Regional and habitat variability in azadirachtin content of Indian neem (*Azadirachta indica* A. Jusieu)

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Azadirachtin content in the seeds of neem (*Azadirachta indica* A. Jusieu) collected from different regions of India was studied. The concentration of azadirachtin varied from 200 to 16,000 ppm (μg/g of the seed kernel). Azadirachtin content was found to be affected by climate and habitat. Annual variation in azadirachtin content was significant. The highest azadirachtin content was recorded in the neem tree populations growing in the southern part of India.

Keywords: Azadirachtin, neem, regional and habitat variability, seed kernel.

NEEM (*Azadirachta indica* A. Jusieu) is an evergreen, multipurpose tree found in the Indian subcontinent and Southeast Asian countries. Every part of the tree is useful in one way or the other[1]. The seed is an important source of vegetable oil and biopesticidal compounds. Neem seed kernel consists of azadirachtin[2,3] and other limonoids such as nimbin, salanin[4] and meliantriol[5,6]. However, azadirachtin is the most important limonoid and is known to possess antifeedant, attractant, repellant[7–11], growth disrupting[12–15] and larvicidal properties[16,17] against a large number of pests.

It is essential to understand the regional and habitat variations in azadirachtin content of neem trees for identification of region-specific elite trees and also to understand the factors affecting the synthesis of azadirachtin. Limited studies carried out on azadirachtin variation suggest that azadirachtin content is influenced by climatic conditions. Ermel *et al.*[18,19] assessed, for the first time, the wide variability of azadirachtin content in neem seeds of different countries and found that the highest yield of azadirachtin content per seed kernel is not restricted to a specific country but is distributed in single trees of different origin. Few reports are available on azadirachtin content variation in different ecotypes and provinces of India[20–23] and Australia[24]. However, all these studies were carried out on a small number of samples and not on the basis of extensive surveys.

A National Network on Integrated Development of Neem involving 11 institutions located in different parts of India was launched by the National Oilseeds and Vegetable Oil Development Board, Gurgaon, Ministry of Agriculture, Government of India in 1999 on 'Integrated Development of Neem'. In the first phase of the project, i.e. from 1999 to 2003, one of the objectives of the network was the systematic collection and characterization of neem germplasm from almost every part of India. Chemical evaluation of 1501 accessions collected from different parts of India (Figure 1) under this network was done to assess the extent of diversity existing in India for identification of neem trees yielding high azadirachtin/oil content and variable fatty acid profile, which can be utilized for selection of elite trees for plantation and genetic improvement programmes. This article presents the findings of the study for azadirachtin variability observed in germplasm growing in different regions and habitats of India.

Experimental details

General

Estimation of azadirachtin was done using a Waters LC Module I quaternary automated liquid chromatograph equipped with an autoinjector, high-sensitivity tunable UV and photodiode array detectors, and Novapak RP-18

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column (3.9 mm × 150 mm). The chromatograms and data were acquired and processed with the Waters Millennium 2010 Chromatography Manager version 2.1 software. HPLC-grade acetonitrile was procured from Merck (India). Ethanol was obtained by distilling the spirit. Azadirachtin standard (96%) was procured from Trifolio-M (Germany). Samples were prepared in Borosil screw-capped centrifuge tubes (15 ml). A thermostatic serological water bath was used for heating the samples. A REMI Revolutionary Research Centrifuge (Model R-23) which could accommodate 36 tubes was used for centrifugation of the samples. The samples were then filtered through Swinnex polypropylene 25-mm filter holders (Millipore) containing Durapore 0.22 µm filters. Azadirachtin samples were filtered into Waters autosampler vials (4 ml). The mobile phase for HPLC was filtered through a Millipore sample clarification kit fitted with Durapore 0.45 µm, 47 mm filters (Millipore). Separation of azadirachtin was achieved using acetonitrile : water (40 : 60) @ 1 ml min^{-1} and the peaks monitored at 214 nm.

Collection of neem seeds

Neem seeds were collected from 1501 candidate plus trees selected from 12 states of India (Figure 1). The seeds were collected during 1999–2001. Fully ripe, yellow fruits were collected directly from branches of individual trees. Fruits were depulped manually and washed thoroughly with clean water to remove traces of pulp from the seed coat. The depulped and washed seeds were dried in shade before packing them in cotton bags. Seed samples of individual trees were packed with an identity tag in muslin bags. One set of the seeds was sent to the National Bureau of Plant Genetic Resources, New Delhi for cryopreservation and another to The Energy and Resources Institute (TERI), New Delhi for chemical evaluation.

Figure 1. Map of India representing the extent of collection from different parts as shaded area (figures report number of samples).

Chemical analysis

Azadirachtin content of neem seeds was determined according to the method standardized in TERI's laboratory[25]. One gram of seed kernel powder was taken in a 15 ml centrifuge tube. Distilled ethanol (6 ml) was added to each tube. The tubes were screw-capped and left overnight in the solvent. The tubes were then centrifuged at 5000 rpm for 10 min. The supernatant was transferred into a new tube and the residue was re-extracted twice with ethanol (2 × 6 ml). The pooled extracts were combined and the final volume was made up to 25 ml in a volumetric flask. A part of this sample (4 ml) was filtered into an autosampler vial through a 0.22 µm membrane in a Swinnex filter holder. The vials were then tightly capped. The sample (10 µl) was injected into the HPLC using an autoinjector. Separation of azadirachtin was achieved on NOVAPAK RP-18 column (3.9 mm × 150 mm) using acetonitrile : water (40 : 60) @ 1 ml/min and the peaks were monitored at 214 nm. On-line degassing was done with helium using an on-line degasser. Azadirachtin content was estimated using calibration curves. A standard solution of azadirachtin (1000 µg/ml) was prepared by dissolving 10 mg of the compound in 10 ml of HPLC-grade acetonitrile. Serial dilutions were made in the range of 100–10 µg/ml to plot the calibration curve. The standard solutions were stored at –20°C. The value of azadirachtin content was calculated based on calibration and expressed as ppm (µg/g of the kernel weight).

Statistical analysis

The azadirachtin sample was clustered into different groups on the basis of geographical region of collection, climatic conditions prevailing in the collection site, year of collection, and growth periods available for the tree for analysing these results statistically. Data were analysed by employing one-way ANOVA and Duncan Multiple Range Test (DMRT) at 5% significance level.

Results and discussion

Regional variation

Azadirachtin content in the seeds collected during 1999–2001 from the different states of India (Figure 1) revealed large, overall variations ranging from 200 to 16,000 ppm

(μg/g) of the seed kernel. Such a wide variability is expected due to the environmental, genotypic and interaction of genotype and environment effect. Average azadirachtin content of these accessions was 3043 ppm (μg/g of the seed kernel). About 27 of the 1500 samples recorded more than 10,000 ppm (μg/g) of azadirachtin content, which was well above the national average of 3043 ppm (μg/g of the seed kernel). A majority of these samples were from the Deccan Plateau region. State-wise compilation of the average azadirachtin content for all the three years of collection reveals that the southern peninsular states, viz. Tamil Nadu, Karnataka and Andhra Pradesh have comparatively higher yields of azadirachtin than the other states (Figure 2).

Based on the broad physico-geographical regions, climate and soil type, all the states from where the neem seeds were collected, can be grouped into four broad categories. The first group comprises of Punjab (PUNJ), Delhi (DEL) and Uttar Pradesh (UP), which lie predominantly in the Indo-Gangetic region of the Northern Plains and have alluvium-type of soil. The second group comprising Haryana (HAR), Rajasthan (RJ) and Gujarat (GUJ), lies in Western Plains and Kutch Peninsula with hot arid climate. The third group comprising Orissa (ORIS) and Madhya Pradesh (MP), lies predominantly in Eastern Ghats and Central Highlands having hot subhumid climate and red loamy soil. The fourth group comprising Maharashtra (MH), Karnataka (KART), Andhra Pradesh (AP) and Tamil Nadu (TN), lies in Deccan Plateau with hot semiarid climate and red black to red loamy soil.

The hierarchical cluster diagram (Figure 3) clearly demonstrates the effect of climatic conditions on azadirachtin content. Cluster analysis divides the states into two distinct groups for azadirachtin content. States in the Deccan Plateau form one group, while the rest form another group. Punjab, Orissa, Delhi and Uttar Pradesh clustered as close groups. These states lie predominantly in the Northern Plain, except Orissa which is situated in the Eastern Ghats. States positioned in the Western Plains, viz. Rajasthan and Haryana, segregated together in the dendogram, while Gujarat stands separately. Thus further supporting the finding that geographical locations are important for azadirachtin content. Thus, on the basis of average azadirachtin content we can conclude that neem trees growing in states in the Deccan Plateau region yield higher azadirachtin content compared to other states.

In order to see the distribution of azadirachtin content within the samples obtained from each state, frequency histograms with cumulative percentage were prepared (Figure 4 *a–l*). These histograms further reveal the effect of climate and geographical location on the distribution of azadirachtin content. In Rajasthan, Haryana and Gujarat, nearly 70% of the sample were in the range <1000–2000 ppm azadirachtin content, while nearly 20% were in the range 2000–4000 ppm and only 10% of samples recorded >4000 ppm. These states have characteristically desert type of climate.

The distribution was only comparatively better in Delhi, Punjab and Uttar Pradesh, where 50% of the samples were in the range <1000–2000 ppm, 30% in the range of 2000–4000 ppm, while only 20% recorded >4000 ppm azadirachtin content. Thus, these states were slightly better than Rajasthan, Haryana and Gujarat in terms of number of samples yielding azadirachtin content more than 2000 ppm.

States in the Deccan Plateau demonstrated their supremacy with 50–70% of samples recording >4000 ppm of azadirachtin against 10–20% for the rest of the states. Further, nearly 40% of samples from Karnataka and Andhra Pradesh recorded >8000 ppm of azadirachtin, which is a high value. Thus, the pattern obtained with frequency histograms clearly demonstrated the influence of agroclimatic conditions on the synthesis of azadirachtin in neem seed.

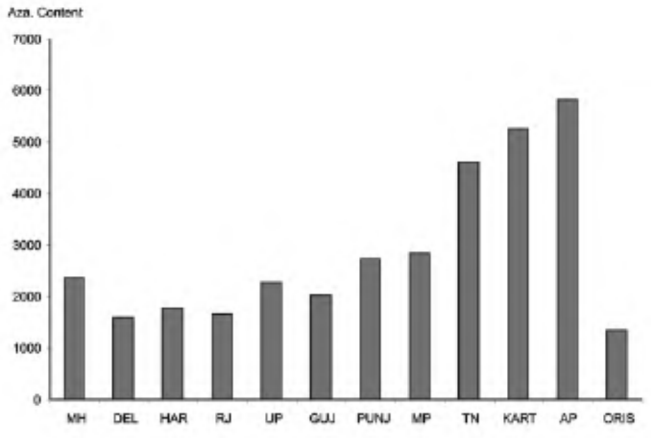

Figure 2. Average azadirachtin content (μg/g of seed kernel) in neem seeds collected from different states of India.

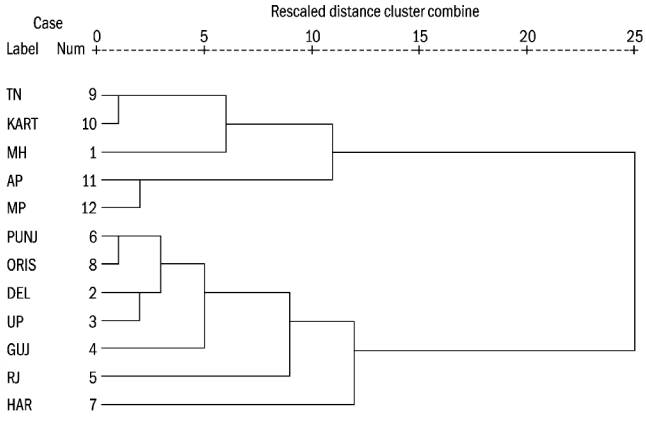

Figure 3. Dendrogram using average linkage (between groups) prepared with average azadirachtin content obtained for different states of India.

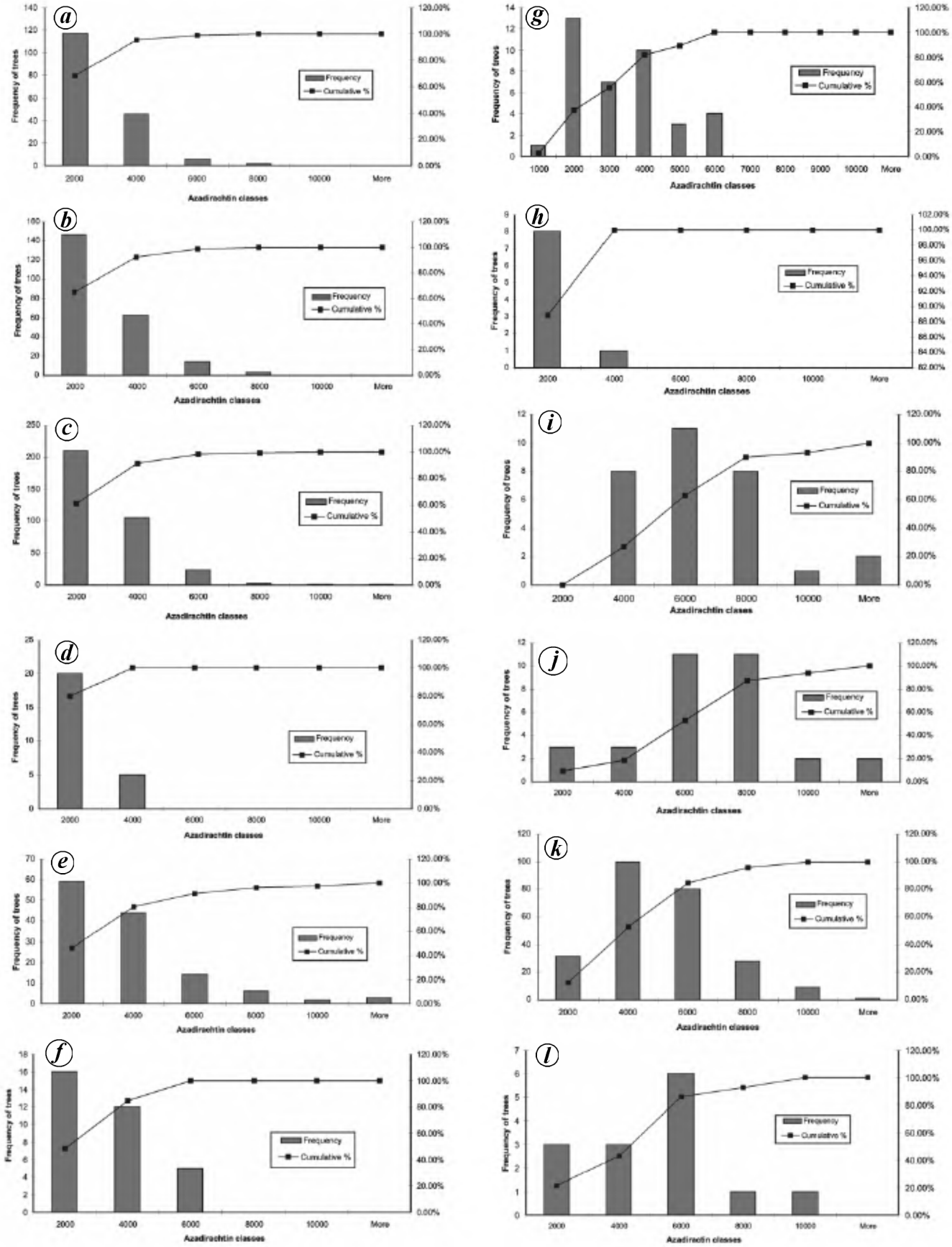

Figure 4. Frequency histogram for azadirachtin content (μg/g of the kernel) for (*a*) Rajasthan, (*b*) Haryana, (*c*) Gujarat, (*d*) Delhi, (*e*) Punjab, (*f*) Uttar Pradesh, (*g*) Madhya Pradesh, (*h*) Orissa, (*i*) Karnataka, (*j*) Andhra Pradesh, (*k*) Tamil Nadu, (*l*) Maharashtra.

Variations due to habitat

On the basis of geographical features, the samples fall into four categories, viz. Indo-Gangetic Plains, Deccan Plateau, the Eastern Ghats and Central Highlands, and Western Plains (desert). ANOVA, performed for ranking azadirachtin content based on geographical features, revealed that azadirachtin content of seeds obtained from neem trees growing in the Deccan plateau ranked first with an average of 4743 ppm, while trees growing in the Indo-Gangetic Plains, Eastern Ghats and Central Highlands ranked second with average azadirachtin content of 2373 and 2427 ppm respectively (Table 1). Trees growing in the desert region recorded the lowest azadirachtin content with an average of 1656 ppm.

Effect of climatic conditions

Rainfall and temperature are the two critical factors affecting the production of secondary metabolites. Therefore, the effect of climatic conditions and azadirachtin content was studied. The samples could be grouped into four distinct climatic classes: hot semi-arid with mild winter; hot sub-humid, hot arid and hot semi-arid with cold winter. The temperature in northern India, which encompasses hot arid and hot semi-arid with cold winter, remains quite low (15–20°C) during winter, while southern India, which includes hot semi-arid with mild winter and hot sub-humid, enjoys moderate climate, as the temperature is above 20°C during winters. Significant differences were observed in azadirachtin content of samples collected from hot semi-arid with mild winter climate. However, samples from hot sub-humid, hot arid and hot semi-arid with cold winter type of climate were found to be statistically lower in terms of azadirachtin synthesis compared to samples from hot semi-arid with mild winter (Table 2). It is important to mention here that hot semi-arid with mild winter type of climate is prevalent in the states of Deccan Plateau, viz. Tamil Nadu, Andhra Pradesh, Karnataka and Maharashtra. Trees growing in hot semi-arid but with cold winter recorded lower average azadirachtin content. Thus moderate climatic conditions are found to be favourable for azadirachtin synthesis, whereas extreme climatic conditions are found to be unfavourable for getting higher azadirachtin yield.

Table 1. Results of statistical analysis of variations in azadirachtin content due to habitat

Geographical features	Average azadirachtin content ppm (µg/g of the kernel)	DMRT ranking
Indo-Gangetic Plains	2373.1 ± 577.36	b
Desert	1656.90 ± 772.92	c
Central Highlands and Eastern Ghats	2427.36 ± 732.59	b
Deccan Plateau	4743.6 ± 989.93	a

LSD = 709.457; F = 12.359; P = 0.0000.

Effect of growth period

Growth period available for a plant species affects the total photosynthetic output and thus production of secondary metabolites. In neem, flower initiation starts in February–March, flowers bloom in April and fruits ripen in July–August. In order to study the effect of growth period on azadirachtin content, the samples were divided into three classes on the basis of growth period with <90, 90–150 and 150–180 days of growth period, and data for these classes were analysed statistically. It is evident from statistical analysis (Table 3) that synthesis of azadirachtin was significantly high in trees growing during 90–150 days of growth period. This was closely followed by 150–180 days of growth period, while shorter growth period (<90) was found to be least favourable for optimum production of azadirachtin. Thus 90–150 days growth period was found optimum for synthesis of azadirachtin. This indicates that shorter growth periods are not favourable for yielding high azadirachtin content.

Annual variations

Since the seeds were collected over a period of three years, it is imperative to study the annual variation of average azadirachtin content for different states. The average azadirachtin content for the years 2000 and 2001 is given in Figure 5. In some states there was significant increase

Table 2. Results of statistical analysis of the effect of the climatic conditions on azadirachtin content

Climatic conditions	Average azadirachtin content (µg/g of the kernel)	SD	DMRT ranking
Hot semi-arid with mild winter	4132.56	657.76	a
Hot sub-humid	2627.04	870.58	b
Hot arid	1902.49	981.82	b
Hot semi-arid with cold winter	2686.64	1187.87	b

F = 9.024; P = 0.001; LSD = 891.978.

Table 3. Results of statistical analysis of the effect of growth period on azadirachtin content

Growing days	Average azadirachtin content (µg/g of the kernel)	DMRT ranking
<90	1576 ± 1056.40	b
90–150	3164.9 ± 1446.25	a
150–180	2376.9 ± 890.15	ab

LSD = 1059.56; F = 4.729; P = 0.0174.

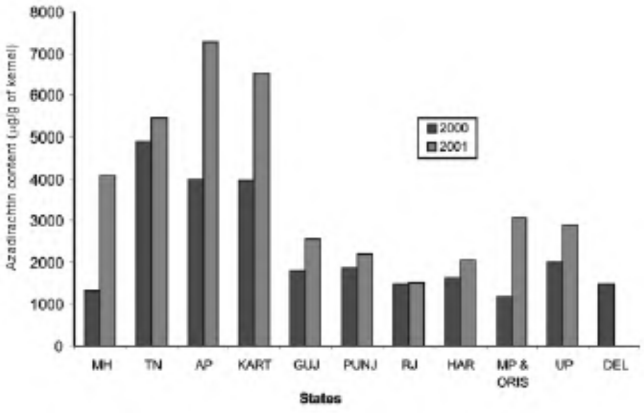

Figure 5. Annual variations in average azadirachtin content in neem trees growing in different states of India.

in azadirachtin content in 2001 compared to 2000. States in Deccan Plateau region, however, continued to yield high azadirachtin in comparison to other states during both the years.

Production of secondary metabolite is governed by both genetic and environmental factors[26]. Few studies have been carried out in India and abroad prior to this study to find the variability of azadirachtin content in neem trees. Ermel *et al.*[19] assessed the wide variability of azadirachtin content in neem seeds of different countries and found that the highest yield of azadirachtin per seed kernel is not restricted to a specific country, but is distributed in single trees of different origin. In a study carried out in India, neem ecotypes showed varying azadirachtin content (0.14–1.66%)[20]. However, both the studies were based on few samples collected from different regions. In region-specific studies, large variations in the azadirachtin content (2895–7525 ppm) have been reported in neem kernels of different ecotypes in Tamil Nadu[23]. However, frequency of distribution was not mentioned. Azadirachtin levels in seeds from plants grown in six ecotypes of Northern Australia ranged from 0.35 to 0.89% of the dried kernel[24]. Studies on azadirachtin content in seeds from trees growing in different parts of Australia[24] and from Andhra Pradesh, India[27,28] also indicate annual variations.

The present findings are based on extensive analysis carried out on a large number of samples collected from different eco-regions of India. A small proportion of samples recorded distinctly high azadirachtin content, i.e. more than 1% from different regions. Trees yielding high azadirachtin content are extremely valuable for plantation programmes. The study further reveals that climatic and geographical conditions in the Deccan Plateau region, India are most suitable for greater synthesis of azadirachtin in neem trees.

India has a production potential of 660,000 tonnes of neem seeds[29]. Looking at the growing demand for biopesticides in integrated pest management, and organic mode of agriculture, more neem trees should be grown as block plantations or as agroforestry component in farmers' fields to increase the availability of seeds. On an average, one tree yields 30–50 kg seeds/yr. Its productivity can be further increased by selecting superior genotypes. Therefore, the present findings are useful in identifying neem trees with high azadirachtin content to make the plant economically more attractive, and also to characterize and catalogue the existing gene pool of neem trees growing in different states of India for identification of elite genotypes at the national as well as regional/state levels.

This variability is important not only for better utilization of this resource, but also to study diversity. High azadirachtin-bearing trees need to be conserved and multiplied for better utilization. Performance of these trees over a longer period of time, and under different agroclimatic conditions by multilocation trials will be evaluated in the second phase of this study.

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