Variation of Azadirachtin Content during Growth and Storage of Neem (*Azadirachta indica*) Seeds

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Variation of azadirachtin content during the growth and storage of neem seeds has been studied by reversed-phase high-performance liquid chromatography using anisole as internal standard. Samples were collected every fortnight from the initial stage of buds to ripened fruit stage and analyzed using an efficient sample preparation protocol developed for this purpose. Results indicated that azadirachtin appears only after the 9th week, gradually reaches the maximum of 0.38 ± 0.06% (w/w, on a dry weight basis) around the 17th week, and decreases to 0.29 ± 0.03% (w/w) by the 19th week. Thus, the fruits can be profitably harvested in the 17th week of development (when the neem fruit turns from green to yellow) for better yield of azadirachtin. Application of the method for storage studies of neem seeds indicated that azadirachtin was unstable under normal conditions of storage of the seeds, with azadirachtin content reducing to about 68% of the original level in a period of 4 months in the dark and to 55% in daylight.

Keywords: *Azadirachtin; anisole; internal standard; neem seeds; Azadirachta indica; quantitation; sample preparation; reversed-phase high-performance liquid chromatography*

INTRODUCTION

The neem tree (*Azadirachta indica* A. Juss) has fascinated scientists from various fields of research as its secondary metabolites find use in agriculture, medicine, cosmetics, etc. (National Research Council, 1992). The isolation of azadirachtin (Figure 1) from neem seeds (Butterworth and Morgan, 1968) has gained worldwide attention in view of the insect-antifeedant and growth-inhibiting properties of the compound (Jacobson, 1989). The neem tree, which yields about 30–40 kg/year of neem seeds, is widely distributed in South Asia, Southeast Asia, and other tropical areas. Azadirachtin, a tetranortriterpenoid (limonoid), present in neem seeds to the extent of 0.2–0.6% (Govindachari et al., 1992), is highly potent at low concentrations against over 200 agricultural pests (Mordue and Blackwell, 1993) and is ecofriendly (Stark, 1992). These properties of azadirachtin make it a viable alternative to the toxic synthetic pesticides. Azadirachtin occurs in neem seeds along with several other limonoids, most of which are inactive as pest control agents. Its solubility, stability, and safety are expected to be superior if azadirachtin could be obtained in a purer form. Extensive world effort is therefore directed toward extraction, separation, and purification of azadirachtin from the neem seeds, which are the only source of the compound. The economics of the various processes and the purity of the product naturally depend on the initial concentration of azadirachtin in the neem seeds. The azadirachtin content of neem is found to vary depending on the geographical origin of the seed (Ermel et al., 1984). Azadirachtin was found to be maximum in seeds from India (0.35%) and Togo (0.4%) compared to seeds from Sudan (0.19%) and Niger (0.15%) (Ermel et al., 1986), indicating the involvement of genetic and environmental factors of different provenances and tree variants. Further, azadirachtin content in neem seeds is influenced by temperature, humidity, and UV light (Ermel et al., 1986). The seeds that fall on the ground are exposed to sunlight, which could lead to a decrease in the azadirachtin content of neem seeds. Moreover, collection of neem seeds is normally from the ground, and the seeds are usually processed much later; no data are available on the change of azadirachtin content during storage. The above factors prompted us to investigate the variation of azadirachtin during growth of the neem seed to determine the best period for harvesting the neem fruits and the methods of storage for better yields of azadirachtin.

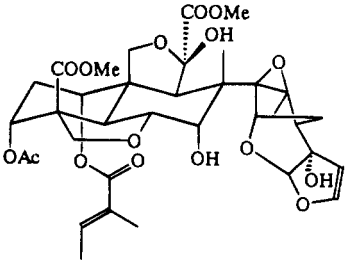

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Figure 1. Structure of azadirachtin.

In this paper, we report a detailed investigation on the variation of azadirachtin content during growth of neem seeds with a statistical approach to minimize the variations among trees. We also discuss an efficient sample preparation protocol and the change in azadirachtin content of neem seeds under normal conditions of storage.

MATERIALS AND METHODS

Plant Material and Sampling. Neem tissue from the stage of buds to ripened fruits was collected every fortnight from week 1 to week 19 (March to July) from Bellal village near Bangalore, India. Ten trees (approximate age 20–25 years) were selected at random, and samples were collected in polythene bags. Soon after the collection, the samples were stored at −70 °C; for analysis, they were thawed to room temperature. Samples from each tree were divided into five portions and subjected to sample preparation and analysis.

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Equipment and Chemicals. The HPLC system consisted of two 510 reciprocating pumps, a U6K loop injector, a 484 tunable wavelength absorbance detector at 217 nm, and a Maxima 820 FC system controller and data processor, all from Waters Associates, Milford, MA; C_{18} Sep-Pak cartridges were also obtained from Waters Associates. A sonicator (VC-600 Model; 60 W; 20 kHz; dual output) was procured from Sonics and Materials, Inc., Danbury, CT. The Remi centrifuge (Model R23) was from Remi Sales and Co., India, and the Milli Q water purification system was from Millipore, Bedford, MA.

Anisole from SD Fine Chemicals, India, was used without further purification. Solvents for extraction and HPLC were obtained from E. Merck (India) and were distilled before use. Water for HPLC was purified using the Milli Q water purification system.

Sample Preparation. A stock solution of anisole was prepared in methanol to obtain a concentration of 20 mg/L of anisole (S-1).

Approximately 5 g of tissue was crushed using a pestle and motar and transferred to a beaker (exact weight of the tissue transferred was noted); 12.5 mL of S-1 was added and sonicated for 30 min (50% duty cycle, 5 A). The temperature during sonication was maintained between 25 and 30 °C using a cold water bath. The sample was centrifuged for 10 min at 4000 rpm (g = 1254). The centrifugate was collected in a 25 mL volumetric flask, and the residue was again mixed with 8 mL of methanol and centrifuged. The centrifugates were pooled in the 25 mL volumetric flask and made up to the mark with 90% aqueous methanol. The extract was filtered, and 1 mL of the filtrate was passed through a C_{18} Sep-Pak cartridge. The Sep-Pak cartridge was eluted with 90% aqueous methanol, and the combined eluant was made up to 5 mL in a volumetric flask. The injection volume was 10 μL (internal standard, 20 ng).

Storage Studies. For storage studies, neem seeds were analyzed for azadirachtin content and the seeds were stored in polythene bags at room temperature (20–35 °C), one set in the dark and the other in daylight. They were again analyzed for azadirachtin after 1 and 4 months. Sample preparation seeds (10 g) were extracted with 80% aqueous methanol (25 mL), and the centrifugates were pooled and extracted with n-hexane (3 × 50 mL) to remove oil. The aqueous methanol layer was collected in a 50 mL volumetric flask and made up to the mark with 80% aqueous methanol. One milliliter of sample solution and 1 mL of anisole solution (containing 200 μg of anisole in methanol) were mixed and passed through a C_{18} Sep-Pak cartridge. The Sep-Pak was eluted with 5 mL of 80% aqueous methanol, and the combined eluant was made up to 10 mL in a volumetric flask. Ten microliters was injected onto the HPLC column. The values reported are the mean of three samples in each case.

HPLC Analysis. The mobile phase system used for analysis was acetonitrile–water (40:60), and the flow rate was 1 mL/min for a run time of 5 min; a gradient to 100% acetonitrile for 5 min at 1 mL/min was used to clean the column. A Waters Novapak C_{18} column (4.6 mm × 15 cm, packed with 4 μm of octadecylsilica) was used for the analysis. The detector response (217 nm) was recorded using a Waters 820 FC data processor. The column was regenerated (Snyder and Dolan, 1989) after every 50 samples, and the efficiency of the column was checked by determining plate count using naphthalene (n = 14 000) as described earlier (Shobha and Ravindranath, 1991).

Dry Weight of Neem Tissue. Approximately 5 g of tissue (buds, flowers, and fruits) was taken in a Petri dish and the weight of tissue noted. The Petri dish was then placed in an oven at 110 °C and dried to constant weight (8 h).

RESULTS AND DISCUSSION

Quantitation of the azadirachtin using anisole as internal standard gives accurate and consistent results, as elaborated elsewhere (Thejavathi et al., 1995). Using this method, it has now been possible to follow the variation of azadirachtin content during growth and storage, very useful information in view of the importance of the compound as a valuable biopesticide. Ten mature trees (20–25 years old) were selected at one farm to minimize changes due to age and geographical and climatic variation, and samples were collected fortnightly from week 1 to week 19, after which time, the ripened fruits fell from the tree. Five samples from each tree were collected by random selection to minimize variation due to sunshine and shade, giving rise to 500 samples for analysis. The moisture content of the tissues was determined by drying a portion of the tissues at 110 °C to constant weight, and the azadirachtin content was determined on a dry weight basis using the method noted above (expressed as % w/w).

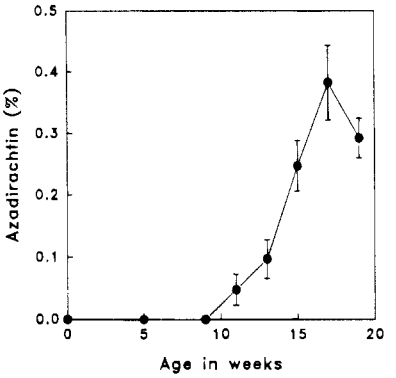

Figure 2. Variation of azadirachtin during growth of neem seeds.

In view of the large number of samples, a rapid and reliable method of sample preparation needed to be developed. Due to the high water content of the tissues, it was necessary to use a water-miscible solvent. Taking advantage of our earlier finding that azadirachtin is stable to sonication in methanol, we developed a sample preparation protocol as described under Materials and Methods. Use of 90% methanol helped removal of lipids by solid-phase extraction (Hull et al., 1993). A known quantity of anisole was added to the tissues at the beginning of the protocol to minimize losses due to extraction and sample cleanup procedures. The stability of azadirachtin and anisole to the sample preparation protocol has been established by parallel experiments and by monitoring the relative ratios of the analyte and internal standard in the mixtures, before and after the sample preparation regime.

The azadirachtin content of samples from each tree and each fortnight was calculated, and the mean and standard deviation of the azadirachtin content among the 10 selected trees were determined. Values beyond the mean ± the standard deviation were rejected to give a confidence level of 95%. The variation of the azadirachtin content during growth is plotted against the age of the tissue, beginning from budding stage (denoted week 1). Most fruits ripened and dropped from the trees in the 20th week, when the analysis was terminated. The results of the above investigation, as summarized in Figure 2, clearly indicated that no azadirachtin is formed in the seed until the 9th week. There is a slow increase in azadirachtin concentration during the third month and rapid accumulation of azadirachtin during the subsequent 1 month period. The azadirachtin content reached a maximum of 0.38 ± 0.06% (dry weight basis) during the 17th week, when the fruits turned from green to yellow, and subsequently fell during the following fortnight to 0.28 ± 0.03%. Investigation of the limonoid composition of the neem seeds during the

second, third, and fourth months may therefore lead to interesting information on the biogenesis of azadirachtin and other limonoids.

Interestingly, the stage at which the green fruits turn yellow is also known to be the period of maximum viability of the neem seed (Maithani et al., 1989). It may be of interest to examine the possible correlation between the azadirachtin content and neem seed viability.

We had also monitored the change in azadirachtin content in the neem seeds under normal conditions of storage, namely, room temperature (20–35 °C) and 50–60% relative humidity. One set of samples was stored in the dark and the other in daylight. The percentage of azadirachtin decreased from the initial 0.22% to 0.20% (about 10% loss) in 1 month and to 0.12% (45% loss) in 4 months in the samples stored in daylight. Even in the samples kept in the dark, the azadirachtin percentage dropped to 0.21 in one month and to 0.15 in 4 months, corresponding to 5% and 32% losses, respectively. It is, therefore, recommended that the fruits be harvested when the color turns from green to yellow and processed as quickly as possible for maximum recovery of azadirachtin.

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LITERATURE CITED

Butterworth, J. H.; Morgan, E. D. Isolation of a substance that suppresses feeding in locusts. *Chem Commun.* **1968**, 23–24.

Ermel, L.; Pahlich, E.; Schmutterer, H. Comparison of the azadirachtin content of neem seeds from ecotypes of Asian and African origin. *Proc. 2nd Int. Neem Conf.* **1984**, 91–94.

Ermel, K.; Pahlich, E.; Schmutterer, H. Azadirachtin content of neem kernels from different geographical locations, and its dependence on temperature, relative humidity, and light. *Proc. 3rd Int. Neem Conf.* **1986**, 171–184.

Govindachari, T. R.; Sandhya, G.; Raj, S. P. G. Azadirachtins H and I: two new tetranortriterpenoids from *Azadirachta indica*. *J. Nat. Prod.* **1992**, *55*, 596–601.

Hull, C. J.; Dutton, W. R.; Switzer, B. S. Quantitation of azadirachtins in insecticidal formulations by high-performance liquid chromatography. *J. Chromatogr.* **1993**, 300–304.

Jacobson, M., Ed. *Focus on Phytochemical Pesticides, Vol. 1, The Neem Tree*; CRC Press: Boca Raton, FL, 1989.

Maithani, G. P.; Bahuguna, V. K.; Rawat, M. M. S.; Sood, O. P. Fruit maturity and interrelated effects of temperature and container on longevity of neem (*Azadirachta indica*) seeds. *Indian For.* **1989**, 89–97.

Mordue, A. J.; Blackwell, A. Azadirachtin: an update. *J. Insect Physiol.* **1993**, *39*, 903–924.

National Research Council. *Neem: A Tree For Solving Global Problems*; National Academy Press: Washington, DC, 1992.

Shobha, S. V.; Ravindranath, B. Supercritical carbon dioxide and solvent extraction of the phenolic lipids of cashew nut (*Anacardium occidentale*) shells. *J. Agric. Food Chem.* **1991**, *39*, 2214–2217.

Snyder, R. L.; Dolan, W. J. *Trouble Shooting LC Systems*; Humana Press: Totowa, NJ, 1989; 448 pp.

Stark, J. D.; Wong, T. T. Y.; Vargas, R. I.; Thalman, R. K. Survival, longevity and reproduction of tephritid parasitoids (Hymenoptera: Braconidae) reared from fruit flies exposed to azadirachtin. *J. Econ. Entomol.* **1992**, *85*, 1125–1129.

Thejavathi, R.; Yakkundi, S. R.; Ravindranath, B. Determination of azadirachtin by reversed-phase high-performance liquid chromatography using anisole as internal standard. *J. Chromatogr.* **1995**, in press.

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