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RESEARCH ARTICLE

EFFICIENT PROTOCOL FOR INDIRECT SHOOT REGENERATION FROM VARIOUS EXPLANTS OF *MALLOTUS PHILIPPENESIS* – AN ENDANGERED PLANT

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ABSTRACT

A micropropagation protocol is established for conservation of critically threatened wood tree species *Mallotus philippenesis.* The indirect shoot regeneration from leaf explants protocol developed in this study provides a basis for germplasm conservation and further investigation of medicinally active constituents of the elite medicinal plant. In the present experiment different explants such as leaves, cotyledons, root and in-vitro raised vegetative parts were used for callus induction. Leaves found to be the best explants for indirect shoot regeneration. MS media supplemented with 2-4D, BAP, KI responded best for callus formation. At higher concentration of growth hormones negative response was also observed. Indirect shoot organogenesis was achieved from the callus using BAP and 2-4D when highest number of shoots (2.0 ± 0.33) with maximum frequency (49.0±0.88) was regenerated. All the in- vitro raised shoots with a maximum length of (5.8±0.35) were transferred to rooting medium supplemented with different concentrations of auxins such as IBA and NAA. The best rooting response was observed on 1.0mg/l IBA with 1.0mg/l NAA. The well rooted plantlets were transferred to plastic cups containing soil+vermiculite + sand in 1: 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability.

Key words: Mallotus philippenesis, Leaf explants, Callus induction, Plant regeneration.

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INTRODUCTION

Micropropagation is an alternative to the conventional methods of vegetative propagation with the objective of enhancing the rate of multiplication. Plant tissue culture has been viewed as an important technology for enhancing the capability of selected elite high yielding varieties, so as to boost production and productivity (Kaur, 1998). *Mallotus philippenesis* (also called sindoori) belongs to the family of euphorbiaceae is a perennial tree plant being about 10-12 meters tall. It is one of the common plants used in Indian system of medicine. Various parts of the plant are used in the treatment of skin problem, bronchitis, antifungal tape worm eye-disease, cancer, diabetes, diarrhea, jaundice, malaria, urinogenital infection etc. In dispersing swellings of the joints from acute rheumatism and of the testes from suppressed gonorrhea. It also shows anti-oxidant, intertidal /pestecidal, anti-microfilaria, anti-lithic, heptoprotective activities etc. (Zafar, 1993). Mallotus is highly cross-pollinated and variations among the same species are limited. However, it is now well documented that some selections are rare and possess beneficial characteristics such as high yield, high oil content, drought resistance, photoperiod insensitivity, resistance/tolerance to major insect pests and diseases. This opens up the opportunities of breeding for hybrids. The current requirement in our country is to mitigate fatty oil import and produce our own cosmetics and pant-varnish through large scale cultivation of crops like *Mallotus*.

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Limitations for such activity are non-availability of quality planting materials seed, seedlings (Agro-Forestry Tree Data Base Literatures, 2009). Currently the plant is facing a threat of extinction due to destructive harvesting of plant parts for medicinal use as well as devastation of its natural habitat by deforestation. Besides, the conventional vegetative propagation methods for mass multiplication of this tree species are hampered due to low rates of seed germination (only-30%). Many rare and endangered plant species are propagated *in-vitro* because they do not respond well to conventional methods of propagation (Jaya Sharma, 2012). The present study was aimed to develop an effective protocol for indirect shoot regeneration and understand the effect of different plant growth regulators at various concentrations on *in-vitro* callus induction and indirect plant regeneration of *Mallotus philippenesis.*

MATERIALS AND METHODS

The research was conducted at the Department of Botany. Sarojini Naidu Govt. Girls Post Graduate (Autonomous) College Shivaji Nagar, Bhopal Madhya Pradesh (India).

Collection and authentication of plant material: The seeds were collected in the month of March, 2011 from mature tree growing inside the Botanical garden of BHEL College Bhopal and The plant were identified by Botanical survey of India, CRC (BSI) Allahabad, where voucher specimen code (1370-158-696) was deposited.

Surface sterilization procedure: Seeds were thoroughly washed under running tap water for 30 min then treated with 5% tween-20 for 5 minutes with constant stirring followed by 3-4 rinses in sterile distilled water and further treated with an antifungal agent (Bavistin) for 2 hours and were further with detergent for 10 min and rinsed 4-5 times tap water. Further sterilization procedures were carried out inside laminar air flow chamber, where seeds were surface sterilization through single dip in 70% (v/v) for half minute followed by three times rinses in sterile distilled water. There after mercuric chloride (0.1%) treatment was given to explants for 8 minutes followed by four times rinsed in sterile distilled water. Thereafter seeds were carefully transferred to be placed over sterile Petri dishes & were then inoculated into the culture establishment medium (Murashige, 1962), using sterile forceps under aseptic conditions. The seeds placed horizontally on the culture medium. The seedlings raised from the *in-vitro* cultures are used as explants for further experiment.

Selection of explants: Leaves, Cotyledons', Roots of 25 days old *in - vitro* raised seedlings were selected as explants for callus induction and indirect shoot initiation. Explants segment of small pieces were excised aseptically. All procedure is carryout in laminar air flow chamber.

Chemicals: Leaves, coteledons, roots induced from seedlings were cultured on MS basal medium supplemented with 3 % (w/v) sucrose (Sd-fine Chemicals, India) for callus induction. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.7 with 1N NaOH or 1N HCl before gelling with 0.8 % (w/v) agar. In all the experiments, the chemicals used were of analytical grade (Merck and SD-fine Chemicals, India). The medium was dispensed into culture vessels (Borosil, Mumbai, India) and autoclaved at 105 kPa at 125°C for 15 minute. The surface sterilized explants were placed vertically on the culture medium. All the cultures were incubated at 25±2°C under 16h light/8h dark photoperiod with irradiance of 45 - 50 µ mol/ m²/s photo synthetically active radiation (PAR) provided by cool white fluorescent tubes (Philip, India) and with 60 - 65 % relative humidity. All subsequent subcultures were done at four weeks intervals. Culture media consisted of MS (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Himedia, India) was evaluated for their effects on *in-vitro* growth and development of *M. philippeneesis*. For induction of shoots, explants were cultured on MS medium supplemented with different concentration of cytokinins, including BAP (mg/l), NAA (mg/l) and Kinetin (mg/l) either individually or in combination. Application of tissue culture to plant conservation in India has been largely restricted to economically important species However, the approach could usefully be extended to conserve all threatened plants so that vital biodiversity and the ecological network it sustains can be preserved (Jiten Chandra Dang, 2011). *Mallotus philippenesis* is categorized as a rare and endangered species and is on the of Endangered species for central eco region (Kadam, 2007).

Callus induction: Leaves, cotyledons, roots were excised and inoculated by horizontal orientation on the culture medium containing different concentration of BAP (.0.5-7 mg/l.), 24D (0.5-7.0mg/l), and KN (0.1-3.0mg/l). Explants were assigned randomly to each treatment and culture were kept under 16 h light/day photoperiod at 25±2°C.callus induction was assessed after process same different media composition for further experiment.

Shoot formation: Sub culturing of callus was carried out at regular intervals of thirty days. Visual observations of the cultures were taken for every transfer and the effects of different treatments were quantified on the basis of percentage of cultures showing response.

Root formation: Regenerated multiple shoots were separated (3-5 cm.) and inoculated in different rooting medium.

Acclimatization and hardening: Well rooted plantlets were separated from the culture tubes, washed and transferred to plastic cups containing soil + vermiculite + sand in 1:1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions. Rooted shoots showed the maximum percentage of survival.

Observations: Effect of various growth regulators (2-4D, BAP, KI) were observed. The different parts of in vitro raised seedlings (leaves, cotyledons, roots) of callus inductions and callus formations are recorded in further results. Leaves responded well for callus induction, which show friable grayish green fast growing callus, cotyledons show hard green with white border, root callus is white watery calli with watery exudates.

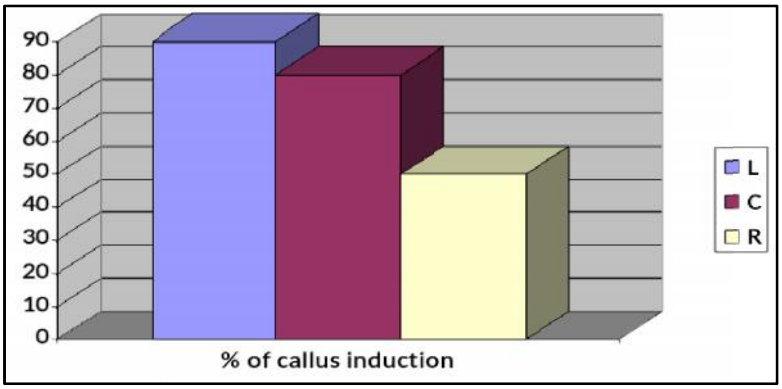
RESULTS AND DISCUSSION

Callus is dedifferentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue. Callus tissue is a good source of genetic variability and shoots formation. In vitro raised explants are the most suitable explants for callus induction. Among the different explants tested leaf explants was found to be best for callus initiation. Highest callus induction is shown in leaves (90%) with 2.0 mg/l 2 4-D or 3.0 mg/l BAP. MS medium supplemented with different levels of BAP, KN, 2 4 D were tried to induce callus induction from in vitro seedling segments of *Mallotus philippenesis*. Although leaf explants showed callus induction on higher concentration of BAP (4.0BAP mg/l) or (2.0 24D mg/l). However lower concentration of growth regulators failed to induce callus formation. callus tissue is a good source of genetic variability and adventitious shoot formation. From the two month old in vitro grown seedling plants the leaves are excised and are used as explants for the callus induction.

Explants segments (0.5-1.0cm) were inoculated on MS medium fortified with different concentration of auxins (2,4 D) singly and in combination with cytokinin's (BAP and Kinetin) gave varied callusing response.(Table 1). NAA, 2, 4-D along with BA were observed to be potent hormonal combination for profuse callus production from leaf explants. In which light green to dark green colour, nodular to fragile callus was formed. Similar callusing response was noted in *Justicia genderussa Diathus caryophyllus* (Agastian, 2006 and Rupali Mehta, 2007). Callus induction is a prerequisite for adventitious shoot formation and also for the other in vitro genetic improvement including induction of somaclonal variations and embryoids. Among the growth regulators tested BA induced maximum frequency for shoot regeneration in the present study BA along with NAA exhibited better morphogenesis. These findings were in line with previous reports in *Asteracantha longifolia* (Panigrahi, 2007).

Table 1. Morphological based percentage of callus induction from different explants

S.no	*Explants*	*% of callus induction*	*Colour and quality of callus*
1.	L	90%	Friable grayish green fast growing callus
2.	C	80%	Hard green with white border
3.	R	50%	White watery calli with watery exudates.



Graph 1. Percentage of callus induction from different explants

Plant growth regulator (mg/			Media	% of callus induction			Nature of callus
Concentration			Media	L	C	R	
24D	BAP	KI	M1	10	10	10	No response
0.5	0.5	0	M2	10	30	30	No response
1.0	0.5	0.5	M3	10	50	40	Light green, compact
2.0	0.5	0.5	M4	20	70	40	Light green, compact
3.0	0.5	0.5	M5	50	80	50	Light green, fragile
4.0	0.5	0.5	M6	60	75	50	Light green, fragile
4.0	1.0	0.0	M7	70	60	40	Friable light green
3.0	2.0	0.0	M8	80	50	20	Friable Grayish
2.0	3.0	0.0	M9	90	40	10	Green, compact
2.0	4.0	0.0	M10	70	40	10	Green, nodular, organogenic
3.0	5.0	0.0	M11	50	30	10	Creamish brown, fragile
4.0	6.0	0.0	M12	30	10	10	Brown, nodular
5.0	6.0	1.0	M13	30	10	10	Brown, nodular
6.0	7.0	1.0	M14	10	10	05	Light brown, organogenic
8.0	7.0	1.0	M15	10	05	05	Light brown, organogenic

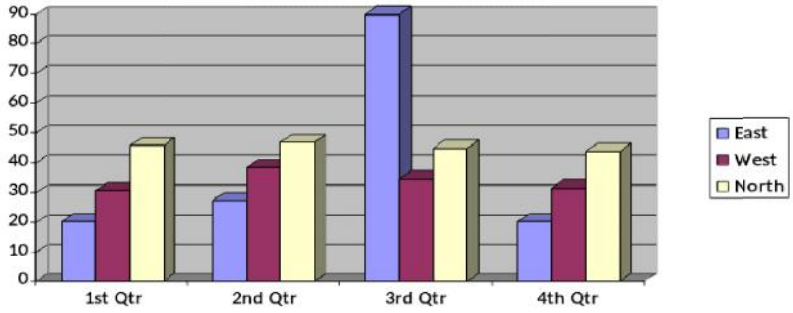


Table 1. Effect of different concentrations of plant growth regulators added to MS medium on induction of callus from leaf of *in vitro* grown *Mallotus philippenesis*

Plant growth regulator (mg/			Media	Degree of callus formation	Nature of callus
24D	BAP	KI	M1	C	No response
0.5	0.5	0	M2	C	No response
1.0	0.5	0.5	M3	C+	Light green, compact
2.0	0.5	0.5	M4	C+	Light green, compact
3.0	0.5	0.5	M5	C++	Light green, fragile
4.0	0.5	0.5	M6	C++	Light green, fragile
4.0	1.0	0.0	M7	C+++	Friable light green
3.0	2.0	0.0	M8	C+++	Friable Grayish
2.0	3.0	0.0	M9	C+++	Green, compact
2.0	4.0	0.0	M10	C+++	Green, nodular, organogenic
3.0	5.0	0.0	M11	C+++	Creamish brown, fragile
4.0	6.0	0.0	M12	C++	Brown, nodular
5.0	6.0	1.0	M13	C+	Brown, nodular
6.0	7.0	1.0	M14	C+	Light brown, organogenic
8.0	7.0	1.0	M15	C+	Light brown, organogenic

Observation: After 4 weeks; C- No response C+- Poor callus; C++ - Moderate callus C+++ - Profuse callus.

A. In-vitro raised seedling B. Callus induction C. Shoot induction D-E. Root induction F. Acclimatization and hardening. G. Survival plant (After 6 month)

Table 2. Indirect shoot organogenesis from callus obtained through leaf explants of *M.Philippenesis*

Plant growth regulator (mg/L)	Media	Regeneration frequency (%)	Mean number of shoots/ callus	Mean shoot length (cm)
0.5 BAP+0.1 NAA	S1	14.0±0.08	1.5±0.14	2.6±0.14
1.0 BAP+0.5 NAA	S2	40.0±0.54	3.8±0.44	4.7±0.24
2.0 BAP+1.0 NAA	S3	49.0±0.88	2.0±0.33	5.8±0.35
2.0 BAP+1.0 NAA+1.0 KI	S4	26.0±1.45	1.6±0.22	2.2±0.21
3.0 BAP+2.0 NAA+2.0KI	S5	15.0±0.25	1.05±0.15	0.5±0.11

Table 3. Root organogenesis of *in vitro* derived shoot

Plant growth regulator (mg/L)	Media	Mean number of roots /shoot	Mean length of root (cm)	Morphology of root
10g/ sucrose	R1	-	-	-
1mg/lIBA+20g/ sucrose+100mg/l AC	R2	2.0±0.55	2.2±0.44	Thin, short
1mg/l NAA+1mg/l IBA+20g/ sucrose+200mg/l AC	R3	5.5±0.44	5.2±0.85	Thin, long
2mg/l NAA+2mg/l IBA+40g/ sucrose+400mg/l AC	R4	3.0±1.07	2.5±1.44	Thin, long

Indirect shoot regeneration through callus phase obtained from leaf explants was earlier reported in many plants like *Spillanthus acmella* (Saritha, 2007), and *Justicia gendarussa* (Agastian, 2006). The effectiveness of 2,4-D and NAA in combination with cytokinins in inducing callus might be due to their role in DNA synthesis and mitosis (Murashige, 1962). In 2, 4-D supplemented medium light brown to dark brown colour fragile callus formation takes place. Brown colour of the callus showed sensitivity of plant tissues to 2, 4-D. This is in agreement with earlier reports in *Ipomea aqualtica* (Nagendra Prasad, 2006). Plant parts especially leaves are desirable explants for in vitro improvement because of regeneration from these explants would preserve the genetic homozygosis of the parent genotype. The presence of cytokinin along with auxin is necessary for indirect adventitious shoot induction .the induction of callus and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators such as BA, KN, NAA, in the culture medium. Among the growth regulators tested BAP (2.0 mg/l) and (1.0 mg/l) induced maximum frequency of shoot regeneration (Table 2).

Root initiation in the 6-8 week old shoots were cultured on half strength MS basal medium supplemented with sucrose and different concentration of PGRs were tested IBA and NAA with active charcoal. The complete rooted plantlets were washed to free from agar and dipped in 0.2% bavistin fungicide for 5-10 min and potted in small plastic containers containing sterilized soil rite, the plantlets were covered with polythene bags to maintain high humidity. These were acclimatized at 25±2°C under 16 photoperiod and watered regularly. After 3-4 weeks the polythene bags were removed and established plantlets were transplanted to earthen pots in a greenhouse (Table-3).Best root formation occurs in MS media supplemented with growth regulator 1mg/l NAA, IBA 20 gram, 200 mg/l AC (activated charcoal) at (5.5±0.44) number of root and best length of root at 5.2±0.85.well rooted plantlets were separated from the culture tubes, washed and transferred to polybags containing soil+ vermiculite + sand in 1:1:1 ratio. For hardening well developed and elongated shoots were transferred to MS Medium supplemented with NAA, IBA and activated charcoal to active rooting to the shoots. The parameters for root induction is shown in (Table 3).

Finally the hardened plantlets were transferred to field conditions. Rooted shoots the maximum % of survival.

Conclusions

A callus culture system offer many advantages as a model system for several biological investigations. Hence, the present study of indirect plant regeneration via callus phase is most effective in micropropagation and conservation of this economically and medicinally important medicinal tree *M.philippenesis*. In this study we reported for first time a protocol for the successful callus induction direct regeneration from leaf explants and regenerated through callus in *Mallotus philippenesis* which would provide more source of medicine. Sehgal and Abbas (1996) induced triploid plants from the endosperm culture of *Mallotus philippenesis*. The frequency of callus induction and regeneration from leaf explants described here was high enough to encourage us to carry out conservation of plant, genetic transformation and improvement of oil and secondary metabolites quality and quantity.

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