Mesopotamia J. of Agric (ISSN 1815-316 X) Vol. ( 34 ) No. ( 1 )
2006

# A PROTOCOL FOR SHOOT REGENERATION FROM LEAVES PETIOLES TISSUE CULTURE OF NEEM TREES ( *Melia azedarach* )

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## ABSTRACT

Shoots were regenerated from petioles – derived callus on solidified Murashige and Skoog (MS) medium supplemented with 1.5 mg / l BA only and MS medium containing 4.0 mg / l IBA and 1.0 mg / l BA . The percentage of shoot formation was 30.0 % . The regeneration frequency was reasonable to obtained sufficient shoots by this method . This simple protocol is the first report in tissue culture of this woody plant which encourage other researchers to worke other studies in tissue culture of neem . Moreover , callus culture will be useful to obtain a wide range of industrial plant products .

## INTRODUCTION

Sabahbah ( *Melia azedarach* L.*)* is a fast growing , deciduous tree, belonging to the family Meliaceae which contains other species of neem trees, *Azadirachta indica* ( Allan *et al.* ,1999) . This woody tree is widely exploited for its effects on insects ( Kearnay *et al.* , 1994) . Current interests in its use as a natural insecticide is concentrated on compounds that exhibit antifeedant and insect growth regulatory effects ( Mordue & Blackwell , 1993) . Most of the active principles from neem fruits as well as from seeds , stem and root bark are tetranortriterpnoids , although biologically active diterpenoids , triterpenoids constituents are also present . More than three hundred compounds have been isolated from various parts of the tree ( Kumar *et al.,* 1996) . and most attention has been directed towards the major principle , Azadiractin . Several studies have centered on *A. indica* callus induction and organogenesis ( Sanyal *et al.,* 1981 ; Schultz , 1984 ; Ramesh & Padhya , 1988) Ramesh and Padhya (1990) studied the production of adventitious shoot buds from leaf discs on Wood and Braun medium supplemented with Kinetin , Benzyl adenine and adenine sulphate . They reported that although there was no growth on basal medium , addition of kinetin or benzyl adenine alone resulted in callus production . While addition of both at concentration of 4mM each resulted in shoot formation . Similar results were found by culturing nodal segments and leaf discs of *A. indica* on MS ( Murashige & Skoog ,1962) medium supplemented with 1.0 mg/L benzyl adenine and 0.5 mg/L kinetin ( Subramani *et al*., 1993) . In later studies , Eeswara (1997) failed to obtain shoot formation in any of the above combinations . The possibility of callus initiation and shoots regeneration of *Melia azedarach* was investigated in this study and according to our knowledge there are no published reports on tissue culture of *Melia azedarach* in our country, The paper represent the

Received 22/11/2005 accepted 15/3/2006

Mesopotamia J. of Agric (ISSN 1815-316 X) Vol. ( 34 ) No. ( 1 ) 2006

first report on tissue culture of *Melia azedarach.*

## MATERIALS AND METHODS

**Collection of plant materials :** Trees of *Melia azedarach*, grown in Mosul University's park (Fig.1,A) were used as a source of plant material which include young leaves and its petioles .

**Surface sterilization of specimens :** Young leaves were picked from Sabahbah trees (15 years old ) , they were thoroughly washed with tap water before surface sterilization . The petioles were cut into segments of 1 cm length while the leaves cut into pieces of 0.5 cm .These explants were surface sterilized according to Eeswara *et. al*., (1997) with some modification , by immersion into 0.1 % HgCl2 solution for 5 min. and washed with sterile water . These treated explants were immersed in 3% commercially available sodium hypochlorite solution for 30 min.,then washed carefully with sterile water

**Callus initiation :** Sterilized leaves and petioles of *Melia azedarach* were cultured on the surface of 15 ml of agar –solidified MS ( Murashige & Skoog ,1962) medium supplemented with different combinations of Naphthalen acetic acid (NAA) with Benzyladenine (BA) or 2,4-dichlorophenoxy acetic acid (2,4-D) with Kinetin . Also Indole butyric acid (IBA) was used . Many initiation media were tested for callus formation from leaf and petiole segments as indicated below :

**NAA and BA combinations**

MSO ( Hormone - free) control
MS + 1.5 mg/l BA
MS + 0.9 mg/l NAA
MS + 0.2 mg/l NAA + 0.1 mg/l BA
MS + 2.0 mg/l NAA + 2.0 mg / l BA
MS +1.0 mg/l NAA + 2.0 mg l BA

**2,4-D and Kinetin combinations**

MS +0.1 mg /l 2,4 - D + 0.2 mg /l Kin.
MS + 0.5 mg /l 2,4 - D + 1.0 mg /l kin.
MS + 1.0 mg /l 2,4 - D + 1.0 mg /l kin.
MS + 1.0 mg /l 2.4 - D + 2.0 mg /l Kin.
MS + 2.0 mg / l 2,4 - D + 4.0 mg /l Kin

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**IBA and BA combinations**

MS + 0.1 mg /l IBA
MS + 2.0 mg /l IBA + 1.0 mg /L BA
MS + 4.0 mg /l IBA + 1.0 mg /L BA

Plant specimens were kept in culture room at 25 ± 1°C , 1500 Lux white fluorescent tube for 16 hrs . light 8 hrs. dark .Other group of samples were incubated at same condition in the dark .

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## RESULTS AND DISCUSSION

This paper indicates the response of *Melia azedarach* tree to plant tissue culture technique throughout the good response of leaves and petioles explants to callus formation on MS medium supplemented with various combination of different auxins (NAA , IBA and 2,4-D )and cytokinines( BA and kinetin) in the dark .

Various concentrations of BA and NAA were helpful in inducing callus initiation from leaves and petioles explants ( Table l ) .

This good response of explants for callus formation may be due to the efficient selection of cells in an explants (Al-Mallah , & Hassan ,1994 ) coupled with compatibility between the endogenous hormonal levels and the addition of growth regulator to the induction medium .

Table 1 : Callus formation from leaves and petioles explants of M*elia azedarach* on agar-solidified MS medium supplemented with NAA and BA

| Medium (mg /L) | Cult. Segment / Init. Segment | | Callus formation (%) | |
|---|---|---|---|---|
| | Leaves | Petioles | Leaves | Petioles |
| MS + 1.5 mg/L BA | 40*/20** | 40/33 | 50 | 82.5 |
| MS + 0.2 mg/LNAA + 0.1 mg/L BA | 30/0 | 30/0 | 0.0 | 0.0 |
| MS + 0.9 mg/LNAA | 30/0 | 30/0 | 0.0 | 0.0 |
| MS + 1.0 mg/LNAA + 2.0 mg/L BA | 40/32 | 40/21 | 80.0 | 52.5 |
| MS + 2.0 mg/LNAA + 2.0 mg/L BA | 30/3 | 30/2 | 10 | 6.6 |
| MSO (control) | 30/0 | 30/0 | 0 | 0 |

* Cult . :cultured segments , **Init .:Initiated segments

The obtained results indicate clearly that MS medium supplemented only with 1.5 mg /l BA sustained callus induction . The petiole explants started callus formation after two weeks of culture and the percent of callus initiation was 82.5 .It was obvious that increasing NAA level inhibited callus formation from 82.5% to 6.4 % ( Table 1 ) .These results confirm that this plant have enough levels of endogenous hormones and does not require the addition of high levels of exogenous growth regulators (Wala & Jasrai , 2003 ).Subramani *et al.* (1993) noticed that agar solidified MS medium supplemented with 3.5 mg /l BA was proper for callus initiation in neem. The results indicated that replacing NAA with IBA seems to be very useful to callus initiation as the induction of callus increased (Table 2 ) .

The petiole explants began callus induction within twelve days(Fig.l ,B ). While leaves explants started callus formation after twelve days on agar solidified MS medium containing IBA 4.0 mg /L and BA 1.0 mg / L (Fig.l ,C) .The results showed that this medium was the best one for callus growth, so it was used for the subculturing and maintenance of callus every 4 weeks interval . Callus was friable and yellow light brown in colour (Fig.l,D). Previous study (Allan *et at* .,1994) showed that agar solidified MS medium supplemented with



IBA 4.0 mg / L and BA 1.0 mg / L was suitablefor callus initiation from leaves explants of *Azdirachta indica* .

Table 2 : Initiation of callus from leaves and petioles of M*elia azedarach* on agar solidified MS medium supplemented with IBA and BA

| Medium | Cult. Segment / Init. Segment | | Callus formation % | |
|---|---|---|---|---|
| | Leaves | Petioles | Leaves | Petioles |
| MS + 0.1 mg/l IBA | 30/0 | 30/0 | 0.0 | 0.0 |
| MS + 2.0 mg/l IBA + 1.0 mg/L BA | 30/1 | 30/2 | 3.3 | 6.6 |
| MS + 4.0 mg/l IBA +1.0 mg /l BA | 50/21 | 50/42 | 42 | 84 |
| MSO( control) | 20/0 | 20/0 | 0.0 | 0.0 |

A

B

C

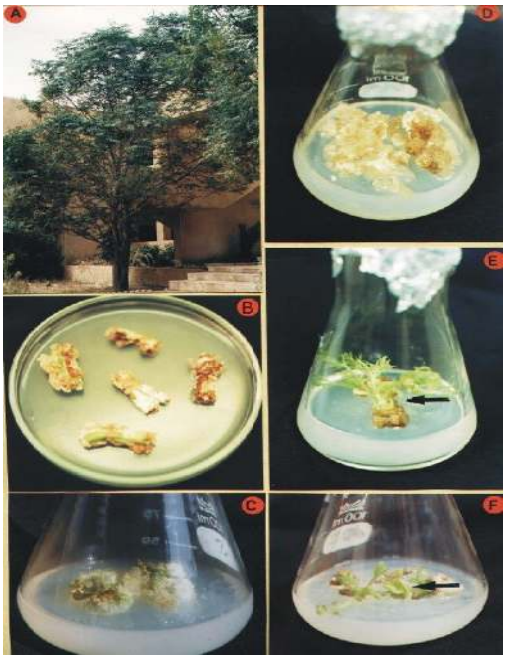

Fig .1: Shoot regeneration from leaves and petioles tissue culture of *Melia azedarach* L.

(A) : *Melia azedarach* tree used as a source of plant material ,(B) : Petioles explants of *Melia azedarach* after twelve days of culture on MS medium supplemented with IBA 4.0 mg / L and BA 1.0 mg /L ,(C) : Leaves explants after twelve days of culture on MS medium supplemented with IBA 4.0 mg / L and BA 1.0 mg /L. ,(D) : Callus of *Melia azedarach* after 30 days on( MS + IBA 4.0 mg / L + BA 1.0 mg /L) ., (E) : Shoots formation from Petioles arrowed derived callus on MS medium supplemented with 1.5 mg / L BA. ,(F) : Shoots formation arrowed from Petioles explants on ( MS + 4.0 mg / L IBA + 1.0 mg /L BA) .

Moreover , 2,4-D and Kinetin can be used in callus initiation of *Melia Azedarach* since callus was continuously induced at ratio of 60.0% (Table 3) .

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Table 3 : Callus formation from leaves and petioles of *Melia Azedarach* on agar solidified MS medium supplemented with different concentration of 2,4-D and kinetin

| Medium (mg /l) | Cult. Segment / Init. Segment | | Callus formation % | |
|---|---|---|---|---|
| | Leaves | Petioles | Leaves | Petioles |
| MS + 0.1 mg/l 2,4 - D + 0.2 mg /l kin | 30/0 | 30/0 | 0.0 | 0.0 |
| MS + 0.5 mg /l 2,4 - D + 1.0 mg /l kin | 40/2 | 40/3 | 5.0 | 7.5 |
| MS + 1.0 mg /L 2,4 - D + 1.0 mg /L ki | 30/0 | 30/0 | 0.0 | 0.0 |
| MS + 1.0 mg /l 2,4 - D + 2.0 mg /l kin | 30/1 | 30/3 | 3.3 | 10.0 |
| MS + 2.0 mg /l 2,4 - D + 4.0 mg /l kin | 30/8 | 30/18 | 26.6 | 60.0 |
| MSO( control) | 30/0 | 30/0 | 0.0 | 0.0 |

This result express the capability of this tree to invitro culture . As previously published with *Azadirachta indica* Nirmalakumari *et al.* (1996) indicated that MS medium containing 2.0 mg / L 2,4-D and 0.5 mg /l kin. promoted callus formation from cotyledones and leaves explants .

All the above results of callus initiation occurred in the dark .Conditions of 8 hrs. dark 16 hrs. Light was not suitable for all tested materials and we do not notice any sign for callus induction .Wewetzer (1998) indicated that the dark was preferable for callus initiation of *Azaderacta indica* .

**Plant regeneration:**The results showed that among all the different agar solidified MS media used , those supplemented with 1.5 mg /l BA only and MS medium containing 4.0 mg / l IBA and 1.0 mg / l BA stimulated shoots formation from petioles derived callus of *Melia Azedarach* .(Fig.1,E)and from petiole explants (Fig.1,F) and shoot formation reached 30% , and 6.0 % respectively (Table 4 ) .

Table 4 : Shoot regeneration from petioles-derived callus of *Melia azedarach* on agar solidified MS medium supplemented with IBA and BA

| Regeneration medium | No. of cultured explants | No. of shoots | shoot regeneration (%) |
|---|---|---|---|
| MS + 1.5mg/l BA | 40 | 12 | 30.0 |
| MS + 4.0 mg/l IBA +1.0 mg /l BA | 50 | 3 | 6.0 |

It was observed that there are different factors influencing tissue growth and differentiation , these factors include explant source and the addition of growth regulators (Mihaljevi *et al* ., 2002)

In conclusion this finding represents a simple protocol for callus induction and shoot formation from leaves and petioles of *Melia azedarach* . This will enhance tissue cultures works on this woody plant by other researchers and promote other workers to start extracting Azadirachtin and other secondary metabolites from callus cultures of this important tree ,or regenerating transformed *Melia azedarach* plants as happened with medicinal plant *Solanum nigrum* (AL-Mallah &Salih ,



2005). And for future work electrostimulation can be applied to increase probably growth of callus , since electrostimulation was found to enhance growth of callus of tobacco (Al-Mallah ,1993),and black night shad *S .nigrum* (Al-Mallah &Salih ,2003) .

## طريقة بسيطة للحصول على الافرع الخضرية من المزارع النسيجية لاعناق اوراق اشجار النيم (*Melia azedarach*)

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### الخلاصة

تمكنت الدراسة الحالية من الحصول على الافرع الخضرية من كالس أعناق الاوراق لاشجار السبحبح *Melia azedarach* في وسط موراشيج وسكوك (MS ) الصلب المدعم باضافة ١.٥ ملغم / لتر بنزايل أدنين ( BA ) ووسط (MS) الصلب المدعم باضافة ٤.٠ ملغم / لتر (IBA) و١.٠ ملغم / لتر (BA) وأظهرت النتائج تمايز مزارع الكالس وبلغت نسبة تكون الافرع الخضرية ٣٠% فيMS% و٦.٠ على التوالي . ومن المحتمل ان تكون هذه الدراسة الاولى في مجال الزراعة النسيجية لاشجار السبحبح في العراق .ان هذه الدراسة تشجع العديد من الدراسات في مجال الزراعة النسيجية لهذه الاشجار او تلك المتعلقة بالحصول على النواتج الايضية او بالتحول الوراثي لهذه الاشجار المنتشرة في البيئة العراقية .

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