Effect of explant source and growth regulators on *in vitro* callus induction and organogenesis of *Melia azedarach* L. trees

تأثير مصدر الجزء النباتي ومنظمات النمو في استحثاث الكالس وتوليد الأعضاء في أشجار السبحبح *Melia azedarach* L. خارج الجسم الحي

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Abstract

***Melia azedarach* L. is one of the important plants because it's a good source of natural compounds that have insecticide and antimicrobial effect. The main aim of this research is to investigate the effect of explants source and plant growth regulators on *in vitro* callus induction and regeneration of organs from it. Callus was induced from nodes, internodes from one-year-old seedlings and seeds of *Melia* plant by culturing them on MS medium supplemented with α-naphthalene acetic acid (NAA) 0.0, 0.1, 0.2, 0.3, or 0.4 mg/L and 6- benzyl adenine (BA) 0.0, 1.0, 2.0, 3.0, or 4.0 mg/L, then shoot regeneration from callus was occurred. Results showed that there was a different response from explants towards callus induction and adventitious shoots formation according to plant growth regulators combination. Seeds gave superior percentage for callus induction 24.4% compared with node and internode 15.6, 12.8% respectively. Combination of 0.3 mg/L NAA + 3.0 mg/L BA was the best for callus induction in all explants 86.6% . Shoot regeneration was achieved in 0.3 mg/L NAA + 4.0 mg/L BA and 0.4 mg/L NAA + 4.0 mg/L BA for callus from seeds and internodes respectively, while the combination 0.3 mg/L NAA + 3.0 mg/L BA or 0.4 mg/L NAA + 3.0 mg/L BA was the best for node callus. The shoots were rooted well in MS + 0.25 mg/L NAA . Rooted plantlets were acclimatized in small plastic pots filled with peat moss: river soil (1 :1 v/v), then transferred to the soil.**

Key words: Organogenesis, BA, NAA, *in vitro*, callus, explants, *Melia azedarach*

المستخلص

تعد أشجار السبحبح *Melia azedarach* L. من النباتات المهمة كونها مصدرا جيدا للمركبات الطبيعية ذات التأثير الكامن كمضادات حشرية أو مضادات ميكروبية. ان الهدف الرئيسي من هذا البحث هو دراسة تأثير مصدر الجزء النباتي المستخدم ومنظمات النمو في استحثاث الكالس وتوليد الأعضاء منه خارج الجسم الحي، إذ تم تحفيز إنتاج الكالس من عقد وسلاميات مأخوذة من شتلات بعمر سنة واحدة وبذور من أشجار السبحبح بزراعتها على الوسط الغذائي MS المجهز بتوليفات من منظمي النمو NAA0.0 و 0.1 و0.2 و 0.3 و 0.4 ملغم/لتر و BA 0.0 و 1.0 و 2.0 و 3.0 و 4.0 ملغم/لتر، ومن ثم إخلاف الأفرع العرضية منه. أظهرت النتائج اختلاف استجابة الأجزاء النباتية المستعملة في الدراسة في تنشئة الكالس وتوليد الأفرع العرضية باختلاف توليفة منظمي النمو المتضمنة في الوسط الغذائي ، فقد أعطت البذور أعلى نسبة لتكوين الكالس بلغت 24.4% مقارنة بالعقد والسلامية 15.6 ، 12.8% على التوالي. وفيما يخص تأثير توليفة منظمي النمو فقد كانت التوليفة 0.3 ملغم/لتر NAA + 4.0 ملغم/لتر BA هي الأفضل لتكوين الكالس في جميع الأجزاء النباتية إذ أعطت أعلى نسبة بلغت 86.6%. وأعطى كالس البذور والسلاميات أكبر عدد من الأفرع العرضية عند استخدام التوليفتين 0.3 ملغم/لتر NAA + 4.0ملغم/لترBA و 0.4 ملغم/لتر NAA + 4.0 ملغم/لتر BA على التولي، في حين أعطى كالس العقد أكبر عدد عند تضمين الوسط بـ 0.3 ملغم/لتر NAA + 3.0 ملغم/لتر BA أو 0.4 ملغم/لترNAA + 3.0 ملغم/لتر BA. تم تجذير الأفرع العرضية المتكونة بزراعتها في وسط MS المجهز بـ 0.25 ملغم/لتر NAA. وتمت أقلمة الأفرع المجذرة بزراعتها في أصص بلاستيكية صغيرة حاوية على وسط مكون من بيتموس وتربة نهرية بنسبة (1:1 حجم/حجم) ونقلت بعدها إلى التربة.

الكلمات المفتاحية: توليد الأعضاء، NAA،BA ،خارج الجسم الحي، الكالس، مزدرع، نبات السبحبح

Introduction

Melia azedarach, "chinaberry" or "persian lilac" is known in Iraq as 'Sabahbah tree',which belongs to the family Meliaceae. It is an Asiatic multipurpose tree, of worldwide cultivation, mainly for its ornamental beauty and landscape value. It withstands cooler climates better than its related tree "neem" *Azidarachta indica* A.

Juss. and it is a fast-growing species with long lasting wood, used as a component of agro forestry systems with inter cropping annual species [1]. Moreover, Chinaberry is also important in timber production and a good source of natural compounds such as azadirachtin - which is found only in seeds- with potent insecticide and antimicrobial action [2,3].

Propagation of *Melia azedarach* by conventional methods of breeding is time-consuming and often inefficient owing to low-seed set, poor germination and great variability. The advantage of using tissue culture technique to propagate this plant can be applied in order to overcome these barriers and may prove to be a short cut in reducing the normal life cycle which may take several months [4].

In vitro shoot regeneration of *Melia azedarach* L. has been previously established by[5,6,7,8,9] from an organogenesis pathway via callus formation. The development of suitable procedures for plant regeneration through organogenesis is one of the main prerequisites for the potential applications of clonal propagation, genetic transformation and *in vitro* preservation of germplasm of woody plants [10].

Callus is defined as tissues constituted by differentiated cells, which develop in response to a chemical or physical lesion, under determinate hormonal conditions. It can be obtained from a tissue fragment and have the ability to differentiate into tissues, organs and even embryos,which being able to regenerate whole plants [11]. Several internal and external factors appear to influence callus induction and growth like the exogenous supply of growth regulators which was frequently necessary in organogenesis [12]. This necessity refers to the type, concentration, ratio of auxin/cytokinin and the source of explants. According to [13] , the combination of auxins and cytokinins promote cellular differentiation and also organogenesis. Among the growth regulators used in callus induction, 2,4-D(2,4-dichlorophenoxyacetic acid), NAA (1-naphthaleneacetic acid), BAP (6-benzylaminopurine) and TDZ (thidiazuron) are the most important.

Also the knowledge of the effect of explant source on regeneration is very important, not only for *Melia* but also for other plants. This study was intended to determine the optimal combinations of plant growth regulators for callus induction and plant regeneration capacity of *Melia azedarach in vitro* using different types of explants.

Materials and Methods

This research was carried out at the Plant Tissue Culture Laboratory, Department of Plant Production Techniques, AL-Musaib Technical College to initiate callus and induce shoots and roots in Persian lilac (*Melia azedarach* L.). The experimental evaluation were conducted on three different explants with performed different concentration of growth regulators.

Preparation of plant materials

Various parts such as seeds, nodes and internodes were used as explants for callus induction and shoot regeneration. Mature fruits of chinaberry *Melia azedarach* were collected from 15 years old trees with desirable forestry characteristics (fast growing, straight stem, healthy), growing in the garden of AL-Musaib Technical College. The hard coat and endocarp of the chinaberry fruit were removed in a mechanical way by broking them handily using a hard tool and the seeds were washed under running tap water and they were used as a source of explants. Seeds were surface-sterilized according to [14] in 70% (v/v) ethanol for 3 minutes, then 2% (v/v) Clorox 6% NaOCl solution for 30 minutes, and finally, they were rinsed three times in sterile distilled water under aseptic conditions to remove the traces of sterilant.

Nodes and internodes were taken from a one-year-old seedling of Melia and Sterilized with 0.1% (w/v) sterile solution of mercuric chloride for 5 minutes, followed by washing three times with sterile distilled water [15].

Callus induction

For callus induction from seeds, nodes and internodes, they were cultured on MS medium [16] containing 3% (w/v) sucrose, 0.7% (w/v) agar supplemented with naphthalene acetic acid (NAA) at concentrations 0.0, 0.1, 0.2 , 0.3 or 0.4 mg/L in combination with benzyl adenine (BA) at concentrations 0.0, 1, 2 , 3 or 4 mg /L. The pH of the medium was adjusted to 5.7 before autoclaving. Jars and tubes containing the medium was then autoclaved at 121 C^{o} and 1.04 kg/cm^{2} pressure for 20 minutes. All the cultures were incubated under a 16 hrs photoperiod (1000 Lux provided by cool-white and day light fluorescent lamps) in growth room at 27 C^{o} for 4 weeks. After 4 weeks of incubation callus induction frequency was calculated using the following formula:

$$\text{Frequency of callus induction (\%)} = \frac{\text{Number of explants induced callus}}{\text{Total number of explants}} \times 100$$

Also, degree of callusing **(**the amount of callus obtained from different explants) was estimated by scoring system and fresh weight parameter.

Regeneration

After callus induction from the explants, The shoot regeneration was attempted by transferring 4-weeks-old callus into a fresh MS medium supplemented with the same plant growth regulators concentrations for testing callus shoot regeneration potential. Data were scored in terms of shoot regeneration percentage and number of shoots. The percentages of shoot regeneration calculated using following formula:

$$\text{Percentage of callus producing shoot} = \frac{\text{Number of culture induced shoot}}{\text{Total number of explants inoculated}} \times 100$$

Root formation and acclimatization

The developed shoots were then rooted on MS medium containing 0.25 mg/L NAA only according to[4] . Rooted shoots were taken out from culture vessels, and the roots were gently washed with sterile distilled water to remove the adhering agar. The regenerated plantlets were transferred to pots containing a mixture of soil and peat moss (1:1).To harden plantlets, initially high humidity was maintained by covering the pots with inverted plastic beakers to prevent the loss of water for one week, then the beakers were pored for gas exchange . After two weeks, beakers were removed and the plantlets were exposed gradually to light for acclimatization. The hardened plantlets 30 days were then transferred to small plastic pots filled with garden soil+ Peat moss (1:1 v/v) and shifted to the glasshouse.

Experimental design and data analysis

A completely randomized design with 10 replicates was used for the experiment. The experiments were repeated two times and the data for each parameter were subjected to analysis of variance (ANOVA). Significant differences were assessed using L.S.D.test ($P < 0.05$) [17].

Results and Discussion

Results in (Table 1 and 2) shows the effects of BA and NAA concentrations and explant types, on callus induction (%), shoot regeneration percentage(%) and shoot number/ explant.

Callus induction

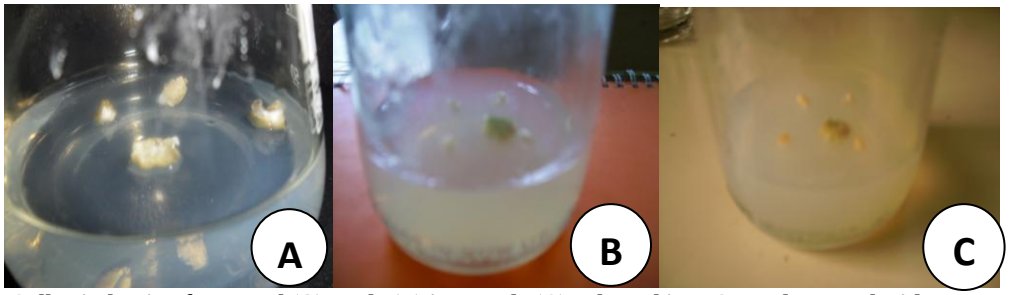
There were considerable differences between explant types and between BA and NAA treatments in the formation of callus Table (1) . Callus which was obtained from all types of explants was depending on plant growth regulators in MS medium but variations were existed between different types of explants in their ability to form callus. Among the tested combinations of NAA and BA for callus induction of different explants(seed, node and internode), data showed that 0.3 and 0.4 mg/L from NAA were the best for callus induction(37.32 and 33.30% respectively , whereas 4.0 mg/L BA gave a significant difference 36.62% than other combinations. Data also showed that 0.3 mg/L NAA + 4.0 mg/L BA gave rapid and maximum *in vitro* response 86.6 %. As results, significant difference was found in callus induction among the three explants of *Melia* in which the frequency 24.4 % of callus induction Table (1) was observed maximum in seed than node and internode 15.6 and 12.8 % respectively.The kind and concentration of growth regulators and kind of explants are the most important factors in production of callus [12].

Seed explants produced considerably more callus than node and internode explants Figure (1), indicating that the source of explant is an important factor in determining the rate of success in such tissue culture experiments. This may also suggest that levels of endogenous hormones or their sensitivity might vary between organs. Many other authors reported that callus induction were dependent on the explant source in *Melia azedarach* [4,14] and in neem tree *Azaderachta indica* [18,19].

The interaction between type of explant and plant growth combination was shown in Table (1). This interaction showed a significant effect on callus induction percentage ($p<0.05$) .Seeds cultured in MS + (0.3 mg/L NAA + 4.0 mg/L BA) gave maximum *in vitro* response 100 % followed by the combination 0.4 mg/L NAA + 3.0 mg/L BA which gave 90% as compared with other combinations, whereas all explants that cultured in MS medium without any addition of growth regulators did not respond to form callus.

Table (1): Effects of different combination of NAA and BA in MS medium on Frequency of callus induction (%) from different plant parts of *Melia azedarach*.

Growthregulators mg/L		Explant type			Mean of NAA×BA	Mean of NAA	Mean of BA
NAA	BA	Seed	Node	Internode			
	0.0	0.0	0.0	0.0	0.0		0.0
	1.0	0.0	0.0	0.0	0.0		10.0
0.0	2.0	0.0	0.0	0.0	0.0	0.0	15.32
	3.0	0.0	0.0	0.0	0.0		25.98
	4.0	0.0	0.0	0.0	0.0		36.62
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	0.0	0.0		
	2.0	0.0	0.0	0.0	0.0		
0.1	3.0	30.0	10.0	0.0	13.3	5.98	
	4.0	0.0	0.0	50.0	16.6		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	0.0	0.0		
0.2	2.0	0.0	0.0	0.0	0.0	11.32	
	3.0	60.0	40.0	0.0	33.3		
	4.0	0.0	70.0	0.0	23.4		
	0.0	0.0	0.0	0.0	0.0		
	1.0	40.0	50.0	0.0	30.0		
0.3	2.0	60.0	0.0	0.0	20.0	37.32	
	3.0	80.0	70.0	0.0	50.0		
	4.0	100.0	80.0	80.0	86.6		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	60.0	20.0		
0.4	2.0	70.0	60.0	40.0	56.6	33.3	
	3.0	90.0	10.0	0.0	33.3		
	4.0	80.0	0.0	90.0	56.6		
Mean of Explant		24.4	15.6	12.8			
L.S.D 0.05	Explants=7.40, NAA=10.19, BA=10.19, NAA×BA=19.11 Explant × NAA × BA =42.22						



Fig(1):Callus induction from seed (A), node (B) internode (C) cultured in MS supplemented with 0.3 mg/L NAA + 4 mg/L BA.

Shoot regeneration

The frequency of adventitious shoot regeneration was depending on the type of explants and concentration of growth regulators added to the regeneration medium Table (2). In assessing the shoot-forming abilities of three different types of explants, the results clearly indicated that the shoot production percentage on different

explant types was variable. Among the 3 explant type evaluated for shooting response, the maximum percentage 10.4% was observed on callus derived from node, whereas it was minimum 5.2% for callus derived from internodes. Plant regeneration in *Melia* was reported from callus derived from internode, leaf and axillary bud [4], leaf [8] and nodular stem sections [20].

The node, internodes and seed explants were prepared and inoculated on Ms medium supplemented with various plant growth regulator combinations as described earlier in materials and methods. Within 45 days of culture, the highest frequency of shoot regeneration 33.3 % was observed in MS medium containing NAA (0.3 mg/L) + BA (4.0 mg/L) or NAA(0.4 mg/L) + BA (4.0 mg/L) (Table 2). Callus derived from seed and internode cultured on MS + NAA(0.3 mg/L) + BA (4.0 mg/L) and MS + NAA(0.4 mg/L) + BA (4.0 mg/L) gave the highest shoot formation percentage 100 % , While Callus derived from node gave maximum 60% when cultured on MS + NAA(0.3 mg/L) + BA (3.0 mg/L) or MS + NAA(0.4 mg/L) + BA (3.0 mg/L).

Table (2): Effects of different combination of NAA and BA in MS medium on shoot regeneration percentage from different plant parts of *Melia azedarach*.

Growth regulators (mg/L)		Explant type			Mean of NAA×BA	Mean ofNAA	Mean of BA
NAA	BA	Seed	Node	Internode			
	0.0	0.0	0.0	0.0	0.0		0.0
	1.0	0.0	0.0	0.0	0.0		2.67
0.0	2.0	0.0	0.0	0.0	0.0	0.0	6.0
	3.0	0.0	0.0	0.0	0.0		15.32
	4.0	0.0	0.0	0.0	0.0		15.32
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	0.0	0.0		
	2.0	0.0	0.0	0.0	0.0		
0.1	3.0	40.0	20.0	0.0	20.0	4.0	
	4.0	0.0	0.0	0.0	0.0		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	0.0	0.0		
0.2	2.0	0.0	0.0	0.0	0.0	5.32	
	3.0	0.0	50.0	0.0	16.6		
	4.0	0.0	30.0	0.0	10.0		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	10.0	0.0	3.33		
0.3	2.0	0.0	0.0	0.0	0.0	11.33	
	3.0	0.0	60.0	0.0	20.0		
	4.0	100.0	0.0	0.0	33.3		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	30.0	10.0		
0.4	2.0	60.0	30.0	0.0	30.0	18.66	
	3.0	0.0	60.0	0.0	20.0		
	4.0	0.0	0.0	100.0	33.3		
Mean of Explant		8.0	10.4	5.2			
L.S.D (0.05)		Explants=3.16, NAA=6.24, BA=6.24, NAA×BA=11.03 Explant × NAA × BA =38.70					

The results showed that there was a great deal of variation among explants in shoot number/ callus Table (3). The maximum number of shoots 0.84, 0.80 was observed on callus derived from seed and node respectively, whereas it was minimum 0.72 on callus derived from internode. Also, the number of shoot per callus varied with the combination of plant growth regulators used in the medium. It was observed that MS + (0.3 mg/L) NAA + (4.0 mg/L) BA or MS + (0.4 mg/L) NAA + (4.0 mg/L) BA or MS+ (0.4 mg/L) NAA + (2.0 mg/L) BA gave the maximum (3.33 shoots), whereas it was minimum at other hormonal combinations.

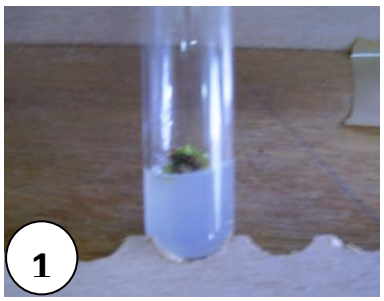
In seed explants the maximum average number of shoots was 10 on medium containing (0.3 mg/L) NAA + (4.0mg/L) BA (Fig. 2) followed 6 shoots on medium containing (4 mg/L) NAA + (2.0 mg/L) BA, while in case of node explant maximum average number of shoots (6) was obtained on medium containing (0.3 mg/L) NAA + (3.0 mg/L) BA followed by MS medium containing (0.2 mg/L) NAA + (3.0 mg/L) BA (5 shoots). In case of internode explant significant differences were recorded for shoot regeneration in all NAA and BA

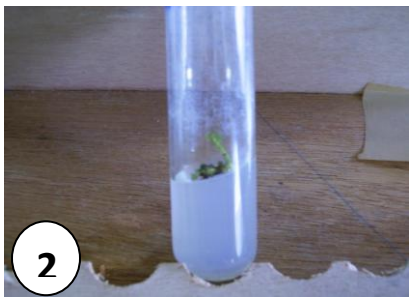
treatments in which the highest number of shoots per explant regenerated 10 shoots in MS medium supplemented with (0.4 mg/L) NAA + (4.0 mg/L) BA. In general, maximum number of shoots 10 shoots were obtained using callus from seed explants cultured on MS supplemented with (0.3 mg/L) NAA + (4.0 mg/L) BA and callus from internode explants cultured on MS supplemented with (0.4 mg/L) NAA + (4.0mg/L) BA comparing with the other interaction between callus source and growth regulators combinations. Cytokinins and auxins are usually known to promote the formation of callus and shoots in many excited and *in vitro* cultured explants. Proper type and concentrations of these hormones are different for each explants, NAA did not stimulate shoot formation in high concentrations[21].

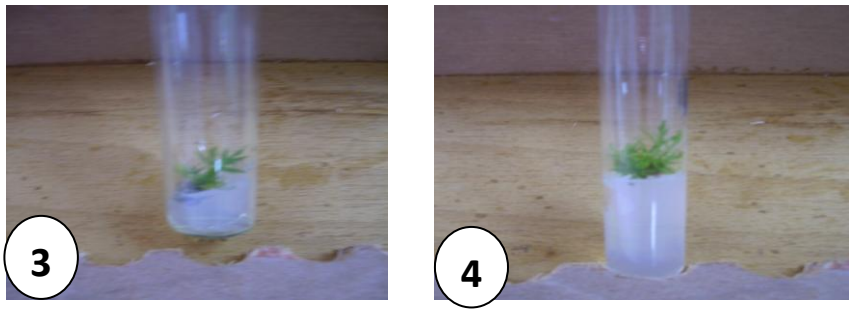
Researchers found that the addition of BA in suitable concentrations could be stimulate shoot formation from callus in ornamental plants[22].

Table (3): Effects of different combination of NAA with BA in MS medium on shoot per callus derived from different plant parts of *Melia azedarach*.

Growth regulators (mg/L)		Explant type			Mean of NAA×BA	Mean of NAA	Mean of BA
NAA	BA	Seed	Node	Internode			
	0.0	0.0	0.0	0.0	0.0		0.0
	1.0	0.0	0.0	0.0	0.0		0.0
0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.53
	3.0	0.0	0.0	0.0	0.0		1.40
	4.0	0.0	0.0	0.0	0.0		1.40
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	0.0	0.0		
	2.0	0.0	0.0	0.0	0.0		
0.1	3.0	5.0	1.0	0.0	2.0	0.4	
	4.0	0.0	0.0	0.0	0.0		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	0.0	0.0		
0.2	2.0	0.0	0.0	0.0	0.0	0.39	
	3.0	0.0	5.0	0.0	1.66		
	4.0	0.0	1.0	0.0	0.33		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	1.0	0.0	0.33		
0.3	2.0	0.0	0.0	0.0	0.0	1.13	
	3.0	0.0	6.0	0.0	2.0		
	4.0	10.0	0.0	0.0	3.33		
	0.0	0.0	0.0	0.0	0.0		
	1.0	0.0	0.0	3.0	1.0		
0.4	2.0	6.0	2.0	0.0	2.66	1.66	
	3.0	0.0	4.0	0.0	1.33		
	4.0	0.0	0.0	10.0	3.33		
Mean of Explant		0.84	0.80	0.72			
L.S.D (0.05)		Explants=0.03, NAA=0.47, BA=0.47, NAA×BA=0.66 Explant × NAA × BA =1.51					







Fig(2): Steps of shoot regeneration from seed callus cultured in MS supplemented with 0.3 mg/L NAA + 4.0 mg/L BA.

Rooting

For rooting, the fully regenerated shoots were excised and placed on MS medium supplemented with 0.25 mg /L NAA according to[4] for roots development which was noted 3 weeks later. The plantlets with fully developed roots were transferred to pots and grown in a growth chamber for hardening. Plantlets were finally transferred to the field successfully.

Conclusion

This study showed that explant types and plant growth regulators combinations are a key factors regulating callus induction and shoot formation. Seeds and node of *Melia azedarach* were found more responsive for regeneration via callus as compared to internode.

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