13. Fishwild, D. M., Wu, H. M., Carroll, S. F. and Bernhard, S., *Clin. Exp. Immunol.*, 1994, **97**, 10–18.
14. Masuda, K., Takahashi, K., Hirano, K. and Takagishi, Y., *Tumour Biol.*, 1994, **15**, 175–183.
15. McIntyre, G. D., Scott, C. F. Jr., Ritz, J., Blatter, W. A. and Lambert, J. M., *Bioconjug. Chem.*, 1994, **5**, 88–97.
16. Cathran, D. C., *Nephron*, 1988, **48**, 142–148.
17. Matsukawa, W., Hara, S., Yoshida, F., Suzuki, N., Atsushi, F., Yawaza, Y., Sakamoto, N. and Matsuo, S., *J. Lab. Clin. Med.*, 1992, **119**, 116–123.

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Seasonal variation in azadirachtins in seeds of *Azadirachta indica*

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Neem produces seeds in rainy season. Phenotypes that produced seeds out-of-season in November–December were identified. Seeds from the normal and out-of-season (winter) were screened for qualitative and quantitative variations in azadirachtin content. Monsoon seeds yielded higher (1.53%) azadirachtin-rich fraction as compared to the winter season seeds (1.26%). Azadirachtin A, B and F were analysed by HPLC using an improved solvent-eluting system for better resolution. Azadirachtin A was the major metabolite in the rainy season seeds, as compared to azadirachtin B and F, however, azadirachtin A and B were in nearly equal proportions in the winter seeds. Concentration of azadirachtin F increased more than two-fold in winter seeds as compared to that of rainy ones. The sum of the peak area of azadirachtins A, B and F was higher in the seeds produced in winter. Winter stress appears to favour synthesis of azadirachtin B and F. Such phenotypes could be a material of choice for clonal multiplication of neem.

AZADIRACHTA INDICA A. Juss (Neem) of family Meliaceae is native to the Indian subcontinent. Neem, today, is grown in many Asian countries and in tropical regions of the western hemisphere. Leaves and seeds of this tree have been traditionally used for centuries for treatment of human ailments and to control pests[1]. Azadirachtin ($C_{35}H_{44}O_{16}$), a tetranortriterpenoid from neem kernel has been rated as the most potent naturally occurring insect feeding deterrent[2], and has generated wide academic interest. Neem derivatives affect more than 200 insect and mite species[3], several species of nematodes, fungi including aflatoxin-producing *Aspergillus flavus*[4]. Azadirachtins function as natural insect control agents because these possess both protectant (antifeedant) and toxic (insect growth regulatory) properties[5]. The principal compound for which extensive structural and biological studies have been carried out is azadirachtin A (ref. 6). Rembold[6] isolated six related compounds (azadirachtins B-G) while Govindachari *et al.*[7] reported five compounds (azadirachtins A, B, D, H and I) from neem kernels. Presently, nine azadirachtins have been isolated from neem seed extracts and their structures have been elucidated[8]. Isolation and purification of azadirachtin have been perfected by flash chromatography, normal-phase and reverse-phase preparative and analytical high performance liquid chromatography[7].

Neem produces seeds in India in monsoon months. However, in areas with many neem trees there are always some plants which set flower (and fruits) out of season[9]. Literature survey revealed that there has been no study on variation in azadirachtin content in neem seeds produced in off season. In the present study, we report variation in azadirachtins A, B and F in neem seeds produced in season and the ones out-of-season.

Neem seeds were collected from 10-year-old trees growing at Biomass Research Centre, Banthra of National Botanical Research Institute, Lucknow. Samples were collected from five different trees of Lucknow provenance. Each sample consisted of 100 seeds collected from different branches. Three phenotypes were located in and around Lucknow that produced seeds in winter (November to December). Phenotypes that produced seeds in both seasons were identified and one hundred yellow to greenish-yellow seeds from each of these three trees were collected in July and late November 1995. Powdered neem seeds (10 g) were defatted with hexane at 25°C using a Kinematica PT-MR 6000 Polytron homogenizer and extracted three times with 90% ethanol worked up in a manner similar to that for hexane extraction. The enriched azadirachtin content from the ethanolic extract was isolated following the method of Govindachari *et al.*[10].

Analyses were performed on a Waters liquid chromatograph equipped with a Waters automated gradient controller, a Waters 501 solvent delivery system, a Rheodyne 7125 sample injector fitted with a 20 µl loop, a Waters 484 variable absorbance detector, and a Waters 740 data module. A reverse-phase analytical C_{18} column (3.9 × 300 mm) equipped with a Waters precolumn was used. Azadirachtins were detected at 220 nm. Mobile phase consisted of an isocratic mixture of acetonitrile–methanol–water (23:22:55) at flow rate of 1.0 ml min^{-1}. The results are presented as per cent weight basis, and the same were quantified using

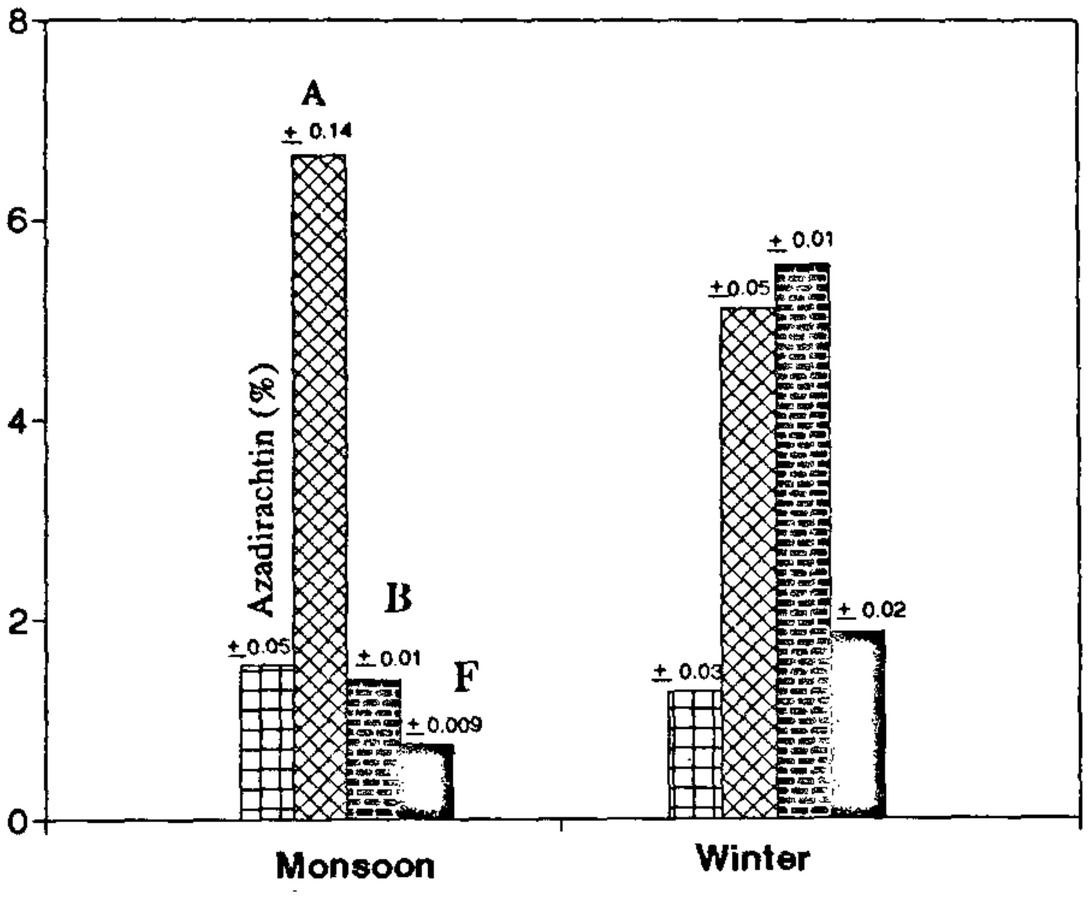

Figure 1. Seasonal variations in azadirachtin (%) and azadirachtins A, B and F mg/10 g of neem seeds.

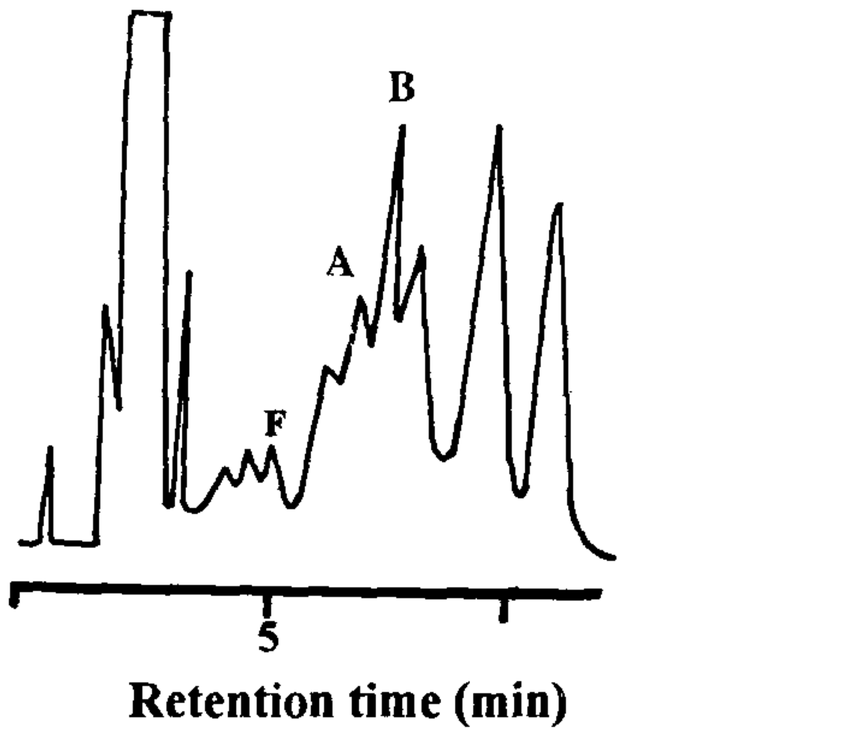

Figure 2. HPLC chromatogram showing separation of azadirachtin A, B and F from neem seeds.

external standards of azadirachtins A, B and F (Trifolio-M GmbH, Lahnau, Germany).

Neem seeds were collected in two different seasons: Monsoon (July) and early winter (November) from the identified phenotypes and screened for qualitative and quantitative variations in azadirachtin content. The monsoon season seeds yielded 1.53% of crude azadirachtin enriched fraction while the winter seeds yielded 1.26% (Figure 1).

Qualitative analysis of azadirachtins A, B and F was made by using reverse-phase analytical HPLC. Chromatographic separation was improved from the earlier reported studies. A combination of acetonitrile–methanol–water (23:22:55 v/v) in an isocratic mixture at flow rate of 1.0 ml min^{-1} gave better resolution than aqueous acetonitrile or aqueous methanol. The peaks of azadirachtins A and B were easily resolved with 7.65 min and 8.37 min retention times respectively. Azadirachtin F appeared at 5.47 min (Figure 2). Yamasaki *et al.*[11] have isolated and purified azadirachtin by normal-phase and reverse-phase HPLC and reported that aqueous acetonitrile achieved better resolution of azadirachtin in shorter time than aqueous methanol while Govindachari *et al.*[7] purified azadirachtins on preparative HPLC and resolved on an analytical HPLC with aqueous methanol. However, we found the proposed solvent improved separation of azadirachtins A and B.

Relative proportions of azadirachtins A, B and F in seeds from two different seasons are shown in Figure 1. Azadirachtin A was the major metabolite as compared to azadirachtins B and F in the rainy season seeds. The concentration of azadirachtin A was 6.6 mg as compared to 1.38 mg of azadirachtin B and 0.75 mg of azadirachtin F per 10 g of dry seeds respectively. On the other hand, azadirachtin A (5.10 mg) was nearly in equal proportion to azadirachtin B (5.56 mg) in the winter seeds. The concentration of azadirachtin F increased more than two-fold in winter seeds (1.86 mg) as compared to the rainy (0.75 mg/10 g) ones. The sum of the peak area of azadirachtins (A, B and F) was higher in the seeds produced in winter months than that of rainy season.

Azadirachtins A and B are the major metabolites in neem oil as compared to azadirachtins C to I (refs. 6, 7). Azadirachtin A has been reported to occur five times more than B, while other azadirachtin isomers (C-G) are only one part each to hundred parts of azadirachtin A (ref. 6). However, we found that there was a significant increase in the synthesis of azadirachtin B and F in winter seeds. Azadirachtins A and B, the main metabolites of neem oil, are well known for their insect antifeedant and growth regulating actions. Azadirachtin F derived from the azadirachtin B structure has similar biological activity as the main compounds such as azadirachtin A and B (ref. 8). Accordingly winter seeds may have better potential for pest management preparations. These phenotypes form excellent base for breeding and for propagating clones.

1. Koul, O., Isman, M. B. and Ketkar, C. M., *Can. J. Bot.*, 1990, **68**, 1–11.
2. Kubo, I., Matsumoto, T. and Matsumoto, A., *Tetrahedron Lett.*, 1984, **25**, 4729.
3. Saxena, R. C., *Global Pesticide Compaigner*, 1994, **4**, 3–5.
4. Abdul, A. K., Saxena, R. C., Boncodin, M. E. M., Krishnamurthy, V. and Seshu, D. V., *J. Econ. Entomol.*, 1989, **82**, 1219–1223.
5. Isman, M. B., Koul, O., Luczynski, A. and Kaminski, J., *J. Agric. Food Chem.*, 1990, **38**, 1406–1411.
6. Rembold, H., in *Economic and Medicinal Plant Research* (eds Wagner, H. and Norman, R.), Academic Press, New York, 1989, vol. 3, pp. 57.

7. Govindachari, T. R., Sandhya, G. and Ganesh, R. S. P., *J. Nat. Prod.*, 1992, **55**, 596–601.
8. Rembold, H. and Puhlmann, I., in *The Neem Tree, Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes* (ed Schmuttere, H.), VCH Verlagsgesellschaft, Weinheim, Federal Republic Germany, 1995, pp. 224.
9. Schmutterer, H., in *The Neem Tree, Source of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes* (ed Schmuttere, H.), VCH Verlagsgesellschaft, Weinheim, Federal Republic Germany, 1995, pp. 28.
10. Govindachari, T. R., Sandhya, G. and Ganesh, R. S. P., *Chromatographia*, 1991, **31**, 303.
11. Yamasaki, R. B., Klocke, J. A., Lee, S. M., Stone, G. A. and Darlington, M. V., *J. Chromatogr.*, 1986, **356**, 220–226.

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Effect of salinity on amino acid composition of the marine fungus *Cirrenalia pygmea*

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***Cirrenalia pygmea*, a mangrove fungus, was grown at various salinities and its amino acid composition determined. Higher salinity led to an increase in the amino acid pool size and the number of amino acids produced. Acidic amino acids were present in higher concentrations at 6.9‰ and 20.7‰ salinities. Gln, His, Thr, Arg and Val were present only when the fungus was exposed to high salinity conditions. The concentration of Gly increased with salinity. Arginine and ornithine were present in low salinity, but in high concentrations at higher salinities. Proline and Dragendorff-positive compounds were absent.**

CIRRENALIA PYGMEA Kohlmeyer, a marine fungus, occurs as a saprophyte on bark and wood of mangrove roots and on calcareous linings of empty shipworm tubes[1]. Although ecological studies on mangrove fungi have been carried out[2], there is little work on the physiology of this group of marine fungi[3]. Our earlier studies on the physiology of *C. pygmea* showed that the fatty acid profile[4] and the melanin content of the hyphae are altered by salinity[5]. Here we report the influence of salinity on the amino acid composition of this fungus.

A single conidial isolate of *C. pygmea* isolated from prop roots of *Rhizophora mucronata* Poiret was grown on cellophane disks[6] overlying malt agar medium (pH 8.0) made up with undiluted, filtered and aged sea water[1] of 34.5‰ salinity or sea water diluted with deionized water. The cultures were incubated at 30°C for 8 days. The free amino acid pool of the mycelium was analysed by HPLC after derivatization with *o*-pthaldialdehyde[7]. The HPLC had an Isco C18 Reverse Phase column and 0.1 M acetic acid buffer and methanol : tetrahydrofuran (97:3 v/v) as solvents in a gradient. The rate of flow was 1.5 ml/min. The detector was Isco V, 9 µl flow cell 7L-2 fluorescence. An excitation filter of 305–395 nm and an emission filter of 430–470 nm were used. The amino acids were identified by comparing their retention time and peak areas with those of standards obtained from Sigma, USA. Dragendorff-positive compounds was tested by thin layer chromatography[8]. The plates were developed in ethanol : ammoniacal solution : water (85:12:13)[9]. Betaine standard was used and visualized with Dragendorff reagent sprays[10].

The amino acid pool size and composition of *C. pygmea* were correlated by salinity (Table 1). The pool size increased from 5.4 to 7 times with the increase in salinity. The number of amino acids increased from 13 to 18 when the fungus was grown on high saline medium. These results were similar to those obtained for *Aphanothece halophytica*, a halophilic cyanobacterium[11]. Gln, His, Thr, Arg and Val were absent in the mycelium grown on 6.9‰ salinity. However, these amino acids were present in high concentrations in the

Table 1. Free amino acid composition (µg/g dry weight) of *C. pygmea* grown in different sea water concentrations

	Salinity (‰)		
Amino acids	6.9	20.7	34.5
Asp	30	650	1410
Glu	680	3940	430
Asn	550	1810	2400
Ser	10	440	440
Gln	nd	2220	70
His	nd	360	720
Gly	190	680	4010
Thr	nd	880	330
Arg	nd	100	1120
Ala	920	3460	300
Tyr	280	2150	3840
Met	980	2520	5370
Val	nd	120	140
Phe	20	60	1000
Ile	260	940	2120
Leu	trace	740	2430
Lys	trace	trace	420
Ornithine	trace	trace	300
Total	3920	21070	26850

nd = not detected; Analysis by HPLC; Figures are mean of 2 values.