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Effect of Environmental Factors on Successful *in vitro* Morphogenesis of *Madhuca longifolia*

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Authors' contributions

This work was carried out in collaboration among all authors. Author FS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors DB and SS managed the analyses of the study. Authors SP and TG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Madhuca longifolia is a commercially important tree species commonly known as mahua. The livelihood of large populations of tribal people depends on collection of its flowers and seeds. Almost all the parts of Mahua are utilized in diversified uses like in industry as artificial sweetner, biodiesel, food products, in soap industry etc. In the present study, a successful attempt was made to establish *in vitro* cultures of Mahua from nodal segments and factors influencing *in vitro* morphogenesis were evaluated as propagation through seeds and cuttings encounters problems. Axillary bud break (64.44%) was successfully achieved by culturing nodal segments on Murashige and Skoogs (MS) medium supplemented with 3 mg l^{-1}Benzyladenine (BA) in nodal explants collected during the months ofJuly-September (rainy season).Shoot multiplication with maximum number of shoots, maximum number of leaves and longest shoots was achieved on MS medium supplemented with 3 mg l^{-1} BA when a subculture cycle of 30 days was followed. On MS medium supplemented with 2 mg l^{-1} Indole-3-Butyric Acid (IBA), *in vitro* excised shoots were successfully rooted (55.55%) after 40 days. A two step method was employed for successful hardening of rooted plantlets. Firstly, the plantlets were transferred for one week in 1/2 strength of MS liquid medium.

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Then, the plantlets were transferred to root trainers containing soilrite soaked with inorganic salts of ½ strength MS medium. The hardened plantlets were acclimatized firstly in a mist chamber and then in polybags in shade house. The present study provides an effective means for *in vitro* shoot regeneration and plantlet formation through nodal segments of *Madhuca longifolia*, a commercially important tropical tree with multifarious uses.

Keywords: In vitro; mahua; micropropagation; morphogenesis; nodal segment; season; sprouting.

ABBREVIATION

ANOVA : Analysis of Variance
BA : N6- benzyladenine
CD : Critical Difference
CV : Coefficient of Variance
DF : Degree of Freedom
FYM : Farmyard Manure
IAA : Indole -3-Acetic Acid
IBA : Indole-3-Butyric Acid
MS : Murashige and Skoogs (1962) medium
NAA : 1-Napthalene Acetic Acid
SE : Standard Error

1. INTRODUCTION

Madhuca longifolia var. *latifolia* is commonly known as Indian Butter tree or 'Mahua' and it belongs to Family *Sapotaceae*. The species is predominantly distributed in central, southern and northern regions of India, Burma and Sri Lanka. Trees of mahua can be found in dry sal and teak forests and dry mixed deciduous forests. This tree is found in central parts of India and sub mountainous parts of the Himalayan region [1]. From the flowers and seeds of one mature tree, an individual can generate income of about Indian Rs. 1500 (20.35 USD). This is in addition to other tangible and intangible benefits from it [2]. Three major requirements of life viz., food, fodder and fuel are provided by this versatile and multipurpose forest species [3].

Mahua occupies important place in the diet and economy of tribal people. The corolla of mahua flowers are edible and form an important constituent of tribal diet. It is a multipurpose forest tree and more than 70 % of tribal population is engaged in collection, drying and sellings of mahua flowers [4]. During recent years renewed activities can be observed in the field of alternative fuels, due to rapid decrease in world petroleum reserves. Mahua oil is one of the main alternative sources being explored for biodiesel [5-7].

Mahua is most commonly propagated through seeds. However, the seeds are recalcitrant and susceptible to fungal attack [8]. Fresh seed has a high percentage of fertility, but the seed quickly loses its viability during storage and is much subject to insect and fungus attack. Fruits fall on the ground after maturation in July (monsoon period) and seeds are exposed after decay of fleshy covering. Wild animals disperse the seeds by eating the fruits. The season for seed collection is short and in the absence of organized harvesting, a considerable portion of crop is lost. Viability of *Madhuca longifolia* seeds cannot be maintained in long-term storage. The freshly mature seeds are desiccation-sensitive (recalcitrant) and germination percentage starts to decline below 35% moisture content. Seeds are also chilling sensitive, damage may occur even at 15°C.

Vegetative propagation through stem cuttings was unsuccessful [9]. The failure of rooting in stem cuttings of mahua is attributed to anatomical and physiological barriers that hindered the rooting. Vegetative propagation method for mahua multiplication has been developed with limited success through grafting and air layering [10]. As a result, opportunities for its propagation and improvement are limited. Micropropagation has a great potential in cloning of selected improved trees for raising new plantations to increase forest productivity [11-12]. Limited literature is available for *in vitro* propagation of mahua [13-16].

The present study deals with factors affecting successful *in vitro* culture establishment and plant regeneration via micropropagation method in *Madhuca longifolia* var. *latifolia* through axillary bud proliferation of nodal segments collected during different months of the year.

2. MATERIALS AND METHODS

2.1 Surface Sterilization of Nodal Segments

Ripe fruits were collected from selected phenotypically superior trees of *Madhuca longifolia* located in Sarguja district of Chhattisgarh state, India. Fruit pulp was removed manually and seeds were dissected out. They were thoroughly washed with water and then

sown in polythene bags containing mixture of soil, sand and FYM. Nodal segments from these seedlings were used as explants. The explants were excised from germinated seedlings every month throughout the year. They were washed for 15-20 min with 0.5% aqueous solution of Cetrimide (SRL, Pvt. Ltd. Maharashtra, India). Subsequently, these explants were continuously washed with distilled water so as to remove soap solution followed by treatment with 0.5% aqueous solution of Bavistin ®, a systemic carbendazim fungicide (BASF, India Ltd, Mumbai, India) and 0.5% solution of streptomycin. Under aseptic conditions, the buds were surface sterilized with (0.1%) aqueous mercuric chloride ($HgCl_2$) solution for 5 min. They were thoroughly rinsed with sterile distilled water to remove traces of sterilizing agent. The sterilized buds were inoculated on MS medium supplemented with 3 mg l^{-1}BA.

2.2 *In vitro* Shoot Multiplication

Sprouted buds with 1-2 number of shoots was inoculated on MS (Murashige and Skoog, 1962) semi-solid medium supplemented with 3 mg l^{-1} concentration of different cytokinins (BA, Kinetin and Zeatin).

2.3 *In vitro* Rooting and Hardening of Plantlets

In vitro raised shoots measuring 5-10 cm growing on multiplication medium were excised and cultured on MS, medium supplemented with various concentrations of different auxins (control, IBA, IAA and NAA). Rooting was initiated after 15-20 days and completed in 40 days.The rooted shoots were transferred to 1/2 strength of MS liquid medium on filter paper bridges. After 10-15 days, plantlets were transferred to root trainers containing autoclaved soilrite soaked with inorganic salts of ½ strength of MS medium.

The inorganic salts were obtained from HiMedia laboratories Pvt. Ltd., India and plant growth regulators and vitamins from SRL Laboratories Pvt. Ltd., India. The medium was fortified with 3 % sucrose and solidification of medium was achieved through 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving for 15 min at 1.06 kg cm^{-2} (121ºC).

2.4 Culture Condition

The cultures were maintained at 25 ± 2°C with light and dark cycle of 16 and 8 hours photoperiod provided by cool white 40 W fluorescent tubes (Philips, India) (100–140 µmol m−2 s−1).

3. EXPERIMENTAL OBSERVATIONS AND STATISTICAL ANALYSIS

The data were analyzed with OPSTAT statistical package according to a completely randomized design. The data recorded for various parameters during the present study were subjected to single factor analysis of variance (ANOVA). F-test was used to ascertain the significance of the data. For comparing means of various treatments Critical Difference (C.D.) was computed at *P* = .05 [17]. For culture establishment and *in vitro* shoot multiplication, 15 explants per treatment were inoculated in three replications. For *in vitro* rooting, 9 shoots per treatment were inoculated in three replications. The observations for sprouting percentage, number of shoot formation, number of leaf and shoot length were recorded 30 days after inoculation. Data of rooting percentage was recorded after 40 days of inoculation.

4. RESULTS

4.1 *In vitro* Axillary Bud Break

Sprouting in the buds of *Madhuca longifolia* was initiated after 15-20 days of inoculation and the data on axillary bud break was evaluated after 30 days of inoculation. Highly significant effect of month in which explants were collected was observed for sprouting percentage, number of shoots, number of leaves and shoot length (cm) (Table 1, Fig. 1 a-d). The data of Tables 2 and 3 represents the mean of all the parameters of growth.

4.1.1 Sprouting percentage

Significant effect of months (August) was observed for axillary bud proliferation (sprouting)through nodal segments of *Madhuca longifolia* after 30 days of inoculation. The maximum sprouting (64.44%) was observed in the month of August followed by July (42.11%). Therefore, July and August (rainy season) were the best months to initiate *in vitro* cultures of mahua. In the hot months (summer) of May (6.67%) and June (2.22%) minimum sprouting of buds was achieved (Table 2).

4.1.2 Number of shoots per nodal segment

Significant effect of months (August) was observed for shoot formation in the nodal segments of *Madhuca longifolia* after 30 days of inoculation. The maximum numbers of shoots (2.98) were obtained in the month of August. Shoot formation in the nodal segments inoculated in the months of May- June was reduced (Table 2).

4.1.3 Number of leaves

Significant effect of months (August) was observed for leaf formation in the shoots of *Madhuca longifolia* after 30 days of inoculation. The maximum number of leaves (3.82) were obtained in the month of August followed by the months of November (2.56), October (2.47) and December (2.04). Minimum number of leaves (0.36) was formed on the shoots of mahua in the month of May (Table 2).

4.1.4 Shoot length (cm)

Significant effect of months was observed for shoot length in *Madhuca longifolia* after 30 days of inoculation. The maximum shoot length (5.46 cm) was obtained in the month of August followed by the months of July (4.08 cm) and October (2.65 cm). Minimum shoot length (0.40 cm) was obtained in the month of June (Table 2).

4.2 *In vitro* Shoot Multiplication

Multiple shoots were obtained from single nodal segments of mahua on MS medium supplemented with different cytokinins (Fig. 2 a-c). Out of the three cytokinins, BA was found to be the best cytokinin for shoot multiplication. Maximum shoot formation with maximum number of leaves and shoot length was obtained on MS medium supplemented with 3mgl^{-1}BA. In the data presented in Table 3, different cytokinins show significant effect for number of shoots. The maximum number (2.80) of shoots were obtained on MS medium supplemented with BA which was more than 1.33 and 1.60 number of shoots multiplied on MS medium supplemented with kinetin and zeatin respectively. Cytokinins also exhibited significant differences for number of leaves formed on shoots of mahua with maximum shoot length. The maximum (4.35) number of leaves and shoot length (4.78 cm) was obtained on medium containing 3 mg l^{-1} BA which was statistically on par with the other cytokinins (kinetin or zeatin) (Table 4).

4.3 *In vitro* Rooting and Hardening

The regenerated shoots were transferred to rooting media for root induction. MS media containing 0.5, 1.0 or 2.0 mgl^{-1} IBA, IAA or NAA were tested. After 40 days of inoculation, 55.55% rooting was obtained in the shoots inoculated on 2 mgl^{-1} IBA. (Table 5, Fig 3 a-f). No rooting was obtained on other auxins (IAA or NAA).

Table 1.Analysis of variance for sprouting and growth behavior of nodal segments of *Madhuca longifolia* in different months

Parameters	Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Sprouting (%)	Replication	2	92.718			
	Months	11	10,074.97	915.906	4.832	0.00083
	Error	22	4,170.38	189.563		
	Total	35	14,338.07			
Numbers of shoot	Replication	2	0.582			
	Months	11	19.361	1.76	3.976	0.00285
	Error	22	9.74	0.443		
	Total	35	29.683			
Numbers of leaf	Replication	2	0.543			
	Months	11	32.01	2.91	3.526	0.00573
	Error	22	18.157	0.825		
	Total	35	50.711			
Shoot length (cm)	Replication	2	1.159			
	Months	11	69.561	6.324	7.537	0.00003
	Error	22	18.458	0.839		
	Total	35	89.178			

Table 2. Effect of different months on response of axillary bud proliferation in nodal segments of *Madhuca longifolia.* The cultures were scored 30 days after inoculation

Months	Sprouting (%)		Number of shoots		Number of leaves		Shoot length (cm)	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
July	42.11	4.39	1.11	0.11	1.56	0.28	4.08	0.59
August	64.44	5.88	2.98	0.10	3.82	0.36	5.46	0.58
September	35.56	5.88	1.44	0.25	1.76	0.25	2.64	0.19
October	31.11	13.52	1.82	0.53	2.47	0.38	2.65	0.33
November	20.00	10.18	1.75	0.52	2.56	0.92	2.07	0.26
December	22.22	9.69	1.40	0.57	2.04	0.91	2.21	0.51
January	17.78	9.69	0.85	0.55	1.09	0.60	1.46	0.83
February	17.78	9.69	0.73	0.42	1.33	0.20	1.60	0.81
March	13.33	3.85	0.89	0.11	1.07	0.07	1.59	0.23
April	8.89	4.44	0.45	0.22	1.09	0.58	1.09	0.55
May	6.67	3.85	0.33	0.33	0.36	0.36	0.50	0.50
June	2.22	2.22	0.33	0.33	0.40	0.40	0.40	0.40
C.D. (0.05)	23.46		1.13		1.55		1.56	
SE(m)	7.95		0.38		0.53		0.53	
SE(d)	11.24		0.54		0.74		0.75	
C.V.	58.57		56.71		55.80		42.68	

Table 3. Effect of season of collection of nodal explants of *Madhuca longifolia* on *in vitro* morphogenetic response

Months	Sprouting (%)	Type of response (After 30 days)**
July	42.11	More response for bud break and develop healthy shoots
August	64.44	Maximum morphogenetic response and healthy shoot buds formed
September	35.56	Healthy shoot buds formed
October	31.11	As above
November	20.00	Few shoot buds sprouted from the axil
December	22.22	As above
January	17.78	As above but did not support healthy growth
February	17.78	As above
March	13.33	Nodal segments turned brown and necrotic with fungal contamination and phenolic exudation
April	8.89	Excessive browning of few explants and become necrotic
May	6.67	Excessive browning of maximum explants and become necrotic
June	2.22	As above

*** On MS medium supplemented with sucrose (3%) and 3 mg l^{-1} BA*
Data represents the mean of three replications

Table 4. Effect of different cytokinins on *in vitro* shoot multiplication in *Madhuca longifolia*

Treatment	Number of shoot	Number of leaves	Shoot length (cm)
BA	2.80±0.12**	4.35±0.15**	4.78±0.22**
Kinetin	1.33±0.24	2.84±0.10	3.44±0.11
Zeatin	1.60±0.12	2.93±0.18	2.67±0.19
C.D.(0.05)	0.56	0.72	0.82
SE(m)	0.14	0.18	0.20
SE(d)	0.20	0.25	0.29
C.V.	12.58	9.17	9.72
DF	2.00	2.00	2.00
Sum of Squares	3.66	4.28	6.83
Mean Squares	1.83	2.14	3.42
F-Calculated	31.69	22.35	27.44
Significance	0.00	0.01	0.00

*** The growth of shoot culture is healthy*

Table 5. Effects of different types of auxins in different concentrations on *in vitro* rooting in *Madhuca longifolia* after 40 days of inoculation

Auxins	Concentration (mgl^{-1})	Days of rooting response	Mean percentage of rooting and morphology of cultures
IAA	0.5	60	Callusing at the basal end and slight browning of shoots at base
	1.00	60	As above
	2.00	60	As above
IBA	0.5	40	33.33 %
	1.00	40	44.44 %
	2.00	40	55.55%
NAA	0.5	60	Callusing at the basal end and slight browning of shoots at base
	1.00	60	As above
	2.00	60	As above

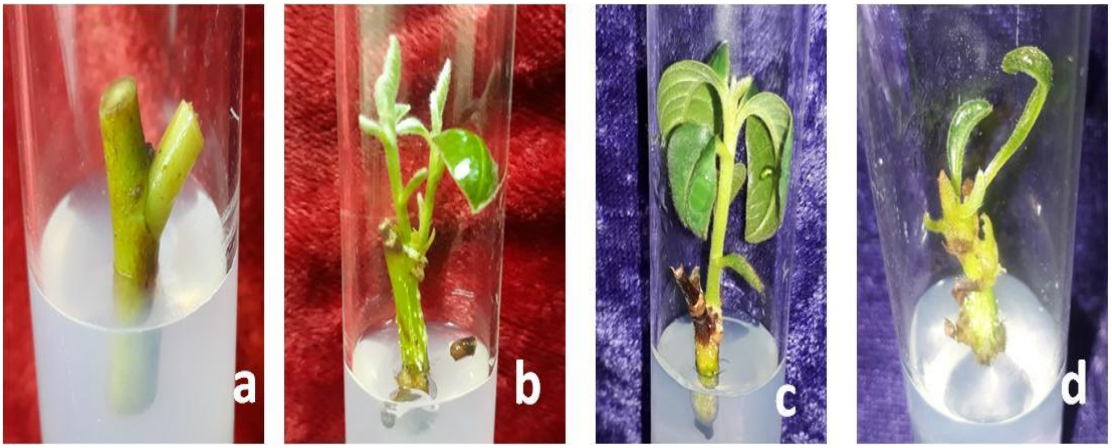

Fig. 1. In vitro culture establishment of Madhuca longifolia after 30-40 days of inoculation: (a) Inoculation of nodal segment, (b) Shoot formation in the month of July, (c) Shoot formation in the month of August, (d) Shoot formation in the month of March

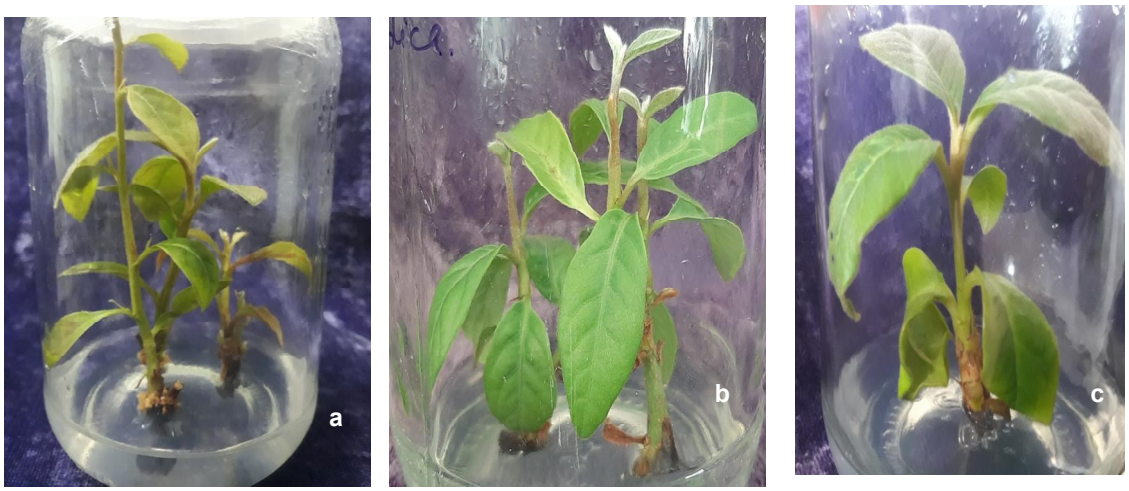

Fig. 2.*In vitro* shoot multiplication in *Madhuca longifolia* on MS medium supplemented with (a) 3 mgl^{-1} BA, (b) 3 mgl^{-1} Kinetin, (c) 3 mgl^{-1} Zeatin after 40 days of inoculation

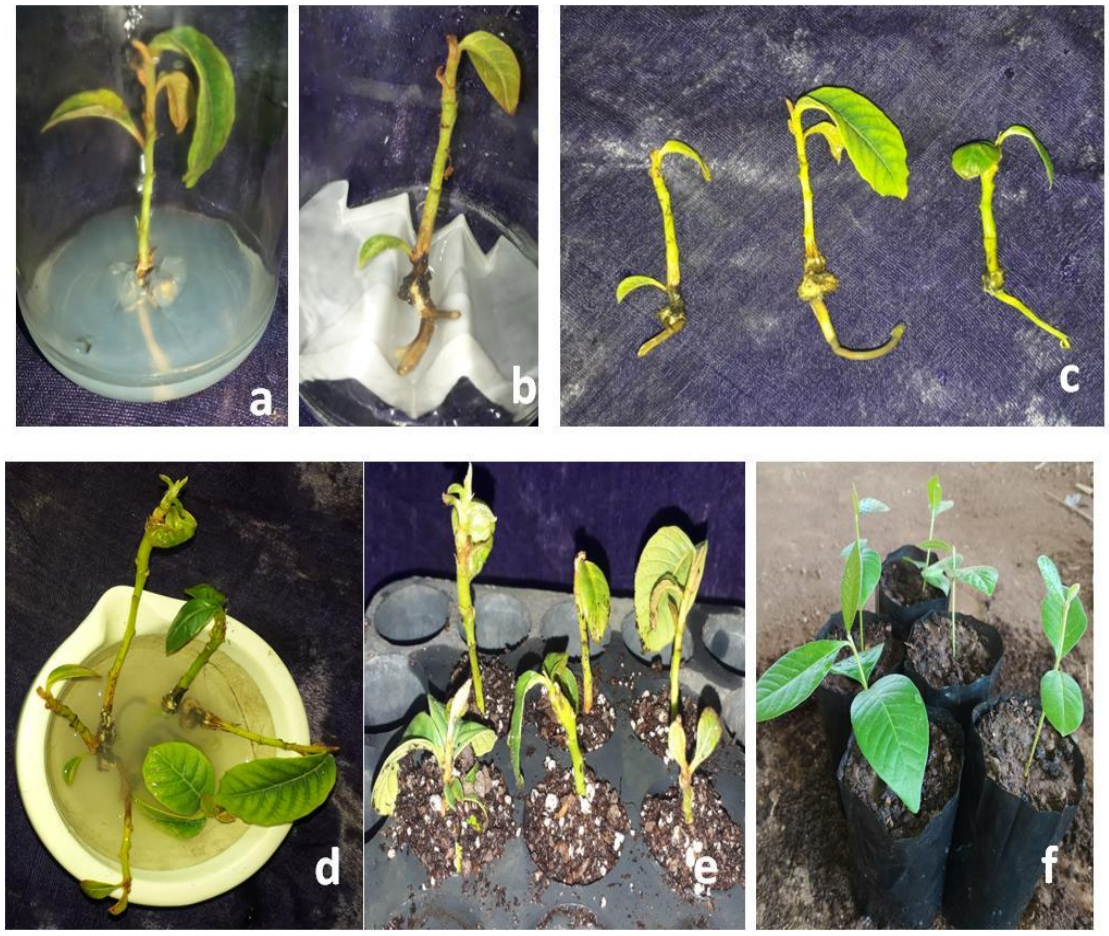

Fig. 3.*In vitro* rooting and hardening in *Madhuca longifolia* (a) rooting on MS medium supplemented with 2 mg l^{-1} IBA, (b) transfer of shoots on ½ strength of MS medium, (c) rooted plantlets, (d) treatment of plantlets with 0.2 % bavistin, (e) hardening of plantlets in soilrite in root trainers, (f) transfer of plants to in shade house

The hardening of rooted plantlets was started by transferring to the plants to 1/2 strength of MS liquid medium. After 10-15 days, the plantlets were washed thoroughly with tap water after they attained a height of 8 to 9 cm, to remove the adhering agar from the roots and were transferred to root trainer containing soilrite. The plantlets were irrigated only with ½ MS solution containing macro and micro nutrients devoid of iron and kept in culture room for 3-4 weeks and then transferred to polythene bags. The rooted plantlets were planted in polybags containing sand + soil + FYM (1:1:1) and maintained in a temperature controlled mist chamber with 28 ± 2^0C temperature and relative humidity of 75%.After 20 -25 days the plantlets were transferred to shade house for acclimatization to outside environmental conditions.

5. DISCUSSION

Mahua is an useful tropical tree growing naturally in deciduous forests, all over the world, especially Asian and Australian countries[18]. This plant is economically important because of its role, in yielding county liquor from edible succulent corolla and oil from the seeds for many purposes including biodiesel[19].Every part of mahua plant possesses some medicinal properties due to presence of some bio-active compounds, either in small or large proportion [20]. Pure plantations of this species have already been tried in Madhya Pradesh, Maharashtra, Tamil Nadu and Punjab (India) by stump planting [21].

Fresh seeds of mahua have high percentage of fertility, but the seed quickly loses its vitality

during storage and is much subject to insect and fungus attacks [10]. The vegetative propagation methods for mahua multiplication through grafting and air layring have been reported with varying success [22-25,10]. But there are only very few papers on propagation methods through plant tissue culture for mahua [13-16].

In the present paper, it was found that successful *in vitro* culture initiation and culture establishment was significantly affected by season (month) of explant collection. When the nodal segments were collected during the months from March to June (summer season), the axillary bud break obtained was minimum and the nodal segments turned brown and necrotic with fungal contamination and phenolic exudation. In the nodal segments collected during July-September (rainy season), maximum morphogenetic response was obtained. This was followed by months of October- February (winter season). The active growing season of mahua is rainy season when new flush of semi evergreen or evergreen leaves is obtained. It is a well known fact that active growing season is most effective for initiation of *in vitro* cultures and establishment of contamination free aseptic cultures [26]. During the active growth period in rainy season, the environmental conditions of temperature and humidity are most favourable. This may be the reason for best response in terms of aseptic culture initiation and establishment in rainy season (July to September). Similar findings have been reported in *Cinnamomum tamala* [27].

There are reports that the percent of bud break is greatly influence by the different seasons in which the explants were inoculated. Significant effect of explanting season on percent bud break was noticed in *Celastrus paniculatus* and highest percentage of bud break (90%) was recorded in the explants cultured between April to July [28]. Similar results were also obtained in *Tylophora indica* [29] and in *Aegle marmelos* [30].

In the present study, buds were effectively proliferated on the medium containing BA. Similarly, Rout and Das [13] reported that for both apical and axillary meristems, BA was found to be more effective for bud proliferation than kinetin. Our results are in contradiction to Bansal and Chibbar [15] where bud break was obtained on medium supplemented with kinetin. The beneficiary role of BA on bud break has been reported for many other tree species e.g. *Wrightia tomentosa* [31], *Clerodendrum colebrokiianum* [32] and *Crataeva nurvala* [33]. Presence of cytokinin in the medium leads to the promotion of bud differentiation and development. BA is one of the cytokinins, which is usually used in the culture establishment of a wide range of plant species.

Combination of BA and NAA were needed for shoot multiplication and 2.30 numbers of shoots were obtained in Mahua [13]. But in the present study 2.8 number of shoots were obtained on medium containing only BA without NAA. Similarly for other species also Kn and zeatin have been found less effective than BA for shoot multiplication, viz., in *Uapaca kirkiana* [34] and *Lens culinaris* [35]. Superiority of BA over Kn has been reported and discussed in relation to shoot multiplication of trees by Bonga and Von Aderkas [36]. The superiority of BA over other sources of cytokinins has been reported earlier and attributed to the group localized at N_6 position of the cytokinins [37-38]. BA, because of its artificial nature, does not get readily metabolized (degraded) in the plant tissue [39].

In earlier publications on mahua, *in vitro* rooting was tried using different auxins and IBA was screened out as the most suitable auxin for rooting [13,15]. Similarly, in the present study, rooting was observed only on IBA supplemented medium. In plants, auxins endogenous or applied exogenously are involved in cell division, elongation and in cell wall synthesis. Roots are mostly induced in the presence of suitable auxins in the medium during *in vitro* propagation of any species. The auxins stimulate root development by inducing root initials that differentiate cells of the young secondary phloem, cambium and pith tissue [40]. In literature also there are reports of IBA being more effective than IAA and NAA [41]. IBA is reported to be more effective as it efficiently induces lateral roots at concentrations that only minimally inhibit root elongation [42]. Therefore, IBA is also commercially employed [43]. After step wise gradual successful hardening and acclimatization, plants are growing well in polybags.

6. CONCLUSION

The nodal segments collected in different seasons (months) differed in their *in vitro* response to shoot proliferation and culture establishment. Rainy season was found to be most suitable for *in vitro* culture establishment of *Madhuca longifolia*. Different cytokinins were also screened and benzyladenine was found to be effective cytokinin for *in vitro* shoot induction

and multiplication. IBA at 2 mg l^{-1} was found to be most suitable auxin for rooting of mahua shoots. Thus, the present paper reports efficient method for *in vitro* shoot regeneration and plantlet production of mahua.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers because we do not intend to use these productsas an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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