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Phytochemical evaluation and Total Phenol quantification of callus leaf explants of Pittosporum dasycaulon Miq.

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ABSTRACT

The present investigation was aimed to evaluate the effect of growth regulators on induction of callus from leaf segments of Pittosporum dasycaulon. The surface sterilized young leaves were inoculated on Murashige and Skoog (MS) medium fortified with different concentration and combination of growth regulators for induction of callus. The phytochemical constituents were analyzed in the different solvent extracts using standard methods. Maximum callus induction was observed on MS medium supplemented with NAA (0.5mg/L) + BAP (2 mg/L).The preliminary phytochemical screening of leaf and leaf derived callus revealed the presence of flavonoids, phenols, steroids,tannins and saponin glycosides. Water extract of leaf and leaf derived callus was quantified for the total phenols and phenol content in leaf extract is 0.41mg/g and callus is 0.28mg/g expressed as catechol equivalent. The developed protocol is useful for large scale production of callus from leaf explants which may be useful for isolation of important bioactive compounds for the treatment of various diseases.

Keywords - Callus induction, Invitro culture, Phenol Quantification, Phytochemical studies, Pittosporum dasycaulon.

1. Introduction

Medicinal plants are the source of important therapeutic aid for reducing human ailments [1]. More or less 85% pharmaceutical formulations include the utilization of plants or plant concentrates [2]. Researchers in the field of biotechnology have great interest in these plants as most of the drug industries now depend on plants for the production of pharmaceutical compounds [3].

Plant tissue culture has been identified as an excellent surrogate method to overcome the problems connected with utilization and conservation of medicinal plants[4].Plant tissue culture offers an alternative to produce bioactive secondary metabolites under a controlled environment, independent of seasonal and geographical conditions[5]. A Callus culture system offers many advantages as a model system for several biological investigations[6].Thus, plant tissue culture considered an important strategy for *in vitro* production of bioactive compounds for drug and food industries[7].

Pittosporum dasycaulon, is an endemic and endangered [8] tree of the Western Ghats has profound medicinal properties shows limited distribution. *Pittosporum dasycaulon* belongs to the family Pittosporaceae. The family is represented by 11 genera and about 250 species, which are distributed in the tropical and subtropical regions of Africa, Asia, Australia and Pacific islands. Of the various genus of the family Pittosporaceae, *Pittosporum* alone is found in India. Its distribution in India is mainly in the rain forests at altitudes between 500 to 2800m and for the most part concentrated in the Himalayan and the Western Ghats regions. About 300 species of *Pittosporum* is reported out of which only eleven are observed in India. Most of the species are useful in traditional Chinese medicine for their sedative and cough relieving effects. Several active phytochemical compounds such as triterpenoid, saponins, carotenoids, and essential oils were isolated from *Pittosporum*[9].The antileukemia properties of *Pittosporum* had been reported recently [10]. The biological properties of *Pittosporum* has been mainly attributed to the presence of a number of volatile mono and sesquiterpenes in leaves [11].

Pittosporum dasycaulon, is commonly known as “Kasumaram". The plant is a small tree up to 8m tall. It is a small evergreen tree. The bark is brownish and lenticellate. Leaves are simple, alternate, spiral and elliptic to elliptic-oblanceolate in shape. The inflorescence is terminal, umbels of short racemes. The fruit is a loculicidal capsule, seeds will be orange to reddish in colour. The plant is self-incompatible and an out crosser. The stem bark of *P.dasycaulon* has been used in folk medicine as an antibacterial and antifungal agent to treat infection [12]. Due to its high medicinal value, the plants are harvested in an excessive manner and thus *P. dasycaulon* needs conservation [13]. Further, no report is available regarding the callus induction studies of *Pittosporum dasycaulon*.

Therefore, the present study aims to develop the efficient protocol for callus induction from leaf explants and to screen the phytoconstituents present in water extract of leaf and leaf derived callus of *Pittosporum dasycaulon.*

2. Materials and methods

The tender leaf explants were collected for culture from the mature plant grown and maintained in the medicinal garden at Vimala College, Thrissur .

2.1 Callus induction

Leaf explants of *P. dasycaulon* were washed under running tap water (10 min) to remove dust particles then followed by washing with teepol. Explants were rinsed with distilled water for 2-3 times. Finally, the explants were sterilized with 0.1% mercuric chloride for 7min followed by washing with double distilled water for five times. Explants were cultured in full strength MS medium(0.8% w/v agar) (Murashige and Skoog, 1962) 3% (w/v) sucrose and supplemented with auxins (1, 2, 3 and 4 mg/L of 2, 4-D), (0.5, 1 mg/L of NAA) and Cytokinins (0.5, 1, 2, 3 and 4 mg/L of BAP) alone and in combinations. The leaf explants were incubated at 25±2°C with a 16/8 h light/dark cycle. Calli formed were transferred onto a new fresh media at every 3 weeks intervals. Callus induction efficiency is calculated as callus index i.e,% of Cultures responding x callus intensity. Callus intensity is recorded as 0.5 (1-100mg), 1 (101-200mg), 1.5 (201-300mg), 2 (301-400mg).

2.2 Preparation of Plant extracts

Preparation for plant extracts 10 gms of shade dried leaf and callus were grinded using mortar and pestle in distilled water. The extracts were filtered through Whatman No. 1 filter paper.

2.3 Preliminary phytochemical analysis

The phytochemical analysis of water extract of callus and leaf were carried out to study the presence and absence of primary and secondary metabolites using standard conventional protocols [14,15].

2.4 Quantification of total phenols

Quantitative analysis of total phenol was estimated in leaf and callus water extract using Bray and Thorpe method[14]. Colchicine is used as standard. Total phenol values are expressed in terms of colchicines equivalent mg/g of sample. The estimations were repeated two times with triplicates and average values taken and read at 650 nm in systronics spectrophotometer.

2.5 Statistical Analysis

All the experiment done is replicated thrice and mean±SD value depicted.

3. Results and discussion

Callus is an unorganized mass of cells. The morphogenetic response of the explants is mainly based on the type and concentration of the hormone used [7]. The explants cultured on MS medium supplemented with different concentration of cytokinins and auxins of BAP, 2,4 D and NAA showed varied response for callusing. Callus induction was observed on MS media supplemented with 1 mg/L of 2, 4-D, 1mg/L NAA and 1mg/L BAP (Table 1).

After eight weeks of cultures maximum callus growth was observed in 1 mg/L of BAP. There was no callus induction when basal medium used without any hormones.

Leaf explants were cultured on MS medium with two hormonal combinations with varying concentrations. Nine different media were tried by varying the concentration of BAP and NAA, BAP and 2,4-D (Table 1). When concentration of BAP kept fixed at 0.5 mg/L and varying the concentration of 2,4-D (2-4mg/L) the rate of callus induction was poor (Fig a). BAP varied between (2-4mg/L) and 2,4-D kept constant(1mg/L), callus induction rate was low (Fig b). BAP 2mg/L and NAA 0.5 mg/L white coloured callus with a high rate of callus induction was observed (Fig c). Morphology of the callus in the BAP and NAA containing culture media showed the formation of fresh nodular green coloured where as the BAP and 2,4-D media showed the formation friable whitish callus. Huge nodular green coloured callus induced in MS+BAP 2 mg/L +NAA 0.5mg/L is used for phytochemical studies.

Table1.Effect of auxins and cytokinins on callus induction from leaf explants of *P. dasycaulon* Miq.

Growth regulators cytokinins	auxins	**Callus index**	**Response after one month**
BAP(1mg/L)	-	39±2.8	Shows callus growth on cut ends.
-	NAA 1mg/L	28±1.7	Show callus growth on cut ends
-	2,4-D 1mg/L	35±3.09	White fragile callus
BAP(2mg/ml)	24D(1mg/L)	26±2.5	Browning prominent ,with low rate of callusing
BAP(3mg/ml)	24D(1mg/L)	31±1.4	Fragile callus on entire surface
BAP(4mg/ml)	24D(1mg/L)	58±2.3	Fragile callus with low rate of callusing
BAP(0.5mg/ml)	24D(2mg/L)	31±1.4	Prominent browning with very poor callusing
BAP(0.5mg/ml)	24D(3mg/L)	45±3.1	Prominent browning with very poor callusing
BAP(0.5mg/ml)	24D(4mg/L)	44±3.3	Prominent browning with very poor callusing
BAP(2mg/ml)	NAA(0.5mg/L)	90±0.85	Green nodular coloured callus, bulk in appearance
BAP(3mg/ml)	NAA(0.5mg/L)	41±2.4	Green nodular coloured callus low rate of callus
BAP(4mg/ml)	NAA(0.5mg/L)	21±0.6	No pronounced changes little callus

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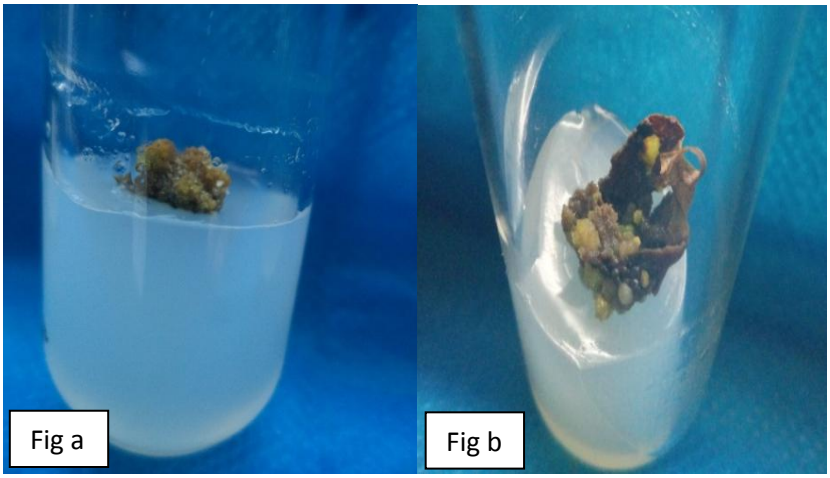

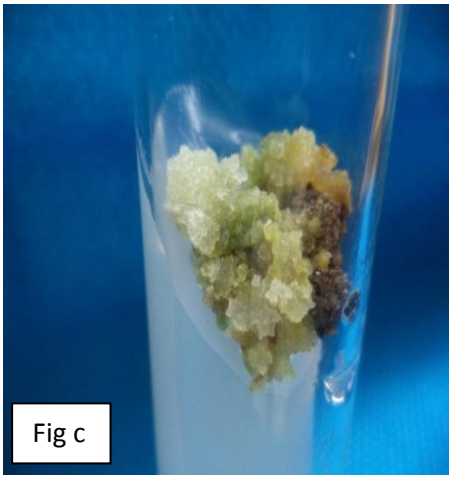

Fig. a-c Callus from leaf explants of *P. dasycaulon* on MS medium a) 0.5mg/L BAP+ 2 mg/L2,4-D b) 4 mg/L BAP+ 1 mg/L2,4-Dc) 2 mg/L BAP+ 0.5 mg/LNAA

Medicinally active constituents of plant tissues are extracted with a broad spectrum of organic solvents of choice through standard procedures [16]. The secondary metabolites produced by the plants play an important role in plant defence against prey, microorganisms, stress as well as interspecies protections. These secondary metabolites have been used as a drug from the time immemorial, hence screening of phytochemicals serve as the initial steps in predicting the potential active compounds in the plant extracts [17]. Chandra and Kaneria [18] have reported that the accumulation of secondary metabolites may vary in different plant parts and leaf is one such plant

parts which have highest accumulation of metabolites [19].Phytochemical studies revealed the presence of phenols, tannins, steroids and saponin glycosides, carbohydrates, starch, sugar proteins (table 2).

Table 2. Preliminary Phytochemical Screening of the leaf and callus extracts of *P. dasycaulon* Miq.

Plant Constituents	**Test /Reagent**	**Leaf**	**Callus**	**Observations**
Carbohydrates	Fehling Test	+	+	Red ppt
Starch	Iodine Test	++	+	Blue colouration
Sugar	Anthrone Reagent Test	+	+	Blue black colouration
Proteins	Biuret Test:	+	+	Violet or pink colour appears.
Amino Acids	Ninhydrin Test:	--	+	No violet or purple colour
Fixed oils and Fat	Filter Paper Test	++	-	oil stains on filter paper
Alkaloid	Ammoniacal Test	--	-	No creamish ppt
Steroid	Salkowski reaction	++	+	Chloroform layer appears red and acid layer shows greenish yellow fluorescence.
	Liebermann Burchard Test	+++	+	Blue green ring
Cardiac Glycosides	Legal Test	--	-	No pink to red colour appears
Anthraquinone Glycosides	Borntrager'stest	--	-	Ammoniacal layer do not turns pink or red.
Saponin Glycosides	Foam test:	++	+	Froth appearance
Phenols and Tannins	Folin Test	+++	++	Blue colouration
Flavonoids	Ethylacetate Test	++	+	No yellow colouration

The total phenol content plays a very important role in the protection of the plants against the deleterious effects of UV rays and also against certain phytopathogenic microorganisms [20]. Most of secondary metabolites are derivative of phenolic compound therefore TPC was determined. In the present study phenol was qualitatively detected by the presence of blue colouration by the Folin test. The callus and leaf extract prepared were analysed quantitatively for the phenol. The results of quantitative estimation of phenols are tabulated in table 3. It is also well known that phenolic compounds contribute to the quality and nutritional value and also provide health beneficial effects [21].

Table 3. Total Phenols content leaf and callus extracts of *P.dasycaulon* Miq.

Sample	Phenol concentration
Leaf Extract	0.41±0.15 mg/g
Callus Extract (2 mg/L BAP+ 0.5 mg/L NAA)	0.28±0.64 mg/g

4. Conclusion

The present study effort is made to standardize a protocol for the large scale callus induction from the leaf explants of *Pittosporum dasycaulon*. The preliminary phytochemical screening of water extracts of leaf and leaf derived callus showed the presence of phenols, flavonoids, steroid and tannins. This study will provide an efficient protocol for rapid induction of callus for the production of bioactive compounds. There will be possibilities of making plant cell factories for the enhanced production of bioactive compounds, which may prevent the exploitation of natural plant resources.

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