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Neem (*Azadirachta indica* A. Jusieu) Biodiversity in India for Bioresource: Azadirachtin - An Important Biopesticide

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Abstract : *Azadirachta indica* A. Juss., or neem, is a multipurpose tropical tree belonging to the family Meliaceae. The species is of commercial importance, primarily due to its medicinal and biopesticidal properties. With this view studies were conducted to assess the biodiversity in neem for Azadirachtin in Gujarat as well as in other states of India.

Three hundred and sixty seven seed samples collected, from four different agro-ecological zones of Gujarat state of India, were assessed for the azadirachtin content. These studies included a general survey on the region and on annual average variation in azadirachtin level in three consecutive years (2000, 2001, and 2002). More than hundred trees were selected for azadirachtin content in each year. Individual trees exhibited tremendous variation in their azadirachtin content and it ranged from 142 ppm to 9527 ppm (µg/g of the kernel). The data were analysed by clustering the observations on the basis of agro-ecological zones, year of collection, and girth classes. Significant differences in mean azadirachtin content were observed in different zones as per ANOVA analysis at 5% level. Zone AER 5B recorded significantly higher azadirachtin contents as compared to other three zones. Highly significant results were also observed on collection year basis. Present investigations also revealed that average azadirachtin content is not significantly influenced by age of the tree.

Azadirachtin content in the seeds of neem collected from different regions of India was also studied. The concentration of azadirachtin varied from 200 to 16,000 ppm (mg/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.

Key words : Agro-ecological zones, Girth-class, Seed, Tetranortriterpenoid, Variability

Introduction

In India, Neem (*Azadirachta indica* A. Juss) has long been recognized for its multifarious properties ranging from pharmaceuticals, pesticidal to religious purposes. However, it gained tremendous importance at the global level after identification of its pesticidal property against locust, by Late Dr. Pradhan in 1960s, and further after characterization of 'azadirachtin' by – Zanno *et al*. (1975) – as an active principle present in the Neem seed kernel. Azadirachtin (C_{35} H_{44} O_{16}) a tetranortriterpenoid, has been rated as the most potent naturally occurring insecticide (Schroeder and Nakanishi, 1987) among all the limonoids found in Neem seed kernel. It is found in different part of the Neem tree. But it is concentrated in seed kernel of mature fruits (Schmutterer, 1981). Various studies have been undertaken on bioefficacy on Neem seed extracts on more than 400 insect pests (Schmutterer and Singh, 1995). Studies have also been carried out on the azadirachtin variation in trees growing in different climatic conditions (Ermel *et al.*, 1984 and 1986; Rengasamy *et al,.* 1993; Gupta *et al.,* 1998; Bally *et al.,* 1996; Kumar *et al.*, 1995). Ermel *et al.*, 1986 assessed the wide variability of azadirachtin contents 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. Azadirachtin content variation has also been reported in different ecotypes and provenances (Rengasamy *et al.,* 1993; Gupta *et al.*, 1998; Sidhu *et al*., 2003). The azadirachtin content was found to be affected by climate, soil type, and altitude. Till now, very little has been published on azadirachtin variation in seeds growing in different agro climatic zones in India. Moreover, all these studies have been carried on a limited number of samples, and not

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on the basis of extensive surveys. It is highly essential to understand the geographical variations in seeds, growing in different parts, for identification of region specific plus trees. It is further important to know the range of azadirachtin variation and the possible factors causing this variation, if one is interested in selection and clonal propagation of high azadirachtin containing planting stock.

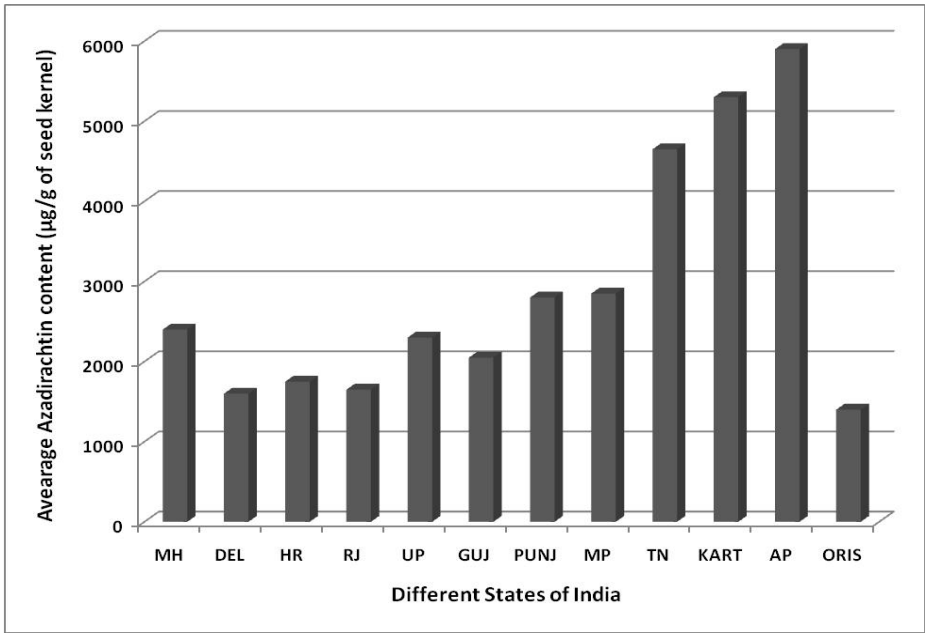
A network on 'Integrated Development of Neem' was created by NOVOD Board (National Oilseeds and Vegetable Oils Development Board), Ministry of Agriculture, India in 1999 for collection, conservation, phonological and chemical evaluation, and mass propagation of Neem trees in India. Under this network, seeds collected from different states of India were evaluated for their chemical constituents. These studies revealed Azadirachtin content in the seeds of neem collected from different regions of India varied from 200 to 16,000 ppm (mg/g of the seed kernel). Azadirachtin content was found to be affected by climate and habitat. Annual variation in azadirachtin content was also significant (Kaushik *et al.*, 2007).

Present paper is a part of the study carried out under above network programme to screen large number of neem seed samples for azadirachtin content from different states of India and a detailed study carried out with the samples collected from different agroclimatic zones of Gujarat.

Materials and Methods

a) Collection of neem seeds from Different States of India: Neem seeds were collected from 1501 candidate plus trees selected from 12 states of India. The seeds were collected during 1999, 2000 and 2001. Fully ripe yellow fruits were collected directly from branches of individual trees. Fruits were depulped manually by hand and washed thoroughly with clean water to remove the 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. Seed samples were collected and supplied to TERI by different Institutes/Organizations working under Neem Network Project sponsored by NOVOD.

b) Seed Collection from five agro-ecological zones of Gujarat: Gujarat is divided into five agro-ecological zones (AER 2, 4, 5, 6, and 19) as shown in map of Gujarat (Figure 2). Since zone AER 5 is a large area and it is further divided into two zones (AER 5A and AER 5B) on the basis different climatic conditions, brief characteristics of these zones are given in Table 1 (along with the characteristics of the districts belonging to these zones). A general survey of Neem trees growing in different agroclimatic zones of Gujarat was carried out for identification of candidate trees on the basis of morphological characters. Seed samples were collected from these individual candidate trees, growing in four



Source: Kaushik et al., 2007

Figure 1: Avearge Azadiractin contents recorded in the samples collected from different states of India

Table 1: Agro-ecological zones of Gujarat state

S.No	Agro-Ecoregion	Characteristics
1	AER- 2	Hot, arid ecoregion with desert and saline soils
2	AER- 4	Hot semi-arid ecoregion with alluvium-derived Soils
3	AER- 5	Hot semi-arid ecoregion with medium, deep black soils
4	AER- 6	Hot semi-arid ecoregion with shallow and medium black soils.
5	AER- 19	Hot humid ecoregion with red, lateritic and laterite-derived soils

** ARE 6 and AER 19 are relatively very small areas and Neem plantations are rarely seen, therefore, no collection from this area could be done due to non–availability of neem plantations.*

different agro climatic zones, in last week of June for three consecutive years 2000, 2001, and 2002. Since it was difficult to know the actual age of the trees, their GBH (girth at breast height) were measured to get an idea about the variation due to age of a tree. A total of 367 trees belonging to AER 2, AER 4, AER 5A, and AER 5B were collected for assessment of azadirachtin content in their seed kernel. Based on the GBH, the age of the trees ranged from 10 years to 60 years.

c) Equipment and material: Azadirachtin estimation was performed using a Waters LC Module I Quaternary Automated Liquid Chromatograph equipped with autoinjector, high-sensitivity tunable UV and photodiode array detectors, and Novapak RP-18 column (3.9 mm x 150 mm). The chromatograms and data were acquired and processed with the Waters Millennium 2010 Chromatography Manager version 2.1 software. The photodiode array spectrum was recorded on a Waters 996 Photodiode Array Detector. HPLC grade acetonitrile was procured from Merck (India). Ethanol was obtained by distilling spirit. Azadirachtin standard (96 %) was procured from Trifolio-M (Germany). The 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. Samples were filtered through Swinnex polypropylene 25-mm filter holders (Millipore, USA) 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, USA).

d) Azadirachtin analysis: Azadiracthin content of the neem seeds was determined as per the method standardized in TERI's laboratory (Kaushik 2002). One gram of seed kernel powder was taken in 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 minutes. The supernatant was transferred into new tube and the residue was re-extracted twice with ethanol (2x6 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. The

separation of azadirachtin was achieved on NOVAPAK RP-18 column (3.9 mm x 150 mm) using acetonitrile–water (40:60) @1 mL/ min and the peaks were monitored at 214 nm. Online degassing was done with helium by using an online 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 were calculated based on the calibration and are expressed as ppm (µg/g of the kernel weight).

e) Statistical analysis: The azadirachtin sample was clustered into different groups on basis of year of collection, agro–ecological zone, and tree girth for analysing these results statistically. The data was analysed by employing one way ANOVA and Duncan Multiple Range Test (DMRT) at 5% significance level.

Results

Present studies were divided into two parts. First part of studies covers only on average Azadirachtin variation in different states of India. Second part of the studies focused on Gujarat states with an idea to find out some other factors such as year, agro-climatic zones and stem girth classes (indirect way of studying age effect) affecting azadirachtin contents.

A. Studies covering different states of India

Azadiractin variations in different states of India: The azadirachtin content in the seeds collected during 1999, 2000 and 2001 from different states of India (Fig. 1) revealed large, overall variations ranging from 200 to 16000 ppm (µg/g) of the seed kernel. Such type of wide variability is expected due to the genotypic effect. Average azadirachtin content of all these accessions was 3043 ppm (µg/g) of the seed kernel. About 27 samples of 1500 samples recorded more than 10000 ppm 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 Deccan plateau regions. The state– wise compilation of the average azadirachtin content for all the three years collection reveals that the southern peninsular states viz. Tamil Nadu, Karnataka and Andhra Pradesh have comparatively higher yields of azadirachtin as compared to other states (Figure 1).

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 five broad groups. First group comprising Punjab (PUNJ), Delhi (DEL) and Uttar Pradesh (UP), lie predominantly in the Indo-Gangetic regions of Northern Plains having alluvium type of soil. The second group consisting of Harayana (HAR), Rajasthan (RJ) and Gujarat (GUJ), lies in Western Plains and with Kutch Peninsula with hot arid climate. The third group having Orissa (ORIS) and Madhya Pradesh (MP), lies predominantly in Eastern Ghats and Central Highlands having hot sub-humid climate and red loamy soil. The fourth group consisting of Maharasthra (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.

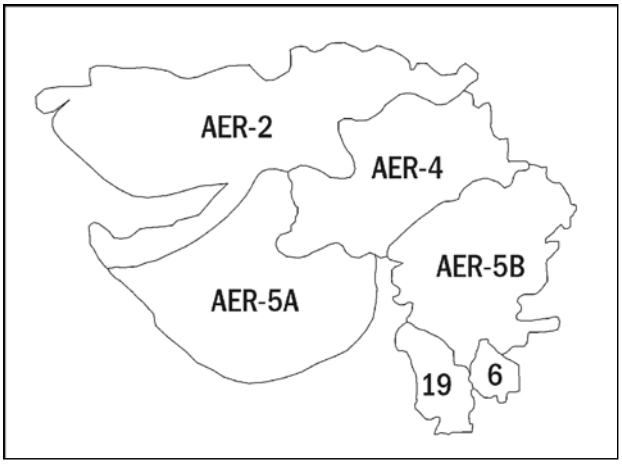


Figure 2: Agro - ecological zones of Gujarat

B. Detailed studies of Gujarat State

Variations within Gujarat: A large variation was recorded in azadirachtin level in 367 seed samples collected from different agro–climatic zones of Gujarat. The azadirachtin content ranged from 142 ppm to 9527 ppm (µg/g of the kernel) and an overall average of the whole population was 2426 ppm. In order to view the frequency distribution of trees, they were clustered into ten different classes having an interval of 1000 ppm, ranging from 0–1000 ppm to 9000–10000 ppm. Figure 2 shows the number of tress distributed in different azadirachtin classes as recorded in 2000, 2001, and 2002. This graph clearly indicates that majority of trees fall within a range of 1000 – 3000 ppm level, irrespective of the year of collection. Sixteen trees recorded above 6000 ppm azadirachtin, which is far above the average azadirachtin content recorded for the state.

Variations on the basis of agroclimatic zones of Gujarat: After intensive survey, in collaboration with Gujarat Forest Department, some areas have been

identified in Gujarat for Neem seed collection. Survey was conducted in all five AER zones of the state. However, good plantations are available only in AER 2, AER 4, and AER 5. AER 5 zone is a large area and hence it is divided into two-sub zone AER5A and AER5B (Figure 2). When azadirachtin levels were statistically analysed on the basis of agroclimatic zones, the average azadirachtin level of four agro climatic zones were significantly different at 5% level. Maximum average azadirachtin content –3347 ppm – was recorded in zone AER 5B and minimum – 2037 ppm – was in AER 4 while AER2, AER4, and AER5A were not significantly different for mean azadirachtin levels, as analysed by DMRT at 5% level (Table 2).

Variations due to age: To evaluate the impact of age on biosynthesis of azadirachtin in seed kernel, the data was clustered into six groups on the basis of girth classes as shown in Table 3. No significant differences were observed in all six classes, as analysed by one–way ANOVA and DMRT. Thus all the girth classes were found to be statistically at par. This indicates that age does not play a significant role in azadirachtin synthesis. However, highest mean average of 2813 ppm was recorded in 151–200 cm girth class, which was the middle-aged tree.

Annual variations: The data was also analyzed on the basis of seed samples collected in three different years that is 2000, 2001, and 2002. A frequency curve

Table 2: Annual variations in average azadirachtin content in seed samples collected from Gujarat in three consecutive years

Year	Number of samples	Azadirachtin value in ppm (mg/g of the kernel)	Standard Error
2000	147	1792.16[a]*	70.9
2001	119	2637.20[b]	145.4
2002	101	3098.69[c]	200.8
Total	**367**	**2425.73**	**82.8**

** DMRT ranking at 5% level*

Table 3: Variation in average azadirachtin contents observed in four AER zones of Gujarat

AER zones	Description	Number of sa
AER 2	340 mm, Hot arid ecoregion with desert and saline soils, gray brown deltic alluvium, arid	131
AER 4	735 mm, Hot semi-arid ecoregion with alluvium derived soils, gray brown coastal alluvium, arid semi arid	86
AER 5A	537 mm, Hot semi-arid ecoregion with medium, deep black soils, medium black calcareous, semi arid	128
AER 5B	974 mm, Hot semi-arid ecoregion with medium, deep black soils, deep black coastal alluvium, sub-humid semi arid	22
	Total	367

** DMRT ranking at 5% level*

Table 4: Variations in average azadirachtin contents in seed samples collected from Gujarat on the basis of girth classes

Girth class (cm)	Number of samples	Azadirachtin in ppm (mg/g of the kernel) Mean ± SE*
(50 –140)	103	2499.01 ± 165.2[a]
(101–150)	153	2228.96 ± 121.4[a]
(151–200)	69	2813.46 ± 200.7[a]
(201–250)	23	2297.17 ± 257.5[a]
(251–300)	4	1974.75 ± 474.1[a]
Total	**352**	**2424.13** ±0**84.0**[0]

** DMRT ranking at 5% level*

of Neem tree on the basis of azadirachtin content recorded in three different years is shown in figure 3. The result indicates that azadirachtin levels increased every year, starting from 2000. The mean value of azadirachtin was 1792 ppm in 2000, which increased to 2637 in the next year. It further increased to 3099 ppm in 2002. One way ANOVA analysis indicates that the results are highly significant (Table 4). DMRT test separated all three means to three significantly different classes. It is worth mentioning here that 2000 to 2002 were drought years in Gujarat and in many other parts of India. Thus from these findings it appears that stress conditions resulted in increased azadirachtin levels.

Discussion

Azadirachtin variation between individual trees and between different ecotypes has been studied by many scientists. Ermel *et al.* (1986) observed that individual trees growing in the same environment exhibit significant difference in their azadirachtin level. He also found that highest azadirachtin was not restricted to specific ecotype but it was from single tree from different origins. This was in contrast to an earlier report by Schmutterer and Zebitz (1984) where they found marked differences in yield of azadirachtin in seeds collected from different sources. Our investigation also exhibits that individual genotypes exhibit large variations. 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. Similarly in Gujarat state also It is clearly demonstrated that agro–climatic conditions play an important role in azadirachtin synthesis. AER 5B zone seems to the best area for growing plants for azadirachtin extraction in Gujarat.

A good understanding of azadirachtin production is necessary for establishing viable commercial industry. It is necessary to study in detail, the variations due to season, year and age. Little is published in this regard. Sindu and Behl (1996) and Bally *et al*. (1996) have reported seasonal variation and annual azadirachtin fluctuations. Present investigations also indicate that average annual azadirachtin varies significantly in all three consecutive years. The reasons of fluctuation in azadirachtin content are likely to be due to climatic and nutritional factors. Present investigations clearly indicate that azadirachtin levels are not greatly influenced by the tree age. However, present patterns of azadirachtin levels based on girth classes gives an indication that highest azadirachtin can be extracted from middle girth class trees.

The productivity of neem seeds and azadirachtin content can be increased by selecting high yielding genotypes and growing them in best agro-climatic zones. Present studies are helpful not only for better utilization of this resource, but also to study diversity and conservation of genetic resources for future needs.

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