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RESEARCH ARTICLE

Studies on Optimization of Medium in Induction and Regeneration of Callus and Shoot from *Costus igneus* and its Phytochemical Profile

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Abstract

Costus igneus is an important medicinal herb which is known to reduce the levels of insulin in the case of Diabetes mellitus. Efficient protocol for micropropagation using various explants is developed and the observations on different parameters are recorded. The surface sterilization of the explants at various time intervals and varying concentration of 70% ethanol, sodium hypochlorite and mercuric chloride was optimized in order to reduce the overall contamination levels during the study. This plant was effectively micropropagated using various explants with the standardized protocol in both standard MS medium and LS medium at various hormone levels. The leaf, node, internode, rhizome were the explants used for the study. Callus was induced on the 4th d in MS medium and on the 3rd d in LS medium at the hormone concentration of BAP (0.4 mg/L) + KIN (0.2 mg/L) + NAA (0.1 mg/L) + IAA (0.2 mg/L) + IBA (0.2 mg/L). Shoot regeneration using the nodal and internodal explants was observed in MS and LS medium with BAP (0.4 mg/L) + KIN (0.25 mg/L) + NAA (0.1 mg/L) + IAA (0.2 mg/L). Qualitative and quantitative phytochemical analysis of hexane, ethyl acetate, diethyl ether, acetone and methanol extracts of wild plant and callus were carried out with various reagents. Methanolic extract was found to have more total phenolic and flavonoid content in wild plant and callus.

Keywords: *Costus igneus*, micropropagation, callus induction, shoot regeneration, phytochemical analysis.

Introduction

Costus igneus is a medicinal plant native to Brazil and falls under the family *Costaceae.* It is used in India to control blood glucose level and is known that diabetic people eat one leaf daily to keep their blood glucose low. Leaves of *Costus igneus* is known to be effectively used for treating diabetes by the tribal people of Kolli Hills of Namakkal district, Tamil Nadu (Elavarasi and Saravanan, 2012). Leaf extract of *C. igneus* has been reported to have antidiabetic activity in diabetic rats (Bhat *et al.,* 2010). Recently, much research work is in progress to evaluate the potential of the plant in other disorders also. The plant is also known to possess various other pharmacological properties such as hypolipidemic, diuretic, antioxidant, antimicrobial, hypoglycaemic etc. Similar properties are observed in other *Costus* species also (Gireesh *et al.,* 2009). To obtain a large scale production of plants with specific traits in short time, the method of micropropagation or *in vitro* regeneration is applied (Pierik, 1987). Medicinal plants are of great interest as pharmaceutical industries depend in part on plants for the production of secondary compounds (Molnar, 2011). The main objective of the present study is to standardize the protocol to obtain callus from *Costus igneus* in short duration with varied levels of hormone in both MS and LS medium.

Materials and methods

Collection of plant material: The healthy and disease free plant was selected from the Laboratory Garden in National Agro Foundation (Anna University Campus, Taramani), Chennai, Tamil Nadu in the month of January 2015 which was authenticated by Dr. K. Balasubramanian (Advisor, Plant Tissue Culture Laboratory, National Agro Foundation).

Preparation of tissue culture media: Recommended Laboratory techniques (Purvis, 1966; Tuite, 1969) were followed for the preparation of media, inoculation and maintenance of cultures.

Optimization of surface sterilization: The sterilized explants were placed under aseptic condition in the autoclaved sterilized glass bottles under the laminar air flow chamber with different concentrations of the surface sterilization chemicals and varying time intervals to observe the percentage of contamination.

Calculation for percentage of contamination: The percentage rate on each sterilization chemicals formulation was calculated using the following equation:

$$Contamination\ \% = \frac{Number\ of\ bottles\ having\ microbial\ growth}{Total\ number\ of\ bottles\ inoculated} \times 100$$

Inoculation of explants: The dried sterile explants were cut into small pieces (0.5 cm-1 cm), inoculated in MS and LS medium and incubated at 25°C.

Callus induction in MS and LS medium: For the callus induction in MS and LS medium, healthy and disease free young shoot nodes, internodes, leaf and rhizome were selected as explants from the mother plant and surface sterilized. Explants were inoculated on the solid medium supplemented with BAP (0.4 mg/L) + KIN (0.2 mg/L) + NAA (0.1 mg/L) + Sucrose (30 g/L), BAP (04 mg/L) + KIN (0.2 mg/L) + NAA (0.1 mg/L) + IAA (0.2 mg/L) + IBA (0.2 mg/L) + Sucrose (30 g/L), BAP (0.4 mg/L) + KIN (0.2 mg/L) + NAA (0.1 mg/L) + IAA (0.2 mg/L) + Sucrose (30 g/L) with pH 5.7. The callus obtained was subcultured, harvested and used for further studies.

Calculation of response percentage: The response percentage rate on each media formulation was calculated using the following equation:

$$Response\,\% = \frac{Number\ of\ explants\ showing\ response}{Total\ number\ of\ explants\ inoculated} \times 100$$

Shoot regeneration (in vitro): For shoot regeneration in MS and LS medium, healthy, fresh, disease free young nodes and internodes were collected as explants from the mother plant and surface sterilized. The medium was supplemented with varying concentration of KIN (0.2-0.3 mg/L), BAP (0.2 mg/L), NAA (0.1 mg/L), IAA (0.2 mg/L), IBA (0.2 mg/L) at pH 5.7. The inoculated bottles were incubated under dark condition at 25°C for 2 d and then transferred into light condition.

Preparation of wild plant and callus extract: The wild plant and callus of *Costus igneus* was collected and shade dried. The dried plant material and callus was ground into fine powder from which 30 g of the powder was taken for solvent extraction. It was sequentially extracted with 90 mL of hexane, diethyl ether, ethyl acetate, acetone and finally with methanol in the same order as mentioned based on the solvent polarity. The extract was collected after 48 h.

Qualitative phytochemical analysis: Chemical tests for screening and identification of bioactive chemical constituents present in the extracts were carried out using the standard procedures (Harbome, 1973; Trease and Evans, 1983; Zafar and Mujeeb, 2002; Sharad *et al*., 2013) for the detection of alkaloids, flavonoids, phenols, saponins and terpenoids.

Quantitative phytochemical estimation
Determination of total phenolic content: Initially gallic acid (1 mg/mL) at various concentrations as 10, 20, 30, 40, 50, 60 and 70 µL was taken and the final volume was made to 1 mL using methanol (Mace, 1963). Then 0.1 mL of extracts of wild plant and callus (1 mg/mL) was also taken in the test tubes and made up to 1 mL using methanol. Then, 0.1 mL of 20% Na_2CO_3 and 0.1 mL of Folin-ciocalteu reagent (diluted tenfold) was added with time interval of 5 min which was incubated in darkness for 30 min. The absorbance of the reaction mixtures was measured at 765 nm. Measurements of every sample were taken and the results were expressed as mg Gallic Acid Equivalents (GAE)/g dried weight of plant extract and calculated based on the following equation:

C = c.V/m

Where, C=Total content of phenol compounds, mg/g plant extract in gallic acid equivalent, c=the concentration of gallic acid established from the calibration curve, mg/mL, V=the volume of the extract, mL, m=the weight of pure plant methanol extract

Determination of total flavonoids content: Plant extract and methanol quercetin standard (1 mg/mL) in various concentrations of 10, 20, 30, 40, 50, 60 and 70 µL was prepared to have 1 mL of final volume with methanol. Then, after adding 0.3 mL of 5% of $NaNO_2$, 0.3 mL of 10% aluminium chloride and 0.3 mL of 0.1 M NaOH to the reaction mixture with time interval of 5 min and 6 min was added respectively and incubated at room temperature for 30 min (Mudasir *et al.,* 2012). Finally the absorbance was noted at 510 nm to determine the total flavonoids present in the extract using the following equation:

$$\text{Total flavonoid content} = \frac{R \times D \times F \times V \times 100}{W}$$

Where, R=Result obtained from the standard curve D.F - Dilution factor, V-Volume of stock solution, 100-For 100 g dried plant, W=Weight of plant used in the experiment.

Results and discussion

Optimization of surface sterilization: The level of contamination was reduced when the explants were surface sterilized with 70% ethanol, 0.05% $HgCl_2$ and 0.2% N_aOCl 4 min each. Percentage of contamination was brought down to 0% from 100% (Table 1). Sterilization of leaf explants using 70% ethanol, 0.05% $HgCl_2$ and 0.2% N_aOCl at various time intervals reduced the contamination from 100% to 10% (Arun *et al.,* 2011).

Optimization of hormone levels to induce callus in MS and LS medium: The callus was best induced in hormone concentration of BAP (0.4 mg/L) + KIN (0.2 mg/L) + NAA (0.1 mg/L) + IAA (0.2 mg/L) and BAP (0.4 mg/L) + KIN (0.2 mg/L) + NAA (0.1 mg/L) + IBA (0.2 mg/L) on 4^{th} d in MS and 3^{rd} d in LS medium. Response percentage of node in MS medium with 77.77% and internode in LS medium with 87.9% was found to be maximum amongst all the explants in various concentrations of hormones (Table 2; Fig.1a-h and 2a-h).

Table 1. Optimization of surface sterilization.

Compound name	Sterilization time	% Contamination
70% Ethanol	2 MIN	100
0.1 % Sodium hypochlorite	2 MIN	100
0.01% Mercuric chloride	2 MIN	100
70% EtOH + 0.1% NaOCl + 0.01% $HgCl_2$	3 MIN	100
0.2% Sodium hypochlorite	3 MIN	100
0.03% Mercuric chloride	3 MIN	75
70% EtOH + 0.2% NaOCl + 0.03% $HgCl_2$	3 MIN	75
70% EtOH + 0.2% NaOCl + 0.03% $HgCl_2$	5 MIN	50
70% EtOH + 0.5% NaOCl + 0.03% $HgCl_2$	4 MIN	20
70% EtOH + 0.5% NaOCl + 0.05% $HgCl_2$	4 MIN	NIL

Table 2. Callus response of various explants in MS and LS medium.

Hormone conc. (mg/L)	Callus induction /Type	No. of days		Explant Type	Callus response %		Nature of response	
		MS medium	LS medium		MS medium	LS medium	MS Medium	LS Medium
BAP+KIN+ NAA (0.4)+(0.2)+(0.1)	NO	NIL	NIL	Internode Leaf Node	-	-	-	-
BAP+KIN+NAA+IAA+IBA (0.4)+(0.2)+(0.1) +(0.2)+(0.2)	NO	NIL	NIL	Node Internode Leaf	-	-	-	-
BAP+KIN +NAA+IAA (0.4)+ (0.2)+(0.1)+(0.2)	YES (Hard Callus)	4	3	Node, Leaf, Internode and Rhizome	Node- 77.77% Internode- 56% Rhizome- 50% Leaf- 41.25%	Leaf- 45% Internode- 83.3%	Proliferation with pigmented callus	Proliferation with pigmented callus
BAP+KIN+NAA +IBA (0.4)+(0.2)+(0.1) +(0.2)	YES (Hard Callus)	8	7	Node and Internode	Node- 76.67% Internode- 59.6%	Node- 73.55% Internode- 87.9%	Pustules with light green colour	Pustules with light green colour

Fig. 1. Callus from MS medium.

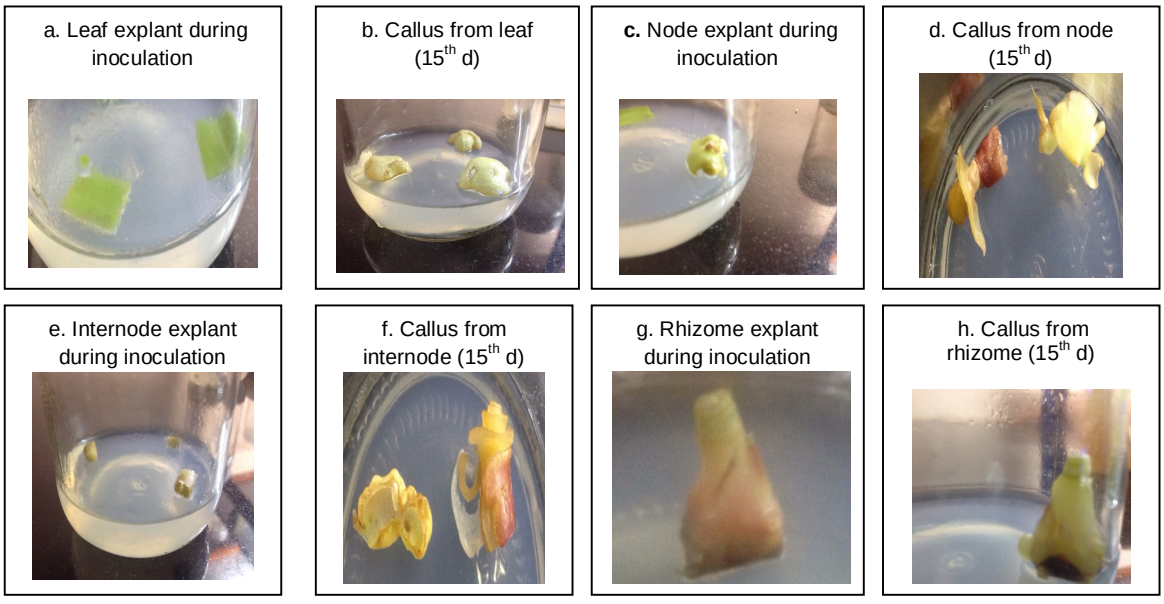


Fig. 2. Callus from LS medium.

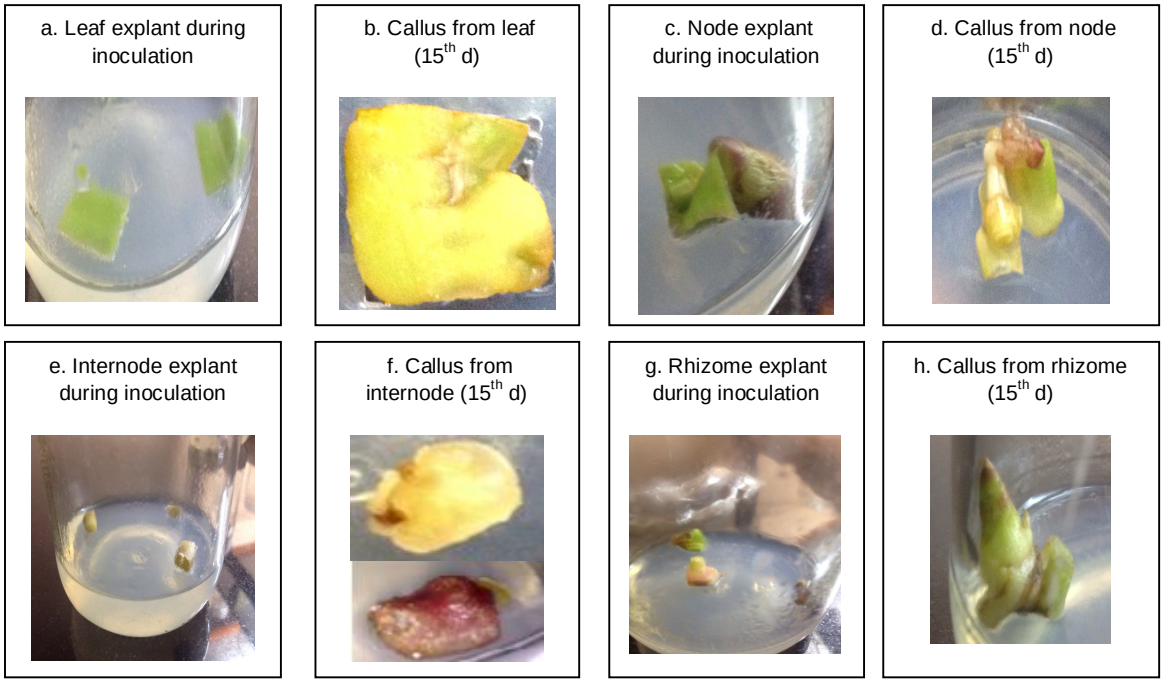
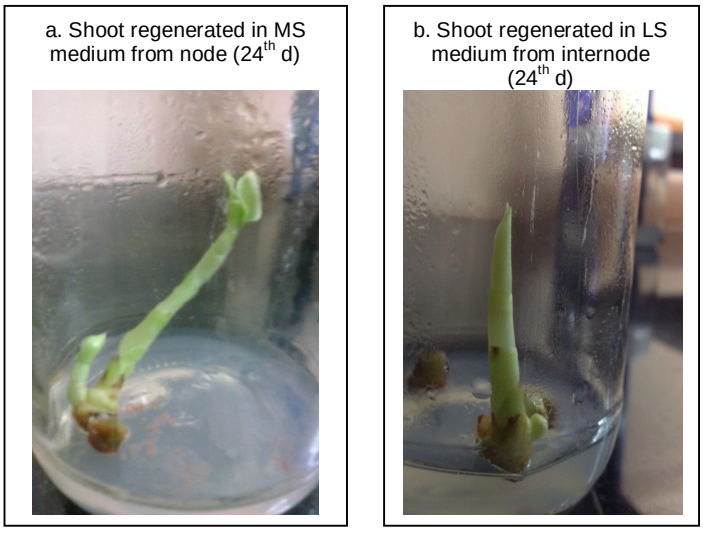


Table 3. Shoot Regeneration in MS and LS medium with various hormone concentrations.

S.No	Hormone concentration (mg/L)								Explants		Shoot growth (cm)		
	MS medium				LS medium				MS medium	LS medium	MS medium	LS medium	Number of days
	BAP	KIN	NAA	IAA	BAP	KIN	NAA	IBA					
1	0.4	0.2	0.1	0.2	0.4	0.2	0.1	0.2			-	-	-
											2.1	1.8	4
2	0.4	0.25	0.1	0.2	0.4	0.25	0.1	0.2	Node	Internode	6.6	5.0	14
											12.0	8.0	24
3	0.4	0.3	0.1	0.2	0.4	0.3	0.1	0.2			-	-	-

Fig. 3. Shoot regeneration from fom LS medium.



Shoot regeneration (in vitro): Shoot growth was observed in direct shoot medium of varying concentration after 4th and 5th d in both LS and MS medium and the length of the shoot developed was recorded at various time intervals. The explants were selected based on the maximum callus response shown in the medium as calculated before. Though the initial shoot growth observed were similar in both the medium, the overall growth till the entire observation period was well recorded in MS medium. The shoot length was calculated for the same (Table 3 and Fig. 3). Based on the callus response in MS and LS medium, explants such as node and internode were selected for regeneration in shooting medium at various hormone levels. The shoot length of *Costus pictus* was maximum in medium supplemented with BAP, IAA, IBA and KIN in MS medium (Sanjay *et al.,* 2013). In our study, shoot growth was observed at hormone concentration of BAP (0.2 mg/L) + KIN (0.25 mg/L) + NAA (0.1 mg/L) + IAA (0.2 mg/L) in MS medium and BAP (0.2 mg/L) + KIN (0.25 mg/L) + NAA (0.1 mg/L) + IBA (0.2 mg/L) in LS medium. Though response from both the medium was satisfactory, shoot grown on MS medium had better survival rate.

Table 4. Qualitative phytochemical analysis of wild plant and callus obtained from MS and LS medium.

Extract tested		Phytochemical compounds													
		Alkaloids				Phenols		Flavonoids				Terpenoids		Saponins	
		Mayer's test		Iodine test				Alkaline test		Ammonia test					
		MS	LS	MS	LS	MS	LS	MS	LS	MS	LS	MS	LS	MS	LS
Wild plant	Hex	-	-	-	-	-	-	-	-	-	-	-	-	++	++
	Dee	+	+	-	-	+	+	-	-	-	-	++	++	+	+
	Ea	+	+	+	+	++	++	+	+	+	+	+	+	-	-
	Ace	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	Met	++	++	+	+	++	++	++	++	+	+	+	+	-	-
Callus	Hex	-	-	-	-	-	-	-	-	-	-	-	-	++	++
	Dee	+	+	-	-	+	+	++	++	+	+	+	+	+	+
	Ea	+	+	+	+	+	+	+	+	++	++	+	+	-	-
	Ace	+	+	+	+	+	+	++	++	+	+	+	+	-	-
	Met	++	++	+	+	++	++	++	++	+	+	+	+	-	-

Table 5. Total phenolic content in MS and LS medium.

Sample		Phenol content (mg/g)	
		MS medium	LS medium
Wild plant	DEE	35.7	35.7
	EA	41	41
	ACE	23	23
	MET	82.8	82.8
Callus	DEE	52	47
	EA	65.8	51.8
	ACE	39	33.1
	MET	193.6	133.6

Table 6. Total flavonoid content in MS and LS medium.

Sample		Flavonoid content (mg/g)	
		MS medium	LS medium
Wild plant	DEE	-	-
	EA	23	21
	ACE	19	14
	MET	46.9	51.9
Callus	DEE	-	-
	EA	29	23
	ACE	28.1	21
	MET	72	56.7

Phytochemical analysis

Qualitative tests: The different solvent extract of wild plant and callus were screened for the comparative phytochemical constituents and the results were recorded (Table 4).

Quantitative tests: The different solvent extract of wild plant and callus were screened for the total phenol and flavonoid content and the results were recorded (Table 5 and 6). Wild plant and callus (MS and LS medium) extracted with different solvents in preliminary screening indicated the presence of high content of phytochemicals like phenols, alkaloids, flavonoids and terpenoids in methanolic extracts. Sequential screening for phytochemicals of *C. igneus* leaves revealed that it is rich in protein, iron, and antioxidant components such as ascorbic acid, α-tocopherol, β-carotene, terpenoids, steroids and flavonoids (Devi and Urooj, 2010; Shankarappa *et al.*, 2011).

Conclusion

Based on the results obtained, it was concluded that the optimized sterilization procedure adopted for explants of *C. igneus* was found to be effective at lower concentrations of chemicals used without affecting the morphological features of explants used. Callus induction in LS medium was rapid, utilizing minimal nutrients. Though callus was induced initially in LS medium, callus growth was observed to be better in MS medium for longer time, which inferred the importance of nutrient concentration in the growth of callus and the same observation was recorded for shoot growth also. Phytochemicals present in the plant and callus were analyzed qualitatively and quantitatively. Further studies are being carried out for the isolation and identification of active constituents from the solvent extracts of both mother plant and its callus.

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