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Callus induction from *Ipomoea aquatica* Forsk. leaf and its antioxidant activity

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Callus from the leaves of *Ipomoea aquatica* Forsk. was initiated on Murashige and Skoog's basal media supplemented with various combinations of auxins 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole butyric acid (IBA) with kinetin/6-benzyl aminopurine (BA). Callus production was observed in all the media but with varied mass. Highest percentage of callus response was obtained in combination of NAA (1.5 mg L^{-1}) with kinetin (0.5 mg L^{-1}). The friable callus was white in NAA supplemented and brown in 2,4-D and kinetin supplemented media. Three distinct phases viz., lag, exponential or liner and log phase were observed in the growth of callus. Antioxidant activities were analyzed by DPPH, TBARS and metal chelating method. Hyper antioxidant activity was observed in 1-month-old callus produced by NAA in combination with kinetin. The EC_{50} value of callus extract was 38 ± 3.05 and 54 ± 3.60 by DPPH and TBARS methods, respectively as against 58 ± 2.6 and 64 ±1.2 in *in vivo* plant material, however, no metal chelating activity was found in both.

Keywords*:* antioxidant activity, callus induction, *Ipomoea aquatica*, leaf explant
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Introduction

Ipomoea aquatica Forsk. commonly known as water spinach belongs to the family *Convolvulaceae.* It is a tender, trailing or floating perennial aquatic plant, found on moist soil along the margins of fresh water, ditches, marshes and wet rice fields. It is commercially cultivated as an edible green leafy vegetable in Hong Kong, Taiwan and China[1] and also in rural India[2]. *I. aquatica* is one of the richest sources of carotenoids and chlorophylls[3]. The leaves contain adequate quantities of most of the essential amino acids like aspartic acid, glycine, alanine, etc. in accordance with the WHO recommended pattern for an ideal dietary protein[4]. This can be compared with conventional food crops such as soybeans or whole egg, indicating its potential for utilization as food supplements[5].

Ayurveda has identified many medicinal properties of this plant and it is effectively used against nosebleed and high blood pressure[6,7]. Further, leaf extract could also be used to reduce blood sugar level[8,9] and as an antibiotic against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, etc[10].The floral buds are used as an anthelmintic[11].

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Leafy vegetables are extensively investigated as a newer source of natural antioxidant and for other bioactive compounds of human health benefits[12]. Further, plant cell culture has been tried tirelessly for increased production of antioxidant principles.[13] The earlier work on *I. aquatica* has explained the isolation and characterization of antioxidant compound, 7-O-β-D-glucopyranosyl-dihydroquercetin-3-O-α-D-glucopyranoside[14]. Due to its seasonal availability and endemic distribution the present investigation was focused to obtain callus from leaves for increased production of antioxidant principle and to provide the perennial source for the bioactive compounds of health benefit.

Materials and Methods

Plant Material

Healthy plants of *Ipomoea aquatica* Forsk. were collected around Myosre city, Karnataka Sate, India. The young leaves (5-7 cm) were washed with liquid detergent (Teepol, Qualigens, India) and disinfected with 10% Savlon (Johnson & Johnson Ltd, India) for 1 min followed by washings with distilled water thrice. The leaves were then surface sterilized with Bavistin (1% w/v) for 3 min and with mercuric chloride (0.1% w/v) for 1 min followed by five washings with sterile distilled water under aseptic conditions in a sterile flask.

Callus Cultures

Leaf segments (1.0-1.5 cm) were inoculated onto Borosil culture tubes (25 × 100 mm) placing abaxial side in contact with Murashige and Skoog[15] basal medium supplemented with various combinations and concentrations of auxins 2,4-D (0.5-2.5 mg L^{-1}), IAA (0.5-2.5 mg L^{-1}), NAA (1-2.5 mg L^{-1}), IBA (0.5-2.5 mg L^{-1}) and cytokinin, kinetin (0.5-2.5 mg L^{-1}) or BA (0.5-2.5 mg L^{-1}) with 3% (w/v) sucrose. All the growth regulators were procured from Sigma Chemicals (USA). The medium was gelled with 0.8 % (w/v) agar-agar (Qualigens, India). The *p*H of the medium was adjusted to 5.8 before autoclaving at 15 p.s.i. at 121°C for 20 min. The cultures were incubated at 25 ± 1°C under 16/8 h photoperiod with 35αE $^{-2}$ S^{-1} illumination with cool white fluorescent lights. All the experiments were repeated three times and SEs was calculated.

Callus Growth

The same callus medium was used for callus initiation and subcultured (30 days). The growth rate of callus was determined at regular intervals (10, 15, 21, 30 and 45 d).

Extraction from Callus Cultures

A known quantity of one-month fresh calluses was taken and oven dried at 60°C to constant weight. The callus was finely ground and extracted with MeOH using soxhlet apparatus for 8 h and solvent was removed by distillation. The crude extract obtained was used for antioxidant activity studies.

Determination of Antioxidant Activity

The antioxidant activity of methanolic extract of leaf and its callus extract was determined by three methods.

DPPH Radical Scavenging Activity

Antioxidant activity of callus and leaf extract was examined with respect to free radical scavenging activity using a stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). DPPH scavenging assay employed in the present study was a modification of the procedure of Moon and Terao.[16] About 0.2 mL of test sample at different concentrations was mixed with 0.8 mL of Tris-HCl buffer (*p*H-7.4) to which 1 mL DPPH (500 μ*M* in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV-visible Spectrophotometer (UV-160A, Shimadzu Co, Japan). All the samples were tested in triplicates with BHA as a positive control. EC_{50} represented 50% of the radicals scavenged by the test sample.

Lipid Peroxidation Assay

Lipid peroxidation inhibitory activity of callus and leaf extract was carried out according to the method of Duh and Yen.[17] Egg lecithin (3 mg L^{-1} phosphate buffer, *p*H 7.4) was sonicated in dr. Hielscher GmbH, UP 50H ultraschallprozessor. The test samples (100 μL) of different concentration were added to 1 mL of liposome mixture, control was without test sample. Lipid peroxidation was induced by adding 10 μL $FeCl_3$ (400 m*M*) plus 10 μL L-ascorbic acid (200 m*M*). After incubation for 1 h at 37°C, the reaction was stopped by adding 2 mL of 0.25 N HCl containing 15% TCA and 0.375% TBA and the reaction mixture was boiled for 15 min then cooled, centrifuged and absorbance of the supernatant was measured at 532 nm.

Metal Chelating Activity

The ferrous ion chelating activity was determined by the method of Dinis[18]. Test samples at different concentrations were mixed with 2 m*M* $FeCl_2$. $4H_2O$ and 5 m*M* ferrozine at a ratio of 10:1:2 and mixture was shaken. After 10 min, the Fe^{++} was monitored by measuring the formation of ferrous ion-ferrozine complex at 562 nm.

Results and Discussion

Callus Growth

The callus growth expressed three distinct phases (Fig. 1). A lag phase of 10 d where 10% growth in callus mass was noticed followed by exponential or linear phase with a rapid and significant increase (75

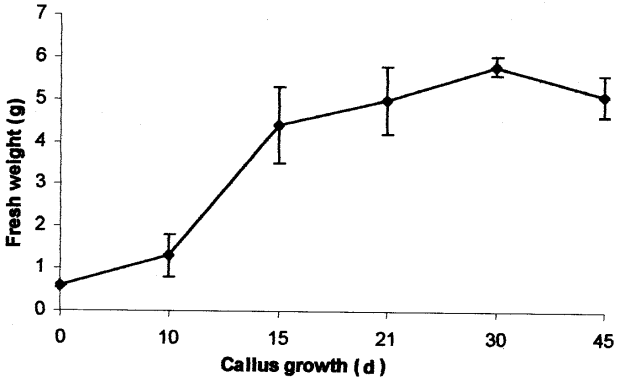

Fig. 1—Callus growth curve of *I. aquatica* leaf

%) in formation of callus during 10-15 d of culture representing early linear phase. It recorded a growth rate of 15% during late linear growth phase extending from 15th to 30th d of culturing. The growth declined after 30 d representing log phase. Depletion of nutrients, accumulation of toxic products, and other limiting factors might have led to cell death and eventually decline in growth[19]. Hence, callus was subcultured at 30 d interval.

Antioxidant Activity

The amount of sample needed to decrease the initial DPPH concentration (EC_{50}) by 50% is a parameter widely used to measure the antioxidant activity. Higher the EC_{50} value, lower the antioxidant activity. Antioxidant activity (EC_{50}) was low (73 µg L^{-1}) in callus during log phase i.e at 10 d. There was an increase in antioxidant activity (38 µg L^{-1}) in callus obtained during linear phase (i.e up to 30 d), which is characterized by accumulation of callus mass indicating the active phase of callus growth. After 30 d there was a decrease in antioxidant activity (104 µg L^{-1}). The increase and decrease in antioxidant activity corresponds to the different stages of growth phase of callus (Table 1). Maximum accumulation of secondary metabolites probably might be the reason for the antioxidant activity during active growth phase of callus upon 1st subculture as recorded in callus of many plants[19].

Callus Induction

All the combinations of NAA, 2,4-D, with kinetin and BA produced callus with a variable response (Tables 2 and 3). Optimum concentrations of auxins and cytokinins, which initiated callus with high percentage of response, were selected for further studies. Use of different concentrations of IAA and IBA to produce callus was not encouraging as they produced negligible amount of callus (data not shown). Percentage of callus induction was very low (4%) in basal media. The highest response (80%) in callus formation was observed in basal media, supplemented with 1.5 mg L^{-1} of NAA and 0.5 mg L^{-1} kinetin (Table 2). Callus formed primarily at the margins of green leaf disc after 10 days of culturing but with more callus formation near vascular tissue due to the availability of more nutrients around the conducting tissue[20]. Growth of callus increased significantly with the increase in incubation period up

Table 1—Antioxidant activity of callus extracts at different stages of growth

Days	% DPPH scavenging activity (EC_{50} value in µg mL^{-1}l) [a]
10	73 ± 5.03
30	38 ± 3.05
45	104 ± 4.16

[a] Values are mean ± standard deviation of three replicate analysis
EC_{50} = Concentration of test sample required to inhibit 50% of DPPH radicals

Table 2—Effect of different concentration of NAA with kinetin and BA on callus induction

Auxin (mg L^{-1})	Cytokinins (mg L^{-1})		% Callus response	Type of callus
NAA	Kinetin	BA		
1.0	0.5	-	42	White, friable
1.5	0.5	-	80	White, friable
2.0	0.5	-	74	White, friable
2.5	0.5	-	70	White, friable
1.0	1.0	-	57	White, friable
1.5	1.0	-	54	White, friable
2.0	1.0	-	45	White, friable
2.5	1.0	-	38	White, friable
1.0	-	1.0	30	White, friable
1.5	-	1.0	27	White, friable
2.0	-	1.0	24	White, friable
2.5	-	1.0	25	White, friable
1.0	-	1.5	44	White, friable
1.5	-	1.5	52	White, friable
2.0	-	1.5	36	White, friable
2.5	-	1.5	35	White, friable
Basal media	-	-	4	White, friable

Explant: leaves
Media: MS + 3%(w/v) sucrose

Table 3—Effect of different concentration of 2,4-D with kinetin and BA on callus induction

Auxin (mg L^{-1})	Cytokinins (mg L^{-1})		% Callus response	Type of callus
2,4-D	Kinetin	BA		
0.5	0.5	-	36	Brown, friable
1.0	0.5	-	25	Brown, friable
1.5	0.5	-	22	Brown, friable
2.0	0.5	-	26	Brown, friable
2.5	0.5	-	22	Brown, friable
0.5	1.0	-	33	Brown, friable
1.0	1.0	-	27	Brown, friable
1.5	1.0	-	20	Brown, friable
2.0	1.0	-	18	Brown, friable
2.5	1.0	-	16	Brown, friable
0.5	-	1.0	23	Brown, friable
1.0	-	1.0	20	Brown, friable
1.5	-	1.0	25	Brown, friable
2.0	-	1.0	19	Brown, friable
2.5	-	1.0	23	Brown, friable

Explant: leaves
Media: MS + 3%(w/v) sucrose

to 30 d and covered the entire surface of the explant. In general, it was observed that NAA was the best source of auxin for callus induction either with kinetin (38-80%) or BAP (24-52%; Table 2), than 2,4-D with kinetin (16-36%) or BA (19-25%; Table 3). Brown colour of the callus showed sensitivity of 2,4-D to plant tissues. With further increase in concentration of kinetin with NAA, there was a low percentage of callus initiation (Table 2). The callus produced by 2,4-D was dark brown in contrast to white in NAA. The colour being mainly influenced by location of phenolic secondary metabolites in cells. NAA may induce localized accumulation of phenolics in vacuoles making the callus to appear white. If the accumulation of the phenolics is in the cytoplasm, it undergoes oxidation and polymerization and the oxidized products appear brown[21].

One-month-old callus induced on different media was subjected to antioxidant activity by DPPH method (Tables 4 and 5). Media containing NAA (1-2.5 mg L^{-1}) with kinetin (0.5 mg L^{-1}) produced high antioxidant activity (88-63%) followed by 2,4-D (30-40%). Increase in 2,4-D concentration from 1.0 to 2.5 mg L^{-1} had no significant improvement for antioxidant activity. Media with NAA and increase in kinetin concentration from 0.5 to 1.0 mg L^{-1} had adverse effect, where antioxidant activity was also found to be low (10-15%).

Since NAA (1.5 mg L^{-1}) with kinetin (0.5 mg L^{-1}) produced maximum amount of callusing with good percentage of antioxidant activity compared to other calluses, it was further tested for antioxidant property by TBARS and metal chelating methods along with crude methanolic extract of leaves from *in vivo* plant (Table 6). In both DPPH and TBARS methods, the

Table 4—DPPH radical scavenging activity of callus extracts obtained from different concentrations of NAA with kinetin and BA

Auxin (mg L^{-1})	Cytokinins (mg L^{-1})		% DPPH radical scavenging activity[a]
NAA	Kinetin	BA	
1.0	0.5	-	72 ± 3.05 [b]
1.5	0.5	-	88 ± 2.08
2.0	0.5	-	70 ± 4.04
2.5	0.5	-	63 ± 2.08
1.0	1.0	-	10 ± 4.50
1.5	1.0	-	13 ± 0.37
2.0	1.0	-	15 ± 2.08
2.5	1.0	-	12 ± 1.52
1.0	-	1.0	29 ± 1.15
1.5	-	1.0	20 ± 2.51
2.0	-	1.0	16 ± 1.5
2.5	-	1.0	15 ± 2.0
1.0	-	1.5	22 ± 0.57
1.5	-	1.5	32 ± 0.57
2.0	-	1.5	20 ± 2.0
2.5	-	1.5	14 ± 2.6
Basal media			6 ± 1.1

[a] Concentration of test sample was 100 μg mL^{-1}
[b] Values are mean ± standard deviation of three replicate analysis
Explant: leaves
Media: MS + 3 %(w/v) sucrose

Table 5—DPPH radical scavenging activity of callus extracts obtained from different concentrations of 2,4-D with kinetin and BA

Auxin (mg L^{-1})	Cytokinins (mg L^{-1})		% DPPH radical scavenging activity[a]
2,4-D	Kinetin	BA	
0.5	0.5	-	40 ± 1.52
1.0	0.5	-	38 ± 2.0
1.5	0.5	-	38 ± 1.52
2.0	0.5	-	35 ± 4.58
2.5	0.5	-	30 ± 3.21
0.5	1.0	-	46 ± 1.15
1.0	1.0	-	35 ± 1.52
1.5	1.0	-	23 ± 1.0
0.5	-	1.0	24 ± 1.52
1.0	-	1.0	26 ± 2.0
1.5	-	1.0	23 ± 1.5
2.0	-	1.0	20 ± 4.1
2.5	-	1.0	23 ± 1.7

[a] Concentration of test sample was 100 μg mL^{-1}
[b] Values are mean ± standard deviation of three replicate analysis
Explant: leaves
Media: MS + 3% (w/v) sucrose

Table 6—Antioxidant potential of methanolic extracts of leaf and its callus

Compound	EC_{50} value (μg mL^{-1}) [a]		
	DPPH radical scavenging activity	Lipid peroxidation inhibitory activity (TBARS)	Metal chelating activity
Methanol extract of leaves	58 ± 2.6	64.4 ± 1.2	-
Callus extract	38 ± 3.05	54 ± 3.60	-

[a] Values are mean ± standard deviation of three replicate analysis
EC_{50} = Concentration of test sample required to inhibit 50% of DPPH radicals

noticeable antioxidant effect was observed in callus extract with an EC_{50} value of 38 ± 3.05 and 54 ± 3.60, respectively in comparison to methanolic extract from leaves showed antioxidant activity of 58 ± 2.6 and 64 ± 1.2 by DPPH and TBARS methods of assay. However, in the present study both callus and methanolic extracts of *in vivo* plants did not show any metal chelating activity. Hyper antioxidant activity (88%, Table 4) was observed in callus obtained with NAA (1.5 mg L^{-1}) with kinetin (0.5 mg L^{-1}). Addition of sugars, phosphates, nitrate and calcium, which was performed in callus probably might have helped in hyper antioxidant activity as observed in many plant callus[22,23]. The antioxidant activity of callus and leaf as expressed by DPPH and TBARS methods indicated that the antioxidant mechanism was due to their H^+ atom donating ability to peroxy radicals, thus inhibiting the oxidation of fatty acid chain termination[24]. Further hyper antioxidant activity of callus encourages *in vitro* mass production of bioactive compounds of health benefits from *I. aquatica*.

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