RESEARCH ARTICLE

Sterilization Procedure and Callus Regeneration in Black Turmeric (*Curcuma caesia*)

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Abstract

Sterilization procedure, media composition, explants selection and control of physical environment are critical for successful cultures and callus induction with surface sterilization being very challenging in most plants. Five different sterilization methods were evaluated to come up with the best for subsequent use to establish an in vitro regeneration method for the induction of callus in *Curcuma caesia* using excised leaf and rhizome explants. Murashige and Skoog (MS) media supplemented with various concentration of 2,4-Dichlorophenoxy acetic acid (2,4-D)/Indole-3-acetic acid (IAA) (0.5–5.0 mg/L), singly or in combination with benzyl aminopurine (BAP)/kinetin (KIN) (0.1–5.0 mg/L), 0.3% sucrose and 0.08% agar were used. The result of the sterilization procedures showed 15% $NaHClO_3$ (5 min)+ 70% ethanol (30 sec) + 0.1% $HgCl_2$ (5 min) to be the most effective in controlling contamination in *C. caesia* among all the treatments tested. The response to callus induction was found to depend on the type of explants used and growth regulators combination. Leaf explants gave the highest percentage of callus induction. Highest percentage of callus induction (66.70%) was obtained in the growth regulator combination of 2, 4-D (0.5 mg/L) + BAP (0.1 mg/L) and least (14.29%) in IAA (2.0 mg/L) + BAP (0.5 mg/L). An equal and higher concentration of 2, 4-D + BAP of 5.0 mg/L each also provided a better result (40.00%). No callus was obtained in all the single concentration of 2, 4-D used.

Keywords: Callus, Contamination, *Curcuma caesia*, Growth regulators, Sterilization.

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Introduction

Black turmeric, *Curcuma caesia* is a very important medicinal plant of greater value and curative properties due to its curcumin and phytochemical content, but less known as compare to other species of *Curcuma*. It is one of about 133 available species of *Curcuma* (Prasad and Aggarwal, 2011), and few reports are available on the plants (Paliwal *et al*., 2011). It is described as excellent species with diverse potential medicinal uses and properties (Abubakar and Pudake, 2014), though is under threat of extinction due to its high demand, unfavorable climatic changes, environmental degradation, and overexploitation without ensuring its regeneration (Mannangatti and Narayanasamy, 2008). As the plant is propagated by underground rhizomes which go into a dormant stage from October to early March, and only sprout during monsoon, its multiplication and growth rate is very low posing a problem for large scale commercial production. Besides, a large amount of edible part (rhizome) is also stored for stock purpose for the next season planting, thereby-making germplasm maintenance by annual planting, an expensive and labor-intensive activity for the marginal farmers (Jala, 2013; Behera *et al*., 2010). Maximum multiplication achievable annually is only 5- fold (Hashemy, 2009). Shirgurkar *et al*., (2011) indicated the need for turmeric plant rapid multiplication technique. Micropropagation is a good alternative to achieving large scale multiplication of black turmeric given the limitation of vegetative propagation. Past research has revealed micropropagation as an important tool for medicinal compound production and many studies carried out proved its role for the propagation of clonal individuals in commercial scale and also for metabolites production (Cousins, 2008).

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The use of rhizome bud in tissue culture of turmeric plants was the most studied, and benzyl amino purine (BAP) supplemented Murashige and Skoog media have been widely used. BAP alone or in combination with other growth regulators were used for callus production as well as multiple shoot production in common turmeric (*Curcuma longa*) (Parthasarathy and Sasikumar, 2006).

Sterilization is a procedure employed to make explants contamination free before use in culture. It is a challenging process that requires a lot of efforts (Srivasatava *et al.,* 2010). Surface sterilization of explants is very critical and need to be carried out thoroughly before inoculation on media (Sundram *et al.,* 2012). It is most essential in obtaining clean plantlets free from contaminants and subsequently increases the survival rate of explants (Srivasatava *et al.,* 2010) as

the explants surface carried a large number of microbes (Sundram *et al.,* 2012) that include fungi, bacteria, and yeast (Leifert *et al.,* 1994). Several factors such as plant species, age, types of explants, as well as prevailing weather condition govern the success of sterilization. If contamination is not overcome successfully, it leads to waste of resources and time (Srivasatava *et al.,* 2010). Various chemicals such as mercury chloride ($HgCl_2$), ethanol, and sodium hypochlorite ($NaHClO_3$) have been reported as effective in achieving asepsis in a number of Zingeberaceae (Sundram *et al.,* 2012). Ethanol is phytotoxic to plant as well as a powerful sterilizing agent, so exposition is only for a few minutes (Afolabi, *et al.,* 2009). $HgCl_2$ is a good and strong sterilizing agent and important in controlling both bacteria and fungi. It reacts with protein and enzymes presence on the cell membrane and cytoplasm of microbes giving effective result (Ghopal, *et al.,* 1998; Sundram, *et al.,* 2012). $NaHClO_3$ has bactericidal activity (Nakagarwara *et. al.,* 1998).

The research was, therefore carried out to establish the best sterilization procedure and standardize the growth regulators on enhancing the frequency of callus regeneration from different explants.

Materials and methods

The experiment was conducted at the plant tissue culture laboratory, Lovely Professional University, Phagwara, Punjab, India (31.25°North latitude, 75.70°East longitude, and altitude of 105.5 m above sea level). The black turmeric plants were sourced from Jagatpur village (31.14° North latitude and 75.93° East and altitude 254 m above sea level) and brought to the university where it was transplanted. The leaves and rhizomes of the plants were brought to the laboratory and were washed thoroughly under running tap water to remove all the earthy particles that cling to it. Rhizome buds were removed using a sharp scalpel and leave reduced to the appropriate size and washed again before taking to the laminar hood chamber for surface sterilization and subsequent inoculation.

To establish an effective procedure for the successful surface sterilization of the rhizome of black turmeric, five (5) different treatments were used:

- *S1:* 70% ethanol (30 sec) + 0.1% $HgCl_2$ (5 min)
- *S2:* 70% ethanol (30 sec) + 15% $NaHClO_3$ (containing 3 drops of tween solution) (5 min)
- *S3:* 15% $NaHClO_3$ (containing 3 drops of tween solution) (5 min) + 0.1% $HgCl_2$ (5 min)
- *S4:* 15% $NaHClO_3$ (5 min)+ 70% ethanol (30 sec) + 0.1% $HgCl_2$ (5 min)
- *S5:* 0.2% Streptomycin (15 min) + 70% ethanol (30 sec) + 0.1% $HgCl_2$ (5 min)

Double distilled water was used to rinse the explants (four times) after successive use of chemicals and at final stage it was rinsed 5 times before inoculating in to the MS media supplemented with various growth regulators.

Surface sterilization of the leaf was carried out in autoclaved bottle jar using 70% ethanol for 30 seconds and subsequently rinsed four times with double distilled water. This was followed by 0.1% $HgCl_2$ solution for 5 minutes and rinsed five times with double distilled water to remove the traces of $HgCl_2$.

Murashige and Skoog (MS) media supplemented with various single or combination of 2,4-Dichlorophenoxy acetic acid (2,4-D), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Naphthalene acetic acid(NAA), Benzyl amino purine (BAP) and Kinetin (KN) were used for the culture. The media were also supplemented with 30 g/L sucrose. The pH of the medium was adjusted to 5.8 before adding agar (8 g/L). Autoclaving was done at 121°C and 15 PSI pressure for 20 min. The media combinations also served as the experimental treatments.

Records for contamination were taken after the inoculation at two (2) days intervals up to the 16 days, and the percentages of contaminated cultures were obtained. The percentages of the contaminant-free cultures after the 16 days were also calculated and tabulated and expressed using a bar chart. The number of media combination that induced calluses were also recorded and expressed in percentage. The number of weeks taken to callus, nature, and morphology were observed and recorded. Analysis of variance (ANOVA) was carried out using GenStat analytical software (VSN International. 2014) using one way ANOVA in a completely randomized design (CRD) and means separated using Fischer protected least significance difference (LSD).

Results and discussion

The result of sterilization procedures (Table 1), revealed S4(15% $NaHClO_3$ (5 min)+ 70% ethanol (30s) + 0.1% $HgCl_2$ (5 min) to be the most effective in controlling contamination in *C. caesia* among all the treatments studied, with 85% contaminants free cultures after 16 days of inoculation and the use of S5 (0.2% streptomycin (15 min) + 70% ethanol (30s) + 0.1% $HgCl_2$ (5 min) produced 55% contaminants free cultures. In the case of S3(15% $NaHClO_3$ [containing three drops of tween solution) (5 min) + 0.1% $HgCl_2$ (5min) there were 50% contaminants free cultures after 16 days of the culture. S1 [70% ethanol (30s) + 0.1% $HgCl_2$ (5min)] and S2 (70% ethanol (30s) + 15% $NaHClO_3$ (containing 3 drops of tween solution) (5 min) proved inefficient in sterilization of *Curcuma caesia* as no single culture remained without contamination after 16 days. Gosh *et al.,* (2013) recorded the best sterilization in *C. longa* at 6 min exposure to 0.1% $HgCl_2$ with 60% contaminant free after 10 days of culture which showed the use of S4 and S5 as obtained in this study better options. The higher survival rate of healthy explants (77%) in *C .mangga* was obtained using 0.3% $HgCl_2$ for 5 min in the fourth week of culture (Sundram *et al.,* 2012). The use of 0.1% $HgCl_2$ for 5 min in the sterilization of nodal explants of *Aconitum heterophyllum* was found to be efficient and produced 100% healthy plant, and 7.5% H_2O_2 produced 90% aseptic seed germination (Srivasatava *et al.,* 2010). In a separate finding, exposure of explants from *C. longa* to

Table 1: Effects of different sterilization methods on the rhizome explants of *C. caesia*

Sterilization Protocols	*Number of inoculated media*	*Percentage (%) of contaminated cultures*								*Percentage of contaminant free culture after 16 days (%)*
		Day 2	*Day 4*	*Day 6*	*Day 8*	*Day 10*	*Day 12*	*Day 14*	*Day 16*	
S1	20	0	0	10	20	0	50	20	0	0
S2	20	0	30	30	30	0	0	10	0	0
S3	20	0	0	20	10	0	10	10	0	50
S4	20	0	0	0	5	0	0	0	10	85
S5	20	0	0	0	0	0	0	0	45	55

Key: S1: 70% Ethanol (30 sec) + 0.1% HgCl2 (5 min), S2: 70% Ethanol (30 sec) + 15% NaHClO3 (containing 3 drops of tween solution) (5 min), S3: 15% NaHClO3 (containing 3 drops of tween solution) (5 min) + 0.1% HgCl2, (5 min), S4: 15% NaHClO3 (5 min)+ 70% Ethanol (30s) + 0.1% HgCl2 (5 min), S5: 0.2% Streptomycin (15 min) + 70% Ethanol (30 sec) + 0.1% HgCl2 (5 min)

0.1% $HgCl_2$ for 8 and 10 minutes eliminated contamination up to 80% and 90% respectively; however these lead to the browning and death of the explants, whereas less than 5 minutes exposure time resulted in 100% contaminations (Gosh *et al.,* 2013). Sundram *et al.,* (2012) reported the use of 0.5% $HgCl_2$ for 10 minutes in addition to Clorox and ethanol to have been most effective in controlling contamination, however, lead to death of 83% of the cultured explants, and they also recorded the highest contamination (80%) where 0.1% $HgCl_2$ was used for 30 sec. The use of NaOCl and H_2O_2 on the one hand, and $HgCl_2$ and NaOCl as indicated by Srivasatava *et al.,* (2010) did not give acceptable result in *Aconitum heterophyllum* even at increased time and concentration for nodal and seed explants, respectively. In comparison to the reported findings on sterilization of *Curcuma* and other related species (Gosh *et al.,* 2013; Sundram *et al.,* 2012; Srivastava *et al.,* 2010), this took the longest time of observation (16 days) and recorded the better result as all survived without contamination. And in addition, in all the five sets of sterilization procedures used, there was no case of browning recorded indicating no toxicity.

The result of the callus induction (Table 2) showed the range of variation between the leaf and rhizome explants, in terms of the nature of callusing and percentage of callus induction. There was variation in the percentage of callus induction across the treatments. Highest callus induction (66.70%) was recorded in the growth regulator combination of 2,4-D (0.5 mg/L) + BAP (0.1 mg/L). The combination of 2,4-D + KIN at 0.5 and 0.1 mg/L concentration respectively promoted callus induction comparable to what was obtained when BAP was used in place of KIN (50.00%) and were statistically

Table 2: Effect of MS media supplemented with different growth regulators on the percentages of callus induction, duration and morphology

Combination of growth regulators	*Type of explants*	*% of calluses induced*	*Duration for callus initiation (weeks)*	*Degree of response*	*Callus morphology*
2,4-D (0.5mg/L) + BAP (0.1 mg/L)	Leaf	66.70a	5	Profuse callusing	White, delicate and globular
2,4-D (0.5 mg/L) + KIN (0.1 mg/L)	Rhizome	50.00a	5	Profuse callusing	White, friable, and yellow interspersed
2,4-D (1.0 mg/L) + BAP (0.5 mg/L)	Rhizome	25.00b	8	More callusing	White to slightly yellow and compact
IAA (2.0 mg/L) +KIN (1.0 mg/L)	Rhizome	14.29b	8	Slight callusing	White, delicate and globular
2,4-D (5.0 mg/L) + BAP (5.0 mg/L)	Rhizome	40.00ab	6	Profuse callusing	White globular at the top, and slightly yellow at the bottom

Means with different letter within the same column are statistically different (p <0.05)



Fig. 1: Calluses obtained using rhizomes explants in black turmeric



Figs 2A and B: (A) Black turmeric plants and (B) rhizomes parts

similar and differed from all others (p <0.05). Equal and higher concentration 2,4-D + BAP of 5 mg/L each, responded well (40.00%) to callus induction in contrast to the finding of Taha *et al*., (2013) who reported higher concentration of 2,4-D up to 4 mg/L in combination with 0.2 mg/L BAP to have performed less in the in vitro culture of Zingiberofficinale using leaves explants. Similarly, in *C. zedoaria*, a higher concentration of 2,4-D gave a lower response with the better performance reported in 1.0 mg/L 2,4-D in combination with 1.0 mg/L BAP and lower (Tuan *et al*., 2011). This variation, however, might not be unconnected to several factors such as genotype and its interaction with the growth environment, media composition, growth regulators, etc. (Abubakar *et al*., 2018; Pandey et al., 2003). The least percentage (14.29%) in callus induction was found in IAA (2.0 mg/L) + BAP (0.5 mg/L). Though, Jamil *et al*., (2007) obtained the best result (98%) in IAA and BAP combination, yet replacing 2, 4-D with other auxins as well as increasing or reducing its concentration was reported to pose a negative effect in the percentage of callus induction (Sundram *et al*., 2012).

The response to callus induction showed a dependence upon the type of explants used. The highest percentage of callus induction (66.70%) was obtained in the media inoculated with the leaf explants. The report of Sultana *et al.,* (2009) was in agreement with this. They successfully induced callus from Suruchi and Bari ada-1 varieties of ginger in 1 mg/L 2, 4-D, 0.5 and 0.75mg/L Dicamba supplemented MS media and obtained the best callus induction (67.77%) using the leaf explants overshoot tip and root.

All the successful induction of callus in this study were obtained in 2, 4-D + BAP supplemented MS media. 2, 4-D alone failed to induce calluses at all the concentration tested (0.5, 1….3 mg/L) which was in line with the report of Zuraida *et al.,* (2014) in *C. caesia* and also with the finding of Miachir *et al.,* (2004) and Tuan *et al.,* (2011) in *C. zedoaria*, however, in contradiction to the finding of Saensouk, (2011) were

2,4-D (2.0 mg/L) alone was reported to have given the best result of callus induction (95%) using the leaf explants of *Cornukaempferia aurantiflora*. Prakash *et al.,* (2004) through their study have shown 1-2 mg/L 2, 4-D as an effective plant growth regulator in callus induction.

There was a little variation in the callus morphology obtained across the treatments. Callus obtained from leaf explants was delicate, white and friable. White globular with slight yellow interspersed friable calluses was generated in all the remaining treatments. The degree of callus induction was observed to have been slight in the IAA + BAP combination, and more in the 2, 4-D (1.0 mg/L) + BAP (0.5 mg/L). There were profuse degrees of callusing in the rest of the treatments. Different types of auxin showed a different response to callus induction in term of morphology and percentage. Variation in morphology, whitish with a mixed friable and globular at higher concentration and whitish and globular at lower concentration of IAA or NAA was reported in *C. manga* where higher concentration of 2,4-D (8mg/L) produced callus relatively brownish and dry in appearance (Sundram *et al.,* 2012), and in another separate finding reported by Jamil *et al.,* (2007), remarkable callus, delicate and creamy morphology with faster growth was induced in the MS media supplemented with a combination of IAA (0.1 mg/L) and BAP (1.0 mg/L).

Duration of callus induction differs between the treatments however, were all statistically the similar. The earliest result was obtained in the 5th week of culture in 2,4-D 0.5 mg/l +BAP 0.1 mg/L, and 2,4-D (0.5 mg/L) + KIN (0.1 mg/L) combinations followed by 2,4-D and BAP (5.0 mg/L each) in the 6th week and 2,4-D (1.0 mg/L) + BAP 0.5 mg/L) and IAA (2.0 mg/L) + BAP (1.0 mg/L) in the 8th week of inoculation. All the responses in term of a number of days from a culture obtained in this study were earlier than what was obtained from *C. caesia* inoculated on woody plant medium supplemented with 2.0 mg/L 2,4-D + 5.0 mg/L BAP (20%) as reported by Zuraida *et al.,*(2014) where the best callus (20%) was achieved after 70 days of culture.

The shoot could not be regenerated from the callus in all the various combination of plant growth regulators tried. BAP was tested singly and in combination with IBA/NAA so also KIN singly and in combination with IBA/NAA all without any success. Review of the literature indicated the addition of NAA, IBA or IAA in the culture medium to be effective in improving response in a number of species in terms of shoot growth (Behera *et al.,* 2010). BAP alone or in combination with other growth regulators were found to have induced multiple shoots (Parthasarathy and Sasikumar, 2006). Hashemy *et al.,* (2009) reported Kinetin at the concentration of 0.2 mg/L in the presence of 2 mg/L of NAA as the most favorable for the formation of shoots in *Curcuma longa.* The results obtained by Srirat *et al.,* (2008) for the *in vitro* shoots propagation of *Curcuma longa* using rhizome buds as explants showed MS medium containing 60 g/L of sucrose and 5 mg/L of thidiazuron (TDZ) to have induced shoots. Axillary rhizome buds in BAP (2.0 mg/L) + NAA (1.0 mg/L) and BAP (1.0 mg/L) + 2, 4-D (4.0 mg/L) supplemented MS media produced shoots within 4 weeks of culture as well as spontaneous rooting in BAP + IAA/NAA (Sarma, *et al.,* 2011). A maximum number of shoots formed per bud on the medium containing 13.3 µM BA within 15 days was obtained by Nayak and Naik, (2006). Rahman, *et al.,* (2005) obtained the best shoot proliferation in *Kaempferia galangal* on 1.0 mg/L BA+ 0.1 mg/L NAA supplemented MS media and also reported IBA among the three types of auxins (IAA, IBA, and NAA) as the most effective in shoot induction and proliferation. Yet, these were all different species, though even in the same species such may be found as a successful response is largely determined by environment and media composition.

Conclusion

Curcuma caesia is an important species of turmeric with significance medicinal property with its curcumin content comparably higher among all turmeric species and distinguishable by its unique bluish rhizome and aromatic nature, emitting a sweet smell. Micropropagation was found to be one of the best strategies for medicinal compound production, and also forms the basis for genetic transformation. These aids in quality improvement and also ensure rapid multiplication. Rhizome bud and leaf explants were shown to be responsive to callus generation. 2, 4-D was found to be the most effective auxins in callus induction; however, it needs to be used in combination with any of BAP or KIN.

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