A Protocol for Rapid Clonal Propagation and Microrhizome Production of *Curcuma caesia* Roxb. (Zingiberaceae): A Critically Endangerd Medicinal Plant of North East India

Indrani Sarma, A.C. Deka, T.C. Sarma

10.18805/IJARe.A-5338

ABSTRACT

Background: *Curcuma caesia* Roxb. (Zingiberaceae) is a rare, critically endangered medicinal plant of Northeast India. The rhizome of the plant is famous for its significant medicinal properties.

Methods: Various concentrations of plant growth regulators in Murashige and Skoog (MS) medium were tried in the study using rhizome bud as explant for development of an efficient cost effective protocol for *in vitro* mass multiplication and microrhizome induction of *Curcuma caesia*.

Result: Shoot multiplication and plant generation was achieved from freshly sprouted shoots of *Curcuma caesia* on Murashige and Skoog's medium supplemented with different phytohormones. The best response for shoot multiplication (23.80±0.51shoots/explant) was obtained within 8 weeks in MS medium supplemented with BAP (1.0 mgl^{-1}) and 2,4-D (0.25 mgl^{-1}). Microrhizomes were induced at the base of the *in vitro* derived shoots upon transferred to medium containing various combinations and concentrations of sucrose and BAP and grown under varying photoperiod. Half strength of MS basal medium containing BAP (1.0 mg/l) and 9% sucrose was found to be optimum for induction of large sized microrhizome within 45 days of incubation under 16 hrs of photoperiod.

Key words: *Curcuma caesia*, Micropropagation, Microrhizome induction.

INTRODUCTION

Curcuma caesia Roxb. (Zingiberaceae), perennial aromatic herb with high medicinal potentialities is under the category of Critically Endangerd species as per the IUCN Red List of threatened species (Das and Saikia, 2019). It is commonly known as 'kolahaldhi'(Black turmeric) in Assam. This plant has diverse medicinal potentialities (Abubakar and Pudake, 2014).The rhizome is pungent, fragrant, heating, appetizer, antihelmintic, alexiteric and also claimed to be ethno medicinally useful in asthma, tuberculous glands of the neck, enlargement of the spleen *etc* (Thingujam and Nayak, 2019). The rhizomes are also used in the treatment of smooth muscle relaxant activity (Arulmozhi *et al*., 2006) haemorrhoids, leprosy, cancer, epilepsy, menstrual disorder, Alzheimer's disease (Sasikumar, 2005, Behar *et al*., 2013).

The indiscriminate exploitation of the species from natural habitat leads to make this species under the threat of extinction (Mannangatti and Narayanasamy, 2008). Moreover, Zingiberaceae plants are most frequently infected with various pathogens like *Pythium* species causing rhizome rot disease, *Colletotrichum* species causing the leaf spot *etc*. resulting reduction of the yield and the quality of the product and even causing the death of the plant (Dixit *et al*., 2000). To overcome, the limitation of conventional method of propagation *in vitro* propagation is the only method for conservation and large scale production of this endangered genotype in order to prevent their potential extinction.

Although *in vitro* plant propagation has been accomplished in other *Curcuma* species (Khumaida *et al*., 2019) however, little work has been reported on *Curcuma caesia* (Shahinozzaman *et al*., 2013).

Moreover, some work has been reported in other species of *Curcuma* for *in vitro* formation of storage organs such as micro rhizomes (Cheethaparambil *et al*., 2014) but not reported in *Curcuma caesia* till date. As these kinds of propagules can be directly transferred to the field without any acclimatization and hardening procedures it facilitate for easy transportation and mass scale production. The present investigation was carried out to establish a standard protocol for *in vitro* multiplication of *Curcuma caesia* and to study the factors affecting for micro rhizome induction of the species.

GC-MS analysis for essential oil and RAPD pattern of *Curcuma caesia* of both conventionally propagated and tissue cultured plant material also helped for qualitative analysis of the plantlets.

The Energy and Resources Institute, North Eastern Regional Centre, Guwahati-781 036, Assam, India.

Corresponding Author: Indrani Sarma, The Energy and Resources Institute, North Eastern Regional Centre, Guwahati-781 036, Assam, India. Email: indranisarma@gmail.com

How to cite this article: Sarma, I., Deka, A.C. and Sarma, T.C. (2021). A Protocol for Rapid Clonal Propagation and Microrhizome Production of *Curcuma caesia* Roxb. (Zingiberaceae): A Critically Endangerd Medicinal Plant of North East India. Indian Journal of Agricultural Research. 55(1): 13-22. DOI: 10.18805/IJARe.A-5338.

Submitted: 19-07-2019 **Accepted:** 18-04-2020 **Online:** 10-11-2020

MATERIALS AND METHODS

Rhizomes of *C. caesia* Roxb. collected and grown in the experimental garden of Department of Botany, Gauhati University, Guwahati. Dormant rhizome axillary buds were excised from the clean rhizome, which were used as the source of explants. Freshly collected rhizomes were cleaned with running tap water and immature buds were excised with sharp blade and washed with detergent (Tween-20, 0.1% v/v) for 15 min and subsequently rinsed with clean water. Explants were then surface sterilized in disinfectant (0.1% $HgCl_2$ to which two - three drops of Tween-20 were added) for 10 minutes after that rinsed five-six times with sterile distilled water and aseptically cultured on shoot induction medium to obtain contamination free cultures. MS(Murashige and Skoog, 1962) basal media supplemented with 3% sucrose and 2.2g/l Gelrite modified with various concentrations of N6-Benzyl-aminopurine (BAP 0.05 to 3.0 mg/l) in combination with Kinetin (Kn; 0.5- 2.0 mg/l), Indole-3-acetic acid (IAA; 0.5-1.0 mg/l), α-naphthaleneacetic acid (NAA; 0.5-1.0 mg/l) and 2, 4-D (0.5-4.0 mg/l) were used for establishment of the culture. Laboratory reagent-grade sucrose was replaced by locally available commercial sugar as carbon source for reducing the cost of the media.

The pH of the media was adjusted to 5.8 and autoclaved at 121°C with 15 lbs pressure for 15 minutes. Excised buds were inoculated into culture media and incubated at 25±2°C under 16 hours of photoperiod. Most of the cultures sprouted within 7-12 days of inoculation and the emerged shoots (1.0-2.0 cm) were sub cultured in the same media for further multiplication. A total of 20 explants were used for each treatments and the number of shoots per explant was recorded after four weeks of inoculation. Each set of experiment was repeated thrice.

The *in vitro* established clumps of microshoots were sub cultured at 15 days interval for 5 cycles. Fully grown shoots were separated and transferred to rooting media containing Indole-3-acetic acid (IAA; 0.005 - 2.5 mgl^{-1}), α - naphthalene acetic acid (NAA; 0.005 - 2.5 mgl^{-1}), 2% and 3% sucrose (market sugar). Rooted shoots were transferred to ½ strength MS basal medium without growth regulator for 10 days. The plantlets were washed thoroughly with sterile distilled water and then transferred to potting mixture (soilrite and compost, 10: 1, v/v) and kept in the growth chamber for 7 days. The environment of the growth chamber was maintained initially at 80% R.H., 24°C temperature, 12 hrs photo-period and gradually adjusted with the slight alteration of the environment to reach as similar to the outside. Finally, *in vitro* acclimatized plantlets were transferred to the soil in the earthen pots and ultimately planted in the nursery for hardening.

Microrhizome Induction

After separating the fully grown shoots originated from the rhizome the micro shoots were divided into small parts and again transferred to fresh medium devoid of growth hormones and cultured for another four weeks to avoid carryover effects of growth regulators and then cultured in freshly prepared medium for micro rhizome induction. Aseptic *in vitro* grown shoots approximately 4-5 cm long were derived from the established culture of *C. caesia* were used as explants for induction of micro rhizomes. MS medium supplemented with BAP (0.5 to 5.0 mgl^{-1}), Kn(0.5-5.0 mgl^{-1}), NAA(0.5 to 1 mgl^{-1}) and IAA(0.5 to 1 mgl^{-1}) along with sucrose (3%, 6% and 9%) was used for the study of micro rhizome induction and cultures were incubated at 25±2°C. Varying photoperiods (16 hrs, 8 hrs, 4 hrs and 0 hr dark) were tried to test their effect on micro-rhizome formation. Each treatment was done on 15 replicates and repeated for 3 times. Data on the percentage of rhizome formation in each replication, rhizome weight and number of buds per rhizome were recorded after 45 days of culture. The micro rhizomes were harvested aseptically and were repeatedly washed in running tap water and air-dried prior to storage in soil. Micro rhizomes were then stored in poly bags containing sterile sand. Poly bags were kept in net house (30°C) and growth initiation percentages of these micro rhizomes were also recorded.

The recorded data was subjected to statistical analysis by using one way ANOVA using Duncan's Multiple Range Test (DMRT; Duncan, 1955). The bio-active compounds of the rhizome of conventionally propagated and tissue cultured plants were studied by GC-MS analysis according to standard methods with slight modifications.

The protocol of Doyle and Doyle (1987) was followed for isolation and purification of genomic DNA from both micro propagated and field grown mother plants. The protocol for RAPD analysis was adapted from that of Williams *et al*. (1990) and Panda *et al.* (2007) to study the banding pattern of DNA.

RESULTS AND DISCUSSION

In the present study, the morphogenetic responses vary with respect to basal media as well as to different growth regulators. MS medium was found suitable for the micro propagation of *Curcuma caesia* which also reported earlier for other *Curcuma* species (Yasuda *et al*., 1987). The present study revealed that an unbalanced nutrient medium reacted poorly, regardless of the shoot multiplication potential of the explant or optimum plant growth regulator (PGR) levels. By optimizing basic macro and micronutrients and balance of growth regulators, the shoot initiating ability of the explant was enhanced. The culture medium with a suitable amount of cytokinin and auxins played an important role during the early stages of initiation and establishment of rhizome axillary buds of *C. caesia*. The shoot proliferation capacity of *C. caesia* depended on the concentration of cytokine like BAP. BAP alone or in combination with other growth regulators induced shoot multiplication (Abubakar and Pudake, 2019; Parthasarathy and Sasikumar, 2006). The positive effect of BAP on multiple shoot regeneration was also observed in ginger (*Zingiber officinale* Rosc.) (Balachandran *et al*., 1990). The synergistic effect of higher

Table 1: Effect of different modified basal media tested for multiple shoot regeneration of *Curcuma caesia* from axillary buds of rhizome.

Concentration (mg/l) media	Days required for multiple shoot initiation	% of explant with multiple shoots	Shoots/ explant	Number of leaves/ shoot	Shoot length (cm)
BAP0	25.0	16.20±0.58h	2.00±0.00e	1.60±0.24cd	2.70±0.09f
BAP 0.05	21.8	38.60±0.68e	2.20±0.37de	1.00±0.00d	3.00±0.13ef
BAP 0.5	17.6	44.40±0.68d	3.00±0.00cd	1.60±0.24cd	3.20±0.09e
BAP 1	17.0	52.20±0.73c	3.40±0.24c	2.20±0.20bc	3.60±0.10d
BAP 1.5	13.0	63.20±0.51a	8.00±0.00a	4.00±0.41a	4.40±0.20b
BAP 2	15.0	58.60±0.51b	4.40±0.24b	2.80±0.20b	5.30±0.07a
BAP 2.5	19.8	50.60±0.51c	5.00±0.32b	2.20±0.20bc	4.00±0.17c
BAP 3	19.0	31.00±0.32f	4.20±0.37b	2.20±0.20bc	3.60±0.07d
BAP 5	21.0	24.20±0.66g	2.60±0.24cde	2.00±0.00c	3.10±0.04e
Kn0.05	24.0	30.00±0.71d	2.20±0.20bc	1.00±0.00c	3.30±0.07c
Kn0.5	23.4	33.60±1.21c	4.00±0.00c	2.00±0.00c	3.70±0.05b
Kn1.0	18.0	46.20±0.37a	7.60±0.24a	3.40±0.24a	4.00±0.00a
Kn1.5	19.2	40.40±0.51b	6.20±0.21a	3.20±0.20ab	3.70±0.04b
Kn2	22.6	30.80±0.37d	2.20±0.20bc	2.00±0.00b	3.40±0.04c
Kn2.5	25.8	26.00±0.32e	2.80±0.37b	2.00±0.00b	3.00±0.12d
Kn3	25.0	22.60±0.51f	2.00±0.00c	2.00±0.00c	2.30±0.08e
Kn5	27.4	18.00±0.45g	1.00±0.00d	2.00±0.00b	2.20±0.07e
2,4-D0.05	21.4	30.20±1.39e	1.00±0.00d	1.00±0.00d	3.00±0.16c
2,4-D 0.5	16.6	39.00±1.05c	3.00±0.00d	2.86±0.21c	3.66±0.04b
2,4-D 1	15.5	47.40±0.51a	6.00±0.32a	3.00±0.24a	3.70±0.15a
2,4-D 1.5	19.4	46.00±0.71a	4.60±0.24ab	2.20±0.20ab	3.50±0.03b
2,4-D 2	20.0	44.00±0.71b	3.20±0.20bc	2.20±0.20ab	3.20±0.07c
2,4-D 2.5	22.0	33.20±0.37d	3.00±0.00c	2.00±0.00bc	2.70±0.07d
2,4-D 3	23.8	29.00±0.71f	3.00±0.00c	1.00±0.00d	2.60±0.04d
2,4-D 5	26.0	24.00±0.32g	1.00±0.00d	1.00±0.00d	2.30±0.03e
BAP 0.5+ Kn 0.05	25.0	33.00±0.45g	2.00±0.00e	1.80±0.37cd	3.20±0.07c
BAP 0.5+ Kn 0.1	22.6	42.20±0.37e	5.20±0.37d	2.00±0.00bc	3.70±0.14b
BAP 1+ Kn 0.05	21.0	62.40±1.21b	9.00±0.71b	3.20±0.20a	4.00±0.12a
BAP 1+ Kn 0.5	16.6	66.00±0.45a	12.80±0.51a	3.00±0.00a	4.70±0.07a
BAP 1.5+ Kn 0.5	21.4	54.40±0.51c	7.00±0.55c	2.60±0.24ab	3.00±0.07c
BAP 2+ Kn 0.5	19.0	47.20±0.37d	5.20±0.20d	2.00±0.00bc	2.70±0.09d
BAP 2+ Kn 1	22.0	40.00±0.55f	3.20±0.37e	1.40±0.24cd	2.40±0.03e
BAP 2.5+ Kn 1	24.4	30.00±1.00h	3.00±0.00e	1.80±0.20cd	2.00±0.07f
BAP 3+ Kn 1	24.0	26.00±0.71i	2.00±0.00e	1.20±0.20d	2.00±0.07f
BAP0.5+IAA0.05	21.0	40.20±0.66d	4.00±0.32d	1.20±0.20c	3.40±0.07d
BAP 1+IAA0.5	14.0	64.20±0.37b	12.60±1.03b	3.00±0.00ab	4.30±0.12b
BAP 1.5+IAA0.5	19.0	53.00±0.71c	10.00±0.71c	3.00±0.45ab	4.00±0.04c
BAP 2+IAA0.5	12.4	76.40±0.68a	19.20±0.51a	4.00±0.00a	5.70±0.07a
BAP 2+IAA1	19.8	40.40±0.81d	4.60±0.51d	1.80±0.37c	3.30±0.07d
BAP 2.5+IAA1.5	18.6	36.60±1.21e	3.00±0.00de	1.75±0.25b	3.00±0.07e
BAP 5+IAA2.5	22.0	20.00±0.71f	2.00±0.00e	1.00±0.00c	3.00±0.13e
BAP0.05+2,4-D0.05	23.0	40.20±1.66h	2.00±0.00g	1.20±0.20d	3.00±0.14d
BAP0.5+2,4-D0.5	20.6	46.00±1.32f	6.20±0.20d	2.00±0.00bcd	4.10±0.03c
BAP1+2,4-D0.5	19.8	70.60±1.51b	19.20±0.37b	2.00±0.32bcd	5.30±0.04b
BAP1+2,4-D 0.25	15.2	84.40±1.24a	23.80±0.51a	3.60±0.40a	5.44±0.04a
BAP2+2,4-D0.5	17.6	68.20±1.37c	18.00±0.32bc	3.40±0.24a	4.00±0.07c
BAP2+2,4-D1	19.0	60.00±1.58d	12.20±0.37c	2.60±0.24b	3.20±0.07d
BAP2.5+2,4-D1.5	19.8	50.40±1.68e	5.00±0.32e	2.20±0.20bc	3.00±0.07d
BAP3+2,4-D1	18.0	42.60±1.51f	3.00±0.00f	2.00±0.00bcd	2.40±0.21e
BAP5+2,4-D1	20.2	36.20±1.66g	1.00±0.00h	1.60±0.24cd	2.00±0.00f

Table 1: Continue...

Table 1: Continue...

BAP5+2,4-D2.5	21.0	22.00±1.71i	1.00±0.00h	1.60±0.40cd	2.00±0.00f
BAP0.5+NAA0.05	13.2	41.80±1.37e	2.00±0.00e	1.00±0.00c	3.80±0.13d
BAP1+NAA0.5	12.0	81.20±1.58a	18.00±0.00a	4.40±0.40a	6.30±0.07a
BAP1.5+NAA0.5	12.6	78.20±1.37b	14.40±0.24b	3.00±0.00b	4.60±0.07b
BAP2+NAA0.5	13.0	53.20±1.66c	11.20±0.45c	3.00±0.00b	4.20±0.04c
BAP2.5+NAA1	13.2	50.00±1.32d	5.80±0.37d	3.00±0.00b	3.00±0.11e
BAP3+NAA1	16.4	41.20±1.37e	3.20±0.37e	2.00±0.00c	2.70±0.07f
BAP5+NAA2	18.8	30.00±1.32f	2.00±0.00e	2.00±0.00c	2.30±0.08g
Kn0.5+NAA 0.05	25.4	38.00±0.45e	2.40±0.24ef	1.40±0.24e	3.70±0.03e
Kn 1.0+NAA 0.5	20.0	46.00±0.71c	10.00±0.32ab	3.20±0.20c	4.00±0.07d
Kn 1.5+NAA 0.5	17.0	50.80±0.86b	10.20±0.37ab	4.20±0.20b	4.60±0.07b
Kn 2.0+NAA 0.5	16.2	58.20±1.00a	16.00±0.45a	4.60±0.32a	5.40±0.12a
Kn 2.0+NAA 1.0	19.6	53.00±0.71a	9.40±0.51b	2.20±0.20d	4.20±0.07c
Kn 2.5+NAA 1.0	20.6	40.20±0.66d	6.60±0.51c	2.00±0.00de	3.40±0.07g
Kn 3.0+NAA 1.0	22.8	39.60±0.51de	5.00±0.45d	2.00±0.00de	3.50±0.07ef
Kn 4.0+NAA 1.0	21.0	34.00±0.71f	3.20±0.20e	2.20±0.20d	3.20±0.07g
Kn 5.0+NAA 1.0	24.0	27.00±0.71g	2.40±0.24ef	2.00±0.32de	2.30±0.07h
Kn 5.0+NAA 2.0	24.6	20.00±0.71h	2.00±0.00f	1.00±0.00e	2.00±0.03i
Kn 0.5+IAA0.025	27.0	29.60±0.51g	2.60±0.24fgh	1.20±0.20f	3.30±0.24ef
Kn 0.5+IAA0.05	23.4	40.00±0.71e	9.40±0.51d	2.20±0.20cd	3.80±0.07d
Kn 1.0+IAA0.05	17.0	60.00±1.21a	15.80±0.40a	4.20±0.20a	5.00±0.07b
Kn 1.0+IAA2.0	19.0	50.40±0.75c	14.60±0.37c	2.00±0.00c	3.60±0.07de
Kn 1.0+IAA4.0	22.2	43.20±0.86d	5.60±0.51e	1.80±0.20de	3.30±0.09ef
Kn 2.0+IAA0.5	21.0	56.00±1.39b	15.00±0.00b	3.40±0.24b	5.60±0.11a
Kn 2.0+IAA1.0	19.6	53.20±0.58bc	14.00±0.32c	3.00±0.24b	4.40±0.13c
Kn 2.5+IAA1.0	23.0	38.20±0.37e	3.40±0.24fg	1.20±0.20f	3.00±0.07fg
Kn 3.0+IAA1.0	25.0	34.40±0.68f	3.00±0.00efg	1.40±0.24ef	2.70±0.04g
Kn 5.0+IAA1.0	26.6	26.80±0.37h	2.00±0.00g	1.00±0.00f	2.30±0.07h
Kn 5.0+IAA2.0	26.0	19.40±0.51i	1.00±0.00h	1.00±0.00f	2.00±0.13h
Kn 0.5+2,4-D 0.05	27.6	30.20±0.58g	2.40±0.24g	1.00±0.00f	3.30±0.03g
Kn 0.5+2,4-D 0.5	24.2	33.60±0.51f	3.60±0.24f	1.60±0.24e	3.80±0.07e
Kn 1.0+2,4-D 0.5	19.0	60.00±0.00b	13.00±0.00b	4.00±0.00b	5.60±0.03b
Kn 1.0+2,4-D 2.0	21.6	56.40±0.68c	9.80±0.86d	3.20±0.20b	5.10±0.07c
Kn 1.0+2,4-D 4.0	22.0	52.20±0.86d	7.60±0.51e	2.80±0.20bc	4.40±0.12d
Kn 1.5+2,4-D 0.5	16.8	62.80±0.37a	15.20±0.58a	4.80±0.20a	6.20±0.07a
Kn 2.0+2,4-D 1.0	18.8	57.40±0.51c	12.00±0.55c	2.80±0.20bc	5.00±0.04c
Kn 2.5+2,4-D 1.0	21.0	40.40±0.68e	5.60±0.24ef	2.40±0.24cd	3.70±0.07e
Kn 3.0+2,4-D 1.0	21.6	32.40±1.21f	3.60±0.24f	2.00±0.00de	3.50±0.07f
Kn 5.0+2,4-D 1.0	23.0	30.00±0.71g	2.20±0.20g	1.80±0.20e	2.40±0.04h
Kn 5.0+2,4-D 2.0	26.0	26.20±0.37h	2.00±0.00g	1.80±0.20e	2.20±0.04i
S.Ed.±		1.04	0.37	0.37	0.13
$CD_{0.05}$		2.11	0.76	0.75	0.26

concentration of cytokinin with lower concentration of auxin induced better shoot organogenic response as reported by various workers in *Curcuma longa* and other plant species (Isah, 2019).

The effect of 1.0 mgl^{-1} BAP with 0.25 mgl^{-1} 2, 4-D on adventitious shoot proliferation was observed in *C. caesia* (Table 1). It was observed that the inclusion of 1.0 mgl^{-1} BAP and 0.25 mgl^{-1} 2,4-D in the culture medium led to the highest number of shoot per explant (23.80±0.51) and shoot length (5.44±0.04 cm) as compared to that of other treatments within 8 weeks of culture (Table 1, Plate e-h). A maximum of (84.40±1.24%) of shoots shows multiplication under this combination of medium.

The synergistic effect of BAP (1.0 mgl^{-1}) in combination with 2,4-D (0.25 mgl^{-1}) on promotion of *C. caesia* shoot cultures is in agreement with the observations of other workers in *Curcuma longa* (Zapata *et al*., 2003) and in *Zingiber officinale* (Khatun *et al*., 2003).

This is followed by the MS media modified with BAP (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) showed (81.20±1.58%) shoot

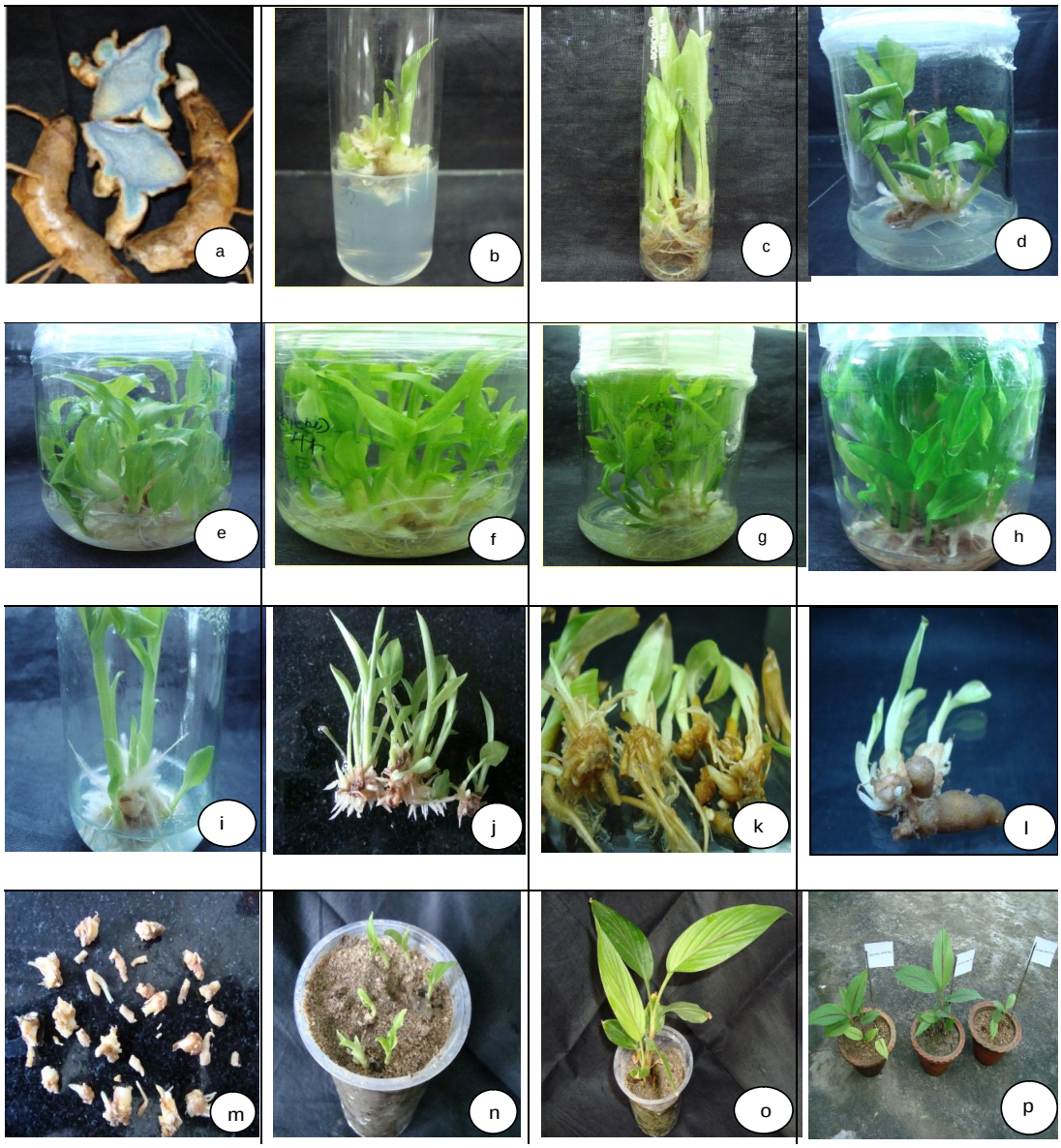


Plate 1: Micropropagation and microrhizome induction of *Curcuma caesia* on MS medium; (a) Rhizome of *Curcuma caesia* (b) Multiple shoots initiation with BAP (1.00mgl^{-1}) and NAA0.5 mgl^{-1}) after 2 weeks of culture; (c, d) Shoot multiplication with BAP (2.0mgl^{-1}) and IAA 0.5 mgl $^{-1}$ after 3 weeks of culture; (e,f,g,h) Shoot multiplication with after 4 weeks of culture) BAP (1.0mgl^{-1}) and 2, 4-D 0.25 mgl^{-1}); (I, j) Rooting after 3 weeks in ½ MS with NAA 1.5 mgl^{-1} and Sucrose 20gl^{-1} (k,l) Microrhizomes bunch induced in half strength MS with BAP 1.0 mgl^{-1} and Sucrose 90gl^{-1} (m) Separated microrhizomes; (n) Sprouted microrhizomes in poly beg; (o) Microrhizome originated plantlets (from poly beg); (p) Separated plantlets transferred to earthen pots.

multiplication (Table 1, Plate b). However, Balachandran *et al*. (1990) and Panda (2007) had reported maximum shoot formation of *Curcuma longa* in the MS medium supplemented with BAP (3 mgl^{-1}). In this study a comparatively lower response was recorded when BAP was added alone in the medium which indicates that the addition of 2, 4-D in the culture medium results in high morphogenetic ability of the cultures of *C. caesia*. However, higher concentration of BAP is found to be inhibitory with or without the supplements of auxin. Stanley and Keng (2007) also support the same result in *Zingiber zerumbet*. Higher concentration of BAP (5.0 mgl^{-1}) with NAA (2.0 mgl^{-1}) showed callusing of the explants with fewer numbers of shoots (2.00±0.00 shoot/explants). In such cultures shoots were stunted with a mean shoot length of 2.30±0.08 cm. However, the higher concentrations of BAP reduced the frequency of shoot induction which is in agreement with the studies in *Zingiber officinale* (Kambaska and Santilata 2009) and in *Kaempferia rotunda* (Bejoy *et al*., 2006).

In the present study, BAP showed better results as compared to Kn (Table 1). But higher concentration of BAP and Kn exhibited negative effect on shoot regeneration in *C.*

Table 2: Rooting respose in *Curcuma caesia*.

Sucrose concentration	Concentration (mg/l)			No shoot of responded for rooting	Rooting %	Days to root induction	Root/ shootlet	Root length (cm)
	BAP	KN	NAA					
MS+Sucrose 30 g/l	0	0	0	3.00±0.32 h	25.00±0.45k	30.00±0.71a	4.00±0.32ij	1.70±0.07l
MS+Sucrose 90 g/l	0	0	0	6.00±0.32ef	50.00±0.32h	20.00±0.32c	7.00±0.32fg	2.70±0.13ghi
½ MS+Sucrose 30 g/l	0	0	0	7.00±0.32de	58.00±0.55g	18.00±0.45d	11.00±0.45bc	3.20±0.03cde
½ MS+Sucrose 30 g/l	2.0	0	0.5	5.00±0.32fg	41.00±0.63i	17.00±0.45de	8.00±0.32ef	3.10±0.12cdef
½ MS+Sucrose 60 g/l	5.0	0	0.5	6.00±0.32ef	50.00±0.45h	15.00±0.32fg	4.00±0.32ij	2.50±0.16ij
½ MS+Sucrose 60 g/l	5.0	0	1.0	7.00±0.32de	58.00±0.32g	15.00±0.71fg	8.00±0.32ef	2.80±0.03fghi
½ MS+Sucrose 20 g/l	0	0	1.5	11.00±0.45a	91.00±0.32a	10.00±0.32i	14.00±0.45a	4.00±0.24a
½ MS+Sucrose 90 g/l	2.0	0	0	6.00±0.32ef	50.00±0.32h	13.00±0.32hi	10.00±0.32cd	2.60±0.07hi
MS+Sucrose 30 g/l	1.0	0	0	3.00±0.32 h	25.00±0.32k	17.00±0.32de	6.00±0.32gh	2.00±0.03kl
MS+Sucrose 60 g/l	5.0	0	0	9.00±0.55bc	70.00±0.32e	14.00±0.45gh	11.00±0.32bc	3.20±0.0 8cde
S.Ed.±				0.48	0.72	0.67	0.49	0.16
CD 0.05				0.96	1.43	1.34	0.97	0.32

caesia. It has also been reported earlier that higher concentration of plant growth regulators was not suitable for *in vitro* culture of Zingiberaceae species (Stanly and Keng, 2007). The results of the present study confirm the importance of the PGR in the dedifferentiation process of *C. caesia*. Although positive response was observed in the control culture (without PGR) but it was very low compared to the other treatments. It has been observed that market sugar also effective for shoot multiplication of *C. caesia*.

Splitting and removal of induced shoots and transferring 2 to 3 shoots per culture favoured higher number of shoots than that of dividing into small parts. Moreover, retention of the explants with proliferated shoots in the initiation medium for more than 8 weeks resulted in decline as indicated by shoot necrosis and premature leaf browning. Shoot tip necrosis was also observed in few treatments with higher concentrations of cytokinins. BAP not only enhanced shoot growth but also supported root induction (Khatun *et al*, 2003). Shoot multiplication accompanied by simultaneous rooting of the shoots was seen after third sub culturing on PGR-free media. Spontaneous rooting was also recorded on the MS basal media supplemented with BAP along with IAA or NAA like other *Zingibers*, *viz.* turmeric and ginger (Chan and Thong, 2004), *Alpinia galanga* (Borthakur *et al*., 1999) and in *Coccinia indica* (Borah *et al*., 2019). Although the rhizome bud of *C. caesia* produced shoots and roots simultaneously on most of the media tested for shoot multiplication, however to develop a healthy root system, fully grown shoots (4-6 cm) after removing all the roots were transferred to MS medium supplemented with BAP, IAA or NAA. MS (half strength) medium enriched with NAA (1.5 mgl^{-1}) and reduced level of sucrose (20 gl^{-1}) responded for (91.00±0.32%) rooting in *C. caesia*, which produced a mean of (14.00±0.45) number of roots per shoot within a short period of (10.00±0.32) days of culture (Table 2).

The rooted plantlets of *C. caesia* obtained from *in vitro* propagation were sequentially acclimatized (hardened). After hardening the plantlets were transferred in field conditions.

Microrhizome Induction

Fully grown *in vitro* raised plantlets were trimmed aseptically and transferred to microrhizome induction medium. After 20-25 days of incubation in microrhizome induction medium containing various phytohormones and different level of sucrose, the swelling of shoot bases were observed, followed by appearance of microrhizomes at the base within 30-45 days of incubation (Plate k). It was observed that sucrose plays a significant role in the size and number of microrhizomes in *C. caesia* and it was observed that 3% sucrose reduced both number and size of the microrhizomes even by increasing the concentration of BAP from the range of 0.5 to 5 mgl^{-1} or by increasing or decreasing the duration of the photoperiod. In the present study 9% sucrose was found to be optimum for the production of microrhizomes on ½ strength MS basal medium supplemented with BAP (1.0 mgl^{-1}) after 4 weeks of culture. Healthy, large sized and maximum number (16.00±0.45) of microrhizomes with an average weight of (1.77±0.03) g were obtained in this combination under 16 hours of photoperiod (Table-3, Plate-l). Both 6% and 9% sucrose gave good response in case of induction of healthy microrhizome in *C. caesia*. The results of the present investigation supports the report of Shirgurkar *et al*. (2001) and Nayak (2000) who obtained optimum micro-rhizome induction in *Curcuma longa* using 6-9% sucrose. The enhanced rate of *in vitro* organ formation with increasing concentration of sucrose may be attributed to the presence of high carbon energy in the form of sucrose since storage organs mostly store carbohydrates (Nayak, 2000).

BAP had significant effects on *in vitro* microrhizome induction of *C. caesia* (Table 3). Among different combinations of PGRs, 1.0 mgl^{-1} BAP exhibited a higher per cent of healthy microrhizomes (73.80±0.37%) induction. Our studies agree with Nayak (2000) in *C. aromatica* Salisb. for enhancement of microrhizome production.

In the present study half strength of MS is found to be more suitable in comparison to full strength of MS salts producing a large number and comparatively larger sized

Table 3: Effect of MS media modified with various concentration of sucrose, phytohormones and photoperiod on microrhizome formation in *Curcuma caesia* Roxb. (After 45 days of culture).

Media (mg/l)	Photo period (hr)	Number of explant with microrhizome	% explant forming microrhizome	Weight of rhizome (g)	Number of buds/ rhizome
Sucrose 30 g/l	16	-	-	-	-
Sucrose 60g/l	0	3.20±0.00	16.00±0.45	0.01±0.00	1.60±0.24
Sucrose 90g/l	8	3.60±0.32	18.00±0.32	0.29±0.00	3.20±0.20
½ MS+Sucrose 30 g/l	0	-	-	-	-
½ MS+Sucrose 60 g/l	4	4.00±0.00	20.00±0.71	0.02±0.00	2.60±0.40
½ MS+Sucrose 60 g/l +BAP1.0+NAA0.5	16	5.20±0.00	26.00±0.84	0.14±0.00	2.00±0.00
½ MS+Sucrose 30 g/l +BAP2.0+NAA0.5	16	-	-	-	-
½ MS+Sucrose 30 g/l +BAP2.0+NAA0.5	8	6.00±0.00	30.00±1.05	0.01±0.00	2.00±0.32
½ MS+Sucrose 60 g/l +BAP3.0+NAA0.5	0	6.60±0.32	33.00±0.7	0.20±0.00	2.80±0.37
½ MS+Sucrose 60 g/l +BAP5.0+NAA0.5	0	9.40±0.24	47.00±0.7	0.80±0.00	2.60±0.24
½ MS+Sucrose 60 g/l +BAP5.0+NAA1.0	0	10.00±0.32	40.00±0.32	0.18±0.01	3.00±0.32
½ MS+Sucrose 30 g/l +BAP5.0	16	6.00±0.32	30.00±0.84	0.23±0.00	2.20±0.37
½ MS+Sucrose 90 g/l +BAP1.0	16	14.60±0.32	73.00±0.37	1.77±0.03	16.60±0.45
½ MS+Sucrose 90 g/l +BAP2.0	16	8.00±0.32	40.00±0.45	1.47±0.03	7.00±0.32
MS+Sucrose 90 g/l +BAP1.0	16	3.80±0.37	19.00±0.32	0.75±0.00	1.80±0.37
MS+Sucrose 30 g/l +BAP1.0	8	5.20±0.40	26.00±0.71	0.21±0.00	2.60±0.24
MS+Sucrose 30 g/l +BAP2.0	8	9.60±0.68	48.00±0.32	0.32±0.00	2.60±0.24
MS+Sucrose 60 g/l +BAP3.0	4	11.20±0.32	56.00±0.55	0.42±0.00	6.00±0.32
MS+Sucrose 60 g/l +BAP5.0	4	12.00±0.00	60.00±0.89	0.16±0.00	2.00±0.00
MS+Sucrose 60 g/l +BAP0.5+NAA0.05	16	14.00±0.37	70.00±0.55	1.05±0.00	15.00±0.71
MS+Sucrose 60 g/l +BAP1.0+NAA0.5	8	9.00±0.32	45.00±0.71	0.01±0.00	2.80±0.20
MS+Sucrose 90 g/l +BAP2.0+NAA0.5	16	6.00±0.32	30.00±0.55	0.17±0.00	1.80±0.20
MS+Sucrose 60 g/l +BAP2.0+NAA1.0	8	7.60±0.40	38.00±0.45	0.41±0.00	3.60±0.24
MS+Sucrose 60 g/l +BAP3.0+NAA1.0	8	9.20±0.37	46.00±0.55	0.31±0.00	6.20±0.37
MS+Sucrose 90 g/l +BAP5.0+NAA1.0	4	8.00±0.71	40.00±0.24	0.02±0.00	7.00±0.45
MS+Sucrose 90 g/l +Kn 0.5	16	-	-	-	-
MS+Sucrose 60 g/l +Kn 1.0	8	4.40±0.37	22.00±0.32	0.21±0.00	3.20±0.37
MS+Sucrose 90 g/l +Kn 2.0	8	7.60±0.32	38.00±0.71	0.86±0.00	5.60±0.24
MS+Sucrose 90 g/l +Kn 3.0	4	6.06±0.24	30.30±0.32	0.67±0.00	7.40±0.24
MS+Sucrose 120 g/l +Kn 1.0+NAA0.5	16	3.60±0.32	18.00±0.71	0.44±0.00	3.00±0.00
MS+Sucrose 90 g/l +Kn 1.0+NAA0.5	16	8.40±0.20	42.00±0.55	1.09±0.00	5.40±0.24
MS+Sucrose 60 g/l +Kn 1.0+NAA0.5	16	4.80±0.37	22.00±0.45	0.67±0.00	4.20±0.20
MS+Sucrose 60 g/l +Kn 2.0+NAA1.0	8	9.20.40	46.00±0.32	0.67±0.00	5.60±0.24
MS+Sucrose 60 g/l +Kn 3.0+NAA1.0	8	4.60±0.32	23.00±0.32	0.41±0.00	3.80±0.37
MS+Sucrose 30 g/l +Kn 5.0+NAA1.0	4	-	-	-	-
S.Ed.±		0.45	0.77	0.01	0.42
$CD_{0.05}$		0.90	1.52	0.02	0.83

rhizomes (Table 3, Plate I). And the similar result was found by Shirgurkar *et al*. (2001) in turmeric. While Islam *et al*. (2004), Nayak (2000) and Sunitibala (2001) used full strength of MS basal medium for microrhizome induction in *C. longa*. Sharma and Singh (1995) also found full strength of MS basal medium along with 7.5% sucrose and 35.2 µM BA optimal for the production of *in vitro* microrhizome in ginger. But incubation under complete darkness reduced the number and size of the microrhizomes significantly. In the present study, Kn alone or in presence of NAA induces very small sized (0.67±0.00 g) microrhizomes. However, Sunitibala *et al*. (2001) reported that Kn (1.0 mgl^{-1}) is better suited for *in vitro* rhizome induction in *C. longa* L.

Microrhizome evaluation

In vitro produced microrhizomes were isolated after 60 days and harvested microrhizomes were directly transferred to the sand-soil bed for plantlet development. Plants developed from three different sizes of microrhizomes [0.5 - 1.0 cm (small), 1.1, 2.0 cm (medium) and > 2.0 cm (large)] (Table 4) were evaluated using various morphological characters. Germination, survival rate and morphological characters varied depending upon the sizes of microrhizomes. Plants

Table 4: Morphological parameters of *Curcuma caesia* (Roxb.) plants regenerated from different size of microrhizomes under *in vivo* condition (after 60 days of culture).

Microrhizome size cm (SE)	Survival rate (%)	Number of shoots	Shoots length (cm)	Number of roots	Roots length (cm)	Number of leaf	Leaf length (cm)	Leaf width (cm)
0.5 - 1.0	33.00±0.71c	0.60±0.24b	5.60±0.22c	3.00±0.32c	3.30±0.27c	1.20±0.20b	3.10±0.12c	1.60±0.08c
1.1 - 2.0	60.00±0.55b	1.20±0.20b	7.20±0.19b	5.40±0.24l	6.70±0.32b	2.60±0.24a	4.90±0.11b	3.30±0.21b
> 2.1	80.00±0.45a	2.00±0.32a	12.60±0.44a	10.00±0.32a	10.60±0.20a	3.00±0.00a	9.30±0.42a	4.00±0.13a
S.Ed.±	0.56	0.25	0.30	0.29	0.26	0.18	0.25	0.15
$CD_{0.05}$	1.78	0.80	0.95	0.91	0.83	0.56	0.80	0.47

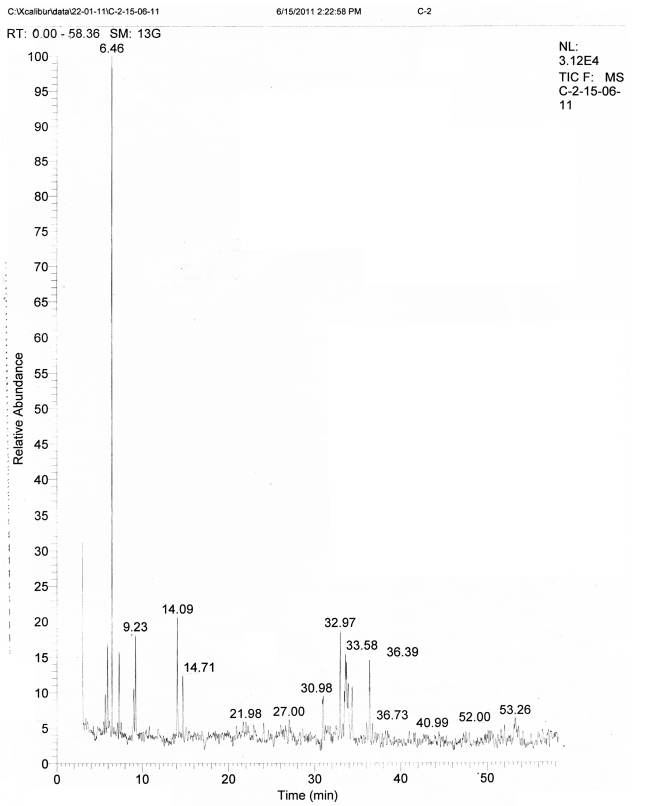


Fig 1: GC-MS Chromatogram of *Curcuma caesia* (field grown).

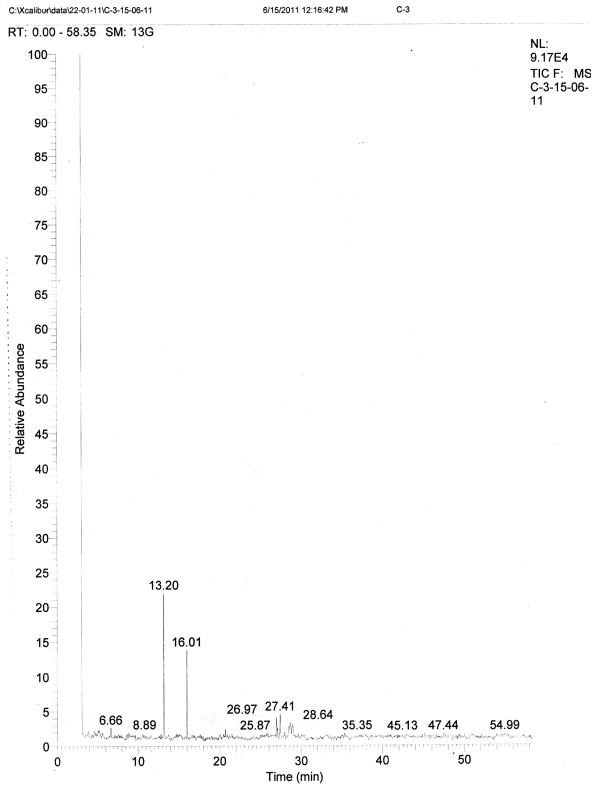


Fig 2: GC-MS Chromatogram of *Curcuma caesia (in vitro).*

regenerated from bigger microrhizomes were found to be more vigorous in terms of their shoot, root and leaf growth parameters (Table 4). Bigger microrhizomes were more competent and vigorous in comparison to smaller rhizomes as also reported earlier by other workers (Islam *et al*., 2004 and Shirgurkar *et al*., 2001). In the present study also a much higher survival rate was obtained because the average weight of microrhizomes was higher, which indicates that large sized microrhizomes is a key factor in achieving commercial success in microrhizome induction in *Curcuma caesia*. Microrhizomes produced in the present investigation were stored under moist conditions at room temperature and more than 80% of the sprouted microrhizomes developed shoots and roots two months after they had been successfully transferred to the field. Sharma and Singh (1995) studies also confirm the same result.

GC-MS analysis of essential oil extracted from tissue culture raised rhizomes revealed the presence of major compounds similar to that of the source (naturally propagated) plants *i.e*., 1, 8-cineole(35.3%), camphor (14.7%), beta-pinene (9.23%), bonieol(5.7%) and α-terpineol (3.3%) as the major compounds (Fig 1 and 2). From the present investigation, it was established that in *Curcuma caesia*, camphor is one of the specific component which gives intense camphoreous odour.

Even though micropropagated plants produced more leaves, grew faster and had more multiplication response than their vegetative counterparts, there was no significant variation in plant morphology and essential oil content as indicated by GC-MS study. Micropropagated plants of both the species were genetically uniform to donor plants, as indicated by random amplified polymorphic DNA (RAPD) analysis (Plate 2).

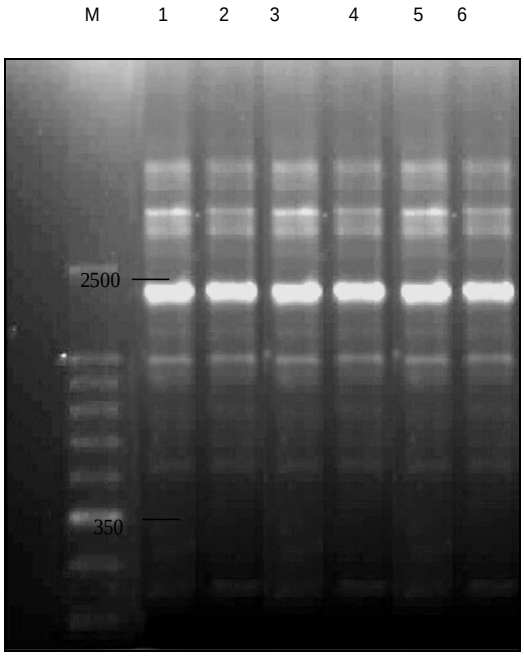


Plate 2: RAPD profile of *Curcuma caesia* using OPA09, M-350bp, Lane-Motherrhizome, Lane 2,3 *in vitro* plantlets, Lane 4,5,6 *in vitro* regenerated hardened plantlets.

CONCLUSION

It may be concluded that the protocol developed from this study will be useful for rapid *in vitro* propagation of the disease- free healthy and large sized microrhizomes of *Curcuma caesia* in lesser time. The procedure described here ensures 10 fold production of plantlets. The developed protocol also highlights the control of end pathogens as well as shoots multiplication that can be exploited for mass propagation to ensure the seasonal independent availability of material and also for germplasm conservation and for the utilization.

REFERENCES

Abubakar, A.S. and Pudake, R.M. (2014). *Curcuma caesia*: A wonder herb with medicinal properties (review). Indian J. Scholarly Res. 3(6): 1-4

Abubakar, A.S. and Pudake, R.M (2019). Sterilization procedure and callus regeneration in black turmeric (*Curcuma caesia*). Agricultural Science Digest. 39(2): 96-101.

Arulmozhi, D.K., Sridhar, N., Veeranjaneyulu, A. and Arora, K.S. (2006). Preliminary Mechanistic studies on the smooth muscle relaxant effect of hydroacloholic extract of *Curcuma caesia.* Journal of Herbal Pharmacotherapy. 6: 3-4.

Balachandran, S.M., Bhat, S.R. and Chandel, K.P.S. (1990). *In vitro* clonal multiplication of turmeric (*Curcuma* sp.) and ginger (*Zingiber officinale* Rosc.). Plant Cell Rep. 8: 521-524.

Behar, N., Tiwari, K.L. and Jadhav, S.K. (2013). Comparative phytochemical screening of bioactive compounds in *Curcuma caesia* Roxb. and *Curcuma longa*. Research Journal of Medicinal Plant. 7(2): 113-118.

Behura, S., Sahoo, S. and Srivastava, V.K. (2002). Major constituents in leaf essential oils of *Curcuma longa* L. and *Curcuma aromatica* Salisb. Curr. Sci. 83: 1312-1313.

Bejoy, M., Dan, M. and Anish, N.P. (2006). Factors affecting the *in vitro* multiplication of the endemic zingiber *Curcuma haritha* Mangaly and Sabu. Asian Journal of Plant Sciences. 5(5): 847-853.

Borah, A.R., Anbumalarmathi, J. and Sharmili, A.S. (2019). *In vitro* propagation of *Coccinia indica* (L.) Voigt. from internodal segments. Indian Journal of Agricultural Research. 53: 202-207.

Borthakur, M., Hazarika, J. and Singh, R.S. (1999). A protocol for micropropagation of *Alpinia galanga* L., Plant Cell, Tissue and Organ Culture. 55: 231-233.

Chan, L.K. and Thong, W.H. (2004). *In vitro* propagation of Zingiberaceae species with medicinal properties. J. Plant Biotechnology. 6: 181-188.

Cheethaparambil, A., Pillai, G.S. and Balachandran, I. (2014). *In vitro* microrhizome and minirhizome production in turmeric (*Curcuma longa* L.) cultivar Alleppey Supreme and its comparative anatomical and histochemical analysis. International Journal of Current Microbiology and Applied Sciences. 3(3): 535-542.

Das, K. and Saikia, L.R. (2019). Phenological studies of *Curcuma caesia* Roxb. and *Curcuma aromatica* Salisb. of Zingiberaceae from upper Brahmaputra Valley, Assam, India. International Journal of scientific and Technology Research. 8(7): 607-609.

Dixit, D., Srivastava, N.K. (2000). Distribution of photosynthetically fixed 14-CO_2 into curcumin and essential oil in relation to primary metabolites in developing turmeric (*Curcuma longa*) leaves. Plant Sci. 152: 165-71.

Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin. 19: 11-15.

Duncan, D.B. (1955). Multiple range and multiple F-tests. Biometrics. 11: 1-42.

Isah, T. (2019). De novo *in vitro* shoot morphogenesis from shoot tip-induced callus cultures of *Gymnema sylvestre* (Retz.) R.Br. ex Sm. Biol. Res. 52(3). https://doi.org/10.1186/s40659-019-0211-1.

Islam, M.A., Kloppstech, K. and Jacobsen, H.J. (2004). Efficient Procedure for *In vitro* Microrhizome Induction in *Curcuma longa* (Zingiberaceae) - A Medicinal Plant of Tropical Asia. Plant Tissue Cult. 14(2): 123-134.

Kambaska, K.B. and Santilata, S. (2009). Effect of plant growth regulator on micropropagtion of ginger (*Zingiber officinale* Rosc.) cv- Suprava and Suruchi. Journal of Agricultural Technology. 5(2): 271-280.

Khatun, A., Nasrin, S. and Hossain, M.T. (2003). Large Scale Multiplication of Ginger (*Zingiber officinale* Rosc.) from Shoot-tip Culture. Journal of Biological Sciences. 3(1): 59-64.

Khumaida, N., Ardie, S.W., Setiadi, A., Artiningsih, L.N. (2019). *In vitro* multiplication and acclimatization of black galingale (*Curcuma Aeruginosa* Roxb.) Journal of Applied Pharma--ceutical Science. 9(4): 110-116.

Mannangatti, K. and Narayanasamy, M. (2008). Anti-fungal protein from *Curcuma caesia* Roxb. J. Biotechnol. S136-90.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473-479.

Nayak, S. (2000). *In vitro* multiplication and microrhizome induction in *Curcuma aromatica* Salisb. Journal Plant Growth Regulation. 32(1): 41-47.

Panda, M., Mohanty, S. Subudhi, E., Acharya, L., Nayak, S. (2007). Assessment of stability of micropropagated plants of *Curcuma longa* L., by cytophotometry and RAPD analyses, International Journal of Integrative Biology. 1(3): 189-195.

Parthasarathy, V.A. and Sasikumar, B. (2006). Biotechnology of *Curcuma*, CAB Reviews: Perspectives in Agric, Vet. Sci., Nut. and Nat. Resources. 1(20): 1-9.

Shahinozzaman, M., Muhammad, F.M., Muhammad, O.F., Azad, M.A.K. and Amin, M.N. (2013). Micropropagation of black turmeric (*Curcuma caesia* Roxb.) through *in vitro* culture of rhizome bud explants. Journal of Central European Agriculture. 14(3): 110-115.

Sharma, T.R. and Singh, B.M. (1995). *In vitro* microrhizome production in *Zingiber officinale* Rosc. Plant Cell Rep. 15: 274-277.

Sasikumar, B. (2005). Genetic resource of *Curcuma*: diversity, characterization and utilization. Plant Genet Resource. 3: 230-251.

Shirgurkar, M.V., John, C.K., Nadgauda, R.S. (2001). Factors affecting *in vitro* micro-rhizome production in turmeric. Plant Cell, Tissue and Organ Cult. 64(1): 5-11.

Stanly, C. and Keng, C.L. (2007). Micropropagation of *Curcuma zedoaria* Rosc. and *Zingiber zerumbet* Smith. Biotechno--logy. 6(4): 555-560

Sunitibala, H., Damayanti, M. Sharma, G.J. (2001). *In vitro* propagation and rhizome formation in *Curcuma longa* Linn. Cytobios. 105(409): 71-82.

Thingujam, V. and Nayak, S. (2019). Dynamics of Curcumin content in *Curcuma caesia* Roxb. rhizomes with different storage conditions and durations. International Journal of Chemical Studies. 7(3): 1733-1735.

Williams, J.G.K. *et al*. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nuclic Acid Research. 18: 65.

Yasuda, K., Tsuda, T., Shimizu, H. and Sugaya, A. (1987). Multiplication of *Curcuma* species by tissue culture. Plant. Med. 54: 75-78.

Zapata, E.V., Morales, G.S., Lauzardo, A.N.H., Bonfil, B.M., Tapia, G.T., Sánchez, A.D.J., Valle, M.V.D., Aparicio, A.J. (2003). *In vitro* regeneration and acclimatization of plants of Turmeric (*Curcuma longa* L.) in a hydroponic system. Biotecnología Aplicada. 20: 25-31.