CHAPTER 2

*In vitro* regeneration of *Curcuma* species:
*C.longa, C.aromatica, C.amada* and *C.zedoaria*
2.1 Introduction

Plant tissue culture is an important tool in the studies of plant physiology, genetics, biochemistry and molecular biology of plant cells. Use of tissue culture is effective in both fundamental and applied aspects. Long term callus culture and subsequent plant regeneration is of prime importance in successful application of tissue culture to crop improvement. The in vitro morphogenic response is often influenced by explant type and size, composition of the media, culture conditions and genotype of the plant species (Murashige and Skoog, 1962; Gamborg et al 1974; Bajaj and Nitsch, 1975).

The genus *Curcuma* belongs to the family Zingeberaceae and includes species *C.longa*, *C.amada*, *C.aromatica*, and *C.zedoaria*, which are of high medicinal importance. *C.longa*, commonly known as turmeric, has been used as aromatic ingredient for cooking. Curcumin extracted from *C.longa* rhizome is an anti-inflammatory agent (Ammon et al 1993) and has anti-carcinogenic properties (Piper et al 1998). *C.aromatica*, a closely related species of *C.longa*, also contains curcumin. Powdered rhizome of this species is of high caloric value and used as a substitute for baby food (Moral and Suhrawardy, 1999). Rhizomes of *C.amada* have special aroma like mango and commonly known as mango ginger. It is used to make chutney and pickles and is a popular condiment in eastern India (Middleditch, 1753). Rhizomes of *C.zedoaria* are eaten as vegetable (Middleditch, 1753).

*Curcuma longa* Linn (turmeric) is a perennial rhizomatous herb and is believed to be originated in India as wild turmeric and is found to be growing in the hilly regions of Western and Eastern ghats. Turmeric is propagated vegetatively from sprouted rhizomes but heterozygosity and lack of seed set of this species hinder breeding efforts by conventional techniques. Therefore a rapid multiplication method is necessary to provide enough planting material especially for the newly developed lines that are available small quantities. The type of explants that has been reported in *C.longa* for tissue culture are axillary buds (Nadgauda et al 1978; Balachandran et al 1990; Sit & Tiwari 1997), shoots (Salvi et al 2001; Prathanturarug et al 2003), young inflorescence (Salvi et al 2000), rhizome buds (Rahman et al 2004; Salvi et al 2002).
In the present thesis, different accessions of 4 *Curcuma* species of *C. longa*, *C. amada*, *C. aromatica* and *C. zedoaria* collected from different places of West Bengal were propagated in our experimental garden. Of these, *C. longa*, *C. amada* and *C. zedoaria* (v1, v7, v12) collected from Thekua, Medinipore and *C. aromatica* (v10) from Malda were used for *in vitro* plant regeneration. For this purpose MS medium supplemented with different plant growth regulators were used to propagate the plants *in vitro*. Successful results were obtained in MS medium with 4mg/l NAA and 5mg/l KIN from node and rhizome explants of *C. amada*. *C. longa* and *C. aromatica* showed good results from nodal explant and *C. zedoaria* showed micropropagation from rhizome explants. Profuse callusing was obtained from leaf sheath of *C. longa* in MS medium containing 0.5mg/l 2,4D and 1mg/l BAP. After 70 days in culture, root initiation was found from these calli and shoot regeneration was obtained from these calli when subcultured in MS medium supplemented with NAA/KIN. The plantlets were healthy and responded very well to hardening.

In all the accessions (v1, v7, v10, v12) *in vitro* propagation was very successful and gave reproducible results.
2.2 Materials and Methods:

2.2.1 Plant material

Rhizomes of *Curcuma* species namely *C. longa* Linn, *C. amada* Roxb, *C. Zedoaria* Roscoe, *C. aromatica* Salisb were procured from local farmers of different regions of West Bengal and propagated in our experimental garden.
2.2.2 Source of explants

Fresh rhizomes, young nodes and young leaves collected from our experimental garden were used as explants.

2.2.3 Surface sterilization of explants

Portions of fresh rhizome, young nodes and pieces of young leaves were sterilized in freshly prepared

a. 0.1% HgCl₂ solution (Qualigen fine chemicals, A Division of Glaxo India Ltd). 100ml distilled water was autoclaved in a conical flask and 100mg HgCl₂ was added to it just before sterilization of explants. Young nodes (2-3cm long) and fresh rhizomes (2-3cm long) of 4 *Curcuma* sp were sterilized in 0.1% HgCl₂ solution for 20-30min with vigorous shaking and then washed in sterile distilled water five- six times to remove the HgCl₂. These explants were inoculated in sterile agar-sucrose medium for regeneration.

b. 20% commercial bleach solution (Robin liquid bleach, Reckitt and Coleman of India Ltd) was also used as a sterilant but this did not give good result.

2.2.4 Preparation of agar-sucrose medium:

Cleaning of glass goods

Glass goods were immersed in 1% solution of detergent, Teepol (tissue culture grade) overnight and then brushed thoroughly and washed in running water to remove detergent. Next the glass goods were washed in distilled water at least 2-3 times. After drying in hot air oven these were used for preparation of medium.

Preparation of agar sucrose medium

Agar-sucrose medium contains 0.9% agar agar (Sigma Chemical Company, Tissue culture grade) and 0.3% sucrose (Sisco Research Laboratories Pvt Ltd, Mumbai, India). 3g of sucrose was dissolved in 80ml distilled water and volume was made up to 100ml. This was then poured in a 200ml conical flask and 0.9g agar agar was added to it and melted in a microwave with intermittent shaking to dissolve agar uniformly.
10ml agar-sucrose medium was poured in each culture tube and plugged with non-absorbent cotton wrapped in gauze cloth. Four culture tubes were secured with rubber band and then wrapped in brown paper and placed in aluminium can. Forceps, scissors were wrapped separately with paper. All these were autoclaved at 20lb/sq.inch pressure for 20min for sterilization. Autoclaved culture tubes were placed vertically in aluminium can in culture room for cooling medium to make stabs.

2.2.5 Preparation of nutrient medium:

MS nutrient medium (Murashige & Skoog 1962) supplemented with different concentration of auxins (α-naphthalene acetic acid, NAA; 2,4D) and cytokinins (6-furfuryl amino purine, KIN; BAP) were used for plant regeneration and callus induction. Lyophilized MS medium was purchased from Himedia Laboratories Pvt. Ltd., Bombay, India. In 1litre MS medium, 440mg CaCl₂, 30g sucrose and 9g agar agar (0.9%) were added. The composition of MS medium is given in the next page.

The MS nutrient medium along with required amount of CaCl₂ and sucrose (440mg and 30g respectively per litre) was dissolved and volume up to 1L with distilled water. Then required amount of auxins and cytokinins were added in different concentrations to prepare different combinations of media. In all combinations of MS medium the pH was adjusted between 5.6 to 5.8 using 0.1N KOH and 0.1N HCl. The liquid medium was solidified with 0.9% agar agar. After dissolving and melting the agar, the medium was poured in cultured tubes (~20ml/tube), plugged with non absorbent cotton, wrapped with brown paper and autoclaved for 20min at 20lb/sq. inch pressure for sterilization. Glass petridishes, scalpel, needle, forceps required for inoculation were also sterilized. The autoclaved hot medium in culture tubes were kept in slanting position for solidification.
**COMPOSITION OF MS MEDIUM (1 Litre)**

<table>
<thead>
<tr>
<th>Composition</th>
<th>Amount (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macro-nutrients:</strong></td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>440</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>370</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>1900</td>
</tr>
<tr>
<td><strong>Micro-nutrients:</strong></td>
<td></td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>8.6</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td>AlCl$_3$</td>
<td>0.025</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>6.2</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Iron Sources:</strong></td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>7.85</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>37.25</td>
</tr>
<tr>
<td><strong>Carbohydrate Source:</strong></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>$30 \times 10^3$</td>
</tr>
<tr>
<td><strong>Vitamins:</strong></td>
<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>AMINO ACID SOURCE</strong></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
</tbody>
</table>
2.2.6 Preparation of stock solution of hormones:

1. Auxin:

Powdered or crystal forms of NAA, 2-4D were purchased from Sigma Chemical Company (USA). 10mg of any of the auxins were dissolved in 1ml of absolute ethyl alcohol in screw capped tube and then volume was made up to 10ml with sterile distilled water. The final concentration of stock solution of auxin was 1mg/ml.

2. Cytokinin:

Powdered or crystal forms of KIN, BAP were purchased from Sigma Chemical Company (USA). 10mg of any of the cytokinins were taken in a screw capped tube and 1N HCl was added to it drop by drop until it dissolved completely and then volume was made up to 10ml with sterile distilled water. The final concentration of stock solution of cytokinin was 1mg/ml.

The hormones were stored at 4°C temperature.

2.2.7 Inoculation of explants for plant regeneration

Young nodes and rhizomes of 2-3cm in length collected from field grown plants of C.longa, C.amada, C.zedoaria and C.aromatica were used as explants. These were sterilized superficially in 0.1% HgCl₂ and inoculated in MS medium supplemented with different concentrations of plant growth regulators (NAA/KIN). Each experiment was set up with 5 replicates and repeated three times.

2.2.8 Culture conditions

The cultures were maintained in tissue culture room on glass racks under Philips fluorescent day light tubes (emitting 32 x 10⁸ moles m⁻² s⁻¹) for 16/8hrs light/dark period. The culture room was maintained with a temperature of 22-25°C and a relative humidity of 55-60%.

2.2.9 Shoot and root initiation

Root and shoot initiation was observed in the same medium on subculturing for 30 days and these cultures were maintained for 4 passages
in the same medium with same concentrations of plant growth regulators in the culture room conditions.

2.2.10 Transfer of regenerated plantlets to the field:

The rooted plantlets were made agar free by thorough washing and maintained aseptically in sterile half strength liquid MS medium (Murashige & Skoog, 1962) without sucrose for 7-8 days under identical culture conditions. These were then transferred to sterile sandy soil in small pots. The pots were covered with polythene bags with small holes, to avoid excess evaporation of water and maintained in culture room conditions for 10 days. Gradually they were exposed to the sun by increasing the time of exposure from 1hr. to about 5-6hr for 8-10 days and finally, the pots were kept in the garden.

2.2.11 Inoculation of explants for induction of callus:

1cm sections of leaf, root and leaf sheath were cut from 45 days old in vitro grown plants. The explants were inoculated aseptically in MS medium supplemented with different concentrations of 2,4D and KIN, 2,4D alone and in combination with 2,4D and BAP.

The explants were placed in a slant position on the medium in the culture tubes. Leaf discs were placed with their dorsal side touching the media. Each experiment was set up with five replicates and repeated 3 times.

Culture conditions:

The culture conditions were exactly the same as described in Chapter 2.2.8.

2.2.12 Initiation of callus:

Callus was formed from the root and leaf sheath explants at the end of the first passage. These were subcultured to the second passage after 30 days, in the same medium with same concentrations of hormones.
2.2.13 Regeneration from callus:

After 2nd passage, calli were transferred to the MS medium with different concentrations of NAA and KIN for the regeneration of plantlets and maintained under the same culture conditions for 30 days. Shoots were initiated from leaf sheath grown callus in MS medium with 4mg/l NAA and 5mg/l KIN. After 4th passage these regenerated plantlets were transferred to the liquid medium with same hormone combinations for hardening of roots and maintained in culture room conditions for 15 days and after that these were transferred to the soil for field trial.

2.2.14 Statistical Analysis:

All the data obtained for regeneration in 4 Curcuma species (i.e. no. of shoots and roots emerging) from different combinations of hormones were statistically analyzed using Duncan’s Multiple Range Test (Gomez & Gomez, 1984). Duncan’s Multiple Range Test (DMRT) is a very useful statistical method that compares all possible pairs of treatment means (mean no. of shoots & roots obtained from different treatments or hormone combinations) which were not pre-assigned and were taken arbitrarily. The steps required in carrying out DMRT has been included in the results (Section 2.3.2). A sample of calculation has been included in the result section.
2.3 Results:

In vitro regeneration was attempted in 4 species of Curcuma, namely Curcuma longa, C.amada, C.aromatica and C.zedoaria. C.longa, C.amada, and C.zedoaria collected from Thekua, Medinipore and C.aromatica from Malda were propagated in our experimental garden and were used for in vitro plant regeneration. Slices of young nodes (2-3cm) and rhizomes (2-3cm) were inoculated in culture medium to regenerate plantlets. The experiments were carried out in agar sucrose medium and in MS medium with different concentrations of NAA (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0mg/l) and kinetin (KIN) (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0mg/l). The same hormone concentrations were maintained for two subcultures each of 30 days duration. The explants on agar sucrose medium showed only small bud like growth. In the 1st passage in MS medium with different concentrations of hormones, shoot was initiated both from node and rhizome explants and at the 2nd passage roots were also initiated. At the end of 3rd passage plantlets multiplied in solid medium and were transferred aseptically to liquid MS medium with the same hormone combinations on filter paper bridge for hardening of roots (Fig 2.1c). After two weeks, they were transferred to sterile soil and maintained in culture room conditions with occasional exposure to the external environment and finally transferred to pots containing garden soil. Among the combinations tested, only 4 combinations were found to give reproducible results and the best result was obtained in MS medium with 4 mg/l NAA and 5 mg/l KIN in case of all the explants. After 20 days of inoculation of explants, shoots appeared and in the 2nd passage roots also appeared. In the best hormone concentration C.longa produced maximum 5-7 shoots/node (Table 2.1) (Figs. 1a, b, c, d) and C.aromatica 4-5 shoots/node (Fig 2.5a, b, c, d) (Table 2.2). Whereas rhizome explants of C.longa and C.aromatica produced only small buds. C.amada produced maximum 3-4 shoots/node (Table 2.3) (Fig 2.2a, b, c, d) and 5-6 shoots/rhizome (Histogram 2.1) (fig 2.4a, b, c) in MS medium with 4 mg/l NAA and 5 mg/l KIN. In C.zedoaria, maximum (3 to 5) shoots (Fig 2.5a, b, c, d) were regenerated (Histogram 2.1) from each rhizome explant. Microrhizome induction was also observed in C.zedoaria in MS medium with 4mg/l NAA and 5mg/l KIN (Fig 2.5b). In all hormone combinations, five replicates were set up. The data obtained in the above experiment was statistically analyzed using Duncan's Multiple Range Test, DMRT. The
concentrations of NAA/KIN which produced considerable number of regenerated plants were statistically analyzed.

**TABLE – 2.1** Shoot and root initiation of *C. longa* (mean ± S.E.) from node in 3rd passage in different concentration of NAA/KIN in MS medium using Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Concentration of NAA/KIN (mg/l)</th>
<th>No. of regenerated shoots per explant (mean ± S.E.)</th>
<th>No. of regenerated roots per explant (mean ± S.E.)</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>4 / 5</td>
<td>6.4 ± 0.83</td>
<td>14.0 ± 1.73</td>
<td>1</td>
</tr>
<tr>
<td>T2</td>
<td>4 / 4</td>
<td>1.4 ± 0.24</td>
<td>5.4 ± 1.66</td>
<td>2</td>
</tr>
<tr>
<td>T1</td>
<td>4 / 3</td>
<td>1.2 ± 0.37</td>
<td>4.2 ± 1.28</td>
<td>2</td>
</tr>
<tr>
<td>T4</td>
<td>5 / 3</td>
<td>0.8 ± 0.37</td>
<td>3.4 ± 0.51</td>
<td>2</td>
</tr>
</tbody>
</table>

For each treatment mean values followed by different letters are significantly different according to Duncan’s multiple range test (P≤0.05).

From Table 2.1, it is evident that the best result of regeneration is obtained with 4 mg/l of NAA and 5 mg/l of KIN.

The results obtained for regeneration in different concentrations of NAA/KIN for *C. longa* were statistically analyzed by Duncan’s Multiple Range Test and the steps are included here.

**2.3.1 Duncan’s Multiple Range Test:**

Here different hormone concentrations are denoted as treatments:

- Treatment no. 1 — T1 — 4 mg/l of NAA, 3 mg/l of KIN
- Treatment no. 2 — T2 — 4 mg/l of NAA, 4 mg/l of KIN
- Treatment no. 3 — T3 — 4 mg/l of NAA, 5 mg/l of KIN
- Treatment no. 4 — T4 — 5 mg/l of NAA, 3 mg/l of KIN

Only those hormone concentrations prompting considerable number of plantlets were considered for statistical analysis.
Raw data of plant regeneration for different treatments (concentrations of NAA/KIN):

<table>
<thead>
<tr>
<th>Treatment (NAA/KIN in mg/l)</th>
<th>Replica</th>
<th>Number of shoot</th>
<th>Total</th>
<th>Treatment Mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_1$ (NAA/KIN)</td>
<td>1</td>
<td>2</td>
<td>6.0</td>
<td>1.2</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_2$ (NAA/KIN)</td>
<td>1</td>
<td>1</td>
<td>7.0</td>
<td>1.4</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_3$ (NAA/KIN)</td>
<td>1</td>
<td>4</td>
<td>32</td>
<td>6.4</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The treatment totals are:

\[
\text{Sum } T_1 = 6 \quad \text{Sum } T_2 = 7 \quad \text{Sum } T_3 = 32 \quad \text{Sum } T_4 = 4
\]

Grand total, \( G \) = Sum of treatment totals

\[
= \text{Sum } T_1 + \text{Sum } T_2 + \ldots \ldots + \text{Sum } T_4
\]

\[
= 6 + 7 + 32 + 4 = 49
\]

Correction factor, C.F. = \( G^2/n \) where \( n \) is the total number of experimental plots,
i.e., \( n = R \times T \) where \( R \) is the number of replica and \( T \) is the number of treatments.

\[
\therefore \quad \text{C.F.} = \frac{G^2}{R \times T}
\]

\[
= \frac{49 \times 49}{5 \times 4} \quad (R = 5, \, T = 4)
\]

\[
= \frac{49 \times 49}{5 \times 4} = 120.05
\]

The sums of squares of the measurements of each plot are:

\[
\text{TSS}_1 = 2^2 + 0^2 + 2^2 + 1^2 + 1^2 = 10
\]

\[
\text{TSS}_2 = 1^2 + 2^2 + 1^2 + 1^2 + 2^2 = 11
\]

\[
\text{TSS}_3 = 4^2 + 8^2 + 7^2 + 5^2 + 8^2 = 218
\]

\[
\text{TSS}_4 = 2^2 + 1^2 + 1^2 + 0^2 + 0^2 = 6
\]

Total sums of squares,
\[ TSS = (TSS_1 + TSS_2 + TSS_3 + TSS_4) - CF \]
\[ = (10 + 11 + 218 + 6) - 120.05 \]
\[ = 124.95 \]

Sum of squares of Treatment Totals,
\[ TRRS = 6^2 + 7^2 + 32^2 + 4^2 \]
\[ = 1125 \]

Treatment sum of squares,
\[ TRSS = \frac{TRSS_1}{R} - C. F. \]
\[ = \frac{1125}{5} - 120.05 \]
\[ = 104.95 \]

Error sum of squares,
\[ ESS = TSS - TRSS_1 \]
\[ = 124.95 - 104.95 = 20 \]

Error mean square,
\[ EMS = \frac{ESS}{T(R-1)} \]
\[ = \frac{20}{4(5-1)} = 1.25 \]

Standard error of mean difference,
\[ S_d = \sqrt{\frac{2 \times EMS}{R}} \]
\[ = \sqrt{\frac{2 \times 1.25}{5}} \]
\[ = 0.70 \text{ shoots per explant} \]

Then, the \((T-1) = (4-1) = 3\) values of the shortest significant ranges are computed as:
\[ R_p = \frac{(r_p) (S_d)}{\sqrt{2}} \text{ for } p = 2, 3, 4 (T) \]
Where \( r_p \) values are the tabular values of the significant studentized ranges obtained from Appendix F (table reproduced from Principles and Procedures of Statistics by R. G. D. Steel and J. H. Torrie, 1960, Mc. Graw Hill Book Co. Inc., New York), and \( p \) is the distance in rank between the pairs of treatment means to be compared (i.e., \( p = 2 \) for the two means with consecutive ranking and \( p = T \) for the highest and lowest means).

The error degree of freedom = \( T (R-1) = 4 (5-1) = 16 \)

Then, the \( r_p \) values with error degree of freedom 16 at the 5% level of significance are obtained from Table of significant studentized ranges and the corresponding values of \( R_p \) are tabulated as follows:

<table>
<thead>
<tr>
<th>( p )</th>
<th>5% level for d.f. 16 ( r_p(0.5) )</th>
<th>( R_p = \frac{r_p (s_d)}{\sqrt{2}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.0</td>
<td>1.48 (4th)</td>
</tr>
<tr>
<td>3</td>
<td>3.15</td>
<td>1.56 (3rd)</td>
</tr>
<tr>
<td>4</td>
<td>3.23</td>
<td>1.60 (2nd)</td>
</tr>
<tr>
<td>5</td>
<td>3.30</td>
<td>1.63 (1st)</td>
</tr>
</tbody>
</table>

The treatments were then ranked according to the descending order of their corresponding means. Then, all the treatment means that do not differ significantly from each other were identified and grouped together as follows:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_3 )</td>
<td>6.4(^a)</td>
<td>1</td>
</tr>
<tr>
<td>( T_2 )</td>
<td>1.4(^b)</td>
<td>2</td>
</tr>
<tr>
<td>( T_1 )</td>
<td>1.2(^b)</td>
<td>3</td>
</tr>
<tr>
<td>( T_4 )</td>
<td>0.8(^b)</td>
<td>4</td>
</tr>
</tbody>
</table>

**Step I**: Difference between the largest treatment mean and largest \( R_p \)

\[ = 6.4 - 1.63 = 4.77 \]
This value is greater than the treatment means of all other treatments (T₁, T₂ and T₄). Hence, the treatment mean of T₃ is significantly different from all other treatment means, and this is denoted by superscript ‘a’ beside the treatment mean of 6.4 of T₃.

**Step II**: Difference between the 2nd largest treatment mean and 2nd largest \( R_p \)

\[
= 1.4 - 1.6 = -0.2
\]

This value is less than all the rest treatment means. Hence, T₂, T₁ and T₄ falls under the same group.

**Step III**: Of the treatment means of T₂, T₁, and T₄ which are falling under the same group, we compute the range (which is the difference of the maximum and minimum within this group) as: 1.4 – 0.8 = 0.6.

For this group, \( p = 4 \) and \( R_p = 1.6 \)

Now, 0.2 < 1.6 [\( R_p \) at \( p = 4 \)].

Hence, the treatments T₂, T₁ and T₄ do not have significantly different means and are denoted by superscript ‘b’ beside the mean values corresponding to the treatments T₂, T₁ and T₄.

**Table -2.2** Comparison of shoot and root initiation of *C. aromatica* (mean ± S.E.) from node in 3rd passage different concentration of NAA/KIN in MS medium using Duncan's multiple range test.

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Concentration of NAA/KIN (mg/L)</th>
<th>No.of regener-ated shoots per explant (mean ± SE)</th>
<th>No of regener-ated roots per explant (mean ± SE)</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₃</td>
<td>4 / 5</td>
<td>4.2±0.19</td>
<td>17.8± 1.15</td>
<td>1</td>
</tr>
<tr>
<td>T₂</td>
<td>4 / 4</td>
<td>1.6±0.24</td>
<td>7.2± 1.65</td>
<td>2</td>
</tr>
<tr>
<td>T₁</td>
<td>4 / 3</td>
<td>1.2±0.37</td>
<td>5.4± 1.50</td>
<td>2</td>
</tr>
<tr>
<td>T₄</td>
<td>5 / 3</td>
<td>1.6±0.24</td>
<td>4.2± 1.35</td>
<td>2</td>
</tr>
</tbody>
</table>

For each treatment mean values followed by different letters are significantly different according to Duncan's multiple range test (\( P \leq 0.05 \)).
**Fig. 2.1a** Root and shoot initiation from node of *C. longa* in MS medium with 4mg/l NAA and 5mg/l KIN after 45 days of inoculation.

**Fig 2.1b** Regenerated plantlets from nodal explant of *C. longa* in liquid medium for hardening after 3rd passages.

**Fig 2.1c** Regenerated plantlets transferred to sterile soil.

**Fig 2.1d** Plant grown in the next season from *in vitro* regenerated plants (2nd generation).
**Fig 2.2a** Initiation of shoots and roots from nodal explant of *C.amada* in MS medium with 4mg/l NAA and 5mg/l KIN after 45 days of inoculation of explant.

**Fig 2.2b** Regenerated plantlets in liquid medium for hardening.

**Fig 2.2c** *In vitro* grown plantlets in sterile soil.

**Fig 2.2d** *In vitro* grown plant in garden soil after one year.
Fig 2.3a Initiation of green shoot bud and root from node of *C. aromatica* in MS medium with 4mg/l NAA and 5mg/l KIN after 25 days of inoculation of explant.

Fig 2.3b Regenerated plantlets in liquid medium after 90 days for hardening.

Fig 2.3c *In vitro* grown plantlets in sterile soil.

Fig 2.3d *In vitro* grown plant in garden soil after one year.
**Fig 2.4a** Shoot initiation from rhizome explant of *C.amada* in MS medium with 4mg/l NAA and 5mg/l KIN after 30 days of inoculation of explant.

**Fig 2.4b.** Regenerated *C.amada* plantlets from rhizome explant in liquid MS medium after 3rd passages.

**Fig 2.4c.** *In vitro* grown plant in soil.
**Fig 2.5a.** Initiation of root from rhizome explant of *C.zedoaria* in MS medium with 4mg/l NAA and 5mg/l KIN after 30 days of inoculation.

**Fig 2.5b.** Regenerated plantlets with microrhizome in liquid MS medium after 3rd passage.

**Fig 2.5c.** *In vitro* regenerated plantlets in sterile soil.

**Fig 2.5d.** Profusely grown plants in next year from *in vitro* grown plants.
Table 2.3 Comparison of shoot and root initiation of *C. amada* (mean ± SE) from node in 3rd passage in different concentration of NAA/KIN in MS medium using Duncan's multiple range test.

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Concentration of NAA/KIN (mg/l)</th>
<th>No. of regenerated shoots per explant (mean ± SE)</th>
<th>No. of regenerated roots per explant (mean ± SE)</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>4 / 5</td>
<td>4.0 ± 0.44</td>
<td>38.0 ± 2.35</td>
<td>1</td>
</tr>
<tr>
<td>T2</td>
<td>4 / 4</td>
<td>1.6b ± 0.24</td>
<td>7.6b ± 1.35</td>
<td>2</td>
</tr>
<tr>
<td>T1</td>
<td>4 / 3</td>
<td>1.2b ± 0.19</td>
<td>4.0b ± 0.83</td>
<td>2</td>
</tr>
<tr>
<td>T4</td>
<td>5 / 3</td>
<td>0.8b ± 0.19</td>
<td>3.6b ± 1.2</td>
<td>2</td>
</tr>
</tbody>
</table>

For each treatment mean values followed by different letters are significantly different according to Duncan's multiple range test (P<0.05).

**Histogram 2.1**— Histogram showing comparative shoot regeneration from rhizome explant in *C. amada* and *C. zedoaria* (mean ± SE) in different concentration of NAA / KIN in MS medium.

![Histogram showing comparative shoot regeneration](image)

The concentration and ratio of NAA (mg/l) and kinetin (mg/l) were 4/3 (T1), 4/4 (T2), 4/5 (T3) and 5/3 (T4) respectively.
2.3.2 Callus induction in *C. longa*

Callus initiation was tried in *C. longa* in MS medium from different explants. Root, leaf sheath and leaf disc (2-3cm long) of *in vitro* grown plant were cultured in MS medium with different concentrations of 2-4D and KIN, 2-4D alone and in combination with 2-4D and BAP. Callus did not develop from leaf disc explant. Small, whitish and soft callus was initiated from root explant (Fig 2.6a) in MS medium with 2-4D/KIN (3,4,4/2,3,4 mg/l) but leaf sheath explant showed soft callus (Fig 2.6a). After 2nd passage there was no change in the calli initiated from root explant and were gradually dried. Leaf sheath explant showed profuse amount of soft, light green callus with small hair like roots after 45 days of inoculation in MS medium with 2-4D/BAPmg/l (6.5/1.0) whereas moderate amount of callus (Fig 2.6c) without root and shoot initiated in other two hormone combinations (2-4D/BAP mg/l) (0.5/0.5) & (1.0/1.0). Maximum (3.5-4g) callus was developed from leaf sheath after 60 days of inoculation of explant in MS medium with 0.5mg/l 2-4D and 1mg/l BAP (Fig) (Histogram 2.2).

**Histogram 2.2**- Histogram representing no. of plant regeneration (mean ± SE) from callus of *C. longa* in different concentration of 2,4-D/BAP (mg/l) in MS medium.

The concentration and ratio of 2,4-D (mg/l) and BAP (mg/l) were 0.5/0.5 (A1), 0.5/1 (A2) and 1/1 (A3) respectively.

The calli with small hair like roots and green shoots from leaf sheath explant with different concentrations of 2-4D/BAP were subcultured
**Fig 2.6a** Left: Callus initiation from leaf sheath explant in MS medium with 0.5mg/l 2,4D, 1mg/l BAP; Right: Callus initiation from root explant in MS medium with 2,4D(4mg/l) and KIN(4mg/l) after 30 days of inoculation.

**Fig 2.6b** Left: Callus initiation from leaf sheath explant in MS medium with 0.5mg/l 2,4D and 0.5mg/l BAP after 45 days inoculation. Right: Callus initiation from leaf sheath explant in MS medium with 1mg/l 2,4D and 1mg/l BAP after 45 days of inoculation.

**Fig 2.6c** Profuse callus with hair like root from leaf sheath explant in MS medium with 0.5mg/l 2,4D and 1mg/l BAP after 45 days of inoculation.

**Fig 2.6d** Callus proliferation from leaf sheath explant in MS medium with 0.5mg/l 2,4D and 1mg/l BAP after 45 days of inoculation.
**Fig 2.7a** *In vitro* regeneration of *C. longa* from callus [following first inoculation in MS medium supplemented with 2,4D (0.5mg/l) and BAP (0.5mg/l) and subcultured in MS with 4mg/l NAA and 5mg/l KIN].

**Fig 2.7b** *In vitro* regeneration of *C. longa* from callus [following first inoculation in MS medium supplemented with 2,4D (1mg/l) and BAP (1mg/l) and subcultured in MS with 4mg/l NAA and 5mg/l KIN].

**Fig 2.7c** *In vitro* regeneration of *C. longa* from callus [following first inoculation in MS medium supplemented with 2,4D (0.5mg/l) and BAP (1mg/l) and subcultured in MS with 4mg/l NAA and 5mg/l KIN].
Fig 2.7a

Fig 2.7b

Fig 2.7c
aseptically to MS medium with 4mg/l NAA and 5mg/l KIN. After 20 days maximum 6-8 shoots (Histogram 2.3) (Fig 2.7a,b,c) were regenerated from the calli that was initiated in 0.5mg/l 2,4-D and 1mg/l BAP. These regenerated plantlets were maintained in culture room in liquid medium for hardening of roots for 15 days and then it was transferred to the autoclaved soil and finally to the garden.

**Histogram 2.3**—Histogram showing comparative no. of shoot initiation from leaf sheath derived callus of *C. longa* (mean ± SE) cultured in MS medium supplemented with different concentration of 2,4- D/BAP (mg/l) for 60days and then subculturing in MS medium containing 4mg/l NAA and 5mg/l KIN.

The concentration and ratio of 2,4-D (mg/l) and BAP (mg/l) were 0.5/0.5 (A1), 0.5/1 (A2) and 1/1 (A3) respectively.

### 2.3.3 Survival of plantlets in the garden:

In *C. longa*, *C. amada*, *C. aromatic* and *C. zedoaria* both roots and shoots appeared in the MS medium with same hormone combinations. At the end of 3rd passage rooted plantlets were subcultered in liquid medium for hardening. After 15 days these plantlets were transferred to the sterile soil and finally to the garden. Few plantlets died due to shock and fungal contamination during transfer from medium to the sterile soil. But it is very interesting that the plantlets which survived in the small pots in sterile soil also survived in the field (Table 2.5) and produced rhizomes and plants in the next year.
Table 2.4: No. of plantlets and their survival in the garden

<table>
<thead>
<tr>
<th>Type of Explant</th>
<th>No. of plants regenerated</th>
<th>plants survived in sterile soil</th>
<th>Survival in garden</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. longa (node)</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>C. longa (callus)</td>
<td>8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>C. amada (rhizome)</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>C. amada (node)</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>C. aromatica (node)</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>C. zedoaria (rhizome)</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
2.4 DISCUSSION

There are earlier reports of direct plant regeneration from leaf base callus along with shoot tips in *C. longa* in MS medium with dicamba, picloram (2mg/l) or 5mg/l NAA in combination with BAP (0.5mg/l) (Salvi et al 2001). Green shoots were noted after transfer of calluses in MS medium with BAP (5mg/l) in combination with 2,4-D (0.5mg/l). Maximum 12 shoots/tube developed after transfer of these calluses on MS medium containing KIN (1mg/l) and roots were developed in phytohormone free medium. Calli were obtained from shoot tips of *C. longa* in MS medium with 2.5 to 3.0 mg/l of 2,4-D. Plantlets from callus were successfully regenerated in MS medium supplemented with standard growth hormones and ultimately set roots (Sunitibala et. al. 2001). In the present investigation profuse callusing was obtained from *C. longa* leaf sheaths cultured in MS medium supplemented with 0.5mg/l 2,4-D and 1mg/l BAP. The calli thus formed produced shoots and roots after 20 days of culture in MS medium with 4mg/l NAA and 5mg/l KIN. The plantlets were healthy and were gradually hardened to soil conditions.

Mrudul et al. (2001) observed *in vitro* microrhizome production in *C. longa* from sprouted buds of small rhizome portions. According to these authors, BAP had an inhibitory effect on microrhizome production whereas Rahman et. al. (2004) obtained shoot multiplication and microrhizome production from freshly sprouted rhizome buds collected from the field grown plants in MS medium supplemented with 2.0 mg/l BAP. On the other hand, Meenakshi et. al. (2001) found little success in *in vitro* shoot production in *C. longa*, while direct regeneration of shoots were observed from immature inflorescence of *C. longa* (Salvi et. al. 2000). They cultured inflorescence (5mm) in MS medium with BAP(5 or 10mg/l) in combination with 2,4-D (0.2mg/l) or NAA (0.1mg/l) and TDZ (1 or 2mg/l) in combination with IAA (0.1mg/l) and after 4 weeks shoot primordia emerged from the surface of the explant in all cases. Among the different hormone combinations, BAP(5mg/l) and 2,4-D (0.2mg/l) showed highest frequency of regeneration (98%). Salvi et. al. (2002) noted *in vitro* regeneration in turmeric from young vegetative buds from sprouting rhizomes (1-1.5cm) in MS medium with streptomycin sulphate. 2 to 4 plantlets developed from each
explant with 2 to 4 roots after 6 to 8 weeks of subculture to MS medium with BAP (10μM) & NAA (1μM). Prathanturarug et. al. (2003) reported high frequency of shoot multiplication in C. longa using thidiazuron. They used both undivided and divided terminal buds as explants in MS medium supplemented with TDZ (0.23-18.17μM). On subculturing after 4 weeks in MS basal, 13.25 ± 0.70 shoots regenerated from undivided and 11.3 ± 0.86 shoots from divided explant. A maximum number of 18.22 ± 0.62 shoots were obtained in MS basal after 12 weeks. In the present investigation 5 to 7 shoots were regenerated from nodal explant of C. longa in MS medium with 4mg/l NAA and 5mg/l KIN.

Microrhizome was obtained in vitro from shoot meristem of C. zedoaria in MS media with BAP(Mello et. al. 2000). The present author observed microrhizome induction in C. zedoaria from rhizome explant in MS medium supplemented with 4mg/l NAA and 5mg/l KIN.

Nayak (2000) reported shoot multiplication and plant regeneration from sprouted buds of C. aromatica in MS medium with BAP alone (1-7mg/l) or in combinations of BAP (1-5mg/l) and kinetin (0.5-1.0mg/l). In 5mg/l BAP, maximum number of shoots and roots emerged and the regenerated plants grew profusely after transfer to the liquid medium. In the present dissertation it was found that about 4 to 5 shoots were regenerated from nodal explant in MS medium supplemented with 4mg/l NAA and 5mg/l KIN.

Nayak (2000) found about 3 to 5 lateral shoots of C. amada inoculated in MS medium with 3mg/l BAP and 0.5mg/l NAA from freshly sprouted buds. Microrhizomes (78%) were induced at the base of the shoots grown in vitro after 30 days of transfer to liquid MS medium with BAP (5mg/l), sucrose (80g/l) at 16hr dark period. Prakash et. al. (2004) reported efficient plantlet regeneration in C. amada from rhizomes and leaf sheath explants. MS medium with 4.44μM BAP and 1.08μM NAA maximum number of shoots (16.4 ± 0.3) were regenerated from each explant. In the present thesis 3 to 4 shoots from nodal explant and 5 to 6 shoots from rhizome.
explant of *C.amada* were produced in MS medium with 4mg./l NAA and 5mg./l KIN.

Miachir et. al. (2004) has reported micropropagation and callogenesis of *C.zedoaria* from rhizome bud meristems in MS medium with 2mg/l BAP and 30g/l sucrose. They observed micropropagation of *C.zedoaria* was BAP dependent and was inhibited by NAA. According to these authors micropropagation rate was 72.3% from rhizome bud explants after 30 days of inoculation. Mello et. al. (2000) inoculated shoot meristem in MS medium with 2mg/l BAP and 30g/l sucrose and a number of plants were developed from each explant. In the present investigation shoot (3 to 5) regeneration was successful from rhizome explant in *C.zedoaria* in MS medium with 4mg/l NAA and 5mg/l KIN.
2.5 SUMMARY

Four species of *Curcuma*, namely *Curcuma longa*, *C.aromatica*, *C.zedoaria* and *C.amada* were collected from different parts of West Bengal, India and propagated in our experimental garden. *In vitro* regeneration of *C.longa* and *C.aromatica* was carried out from nodal explants and that of *C.zedoaria* from rhizome explant. Shoots were successfully regenerated from both nodes and rhizomes of *C.amada*. Plants regenerated *in vitro* produced rhizomes when planted in pots containing sterile soil. Callus culture was established from leaf sheath of *C.longa* in MS medium with 0.5mg/l 2,4-D and 1mg/l BAP. Shoots and roots regenerated from these calli when subcultured in MS medium containing 4mg/l NAA and 5mg/l KIN. In the present dissertation the technique of *in vitro* regeneration of the 4 species of *Curcuma* described are reproducible and always produced healthy plants that survived in the hardening conditions.