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Callus induction in rhizome of *Curcuma caesia*: A medicinal plant

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Abstract
Kali haldi (*Curcuma caesia*) is a medicinal plant. The rhizome of the plant traditionally known for treating various diseases such as leukoderma, asthma, tumours, piles, leprosy, epilepsy, cancer by the tribal peoples. The medicinal properties of Kali haldi might be various phytochemical constituents present inside the plant. In present investigation an attempt was taken to induce callus for its *in vitro* propagation and phytochemical extraction. For inducing callus, MS media supplemented with various combinations and concentrations of 2, 4-D, BAP, IAA and NAA was used. The most suitable media composition on which 90.28% callus generated was MS medium added with $CaCl_2$ and supplemented with 2,4D (1.0/ mgl^{-1}) and NAA (0.5/mgl^{-1}) phytohormones.

Keywords: *Curcuma caesia, in vitro*, callus, MS media, 2, 4-D, NAA, BAP & $CaCl_2$

Introduction
Curcuma caesia Roxb, a member of family *Zingiberaceae* is a perennial herbs characterized by dark brown midrib of leaf and bluish black rhizome. It is native to North – East and Central India and also found in Bangladesh as a wild species. It is commonly known as Kali Haldi or Black turmeric (Bharalee *et al.*, 2005, Muhammad *et al.*, 2013; Zuraida *et al.*, 2014 & Singh *et al.*, 2015) [8, 5, 1, 14]. In common language of Indian Subcontinent, it is also known by varied names such as Kalahaldhi in Assam (Bharalee *et al.*, 2005; Muhammad *et al.*, 2013 & Zuraida *et al.*, 2014) [5, 8, 1] Yaingang Amuba or Yaimu in Manipuri; Kariarishina or Narn Kachora in Kannada, Aihang or Ailaihand in Mizoram, Kaalo Haledo in Nepalese (Baghel *et al.*, 2013) [10]. Its cultivation and habitat is localized and restricted as mentioned in the Indian Biodiversity Portal (https://indiabiodiversity.org)
Its rhizome is used in treating various ailments and metabolic disorders such as leukoderma (Zuraida *et al.*, 2014 ; Bharalee *et al.*, 2005) [1, 8] asthma (Muhammad *et al.*, 2013; Zuraida *et al.*, 2014 & Singh *et al.*, 2015) [5, 1, 14], tumors, piles (Bharalee *et al.*, 2005, Muhammad *et al.*, 2013; Zuraida *et al.*, 2014) [1, 8, 5], bronchitis, leprosy (Muhammad *et al.*, 2013) [8], epilepsy (Muhammad *et al.*, 2013; Zuraida *et al.*, 2014) [1, 5], cancer (Muhammad *et al.*, 2013; Zuraida *et al.*, 2014) [1, 5], cough and Cold in children (Singh *et al.*, 2015) [14].
A plethora of reports indicated that medicinal properties are due to the phytochemical constituents present inside the plant (Reshma *et al.*, 2013; Baghel *et al.*, 2013, Bimal Dutta 2015) [8, 10, 2]. These properties make the plant economically important and precious for us. However its restricted availability and excessive exploitation makes the plant threatened. (Muhammad *et al.*, 2013; Singh *et al.*, 2015; Ranemma *et al.*, 2017, Jose *et al.*, 2015) [5, 14, 3, 13]. So an initiative has made to induce callus in *Curcuma caesia* for its *in vitro* propagation and used for phytochemical extraction.

Materials and Method
The plant material is Kali haldi and obtained from the Botanical Garden, Bihar Agricultural University, Sabour; Bhagalpur, Bihar, India. Germinating rhizome and leaf of the plant were used as explants for the experiment.

Preparation of media
Media composition
MS media products of HIMEDIA such as PT011,TS-1OO4,VP021,PT021 and PT018 along with 3 % sucrose and 0.7% agar was used in all experiment and also added $CaCl_2$ (1.47 gml^{-1}) as mentioned in Table-1.

Preparation of plant growth regulators

Plant growth regulator product of Himedia solutions of BAP, NAA and IAA were used while 2, 4-D (Himedia) was prepared as mentioned in the protocol and their stock solutions stored at 4^0 C.

Preparation and sterilization of media

Sterilization of glassware and surgical instruments

The experimental items such as glassware, spatula, and knife/blade were washed under running tap water after that rinsed with distilled water. Then these items along with, cotton plugged, tissue paper and adequate amount of distilled water were subjected to autoclave at 15 psi for 20 minutes. Then all autoclaved items were put inside the Laminar Air Flow.

Sterilization of inoculation chamber

Prior to inoculation work the surface of laminar airflow was cleaned with spirit or ethyl alcohol.

Sterilisation of explant

Plant of *C. caesia* was uprooted and cleaned several times under running tap water. Then targeted plant part *i.e*. germinating rhizome and leaf used as explants were detached and washed again 2 to 3 times under running tap water then put together inside a fresh washed bottle. Then kept the bottle under running tap water in the laboratory for one and half hour (Zuraida *et.al*. 2014) [1]. followed by washing with commercial laboratory detergent (Decon 5%, v/v) and rinsed with tap water again 2 or 3 rinse of distilled water were also given. Then the materials are put into cleaned, fresh and sterilised bottle then taken inside laminar hood for further sterilisation.

Further explants were taken out and dipped into 70% Ethyl alcohol for 30 seconds, followed by 2-3 times washing by sterile water. After these operations, explants were surface sterilised with fresh prepared 0.1% (w/v) aqueous solution of Mercuric Chloride ($HgCl_2$) for 5 to 10 minutes (Muhammad *et. al*., 2013) [5]. Then it was thoroughly washed for 3-4 times with sterile distilled water to remove traces of $HgCl_2$ and put on sterilized autoclaved tissue paper to absorb water.

Inoculation of explants

The explants were cut into suitable size (~1-2 cm) then transferred to culture bottle/ tubes containing culture medium. During the inoculation, mouth of the culture bottle was quick flamed then tightly capped with sterile cotton plugged. After inoculation mentioned media code, date of inoculation etc on the cultured bottles and transferred to culture growth room and observations were taken at regular interval. Culture growth room has controlled condition such as temperature of 25 ± 2^0 and relative humidity (RH) 50 to 80 % with continuous illumination of white fluorescent light intensity of 2000 lux.

Experimental make-up for callus induction

For callus induction, germinating rhizome (E1) and leaf (E2) explants were cultured on 50 different media composition.

Table 1: Media Compositions used for callus induction in *Curcuma caesia.*

Sl No.	Media Name	Media Compositions
1.	M1.	MS modified (without Ammonium Nitrate)
2.	M2.	MS+$Cacl_2$ +BAP(1.0/ mgl^{-1}) + IAA (0.1+/ mgl^{-1})
3.	M3.	MS+$Cacl_2$ +BAP(1.0/ mgl^{-1}) + IAA (0.2 / mgl^{-1})
4.	M4.	MS+$Cacl_2$ +BAP(1.0/ mgl^{-1}) + IAA (0.5 / mgl^{-1})
5.	M5.	MS+$Cacl_2$ +BAP(1.0/ mgl^{-1}) + IAA (1.0 / mgl^{-1})
6.	M6.	MS+$Cacl_2$ +BAP(2.0/ mgl^{-1}) + IAA (0.1 / mgl^{-1})
7.	M7.	MS+$Cacl_2$ +BAP (2.0/ mgl^{-1}) + IAA (0.2 / mgl^{-1})
8.	M8.	MS+$Cacl_2$ +BAP(2.0/ mgl^{-1}) + IAA (0.5 / mgl^{-1})
9.	M9.	MS+$Cacl_2$ +BAP(2.0/ mgl^{-1}) + IAA (1.0 / mgl^{-1})
10.	M10.	MS+$Cacl_2$ +BAP(3.0/ mgl^{-1}) + IAA (0.1 / mgl^{-1})
11.	M11.	MS +$Cacl_2$+BAP(3.0/ mgl^{-1}) + IAA (0.2 / mgl^{-1})
12.	M12.	MS +$Cacl_2$+BAP(3.0/ mgl^{-1}) + IAA (0.5 / mgl^{-1})
13.	M13.	MS+$Cacl_2$ +BAP(3.0/ mgl^{-1}) + IAA (1.0 / mgl^{-1})
14.	M14.	MS+$Cacl_2$ +BAP(4.0/ mgl^{-1}) + IAA (0.1 / mgl^{-1})
15.	M15.	MS+$Cacl_2$ +BAP(4.0/ mgl^{-1}) + IAA (0.2 / mgl^{-1})
16.	M16.	MS+$Cacl_2$ +BAP(4.0/ mgl^{-1}) + IAA (0.5 / mgl^{-1})
17.	M17.	MS+$Cacl_2$ +BAP(4.0/ mgl^{-1}) + IAA (1.0 / mgl^{-1})
18.	M18.	MS+$Cacl_2$ +BAP(5.0/ mgl^{-1}) + IAA (0.1/ mgl^{-1})
19.	M19.	MS+$Cacl_2$ +BAP(5.0/ mgl^{-1}) + IAA (0.2 / mgl^{-1})
20.	M20.	MS+$Cacl_2$ +BAP(5.0/ mgl^{-1}) + IAA (0.5 / mgl^{-1})
21.	M21.	MS+$Cacl_2$ +BAP(5.0/ mgl^{-1}) + IAA (1.0 / mgl^{-1})
22.	M22.	MS +Cacl2 +2,4D (1.0/ mgl^{-1}) + NAA (0.5 / mgl^{-1})
23.	M23	MS +2,4D (1.0/ mgl^{-1}) + BAP (1.0 / mgl^{-1})
24.	M24	MS +2,4D (1.0/ mgl^{-1}) + BAP (3.0 / mgl^{-1})
25.	M25	MS +2,4D (1.0/ mgl^{-1}) + BAP (5.0 / mgl^{-1})
26.	M26	MS +2,4D (2.0/ mgl^{-1}) + BAP (1.0 / mgl^{-1})
27.	M27	MS +2,4D (2.0/ mgl^{-1}) + BAP (3.0 / mgl^{-1})
28.	M28	MS +2,4D (2.0/ mgl^{-1}) + BAP (5.0 / mgl^{-1})
29.	M29	MS +2,4D (3.0/ mgl^{-1}) + BAP (1.0 / mgl^{-1})
30.	M30	MS +2,4D (3.0/ mgl^{-1}) + BAP (3.0 / mgl^{-1})
31.	M31	MS +2,4D (3.0/ mgl^{-1}) + BAP (5.0 / mgl^{-1})
32.	M32	MS + $CaCl_2$+2,4D(0.025mgl^{-1})
33.	M33	MS + $CaCl_2$+2,4D(0.05mgl^{-1})
34.	M34	MS + $CaCl_2$+2,4D(0.1mgl^{-1})
35.	M35	MS + $CaCl_2$+2,4D(0.15mgl^{-1})

36.	M36	MS + $CaCl_2$+2,4D(0.2mgl^{-1})
37	M37	MS + $CaCl_2$+2,4D(0.25mgl^{-1})
38.	M38	MS + $CaCl_2$+2,4D(0.3mgl^{-1})
39.	M39	MS + $CaCl_2$+2,4D(0.4mgl^{-1})
40.	M40	MS + $CaCl_2$+2,4D(0.5mgl^{-1})
41.	M41	MS+$Cacl_2$ +2,4D (3.0/ mgl^{-1}) + BAP (1.0 / mgl^{-1})
42.	M42	MS+$Cacl_2$ +2,4D (3.0/ mgl^{-1}) + BAP (2.0 / mgl^{-1})
43.	M43	MS+$Cacl_2$ +2,4D (5.0/ mgl^{-1}) + BAP (1.0 / mgl^{-1})
44.	M44	MS+$Cacl_2$ +2,4D (5.0/ mgl^{-1}) + BAP (2.0 / mgl^{-1})
45.	M45	MS+$Cacl_2$ +2,4D (2.25/ mgl^{-1}) + BAP (1.0 / mgl^{-1})
46.	M46	MS+$Cacl_2$ +2,4D (2.25/ mgl^{-1}) + BAP (2.0 / mgl^{-1})
47.	M47	MS+$Cacl_2$ +2,4D (4.50/ mgl^{-1}) + BAP (2.0 / mgl^{-1})
48	M48	MS+$Cacl_2$ +2,4D (4.50/ mgl^{-1}) + BAP (1.0 / mgl^{-1})
49	M49	MS +2,4D (3.0/ mgl^{-1}) + BAP (2.0 / mgl^{-1})
50	M50	MS +2,4D (5.0/ mgl^{-1}) + BAP (2.0 / mgl^{-1})

Callus induction: The cultured explants were initially kept in dark then under a photoperiod of 16/18 hours. After three weeks of culture, calli proliferated from the explants and the obtained calli were subculture to the fresh medium.

Observations

Observations related to nature, colour, growth and frequency of callus were taken in accordance to Tables. 2 and Formula given below:

Table 2: Observation parameter for tissue culture responses

Symbol used	Explanation	Measurement of growth		
		Callus (in cm)	Shoot (height in cm)	Root (length in cm)
-	No growth	-	-	-
+	Low growth	Less than 0.5	Less than 0.5	Less than 0.5
+++	Good growth	1.0-2.0	1.0-2.0	1.0-2.0

$$Callus\ inductions\ Frequency = \frac{No.of\ explants\ producing\ callus}{Total\ No.of\ explants\ on\ callus\ induction\ media} X100$$

Statistical analysis

The experiment was conducted in completely randomized design (CRD) and repeated thrice with a minimum of 18-20 cultures per treatment. Data were analyzed by one way analysis of variance (ANOVA) using OP Stat. The results were expressed as a mean $\pm$ SE.

Result

Callogenesis: The initiation of Callogenesis was observed after 23 to 29 days of inoculation.

Callus developed from the cut end of the explants which was in contact with the medium and gradually extended over entire surface of the explants as a result of cell division in epidermal and sub-epidermal regions. Data of Callogenesis is presented in Table 3 and 4 and graphical representation in Figure 1 (a) and (b).

An optimised media composition is a prerequisite for any response of explants to plant growth regulators. Among the fifty media only seven media showed Callogenesis from explants. It was found that highest mean value of Callogenesis percentage of both explant (E1 and E2) were 90.28% and 78.50% respectively on medium M22. Callus growth on media M32, M33 & M34 was poor whereas on media M22, M53 & M54 growth varied from good to very good. On medium M22 callus colour was light Green whereas on medium M53 colour was pink. Nature of callus was compact on medium M53, M54 while fragile on medium M22.

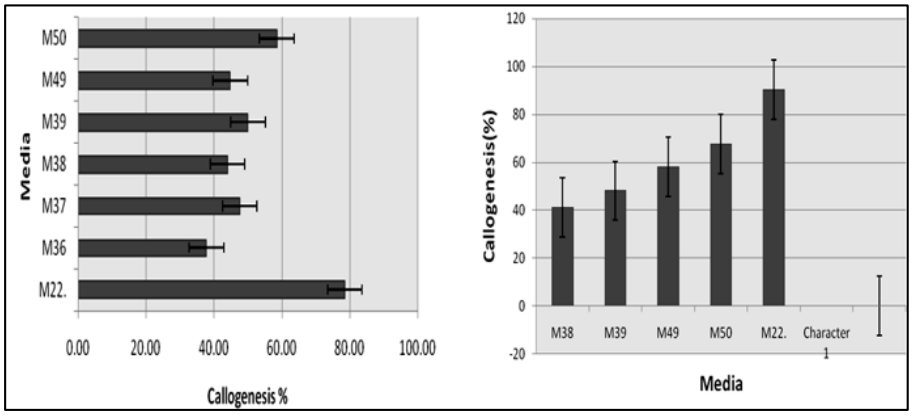

Fig 1 (a), (b): Callogenesis percentage of leaf and rhizome explant of *Curcuma caesia* on different media

Table 3: Callogenesis percentage of cultured rhizomes (E1) and leaves (E2) explants of *Curcuma caesia* with respect to the effect of different media

Sl. No.	Name of media	E1	E2	Mean	SD	SE(m)	CD	CV (%)
		Mean	Mean					
1	M_{22}	90.28 ± 1.74	78.49 ± 1.43	84.39	6.91	2.61	6.71	8.19
2	M_{36}	28.88 ± 4.44	37.77 ± 2.22	33.33	5.27	1.99	5.11	15.8
3	M_{37}	37.89 ± 2.75	47.62± 2.38	42.76	6.04	2.28	5.86	14.12
4	M_{38}	41.06 ± 1.78	40.62± 2.89	40.83	2.61	0.98	2.53	6.38
5	M_{39}	48.14 ± 1.85	44.81 ± 3.61	49.07	2.47	0.93	2.4	5.04

6	M_{49}	58.18 ± 1.82	49.99± 3.20	51.5	7.7	2.9	7.47	14.94
7	M_{50}	67.51 ± 0.85	58.51 ± 1.48	63.02	5.27	1.99	5.12	8.37
GM	53.14	51.6						
C.D.	7.42	7.9						
SE(m)	2.42	2.58						
SE(d)	3.42	3.65						
C.V. (%)	7.89	8.66						

Table 4: Growth, colour and nature of callus of *Curcuma caesia* observed in the different media

S. No	Media name	Growth nature of callus	Colour of callus	Nature of callus	Days taken
1	M_{22}	+++	Greenish Creamy	Fragile	29
2	M_{36}	+	Milky White	Friable	23
3	M_{37}	+	Milky White	Friable	23
4	M_{38}	+	Milky White	Friable	23
5	M_{39}	+	Milky White	Friable	23
6	M_{49}	++	Pinkish White	Compact	23
7	M_{50}	++	Pinkish White	Compact	23

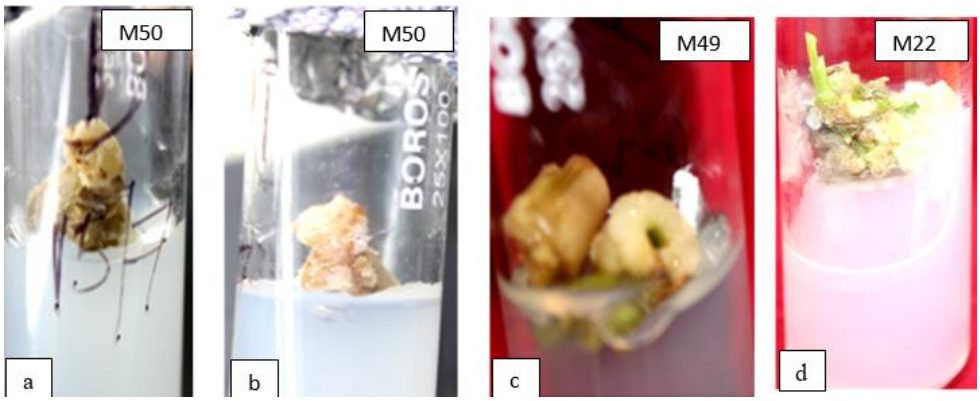

Fig 2 (a to b): *Curcuma caesia* at vegetative and flowering stage

Fig 3(a to b): *Curcuma caesia* at vegetative and flowering stage

Discussion

In present study callus induction was observed when growing rhizome and leaf were used as explant. In this context reporting of earlier workers *viz.* Zuraida *et al.*, 2014 [1]. have reported that callus was induced when sprouted rhizome used as explant and cultured on Woody Plant Medium (WPM), supplemented with BAP (5 mg/l) and 2,4-D (2 mg/l).

Similarly Singh *et al*. 2015 [14]. also reported that callus was obtained when they used rhizome as an explant and cultured on MS-medium supplemented with 2, 4 D (2.7-3.4 mg/l).

Jose *et al*. 2014 [12]. also obtained callus when rhizome bud and leaf explant was cultured on MS Medium supplemented with 2, 4-D (6.7 uM) and NAA (2.7 uM). They obtained white, friable callus after 70 days of culture in 84 and 71 %.

In this series, report of Mei Fong, Yin 2015 was also mentioned. He has claimed that he has induced callus from leaf and root explants with percentage of 94.64 % and 100.00 % respectively in the combination of 2.0 mg/L BAP with 0.5 mg/L 2, 4-D.

In comparison to the above mentioned earlier works, in the present study, the best callus induction percentage was observed in three media composition *viz;* M22,M49 and M50 (Table 1) using germinating rhizome and leaf as explant and cultured on different combinations and concentrations of 2,4-D, BAP and NAA. The explant rhizome performed better than leaf for the development of callus, since callus induction percentage by rhizome on the medium M22, M49, M50 was 90.28%, 58. 18% and 67.51% respectively whereas in case of leaf it was 78.49%, 49.99 % and 58.51% on their corresponding medium. It means the callus induction percentage of rhizome was higher than the leaf percentage so rhizome was better explant than leaf. This was also supported

by report of the previous workers namely Zuraida *et al.*, 2014 [1]. and Singh *et al*. 2015 [14]. used rhizome whereas, Jose *et al*. 2014 [12]. used rhizome bud and leaf as explant and obtained more callus percentage (84%) in rhizome bud than leaf. Therefore rhizome is the better explant than leaf in *Curcuma caesia* since more amounts of growing tissues are present in the rhizome.

Now as far as growth of callus with respect to media is concern, it was observed that growth of callus was vigorous on M22 medium and moderate on M49 and M50.

Jose *et al*. 2014 [12]. reported 84.71% of callus on MS medium supplemented with 2, 4-D (6.7 uM) and NAA (2.7 uM) whereas in present study 90.28% callus was obtained on the same phytohormones combination but concentrations of 2, 4-D and NAA was 1.0 mg l^{-1} and 0.5 mg l^{-1} respectively in MS basal medium added with 1.47 gm l^{-1} $CaCl_2$. Therefore MS medium added with $CaCl_2$ enhances the callus induction percentage.

Considering the references of media compositions, Zuraida *et al.*, 2014 [1]. have induced callus on WPM when supplemented with BAP (5mg l^{-1}) and 2,4-D (2 mg l^{-1}) whereas in present study 67.51% callus of good growth was obtained on BAP (2mg l^{-1}) and 2,4-D (3.0 mg l^{-1} or 5.0 mg l^{-1}) phytohormones in MS basal medium. It means, amount of auxin imparts remarkable impact on callus induction. However one report of Mei Fong, Yin 2015 seems to be contradictory as his reporting percentage of callus formation was neither matched nor near to the previous workers on the above discussed phytohormones combination.

Likewise, reporting percentage of callus formation by Singh *et al*. 2015 [14]. is also seems to be contradictory to the present study and reports of Zuraida *et al.*, 2014 [1]. Since we did not, obtained recordable growth and substantial amount of callus when used 2, 4–D alone with MS medium or 2, 4–D alone with MS medium added with $CaCl_2$

Finally, in the present study only three media composition were found suitable for the callus induction in *Curcuma caesia* which are M22 [MS +Cacl2 +2,4D (1.0/ mgl^{-1}) + NAA (0.5/mgl^{-1})], M49 [MS+2,4D (3.0/mgl^{-1}) + BAP (2.0/mgl^{-1})] and M50 [MS+2, 4D (5.0/ mgl^{-1}) + BAP (2.0/mgl^{-1})].

Conclusion

Therefore the present work reveals that growing or sprouted rhizome is the best for callus induction or *in vitro* propagation in *Curcuma caesia* and three media composition namely MS +Cacl2 +2,4D (1.0/ mgl^{-1}) + NAA (0. 5 / mgl^{-1}), MS +2,4D (3.0/ mgl^{-1}) + BAP (2.0 / mgl^{-1}) and MS +2,4D (5.0/ $mgl^{-}1$) + BAP (2.0 / $mgl^{-}1$) were found suitable for the callus induction in *Curcuma caesia.* MS medium added with $CaCl_2$ and supplemented with 2,4D (1.0/ mgl^{-1}) and NAA (0.5/mgl^{-1}) phytohormones was the most suitable media composition on which maximum callus generated and the callus generated on the media may also use for *in vitro* propagation through somatic embryogenesis as its colour is light green.

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