Ecomorphometric markers reflect variations in Azadirachtin-A content of *Azadirachta indica* A. Juss (Meliaceae) in select regions of Andhra Pradesh, India

Sai Jyothi Pattnaik, N. D. Raghupati Rao and Parvathi Chary*

Department of Biosciences, Sri Sathya Sai Institute of Higher Learning, Prashanthi Nilayam Campus, Puttaparthi 515 134, India

Ecomorphometric markers reflect quantitative variations in the potent antifeedant, azadirachtin-A from neem. Fifty accessions from five ecogeographically distinct regions in Andhra Pradesh, India were studied. Significant disparity in azadirachtin-A density, 100 seed weight and total azadirachtin-A was observed in accessions between the extremely-arid-alkaline and delta-wet regions. Statistical tools such as Linear Multiple Regression Analysis, Duncan's New Multiple Range Test and Pearson Correlation were used to find out the relationship between independent variables (leaf, fruit/seed and soil parameters) and dependent variables (azadirachtin-A density and total azadirachtin-A content).

Keywords: Azadirachtin-A, extraction, ecomorphometry, HPLC, neem.

THE medicinal value of *Azadirachta indica* A. Juss (neem), family Meliaceae dates back to the Vedic times, as mentioned in Ayurveda as 'Sarva-roga-nivarini'[1]. It has also found a place in the Unani system of medicine because of its multi-faceted applications[2]. Neem-based products constitute a broad spectrum of pesticides with a variety of functions. For instance, azadirachtin-A acts as an insect repellent, antifeedant, affects hormonal balance and disrupts the metamorphosis of several insect larvae. Detailed chemical analysis shows that it mimics hormones and thus blocks the receptors for ecdysteroids, which are needed for larval development[3].

Neem is usually grown in dry areas, but often cultivated as a medicinal plant in wet regions[4]. Given the wide distribution of neem and in spite of the awareness of its biodiversity, the impetus for exploration into ecosystem monitoring, DNA fingerprinting and development of a comprehensive information databank is barely in its infancy. With specific reference to azadirachtin-A content there are reports showing seasonal variations[5], differential regulation in the flowering and fruiting cycle[6], impact of ecotypes in relation to various districts of a state or in relation to the agro-ecological regions of India[7–9]. However, little is known about the genetic factors influencing the wide range in azadirachtin-A content among neem trees. This broad variability could be a result of both environmental and genetic factors[2,8]. Thus, genetic and phenotypical variability of neem can be studied through morphological parameters[10,11].

Keeping in view the immense potential of azadirachtin-A, the present investigation was undertaken to explore if ecomorphological parameters could serve as reliable biomarkers in determining the variations in this biopesticide.

Materials and methods

Sample collection

Neem trees were chosen from five ecogeographically different regions encompassing soils of varied pH, from the Deccan plateau, Andhra Pradesh, India (Table 1). Mature unipinnately compound leaves, fruits and soil samples were collected from 50 selected accessions during three consecutive fruiting seasons. The leaves (10–15) from each tree were collected from the lowermost layer of the canopy and placed in large, clean, airtight plastic bags and stored at 4°C to prevent etiolation and shrivelling.

The disease-free, mature, ripe (deep yellow to light yellow) fruits (100) were collected to determine seed weight. The seeds were then manually depulped, cleaned under running tap water and then air-dried. The mesocarp adhering to the hard epicarp was removed by scrubbing the seed on a rough surface, usually using a sieve or sand paper. The dry seeds were packed in separate plastic covers, labelled and stored at 4°C to prevent microbial growth, aflatoxin contamination and azadirachtin-A degradation.

About 1 kg of topsoil was collected from the soil supporting each neem tree at about a distance of 75–100 cm for soil analysis.

Azadirachtin-A extraction and HPLC analysis

Azadirachtin-A from seed kernels was extracted for HPLC analysis[12]. First, seed kernels were made into a fine pow-

*For correspondence. (e-mail: pchary2000@yahoo.com)

Table 1. Meteorological data from five different ecological regions in Andhra Pradesh, India

District	Area	Annual rainfall (mm)	Latitude	Longitude	Crops
Krishna	Semi-arid	900	16°–47′09″N	80°51′11″E	Groundnut, red gram and mango
Adilabad	Semi-arid/arid	1200	9°03′06″	79°29′14″	Castor, sugarcane, jowar, sunflower and turmeric
Anantapur	Extremely arid alkaline	668	140°	77°E	Groundnut, sugarcane and mango
East–Godavari	Delta-wet	1000	17°06′58″	82°14′47″	Paddy, groundnut, cotton, chillies, red gram and green gram
Khammam	Arid	1000	17°32′41″	80°37′30″	Groundnut, chillies, cotton, red gram and green gram

der using a sample mill. Next, by repeated extraction with *n*-hexane, fat was completely removed from the samples and azadirachtin-A was subsequently extracted in methanol. The residue was air-dried and subsequently the precipitate was ground in methanol. An aliquot of 20 µl of this extract was utilized for quantifying azadirachtin-A using HPLC with a differential refractometer. C8 columns (15 cm × 0.46 cm) were used with water, methanol and acetonitrile in the ratio 50 : 35 : 15 as the mobile phase with a flow rate of 1 ml/min azadirachtin-A was detected at 215 nm.

Estimation of soil elements

Qualitative soil parameters, namely colour and texture and quantitative soil parameters such as pH, electrical conductivity (EC), potassium oxide kg ha^{-1}, phosphorus pentoxide (kg ha^{-1}), zinc (ppm), manganese (ppm), iron (ppm) and copper (ppm) were determined. Soil colour and texture were essentially categorized as specified by Tiwari[13]. Seven soil colours and five soil textures were included in this study. 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 1411.8 × 106 m hos/cm at 25°C served as a standard. Soil phosphorus was estimated using sodium bicarbonate (Olsen's reagent) as standard. 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[14]. In essence, standard methods were followed for estimating all the above soil microelements and macroelements[14–16].

Statistical analysis

A total of 25 parameters, including the two dependent (azadirachtin-A density expressed as mg/g seed weight and total azadirachtin-A expressed as mg/g 100 seed wt) and 23 independent variables (fruit/seed, leaf and soil parameters) were identified for this study. Fruit/seed parameters included five independent variables, namely hundred seed weight (S_WT), fruit length (F_LEN), fruit diameter (F_DIA), seed length (S_LEN) and seed diameter (S_DIA). Seed size (length was diameter) was determined using a vernier calipers. Leaf parameters included nine independent variables, namely number of leaflets (NNLLETS), average leaflet length (LEN_LLET), average leaflet width (WID_LLET), upper lobe distance (ULD), lower lobe distance (LLD), no. of serrations in the upper lobe (ULC) and in the lower lobe (LLC), no. of marginal undulations (MUNDL) and angle of leaflet tip (Ang LLET). Soil parameters consisted of soil colour, texture, percentage moisture, pH, EC, K_2O, P_2O_5, Zn, Mn, Fe, and Cu.

The relationship of independent variables with dependent variables was evaluated with Linear Multiple Regression Analysis (LMRA), Duncan's New Multiple Range Test (DMRT), Pearson Correlation Analysis (PCA), and descriptive statistics (DS) was performed[17] using SPSS package version 10.0 for Windows®.

Results

The ecomorphological study on 50 accessions from five ecogeographically distinct regions (Table 1) was subjected to statistical analysis such as DS, PCA, LMRA and DMRT.

Quantitative variations in azadirachtin-A and total azadirachtin-A

HPLC analysis of azadirachtin-A density from seed kernels ranged from 0.46 to 3.83 mg/g seed wt, obtained from 50 accessions represented by five ecogeographically distinct regions of Andhra Pradesh. The weight of 100 seeds and total azadirachtin-A in these accessions ranged from 12.26 to 36.24 g and 7.82 to 63.60 mg/g 100 seed wt respectively (Table 2).

These samples were segregated into high and low azadirachtin-A containing chemotypes within each of the ecological regions, namely semi-arid, semi-arid/arid, extremely-arid-alkaline, delta-wet and arid. Among these regions, accessions growing in extremely-arid-alkaline region exhibited highest azadirachtin-A density, 100 seed weight and total azadirachtin-A, compared to those from the delta-wet region which exhibited the lowest values (Table 2).

Table 2. Azadirachtin-A density, 100 seed weight and total azadirachtin-A in accessions from five different ecological regions

Parameter	Krishna (semi-arid)	Adilabad (semi-arid/arid)	Khammam (arid)	East Godavari (delta-wet)	Anantapur (extremely-arid-alkaline)	Percentage increase (from East Godavari to Anantapur)
Azadirachtin-A density (mg/g seed wt)	1.61 (0.56–3.83)	1.56 (0.94–3.11)	1.52 (1.18–1.89)	1.03 (0.46–1.97)	1.98 (0.95–2.60)	92.20
100 seed wt (g)	16.10 (13.08–20.48)	18.54 (12.73–22.42)	20.96 (12.26–29.00)	23.88 (16.82–36.24)	19.43 (14.00–27.76)	22.90
Total azadirachtin-A (mg/100 seed wt g)	25.24 (7.82–50.15)	28.84 (17.15–54.88)	31.84 (15.74–50.60)	27.27 (8.81–63.60)	37.65 (13.64–51.58)	36.06

Data in parenthesis indicates the range.

Statistical analysis

PCA: Correlation studies were conducted involving 25 parameters that originated from various sources. These included the independent variables (leaf, fruit/seed and soil parameters) and dependent variables (azadirachtin-A density and total azadirachtin-A).

Correlations among five ecological regions that were analysed as an entity and separately, showed that azadirachtin-A density was highly correlated with total azadirachtin-A, in general. In addition, although several independent variables exhibited high positive correlation within their parameter groups as expected, there were some that showed negative correlation. This was of significance in view of the LMRA studies that were to follow, for understanding and predicting the relationships between the independent and dependent variables through regression.

Pearson correlation that indicated relationships at the ** level ($P > 0.01$) alone was considered. This was studied between all the variables, irrespective of their parameter groups like fruit/seed, leaf, soil and dependent variables (azadirachtin-A density and total azadirachtin-A) which were analysed in the five ecological regions, either together or separately. The number of significant inter-parameter relationships was observed to be high (31) in the correlation studies that involved all the accessions irrespective of their ecological origin, followed by those from the semi-arid (10), arid (10), delta-wet (8), semi-arid/arid (7) and extremely arid alkaline (6) regions (data not shown).

The correlation analysis further suggested a need for evaluating the data in the light of regression studies. Among the numerous combinations (72) of various parameters observed in the correlation, special attention was drawn to eight parameters, which were found significant in LMRA studies. As detailed below, only those among the eight parameters that were found not to be correlated with each other, were considered for further analysis (Table 3).

S_DIA was not correlated with any of the parameters, irrespective of their groups (leaf, fruit/seed and soil) and their ecological origin, namely either the five ecological regions together or separately. K_2O was correlated with LLD (in semi-arid/arid region). Zn was correlated with ULC and Mn (in all regions) and with F_DIA (in arid region). ULD was correlated with LLD and WID_LLET (in all regions), LEN_LLET (in semi-arid region), WID_LLET (in semi-arid/arid), ULC (in extremely-arid-alkaline), WID_LLET (in delta-wet and arid). MOS was correlated with Mn (in all regions and semi-arid), with Fe (in delta-wet) and Cu (in arid region). pH was correlated with Mn, Fe and Cu in all regions, with Fe and Ang LLET (in extremely-arid-alkaline), with Mn and Fe (in delta-wet) and Fe (in arid). P_2O_5 was correlated with Cu and 100 seed weight (in all regions), and with EC (in arid). LLC was correlated with NNLLETS, LLD and ULC (in all regions), with WID_LLET, LLD and ULC (in semi-arid), with NNLLETS and EC (in semi-arid/arid; Table 3).

LMRA: The LMRA using the backward method of execution with the SPSS version 10.0 for Windows® indicated 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.

The LMRA studies were classified under two broad groups based on the dependent variables: (a) azadirachtin-A density and (b) total azadirachtin-A. The relationships with each of these dependent variables were studied with the three groups encompassed by 23 independent variables (of fruit/seed, leaf and soil), separately and together. Furthermore, the analysis was performed with all the accessions in the five ecological regions together and also separately (Tables 4–6).

The independent variables were related to the dependent variables either positively or negatively: (a) at the *** level of significance ($P > 0.001$) being + () or – (), (b) at the ** level of significance ($P > 0.01$) being + () or – (), and (c) at the * level of significance ($P > 0.05$) being + () or – () (Tables 4 and 5).

Table 3. Comprehensive representation of parameters that were significantly correlated at $P > 0.01$ to $P < 0.05$ level (**) and $P > 0.05$ to $P < 0.1$ level (*) from all accessions together and accessions from five ecogeographically different regions

Parameters significant in LMRA within various districts	Interactions (+ or –) of significant LMRA parameters with AZA DEN	Parameters eliminated due to correlation with other significant variables in LMRA	All districts	Krishna (semi-arid)	Adilabad (semi-arid/arid)	Anantapur (extremely-arid-alkaline)	East Godavari (delta-wet)	Khammam (arid)
Cu	+	Eliminated	SD_WT, MUNDL, pH, P_2O_5, Mn, Fe	–	–	–	–	MOS
K_2O	–	Retained	–	–	LLD	–	–	–
Zn	–	Retained	ULC, Mn	–-	–	–	–	F_DIA
ULD	–	Retained	LLD, WID_LLET	LEN_LLET	WID_LLET	ULC	WID_LLET	WID_LLET
MOS	–	Retained	Mn	Mn	–	–	Fe	Cu
pH	+	Retained	Mn, Fe, Cu	–	–	Fe, Ang LLET	Mn, Fe	Fe
P_2O_5	–	Retained	Cu, SD_WT	EC	–	–	–	–
Ang LLET	–	Eliminated	NNLLETS, LLD, MUNDL		–	PH	–	–
LLC	+	Retained	NNLLETS, LLD, ULC	WID_LLET, LLD, ULC	NNLLETS, EC	–	–	–
Mn	–	Eliminated	Fe, Cu	MOS	EC	TOT AZA	Fe, pH	–
S_DIA	–	Retained	–	–	–	–	–	–
SD_WT	+	Eliminated	TOT AZA, S_LEN, Cu, F_LEN, P_2O_5	–	–	–	–	TOT AZA

Total azadirachtin-A (TOT AZA), hundred seed weight (SD_WT), fruit length (F_LEN), fruit diameter (F_DIA), seed length (S_LEN), seed diameter (S_DIA), number of leaflets (NNLLETS), average leaflet length (LEN_LLET), average leaflet width (WID_LLET), upper lobe distance (ULD), lower lobe distance (LLD), no. of serrations in the upper lobe (ULC); no. of serrations in the in the lower lobe (LLC), no. of marginal undulations (MUNDL), angle of leaflet tip (Ang LLET), pH, electric conductance (EC), potassium oxide (K_2O), phosphorus pentoxide (P_2O_5), percentage moisture (MOS), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu).

Table 4. LMRA of independent variables with azadirachtin-A density as dependent variable

District	Parameter group	Significant parameters from parameter group	β-constant	Adjusted R^2	F-statistics
All	All	ULC, Ang LLET, K_2O, Zn □	4.45 (6.79)***	0.25	5.13***
	Leaf	MUNDL	2.08 (7.97)***	0.07	4.86**
	Fruit/seed	S_LEN◊	3.38 (3.52)***	0.05	3.71*
	Soil	K_2O◊, Zn, Fe ◊	2.7 (6.68)***	0.14	3.57**
Krishna	All	F_DIA, S_DIA ♦, LLC◊, Zn, Cu ♦	4.14 (–1.19)	0.74	6.09*
	Leaf	–	–	–	–
	Fruit/seed	–	–	–	–
	Soil	EC•, pH•, P_2O_5, K_2O, MOS ◊, Mn♦, Fe♦, Cu•	–60.36 (–23.70)**	0.99	200.90*
Adilabad	All	F_LEN■, S_LEN◊, ULD■, LLD•, ULC■, Ang LLET•, Mn, Cu ■	–6.61 (–118.09)***	1	11131.32***
	Leaf	NNLETS♦, ULD•	–9.78 (–2.49)**	0.45	4.60*
	Fruit/seed	F_LEN•, S_LEN, S_DIA	9.02 (3.11)**	0.57	5.01**
	Soil	Zn, Cu ■	1.09 (3.11)**	0.74	14.11***
Anantapur	All	MOS■, Cu■	6.12 (7.87)***	0.77	16.50***
	Leaf	NNLETS□, LEN_LLET■, LLD□, ULC□, LLC■	5.71 (5.51)***	0.9	18.04***
	Fruit/seed	–	–	–	–
	Soil	EC◊, pH, K_2O, MOS, Zn ♦, Mn•, Fe, Cu	58.65 (35.57)**	0.99	868.95**
East Godavari	All	F_DIA•, S_LEN, SD_WT •, ULD, LLC, MUNDL, Cu	4.21 (9.04)	0.96	31.19**
	Leaf	–	–	–	–
	Fruit/seed	–	–	–	–
	Soil	P_2O_5■, pH■, MOS■, Zn•	14.87 (8.06)***	0.87	18.54***
Khammam	All	LEN_LET•, ULD■, Ang LLET•, pH, K_2O, Mn	3.14 (13.69)***	0.93	22.15**
	Leaf	ULD◊, Ang LLET◊	2.51 (5.85)***	0.28	2.75
	Fruit/seed	–	–	–	–
	Soil	EC♦, K_2O◊, MOS♦, Cu◊	2.25 (7.31)***	0.28	2.75

Independent variables were related to dependent variables either positively or negatively: (a) significance at $P > 0.001$ (***) level being +ve (■) or –ve (□); (b) at $P > 0.01$ level (**) being +ve (•) or –ve () and (c) at $P > 0.05$ level (*) being +ve (♦) or (◊).

Table 5. LMRA of independent variables with total azadirachtin-A as dependent variable

District	Parameter group	Significant parameters from parameter group	β-constant	Adjusted R^2	F-statistics
All	All	SD_WT■, ULC, Ang LLET, K $_2$O, Zn □	55.10 (4.32)***	0.31	5.40***
	Leaf	NNLETS	57.78 (4.20)***	0.06	4.10**
	Fruit/Seed	S_DIA•	(–4.89) (–0.30)	0.07	4.51**
	Soil	K_2O◊, Zn, Fe, Cu •	43.34 (5.34)***	0.14	3.03**
Krishna	All	–	–	–	–
	Leaf	LEN_LLET, ULD •, LLD◊, Ang LLET	21.13 (0.73)	0.55	3.78*
	Fruit/seed	–	–	–	–
	Soil	P_2O_5◊, pH•, K_2O, MOS, Z n, Mn •, Cu•	(–595.14) (–5.42)**	0.95	23.50**
Adilabad	All	Cu•	7.11 (0.97)	0.51	10.21**
	Leaf	–	–	–	–
	Fruit/seed	F_LEN•, S_LEN◊, SD_WT◊	111.41 (1.82)	0.43	3.29*
	Soil	P_2O_5□, pH■, K_2O•, MOS□, Zn□, Mn□, Cu■	322.00 (17.55)***	0.99	1160.27***
Anantapur	All	–	–	–	–
	Leaf	LEN_LLET♦, WID_LLET•, ULD, LLD, LLC •, Ang LLET•	(–418.13) (–2.97)*	0.42	2.09
	Fruit/seed	–	–	–	–
	Soil	K_2O, pH □, MOS, Fe ◊, Cu	1090.60 (5.45)***	0.85	11.19**
East Godavari	All	F_DIA♦, S_LEN, SD_WT •, ULD, LLD •, LLC♦, MNDL, Cu ♦	47.36 (21.00)**	0.99	1031.88**
	Leaf	NNLETS, LEN_LLET •, ULD, LLC , MNDL ◊	62.26 (1.31)	0.69	5.02*
	Fruit/seed	–	–	–	–
	Soil	EC□, pH□, P_2O_5□, K_2O, Zn □, Mn□, Fe■, Cu■	238.36 (293.53)***	1	60111.91***
Khammam	All	F_LEN■, LLC•, K_2O□, Mn◊	(–25.83) (–1.82)	0.88	17.64***
	Leaf	–	–	–	–
	Fruit/seed	F_LEN•	(–28.39) (–1.39)	0.47	8.89**
	Soil	Fe♦, pH♦	(–88.60) (–1.45)	0.19	2.05

Independent variables were related to dependent variables either positively or negatively: (a) significance at $P > 0.001$ (***) level being +ve (■) or –ve (□); (b) at $P > 0.01$ level (**) being +ve (•) or –ve () and (c) at $P > 0.05$ level (*) being +ve (♦) or (◊).

In general, the best specification yielded the highest values for the adjusted R^2 and F-statistics, the latter with a significance value at the *** level ($P > 0.001$). In addition, the β-constant values for all the best specifications reflected a strong positive relationship (with significance value at *** level ($P > 0.001$) in general) with both the dependent variables (azadirachtin-A density and total azadirachtin-A; Table 4).

In order to determine the hierarchy of the parameters that were considered significant in LMRA, a tabulation (Table 6) was first made that indicated the number of times each independent variable was found related either at the *** or ** or * level to the dependent variables, azadirachtin-A density or total azadirachtin-A. Each variable was scored out of a total of 12 possibilities (six being from the 'all parameter group' and six from 'the individual parameter group', with all the accessions from all the districts together and accessions from each of the districts individually (Tables 4 and 5).

Potassium oxide was considered the most significant parameter by LMRA that was common to both the dependent variables, based on this scoring system (Table 6). This element was found related to azadirachtin-A density negatively in six combinations (Tables 4 and 6), and found negatively related to total azadirachtin-A in five combinations (Tables 5 and 6) and it could be represented as K_2O (11–, 2+). In contrast, LLC, which was found to show a cumulative positive relationship to both the dependent variables, exhibited four positive and three negative interactions with azadirachtin-A density and total azadirachtin-A together and could be represented as LLC (4+, 3–). These two parameters represent the two ends of the spectrum for the variables found significant in LMRA. Encompassed between these two parameters are: Zn (8–, 2+), ULD (5–, 4+), pH (5+, 4–), MOS (5–, 3+) and P_2O_5 (4–, 1+). S_DIA (1– **, or 1+ *) was the only parameter found to be significant in LMRA with azadirachtin-A density alone, with a significant adjusted R^2 value (0.74 and 0.57 as shown in Table 4). Although, S_DIA showed significant relationship with total azadirachtin-A, its adjusted R^2 value was insignificant (0.07 as shown in Table 5).

DS: Results from DS showed that a positive relationship was recorded between the azadirachtin-A density and the independent variable, pH that was significant in LMRA. However, a negative relationship was recorded between the azadirachtin-A density and K_2O, ULD, LLC, S_DIA, Zn, percentage MOS and P_2O_5. Thus, in general, there was 87.5% concurrence with the LMRA results that were predominantly negative with relation to azadirachtin-A density (Table 7).

Soil colour and texture in relationship to both dependent and independent variables: The impact of qualitative para-

Table 6. Significant independent variables from LMRA with azadirachtin-A density and total azadirachtin-A as dependent variables

		Azadirachtin-A density Group A (from Table 4) I number of times*		Total azadirachtin-A Group A (from Table 5) II number of times*	
Parameter group	Parameter	+	–	+	–
Leaf	NNLLETS	1	1	–	2
	LEN_LLET	2	–	2	1
	WID_LLET	–	–	1	–
	ULD	3	2	1	3
	LLD	1	1	1	2
	ULC	1	2	–	1
	LLC	1	2	3	1
	MUNDL	–	2	–	2
	Ang LLET	2	2	–	3
Fruit/seed	SD_WT	1	–	2	1
	F_LEN	2	–	2	–
	F_DIA	1	1	1	–
	S_LEN	–	4	–	2
	S_DIA	1	1	1	–
Soil	pH	2	2	3	2
	EC	2	1	–	1
	MOS	3	2	–	3
	P_2O_5	1	1	–	3
	Zn	2	3	–	5
	Mn	2	1	1	3
	Fe	1	2	2	2
	Cu	5	3	6	1
	K_2O	–	6	2	5

*Number of times the parameter appeared in LMRA with any of the dependent variables in Tables 4 and 5.

Table 7. Comprehensive representation of relationship of significant variables of LMRA with azadirachtin-A density and their concurrence with descriptive independent statistics, soil colour and soil texture from accessions growing in five different ecogeographical regions

Significant parameters from LMRA	Relationship of significant independent variables with azadirachtin-A density			
	LMRA	Descriptive statistics (DS)	Soil colour	Soil texture
LLC	+	–	–	–
ULD	–	–	+	–
S_DIA	–	–	+	–
K_2O	–	–	–	–
Zn	–	–	–	+
MOS	–	–	–	–
PH	+	+	+	+
P_2O_5	–	–	–	–
% concurrence	N/A	87.50	62.50	62.50

The % concurrence of each of the three tools (DS, soil colour and soil texture) compared with LMRA individually was 87.5, 62.5 and 62.5 respectively.
The % concurrence across the board compared with all the four tools (LMRA, DS, soil colour and soil texture) was 50% (four out of eight parameters exhibited the same results with each of the four tools).

meters such as soil colour and texture on distribution of neem accessions indicated quantitative variations in both the dependent variables such as azadirachtin-A density and total azadirachtin-A and also on several independent variables such as those found significant in LMRA (K_2O, Zn, P_2O_5 and S_DIA being negatively significant and MOS, ULD, LLC, and pH, being positively significant; Table 7).

Out of the 50 accessions studied from the five regions, those represented in light reddish-brown soils (13 out of 50) and dark brown soils (26 out of 50) accounted for

80% of the total accessions. Their corresponding textures (especially those including accessions from the extremely-arid-alkaline and the delta-wet regions) predominantly included silty clay/sandy loam (24 out of 50 and 15 out of 50) and clayey (6 out of 50) soils respectively, which encompassed 90% of the total ecological regions.

In essence, with specific reference to the location of the maximum number of accessions from the two regions of interest that formed the ends of the spectrum, especially in terms of azadirachtin-A density, accessions from extremely-arid-alkaline regions thrived in light reddish-brown soils with silty clay texture, whereas in contrast, accessions from delta-wet soils thrived in dark brown soils with clayey texture (Table 2).

Not only was the average azadirachtin-A density value the highest in extremely-arid-alkaline regions, but even the individual accession value for azadirachtin-A density in this region was the highest (2.75 mg/g). It showed a significant disparity with the corresponding highest value of the accession from the delta-wet region (2.1 mg/g) that incidentally also had the lowest average azadirachtin-A density value (Table 2).

The highest values and highest accession numbers for the parameters that were found significant in LMRA were compared between the accessions from extremely-arid-alkaline regions versus those from delta-wet regions. The maximum values for these parameters and also the maximum number of accessions from extremely-arid-alkaline soils were found in light reddish-brown and silty clay-textured soils. Thus, soil colour and texture in which these accessions were found, were the same as those exhibited by accessions having the highest individual azadirachtin-A density value (2.75 mg/g). In contrast, the delta-wet region showed the highest values and highest number of accessions for each of the above parameters in soils that were dark brown in colour and clayey in texture. Soil colour and texture in which these accessions were found, were also the same as those exhibited by accessions having the highest individual azadirachtin-A density value (2.1 mg/g).

The highest values for percentage MOS, S_DIA and pH represented by an accession from the extremely-arid-alkaline region were 0.0032, 0.76 cm and 8.4 respectively, whereas the corresponding highest values of an accession from delta-wet region were 0.0085, 0.80 cm and 8.25 respectively (raw data not shown).

On investigating the relationship of the eight independent variables that were significant in LMRA, with azadirachtin-A density that was obtained from accessions growing in soils with various colours, the following results were observed. ULD, S_DIA and pH were positively related to azadirachtin-A density. However, LLC, K_2O, Zn, MOS and P_2O_5 were negatively related to azadirachtin-A density (Table 7).

Furthermore, the relationship of the significant independent variables from LMRA with azadirachtin-A density that was obtained from accessions growing in soils of various textures gave the following results: S_DIA, Zn and pH were positively related to azadirachtin-A density. However, LLC, ULD, K_2O, MOS and P_2O_5 were negatively related to azadirachtin-A density (Table 7).

DMRT: In this study, DMRT was utilized to observe if there was any significant disparity in values between accessions from any two regions (among the five ecological regions) for any of the 25 ecomorphological parameters studied.

DMRT indicated definite morphological differentiation among all the ecogeographically different regions. As shown in Table 8, 48% of the total parameters studied differed significantly between the extremely-arid-alkaline and delta-wet regions, followed by 32% difference between extremely arid-alkaline and arid regions, and also

Table 8. DMRT for percentage variations between the areas in the number of significant independent variables in LMRA from all samples of the five regions under study

Ecogeographical region	Parameters significantly different between two regions	No. of parameters: Differing	No. of parameters: Total studied	Percentage of parameters significantly different between ecogeographical regions
Semi-arid and semi-arid/arid	F_DIA	1	25	4
Semi-arid and extremely-arid alkaline	MUNDL, Fe, NNLLETS, Ang LLET	4	25	16
Semi-arid and delta-wet	S_WT, Ang LLET, LLC, F_LEN, S_LEN, ULC, Cu, P_2O_5	8	25	32
Semi-arid and arid	F_LEN, S_LEN, F_DIA, Cu, P_2O_5, pH	6	25	24
Semi-arid/arid and extremely-arid-alkaline	NNLLETS, K_2O	2	25	8
Semi-arid/arid and delta wet	S_WT, MUNDL, LLC, ULC, Cu, K_2O, P_2O_5	7	25	28
Semi-arid/arid and arid	ULC, pH, Cu	3	25	12
Extremely-arid alkaline and delta-wet	S_WT, MUNDL, LLC, S_LEN, Cu, P_2O_5, pH, NNLETS, Ang LLET, AZA DEN, Fe, MOS	12	25	48
Extremely-arid alkaline–arid	F_LEN, S_LEN, Fe, Ang LLET, pH, Cu, P_2O_5, K_2O	8	25	32
Delta wet-arid	MUNDL, F_DIA, K_2O, pH	4	25	16

between semi-arid/arid and delta-wet regions, and with 28% difference between semi-arid/arid and delta-wet regions. The remaining eight combinations showed disparity within each of the paired regions ranging from 4 to 24%.

Furthermore, among the ten combinations, each constituted two areas individually for scoring. Out of each pair of regions that were examined for all the 25 variables under study, the extremely-arid-alkaline and delta-wet region combination alone showed azadirachtin-A values to be significantly different. Of interest is the fact that where there were differences of 48 and 32%, it included the parameters S_LEN, Cu and P_2O_5. These parameters were also significant in LMRA studies (Table 8).

Discussion

The present investigation involves the selection of elite neem trees with high levels of the potent biopesticide azadirachtin-A using ecomorphometric characters as qualitative markers to reflect quantitative variations in this tetranortriterpenoid, mainly using statistical tools.

Habitat-related variations in azadirachtin-A content

Quantitative variations of the potent biopesticide azadirachtin-A in the seed kernels of neem are attributed not only to the genetic aspects, but also to the ecological and geographical factors[18]. Among countries like Myanmar, Thailand and India, neem ecotypes found in India showed the highest azadirachtin-A content[9]. Furthermore, among the various states in India, Andhra Pradesh showed the highest azadirachtin-A values, with Anantapur District representing elite trees with maximum value of this biopesticide[4,9,19].

Our present study involved the choice of five different ecogeographical regions. The range encompassed from extremely-arid-alkaline, semi-arid, semi-arid/arid, arid to delta-wet regions. They included a total of 50 accessions from these five regions. A wide range of neem trees was recorded in terms of azadirachtin-A density, 100 seed weight and total azadirachtin-A. Furthermore, between the accessions studied from the extremely-arid-alkaline and delta-wet regions which formed the two ends of the spectrum, a significant difference in the average values of the above three parameters was observed. This included their respective increase by 92.2, 22.9 and 38.06% respectively.

From our earlier findings in the two micropopulations within the extremely-arid-alkaline region of Puttaparthi, Andhra Pradesh, a direct relationship between soil pH and azadirachtin-A density was suggested (manuscript under preparation). Similar reports on neem trees studied from six districts in West Bengal with a varying range of soil pH (5.25 to 6.43), suggested a correlation with azadirachtin-A content. Essentially, trees with high azadirachtin-A density (2%) thrived best in soils with highest pH (6.43) among the areas studied. Although our findings among the five districts of Andhra Pradesh also exhibited highest azadirachtin-A content (2.75 mg/g) from trees grown in extremely-arid-alkaline soils with highest soil pH (8.05), the results varied from those observed in West Bengal. As shown by Roy and Ghosh (pers. commun.), new alluvial soils of Burdwan and Nadia bore trees that yielded highest azadirachtin-A. These soils were of acidic pH (6.35–6.43). This was in contrast to the trees grown in alkaline soils (pH 8.05) in Andhra Pradesh that exhibited maximum azadirachtin-A in their seed kernels (present study). Nevertheless, in both the studies, areas exhibiting highest soil pH have trees which yield maximum azadirachtin-A. It is quite plausible that if accessions from West Bengal soils with higher pH ranges were chosen, it would probably yield higher azadirachtin-A-yielding trees than those reported (pers. commun.).

Selection of ecomorphological biomarkers using statistical tools

In general, the significant independent variables showed stronger negative relationship than a positive one, with the two dependent variables (66.2 vs 33.8%; Table 6, I, II). Among all the negative correlations available, the relationship of pH of soil with Mn and Fe suggested that with an increase in 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[20,21]. Also, studies regarding morphological characters such as S_DIA have been proven to show variations in these components in neem[10,11]. Based on the results obtained from the present study and also from our earlier work on two micropopulations from the extremely-arid-alkaline region, the biomarkers S_DIA and P_2O_5 were commonly and significantly related to azadirachtin-A density. Thus these markers can be used to study the variation in the dependent variable.

DMRT studies showed that the maximum number of ecomorphological parameters varying between two regions that include extremely-arid-alkaline and delta-wet regions, amounted to 48%. These two regions are farthest apart, thus accounting for the role of geographical factors. However, only 16% of the variables was significantly different between the two regions of extremely-arid-alkaline and semi-arid soil, although they are equally far apart. This three-fold difference (48 versus 16%) between the two scenarios could be attributed to the role of soil pH, percentage soil moisture, colour and texture of soils. Clayey soils of the delta-wet regions with their higher water-retention capability versus silty clay soils of the semi-arid regions with greater water-percolating ability, could influence this threefold increase. There are similar instances where the impact of separation by geographical distances is reflected on the size and form of *Lutzomyia quinquefer*[22]. This relation suggests that the amount of morphological divergence could be predicted by the level of geographic isolation, without the need to infer from climatic or ecological differences[22].

The disparity in DMRT values varied among the remaining combinations (eight) ranging from 4 to 24%. These variations in ecomorphological parameters could predominantly be due to ecological factors alone because they are not separated by significant geographical isolation.

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

Received 29 December 2004; revised accepted 6 April 2006