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RESEARCH ARTICLE

A comprehensive study on characterization of elite Neem chemotypes through mycofloral, tissue-cultural, ecomorphological and molecular analyses using azadirachtin-A as a biomarker

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Abstract Azadirachtin-A (Aza-A), a tetranortriterpenoid, found in minuscule amounts in the Neem seed-kernels, has proved to be a potent biopesticide. Given the vast biodiversity of *Azadirachta indica* (Neem) in India, this study is an overview of four main aspects that corroborate with each other in identifying elite Neem chemotypes based on their Aza-A content. These biomarkers included mycofloral, tissue-cultural, ecomorphometrical and molecular analyses on accessions from five ecogeographically different regions in Andhra Pradesh, India, which high-lighted the characteristics of trees that yielded the highest Aza-A. In essence, extremely-arid-alkaline regions with maximum soil pH (8.05) yielded trees with the highest amount of this biopesticide. Likewise, both VAM and soil fungal diversity and frequency exhibited maximal values in their rhizosphere, whereas it exhibited the least values for percentage moisture and also for several micronutrients measured (P_2O_5, Zn, Fe and Cu). *In vitro* studies on seeds with high versus low Aza-A content gave sturdier seedlings in the former; with profusely coiled roots and fibirillar foliage in tissue-culture; in addition to these seeds being more viable. Furthermore, their cotyledons alone exhibited significant amount of Aza-A, as measured by HPLC. Besides this significant difference, the impact of growth factors culminated not only in the variations of several secondary metabolites, but also differences in DNA patterns from various parts of a single *in vitro* plant. Ecomorphometric analyses clearly indicated that at least eight parameters (seed diameter, soil pH, percentage moisture, K_2O, P_2O_5, Zn, lower lobe serrations and upper-lobe-distance of leaves) were significantly related to the quantitative variations in Aza-A. Finally, PCR analyses exhibited a habitat-based molecular concordance of ISSR and FISSR profiles with Aza-A content among the Neem chemotypes. Their relatedness was based on dendrograms constructed by UPGMA algorithms using similarity-index-values.

Keywords *Azadirachta indica* A *Juss* · Azadirachtin-A · Ecomorphology · HPLC · *In-vitro* cultures · ISSR-FISSR-RAPD · Soil-fungi · VAM-fungi

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Introduction

Azadirachta indica A *Juss* (Neem, Family: *Meliaceae*), from as early as the Vedic times has been aptly referred to as the "Sarva Roga Nivarini" in the *Charaka Samhita*. It has held the interest of phytochemists all over the world for its rich source of alkaloids and terpenoids. However, it was only in the past half-a-century that this tree has come into prominence since Morgan's isolation of azadirachtin-A (Aza-A) from its seed-kernels (Butterworth and Morgan 1968). It is effective as an antifeedant and as an ecdysics inhibitor against several hundred insect species, even when applied in minuscule quantities. Furthermore, it took another two decades to establish the structure of this eco-friendly, biopesticide through a co-operative effort of several laboratories, when the compound was crystallized and studied using NMR spectroscopy and X-ray diffraction (Turner et al. 1987; Kraus 1995; Kabaleeswaran et al. 1994). Aza-A is a tetranortriterpinoid, widely used in empirical Ayurvedic medicine (Shastry and Chary 2004; Rao and Chary 2009). There is, however, an innate possibility that if fresh and ripe Neem-seed kernels are not used for oil-production and especially the terpenoids,

the hazardous infection by aflotoxin-producing strains of the fungus *Aspergillus flavus* is imminent (Schmutterer 1990, 1995). It therefore becomes significant to study the soil and VAM fungi in the Neem rhizosphere and its possible impact on Aza-A (Rao and Chary 2007a).

Aza-A, which is the most important active principle in Neem-seed-kernels varies considerably in quantity because of environmental factors and possibly also for genetic reasons. Therefore, to obtain trees with high Aza-A content, a preferential selection procedure is often recommended. This initial selection calls for *in vitro* studies (Kota et al. 2006) followed by clonal propagation of high Aza-A yielding varieties (Venkateswarlu et al. 1996). Finally, ecomorphometric and molecular studies become essential to identify these elite chemotypes.

Our laboratory has made a detailed study on ecomorphometric and molecular biomarkers to identify trees that yield high levels of Aza-A. This was performed both in a micro-population and also in different eco-geographical regions in Andhra Pradesh, India which incidentally is reported to have Neem trees with high Aza-A content (Anand et al. 2006, 2007; Pattnaik et al. 2006; Rao and Chary 2007a, b).

Materials and methods

Microelement and microfloral analysis of Neem rhizosphere

The microelemental studies involving the estimation of all the soil parameters namely pH, EC, K_2O (Kg hec^{-1}), P_2O_5 (Kg hec^{-1}), Zn (in ppm), Mn (in ppm), Fe (in ppm) and Cu (in ppm) were performed using standard methods. In addition, water holding capacity (H_2O_HOLD) and water concentration ((H_2O_CONC) were also estimated. To estimate soil pH, soil water suspensions were prepared. An automated digital pH meter was utilized for pH measurement with solutions from buffer tablets as standards. For EC measurement, potassium chloride solution with 1,411.8×106 m hos/cm at 25 °C served as a standard (Wantanabe and Olsen 1965). Soil phosphorous was estimated by using sodium bicarbonate (Olsen's reagent) as a standard. These procedures are elaborated in Rao and Chary (2007a). The amount of soil potassium was estimated by flame photometry. Cu, Zn, Mn, and Fe microelements were estimated essentially by the method of Lindsay and Norvel (1978). In essence, standard methods were followed for estimating all the above soil micro elements and macro elements (Muralikrishna 1997).

Soil and VAM fungi were examined using classical mycological techniques (Benthlenfalvay and Lindermann 1992; Gerdemann and Trappe 1974; Rao and Gupta 1999).

In vitro studies

The techniques followed in this study are elaborated by Kota et al. (2006).

a. *Growth regulators utilized*: Extreme care was taken to conduct *in vitro* studies in sterile conditions. The various growth regulators used in Murashige and Skoog medium (MS) (1962) were 1) 1 mg/L NAA, 2) 2 mg/L NAA, and 3) 1 mg/L NAA + 0.5 mg/L Kn, 4) 2 mg/L NAA + 0.5 mg/L Kn (Fig. 1). Different explants such as leaf, hypocotyl, epicotyl, cotyledons, and root were selected from *in vitro* grown seedlings from callus induction. Explants (0.5–1.0 cm) were grown on MS medium supplemented with naphthalene-acetic acid (NAA; 1.0–20 mg/l) and kinetin (Kn; 0.5 mg/l). The medium was supplemented with 3 % (w/v) sucrose as a carbon source and the pH was adjusted to 5.7. The seeds were surface-sterlized by treating with 2 % $HgCl_2$ and Triton X 100 (in 100 ml H_2O) fro 20 min with gentle agitation (Razdan 1993). The seeds were then thoroughly washed with sterile distilled water thrice prior to use for *in vitro* germination studies. Sterilized seeds were cultured on MS medium. In order to prevent bacterial and fungal contamination, after sterilizing and cooling the MS medium, 20 ppm diethane fungicide, 50 ppm captom and 62.5 mg of cefotaxime were added.
b. *Analysis of secondary metabolites:* Standard biochemical methods were employed for the detection of secondary metabolites. Seeds were pulvarized using a sample mill. By repeated treatment with n-hexane, fat was completely removed and Aza-A was subsequently extracted in methanol (Ubel et al. 1979). An aliquot of 20 µl of this extract was utilized for quantitation using a differential refractometer (Fig. 2Ia to Id). Other secondary metabolites were also analyzed from this extract using TLC. They included the 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 Thin Layer Chromatography by different solvent systems. Specific spray agents were used to identify individual compounds. For esters, hydroxylamine ferric chloride, for acids, bromocresol green, for flavanoids, aluminum chloride, for 1,2 diols, metaperiodate-benzidine and for steroids, acetic anhydride and concentrated sulphuric acid was used. Essentially common laboratory methods were implemented (Malik et al. 1998; Stahl 1973; Jork et al. 1990).
c. *Methods for molecular analysis in Tissue Culture samples*: *In vitro* raised seedlings and callus tissue were

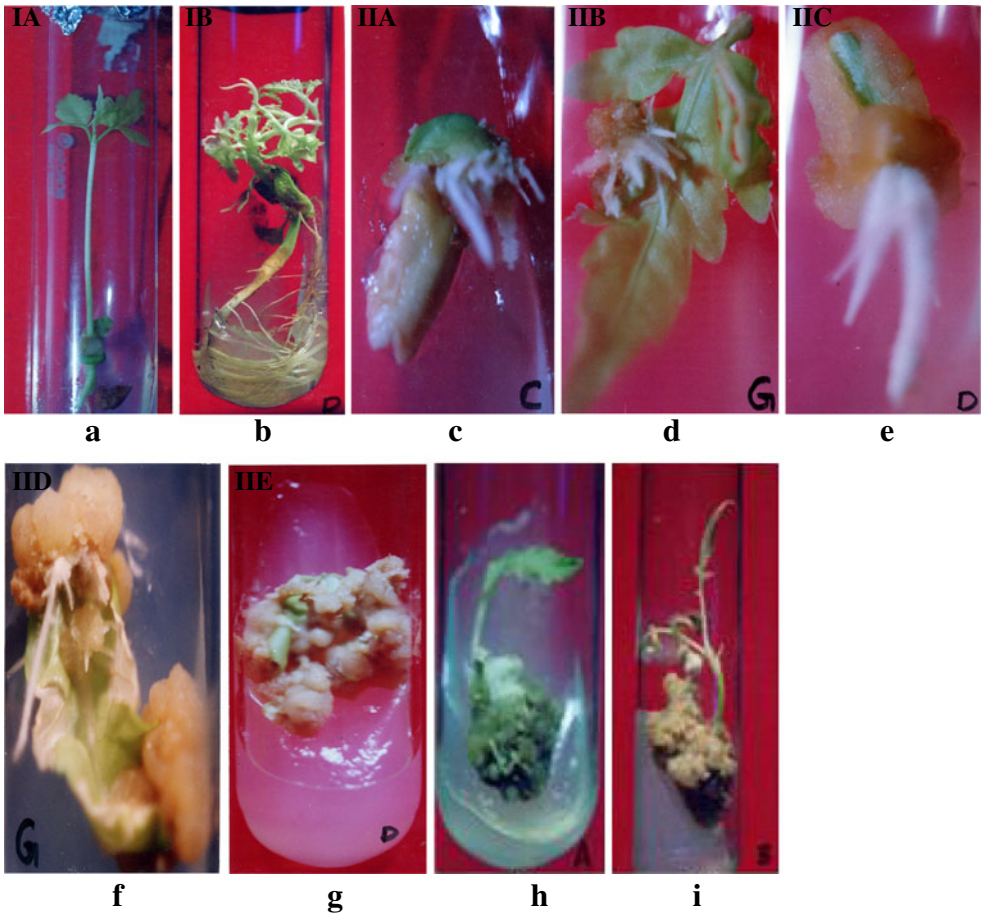

Fig. 1 **a** *In vitro* germination of *Azadirachta indica* (low azadirachtin-A) seeds with cold treatment. (4 °C), after 45 days. **b** *In vitro* germination of *Azadirachta indica* (high azadirachta-A) seeds with cold treatment (4 °C), after 45 days. **c** Callus induction from cotyledon explant of *Azadirachta indica* (high azadirachtin-A) seedlings on MS+ 2 mg/L NAA, after 15 days. **d** Callus induction from leaf explant of *Azadirachta indica* (low azadirachtin-A) seedlings on MS+ 1 mg/L NAA + 0.5 mg/L Kn, after 15 days. **e** Callus induction from hypocotyl explant of *Azadirachta indica* (low azadirachtin-A) seedlings on MS + 1 mg/L NAA + 0.5 mg/L Kn, after 15 days. **f** Callus induction from leaf explant of *Azadirachta indica* (low azadirachtin-A) seedlings on MS + 2 mg/L NAA + 0.5 mg/L Kn, after 30 days. **g** Callus induction from leaf explant of *Azadirachta indica* (high azadirachtin-A) seedlings on MS + 2 mg/L NAA + 0.5 mg/L Kn. **h** Shoot from callus of leaf explant of *Azadirachta indica* (high azadirachtin-A) seedlings on MS + 2 mg/L NAA. **i** Shoot from callus of leaf explant of *Azadirachta indica* (low azadirachtin-A) seedlings on MS + 2 mg/L NAA + 0.5 mg/L Kn

stored at −20 °C for DNA extraction. For molecular analysis, clean genomic DNA free of phenolics was extracted from 1) tender Neem leaves of trees, 2) *in vitro* seedlings and 3) calli by using the method of Doyle and Doyle (1990). Polymerase Chain Reaction (PCR) amplification reactions were performed using Taq polymerase (Finnzymes, Finland). Random Amplified Polymorphic DNA (RAPD) analyses were performed using two RAPD primers (Operon Life Technologies, Alameda, California, USA) and four ISSR primers (Biotool Techno-concepts, India) with the respective sequences: RAPD primers: OPA-09-(5′ GGGTAACGGG3′) and OPG-11-(5′TGCCCGTCGT3′) and ISSR primers: (A) (CT) 9-A, (B) (CA)8G, (C) (CI) 9-RA and (D) (CA)8-RG) (Jones et al. 1997) (Fig. 2IIa to IIg)

Ecomorphometric analysis

The techniques employed in this analysis, essentially followed those of Pattnaik et al. (2006); Anand et al. (2006).

1) The following tools were utilized from SPSS version 10.0 for ecomorphometric analysis:
   a) Descriptive statistics
   b) Analysis of Variance (ANOVA)
   c) Pearson Correlation studies
   d) Linear Multiple Regression Analysis (LMRA)

   LMRA studies were performed with Aza-A and total Aza-A being analyzed sequentially. The LMRA using the backward method of execution with the SPSS version 10.0 for Windows® indicat-

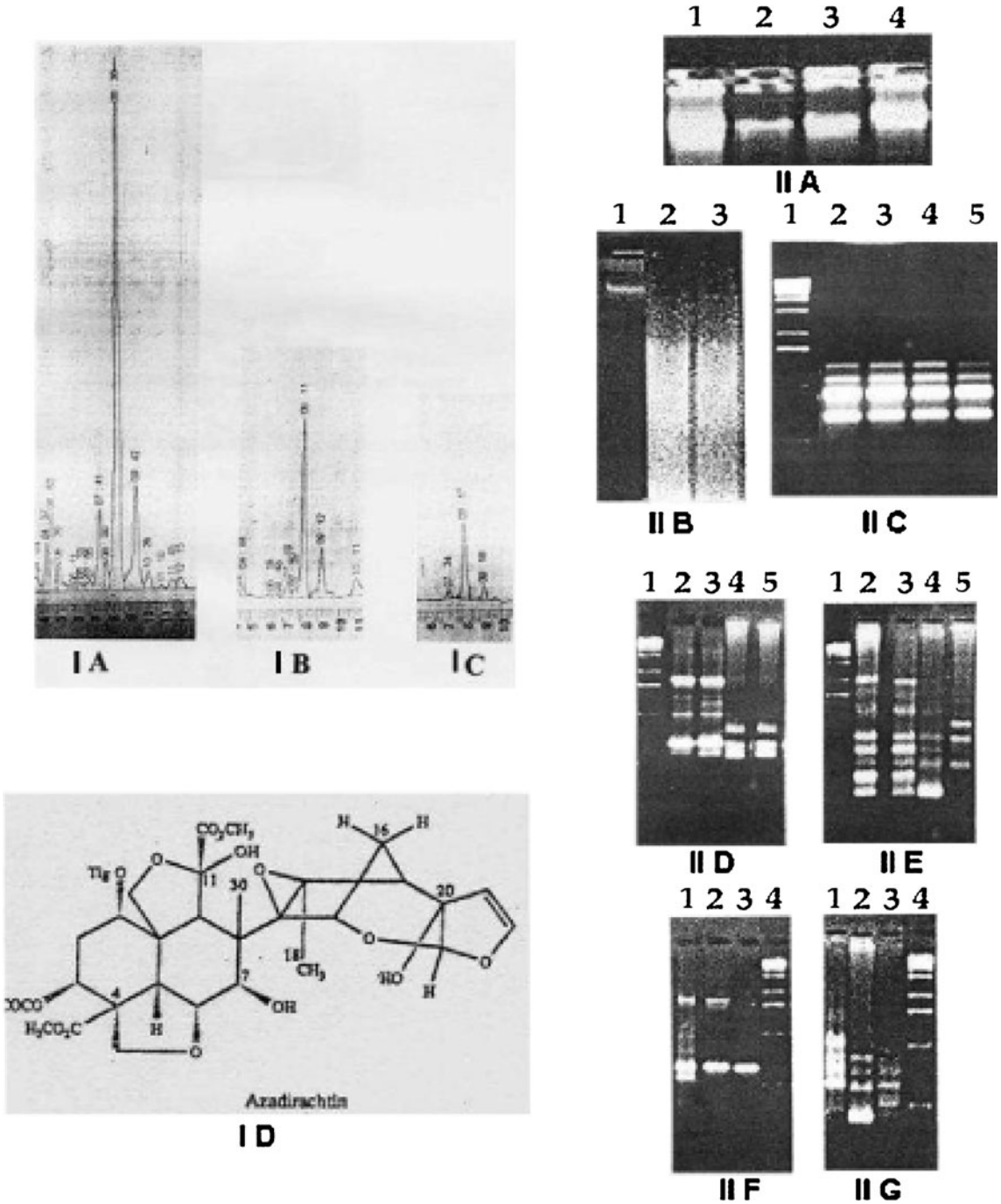

Fig. 2 **Ia–c** HPLC analysis of the crude extract of Neem seed kernels. Distinct peaks were observed at 218 nm. **a**, **b** and **c** represent standard, high and low quantities of azadirachtin-A respectively (*left* to *right*). **Id** Chemical structure of azadirachtin-A. **IIa** Genomic DNA extracted by a modified CTAB method on silica gel dried leaves (ground with or without dry ice or liquid nitrogen) of Neem trees bearing low azadirachtin-A containing seeds (1, 2 lanes) and high azadirachtin-A containing seeds (3, 4 lanes). **IIb** Restriction endonuclease digest-uncut genomic DNA from tender leaves obtained from high azadirachtin-A yielding accessions (*lane 1*), restriction digest with *Eco R1* (*lane 2*) and *Bam H1* (*lane 3*). **IIC** Amplification of genomic DNA from low and high azadirachtin-A seed bearing trees using ISSR markers. Four different primers were used [(A) (CT) $_9$-A, (B) $(CA)_8$G, (C) $(CI)_9$-RA and (D) $(CA)_8$-RG]. Lane 1 is Lambda marker. Lanes 2 and 3 include samples from trees bearing seeds containing low and high azadirachtin-A. Lanes 4 and 5 include samples from trees bearing seeds containing low azadirachtin-A. **IId** to **IIg** Amplification of genomic DNA from trees from low and high azadirachtin-A containing seeds using two different RAPD primers. [Panels **IId** and **IIf** represent genomic DNA amplified by RAPD primer OPA-09]. [Panels **IIe** and **IIg** represent genomic DNA amplified by RAPD primer OPA-11]. **IId** and **IIe**: Lane 1 is Lambda marker, lanes 2 and 3 are amplified genomic DNA obtained from trees bearing low azadirachtin-A containing seeds, whereas, lanes 4 and 5 are amplified genomic DNA obtained from trees bearing high azadirachtin-A containing seeds. **IIf** and **IIg** lanes 1 through 3 represent amplified genomic DNA from tender leaves of Neem trees bearing high azadirachtin-A containing seeds, *in vitro* seedling from a seed of the same tree and callus from the leaf explant of the seedling

ed relationships at *** level ($P>0.001$), at ** level ($P>0.01$) or at * level ($P>0.05$). This method followed a preprogrammed-careful-process of exclusion of the parameters. It progressively removed in a stepwise manner the independent variables that were insignificant, thus leading to a specification in the end, which as a unit, best explained the dependent variable.

2) Duncan's Multiple Range Test (DMRT) was performed by using QBASIC program in MS-DOS. It follows a preprogrammed process of organizing the data into ascending-order-data-sets in a hierarchical order, taking into consideration the accessions from each of the ecogeographically different regions that are not separated in their respective regions from which they originated (Brigges and Walters 1997).
3) Graphs and plots: These were analyzed using Microsoft Excel®.

Molecular analysis

Molecular studies that were employed in this section are detailed in Rao and Chary (2007b); Anand et al. (2007).

a) *Primers*: Initially 20 ISSR primers were used for amplification of genomic DNA. Their sequences were 1) 5′-(CT)8 T-3′ 2)5′-(CA)8 G-3′ 3)5′-(GA)7A-3′ 4) 5′-(GATA)2 ¬(GACA)2-3′ 5)5′-RYA CRY RCA R (TG)7-3′ 6)5′-YGY RAY (GA)8-3′ 7)5′-(GA)8 RGY-3′ 8)5′-CRT AY (GT)9-3′ 9)5′-ARR TYC (AGC)3 AG-3′ 10) 5′-RAT YTA (TTA)3 TT-3′ 11)5′-AT (AAT)43′ 12) 5′-GA (ATT)4-3′ 13) 5′¬- G (ATT)4-3′ 14) 5′-CT (AAT) 43′ 15) 5′-A3 (AAT)4-3′ 16)5′-(GA)8 C-3′ 17)5′-T3 (ATT)4-3′ 18)5′-TC (ATT)4-3′ 19) 5′-CGA (ATT)4-3′ 20)5′-T (GT)9-3′

Among these primers about half of them were 3′ anchored and the other half were 5′ anchored, where Y = C/T and R = A/G. Out of these 20 primers (Metabion GmbH, Germany), 11 of them gave polymorphic bands of which 6 produced as many as thirteen bands per primer. A total of 119 bands were polymorphic. These primers were a)5′-YGY RAY (GA)8-3′ b)5′-(GA)8 RGY-3′ c)5′-CRT AY (GT)9-3′ d)5′-ARR TYC (AGC)3 AG-3′ e)5′-(GA) 8 C-3' f)5′-T (GT)9-3′. These six ISSR primers that exhibited maximum amplification were also used for the Fluorescent ISSR-PCReactions.

b) *DNA extraction:* Plant genomic DNA was extracted essentially by standard methods (Doyle and Doyle 1987, 1990) by using silica gel dried tender leaves. The uniqueness of our method involved the cryogenic-free method of DNA extraction with 3 % Polyvynylpolyphenol (PVPP) in the aqueous extraction buffer (Fig. 2IIa).

c) *Restriction Endonucleases:* Two restriction enzymes Eco*RI* and Bam*HI* were utilized to digest the genomic DNA in order to ensure the purity of the DNA extracted. This method was considered ideal because the residual CTAB could interfere with the spectroscopic observations. Undigested DNA was used as control in the adjacent lane (Fig. 2IIb).

d) *Inter Simple Sequence Repeats (ISSR) and Flourescent ISSR (FISSR) amplifications*: The PCR reactions were carried out using Finnzymes—a DNA amplification kit with DyNAzymeTM II DNA polymerase which was stored at −20 °C. The primers were provided as lyophilized powders in vials and were stored at −20 °C. Pure undegraded genomic DNA was amplified using ISSR primers. Each PCReaction was performed in a total volume of 20 mL containing 5 mL of DNA (1 ng/mL), 10× buffer 2 mL of 25 mM $MgCl_2$, 0.5 mL 10 mM dNTP, 1 ml (0.8 mM) ISSR primer. To this, 0.5 mL of Taq polymearse (1unit/mL) and 11 mL of water were added, making it to a total of 20 ml. The DNA samples were amplified using the following thermal profiles: 1) One cycle at 94 °C for 5 min, followed by; 2) 35 cycles at 94 °C for 30 s at 42 °C for 45 s; and at 72 °C for 2 min, followed by 3) a final extension at 72 °C for 7 min. The reaction was terminated by indefinitely holding temperature at 4 °C. The samples were electrophoresed on 2 % agarose gels, stained with ethidium bromide and viewed by using the gel documentation system (Fig. 3a).

The amplification of FISSR-PCR primers was carried out using the ISSR primers, which were tagged with a fluorescent dye and their amplification products were subsequently electrophoresed on PAGE gels to determine polymorphism among the various DNA samples from different eco/chemotypes. The reactions were carried out in 7 mL volume involving 7 ng genomic DNA, 1XPCR buffer (1.5 mM $MgCl_2$, 0.8 mM primer, 25 mM each of dCTP, dGTP, dTTP and dATP), fluorescent 0.4 mM dUTP (Tamara, Perkin Elmer) and 0.4 units of Taq Gold DNA polymerase (Perkin Elmer) and Taq DNA polymerase (MBI Fermentas) for di and trinucelotide anchored primers. The thermal profiles for amplification of DNA samples using FISSR primers were similar to the conditions used with ISSR primers. The amplified products were run on 5 % polyacrylamide gel with 7 M urea, on an ABI 377 automated sequencer at 3,000 kV for 7 h. The data was analyzed using the GENESCAN™ analysis software. In essence PCR amplification using ISSR and FISSR markers were performed by the methods detailed by Nagaraju et al. 2002. Agarose gel electrophoresis and data analysis followed standard methods (Dangi et al. 2004) (Fig. 3a and b).

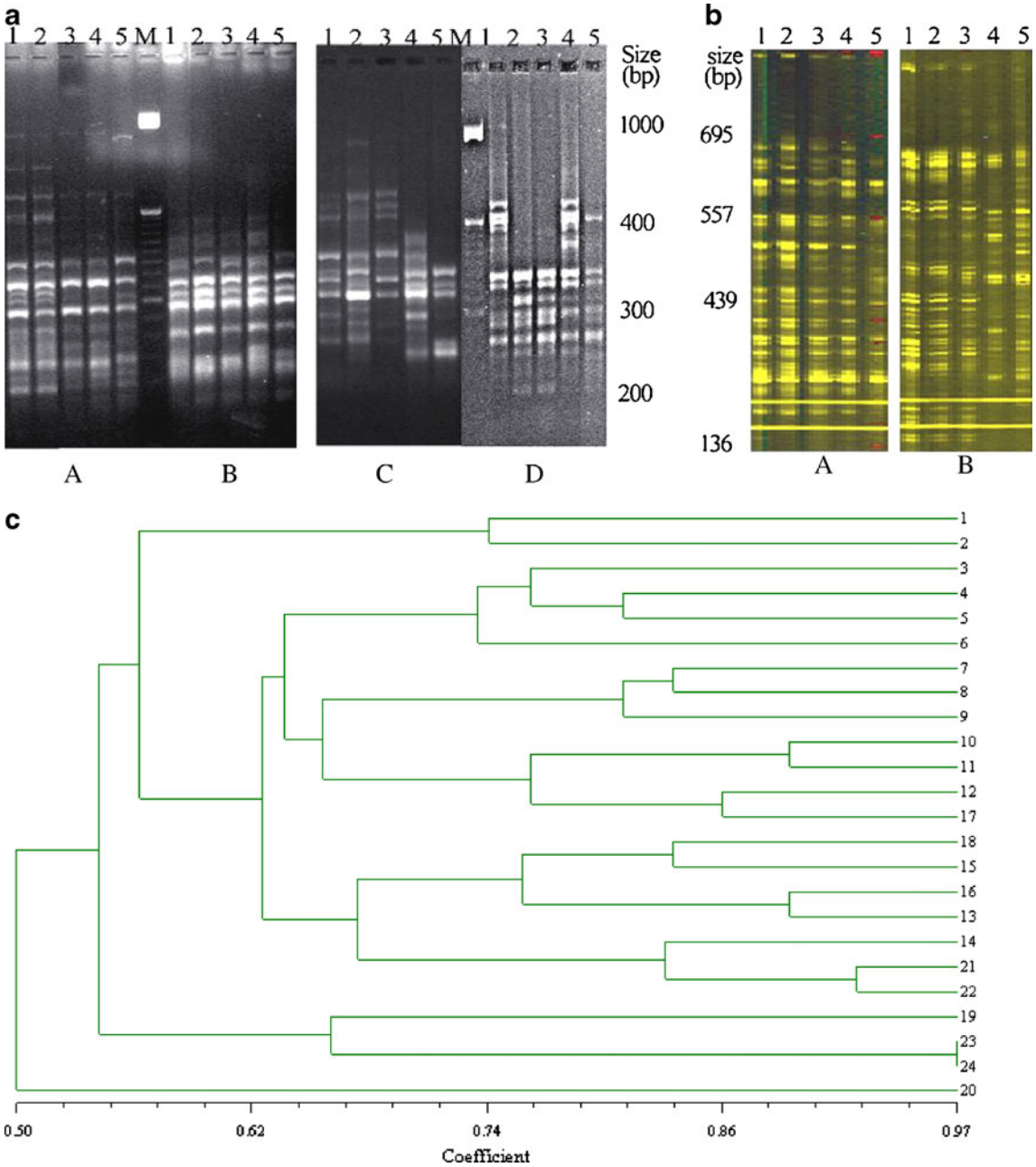

Fig. 3 **a**, **b** represent amplified DNA using ISSR markers and FISSR markers respectively. **c** represents the dendrogram. **a** *(Agarose gels)* PCR amplification of genomic DNA was obtained from tender leaves of accessions growing in five ecogeographically different regions that included semiarid, semiarid/arid, extremely arid alkaline, arid and delta wet regions using two ISSR primers: 1) $^{5'}$ -YGY RAY Y-$(GA)_8$-$^{3'}$ and 2) $^{5'}$-ARR TY $(CAG)_4$-$^{3'}$. Panels *a*, *b*, *c* and *d* included the five DNA samples each, in the order mentioned above that encompass accessions from high (panels *a*, *c*) and low (panels *b*, *d*) azadirachtin-A yielding chemotypes as earlier determined by HPLC analysis. DNA profiles shown in panels *a* and *b* were amplified by the ISSR primer 1, whereas, DNA profiles shown in panels C and D were amplified by ISSR primer 2. **b** *(Polyacrylamide gels)* PCR Amplification of genomic DNA obtained from tender leaves of accessions growing in five ecogeographically different regions included semiarid, semiarid/arid, extremely arid alkaline, arid and delta wet regions using FISSR primer 2: $^{5'}$-ARR TY $(CAG)_4$-$^{3'}$. Panels *a* and *b* represent sets of five DNA profiles from high and low azadirachtin-A yielding chemotypes from five different regions. PAGE gels were automatically analysed by Genescan™ software. *Note: Amplified products were clear and crisp in both agarose and polyacrylamide gels. **c** Dendrograms derived from an unweighted pair group method analysis (UPGMA) based on ISSR and FISSR profiles. The tree plot (of ISSR primers) shown above utilized the data of five ecogeographically different regions in Andhra Pradesh, India. *Note that the data from Table 11 is supportive of **c**

DNA banding patterns generated by these ISSR and FISSR markers were scored for the presence or absence of monomorphic and polymorphic bands that was in turn used to generate the Jaccard's similarity coefficient. The statistical analysis was performed using the NTSYS software (version 1.5). A dendrogram was generated by UPGMA (Sneath and Sokal 1973) in order to group ecads into discrete clusters (Fig. 3c).

Results and discussion

Mycofloral biomarkers

This study is an overview of the four main aspects that were dealt with, in order to readily identify the elite Neem chemotypes based on their Aza-A content, a biopesticide that is present in miniscule quantities in the Neem seed kernels. The soil and fungal aspects, tissue culture studies, ecomorphometric and molecular aspects, all corroborate toward the same goal of identifying an elite chemotype of Neem bearing its Aza-A content as the predominant source.

Mycofloral biomarkers of soil and Vesicular Arbuscular Mycorrhizal (VAM) fungi were utilized to reflect quantitative variations in azadirachtin-A from Neem chemotypes thriving in various soils. These mycoflora from five ecogeographically different regions in Andhra Pradesh, India exhibited a clear dependence on soil parameters and were related to the potent biopesticide Aza-A. The five areas ranged from delta-wet to extremely-arid-alkaline regions (with arid, semi-arid and semi-arid/arid regions in between) based on the pH and percentage moisture of the soil.

The estimation of mycoflora and the various soil microelements indicated that the harsh environment of the extremely-arid-alkaline region with maximum soil pH (8.05) exhibited the maximum fungal diversity and frequency (15 out of 17 identified fungal species and 1.82×10^6 colonies/g soil) and also maximum number of VAM fungal genera (3) colonizing as the maximum number of different species (6). The soil showed the lowest values for percentage moisture and for most of the microelements (Zn, Fe, Cu, Mn, P_2O_5 and K_2O).

Thus, soil Fe, Cu, Mn, and Zn showed an inverse relationship with pH. This could be attributed to the fact that, with specific reference to Cu and Fe in their respective stable ionic states (Cu^{2+} and Fe^{3+}), exist as available ions in the aqueous phase for detection in a particular soil suspension with a neutral or near neutral pH. These serve as co-factors in plant metabolism. However, when the pH becomes alkaline these ions transform into their respective hydroxides and precipitate. This results in unavailability of these ions for detection (Zekri and Obereza 2003). Our results showed similar findings as indicated by a 10-fold decrease in Fe (in ppm), 3-fold decrease in Cu (in ppm) and P_2O_5 (kg ha^{-1}) and a two-fold decrease in Mn (in ppm), and Zn (in ppm) in the extremely-arid-alkaline soils when compared to the corresponding values in the delta-wet regions. This could be a result of a change in pH from 7.05 in delta-wet regions to a pH of 8.05 in the extremely-arid-alkaline regions.

The high soil-fungal-diversity in extremely-arid-alkaline soils could be a result of the fact that lesser the water logging capacity of the soil, better colonization of fungi. Furthermore, the more arid the soil, the less availability of phosphate to the organism. This is because the phosphorous is bound to the soil particles and therefore not available to the plants. The presence of VAM in such soils helps in loosening the phosphorous and makes it available to the plants, thus allowing the facilitation and mobilization of nutrients. In essence, VAM diversity is greater in extremely-arid soil as supported by our results. These VAM fungi trap the available water by efficiently extending their microrrhizal-hyphae through the soil particles and subsequently absorbing them.

In contrast, the fertile environment of the delta-wet region with the lowest soil pH (7.05) exhibited a moderate fungal diversity with lowest fungal frequency (nine out of 17 identified fungal species and 5.46×10^5 colonies/g of soil), and with a single VAM fungal genus constituting four species. This soil showed the highest values for percentage moisture and for most of the microelements analyzed.

Thus, our study shows that with a unit increase in pH from 7.05 (delta-wet region) to 8.05 (extremely-arid-alkaline), the diversity of soil fungi increased from nine to 15 species. There are studies to support this fact that, dominance of certain soil fungal genera at a particular location is influenced by soil climate (Scalbert 1991). As shown in our present study, the saline-arid-parched pocket, a subset of the extremely-arid-alkaline soil exhibited three unique fungal species namely, *Monilia sitophila*, *Aspergillus versicolor* and *Paecilomyces fusisporus*, whereas, the delta-wet region exhibited *Rhizopus nigricans* as its unique species. Overall, in the five regions studied, three VAM genera with nine species were observed, with *Glomus* being the predominant genus.

The estimated value of the secondary metabolite, Aza-A density and total Aza-A were highest in the Neem trees from the extremely-arid-alkaline regions (1.98 mg/g and 37.68 mg/100seed weight in g respectively) and lowest from Neem growing in the delta-wet regions(1.03 mg/g and 27.27 mg/100seed weight in g respectively). Since the VAM and soil fungal diversity and frequency along with soil pH, percentage moisture and microelements are clearly distinct between the two regions, these factors could serve

Table 1 Mean values of soil parameters from five different ecological regions of Andhra Pradesh

Districts	Area	PH	EC	P_2O_5	K_2O	% moisture	Zn	Mn	Fe	Cu
Krishna	Semi arid	7.715	0.65	52.7	341	0.00199	2.060	12.456	8.972	0.932
Adilabad	Semi arid/arid	7.868	0.36	62.4	269.9	0.00211	3.686	12.64	5.676	0.888
Anantapur	Extremely arid alkaline	8.055	0.39	58.4	397.3	0.00086	1.860	11.716	1.426	0.638
East-Godavari	Delta-wet	7.050	0.53	117	422.2	0.01204	2.076	18.233	10.57	1.538
Khammam	Arid	7.200	0.50	117	257	0.00168	2.990	15.58	8.280	1.308

P_2O_5 and K_2O are expressed in kg ha^{-1}. Zn, Mn, Fe, and Cu are expressed in ppm

EC Electrical conductivity, DMRT Statistical tool was used to compare the values of each parameter between the five regions in order to see the difference was significant or not

as good biomarkers to determine the variations in azadirachtin-A content, leading to the selection of elite Neem trees for this biopesticide (Tables 1, 2, 3, 4 and 5).

Tissue-culture studies as biomarkers

With the above findings on soil mycoflora and microelements and their influence on azadirachtin-A from Neem-seed-kernels as a prelude, our work progressed to determine the following aspects in terms of *in vitro* studies. Correlations were made between tissue culture findings and the role of categorization of Neem chemotypes into high and low Aza-A yielding varieties. The impact of growth factors on biochemical and molecular variations in Neem as elucidated by secondary metabolites and RAPD profiles were examined.

The effect of growth hormones on various explants

Ecological and nutritional factors strongly influence the somatic and genetic variations in Neem. Variations in callus growth utilizing different combinations of hormones often result in differences in their chemical nature (Shrikhande et al. 1997). Plantlet regeneration from calli of leaves was observed when the growth medium was supplemented with BAP in different concentrations (Narayan and Jaiswal 1985). Callus growth was also observed from leaves and stem of Neem in the medium supported with 2,4-D and

Table 2 Fungal colony frequency and% diversity per gram of soil

Fungal species	Anantapur (Extremely-Arid-Alkaline)	East Godavari (Delta-wet)	Khammam (Arid)	Krishna (Semi-arid)	Adilabad (Semi/Arid-arid)
Aspergillus niger	4.6×10^5 (25.3)	1.5×10^5 (252.47)	3.0×10^5 (52.17)	0.83×10^5 (10.21)	7.0×10^5 (57.57)
A. nidulans	1.5×10^5 (8.25)	0.66×10^5 (12.09)	0.25×10^5 (4.35)	0.5×10^5 (6.13)	0.33×10^5 (2.71)
A. versicolor	0.25×10^5 (1.80)				
A. fumigatus	0.25×10^5 (1.38)		0.25×10^5 (4.35)		
Alternaria tenuis	2.0×10^5 (11.00)	1.0×10^5 (18.32)			0.66×10^5 (5.43)
A. alternata	3.25×10^5 (17.88)	1.16×10^5 (21.25)	0.25×10^5 (4.35)		0.66×10^5 (5.43)
Cladosporium	1.41×10^5 (7.76)		0.25×10^5 (4.35)		
Cladosporium spp.	0.5×10^5 (2.75)	0.16×10^5 (2.93)			
Cephalospora spp.	1.0×10^5 (5.5)				0.33×10^5 (2.71)
Candida albicans	1.75×10^5 (9.63)		0.25×10^5 (4.35)		
Fusarium oxysporum	0.75×10^5 (1.13)	0.5×10^5 (9.16)	0.5×10^5 (8.70)	0.33×10^5 (4.08)	0.66×10^5 (5.43)
Pestolatia monorhinca	0.25×10^5 (1.35)		0.75×10^5 (13.04)	6.5×10^5 (79.66)	2.5×10^5 (20.56)
Paecilomyces	0.17×10^5 (0.94)				
Monilia sitophila	0.25×10^5 (1.38)				
Nigrospora oryzae	0.25×10^5 (1.38)	0.16×10^5 (2.93)			
Aspergillus flavus		0.16×10^5 (2.93)	0.25×10^5 (4.35)		
Rhizopus nigricans		0.16×10^5 (2.93)			
Freq of total fungi/g of soil	1.82×10^6	5.46×10^5	5.75×10^5	8.16×10^5	1.22×10^6

Table 3 VAM spores/100 g of soil and species number in parenthesis

Anantapur (Extremely arid-alkaline)	East Godavaru (Delta-wet)	Khammam (Arid)	Krishna (semi-arid)	Adilabad (Semi/Arid-arid)
220 (6)	150 (4)	120 (3)	200 (3)	150 (4)

IAA (Rao et al. 1988). Studies on parallel lines were carried out in the present investigation.

Micro-populations of Neem from extremely-arid-alkaline soils grouped into high (1.0 mg/g) and low Aza-A (0.4 mg/gm respectively) containing served as mother plants. The leaf explants from the seedlings of high Aza-A containing seeds exhibited the best *in vitro* response between 15 to 30 days in terms of various stages of callus metamorphosis in the MS medium that included four growth hormonal combinations of naphthelene acetic acid (NAA) with and without kinetin (Kn). A similar response in the juvenile leaf explants of the seedlings from low Aza-A seeds showed that Kn was essential in addition to NAA in the medium. Calli from most explants showed root differentiation after 30 days, whereas, only the calli obtained from the leaf explants also redifferentiated into shoots (Tables 6, 7, 8, and 9).

Increase in callus biomass was also observed from leaf explants by a) changing growth-hormones in the medium or b) by cold-treatment. Our data showed that the presence of Kn in the media resulted in the improvement of callus biomass and organ differentiation.

Aza-A, because of its potent antifeedant and antipesticidal properties, provides resistance and protection to the seed against seed-borne fungi like *Aspergillus sp.* (Spory and Kleeberg 2000). Our studies showed that seeds obtained from the high Aza-A yielding tree were less susceptible to fungal infections on storage. The present study indicates that the seeds with high Aza-A were viable even after long periods of storage at 4 °C and germinated into sturdy seedlings *in vitro*. However, seeds from low Aza-A yielding tree exhibited very poor viability (Fig. 1).

Biochemical implications

Biochemical studies on calli and various explants revealed detectable amounts of azadirachtin-A only in the cotyledons of the seedlings from high Aza-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 Aza-A containing seeds with a distinct disparity in their levels. In general, the increase in levels of saponins indicates the presence of a healthy plant (Osbourn 1996). On the other hand, presence of tannins offers anti-microbial properties (Scalbert 1991). High levels of these compounds generally serve as biomarkers to indicate the resistance of the plant to pathogenesis. The allelopathic nature of saponins, which has 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 analyzed in various explants. This was supported by polymorphism in DNA amplification studies at the molecular level (Table 9; Fig. 2IIa to IIc).

Table 4 VAM species from five ecologically different regions in Andhra Pradesh

Districts Vam species	Krishna Semiarid	Adilabad Semiarid/arid	Anantapur Extremely arid alkaline	East-Godavari Delta-wet	Khammam Arid
1. *Glomus mossae*	(+)	(+)	(+)		(+)
2. *Glomus microcarpum*	(+)				
3. *Glomus macrocarpum*	(+)		(+)	(+)	
4. *Glomus constrictum*		(+)			(+)
5. *Glomus fasciculatum*		(+)	(+)	(+)	(+)
6. *Glomus albida*				(+)	
7. *Glomus multisubstance*				(+)	
8. *Glomus deserticala*			(+)		
9. *Gigaspora margarita*		(+)	(+)		
10. *Acaulospora species*			(+)		

Table 5 Range and average values of azadirachtin-A density, 100 seed weight and total azadirachtin-A from the entire sample size and the percentage increase of azadirachtin-A density, 100 seed weight and total azadirachtin-A in the accessions from five ecodiverse environments together and separately

Parameters	All Districts	Krishna (Semi arid)	Adilabad (Semi arid/arid)	Khammam (Arid)	East-Godavari (Delta-wet)	Anantapur (Extremely arid alkaline)	% Increase (From East Godavari to Anantapur)
Range of azadirachtin-A density	0.46–3.83 mg/g. seed wt.	0.56–3.83 mg/g. seed wt.	0.94–3.11 mg/g. seed wt.	1.18–1.89 mg/g. seed wt.	0.46–1.97 mg/g. seed wt.	0.95–2.60 mg/g. seed wt.	–
Average of azadirachtin-A density	1.54 mg/g. seed wt.	1.61 mg/g. seed wt.	1.56 mg/g. seed wt.	1.52 mg/g. seed wt.	1.03 mg/g. seed wt.	1.98 mg/g. seed wt.	92.2 %
Range of 100 seed weight in g	12.26–36.24 g.	13.08–20.48 g.	12.73–22.42 g.	12.26–29.00 g.	16.82–36.24 g.	14.00–27.76 g.	–
Average 100 seed weight in g	19.78 g.	16.10 g.	18.54 g.	20.96 g.	23.88 g.	19.43 g.	22.9 %
Range of total azadirachtin-A	7.82–63.60 mg/ 100 seed wt. in g.	7.82–50.15 mg/ 100 seed wt. in g.	17.15–54.88 mg/ 100 seed wt. in g.	15,74–50.60 mg/ 100 seed wt. in g.	8.81–63.60 mg/ 100 seed wt. in g.	13.64–51.58 mg/ 100 seed wt. in g.	–
Average of total azadirachtin-A	30.17 mg/ 100 seed wt. in g.	25.24 mg/ 100 seed wt. in g.	28.84 mg/ 100 seed wt. in g.	31.84 mg/ 100 seed wt. in g.	27.27 mg/ 100 seed wt. in g.	37.65 mg/ 100 seed wt. in g.	38.06 %

Impact of DNA fingerprinting studies

Genomic DNA amplification studies showed polymorphism not only between the high and low azadirachtin-A yielding trees but also within that of DNA from the tender leaves, *in vitro* seedlings of high azadirachtin-A yielding seeds and calli from their leaf explants (Fig. 2IId to IIg). Similar to our observations on molecular polymorphism within Neem chemotypes, there are reports in the black and red spruce varieties, within a species, where the variation is attributed to the interaction between the plantlets and the microenvironment (Singh et al. 1999; Nkongolo et al. 2002). Also, reports of RAPD profiles of plantlets generated from callus of the somatic hybrids of *Asparagus officinalis* and *Asparagus marcowani* differed from the parental lines but, no differences were observed in the morphological characters (Kunitaki et al. 1996).

Furthermore, parallel to our findings of a distinct DNA polymorphism between *in vitro* seedlings and calli from their various explants, there are examples wherein the genetic constitution of the callus is clearly different from the explants' source; possibly because of growth hormones. For example, *Geraniums* are known to show somaclonal variations in plants obtained from in vivo roots or petiole cuttings (Skirvin and Janicke 1976). Similar variations have been observed in pineapple and potato (Razdan 1993).

Thus, environmental and molecular influences on plants have a great impact on growth in terms of biomass, on biochemical aspects in terms of secondary metabolites and at the genomic level in terms of differentiation of DNA fingerprinting patterns.

Our *in vitro*, biochemical and molecular studies on Neem highlight the role of both macro and micro-environments on the genetic constitution of the individual tissues that could easily distinguish Neem trees with quantitative variations in Aza-A. This could serve as a useful biomarker to identify elite Neem varieties with high Aza-A content.

Ecomorphological biomarkers

Plant-soil interactions with a broad range of ecomorphological diversity calls for a need to readily identify chemotypes of elite varieties, laying specific emphasis on economically important secondary metabolites. Morphometric studies have been done on various organisms such as: a) variations in flowering morphology of *Rizhophora mangal* and geographical differences (Ceaser et al. 1998), b) classificatory schemes proposed for the *Ixeris chinensis* complex based on morphological characters (Whang et al. 2002) and c) correlation between morphological characters such as leaf margin and canopy of the tree with environmental characters such as temperature (Burnham et al. 2001).

Table 6 Cumulative representation of various explants' responses from *high* azadirachtin-A containing seeds in the media supplemented with different growth regulators

Type of response	Root	Hypo	Coty	Epi	Leaf	Root	Hypo	Coty	Epi	Leaf
EE		2,4		2						
CI	4	1,2	1		1,2,3		1		2	
CG		3	4	1	2,3,4		2,3,4	4	4,1	1,2,3,4
CR			2		1,2,3	1,4		2,1		1,2,3
CT							4			1,2,3,4

However, to date there are only meagre records to show the implementation of statistical tools for the detailed analysis of the ecomorphological markers in relationship to Aza-A in Neem. Toward the goal of selection of elite Neem trees with high levels of Aza-A, ecomorphometric characters have been used as qualitative markers to reflect the quantitative variations in this tetranortriterpenoid mainly by using statistical tools supplied by the SPSS package version 10.0. Our investigations have helped in the identification of ecomorphometric markers that could determine high Aza-A yielding chemotypes. This consisted of two studies that included the same regions chosen for mycofloral survey and molecular analyses (sections "Mycofloral biomarkers" and "Molecular biomarkers").

Habitat-related variations in Aza-A content

While the accessions suggested a clear categorization into high and low Aza-A yielding chemotypes in the two micro-environments of the first study; the greatest disparity in average Aza-A density, 100 seed weight and total Aza-A was observed in accessions between the extremely-arid-alkaline (pH 8.05) and delta-wet (pH 7.05) regions in the second study; with the maximum values in the former, thus strengthening the impact of ecomorphometric analyses.

Similar reports on Neem trees from six West Bengal districts with a varying range of soil pH (5.25 to 6.43) suggested a correlation with Aza-A content. Essentially, trees with high Aza-A density (2 %) thrived best in soils with the highest pH (6.43) among the areas studied.

In general, seed weight becomes an important parameter, to identify Neem trees for commercial and industrial purposes. Total Aza-A is dependent on both Aza-A density and hundred seed weight (Aza-A density is mg of the biopesticide/g dry weight of seed; and total Aza-A is a product of Aza-A density and 100 seed weight expressed in mg/100 seed weight in gm). But, it is also possible that total Aza-A could be independent of azadirachtin-A density, given that two accessions have the same azadirachtin-A density, but vary dramatically in their fruit yield as measured by hundred seed weight in g. For example, in each of our two studies, at least two samples had the same Aza-A density values. However, their 100 seed weight differed by at least 40 %, which was in turn reflected in the increase of the total Aza-A by at least a corresponding value of 40 %.

Impact of statistical analyses for determining ecomorphometric biomarkers

The LMRA of at least 22 independent variables which included three parameter groups (leaf, fruit/seed and soil) were studied together and separately with each of the two dependent variables (Aza-A density and total Aza-A). Based on the LMRA studies, the nine significant parameters (from the two microenvironments) and the seven

Table 7 Cumulative representation of various explants' responses from *low* azadirachtin-A containing seeds in the media supplemented with different growth regulators

Type of response	After 15 Days					After 30 Days				
	Root	Hypo	Coty	Epi	Leaf	Root	Hypo	Coty	Epi	Leaf
EE	2	4		3,4						
CI	1,3		1	2	3			1	4	
CG		2	1		4		3	2,3	3	3,4
CR		3		1			1	3	1	3,4
CT										

The abbreviations and numbers are for Tables 6 and 7

1–1 mg/L NAA, 2–2 mg/L NAA, 3–1 mg/L NAA + 0.5 mg/L Kn, 4–2 mg/L NAA + 0.5 mg/L Kn

EE Explant expansion, *CI* Callus initiation, *CG* Callus growth, *CR* Root from callus, *CT* Callus growth continued, *Hypo* Hypocotyl, *Epi* Epicotyl, *Coty* Cotyledon

Table 8 Cumulative representation of percentage influence of growth factors on the explants derived from the seedlings of the seeds from high and low azadirachtin-A groups

Hormone type									
		After 15 Days				After 30 Days			
Explant source		1	2	3	4	1	2	3	4
High azadirachtin-A containing seeds	Individual influence	25 %	35 %	20 %	20 %	29.2 %	25 %	16.6 %	29.2 %
	Combined Influence	60 %		40 %		54.2 %		45.8 %	
Low azadirachtin-A containing seeds	Individual influence	28.6 %	21.4 %	28.6 %	21.4 %	23.1 %	7.7 %	46.2 %	23.1 %
	Combined Influence	50 %		50 %		30.7 %		69.3 %	

Percentage influence for each of the hormone was calculated by counting the total number of responses in 15 or 30 days. Then the total number of responses per each hormone was counted and the percentage was determined. (E.g., for 15 days, Hormone 1=5/20X100=25 %)

significant parameters (from the five ecogeographical regions) examined together as a unit could explain the variations in both the dependent variables. These parameters showed a stronger negative relationship than a positive one to the two dependent variables in general.

Of all the negative correlations available, the relationship of pH of the soil with Mn and Fe suggested that with an increase in the pH of the soil, there would be a decrease in the available soil Mn (in ppm) and Fe (in ppm). There is evidence for definite negative relationship of pH with Zn, Cu, Mn and Fe as described earlier (Benthlenfalvay and Lindermann 1992; Zekri and Obereza 2003). The LMRA studies further showed a negative relationship of Mn with both Aza-A density and total Aza-A. Also, earlier studies regarding morphological characters such as seed diameter (S_DIA) (−) and seed length (S_LEN) (+) have been proven to show variations in relationship to these dependent components in Neem (Kumaran et al. 1993; Sinduveerendra 1995). Based on the results obtained from our two studies, the biomarkers S_DIA (−), pH (+), percentage moisture (% MOS) (−) and P_2O_5 (−) were commonly and significantly related to Aza-A density. Thus these markers can be used to study the variation in the dependent variable.

Out of at least eight independent variables found significant in LMRA in both the studies, none of which were correlated with each other, a minimum of two-third of these showed concurrence with data obtained from descriptive statistics (DS), and graph plots of these values segregated based on soil colour and texture. ANOVA showed a significant difference between groups rather than within groups of the two microenvironments using, a) habitat, b) pointedness and c) bitterness of leaflets chosen as criteria, for all the independent parameters found significant in LMRA studies.

The DMRT performed on the larger population encompassing the five ecogeographically distinct regions showed that about 50 % of the independent variables tested, differed significantly between the extremely-arid-alkaline and delta-wet regions; Aza-A density being one of the predominant factors. In essence, for each of these five regions, a number is allocated based upon their values of a particular parameter. Next, the program determined if there was a statistical difference between every combination of the five regions, amounting to a total of ten combinations. This was recorded for each of the 25 variables that included leaf, fruit/seed, soil, Aza-A density and total azadirachtin-

Table 9 Secondary metabolites isolated from seed and callus obtained from various explants of *in vitro* raisedseedlings of Azadirachta indica

(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

Secondary metabilite	Callus										Seed	
	Root		Hypocotyl		Cotyledon		Epicotyl		Leaves			
	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. Furthermore, these findings were tabulated wherein each of the ten combinations of two areas each are scored for the total number of variables that were significantly different between the areas (Table 10).

Molecular biomarkers

The quantitative variations in Aza-A are attributed to both ecogeographical and genetic factors (Ermel 1995). As a robust alternative to traditional methods, RAPD and ISSR primers were recently used to resolve the genetic diversity more precisely (Awasthi et al. 2004; Ammiraju et al. 2001). Our molecular studies using RAPD, ISSR and FISSR primers showed that DNA fingerprinting patterns catogorized Neem accessions according to the quantitative variations in Aza-A density. The accessions that exhibited high and low Aza-A were clearly segregated into groups that distinctly overlapped with the clusters generated, both based on their azadirachtin-A content and also with respect to their habitat (Table 11; Fig. 3a to c). However, because of the complexity of the genome, little correlation can be drawn between the polymorphic pattern of the amplified DNA and this secondary metabolite. Thus, the status of the molecular studies to date on Neem stands at the cataloguing level of high versus low Aza-A containing trees.

In addition to the DNA profiles exhibiting an ecological concordance with the chemotypes, across all the ecogeographic regions under study, the relatedness of the accessions from Sydney (Australian continent's east coast, Southern hemisphere) and the low Aza-A yielding chemotypes from the delta-wet (Indian sub-continent's east coast, Northern hemisphere) regions further suggested a possible environmental influence (irrespective of the hemispheres) on the molecular segregation. As shown in the dendrogram, the samples from the two regions indicated a high similarity, suggesting the powerful impact of ecological factors (both being on the east coast of their respective continents) on molecular patterns. Thus, this molecular marker technique could be used to recognize the elite varieties of tree species for desirable traits (Fig. 3).

Similar findings have been observed independently in two different plants namely, *Chrysanthemum* and *Capsella bursa-pastoris*. DNA fingerprinting of different *Chrysanthemum* cultivars analyzed by UPGMA segregated them into two major groupings that were consistent with their morphological differences and geographical distribution. (Sehrawat et al. 1997). Likewise, seven populations of *Capsella bursa-pastoris* were divided into two groups based on morphological characters. This segregation was in concurrence with that obtained from DNA fingerprinting patterns using three RAPD markers. These molecular markers may be linked to significant morphological characters such as leaf size and date of flowering, which are important criteria used to distinguish these two groups of *Capsella bursa-pastoris* (Yang et al. 1998).

Table 10 Duncan's Multiple Range test for the percentage variations between the areas in the number of significant independent variables in the LMRA from all the samples of the five regions under study

S. no	Ecogeographical regions	Parameters significantly different between two regions	No. of parameters		% of parameters significantly different between ecogeographical regions
			Differing	Total studied	
1	Semiarid-semiarid/arid	F_DIA	1	25	4
2	Semiarid-exremely and alkaline	MNDL, Fe, NNLETS, Ang LLET	4	25	16
3	Semiarid/delta wet	S_WT, Ang LLET, LLC, F_LEN, S_LEN, ULC, Cu, P_2O_5	8	25	32
4	Semiarid-arid	F_LEN, S_LEN, F_DIA, Cu, P_2O_5, pH	6	25	24
5	Semiarid/arid-extremely arid alkaline	NNLETS, K_2O	2	25	8
6	Semiarid/arid-delta wet	S_WT, MNDL, LLC, ULC, Cu, K_2O, P_2O_5	7	25	28
7	Semiarid/arid-arid	ULC, pH, Cu	3	25	12
8	Extremely arid alkaline-delta wet	S_WT, MNDL, LLC, S_LEN, CU, P_2O_5, Ph, NNLETS, Ang LLET, AZA DEN, Fe, MOS	12	25	48
9	Extremely arid alkaline—arid	F_LEN, S_LEN, Fe, Ang LLET, AZA DEN, Fe, MOS	8	25	32
10	Delta wet-arid	MNDL, F_DIA, K_2O, Ph	4	25	16

TOT AZA Total azadirachtin-A, *S_WT* Hundred seed weight, *F_LEN* Fruit length, *F_DIA* Fruit diameter, *S_LEN* Seed length, *S_DIA* Seed diameter, *NNLLETS* Number of leaflets, *LEN_LLET* Average leaflet length, *WID_LLET* Average leaflet width, *ULD* Upper lobe distance, *LLD* Lower lobe distance, *ULC* No. of serrations in the upper lobe, *LLC* No. of serrations in the in the lower lobe, *MUNDL* No. of marginal undulations, *Ang LLET* Angle of leaflet tip, *EC* pH,Electric conductance, *K_2O* Potassium oxide, *P_2O_5* Phosphorous pentoxide, *MOS* Percentage moisture, *Zn* Zinc, *Mn* Manganese, *Fe* Iron, *Cu* Copper

Table 11 Difference in the azadirachitn-A density, 100 seed weight and total aadirachtin-A content between the groups G Ia and GIb and GII a and GIIb

Accession no	EG Lo 20	Aus Lo? 24	Aus Lo? 23	EG Lo 19	Aus Lo? 22	Aus Lo? 21	Kr Lo 14	Kr Lo 13	Ad Lo 16	Ad Lo 15	Km Lo 18	Km Lo 17	Atp Lo 12
Azadensity (mg/gm seed wt)	0.46	–	–	0.54	–	–	0.81	0.94	1.06	0.94	1.18	1.28	0.95
100 seed wt (gms)	20.40	–	–	16.82	–	–	20.48	14.08	18.52	20.17	24.10	12.26	23.67
Total aza (mg/ 100 seed wt in g.)	9.38	–	–	9.08	–	–	16.59	13.24	19.63	18.96	28.44	15.69	22.49
Habitat	DW	C	C	DW	C	C	SA	SA	SA/A	SA/A	A	A	EAA
Group	G IIb	G IIb	G IIb	G IIb	G IIb	G IIb	G IIa	G IIa	G IIa	G IIa	G IIa	G IIa	G IIa
Accession no	Atp Lo 11	EG Hi 10	EG Hi 9	Km Hi 8	Km Hi 7	Ad Hi 6	Ad Hi 5	Kr Hi 4	Kr Hi 3	Atp Hi 2	Atp Hi 1		
Azadensity (mg/gm seed wt)	0.97	1.36	1.58	1.65	1.66	1.73	2.22	2.17	2.24	2.43	2.60		
100 seed wt (gms)	14.0	26.50	21.10	16.14	21.54	22.42	17.55	15.88	20.03	16.43	17.26		
Total aza (mg/ 100 seed wt in g.)	13.58	36.04	33.34	26.63	35.76	38.79	38.96	34.46	44.87	39.92	44.88		
Habitat	EAA	DW	DW	A	A	SA/A	SA/A	SA	SA	EAA	EAA		
Group	G Ib	G Ib	G Ia	G Ia	G Ia	G Ia	G Ia	G Ia	G Ia	G Ia	G Ia		

Kr Krishna, *Ad* Adilabad, *Atp* Anantapur, *EG* East Godavari, *Km* Khammam, *Aus* Australia, *SA* Semi Arid, *SA/A* Semi Arid/Arid, *EAA* Extremely Arid Alkaline, *DW* Delta-wet, *A* Arid, *C* Coastal

This that Table 11 is supportive of Fig. 3

Conclusion

Charaka-Samhita stands testimony to the role of Neem in ancient medicine. But, inspite of its antiquity it is only in the past two decades that the interest in Neem research has taken an impetus (Sinduveerendra 1995; Tewari 1992). About 300 liminoids of economic importance have been isolated from their seed kernels (Kumar and Parmar 1997). Many of these compounds are triterpenoids, which are derived from the tetracyclic triterpene tirucallol (Govindachari 1992). Of these secondary metabolites, several of them have been proven to have potentially biopesticidal or antifeedant properties. Among these, nimbin, nimbidin, salanin, and Aza-A have been studied in some depth. Out of these liminoids, Aza-A, a tetranortriterpeniod is the most potent, environment friendly, and biodegradable pesticide, with antifeedent and growth inhibiting properties (Yakkundi et al. 1995). However, the biochemical pathway of Aza-A is poorly understood, with the last identified enzyme being squalene synthase that leads to the formation of squalene (Govindachari et al. 1995). This compound forms the source for several major compounds including steroids, triterpenoids, tetraterpenoids, tetranortriterpenoids and numerous other complex molecules. Therefore, the need to study the various steps in the metabolic pathway becomes necessary.

Variations in Aza-A have been observed in Neem trees based on seasonal variations (Sidhu and Behl 1996), differential regulation in flowering and fruiting cycle (Rengaswamy and Parmar 1995), impact on ecotypes in relation to various districts of a state or in relation to the agroecological regions of India (Kumar and Parmar 1997; Rengaswamy and Parmar 1995; Venkateswarlu et al. 1996). Elite chemotypes with higher Aza-A were clonally propagated (Venkateswarlu et al. 1996). Following this, recent biomarker studies using both biochemical and molecular techniques have been attempted on Neem. Isozyme marker studies to assess the genetic variation of Neem ecotypes from India and Thailand have shown that Indian Neem varieties differed widely from those introduced from Thailand (Wickramasinghe and Simons 1994). A variety of DNA fingerprinting techniques are available, most of which use PCR to detect polymorphism in trees through the identification and amplification of fragments (Vos et al. 1995; Jones et al. 1997). The AFLP (Amplified Fragment Length Polymorphism) markers have been used to show that Indian Neem germplasm constituted a broad genetic range compared to the Thailand lines (Singh et al. 1999). The RAPD (Random Amplified Polymorphic DNA) markers have also been used to assess the genetic diversity in Neem populations (Farooque et al. 1998).

To date, molecular studies on Neem only involved variations in ecotypes but not in relationship with Aza-A.

Our recent studies **alone** suggest molecular cataloguing of Neem trees based on the quantitative variations in Aza-A from five ecogeographically distinct regions in Andhra Pradesh, using RAPD and ISSR biomarkers (Rao and Chary 2007b, 2009). However, because of the complexity of the genome, little correlation can be drawn between the polymorphic pattern of the amplified DNA and this secondary metabolite.

The gene coding for squalene synthase has been cloned from *Nicotiana tobaccum* and its expression studied (Deverenne et al. 1998). Given that squalene synthase is the key enzyme known to date involved in pathways that result in the formation of both steroids (cholesterol) and terpenoids, it becomes relevant to dwell in greater detail on this enzyme. Thus the choice of using squalene synthase as one of the molecular biomarkers for understanding differential gene expression of various components, with specific reference to Aza-A in future studies becomes pertinent (Shastry and Chary 2004). Towards this goal, once the above genes are identified and sequenced, hybridization probes can be made to serve as markers to distinguish between high and low Aza-A containing trees at the transcription level.

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