



***In vitro* callus induction protocols of *Mesua ferrea* (a slow growing medicinal tree) using two type explants & different concentrations of PGRs**

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Abstract: Young stem and leaf explants of *Mesua ferrea* were cultured after surface sterilization on woody plant medium (WPM) and Murashige and Skoog's (MS) medium supplemented with different plant growth regulators (PGRs) cytokinins and auxins, individually and in various combinations. Leaf segments proved to be the best explant for callus induction compared to stem explants. Different treatments were employed for surface sterilization of explants. Stem and leaf segments cultured on WPM and MS medium supplemented with different concentrations of PGRs (1.0 mg/l 2, 4-D with 2.0 or 3.0 mg/l BAP) led to maximum callus induction but induction time was reduced in case of WPM. This is the first successful attempt to establish consistent callus formation protocols from leaf and stem segments of *Mesua ferrea*. This study may contribute in conservation management of this native plant species found especially in wet zone of Sri Lanka and India.

Keywords: Callus induction, *Mesua ferrea*, plant growth regulators, cytokinins, auxins

Introduction

Mesua ferrea is an important member of the family Clusiaceae (Guttiferae). The plant is named after the heaviness of its timber and cultivated in tropical climates. It is placed in the priority list of medicinal plants. It is native plant of Sri Lanka but also cultivated in Assam, Southern Nepal, Indochina and the Malay Peninsula. It has been used for centuries in folklore and traditional system of medicine for several indications such as rheumatism, cough, dysentery, vomiting, sore throat, fever, itching etc. From the time immemorial *Mesua ferrea* is propagated by seed. The seeds, however, have short viability and are prone to microbial attacks. The tree bears fertile seeds in 15-20 years. Due to over exploitation and misuse of medicinal plants we are facing the problem of losing our precious plant resource for future. This situation calls for effective and in time conservation measures to enrich our lives with the services of plants. In this regard various research groups across the world have attempted conservation of plants to protect biodiversity (Malik *et al.*, 2005. Nadeem *et al.*, 2000. Prabia *et al.*, 2007. Ray and Bhattacharya, 2008). Different techniques for conservation of plants have been practiced worldwide, the most important being tissue culture (Prabia *et al.*, 2007), as it produces multiple copies of a plant species with in limited time and space and enhancement of biochemical status without destroying the plant. Various *in vivo* studies have been carried on *Mesua ferrea* but no

research has been on its tissue culture aspects. The objectives of this study were to investigate the best surface sterilization method, selection of suitable explants and plant growth regulators on callogenesis in *Mesua ferrea*.

Material and Methods

This work was carried out in tissue culture laboratory, Department of Botany, C.C.S. University, Meerut.

Plant Material

The plant materials were collected from Botanical garden of Department of Botany, C.C.S. University Meerut. Young nodal stem plant (soft, non woody and green in colour) and leaf explants (soft and reddish green in colour) were selected as an explant source.

Media Preparation

The potential of two different PGRs auxins (1.0-4.0 mg/l 2, 4-D, IAA or IBA) and cytokinins (1.0-4.0 mg/l Kn or BAP) were analyzed for the induction of callus. The basal nutrient media MS (Murashige and Skoog, 1962) and WPM (Lloyd and McCrown, 1980) supplemented with different combinations of PGRs (auxin and cytokinin) were used. The effect of GA₃ (1×10⁻⁶mM) and Calliterpenone (1×10⁻⁶mM) were also studied on callus as calliterpenone is reported to exhibit GA like activity (Maji *et al.*, 2012).

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Explant surface sterilization

Freshly harvested young stem and leaves were cleaned and dead/decaying parts separated, that were washed with the running tap water for 30 minutes to remove the dust particles. The explants were surface sterilized with Tween-20 and finally with 0.1% HgCl₂ for 5 minute under laminar air flow.

Inoculation of ex-plants

Stem and leaf segments about 1-1.5 cm. were prepared aseptically and were implanted vertically on MS and WPM prepared with different concentrations of auxins and cytokinins, singly or in combination, for callus induction. The medium containing 3% sucrose was solidified with 8% agar (including different concentration of PGRs). The pH of the media was adjusted with 1N NaOH or 1 N HCl solutions prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 121°C and 15 psi

for 20-25min. The cultures were incubated under controlled humidity. For each experiment a minimum of 6 replicates were taken and experiments were repeated thrice. The flasks containing culture media of different concentrations of PGRs were put into laminar air flow for 1 hour.

All the cultures from different explants were maintained in controlled environment of growth room under illumination, provided by white fluorescent tube lights with a photoperiod of 16h. Cultures were visited regularly to observe their response to tissue culture.

Data were recorded after every week and the values are presented as scores as +, ++, +++, - representing low, medium, good and no response, respectively.

Table 1: Influence of various PGRs on callus induction in *Mesua ferrea* leaf / stem explants

S.No	MS+3%SUCROSE+(mg/l)PGRs	Colour Of Callus	Type Of Callus	Day Of Response	Degree Of Response
1	1.0 2,4-D	Pinkish Green	Compact	35 days	++
2	2.0 2,4-D	Pinkish Green	Compact	35 days	++
3	3.0 2,4-D	Pinkish Green	Compact	35 days	+
4	4.0 2,4-D	Pinkish Green	Compact	35 days	+
5	1.0 BAP	-	-	-	-
6	2.0 BAP	-	-	-	-
7	3.0 BAP	-	-	-	-
8	4.0 BAP	-	-	-	-
9	1.0 Kn	-	-	-	-
10	2.0 Kn	-	-	-	-
11	3.0 Kn	-	-	-	-
12	4.0 Kn	-	-	-	-
13	1.0 2,4-D + 1.0 BAP	Greenish white	Compact	30 days	++
14	1.0 2,4-D + 2.0 BAP	Greenish white	Compact	30 days	+++
15	1.0 2,4-D + 3.0 BAP	Greenish white	Compact	30 days	+++
16	1.0 2,4-D + 4.0 BAP	Greenish white	Compact	30 days	++
17	1.0 2,4-D + 1.0 Kn	Green	Compact	35 days	+
18	1.0 2,4-D + 2.0 Kn	Green	Compact	35 days	+
19	1.0 2,4-D + 3.0 Kn	Green	Compact	35 days	+
20	1.0 2,4-D + 4.0 Kn	Green	Compact	35 days	+
21	1.0 IAA	-	-	-	-
22	2.0 IAA	-	-	-	-
23	3.0 IAA	-	-	-	-
24	4.0 IAA	-	-	-	-
25	1.0 IBA	-	-	-	-
26	2.0 IBA	-	-	-	-
27	3.0 IBA	-	-	-	-
28	4.0 IBA	-	-	-	-
29	1.0 2,4-D+0.1 Kn	Green	Compact	30	+
30	2.0 2,4-D+0.1 Kn	Green	Compact	30	+
31	3.0 2,4-D+0.1 Kn	Green	Compact	30	+
32	4.0 2,4-D+0.1 Kn	Green	Compact	30	+
33	1.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
34	2.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
35	3.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
36	4.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
36	1.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-
38	2.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-
39	3.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-
40	4.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-

+++ Good response; ++ Medium response; + Low response; - No response

2,4-D (2,4-Dichlorophenoxy Acetic Acid); BAP (Benzyl Amino Purine); Kn (Kinetin); IAA(Indole Acetic Acid); IBA (Indole Buteric Acid); CT (Calliterpenone); GA (Gibberlic Acid).

Table 2: influence of various PGRs on callus induction in *Mesua ferrea* leaf/stem explants

S.No	WPM+3% SUCROSE+(mg/l)PGRs	Colour Of Callus	Type Of Callus	Day Of Response	Degree Of Response
1	1.0 2,4-D	Whitish Green	Compact	30 days	++
2	2.0 2,4-D	Whitish Green	Compact	30 days	++
3	3.0 2,4-D	Whitish Green	Compact	30 days	++
4	4.0 2,4-D	Whitish Green	Compact	30 days	++
5	1.0 BAP	-	-	-	-
6	2.0 BAP	-	-	-	-
7	3.0 BAP	-	-	-	-
8	4.0 BAP	-	-	-	-
9	1.0 Kn	-	-	-	-
10	2.0 Kn	-	-	-	-
11	3.0 Kn	-	-	-	-
12	4.0 Kn	-	-	-	-
13	1.0 2,4-D + 1.0 BAP	Greenish white	Compact	25 days	+++
14	1.0 2,4-D + 2.0 BAP	Greenish white	Compact	25 days	+++
15	1.0 2,4-D + 3.0 BAP	Greenish white	Compact	25 days	+++
16	1.0 2,4-D + 4.0 BAP	Greenish white	Compact	25 days	++
17	1.0 2,4-D + 1.0 Kn	Green	Compact	30 days	+
18	1.0 2,4-D + 2.0 Kn	Green	Compact	30 days	+
19	1.0 2,4-D + 3.0 Kn	Green	Compact	30 days	+
20	1.0 2,4-D + 4.0 Kn	Green	Compact	30 days	+
21	1.0 IAA	-	-	-	-
22	2.0 IAA	-	-	-	-
23	3.0 IAA	-	-	-	-
24	4.0 IAA	-	-	-	-
25	1.0 IBA	-	-	-	-
26	2.0 IBA	-	-	-	-
27	3.0 IBA	-	-	-	-
28	4.0 IBA	-	-	-	-
29	1.0 2,4-D+0.1 Kn	Green	Compact	30	++
30	2.0 2,4-D+0.1 Kn	Green	Compact	30	++
31	3.0 2,4-D+0.1 Kn	Green	Compact	30	++
32	4.0 2,4-D+0.1 Kn	Green	Compact	30	++
33	1.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
34	2.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
35	3.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
36	4.0 2,4-D+10 ⁻⁶ mM(CT)	-	-	-	-
36	1.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-
38	2.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-
39	3.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-
40	4.0 2,4-D+10 ⁻⁶ mM(GA)	-	-	-	-

+++ Good response;
 ++ Medium response;
 + Low response;
 - No response

2,4-D (2,4-Dichlorophenoxy Acetic Acid); BAP(Benzyl Amino Purine); Kn (Kinetin); IAA(Indole Acetic Acid); IBA (Indole Butyric Acid); CT (Calliterpenone); GA (Gibberlic Acid).

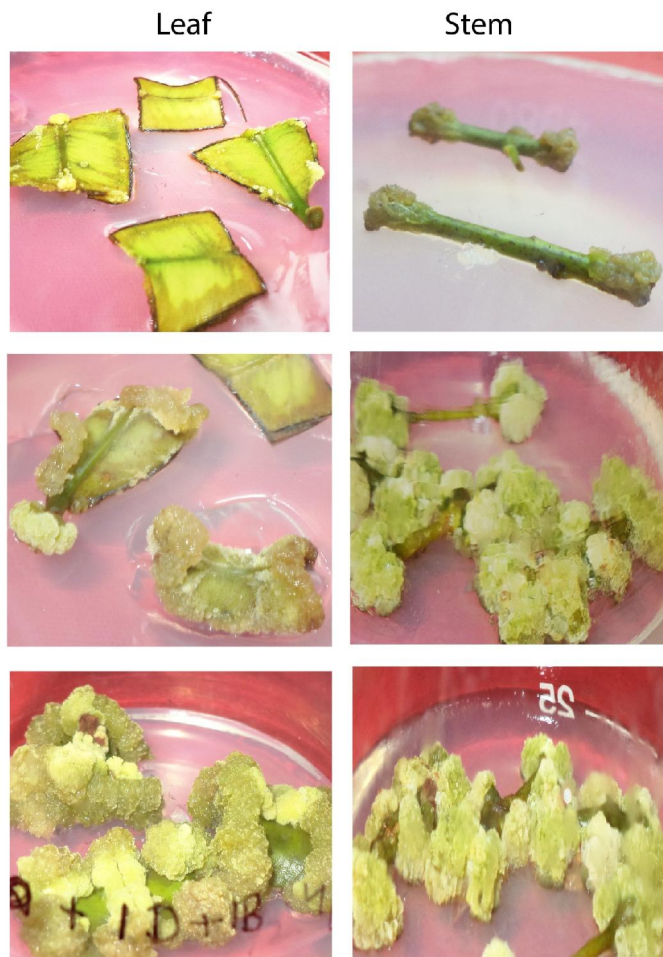
Young leaf (5-7 cm.in length, 1-2 cm. in diameter and reddish green in colour) segments found the best source of explants for callus induction that showed response as compare with nodal stem explants(green non woody and 1-1.5 cm in length).

Result and Discussion

Both auxins and cytokinins are major growth regulators that have profound influence on various phenomenon of cell division, callus induction and regeneration (Munazir *et al*; 2000. Tang *et al*; 2000). Different explants such as leaf, stem are used for callus induction on the woody plant medium as well as on MS medium with different combinations of auxin and cytokinin. Different concentration of different auxins (2,4-D, IAA and IBA) are used on WPM and MS medium but callus induction was started only with different concentration of 2,4-D (1,2,3 and 4.0 mg/l 2,4-D) on both nutrient medium. The colour of the callus was pinkish

green in case of MS medium and whitish green in case of WPM. It indicates that the plant cells undergo stress when inoculated on MS medium. Different concentrations of BAP or Kn with WPM and MS, without auxin were used, hence concluded that auxin and cytokinin both are necessary for callus induction in place of simple BAP and Kn. Beside it 10^{-6} mM CT and GA was used but no callus induction was started. Calliterpenone (CT) is a phyllocladane diterpenoid used as a growth promoter isolated from the cultivated plant *Callicarpa macrophylla* (Kalra et al., 2010; Singh et al., 2011; Maji et al., 2012). In the present study, maximum callus inductions were observed when WPM + 1.0 (mg/l), 24-D + 2.0 (mg/l) BAP, MS + 1.0 (mg/l), 24-D + 2.0 (mg/l) BAP, WPM + 1.0 (mg/l), 24-D + 3.0 (mg/l) BAP and MS + 1.0 (mg/l), 24-D + 2.0 (mg/l) BAP (Table.1&2) nutrient media are used. The rest of combinations of auxins and cytokinins were capable of producing more or less poor results. The control treatment (WPM and MS medium without any hormone) was unable to induce callus. In both medium MS and WPM nature, type of colour of the callus was same but induction time reduced in case of WPM. It was supposed to be the presence of higher N content in woody plant medium. Rapid callus induction and its proliferation are vital to tissue culture as those calli are of no use which fails to proliferate (munazir et al, 2000). It was observed that when WPM and MS were supplemented with 1.0 (mg/l), 24D +2.0 and 3.0 (mg/l) BAP on nutrient medium, it did not only result in maximum callus induction but also in callus proliferation.

Different stages of callus induction in *Mesua ferrea*



Conclusion

In vitro culture of plants has gained importance during recent years because this technique can be used for the rapid multiplication and *exsitu* conservation of some plants. To the best of our knowledge, no report is available about micro-propagation of *Mesua ferrea*. The aim of the present work was to determine the culture conditions for micro-propagation of this plant. The present study discovers leaf and nodal segment as an effective explants which expressed maximum callus in MS as well as in WPM with different concentration of PGRs.

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