

## Reproductive behaviour and genetic variability in geographically isolated populations of *Rhododendron arboreum* (Ericaceae)

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*Rhododendron arboreum* ssp. *arboreum* Smith and *R. arboreum* ssp. *nilagiricum* (Zenker) Tagg (Ericaceae) are geographically disjunct populations distributed in Himalaya and tropical montane forests of Nilgiri Hills in south India respectively. Studies on the reproductive behaviour of these disjunct populations revealed that they are self- as well as cross-compatible and reciprocal crosses between them yielded F<sub>1</sub> hybrids, indicating the absence of any pre- or post-fertilization barriers. The levels of genetic variability and the relationships within and among the disjunct, but reproductively compatible populations, were evaluated by employing RAPD assay. Random amplification of polymorphic DNA revealed higher genetic variability among individuals of temperate rhododendron populations than among those of tropical rhododendron. A dendrogram constructed on the basis of 290 amplification products showed the separation of 18 individuals in two distinct clusters that correspond to ssp. *arboreum* and ssp. *nilagiricum*. On the basis of the present study it is suggested that the levels of genetic variability among populations of tropical rhododendron could potentially be enhanced by breeding them with reproductively compatible temperate counterparts. This could possibly increase the ecological amplitude and range of cultivation of the hybrid rhododendron. We also conclude that the rhododendron populations of south India characterized by their narrow niche width need to be conserved *in situ*.

*RHODODENDRON arboreum* Smith (Ericaceae) is one of the 80 species of the genus *Rhododendron* in India<sup>1</sup> with a widespread distribution in mountain ranges of India and Sri Lanka. The species is represented by *R. arboreum* ssp. *arboreum* in broad-leaf close canopy forests of temperate Himalaya, whereas *R. arboreum* ssp. *nilagiricum* (Zenker) Tagg is restricted to hills of south India, occupying open areas like shrub-savanna or the margins of tropical montane (shola) forests<sup>2</sup>. The distribution of *R. arboreum* also extends to the uplands of Sri Lanka as *R. arboreum* ssp. *zeylanicum*. This kind of geographical distribution has

been attributed to the Pleistocene glaciation (Ice Age), which pushed down these taxa from higher latitudes in the Himalaya to lower latitudes in south India and after the glacial retreat these taxa got restricted to cooler hill tops in south India<sup>3</sup>. Majority of the genera of Himalayan origin such as *Rhododendron*, *Cotoneaster*, *Gaultheria*, *Geranium* and *Mahonia* are represented by only one species in the hills of south India and show relatively high rate of endemism in south Indian montane forest flora<sup>4</sup>. Geographically isolated populations have often been associated with increased genetic drift and inbreeding, leading to an increased homozygosity within populations which makes them susceptible to biotic and abiotic stresses<sup>5</sup>. Such populations need to be protected by adopting suitable *ex situ* and/or *in situ* conservation strategies. For successful implementation of conservation management plan, it is mandatory not only to evaluate the reproductive behaviour but also to quantify the genetic distances within and across isolated populations using molecular markers. Analysis of the genetic structure of isolated populations has of late received greater attention because of its role in their conservation<sup>6</sup>. Among molecular markers, the random amplified polymorphic DNA (RAPD) assay has been used for the evaluation of genetic variability among the closely related rhododendron cultivars and their hybrids<sup>7,8</sup>. In this paper we report the breeding behaviour, the levels of genetic diversity and the relationships within and between geographically disjunct populations of Indian temperate and tropical rhododendrons.

Table 1 presents the sampling sites and the codes of the natural populations of *R. arboreum* ssp. *arboreum* (Mussoorie, Himalaya) and ssp. *nilagiricum* (Mount Koilmund, Nilgiri Hills) analysed in the present study. However, the populations of *R. arboreum* ssp. *zeylanicum* found in the uplands of Sri Lanka were not included in the present investigations and the studies were confined to Indian populations only. The leaf material for DNA analysis was collected randomly from three sampling sites each at Mussoorie (M1, M2 and M3) and Mount Koilmund (N1, N2 and N3). The sampling sites in each region were separated by a minimum distance of 5 km. At each sampling site the samples were collected randomly from three individuals (a, b and c) separated by a minimum distance of 100 m or more.

**Table 1.** Natural populations of *R. arboreum* ssp. *arboreum* and *R. arboreum* ssp. *nilagiricum* analysed in the present study

Geographical region	Area	Sampling site	Individual code
Himalaya	Mussoorie	M1	M1a, M1b, M1c
		M2	M2a, M2b, M2c
		M3	M3a, M3b, M3c
Nilgiri Hills	Mount Koilmund	N1	N1a, N1b, N1c
		N2	N2a, N2b, N2c
		N3	N3a, N3b, N3c

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Genomic DNA was extracted from young leaf tissue following the procedure described by Saghai-Marooof *et al.*<sup>9</sup>. Synthetic decamer primers from Operon Technologies Inc., Alameda, Calif. (Kits P, Q and W) were used for amplification reactions using the standard protocol of Williams *et al.*<sup>10</sup> with minor modifications as described by Jain *et al.*<sup>11</sup>. Results of the RAPD assay represented a consensus of two replicates. For each individual, only reproducible and unambiguously scorable bands in both the replicates were considered. Data were analysed using NTSYS-pc, version 1.50 (Exeter Software, NY)<sup>12</sup>. Pair-wise comparisons of individuals, based on both unique and shared amplification products were used for estimating genetic similarity by employing Jaccard's coefficient of similarity. Cluster analyses were conducted on similarity estimates using the unweighted pair-group method with arithmetic averages (UPGMA) and the resulting clusters were expressed as a dendrogram representing genetic relationships among individuals of geographically isolated populations. Genetic distance (GD) was calculated as:  $GD = 1 - GS$  (genetic similarity).

Field-pollinations were performed at Mussoorie and Mount Koilmund during February–March (1995–1997) to determine the reproductive behaviour of temperate and tropical rhododendron populations. Two sets of experiments were conducted. In the first set, flowers were merely tagged and allowed to pollinate naturally. In the second set, manual pollinations were carried out for: (a) selfing, using pollen from the same flower, (b) cross-pollinations, using pollen from different individuals separated by a minimum distance of 100 m, and (c) reciprocal crosses between temperate and tropical populations. A total of 215–240 pollinations were carried out for each set of experiment with a minimum of 25 pollinations on a single tree. For reciprocal crosses, pollen samples collected from different sites were stored desiccated over dry silica gel. Fluorochromatic reaction (FCR) test<sup>13</sup> was performed to determine the viability of the pollen samples before using them for field pollinations. Following different pollination treatments, fruits were harvested after about four to six months, transferred to the butter paper bags and allowed to dehisce at room temperature ( $28 \pm 2^\circ\text{C}$ ). Seeds obtained for each set of experiments were stored desiccated at  $4^\circ\text{C}$ . For calculating seed/ovule ratio, five to six mature ovaries and fruits were collected from each of the nine trees of the temperate and tropical populations used for manual pollinations. This ratio is represented by the mean number of ovules and seeds harvested from 45 to 50 ovaries and fruits, respectively. Per cent fruit-set and seed/ovule ratio obtained through different manual pollination treatments were compared with those achieved through natural pollinations.

Seed germination tests were conducted using 10 replicates of 50 seeds each for different treatments. Seeds were soaked for 2–3 h in sterile distilled water and plated in 9 cm petri plates lined with two layers of moistened filter

paper. The plated seed samples were given chilling treatment at  $4 \pm 2^\circ\text{C}$  for 24 h. For germination, seeds were kept in sterile growth chamber (16 h day,  $20 \pm 2^\circ\text{C}$ ). The filter papers in the petri plates were kept moistened continuously with sterile distilled water during the entire period of the experiment. A seed was treated as germinated when its radicle protruded through the seed coat and attained at least twice the length of the seed.

To determine, if there are differences within and between temperate and tropical populations in the pollen adherence and germination of self- and cross-pollen on stigmas, 18 pollinated pistils each from temperate and tropical populations (two pistils/tree) for each set of experiment were fixed in Carnoy's fixative, one, two and seven days after pollinations. Fixed pistils were cleared overnight in 4 N NaOH and mounted in decolourized aniline blue with one drop of 50 per cent glycerine. The preparations were observed under fluorescence microscope (Nikon Optiphot) for *in vivo* pollen germination and pollen tube growth. The micropylar ends of the ovules were examined for the entry of pollen tubes.

Duncan's new multiple range test<sup>14</sup> was employed to make comparisons among different values (seed/ovule ratio, per cent fruit-set, and per cent seed germination) obtained through natural and manual pollination treatments in *R. arboreum* ssp. *arboreum* and ssp. *nilagiricum*.

The levels of polymorphism within and across the sampled individuals representing populations of ssp. *arboreum* and ssp. *nilagiricum* were detected by using randomly selected 30 decamer primers. Table 2 gives the details of the total number of amplification and polymorphic products generated, per cent polymorphism observed and mean number of polymorphic products/primer within and across the individuals of tropical and temperate rhododendron populations. The 30 primers used for the RAPD assay differed greatly in their efficacy in generating both the number and size of the amplified products which revealed polymorphism. The number of scorable bands amplified by each primer varied from 5 (OPW-4) to 16 (OPP-8), ranging in size from about 0.3 to less than 3.0 kb. No correlation was observed between the PCR yield of detectable amplified products and the GC content of a primer. Primer OPQ-7 having 70 per cent GC content produced 12 amplification products, while primer OPP-6 having similar GC content, generated only 7 amplification

**Table 2.** Genetic polymorphism within and across *R. arboreum* ssp. *arboreum* (Ra) and ssp. *nilagiricum* (Rn) revealed by RAPD assay using 30 decamer primers

	No. of amplification products	Polymorphic products		Mean no. of polymorphic products/primer
		No.	%	
Among Ra	238	121	50.84	4.0
Among Rn	217	74	34.10	3.1
Across Ra and Rn	290	165	56.89	5.5

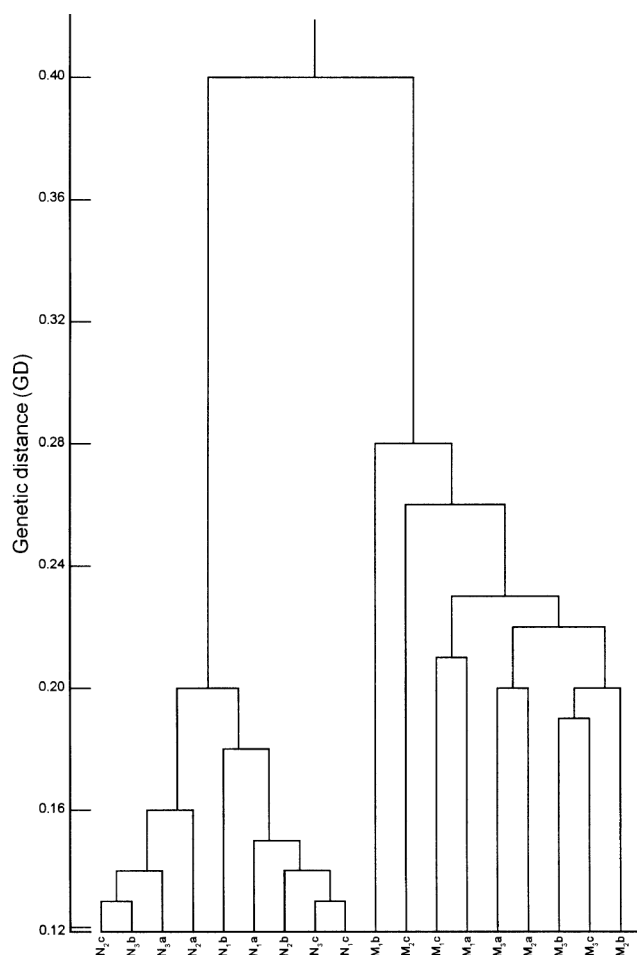
products. All the primers tested revealed polymorphism across individuals which varied from 20 per cent (OPP-4) to 90.66 per cent (OPP-1 and OPQ-10). Out of 30 primers tested, 22 revealed higher genetic variability among the individuals from Himalaya, compared to those from the tropical populations. The only exception was the primer (OPQ-5) which generated higher per cent polymorphism across the individuals from Nilgiri Hills. Seven primers did not show any variation in per cent polymorphism across the individuals of both the temperate and tropical populations. Out of 290 amplification products, 50.84 per cent were polymorphic among *ssp. arboreum* individuals, while only 34.10 per cent were polymorphic among individuals of *ssp. nilagiricum* with an average of 4.0 and 3.1 polymorphic products/primer respectively.

For the estimation of genetic distance (GD), use of at least 50 different polymorphic loci has been recommended<sup>15</sup>. In the present study, 165 unambiguous RAPDs were used to construct an integrated dendrogram (Figure 1). All the nine individuals from the Nilgiri Hills were grouped in one cluster while, individuals belonging to Himalaya formed another cluster. Both the clusters were quite distinct with a GD estimate of 0.40. Four individuals from Nilgiri Hills, i.e. N1c, N2c, N3b and N3c exhibited a minimum GD of 0.13 amongst them but these individuals could still be differentiated from each other by 8 primers out of 30 tested. Among all the tropical individuals, N1b exhibited maximum GD of 0.18. Generally, the GD estimates across the individuals from Nilgiri Hills were lower than those of the individuals from Himalaya which exhibited extensive genetic variability not only between the individuals from different sampling sites but also amongst them. The GD estimates varied from 0.20 for the most closely related temperate individuals, i.e. M3b and M3c to 0.28 for the most distantly related M1b individual.

FCR scores of all the pollen samples, used for manual pollinations, averaged between 75 and 80 per cent (data not shown). The number of pollen grains adhering to self- and cross-pollinated stigmas did not differ significantly among pollination treatments of individuals from temperate and tropical regions. Similarly, the percentage of pollen grains that had germinated (averaged 50–60 per cent) on stigmas showed a similar trend for different pollination treatments. In all the treatments, one to three pollen tubes were observed from each tetrad, showing profuse germination on the stigmas. These tubes could be traced up to the base of the stigmatic region 24 h after pollination. Two days after different pollination treatments, a large number of pollen tubes traversed from half to 2/3rd lengths of the styles of which a few could be traced down in the ovaries after seven days. Though pollen tube growth rates varied, they did not show any correlation with the type of pollination treatments.

Table 3 presents the details of seed/ovule ratio, per cent fruit-set and per cent seed germination obtained through

manual and natural pollinations. The notable difference observed in the reproductive behaviour of temperate and tropical rhododendrons was in the number of ovules/ovary with the former characterized by a significantly higher number (average 1030) when compared with the latter (average 700). However, differences in values of seed/ovule ratio and per cent fruit-set between *ssp. arboreum* and *ssp. nilagiricum* were statistically insignificant ( $P < 0.05$ ). Similarly, the differences in values of seed/ovule ratio, per cent fruit-set and per cent seed germination between natural and manual pollinations in *ssp. arboreum* and *ssp. nilagiricum* were statistically insignificant ( $P < 0.05$ ). Even different pollination treatments (self- and cross-pollinations and reciprocal crosses) in temperate and tropical populations did not show any significant effect on seed/ovule ratio, per cent fruit-set and per cent seed germination. However, irrespective of the pollination treatment, significantly low per cent seed germination



**Figure 1.** Dendrogram illustrating genetic relationships among geographically disjunct populations of temperate and tropical rhododendrons generated by the UPGMA cluster analysis (NTSYS-pc) of Jaccard's genetic similarity (GS) coefficients calculated from 290 amplification products produced by 30 primers. M1a, M1b, M1c, M2a, M2b, M2c, M3a, M3b and M3c represent individuals from temperate Himalaya whereas, N1a, N1b, N1c, N2a, N2b, N2c, N3a, N3b and N3c are the individuals from Nilgiri Hills.

**Table 3.** Details of per cent fruit-set, seed/ovule ratio and per cent seed germination obtained through natural and manual pollinations<sup>†</sup>

Treatment	No. of pollinated pistils	Seed/ovule ratio	Fruit-set (%)	Germination (%) (mean $\pm$ SD)
<b>Natural pollination</b>				
<i>R. arboreum</i> ssp. <i>arboreum</i>	230	0.63 <sup>a</sup>	76.65 <sup>a</sup>	67.55 $\pm$ 1.50 <sup>a</sup>
<i>R. arboreum</i> ssp. <i>nilagiricum</i>	218	0.65 <sup>a</sup>	78.26 <sup>a</sup>	48.26 $\pm$ 2.16 <sup>b</sup>
<b>Manual pollination</b>				
Self-pollination				
<i>R. arboreum</i> ssp. <i>arboreum</i>	220	0.64 <sup>a</sup>	77.50 <sup>a</sup>	69.48 $\pm$ 4.72 <sup>a</sup>
<i>R. arboreum</i> ssp. <i>nilagiricum</i>	224	0.66 <sup>a</sup>	77.68 <sup>a</sup>	46.92 $\pm$ 3.79 <sup>b</sup>
Cross-pollination				
ssp. <i>arboreum</i> $\times$ ssp. <i>arboreum</i>	235	0.65 <sup>a</sup>	78.20 <sup>a</sup>	70.80 $\pm$ 3.13 <sup>a</sup>
ssp. <i>nilagiricum</i> $\times$ ssp. <i>nilagiricum</i>	227	0.64 <sup>a</sup>	79.35 <sup>a</sup>	50.85 $\pm$ 2.93 <sup>b</sup>
Reciprocal crosses				
ssp. <i>arboreum</i> $\times$ ssp. <i>nilagiricum</i>	240	0.65 <sup>a</sup>	79.50 <sup>a</sup>	69.21 $\pm$ 3.01 <sup>a</sup>
ssp. <i>nilagiricum</i> $\times$ ssp. <i>arboreum</i>	228	0.66 <sup>a</sup>	77.62 <sup>a</sup>	48.16 $\pm$ 2.45 <sup>b</sup>

<sup>†</sup>Values within any column followed by the same letter are not significantly different at  $P < 0.05$ .

( $P < 0.05$ ) was observed when the female parent was an individual from tropical rhododendron population.

F<sub>1</sub> hybrids obtained from crossing tropical and temperate rhododendrons were also analysed by RAPD assay to confirm their hybrid nature. Out of the five primers tested, three (OPW-11, OPW-12 and OPW-15) generated amplification products which were specific to both the parents. Unique amplification products, not present in either of the parents, were not detected in the hybrids with any of the primers tested. Collectively, results from various pollination treatments suggest that temperate and tropical rhododendron populations are self- and cross-compatible and indicated lack of crossability barriers between geographically disjunct populations.

Many studies have reported a significant variation in the levels of genetic polymorphism in geographically isolated populations which has been largely attributed to the interplay of their breeding behaviours and population dynamics<sup>16</sup>. Earlier reports on disjunct populations of *R. arboreum* found in the Indian subcontinent focused mainly on their phylogeographical distribution<sup>3,4</sup>. In the present investigations we observed that despite the geographical isolation of populations of temperate and tropical regions, they were still reproductively compatible, which is in agreement with similar reports on several other rhododendron species<sup>17</sup>. Only in a few rhododendron species, i.e. *R. ellipticum*, *R. championae* and *R. amamiense*, self-incompatibility has been reported as a post-zygotic mechanism<sup>18</sup>. The study indicated that neither pollen availability nor pollination mechanisms limited seed/ovule ratios and fruit-set in these populations. Self- and out-crossing nature in temperate and tropical rhododendron populations suggests complex evolutionary responses to the selective forces influencing plant fitness.

Wyatt<sup>19</sup> has suggested that in isolated populations, self-fertilizing individuals may be at a selective advantage if

reproductive success by outcrossing is restricted because of inadequate cross-pollination. In many plant species, extensive genetic diversity has been correlated with higher outcrossing rates while, self-compatible populations with restricted geographical distribution favouring self-pollination has been considered responsible for lower levels of genetic variation<sup>20</sup>. Barrett and Husband<sup>5</sup> also reported lesser genetic polymorphism in *Eichhornia paniculata* populations isolated from the main range which has been attributed to an apparent shift from predominant outbreeding in continental populations to inbreeding in disjunct populations. These studies and the present investigations suggest that the low level of genetic variability in isolated populations of tropical rhododendron could be attributed to their restricted distribution and its consequent effect on their breeding behaviour. It is quite likely that populations of tropical rhododendron favour self-pollination to ensure some reproduction in the relative absence of potential mates.

Restricted geographical distribution and low levels of genetic polymorphism in tropical rhododendron populations do indicate that these populations have developed characteristics akin to colonizing plant species which got separated from the main genetic stock (see also Barrett and Shore<sup>21</sup>). Pandit and Babu<sup>22</sup> have reported that facultative inbreeding in plant populations with small sizes and narrow niche width are susceptible to extinctions through environmental perturbations and anthropogenic pressures. On the basis of morphological and cytogenetical studies on such spatio-temporally isolated populations of rhododendron and some other Himalayan genera found in the hills of south India, Kumar<sup>23,24</sup> suggested that these populations could be representing the remnants of the Himalayan vegetation driven down south, during the recent glacial epoch, where they evolved independently in the changed environmental conditions and different diurnal

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<sup>11,25</sup>. 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, <sup>1</sup>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<sup>1</sup>. The technical endosulfan is a mixture of two stereoisomers<sup>2</sup>, 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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rhythms. In view of these previous studies and the present investigations, the *in situ* conservation of the tropical rhododendron population assumes greater 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, <sup>1</sup>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<sup>1</sup>. The technical endosulfan is a mixture of two stereoisomers<sup>2</sup>, 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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