In vitro response of select regions of *Azadirachta indica* A. Juss (Meliaceae) as elucidated by biochemical and molecular variations

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Micro-populations of neem from alkaline and neutral soils were grouped into high and low azadirachtin-A containing plants, respectively. The leaf explants from the seedlings of high azadirachtin-A containing seeds exhibited the best callus induction response on MS medium supplemented with NAA, whereas leaf explant of the seedlings from low azadirachtin-A seeds showed similar response on MS medium with Kn in addition to NAA. Calli from most of the explants showed root differentiation after 30 days, whereas the callus obtained from the leaf explants alone also redifferentiated into shoots. Callus biomass from leaf explants was increased by cold-treatment. Biochemical studies revealed detectable amounts of azadirachtin-A only in cotyledons of the seedlings from high azadirachtin-A containing seeds. Other secondary metabolites such as steroids and saponins were detected from calli of several explants from the seedlings of both high and low azadirachtin-A containing seeds. Genomic DNA amplification studies showed polymorphism not only between the two azadirachtin-A groups but also within that of DNA from the tender leaves, *in vitro* seedlings of high azadirachtin-A yielding seeds and calli of leaf explants.

Keywords: Azadirachtin-A, growth factors, *in vitro* cultures, molecular analysis, neem.

AZADIRACHTA indica A. Juss, commonly referred to as neem, is of great importance in agriculture owing to its antifeedant and insect-repelling properties. This is predominantly contributed by secondary metabolites (tetranortriterpenoids) such as azadirachtin-A, nimbin and nimbidin[1]. In general, the yield of these secondary metabolites is genetically determined, but that of azadirachtin-A also specifically varies with edaphic and climatic factors, maturity of the seeds, and length of storage and post-harvest handling, etc.[2,3]. The selection of elite neem trees with high azadirachtin-A content in seed kernels has been emphasized[3–6]. In order to improve the yield of azadirachtin-A per gram biomass of tissue, given the fact that it is present in such small quantities in nature, tissue-culture studies could offer a solution to this problem[7].

Besides the identification of secondary metabolites such as azadirachtin-A, it is of equal importance to determine other compounds such as steroids and saponins that could throw light indirectly on the biochemical pathway of these secondary metabolites. Although tissue cultural techniques for the propagation and production of insecticidal plant constituents, including terpenoids are being widely investigated, the genetic stability of the plantlets regenerated from the callus is unpredictable to date.

Molecular techniques like Amplified Fragment Length polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) and Random Fragment Length Polymorphism (RFLP) banding patterns are recently being used to assess neem ecotypes for genetic diversity[8–10].

The current investigation aims to estimate the amount of azadirachtin-*A in vitro* raised callus and determine the genetic variation in neem trees.

Materials and methods

Seeds from high and low azadirachtin-A yielding neem trees grown in alkaline and neutral soils were selected for the present study. Different explants such as leaf, hypocotyl, epicotyl, cotyledons and root were selected from *in vitro* grown seedlings for callus induction. Explants (0.5–1.0 cm) were grown on MS medium supplemented with naphthaleneacetic acid (NAA; 1.0–2.0 mg/l) and kinetin (Kn; 0.5 mg/l). The medium was supplemented with 3% (w/v) sucrose as a carbon source. The pH of the medium was adjusted to 5.7 before autoclaving at a pressure of 1.06 kg cm^{-2} and 120°C for 15 min. All cultures were incubated at 25 ± 2°C, 16 h photoperiod by cool white fluorescent light (35 mEm^{-2} s^{-1}).

The seeds were surface-sterilized by treating with 2% $HgCl_2$ and Triton X 100 (in 100 ml H_2O) for 20 min with gentle agitation[11]. The seeds were then thoroughly washed with sterile distilled water thrice prior to use for *in vitro*

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germination studies. Sterilized seeds were cultured on Murashige and Skoog (MS; 1969) basal medium. In order to prevent bacterial and fungal contamination, after sterilizing and cooling the MS medium, 20 ppm diethane fungicide, 50 ppm capton and 62.5 mg of cefotaxime were added.

Seeds were made into a fine powder using a sample mill. By repeated treatment with *n*-hexane, fat was completely removed from the samples and azadirachtin-A was subsequently extracted in methanol[12]. An aliquot of 20 µl of this extract was utilized for quantifying azadirachtin-A using a differential refractometer. Furthermore, several other secondary metabolites were also analysed from this extract using Thin Layer Chromatography (TLC). They included identification of steroids, 1,2 diols, flavonoids, esters and acids. To estimate these secondary metabolites, equal amounts of calli from all the explants were ground in acetone individually and separated on TLC by different solvent systems. Then, specific spray agents were utilized to identify individual compounds. For esters, hydroxylamine ferric chloride was used. For acids, bromocresol green, for flavanoids, aluminum chloride, for 1,2 diols, metaperiodate-benzidine and for steroids, acetic anhydride and concentrated sulphuric acid were used. Essentially laboratory methods as elucidated by Stahl[13] and Jork[14] were implemented.

In vitro raised seedlings and callus tissue were stored at –20°C for DNA extraction. For molecular analysis, clean genomic DNA free from phenolics was extracted from (a) tender neem leaves from trees, (b) *in vitro* seedlings and (c) calli grown from various explants, using a modified method of Doyle and Doyle[15]. PCR amplification reactions were performed using taq polymerase obtained from FINNZYMES®. RAPD analyses were performed using two RAPD primers (Operon Life Technologies, Alameda, California) and four ISSR primers (Biotool Technoconcepts, India) with the respective sequences: RAPD primers – OPA-09-($^{5'}$GGGTAACGGG$^{3'}$) and OPG-11-($^{5'}$TGCCCG-TCGT$^{3'}$) and ISSR primers – (A) $(CT)_9$-A, (B) $(CA)_8$G, (C) $(CI)_9$-RA and (D) $(CA)_8$-RG).

Results

In vitro studies on seedlings from high and low azadirachtin-A containing seeds

The result of cold treatment on i*n vitro* germination experiments suggested that high azadirachtin-A containing seeds grew slower than low azadirachtin-A containing seeds, bearing lesser leaves, which were light green in colour (Figure 1 *a*). Cold treatment of high azadirachtin-A containing seeds at 4°C for 45 days resulted in seedlings that grew slowly, which developed into a sturdy plantlet supported by profusely coiled roots and exhibited fibrillar foliage (Figure 1 *b*).

The presence of growth hormones in the MS medium had a significant effect on explants. As shown in Figure 1 *c–f*, callus from cotyledon, leaf and hypocotyl explants showed production of roots between 15 and 30 days when grown in MS medium supplemented with NAA alone or NAA in combination with Kn. Of all explants from the seedlings of high azadirachtin-A containing seeds, leaf explants exhibited the best response between 15 and 30 days in terms of callus initiation, callus growth, root initiation from callus and subsequent continuous callus growth in all four hormonal combinations (Table 1). Hypocotyl explant from seedlings of high azadirachtin-A containing seeds also showed continuous growth even after 30 days in 2 mg/l NAA + 0.5 mg/l Kn. None of the other explants from the seedlings of high azadirachtin-A containing seeds showed continuous callus growth after 30 days (Table 1). However, the response of explants from the seedlings of low azadirachtin-A containing seeds was not significant. It was observed that none of the explants from the seedlings of low azadirachtin-A containing seeds showed continuous callus growth after 30 days. While the explants from the seedling of high azadirachtin-A containing seeds showed varied responses by having universality in their choice of media, explants derived from seedlings of low azadirachtin-A containing seeds showed a choice of only one or two different hormonal specifications (Table 2).

The phenomenon of redifferentiation into both roots and shoots was observed only from the calli of leaf explants of both the seedlings from high and low azadirachtin-A containing seeds. Root redifferentiation alone was observed after 30 days from all the five explants obtained from the seedlings of high azadirachtin-A containing seeds, by utilizing four growth hormonal specifications in their respective growth media, whereas, only leaf, epicotyl and hypocotyl explants from the seedlings of the low azadirachtin-A containing seeds showed a similar response (Figure 1 *h* and *i*; Table 3).

The impact of growth hormones as well as cold treatment resulted in increased callus biomass. First, extensive callus growth was obtained from leaf explant of seedlings of both the azadirachtin-A containing groups in 2 mg/l NAA + 0.5 mg/l Kn. The transfer of callus from 1 to 2 mg/l NAA alone was sufficient for improving callus biomass and subsequent shoot redifferentiation from the leaf explants of seedlings of high azadirachtin-A containing plants, whereas addition of kinetin was essential for a similar response from leaf explants of seedlings from the low azadirachtin-A containing seeds. A second method that improved callus biomass was to subject the seeds to cold treatment (at 4°C for 45 days). Callus growth was significantly increased from leaf explant obtained from seedlings of high azadirachtin-A containing seeds (Figure 1 *g*). The seedlings obtained after cold treatment were also sturdier with copious production of highly coiled roots and with the formation of thick fibrillar leaves (Figure 1 *b*).

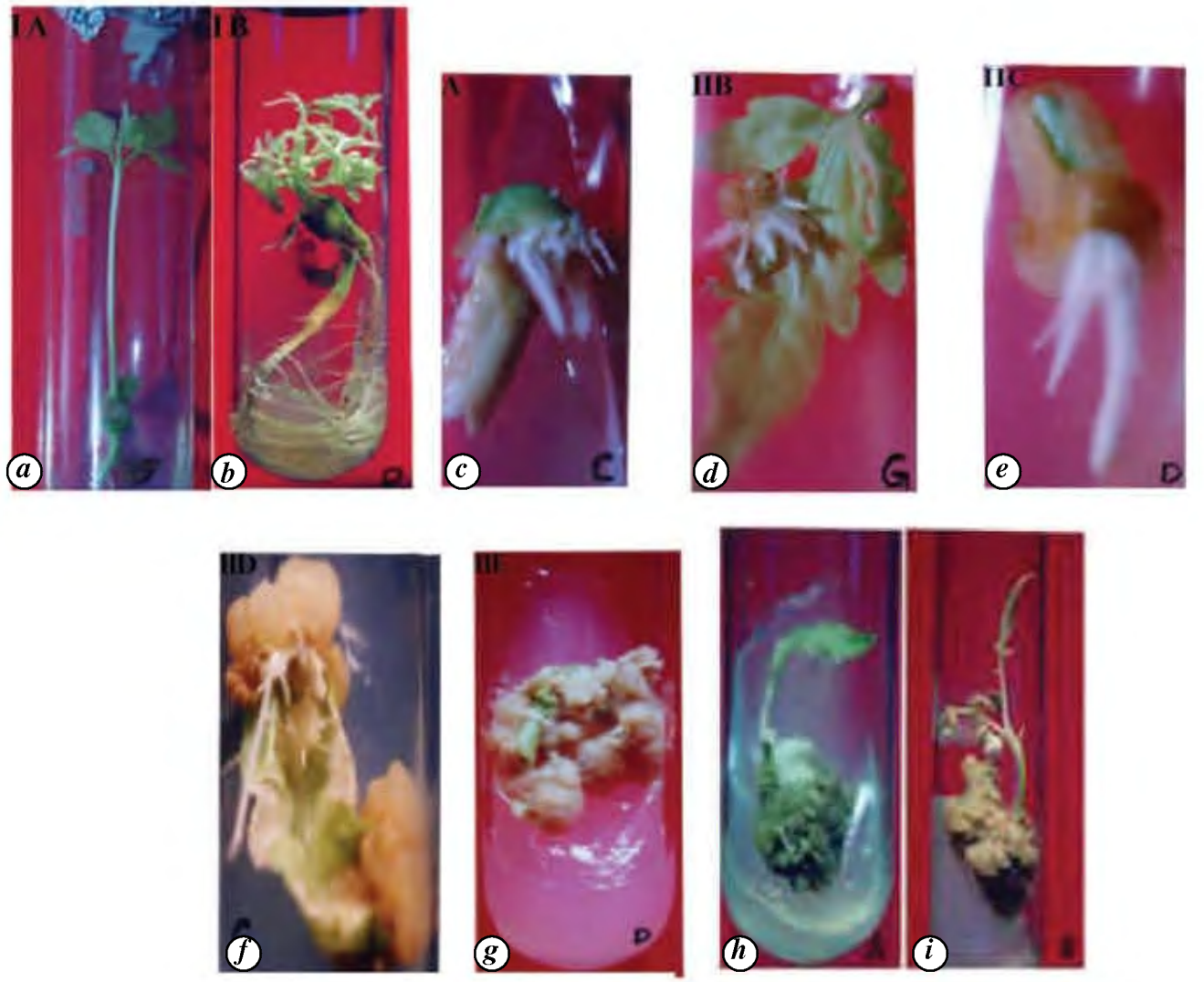

Figure 1. ***a***, ***b***, *In vitro* germination of *Azadirachta indica*: (***a***) High azadirachtin-A seeds without cold treatment, and (***b***) Low azadirachtin-A seeds with cold treatment (4°C) after 45 days. ***c***, Callus induction from cotyledon explant of *A. indica* (high azadirachtin-A) seedlings on MS + 2 mg/l NAA after 15 days. ***d***, ***e***, Callus induction from hypocotyl explant of *A. indica* (***d***) High azadirachtin-A seedlings on MS + 1 mg/l NAA + 0.5 mg/l Kn, after 15 days and (***e***) Low azadirachtin-A seedlings on MS + 1 mg/l NAA + 0.5 mg/l Kn after 15 days. ***f***, ***g***, Callus induction from leaf explant of *A. indica*: (***f***) Low azadirachtin-A seedlings on MS + 2 mg/l NAA + 0.5 mg/l Kn after 30 days and (***g***) High azadirachtin-A seedlings on MS + 2 mg/l NAA + 0.5 mg/l Kn. ***h***, ***i***, Shoot from callus of leaf explant of *A. indica*: (***h***) High azadirachtin-A seedlings on MS + 2 mg/l NAA and (***i***) Low azadirachtin-A seedlings on MS + 2 mg/l NAA + 0.5 mg/l Kn.

Table 1. Response of explants from high azadirachtin A seeds of *Azadirachta indica* grown on MS medium supplemented with NAA and Kn after 15 and 30 days

Plant growth regulator (mg/l)		After 15 days					After 30 days				
		Root	Hypocotyl	Cotyledon	Epicotyl	Leaf	Root	Hypocotyl	Cotyledon	Epicotyl	Leaf
NAA	Kn										
1.0	0		CI	CI	CG	CI, CR	CR	CI	CR	CG	CG, CR, CT
2.0	0		EE, CI	CR	EE	CI, CG, CR		CG	CR	CI	CG, CR, CT
1.0	0.5		CG			CI ,CG, CR		CG			CG, CR, CT
2.0	0.5	CI	EE	CG		CG	CR	CG, CT	CG	CG	CG, CT

EE, Explant expansion; CI, Callus initiation; CG, Callus growth; CR, Root initiation from callus; CT, Callus growth continued.

Table 2. Response of explants from low azadirachtin-A containing seeds of *A. indica* grown on MS medium supplemented with NAA and Kn after 15 and 30 days

Plant growth regulator (mg/l)		After 15 days					After 30 days				
		Root	Hypocotyl	Cotyledon	Epicotyl	Leaf	Root	Hypocotyl	Cotyledon	Epicotyl	Leaf
NAA	Kn										
1.0	0	CI		CI, CG	CR		CR	CI	CR		
2.0	0	EE	CG		CI			CG			
1.0	0.5	CI	CR		EE	CI	CG	CG, CR	CG		CG, CR
2.0	0.5		EE		EE	CG			CI		CG, CR

EE, Explant expansion; CI, Callus initiation; CG, Callus growth; CR, Root initiation from callus; CT, Callus growth continued.

Table 3. Organogenesis response of callus derived from seed kernels of *A. indica* grown on MS medium supplemented with NAA and Kn after 15 and 30 days

Plant growth regulator (mg/l)		High azadirachtin seed										Low azadirachtin seed									
		R		H		C		E		L		R		H		C		E		L	
NAA	Kn	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d	15 d	30 d
1.0	0.0		R						R	R, S	R							R	R		
2.0	0.0						R			R	R										
1.0	0.5									R	R			R	R						R, S
2.0	0.5		R	R																	R

Different explants used for callus growth and subsequent organogenesis: Explants – R, Root; H, Hypocotyl; C, Cotyledon; E, Epicotyl; L, Leaf. Organogenesis – R, Root; S, Shoot.

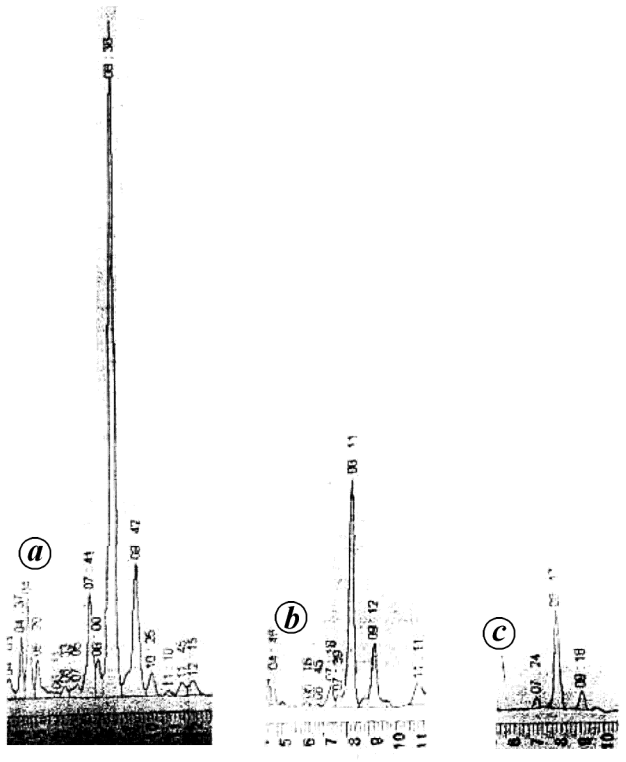

Figure 2. HPLC analysis of crude extract of neem seed kernels. ***a***, Standard; ***b***, High quantity of azadirachtin-A; ***c***, Low quantity of azadirachtin-A.

Screening of secondary metabolites

Secondary metabolites play an important role in protecting plants against invaders and warding-off diseases. Although azadirachtin-A, a potent biopesticide is normally estimated from neem seed kernels (Figure 2 *a–c*), other secondary metabolites also offer defence to plants. Azadirachtin-A was detected in measurable amounts only in cotyledons from the seedlings of the high azadirachtin-A yielding seeds. However, our study and as well as others determined that it also existed in negligible amounts in the calli from various explants of the seedlings from high and low azadirachtin-A containing seeds[16] (Figure 2 *a–c*; Table 4).

Our experiments exhibited higher levels of steroids in low azadirachtin-A containing seeds and in calli from explants of leaves and cotyledons from the same plant. However, the levels of steroids in the calli derived from epicotyl, hypocotyl and roots from the seedlings of high azadirachtin-A containing seeds were higher as shown by TLC analysis (Table 4). Saponins (steroids + 1,2-diols) measured in the various calli of explants from the seedlings of high and low azadirachtin-A containing seeds were in concurrence with the results of those obtained on steroids. As saponins are clear indicators to determine the resistance of a plant, the presence of high levels of this metabolite in neem plants resulted in sturdy growth,

Table 4. Secondary metabolites isolated from seed and callus obtained from various explants of *in vitro* raised seedlings of *A. indica*

	Callus										Seed	
	Root		Hypocotyl		Cotyledon		Epicotyl		Leaves			
Secondary metabilite	A	B	A	B	A	B	A	B	A	B	A	B
Steriods	++	+	++	+	+	++	++	+	+	++	+	++
1,2-Diols	++	+	++	+	+	++	++	+	+	++	–	–
Sapoinins	++	+	++	+	+	++	++	+	+	++	NA	NA
Azadirachtin-A	–	–	–	–	++	–	–	–	–	–	–	–
Flavanoids	X	X	X	X	X	X	X	X	X	X	X	X
Esters	X	X	X	X	X	X	X	X	X	X	X	X
Acids	X	X	X	X	X	X	X	X	X	X	X	X

A, Explant from a seedling from high azadirachtin-A containing seed; B, Explant from a seedling from low azadirachtin-A containing seed; +, Present in moderate amounts; ++, Present in significant amounts; X, No reaction ; –, Negligible; NA, Not applicable.

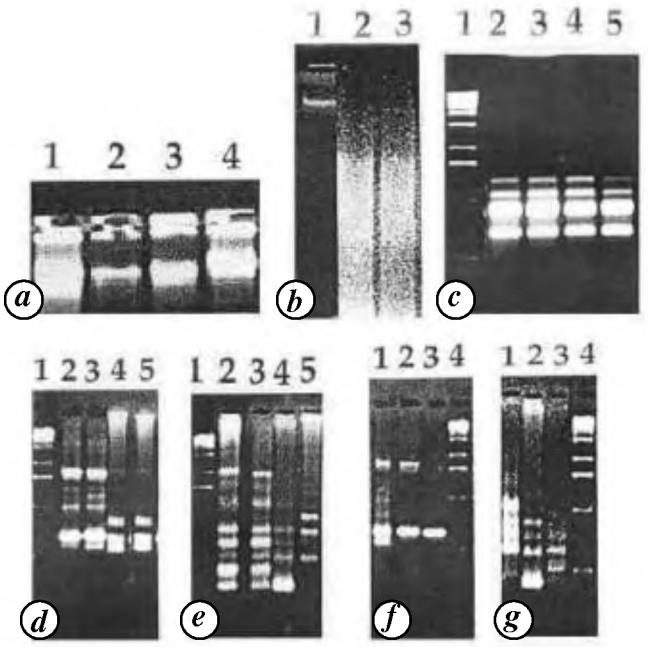

Figure 3. ***a***, Genomic DNA from *A. indica* leaf extraction by CTAB method. Lanes 1 and 2, High azadirachtin-A containing seed; lanes 3 and 4, Low azadirachtin-A containing seed. ***b***, Restriction endonuclease digestion. Lane 1, Lambda marker; lanes 2 and 3, High azadirachtin-A seed; lanes 4 and 5, Low azadirachtin-A seed. ***c***, Amplification of genomic DNA *A. indica* using ISSR markers. Lane 1, Lambda marker; lanes 2 and 3, High azadirachtin-A seed; lanes 4 and 5, Low azadirachtin-A seed. ***d***, ***f***, Genomic DNA amplified by primer OPA-09. ***e***, ***g***, Genomic DNA amplified by primer OPA-11. ***d***, ***e***, Lane 1, Lambda marker; lanes 2 and 3, High azadirachtin-A seed; lanes 4 and 5, Low azadirachtin-A seed. ***f***, ***g***, Lane 1, Genomic DNA of tender leaves of neem tree of high azadirachtin-A; lane 2, Genomic DNA *in vitro* seedling of neem of high azadirachtin-A, lane 3, Callus from leaf explant of neem of high azadirachtin-A; lane 4, Lambda marker.

as shown in the present study. Flavanoids, esters and acids were not present in detectable amounts in all the explant-derived calli from seedlings of the two azadirachtin-A seed groups (Table 4).

Molecular analysis

The genomic DNA was extracted from callus using CTAB method with liquid nitrogen to avoid possible contamination (Figure 3 *a*, *f*, *g*). All DNA samples were found to be free from phenolics and polysaccharide contamination. This DNA was digested with restriction endonuleases such as *Eco*RI and *Bam*HI to check its purity (Figure 3 *b*).

Genomic DNA obtained from leaves dried by silica gel was the best suited for DNA amplification by using RAPD and ISSR markers. Out of six RAPD and four ISSR primer combinations used for PCR amplification, a total of 60 bands were scored of which 48.3% were polymorphic. The primer OPA-09- ($^{5'}$GGGTAACGGG$^{3'}$) gave 11 scorable bands and the primer OPG-11-($^{5'}$TGCCCGTCGT$^{3'}$) gave six scorable bands (Figure 3 *d* and *e*). ISSR markers in contrast, showed only monomorphic bands (Figure 3 *c*). The amplicons ranged between 500 bp and 1.5 kb.

Two significant findings were noted: (i) A distinct variation in DNA pattern was observed between high and low azadirachtin-A yielding neem trees. (ii) Furthermore, DNA amplification patterns using RAPD primers also showed polymorphism within DNA from tender leaves, *in vitro* seedlings and calli from explants.

Discussion

Studies on the growth of immature cotyledonary tissue of *A. indica in vitro* supported by 0.5 mg/l NAA and 1 mg/l BAP showed variations in the nature of the calli[17]. Plantlet regeneration from callus of leaves was observed in growth medium supplemented with BAP in different concentrations[18]. Callus growth was also observed from leaves and stem of neem in the medium supported with 2,4-D and IAA[19]. Studies on parallel lines were carried out in the present investigation. A synergistic effect of Kn was observed with NAA in the present study for improving callus production and also organogenesis.

Azadirachtin-A, because of its potent antifeedant and antipesticidal properties, is known to offer resistance and protection to the seed against seed-borne fungi like the *Aspergillus* sps.[20]. Our study showed that seeds obtained from the high azadirachtin-A yielding trees were less susceptible to fungal infections on storage. About 100 seeds from each of the two categories were examined for fungal infection and viability, after 30 days of storage. Only less than 5% of seeds from the high azadirachtin-A yielding tree was infected with *Aspergillus* sps. and all the healthy seeds were viable. In contrast, more than 20% of seeds from low Azadirachtin-A yielding trees were infected with the fungus, with a corresponding drop in viability by 20%. The present study has also shown that the seeds with high azadirachtin-A were viable even after long periods of storage at 4°C, which germinated into sturdy seedlings *in vitro* (Figure 1 *b*). However, seeds from low azadirachtin-A yielding trees exhibited not only poor viability but also sprouting of comparatively weaker seedlings.

The distinct disparity in steroid levels among calli obtained from various explants could suggest somaclonal variations. In contrast, saponins are generally absent in roots. But their existence in high and low levels in the calli of root explants from seedlings of high and low azadirachtin-A containing seeds respectively, also suggested the possibility of somaclonal variations. In general, increase in the level of saponins indicates the presence of a healthy plant. Furthermore, a high level generally serves as a biomarker to indicate the resistance of the plant to pathogenesis. There are several reports that suggest the biological importance of saponins. Predominantly, saponins serve as plant defences[21]. They function as preformed antimicrobial compounds and act as frontline plant defences against fungal attack[22]. They also serve as mollucscicides and fungicides, while some of them are toxic to fish. The allelopathic nature of saponins, which have both a nonpolar moiety and a polar 1,2-diol moiety, is highly beneficial in solubulizing phenols, which are nonpolar in nature. In turn, solubulization of phenols in the soil directly underneath a particular plant repels other seeds from germinating in the same environment.

Thus, our observations exhibited differential expression of various secondary metabolites in neem with further variations of each of the biochemicals analysed in various explants. This was supported by polymorphism in DNA amplification studies at the molecular level.

The present investigation involved molecular analysis using DNA fingerprinting studies on genomic DNA extracted from tender leaves obtained from high and low azadirachtin-A yielding neem trees. The DNA polymorphism observed between neem samples collected from the two microenvironments could suggest a genetic variation, similar to reports of Singh *et al.*[8]. Molecular studies using RAPD primers have been used to examine genetic variation within a species, to analyse particular genotypes, for cultivar identification and to study the clonal structure of several tree species[23–25]. High variations in the RAPD patterns have also been reported in the black and red spruce varieties, where the variation is attributed to interaction between the plantlets and the microenvironment[26]. Thus, RAPD analysis, which is generally considered a very good starting point for studies of species[27], has proved true in our present investigation also (Figure 3 *d–g*).

Similar to the present study, RAPD analysis and microsatellite studies on *Brassica juncea* cultivars have revealed the success of the techniques for studying interspecific and variety-specific polymorphism between cultivars[28]. Likewise, in *Eucalyptus* species, the outcrossing rate in an open pollinated breeding programme was studied using molecular markers. Eleven open-pollinated arrays of 24 individuals were used for this study. The analysis involved RAPD and AFLP techniques that served in detecting dominant markers for estimating the outcross rate in breeding natural populations of these forest trees[29]. In essence, the polymorphism exhibited by neem plants in the present study may be attributed to the high degree of cross-pollination and micro-gene complex to adapt to the wild or domesticated environment.

Another aspect of molecular studies involved variations in DNA fingerprinting patterns in tissue culture samples that may in turn be attributed to the microenvironment in which the plants are grown. Our data showed a distinct DNA polymorphism between tender leaves, *in vitro* seedlings and calli from various explants. Similar reports of RAPD profiles of plantlets generated from the callus of somatic hybrids of *Asparagus officinalis* and *Asparagus marcowani* differed from the parental lines[9]. Although the RAPD profiles were found to be varying, no differences were observed in the morphological characters[9]. There are further examples wherein the genetic constitution of the callus is clearly different from the source of the explants as a result of growth regulators. For example, geraniums are known to show somaclonal variations obtained from *in vivo* root or petiole cutting[30]. Similar variations have been observed in pineapple and potato[11].

In conclusion, the present investigation elucidates the impact of growth factors on biochemical and molecular variations in select neem accessions *in vitro*. It is thus suggestive that nutritional factors could influence the somatic and genetic variations in neem. Furthermore, increased levels of secondary metabolites such as Azadirachtin-A, steroids and saponins could serve as a frontline defence and offer plant resistance against fungi and insects.

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ACKNOWLEDGEMENTS. We thank Prof. P. V. Bhiravamurty, Sri Sathya Sai Institute of Higher Learning, Puttaparthi and Prof. T. N. Lakhanpal, University of Himachal Pradesh, Simla, for encouragement. P.C. thanks the Department of Biotechnology, New Delhi for financial support.

Received 29 November 2004; revised accepted 6 April 2006