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RESEARCH ARTICLE

Development of In-vitro Protocol to Enhance Mass Multiplication and Acclimatization of Black Turmeric (*Curcuma caesia* Roxb)

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Abstract

Curcuma caesia Roxb., commonly known as black turmeric, faces challenges due to low propagation rates and difficulties in acclimatization. This study aims to develop an efficient *in-vitro* protocol to enhance the mass multiplication and successful acclimatization of black turmeric. In this study, ½MS medium with varying concentrations of BAP alone and in combinations with NAA was used for shoot induction from rhizome bud explants. The highest shoot induction of 57.77% with a mean number of 6.66 ± 0.88 shoots per explant, was observed with ½MS medium supplemented with 3.0 mgl^{-1} BAP. For shoot multiplication and elongation, explants were transferred to MS medium with different combinations of BAP, NAA, and Kinetin, achieving maximum shoot multiplication of 37.75% and elongation of 4 to 5 cm with 4.0 mgl^{-1} BAP and 1.5 mgl^{-1} NAA. Root induction was successfully initiated on MS medium fortified with IAA and IBA, with the highest root number of 8.35 ± 0.34 and length of 6.66 ± 0.35 cm observed in 0.5 mgl^{-1} IAA. After root induction, plantlets were acclimatized using a pot mixture of soil, sand, compost, cocopeat, and vermicompost in a 1:1:1 ratio, achieving an 86.66% survival rate in the Cocopeat: vermicompost: soil mixture.

Keywords: *In-vitro*, Black turmeric, Acclimatization, Mass multiplication, Root induction.

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Introduction

Medicinal plants have contributed significantly to the evolution of the ancient Indian medical system. Our country is a veritable treasure trove for medicinal plant genetic diversity (Lakshman, 2016). Medicinal herbs are becoming increasingly popular in both developing and emerging nations, with the majority of trade substances still sourced from forests and a small number of species cultivated (Pathak *et al.,* 2024). Black turmeric scientifically known as *Curcuma caesia* Roxb, is a rhizomatous aromatic herb of enormous therapeutic and commercial significance, belonging to the Zingiberaceae family (Sahu *et al.,* 2016; Mahanta *et al.,* 2023). Native to central and northeastern India, this relatively less-known medicinal herb is valued for its healing properties throughout Asia (Singh *et al*., 2015; Bara, 2024). In India, it is generally found in forest areas of Chhattisgarh, Madhya Pradesh, Odisha, Uttar Pradesh, and West Bengal (Vidya *et al.,* 2023). This plant holds significant social, economic, and medicinal value. In West Bengal, its rhizome is used during Kali puja, earning it the name Kali Haldi. "Kali" is derived from the word "kala," meaning black, which is why the plant is known as black turmeric in English (Mahato and Sharma, 2018). The underground rhizomes of this species have various applications. This herb is becoming highly economically significant for local

tribes and other communities because of its versatile use as an organic food preservative, food dye, cosmetics, spice, pharmaceutical and therapeutic agent (Benya *et al.,* 2023). Rhizome extracts are used to treat a range of conditions including piles, leprosy, bronchitis, asthma, cancer, epilepsy, fever, wounds, impotence, fertility issues, toothaches, and vomiting. Additionally, they are beneficial for treating leucoderma, tuberculosis, and spleen enlargement. The rhizome can also serve as a tonic for the heart and brain and is used as an expectorant, as well as for alleviating pain, inflammation, bruises, and sprains (Venugopal *et al*., 2017; Ibrahim *et al*., 2023). Due to the essential oil present, the interior of the rhizome is bluish-black and has a distinct pleasant aroma (Das *et al.,* 2013; Borah *et al.,* 2020).

However, the natural habitats of *C. caesia* are under threat due to various human activities, including over-exploitation for traditional medicine, as well as industrialization and urban expansion (Ibrahim *et al*., 2023). Reproduction of the plant occurs solely through its rhizomes, but cultivation efforts are hindered by challenges related to obtaining rhizomes of adequate quality and issues pertaining to seasonal constraints (Benya *et al*., 2023). The conventional method of propagating *C. caesia* involves certain limitations. Notably, it necessitates setting aside 10 to 20% of the harvested produce for replanting in the subsequent growing season (Shahinozzaman *et al.,* 2013). Additionally, this approach raises concerns regarding the transmission of soil-borne diseases from one crop cycle to the next and across different geographic locations. Rhizomes are particularly vulnerable to rhizome rot, which is caused by various bacteria and fungi, and they are also susceptible to infestations by insects (Bharalee *et al*., 2005). These challenges further complicate the cultivation and sustainable management of *C. caesia* (Bharalee *et al.,* 2005). The Central Forest Department of India has classified *C. caesia*, also known as black turmeric, as an endangered species, primarily due to the threats posed by biopiracy. The designation as endangered underscores the urgent requirement for protective measures against the exploitation and unsustainable harvesting of *C. caesia*, to preserve its ecological and medicinal value (Venugopal *et al.,* 2017). Hence, considering the current status and future prospects of *C. caesia*, an advanced method for its rapid and mass propagation has been developed through the use of *in-vitro* tissue culture techniques. Previously, many studies relevant to the micropropagation of *C. caesia* via direct and indirect organogenesis, have been conducted (Zuraida, 2013; Jose and Thomas, 2015; Singh *et al*., 2015; Sharma *et al*., 2021; Haida *et al*., 2022). Our study involved the alteration of basal medium formulation, Plant growth regulator and potting medium for acclimatization. Therefore, the aim of our study is to develop an improved mass multiplication protocol of *C. caesia* with suitable alteration of basal medium formulation, auxin and cytokinin concentration and acclimatization medium.

Materials and Methods

Plant materials and culture conditions

C. caesia germplasm, available at the medicinal garden of the Department of Plant Physiology at the College of Agriculture (JNKVV), Jabalpur, Madhya Pradesh was utilized as mother plants to establish the fresh cultures, free from the pathogens. Experiments were conducted at the Plant Tissue Culture and Transgenic Laboratory of the Biotechnology Center. For effective sterilization, rhizome bud explants (1–5 cm), were first washed with tap water for 30 min., followed by treatment with 5 to 6 drops of Tween-20 and rinsing with double distilled water multiple times. Subsequently, they were immersed in 1% (w/v) bavistin for 30 minutes and then treated with 0.1% (w/v) $HgCl_2$ for 3 minutes. The experimental media consist of Murashige and Skoog (½ MS) media along with different plant growth regulators (PGRs) (Sigma-Aldrich, Saint Louis, MO, USA) combinations, supplemented with 0.8% agar and 3% (w/v) sucrose (Himedia Laboratories, Mumbai, India). The pH of the media was carefully adjusted to 5.8 using either 1 N NaOH or 1 N HCl prior to autoclaving at 121°C at 15 psi pressure for 20 minutes. Approx 25 mL of ½ media was filled into culture tubes (25x150 mm; borosil, Mumbai, India) for inoculation. The culture tubes were properly sealed with the help of parafilm. All the cultures were incubated at a temperature of 25 ± 2°C with a 16/8 h photoperiod with a light intensity of 40 $\mu Em^{-2} s^{-1}$ using white fluorescent light (Phillips, India). On the same medium, the explants were subcultured after 21 to 24 days intervals.

In-vitro shooting

The rhizome bud explants were inoculated on ½ MS media supplemented with varying concentrations of BAP and NAA to observe the best initiation of shoots. The concentration of BAP varied in the range of 0.5 to 5.0 mg/l, and NAA was added in combination with concentrations ranging from 0.5 to 1.5 mg/l. For shoot multiplication, initiated shoots were carefully transferred to ½ MS medium supplemented with varying concentrations of BAP, NAA, and kinetin. After inoculation data on the number of days for shoot initiation, the mean number of shoots per explant and the percentage of explants who responded were recorded regularly.

In-vitro rooting of initiated shoots

To induce root growth, well-developed and healthy *in-vitro* shoots were used for sub-culturing. The rooting pattern was examined using ½ MS media (3% sucrose and 0.8% agar) supplemented with various auxin combinations, such as IAA and IBA, at varied concentrations. Approx 50 ml of media was filled into culture bottles for inoculation. The culture bottles were properly sealed with the help of parafilm. Data on the number of days for root initiation, mean number of roots per explant and mean length of roots per explant

were recorded regularly. Each experiment was carried out in triplicate under controlled conditions.

Acclimatization to ex-vitro conditions of the regenerated plants

For acclimatization of tissue culture raised plantlets of black turmeric, and plantlets with well-developed shoots and roots were removed from culture bottles and rinsed with running tap water in order to remove the traces of adhering medium from the roots of plantlets. After that, plantlets were dipped in 0.05% solution of bavistin and then rooted plantlets were transferred to a pot containing different concentrations of media pot mixtures (compost, sand, cocopeat, vermicompost, and soil) and covered with semi-transparent bags and kept at 28°C for 7 to 8 days. After the completion of the seven-day period, periodically the cover was taken off for three hours at first, then six and twelve hours over the course of the following three days. During the night the cover was taken off and lights were also switched off for the next three days. The time duration of plantlets without any cover is gradually extended, and after 15 days, they were moved outside into the shadow. For the next ten days, the plants are exposed to the sun in order to help them become acclimated to the natural environment.

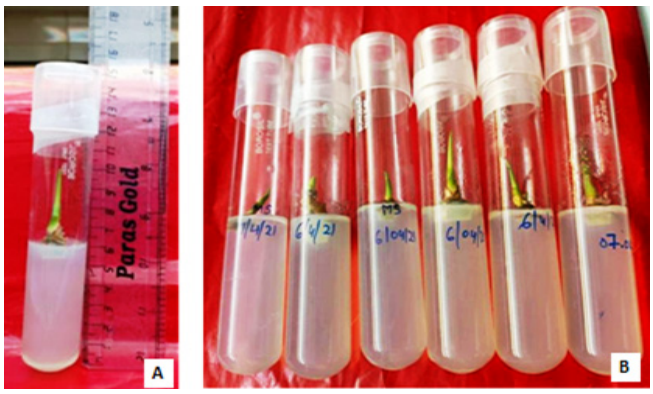

Fig. 1: Shoot initiation from rhizome bud explants of *C. caesia* Roxb. (A) Rhizome bud initiation in MS medium supplemented with BAP 3 mg l^{-1} after two weeks of inoculation (B) Observation of shooting in test tubes

Results and Discussions

The present study aimed to standardize an *in-vitro* mass multiplication protocol for *C. caesia*, focusing on both shoot and root induction by using rhizome buds as explants. The optimal disinfection treatment was determined to be the application of 0.1% (w/v) $HgCl_2$ for 3 minutes. At this concentration, no contamination was observed in the inoculated explants. There are many reports in which $HgCl_2$ has been found effective in sterilizing explants in *C. caesia* (Shahinozzaman *et al.,* 2013; Abubakar *et al.,* 2019; Shashikant *et al.,* 2019) and *C. longa* (Sinchana *et al.,* 2020). In the present investigation, it was found that the rate of contamination was high with lower concentrations of mercuric chloride, while the number of non-viable explants increased at higher concentrations. Out of 30 inoculated explants, 26 (86.66%) explants were survive at 0.1% $HgCl_2$ concentration whereas at other concentration survival rate was observed low. Therefore, for the purpose of sterilizing explants in *C.*

Table 1: Culture response on different hormone concentrations with various treatment combinations

S. No.	Growth regulators	Concentration (mg l^{-1})	No of days for shoot initiation (days)	Mean no. of shoots per explants (*)	Explants responded/100 explants (%)
1	1/2 MS+BAP	0.5	14-15	4.66 ± 0.66 (12.46)	31.34 ± 4.3 (34.04)
2	1/2 MS+BAP	1.0	13-14	5.33 ± 0.66 (13.34)	35.67 ± 4.3 (36. 67)
3	1/2 MS+BAP	1.5	13-14	6.33 ± 0.66 (14.57)	37.77 ± 4.4 (49.47)
4	1/2 MS+BAP	2.0	12-13	6.33 ± 0.88 (14.57)	42.22 ± 5.8 (40.52)
5	1/2 MS+BAP	2.5	12-13	6.66 ± 0.88 (14.95)	44.44 ± 5.8 (41.80)
6	1/2 MS+BAP	3.0	10-11	8.66 ± 0.88 (17.11)	57.77 ± 5.8 (37.92)
7	1/2 MS+BAP	3.5	12-13	7.33 ± 0.88 (15.70)	48.88 ± 5.5 (44.35)
8	1/2 MS+BAP	4.0	13-14	7.66 ± 0.66 (16.06)	51.10 ± 4.4 (45. 63)
9	1/2 MS+BAP	4.5	14-15	6.66 ± 0.87 (14.95)	44.40 ± 5.3 (41.78)
10	1/2 MS+BAP	5.0	14-15	5.34 ± 0.33 (13.36)	35.55 ± 2.3 (36. 60)
11	1/2 MS+BAP+NAA	3.0+0.5	13-14	5.67 ± 0.85 (13.77)	37.89 ± 5.6 (37.99)
12	1/2 MS+BAP+NAA	3.0+1.0	11-12	8.31 ± 0.60 (16.75)	55.50 ± 4.0 (48.15)
13	1/2 MS+BAP+NAA	3.0+1.5	12-13	6.38 ± 0.28 (14. 63)	42.22 ± 2.7 (40.52)
14	Control (MS)	0	0	0	0
	CD (5%)			2.16	14.33
	SE(m)			0.74	4.90
	SE(d)			1.04	6.93
	C.V.			19.51	19.55

*Data expressed as Mean ± SE from 3 replicates
Values in parenthesis are Arcsine transformed values

caesia, a concentration of 0.1% mercuric chloride for 3 min is highly effective and produces the highest viable explants.

Shoot induction in C. caesia

An optimal selection process for suitable micropropagation protocol involves the selection of proper explants, and appropriate combinations of the PGRs in optimum ratio, as these factors have a direct impact on the shoot and root induction of the plantlets (Da *et al.*, 2022). For direct shoot induction using rhizome bud as explants, various concentrations of BAP alone or combined with NAA on ½ MS medium was tested (Table 1). MS medium without growth regulators was used as a control. After following sterilization steps, explants were vertically placed on the growth medium with different combinations of BAP (0.5–5.0 mgl^{-1}) and NAA (0.5–1.5 mgl^{-1}). Maximum shoot induction *i.e*, 57.77%, was observed in ½ MS medium supplemented with 3.0 mgl^{-1} BAP (Fig. 1), followed by 55.50% in ½ MS medium supplemented with 3.0 mgl^{-1} BAP + 1.0 mgl^{-1} NAA, after three weeks of incubation (Table 1). Shoot induction gradually increased with BAP concentrations up to 3 mg/l, but decreased substantially at 3.5 mg/l. Cytokinin and auxin are the most widely used and studied hormones in organ regeneration. Several previous studies have supported the role of BAP and NAA in shoot induction in *C. caesia* (Ghosh *et al.,* 2013; Shahinozzaman *et al.,* 2013; Behara *et al.,* 2014; Sarma and Deka, 2020). In a similar way various combinations of BAP, Kinetin, IAA, and NAA were used to induce shoots in *C. zedoaria* (Jena *et al.,* 2020).

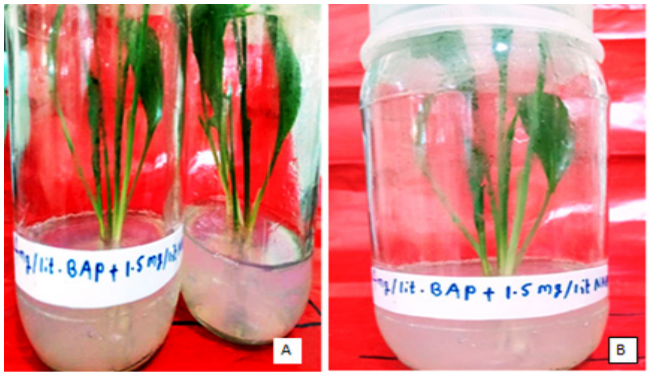

Fig. 2: Multiplication and shoots elongation in *C. caesia* Roxb. (A-B) Observation of shoots elongation in MS medium supplemented with 4.0 mg l^{-1} BAP + 1.5 mg l^{-1} NAA

Multiplication and elongation of shoots

After four weeks of incubation, regenerated shoots were sub-cultured on MS media supplemented with different combinations of BAP, NAA, and Kinetin to promote shoot multiplication and elongation. The highest mean shoot number, 5.66 ± 0.35 with 37.75% explants responding was achieved in ½ MS supplemented with 4.0 mg/l BAP and 1.5 mg/l NAA, followed by 4.66 ± 0.67 shoots with 31.10% of explants responded in 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin, while the lowest 2.63 ± 0.30 was with 4.0 mg/l BAP and 2.0 mg/l Kinetin. These results indicate that, increasing NAA concentration initially boosted shoot multiplication, but after 1.5 mg/l, it decreased gradually. For shoot elongation, a maximum 4 to 5 cm length of shoots was observed with 4.0 mg/l BAP + 1.5 mg/l NAA (Fig. 2), followed by 3-4 cm length of shoots with 1.0 mg/l BAP + 0.5 mg/l Kinetin (Table 2). Our results have been supported by previous research in *C.*

Table 2: Effect of different plant growth regulators with ½ MS medium supplement with different concentration of hormones on *in-vitro* shoot multiplication and elongation

S. No.	Growth regulators	Concentration (mg l^{-1})	Mean no. of shoots per explants	Shoot elongation per explants	Explants responded (%)
1	1/2 MS+BAP+NAA	4.0+0.5	3.34 ± 0.65 (10.53)	++	22.21 ± 4.4 (28.11)
2	1/2 MS+BAP+NAA	4.0+1.0	4.35 ± 0.34 (12.03)	+++	28.88 ± 2.2 (32.50)
3	1/2MS+BAP+NAA	4.0+1.5	5.66 ± 0.35 (13.76)	+++++	37.75 ± 2.5 (37.90)
4	1/2 MS+BAP+NAA	4.0+2.0	4.0 ± 0.57 (11.53)	+++	26.65 ± 3.8 (31.08)
5	1/2 MS+BAP+KIN	1.0+0.5	4.66 ± 0.67 (12.46)	++++	31.10 ± 4.5 (33.89)
6	1/2 MS+BAP+KIN	2.0+1.0	3.67 ± 0.34 (11.04)	+++	24.44 ± 2.3 (29. 62)
7	1/2 MS+BAP+KIN	3.0+1.5	4.30 ± 0.29 (11.96)	++	28.88 ± 2.6 (32.50)
8	1/2 MS+BAP+KIN	4.0+2.0	2.63 ± 0.30 (9.33)	++	17.71 ± 2.4 (24.88)
9	Control	0	0	0	0
	CD (5%)		1.42		9.50
	SE(m)		0.47		3.14
	SE(d)		0.66		4.44
	V.		19.99		20.00

*Data expressed as Mean ± SE from 3 replicates
Values in parenthesis are Arcsine transformed values
(Where, **+** represents less than 1 cm of shoots elongation; **++** represents 1-2 cm of shoots elongation; **+++** represents 2-3 cm shoots elongation; **++++** represents 3-4 cm of shoots elongation and **+++++** represents 4-5 cm of shoot elongation.)

Table 3: Effect of different treatments on root initiation of *C. caesia* Roxb.

S. No.	Growth regulators	Concentration (mg l^{-1})	Mean no. of roots per explants	Number of days for root initiation (days)	Mean length of roots per explants (cm)
1	1/2 MS+IAA	0.1	5.34 ± 0.25 (13.36)	13-14	4.67 ± 0.34 (12.48)
2	1/2 MS+IAA	0.5	8.35 ± 0.34 (16.79)	11-12	6.66 ± 0.35 (14.95)
3	1/2 MS+IAA	1.0	6.33 ± 0.33 (14.57)	12-13	5.30 ± 0.36 (13.30)
4	1/2 MS+IAA	1.5	4.66 ± 0.67 (12.46)	13-14	5.00 ± 0.58 (12.92)
5	1/2 MS+IAA	2.0	3.33 ± 0.35 (10.51)	13-14	4.30 ± 0.33 (11.96)
6	1/2 MS+IBA	0.5	6.34 ± 0.34 (14.58)	12-13	5.66 ± 0.34 (13.76)
7	1/2 MS+IBA	1.0	4.65 ± 0.30 (12.45)	13-14	4.34 ± 0.35 (12.02)
8	1/2 MS+IBA	1.5	5.0 ± 0.57 (12.92)	15-16	3.65 ± 0.30 (11.01)
9	1/2 MS+IBA	2.0	3.34 ± 0.40 (10.53)	16-17	3.00 ± 0.57 (9.97)
10	Control	0	0	0	0
	CD (5%)		1.25		1.33
	SE(m)		0.41		0.44
	SE(d)		0.58		0.62
	CV		13.69		16.23

*Data expressed as Mean ± SE from 3 replicates
Values in parenthesis are Arcsine transformed values

Fig. 3: Root induction from the shoot of *C. caesia* Roxb. (A) Root initiation in shoots of *C. caesia* Roxb. (B-C) Observation of number and length of roots (in cm) after 21 days of inoculation with the help of a scale

Fig. 4: Acclimatized plants of black turmeric in the pot having the mixture of cocopeat: soil: vermicompost (1:1:1) after 40 days

caesia (Bharalee *et al.,* 2005; Fong and Sani, 2019), *C. amada* (Prakash *et al.,* 2004; Ferdous *et al.,* 2012), and *C. longa* (Islam *et al.,* 2004; Kambaska *et al.,* 2010). According to Theanphong *et al.* (2010), cytokinin and auxin increased the number of shoots multiplied and the length of shoots elongated in MS medium, but at higher cytokinin concentrations, shoot multiplication increased while shoot elongation decreased.

Root Initiation

Based on the observations significant impact of different concentrations of IAA and IBA on root initiation in inoculated shoots of *C. caesia* was observed supplemented with ½ MS medium (Table 3). The highest number of roots 8.35 was obtained with 0.5 mg/l IAA in ½ MS medium. The length of roots 6.66 cm was observed in shoots inoculated with ½ MS and 0.5 mg/l IAA, followed by 6.34 cm and 5.66 cm with 0.5 mg/l IBA (Fig. 3). Auxins, particularly IAA and IBA, play an important role in rhizogenesis from regenerated shoots of various plants. In previous studies on *C. caesia*, Bharalee *et al.,* (2005) used IAA, Shahinozzaman *et al.,* (2013) used IBA

Table 4: Effect of different hardening media compositions on the percent survival of the plants

S. No.	*Media mixture*	*Ratio*	*Rate of survival of plantlets (%)*	*Response of growth of plant*
1	Control (soil)	0	20	*
2	Cocopeat: Soil: Compost	1:1:1	70	**
3	Cocopeat: Soil: Vermicompost	1:1:1	90	***
4	Cocopeat: Soil: Sand	1:1:1	50	**

* Denotes the growth of plants. * - Good growth, **- Best growth, ***- Excellent growth.

and NAA, Ghosh *et al.,* (2013) used IBA, and Tan (2016) used IAA, IBA, and In NAA, Haida *et al*., (2022) used IAA and IBA for root induction. During our investigation, only IAA and IBA were employed for root induction, and it was discovered that as the concentrations of IAA and IBA increased, root induction in *C. caesia* decreased.

Hardening of In-vitro plantlets

For acclimatization, *in-vitro* regenerated plantlets were transplanted into pots containing various hardening media mixtures of sand, soil, compost, vermicompost, and cocopeat in a 1:1:1 ratio (Table 4). Optimal growth and survival rates (90%) were observed in the mixture with vermicompost, soil, and cocopeat in a 1:1:1 ratio followed by 70% survival at 1:1:1 ratio of cocopeat: soil: compost. Minimum *i.e*. 20% survival was observed in control containing only soil. The plants were gradually acclimatized to the natural environment by exposing them to sunlight over the following 10 days and after this plants were ready to transplant into the field (Fig. 4). In previous studies, Shahinozzaman *et al.,* (2013) used compost and soil mixture for hardening *C. caesia*, while Sinchana *et al.,* (2020) employed a cocopeat and perlite mixture for *C. longa*.

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