CALLUS INDUCTION AND PLANT REGENERATION FROM ANTHERS OF *AZADIRACHTA INDICA*

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DHILLON, R. S., SINGH, S., SEHRAWAT, A. R., ARYA, S. & HUSSAIN, Z. 2005. Callus induction and plant regeneration from anthers of *Azadirachta indica*. The combination of 4.0 ppm α-naphthaleneacetic acid (NAA) with 2.56 ppm kinetin in Murashige and Skoog (MS) basal medium was found to be the best for callus formation from yellow-coloured neem (*Azadirachta indiea*) anthers (a day prior to anthesis) after 30 to 35 days of incubation. The green nodular callus developed into shoots/shoot buds on MS medium supplemented with 1.0 ppm of benzylaminopurine (BAP). Prolonged incubation on this medium increased the length of shoots with green shiny leaves. The addition of 0.25 mg l^{-1} silver nitrate produced longer shoots with good growth and bigger and broader leaves; induction of new buds appeared after three months of incubation. The excised shoots developed healthy roots upon transfer to half-strength MS medium containing 1.0 ppm indole-3-acetic acid (IAA) alone or in combination with 0.01 ppm kinetin (which responded better) after 15 to 20 days of incubation.

Key words: Neem – growth regulators – anther culture – plantlet

DHILLON, R. S., SINGH, S., SEHRAWAT, A. R., ARYA, S. & HUSSAIN, Z. 2005. Keberkesanan tinggi aruhan kalus dan pertumbuhan semula tanaman daripada anter *Azadirachta indica*. Kombinasi asid α-naftaleneasetik (NAA) dengan kinetin dalam medium asas MS didapati paling baik bagi pembentukan kalus daripada anter *Azadirachta indica* yang berwarna kuning (sehari sebelum antesis) selepas 30 hingga

35 hari pengeraman. Struktur nodul hijau terbentuk dalam kalus setelah satu subkultur atas medium asas MS baru yang mengandungi 4.0 ppm NAA dan 2.56 ppm kinetin. Kalus nodul hijau membentuk pucuk/tunas pucuk di atas medium asas yang ditambah 1 ppm benzilaminopurin (BAP). Pengeraman yang lama di atas medium ini menghasilkan pucuk yang lebih panjang serta berdaun hijau dan berkilat. Tambahan 0.25 mg/l argentum nitrat menghasilkan pucuk yang lebih panjang dan tumbuh dengan baik serta mempunyai daun yang lebih lebar dan besar; aruhan pucuk baru muncul selepas tiga bulan pengeraman. Pucuk yang dipotong membentuk akar yang sihat apabila dipindahkan ke medium MS yang separuh kekuatannya dan mengandungi 1.0 ppm asid indol-3-asetik (IAA) sama ada secara berasingan atau bersama 0.01 ppm kinetin.

Introduction

Neem (*Azadirachta indica*), commonly known as margosa or Indian lilac, belongs to the family Meliaceae, and is a native of the Indian subcontinent and Myanmar. However, this species has spread to many Asian and African countries as well as most of the other warmer parts of the world. Much research has been conducted worldwide to study its medicinal properties and the biological activities of ingredients found in its seeds, leaves and bark against a wide range of pests and pathogens. Neem has also been identified as the best alternative to toxic pesticides due to its quick biodegradability, non-residual effects for agricultural produce and low toxicity (Cooper 1999).

The callus cultures of neem have been studied for the production of secondary metabolites which possess no acute mammalian toxicity and are biodegradable (Wewetzer 1998). These studies have led to the laboratory production of azadirachtin, nimbidin and other important bioactive substances. The demand for neem tree is thus increasing, causing concern for its continuous existence, especially since neem is also difficult and slow to propagate vegetatively. In addition, neem seeds remain viable only for a short period. To produce a large number of plants, *in vitro* culture techniques have proven to be successful.

There are several reports on neem regeneration from different explants including anthers (Schulz 1984, Narayan & Jaiswal 1985, Ramesh & Padhya 1990, Gautam *et al.* 1993, Venkateswarlu *et al.* 1998). This study describes a simple protocol for the production of a large number of neem plantlets with roots from anther derived callus.

Materials and methods

Floral buds were collected from trees at the CCS Haryana Agricultural University, Hisar, India. The buds containing mature anthers (one day prior to anthesis) were surface sterilized with absolute alcohol for 10 s, and subsequently with 0.1% of mercuric chloride for 5 to 7 min. The buds were then rinsed five or six times with sterile distilled water. Anthers (yellow-coloured) were excised and inoculated under aseptic conditions on Murashige and Skoog (MS) basal medium (Murashige & Skoog 1962) supplemented with different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA) and kinetin (Kn) (Table 1). Eight culture flasks containing twenty anthers each formed one experimental group. This was repeated three times.

Table 1 Callus induction from anthers of *Azadirachta indica* on MS medium supplemented with different growth regulators

Growth regulators (ppm)	Callus induction	Callus growth	Averege No. of anthers that produced callus (mean ± SE)
NAA (2.0)+kinetin (1.0)	+	++	0.29 ± 0.015
NAA (4.0)+kinetin (2.56)	+	+++	0.37 ± 0.019
2,4 D (0.5)+kinetin (1.0)	0	-	
2,4 D (1.0)	0	-	
NAA (1.0)	0	-	
Kinetin (1.5)	0	-	

+ = callus induction
++ = good callus growth
+++ = very good callus growth
NAA = α-naphthaleneacetic acid
2,4-D = 2,4-dichlorophenoxyacetic acid

Table 2 Induction of shoot buds and development of buds into shoots on MS medium supplemented with different growth regulators

Growth regulators (ppm)	Callus growth	No. of shoots/ callus piece	Averege No. of shoots / callus (mean ± SE)
BAP			
1.0	++++	0–6	3.8 ± 0.03
1.5	+++	-	-
2.0	+++	-	-
3.0	+++	-	-
3.5	+++	-	-
Kinetin			
1.0	+	-	-
1.5	+	-	-
2.0	+	-	-
IAA (3.0) + BAP(1.5)	++	-	-
BAP (1.0)+ GA_3 (0.1) + CH(300 mg l^{-1})	+++	-	-

+ = little callus growth
++ = good callus growth
+++ = very good callus growth
++++ = best granular green callus
BAP = benzylaminopurine
IAA = indole-3-acetic acid
GA_3 = gibberellic acid
CH = casein hydrolysate

For the regeneration study, four small pieces of callus per culture flask were subcultured on MS medium with various concentrations and combinations of benzylaminopurine (BAP), kinetin (Kn), indole-3-acetic acid (IAA), gibberellic acid (GA_3) and casein hydrolysate (CH) (Table 2). Twelve such flasks formed one

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experiment. Each experiment was repeated three times. In total, 144 callus pieces were studied for each regeneration medium. All cultures were maintained at 25 ± 1 °C with 16-h photoperiod (250 μE $m^{-2}s^{-1}$ light intensity). In another set of experiments different concentrations of silver nitrate (0.05 to 0.40 mg l^{-1}) were added to the MS medium containing 1.0 ppm BAP.

Results and discussion

Callus initiation

The yellow-coloured anthers became black, shrunken and dried within 15 days of incubation on MS medium supplemented with 2.0 ppm NAA + 1.0 ppm kinetin, or with 4.0 ppm NAA + 2.56 ppm kinetin. However, callus induction occurred from the central parts of these black dried anthers after 30 to 35 days of incubation on both of the above media.

The average callus inductions were 29% and 37% under these two treatments, the maximum being 40%. The callus was friable and yellowish white, although the amount of callus formed and its morphological characteristics varied with the different concentrations of NAA and kinetin used. Gautam *et al.* (1993) reported slight swelling of anthers in *Azadirachta indica*, but in the present study no swelling was observed. Our results also showed that combinations of NAA with kinetin were the most suitable for callus induction and this conforms with the results of Gautam *et al.* (1993) and also those of Rao and De (1987) in *Peltophorum pterocarpum*. In the growth studies, the combination of MS with 4.0 ppm NAA and 2.56 ppm kinetin produced the best callus growth for further regeneration. The callus became greenish white, granular and increased in size after the first subculture on this medium.

Shoot regeneration

Green calli growing on the MS medium supplemented with 4.0 ppm NAA and 2.56 ppm kinetin were transferred onto the MS medium supplemented with different concentrations of BAP or kinetin or BAP in combination with IAA or gibberellin (GA_3) (Table 2). Only calli growing on MS medium containing 1.0 ppm BAP for 45 to 50 days in 16-h photoperiod (250 mE m^{-2} s^{-1} intensity) differentiated into small shoots. Differentiating calli increased in volume and became highly nodulated. No external morphogenesis response was observed in calli incubated in media supplemented with BAP at concentrations higher than 1.0 ppm, in kinetin at any concentration or in the combinations of BAP with IAA, GA_3 and CH (Table 2).

The present findings are in close agreement with the results of Narayan and Jaiswal (1985), Venkateswarlu *et al.* (1998), and Ayed and Fattah (1999). However, Gautam *et al.* (1993) did not observe shoot regeneration when using medium containing BAP alone. In this study, calli growing on 2.0 ppm NAA with 1.0 ppm

kinetin were also observed to develop shoots after being transferred to MS medium containing 1.0 ppm BAP. But the shoots appeared feeble compared with those developed from calli grown in 4.0 ppm NAA and 2.56 ppm kinetin. Thus further subcultures were made to grow calli on MS medium supplemented with the latter concentrations of NAA and kinetin for subsequent experiments. Although shoots could be induced by 30 to 35 days, there was an increase in shoot length with longer incubation time.

To observe whether silver nitrate could promote the further growth of shoots, different concentrations (0.05 to 0.40 mg l^{-1}) were added in a separate set of experiments to the MS medium containing 1.0 ppm BAP. Only the addition of 0.25 mg l^{-1} silver nitrate showed a positive response with elongation of shoot with good growth and bigger and broader leaves. In addition new buds were induced after three months of incubation. Vain *et al.* (1989) reported similar results in cereals. The calli in this medium remained green until the shoot buds elongated into healthy shoots after which the calli turned blackish brown. Polyvinyl pyrrolidone (PVP), which is normally added to check browning of calli, was not added as there was no interference of shoot growth at the early stage.

Root formation

Single shoots were excised and transferred into culture tubes containing half-strength MS medium and 1.0 ppm IAA or in combination with 0.01 ppm kinetin. The tubes were covered with black paper from the upper surface of the medium towards the base of the tube and kept in the light for 16-h photoperiod (250 $\mu E\ m^{-2}\ s^{-1}$ intensity). Root initiation was observed after 15 to 20 days of incubation on both media but the medium with the combination of 0.01 ppm kinetin was more responsive. Prolonged incubation (30 to 35 days) produced healthy roots in more than 85% cultured shoots. Kaur *et al.* (1998) also reported the role of IAA in inducing roots of *Acacia catechu*. Both kinds of shoots, i.e. those grown in 1.0 ppm BAP only and those with the addition of 0.25 mg l^{-1} silver nitrate (which were healthier), could develop roots in the rooting media.

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