardous chemical[5]. Owing to its extensive usage, endosulfan residues are commonly found in the environment[6]. Microbial degradation of endosulfan may play an important role in detoxifying the endosulfan-contaminated sites in the environment. There are a few reports on degradation of endosulfan by different groups of microorganisms[7–9]. However, recent reports indicated that microbial conversion of endosulfan to endosulfan diol (Figure 1 *c*) by hydrolytic pathway is a detoxification process whereas endosulfan sulfate (Figure 1 *d*) was found to be a terminal degradation product[6,10]. In this study, we report the degradation of endosulfan by a soil fungus *M. thermo-hyalospora* MTCC 1384 in culture medium under laboratory conditions.

The fungus, *M. thermo-hyalospora* MTCC 1384 was obtained from the Department of Microbiology, Bangalore University, India. The fungus was grown and maintained in Czapek-Dox nutrient medium containing sucrose 30 g, sodium nitrate 3 g, dipotassium phosphate 1 g, magnesium sulfate 0.5 g, potassium chloride 0.5 g, ferrous sulfate 0.01 g, distilled water 1000 ml, final pH 7.0. Technical grade endosulfan was supplied by Recon Ltd, Bangalore, India. This was further purified by silica gel column chromatography (99 + % purity). All other chemicals used were of the highest analytical grade.

The fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml of Czapek-Dox agar and incubated for three days at 28 ± 2°C. After incubation, the mycelia were collected from the agar medium in sterile water, homogenized and filtered through sterile muslin cloth. Then enumeration of mycelial suspension was done by dilution plate technique and 2×10^5/ml of mycelial inoculum were added to each experimental flask.

The cultures were grown in carbon-deficient (0.1% sucrose) Czapek-Dox broth in a Newtronic orbital shaking incubator (100 rpm) at 28 ± 2°C for 48 h. After the preliminary incubation, each flask received 28 µl of endosulfan dissolved in acetone to give a final concentration of 5 ppm in the culture medium. Cultures were incubated further for different growth periods of up to 20 days after addition of endosulfan. A set of heat killed control samples were prepared by autoclaving the cultures before adding endosulfan and were incubated along with the live culture for the same period of time. Appropriate control experiments were carried out with uninoculated medium. At intervals, samples in triplicate from control, heat-killed control and live cultures were removed and acidified to pH 2 with 1N HCl and then extracted with equal volume of hexane–acetone mixture (8 : 2 v/v). For large scale degradation studies, the organisms were grown in 100 ml carbon-deficient Czapek-Dox broth in 500 ml Erlenmeyer flasks along with uninoculated controls, for eight days and were extracted following the above-mentioned method. The solvent extracts were dried over anhydrous sodium sulfate and evaporated to near dryness in a rotary-evaporator. It was redissolved in a suitable organic solvent for further analysis.

The metabolites were separated by column chromatography on silica gel columns using graded concentrations of ethyl acetate (1–20%) in hexane as eluents. Different fractions were examined by thin layer chromatography (TLC) using 0.25 mm silica gel G plates and hexane–chloroform–acetone (9 : 3 : 1 v/v/v) as the mobile phase[7]. The TLC plates were sprayed with ortho toluidine (3% solution in acetone) and exposed to sunlight for 15 min for the detection of endosulfan and its metabolites.

Quantitative analysis of endosulfan and its metabolites was done by gas chromatography-Chemito Model 3685 GC equipped with a ^{63}Ni electron capture detector, by using a glass column (8 inches length × 0.25 inch diameter) packed with 10% DC–200 + 15% QF-1 on Gas-

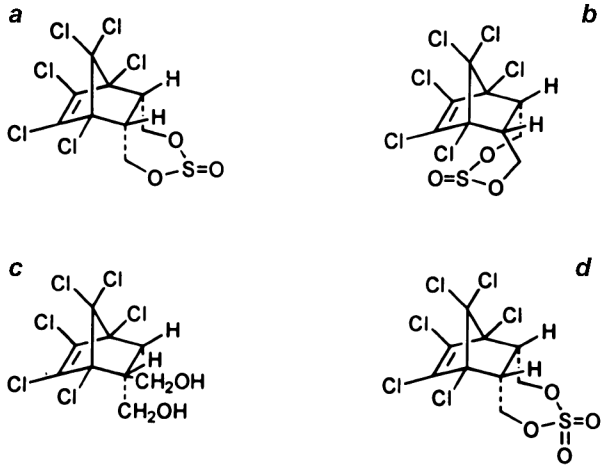

Figure 1. Endosulfan isomers and its metabolites; *a*, α-endosulfan; *b*, β-endosulfan; *c*, endodiol and *d*, endosulfate.

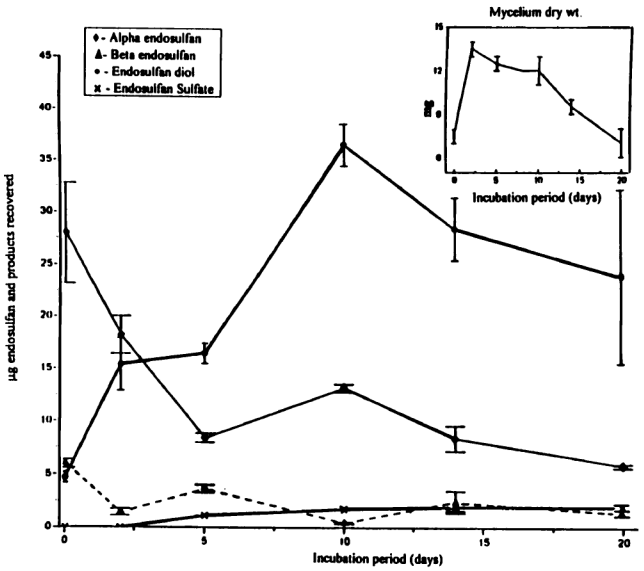

Figure 2. Time course of mycelial mass and endosulfan degradation by *Mucor thermo-hyalospora*.

chrom Q HP. The operating conditions were as follows: The column temperature was 200°C. Injection port and detector temperatures were 220°C and 270°C respectively. Nitrogen was used as a carrier gas at a flow rate of 24 ml/min. Infrared (IR) spectra were recorded in neat or nujol mull on a Perkin Elmer model 397 IR spectrometer. ^{1}H-nuclear magnetic resonance (NMR) spectra were recorded on a JEOL-FX–90 Q or Brucker WH-270 MHz spectrometer. Mass spectra (MS) were recorded on a JEOL–JMS-DX 303 instrument with a JMA-DA 5000 data system.

There was faster disappearance of both isomers of endosulfan by *M. thermo-hyalospora* with the formation of metabolic products over the incubation period (Figure 2). GLC-ECD analysis of hexane extracts and TLC studies revealed the formation of two metabolites (say M_1 and M_2) with retention time (R_t) of 5.20, 23.30 min and the R_f value of 0.24, 0.50 respectively. These matched with the R_f times and R_f values of the reference standards of endosulfan diol and endosulfan sulfate respectively. The spectral analysis showed that the metabolite (M_1, R_f 0.24) had the following characteristics: The IR spectrum (nujol) showed absorption at 3200 cm^{-1} (hydroxyl group) and 1590 (double bond). The ^{1}H NMR ($CDCl_3$) showed δ : 3.9 (2H, *d*, C<u>H</u>$_2$–OH), 3.6 (2H, *t*, C<u>H</u>–CH$_2$), 3.2 (2H, *d*, CH$_2$–OH) and 2.35 (2H, *bs*, OH). The MS data (*m/z*) for this compound were: 360 (M^+), 342 (M^+–H_2O), 324 (M^+–H_2O–H_2O), 277 (M^+–$CHCl_2$) and 229 (M^+–$C_2H_5O_2Cl_2$). For the high resolution mass spectrum (HRMS), $C_9H_8Cl_6O_2$ requires *m/z* 360.9108 and the present compound (M_1) showed a *m/z* of 360.8802. The metabolite (M_2, R_f 0.50) had the following spectral characteristics: The IR spectrum (neat) showed absorption at 1700 cm^{-1} (sulfate group) and 1600 cm^{-1}(double bond). The ^{1}H NMR ($CDCl_3$) showed δ : 4.55 (4H, *q*, 2C<u>H</u>$_2$–O) and 3.5 (2H, *bs*, 2C–H). The MS data showed (*m/z*): 422 (M^+), 387 (M^+–Cl), 358 (M^+–SO_2) and 272 (M^+–$CH_4Cl_2SO_2$). For the HRMS, $C_9H_6Cl_6O_4S$ requires 422.9629 and M_2 had *m/z* of 422.9304.

In the present investigation, *M. thermo-hyalospora* was found to be capable of transforming endosulfan. The substrate disappearance followed by increase in product formation indicated the degradation of endosulfan by this organism (Figure 2). Further, spectral and analytical studies confirmed the formation of endosulfan diol as the major metabolites followed by the production of endosulfan sulfate. Martens[7] reported that most of soil fungi produced endosulfan sulfate as the major metabolites of endosulfan through an oxidative pathway. According to an earlier report the endosulfan sulfate is as toxic and persistent as the parent compound[11]. However, the formation of endosulfan diol and endosulfan hydroxy-ether constitutes detoxification[2,12]. In the present investigation, the fungal strain *M. thermo-hyalospora* MTCC 1384 was found to be efficient in bringing transformation of endosulfan in carbon-deficient medium with the formation of non-toxic endosulfan diol as a major metabolite (more than 80% of applied endosulfan) and also production of insignificant amount of endosulfan sulfate. El Zorgani and Omer[13] reported the transformation of endosulfan to endosulfan diol (only 30% of added endosulfan) by *Aspergillus niger*, while, Katayama and Matsumura[9] observed that the major enzyme system in *Trichoderma harzianum* was oxidative in nature and was responsible for degradation of endosulfan to endosulfan sulfate. Further, they hypothesized that some microorganisms have a hydrolytic enzyme such as sulfatase, that could mediate biotransformation of endosulfan to endosulfan diol indirectly. In an experiment, Kullman and Matsumura[6] found transformation of endosulfan by a white rot fungus, *Phanerochaete chrysosporium* through oxidative and hydrolytic pathways producing more endosulfan sulfate than endosulfan diol. Our study showed the formation of endosulfan diol as the major metabolites of endosulfan by the fungus *M. thermo-hyalospora* MTCC 1384. This organism therefore, will be more useful in detoxification process, since the endosulfan diol can be detoxified further to harmless compounds.

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