Field evaluation of chlorophyll meter for screening groundnut (Arachis hypogaea L.) genotypes tolerant to iron-deficiency chlorosis

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Groundnut, an important oilseed crop of India, frequently suffers from iron-deficiency chlorosis which may become so severe that the entire plant becomes yellow, papery-white, and may even die resulting in severe yield losses. As groundnut genotypes differ in their ability to utilize iron, selection of Fe-efficient genotypes which can tolerate Fe-deficiency chlorosis is the solution to this problem. Presently no field instrument is used to measure the intensity of chlorosis and only visual diagnosis, using visual chlorotic rating (VCR) and chlorophyll content determination method is widely used for selecting genotypes tolerant to iron-deficiency chlorosis in the field. Therefore, the present investigation was carried out to explore the possibility of using a chlorophyll meter (SPAD) for rapid and in situ screening of groundnut genotypes for their tolerance to iron chlorosis. The study showed that correlation between SPAD readings and chlorophyll content was positive and highly significant with r value of 0.94** for chlorophyll a, 0.90** for chlorophyll b, and 0.93** for total chlorophyll. This has clearly indicated a closer relationship of these traits with SPAD reading, i.e. higher the SPAD reading, higher will be the chlorophyll pigments and vice versa. The regression lines showed that these variables have a linear relationship with each other. On the basis of the linear relationship regression equations were developed from which it is possible to predict the chlorophyll content and VCR of the leaves. Thus the chlorophyll meter is an efficient and speedy equipment for screening genotypes for their tolerance to iron-deficiency chlorosis.

PHOTOSYNTHESIS is the most important biochemical process occurring in plants and chlorophyll is the key pigment involved in it. Groundnut, an important oilseed crop, frequently suffers from iron-deficiency chlorosis in most parts of India, particularly in calcareous, alkaline and marginal soils\textsuperscript{1–3}. Singh and Joshi\textsuperscript{4} reported mild to severe yield losses depending upon the intensity of chlorosis. Many times chlorosis becomes so severe that the entire plant becomes yellow, papery-white, with reduced photosynthetic activity and ultimate death. Soil and foliar application of iron containing fertilizers helps the plants recover from chlorosis but their effect does not persist for long in the field and requires frequent applications\textsuperscript{5}. The selection of Fe-efficient genotypes which can tolerate Fe-deficiency chlorosis could be a potential alternative solution to this problem. As groundnut genotypes differ in their response to Fe stress\textsuperscript{2,6}, visual chlorotic rating (VCR) method followed by chlorophyll estimation is used for measuring the...
intensity of chlorosis. As the estimation of chlorophyll content is laborious and time consuming, now-a-days the chlorophyll meter is being used for fast estimation of chlorophyll in the field. The present investigation was carried out to explore the possibility of using the chlorophyll meter for rapid field screening of groundnut genotypes for their tolerance to iron chlorosis.

Thirty advanced breeding genotypes of groundnut were grown in a randomized block design with three replications during rabi–summer 1999 at the National Research Centre for Groundnut (NRCG), Junagadh. The plot size for each genotype was 5 m × 0.45 m. The soil of the experimental plot had a pH of 7.9, contained 29.6% calcium carbonate, 0.8% organic carbon, 0.06% total nitrogen, 6 ppm available P (Olsen’s), and 1.35 ppm available Fe. This soil was thus suitable for screening groundnut germplasm to ascertain their tolerance for iron-deficiency chlorosis. For comparison, one iron chlorosis tolerant (I1) and three iron chlorosis susceptible checks (I2, VRI 3 and ICG 7887; Figure 1) were included in the experiment based on the results of earlier studies. The recommended package of practice was followed to grow the crop. The first fully opened leaf of the main axis, from 10 randomly selected plants of each genotype was collected and read for chlorophyll using the chlorophyll meter (SPAD-502 Minolta, Japan) and estimated for chlorophylls a and b and total chlorophyll (TC) content following Arnon. Based on chlorophyll contents and VCR as per Singh and Chaudhari, the genotypes were put into three categories: (i) Tolerant – showing dark green leaves, rare appearance of chlorotic plants and VCR less than 2.00 and TC content more than 6.00 mg/g dry weight of leaves; (ii) Moderately tolerant – green leaves with VCR ranging between 2.01 and 5.00, TC content ranging from 3.00 to 6.00 mg/g dry weight of leaves and chlorophyll contents ranging from 2.00 to 3.00 mg/g dry weight basis; and (iii) Susceptible – showing chlorotic plants with VCR ranging from 5.01 to 8.50, TC content ranging from 1.00 to 3.00 mg/g dry weight of leaves and chlorophyll contents ranging from 1.00 to 2.00 mg/g dry weight basis.

Table 1. Mean SPAD reading, visual chlorotic rating, chlorophyll a, chlorophyll b and total chlorophyll contents (mg/g dry weight basis), and pod yield in advanced breeding genotypes of groundnut.

<table>
<thead>
<tr>
<th>Entry name</th>
<th>Pedigree of the genotypes</th>
<th>SPAD reading</th>
<th>VCR</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
<th>Total chlorophyll</th>
<th>Pod yield (g/plant)</th>
</tr>
</thead>
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<tr>
<td>PBS 12115</td>
<td>Kisan × NC Ac 17133</td>
<td>32.54</td>
<td>1.75</td>
<td>5.72</td>
<td>1.83</td>
<td>7.55</td>
<td>8.86</td>
</tr>
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<td>PBS 12118</td>
<td>V(G)E5 × B 227</td>
<td>25.09</td>
<td>2.50</td>
<td>5.01</td>
<td>1.46</td>
<td>6.47</td>
<td>10.61</td>
</tr>
<tr>
<td>PBS 12120</td>
<td>V(G)E5 × B 227</td>
<td>26.06</td>
<td>2.50</td>
<td>4.39</td>
<td>1.23</td>
<td>5.62</td>
<td>8.86</td>
</tr>
<tr>
<td>PBS 12124</td>
<td>TMV 2 × PI 337409</td>
<td>28.96</td>
<td>2.42</td>
<td>5.04</td>
<td>1.47</td>
<td>6.51</td>
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<tr>
<td>PBS 12126</td>
<td>Latur 33 × PI 405132</td>
<td>27.40</td>
<td>2.17</td>
<td>5.42</td>
<td>1.60</td>
<td>7.02</td>
<td>13.30</td>
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<tr>
<td>PBS 14016</td>
<td>CGC 3 × JL 24</td>
<td>22.01</td>
<td>3.08</td>
<td>3.96</td>
<td>1.19</td>
<td>5.15</td>
<td>13.87</td>
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<td>PBS 14021</td>
<td>TMV 7 × Chico</td>
<td>25.78</td>
<td>3.17</td>
<td>4.45</td>
<td>1.30</td>
<td>5.75</td>
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<tr>
<td>PBS 19003</td>
<td>M 13 × PI 314817</td>
<td>25.45</td>
<td>3.17</td>
<td>4.28</td>
<td>1.25</td>
<td>5.53</td>
<td>9.47</td>
</tr>
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<td>PBS 21031</td>
<td>TMV 10 × PI 405132</td>
<td>29.63</td>
<td>1.83</td>
<td>5.69</td>
<td>1.76</td>
<td>7.45</td>
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<td>PBS 21052</td>
<td>C1 IV (Selection)</td>
<td>24.61</td>
<td>3.08</td>
<td>3.95</td>
<td>1.22</td>
<td>5.17</td>
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<td>PBS 21063</td>
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<td>PBS 22031</td>
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<td>27.43</td>
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<td>1.88</td>
<td>8.00</td>
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<td>PBS 24001</td>
<td>Chandra × Chico</td>
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<td>PBS 24002</td>
<td>GG 11 × Robut 33-1</td>
<td>27.58</td>
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<td>5.68</td>
<td>1.86</td>
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<td>GG 11 × Robut 33-1</td>
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<td>PBS 24004</td>
<td>Latur 33 × Tritur</td>
<td>32.59</td>
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<td>PBS 24006</td>
<td>M 13 × Robut 33-1</td>
<td>36.35</td>
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<td>M 13 × Robut 33-1</td>
<td>29.93</td>
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<td>1.43</td>
<td>6.91</td>
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<td>PBS 24040</td>
<td>Latur 33 × Tritur</td>
<td>35.71</td>
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<td>M 13 × NC Ac 17278</td>
<td>33.96</td>
<td>2.00</td>
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<td>1.88</td>
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<td>Sel 28 a</td>
<td>Dh 3-30 × NC Ac 2214</td>
<td>26.01</td>
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<td>FSD-1</td>
<td>GAU 1 × GG 2</td>
<td>29.75</td>
<td>2.75</td>
<td>5.15</td>
<td>1.54</td>
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<td>GAU 1 × GG 2</td>
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<td>FSD-66</td>
<td>GAU 1 × GG 2</td>
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<td>PBS 20100  (RC)</td>
<td>I1</td>
<td>41.83</td>
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<td>24.28</td>
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<td>1.26</td>
<td>5.52</td>
<td>8.20</td>
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<tr>
<td>PBS 20511 (SC)</td>
<td>VRI 3</td>
<td>24.05</td>
<td>3.16</td>
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<td>1.18</td>
<td>5.24</td>
<td>6.79</td>
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<td>PBS 20055 (SC)</td>
<td>ICG 7887</td>
<td>21.53</td>
<td>3.58</td>
<td>4.17</td>
<td>1.25</td>
<td>5.42</td>
<td>10.22</td>
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</table>

RC, Resistant check; SC, Susceptible check.
Table 2. Correlation coefficients and regression equations between SPAD reading and chlorophyll \(a\), chlorophyll \(b\), total chlorophyll, and visual chlorotic rating

<table>
<thead>
<tr>
<th>Characters</th>
<th>30 DAE</th>
<th>45 DAE</th>
<th>60 DAE</th>
<th>75 DAE</th>
<th>Over means</th>
<th>Regression equations</th>
</tr>
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<tr>
<td>Chlorophyll (a)</td>
<td>0.77**</td>
<td>0.89**</td>
<td>0.77**</td>
<td>0.92**</td>
<td>0.94**</td>
<td>(Y = -0.30 + 0.190X)</td>
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<td>Chlorophyll (b)</td>
<td>0.69**</td>
<td>0.62**</td>
<td>0.77**</td>
<td>0.87**</td>
<td>0.90**</td>
<td>(Y = -0.23 + 0.062X)</td>
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<tr>
<td>Total chlorophyll</td>
<td>0.75**</td>
<td>0.79**</td>
<td>0.78**</td>
<td>0.92**</td>
<td>0.93**</td>
<td>(Y = -0.60 + 0.087X)</td>
</tr>
<tr>
<td>VCR</td>
<td>-0.67**</td>
<td>-0.64**</td>
<td>-0.77**</td>
<td>-0.60**</td>
<td>-0.87**</td>
<td>(Y = 5.50 - 0.110X)</td>
</tr>
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</table>

**Significant at 1 per cent level; \(Y\), Predicted value of chlorophylls \(a\) and \(b\), TC, and VCR; X, Value of chlorophyll meter (SPAD reading).

Figure 2. Relationship between (a), SPAD reading and chlorophyll \(a\) (mg/g); (b), SPAD reading and chlorophyll \(b\) (mg/g); (c), SPAD reading and total chlorophyll (mg/g); (d), SPAD reading and visual chlorotic rating. \(R^2\), Coefficient of determination.

2.75 and TC content 6.5-8 mg/g, and (iii) susceptible – light green to yellow, VCR more than 2.75 and TC content less than 6.00 mg/g, and plant showing some interveinal to complete chlorosis (a typical symptom of Fe-deficiency). The crop was harvested at maturity and pod and haulm yields were recorded. Data were analysed statistically.

The analysis of variance revealed significant mean squares for all the traits under study, viz. chlorophyll \(a\), chlorophyll \(b\), TC content, VCR and SPAD readings, recorded at various crop growth stages and also for pod and haulm yields. This indicated the substantial genetic variability present in the test materials for their response to Fe deficiency chlorosis related traits. The VCR scores, chlorophyll contents, SPAD reading, pod and haulm yields for various groundnut genotypes are presented in Table 1.

The TC content and SPAD reading were highest at 9.86 mg/g dry weight of leaves and 41.83, respectively in the tolerant check I\(_1\) (PBS 20100); the lowest SPAD reading of 21.53 was recorded in one of the susceptible checks ICG 7887 (PBS 20055). The genotypes ‘PBS 21063’, ‘23003’, ‘24004’, ‘24040’, and ‘FSD 66’ which fell under the tolerant category showed 9.21, 8.00, 8.55, 8.48, and 8.29 mg/g TC and 37.85, 31.98, 32.59, 35.71 and 33.25 SPAD reading, respectively. All these genotypes showed a SPAD reading of more than 30. The genotypes ‘PBS 12115’, ‘12124’, ‘12126’, ‘21031’, ‘22017’, ‘24001’, ‘24002’, ‘24003’, ‘24006’, ‘24030’, ‘29030’, ‘FSD-1’, and ‘FSD-61’ were categorized as moderately tolerant. The genotypes ‘PBS ’14016’, ‘14021’, ‘22023’ and FSD-37 which were highly sensitive to chlorosis showed SPAD...
reading between 22.0 and 25.8 and chlorophyll content below 6 mg/g. The lowest TC content of 5.15 mg/g was recorded in ‘PBS 14016’ with a SPAD reading 22.01.

Correlation coefficients ($r$) and regression equations between SPAD reading and chlorophyll contents and VCR are presented in Table 2. Correlation coefficients between the SPAD reading and chlorophyll contents were highly significant at all the stages of sampling. Over the mean of four samples recorded at 30, 45, 60 and 75 DAE, the $r$ values between the SPAD reading and chlorophyll $a$ (0.94**), chlorophyll $b$ (0.90**), and TC content (0.93**) were very high, positive and significant, indicating closer relationship of these traits with the SPAD reading, i.e. higher the SPAD reading higher will be the chlorophyll pigments and vice versa. The regression lines (Figure 2 a–d) showed that these variables are linearly related with each other. On the basis of the linear relationship, regression equations were developed (Table 2). Further, to test the consistency in ranking of genotypes with respect to the SPAD reading and estimated chlorophyll contents, the rank correlation coefficients ($r_d$) were calculated. It was found that the value of $r_d$ between SPAD and chlorophyll $a$, $b$ and TC content was very high and positive (0.94, 0.91 and 0.94, respectively), showing similar rankings of different genotypes. In the present investigation, the tolerant genotypes showed SPAD reading more than 30 and total chlorophyll content more than 8.0 mg/g dry weight of leaves and VCR below 2.0. On the other hand, genotypes with SPAD reading below 25, TC content less than 6.0 mg/g, and VCR more than 2.75 were found to be sensitive to iron-chlorosis. On the basis of the regression equations given here, it is convenient for researchers to predict chlorophyll content and VCR in the plants for categorization of genotypes.

The use of the SPAD meter in nitrogen management of tall fescue8 (Festuca arundinacea) and switch grass9 (Panicum virgatum) has been demonstrated. The present study clearly shows that the chlorophyll meter (SPAD) is an efficient and speedy equipment for chlorophyll estimation in groundnut and can be used for screening genotypes having higher efficiency of iron utilization and thus selecting iron-chlorosis tolerant genotypes.


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Replacement of live-food with refrigerated-plankton food for Cyprinus carpio (L.) larvae cultured with three different types of biological filters

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Common carp, Cyprinus carpio (L.) larvae were cultured with either live-food or refrigerated-plankton food in the recirculating system. Three types of biological filters: (i) pebbles, foam and weed (Lemna major) filter system (PFWS), (ii) pebbles and foam filter system (PFS), and (iii) only weed (Lemna major) filter system (WS) were used for each feeding scheme. After 40 days of culture, a significantly higher ($P < 0.01$) rate of survival was obtained with the live-food system (LFS) than the refrigerated-plankton food system (RPFS) regardless of filtration type. The final average weight was influenced by both filtration unit and food. Significantly higher ($P < 0.05$) values for average weight (130 ± 1.5 mg), specific growth rate (4.69 ± 0.01), and RNA/DNA ratio (5.60 ± 0.13) were obtained in the PFWS of live-food treatment. Food was more efficiently utilized in the LFS as indicated by the significantly lower ($P < 0.05$) values of food conversion ratio (0.93 to 1.60) compared to the RPFS (3.87 to 4.91). Amylase (0.09–0.162 mg maltose/mg protein/h) and proteolytic enzyme (2.52–4.70 mg tyrosine/mg protein/h) activities were significantly higher in the LFS than the RPFS. Significantly higher values for ammonia (0.014 ± 0.031 mg/l), phosphate (0.150–0.157 mg/l) and COD (197–207 mg/l) were observed in the PFS than for the other two filter systems. Results indicate that use of Lemna major alone or in conjugation with pebbles and foam helped in the maintenance of improved water quality in the culture system which resulted in the better performance of carp larvae.

Common carp, Cyprinus carpio (L.) is an important commercial fish in India and is widely used for compos-

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A multidisciplinary approach, such as a study of digestive physiology and zootechnical performance, is essential to gain complete control over larviculture as well as for sustainable aquaculture development. The present investigation aims to study the effect of live-food and refrigerated-plankton food on the performance of common carp larvae and on the maintenance of water quality with three different biological filtration units over a 40-day culture period.

Carp larvae (6-day-old, 1.2 ± 0.07 mg) were cultured with two feeding regimes, the live-food system (LFS) and the refrigerated-plankton food system (RPFS) and with three types of biological filters – (i) pebbles, foam and weeds system (Lemna major) (PFWS), (ii) pebbles and foam system (PFS) and (iii) weeds system (Lemna major) (WS) in recirculating system. The recirculating system consisted of two parts: the upper part had a square glass aquarium (15 l each) and the lower part had a bigger (55 l) rectangular glass aquarium. The lower part was divided into two compartments of 45 l and 10 l by a glass partition. A biological filter was used in the first, bigger chamber and through a channel the filtered water was brought into the smaller chamber from which it was pumped into the fish culture units with the help of a water pump.

In the PFS, a 20 cm layer of pebbles was overlaid by a 20 cm layer of foam (400 mm). The two layers were separated by a nylon net. In the PFWS system, an additional bed of Lemna major (30 cm × 45 cm, 100 g wet weight) was kept under the pebbles and foam layers. In the third one, only a layer of Lemna major (30 cm × 45 cm, 100 g wet weight) was maintained. Water flow rate used in the fish aquarium was 0.188 l/min. The duration of water circulation was 6 h per day.

Larvae were stocked at the rate of 125/15 l aquarium. Three replicates were maintained for each group (3 replicates × 3 types of filters × 2 types of food). In the LFS, plankton was supplied at the rate of 120 mg/aquarium (dry weight). In the RPFS, the same amount of plankton was kept in the refrigerator at 4°C for 12 h and then directly supplied to the larvae. Qualitative analysis of plankton samples showed that Ceriodaphnia spp. was the dominant species contributing about 70% of the total plankton population. Other species were Mesocyclops spp. (10%), Brachionus spp. (18%) and the rest were phytoplanктon. Water quality parameters were monitored at 10-day intervals throughout the experimental period. Water temperature ranged from 23 to 25.5°C throughout the culture period. The pH of water was measured with a pH meter (HANNA model Hi 8424). Dissolved oxygen was determined with a dissolved oxygen meter (Orion model 810). Ammonia, nitrite, phosphate and COD were measured according to standard methods1. After 40 days of growing, larvae were harvested, counted and weighed. In order to study the amylase and proteolytic activity, larvae were sampled at 8 am before morning feeding at the end of the experiment (after 40 days). One larva was sacrificed for each sample. Three replicates were used for each treatment. Dissections under microscope were conducted on a glass plate maintained at 0°C. The digestive tract was homogenized in 10 volumes (v/w) of ice-cold distilled water and centrifuged at 4°C at 10,000 g. The supernatants were taken for analysis.

Amylase activity was assayed according to the method in which the increase in reducing power of buffered starch solution is measured5. The incubation mixture consisted of 1 ml of 10% starch solution, 1 ml of 0.1 M phosphate buffer (pH 7.0), 1 ml of 10% NaCl and 1 ml of enzyme solution. After 1 h of incubation, reaction was stopped by the addition of 0.5 ml 3,5-dinitro-salicylic acid and absorbance was measured at 540 nm. Amylase activity was expressed in terms of mg of maltose liberated per mg of tissue protein per h at 37°C.

Proteolytic enzyme activity was measured by using a casein substrate1,4. The reaction mixture consisted of 1 ml of substrate solution, 1 ml of 0.1 M phosphate buffer (pH 7.6), 1 ml of calcium chloride and 1 ml of crude enzyme extract. Reaction was stopped after 1 h of incubation with 3 ml of 5% TCA solution. After standing for 10 min, the precipitate was removed by centrifugation and the supernatant was stained with diluted Folin–ciocalteu reagent and absorbance was measured at 650 nm. The results were calculated from a standard curve of tyrosine. Protein was estimated by the standard method3. The results are given in mg of tyrosine per mg of protein per h at 37°C as the specific activity. The nucleic acids were determined by pentose analysis6. RNA and DNA were determined by orcinol reaction and diphenylamine reaction, respectively.

Food conversion ratio (FCR) and the specific growth rate (SGR) were calculated according to the following equations: FCR = Wf/Wi; SGR = 100 × (ln W2 − ln W1)/t, where W1 and W2 are the initial and final weights of larvae, Wf is the dry weight of food and t is the time in days. Differences in fish growth, survival, enzyme activity and water quality parameters were evaluated by analysis of variance, least square difference test and regression analysis (with a computer using the SPSS programme). The level of significance was accepted at P < 0.05.

Water temperature ranged from 23 to 25.5°C throughout the culture period. The pH gradually increased over time in all culture systems except the PFWS of the LFS (Figure 1 a). Significantly higher (P < 0.05) level of dissolved oxygen was observed in the LFS than the RPFS regardless of filtration units. Highest mean value for ammonia (0.031 ± 0.012 mg/l) was obtained in the PFS of the RPFS. Nitrite levels were significantly higher (P < 0.05) in the beginning of the study than dur-
ing the remaining culture period (Figure 1b) regardless of culture systems and food. Unlike nitrites, values for phosphate and COD were minimum in the beginning of the study and increased over time (Figure 1b). Mean values of phosphate were significantly higher \((P < 0.05)\) in the PFS of both feeding schemes (LFS: \(0.150 \pm 0.024 \text{ mg/l} \), RPFS: \(0.157 \pm 0.024 \text{ mg/l} \)). Similarly, mean COD level was significantly higher \((P < 0.05)\) in the PFS \((197 \pm 35 \text{ to } 207 \pm 41 \text{ mg/l})\) of both feeding regimes than the remaining systems.

There was no significant difference \((P > 0.05)\) in the survival of \textit{Cyprinus carpio} larvae among the PFWS and WS of the LFS as well as PFWS and WS of the RPFS. Highest rate of survival \((85\%)\) was obtained in PFWS and WS of the live-food fed group (Table 1). Significantly higher \((P < 0.01)\) average weight \((130 \pm 1.5 \text{ mg})\) was obtained in the PFWS of LFS. Food conversion ratio showed the minimum and maximum values in the PFWS \((0.93 \pm 0.01)\) of LFS and PFS \((4.91 \pm 0.5)\) of RPFS, respectively. Highest SGR value was obtained in the PFWS of the LFS (Table 1). RNA/DNA ratio was maximum and minimum in PFWS of the LFS \((5.60 \pm 0.13)\) and PFS of the RPFS \((3.48 \pm 0.05)\), respectively. RNA/DNA ratio in PFWS of the LFS was 5 to 38% higher than the remaining culture systems.

Amylase activity in the PFS of LFS was significantly \((P < 0.01)\) lower than the other two filtration systems of the same feeding scheme. The enzyme activity in the PFWS of the the LFS was 3.5 to 11-fold higher than the RPFS. Similarly, the specific proteolytic activity \((4.74 \pm 0.24 \text{ mg tyrosine/mg protein/h})\) was significantly higher \((P < 0.05)\) in the PFWS of the LFS. Specific proteolytic activity in this treatment was 3.4 to 7-fold higher than the RPFS (Table 1). Both the amylase and proteolytic activities showed direct relationship (Figure 2a and b) with the average weight of fish regardless of culture systems and feeding regimes.

Feeding of \textit{C. carpio} larvae with live-food led to a better survival rate than the larvae fed with refrigerated-plankton, cultured with similar filtration units in the recirculating systems. The final average weight and the specific growth rate were also significantly \((P < 0.05)\) higher in the live-food treatment than the refrigerated one. In African catfish, significantly higher \((P < 0.05)\) final mean weight and specific growth rate were obtained in the group fed with live \textit{Artemia} than the group fed with frozen ones, but there was no significant difference \((P > 0.05)\) in the survival rate\(^7\). Asian seabass, \textit{Lates calcarifer} (Bloch) showed significantly higher SGR and survival when fed with live \textit{Moina microcopa} than frozen ones\(^8\). The denaturation of vitamins and proteins, or lipid oxidation eventually aggravated by the thawing procedure and the thawing duration may explain inferior results obtained with a diet of frozen food organisms. The loss of essential nutrients during thaw-
Figure 1b. Mean values for ammonia, nitrite, phosphate and COD in recirculating systems during various days of culture of common carp with three biological systems of two feeding regimes. Each point represents three replicates.
Table 1. Mean and SE of final average weight, percentage survival, feed conversion ratio (FCR), specific growth rate (SGR), RNA/DNA ratio, amylase and proteolytic enzyme activities for common carp fed live-food and refrigerated-plankton food with three types of biological filters in recirculating systems

<table>
<thead>
<tr>
<th></th>
<th>PFWS</th>
<th>WS</th>
<th>PFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average weight (mg)</td>
<td>LFS 1.130 ± 1.5\textsuperscript{a}</td>
<td>86.33 ± 0.57\textsuperscript{b}</td>
<td>76.33 ± 0.33\textsuperscript{c}</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>LFS 11.85 ± 1.45\textsuperscript{a}</td>
<td>86.33 ± 0.57\textsuperscript{b}</td>
<td>76.33 ± 0.33\textsuperscript{c}</td>
</tr>
<tr>
<td>FCR</td>
<td>LFS 10.93 ± 0.01\textsuperscript{a}</td>
<td>11.29 ± 0.01\textsuperscript{d}</td>
<td>11.60 ± 0.07\textsuperscript{d}</td>
</tr>
<tr>
<td>SGR</td>
<td>LFS 10.93 ± 0.01\textsuperscript{a}</td>
<td>11.29 ± 0.01\textsuperscript{d}</td>
<td>11.60 ± 0.07\textsuperscript{d}</td>
</tr>
<tr>
<td>RNA/DNA ratio</td>
<td>LFS 15.60 ± 0.13\textsuperscript{a}</td>
<td>15.33 ± 0.03\textsuperscript{b}</td>
<td>14.90 ± 0.05\textsuperscript{c}</td>
</tr>
<tr>
<td>Amylase (mg maltose/mg protein)</td>
<td>LFS 0.162 ± 0.01\textsuperscript{a}</td>
<td>0.150 ± 0.005\textsuperscript{b}</td>
<td>0.109 ± 0.002\textsuperscript{c}</td>
</tr>
<tr>
<td>Proteolytic enzyme (mg tyrosine/mg protein/h)</td>
<td>LFS 14.74 ± 0.24\textsuperscript{a}</td>
<td>13.30 ± 0.08\textsuperscript{b}</td>
<td>12.52 ± 0.21\textsuperscript{c}</td>
</tr>
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Means not sharing the same letter for each parameter are significantly different (P < 0.05).

Figure 2. Relationship between a, final average weight and amylase activity; and b, final average weight and specific proteolytic enzyme activity in common carp.

Figure 3. Relationship between RNA/DNA ratio and growth rate (mg/day) of common carp.

Average weight and RNA/DNA ratio of larvae were significantly different (P < 0.05) among three filtration systems of both the LFS and RPFS. It seems that survival of larvae was mainly influenced by the quality of food but the weight gain and RNA/DNA ratio were influenced by both the food and the quality of water. RNA/DNA ratio showed a positive relation with growth (Figure 3). Similar results were also obtained in salmonids\textsuperscript{10}. Significantly lower (P < 0.05) values for ammonia, nitrite, phosphate and COD prevailed in the PFWS which resulted in better performance of larvae in this system. In fish subjected to the stress of high environmental temperatures\textsuperscript{11} or heavy metal salts\textsuperscript{12}, the ratios between these two nucleic acids reduced.

The results of this study indicate that the use of duckweed *Lemna major* as a biological filter in conjugation with the simple pebbles and foam filter helped in the maintenance of water quality in the intensive culture of *C. carpio* larvae which resulted in better performance of.
larvae. The results also showed the poor growth of lar-
vae with refrigerated-plankton food. Further study is
needed in this regard.

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NMR studies of a neurotoxin
(candoxin) from Bungarus candidus –
Presence of a predominantly b-sheet
structure

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Complete sequence-specific 1H resonance assignments of a neurotoxin, candoxin, from Bungarus candidus has been reported. Qualitative interpreta-
tion of the NOEs, chemical shift indices and deuterium exchange rates indicate a highly ordered and rigid conformation for this protein with an extended conformation for more than 50% of the residues and the complete absence of a-helical segments.

TOXICITY of snake venom arises from a complex mixture of ingredients. The toxins target ion channels, re-
ceptors and enzymes. One class of these molecules is neurotoxins which bind to cholinergic receptors leading to the closure of the ion channel, thus blocking neurotransmission1–3. Neurotoxins can be broadly classified into three groups: short, long and cytotoxins. Short neurotoxins are 58–62 residues long and have four disulfide bridges, while long neurotoxins have 65–74 residues and five disulfide bridges. In both cases the structure consists of three loops protruding from a central core. Most of the sequence heterogeneity among the long neurotoxins is in the first loop and in the C-terminal tail. Structures of several neurotoxins have been solved by crystallographic and NMR methods4–8. Though the overall topologies are similar, the function and interaction of each neurotoxin is unique9. Recently we have isolated and purified a weak neurotoxin, candoxin, from Bungarus candidus (Malayan Krait) venom. Candoxin differs from other known long neurotoxins in that it acts on both pre- and post-synaptic sites.

Here, we describe the complete sequence specific 1H NMR assignments of this toxin and its secondary structure. Protein concentration of 4.5 mM at pH 3.0 (99.9% 2H2O and 90% H2O/10% 2H2O) in Shigemi tubes were used wherein smaller volumes are required. NMR experiments carried out on a Varian Unity + 600 MHz NMR spectrometer are: two quantum filtered correlated spectroscopy10 (2QF-COSY), three quantum filtered correlated spectroscopy11 (3QF-
COSY), clean total correlation spectroscopy12 (clean TOCSY) (τm = 80 ms), nuclear Overhauser enhancement spectroscopy13 (NOESY) (τm = 50, 75, 150 and 200 ms) and watergate NOESY14 (τm = 100, 150 and 200 ms). Relayed COSY15 was done on a Bruker AMX
500 MHz NMR spectrometer. Deuterium exchange studies were carried out by recording a series of 1D and 2D TOCSY spectra, 12 m after the lyophilized and fully protonated toxin was dissolved in 99.9% 2H2O. Sections of TOCSY and NOESY spectra in the H3–H3 region are shown in Figures 1 and 2, respectively.

Candoxin has a single polypeptide chain containing 66 amino acid residues with the sequence: MKC5
C6NFTDC11 RAGELKVC19 ASGEKYC24 FKESWREA-
RGTRIERGC27 AAT27 PKGSVYGLYVL29 C59–60 TTDD
C65N.

In the 1D 1H NMR spectrum, we observe several up-
field shifted methyl resonances, downfield shifted Hα
resonances and a highly dispersed Hβ region, which indicates a highly ordered and rigid conformation.

Amino acids have been classified on the basis of their side chain spin systems and identified using established procedures16. All the five Gly residues could be identified on the basis of their expected Hβ–Hα connectivity.
pattern in the 2QF-COSY spectrum and by their absence in the 3QF COSY spectrum. All the five Ala and four of the five Thr residues have been unambiguously identified in the 2QF-COSY spectrum. The fifth Thr was assigned by sequence-specific resonance assignment. Absence of H^N–H^β cross peaks in 3QF COSY and observation of H^N–H^β relay peaks in TOCSY substantiate the identification of Thr residues. 3 Val, 2 Ile and 3 Leu have been unambiguously identified by the concerted use of 2QF-COSY, 3QF-COSY and TOCSY. There are no overlap problems in identifying protons belonging to these amino acid residues. There are altogether 24 AMX spin systems (3 Ser, 3 Asp, 2 Asn, 10 Cys, 2 Phe, 3 Tyr and 1 Trp). Identification of all 24 pairs of H^α–H^β connectivities was straightforward with the use of 2QF-COSY, 3QF-COSY and TOCSY. In most cases, only one H^α–H^β cross peak has been observed in 2QF-COSY. TOCSY and 3QF-COSY spectra enable identification of the second cross peak. Downfield shifts of both H^β protons in the case of Ser residues helped in distinguishing them from other AMX spin systems. The six aromatic AMX spin systems have been identified from the observation of nOes from H^β protons to the nearest aromatic ring protons. The aromatic ring protons of W31 have been identified from the indole ring H^α, which shows COSY/TOCSY correlation to H^β and NOESY correlation to H^γ and H^δ. Two distinct nOes are observed for W(H^α), which arise from H^γ and H^β of the indole rings. The distinction between the two protons has been achieved from the H^α-H^β cross peak in the TOCSY spectrum. Protons, H^α and H^β are not J-coupled hence do not show mutual interaction in TOCSY, but a strong nOe between these protons is observed (d = 2.84 Å). From a knowledge of the H^β position, the remaining indole protons, H^γ, H^δ and H^ε have been assigned using TOCSY. Once the chemical shift of H^δ proton has been identified, the rest of the Trp ring protons (H^γ, H^ε and H^β) could be identified. Out of the 16 long side-chain residues, 6 could be assigned to 5 Glu and 1 Met from their characteristic connectivity pattern in TOCSY. All the Arg and Lys spin systems have been identified from their characteristic fingerprint in the TOCSY spectrum. The lone Pro spin system could be identified during sequence-specific resonance assignments.

In the ‘fingerprint (H^α–H^N) region’ of 2QF-COSY and TOCSY, one expects 70 H^α–H^β J-correlations (accounting for the absence of H^β in the Pro residue and presence of an extra H^β resonance in the Gly residues). We observe all the expected cross peaks except M1 (H^α-H^N), Gly H^N proton resonances have been distinguished by making use of the relayed COSY. The single Trp (W31) has been chosen as a starting point for sequence-specific resonance assignment using the NOESY spectrum. Intra-residue nOes H^α to H^β and H^β protons and H^α–H^β allow complete identification of the protons of W31. With this knowledge, nOe cross peaks corresponding to sequential distances, d_Ag and d_gN between neighbouring amino acid residues, led to the assignment of H^α and H^β of S30. The S30(H^β) was then identified in the COSY spectrum via the expected H^α–H^β J-connectivity. Similarly we could assign resonances for R32. The fact that the tripeptide segment S30-W31-R32 is unique in the primary sequence of candoxin helps in unambiguous sequence-specific resonance assignment. We could walk on either side of W31. Following this procedure, assignments were obtained from W31 to C47 in one direction and W31 to M1 in the other. A break was encountered in the forward direction due to the presence of P48. Figure 2 shows an illustrative example of the sequential connectivity diagram.

G50 and G22 served as other useful starting points. All the five Gly containing tripeptide sequences, i.e. A13-G14-E15, S21-G22-E23, R35-G36-T37, K49-G50-S51 and F53-G54-L55 are unique. These tripeptides are
easily assigned and sequential assignments could be achieved following the connectivity pathway from these Gly in either direction. Thus, we could achieve unambiguous resonance assignments for all the 66 amino acid residues (Figure 1). The chemical shifts of individual protons thus obtained have been submitted in the BioMagResBank (http://www.bmrb.wisc.edu) under BMRB accession number BMRB-4391.

The \(^1\)H resonance assignments coupled with nOe pattern, chemical shift differences for \(^1\)H from the corresponding random coil values and deuterium exchange rates enable us to comment on the secondary structure of candoxin. In \(\alpha\)-helices sequential \(\Delta\)H–\(\Delta\)H distances (\(d_{\text{SN}}\)) are lesser than 2.8 Å and strong \(d_{\text{SN}}\) nOes are expected. Such \(d_{\text{SN}}\) contacts are not seen in parallel or anti-parallel \(\beta\)-sheets (\(d_{\text{SN}} \times 5\) Å). Further, one expects medium intensity \(d_{\text{NN}}\) (\(i, i+3\)) and \(d_{\text{NN}}\) (\(i, i+4\)) nOes from \(\alpha\)-helical segments. On the other hand, for \(\beta\)-sheet segments strong sequential \(\Delta\)H–\(\Delta\)H nOes \((d_{\text{SN}} \approx 3\) Å) are expected. The complete absence of \(d_{\text{SN}}\) (\(i, i+3\)) and \(d_{\text{SN}}\) (\(i, i+4\)) nOes and the presence of very few \(d_{\text{SN}}\) and \(d_{\text{SN}}\) (\(i, i+2\)) nOes in candoxin indicates the absence of \(\alpha\)-helical segments. Strong sequential \(d_{\text{SN}}\) nOes and weak self \(d_{\text{SN}}\) connectivities suggest extended conformation.

The Chemical Shift Indices (CSI) for \(\Delta\)H are shown in Figure 3a. One finds several sequences of residues having shifts displaced to the downfield side. This is indicative of \(\beta\)-sheet conformation\(^{17-19}\). The breaks probably represent the turns.

Depending on the location in the protein, largely different exchange rates are found for individual \(\Delta\)H. Out of the 65 backbone amide protons we could identify 41 resonances in a freshly prepared \(\text{H}_2\text{O}\) solution. This implies that only 24 protons, exchange within 12 min (fast exchange). Another 23 \(\Delta\)H exchange over a period of 10 h (medium exchange rate). The remaining \(\Delta\)H survive even beyond this period (slow exchange) (Figure 3b). The fast exchanging protons are obviously exposed to the solvent. However, the remaining protons lie in the hydrophobic core of candoxin and some are probably involved in hydrogen bonding.

Thus under experimental conditions, candoxin adopts a highly ordered structure. CSI, nOes and deuterium exchange rates are consistent with each other and indicate that most of the residues are in the \(\beta\)-sheet conformation. There are no \(\alpha\)-helical segments. The disulfide linkages have also been established by NMR, which shows that candoxin has a bridge at the end of loop I (Cys6-Cys11) instead of loop II as found in other neurotoxins. Thus candoxin has a structure distinctly different from other neurotoxins. We are currently working on the 3D structure of candoxin and the results will be published elsewhere.

The NMR spectra were recorded at the National Facility for High Field NMR, located at TIFR, Mumbai and supported by the DST, DBT and CSIR.

Figure 3. a. Plot of chemical shift indices (CSI) for each amino acid residue in candoxin for the \(\Delta\)H protons. +1 and −1 correspond to a low field and high field shift, respectively, related to the corresponding random coil chemical shift values. If the chemical shift is within ±0.1 ppm from the CSI value, then the index for the residue is taken as zero. A higher positive difference is said to have a +1 index and a negative shift −1. An uninterrupted set of four or more +1 or −1 values is usually taken as an indication of a \(\beta\)-strand and \(\alpha\)-helix, respectively. The plot reveals several \(\beta\)-sheet structural elements; b. Exchange rates of amide (\(\Delta\)H) protons in candoxin. The exchange rates have been divided into three categories, slow, medium and fast, as explained in the text. The regime having CSI of +1 (\(\beta\)-sheets) generally shows medium to slow exchange.

RESEARCH COMMUNICATIONS

Excess use of *Momordica charantia* extract may not be safe with respect to thyroid function and lipid peroxidation

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Effects of alcoholic extract of *Momordica charantia* fruits (100, 200, 400 and 500 mg kg\(^{-1}\) body weight day\(^{-1}\) for 15 days) on the alterations in serum thyroxine (T\(_4\)) and triiodothyronine (T\(_3\)) concentrations and on hepatic lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activities were studied in adult male mice. Higher concentrations of *M. charantia* (400 and 500 mg kg\(^{-1}\)) decreased the serum concentrations of T\(_3\) and T\(_4\) and enhanced hepatic LPO with a concomitant decrease in CAT activities, indicating a thyroid inhibitory and peroxidative role of the plant extract. However, with the lower doses (100 and 200 mg kg\(^{-1}\)) these adverse effects were not seen. Although 200 mg kg\(^{-1}\) was found to enhance T\(_3\), T\(_4\) was reduced. Since two higher doses inhibited thyroid hormone concentrations and increased hepatic LPO, we suggest that *M. charantia* fruit extract, when used in excess may prove to be harmful with respect to thyroid function and lipid peroxidation.

BITTERGOUD, *Momordica charantia*, Linn (Family-Cucurbitaceae), commonly known as ‘Karela’ in India is a climbing plant, cultivated throughout southern Asia. This plant is mainly used for the consumption of its fruits as vegetables. While, its antispermatic, hypoglycaemic and antidiabetic properties have been documented from time to time\(^{1–5}\), no study was made with respect to alterations in thyroid hormone(s). In fact, literature on the regulatory role of commonly used consumable plant materials on the thyroid function is meagre\(^{6,9}\), despite the fact that thyroid gland is one of the most important endocrine organs\(^7\), primarily responsible for the regulation of body metabolism. Therefore, in the present study, an attempt has been made to reveal the possible role of *M. charantia* fruit extract in the alteration of two thyroid hormones, triiodothyronine (T\(_3\)) and thyroxine (T\(_4\)). Lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activities were also investigated in order to evaluate the safe nature of the extract.

Diethylene triamine pentaacetic acid (DTPA) was purchased from Sigma Chemical Co, USA and thiobarbituric acid (TBA) was from E. Merck, Germany. Sodium dodecyl sulphate (SDS), pyrogallol and hydrogen peroxide were of reagent grade, obtained from Loba Chemie, Mumbai, India. Radioimmunoassay (RIA) kits for the quantification of T\(_3\) and T\(_4\) levels were supplied by Bhabha Atomic Research Centre (BARC), Mumbai, India.

An alcoholic extract of *Momordica* fruits was prepared according to the method of Shibib et al.\(^8\). In brief, fresh fruits purchased from the local market were thoroughly washed in tap water, cut into pieces and the seeds removed manually. About 1 kg of seedless vegetable was then blended with 1500 ml of 95% ethanol, left at room temperature with occasional shaking for 48 h. The suspension was filtered through a cheesecloth and the filtrate was evaporated at 40–50°C to remove alcohol and to produce a free flowing powder. On the day of experimentation, the desired amount of powder was suspended in distilled water for the final administration.

Three-month-old Swiss albino male mice weighing 32 ± 3 g were used in five groups of 8 each. Groups II, III, IV and V were administered with different doses (100, 200, 400 and 500 mg kg\(^{-1}\) body wt) of *M. charantia* extract by gastric incubation. Two of these doses (200 and 500 mg kg\(^{-1}\)) were taken from earlier studies\(^8,9\). The other two doses were considered because of the fact that our preliminary investigation with 500 mg kg\(^{-1}\) proved to be toxic (data not shown). Group I, receiving 0.1 ml of vehicle (distilled water) served as control. The treatment was continued for 15 days. On the last day of experimentation, blood was collected from each animal and serum was separated by centrifugation and stored at –20°C for the estimation of T\(_3\) and T\(_4\) levels. After exsanguination, the liver was removed, washed twice in phosphate buffered saline and immediately processed for estimation.

Serum concentrations of T\(_3\) and T\(_4\) were estimated by RIA, following the protocol of BARC, as followed earlier in our laboratory\(^5,9\). Lower limits of sensitivity for T\(_3\) and T\(_4\) were 0.07 ng ml\(^{-1}\) and 0.12 ng ml\(^{-1}\), respec-
Inter-assay variation was less than 5% for both the hormones.

Liver was homogenized in 10% (w/v) ice-cold 0.15 M phosphate buffer (pH 7.4) using Potter–Elvehjem teflon homogenizer and the homogenate was centrifuged at 15,000 g at 4°C for 30 min. Assay of LPO was done by the method of Marklund and Marklund, and the amount of malondialdehyde (MDA) formed was measured by taking the absorbance at 532 nm (extinction coefficient 1.52 × 10^5) using Shimadzu UV-160 A spectrophotometer. LPO was finally expressed as the nmole of MDA formed per h per mg protein.

Hepatic SOD activity was assayed according to the method of Marklund and Marklund, and the enzyme activity was expressed as units per mg protein. One unit of this enzyme is defined as the enzyme activity that inhibits autooxidation of pyrogallol by 50%. CAT activity was estimated in the liver homogenate following the method of Marklund and Marklund, and the amount of malondialdehyde (MDA) formed was measured by taking the absorbance at 532 nm (extinction coefficient 1.52 × 10^5) using Shimadzu UV-160 A spectrophotometer. LPO was finally expressed as the nmole of MDA formed per h per mg protein.

The results are summarized in Figure 1 and Table 1. Dose-specific alterations in hepatic LPO and in thyroid hormone concentrations were observed. LPO was significantly high in 400 mg kg⁻¹ body wt (P < 0.05) and in 500 mg kg⁻¹ body wt (P < 0.01) treated animals compared to the control value. CAT activity was also significantly decreased (P < 0.001) by these doses. In these groups, there was also a significant decline in both T₃ and T₄ concentrations (P < 0.001 for both the hormones). However, in 200 mg kg⁻¹ treated group, although T₃ concentration was significantly more, T₄ concentration was reduced (P < 0.001 for both).

The results clearly reveal a dose-dependent alteration in thyroid hormone concentrations following the treatment of M. charantia fruit extract. An increase in T₃ concentration by 200 mg kg⁻¹ body wt of the plant extract and a decrease by the two higher doses (400 and 500 mg kg⁻¹ body wt) suggest that low dose may stimulate the synthesis and/or release of T₃ whereas, higher doses are inhibitory to both the hormones. Although some other plant extracts have already been reported to inhibit thyroid function, the dose-dependent alterations in thyroid hormone concentrations and in LPO as observed presently following M. charantia extract treatment, is an interesting observation.

Out of the two thyroid hormones, T₄ is synthesized by the thyroid gland, whereas T₃, which is metabolically more potent than T₄, is largely produced by peripheral conversion of the latter hormone in the liver and kidney. Therefore, M. charantia-induced alterations in both the thyroid hormones indicate that the plant extract might be acting both at the level of the thyroid gland and at the level of extra-thyroidal tissues, including liver. Interestingly, when hepatic LPO was studied, it was enhanced by the two higher doses of the plant extracts, indicating the hepatotoxic effects of these doses. Changes in the antioxidant enzyme, CAT, also corroborate with that of LPO. Since LPO is a deleterious process, increase in LPO and a concomitant decrease in CAT activity in higher dosed groups, reveal that excess use of the extract could be harmful.

Although other mechanisms of action(s) of M. charantia cannot be ruled out, from the present findings it is evident that chronic use of moderate amounts of alcoholic extract of M. charantia fruit may not cause hepatic damage, but the higher doses may prove to be deleterious, at least with respect to thyroid function and hepatic LPO.

Role of nitrification inhibitors on nitrous oxide emissions in a fertilized alluvial clay loam under different moisture regimes

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Fertilized soil is considered to be a major source of nitrous oxide (N\textsubscript{2}O) in the atmosphere. In a laboratory incubation experiment, emission of N\textsubscript{2}O was studied from a clay loam soil fertilized with urea alone and urea combined with nitrification inhibitors, viz. dicyandiamide (DCD) and thiosulphate, at different moisture regimes. Emission of N\textsubscript{2}O was observed from day 1 and was appreciable during the first 2 weeks and decreased subsequently. Soil at 80% max. water holding capacity (WHC) had highest total N\textsubscript{2}O-N emission followed by soil at field capacity and submergence. Total emissions from control (no N), urea, urea combined with DCD (urea–DCD) and thiosulphate (urea–thiosulphate) were 78.88, 744.39, 415.64 and 654.75 mg N\textsubscript{2}O-N kg\textsuperscript{-1} soil, respectively, at 80% max. WHC, while total emissions from the corresponding treatments under submergence and at field capacity were 31.7, 298.5, 138.4, 272.8 and 54.6, 333.7, 217.8, 313.5 mg N\textsubscript{2}O-N kg\textsuperscript{-1} soil, respectively. Of the applied N, nitrogen lost through total N\textsubscript{2}O emission was 1.06, 0.54 and 0.92% at 80% max. WHC; 0.45, 0.26 and 0.41% at field capacity and 0.43, 0.17 and 0.39% under submergence from urea, urea–DCD and urea–thiosulphate, respectively. Thus, addition of DCD reduced total N\textsubscript{2}O-N emission to the extent of 60, 41.5 and 49.4% under submergence, at field capacity and at 80% max. WHC, respectively, when compared to urea alone, while the corresponding reductions on addition of thiosulphate were 9.6, 7.2 and 13.5%.

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applied in the control. DCD was applied at 10% of nitrogen applied through urea. As DCD also contains nitrogen, the amount of urea was reduced proportionally on combining with DCD, to ensure equal application of N to all nitrogen treatments. Thiosulphate was applied through sodium thiosulphate as S at 10% of nitrogen applied through urea. Fertilizer and inhibitors were added through aqueous solutions to ensure their uniform distribution in the soil. Phosphorous and potassium were applied @ 22.2 mg kg⁻¹ soil (equivalent to recommended levels of 50 kg P₂O₅ and K₂O ha⁻¹) through aqueous solutions of single super phosphate and muriate of potash, respectively, in all treatments. Before putting the beakers for incubation, the soil moisture was adjusted at field capacity (18% w/w), 80% max. WHC (30% w/w) and submergence (2 cm of standing water). Beakers were weighed every two days to find the moisture loss, which was replenished by adding distilled water, whenever detected.

For gas sampling, the incubated soil samples were closed airtight in beakers fitted with rubber corks with additional provision for sampling port. Air samples in the beaker were collected at 0 and 1 h in 10 ml syringes through the sampling ports fitted with rubber septa. The samples were fed to a gas chromatograph (HP 5890 Series II), fitted with an electron capture detector (ECD) and a 6'×½″ stainless steel column (porapak N). The column, injector and detector temperatures were set at 50, 120 and 350°C, respectively. Nitrogen was used as the carrier gas with a flow rate of 14 ml min⁻¹. A compressed zero-air cylinder calibrated at 313 ppbv N₂O (calibrated with a primary N₂O standard of 500 ppbv obtained from National Physical Laboratory, New Delhi) was used as secondary N₂O standard. Total N₂O emission during the study period was calculated by integration of actual N₂O emissions on each sampling day and cumulative N₂O emissions during periods in between sampling days. These cumulative emissions were estimated by multiplication of the average N₂O emission of two successive sampling days by the number of non-sampling days between them. Percentage inhibition of total N₂O emission by nitrification inhibitors was calculated using the following equation:

\[
\% \text{ N}_2\text{O inhibition} = \frac{(\text{TE}_U - \text{TE}_C) - (\text{TE}_I - \text{TE}_C)}{(\text{TE}_U - \text{TE}_C)} \times 100,
\]

where \(\text{TE}_U\) is the total N₂O emission from urea alone; \(\text{TE}_C\) is the total N₂O emission from the control; \(\text{TE}_I\) is the total N₂O emission with the inhibitor.

Soil samples (500 g) were incubated separately in plastic containers (2000 ml capacity) with the above treatments for NH₄ and NO₃ estimations. Soil samples were drawn intermittently and were extracted with 2

![Figure 1. N₂O emission from fertilized soil incubated at different moisture regimes. Bars indicate mean ± SD; DAI, day after incubation.](image)
Table 2. Accumulation of nitrate in soils supplemented with fertilizer-N at different moisture regimes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Moisture regime</th>
<th>Day 2</th>
<th>Day 13</th>
<th>Day 23</th>
<th>Day 27</th>
<th>Day 31</th>
<th>Day 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Submergence</td>
<td>4.06</td>
<td>5.19</td>
<td>5.32</td>
<td>7.16</td>
<td>8.13</td>
<td>9.15</td>
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<td></td>
<td>Field capacity</td>
<td>5.44</td>
<td>10.08</td>
<td>17.72</td>
<td>28.52</td>
<td>39.31</td>
<td>40.2</td>
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<tr>
<td></td>
<td>80% max WHC</td>
<td>4.96</td>
<td>9.18</td>
<td>14.02</td>
<td>20.27</td>
<td>24.13</td>
<td>25.53</td>
</tr>
<tr>
<td>Urea</td>
<td>Submergence</td>
<td>4.2</td>
<td>7.48</td>
<td>9.76</td>
<td>12.22</td>
<td>15.68</td>
<td>18.69</td>
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<td></td>
<td>Field capacity</td>
<td>5.89</td>
<td>14.40</td>
<td>22.91</td>
<td>36.84</td>
<td>50.77</td>
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<td></td>
<td>80% max WHC</td>
<td>6.80</td>
<td>15.6</td>
<td>20.9</td>
<td>32.56</td>
<td>46.1</td>
<td>48.51</td>
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<td>Urea–DCD</td>
<td>Submergence</td>
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<tr>
<td></td>
<td>Field capacity</td>
<td>4.95</td>
<td>10.76</td>
<td>14.57</td>
<td>33.53</td>
<td>47.49</td>
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<td>24.53</td>
<td>43</td>
<td>44.52</td>
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<tr>
<td>Urea–thiosulphate</td>
<td>Submergence</td>
<td>3.78</td>
<td>4.53</td>
<td>6.47</td>
<td>11.81</td>
<td>18.14</td>
<td>22.15</td>
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<tr>
<td></td>
<td>Field capacity</td>
<td>4.43</td>
<td>12.25</td>
<td>21.08</td>
<td>31.5</td>
<td>41.92</td>
<td>45.21</td>
</tr>
<tr>
<td></td>
<td>80% max WHC</td>
<td>4.02</td>
<td>10.4</td>
<td>17.8</td>
<td>29.04</td>
<td>44.9</td>
<td>45.56</td>
</tr>
</tbody>
</table>

Table 3. Accumulation of ammonium in soil supplemented with fertilizer-N at different moisture regimes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Moisture regime</th>
<th>Day 2</th>
<th>Day 13</th>
<th>Day 23</th>
<th>Day 27</th>
<th>Day 31</th>
<th>Day 36</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>Submergence</td>
<td>34.2</td>
<td>30.5</td>
<td>25.2</td>
<td>24.11</td>
<td>22.15</td>
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<td>Field capacity</td>
<td>28.9</td>
<td>26.5</td>
<td>17.8</td>
<td>14.4</td>
<td>10.5</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>80% max WHC</td>
<td>33.5</td>
<td>30.4</td>
<td>20.3</td>
<td>17.6</td>
<td>12.9</td>
<td>8.7</td>
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<tr>
<td>Urea</td>
<td>Submergence</td>
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<td>69.6</td>
<td>64.9</td>
<td>57.1</td>
<td>49.2</td>
<td>40.2</td>
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<tr>
<td></td>
<td>Field capacity</td>
<td>54.6</td>
<td>51.7</td>
<td>48.7</td>
<td>37.3</td>
<td>32.8</td>
<td>27.5</td>
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<tr>
<td></td>
<td>80% max WHC</td>
<td>65.8</td>
<td>60.8</td>
<td>57.9</td>
<td>44.3</td>
<td>39.9</td>
<td>32.1</td>
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<td>Urea–DCD</td>
<td>Submergence</td>
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<td>74.8</td>
<td>68.2</td>
<td>62.1</td>
<td>54.5</td>
<td>45.3</td>
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<tr>
<td></td>
<td>Field capacity</td>
<td>64.7</td>
<td>59.5</td>
<td>53.2</td>
<td>46.5</td>
<td>35.6</td>
<td>32.4</td>
</tr>
<tr>
<td></td>
<td>80% max WHC</td>
<td>72.7</td>
<td>68.6</td>
<td>63.6</td>
<td>44.7</td>
<td>39.4</td>
<td>35.1</td>
</tr>
<tr>
<td>Urea–thiosulphate</td>
<td>Submergence</td>
<td>74.1</td>
<td>71.8</td>
<td>64.5</td>
<td>57.7</td>
<td>51.5</td>
<td>46.2</td>
</tr>
<tr>
<td></td>
<td>Field capacity</td>
<td>61.6</td>
<td>54.9</td>
<td>50.3</td>
<td>38.6</td>
<td>34.9</td>
<td>30.1</td>
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<tr>
<td></td>
<td>80% max WHC</td>
<td>68.9</td>
<td>66.4</td>
<td>53.9</td>
<td>43.2</td>
<td>37.5</td>
<td>32.8</td>
</tr>
</tbody>
</table>

M KCl and distilled water for ammoniacal and nitrate determination by indophenol blue\textsuperscript{16} and phenol disulfonic acid\textsuperscript{17} methods, respectively. The results were subjected to statistical analyses by MSTAT-C\textsuperscript{18}.

\(\text{N}_2\text{O}\) emission started from day 1 after incubation (DAI) with and without fertilizer-N application at all moisture regimes (Figure 1 \(a-c\)). Peak emissions from urea and control were respectively recorded on 3 DAI and 7 DAI at 80% max. WHC, 2 and 3 DAI at field capacity and 5 and 13 DAI, under submergence. On application of urea, mean daily \(\text{N}_2\text{O}\) emission was substantially higher than the control. In the control, mean daily \(\text{N}_2\text{O}\) emission was highest in 80% max. WHC followed by field capacity and submergence. Temporal variation of daily \(\text{N}_2\text{O}\) emission in the control was much lower under submergence (average CV = 95%) than field capacity (av. CV = 149%) and 80% max. WHC (av. CV = 164%). Application of urea at 80% max. WHC led to highest mean daily \(\text{N}_2\text{O}\) emission followed by field capacity and submergence. Temporal variation of daily \(\text{N}_2\text{O}\) emission from urea under different moisture regimes was high but not much different from each other (av. CV = 129–141%).

It has been observed that most of the \(\text{N}_2\text{O}\) at field capacity and 80% max. WHC was released within the first week of incubation (Figure 1 \(a\) and \(b\)), during which hydrolysis of urea took place and \(\text{NH}_4^+\) was formed from urea. Nitrification was high in this period due to presence of appreciable amounts of \(\text{NH}_4^+\) and consequently \(\text{N}_2\text{O}\) may mainly be produced via nitrification. Increase in soil \(\text{NO}_3^-\) at both field capacity and 80% max. WHC during this period indicated that nitrification
was appreciable (Table 2). The earlier appearance of \(\text{N}_2\text{O}\) at field capacity than at 80% max. WHC was due to faster nitrification at field capacity and is confirmed by higher NO\(^-\) accumulation (Table 2). Our results were in conformity with the findings of Billore et al.\(^{19}\), who reported more \(\text{N}_2\text{O}\) emission from fertilizer-treated soil than from natural soil at 50–60% WHC and opined that nitrification plays a major role in \(\text{N}_2\text{O}\) production. At 80% max. WHC, accumulation of NO\(^-\) had supported denitrification, while up to 13 DAI NH\(^+\) was substantial to support nitrification (Tables 2 and 3). At field capacity, higher accumulation of soil NO\(^-\) showed that it was not used up as fast as at 80% max. WHC through denitrification and \(\text{N}_2\text{O}\) was produced mainly via nitrification. Mean daily \(\text{N}_2\text{O}\) emission under submergence was low (Figure 1c) mainly due to slow nitrification, lower NO\(^-\) formation (a precursor of \(\text{N}_2\text{O}\) production via denitrification) and further denitrification of \(\text{N}_2\text{O}\) to \(\text{N}_2\) (ref. 20).

Maximum \(\text{N}_2\text{O}\) emission from urea–DCD and urea–thiosulphate was recorded at 3 and 2 DAI from both 80% max. WHC and field capacity, respectively. Under submergence, peak emissions from urea–DCD and urea–thiosulphate were recorded at 5 and 9 DAI, respectively. On combining DCD with urea, mean daily \(\text{N}_2\text{O}\) emission was found to be appreciably lower than urea alone at all moisture regimes. Mean daily \(\text{N}_2\text{O}\) emission was highest at 80% max. WHC followed by field capacity. Temporal variation of daily \(\text{N}_2\text{O}\) emission from urea–DCD was high (av. CV = 168–176%). Addition of thiosulphate to urea resulted in only a slight reduction in daily mean \(\text{N}_2\text{O}\) emission from urea alone. Temporal variation in daily \(\text{N}_2\text{O}\) emission was high on addition of thiosulphate to urea (av. CV = 129–158%). Addition of DCD and thiosulphate to urea reduced the total \(\text{N}_2\text{O}\)-N emissions, respectively, by 60 and 9.6% under submergence, by 41.5 and 7.2% at field capacity and by 49.4 and 13.5% at 80% max. WHC, from urea alone.

Nitrification inhibitors (DCD and thiosulphate), when mixed with urea reduced mean \(\text{N}_2\text{O}\) emissions due to their inhibitory effects on nitrification. Accumulation of NO\(^-\) in soil was much lower on the addition of the inhibitors to urea (Table 2). \(\text{N}_2\text{O}\) emissions from urea combined with inhibitors at field capacity and 80% max. WHC were mainly observed during the first week of incubation (Figure 1a and b), and decreased subsequently. A sudden decrease in daily \(\text{N}_2\text{O}\) emissions resulted in an overall decrease in total \(\text{N}_2\text{O}\)-N emission, since after 3 DAI low emissions were recorded at field capacity and 80% max. WHC. DCD was more efficient than thiosulphate in reducing total \(\text{N}_2\text{O}\)-N emission from urea at all moisture regime (Table 4). This was due to higher nitrification inhibition by DCD and lower accumulation of NO\(^-\), which is the substrate for denitrification. In grassland and barley fields, McTaggart et al.\(^{21}\) have reported 58–78% reduction of \(\text{N}_2\text{O}\) emission when DCD was mixed with urea. Thiosulphate was less effective probably due to the fact that thiosulphate checks the second step of nitrification, i.e. conversion of nitrite to nitrate\(^{22}\). Thus, it results in accumulation of nitrite, thereby increasing the possibility of \(\text{N}_2\text{O}\) emission by conversion of NH\(^+\) to \(\text{N}_2\text{O}\) and by reduction of nitrite to \(\text{N}_2\).

\(\text{N}_2\text{O}\)-N emission was lowest under submergence and was highest at 80% max. WHC under each treatment (Table 4). Total \(\text{N}_2\text{O}\)-N emission was found to be significantly higher from urea than the control at all moisture regimes. Addition of DCD to urea reduced total \(\text{N}_2\text{O}\)-N emission significantly from urea at all moisture regimes while thiosulphate was able to reduce total \(\text{N}_2\text{O}\)-N emission significantly only at 80% max. WHC, when combined with urea. Total N lost through \(\text{N}_2\text{O}\) emission during 37 days under submergence was 0.43, 0.17 and 0.39% of N applied through urea, urea–DCD and urea–thiosulphate, respectively, while at field capacity and 80% max. WHC, total emissions from the same treatments were 0.44, 0.26, 0.41% and 1.06, 0.54, 0.92% of applied N, respectively. On application of urea to fallow soil, \(\text{N}_2\text{O}\)-N emissions were 0.11–0.14% (ref. 23) and 0.07% (ref. 24) of the total nitrogen applied. Studies in rice and wheat fields have shown that \(\text{N}_2\text{O}\)-N emissions were 0.01–0.55% (refs 25 and 26) and 0.17–0.77% (refs 27 and 28) of total nitrogen applied.

### Table 4. Total emission of nitrous oxide (mean ± SD) from the soil in 37 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Submergence</th>
<th>Field capacity</th>
<th>80% max WHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.64 ± 2.2a</td>
<td>54.56 ± 4.5b</td>
<td>78.88 ± 3.1c</td>
</tr>
<tr>
<td>Urea</td>
<td>298.51 ± 8.54 (844)</td>
<td>333.68 ± 15.45 (512)</td>
<td>744.4 ± 18.3 (844)</td>
</tr>
<tr>
<td>Urea–DCD</td>
<td>138.35 ± 5.9 (337)</td>
<td>217.77 ± 17.4 (299)</td>
<td>415.64 ± 18.8 (427)</td>
</tr>
<tr>
<td>Urea–thiosulphate</td>
<td>272.78 ± 8.1 (762)</td>
<td>313.53 ± 14.1 (475)</td>
<td>654.75 ± 17.5 (730)</td>
</tr>
</tbody>
</table>

Values followed by the same letters are not significantly different from each other at 5% level of significance, according to Duncan’s Multiple Range test; Values in parentheses indicate per cent increase in total \(\text{N}_2\text{O}\) emission over control.
Addition of nitrogen as urea had resulted in substantial increase in N₂O emissions. The increase was highest at 80% max. WHC while it was lowest under submergence. The study indicated that continuous submergence during rice crop would reduce nitrification and accumulation of NO₃⁻, thereby reducing N₂O production. In other crops, where stagnation of water is avoided and crops are grown in aerobic or partially aerobic conditions, N₂O emission may be higher mainly due to high nitrification and to some extent, via denitrification of accumulated NO₃⁻ in periods of water saturation. DCD can reduce N₂O emission appreciably in soils at different moisture regimes, except at submergence, where nitrification inhibitors may not be very efficient in mitigating N₂O emissions as nitrification proceeds slowly under submergence. It has already been established at field scale that from the point of view of crop production, use of DCD is economically feasible. The present study shows that DCD is also capable of mitigating N₂O emissions considerably from the soil at different moisture regimes.

18. MSTAT-C (version 1.41), Crop and Soil Sciences Department, Michigan State University, USA.
During R. V. *Samudra Manthan* cruise no. 127, echo sounding (3.5 kHz) data were collected over the continental shelf off Point Calimere – Pondicherry, Bay of Bengal along shore parallel and perpendicular transects at 10 km intervals. Sediment samples from the mid- and outer continental shelf by van Veen grab on 5 km grid were collected.

The continental shelf is about 80 km wide off Point Calimere and 45 km off Karaikal with the slope of about 00°30’ and shelf break occurs at an average depth of 200 m. Echo profiles revealed the presence of prominent terrace features at – 90 m, – 100 m and –110 m depths and shore parallel ridges at – 60 m, – 70 m, –85 m, –95 m, –115 m and –125 m depths, indicative of former low strandline positions.

The shelf is generally carpeted by clayey sand, sandy clay, sandy silt and silty clay. The sandy ridges are mainly composed of abundant degraded and inarticulated shells, shell fragments and ooids. Calcareous algal concretions are more common in the outer continental shelf area.

Notable geomorphic features picked-up from echo sounding records of the outer continental shelf were dredged. Abundant coral chunks and debris were collected (Figure 1) NE off Karaikal from the irregularly crested ridge at – 125 m depth (Sample no. 8595, Location 11°11.06’N; 80°03.92’E). The scanning of corals revealed that they are freshly broken from the reef during dredging and mostly belong to the genus *Acropora* sp. and *Pocillopora* sp. *Acropora* sp. is a colonial branching species with branchlets of 4 to 7 cm long, 8 to 10 mm thick and the tips slightly pointed. Towards the lower part, the branchlets are fused and finally become massive at the base. The branches of *Pocillopora* sp. are cylindrical at the base and 1 to 1.5 cm broad at the top. Usually, *Pocillopora* sp. grows into colonies of 25 cm height with hemispherical outline. *Acropora* sp. which is generally restricted within 5 m of water depth was selected for radiocarbon (14C) dating and cleaned thoroughly to avoid contamination.

The mineralogy of the coral determined by X-ray diffraction revealed that *Acropora* sp. contains only aragonite (100%), thus confirming no recrystallization of primary aragonite. The coral debris (*Acropora* sp.) was radiocarbon (14C) dated at Birbal Sahni Institute of Palaeobotany, Lucknow following the Radiocarbon Calibration Programme Rev. 3.0.3 of the University of Washington18. Radiocarbon (14C) age was calculated using the half-life value of 5570 ± 30 years.

Colonial growth of tropical corals usually flourish in calm shallow seas with water temperature around 22°C (ref. 19). The observations in the study area suggest that a low sea-level stand around –120 m depth facilitated the thriving of a coral reef at –125 m depth. *Acropora* sp. collected from – 125 m water depth yielded a radiocarbon (14C) age of 18390 ± 220 yr (calibrated) BP.

The continental shelf off Point Calimere – Karaikal is one of the widest in the east coast of India, where former sea-level positions are well preserved. Earlier studies confirm such evidences between –50 m and –130 m depths20,21. In the shelf of the study area, the lowest shelf parallel ridge is recorded at – 125 m (Figure 2).

Worldwide studies indicate that the sea-level retreated maximum between –121 m and – 130 m depth about 18,000 yr BP, i.e. during the LGM22,23. Subsequently, the warm period began and the sea-level rose to the present one with pauses. The coral reefs drilled offshore of Barbados and their age point that the sea-level was at – 121 ± 5 m below the present sea-level during the LGM22. A study along the Atlantic continental shelf of USA confirms the lowest sea-level of the LGM at – 130 m (ref. 23). Similarly, the oxygen (18O) isotope records and 14C dating of deep sea samples from the Bay of Bengal and Arabian Sea suggest a mean age of the LGM as ~ 18,000 yr BP with a standard deviation of 1500 yr (ref. 24). On the basis of oxygen (18O) isotope studies, the sea-level during the LGM has been estimated to be about – 130 m (ref. 25). On comparison of the above documentations with the 14C dating obtained for the relict coral reef off Karaikal, it is surmised that the lowest sea-level corresponding to the LGM was at –120 m depth in the study area. This age of 18,390 ± 220 yr BP attests to an evidence of the lowest sea-level position during the LGM in east coast of India, which is hitherto not recorded by absolute radiometric dating.

A relict coral reef at – 115 m depth from the continental shelf off Mahabalipuram has been dated 14510 ± 190 yr BP (ref. 17). Algal barriers at – 85 m and – 100 m depths off Visakhapatnam have been dated 10790 ± 190 yr BP and 12530 ± 170 yr BP (ref. 16). The relict coral reef off Karaikal (the present study) has yielded an age of 18390 ± 220 yr. BP. Although the
above dates are from widely spaced geographic locations, a tentative rate of sea-level rise was deduced from the maximum age of 18,390 years (off Karaikal from –120 m depth) and the minimum age of 10,790 years (off Visakhapatnam from –85 m depth). It is inferred that in the eastern continental shelf of India, the sea-level rose at the rate of 4.61 m/kyr since the LGM until about 11,000 yr BP (Younger Dryas).

Earlier studies suggested that sea-level fluctuations all along the east coast of India have been caused by a combined effect of neotectonic movements and glacio-eustatic sea-level changes. As the age and LGM (–120 m) level off Karaikal support other reports worldwide, it is envisaged that the continental shelf off Karaikal did not undergo any significant vertical movement after the LGM period.

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