بسم الله الرحمن الرحيم

In the Name of Allah the Most Gracious, the Most Merciful.

فَتَبَسَّمَ ضَاحِكًا مِّن قَوْلِهَا وَقَالَ رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ وَعَلَى وَالِدَيَّ وَأَنْ أَعْمَلَ صَالِحًا تَرْضَاهُ وَأَدْخِلْنِي بِرَحْمَتِكَ فِي عِبَادِكَ الصَّالِحِينَ

So he [Sulaiman (Solomon)] smiled, amused at her speech and said: "My Lord! Inspire me and bestow upon me the power and ability that I may be grateful for Your Favours which You have Bestowed on me and my parents, and that I may do righteous good deeds that will please You, and admit me by your mercy among Your righteous slaves."

Part 19, An-Naml, 19

وَوَصَّيْنَا الْإِنسَانَ بِوَالِدَيْهِ إِحْسَانًا حَمَلَتْهُ أُمُّهُ كُرْهًا وَوَضَعَتْهُ كُرْهًا وَحَمْلُهُ وَفِصَالُهُ ثَلَاثُونَ شَهْرًا حَتَّى إِذَا بَلَغَ أَشُدَّهُ وَبَلَغَ أَرْبَعِينَ سَنَةً قَالَ رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ وَعَلَى وَالِدَيَّ وَأَنْ أَعْمَلَ صَالِحًا تَرْضَاهُ وَأَصْلِحْ لِي فِي ذُرِّيَّتِي إِنِّي تُبْتُ إِلَيْكَ وَإِنِّي مِنَ الْمُسْلِمِينَ.

And we have enjoined on man to be dutiful and kind to his parents. His mother bears him with hardship. And she brings him forth with hardship, and the bearing of him, and the weaning of him is thirty months, till when he attains full strength and reaches forty years, he says: "My lord! Grant me the power and ability that I may be grateful for Your Favour which You have bestowed upon me and upon my parents, and that I may do righteous good deeds, such as please You, and make my offspring good. Truly, I have turned to You in repentance, and truly, I am one of the Muslims (submitting to Your Will).

Part 26, Al-Ahqâf, 15.

VARIABILITY IN GROWTH, OIL AND LIMONOIDS IN NEEM (*AZADIRACHTA INDICA* A. JUSS) FROM DIFFERENT ECOZONES IN SUDAN

By

INTISAR ELNOUR ELTERAIFI

B.Sc. (Hons.) Forestry, Khartoum University, 1991
M.Sc. (Forestry), Khartoum University, 1996

A Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Faculty of Agricultural Sciences
Gezira University
2004

DECLARATION

I, **Intisar Elnour Elteraifi,** hereby declare that the work embodied in this thesis is the result of my own investigations during the three years research undertaken under supervision of Gezira University- Sudan and International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, has not been submitted before for any degree in any other university and it is an original work.

Signed .... 10th June 2004

INTISAR ELNOUR ELTERAIFI (Candidate)

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DEDICATION

To My Father

with Intimate Love and Deep Thanks

Intisar

VARIABILITY IN GROWTH, OIL AND LIMONOIDS IN NEEM (AZADIRACHTA INDICA A. JUSS) FROM DIFFERENT ECOZONES IN SUDAN

By

INTISAR ELNOUR ELTERAIFI

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VARIABILITY IN GROWTH, OIL AND LIMONOIDS IN NEEM (*AZADIRACHTA INDICA* A. JUSS) FROM DIFFERENT ECOZONES IN SUDAN

By

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In The Name Of Allah, The Beneficent, The Merciful.

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Intisar Elnour ELTERAIFI

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ABBREVIATIONS

ABBREVIATIONS	Full Name
ARPPIS	African Regional Postgraduate Program in Insect Science
BCED	Behavioral and Chemical Ecology Department
DRP	Director of Research and Partnership
GLM	General linear model
HPLC	High Performance Liquid Chromatography
ICIPE	International Centre of Insect Physiology and Ecology
IGR	Insect growth regulator
SAS	Statistical analysis system
UV	Ultra violet spectroscopy
DNA	Deoxyribonucleic acid
mg	Milligram (s)
mm	Millimeter (s)
RAPD	Random amplified polymorphic DNA
PCR	Polymerase chain reaction
pH	-log hydrogen ion concentration
rpm	Revolutions per minute
v/v	Volume per unit volume
w/v	Weight per unit volume
µ	Micro
BCE	Behavior & chemical ecology

VARIABILITY IN GROWTH, OIL AND LIMONOIDS IN NEEM (*AZADIRACHTA INDICA* A. JUSS) FROM DIFFERENT ECOZONES IN SUDAN.

BY: INTISAR ELNOUR ELTERAIFI

ABSTRACT

The main objective of this study was to generate requisite information, which would provide the basis of exploiting the neem tree (*Azadirachta indica* A. Juss) in Sudan as a source of safe, 'soft' pesticides, as well as other raw materials (e.g. fertilizers, medicines and cosmetics). The study, therefore, included investigations on seed characteristics, seedling growth, and variability in neem oil and limonoids in seed kernel.

The characteristics of the seeds sampled from ten seed zones, performance of the seedlings at the nursery, and the effects of different irrigation regimes on seedling growth were studied over two seasons at the National Tree Seed Centre (NTSC), Soba-Sudan. Investigations of neem oil and limonoids (azadirachtin, salannin and nimbin) were carried out over two successive seasons at Behavior and Chemical Ecology Department of the International Center of Insect Physiology and Ecology (BCED-ICIPE), Nairobi-Kenya. The effects of the climatic parameters (rainfall, temperature, and relative humidity) were analyzed in relation to the concentration of the limonoids in the seed kernels.

Neem ecotypes showed significant variations in seed weight, size, number of seed per kilogram, and kernel weight. No variations between ecotypes were detected in most of the growth traits measured at the nursery. No significant variations were found in response of various ecotypes to the two irrigation regimes in almost all growth traits measured.

The percentage of oil content in the neem seed kernel was found to constitute about 44.6% of the seed kernel, with no significant variation between ecotypes.

The azadirachtin content in the samples from the various zones ranged from 1.08 to 2.3 mg/g. and from 0.48 to 3.09 mg/g. in seasons 2001 and 2002, respectively. The major climatic factor that had a significant relation to the level of the azadirachtin in the seed kernel was found to be the rainfall. Azadirachtin level increased with the increase in rainfall, with the optimum being 717 mm. Temperature and relative humidity had no direct effect on the azadirachtin content.

Significant variations were observed between the ten zones in the level of the salannin and nimbin in both seasons. Salannin ranged from 1.59 to 3.02 mg/g and 1.05 to 2.82 mg/g and nimbin ranged from 1.39 to 2.11 mg/g and 1.05 to 2.61 mg/g in the two in the first and second seasons respectively. Salannin was affected by rainfall, while nimbin was not affected by any of the climatic factors examined. Relative humidity and temperature had no effect on both salannin and nimbin.

In conclusion, this study showed variations between ecotypes in the contents of azadirachtin, salannin and nimbin in neem seed kernels in relation to climatic factors. The practical implication of this is that the best sources of limonoids in Sudan for bio-pesticides production would be from neem trees growing in humid and semi-humid zones.

الاختلافات في النّموّ، الزّيت و الليمونويدز في شجرة النيم (Azadirachta indica) من المناطق البيئيّة المختلفة في السّودان

انتصار النور الطريفي عبد الرحمن

الخلاصة

هدفت الدّراسة الي توفير المعلومات اللازمة للمساعدة في استغلال شجرة النيم (*Azadirachta indica* A. Juss) في السودان كمصدر للمبيدات المأمونة وقليلة الاثر السالب في البيئة, بالاضافة الي كونها مادة خام لعدة استخدامات أخري كمخصبات تربة، أدوية و مواد التجميل.

اُختيرَتْ عشرة مناطق بذور مختلفة في السودان، حيث جُمِعَتْ البذور من أربعة مواقع في كلّ منطقة. درست صفات البذور, كما درس أداء النّباتات من المناطق المختلفة في المشتل وكذلك آثار فترات الرّيّ المختلفة علي نمو و تطور العينات خلال موسمي 2000 و 2001. كما درست ايضا كميات زيت النيم و كميات الليمونويدز (الأذاديراختين,السالانين و النيمبين) طوال موسمين متتالين. استخدمت تحليلات احصائية متعددة لتقييم آثار العوامل المناخيّة (معدّل المطر، الحرارة و الرّطوبة النّسبيّة) على تركيزات الليمونويدز في لبّ بذور النيم.

أظهرت عينات النيم من المصادر المختلفة اختلافات معنوية في وزن لب البذور، وزن البذور، حجم البذور و عدد البذورفي الكيلوجرام. لم تكشف عينات نباتات النيم من المصادر المختلفة التي درست لمدّة سنة واحدة تحت ظروف المشتل عن أيّ اختلافات معنوية في معظم صفات النّمو. بينما لم تظهر اختلافات هامّة بين تلك المصادر في مدي الاستجابة الي فترات الري المختلفة ، غير انه وُجِدَتْ اختلافات معنوية بين فترتي الريّ علي كلّ سمات النّمو تقريبا بغض النظر عن مصدر البذرة.

أظهرت النتائج أن محتوىّ زيت النيمَ في لبّ بذور النيم كان متشابه احصائيا في كل العينات حيث بلغ 44.6% من وزن لب بذور النيم. غير أن معدل الأذادير اختين أظهرتغيّرات معنوية اعتمادا على معدّل المطر و تراوح محتوىّ الأذادير اختين في العيّنات المختلفة من 1.08 إلى 2.3 ميلليجرام/جرام و من 0.48 إلى 3.09 ميلليجرام/جرام في الموسمين 2001 و 2002 على التوالي. وجد أن معدل المطر الأمثل هو717 ملم حيث تزداد محتويات الاذادراختين مع ازدياد الأمطار الي الأمثل ثم تنخفض بعد ذلك و كان معدّل سقوط المطر هو العامل الرّئيسيّ الذي يؤثر على مستوى الأذادير اختين في لبّ بذور النيم , بينما لم يظهرد للحرارة أو الرّطوبة النّسبيّة أيّ أثر مباشر على محتوىّ الأذادير اختين.

وُجِدَ أن السالانين يتّراوح من 1.594 إلى 3.023 ميلليجرام/جرام ومن 1.045 إلى 2.82 ميلليجرام/جرام في الموسم الأول و الثاني على التّوالي . بينما تراوح النيمبين من 1.389 إلى 2.114 ميلليجرام/جرام ومن 1.048 إلى 2.561 ميلليجرام/جرام في موسمين على التّوالي. ظهر تباين معنوي بين العشرة مناطق في كمّيّات كل من السالانين والنيمبين في كلا من الموسمين. وُجِد أن السالانين يَتَأثر بمعدّل سقوط المطر، بينما لم يَتَأثر النيمبين بأيّ من العوامل المناخية المذكورة لم يكن للرّطوبة النّسبيّة و الحرارة أثر مباشر على كلا من السالانين و النيمبين.

خلصت الدراسة الي وجود اختلافات فيما بين المناطق المختلفة في السّودان في محتوي كل من الأذادير اختين,السالانين و النيمبين في علاقة واضحة بالمؤثرات المناخية. تشير الدراسة الي أن من الأنسب استخدام بذور من المصادر التي تقع في الاقاليم الرطبة و شبه الرّطبة في انتاج و استخلاص المبيدات الطبيعية.

CHAPTER ONE

INTRODUCTION

1.0 Background

The world's forests are retreating rapidly due to the expansion of human activities, climatic changes and reduction of the biodiversity. Deforestation in Africa is reported at the rate of 4.1 million hectares per year. At this rate, it is said that Africa may lose all its forest cover in fifty years' time. Countries in West African region like Benin, Ivory Coast, Ghana, Nigeria and Togo are reported to have lost almost all their forest cover. Sudan forest cover is retreating southwards where about 84% of energy requirements in the form firewood and charcoal (Anonymous, 2001). Trees in the north are only found along the banks of rivers and in valleys (wadis). The number of species and stocking intensity of trees increase with rainfall southwards. Sudan is rich in many valuable indigenous trees such as *Acacia* spp., *Isoberlinia doka, Tectona grandis, Khaya senegalensis, Dalbergia sissoo, Tamarandis indica, Ziziphus spinichristi, Balanites aegyptiaca* and *Terminalia* spp. many others have been introduced in Sudan for different uses like *Eucalyptus* spp., *Prosopis* spp. and *Azadirachta indica.*

1.1 The need for bio-pesticides

With the world's population expected to rise by almost 90 million a year over the next decade, a more productive agriculture is vital to food security and poverty alleviation. Agricultural pests are the major factors in the production losses in tropical and subtropical regions. Use of synthetic pesticides increases the production significantly, but it has resulted in: loss of biodiversity, deleterious effects on non-targeted organisms, development of insect resistance, and increase in spraying intervals and, eventually, environmental pollution. The increasing cost of synthetic insecticides and the growing awareness of hazards associated with their large-scale use have evoked a worldwide interest in pest control agents of plant origin.

In the search for botanical insecticides, the Meliaceae (Mahogany family) has attracted some attention (Arnason, *et. al.* 1993; Isman *et. al.* 1994) in the last three decades.

Different laboratories and research groups have been investigating the insecticidal properties of the Meliaceae in different parts of the world. One of the most promising plants in this family is neem tree (*Azadirachta indica A. Juss).* The ever-increasing emphasis on developing environmentally benign pest control agents has brought neem to the fore.

1.2 Neem as a source of pesticides

The neem tree has been termed as "the most promising of all plants" by National Academy of Science of USA (Anonymous, 1992). It is also identified as "nature's gift to mankind", "the wonder tree", and "the tree for solving global problems" or the "global tree".

There have been many world neem conferences to date (Schmutterer *et al.* 1981; Schmutterer and Ascher 1984; 1987; Singh *et al.* 1993; and the 7th was held in Canada in 1999. This recent surge of interest in this tree is due to the spectacular biological activity of its ingredients found in the bark, leaves and seeds against a wide range of insects, fungi and viruses and other pathogens.

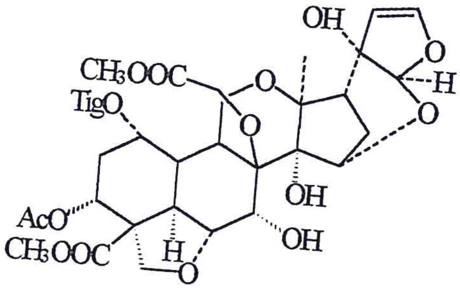
An environmentally benign pesticide, which is defined as one that is selectively toxic doesn't bioaccumulate, and exhibit relat persistence in the environment, is needed for modern inte management programs. Neem insecticides appear to fit this because they have been shown to be selective (Saxena and K Ramesh and Balasubramanian, 1998), with short persistence, and negative impacts on ecosystems than conventional insecticides (Sc 1990). Interest in neem insecticides has grown over the past 20 more pesticides are lost due to environmental and food safety (Koul *et al. 1990;* Schmutterer, 1990; Ascher, 1993).

Many studies have been carried out on the pesticidal and f activities of neem. Little work has been carried out on its ecolog genetic variations, ecotypes and the effect of climatic factors on the of different parts of the neem tree, especially the active ingredient seed.

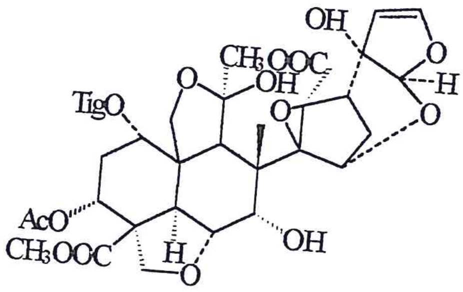
1.3 Active compounds in neem

Biological activities of neem extracts were investigated exte during the past two decades in many parts of the world. Since the investigation of Siddiqui (1942), more than three hundred compounds been isolated from various parts of the tree. Several reviews on constit of neem have been published to date. However, only few pure compo have been tested for biological activity, and little work has been done or structure -activity relationships of the isolated compounds (Rembold Puhlmann, 1995). Most of the active constituents belong tetranortriterpenoid (limonoids) group, but biologically active diterpeno triterpenoids, pentanortriterpenoids, and a small number of nonterpenoi compounds were also isolated (Kraus, 1995; Siddiqui *et al*, 1988). Limonoids are a group of chemically related tetranortriterpenoids present in the order Rutaceae and Meliaceae family plants (Champagne *et al*, 1992 and Hasegawa *et al.* 1997).

Continuing research into the triterpenoids of the Meliaceae still produces compounds of considerable chemical and biological interest. The most famous of these is azadirachtin, which was first isolated by Morgan in 1968 from the seed of *Azadirachta indica*, the Indian neem tree, and showed that it has the formula $C_{35}H_{44}O_{16}$ (Butterworth and Morgan, 1968, 1971). The early work of Morgan showed that the structure was complex, and this culminated in 1975 with proposal of a structure by Nakanishi (Zanno *et al*, 1975). This was followed by other structure proposals by Karus and Ley in 1975 (Taylor, 1986) as shown in (Scheme 1). This compound is now known to affect over 200 species of insects and mites (Saxena, 1989). Although many isomers of azadirachtin, aza-A to aza-H (Rembold, 1989), have been reported in the literature, azadirachtin-A is the most important and it is used as a standard to express the activity of neem seed, extracts and its formulation (Kaushik, 2002). However, azadirachtin-B was found to be the most effective against nematode (*Rotylenchulus reniform*), with (EC_{50} 96.6 ppm) followed by azadirachtin-A (119.1 ppm) and azadirachtin-H (141.2 ppm) (Sharma, *et al.*, 2003).



Azadirachtin (Nakanishi, 1975)



Azadirachtin (Karus, Ley 1985)

Scheme 1. Azadirachtin structure as showed by Nakanishi, 1975, Karus, Lay 1985
Source: D A H Taylor, 1987.

Azadirachtin is reported to have antifeedant, growth disrupting, and ovicidal activity against a variety of insect pests (Saxena *et al.* 1984, Rembold, 1989; Schmutterer, 1990; Schmutterer, 1995).
According to the study of Ermel (1995) there were marked variations in azadirachtin content within and between countries. In a sample from the Sudan azadirachtin content was found to be 2.5 mg/g of kernel. In other countries, Ermel found an average 3.6 mg /g

1.4 Pharmacological activities of neem

The medicinal properties of neem have been known for centuries in India. Recently, scientists have found even more uses for this remarkable tree. The seed, bark and leaves contain compounds with proven antiseptic, antiviral, antipyretic, anti-inflammatory, anti-ulcer and antifungal uses.

Neem preparation has been used to treat blood disorders, hepatitis, eye diseases, cancer, ulcers, constipation, diabetes, indigestion, sleeplessness, stomachache, boils, burns, cholera, gingivitis, malaria, measles, nausea, snakebite, rheumatism and syphilis (Jacobson, 1988). Also, Conrick (1996) reported in details the medicinal uses of neem for various diseases e.g. AIDS, cancer, diabetes, psoriasis, heart disease, herpes, malaria…etc. Amazingly, many of these biological activities are being substantiated by recent research (Khalid *et al.* 1986, Khalid *et al.* 1989, Tepsuwan, *et al.* 2002; Khalid, 2002, Khosla, *et al.*, 2002 and Omar, *et al.*, 2003). Pai *et. al.*, (2004) concluded that the dental gel containing neem leaves extracts has significantly ($P<0.05$) reduced the plaque index and bacterial count than that of the control group.

1.5 Role of neem as a forestry and agro-forestry tree

Beside the importance of the neem tree as a source of safe, 'soft' and environment-friendly pesticides, it has been selected to be among the best for agro-forestry systems for dry and semi dry zones. Its leaf mulch was shown to have soil-water conservation and temperature –reducing properties and also released nutrients through decomposition rate well suited to crop demand. The tree is highly suitable for the improvement of degraded and nutrient-poor soils (Von Maydell, 1986) and is, therefore, considered important in reforestation programs to combat desertification.

1.6 Neem research in Sudan

Neem tree was introduced to Sudan in 1916 and now is naturalized and commonly grown for shade all over the country (Hamza, 1990, Badi *et.*

al., 1989. From early 1930s, it has been planted by the Forest Department on the areas adjacent along the Nile.

Neem research in Sudan started in early sixties during the locust invasion when the researchers noticed that neem trees were the only plants that remained green and healthy while all other plants were completely destroyed by the locust plague (Ahmed, S. A. 2002; personal contact; Schmutterer, 1995).

Currently, many research groups are working in Sudan in collaboration with national and/or internationals organizations and centers. Most of the research work carried out in IPM and pest control use aqueous and other crude extracts both in the field and laboratory.

Recently, the potential of neem products as home-made and commercial insecticides (NeemAzal-T/S and neem oil) in the management of three homopterous pests (*Jacobiasca lybica* (de Berg), *Bemisia tabaci* (Gennadius) and *Aphis gossypii* Gover) were tested in potato (*Solanum tuberosum)* and eggplant (*Solanum melongena*), where increase in the production was observed. No significant difference was found between the two products tested (El Shafie, 2003).

Bashir and Ali (2002) recommended neem seed powder at the rate of 50 kg. per Feddan or 100 kg. neem leave powder per Feddan to be applied during land preparation for the control of termites (*Microtermes thoracalis* Sojost.) on groundnut (*Vigna subterranea* (L.) Verdc.) in the sandy soils of the traditional rain fed sector.

Khalid, (2002) used neem methanol extracts (10 µg/ml) as a successful treatment as anti-cutaneous Lieishmaniasis activity with positive response of 83.3 % of cases in period of 15-37 days.

Aissawi, (1999) reported gradual degradation in neem extracts at 65°C, any pH exceeded 5.3 and 7.0 and minor changes occurs when it exposed to 254 nm UV.

Bashir (1994) observed death and abnormalities on molting of *Epilachna varivestis* larvae with neem oil and methanol extracts of neem seeds obtained from Togo. Methanol extracts at 62.5 ppm was the most effective concentration on the larvae.

Significant effect was found on tomato key pests (aphid, jassid and whitefly) by using neem seed ethanol extracts on the powdery mildew (*Microsphaera palczewskii*) (Osman, 1991).

Malaria is one of the serious health and environmental problems in Sudan, especially in Gezira state. Pharmacological investigations on neem as anti-malarial carried out in Sudan proved that neem could be one of the promising solutions (Khalid *et al*. 1986 and 1989).

Some studies were carried out on the management, silviculture, seed storage, conservation and propagation of the neem tree in Sudan. Elteraifi *et al.* (2001) recommended fresh, clean and air dried seeds for short time storage. Direct sowing has also been recommended by (Elteraifi, 1996). No significant difference between this and transplanting method was found. Drought tolerance and growth evaluations were carried earlier by Khan (1966).

Sudan is a member in the International Neem Network provenances trials under FAO/IPGER through NTSC/ARC. However no research has been carried out in Sudan on provenances selection and genetic evaluation and/or improvement. Moreover, no methodical research work has been carried in quantifying and evaluating the yields of the oil and the limonoids from different seed sources in Sudan.

1.7 Hypothesis of the study

Look in narrow genetic profile will give better chance to find climatic effect on chemical and adaptation makeup, specifically in limonoids and oil, more than samples collected from wide range of genetic and environmental variations.

1.8 Justification and rationale of the study

Sudan is a large country, which extends latitudes between 3' 35′and 22' 35′ north and longitudes 22'38′ east, with a total area of 2,505,813 square kilometers (Fig. 1). The altitude varies from 3000 a.s.l. to 300 m. a.s.l.; the rainfall varies from zero mm in the extreme north and above 1500 mm in the south. Thus, different ecological zones exist which vary from the desert in the north to humid high land savanna in the south.

Neem tree occurs throughout Sudan; its performance is quite good even in the harshest conditions. But till now there is no estimation of the total number of the neem trees in Sudan. Most of the origin plantations were carried out by the colonial officers along the railway and the Nile banks. Then these spread all over the country.

Sudan which basically depends on agriculture imports annually large quantities of synthetic pesticides annually. These pesticides have a negative impact on the environment and human health, in addition to their high cost. A rdworthy observation in an increase in the number of cancer and allergy patients in Sudan, especially in the central region where the Gezira Scheme is located. It is believed that there is a relation between these two problems, but there is no hand scientific evidence yet. This study is an attempt to search for cheap and affordable bio-pesticides sources.

Investigations in growth performance, drought tolerance and genetic make up of the provenances of the neem tree will help in maximizing the benefit from the active ingredients available in it. Quantification and correlation of these compounds with ecological factors will help to clarify the scientific basis of their exploitation.

1.9 Research objectives

As stated in section 1.1 and 1.7, it is clear that the alarming increase in the prevalence of insecticide pollution in Sudan requires great efforts to alleviate the problem. Neem tree, which was chosen for the present investigation, is widely employed in traditional and modern medicine, and in agriculture in the rest of the world. Although neem is found allover the country, information on growth, drought tolerance, genetic variation and levels of chemical constituents of different parts of the tree in different ecological zones of the Sudan is scanty.

1.9.1 Overall Objective

The main objective of this study is to generate requisite information, which would provide the basis of exploiting neem tree in Sudan as a source of safe, 'soft' pesticides, as well as other raw materials.

1.9.2. Specific objectives

1. To investigate the establishment, growth and drought-tolerance of neem ecotypes from different ecological zones under nursery conditions.
2. To quantify and evaluate the levels of the oil, azadirachtin A and B, salannin and nimbin in neem seed kernels from 10 ecotypes in Sudan over two seasons and to establish their relationship, if any, to agro-ecological attributes.

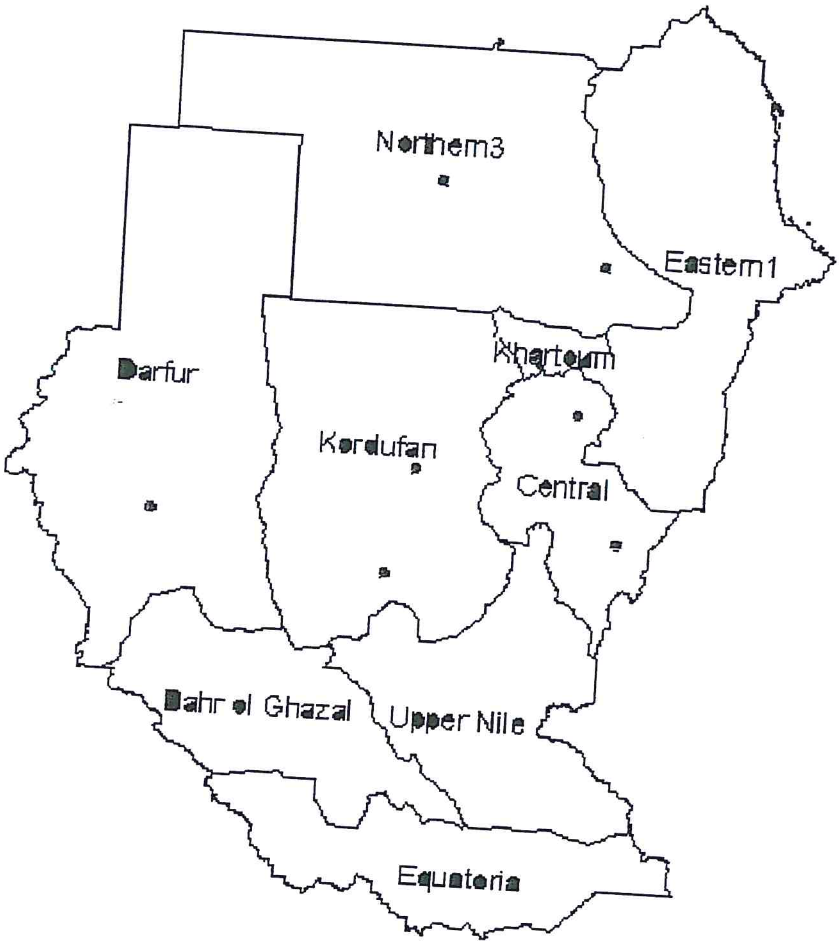


Fig. 1.1. Distribution of the selected seed zones in Sudan

CHAPTER TWO
LITERATURE REVIEW

2.1 Botany of the neem tree

Azadirachta indica A. Juss (Meliaceae) is a fast-growing, small to medium-sized, ever-green tree, which sheds most of its leaves in dry season and then blooms in full foliage (Tewari, 1992). The **Bark** is pale grey-brown and rough. **Leaves** are fresh, glossy green, crowded towards the ends of branches and compound, with 5-8 pairs of leaflets up to 10 cm. **Flowers** are small, fragrant, white or cream-colored, hanging in long few flowered axillary's inflorescence. **Fruits** are oval greenish-yellow berries, 1-2 cm long. **Seeds** are ovate or spherical, testa thin, composed of a shell and a kernel (sometimes 2 or 3 kernels) each about half of the seed's weight. **Roots** grow deep and wide, don't stand water logging (Shumutterer, 1995, Bo Tengnäs, 1995, Albrecht, 1993 and KFRI, 1992) fig. 2.1 **Flowering** in the Sudan occurs in March to September and **fruiting** in April to October (Hamza, 1990). The fruits can be harvested after 5 years and the first timber crop after 5-7 years.

2. 2 Origin and distribution

Neem tree is native to the dry forest zones of the Asian subcontinent. The exact region of origin of *A. indica* is not known. Some authors suggested that it may lie in Myanmar (Burma) and/or parts of southern India (Shumutterer, 1995), while others consider large parts of southeastern and southern Asia from Indonesia to Iran as the origin (Fig 2.2).

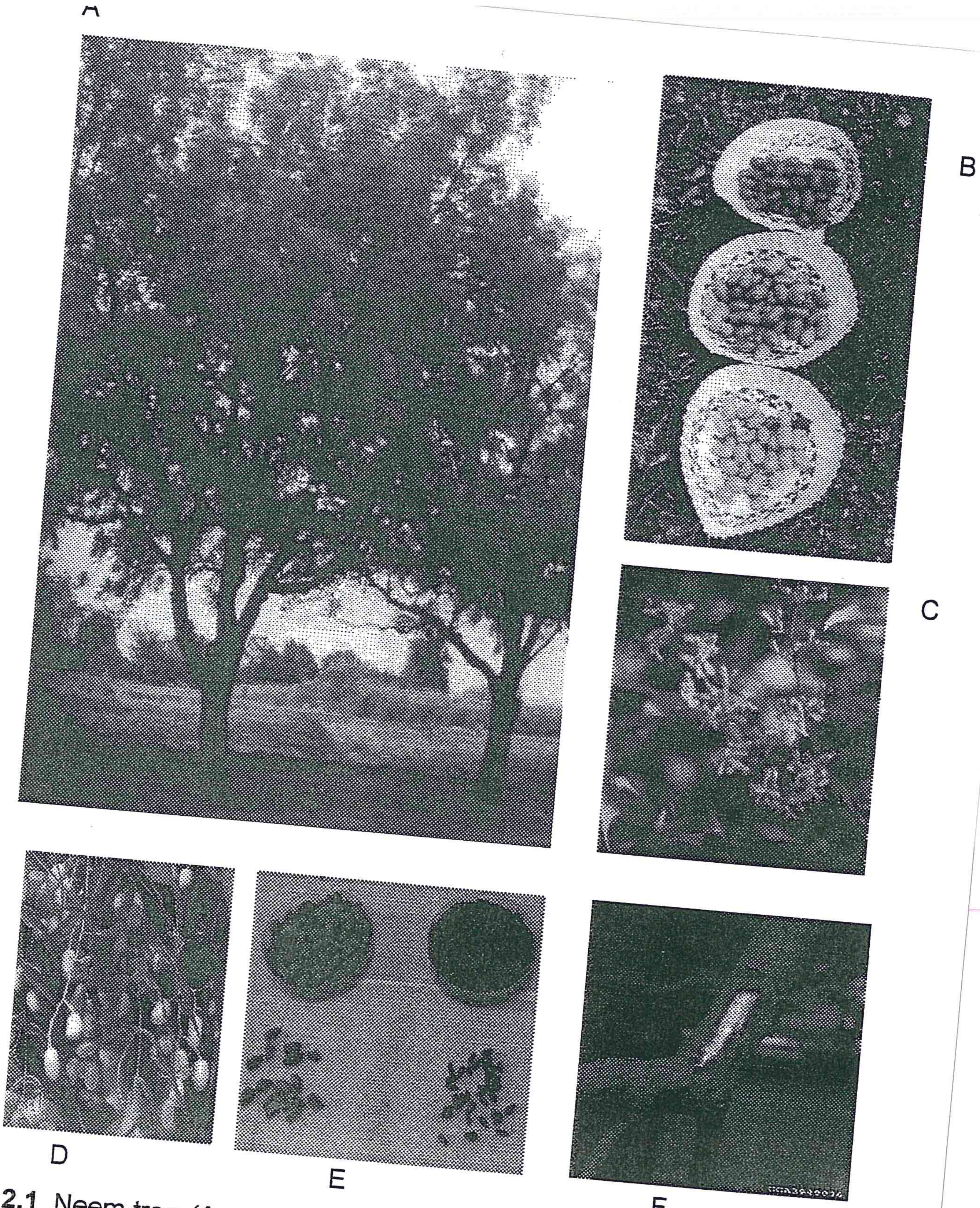


Fig 2.1 Neem tree *(Azadirachta indica* A. Juss.) (A= neem tree, B = Fruits (Green, yellow and greenish-yellow), C = Flowers D= Leaves and ripe fruits, E=Seeds and kernels and F=Bark.)

of India, Pakistan, Sri Lanka, Malaya, Indonesia, Thailand, and Burma (Shumutterer, 1995). It has become widely distributed by introduction into dry, arid and semi arid tropical and subtropical zones. In Africa different routes of spread have been documented between 1919 and 1927 and mainly from India (Anonymous, 1992). It has been grown well in plantations in the Sudan and Sahelian zones of Africa as well as in Sierra Leone, Malawi, Zimbabwe, Tanzania, Zanzibar and the non-Sahelian areas of Nigeria, and Ghana. In Uganda it grows very well in lowland areas, it does well at the Kenyan coast and it is also found around the Lake Victoria (Bo Tengnäs, 1995 and ICRAF, 1995). Today neem is found throughout western, central and eastern Africa (Schmutterer, 1995). One of the biggest plantations of neem is in the Arafat plain near Mecca in Saudi Arabia where it provides shade for pilgrims (Ahmed *et al.* 1989). It has recently been introduced in most of the warmer parts of the world including South and Central America and Australia (Schmutterer, 1995). The tree is now under cultivation in USA , Arizona, California, Florida and Oklahoma; and in Haiti, Colombia, Brazil, Honduras, Ecuador, the Dominican Republic and Argentina (Jacobson, 1986). It is one of the fastest spreading trees and has become pan-tropic.

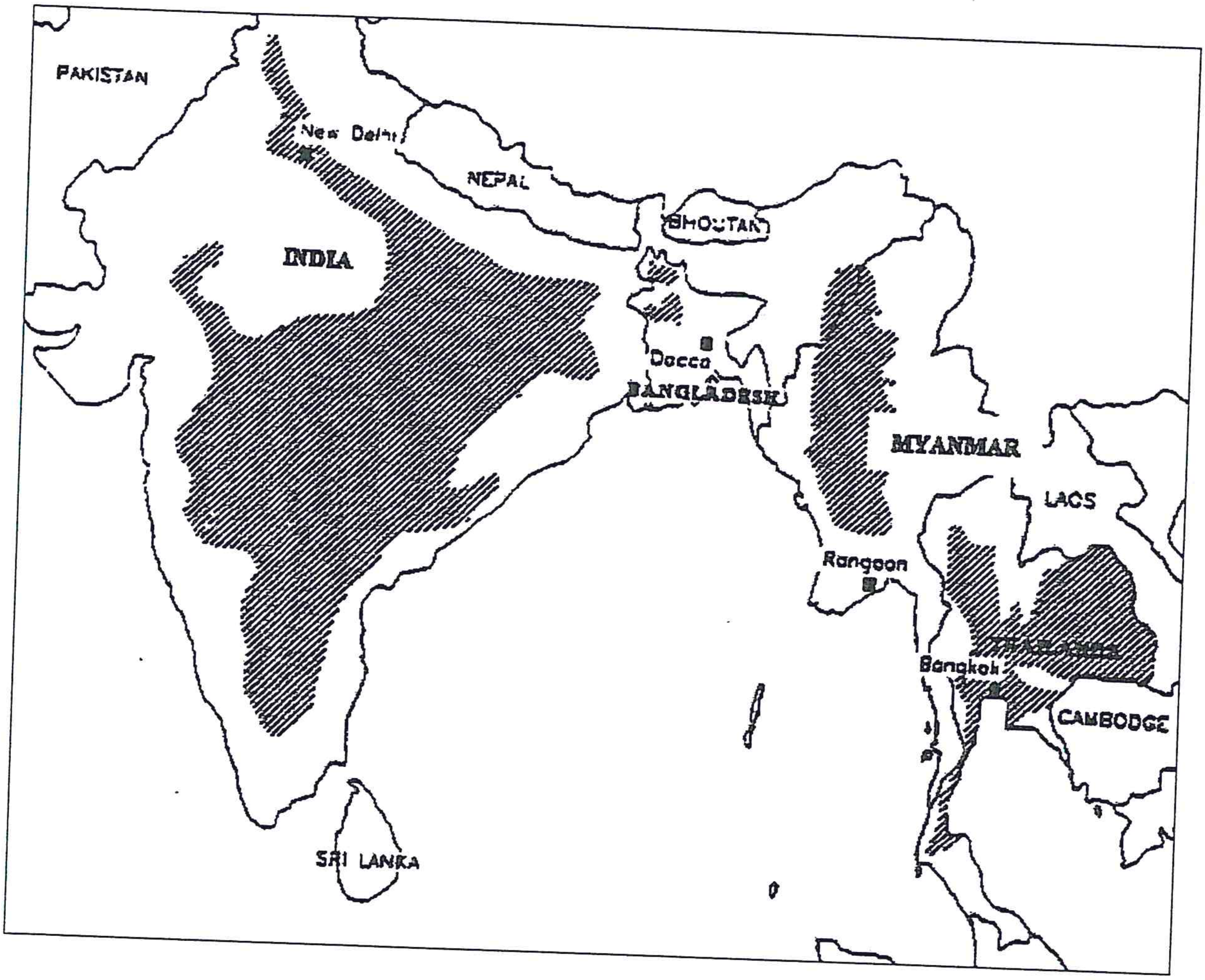


Fig 2.2 Natural distribution of the neem tree (*Azadirachta indica*). *(Source: Kundu, S. 1999).*

2.3 Site requirements

The tree has no particular site requirement. It is very drought tolerant, but it is sensitive to cold weather. Neem tree quickly dies in waterlogged soils, requires light, but tolerates fairly heavy shade during the first few years. It grows at altitudes ranging from zero to 1500 m, mean annual temperature up to 40 °C and rainfall between 400 and 1200 mm. It does well in areas less than 400 mm, and can grow with only 150 mm of rainfall. Neem grows on a wide variety of neutral to alkaline soils but performs better than most species on shallow, stony, sandy soils, or in places where there is a hard calcareous or clay pan not far below the surface. It grows best on soils with a pH of 6.2 to 7. (Schmutterer, 1995).

2.4 Siliviculture of the tree

A. indica trees may start flowering and fruiting at the age of 4-5 years, but economic quantities of seeds are produced only after 10-12 years. Pollination is by insects such as honeybees. Isolated trees may not set fruits, suggesting the occurrence of self-incompatibility. The flowering and fruiting seasons largely depend on location and habitat. In Sudan the peak of the flowering is in March to May, with fruiting in May to July (Hamza, 1990). Fruits ripen in about 12 weeks and are eaten by bats and birds, which distribute the seeds. Yields of fresh fruits per tree range between 37 and 50 kg/year (Koul *et al.* 1989). Neem seeds have a short viability in natural conditions (Anonymous, 1992); it is generally lost within 3 to 4 months after harvest and only 3-4 weeks in the dry areas (Sacandé, 2000). Storage behavior of neem seeds is controversial; it has variously been described as recalcitrant, intermediate and orthodox seed (Elteraifi *et al*, 2001, Tompsett, 1994, Roederer and Bellefontaine, 1989, Maithani *et al.* and 1989, Ezumah, 1986,). In Sudan, Elteraifi (1996) recommended short time storage of shade dried seeds in a ventilated container (cotton bags) at room temperature with high relative humidity.

A neem plantation can be raised by directly sowing seeds or by planting seedlings. Direct sowing of fresh seeds in the shelter of existing vegetation has also proved successful. In Sudan, no significant differences were found between the direct sown and transplanted seedlings in growth parameters and biomass yields (Elteraifi, 1996).

No seed pretreatment is required, although depulping and cleaning of seeds considerably improves the germination rate (Elteraifi *et al.* 2001). Mature fresh seeds germinate within 2-3 weeks with a germination percentage of 75-90%. Neem can also be propagated vegetativly by air layering, root and shoot cuttings, grafting and cutting. Clonal propagation and micropropagation by somatic embryogenesis and organogenesis are also used (Allan, *et al.* 1999) Neem seedlings are usually propagated in a nursery and transplanted to the field, although direct sowing has been successful under conditions of adequate rainfall. Seedlings should be hardened off before being transplanted into the field. Newly transplanted seedlings may benefit from shading. A planting spacing of 1.8 x 1.8 m is recommended (De Jussieu, 1963). Planting density can be 100-300 /ha, but it is commonly 150 /ha. Seedlings respond well to chemical and organic fertilizers, although they may not need fertilizers except on very infertile soils. Neem coppices well and produces root suckers, especially in dry localities. Early growth from coppice is faster than growth from seedlings. It withstands pollarding well, but seed production is adversely affected when trees are lopped for fodder. The practice of de-branching is very common in Sudan before the rainy season which affects seed production.

Weeding in neem plantations in dry areas is essential, as the tree cannot withstand competition, especially from grasses. It is weed and frost sensitive, especially in the seedling stage. The tree is not tolerant of extended flooding or fire.

2.5 Ecology and adaptation

Neem is a fast growing sturdy tree adapted to hot dry regions receiving 300 to 1500 mm of rainfall. It will grow well on poor, stony or shallow soils in places not suitable for cropping. The deep taproot, extensive and powerful root system can extract nutrients from leached soils and return them to the surface via leaf and litter fall. It thrives in tropical climate with pronounced hot, dry winters and hot wet summers. Cool weather slows growth, and frost kills young trees. Areas with poor drainage should be avoided as water logging causes death of the taproot and eventual death of the tree. In high rainfall (3000-4000 mm/year) areas it has failed completely (Shumutterer, 1995). The wide range of habitats and good adaptation to the drier conditions makes the distribution of the tree very wide.

Neem performs well in wide range of soil types; in Asia, neem is found growing in mixed forests, for instance in association with *Acacia spp.* and *Dalbergia sisso* (Benge 1989). In Africa, it is found in evergreen forests and in dry deciduous forests, the major associates are *Albizia spp.*, *Tamarindus indica*, *Tectona grandis*, *Boswellia serrata*, *terminalia tementosa*, *Acacia nilotica*, *Cappris spp.*, *Ziziphus spp.*, *Acacia senegal*, *Prosospis spp.* many others. Neem grows well in low rainfall (130 mm/year) areas in northern and western Sudan, in hilly area with moderate rainfall in Nuba Mountains, along the Nile and water streams all over the country.

2.6 Genetic variation and provenance selection

It is important to recognize the genetic variation of the species and to identify the best seed sources adapted to different ecological zones in order to breed them for efficient uses according to objectives. In forestry, provenance refers to collection of some trees growing in a particular place, and represents a population sample of a defined area (Màtyàs, 1997). Plant population exhibits genetic variation on several geographical levels and such variation exists along ecological factors in latitude, altitude and humidity (Hill *et al*. 1998). Provenance testing is done for very practical reasons: to screen the naturally available genetic variation and to allow selection of the best available types and most adapted for reforestation for further breeding. Population genetic variation in tropical tree species correlates closely with the extent of the geographical range (Hamrick *et al*. 1992).

Ermel *et al* 1987 and Ermel, 1995 found differences in azadirachtin and oil contents of neem seed kernel from different geographical zones. Selection of provenances of high oil and azadirachtin contents will be of great use for appropriate neem plantations as a source of bio-pesticides.

Various ecotypes of neem exhibit variation in several characters (Arora, 1993). Significant provenance variation in height growth of neem was reported among 39 seed sources in India by Rajawat *et al*. (1994). Geographical variation in neem oil, seed size and growth related characters have also been reported (Verendra, 1995; Surendran *et al*. 1993 and Dwivedi, 1993). Provenance variation on morphometeric and phonological

2.7 Neem pests

A. indica has few serious pests. However, several scale insects have been reported to infest it (Lale, 1998), for example *Aonidiella orientalis*, Oriental scale (Homoptera: Diaspidiae) feeding on sap of young branches and young stems, which is the most important pest in Sudan), and *Pulvinaria maxima* (feeding on sap and covering tender shoots and stems) (Schmutterer, 1995). The nymphs of *Helopeltis antonii* also feed on the sap. In India, a shoot borer damages the plant. Occasional infestations by *Micotermes thoracalis* (Termitidae) and *Lorantus* species of insects have been recorded in Sudan and Nigeria, but the attacked tree almost invariably recovers. Rats and porcupines attack and occasionally kill *A. indica* seedlings and trees by gnawing the bark around the base (Schmutterer, 1995).

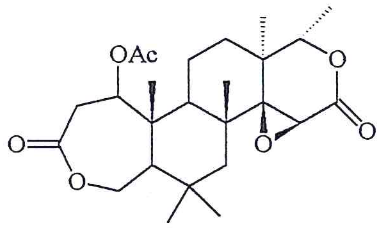
Mistletoes that affect *A. indica* are *Dendrophtoe falcata* and *Tapinanthus* spp. There are no records of fungi attacking *A. indica* in Southeast Asia. In India, *Psuedocercospora subsesessilis* (Moniliaceae) is the most common fungus attacking the leaves, causing the shot-hole effect. *Hendersonula* sp. attacks old neem trees in Sudan (Schmutterer, 1995). Various species of genus *Aspergillus* were recorded from stored neem seeds especially in humid areas in Asia, Africa, southern and central America. In India, the bacterium *Pseudomonas azadirachtae* may damage leaves.

2.8 Limonoids

Limonoids are secondary metabolites produced in plants found in the order Rutales, mostly in the family Meliaceae. In the search for alternative methods of pest control, in recent years, considerable efforts have been expended in the isolation and identification of naturally occurring insect antifeedants. Synthetic pesticides possess quick knockdown effects, but they are often toxic to mammals and nontargeted organisms (Klassen *et al.* 1986); for these reasons safe ecological pesticides that don't leach residues in the environment have great importance (Cook and Baker, 1983). Limonoids are described as modified triterpenes with or derived from a precursor with a 4, 4, 8-trimethyl-17-furnaylsteroid skeleton (Taylor, 1984 and Siddiqui *et al.* 1988). They have high complex structures which preclude application of synthetic compounds and utilization will be limited to the natural products.

Over 300 limonoids had been identified by 1992, and in particular they characterized the members of the family Meliaceae, where they are diverse and abundant (Connolly, 1983; Taylor, 1983; Das *et al.* 1984 and Champagne *et al.* 1992). Limonoids have attracted much attention because of the marked insect antifeedant and growth regulating (IGR) activity of azadirachtin and related highly oxidized C-*seco* limonoids from the neem tree and the chinaberry tree (*Melia azedarch* L.) (Jacobson, 1988; Ascher & Meisner, 1989).

Limonoids are one of bitter principles in citrus juices, where nomilin(1) is considered to be the precursor of all limonoids accumulated in citrus and related species (Hasegawa and Miyake, 1996; Hasegawa *et al.* 1997). Citrolin **(2),** harissonin(3), acetoxylharrisonin **(5)** (Rajab *et al.* 1997) and limonin **(4)** were tested for antifeedant activity (Hassanali, *et al.* 1986). Large quantities of waste citrus by-product may provide a source of limonoids (Klocke and Kubo, 1982). Limonoids are characterized by insect growth regulation, insect antifeedant, and medicinal effects on humans and animals as antibacterial, viral, antifungal properties. Of recent interest, liomomid's possible anticancercinogenic properties are being explored. Further interest in limonoids is generated by their economic impacts on the citrus fruit and juice industry.



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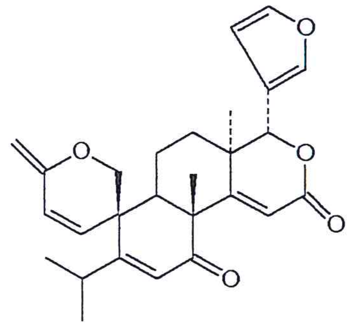

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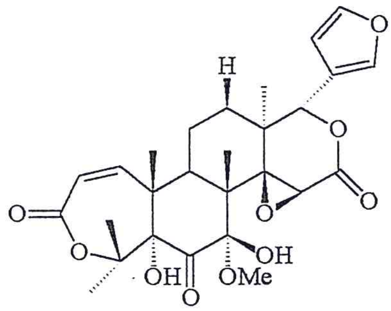

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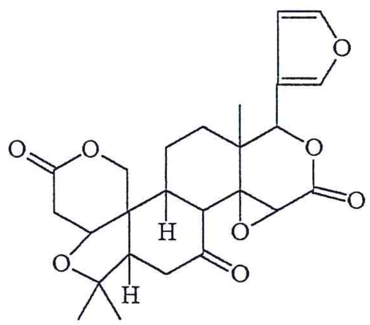

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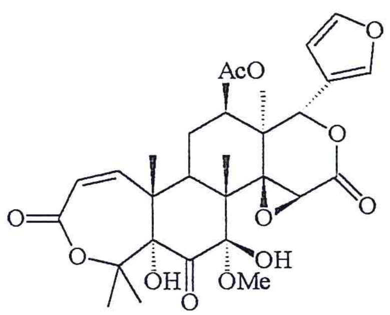


(5)

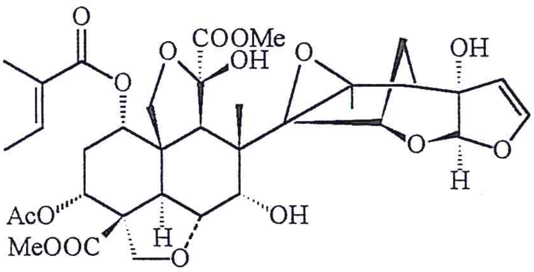
Scheme 2 Structures of: (1) Nomilin. **(2)** Citrolin. **(3)**Harissonin. **(4)** limonin. **(5)** Acetoxylharrisonin.

2.8.1 Limonoids in the neem tree

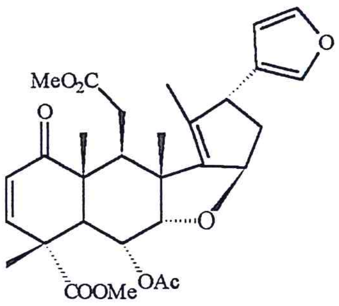
A great deal of attention has been focused on limonoids derived from the neem tree (Henderson *et al.* 1968; Butterworth & Morgan 1968; Zanno *et al.* 1975; Karus *et al.* 1981; Shumutterer, 1990, Singh., 1993; Shumutterer, 1995).

Neem seed kernel comprises azadirachtin (6) and other limonoids such as nimbin (7), salannin (8), 3-deacetylsalannin (9) and meliantriol (10). Recently there is evolution of the limonoids due to their putative function against insects (Das *et al.* 1984, 1987). The biological activity of over 70 limonoids has been reviewed by Champagne *et al.* 1992. Other limonoids have been extracted from neem stem bark and leaves; gedunin (11) and 7-deacetylegedunin (12) (Kraus, 1995).

Andrew *et al.* (1999) reported that; triterpenoids from the seeds of the neem tree can be quantified by using high performance liquid chromatography (HPLC). Eleven of these triterpenoids were determined by these authors and pure compounds including azadirachtin and salannin were obtained from the neem seed extracts. Limonoids have a variety of medicinal effects on animals and humans; antifungal, bactericidal, and antiviral activities (Shumutterer & Ascher 1984; 1987 and Shumutterer, 1995). Over the last two decades information on the biological activities of over hundred other limonoids has been published.



(6)



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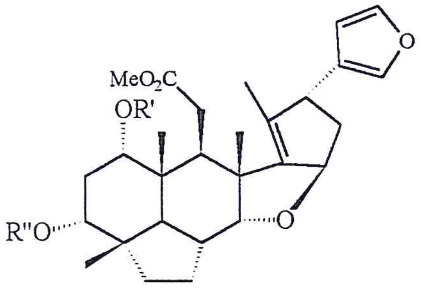
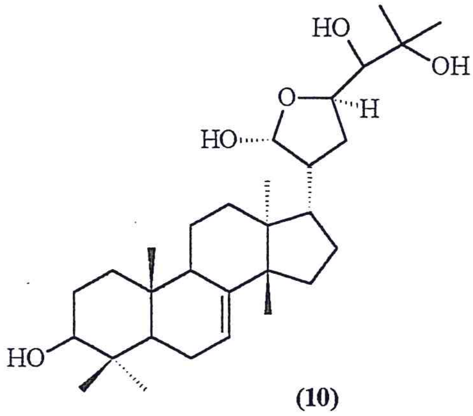


(8) R' = Tig R" = Ac

(9) R' = Tig R" = H



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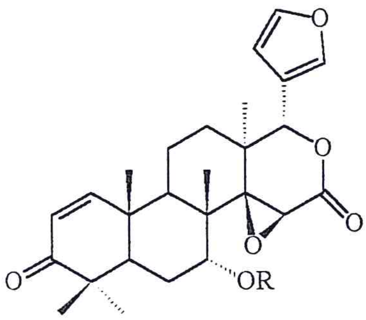

(11) R=Ac

(12) R=H

Scheme 3. Chemical structures of: (6) Azadirachtin. **(7)** Nimbin. **(8)** Salannin **(9)** 3-deacetylsalannin. **(10)** Mmeliantriol. **(11)**. Gedunin. (12) 7-deacetylegedunin.

2. 8.1.1 Azadirachtin

Azadirachtin is the most biologically active compound, which has been a focus of increasingly intense research in the past three decades since its isolation from the neem seeds (Butterworth and Morgan, 1968, 1972). It is a highly oxidized triterpenoid and one of the most potent antifeedant compounds yet discovered. It is also affects the normal growth and development of a wide spectrum of insects (Mordue and Blackwell, 1993; Schumutterer, 1995). Azadirachtin has gained an increasing interest, because of its dual forms of activity: as an anti-feedant at higher concentrations and an insect growth inhibitor (IGI) at low concentrations (Schmutterer and Ascher, 1984 and 1987; Rembold, 1989). Efficacy, low toxicity to mammals and fast degradation makes azadirachtin an environment-friendly agent to control pests. However, its liability to heat, moisture and air has been a matter of concern that led to global efforts to stabilize it (Stark and Walter 1995; Raguraman and Jayaraj, 1994; Stokes and Redfern, 1982; Barnby *et al.* 1989).

It represents about 0.2-0.8% of the seeds by weight, and it is accompanied by a number of other triterpenoids, such as nimbin (Harris *et al.* 1968) and salannin (Henderson *et al.* 1968), which exhibit similar biological properties, to a greater or lesser extent. The neem azadirachtin content of different ecotypes in India lacked a correlation with their bioactivity (Srivastava, *et al.* 1997). The highest content of azadirachtin (10-mg/g seed kernel) was recorded by Shaun *et al.* (1996) in newly ripened seeds. There was some loss of azadirachtin and salannin in storage after harvesting for up to 6 months.

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The insecticidal activity of azadirachtin has been reviewed by Rembold, *et al.* 1987, Rembold 1989, Schmutterer, 1990 and Schmutterer 1995. Presently, enriched azadirachtin is commercially available. However, 100% pure azadirachtin is still not easy to extract. Extracts can be made from leaves and other tissues, but the seeds contain the highest concentrations of the compound. It acts as an insect repellent, feeding inhibitor and insect growth, metamorphosis and reproduction disruptor.

There are many commercial neem insecticides available now in the developed countries for example Margosan-O and Azatin in USA have recently received an exemption from residue tolerance on food crops by U.S.EPA. Several other neem products may soon be available.

Due to small or no side effects on the important beneficial organisms for example Honey-bee (Schmutterer, 1995) or lacewing and coccinellid larvae neem extracts could be an interesting alternative especially for farmers who reject the application of synthetic insecticides. Azadirachtin A is the currently accepted reference ingredient for standardizing neem-based products (Anonymous, 1996).

2.8.1.2 Salannin and nimbin

A considerable number of other active compounds were isolated from neem seed kernels, salannin, salannol, salanolacetate, 3-deacetylsalannin azadiradion, gedunin **(11)**, nimbinin and nimbin (Kraus, 1995). Johnson and Morgan (1997) isolated nimbin and salannin beside ten other triterpenoids from neem kernel extracts. Jarvis *et al* (1999) reported a method to obtain pure azadirachtin and salannin from neem seed kernel.

Most of the compounds in the neem extracts possess insect growth regulating activity (Saxena, 1989). The triterpenoids usually present in large amount in neem seed are salannin and nimbin (Jarvis *et al.* 1997). Nimbin was the first constituent of neem to be isolated (Siddiqui, 1942) and its structure elucidated by Harris *et al.* (1968) and reviewed recently by Kraus (1995).

Nimbin is a large and important group containing some of the complex compounds (Taylor, 1984). The simplest derivative is known as salannin (Henderson *et al.* 1968). Nimbin and salannin are generally considered to be less active feeding (Blaney *et al.* 1990) and growth inhibitors (Simmonds *et al.* 1990) than azadirachtin. However, nimbin showed better bioactivity than salannin and other related compounds. Photo oxidation of nimbin and salannin proceeded much faster than that of azadirachtin (Jarvis *et al.* 1997), and all photoproducts showed some biological activity.

These triterpenoids are extracted either by first grinding the seeds with alcohol, and then removing the oil by partitioning the alcoholic extracts against a more lipophilic solvent (Butterworth and Morgan, 1972) or alternatively grinding the seeds first with hexane or petrol to remove the oil, and then extracting the triterpenoids from the seed matrix using a more polar solvent (Schroeder and Nakanishi, 1987; Johnson and Morgan, 1997).

However, the recent method used involves seed materials being homogenized and extracted by soxhlet, or equivalently, using methanol as an extracting solvent.

2.9 Neem oil

A. indica oil has been produced in Asia on an industrial scale for use in soaps, cosmetics, pharmaceuticals and other non-edible products. Neem kernels contain 30-45% (w/w) oil (Koul *et al.* 1990; Parmar and Ketkar, 1993; Kumar and Parmar, 1996). The seed oil yield is sometimes as high as 50% of the weight of the kernel. Neem oil is valued at about USD 700 per tone (1990). It is one of the most important by-products of the neem tree and has some commercial uses. It is easy to prepare, native farmers can grow the tree and the oil can be extracted using simple devices. Pressing of the neem seed or neem kernel mechanically resulted in two types of products; neem oil and neem cake. Both of the products can be used as a source of pesticides. The dry clean seeds decorticated manually to obtain kernels, which will be extracted with n-Hexane (60-80 °C) in the soxhlet extractor for 8 hours (Kumar and Parmar, 1996). Annual production of neem oil in India averages 83 000 tons (Mapiappn and Saxena, 1983).

There are differences in the formulations between the neem insecticides available in the United States. Stark and Walter (1995) examined the relationship between the neem oil and the effectiveness of neem insecticides. Addition of neem oil increased the efficacy of neem insecticides that did not contain the oil, while removal of neem oil from Margosan O (an insecticide contain 3-5% neem oil) reduced its efficacy 62% (Stark and Walter, 1995). The neem oil and/or a component of the oil influenced insecticidal activity of Azadirachtin, the active ingredient in these neem insecticides.

Stark and Walter (1995) identified six Limonoids; nimbanoids, deacetylnimbin, 6-acetylnimbandiol, deacetysalannin, and two unidentified chemicals believed to be limonoids, in neem oil. They indicated that; neem oil and other oils increase the efficacy of neem insecticides, but a polar component (s) of neem oil also contributes to increased biological activity of neem insecticides.

Variation in neem oil content and its physicochemical characteristics such as color, specific gravity, refractive index, saponification, iodine and acid composition, is reported (Roy and Dutti, 1929; Child and Ramanathan, 1936; Hilditch and Murti, 1939; Skellon, *et al.* 1962). The variation in content of the main active ingredient, Azadirachtin, is also well documented (Ermel, *et al,* 1987; Rengasamy, *et al.* 1993). Variation and specifications for neem oil are prescribed (Anonymous, 1975; The Indian Pharmacopoeia, 1966).

Kumar and Parmar (1996) reported a wide variation in the neem oil content and their physicochemical characteristics (color, specific gravity, refractive index, iodine value, acid value and saponification value), total fatty acid composition, (oleic, stearic, palmitic, linoleic, myristic, arachidic and behenic acids) and the key meliacins (azadirachtin, salannin and nimbin) in 42 neem ecotypes of India.

It is found that Azadirachtin content of the neem oil did not correlate with any of the physicochemical and chemical parameters, but nimbin and salannin contents correlated significantly with each other. (Kumar & Parmar, 1996).

Neem oil is relatively inactive against non-target insects (Shumutterer, 1995 and Singh, 1993).

2.10 Importance and uses of the neem tree

2.10.1 General uses

A. indica is an important multipurpose tree species in many developing countries, including Sudan. It is a naturally renewable resource producing extensive useful biomass. Its genetic improvement and economic exploitation are of great interest. The tree is known for its impressive range of uses in medicine, agriculture (as pesticides), timber and fuel wood, and its role in agroforestry, crop production, air pollution (Anon, 1989; Koul *et al.* 1990; Jacobson, 1986; Singh *et al.* 1993 and Schmutterer, 1995).

A. indica fruit is an important source of food for some wildlife, especially birds and bats, although they digest only the pulp, not the seed. Fruits are eaten fresh or cooked, or prepared as a dessert or lemonade-type drink. The young twigs and flowers are occasionally consumed as vegetables. The leaves, though very bitter, are used as a dry season fodder. It is also used as mulch and green manure. Intercropping of *A. indica* with pearl millet, *Pennisetum glaucum*, has given good results in India.

The large crown of *A. indica* makes it an effective shade tree, planted widely as an avenue tree in towns and villages and along roads in many tropical countries. Because of its low branching, it is a valuable asset for use as a windbreaker. Neem cake can be used as organic manure and in soil amendment. It enhances the efficiency of nitrogen fertilizers by reducing the rate of nitrification and inhibiting soil pests including nematodes, fungi, and insects.

For fuelwood production the neem tree has been identified as one of 233 species suitable for arid and semi-arid regions and one of 145 species suitable in humid tropics. Charcoal made from *A. indica* wood is of excellent quality.

The wood is used to make wardrobes, bookcases and closets, as well as packing cases because its anti-insect quality helps to protect the contents from insect damage.

The main stem of the tree is also widely used to make posts for construction or fencing because the wood is termite resistant. An exudate can be tapped from the trunk by wounding the bark. This high-protein material has a potential as food additive and is widely used in Southeast Asia as 'neem glue'. Tree bark contains 12-14% tannins. This compares favorably with conventional tannin chemicals.

.2.10.2 Uses in pest control

For centuries neem has been known for its medicinal and insecticidal values, one key practical use of neem is in the control of farm and household pests (Shumutterer, 1995). Extracts from neem seeds and leaves make highly effective insecticides: which attack many pestiferous species, are biodegradable, appear unlikely to quickly lose their potency due to build up of genetic resistance in the pests, seem to leave human, vertebrates and beneficial insects unharmed, and their effectiveness equals that of DDT, Dieldrin and other synthetic pesticides. It's bioactivity against insect pests has been particularly investigated in details (Shumutterer, 1995, Singh, 1993, Shumutterer, 1990, Shumutterer and Acher, 1987).

Information on the performance of various neem products against different pests has been flooding the scientific literature up to date . Due to its relative safety to non-target insects and organisms compared to the synthetic insecticides, its use in integrated pest management has been recommended (Parmar, 1993).

2.10.3 Uses in folk and modern medicine

Herbal medicine is the oldest form of therapy practised by mankind over centuries. Its pharmacological properties were so popular that the neem tree virtually played the role of a village dispensary in India, where neem twig is nature's toothbrush to over 500 million people daily. Neem has proved effective against certain fungi, bacteria and viruses that infect humans

Neem oil is a powerful spermicidal and can therefore be used as an inexpensive birth control method. Neem oil-based product, Sensal, is being marketed in India as an intra vaginal contraceptive. Neem oil has been used traditionally as a topical treatment for skin symptoms in both humans and livestock, every part of the tree finds use in one way or other (Ketkar and Ketkar, 1995). Leaf tea is used to treat malaria. However; this can not account for the traditional use of neem leaf extract in the treatment of malaria. The activity may have been due to the presence of gedunin, which was isolated as active factor inhibiting *P. falciparum* (Khalid *et al.* 1989). Recently, antimaliaral activity extracts of certain Meliaceace species has been demonstrated. Extract of leaves of *A. indica, Cedrela savadorensis* Roxb. ex Rottl. and *Chukrasia tabularis;* the bark of *Trichlia glabra;* and the wood of *Cedrela odorata* and *Desoxylum* are reported to have activity against chloroquine sensitve *P. falcipram* clone. Limonoids constituent of these plants, particularly those related to gedunin were found to be responsible for the anti malarial activity experimental.

It has been reported in neem association site that in Kenya and the rest of Africa, neem has being used as a treatment for malaria, diabetes, pneumonia, ulcers, gout and chest related cases with potential healing power in the herbal clinics (Conrick, 1996). Studies on the effect of neem bark and neem leaf extracts show they significantly block production of viral proteins therapy and stopping replication of HIV virus*. Wanzala, *et al.* (2002) mentioned "natural pharmacy" should not be ignored as 80% of the world population depends on it for healthcare, especially for such pandemic HIV/AIDS.

Recently, there are many neem products in the international market including medicine, pesticides and cosmetics. The tree is becoming widely distributed and with considerable commercial value.

2.10.4 Economic and commercial importance

Neem's potential in solving global agricultural, public health, population and environmental pollution problems is quite understood today. It calls for building awareness of its potential and dissemination of neem-based technology whether for pest management, public health, family welfare programs reforestation, or production and commercialization of various neem products for domestic use or exports.

Due to small or no side effects to the important beneficial organisms for example Honey-bee (Shumutterer, 1995) or lacewing and coccinellid larvae neem extracts could be an interesting alternative especially for farmers who reject the application of synthetic insecticides. Azadirachtin A is the currently accepted reference ingredient for standardizing neem-based products (Anonymous, 1996).

* http://www.ajtsc.com/neemand.htm

Under favorable conditions, fresh fruit yield per tree is about 50 Kg per year. Therefore, an increased commercial plantations and agro-forestry involving neem can enhance the economic potential of this tree with positive and large externalities for pesticides, fertilizers, live stocks, dairying and other value-added products. Almost every part of the neem tree viz. roots, leaves, flowers, seeds, trunks and branches has multiple uses. Neem is not only an excellent source of pesticides; it also provides good fodder, fuel and timber. This makes neem tree a very potentially profitable especially for the poor farmers in small scale farms where they can use the tree as shelterbelt and commercial products. Large scale plantations are established in America, Australia, Asia and West Africa.

As a result, demand for neem products, especially the seed as the basic raw material, is going to increase by leaps and bounds. Herein also lays a solution for creating income generation and job opportunities.

There are many commercial neem insecticides available now in the developed countries for example; Margosan-O and Azatin in USA have recently received an exemption from residue tolerance on food crops by U.S.EPA, several other neem products may soon be available.

In Sudan the tree is spreading all over the country but no large scale plantation is taking place.

CHAPTER THREE

MATERIALS AND METHODS

3. General experimental background

This chapter includes two parts as outlined below

Part I, Seed measurements and nursery experiments: it involves two experiments which were conducted in a nursery in Soba, Sudan (latitude 14° 05′ N, longitude 33° 38′ E, alt. 380 m a.s.l), to evaluate the growth of ecotypes and their tolerance to drought. The experiment was conducted over two seasons.

Part II, includes laboratory experiments: which were conducted at the Behavioral and Chemical Ecology Department (BCED) at ICIPE over two successive seasons, on analyses of limonoids and oil yields.

3.1 Biological materials

All neem seed samples in this study were collected from Sudan (Fig. 1.1).

3.1.1 Seed sources

Ten agroecological zones were selected for neem seed collection according to the tree seed zoning systems map (DANIDA/ARC, 1995). These regions cover the whole country except the south (Table 3.1). Seeds were collected over two seasons with collaboration of Forest National Cooperation (FNC) and the Agricultural Research Cooperation (ARC).

3.1.2 Collection and processing of neem seeds

Neem fruits were collected from neem plantations at the selected zones (Table 1). Four sites within each zone were selected randomly and 15-20 trees were marked randomly in each site. In total 2-3 kg of the neem fruits were collected from the marked trees. Collected fruits were then transported to the National Tree Seed Centre, Soba-Sudan, where they were cleaned in the processing room and dried under shade in dry weather. Seed processing was carried in accordance with ISTA (1993) and International Neem Network recommendations (Thomson and Souvannavong, 1994). A sample of 500 g was drawn from each lot using the seed divider and stored at 4°C till it was used in the Behavioral and Chemical Ecology Department (BCED) laboratory at ICIPE-Kenya for part II. The rest of the seed lot was used in Sudan for the nursery experiments (part I).

3.1.3 Seedlings raisings

Clean neem seeds were sown in soil medium (2 clay: 1 sand) in polythene pots (20x25 cm and 15 mm thickness) placed under shade (traditional nursery) at 3-5 seeds per pot. All of the nursery experiments were conducted at the National Tree Centre located at Soba Research Station of the Agricultural Research and Technology Corporation, Sudan.

3.2 Seed measurements

3.2.1 Number of seeds per kilogram

In each season, three hundred seeds were taken at random and divided into 3 replicates. Seed weight per 100 seeds was recorded using an electric balance to three decimal places. Calculation of the number of seeds per kilogram was carried out for each ecotypes according to ISTA (1993).

3.2.2 Seed dimensions

Seed length and width were measured in millimeters by a vernier, 100 seeds were used replicated four times for each seed source.

3.2.3 Seed, kernel and shell weights

Seed weight of 100 seeds replicated three times was measured by using electrical balance. The same seed samples were used to obtain the weight of the shells and kernels.

Table 3.1 Ecological zones for neem seed collections

Seed code	Vegetation type	Agro ecological zone	Latitude N	Longitude E	Altitude (a.s.l.)	Mean annual rainfall (mm)	Mean annual temp. (°C)	Relative humidity %
S1	Vegetation confined to watercourses; *Phonex spp.* & *Acacia spp.*	Desert	19 10’	30 29’	228	12.3	27.3	25
S2	*Acacia tortilis, Maerua crassifolia* & other desert shrubs.	Semi desert	17 34’	33 56’	350	39.4	29.4	25
S3	*Acacia spp.* & other trees planted for shade	Semi desert	15 36’	32 33’	380	162.4	29.9	29
S4	*Acacia mellifera, Acacia nilotica* and other semi desert *spp.* on clay	Semi desert on clay	14 25’	33 29’	405	306.4	28.6	39
S5	*Acacia Senegal, Adansonia digitata* & other semi desert *spp.* on sand	Central *A.senegal* savanna	13 10’	30 14’	570	318.0	27.3	34
S6	*Acacia spp.* on semi desert grass land on sand	Semi desert on sand	‘	‘				
S7	*Acacia seyal, Balanities aegyptiaca* & other *Acacia spp.*	Central to eastern *A.seyal-Balanities* wood land	‘	‘				
S8	*Anogeissus spp., Combretum hartmannium* & *Acacia spp.*	Eastern deciduous *Anogeissus/Comberetum* wood land	11 49’	34 24’	470	712.9	28.3	47
S9	*Khaya senegalensis, Combretum spp.* & *Anogeissus spp.*	Darfur-western Kordofan Deciduous wood land	12 04’	24 53’	655	398.3	27.2	31
S10	Broad-leaved trees; *Anogeissus spp.* & *Combertum spp.*	Nuba mountain hill catena	11 00’	29 43’	500	633.1	28.1	47

*** Source: Meteorology Department, Sudan.**

3.2.4 Moisture content

Samples of 15 seeds replicated 4 times were drawn for moisture content determination in accordance with ISTA (1993) low constant temperature method (103±1°C for 17±1 hrs). The moisture content was calculated on a wet weight basis.

3.3 Nursery Experiments

3.3.1 Experiment 1. Provenances variations

Seedlings for this experiment were prepared at the nursery as previously described (Section 3.1.3) using seed sources from the ten seed zones (seeds from two zones did not provide enough seedlings, and were dropped from further experiments.). Sowing date was 25/7/1999; the polythene pots were pre-watered before the sowing date. After sowing, seedlings were flood irrigated every day for 12 weeks. During this period all silvicultural operations needed were carried out.

3.3.1.1 Experimental design

Seedlings with uniform height were selected after 12 weeks from planting at the nursery and arranged in 4 blocks. Each block was divided into three replicates. Each replicate contained eight provenances; each provenance was represented by 20 seedlings in a Randomized Complete Block Design (4x3x8x20).

3.3.1.2 Seedling morphometrics and data collection

Destructive measurements were conducted every 4 weeks. After removal of the rooting media (by washing roots with water) seedlings were cut at root collar level beneath the cotyledons scars. Measurements carried out are summarized in Table 3.2. The dry weight for shoot and root were obtained after drying at 85±1°C for 48 (ISTA, 1993).

Table 3.2 Morphometeric characters of seed and seedlings measured.

Seedling	Leaf	Seed
- seedling height (SH)	- total no. of leaves per seedling (TL)	- seed length (SL)
- collar diameter (CD)	- leaf length (LL)	- seed diameter (SD)
- root length (RL)	- leaflet ratio (length: widths) (L:W)	- seed weight (SW)
- no. of roots (R No.)		- no. of seed per Kg
- distance between cotyledons and the1st two leaves (DCL)		-kernel weight
- shoot dry weight (SDW)		
- root dry weight (RDW)		
- shoot: root ratio (S: R)		

3.3.2 Experiment 2. Drought tolerance studies

3.3.2.1 Experimental design

This experiment was carried out in seasons 2000-2001 and 2002-2003. After sixteen weeks from the sowing date, ten healthy seedlings of uniform height were chosen from each provenance and transferred out of the nursery to an open and shaded area. Seedlings were divided into 2 blocks; each block was divided into three replicates in a randomized complete block design (2x3x8x10).

Seedlings in block I were watered every day, while the seedlings in block II were watered every 10 days. The day/night temperatures and relative humidity were about 30±3/20±3 °C and 60/75%, respectively. The natural photoperiod was at 12/12 hours daily dark/light (not controlled). The seedlings were harvested on May 25th, 2000 after 27 weeks under the irrigation regimes.

3.3.2.2 Sampling and data collection

After removal of the rooting media (by washing with water), seedlings were cut at root collar level beneath the cotyledons scars and the following measurements were made:

(i) seedling height

(ii) root length

(iii) shoot and root dry weight

The dry weight for shoot and root were obtained by drying at 85±1°C for 48 hours as in accordance with ISTA (1993). Sampling was done at the end of the experiment for all the seedling stock.

3.3.2.3 Data analyses

Data for the seed morphometrics and for experiments 1 and 2 were subjected to analysis of variance (ANOVA) and principal component analysis (PCA) using SAS software (SAS Institute Inc., V8, 1987; Cary, North Carolina, USA). The multiple comparison procedure using Least Significant Difference (LSD) test (P=0.05) was used to compare the provenance means.

3. 4 Laboratory experiments

3. 4.0 General experimental procedures

All recyclable glassware used was washed in hot water and soap and rinsed with distilled water and acetone. The glassware was then dried at 100 °C for one hour. All the solvents, reagents and HPLC grades solvents were obtained from Aldrich Chemical Co. Ltd., England or Merck, Germany.

3. 4. 1 Plant materials

Neem seeds were collected, cleaned, dried and stored as described earlier (Section 3.1.2). Seeds were taken to the chemistry laboratory at International Center of Insect Physiology and Ecology (ICIPE) at Nairobi-Kenya. Neem seeds were crushed with a pestle in a mortar to obtain the kernels. The husks were then removed and the seed kernels were ground in an electric blender to prepare the kernel powder (NSKP) which was used in the analyses.

3. 4. 2 Chemicals

Laboratory-grade reagents and solvents were used in extractions; methanol (CH_3OH) with purity of 99%, hexane (CH_3 $(CH_2)_4CH_3$) with purity of 99.8% together with distilled and deionized water. Appropriate analytical-grade solvents were used in high pressure liquid chromatography (HPLC) acetonitrile (HPLC grade) and distilled and deionized water.

3. 4. 3 Extraction and separation of polar limonoids from non-polar constituents

Extraction was done in two replicates; 12 g of each sample was extracted with 25 ml of methanol in a 250 ml conical flask and stirred magnetically for 2 hrs. The extraction was repeated two times (for 1 hr each). The pooled methanol extract was shaken with 30 ml of hexane and 1.0 ml of distilled water. Extraction with hexane was repeated twice. Two layers were obtained in each case, the upper mainly hexane layer was separated from the lower aqueous methanol layer. The latter was concentrated in *vacuo* (Fig 3.1) to 10 ml and stored at (-15°C) for high performance liquid chromatography (Plate 3.3).

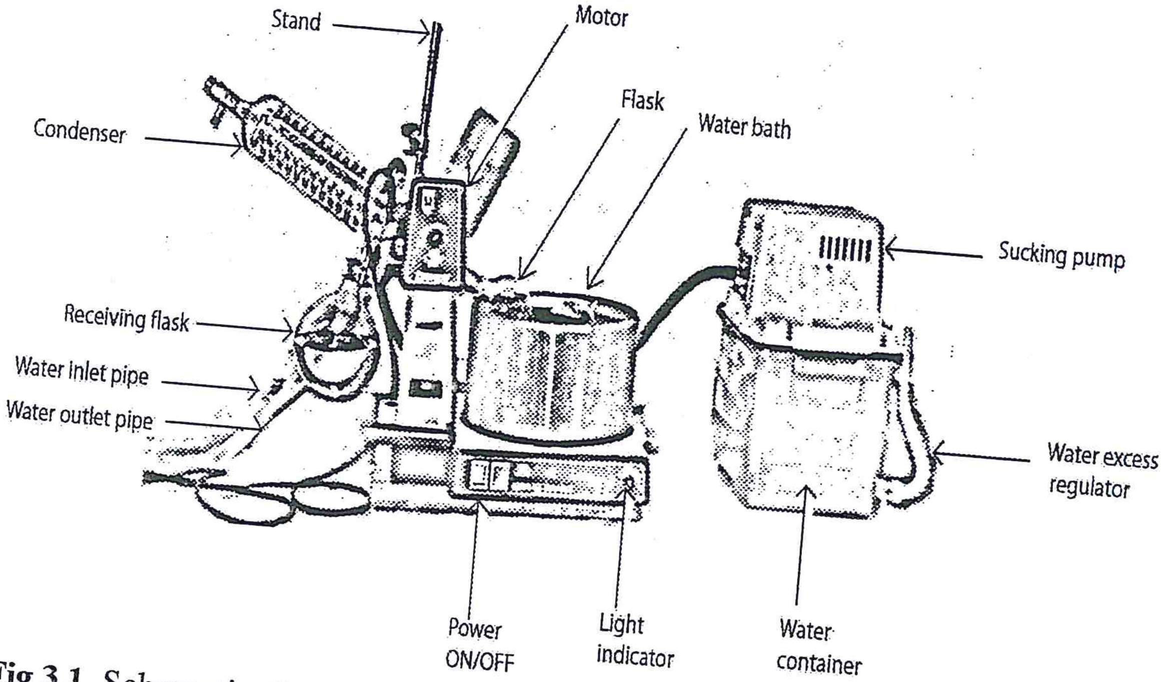


Fig 3.1. Schematic diagram of Rotavapor (Büchi 461, water bath, Switzerland)

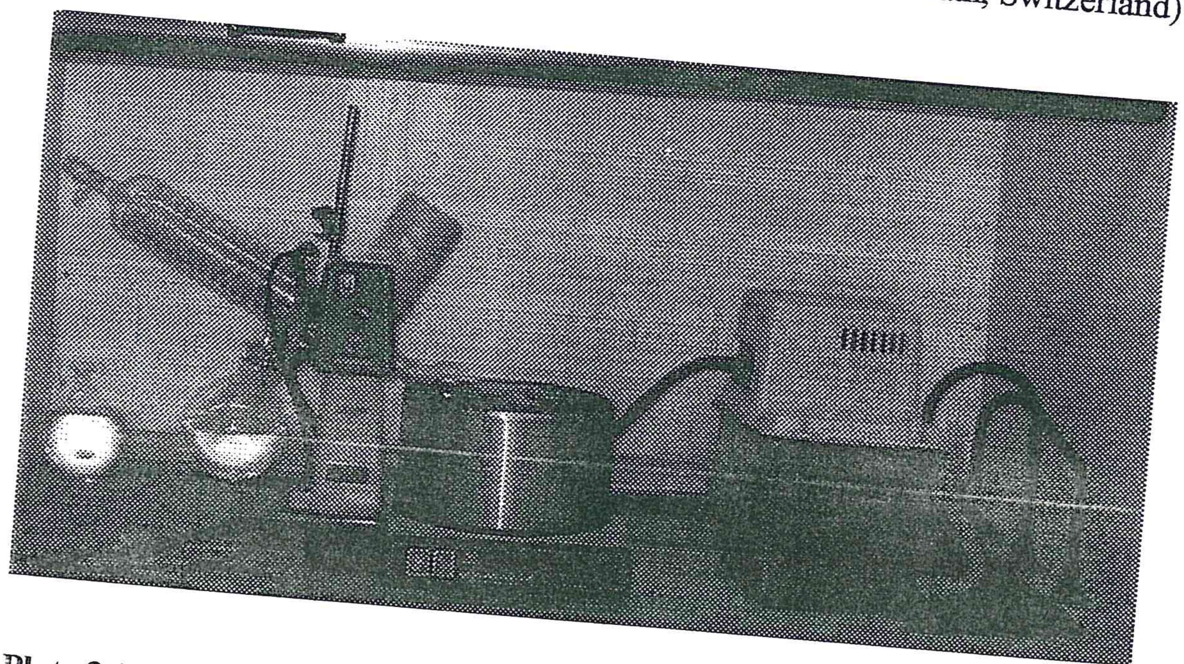


Plate 3.1. Rotavapor (Büchi 461, water bath, Switzerland)

The residual kernel powder was then re-extracted with hexane (three replicates 30 ml each). The upper hexane layers from above were mixed with the hexane layer obtained earlier and concentrated in *vacuo* to give the oil. All oil samples prepared were stored at -15°C.

3. 4. 4 Extraction of the total oil

The oil from the neem seed kernel powder was extracted in a soxhlet apparatus (plate 3.2). Extraction was done in two replicates; three grams of neem seed powder were placed in a thimble and 500 ml of hexane were used to extract the oil. Each sample was replicated twice. Neem oil percentage was calculated by weighing the flask before and after extraction using the following formula:

$$\text{Oil \%} = \frac{wt3 - wt2}{wt1} X100$$

where: $wt1$ = weight of the neem kernel powder

$wt2$ = weight of the empty flask

$wt3$ = weight of the flask with oil

3. 4. 5 Chromatography

Analytical High Performance Liquid Chromatography (HPLC) (Beckman HPLC, System Gold) (plate 3.3) separation was performed on ODS columns, ultrasphere C-18, 250×4.6 mm, programmed as follows: 40% acetonitrile in water (10 min.) to 70% acetonitrile (10 min.) and finally to 100% acetonitrile (5 min.), all at 1 ml/min. The eluents were monitored at 214 nm. Quantification of the limonoids was based on injections of known quantities of standard samples.

The ultra-violet spectra of crude substances were determined using an on-line diode array detector on a running acetonitrile/water solvent system. Each sample was analyzed three times.

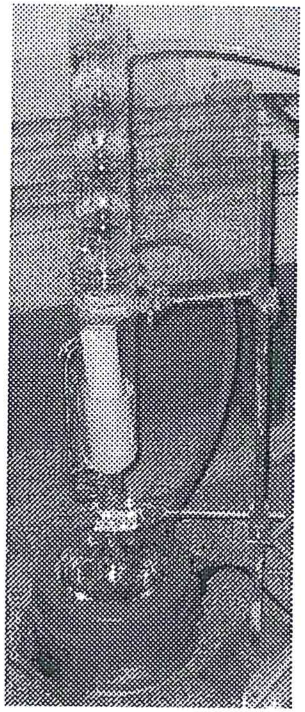


Plate 3.2 Soxhlet apparatus

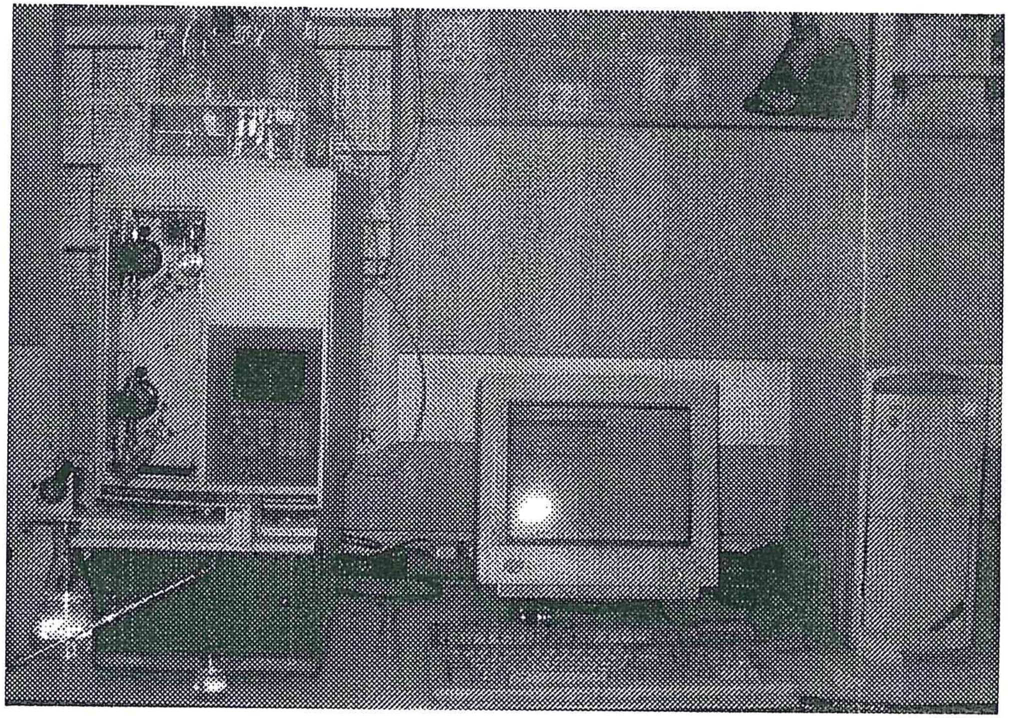


Plate 3.3. Analytical High Performance Liquid Chromatography (Beckman HPLC, System Gold).

3.4.6 Source and preparation of standards

The standard substances azadirachtin A and B (96 % purity), salannin and nimbin were bought from Trifolo-M-GmbH (Lahnau, Federal Republic of Germany). 1.00 µg of each was dissolved in 5 ml of methanol.

3. 4. 7 Calculation of the limonoids contents

The amount of the azadirachtin, salannin and nimbin in dry matter of neem kernel for each replication of a sample is calculated by applying the following formula

$$\chi S = \frac{[\chi D]\times[V]\times Df \times 100}{[W]\times(100 - MC)}$$

χ S	=	limonoid content of the sample [mg/g dry matter]
χ D	=	limonoid content in the dilution [mg/ml]
V	=	total volume of the extract [ml]
Df	=	dilution factor
W	=	sample fresh weight [g]
MC	=	mean weight loss on drying [%]

3.4.8 Meteorological and geographical data

Meteorological (rainfall, temperature and relative humidity) and geographical positioning (GPS) data (latitude, longitude and altitude) for the selected agro-ecological zones were obtained from the Meteorological Department, Sudan (Table 3.1)

3.4.9 Data analyses

Data were analyzed using SAS (SAS Institute Inc., V8, 1987; Cary, North Carolina, USA). Separation of means for the means of the three compounds (azadirachtin , salannin, nimbin) and the combination of the three limonoids were carried out using Honest Significant Difference (HSD) test for equal replications (P=0.05) to compare the limonoid content in the different zones. Correlation and multiple regression analysis were also carried using the same software to find the relations between the meteorological data, geographical position data and the limonoid contents. GLM and PCA procedures were also used.

CHAPTER FOUR
RESULTS AND DISCUSSIONS

4.1.1 Seed characteristics

The statistical parameters (mean and standard error) for the seeds collected form various ecological zones in season 2000-2001 are presented in Tables 4.1, 4.2 and 4.3. There were highly significant differences ($P<0.0001$) between various seed zones in the number of seeds per kilogram, seed length, seed width, seed weight and kernel weight. However, there were no significant differences in these traits between different trees within the same seed zone Table (4.3). These variations could be attributed to the climatic differences between the various ecological zones in Sudan, in addition to soil types and moisture content which play an important role in the number of seed per kilograms. The result confirmed the findings of Kundu (1999) who stated that; highly significant ($P<0.0001$) differences were recorded among ten provenances in neem seed related traits. The same results were found in *B. aegyptiaca* and were attributed to substantial genetic variations within and between progenies (Ladipo,1989).

Seed kernel dry weight was found to be varying from 97.26 mg to 38.22 mg; while that of the seed shell varied from 128.5 mg to 75.29 mg. Seed kernel weight is a critical factor when dealing with the oil and limonoids production. Mahgoub (2002) reported that the study of the seed structure and the seedling morphology revealed strong correlation between the seed, the seedlings, and their respective environments.

Seed weight is a useful parameter in the calculation of the number of seeds/kg and hence the estimation of the seed demands and production per unit area. Generally seed shell was found to have higher contribution to the seed weight compared to the kernel weight. Variations in seed weight, size and growth related characters have also been reported by Verendra (1995); Surendran *et al*. (1993) and Dwivedi (1993).

Table 4.1 Variations in traits of the neem seed collected from various eco-zones in Sudan.

Zone	Seed		
	Length (cm)	Width (cm)	Number of seeds /kg
	Mean ± SE	Mean ± SE	Mean ± SE
S1	1.271 ±0.002ab	0.678 ±0.009ab	5011.550 ±118.223b
S2	1.275 ±0.013ab	0.658 ±0.005ab	4571.090 ±32.898cd
S3	1.264 ±0.017ab	0.701 ±0.058ab	4922.500 ±7.335b
S4	1.216 ±0.019b	0.641 ±0.008ab	5565.090 ±33.512a
S5	1.214 ±0.014b	0.655 ±0.006ab	4922.650 ±24.329b
S6	1.262 ±0.007ab	0.671 ±0.010ab	4891.560 ±30.471bc
S7	1.237 ±0.009b	0.669 ±0.004ab	4699.250 ±32.224bcd
S8	1.312 ±0.016a	0.621 ±0.008b	5514.990 ±51.170a
S9	1.255 ±0.009ab	0.682 ±0.003ab	4505.300 ±59.752
S10	1.258 ±0.028ab	0.720 ±0.014a	4691.690 ±85.325

Means followed by the same letter in the same column are not significantly different at $P<0.05$ (Tukey's HSD test).

Table 4.2 Variations between the weight of neem seed and kernel from ten ecological zones in the Sudan.

Zones	Kernel weight (mg) Mean ± SE	Shell weight (mg) Mean± SE
S1	71.814±1.714 d	121.444±0.756 ab
S2	82.409±0.729 bc	111.613±2.576 c
S3	75.250±2.440 cd	75.293±1.302 f
S4	73.670±1.302 cd	89.678±1.431 e
S5	85.412±2.390 b	110.326±3.030 c
S6	87.271±0.862 b	97.997±1.567 d
S7	73.557±2.398 cd	128.503±3.344 a
S8	38.215±0.889 e	127.620±1.239 a
S9	97.261±3.402 a	118.978±3.489 b
S10	82.399±4.019bc	128.137±1.388a
CV%	7.880	7.169

Means followed by the same letter in the same column are not significantly different at $P<0.05$ (Tukey's HSD test).

Table 4.3 Seed variations between trees within the same site in the Sudan

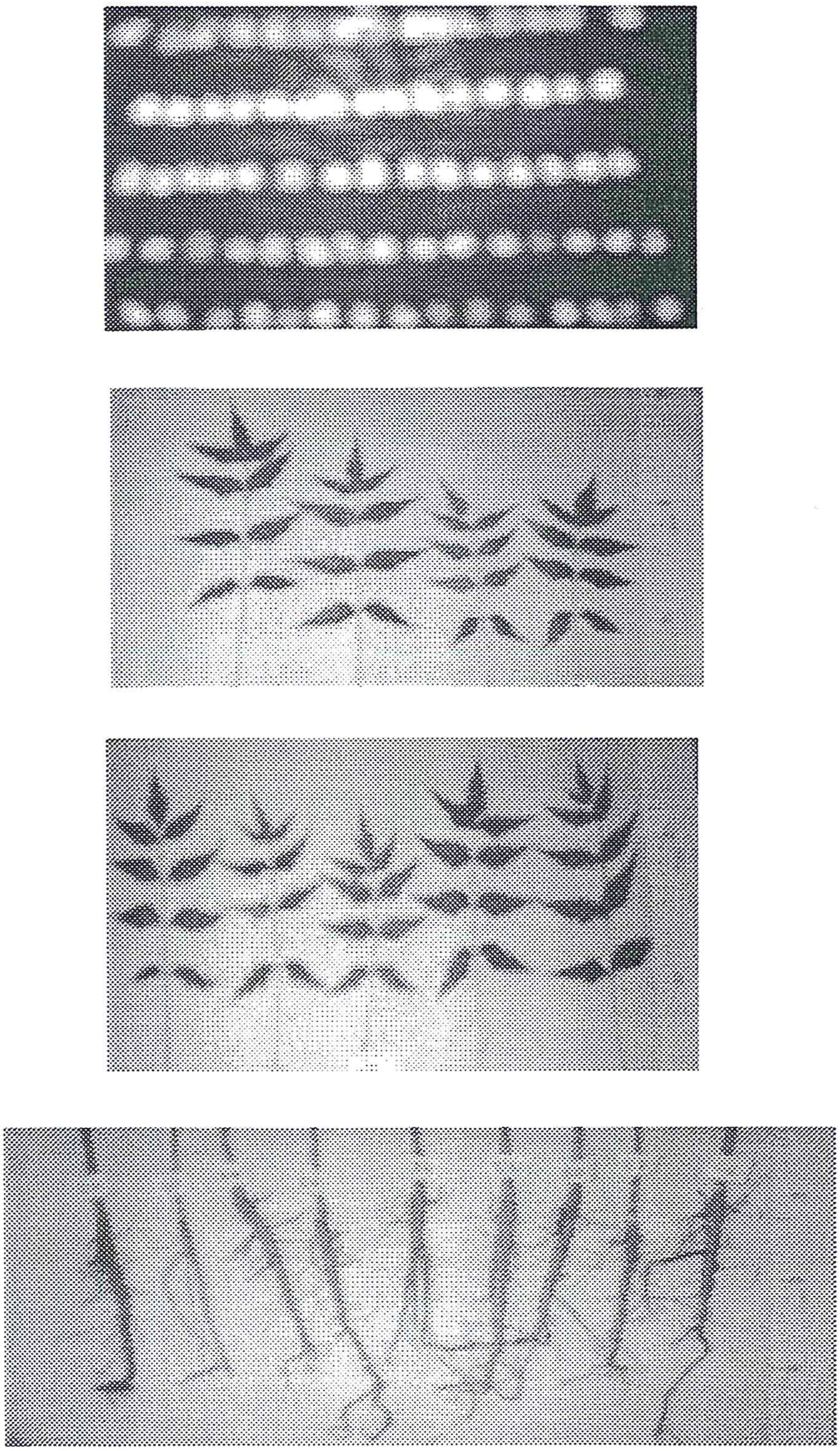
Tree no.	Seed		
	Length (cm) (Mean ± SE)	Seed width (cm) (Mean ± SE)	Number of seed/kg (Mean ± SE)
1	0.630 ±0.005a	1.199 ±0.041a	5832.510 ±170.191a
2	0.642 ±0.005a	1.187 ±0.009a	5444.200 ±33.531a
3	0.614 ±0.039a	1.192 ±0.025a	5621.790 ±50.589a
4	0.617 ±0.004a	1.174 ±0.007a	5742.070 ±64.222a
5	0.629 ±0.003a	1.187 ±0.005a	5675.560 ±83.824a
6	0.628 ±0.006a	1.186 ±0.011a	5584.570 ±80.435a

Means followed by the same letter in the same column are not significantly different at $P<0.05$ (Tukey's HSD test).

4.1.2 Provenance Variations

The statistical parameters (mean and standard error of the mean (SE)) for the measured traits along with statistical significance at the 5% level of probability are presented in tables (4.4 & 4.5). In the fourth destructive measurements (A4) slight significant differences between seed sources in leaf length (P=0.009), root length (P=0.01) and root dry weight (P=0.03) were detected. However, in the fifth destructive measurement, (A5) no significant differences were detected in all measured traits except on the leaf-let ratio (P=0.03). In general, no significant morphological variations between the provenances were observed over most of the growth traits investigated, except the diameter at collar. Little or no significant variation among neem provenances in growth traits was observed, and this may suggest that the genetic profile of neem tree in Sudan is narrow. Furthermore, there was significant age effect in all provenances. However, the variations within the ages were not significantly different except the total number of leaves in the first and last destructive measurements (A1, with P=0.01 and A7 with P=0.02) and root dry weight in A7 (P=0.003), Tables (4.4 and 4.5). Plant height, root length, collar diameter, leaf length, leaflet ratio and dry matter weight are important and easily measured traits for an early evaluation of seed sources before further field work.

Morphological variations in seed and plant traits are easily recognized in neem populations. As shown in Fig. (4.1), slight morphological variations can be observed in the leaves, root length and shoot height of the different seed sources in Sudan. Kundu (1999) reported significant variation among neem provenances in seed and growth traits for an early evaluation of neem seed sources in both the growth chamber and the field from different areas in the world. Variation on morphometeric and pheonological traits of neem provenances have been reported by Dwivedi (1993), Surendran *et al.* (1993), Rajwat *et al.*, (1994), Verendra (1995), Gupta *et al*. (1996) and Kundu (1999).



a

b

c

Fig. 4.1 Morphological variations in some neem provenances in Sudan. a) Seeds ; b) leaves ; c) roots.

Overall, this experiment revealed no significant differences between the neem provenances in most of the early growth traits studied. This result could be attributed to the narrow genetic profile of the tree in the Sudan, and little or no genetic /environment interaction. Other studies found distinct ecotypes differences within India (Rajwat *et al.*, 1994) and between different areas in the world (Ermel, 1995). Geographical variations have also been reported (Verendra, 1995; Surendran *et al.*, 1993; Dwivedi, 1993).

Correlation coefficient (r) among all possible combinations of variables both the biometric traits and geoclimatic data of the seed source are summarized in tables (4.6 and 4.7), respectively. No significant correlations were found within the measured traits, and between the measured traits and geoclimatic data except between the leaf length and altitude which was significant and positive relation at ($P<0.05$). The correlation between temperature and the distance between the cotyledons scars and the first two leaves was significant and negative. Kundu (1999) found strong correlations between numbers of leaves, shoot: root ratio with mean annual rainfall at the field level in neem provenances, which indicates adaptation to water availability.

Table (4.4) Variations between various seed zones in the seedling growth traits for the first age (A1) under nursery conditions in the Sudan.

Trait		S1	S2	S3	S5	S6	S8	S9	S10
Sh.H	Mean	26.211a	38.02a	31.944a	26.023a	32.167a	31.48a	29.68a	38.457a
	±SE	1.878	5.672	2.352	3.508	5.123	1.135	2.672	2.931
R.L	Mean	19.822 a	23.067a	22.8a	21.047a	21.433a	18.253a	20.657a	20.266a
	±SE	1.478	0.958	1.549	0.169	0.89	1.157	1.226	1.607
CD	Mean	3.333a	4.953a	4.211a	4.523a	4.373a	4.247a	4.52a	4.947a
	±SE	0.107	0.226	0.326	0.186	0.484	0.423	0.56	0.251
DCL	Mean	2.945a	2.243	2.411a	2.357a	1.98a	2.143a	2.837a	3.067a
	±SE	0.232	0.125a	0.299	0.209	0.105	0.127	0.088	0.278
Sh.D.Wt	Mean	0.962a	2.095	1.398a	1.203a	1.935a	1.772a	1.543a	1.781a
	±SE	0.122	0.417	0.093	0.136	0.488	0.325	0.383	0.268
R.D.Wt	Mean	0.323a	0.703a	0.559a	0.624a	0.833a	0.805a	0.543a	0.698a
	±SE	0.098	0.016	0.188	0.072	0.112	0.242	0.171	0.11
NR	Mean	1.223a	1.334a	1.333a	1.333a	1.447a	1.447a	1.78a	1.223a
	±SE	0.223	0.334	0.193	0.193	0.223	0.223	0.485	0.223
TL	Mean	10.111b	17.443a	10.934ab	13.557ab	17.223a	16.223ab	11.9ab	15.657ab
	±SE	0.689	1.724	0.657	0.953	2.279	1.223	1.029	1.572
LL	Mean	10.111a	12.8a	10.934a	10.867a	11.39a	10.7a	15.11a	12.344a
	±SE	0.689	1.017	0.657	1.131	1.54	0.669	1.602	0.251
Lt.R	Mean	8.007a	12.628a	8.825a	10.568a	10.794a	9.05a	11.637a	8.781a
	±SE	0.461	1.37	0.871	1.131	1.459	1.75	2.328	0.549

Means followed by the same letter in the same raw are not significantly different at $P<0.05$ (Tukey's HSD test).

Table (4.5). Variations between various seed zones in the seedling growth traits for the last month (A7) under nursery conditions in the Sudan

Trait		S1	S2	S3	S5	S6	S9	S10
Sh.H	Mean	48.539a	57.444a	64.589a	66.467a	68.367a	53.367a	62.656a
	±SE	5.849	4.521	10.81	1.938	6.399	4.189	5.942
R.L	Mean	25.017a	30.222a	27.968a	30.033a	29.79a	53.367a	62.656a
	±SE	2.926	1.073	1.751	2.118	1.161	4.189	5.942
CD	Mean	8.611a	9.478a	9.234a	9.633a	9.767a	6.833a	7.611a
	±SE	0.772	0.529	0.859	0.924	0.906	0.552	0.741
DCL	Mean	3.303a	3.089a	2.989a	2.8a	3.000a	3.300a	3.280a
	±SE	0.253	0.216	0.095	0.208	0.115	0.126	0.109
Sh.D.wt	Mean	5.546a	6.331a	6.517a	5.891a	7.514a	3.846a	5.431a
	±SE	2.098	0.777	1.527	1.288	1.12	0.548	0.697
R..D.wt	Mean	3.281c	5.413abc	5.913ab	5.498abc	6.571a	3.361bc	3.782abc
	±SE	0.504	0.364	0.358	0.638	1.02	0.351	0.428
RN	Mean	1.000a	1.334a	1.668a	1.333a	1.447a	1.334a	1.111a
	±SE	0.000	0.193	0.51	0.333	0.223	0.193	0.111
TL	Mean	25.945b	31.889ab	30.567ab	30.344ab	34.8a	26.122b	31.667ab
	±SE	1.498	0.889	1.484	1.34	2.722	1.83	1.644
LL	Mean		19.756a	19.567a	20.467a	21.567a	18.389a	18.933a
	±SE		0.656	0.731	0.617	1.129	0.964	0.391
Lt.R	Mean		8.58a	10.067a	8.883a	9.744a	8.799a	6.926a
	±SE		0.895	0.669	0.456	2.944	0.956	1.237

Means followed by the same letter in the same raw are not significantly different at $P<0.05$ (Tukey's HSD test).

Table (4.6). Correlation coefficient (r) among the seedling traits (provenance mean basis) of *Azadirachta indica*.

Traits	Sh.H	R.L	CD	DCL	Sh.D.wt.	R.D.Wt.	RN	TL	LL	Lt.R.
Sh.H	1.00									
R.L	0.50***	1.00								
CD	0.77***	0.67***	1.00							
DCL	0.32*	0.11	0.19	1.00						
Sh.D.Wt.	0.91***	0.63***	0.92***	0.29*	1.00					
R.D.Wt.	0.77***	0.74***	0.95***	0.19	0.91***	1.00				
RN	-0.02	-0.07	0.04	0.10	-0.08	0.02	1.00			
TL	-0.08	0.10	-.06	-0.02	-0.06	-0.06	-0.02	1.00		
LL	0.88***	0.65***	0.88***	0.31*	0.92***	0.87***	-0.01	-0.02	1.00	
Lt.R	-0.26	-0.08	-0.2	-0.36**	-0.2	-0.07	0.01	0.09	-0.1	1.00

* Significant, ** highly significant and *** very highly significant

Table (4.7). Simple correlation (r) between traits studied (provenance mean basis) and climatic data of 10 provenances of *Azadirachta indica* in Sudan

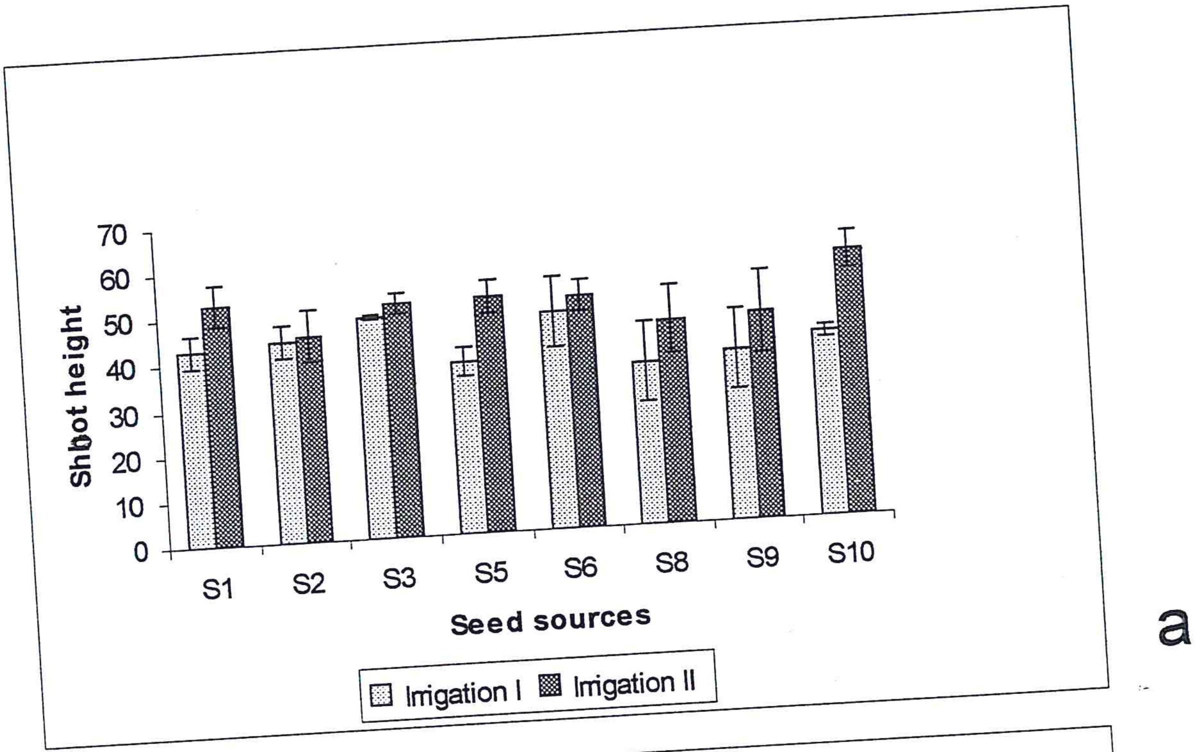
Trait	R.L	Sh. H	CD	DCL	Sh.D.Wt	R.D.Wt.	RN	TL	LL	Lt.R
RF	0.046	0.141	0.040	-0.115	0.055	0.056	-0.086	0.22	0.191	-0.004
Temp.	0.080	0.142	0.087	-0.352*	0.065	0.138	0.245	0.019	-0.036	0.046
RH%	0.003	0.139	0.041	-0.113	0.052	0.051	-0.076	0.225	0.129	-0.037
Altit.	0.210	0.149	0.074	-0.020	0.098	0.096	-0.07	0.022	0.289*	-0.112

*Significant

4.1.3 Effect of Two Types of Irrigation Regimes on the Neem Ecotypes in the Nursery

This experiment was carried out over two successive seasons to investigate the effect of 10 successive drought periods, of 10 days each, in comparison to the daily irrigation, which is the widespread practice in most of the traditional nurseries for raising neem seedlings in Sudan.

Results showed highly significant differences (P<0.0001) between the two regimes of the irrigation adopted in almost all parameters measured; seedling height, root length, shoot dry-weight, root dry-weight and survival percentage in the first- season (2001-2002), and the second-season (2002-2003) as shown in tables (4.8 and 4.9) respectively for the seasons. No variations were found between the seed sources within the same treatment (irrigation regime) in all measured traits (Fig 4.2, 4.3 and 4.5). However, there was a clear morphological difference between the two regimes of irrigation in terms of growth and the color of the leaves (Fig. 4.4).



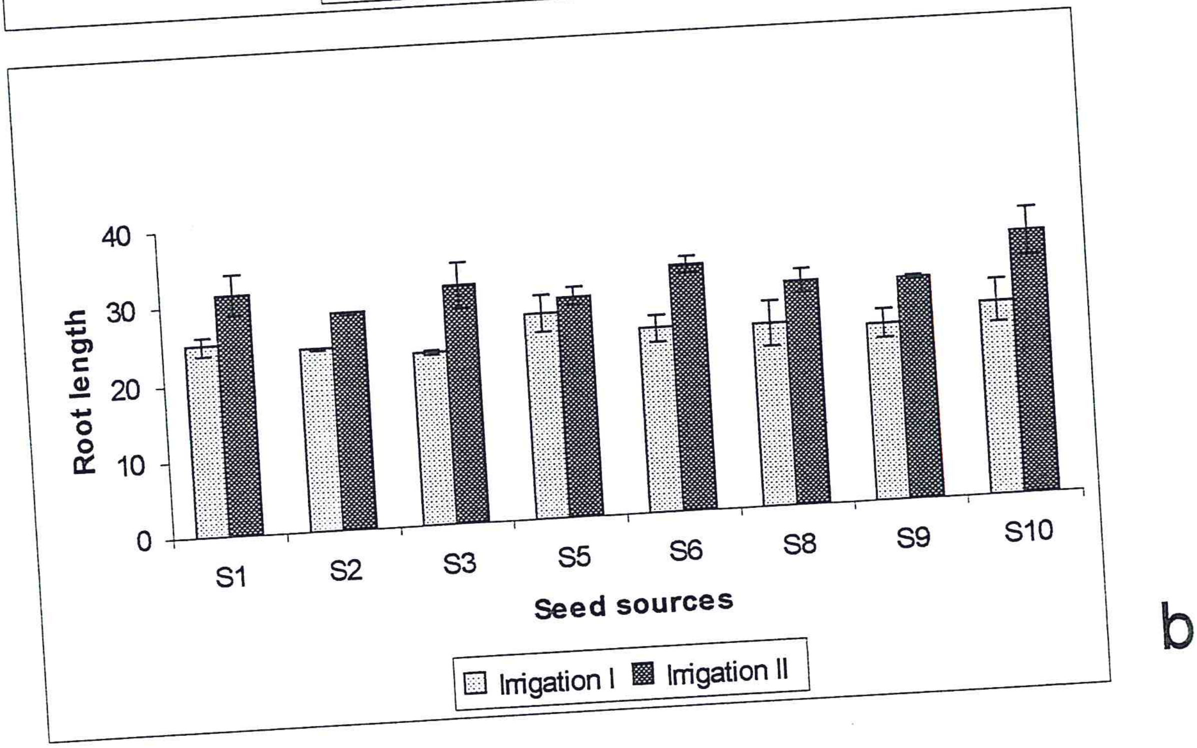
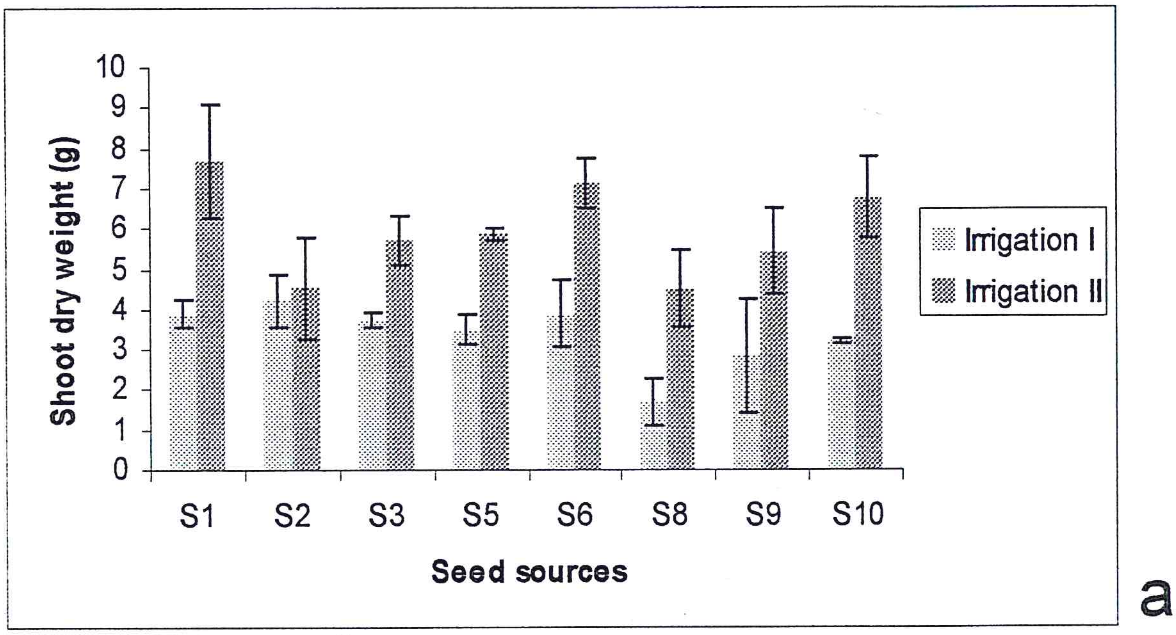


Fig. 4.2 Effects of two Irrigation regimes on: a) Shoot height and b) Root length of neem seedlings from different ecozones.



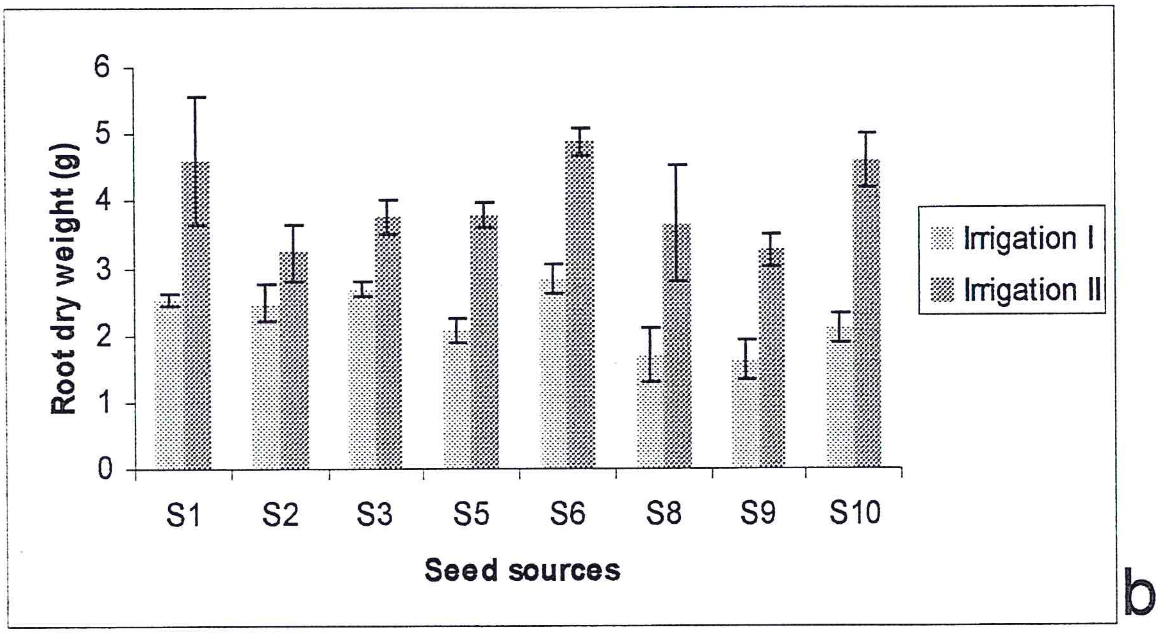
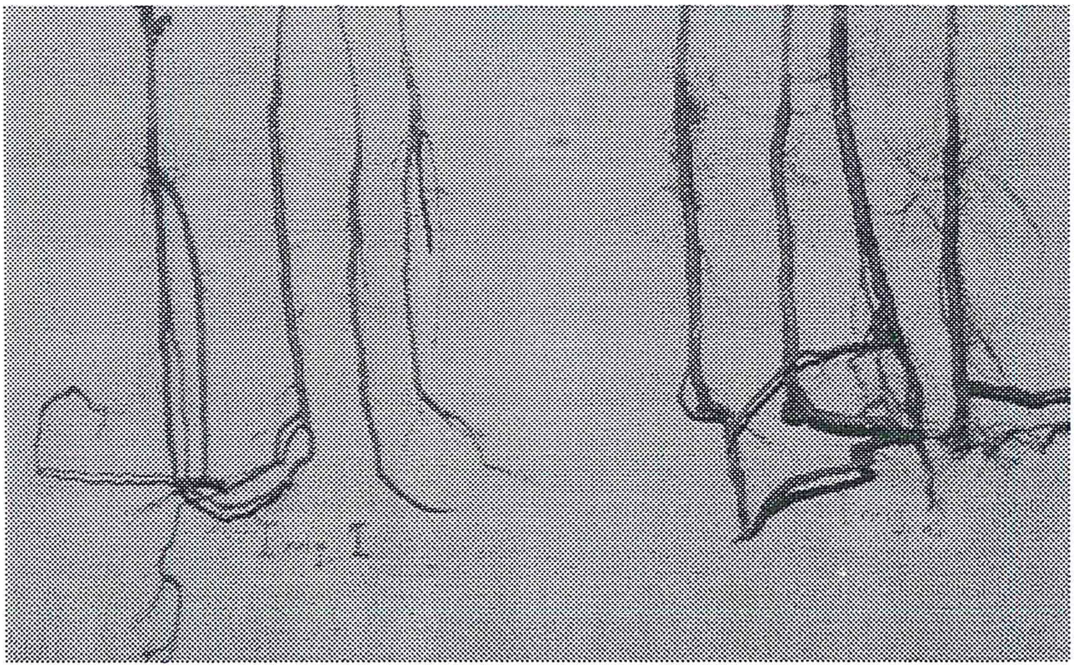


Fig. 4.3 Effects of two Irrigation regimes on: a) Shoot dry weight and b) Root dry weight of neem seedlings from different ecozones.

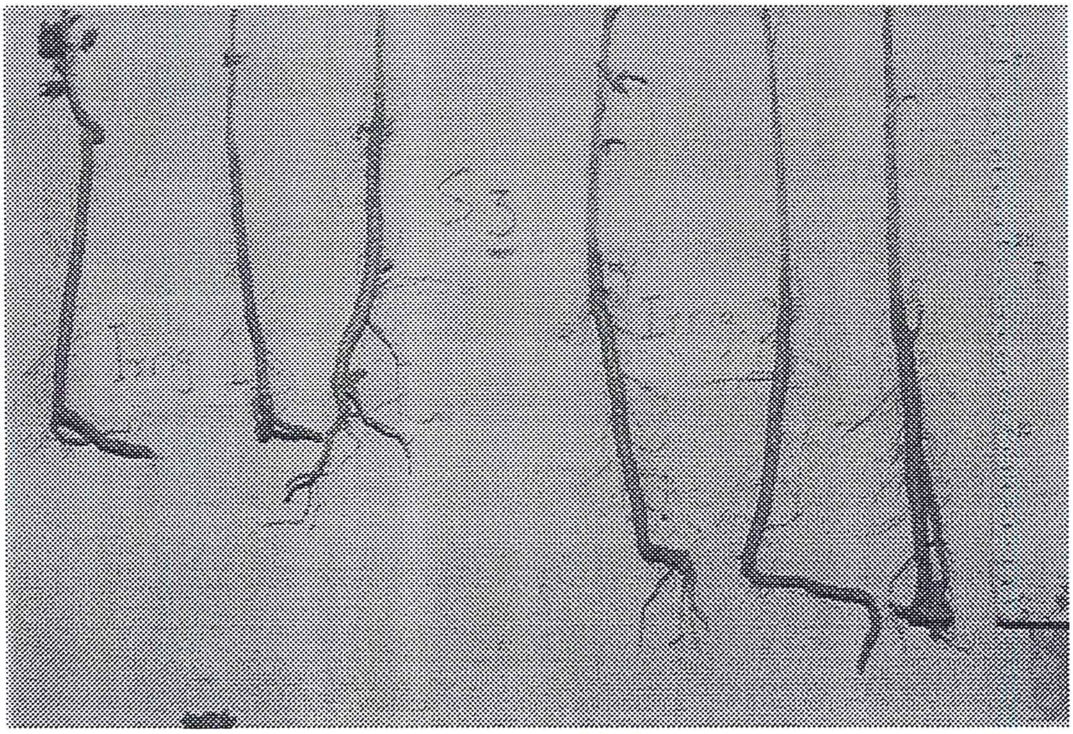
(a)

(b)

Fig. 4.4 Effects of two Irrigation regimes on: neem seedlings from different ecozones: a) Irrigation I and b) Irrigation II



a



b

Fig. 4.5 Morphological variations between the root system of two different seed sources: a) S9 and b) S3.

Table (4.8). Effects of two irrigation regimes on the seedling height, root length, shoot dry weight, root dry weight and survival percentage (season 2001-2002).

Watering regime	Sh. H (cm)	R.L (cm)	Sh. D. t. (g)	R. D. Wt. (g)	Survival %
I	42.03 b	24.32 b	3.37 b	2.25 b	63.33 b
II	50.24 a	30.56 a	5.95 a	3.97 a	94.17 a

Watering regime I = Irrigation every 10 days.
Watering regime II = Irrigation every day

Means followed by the same letter in the same column are not significantly different ($P<0.05$). (Tukey's HSD test).

The Overall effect of the Two Regimes of Irrigation on the Neem Ecotypes

The overall effect of this experiment revealed no morphological and survival variation between the ecotypes studied, although there were slight variations on the biomass, (Table 4.10). These results might be attributed to the limited genetic profile of the neem tree in Sudan. Genetic variations of drought tolerance have been found within tree species with extensive geographic or habitat range by several workers (Bongarten *et al.*, 1986; Abrams *et al.*, 1990; Parker and Pallardy 1991). This result strengthens the previous finding no variations between the neem seed sources in Sudan during the first year of the establishment in the nursery. Neem seedlings are highly tolerant to drought and can survive under harsh conditions with insignificant changes in both the root length and weight.

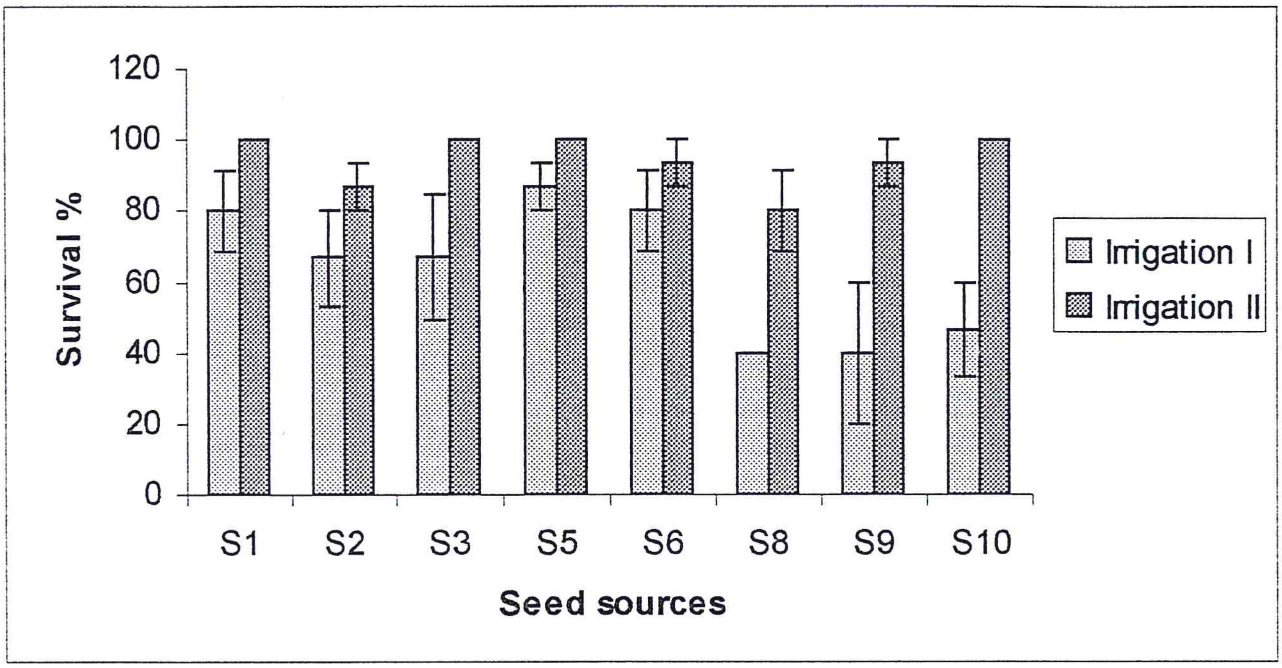


Fig. 4.6 Effect of two irrigation regimes on survival % of different neem ecotypes in Sudan

Table 4.9. Effects of two irrigation regimes on the neem seedling parameters (season 2002-2003).

Watering regime	Sh. H (cm)	R.L (cm)	Sh. D. Wt. (g)	R. D. Wt. (g)	Survival %
I	36.50 a	26.58 b	3.16 b	1.39 b	65.53 b
II	34.02 a	31.35 a	4.47 a	1.75 a	90.14 a

Watering regime I = Irrigation every 10 days
Watering regime II =. Irrigation every day

Means followed by the same letter in the same column are not significantly different (P<0.05). (Tukey's HSD test).

Part Two

Limonoids in the neem seed kernels

4.2.1 Yield of the neem oil

Neem oil was calculated as percentage of the original dry weight of the neem seed kernel. Neem oil percentages are found to vary from 41.1% to 47.4% (at 5% mc). The average yield of the country was found to be 44.6%. No variation was found between the different regions in the oil-contents. The same results were obtained by Ermel (1995) where he found that; the variations in oil-content were less than those in azadirachtin. Moisture-content is an affecting factor on the oil-content, and further relationships are needed to find out the most affecting factors. However, Rengasamy *et al.* (1993) reported that; oils of different ecotypes showed different azadirachtin content and physio-chemical properties in India, while no correlation was found between the neem oil- and its Az-content (Ermel, 1995).

4.2.2 Limonoids in the neem seed kernels

The limonoids results presented in this section are AZ (the sum. of AZ-A + AZ-B), salannin, and nimbin (Fig 4.7). The total amount of the limonoids was the summation of the three limonoids.

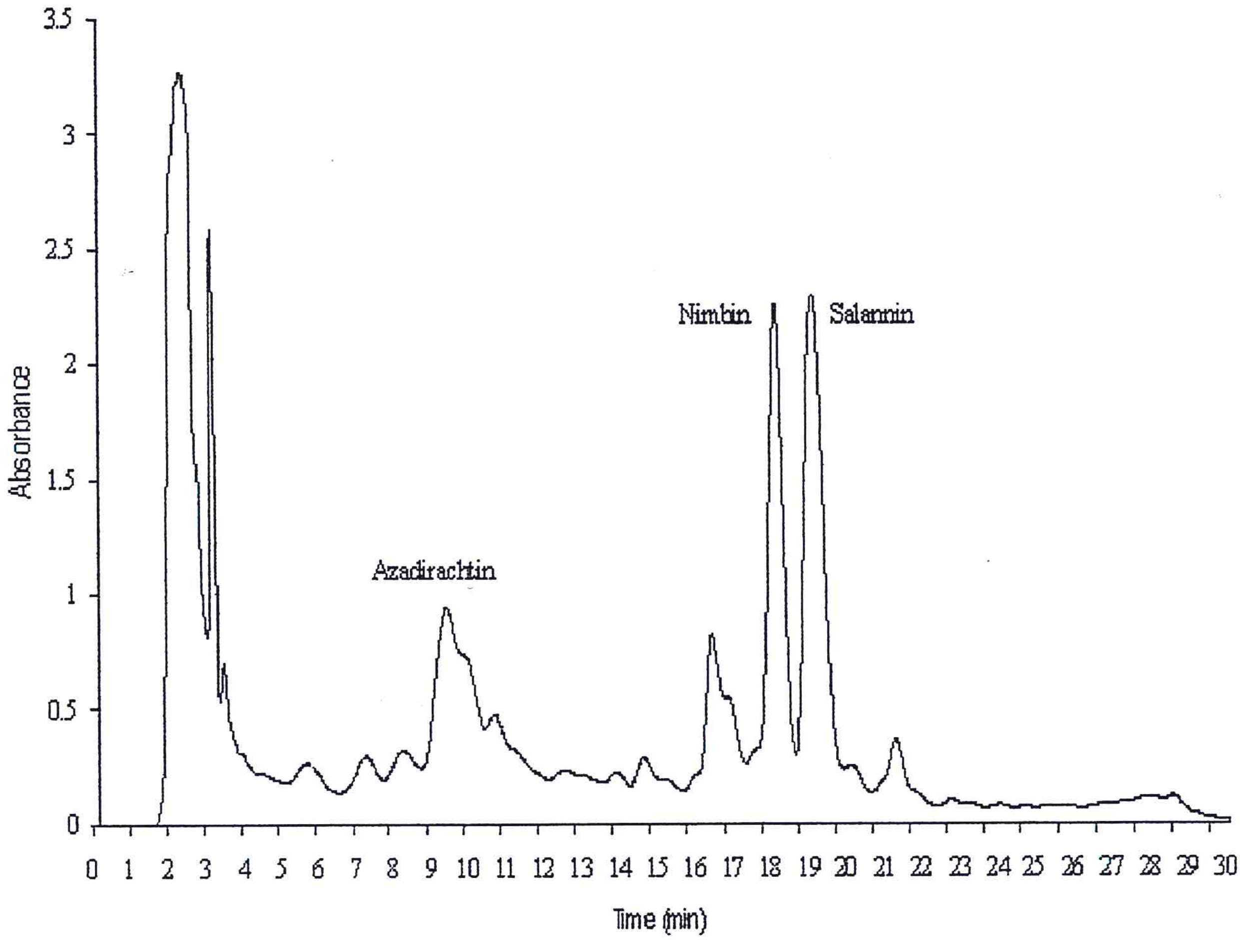
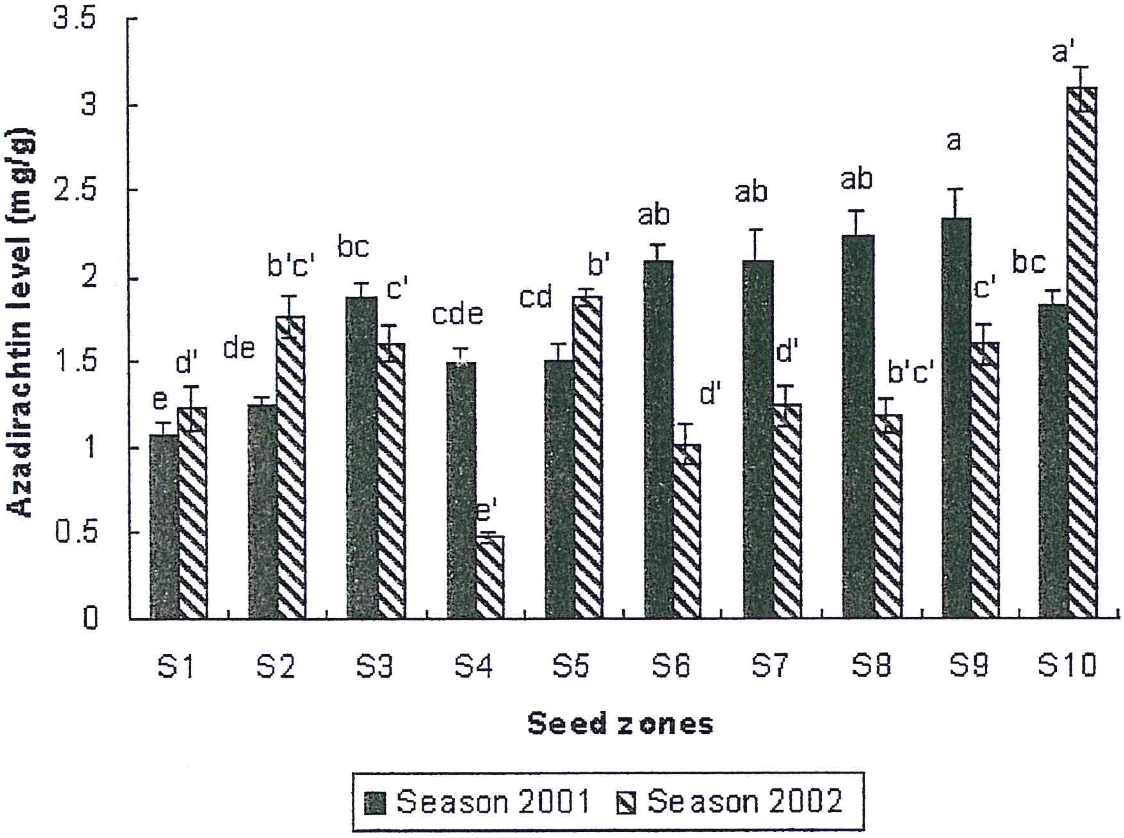


Fig. 4.7 High performance liquid chromatography of the neem seed kernel methanol extract

4.2.2.1 Variation in the AZ- level between zones

Results showed significant ($p<0.0001$) variations in AZ- level between the different zones in the Sudan (Fig. 4.8; CV = 25.65% and CV = 16.96%, for the first- and second- seasons, respectively). AZ-content varied from 1.0771 mg/g to 2.3444 mg/g for season 2001 and from 0.476 mg/g to 3.091 mg/g for season 2002. Moreover, there were significant variations between the different sites within the zones, and between the trees of same the sites. Similar results were reported by (Ermel *et al.*, 1984, and 1987). Ermel *et al.* (1987) determined AZ-contents in NS of different countries and found that the highest yield of AZ-content per NSK is not restricted to a specific country, but it is distributed in trees of different origin. Rengasamy *et al.* (1993) reported that the neem ecotypes of India showed varying AZ-content (0.14 to 1.66%). The present work results were inline with the findings of Ermel (1995) who investigated seed samples from different parts of the world which showed great variations between and within countries. These present work results were also inline with investigations of Gruber (1991) on AZ-content of 47 marked trees from five locations in Nicaragua over a period of 4 years. The latter study revealed the great influence of edaphic and climatic factors on the synthesis and degradation of AZ. In another study (Kumar *et al.*, 1995) variation was established between the ecotypes within the zones, and even between the trees in the same site. AZ-content (2895 to 7525 ppm) has also been observed in the NSK of the different ecotypes of Tamil Nadu (Kumar *et al.*, 1995).

The present work results variations could be attributed to the edaphic and climatic factors in the Sudan. Very few studies have been carried out so far in the world to detect the existing variability of AZ- content in neem trees, and even fewer on salannin, nimbin and oil-content variability line with the present work results. Therefore, it is now concluded that there is great variation between ecotypes within the zones and between the different trees growing in the same site in the Sudan. Total amount of the limonoids also showed similar variations trend (Table 4.9).



Columns with the same letter are not significantly different at $p \leq 0.05$

Fig 4.8. Variation in the azadirachtin level (mg/g) from different zones in Sudan.

Table 4.11 Variations in the total amount of limonoids and azadirachtin (AZ) in neem seed kernels from different zones in Sudan.

Zone	AZ (mg/g)*	Total(mg/g) *
S1	1.0771 ±0.0736[e]	4.1286 ±0.2154[c]
S2	1.2454 ±0.05286[de]	4.4631 ±0.0848[c]
S3	1.8696 ±0.08677[bc]	6.2766 ±0.213[b]
S4	1.4884 ±0.0906[cde]	4.8287 ±0.26[c]
S5	1.5064 ±0.097[cd]	4.8066 ±0.2462[c]
S6	2.0969 ±0.0989[ab]	6.6798 ±0.1276[ab]
S7	2.0995 ±0.1649[ab]	6.6354 ±0.2754[ab]
S8	2.2426 ±0.1544[ab]	6.9247 ±0.2847[ab]
S9	2.3444 ±0.1531[a]	7.1594 ±0.2922[a]
S10	1.8184 ±0.0963[bc]	6.2897 ±0.1636[b]

Means followed by the same letters in the same column are not significantly different ($p<0.05$), Tukey's Student Range (HSD) test.
.* mean± SE

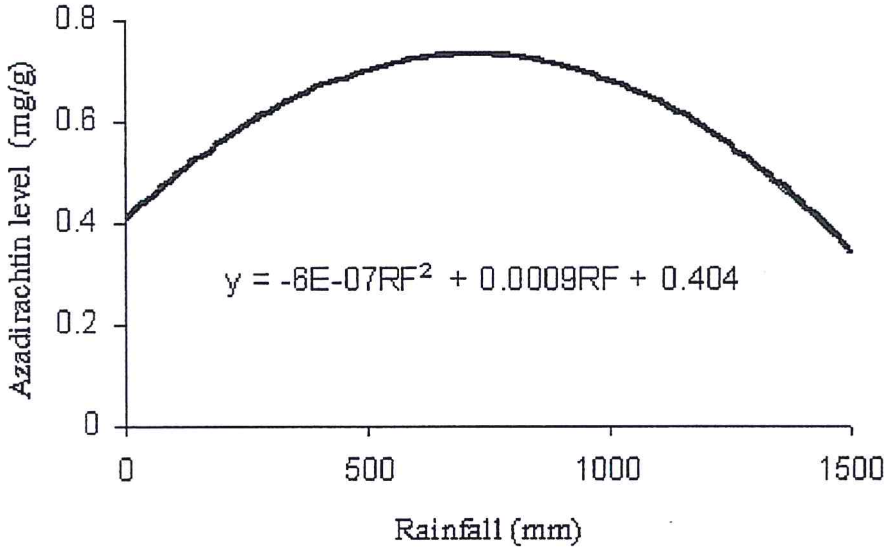
4.2.2.2 Effect of the Meteorological and Geographical Factors on AZ-Level in NSK

Simple and multiple regressions were used to find out the possible relations of meteorological factors with AZ-level (concentration) in NSK collected from 10 different zones. The results showed no significant temperature effect on the level of the AZ in NSK; however, the rainfall was significantly affecting AZ-concentration, and the optimum rainfall was found to be 717.9 mm (Fig. 4.9). There was a significant negative relationship between AZ and latitude (Table 4.10), which is a reflection for the rainfall effect, while altitude showed a significant positive effect on AZ-content (Fig. 4.10). This result coincides with that of Baumgart (1991) in Benin, who found that in areas with a high rainfall, AZ concentrations showed increases. On the contrary, Singh (1987) reported high antifeedant activity of extracts from NSK of trees growing in arid areas in India, when compared with those growing in coastal areas.

The R.H. and temperature showed no significant correlation with AZ-level, but the combination of temperature and rain fall revealed a significant effect on this parameter (P=0.04) (Fig 4.11).

The AZ-content therefore, was found to be affected by rainfall and altitude. Zones with moderately high rainfall (up to 712 mm.) proved to produce NSK rich in the AZ-content; while zones with hot dry weather (mean annual temperature 30°C and 25% R.H.) were found to have lower content. Seasonal and annual variations in the AZ-content were also observed over the country; this is likely to be environmental more than geneticl variations. Similar results were obtained within an investigation in India carried out by Rengasamy *et al.* (1993), who mentioned that ecotypes growing in zones with moderate climate, red and shallow medium soils, and altitudes up to 500 m (a.s.l.) were rich in AZ-content.

Ecotypes growing in high altitude alluvial soils, with extremely hot and cold climates, were poor in AZ-content. These studies lack detailed meteorological correlations to the AZ-contents, and can be taken as indicators only. On the other hand, efforts are underway at CRIDA, Hyderabad, on correlating the different factors, *e.g.* soil type, rainfall, and R.H. with the AZ-content in the seeds for the trees growing in different parts of Andhra Pradesh State (Veerendra, 1995). These studies suggest that there is a need to do the chemical evaluation of the seeds from diverse zones to identify neem trees with high amounts of oil and AZ to make the plant economically more attractive.



The optimal rainfall = 717 mm

Fig. 4.9 The trend of azadirachtin level in neem seed kernels with the Rainfall in Sudan

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Table 4.12 Pearson correlation coefficients of the ecological factors and the limonoids concentrations.

	AZ	Sa	Ni	Total
Temp	-0.006^{NS}	0.095^{NS}	0.099^{NS}	0.002^{NS}
	(0.880)	(0.622)	(0.816)	(0.997)
RF	0.738*	0.786*	0.378^{NS}	0.726*
	(0.037)	(0.021)	(0.356)	(0.042)
R H	0.513^{NS}	0.616^{NS}	0.115^{NS}	0.511^{NS}
	(0.194)	(0.102)	(0.787)	(0.196)
Alt.	0.725*	0.551^{NS}	0.561^{NS}	0.644^{NS}
	(0.042)	(0.157)	(0.148)	(0.085)
Lat.	0.790*	0.765*	-0.487^{NS}	-0.756*
	(0.020)	(0.027)	(0.221)	(0.030)

Values of P are shown between the brackets.

$^{NS:}$ Not significant

* Significant ($P \leq 0.05$)

4.2.2.3 Salannin and nimbin

4.2.2.3.1 Variation in salannin and nimbin between the zones

Salannin and nimbin contents in the tested samples ranged between 1.594 mg/g to 3.023 mg/g and 1.389 mg/g to2.114 mg/g. in the first season (2001) respectively. In the second season (2002), salannin ranged from 1.045 mg/g to 2.82 mg/g, while nimbin ranged from 1.048 mg/g to 2.561 mg/g (Table 4.11). Significant variations were found between different zones in both salannin ($p<0.0001$; CV=11.592) and nimbin ($p<0.0001$; CV=15.422) over the two seasons (Table 4.11). Variations were also observed between sites within the zones and between trees in the same site.

In this study, significant variations were found in salannin- and nimbin- contents in the NSK obtained from the different seed collection zones in the Sudan (Table 4.11). These variations could be attributed to the different ecological factors. Genetic factors could also contribute to the variations, but no detailed studies have been done so far. Variations were observed in AZ content, the main active ingredient in the neem seed kernel extracts from different regions by (Ermel *et al*., 1984, 1987 and Ermel 1995), but no such studies have been done for the other limonoids in the NSK extracts.

This result agrees with Eeswara *et al* (1996) who found variations on meliancin content between trees compared to that between sites on both callus and NSK extracts. Similar results were reported by Ermel *et al*., 1984, 1987 and Ermel 1995 where they showed great variations between and within countries in AZ content.

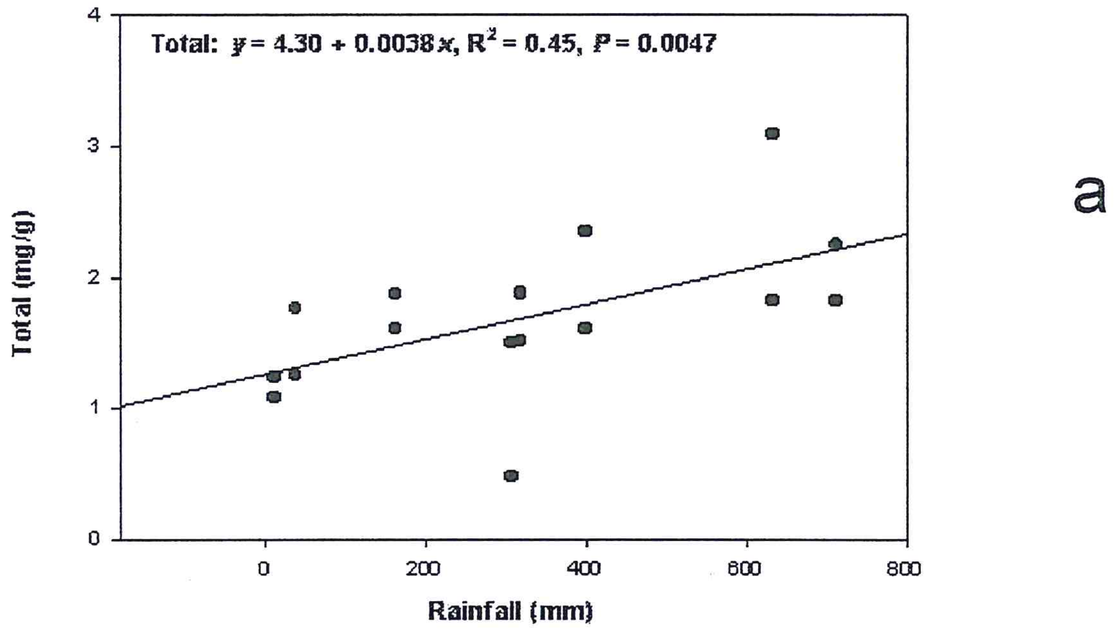
Table.4.13 Variations in salannin and nimbin content (mean ± SE) in different eco-zones in the Sudan.

	Variables (mg/g)			
	Season 2000-2001		Season 2001-2002	
Zones	Salannin	Nimbin	Salannin	Nimbin
1	1.594 ±0.0781[d]	1.457 ±0.085[cd]	1.463 ±0.09d	1.671 ±0.086c
2	1.7922 ±0.0447[d]	1.4255 ±0.0798[cd]	1.998 ±0.117bc	1.553 ±0.117d
3	2.6636 ±0.052[bc]	1.7434 ±0.1153[bc]	1.897 ±0.028c	1.651 ±0.074c
4	1.8905 ±0.0975[d]	1.4498 ±0.1313[cd]	1.045 ±0.084e	1.048 ±0.076e
5	1.9113 ±0.111[d]	1.389 ±0.0695[d]	2.141 ±0.064b	1.682 ±0.886c
6	2.4687 ±0.0578[c]	2.1143 ±0.0428[a]	2.194 ±0.079b	1.836 ±0.058c
7	2.7868 ±0.0898[abc]	1.7491 ±0.0926[bc]	1.409 ±0.128d	1.148 ±0.093e
8	3.0231 ±0.0157[a]	1.6589 ±0.0915[cd]	2.820 ±0.071a	2.561 ±0.140a
9	2.7699 ±0.1079[abc]	2.0451 ±0.1394[ab]	2.042 ±0.141bc	1.494 ±0.134d
10	2.8128 ±0.0851[ab]	1.6586 ±0.1032[cd]	2.700 ±0.122a	2.169 ±0.093b

- Means followed by the same letters in the same column are not significantly different ($p>0.05$), Tukey's Studentized Range (HSD) test.

4.2.2.3.2 Effect of the meteorological factors on the Salannin and Nimbin

Rainfall was found to have significant (P=0.02) and positive correlation with salannin and total amount of limonoids in the NSK (Fig 4.10). Temperature, relative humidity and altitude were found to have no significant correlation with both of the salannin and nimbin (Table 4.12). Salannin content was found to be significantly (P=0.02) affected by cross products of temperature and rainfall (Fig.4.11). Similar results were obtained with AZ which showed positive correlation with the rain fall (Elteraifi, *et al.* unpublished data). The results strengthen the findings of Kumar and Parmar (1997) who reported some variations in yield of the neem oil and key component meliancin (AZ, salannin and nimbin) in relation to ecosystem. Nimbin showed no correlation with any of the studied meteorological factors. No detailed studies have been done on the variations of salannin and nimbin contents and their relation to the ecological factors, but not many studies have been done on AZ (Ermel, 1995). Salannin and nimbin are structurally related to AZ and could similarly be affected although nimbin was not found to be affected by any of the ecological factors.



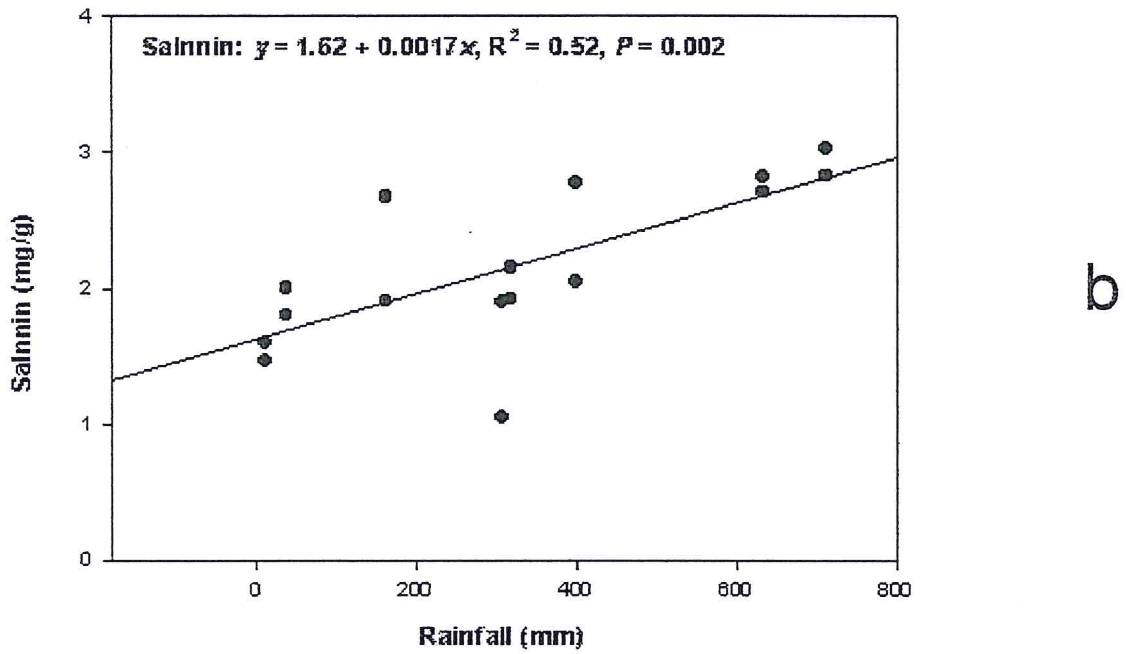
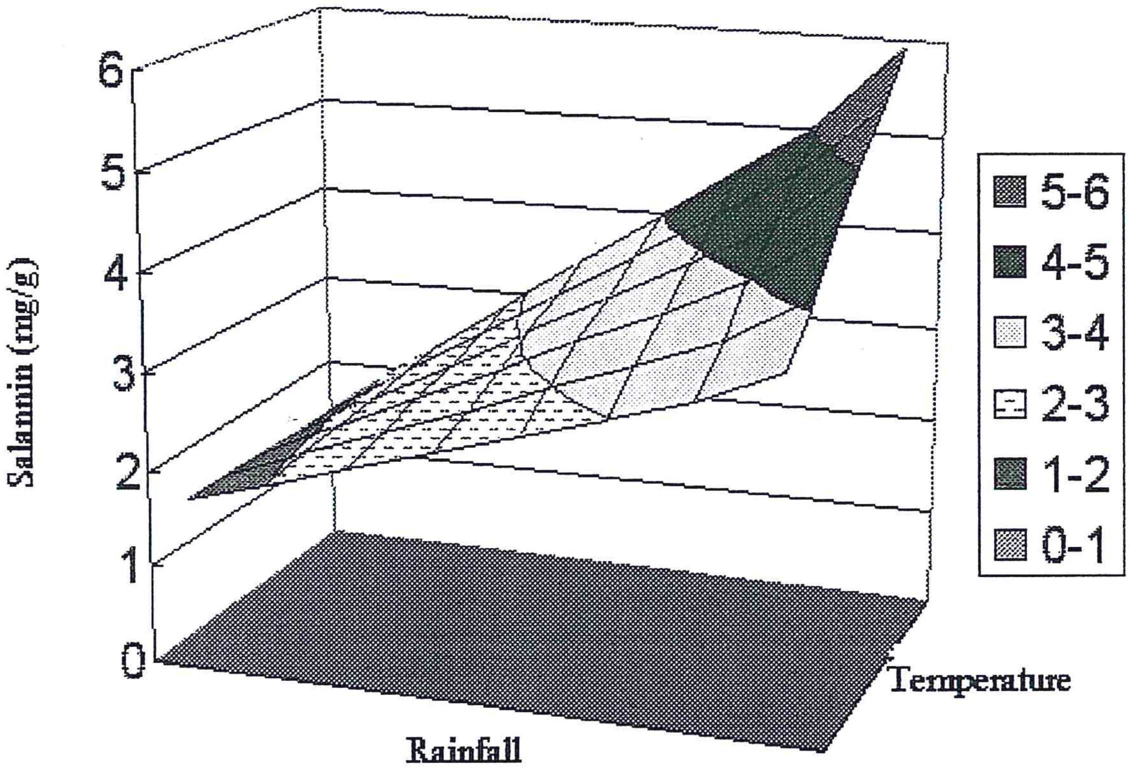


Fig. 4.10 Relationship between the rainfall and limonoids:
a) Total amount of limonoids. b) Salannin.



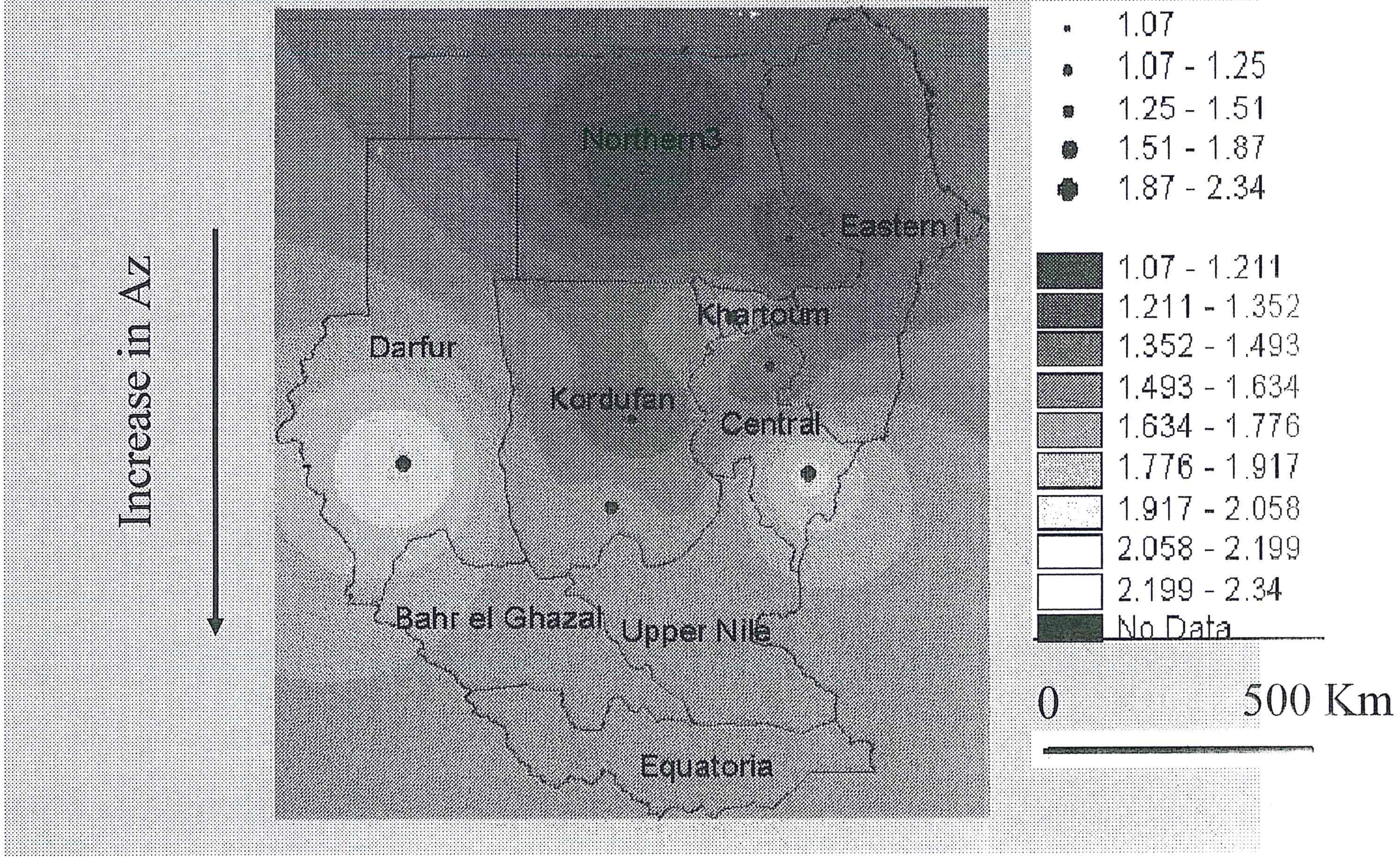
$$Sa=1.74513+6194*10^{-4}*(RF*Temp) \qquad (P=0.02)$$

Fig. 4.11 The effect of rainfall and temperature on the level of salannin.

2.4.3 Mapping and GIS

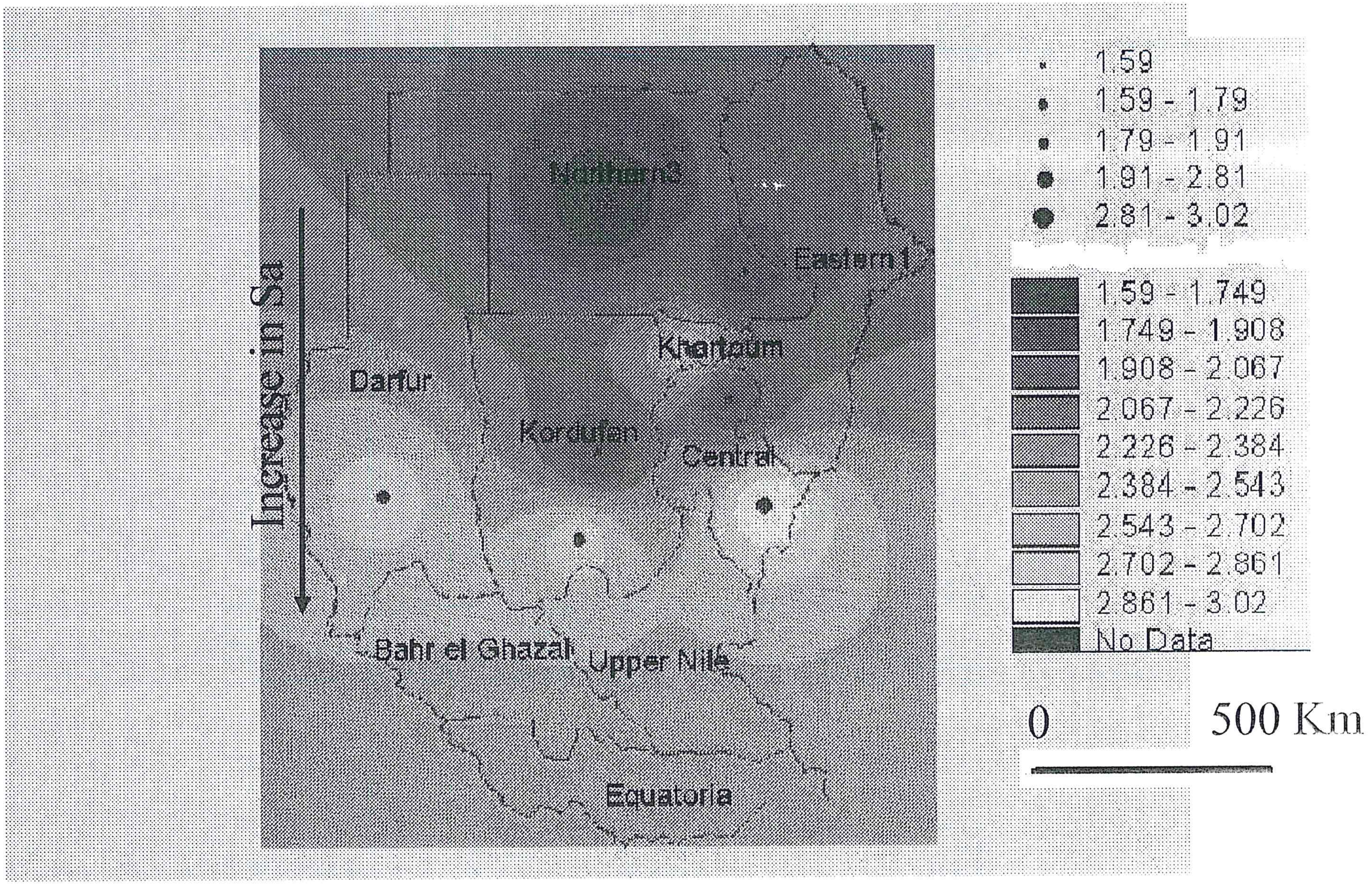
Using the results mentioned above, meteorological and GPS data beside the significant regressions associations' three maps were produced for the predicted concentrations of AZ, salannin and nimbin in the Sudan (Fig 4.11, 4.12 and 4.13). These maps were produced by spatial analysis methods: Inverse distance weight: nearest neighborhood: power (Chang, 2003).

Fig 4.12 Predicted concentration of azadirachtin in Sudan as determined by GIS.



Az = - f (latitude), Except Khartoum

Fig 4.13 Predicted concentration of salannin in Sudan as determined by GIS.



Sa = - (latitude), except Khartoum

4.3 Conclusions

1. The present study showed that morphological variations occur between neem seeds from different provenances, but no significant morphological variations were found in the corresponding seedlings at the nursery level.
2. Nursery neem seedlings from different ecozones did not show any discernible variations in response to two irrigation regimes.
3. No variations were found in oil content in neem kernel of seeds from different ecozones.
4. The amounts of azadirachtin, salannin, nimbin and the total amount of limonoids in the neem seed kernels from different ecozones were significantly different.
5. Azadirachtin was found to be affected by rainfall, increasing with rainfall in a hyperbolic fashion reaching an optimum level at ~717 mm.
6. Salannin levels showed greater variation in different ecozones, which correlated with rainfall.
7. Nimbin showed similar variation between ecozones, but this did not correlate with climatic factors (the reasons for which are unclear).
8. Previous screening of seed kernels from trees located in different continents (drawn from a larger genetic pool) gave limonoid levels that could not be correlated with climatic factors. The present study, which is based on a narrow pool, shows that climatic factors are important set of determinants for the level of limonoids found in neem trees.

4.4 Recommendations and suggestions for further investigations

1. This study showed variations between ecotypes with respect to the contents of azadirachtin and salannin in neem seed kernels in relation to climatic factors. For exploitation of limonoids for bio-pesticides production, the best source of seeds are from neem trees growing in humid and semi-humid zones.
2. Whether these variations result principally from environmental factors or represent interactions between genomic differences and climatic factors need to be resolved by two kinds of studies: (a) screening genetic differences, if any, between neem provenances, and (b) long-term studies of the performance and phytochemical attributes of plants derived from seeds from one provenance growing in other provenances.
3. Neem seedlings in the nurseries proved to be quite resistant during drought periods; therefore, it is recommended that minimum inputs may be used at this stage.
4. Looking at more samples from other ecozones in Sudan (e.g. southern parts) is recommended to extend the screening programme to other climatic zones in the country.
5. Detailed studies on other potentially useful limonoids from neem leaves and bark (e.g. antimalarial constituents such as gedunin) would also help in deciding the best sources of these phytochemicals.

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