Assessment of age and morphometric parameters of seeds on azadirachtin production in neem seed kernels collected from various ecotypes

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Abstract

Azadirachtin is one of the most prominent triterpenoids obtained from neem (Azadirachta indica A. Juss.) seed kernels. Its demand has been on rise in industries due to its immediate application as an ecofriendly, biodegradable biopesticide and various other significant biological activities. Concerted efforts are being made for its extraction in higher quantities in an economically feasible way. However, fulfillment of this objective has been long overdue owing to high heterogeneity in quality and quantity of azadirachtin present in the seed kernels. In the present study, an attempt has been made to study the effect of various parameters, like age and morphometric parameter of seeds, on azadirachtin production in neem trees growing in fifteen different ecotypes. It has been observed that the middle age trees (20-40 years old) produced maximum amount of azadirachtin (4000 µg/g dry weight of kernels) inspite of that the highest kernel weight was observed in trees of youngest age group.

Introduction

Azadirachta indica A. Juss. or Neem is an evergreen, multipurpose tropical tree belonging to the family Meliaceae. Each and every part of the tree possesses numerous medicinal and biopesticidal properties. Azadirachtin, a highly oxidized limonoid (triterpenoid) present prominently in neem seed kernels, is mainly responsible for diverse biological activities. It possesses repellant, antifeedant, larvicidal, growth inhibiting properties against a wide range of pests and, thus, has been well recognized as an environment friendly, biodegradable biopesticide. A lot of heterogeneity is prevalent in neem trees which may be attributed to inherent cross breeding nature of the plant. Apart from this, the seeds have a low shelf life and poor viability period of about 2-3 weeks. In this context, it is important to critically assess all the parameters that affect azadirachtin production.

Much has been written about wide variability of azadirachtin content in neem seeds of different countries or different regions within a country. Kumar and Parmar[1] reported azadirachtin content variation in different ecotypes. Ermel[2] and Venkateswarlu et al.[3] found that these variations in azadirachtin content is due to local environmental conditions such as humidity, rainfall, temperature, or season. However, all the individuals of a particular locality have also been shown to possess variation in azadirachtin content which could be due to climate and ecotype interactions ensuing genetically fixed variations. The azadirachtin content of this mosaic population can be studied through genetically based physiological and morphological markers. Hence, the objective of the present study is to evaluate whether the effect of age of neem trees growing in various ecotypes and morphometric parameter of seeds from trees of different age groups could serve as reliable biomarkers to determine the variation in azadirachtin content of seeds.

Materials and Methods

Plant Material and Sample Collection

For analytical studies, healthy, disease free and ripe (yellowish green to yellow) fruits were collected from fifteen trees (10 to 50 years old) growing in different localities, in and around Delhi (Table 1). Based on the age of tree, the seeds were clustered into three groups: I (trees less than 20 years of age), II (between 20 to 40 years of age) and III (40 years and above). For each age group there were five sample trees. As the fruiting in neem coincides with the rainy season, and the fruits falling on the ground are quickly attacked by mould, therefore, the fruits were directly picked from the trees. The freshly collected fruits were depulped manually and washed thoroughly with clean water

to remove traces of pulp from the seed coat. Seeds enclosed in endocarp were dried in shade at ambient temperature to prevent decomposition of azadirachtin. Dried seeds were stored in aerated bags (jute bags).

Preparation of Extract and Azadirachtin Estimation

At the time of azadirachtin estimation, kernels were excised by cracking the endocarp and finely chopped to avoid loss of azadirachtin by crushing. The size of the pieces is indirectly related to the efficacy of product. The chopped kernels were dried overnight in a hot air oven at 35°C. The dried kernels (50 g) from each sample tree were extracted in HPLC grade methanol (S.D. Fine Chemie) in a soxhlet apparatus at 40°C for 12 h. The extract obtained was further concentrated in a rotary evaporator. Subsequently, the concentrated extract (500 mg) was redissolved in 5 ml methanol and an aliquot of 10 µl of this extract was utilized for quantifying azadirachtin using HPLC. A standard Azadirachtin, procured from Sigma Aldrich, USA (95% pure), was prepared by dissolving 0.5 mg of the compound in 500 µl methanol to prepare a stock concentration of 1 mg/ml.

Estimation of azadirachtin was achieved with HPLC equipped with a UV detector, a 10µl injector loop and printer-plotter-cum integrator. A Zorbax ODS C-18 column with dimensions 150 mm x 4.6 mm was used for analyzing the extract in a mobile phase that consisted of methanol: water in 90:10 ratio. A flow rate of 0.5 ml/min was used and eluted samples were detected at 210 nm.

Statistical Analysis

The intra and inter group variability in azadirachtin content and kernel weight per gram seed weight were analyzed by one way ANOVA. At least hundred seeds were collected from each sample tree to study various parameters.

Results and Discussion

Ecotypes/ ecological/ physiological races arise as a result of genotypic response of an ecospecies to a particular habitat. The different ecotypes of a particular species may differ in their edaphic, biotic or microclimatic requirements and the adaptations that occur are genetically fixed[4]. A considerable variability, with respect to azadirachtin content of seed kernels and other seed related parameters occur among neem populations. Although,

little is known about the genetic factors, this broad variability could be due to both climate and ecotype interaction resulting into genetically based morphological variability.

The variations in the shape and size of seeds (enclosed in endocarp) are shown in figure 1**.** Their various morphometric parameters relative to the age groups are listed in table 1. As can be noticed, group III with oldest trees bore the largest seeds (2.06±0.3 cm^2) and those from youngest trees in age group I the smallest (1.81±0.3 cm^2). Further, the larger seeds have high pulp to low kernel weight ratio than their smaller seed counterparts, thus, showing inverse relationship with regard to these two parameters. As far as the amount of azadirachtin is concerned, variation has been observed even within group populations (Table 1, Fig. 2). A deviation from an expected result is observed when average azadirachtin amount is considered among three groups. Normally, it would be anticipated that the group having maximum average kernel weight would contain maximum azadirachtin but in this study, the maximum production of azadirachtin has been found in group II (4000 µg/g dry weight of kernel) which has the second highest average kernel weight, followed by trees of group III (3400 µg/g dry weight of kernel) and group I (2800 µg/g dry weight of kernel) (Fig. 3). This can be attributed to the genetic and biosynthetic efficacy of group II trees, which are in their prime age, this capability declined with age in the older trees while in the youngest cluster it is still in the developing stage. Middle-aged trees appear biosynthetically more potent and specialized in synthesizing azadirachtin than the other two groups. Youngest group exhibited least average azadirachtin production inspite of maximum kernel weight.

Variations in various morphological characteristics of neem, such as variability in seed length, seed width, seed weight, growing in different agro-climatic conditions has been reported by several workers[1,5]. According to Anand et. al.[6] there are instances where density of azadirachtin was found to be the same between two accessions but their 100 seed weight was differing significantly. Therefore, seed weight becomes an important parameter that needs evaluation before selection of neem trees for commercial and industrial purposes. Particularly, the weight of kernel present in seeds is an important parameter for determining the azadirachtin content as total azadirachtin is dependent on both azadirachtin density and hundred seed weight.

The results of one way ANOVA are presented in tables 2 and 3. The analysis revealed no significant differences in azadirachtin density and kernel weight within and between the three age groups ($P>0.05$ in all the cases). The study shows that apart from age, other factors like genetic constitution of trees play a pivotal role in determining morphometric parameters and chemical composition of the individual trees. In an extensive study Singh et al.[7]

observed large genetic diversity in neem trees collected from different regions of India. They reported an average of 69.8% polymorphism with each AFLP fingerprint, indicating a high marker index. Moreover, the authors noticed that no two accessions shared a similar DNA profile, indicating that it is possible to uniquely fingerprint all the genotypes. AFLP analysis further revealed that the genetic similarity in neem varied from a high of 93% (0.93) to a low of 46% (0.46) within the 41 neem genotypes. Such a wide range in similarity coefficient values suggests that the neem germplasm collection represents a genetically diverse population. One of the major causative factors to high degree of polymorphism observed in neem may be due to its evolutionary status as an out-crossing angiosperm.

Conclusions

The present study revealed the effect of age of trees and morphometric parameters of seeds from different ecotypes on azadirachtin production. From the results it can be concluded that as more than one feature contributes to variability in neem populations, no single factor can be pointed that is responsible for variability in azadirachtin content in different neem accessions.

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Table 1: Variations in morphometric parameters of fifteen neem trees studied from different localities

Group	Sampling Sites**	Size of Seed (cm^2)*	Kernel weight/ gram seed	Pulp weight/ gram seed	No. of Seeds/ gram	% Azadirachtin
I (<20 Years)	**1**	1.77±0.2	0.23±0.02	0.35±0.01	0.81±0.2	0.40
	2	1.35±0.2	0.20±0.01	0.40±0.02	1.01±0.2	0.18
	3	1.60±0.2	0.20±0.02	0.40±0.02	0.92±0.1	0.25
	4	2.28±0.2	0.30±0.02	0.25±0.02	0.65±0.1	0.23
	5	2.04±0.2	0.10±0.02	0.50±0.02	0.63±0.1	0.35
	GROUP I AVERAGE	**1.81±0.3**	**0.21±0.07**	**0.38±0.09**	**0.8±0.2**	**0.28±0.09**
II (20 to <40 Years)	**6**	1.75±0.2	0.35±0.01	0.36±0.02	0.80±0.2	0.50
	7	2.80±0.2	0.20±0.01	0.25±0.02	0.42±0.1	0.56
	8	1.80±0.1	0.10±0.02	0.50±0.02	0.61±0.1	0.12
	9	1.92±0.2	0.15±0.01	0.45±0.01	0.52±0.1	0.40
	10	1.90±0.2	0.20±0.02	0.40±0.02	0.81±0.2	0.42
	GROUP II AVERAGE	**2.03±0.4**	**0.20±0.09**	**0.39±0.1**	**0.63±0.2**	**0.4±0.17**
III (>40 Years)	**11**	2.47±0.2	0.15±0.02	0.45±0.02	0.63±0.1	0.30
	12	1.44±0.1	0.25±0.01	0.35±0.01	1.05±0.2	0.35
	13	2.21±0.2	0.15±0.01	0.45±0.02	0.52±0.1	0.29
	14	2.20±0.2	0.15±0.02	0.35±0.02	0.65±0.1	0.42
	15	1.96±0.2	0.20±0.02	0.40±0.02	0.79±0.2	0.36
	GROUP III AVERAGE	**2.06±0.3**	**0.18±0.04**	**0.40±0.05**	**0.73±0.2**	**0.34±0.05**

** **1- New Delhi Ridge; 2- Dhaula Kuan; 3- Noida; 4- Patparganj; 5- Shahdara; 6- Alipur; 7- Delhi Cantonment; 8- Delhi Gate; 9- Faridabad Border; 10- Old Delhi Ridge; 11- Mall Road; 12- Hauz Khas; 13- Okhla; 14- Indian Agricultural Research Institute; 15- Hinden River**

* **Seed= Kernel + Endocarp**

Table 2: One-Way ANOVA of Variability in Kernel-weight per gram Seed in Intra and Inter Age Groups

Summary

Groups	Count	Sum	Average	Variance
I	5	12.82	2.564	0.23373
II	5	12.54	2.508	0.33837
III	5	12.09	2.418	0.08552

ANOVA

Source of Variation	SS	*df*	MS	F	P-value	F crit
Between Groups	0.054253	2	0.027127	0.123749	0.884714	3.885294
Within Groups	2.63048	12	0.219207			
Total	2.684733	14				

Table 3: One-Way ANOVA of Variability in Azadirachtin content in Intra and Inter Age Groups

Summary

Groups	Count	Sum	Average	Variance
I	5	15.07	3.014	0.23808
II	5	17.68	3.536	0.81638
III	5	16.78	3.356	0.06453

ANOVA

Source of Variation	SS	*df*	MS	F	P-value	F crit
Between Groups	0.70308	2	0.35154	0.942475	0.416698	3.885294
Within Groups	4.47596	12	0.372997			
Total	5.17904	14				

SS: Sum of Squares; *df*: Degree of Freedom; MS: Mean Square

Figure Legends

Figure 1: Morphological variations in the neem seeds clustered into three age groups.

Figure 2: Graphical representation of variation in percentage azadirachtin content in the seeds of different age groups.

Figure 3: Graphical representation of comparative variation in average kernel weight per gram seed and average azadirachtin content (µg) per gram DW of kernels in three age groups.

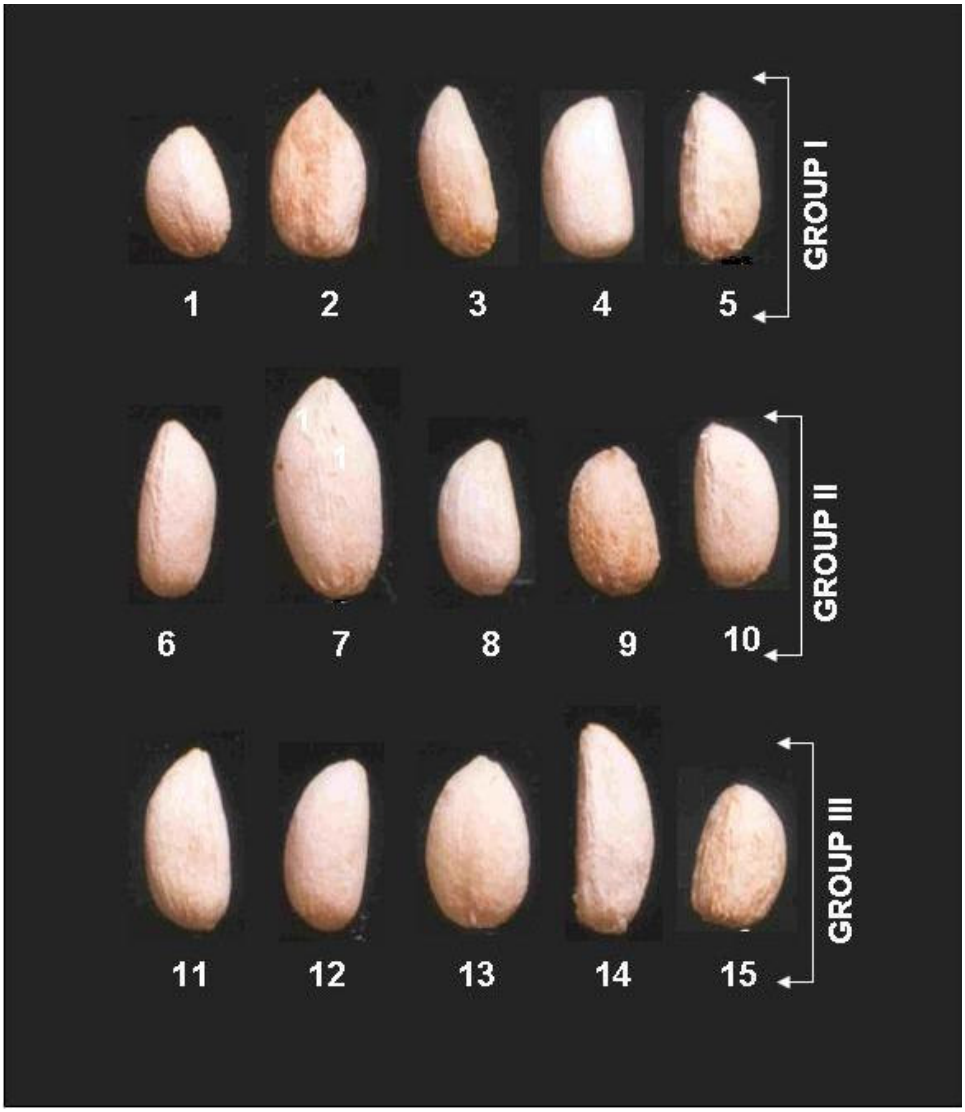

Fig. 1

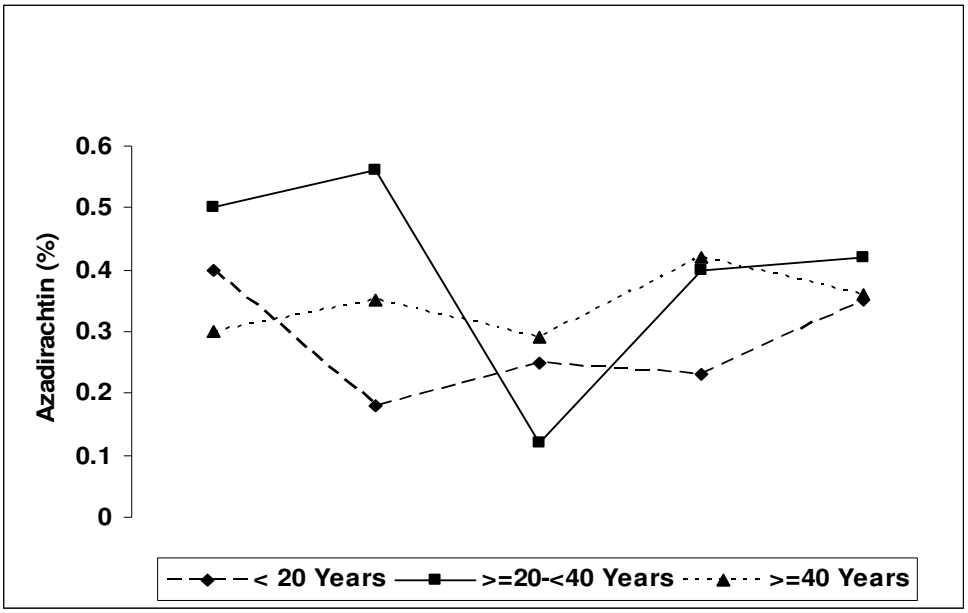
0.6
0.5
0.4
0.3
0.2
0.1
0
Azadirachtin (%)
< 20 Years
>=20-<40 Years
>=40 Years

Fig. 2

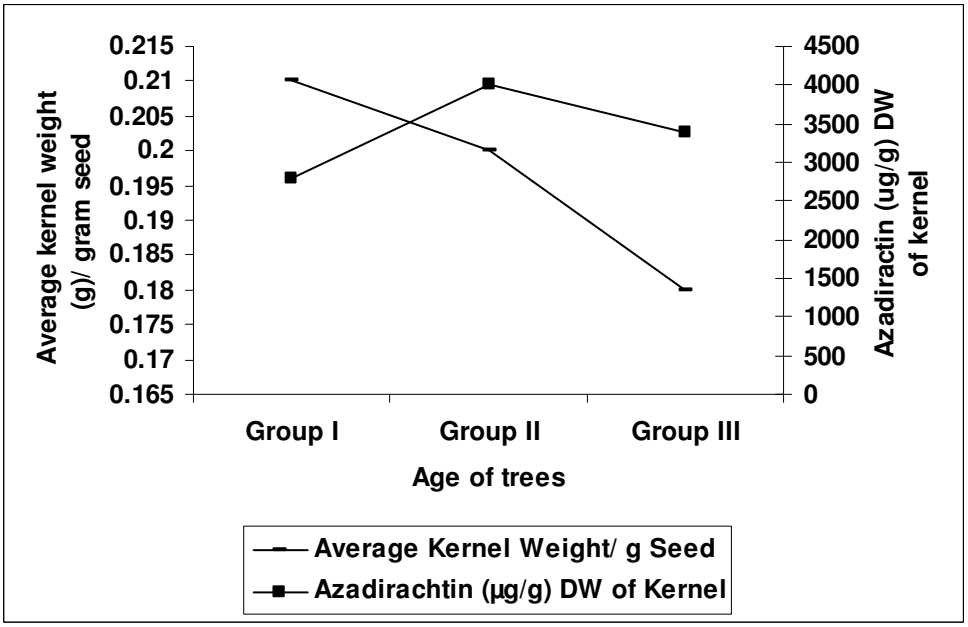
0.215
0.21
0.205
0.2
0.195
0.19
0.185
0.18
0.175
0.17
0.165
Average kernel weight (g)/ gram seed
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Azadiractin (ug/g) DW of kernel
Group I
Group II
Group III
Age of trees
Average Kernel Weight/ g Seed
Azadirachtin (µg/g) DW of Kernel

Fig. 3