In vitro propagation of Curcuma longa L. (Turmeric)

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IN

BIOCHEMISTRY

Submitted by

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DECLARATION

I hereby declare that the thesis entitled **"*In vitro* propagation of Curcuma longa L. (Turmeric)"** submitted to **Institute of Science, Nirma University, Ahmedabad** in partial fulfillment of the requirements for the award of the degree of Masters of Science in Biochemistry. This thesis represents my own work done under the guidance of Dr. Daksha Bhatt, Technical Director, Aranya Agribiotech, Mogar. This dissertation project part/whole shall not be used for presentation or publication without the consent of the dissertation guide.

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"Perseverance, inspiration and motivation have always played a key role the success of and venture". It is not the brain that matters the most, but that which guides it: the heart, the character, the qualities and progressive forces. The wide spectrum of knowledge is difficult to understand without proper guidance.

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Abbreviations

BAP	6-Benzylaminopurine
g	Grams
GA3	Gibberellic acid
HPLC	High performance liquid chromatography
DM	Demineralized water
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
kcal	Kilocalorie
mg	Milligram
MS	Murashige and Skoog
NAA	1-Napthaleneacetic acid
ºC	Degree Celsius
PDA	Photometric diode array
PGRs	Plant growth regulators
pH	Potential of hydrogen
tsp	Tea spoon
WPM	Woody plant media

Abstract

The different plant tissue culture techniques were used to grow the plants *in vitro*. Majorly sterilization process, media, temperature and light intensity are essential in plant tissue culture. The different parts like buds, sprouted shoots, leaves and powder of dried rhizome were used in the experiment. Two types of media MS and WPM were used for clonal multiplication, germination of explants and callus induction. Different growth hormones like IAA, NAA, BAP, GA3 and 2,4-D were used for the project work. Phytohormones like BAP, IAA, NAA and GA3 were used to increase the germination growth and multiplication rate whereas different concentrations of 2,4-D and BAP was used for callus induction. The concentration of chlorophyll varies in each species and it depends on the photosynthetic rate, estimation was done using young and adult leaves. The quality of Curcuma longa L. was determined using HPLC technique and the results were obtained.

1. INTRODUCTION

INTRODUCTION

Turmeric is an herbaceous persistent underground rhizome like plant of zingiberaceae family, native to southwest India. These herb is cultivated all over Asian countries like India, China and other nations with tropical climate. The height of a plant is 3-5 feet, having oblong and pointed leaves with sheath like petiole. The inflorescence is cone-like having length of 10-15 cm and is attached inside a sheathing petiole to a stem. Flowering occurs in monsoon season, fruit is a globular capsule and is very rare (Ahmad et al., 2010). The rhizome has rough surface of skin with segmentations. It is yellow to brown with light orange interior and the powder looks yellowish-orange in color. The length and diameter of rhizome is 2.5-7.5 cm and 2.5 cm with a small tuber branching off. The mother (main) rhizome is ovate or pear shaped, with leaf scars encircled on the upper part and secondary rhizome and roots on its lower part. The secondary rhizomes are 0.5-1.5 cm thick, elongated, ringed or branched (Khalandar et al., 2018).

Turmeric production is becoming pandemic, India ranks 1st in its production. In India majorly it is cultivated in Andhra Pradesh, Odisha, Kerala, Tamil Nadu, West Bengal and Maharashtra. The cultivation of Curcuma longa L. is carried out in 6% of total area under spices. The annual production is around 793,000 tons and area used for its cultivation 181,000 hectares. Rational cropping is done from the month of July to March. Initially process of sowing starts form July while harvesting can be done from December to February. The peak arrival is from March to May. The temperature range should be between 20-35ºC with annual rainfall of 1500mm or more is required for its growth. Mostly clayey soil with high humus content is needed for cultivation, red, black and loamy soil can also be used. The soil pH should range from 4.5-7.5 (Chattopadhyay et al., 2004).

The rhizome is characterized under spices but it also has numerous traditional, medicinal and pharmacological properties. There are about 133 species of Curcuma L. identified worldwide. Its usage dates back nearly 4000 years, in India. It is used in ayurvedic formulations and is considered as antibacterial, antitumor, anti-allergic, analgesic, antiseptic, appetizer, astringent, cardiovascular, diuretic and stimulant (Prasad et al., 2011).

Botanical Name: Curcuma longa L.

Figure No. 1: Habitat of Curcuma longa L. (Turmeric)

Table 1-Taxonomical Classification

Kingdom	*Plantae*	Plants
Sub-kingdom	*Tracheobionta*	Vascular plants
Super-division	*Spermatophyta*	Seed plants
Division	*Magnoliophyta*	Flowering plants
Class	*Liliopsida*	Monocotyledons
Sub-class	*Zingiberidae*	-
Order	*Zingiberales*	-
Family	*Zingiberaceae*	Ginger family
Genus	*Curcuma* L.	-

1.1 Nutritional value

Composition of turmeric powder of about 1tsp (7g)

- **Nutritional data:-**
- Proximate values: water (0.8ml), energy (23.9kcal), protein (0.5g), fats (0.7g), carbohydrate (4.4g)
- Minerals: calcium (12.4mg), magnesium (13mg), phosphorus (18.1mg), potassium (170mg), sodium (2.6mg), zinc (0.3mg)
- Vitamins: ascorbic acid (1.7mg), thiamin (0mg), riboflavin (0mg), niacin (0.3mg), vitaminB6 (0.1mg), folate (2.6mg), vitaminB12 (0mg).
- Lipids: saturated fat (0.2g), monosaturated fat (0.1g), polysaturated (0.1g), cholesterol (0mg)

(Source: Nutritional value listed above was provided by USDA SR-21)

1.2 Economics

The leading producer, consumer and supplier of turmeric in the world is India. The quality of turmeric depends on its curcumin content, Indian turmeric serves the purpose. India accounts 80% of turmeric production and 60% of its export around the world. Major exporting countries include India, Thailand, Taiwan and several Asian, Latin American countries. The importing countries are Japan, Sri Lanka, Iran, UAE, US, UK and Ethiopia. Indian states like Andhra Pradesh, Tamil Nadu, Telangana, Karnataka, Orissa, West Bengal and Maharashtra are majorly involved in turmeric production.

Table 2- Area for cultivation and production of Turmeric in India.

Name of plant	2017-18 (Final Estimate)		2018-2019 (Estimation)	
Turmeric	Area (Ha)	Production (Mt)	Area (Ha)	Production (Mt)
	47888	678,000	44956	476,000

Source: Area for cultivation and production of Horticulture crops-All India, Government of India, Ministry of Agriculture and Farmers Welfare, Department of Agriculture, Cooperation and Farmers Welfare, (2018).

Table 3: Area for cultivation & production of Turmeric in Gujarat state for the year 2016-17.

State	Area (Ha)	Production (Mt)
Gujarat	3552	69247.90

Source: Horticulture in Gujarat, District wise estimated area & production of horticultural crops for the year 2016-17.

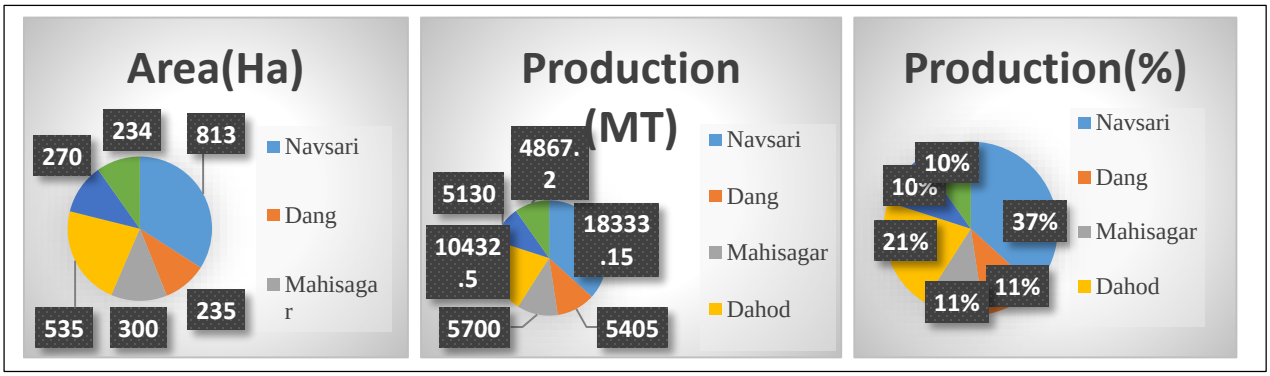

Figure 2: Area covered for cultivation and production of turmeric in Gujarat state.

Thus I hypothesized that *in vitro* clonal propagation and multiplication of Curcuma L. is poor and challenging because of insufficient elite variety. Apart from that rhizome is a slow propagating species and the multiplication rate is very low according to (Sunitibala et al., 2001).

My study includes the following objective to validate the hypothesis.

1. To develop the procedure for *in vitro* shoot multiplication of shoots in turmeric.
2. To study *in vitro* initiation of turmeric from rhizome buds.
3. To study plant regeneration through callus culture.
4. To measure the chlorophyll content in young and adult leaves of *Curcuma longa*.
5. To check the curcumin content in turmeric through HPLC.

2. REVIEW OF LITERATURE

2.1 Tissue culture study

Tissue culture implicates growing new flora in a controlled environment. These technique is used for the production and research work on plants. Tissue culture is a technology which provides massive propagation, rapid clonal multiplication and preservation of plant species which are disease free and genetically modified plants (Naz et al., 2009). The primary requirement is the use of explants, the fragments of tissue from plants. The 'Totipotency' is basic in plant tissue culture. *In vitro* culturing was developed by Haberlandt in 1902. Murashige and Skoog (1962) formulated and developed the nutrient media for culturing horticulture plants.

2.2 Collection of plant

The plant to be collected should be healthy, disease free and having the best quality. It is very crucial to propagate these kind of plant for research purpose. Depending on the desired response the choice of explant tissue varies.

The mother rhizomes are appropriate compared to finger rhizomes, as their overall growth and development rate is higher than finger rhizomes in having plant height, number of leaves along with the length and breadth, and number of roots per plant (Govind et al., 1993).

For *in vitro* culturing the age of explants play a very important role as physiologically younger tissues are more responsive than older ones. Explants size also effect on the response of tissue. It is observed that smaller explants are hard to culture, as larger explants have more nutrition reservoir.

The induction of callus can be done using pieces of the cotyledons, hypocotyl, stem, leaf or embryo. Explants that are used should be aseptically germinated or immature inflorescence (Smith, 2013).

2.3 Micropropagation of rhizome plant

The microrhizome production is the induction of rhizome buds/eyes *in vitro* condition. Swelling of each shoot base and the initiation of microrhizomes at shoot base was seen after 30 days of inoculation (Nayak, 2000).

2.4 Rapid Clonal Multiplication

The successful *in vitro* multiplication and production of microrhizome in Curcuma aromatic L. was firstly reported by Nayak (2000). Turmeric is a vegetatively propagated underground rhizomatic plant. The rate of multiplication is very low providing the yield of 15 to 25 tons/hectare (Balachandran et al., 1990). The zingiberaceae members have buds/eyes, which sprouts under

suitable environmental conditions. Such buds/eyes of rhizomes are used as explant for microshoot induction.

2.5 Factors effecting the micropropagation and rapid clonal multiplication

The basal media, amount of sucrose, concentration of growth regulators and photoperiod plays a chief role in *in vitro* microrhizome production and clonal multiplication.

2.5.1 Basal media

MS media is the widely used media for microtuber propagation. Sometimes growth regulators are nullified from culture media, half strength MS media is used for shoot multiplication, in some laboratories.

It was noted that semi strength MS medium is suitable for turmeric microrhizome production (Shirgurkar et al., 2001). While Nayak (2000) and Sunitabala et al. (2000) used MS medium with its complete constituents for the induction of microtuber in Curcuma aromatic L. and Curcuma longa L. A half strength MS media produced smaller rhizome and in less quantity while full strength MS media produced lower number of rhizomes than half strength but the size was comparatively larger (Islam et al., 2004).

2.5.2 Growth regulators

Skoog and Miller (1957) discovered the formulation for adventitious shoots and roots by altering the ratio of cytokinin and auxin. They stated that high concentration of cytokinin/auxin promotes shoot bud formation and inhibits root formation. A medium with high auxin concentration favours the root formation.

2.5.2.1 Effect of Cytokinins

The most commonly used cytokinins are kinetin and 6-benzyl aminopurine which stimulate growth and development of plants. Chan and Thong (2004) found that MS medium with BAP (2.0 mg/l) and IBA (2.0 mg/l) was suitable for *in vitro* propagation and multiplication of zingiberaceous members.

Desired number of shoots were obtained by using 2.0 mg/l BAP in MS media. Rooting was obtained on half nutrient MS medium with 0.1-1 mg/l BAP. The transplanted survival on the field was more than 70% (Rahaman et al., 2004).

The highest number of shoot propagation was seen on MS media supplemented with 3 mg/l BAP and 0.5 mg/l IBA. Shoot multiplication was strongly obtained using 3 mg/l BAP or with addition of 1mg/l IBA, 0.5 mg/l NAA (Hoang et al., 2005).

2.5.2.2 Effect of Auxins

There are natural as well as synthetic auxins. The commonly used auxins are IAA, IBA and NAA, the concentration required varies. Several studies have been done on using auxins with cytokinin and compare its synergistic effect.

In vitro culture of plants with these growth regulators provides significant large propagules. The synergistic effect of NAA with BAP showed shoot multiplication in Curcuma Amada Roxb., Borthakur and Bordoloi (1992). The addition of NAA or IBA to MS media with 3 mg/l BAP or 1 mg/l Kinetin significantly showed increase in the number of shoots per plant, but depletion in shoot length (Nguyen Hoang et al., 2005).

2.5.3 Photoperiod

Photoperiod also has a significant role in microrhizome germination and development. Wang and Hu (1982) found that 8 hours was better than 16 hours of photoperiod in potato and study by Abbott and Belcher (1986) showed that short day length i.e. 8 hours photoperiod was optimum for Curcuma aromatic L.

Microrhizome initiated in 4 hours photoperiod condition were best, and microrhizome inoculated in dark had higher number of buds formation (Nayak and Naik, 2006).

2.5.4 Temperature

The optimal required temperature for *in vitro* microrhizome propagation is 20ºC with constant temperature than day and night alteration. The cultures were incubated at 25±2ºC for microrhizome formation which was an effective condition (Chirangini and Sharma, 2005).

2.6 Callus Induction

The callus induction can be done by using excised shoot tips, leaf, hypocotyl, cotyledons and embryo can be used. Temperature required is around 25±2ºC under a 16 hours photoperiod. The calli were translocated onto fresh MS medium with growth hormones for the sake of maintenance (Smith, 2013).

2.7 Rooting

The micro propagated shoots were transferred on semi and complete strength MS medium with variable concentrations of IBA i.e 0.5 – 5 mg/l for root development. Mostly the roots use to grow with the shoot itself so there is no need to transfer it to another medium if proper medium is used for the shoot inoculation and growth (Parthadeb Ghosh et al., 2013).

2.7.1Hardening and acclimatization

Acclimatization allows the regenerated plantlets to adapt and withstand the environmental conditions just like a mother plant. A well-developed plant with roots were selected for primary hardening (Shamsudheen et al., 2018). Firstly the plantlets were removed and washed with water to remove adhered agar. The plantlets planted in plastic cup size container having mixture of sand, soil and vermiculate in ratio of 1:2:1. They are kept in green house where humid condition is maintained. The survival rate is 75% after 1 month in shade condition (Parthadeb Ghosh et al., 2013).

3. MATERIALS

AND

METHOD

The dissertation work on **"*In vitro* propagation of *Curcuma longa* L. (Turmeric)"** was carried out at R&D laboratory, Aranya Agribiotech, Mogar, Dist: Anand from December 2018-March 2019.

3.1 Experimental material

Turmeric rhizomes were used for inception of the experiment, it was collected from Assam.

3.2 Plant material

The rhizomes were kept in dark for the shoot/buds to sprout and were used as explants. Two types of explants were used for the experimental work.

(a) Sprouted rhizome shoots

(b) Sprouted rhizome buds

The leaves of plant and dried rhizome powder were also used.

3.3 Phytohormones

Auxins: 2,4-D, NAA, IAA

Cytokinin: BAP

Gibberellin: GA3

3.4 Glassware and equipment

The borosilicate glasswares were used for the experiment. The glasswares were dipped in chromic acid, further washing was carried out in running water. The culture vessels and equipments were sterilized in autoclave at 121ºC before using it.

3.5 Media

In these experimental study Murashige and Skoog, 1962 (MS) media and Lloyd and McCown, 1981 Woody Plant Medium (WPM) media was used. The details of constituents with their concentration is listed in Appendix.

3.6 Methodology

3.6.1 Explant collection

The collection of rhizome shoots was done from Assam. It was grown and cultivated in the field itself so that vegetative buds can be used as explant.

3.6.2 The clonal multiplication of Curcuma longa L. in *in vitro* condition

Pre-treatment of plant material:

The rhizomes were treated with fungicide (bavistin) powder and was kept in dark for 2 months so that the shoots can germinate. The shoot sprouts were collected and washed with water for further

treatment. The shoot sprouts were again treated with fungicide wash for 20 minutes on a rotary shaker and transferred to the laminar hood for disinfection process.

Table 4: Disinfection of Plant material

Sr. no	Treatment 1	Water wash	Treatment 2	Water wash	Treatment 3	Water wash
1.	50% Ethanol (v/v) 1 minute	2 minutes	10% Hypochloride (v/v), 1 minute	2 minutes	0.8% $HgCl_2$ (w/v), 1 minute	3 times, each for 2 minutes

Culture medium and condition:

MS medium and WPM was prepared with 3% sucrose and 0.8% agar for *in vitro* culture. After adjusting the pH between 5.6-5.8 the media was boiled and poured in sterilized glass bottles. The pH was adjusted using NaOH or HCL for alkalinity or acidic conditions. Followed by autoclaving it at 121ºC for 20 minutes at 15 Psi. The medium is stored for 7 days before use to see whether it is contamination free or not.

Preparation of phytohormone stock solution:

Stock solution for 2,4-D, IAA, IBA, GA3 and NAA were prepared by firstly dissolving them. GA3 was dissolved in ethanol while other hormones are dissolved in NaOH (alkaline solution). Once dissolved completely volume is made up. Demineralized water was used for stock and media preparation. Stock solutions were stored at 7-8ºC.

Shoot induction:

The shoot sprouts of rhizome were inoculated in bottles having MS medium with different concentrations of plant growth regulators in the laminar hood where continuous sterile air flows. The inoculated plant was later on transferred to another bottle with different PGRs after 10, 20, 30 days interval for its clonal multiplication. The incubation photoperiod was of 16 hours and the temperature was 25±1ºC.

Table 5: Inoculation of explants in MS and WPM medium with different PGRs concentration

Sr. no	Treatment Code	Basal Media	Growth regulators (mg/l)		
			BAP	NAA	IAA
1.	C	MS	-	-	-
2.	MS1	MS	3	0.1	-
3.	MS2	MS	3	0.5	-
4.	MS3	MS	3	1	-
5.	MS4	MS	4	0.1	-
6.	MS5	MS	4	0.5	-
7.	MS6	MS	4	1	-
8.	MS7	MS	3	-	1
9.	WPM1	WPM	3	0.1	-
10.	WPM2	WPM	3	0.5	-
11.	WPM3	WPM	3	1	-
12.	WPM4	WPM	4	0.1	-
13.	WPM5	WPM	4	0.5	-
14.	WPM6	WPM	4	1	-

3.6.3 *In vitro* initiation of Turmeric from rhizome buds

Surface sterilization of explants:

Turmeric rhizome was collected from field and were washed with water. The rhizomes were dipped in 1% fungicide (bavistin, 1gm/l) for 60 minutes. After that scaly leaves present on rhizome was scraped off. The scraped rhizome was treated with solution of 1% fungicide, 0.25% streptocycline and 2-3 drops of tween-20 for 20 minutes on a rotary shaker. Later on they were transferred to laminar flow cabinet in sterile environment where secondary treatment was provided.

Table 6: Pre-treatment of explant before inoculation

Sr. no	Treatment 1	Water wash	Treatment 2	Water wash	Treatment 3	Water wash
1.	50% Ethanol (v/v) 2 minutes	2 minutes	10% Hypochloride (v/v), 2 minutes	2 minutes	0.8% $HgCl_2$ (w/v), 2 minut	3 times, each for 2 minutes

Inoculation of rhizome buds/eyes for micropropagation:

The explants (rhizome bud/eye) were inoculated in MS media having phytohormones with their varying concentration. The cultures were incubated at 24±1ºC with a photoperiod of 16 hours.

Table 7: Inoculation of explants in MS medium with varying concentration of growth regulators

Sr. no	Basal Media	Growth regulators (mg/l)		
		BAP	GA3	NAA
1.	MS	-	-	-
2.	MS	2	-	-
3.	MS	2	0.5	-
4.	MS	2	-	0.5

3.7 Regeneration of plantlets through callus formation

Plant material: The sprouted vegetative buds were used as explants.

Pre-treatment of plant material:

The buds were dipped in 1% (v/v) fungicide solution for an hour, than after it was thoroughly washed with water. Then they were surface sterilized using 10% hypochloride and 2-3 drops of tween-20 under laminar air flow (sterile conditions).

Callus induction:

The basal part of the plant Curcuma longa L. was used for callus induction. The leaves and roots were removed from the plant segments. The MS media used was supplemented with 2,4-D 1mg/l and BAP in combination with 0.5,1,2,3 mg/l. The sub-culture of callus was done after 30 days and callus forming frequency was calculated after 60 days of culturing.

3.8 Estimation of chlorophyll content in Curcuma longa L. leaves

Plant material: Leaves were used for measuring the chlorophyll.

Procedure: weigh 1g of leaves and grind the tissue to a pulp in a clean mortar with the help of 20ml of 80% of acetone. Centrifuge at 5000rpm for 5 minutes and transfer the supernatant to a flask. Repeat the process until the residue is colorless. Make up the volume to 100ml by using 80% acetone. Take the absorbance reading at 645, 663 and 652nm against the blank (805 acetone). Demineralized water was used for making 80% acetone.

3.9 Measure the curcumin content in turmeric using HPLC

Material and sample preparation: Dried turmeric rhizome powder. Turmeric sample was extracted using methanol. The weighed sample (2g) was dissolved in 30ml of methanol.

Procedure: Formulation standardization was done using curcumin as marker. HPLC analysis was carried out using reverse-phase C_{18} column Luna having 5µm particle size of (phenomenex) using Shimadzu instrument. The injection volume was 20µl (sample) and the flow rate was 1ml/l. Total run time was 30mins, column temperature was 33ºC. Isocratic elution was done by using

benzene and ethyl acetate mixtures. The detection was done by photometric diode array (PDA) detector at the wavelength of 425nm.

3.10 Observation during the experimental work

3.10.1 Percentage of shoot multiplication:

The number of shoots and buds per initial shoot is the multiplication rate. The multiplication cycle is of 4 weeks. The rate of multiplication (ROM) is calculated by dividing the no. of sub-cultured plants form the existing no. of cultures.

$$ROM = \frac{No.of\ existing\ cultures}{No.of\ subcultures\ done}$$

3.10.2 Chlorophyll estimation equation:

The estimation of chlorophyll a, chlorophyll b and total chlorophyll content can be calculated using following equation:

$$mg\ of\ Chlorophyll\ a\ per\ gram\ of\ tissue = \frac{12.7\ (A663) - 2.69\ (A645) \times V}{1000 \times W}$$

$$mg\ of\ Chlorophyll\ b\ per\ gram\ of\ tissue = \frac{22.9\ (A645) - 4.68\ (A663) \times V}{1000 \times W}$$

$$total\ mg\ of\ chlorophyll\ in\ per\ gram\ of\ tissue = \frac{20.2\ (A645) + 8.02\ (A663) \times V}{1000 \times W}$$

4. RESULT AND DISCUSSION

4.1 *In vitro* clonal multiplication of Curcuma longa L. from newly sprouted shoot buds.

The results of the experiments performed with Curcuma longa L. are described in this chapter.

The sprouted shoots of rhizome were inoculated in MS and WPM medium with varying concentration of growth regulators. The buds showed highest multiplication in MS1 treatment code. The shoot multiplication of 99.66% was obtained in this treatment as showed in table 9 and figure 4. The combination of cytokinin (BAP) with auxin (NAA) elicits plant growth and development, which is proved by the experiment. As auxins keeps the lateral buds dormant the shoot growth is promoted and BAP reduces senescence. Lowest multiplication was seen in MS4 and WPM6 (30%).

Table 8: *In vitro* clonal multiplication of turmeric.

Treatment Code	Basal Media	Growth regulators (mg/l)			Mean % Response of Explants±S.D.	Mean No. of shoots/ explant	Mean ROM
		BAP	NAA	IAA			
C	MS	-	-	-	33.33±0.82	1	1
MS1	MS	3	0.1	-	96.66±0.32	6	4.43
MS2	MS	3	0.5	-	90±0.48	4	2.60
MS3	MS	3	1	-	80±0.52	3	1.72
MS4	MS	4	0.1	-	30±0.74	1	1.21
MS5	MS	4	0.5	-	33.33±0.67	1	1.18
MS6	MS	4	1	-	56.66±0.95	2	1.66
MS7	MS	3	-	1	80±0.52	3	1.59
WPM1	WPM	3	0.1	-	66.66±0.82	2	1.57
WPM2	WPM	3	0.5	-	50±0.85	2	1.24
WPM3	WPM	3	1	-	56.66±0.67	2	1.30
WPM4	WPM	4	0.1	-	40±0.63	1	1.27
WPM5	WPM	4	0.5	-	36.66±0.74	1	1.30
WPM6	WPM	4	1	-	30±0.74	1	1.27

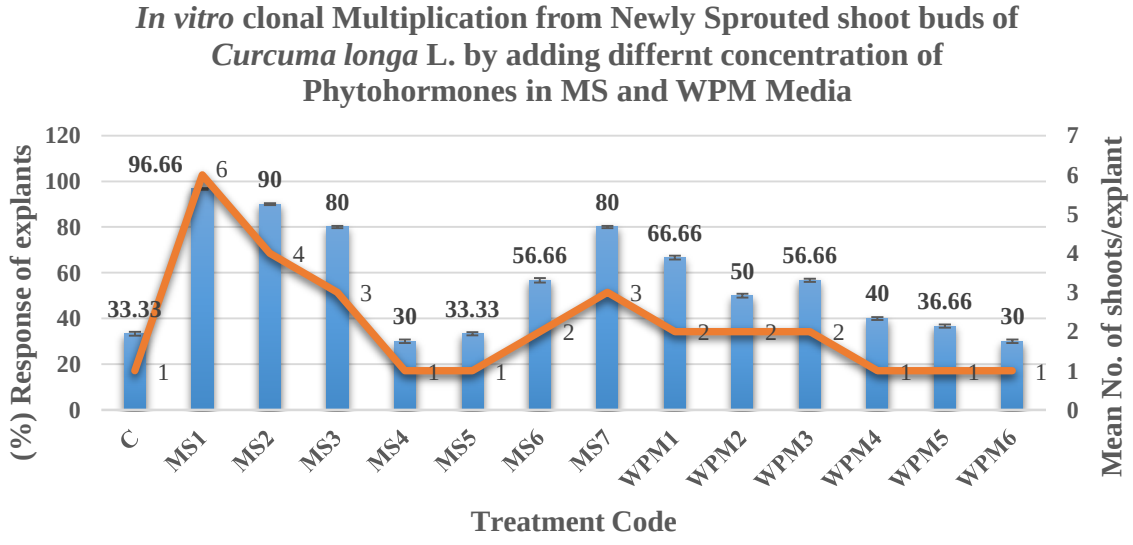

Figure 3: The graph represents *in vitro* clonal multiplication of Curcuma longa L.

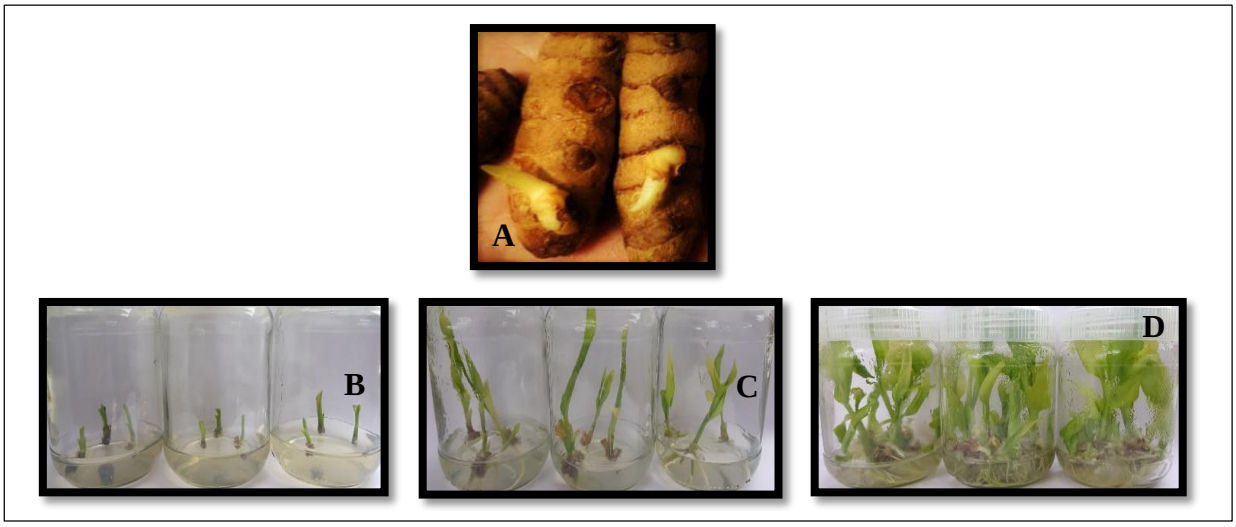

Figure 4: Various steps performed for *in vitro* clonal multiplication **a** sprouted buds of turmeric **b** inoculation of sprouted shoots **c** shoot elongation and growth **d** multiplied shoots

4.2 *In vitro* initiation of Turmeric

The buds of rhizome were pre-treated with fungicide and surface sterilants like fungicide, hypochloride and $HgCl_2$. Further inoculated in different concentrations of phytohormones. The germination was observed best in treatment code MSB3 and the lowest in treatment code C1.

Table 9: The germination of rhizome

Treatment code	Basal media	Growth regulators (mg/l)			Mean	%Response±SD of explants	Avg. shoot length Mean±SD	Days taken for initiation
		BAP	GA3	NAA				
C1	MS	-	-	-	0.6	26.66±0.547	1±0.13	20 to 25
MSB1	MS	2	-	-	1	40±0.701	3.02±0.27	18 to 20
MSB2	MS	2	0.5	-	1.8	60±0.836	5±0.44	12 to 15
MSB3	MS	2	-	0.5	2.6	86.66±0.489	6.18±0.53	8 to 10

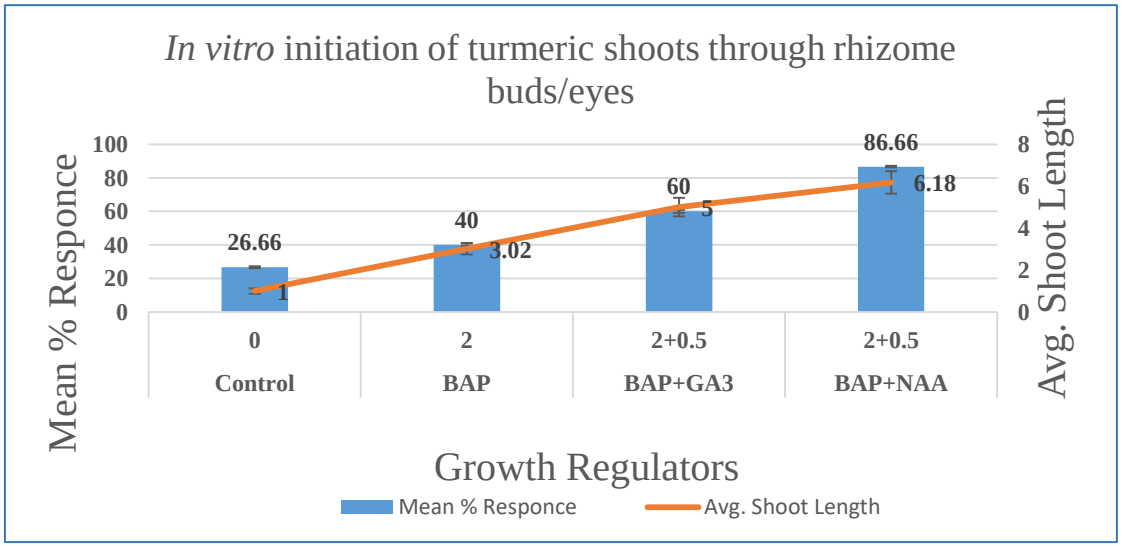

Figure 5: The graph represents *in vitro* initiation of turmeric shoots from buds/eyes.

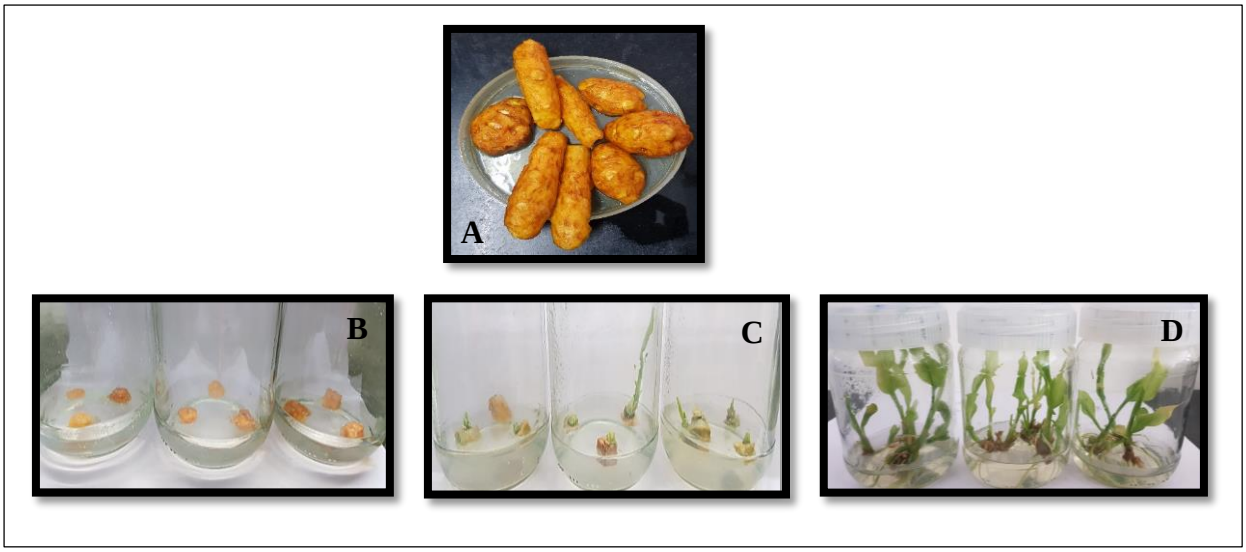

Figure 6: Steps for inoculation of buds/eyes of Curcuma longa L. **a** mother and finger rhizome without epidermis **b** inoculation of buds after treatments **c** sprouting of buds **d** the formation of young plants

4.3 Callus induction through shoot base

Different concentrations and combinations were tried to establish the most appropriate concentration of phytohormones for rejuvenation of plant through somatic embryogenesis. The observation table shows the % of callus induction after four months of culture. The best result was observed in the medium containing 2,4-D (1mg/l) + BAP (5mg/l).

Table 10: The percentage of callus induction and growth of new plantlets

Growth Regulators	Concentration (mg/l)	Mean % Callus Response	SD	No. of regenerated plants
Control	0	10	0.105	0
2,4-D+BAP	1+0.5	10	0.15	1
2,4-D+BAP	1+1	15	0.135	2
2,4-D+BAP	1+3	20	0.27	4
2,4-D+BAP	1+5	28	0.192	5

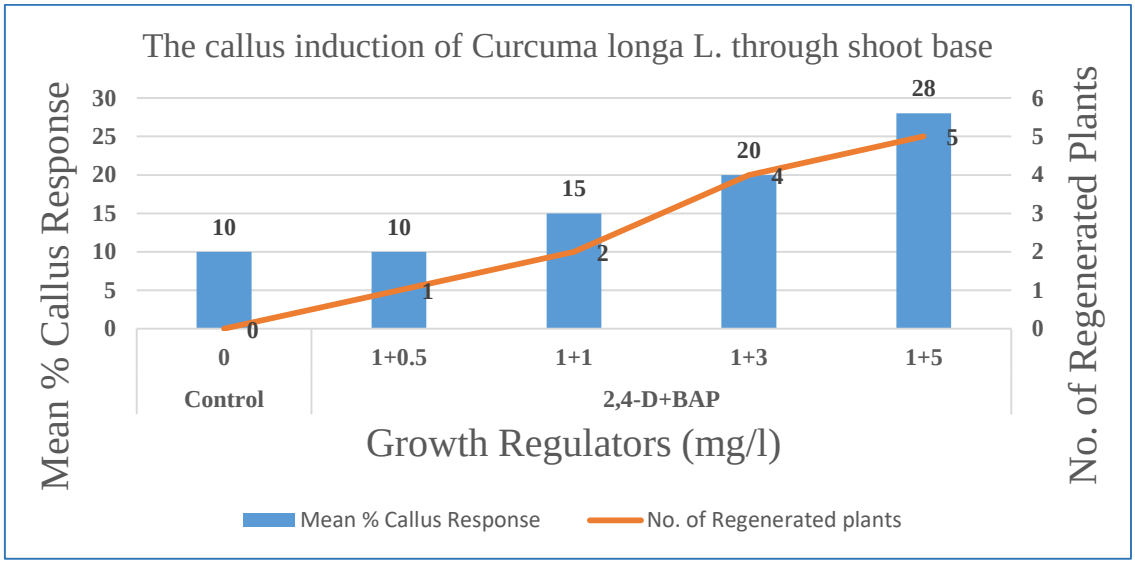

Figure 7: The graph represents callus induction of Curcuma longa L.

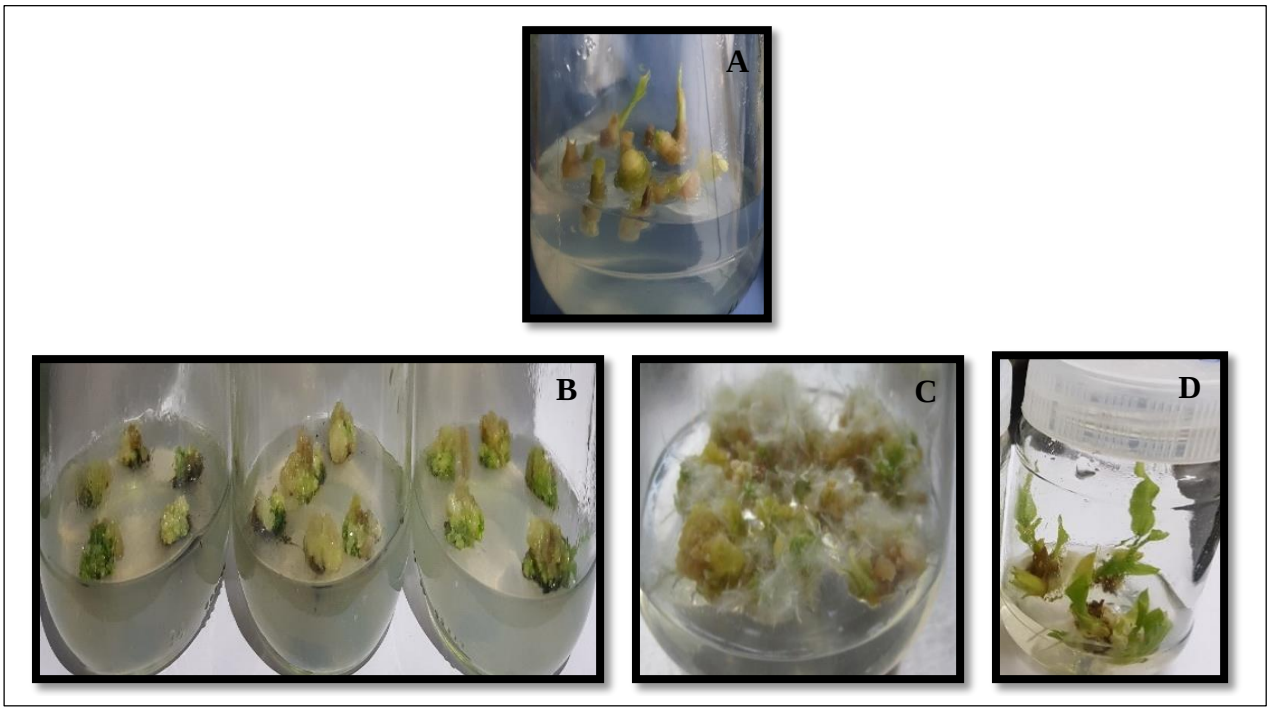

Figure 8: Different stages of callus induction **a** explant inoculation in media **b** three sub-culturing resulted in greenish callus formation **c** formation of somatic embryo **d** development of new shoots

4.4 Chlorophyll estimation of adult and young leaves of turmeric

The leaves were collected and crushed using 80% acetone. Then the absorbance reading was taken at 645, 652 and 663nm. The total chlorophyll content with chlorophyll (a,b) was calculated by using Arnon formula (1949). The results are described in table 12.

Table 11: The content of chlorophyll in *Curcuma longa* leaves

Sr.no	Adult/young plant	Mean of O.D readings		Chl a (mg/l)	Chl b (mg/l)	Total chlorophyll
		645nm	663nm			
1.	Young leaves	0.13453	0.3412	0.26	0.17	0.3
2.	Adult leaves	0.1862	0.0579	2.79	1.87	4.03

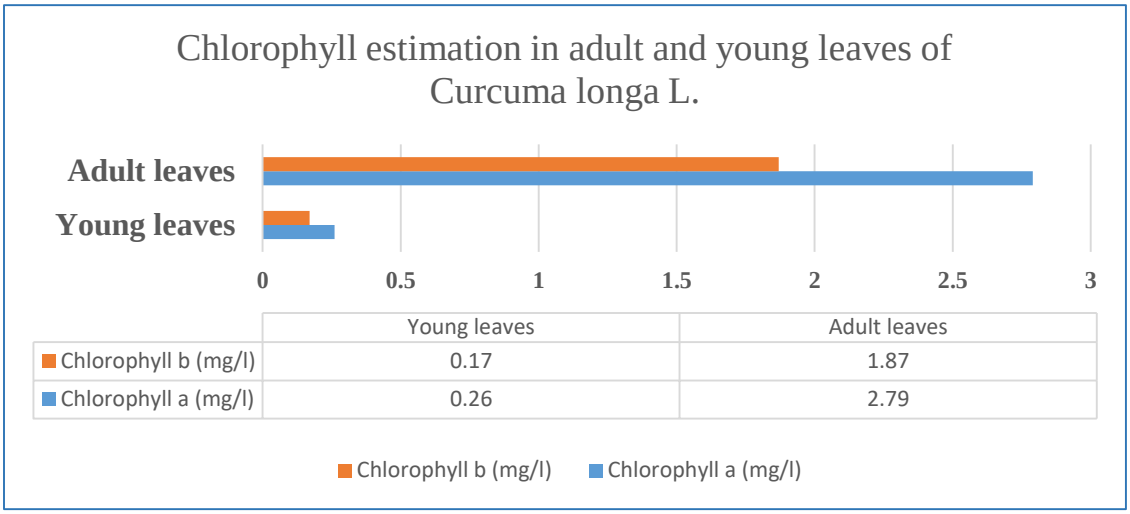

Figure 9: The graph represents the presence of chlorophyll content in adult and young leaves of Curcuma longa L.

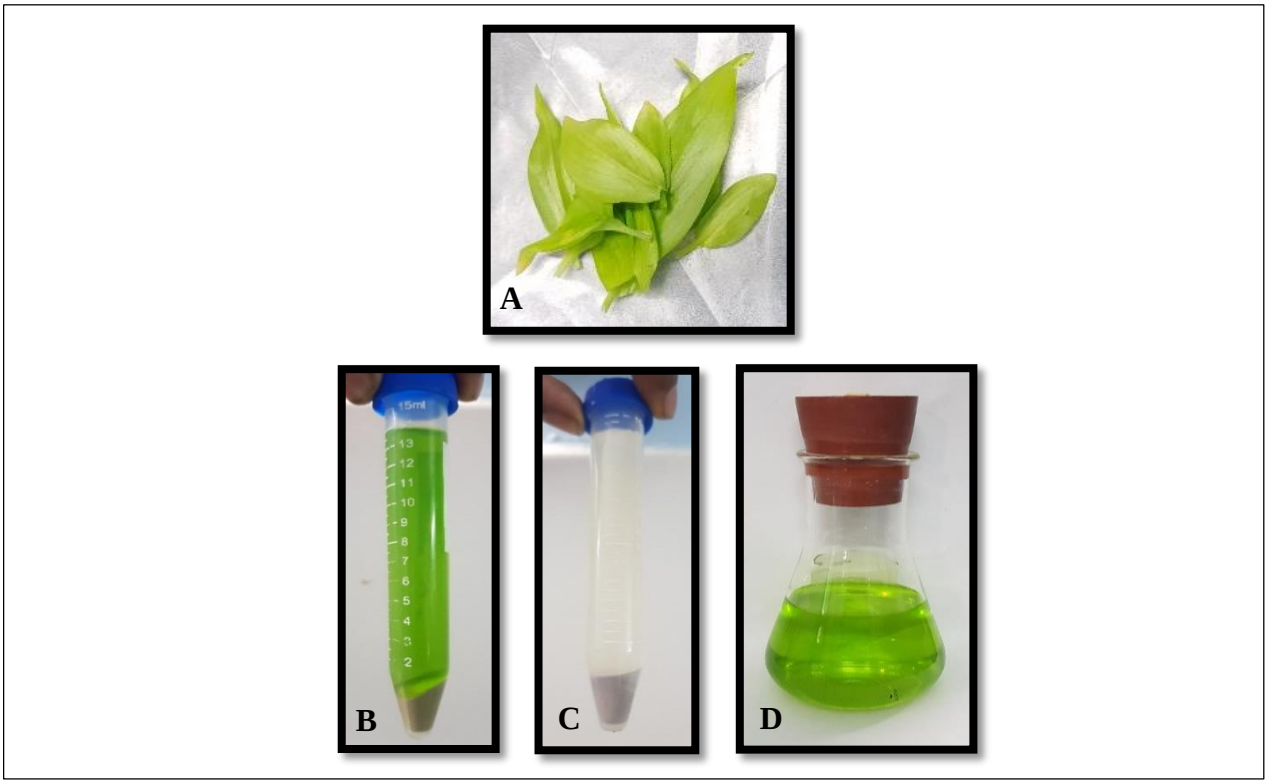

Figure 10: The valuation of chlorophyll in leaves **a** leaves of *Curcuma longa* **b** centrifugation of sample which was crushed in acetone **c** centrifuge till the supernatant becomes colorless **d** the makeup of sample was done to 100ml

4.5 Presence of curcumin in Turmeric rhizome

Pure curcumin was isolated by HPLC. Separation of different compounds was done by elution with benzene and ethyl acetate mixtures. The compounds showed single peaks at retention times of 3.06, 4.315, 9.952, 14.487 minutes. The compounds were identified by comparing the peaks with the standard results. The results showed that turmeric sample contains 2.93% of curcumin by weight with the purity of around 45%.

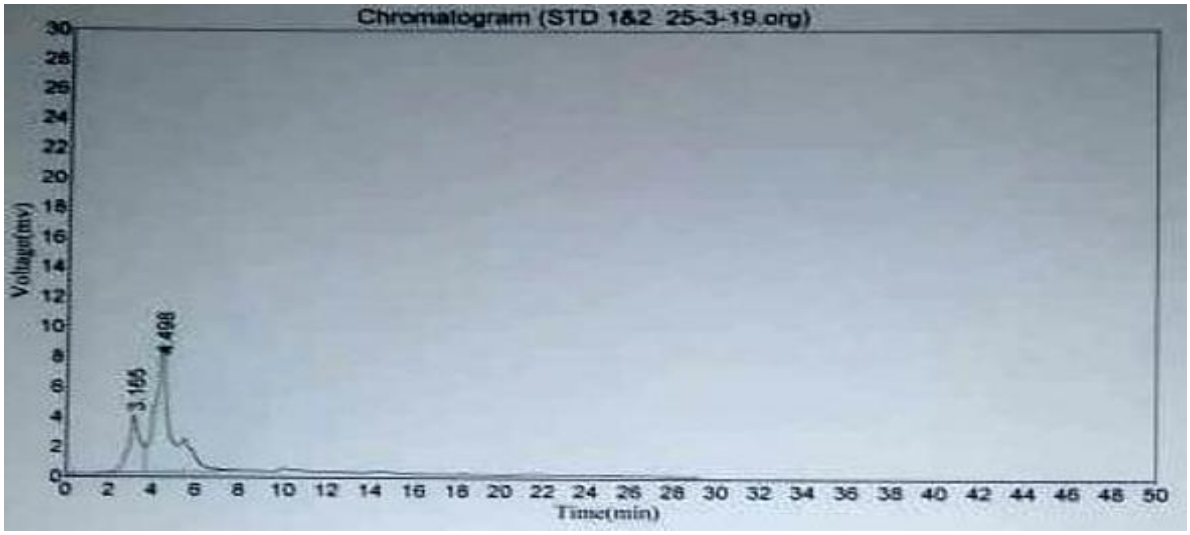

Figure 11: Chromatogram of standard

Table 12: HPLC report of curcumin of standard.

	Peak No.	Retention Time	Height	Area	Concentration (%)	
Std	1	3.165	3,549,756	142,659,391	25	
	2	4.498	8,051,111	450,374,219	75	Curcumin
					100	

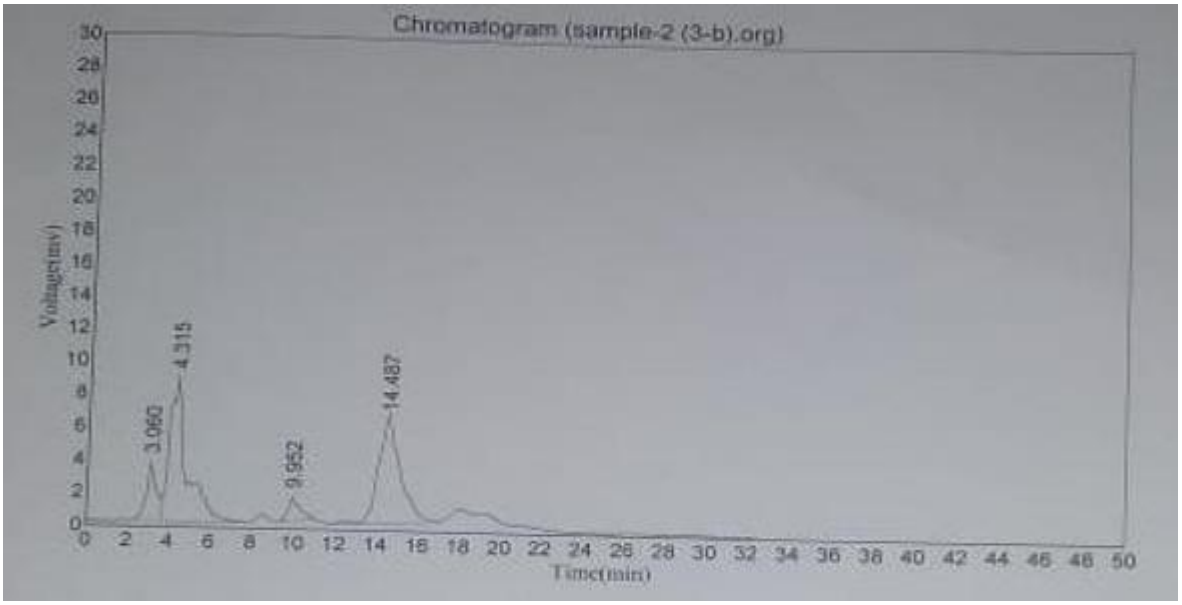

Figure 12: Chromatogram of sample

Table 13: HPLC report of curcumin from sample (powder of turmeric rhizome).

	Peak No.	Retention Time	Height	Area	Concentration (%)	
Std	1	3.06	3,385,644	117,763,141	10	
	2	4.315	8,532,422	501,481,781	45	Curcumin
	3	9.952	1,289,304	53,044,047	4	
	4	14.487	6,416,142	466,326,938	41	
					100	

5. CONCLUSION

Conclusion

The data presented in the thesis shows that MS medium with BAP (3mg/l) + NAA (0.1mg/l) was best for *in vitro* clonal multiplication of Curcuma longa L. while the combination of BAP (2mg/l) + NAA (0.5mg/l) with MS media is good *in vitro* initiation of turmeric. Callus induction was done to increase the production. The combination of 2,4-D (1mg/l) + BAP (5mg/l) generated highest no. of plantlets from callus. The chlorophyll estimation and its comparison was done between initiated plants and greenhouse acclimatized plants. The chlorophyll content varies in adult and younger leaves of plant as photosynthetic rate is high in adult leaves than in younger leaves. Quality check of turmeric was done, the HPLC results showed 2.93% curcumin is present in turmeric with purity of around 45%.

Appendix

Appendix A-Composition of MS basal medium (Murashige and Skoog, 1962).

Constituents of MS basal medium are:

- Macro elements

  Ammonium nitrate (1650 mg/l), Calcium chloride (332.2 mg/l), Magnesium sulphate (180.69 mg/l), Potassium nitrate (1900 mg/l), Potassium phosphate monobasic (170 mg/l).

- Micro elements

  Boric acid (6.2 mg/l), Potassium iodide (0.83 mg/l), Sodium molybdate (0.25 mg/l), Cobalt chloride hexahydrate (0.025 mg/l), Manganese sulphate tetrahydrate (22.3 mg/l), Zinc sulphate heptahydrate (8.6 mg/l), Copper sulphate pentahydrate (0.025 mg/l), Ferric sulphate (27.8 mg/l), EDTA disodium salt dehydrate (37.3 mg/l).

- Vitamins

  Nicotinic acid (0.5 mg/l), Pyridoxine hydrochloric acid (0.5 mg/l), Thiamine hydrochloric acid (0.1 mg/l), Glycine (2 mg/l), Myo-inositol (100 mg/l).

Appendix B-Composition of WPM basal medium (Lloyd and McCown, 1981).

Constituents of WPM basal medium are:

- Macro elements

  Ammonium nitrate (400 mg/l), Calcium chloride (72.5 mg/l), Magnesium sulphate (180.69 mg/l), Potassium sulphate (900 mg/l), Potassium phosphate monobasic (170 mg/l).

- Micro elements

  Boric acid (6.2 mg/l), Manganese sulphate tetrahydrate (22.3 mg/l), Molybdic acid (0.213 mg/l), Zinc sulphate heptahydrate (8.6 mg/l), Copper sulphate pentahydrate (0.025 mg/l), Ferrous sulphate heptahydrate (27.8 mg/l), EDTA disodium salt dehydrate (37.3 mg/l).

- Vitamins

  Nicotinic acid (0.5 mg/l), Pyridoxine hydrochloric acid (0.5 mg/l), Thiamine hydrochloric acid (1 mg/l), Glycine (2 mg/l), Myo-inositol (100 mg/l).

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