RESEARCH PAPER

Plant Signaling & Behavior 6:7, 1-8; July 2011; ©2011 Landes Bioscience

Increased production of azadirachtin from an improved method of androgenic cultures of a medicinal tree *Azadirachta indica* A. Juss

Priyanka Srivastava† and Rakhi Chaturvedi*

Department of Biotechnology; Indian Institute of Technology; Guwahati, Assam, India

†Current address: Division of Biomedical Sciences; School of Bio Sciences and Technology; Vellore Institute of Technology University; Vellore, Tamil Nadu, India

Key words: anther culture, *Azadirachta indica*, azadirachtin, haploid plants, RP-HPLC

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; CH, casein hydrolysate; GA_3, gibberellic acid; IAA, 3-indole acetic acid; Kinetin, 6-furfuryladenine; RP-HPLC, reverse phase high pressure liquid chromatography; MS, mass spectroscopy

Present report is the first direct evidence of azadirachtin production in androgenic haploid cultures of *Azadirachta indica*, a woody medicinal tree. Anther cultures at early-late-uninucleate stage of microspores were established on MS medium with BAP (5 μM), 2,4-D (1 μM) and NAA (1 μM) containing 12% sucrose. The calli, induced, were further multiplied on 2,4-D and Kinetin media. Shoots, differentiated on BAP (2.2 μM) + NAA (0.05 μM) medium, were elongated on MS + BAP (0.5 μM) and multiplied on MS + BAP (1 μM) + CH (250 mg/l). Thereafter, the shoots were rooted on ¼ MS + IBA (0.5 μM). Cytological analysis of the calli and regenerants have confirmed their haploid status with the chromosome number as 2n = x = 12. The haploid cell lines and leaves from in vitro grown plantlets were analyzed for azadirachtin by RP-HPLC and mass spectroscopy. Maximum azadirachtin (728.41 μg/g DW) was detected in calli supporting best shoot proliferation while least (49 μg/g DW) was observed in an undifferentiated line from maintenance medium. This study has brought us a step closer to develop genetically pure lines that could serve as new and attractive alternative ways of homogeneous controlled production of high value compounds, round the year, independent of geographical and climatic barrier.

Introduction

Azadirachta indica A. Juss or Neem (family Meliaceae) is a valuable tropical forest tree which has multiple medicinal, agrochemical and economic benefits due to several bioactive ingredients present in various parts of the tree. The most notable is azadirachtin, a tetranortriterpenoid, which is chiefly present in the seed kernels.[1] Its demand has been on rise in industries due to its immediate application as an ecofriendly, biodegradable biopesticide and various other significant bioactivities.[2,3] The benefits claimed in the papers are not fully utilized because of certain practical limitations. The bottlenecks, with regard to extraction and availability of azadirachtin-related limonoids, lie in unavailability of genetically pure lines due to out-breeding nature of the plant and long reproductive cycle. Improvement by conventional methods is also restricted because of the same reasons. Further, the availability of seeds is limited; the reproductive stage in Neem begins at about three to five years of age, they become fully reproductive after ten.[4] From this time on, the tree yields an average of about 20 kilograms of fruit per year, with maximum production reaching 50 kilograms per year.[5] Of the fruit yield, only about 10% is attributed to seed kernels (after removing the seed coat) and desired biologically active compounds comprise only 10 grams per kilogram of kernel weight. Based on this estimate, an adult Neem tree produces only about 20 grams of pesticide compounds in a season.

In this context, the perspective of raising in vitro haploid plants, thus, offers scores of foreseeable advantages, like shortening of breeding period, production of homozygous diploid (double-haploid) lines in a single step through chromosomal doubling and isolation of valuable recessive traits at sporophytic level which otherwise, remain accumulated and unexpressed in natural heterozygous diploid population. Since the plant cells are totipotent in nature, consequently, the cultured cells will also contain genetic information for the production of bioactive compounds with added potential of increasing yield by manipulating the culture conditions.[6] As cell cultures have a higher rate of metabolism than intact plants due to fast proliferation of cell biomass, thus, production of bioactive compounds can take place within a short cultivation time of 4–5 weeks, in comparison to, conventional way of isolation of compounds from intact plants.

*Correspondence to: Rakhi Chaturvedi; Email: rakhi_chaturvedi@iitg.ernet.in or rakhi_chaturvedi@yahoo.co.uk
Submitted: 02/23/11; Revised: 03/16/10; Accepted: 03/17/11
DOI:

The motivation to work along this line, in the present study, arose from the fact that to date the available reports on azadirachtin production have utilized only diploid tissues like seeds, roots, leaves, etc. from heterozygous plants that result in inconsistency and heterogeneity in chemical makeup of the plant. The haploid cells or tissues of Neem were not explored earlier for metabolite production. It is noteworthy that genetically pure lines of strictly cross-pollinating species, like Neem, are highly desirable to increase the efficiency of selection and for homogeneous, constant production of metabolites. The conventional methods to produce homozygous diploid plants is lengthy and laborious requiring 7–8 recurrent cycles of inbreeding, which is impossible in the case of cross-breeding trees like Neem. However, with in vitro haploids, homozygous diploids can be produced in a single step, thus, cutting down the breeding period to almost half. The present report, therefore, aimed at developing an improved method of androgenesis and evaluating their feasibility of azadirachtin production. All physical and chemical parameters that influence the establishment of anther cultures in vitro are also discussed.

Results and Discussion

Callus induction and multiplication. Flower buds of 2 mm size (**Fig. 1A**) were collected from an elite Neem tree growing at Guwahati (Northeast) India. The anthers bearing early-to-late uninucleate stages of microspores (**Fig. 1B and C**) were cultured on medium to raise androgenic haploids of Neem. At the uninucleate stage, the wall of microspores is thin and it is easy to divert their mode of development from gametophytic to sporophytic. Androgenesis in Neem was earlier reported by Chaturvedi et al.[7] from a tree growing at Delhi (North), India. However, as heterozygosity due to cross pollination and also the environmental conditions of the two regions have great influence on the physiology of parent plant,[8,9] consequently, nutritional requirements of the two plants may vary under the in vitro conditions. This is in agreement with the present observation where the anthers taken from a plant growing in Guwahati, India, needed significant modifications at various steps during androgenic plant development under in vitro conditions, compared to the one, developed by Chaturvedi et al.[7] from the plant growing at Delhi, India. A comparative account to these studies has been tabulated in **Table 1**.

Of the various growth regulator combinations tested, MS medium supplemented with 2,4-D (1 μM), NAA (1 μM) and BAP (5 μM) induced maximum callusing. The percent anther response and the degree of callusing varied with the sucrose concentrations. At its various concentrations, 12% sucrose was observed to be significantly better ($p < 0.05$) for callus induction, followed by 9%, 6% and 3%. Sucrose concentrations above 12% were found to be inhibitory (**Table 2**). Decline in response above this concentration may be explained by disturbed osmoticum of the medium. On MS (with 12% Sucrose) + 2,4-D (1 μM), NAA (1 μM) and BAP (5 μM), more than 85% anther cultures responded for callusing. Compare to this, Chaturvedi et al.[7] obtained callus induction response on the best treatment consisting of 9% sucrose in the medium. In the present study, two clear patterns of callusing were seen on the responding medium: in one, the anther walls burst open and pulled apart due to pressure of callusing microspores, resulting in the release of shiny, globular masses emerged from within the anthers (**Fig. 1D and E**). In a few other cases, callus was developed at the base from anther walls. Only the former cultures were utilized for further experiments and for data calculation. The callusing started after four weeks, and by the eighth week excellent callus growth was observed.

The above combination of growth regulators consisting of 12% sucrose was also tested with B_5 and NLN basal media. While on MS medium callus growth was excellent and the calli were fresh, soft, friable and cream, those on NLN medium were dark brown, friable with very little growth and mostly the calli originated from the anther walls. There was no response on B_5 medium. Among the temperature pretreatments given to cultured anthers, best response was observed in control at 25°C temperature, followed by one day pre-treatments at 12°C and 4°C temperatures (**Table 3**). Temperatures higher than 25°C were found to have adverse effect on callusing. Therefore, further experiments on callus induction were conducted with MS medium and the cultures were continuously incubated at 25°C in dark. Although regular attempts have been made to induce androgenesis in woody trees, success rate is limited, chiefly due to their recalcitrance in culture. Factors that affect androgenesis have been described elaborately by Atanassov et al. Out of the various factors, genotype, environmental conditions, physiological status of the donor plant, developmental stage of microspores (early-to-late uninucleate stages being most crucial), pretreatment of anthers, culture media, carbon source and its concentrations and culture conditions play a vital role in induction of androgenesis. Higher sugar concentration during the induction phase is shown to have suppressive effect on the division of somatic cells, therefore, division from haploid cells (microspores) are favored. Dark incubation, in most cases has been found essential in the initial periods of induction in cultures. Likewise, anthers from the first flush of flowering in the season are suggested to be more responsive than those borne later.[8]

The calli that emerged from inside burst anthers were detached from the parental tissues and transferred to fresh medium with a reduced sucrose concentration of 3% and 16/8 h photoperiod regime. All experiments from now onwards related to regeneration and chemical analysis had been conducted with these microspore derived calli.

The induction medium did not support sustained callus growth even after 20 weeks. However, inclusion of one auxin (2,4-D) and one cytokinin (Kinetin) in the MS medium supported sustained callus growth. In terms of biomass, best response was observed on MS + 2,4-D (1 μM) + Kinetin (10 μM) followed by MS + 2,4-D (0.5 μM) + Kinetin (4.5 μM) and MS + 2,4-D (0.5 μM), in a single growth cycle of 8 weeks. The calli obtained on these combinations were cream, soft, friable and moderate to fast growing. The calli were allowed to multiply on these three media for more than 2 years.

Shoot regeneration, elongation and micropropagation. Calli maintained on above three maintenance media were utilized

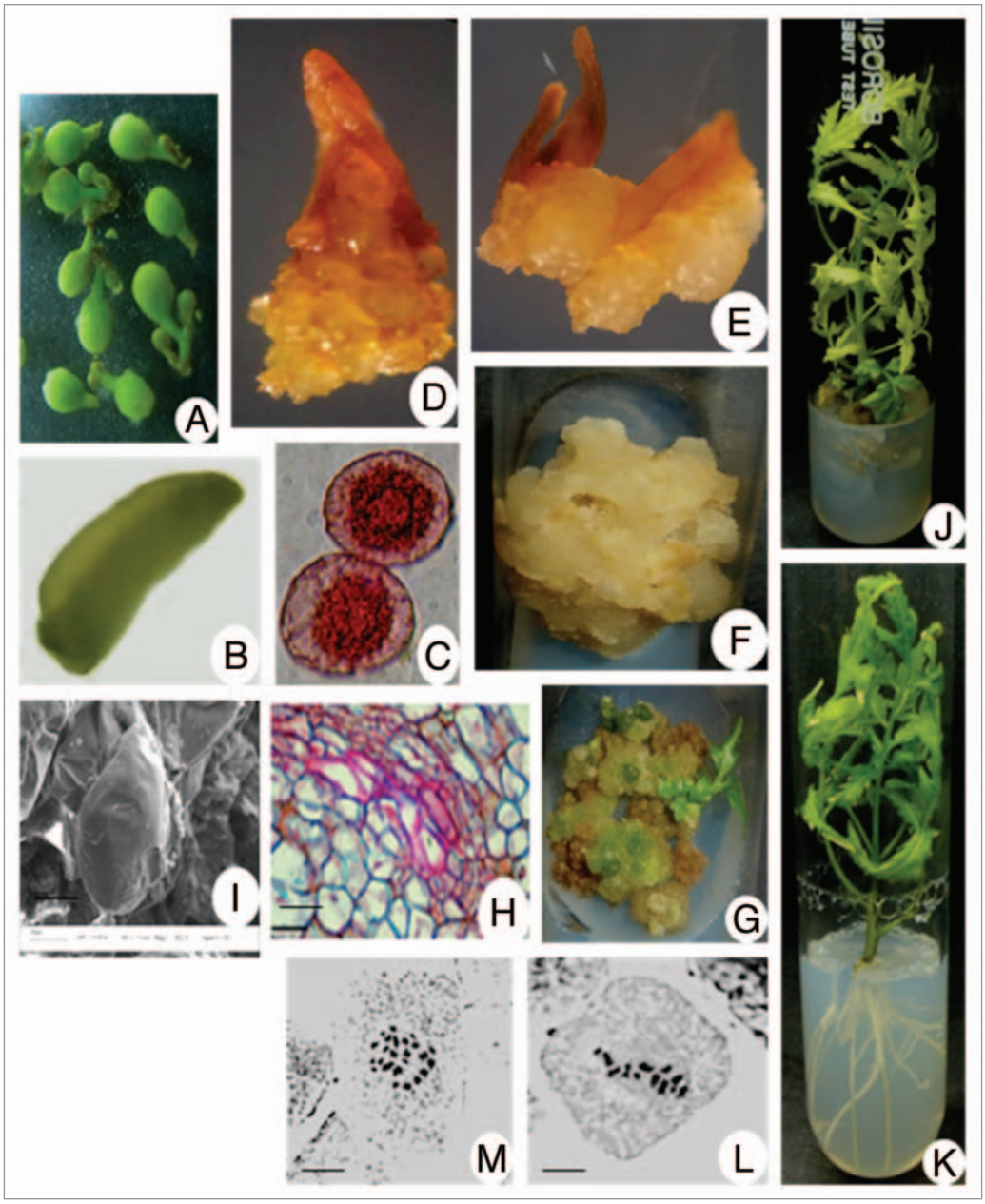


Figure 1. Establishment of anther cultures on MS + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM); Growth Period: 8 weeks. (A) Flower buds of 2 mm size bearing correct stage of microspores (x2.2X). (B) An isolated anther at culture, with uninucleate microspores (x70X). (C) Uninucleate microspores stained with acetocarmine; bar represents 5 μm. (D and E) 8-week-old anther at culture where the anther walls pulled apart with the pressure of callusing microspores (x55X). (F) 8-week-old callus cultures on multiplication medium, MS + 2,4-D (0.5 μM) + Kinetin (4.5 μM) (x1.2X). (G) 6-week-old cultures on MS + BAP (2.2 μM) + NAA (0.05 μM), showing shoot regeneration (x1.2X). (H) Histological section of a regenerating callus showing vascular strands and distinct tracheary elements; bar represents 200 μm. (I) Scanning Electron Micrograph of callus showing well developed nodular structures (x200X). (J) GA_3 (3 μM) pretreated shoot on elongation medium MS + BAP (0.5 μM), after 6 weeks of culture (x1X). (K) A shoot rooted on ¼ MS + IBA (0.5 μM), after 6 weeks (x1X). (L) Cytology of shoot-tip from in vitro haploid plantlet, stained with 2% aceto-carmine, showing haploid number of chromosomes (2n = x = 12); bar represents 10 μm. (M) Cytology of root-tip from seedling, stained with 1% aceto-orcein showing diploid number of chromosomes (2n = 2x = 24); bar represents 20 μm.

for regeneration experiments. MS + 2,4-D (0.5 μM) + Kinetin (4.5 μM) served as the best source (**Fig. 1F**), as it gave maximum percentage of regenerating cultures. Shoot regeneration was achieved on two combinations: MS + BAP (2.5 μM) + IAA (5 μM) + CH (500 mg/l) and MS + BAP (2.2 μM) + NAA (0.05 μM). The callus in the former medium, turned bright-green first and later compact nodular structures was differentiated into shoot-buds after 6 weeks. On an average 27.8% cultures formed 4.5 shoot-buds per explant on this combination. However, considerably better shoot-bud proliferation frequency was achieved in the latter medium. In this case, two step protocol was followed where the calli from multiplication medium were first transferred

Table 1. Comparative account of a previously existing and new improved method of androgenic cultures of *Azadirachta indica* A. Juss

S. No.	Parameters/ Comparison of study	Previously existing method (Chaturvedi et al. 2003)	New improved method (present study)
1.	Material Used	Flower buds collected from an adult Neem tree growing in Delhi, North India.	Flower buds collected from an adult Neem tree growing in Guwahati, North-east India.
2.	Sucrose concentration in induction medium	Emphasized the requirement of 9% sucrose concentration in the induction medium during establishment phase of Neem anther cultures.	In the present investigation, the best callus induction response was obtained in the induction medium with 12% sucrose concentration.
3.	Anther culture pretreatment	No studies performed.	Effect of temperature pretreatments (cold and heat) were studied. The best response was observed in control at 25°C temperature, followed by one day pre-treatments at 12°C and 4°C temperatures; heat treatments had adverse effect.
4.	Culture medium	Calli from multiplication medium were directly transferred to regeneration medium that consisted of only cytokinin (BAP).	In the present case, calli from multiplication medium were first incubated in a combination of one auxin (NAA) and one cytokinins (BAP) at higher concentration to induce shoot-buds in the regeneration medium which consisted of ten times reduced concentration of BAP+NAA.
5.	Shoot regeneration frequency	Maximum shoot regeneration frequency obtained was 75%, with cultures forming an average of 4.5 shoot-buds/explant on MS + BAP (5 μM).	Maximum shoot regeneration frequency obtained was 98.5% with an average of 8.5 shoot-buds/ explant on MS + BAP (2.2 μM) + NAA (0.05 μM).
6.	Shoot elongation medium	Shoots were elongated at ten times lower concentration of regeneration medium consisting of BAP at 0.5 μM.	In the present study, shoot elongation required a pretreatment of GA_3 (3 μM) for two weeks followed by transfer of shoots to elongation medium MS + BAP (0.5 μM).
7.	Outcome of study	Anther cultures established and androgenic plants produced.	An improved method is established to raise androgenic plants. Additionally, androgenic cultures and plantlets were characterized with respect to azadirachtin production.
8.	Reason for different culture medium requirements for the same explants taken from two different trees	1. Different environmental conditions of the two regions, Delhi and Guwahati, where the experimental trees were growing, thus, affected the physiology of the parent plant.	
		2. The tree is highly heterozygous in nature due to strict cross pollination. As a result, the plants growing side by side also show variation due to genetic reasons.	

Table 2. Effect of sucrose concentration on callus induction from anther cultures

Sucrose (g/l)	Total anthers cultured	Anthers responded	Percent response(*)
30 (control)	480	193.82	40.38^{ab}
60	480	252.96	52.7^{b}
90	480	299.28	62.35^{ab}
120	480	409.92	85.4^{a}
150	480	168	35^{c}
180	480	24	5^{d}

Growth Period: 8 weeks. Control: MS + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM) with 3% Sucrose. (*) Those marked with the same letters are not significantly different at $p < 0.05$ according to Duncan's multiple range test.

to high BAP (22 μM) and low NAA (0.5 μM) supplemented medium for 12 weeks, and then subsequently transferred to 10 times lower concentration of BAP (2.2 μM) + NAA (0.05 μM). With this two step method, the shoot-bud regeneration frequency was remarkably improved where 98.5% cultures formed an average of 8.5 shoot-buds per explant, after 6 weeks (**Fig. 1G**). Compare to this, Chaturvedi et al.[7] could be able to obtain regeneration in 75% cultures with an average of 4.5 shoot-buds on the best medium consisted of only cytokinin (BAP) (**Table 1**), whereas in the present case it had to be compounded with an auxin (IAA/NAA). Histological sections of regenerating calli revealed that before the onset of shoot-buds, well organized vascular strands with tracheary elements were developed (**Fig. 1H**) within the calli. These conducting strands later gave rise to nodules as was evident by scanning electron micrographs of the calli (**Fig. 1I**).

Since the shoots did not grow much on regeneration medium, therefore, small shoots were excised, pretreated with 3 μM GA_3 for 14 days and transferred to lower BAP concentration at 0.5 μM. With this method, on an average, shoots attained a length of 4.5 cm with four nodes per shoot after 6 weeks (**Fig. 1J**). Further shoot multiplication was achieved on MS + BAP (1.0 μM) + CH (250 mg/l) via axillary shoot proliferation by culturing single nodal segments. Here shoots grew 6 cm long with six nodes per shoot in 6 weeks. The number of propagules obtained at the end of a multiplication cycle was taken to be the rate of shoot multiplication. Hence, adopting this method, six-fold shoot multiplication was achieved every 6 weeks.

Rooting of shoots and ploidy analysis. For rooting, 3 cm long terminal portions of 6-week-old elongated shoots were cut and transferred to quarter (1/4) strength MS medium supplemented with IBA (0.5 μM). Remaining part of the shoot was utilized for multiplication by nodal segment culture. On ¼ MS + IBA (0.5 μM), root initiation occurred in three weeks directly from the

Table 3. Effect of temperature pretreatments at different duration on callusing from anthers cultured on MS (with 12% sucrose) + 2,4-D (1 μM) + NAA (1 μM) + BAP (5 μM); Growth Period: 8 weeks

Temperature (°C)	Duration (days)	Total anthers cultured	Anthers responded	Percent response
25 (control)	-	480	409.92	85.4
4	1	120	94.44	78.7
	7	120	67.92	56.6
	14	120	51.24	42.7
12	1	120	98.28	81.9
	7	120	68.64	57.2
	14	120	56.76	47.3
32	1	120	58.18	48.48
	7	120	60.00	50.0
	14	120	74.11	61.76
40	1	120	66.32	55.27
	7	120	0	0
	14	120	0	0

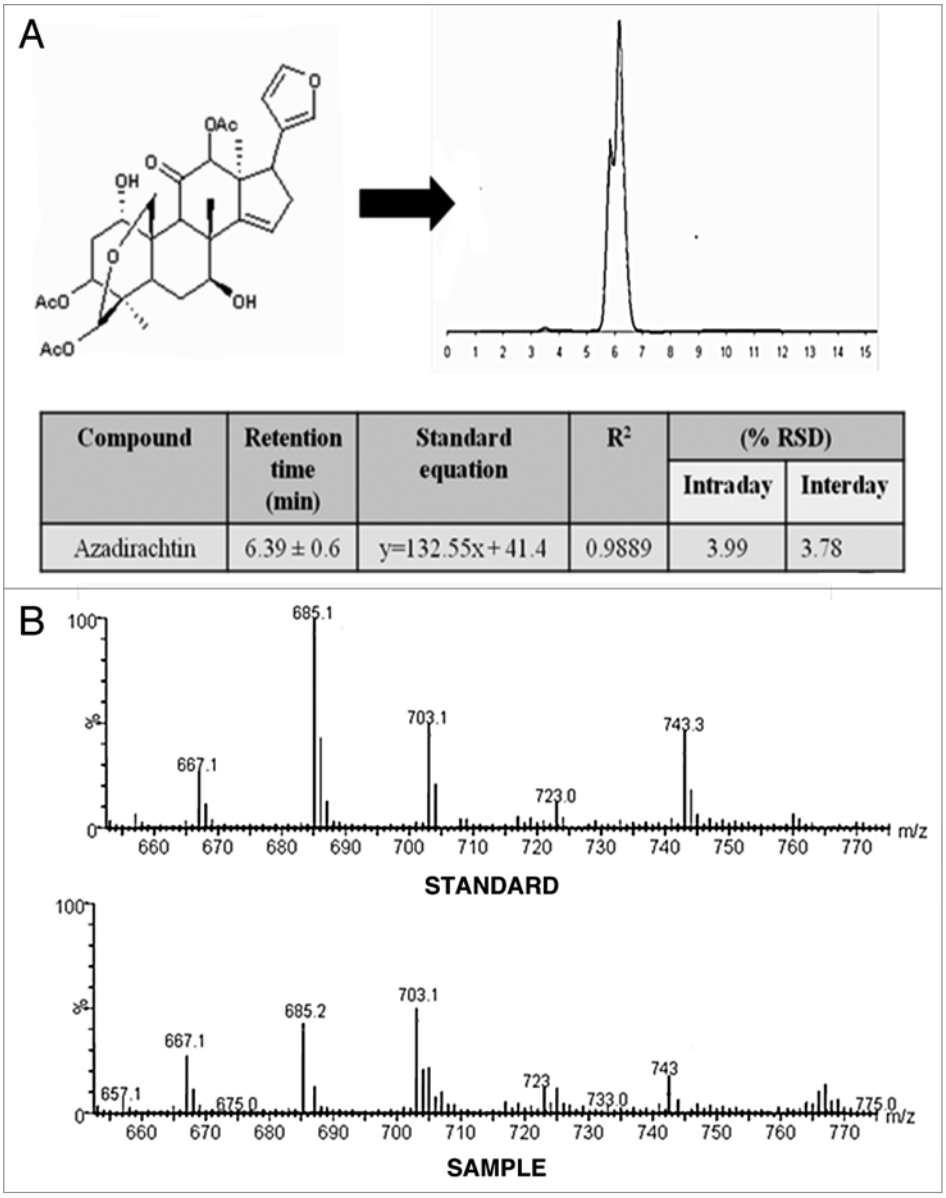
Two-way ANOVA to study the effect of different temperatures and duration of pretreatment on anther cultures.

Summary	Count	Sum	Average	Variance		
Row 1	4	264.35	66.0875	278.7176		
Row 2	4	163.8	40.95	755.93		
Row 3	4	151.76	37.94	705.7011		
Column 1	3	178	59.33333	329.6033		
Column 2	3	186.4	62.13333	317.5433		
Column 3	3	160.24	53.41333	52.82773		
Column 4	3	55.27	18.42333	1018.258		
Source of Variation	**SS**	**df**	**MS**	**F**	**p-value**	**F crit**
Treatment length	1910.981	2	955.4905	3.758117	0.087476	5.143253
Different temperatures	3695.563	3	1231.854	4.8451-05	**0.048185**	4.757063
Error	1525.483	6	254.2472			
Total	7132.027	11				

basal cut end of the shoots and after six weeks 100% cultures developed an average of 10 roots per shoot (**Fig. 1K**).

Cytological analysis of callus cells in induction, multiplication and regeneration media, and shoot-tips from haploid plantlets revealed that majority of the cells were in haploid state (2n = x = 12) (**Fig. 1L**). However, by the time entire plantlets were developed on ¼ MS + IBA (0.5 μM), 40% of the plants maintained their haploid status while the rest were diploids (2n = 2x = 24) or aneuploids (2n = 2x - 2 = 22) or (2n = 2x - 1 = 21) which may arose from haploid cells either spontaneously or due to manipulation of cultures. The mitotic preparation from root-tips of seedlings showed the diploid number of chromosomes in Neem is (2n = 2x = 24) (**Fig. 1M**). It has been established by earlier workers that ploidy levels, lower than the usual ploidy level of a species is quite unstable. In long term cultures, diploidization in haploid calli is much more common than the occurrence of tetraploids in diploid lines.[11] The present study is also in agreement with this finding as majority of the cells maintained their haploid status for a long period but gradually, by the time of plantlet formation, this frequency reduced. It may be attributed to the unstability of cells in haploid state and tendency to undergo autodiploidization through endomitosis to regain their normal status. As a result, the frequency of diploid cells in culture increases with age.[10,12]

Identification of azadirachtin by HPLC and mass spectrometry. The standard and sample extracts, prepared by following the protocol as described in materials and methods, showed elution of azadirachtin, a tetranortriterpenoid, at 6.39 min in HPLC (**Fig. 2A**). Calibration curve for azadirachtin standard showed good linearity at tested concentrations (0.0625 mg/ml to 1 mg/ml) with a correlation coefficient (R^2) of 0.9889. The equation generated from the curve by external standard method (**Fig. 2A**) was used to calculate amount of compounds present in crude sample. For precision, the standard samples at same concentration were analyzed at least five times within the same day and the RSD value obtained was 3.99%. Similarly, for interday variability, where same concentration of the standard compound was run thrice at one day interval, the value was 3.78%. From the standard equation obtained, the amount of azadirachtin in different callus lines was calculated and listed in **Table 4**. Presence of azadirachtin was confirmed in all the callus lines tested. However, it was observed that redifferentiated



Compound	Retention time (min)	Standard equation	R^2	(% RSD)	
				Intraday	Interday
Azadirachtin	6.39 ± 0.6	y=132.55x+41.4	0.9889	3.99	3.78

Figure 2. HPLC chromatogram of standard azadirachtin. (A) A structure of Azadirachtin, a tetranortriterpenoid (left side). Right side of figure shows Azadirachtin peaks eluted at 6.39 min in HPLC. The table below the peak is showing the data generated from azadirachtin standard. (B) Mass spectrometric profile of standard azadirachtin and HPLC eluted sample fraction.

calli on MS + BAP (2.2 μM) + NAA (0.05 μM), possessed highest azadirachtin content (728.41 μg/g DW) while the least was obtained from dedifferentiated line, maintained on MS + 2,4-D (1 μM) + Kinetin (10 μM) (49 μg/g DW). In vitro grown haploid leaves contained 700 μg/g DW of azadirachtin (**Table 4**).

The azadirachtin fractions eluted from HPLC of samples were collected and the fragment characteristics were compared by mass spectra with that of standard azadirachtin procured from Sigma, Aldrich (**Fig. 2B**). Spectra were obtained in full scan mode. Base peak of m/z 685 resulted due to loss of two water molecules [$MH^+ - 2H_2O$], m/z 703 formed due to the loss of one water molecule [$MH^+ - H_2O$] and m/z 743 corresponded to the formation of sodium adduct [$MH^+ + Na^+$]. Presence of similar m/z fragments in both standard compound and sample fractions has further confirmed the presence of azadirachtin.

Assessment of haploid (n) cell lines and their derivatives, as source of azadirachtin has been done for the first time in Neem, in the present study. Till date, the available reports on azadirachtin and related limonoid production have utilized cultures from different somatic parts (heterozygous diploids) like leaves,[13-18] bark,[13] stem[17] and seeds (embryos).[18,19] However, as is the case with the metabolite content under in situ conditions, the same limitation is posed if cultures are established from seeds or other somatic tissues owing to the variability inflicted upon by prevalent heterozygosity in the genus due to cross pollination. The yield range of azadirachtin, obtained in the present study, from haploid lines is much higher (49–728.41 μg/g) than most of these reports. In available reports, while the recorded values of azadirachtin in leaf derived callus was in the range of 4 μg/g to 64 μg/g DW; bark, embryo and stem cultures yielded 44 μg/g DW, 4–8 μg/g DW and 2.7 μg/g DW azadirachtin, respectively. Moreover, variability in content due to heterozygosity is the major issue that needs to be addressed in cases where somatic parts are utilized.

Materials and Methods

Establishment of cultures and callus induction. Healthy flowering twigs of Neem were collected from a mature Neem tree growing near the campus of IIT-Guwahati, during the months of April-May between 5:30 a.m. to 6:30 a.m. The stage of microspore development was checked with acetocarmine squashes. Two mm size flower buds were surface sterilized with 0.1% mercuric chloride solution for 7 min inside the laminar-air-flow cabinet (Saveer Biotech, India) and rinsed thrice with sterile distilled water. The buds were dissected and twenty anthers, from two buds, were cultured in 60 mm x 15 mm pre-sterilized, disposable Petriplates containing 10 ml of medium with or without various combinations and concentrations of growth regulators (2,4-D, NAA, BAP, IAA, Kinetin) and sealed with parafilm (Pechiney, USA). The best responding combination was checked at various sucrose concentrations, 3–18%. Three different basal media MS,[20] B_5,[21] and NLN,[22] were also tested with best sucrose and

Table 4. Evaluation of androgenic lines of Neem for azadirachtin production

Material	Medium	Amount of azadirachtin (μg/g DW)
Dedifferentiated callus	MS + 2,4-D (0.5 μM)	288 ± 0.32
Dedifferentiated callus	MS + 2,4-D (0.5 μM) + Kinetin (4.5 μM)	79 ± 0.3
Dedifferentiated callus	MS + 2,4-D (1 μM) + Kinetin (10 μM)	49 ± 0.2
Redifferentiated callus	MS + BAP (2.5 μM) + IAA (5 μM) + CH + (500 mg/l)	411.38 ± 0.3
Redifferentiated callus	MS + BAP (2.2 μM) + NAA (0.05 μM)	728.41 ± 0.4
In vitro leaves (haploids)	BAP (0.5 μM)	700 ± 0.52
Control I (Leaves parent plant)	-	5435.6 ± 4.0
Control II (Seeds parent plant)	-	6800 ± 4.1

growth regulator combinations. Anthers from each treatment were given a temperature pretreatment of 4°C, 12°C, 32°C and 40°C for 1, 7 and 14 days, respectively, to induce calli. All the cultures were kept in dark until 8 weeks, for callus induction.

Callus multiplication and shoot regeneration. The calli obtained from induction media were further multiplied and maintained on media containing completely different sets of auxin (2,4-D) or/and cytokinin (Kinetin). From now on calli were maintained in diffused light (1,000–1,600 lux) under 16 h photoperiod. For experiments on shoot organogenesis, calli (ca. 0.2 g) from maintenance media were utilized and grown on MS medium supplemented with cytokinins, BAP in combination with auxins like NAA or IAA and additives like CH to obtain shoot regeneration from callus.

Histological studies. For histological sections, regenerating calli were sampled and wax sections were cut to trace the developing vascular strands in the calli. Material was fixed in FAA (5:5:90 v/v/v Formalin: Acetic acid: 70% Ethanol) for 48 h and stored in 70% alcohol. After passing through the tertiary-butyl alcohol (TBA) series for dehydration, the material was infiltrated with paraffin wax (melting point 60°C, E. Merck, Germany) and finally, embedded in pure paraffin wax. Paraffin blocks were mounted on wooden stubs and 8–10 μm thick sections were cut using Rotary Microtome (Leica, Germany), attached with a steel knife. The sections were mounted on microslides, dewaxed and double stained with safranin (1%) and astra-blue (1%).

Scanning electron microscopy. Nodulated calli from shoot regeneration media were fixed in 2.5% glutaraldehyde and dehydrated through a graded alcohol series. After drying, the samples were sputter-coated with gold and observed under a scanning electron microscope (Leo 1430vp, Carl Zeiss, Germany).

Shoot elongation, multiplication and rooting. Individual shoots of 0.5 cm length were detached from calli and given a 14 days pre-treatment of GA_3 at 3 μM concentration before transferring them to a lower concentration of BAP at 0.5 μM for elongation. After attaining sufficient length, further shoot multiplication was achieved on MS medium supplemented with BAP (1.0 μM) and CH (250 mg/l) by employing single node segments. The number of propagules obtained at the end of a multiplication cycle was regarded as the rate of shoot multiplication.

For rooting, individual shoots, measuring 3 cm, with 3–4 nodes, were excised and cultured on ¼ MS (major salts reduced to quarter strength) medium supplemented with IBA at 0.5 μM.

Ploidy analysis. For ploidy analysis, calli from induction, multiplication and regeneration media, root/shoot-tips from in vitro raised plantlets and roots tips from seedlings were excised and pretreated with 0.02% 8-hydroxyquinoline (BDH, India) at 4°C for 4 h. This was followed by fixation in modified Carnoy's fluid containing absolute alcohol, chloroform, glacial acetic acid and methanol (7:3:1:1) for 48 h. The fixed material was warmed in a mixture of nine drops of 1% aceto-orcein (or) 2% aceto-carmine and one drop of 1 N HCl, squashed and observed under Nikon epifluorescence microscope. The cells showing a good separation of chromosomes were photographed.

Selection of haploid lines and preparation of extract. The androgenic calli from different media, leaves from in vitro developed haploid plantlets and leaves (control I) and seeds (control II) from adult parent plant were utilized to check azadirachtin production. The calli cultures were broadly categorized into dedifferentiated (non-regenerating) and redifferentiated (regenerating) lines. These Calli were harvested, washed with distilled water and filtered under vacuum. Thereafter, washed callus lines, leaves and seed samples were dried in an oven at 30°C ± 2°C until a constant weight was achieved. The dried samples were soaked in methanol (analytical grade, Merck, India) overnight followed by sonication for 45 min at 35% amplitude (pulser 5 s on/off cycle). The extract was centrifuged at 10,000 rpm for 15 min and was further fractionated by adding water in 60:40 ratio followed by 50:50 dichloromethane (Merck, India). Dichloromethane fractions were pooled, dried in a rotary evaporator (Buchi R-200, Japan) and further analysed for presence of azadirachtin. Extraction from seeds consisted of an additional step of defattening with hexane prior to extraction with methanol.

Preparation of azadirachtin standard. Stock solution of standard azadirachtin (Sigma-Aldrich, USA) was prepared in methanol (HPLC grade, Merck, India) at a concentration of 1 mg/ml and stored at -20°C. Calibration curve was generated by external standard method. The stock solution was serially diluted to five different concentrations and each concentration was run at least thrice to check the repeatability of results.

Linearity of developed method was checked by running the standard compound at five different concentrations. Precision of developed assay was evaluated by running same concentration of standard compounds at least four times on the same day (intraday) and thrice at one day interval (interday). The values

were calculated in terms of percent relative standard deviation (% RSD) which is calculated as: (Standard Deviation/Mean) x 100.

High performance liquid chromatography. Quantitative estimation of azadirachtin was carried out on Varian Prostar HPLC system (Varian, USA) equipped with a binary pump, UV detector and a 20 µl injection loop. Hypersil BDS RP-C18 column (Thermo, USA) of dimensions 250 x 4.6 mm was used with methanol:water (90:10) (v/v) as the mobile phase at a flow rate of 0.5 ml/min. The eluted samples were detected at 214 nm. The identification of azadirachtin was done by comparing its retention time with authentic standard (Sigma, USA). The crude and standard samples were filtered through 0.22 µm PVDF membrane filters (Millipore, USA) prior to analysis and aliquots of 20 µl of clean solution were injected into the HPLC system. System suitability tests were performed by checking linearity and precision of the developed assay.

Mass spectroscopy. MS detection was carried out on Waters Quadrapole-Tof Premier mass spectrometer with micro channel plate detector (Waters, USA). Samples were analyzed in positive mode with a probe temperature of 400°C and a source block temperature of 150°C. The source was operated with a corona pin voltage of 3.50 kV and a cone voltage of 25 V. The MS data were obtained in full scan mode (mass range 100–1,000 amu). A comparison of mass spectra of the standard compound with that of sample isolated from HPLC, confirmed the presence of azadirachtin.

Statistical analysis. For culture initiation and induction responses, 20 anthers were placed in one Petridish with 24 dishes for each treatment. Data were collected as number of responding anthers relative to total number of anthers cultured. Regeneration in the subcultures of callus was expressed as percent response. Data were subjected to one-way or two-way analysis of variance (ANOVA) and means were calculated using Duncan's multiple range test by SPSS 16.0 software. p-values less than 0.05 were considered statistically significant. Arcsine transformation was done for the percent values before proceeding for ANOVA. For azadirachtin estimation, all results are an average of three separate analysis. Results are represented as mean ± SD.

Conclusions

In our search for novel sources, we believe that haploid lines could be a valuable source of consistent qualitative and quantitative homogeneous metabolite production. These lines, to our knowledge, were never earlier assessed for azadirachtin related metabolite production. This study has brought us a step closer to obtain genetically pure lines (homozygous diploid) of Neem and to diminish variations which are prevalent in the genus due to heterozygosity. As the conventional methods to produce homozygous diploid plants is lengthy and laborious, requiring 7–8 recurrent cycles of inbreeding which is impossible incase of cross-breeding trees like Neem. With in vitro haploids, homozygous diploids can be produced in a single step, cutting down the breeding period to almost half. Our goal is to employ double-haploid lines (homozygous diploid) for identification and purification of compounds in bulk, round the year independent of region and vagaries of climate, by employing superior lines.

Acknowledgements

The authors thank Department of Science and Technology (DST), New Delhi, India, for financial assistance.

References

1. Kaushik N. Determination of azadirachtin and fatty acid methyl esters of *Azadirachta indica* seeds by HPLC and GLC. Anal Bioanal Chem 2002; 374:1199-204.
2. Priyadarsini RV, Murugan RS, Sripriya P, Karunagaran D, Nagini S. The neem limonoids azadirachtin and nimbolide induce cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells. Free Radic Res 2010; 44:624-34.
3. Thoh M, Babajan B, Raghavendra PB, Sureshkumar C, Manna SK. Azadirachtin interacts with retinoic acid receptors and inhibits retinoic acid-mediated biological responses. J Biol Chem 2011; 286:4690-702.
4. Koul O, Isman MB, Ketkar CM. Properties and uses of neem, *Azadirachta indica.* Can J Bot 1990; 68:1-11.
5. National Research Council. Neem: A Tree For Solving Global Problems. National Academy Press, Washington DC 1992.
6. Srivastava P, Chaturvedi R. In vitro androgenesis in tree species: An update and prospect for further research. Biotechnol. Adv 2008; 26:482-91.
7. Chaturvedi R, Razdan MK, Bhojwani SS. Production of haploid plants of neem (*Azadirachta indica* A. Juss.) by anther culture. Plant Cell Rep 2003; 21:531-7.
8. Bhojwani SS, Razdan MK. Plant tissue culture: theory and practice. Amsterdam, Elsevier 1996.
9. Pattnaik SJ, Rao NDR, Chary P. Ecomorphometric markers reflect variations in Azadirachtin-A content of *Azadirachta indica* A. Juss. (Meliaceae) in select regions of Andhra Pradesh, India. Curr Sci 2006; 91:628-36.
10. Atanassov A, Zagorska N, Boyadjiev P, Djilianov D In vitro production of haploid plants. World J Microbiol Biotechnol 1995; 11:400-8.
11. Zagorska NA, Shtereva LA, Kruleva MM, Sotirova VG, Baralieva DL, Dimitrov BD. Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.). III. Characterization of the regenerants. Plant Cell Rep 2004; 22:449-56.
12. Sacristan MD. Karyotypic changes in callus cultures from haploid and diploid plants of *Crepis capillaris* (L.) Wallr. Chromosoma 1971; 33:273-83.
13. Wewetzer A. Callus cultures of *Azadirachta indica* and their potential for the production of azadirachtin. Phytoparasitica 1998; 26:47-52.
14. Allan EJ, Eeswara JP, Mordue AJ, Morgan ED, Stuchbury T. The production of azadirachtn by in vitro tissue culture of neem, *Azadirachta indica*. Pestic Sci 1994; 42:147-52.
15. Kuruvilla T, Komaraiah P, Ramakrishna SV. Enhanced secretion of azadirachtin by permeabilized margosa (*Azadirachta indica*) cells. Indian J Exp Biol 1999; 37:89-91.
16. Kearney ML, Allan EJ, Hooker JE, Mordue AJ. Antifeedant affects of in vitro culture extracts of the neem tree, *Azadirachta indica* against the desert locust (*Schistocera gregaria)*. Plant Cell Tissue Organ Cult 1994; 37:67-71.
17. Sundaram KMS, Curry J. Effect of some UV light absorbers on the photostabilization of azadirachtin, a neem-based biopesticide. Chemosphere 1996; 32:649-59.
18. Srividya N, Sridevi BP, Satyanarayana P. Azadirachtin and nimbin content in in vitro cultured shoots and roots of *Azadirachta indica* A. Juss. Ind J Plant Physiol 1998; 32:129-34.
19. Prakash G, Emmannuel CJSK, Srivastava AK. Variability of azadirachtin in *Azadirachta indica* (neem) and batch kinetics studies of cell suspension culture. Biotechnol Bioprocess Eng 2005; 10:198-204.
20. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 1962; 15:473-97.
21. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 1968; 50:151-8.
22. Lichter RZ. Anther culture of *Brassica napus* in a liquid medium. Pflanzenphysiol 1981; 103:229-37.