The Development of a Commercially-available Neem Seed Kernel Extract as a Soil-applied Systemic Granular Plant Protection Product.

By

Abdiqani Ahmed Farah BSc., MSc., MSc

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Division of Biochemistry and Molecular Biology

Institute of Biomedical & Life Sciences

University of Glasgow

Abstract

Crude extracts of the seed kernels of the neem tree (*Azadirachta indica*) are widely used as plant protection products. The active ingredient (a.i.) of these extracts is azadirachtin A (aza A). aza A is a phytochemical (botanical) complex secondary metabolite which, with it is multiple toxic effects on insects, protects the plant against predation. Aza A is present in only low concentration in neem oil, but makes up 20-50% in the NSKEs extracted by polar solvents from the kernels. However, when used as foliar sprays it is rapidly destroyed by sunlight, and might be more effective if it is used systemically. Therefore the aim of the project was to extend previous work and to prepare a pelleted version of the main commercially-available neem-seed kernel extract, NeemAzal®-Technical (NAT) produced by Trifolio GmbH, in preparation for the expected registration of the product in the UK in 2011.

It was first necessary to purify a quantity of aza A for quantification of the a.i. pelleted material and in soil and plants in the rest of the project. In achieving high purity (over 98%) aza A, reverse phase chromatographic methods were used, and mass spectrometery was used to confirm purity and identification. A final quantity of 6.2 mg of azadirachtin A was obtained from 4 gm of NAT, a yield of 0.15%.

If aza A and the other neem terpenoids are to be used to plant protection, they must have a low phytotoxicity. Effect of NAT on the germination and its ensuing seedling development of two commercially important crops, sugar beet and cabbage was examined. NAT did have an inhibitory effect on seedling growth at 10^{-3} M aza A. In order to explore the inhibitory affect of aza A, the second part of the chapter was to examine effect of aza A on mitosis of onion root tips. The limonoids in concentration of 10^{-3} M adversely affected the mitotic activity of onion root tip cells. This could be failure of microtubules polymerisation into microtubules, or some other biochemical effect. From the findings in this part of the project, it can be concluded that only at a concentration of 10^{-3} M is aza A toxic to plant young seedlings, but in practice this is unlikely to be a significant problem.

The first part of Chapter 4 of the project was to lay the foundations for the behaviour of aza A in soil environment in both powder form and in 2 types of granular formulations. The half-life of azadirachtin in soil from this work was found to be 1.6 days which is consistent with the previous reports. This short half-life of aza A may be problematic in use as a PPP. The short persistence might be overcome by formulating neem materials in granules to achieve environmental stability and biological efficacy of application. The granular formulations used in the project showed controlled release characteristics. The release of azadirachtin into the soil water was in fact delayed by encapsulating it in pellets. Systemic uptake of aza A by roots and subsequent presence in the vascular system of plants was assessed. Aza A was transported and was more stable in the leaf areas of cabbage and sugar beet plants than in the soil, as the half-life was found to be 9 days. The concentration of aza A in the leaf-water was less than 10% of the solution bathing the roots.

The final part of the project, the application of the pelleted NSKE to protect cabbage, in both glass house and field conditions, demonstrated that neem products in pelleted formulations could be used as effective, systemically applied PPP to control pests of cabbage. In the field tests, the protective effect of the neem extract could be shown over a period of at least 5 weeks after addition of the pellets to the soil.

In conclusion, the short soil half-life of the neem a.i., aza A, in PPP could be overcome by a pelleted formulation, the composition of which can delay release of the a.i. The technology allows protection of crops from soil-borne, as well as foliar sucking and biting pest damage by controlled release into the soil to allow uptake into plant vascular system.

Declaration

I hereby declare that this thesis is my own composition, and the work presented herein was performed by myself and that it has not been presented in my previous application for a higher degree.

Abdiqani Ahmed Farah

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List of Figures and tables.

Abbreviations

ACh.	Acetylcholine
AChE	Acetylcholinesterase
a.i	Active ingredient
AMP	Amiprophos-methyl
ANOVA	Analysis of variance
AR	Analytical grade
Aza A	Azadirachtin A
CIPAC	Collaborative International Pesticides Analytical Council
CRF	Cabbage root fly
CSIs	Chitin Synthesis Inhibitors
DCM	Dichloromethane
DEFRA	Department for Environment, Food & Rural Affairs
DDT	Dichlorodiphenyltricholoro ethane
DMSO	Dimethylsulphoxide
DT_{50}	Half-life
EC	Emulsifiable concentrate
EPAC	European Crop Protection Association
EC	European council
EC	European Commission
EEC	European Union Commission
EPPO	European and Mediterranean Plant Protection Organization
GPP	Good plant protection practice

GTP	Guanosine triphosphate
GUS	Groundwater Ubiquity Score
HCl	Hydrochloric acid
HPLC	High performance Liquid Chromatography
hsp60	Heat-shock protein 60
IGR	Insect Growth Regulator
JH	Juvenile hormone
K_d	Dissociation constant,
K_i	Inhibitory constant
K_{oc}	Organic carbon content
Log P_{ow}	Partition between oil and water
MT	Microtubules
MTOCs	Microtubular organizing centers
NAT	NeemAzal® -Technical
NSKE	Neem Seed Kernel Extract
"Neem1"	Hydrophilic pellets
"Neem2"	Hydrophobic pellets
OC	Organochlorine
OP	Organophosphate
PPP	Plant Protection Product
PNS	Peripheral nervous system
PTTH	Prothoracicotropic hormone
RBD	Randomised block design

RT	Retention time
SD	Standard deviation
SE	Standard error of the mean
SANCO	Santé et Consommateurs (Directorate General Health and Consumers; European Commission; Brussels, Belgium)
CNS	Central nervous system
SP	Soluble powder
SAC	Scottish Agricultural College
SCRI	Scottish Crop Research Institute
TIC	Total ion scan
UNDP	United Nations Development Programme
WP	Wetable powder
WHO	World Health Organization

Chapter I: General Introduction and Literature Review.

1.1 Introduction.

Although no neem-based plant protection product has yet been registered for use in the UK, the company, NeemCo., sponsoring the research reported here, hope to achieve registration in 2011 (R. Strang, personal communication). The research therefore aimed at being very much applied. For that reason the test plants for the work, cabbage and sugar beet, were chosen as being an important crop grown throughout the UK, and elsewhere in the world.

An estimate of the value of the brassica crop in the UK in 2004 was over £150 million (Hancock, J, Market Survey for NeemCo Ltd, 2004). Both crops are attacked by a range of insect pests, biting and sucking. As will be described later, azadirachtin A (aza A), the active ingredient of neem-based pesticides, is water-soluble and can act systemically in plants. This may be of importance in protecting plants against both soil pests and foliar pests. Cabbage is much affected by Cabbage Root Fly. The particular shape of the cabbage plant means that it cannot be easily protected by spraying (Figure 1.1), and is best protected by a systemic insecticide. Finally, the plants chosen were suitable for both indoor and field cultivation.

The next sections describe the plants and their main insect pests.

1.2 Brassicas.

Brassicas are geographically widespread throughout the world. The genus has a huge commercial importance, as indicated above for the UK, and contains a number of major vegetables for human consumption, animal fodder and oil seeds. (Lunn, 1988). It's also attacked by wide range of pests (Evans, 2003.).

1.2.1 Taxonomy

Brassica is the Latin name for cabbage. It is a genus of about 300 species of annual, biennial and occasionally perennial herbs with yellow or white flowers. The genus *Brassica* belongs to family *Brassicaceae* (alt. *Cruciferae*), subfamily *Brassicoideae* (Hayman, 1995).

Most botanists agree that almost all of the modern Brassicas were developed from a wild sea kale (*Brassica oleracea* L. subsp. *oleracea*) indigenous to the coastal areas of Western Europe including Great Britain. Several distinct vegetables have been developed from it and are collectively known as "cole crops", including cabbage, cauliflower, broccoli, Brussels sprouts, kale, collard and kohlarabi (Martin, 1997; Phillips, 1993). The traditional methods of determining plant relationships has depended on plant structures. (Vaughan, 1977), but the advent of modern molecular methods has meant that organisms can now be categorised at the fundamental level of their genes. In the following descriptions, based on both classical plant structure methods (Vaughan, 1977) and modern molecular systemics, such as restriction fragment length polymorphisms (RFLPs) (Song *et al*., 1990;) and random amplified polymorpkic DNA (RAPD) (Ananga *et al*.,

2008) taxonomic methods are some of the important Brassica species and their varieties that are more or less grouped together in the Brassica phylogenetic trees (Warwick and Black, 1990).

1.2.2 Brassica oleracea

a) Cabbage

Cabbage is an anglicised word of the French term caboche, meaning head. It has been used to refer to loose-heading (or even no-heading) forms of *Brassica oleracea* as well as to the modern hard-heading type classified as *B. oleracea* subspecies *capitata* (Figure 1.1). Infolding of leaves forms a head, which is demanded by consumers. They are externally green and white internally.

Figure 1.1: Winter Cabbage (*Brassica oleracea, capitata*). (Gardenaction.co.Uk/fruit, 2005).

b) Broccoli and cauliflower

These are two more kinds of *Brassica oleracea*, and because of their similarity both are designated as botanical variety botrytis, from a Greek word meaning a cluster like a bunch of grapes. Broccoli is an Italian word taken from the Latin *brachium*, meaning an arm or branch. Cauliflower comes from the Latin terms *caulis* (cabbage) and *floris* (flower). These cabbages are grown for their thickened, profuse, undeveloped flowers and flower stalks instead of for their leaves. “Clarke” and “Armado” cauliflower (Figure 1.2) are very adaptable varieties popular in the UK. They produce high quality, very deep and heavy curds. The two varieties shown below are the main ones produced commercially in UK.

a: Clarke b: Armado

Figure 1.2 a and b: Two popular varieties of cauliflowers widely grown in UK. (Gardenaction.co.Uk/fruit, 2005).

Broccoli has two distinct forms. One makes a dense, white curd like that of cauliflower and is called “heading broccoli” or “cauliflower broccoli”. The other makes a somewhat branching cluster of green flower buds atop a thick, green flower stalk about 50 cm tall and smaller clusters that arise like sprouts from the stems at the attachments of the leaves.

This form is called “sprouting broccoli”.

The words “broccoli” and “calabrese” describe different varieties of the same vegetable. In general terms, calabrese produces green heads, whereas broccoli produces purple or white heads. One of the most popular and common varieties of broccoli in UK is calabrese, which confusingly is sold in the supermarkets as “broccoli”.

a) Calabrese b) Purple sprouting broccoli

Figure 1.3 a and b: Two popular varieties of broccoli available in the UK. (Gardenaction.co.Uk/fruit, 2005).

c) Kale and collards:

Resemble each other in many respects, but are distinguished one from the other by the forms of their leaves. They are, in effect, primitive cabbages that have been retained unchanged through thousands of years of cultivation. Although more highly developed forms, such as cauliflower, broccoli, and head cabbage, have been developed in the last two thousand years or so, the kales and collards have persisted, although primitive, because of their merits (vigour, hardiness and adoptability) as garden vegetables.

These leafy no-heading cabbages bear the Latin name *Brassica oleracea* var. *acephala*, the last term meaning "without a head." They have many names in many languages, as a

result of their great antiquity and widespread use.

Kale is often called “borecole”, and in some places sometimes called “sprouts”. Kale is a Scottish word derived from *coles* or *caulis*, terms used by the Greeks and Romans in referring to the whole cabbage-like group of plants. The German word *Kohl* has the same origin.

“Collards” is a distortion of *coleworts* or *colewyrts*, Anglo-Saxon terms literally meaning "cabbage plants." They are native to the eastern Mediterranean or to Asia Minor. They have been in cultivation for so long, and have been so widely distributed by prehistoric traders and migrating tribes, that it is not certain which of those two regions is the origin of the species.

d) Kohlrabi and Brussels sprouts

Although kohlrabi (*Brassica oleracea* var. *caulo-rapa*) and Brussels sprouts (*B. oleracea* variety *gemmifera*) appear radically different from each other, they are merely different horticultural forms or races of the same species, *Brassica oleracea*, to which common cabbage, kale, broccoli, and cauliflower belong. They all came from a common parent, "wild cabbage". Kohlrabi Means "cabbage turnip". Kohlrabi is a German word adopted without change into English language, *kohl* meaning cabbage and *rabi* meaning turnip. This cabbage with a turnip-like enlargement of the stem above ground was apparently developed in northern Europe not long before the 16th century. The marrow cabbage from which it probably came is a cold-tender, non-heading plant with a thick succulent stem, while kohlrabi as we know it is a hardy vegetable, evidently developed in a cool climate.

e) Brussels sprouts:

Brussels sprouts require cool climate**.** This variant of the cabbage has been known for about 400 years. The first rough description of it was in 1587, and some famous botanists as late as the 17th century referred to it only as something they had heard about but had never seen. The Brussels sprouts plant is really a tall stemmed cabbage in which many tiny heads ("sprouts") form along the stem at the bases of the leaves instead of making one large head at the top of a short stem (Figure 1.4). After a head of common cabbage is cut from the plant, numerous tiny heads often will grow from the remaining stem. Brussels sprouts need a long, cool growing season, like that of northern Europe and the British Isles. It’s ideally suited to the UK's frosty winters, and Brussels sprouts produce their crops from October to March. By 1800 it was commonly grown in Belgium and France, and by 1850 it was becoming popular in England, where it is in high favour today.

Figure 1.4 a and b: Brussels sprouts. (Gardenaction.co.Uk/fruit, 2005).

1.2.3 *Brassica campestris*.

a) Chinese cabbage (*Brassica pekinensis)* and Chinese mustard *(Brassica chinensis*):

These are similar in their origin, history, and plant characters. These common names are simply modern terms that indicate our impressions of what these two plants are. They are often called by Chinese name *pe-tsai* (cabbage). Both vegetables, in effect, are mild-flavoured mustards. Chinese cabbage has been inaccurately called "celery cabbage" because of the fancied similarity of shape of the head to a bunch of celery, but it is not related to celery in any way.

Some varieties of Chinese mustard have neat leaf blades that are somewhat spoon-shaped, with long, white, erect leaf stalks, all forming a clump so dense that they were long confused with *pe-tsai* by Americans. This type is only one of the remarkable diversity of leaf shapes and growth habits found within the species of Chinese mustard.

1.2.4 *Brassica napus*.

a) Turnip and Rutabagas (Swedes):

Turnip belongs to subspecies of *B. napus* and rutabagas/swedes to *napobrassica.* Much confusion surrounded the origins, and even the identity, of turnips and rutabagas, or swedes, for a long time. They are distinctly different species. Most varieties of turnip are white-fleshed and most varieties of rutabaga/swedes are yellow-fleshed, but there are also white-fleshed rutabagas and yellow-fleshed turnips. Rutabaga leaves are smooth like cabbage leaves, while those of the turnip are somewhat rough, with sparse, stiff hairs over them.

The most significant difference between them, however, is in the make-up of their mechanisms of heredity and the structures of their individual cells. The turnip has 20 chromosomes, while the rutabaga/swedes has 38 (Martin, 1997; Wray, 2005).

1.3 Sugar Beet (*Beta vulgaris* L.).

1.3.1 Biology of Sugar Beet.

Sugar beet (*Beta vulgaris* L.), (Figure 1.5), is a member of the *Chinopodiaceae* family. There are more than 1300 species in a 105 genus of the family (Watson, 1998). The family belongs to order of *Cryophyllates* and class of *Magnoliopsida*. They are dicototyledonous and herbaceous in nature. Sugar beet, and spinach, (*Spinacia olarecea),* are the two major economically important species of the family.

Sugar beet is a large, pale brown root crop, and is a biennial species. However, under certain conditions it can act as an annual (Smith, 1987). Right now, sugar beet constitutes the main sugar crop in temperate regions of the world. Sugar beet market in Europe is determined by European council regulation EC No. 1260／2001. All member states are expected to adopt relevant environmental measures in the sugar sector.

(Märländer, 2003). In the United Kingdom, sugar beet supplies over half of the sugar demands. It is usually grown as a part of rotation with other crops.

Figure 1.5a and b: a) Sugar beet (*Beta vulgaris* L.) b) Seed fascicles each with two to four seeds (FOOD_INFO, 2009).

1.4 Some of the important pests of brassicas and sugar beet in the UK.

a) Sugar beet.

There are about 150 species of insects for which sugar beet is their host, and out of these 40 to 50 can cause economic injury. Among the 50 or so of economic importance, there are a few key ones that are called “primary” or “critically main” pests which occur ubiquitously with high numbers throughout the world. Among them are green peach aphid, (*Myzus persicae L.)*, the cabbage root fly (*Delia radicum* L.), the beet fly, (*Pegomyia betae)*, the flea beetles, (*Chaetocnema tibialis* and *Phyllotrata nemorum* L.) (Godfrey and Mauk. 1993; Lange, 1987; Hills *et al*., 1982). In general, since sugar beet and cabbages share common pests, and the main focus of this work is on cabbage, cabbage pests are discussed in greater detail in the following sections.

b) Cabbage.

1.4.1 Cabbage root fly.

The cabbage root fly (*Delia radicum* L.) (Figure 1.6) is an important pest of swedes and turnips, as well as cabbage. The larvae feed on the root, causing wilting and death of plants. After overwintering in the soil, a small housefly-like adult emerges. After landing on the plant it lays oval, 1mm, white eggs at the base of the plant. When hatched, the larvae feed on the roots 2 to 3 cm beneath the soil surface. As a result, the plant secondary roots are severely damaged, leaving a spindle tap-root that is poor at taking up water and nutrients. In most cases the spindle tap-root itself is damaged and that results in wilting or even death. Sometimes the damage caused allows pathogens to set in, leading to soft rots, which downgrades the quality of the harvest/crop. Another species, the turnip root fly (*D. floratis*. Fall) is common in Scotland and appears in August in between the two generations of cabbage root fly. This fly feeds on the heart of the root by burying itself deep into the soil.

Figure 1.6 a and b: a) Adult and Larvae of cabbage root fly (*Delia radicum* L.), b) Maggots. (Gardenaction.co.Uk/fruit, 2005).

1.4.2 Flea beetles (*Phyllotrata* spp.).

As their common name implies, they have very powerful hind legs that enable them to jump like fleas if disturbed. There are two distinct types of them: striped wing, for example *P. nemorum* L. (Figure 1.7), and collared: *P. cruciferae* Goeze. It's the adult of 1.5 - 3mm in size that causes damage to the crops. In April and May, when the warm weather comes, the adult moves to the crop and feeds on recently germinated plant leaves and shoots, resulting in "shot holding". When the adult lays eggs in soil from the end of May onwards, the hatched larvae feed on either roots or leaf. Nevertheless, larval damage is not so severe as that of the adults (Evans, 2003.; Gardenaction.co.Uk/fruit, 2005).

Figure 1.7: Striped wing flea beetle (*Phyllotreta nemorum* L).
infos.blanquefort.net/blog/agenda/wp-content (Bio, 2007).

1.4.3 Butterflies and Moths.

Although the caterpillars of moths and butterflies feed on swedes and turnips they are not as important as they are to the leafy brassicas crop such as cabbages. Large and small cabbage white butterflies (*Pieris brassicae* L. and *P. rapae* L) (Figure 1.8), often feed on the leaves of crops. Unless there is a large outbreak, their feeding on leaves does not cause

any important damage to the crops.

a) Larvae b) Adult

Figure 1.8: Cabbage white butterflies a) larvae and b) adult (Pieris brassica L.). (Gardenaction.co.Uk/fruit, 2005). www.lepidoptera.ch/.../ PierisBrassicae_W.gif.

The damage caused by diamond back moth (*Plutalla xylostella* L.) (Figure 1. 9) is so severe that they can reduce the young seedlings into bare skeleton, thus almost destroying the crop. The larvae, "cutworms", for example turnip moth (*Agrotis segetum*, Den & Schiff), feed on the roots and lower stems. The damage they cause to the root may not be detected until the harvest, when the large holes as a result of their feeding on it became apparent (Evans, 2003.; Gardenaction.co.Uk/fruit, 2005).

Figure 1.9: The larvae of diamond back moth (*Plutalla xylostella* L.).www.jpmoth.org/.../ L2Plutella_xylostella.jpg.

1.4.4 Aphids.

Aphids are not only serious pest on a large number of crops (arable and horticultural) including brassica plants. As well as affecting the vitality of growing plants by feeding on them, they are also important vectors of plant virus transmission among crops. Peach-potato aphid (*Myzus persicae,* Sulzer) and the cabbage aphid (*Brevicoryne brassicae* L.) (Figure 1.10), are the most widespread and extensively studied aphid species (Scri, 2009; Nisbet *et al*, 1992; Kasprowicza, 2008). The cabbage aphid (*Brevicoryne brassicae* L.) get their name due to distinctive grey-white wax covering their body. Aphids over-winter as eggs on the brassicas weeds or crops. After they hatch in May, they move to the newly planted crops. Bleaching and yellowing of leaves are the first symptoms of aphid infestation (Evans, 2003.). These two species, particularly peach-potato aphid are the most adaptable and are major contributors of spread of potato leafroll virus (PLRV) among the major crops such as potato, sugar beet and tobacco (Nisbet *et al*, 1992; Kasprowicza *et al*., 2008). Intensive application of insecticides has rendered them resistant, to at least three classes of chemical insecticides, as reported by Kasprowicza *et al*, (2008).

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Figure 1.10: Cabbage aphid (*Brevicoryne brassicae* L.). ipm.ncsu.edu/vegetables/ pamphlets/crucifer/ca.jpg.

1.5 Insecticides.

Almost all plants grown either as food crop or ornamental are attacked by insect pests, which destroy approximately one third of the world's food crop during growth, harvest, and storage (Jacobson, 1988). The brunt of insect damage occurs in tropical countries (Iqbal, 1999). Losses are considerably higher in many countries of Asia and Africa than developed (western) world. The monetary loss due to feeding by larvae and adults of pest insects amounts to billions of dollars each year (Copping, 1998; Jacobson, 1988). It was estimated that there are almost 9000 species of insect and mites that infest crops, and most of these are insects that have moved from native vegetation on to the introduced crop. Out of these at least 600 cause crop damage that warrants use of some control measures, either chemical or physical (Klassen, 1981).

From the earliest times, there are references to various means of protecting cultivated plants from insect predators. For at least two thousand years, until the 20th century, the materials used fell into two broad categories: inorganic poisons and plant extracts.

Elemental sulphur appears to have been used to dust crops in ancient Greece and Sumaria, and by the later Middle Ages salts of arsenic, lead, mercury and fluorine were all applied to crops. It was the use of Paris Green, an arsenical compound, in the USA in the late 19th century, which led to the first legislation to control the application of toxic compounds to crop plants. Most of the inorganic compounds are too toxic for modern use, but there is still some commercial application of fluoride salts for crop protection.

Much more relevant to this work is the use of phytochemicals (botanicals), as crude extracts. It is likely that most plants in the course of evolution have developed protective mechanisms against their principal enemies: the insects. These phytochemicals are

usually secondary metabolites, and are often very complex in structure. In the case of a few plants, these are so effective as insect poisons that they have been used, first locally for many hundreds of years, and, more recently, throughout the world. These are summarised in Table 1.1 below. The botanicals have been used over a long period of time. For the sake of completeness, also included in the table are a recently developed group of secondary metabolites which are not produced from plants, but which come from microorganisms, fungal or bacterial. Some of active ingredients listed such as pyrethrin and rotenone, have a long history of use as insecticides, and supported a considerable world trade until recently. Eserine was probably not used as an insecticide until it gave rise to a range of derivates as will be discussed later. Aza A, the main subject of this thesis, has been largely restricted to use in India until relatively recently.

Type	Active Compound	Source	Mode of Action	Point of action
Plant-Derived	Azadirachtin A	*Azadirachta indica*	Growth inhibitor/ antifeedant	Uncertain
	Eserine (Phystostigmine)	*Physostigma venenosum*	Neurotoxin	Acetylcholine estertase inhib.
	Nicotine	*Nicotiana tabacum*	Neurotoxin	nACh receptor Agonist
	Pyrethrin	*Chrysanthemum cinerariaefolium*	Neurotoxin	Na^+ channel Blocker
	Rotenone	*Derris (Lonchocarpus) spp.*	Metabolic Poison	Electron transport chain
	Ryanodine	*Ryania speciosa*	Muscle poison	Ca^{++} channels
	Veratridine	*Veratrum album*	Neurotoxin	Na^+ channel Blocker
Plant	Protease inhibitors	Plant proteins	Inhibition of insect digestive proteases	Proteolytic enzymes/midgut
Defence	Non-protein amino acids of plants	Intermediate/end product of primary metabolism	Poisoning/deterrent	Possibly affect lysozyme activity
compounds	Lectins	Plant proteins	Retard rate of development and reproduction	Multiple binding sites
Micro-	Avermectin	*Streptomyces*	Neurotoxin	Cl^- channel
Organism-		*avermitilis*		Agonist
Derived	"Cry" toxins	*Bacillus thuringiensis*	Stomach poison	Ion channels
	Spinosad	*Saccharphyspora*	Neurotoxin	nACh receptor
		spinosa		Agonist

Table 1.1: A list of the main active compounds from plants and microbial sources used as insecticides (Rockstein, 1978; Peumans and Damme, 1995; Birch *et al*., 1999; Brown, 2005; Schmutterer, 2002; Bell, 2003; Amirhusin *et al*., 2007;).

It is clear from the table that the majority and the most widely used of the phytochemicals act as neurotoxins. This fact explains their success. They have an immediate effect on the insect, by paralysing it. Rotenone is not primarily a neurotoxin, but as an inhibitor of

oxidative phosphorylation, it still acts rapidly. Azadirachtin differs from the rest, as its action is much slower, and had not been fully determined, as will be discussed in a later section.

Recently, less well-known plant defence compounds, such as protease inhibitors, non-protein amino acids and lectins, are being explored as novel pesticides. Plant protease inhibitors (PIs) are proteins that inhibit phytophgous insect midgut digestive proteases, reducing the supply of amino acids important for their growth and development thus leading to insect death (Leo *et al.*, 2002; Lawrence and Koundal, 2002; Pilon *et al.*, 2006; Amirhusin *et al.*, 2007). Plant lectins are proteins that have at least one catalytic domain which reversibly binds to particular mono- or oligo-saccharides (Peumans and Damme, 1995). They are considered to have a role in plant defence against phytophgous insects and thus have become possible means of producing resistance by transgenic methods (Birch *et al.*, 1999). At the moment, it is not clear whether or not the growth and development effect is actually related to carbohydrate-binding activity of the lectin (Sadeghi *et al.*, 2006). Non-protein amino acids, mainly from various legume seeds, were reported to act as antifeedants, and also being toxic to insects which do consume them, by inhibition of lysozyme activity (Bell *et al.*, 1996; Bell, 2003). The loss of this enzyme's activity might be expected to make the insects more liable to bacterial infection.

With the discovery of the first synthetic insecticide DDT (dichlorodiphenyltricholoro ethane) in 1938, the whole crop protection scene was changed. For at least 25 years from the mid-1940s, there was a huge burst of synthetic activity, producing neurotoxic compounds which more or less eliminated all the previously used ones. The main classes of the compound produced were the organochlorines and, later, the organophosphates. It

is estimated that over 500,000 organophosphates have been evaluated as insecticides (Sales *et al*., 2000). Although the organochlorines have largely been phased out, the organophosphates still have an important role in plant protection. However, as these two huge classes of compounds are not derived from phytochemicals, they are not strictly relevant to this review.

Some of the botanicals listed in Table 1.1 have a relatively simple structure, which allowed their synthesis, and which has allowed the development of new classes of insecticides derived from a plant or other natural source. These are listed in Table 1.2.

Natural Compound	Synthetic Derivatives
Eserine	Carbamates
Pyrethrin	Pyrethroids
Nicotine	Neonicotinoids
Juvenile hormone	Analogues
Nereis toxin	Analogues

Table 1.2: The natural compounds which have given rise to synthetic derivatives. (The last two sets of compounds are not from plant sources, but are included for completeness.)

1.5.1 Carbamates.

Carbamates are ester derivatives of methyl and dimethylcarbamic acid, $CH_3HNC(O)OH$ $(CH_3)_2NC(O)OH$ and synthetic derivatives of physostigmine (eserine), which is an alkaloid isolated from the calabar bean, *Physostigma venenosum* (Casida, 1963; Coats, 1994). But all the above carbamates were not toxicologically effective on insects since

they are ionisable. The Geigy company of Switzerland first produced the first insecticidal carbamates- N-dmethyl, such as Carbaryl (Sevin), and N-dimetyl, Pyrolan and Isolan, compounds of which the latter became most popular and effective insecticides. The most widely used carbamate is Aldicarb. These compounds almost all are aromatic (naphtholic and phenolic) and highly effective insecticides used in numerous soil or plant treatments (Sánchez-Brunete and Tadeo, 2003). Members of the group (some such as Carbaryl act systemically in plants) are extensively applied in agricultural. Others are non-systemic such as m-ethoxy. Along with organophosphates, carbamates represented approximately 50%, up until the EU directive 91/414 EEC was enacted in 1991, of the total insecticide and acaricide usage worldwide (Galloway *et al*., 2002 and Villatte & Bachmann, 2002).

The insecticidal mode of action of carbamates like that of the OPs, is well understood and is due to their structural resemblance to ACh . They act as inhibitors of AChE, resulting in nervous system disruption (Casida and Quistad, 1998; Sanchez-Hernandez and Walker, 2000); Yerushalmi and Cohen, 2002). However, unlike organophosphate compounds, the deacylation or hydrolysis is faster with carbamates than OPs, thus inhibition in this instance is of shorter duration and more reversible than OPs (Aprea *et al*., 2002). Nevertheless, according to dissociation constant, K_d ,of carbamates, it's more than enough to inhibit AChE and cause repetitive nerve action and paralysis to insects (Rockstein, 1978). As carbamates are less toxic to humans than the OPs their use has been growing in recent years.

1.5.2 Synthetic Pyrethroids.

The synthetic pyrethroids, the derivatives of one of the oldest organic insecticides (pyrethrin found in pyrethrum flowers of various species of genus *Chrysanthemum* of which species *cinerariaefolium* found most potent) have become one of the most important classes of insecticide, contributing to over 25% of the world insecticide market due to low toxicity to mammals and birds and rapid knockdown effect on insects (O'Brien, 1967; Horia Vais, 2001). Certain of these synthetic analogue compounds such as, Allethrin, Tetramethrin and Resmethrin have found numerous uses in agriculture, veterinary and in urban situations, and in combination with the OPs, have largely replaced the phased out organochlorine insecticide class (Soderlund and Bloomquist, 1989; Best. and Ruthren, 1995 and Fakata *et al*., 1998; Coats, 1990).

Synthetic analogues of the natural insecticide pyrethrum were developed in order to over-come the photolabile nature of the natural pyrethrin by addition of single or multiple halogen atoms within the molecular structure (Coats, 1990; Plapp (Jr), 1981; Ascher, 1986). The class may be divided into two groups. These are the type I pyrethroids (e.g. Permethrin), which lack a cyano moiety in the α-benzylic position, and type II pyrethroids (e.g. Deltamethrin) which have this α-cyano group (Narahashi, 2000).

The generally accepted mode of action of pyrethroids is that they are axonic and act upon sodium channels. They delay or prevent sodium channel from closing, or opening the potassium gate, thus the repolarisation or falling phase action potential is delayed. This results in repetitive or continuous excitation or impulse transmission, convulsion and death (Coats, 1990; (Narahashi, 2000; Plapp (Jr)1981; Toth *et al*., 1990; Brown, 2005).

1.5.3 Non-neurotoxic insecticides.

Although the highly successful neurotoxic insecticides are generally more effective against insects than humans, they attack features of the nerve cells which are common to all nervous systems. They certainly affect other, often beneficial, organisms, and are seen as generally harmful to the environment. Thus for the last two or three decades there has been a push to try to develop insecticides which specifically attack aspects of biology and biochemistry which are unique to insects. These can be classed as insect growth regulators. They have their action by various means such as primary or secondary antifeedant effects, reducing the insect immune response, or interfering with the complex hormonal control of insect growth and development. Part of the action of aza A is to interfere with the development of insects, and so it can be said to fall into this category.

1.5.3.1 Insecticides that target the insect's growth and development

As they are growing in size and developing to adult, insects must shed their hard cuticle periodically in a process called molting. There are neurosecretory hormones that are important in molting processes, and disruption of any of these hormonal cascades cause inactivation of the process. There are some classes of chemicals that target the insect's growth and development processes through either disrupting the hormones or blocking the production of a structural chemical component necessary for exoskeleton. (Brown, 2005; Marx, 1977; SU and Scheffrahn, 1993).

1.5.3.2 Insect Growth Regulators (IGRs)

Insect Growth regulators (IGRs) interfere with the insect's endocrine system in order to inhibit the production of hormones, such as prothoracicotropic hormone (PTTH), juvenile hormone (JH) and eclosion hormones.

Many of the currently available IGRs, such as Fenoxycarb and Hydroprene, mimic JH and a high titer of it in insect body prevents the insect reaching adulthood. (Brown, 2005; Leighton, Marks, and Leighton, 1981; Marx, 1977; Su and Scheffrahn, 1993).

Tebufenozide disrupts the production of the steroid molting hormone, ecdysone, by prothoracic glands. This causes the ecdysone level in the body to drop, which in turn prevents the insect developing into adulthood (Figure 1.11).

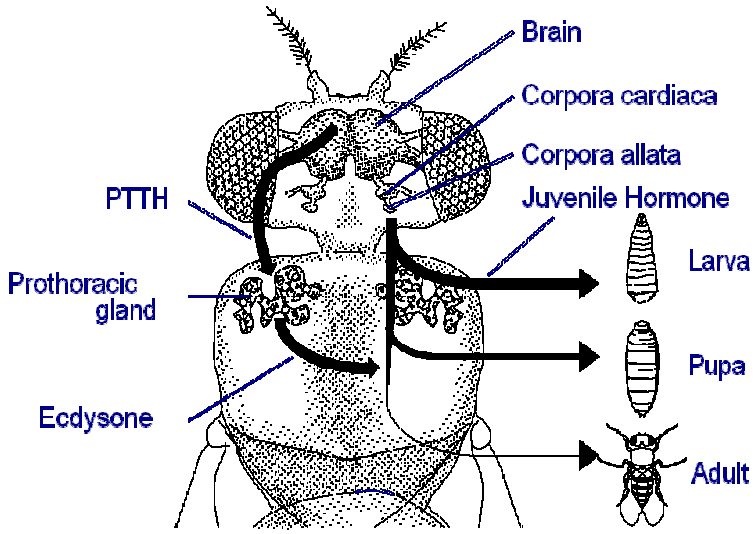


Figure 1.11: Hormonal control of insect growth and development. (cas.bellarmine.edu/.../Hormone&NS.htm.)

1.5.3.3 Chitin Synthesis Inhibitors (CSIs)

Chitin is a polysaccharide component of the outer tough waxy cuticle of insects. The discovery of compounds, the benzoylphenylureas, that inhibit the production of chitin led to a group of insecticides. Treated insects cannot develop the new cuticle required to pass from one instar to the next and so die. (Su and Scheffrhan, 1993; Brown, 2005). Bistrifluron is among several CSIs in the market. Although higher animals which do not synthesize chitin are unaffected, it is toxic to all chitin-making animals, such as crustaceans.

The growth regulatory nature of azadirachtin A will be discussed in a later section (1.6).

1.5.4 Problems associated with pesticide use.

All the most successful insecticides, whether phytochemicals or synthetic compounds, are neurotoxins. Their widespread use over the last 50 years has revolutionised agriculture. Although their problems were obvious from the earliest days of their use, the advantages were considered to outweigh the drawbacks. Their quick knock-down effect particularly appealed to farmers, who found them very cost-effective, and who were generally not concerned with the wider effects (Colosio and Maroni, 2003; Pimentel *et al*., 1992). It was not until 20 years of extensive use of insecticides, that their long-term risk for health and environment become apparent, and public opinion become suspicious of their severe drawbacks. It became more and more clear that a large number of them proved to have been persistent and harmful to non-target animals and beneficial insects (Casida and Quistad, 1998). The drawbacks, such as impact on human and animal health and non-target, beneficial insects, environmental problems and pesticide resistance, then began to

outweigh the advantages.

1.5.5 Impact on public health

Large numbers of synthetic insecticides were registered in the decades following the Second World War, as their registration cost was not difficult and health risk assessment was based only on acute oral and dermal toxicity data. Suddenly there was a fear of pesticides being carcinogenic or producing long-term neurotoxic effects such as Parkinson's Disease, and stringent test methods were introduced to monitor their toxicity, with risk assessment leading to increasing safety requirement, resulting in huge expenditures of money and time, (Casida and Quistad, 1998; Coats, 1994).

Different insecticides' toxicity (acute and chronic exposure) varies according to their mode of actions. Toxicity is mainly dose-dependent, type of chemical and its metabolites in relation to its impact on humans (Skinner *et al*., 1997). According to WHO-UNDP (1989) report there has been about 1 million, mainly occupational, incidents of pesticide poisoning. Even though the long-term health implications associated with exposure to pesticides and their residues are not as severe for the general public, the main source of exposure is either residues in contaminated food (fruit and vegetables.), from drinking water, physical contact or through respiration (Coats, 1994; Ramos et al., 2000; van der Werf, 1996; Skinner *et al*., 1997). There is now a widespread alarm among the general public, rightly or wrongly, about the possible effects of exposure to pesticides. These include: neurotoxic disorders, immunodisfunction, mutagenesis, teratogenesis and carcinogenesis (Ballantyne and Marrs, 2004; Banerjee, 1999; Williams, Bernard, and Krieger, 2003; Bolognesi and Morasso, 2000; Colosio *et al*., 1999; Gómez-Arroyo *et al*.,

2000; Laden *et al*., 2001; Longnecker, Rogan, and Lucier, 1997; Marcello Lotti, 2002; Nishioka *et al*., 1999; van der Werf, 1996; Laura Settimi, 2003; Webster, McKenzie, and Moriarty, 2002; Vale *et al*., 2003). These fears have, of course, had their impact on legislation concerning pesticides.

1.5.6 Environmental impact of pesticides

In addition to the importance of the impact on human health, pesticides have profound ecological effects. Pollution of pesticides in the environment is assessed through three main criteria according to European procedures: soil pollution (result of direct application), water pollution (such as spray drift) and ground water pollution (associated with leaching through soil column), (Ramos *et al*., 2000).

When insecticides are applied to control pests, a considerable amount of it reaches to the soil and affects soil–borne, beneficial fauna and flora. Though the environmental consequence of pesticide depends on the degree of exposure (i.e. dispersion and resulting environmental concentration) and on the toxicological properties of the chemical concerned, there are always potential adverse effects on soil microflora and fauna (Russell, 1973 ref. by van der Werf, 1996).

Pesticides are also harmful to insect predators of target pests, i.e. beneficial insects. Intensive usage of insecticides means that each year around 2.5 million tons of pesticides are applied to agricultural field crops, with only a small proportion reaching the target pests. This has resulted in a dramatic reduction of non-target insect populations and other beneficial invertebrates (EPPO 1994; van der Werf, 1996).

Around the 1950s it was very common to see large numbers of birds dying in field

sprayed with DDT. Seed treatment with pesticides, or eating insects or fish contaminated with insecticides were two factors contributing to the death of birds in the fields. And for those birds which didn't die by the consumption of pesticides, sub-lethal effects such as birth defects and thin egg shells were evident (Hart, 1990; LeBlanc, 1995; Metcalf, 1989). These effects were the main reasons for the banning of DDT throughout most of the world.

Effects to aquatic life were widespread, with fish death as the major indicator. Pesticide contamination and toxicity is now measured in effects to algae, crustaceans and fish toxicity as representative of food chain tropic levels (LeBlanc, 1995; van der Werf, 1996).

1.5.7 Resistance to pesticides

Insects becoming resistant to insecticides have been a problem ever since synthetic insecticides have been introduced and was evident before 1950. According to (Brattston, 1989), in 1988 there were almost 500 insect species resistant to various pesticides, with a large number of them cross-resistant to more than one type. The response of producers to resistance is to increased application rates. This elevates the risk to applicators, increase pesticides residues, hence risk to consumers and to the environment (Brattston, 1989; Heimbach *et al*., 2002; Rotteveel *et al*., 1997; Daly, 2004; Salehzadeh *et al*., 2003; Scott *et al*., 2000). Cross-resistance occurs, for instance, when a single enzyme has mutated to a form resistant to more than one type of pesticide sharing a common detoxification process. Multiple resistance occurs through the co-occurrence of several resistance mechanisms involving several enzymes. Clearly, acetylcholine esterase is the target of

OPs and carbamates, and is a likely candidate for such effects. (Brattston, 1989); (Metcalf, 1989).

Resistance in a population of insects will depend on variants in crucial genes coding for target or detoxification proteins. The axonal sodium channel (target of DDT and the pyrethroids) is an example which has been observed. As insects can undergo rapid generation, the continued use of a particular insecticide will ensure that rapid rise of a resistant population. (Hemmingway *et al*., 2002)

So far there is no general consensus on uniform strategy to prevent resistance occurring (James, 1997). There is a growing realisation that the way forward is the use of integrated pest management programs which aim at long term sustainability through a combination of control regimes, including biological, thereby optimising the efficiency and profitability of crop production, and avoiding the continued use of a single type of insecticide (Council directive, 1991; Metcalf, 1989; Richard, 2000).

1.5.8 The regulation of pesticides and plant protection products

Public attitude and awareness regarding possible effects of pesticide use forced the development of rigorous and comprehensive legislative control to protect human safety and health and the environment and to ensure that products are sold, supplied, stored and used correctly and efficiently (Mike, 2000), and its EU Council Directive 91/414/EEC (adopted in July 1991) which sets out a community-harmonised framework for authorisation, use and control of these products (ECPA, 2000a); (ECPA, 2000b). The basic principle of the directive is the development of a positive list (Annex 1) of active substances through a review program. The basic principle of the review is to protect

human health, wildlife and the environment than the enhancement of crop productivity (ECPA, 1998a; Toyofuku, 2006). The target has been to conduct an appraisal of over 800 existing active substances during a 12- year period (due for completion in July 2003, although now extended until at least 2010) while simultaneously assessing new active substances (ECPA, 2001a).

as a result, it has been forecasted that there might be a discrepancies between the available plant protection products and that which is obtainable (SANCO, 2001). It is a source of great concern to producers, that there will be "gaps" in the pesticides available for specific pests in specific crops. An example of particular relevance to this work is cabbage root fly in Brassicas, which depend on OPs at the moment (Thompson, 2002).

1.6 The potential for neem-seed kernel extracts as plant protection products.

As has been outlined in the previous sections, the current situation is that by 2010, many previously available synthetic insecticides will no longer be available for producers. The public is generally suspicious of the widespread use of pesticides, especially of neurotoxins. Many producers are turning to "organic" methods which almost completely exclude the use of pesticides, except those from the neem tree.(Hammond, and Fuchs, 2000; Peterson and Coats, 2000).

This is the background which should be hopeful for the use of plant extracts, including those from the neem tree. The tree and its active compounds will be described in the following sections.

1.6.1 The Neem Tree.

The Neem Tree, (*Azadirachta indica* L.) is a member of mahogany family (*Meliaceae*). Individual plants can grow to a height of 40 to 80 feet. The leaves are dark green and slender. The tree is a drought-tolerant and thrives in tropics with extended dry season. It copes with long dry seasons by shedding its leaves (Puri, 1999; Schmutterer, 1990a). Flowers are whitish pink. Neem usually flowers from January through April with fruits ripen in June through August, but occasionally second minor flowering may occur from July to October (Puri, 1999; Raju, 1998). Neem produces an ovoid drupe with thin mucilaginous sweet pulp. When matured, the green fruits produced per tree varies, but is estimated between 11-50 kg (Puri, 1999; Schmutterer, 1990a). It is probably indigenous to the Indian sub-continent, but is now widespead in tropical and subtropical areas of Asia, Africa, Australia and South America, and the Pacific Islands. Neem is a traditional source of a wide variety of products including beauty aids, fertilizers, herbs, lumber, pesticides and numerous pharmaceuticals. They are all derived from different parts of the tree such as leaves, bark and the seeds (Puri, 1999; Schmutterer, 1990a; Schmutterer, 2002).

a) Insecticidal Components of Neem.

It was Chopra, (1928), who first drew attention to neem for its insecticidal and insect repellent properties, although they had long been known to the Indian people as a whole. It has taken a long time to investigate the specific components which give that quality. Even though a large part of the investigation focused on its seeds, neem leaves and bark also yield number of active components (Fagoonee, 1986) There was quite a number of

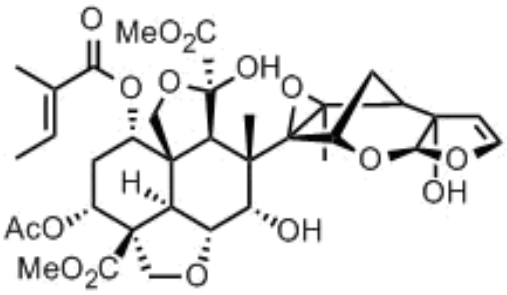
components that have been extracted from the seeds of the neem tree and of the well-characterised compounds below to the complex phytochemicals called limonoids, and more strictly as tetranortriterpenoids (basically C26 compounds; Govindachari, 1992). Among a very large number of such compounds produced by the tree, the one most investigated is azadirachtin A. This is the compound with the clearest anti-insect effect, and is the one defined as the active ingredient of neem-based plant protection products. Much of the work reported in this thesis was concerned with the analysis of azadirachtin A in crude neem-seed extracts, pellets, soil and plants, and with its effect on insect pests. Its physical and chemical characteristics are important in getting the best out of the limonoid as a plant protection agent.

b) The chemistry of Azadirachtin.

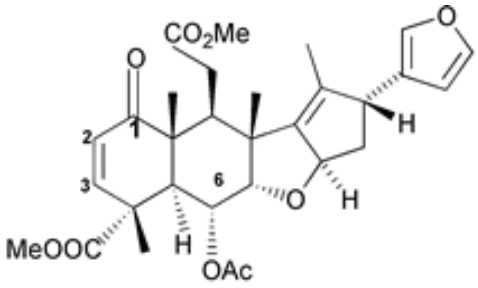
Azadirachtin A is the most important of a mixture of congeners (compounds with a common basic precursor) which are called the azadirachtoids: these are structurally related tetranortriterpinoids classified arbitrarily as azadirachtin A to azadirachtin G (Deota *et al.*, 1999; Rembold *et al.*, 1983). Of those azadirachtin A (C_{35} H_{44} O_{16},), Mr 720 (Fig.1.12) , is the major active component, almost 80% of the total azadirachtoids (Mordue, 1997; Mulla, 1999; Rembold, 1989; Mulla & Su, 1999). Studies made on the functional groups of azadirachtin A have shown that it is highly a oxidized compound containing no fewer than 16 chiral centres and a strong oxygen functionality. The molecule includes an enol ether, an acetone hemiacetal and tetra-substituted oxirane and a variety of carboxylic esters. Furthermore, both secondary and tertiary hydroxyl group and tetrahydrofuran moiety are present (Durand-Reville *et al.*, 2001; Ley *et al.*, 1989).

Due to the large number of oxygen groups, it is a highly polar compound, soluble in water. It hydrolyses in water, the rate of hydrolysis depending on the pH of the medium. It rapidly breaks down in sunlight. It is rapidly biodegradable in soil, with a half-life of a few days. It doesn't easily penetrate into the cuticles of insects (Deota *et al*., 1999), and is not a good contact poison. The extreme complexity of its structure has meant that determining the structure took 17 years (Morgan, 2008). Attempts to synthesize aza A have taken even longer, almost 25 years. Only in 2007 was this finally accomplished by the group headed by Professor Ley *et al*., (2008). Due to its (azadirachtin A) being highly oxidised polar molecule and being highly complex compound (with 16 chiral centres) it has not been possible to develop more potential compounds based on azadirachtin (Deota *et al*., 1999).

Crude extracts of the neem seeds contain many other compounds related to azadirachtin. They do not seem to have much effect on insects, and have not been studied in detail. One is termed Nimbin (C_{30} H_{36} O_9), Mr 540 (Fig. 10). And another is Salannin (C_{34} H_{44} O_{90}) Mr 596 (Fig. 11), and was characterized by the presence of two oxygen bridges at C-6/28 and C-7/14 (Kraus, 2002 ref. by Schmutterer, 2002).



a)



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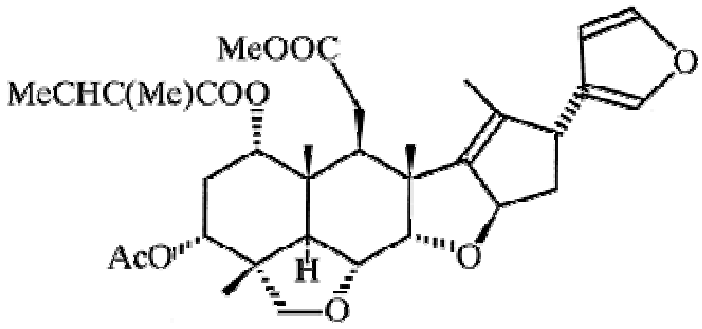


c)

Figure 1.12: Major limonoids present in the seed kernels of *A. indica*: a) azadirachtin A b) nimbin, and c) salannin

c) The Mode of Action.

Like the well-known plant protection products discussed previously, aza A is a phytochemical or botanical: a complex secondary metabolite whose function is to protect the plant against insect predation. Unlike most of the widely used compounds, however, it is not a neurotoxin. At the moment, it is not certain how the limonoid has its effect on insects. It does not seem to have a single well-defined target. Again, unlike the neurotoxins, aza A is generally slow to take effect, i.e. hours and days, rather than minutes. Azadirachtin has multiple modes of action in its activity on insects, and the importance of each can vary between insect orders and even species. There have been several reviews published which outline the use of aza A as a botanical pesticide. Most of them are the results of experiments involving neem use as an insecticide for arthropod pests. Even though the insecticidal efficacy /performance of neem products against most insects is much less immediate than that of synthetic insecticides, in general it has a comparable performance to the other botanical products in terms of reducing pest insect infestations.

The effects of azadirachtin on whole insects may be summarised as follows:

1. Primary antifeedant: some insects make no attempt to feed on plant material treated with aza A, and may starve to death. This sensitivity varies between species. For instance, the desert locust (*Sch. gregaria*) is highly sensitive, but other locusts such as *L. migratoria* are much less sensitive. (It was the observation of the desert locust did not touch neem trees in the Sudan, which started the recent interest in the science of azadirachtin (Schmutterer *et al*., 1984)).

2. Developmental failure.

Immature insects fail to make the transition from one instar to the next. This suggests an effect on hormonal secretion or action (Schmutterer, 2002).

3. Loss of fertility.

This effects mature insects, both male and female, and might be another effect on hormones (Schmutterer, 2002).

4. Secondary anti-feedant effect.

The insect digestive system fails to function properly, and it ceases feeding.

5. General loss of biological fitness .

This may be represented by, for instance, the failure to fly due to poor muscle development, and general loss of resistance to infection (Schmutterer, 2002).

It is not clear if there is a single target which might cause such a range of effects, but it might be associated with protein synthesis (Paranagama *et al*., 1993), or the formation and secretion of hormones (Mordue and Blackwell, 1993), or cell duplication (Schluter, 1987).

At the moment, two possible protein targets have been tentatively identified: tubulin (Salehzadeh *at al*., 2003), and a heat-shock protein from *Drosophila melanogaster* : hsp60 (Robertson *et al*., 2007).

i) Tubulin.

A number of publications have identified an anti-mitotic and anti-meiotic effect of azadirachtin A in various insects (Schluter, 1987; Shimizu, 1998; Linton *et al* 1997).

These studies were consistent with the possibility that azadirachtin A interferes with the process of spindle formation and assembly which is essential for cell division. The

protein responsible for spindle formation is tubulin. Tubulin is a highly conserved protein present in all eukaryotic cells, which is part of the cytoskeleton on the cell, and whose rapid polymerisation and depolymerisation is responsible for cell division, cell movement and axonal transport. Salehzadeh *et al*., (2003) showed that azadirachtin prevented the polymerisation of mammalian tubulin in the same way, but less effectively, than colchicine. They also showed that azadirachtin appeared to displace colchicine from a cellular binding site, which can be presumed to be tubulin.

Many of the observed effects of azadirachtin on insects, listed above, could be accounted for by interfering with the polymerisation of tubulin. By preventing meisis and mitosis, it could cause the loss of fertility and developmental problems. Also, as tubulin is essential for neurosecretion, it could disrupt all the processes which require hormonal control.

ii) Hsp 60.

In 2007 evidence was presented that the heat-shock protein hsp 60 in cultured Drosophila Kc 167 cells could bind to azadirachtin A (Robertson *et al*., 2007). Hsp 60 is a ubiquitous "chaperone" protein. It is not clear what role it might play in the action of azadirachtin, but it might certainly associated with a failure of protein synthesis and release, which could account for some of the effects of aza A.

1.6.2 The formation of microtubules and its inhibitors.

During the life cycle of higher plant cell there is reversible polymerization process of tubulin into microtubules (MT) at a specific times at a specific locations in the cell. This process is, dynamic instability of MT, can be summarized into four distinct stages: stage of polymerization, depolymerization, transition from polymerization-depolymerization

and the reverse phase (Belmont and Mitchison, 1996,). The process, which occurs in different occasions of the cell – polymerization/depolymerization - is controlled by microtubular organizing centers (MTOCs). *In vitro* polymerization of animal microtubule showed that it has a quite a number of binding sites for different purposes one for different antitubulin, such as *colchicines* and *vinblastine*; one for GTP and one for lateral and longitudinal binding required during the formation of microtubules (Dieter and Marme, 1980; Van Eldik, 1988).

1.7 Compounds that induce morphological and cytological effects with the microtubules.

1.7.1 Natural ones.

The loss or disruption of microtubule function should have a severe impact on the individual plant cell division thus growth of the plant as whole. For example, spindle microtubules play a significant role in the cell division and their lose affects nuclear division and the separation of chromosomes. Likewise, lack of cortical microtubules affects the morphogenesis of the cells and tissues (Delye *et al*., 2004; Itoh, 1976)

Apart from the known anti-microtubule herbicides, there are some other compounds that have as the same effect, at least on animal cell, to the microtubules. Colchicine binds to the tubulin dimer and as result inhibits the formation process of microtubules, but only at high concentrations, while Taxol stabilizes microtubules from depolymerization back to individual tubules (Hart and Sabnis, 1976; Montague and Ikuma, 1975.; Salehzadeh et al., 2003). And, of course Plant growth inhibitor *ancymidol* (Montague and Ikuma, 1975.).

1.7.2 Anti-mitotic Herbicides.

Dinitroanalines and Carbamides are the two structural series that interfere with the microtubular systems that has been known for a long time, Phosphoric amides, although structurally dissimilar to the mentioned ones, also show interference with the assembly and function of microtubules (Anthony and Hussey, 1999; Ellis, Taylor, and Hussey, 1994; Fedtke, 1982).

a) Dinitroanalines.

Dinitroaniline herbicides act by inhibiting cell division (mitosis). Specifically, they inhibit microtubulin synthesis necessary in the formation of cell walls and in chromosome movement to daughter cells during mitosis. The cell does not complete division and affected cells remain as single cells with multiple nuclear chromosomes: multi-nucleated cells (Ahrens, 1994). Disruption of cell division process, nucleic acid metabolism and protein synthesis is the main mechanism of action of herbicides (Fedtke, 1982).

b) Phosphoric amides.

Work has been done on the molecular mode action of Amiprophos-*methyl* on algae. After treatment microtubules were virtually absent in the cell, and the cell wall organization was abnormal (Fedtke, 1982). Extensive study was made on the correlations between cellulose microfibril and cortical microtubule using Colchicine, Amiprophos-methyl (APM) and protein synthesis inhibitor cycloheximide. Amiprophos-methyl showed that it neither interferes with the *in vitro* brain tubulin polymerisation, even if the concentration is increased (Wagenbreth and Robinson, 1978). Study showed that after the tubulin synthesis has already started Amiprophos-methyl is still capable of degrading tubulin

mRNA or inhibit the synthesis of ones (Fedtke, 1982).

APM, a phosphoric amide herbicide, was previously reported to inhibit the *in vitro* polymerisation of isolated plant tubulin. APM inhibits competitively the binding of oryzalin to tubulin, indicating the formation of a moderate affinity tubulin-APM complex that may interact with the ends of microtubules. APM concentrations inhibiting tobacco cell growth were within the threshold range of AMP concentrations that depolymerised cellular microtubules, indicating that growth inhibition is caused by microtubules depolymerisation. APM had no apparent effect on microtubules in mouse 3T3 fibroblasts. Because cellular microtubules were depolymerised at APM and oryzalin concentrations below their respective Ki and Kd values, both herbicides are proposed to depolymerise microtubules by a substoichiometric endwise mechanism (Murthy *et al*., 1994).

1.8 Effects of azadirachtin on plants.

Both the potential targets for azadirachtin binding which have been identified, tubulin and hsp 60, are highly conserved in eukaryotic cells. Tubulin at least has well-defined and essential role in many cellular functions. It would seem possible that it would have an antimitotic effect in plants as it does in insects. In fact, azadirachtin has been shown to have a phytotoxic effect, particularly in young plants, which might be due to prevention of cell division. The monomers α- and β-tubulin from plants cells are not the same as those of the animal cells as far as their molecular weight and to the colchicine-binding ability is concerned (Filner and Yadav, 1979). However, as described above, there is a class of herbicides, which act as antimitotic compounds in plants, due to their effect on

tubulin. These will be described in greater detail in Chapter 3 of this thesis. If azadirachtin is to be used systemically on growing plants, it is important to discover if the compound does have an antimitotic effect, and at what concentrations this effect might occur.

1.8.1 Effects of Azadirachtin on the Environment, Animal and Human health.

The main point of the work reported in this thesis was to study the effect of aza A added to soil, and then taken up by plants to act systemically. Being an oxygenated and thus polar compound, aza A, unlike most insecticides, is moderately soluble in water (1-2g. L^{-1}, Daly, 2004, Kleeberg, H, (unpublished results)) and relatively mobile in soil. There is an obvious risk of ground-water contamination, but this is being offset by its rapid biodegradability in soil: azadirachtin has a reported average half-life of 2.3 days (Daly, 2004) in field soil.

Azadirachtin is regarded as being non-toxic to higher animals. In so far as the human health effects of azadirachtin products are concerned, studies of azadirachtin mutagenicity and acute toxicity have shown that it's likely not to pose a significant risk to human health. However, some people have exhibited skin and mucous membrane irritation from neem seed dust (Weinzierl and Henn, 1991).

Rat oral LD_{50} of azadirachtin is >5000 mg/kg. (Miller and Uetz, 1998), placing it in the lowest category of toxicity (IV) of the Pesticide Manual.

Azadirachtin's toxicity towards fish is moderate and is not expected to kill fish under normal use.

Azadirachtin has little or no negative effect on adult beneficial insects. It is reported to be relatively harmless to bees, spiders, ladybirds, parasitoid wasps, and adult butterflies, although in a few trials, negative effects have been noted on immature stages of beneficial species exposed to neem (Banken and Stark, 1997). Neem products are generally thought to be suitable for inclusion into integrated pest management (IPM) programs (Lowery and Isman, 1994a). With other non-target organisms such as birds and fish, azadirachtin is considered to be generally non-toxic (Grunert. 1996; Johnson, 1996a, b; Wan *et al*, 1996; Elangovan *et al*, 2000; Schmutterer , 2002).

1.8.2 Systemic effect of Neem Products.

Gill and Lewis, (1971); Nisbet *et al*., (1993); Osman and Port, (1990), have at different times reported that plants can absorb active neem constituents through roots allow the terpenoid to systemically move upward through the plant through xylem tissues. As Hummel. E and Kleeberg, (2003) and Daly, (2004), reinforced later on, this works best when sufficient quantities are applied to the root zone. At the moment most use of neem formulations are for foliar sprays, but as the limonoid is rapidly destroyed by sunlight, it may be that most of its effect is systemic, even although it is absorbed poorly through the plant cuticle. Daly (2004) showed that the half-life of azadirachtin A was much longer in the leaf water than in the soil. The systemic properties of aza A suggest that applying it to transplants just before planting to the field could be an effective and inexpensive way to control certain pests. Similarly, applying neem with relatively large amounts of water, in directed sprays over the rows of small seedlings, could be a very efficient method of

application. In one study, neem applied through a drip irrigation system significantly reduced lettuce aphids on lettuce by over 50% (Palumbo *et al*., 2001)

In another finding, a newly standardised powder formulation, NeemAzal-PC, intended for hydroponic use, has been used successfully to control aphids (*Aphis fabae*) and bollworm moth (*Heliothis armigera*) on beans (*Phaseolus vulgaris*) (Hummel and Kleeberg, (2003). As much of the intensive growing of high value vegetables such as peppers is done uinder glass in hydroponic systems, this may suggest an important future market for azadirachtin-containing plant protection products.

1.8.3 Practical Problems of Neem Application.

One reason for the slow acceptance of neem PPP is the delayed effect of neem derivatives may irritate the farmers who are used to synthetic neurotoxins/pesticides with immediate knockdown efficacy. Pests such as aphids continue to feed on the treated plants for a considerably time, even though the amount of food ingested by insect is considerable reduced due to the primary and secondary antifeedant effect by neem derivatives (Schmutterer, 1990b)

The effect or field performance of Neem pesticides is indirectly influenced by environmental factors such as temperature. In comparing the pure compound of neem to the commercial formulations, Copping and Menn, (2000) concluded that due to a photodegredation, the commercial formulations of azadirachtin was five times faster than pure compound in breaking down after application.

The application of neem-based pesticides against adult insect does not normally lead to

mortality, but may reduce substantially fecundity of the target insects. As a result, the following generation mass may be reduced below economic threshold level. (Schmutterer, 1987).

As already discussed, the problems of resistance have been present almost from the first years of the use of synthetic insecticides. The misuses of neem pesticides may lead to the target insects develop adaptation after some time and insects will be capable of differentiating between the treated and untreated parts of their host plant (Daly, 2004). Resistance is due to the overuse of a single pesticide with whose action depends on a single molecular target; thus all other pesticides that possess the same mode of action will also fail to be effective (Daborn *et al*., 2002). This is an argument for using a mixture of the azadirachtinoids, rather than pure azadirachtin A. Despite 20 years of use in Europe and USA, there are no reports of resistance to neem-based plant protection products.

If azadirachtin is applied to the soil as a drench, the short half-life will mean that it will disappear rapidly, possibly before being taken up by the plant. The advantage of the pelleted version should be to prolong the active life of the terpenoid in the soil.

1.9 Conclusion.

Although neem-seed extracts with azadirachtin A as their active ingredient are registered in many countries in Europe and in the USA, and have been used for about 20 years, outside India, they remain a niche-product. Although azadirachtin A has many advantages, it has some disadvantages as well. These have been discussed in this Introduction, and are summarised below. Will these products ever be widely used?

1.9.1 Advantages of azadirachtin as a plant protection product:

- Affects almost all pest insect species yet tested
- Non-neurotoxic
- Non-toxic to mammals
- Compatible with beneficial and IPM
- No resistance yet reported
- Short soil half-life (no threat to ground-water)
- Acceptable to organic producers and Soil Association
- Large industry in India to supply extracts

1.9.2 Disadvantages of azadirachtin as a plant protection product:

- Slow in action
- Poor contact insecticide
- Sensitive to lysis by light and water
- No possibility of economic synthesis
- More expensive than synthetic insecticides
- Not registered in UK
- Some phytotoxicity

At the moment there are three developments which are working in favour of greater use of neem extracts with a high level of azadirachtin A. One is the fact that a huge revision of pesticides has been taking place in Europe over the last decade. This has seen the removal of 60% of the previously-employed synthetic insecticides, leaving many crops without adequate insecticidal protection. The other is the growth of organic farming, born to some extent out of a fear of neurotoxic insectides such as the organohosphates. Neem, with no toxicity towards mammals, is well-placed to cater for this growing market. The final point is that with the encouragement of the Pesticide Safety Directorate in York and the collaboration of the leading German neem company, Trifolio GmbH, the CASE-sponsoring company NeemCo, intends to register neem as a benign insecticide in the UK next year (2010).

The relatively high water-solubility of azadirachtin, which allows it to move in the soil, and within plants, is not being exploited by the foliar sprays which currently dominate the market. A drawback of azadirachtin is that it has a very short half-life in soil. It is, however, broken down much more slowly in plants. The work of (Daly, 2004) has shown that a slow-release pellet incorporating neem extracts can be used in soil. It is hoped that this means of delivery can extend the soil half-life and give protection to both soil and foliar pests.

1.10 Aims.

The main aim of the project was to extend the previous work by Daly (2004) and to try to prepare a pelleted version of the main commercially-available neem-seed kernel extract, NeemAzal®-T produced by Trifolio GmbH, in preparation for the expected registration of the product in the UK in 2011.

The previous work had shown that aza A could be applied to soil in pellet form, and was taken up by plants. It did not show how successful this approach was in plant protection. The pellets were made in the laboratory by Daly (2004), not by a commercial specialist. The plants used, nasturtium, were not of any commercial significance.

The aim of the present work was to use commercially-made pellets, and to use plants of commercial significance: cabbages and sugar-beet.

The immediate aims of the project can be outlined in this way:

1. To purify aza A from NeemAzal®-T to greater than 98% w/w purity to act as a quantitative standard to quantify the terpenoid extracted from pellets, plants and soil in the rest of the project.
2. To determine the level of phytotoxicity to germinating and growing plants due to NeemAzal®-T, and to try to determine if the phytotoxicity was due to an antimitotic effect.
3. To use a hydroponic system with known concentrations of aza A in the medium, to follow the uptake of the terpenoid into plants, and the half-life in the plants.
4. To get NeemAzal®-T incorporated into standard commercial pellets by Germains Technology Group (Kings Lynn).

5. To characterise the pellets in terms of their content of aza A, and the release of the terpenoid into soil in laboratory experiments, and, by varying the content of the pellets, to try to delay the release of the active ingredient.
6. To follow the uptake of aza A in the leaves of plants grown in soil containing the pellets.
7. To use the results obtained to examine, in green-house experiments, the effects of the systemic treatment in controlling the main pests of cabbage: aphids, caterpillars, flea beetles, and cabbage root fly.
8. To repeat the above with field experiments on a large scale.

Chapter 2: Quantitative analysis of azadirachtin A.

2. Aims

Much of the work reported here depended on the quantification of aza A, which is the a.i. in the various neem-based PPP available in those countries in which the extracts are registered. It is best estimated by the standard method adopted by CIPAC in 2005 (www.cipac.org) which uses UV absorption at 217 nm in an HPLC method which will be described later. An alternative colorimetric method (Dai *et al*., 1999; Daly, 2004) is not sensitive or specific.

A pure standard of aza A must be obtained to act as a reference. Although available commercially, pure aza A is very expensive (£ 95 per mg; Sigma-Aldrich) and so the first part of this work was to prepare a sample of aza A of the highest purity.

It was first necessary to purify, to a high level, aza A, so that a standard curve could be obtained for subsequent quantitative analyses. It has always been considered difficult and tedious process in isolating and purifying aza A due to its structural similarity to its related limonoids (Strang, R., personal communication; Turner *et al*., (1987); Daly, (2004); Deota *et al*., (2000).

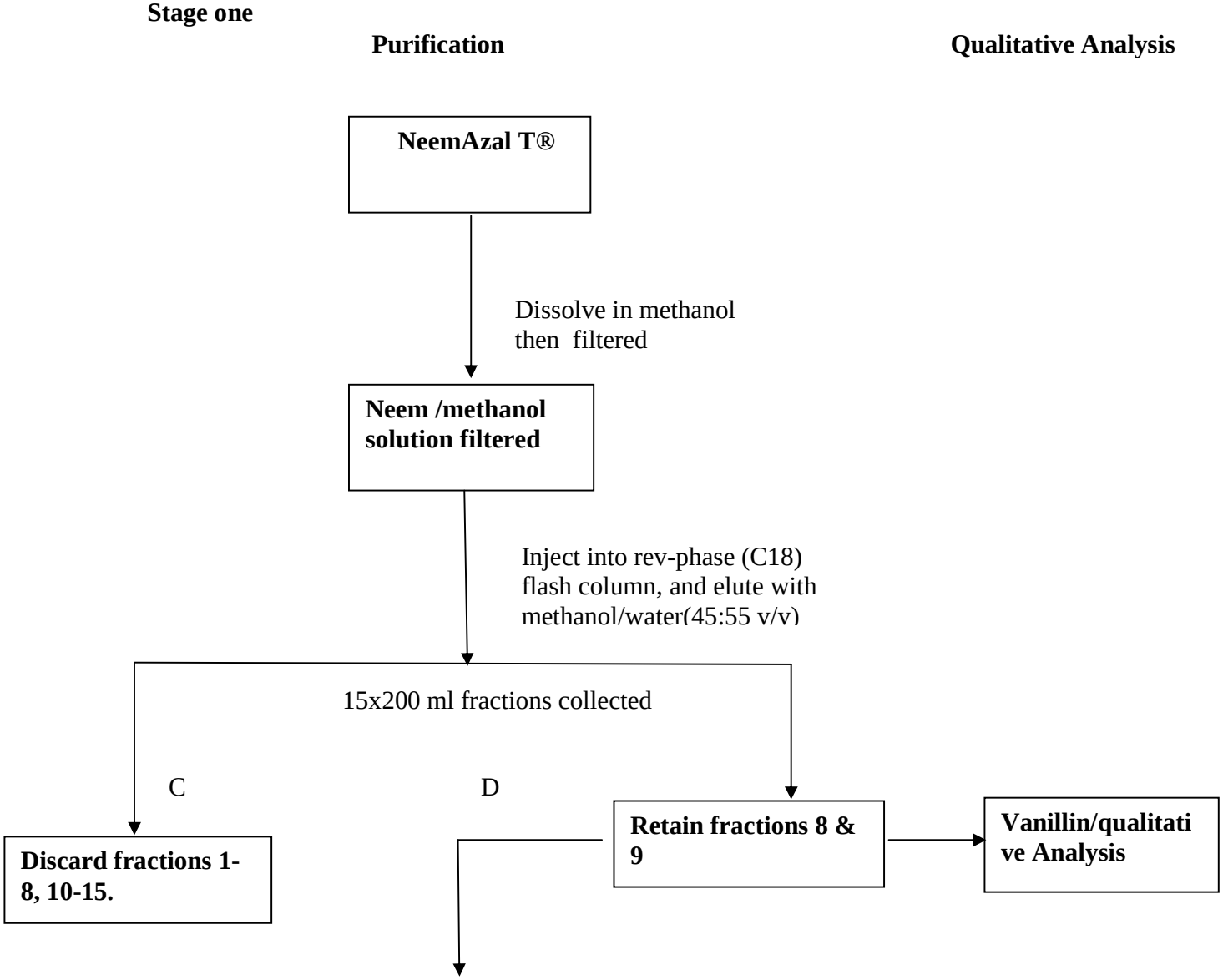
There are two stages in isolating aza A from seed kernels. Even though semi-purified azadirachtin is used as the starting material, it worth stating the two stages it takes to reach the final aza A purity. The preliminary stage is preparative clean up one: It is the stage in which triglycerides, water soluble proteins, and sugars are removed by polar solvents to reach finely-powdered neem seed kernel extract, which contains 20-40% aza A. The second stage (Figure 1.2) is the one employed here and various chromatographic

methods were used. It separates aza A from its congeners, (the other azadirachtoids) such as azadirachtin B which has the almost the same polarity as aza A, to reach refined aza A isolation (Barnby and Klocke, 1987; Morgan and Jarvis, 2001; Schroeder and Nakanishi, 1987; Deota *et al*., 2000).

It is important to mention that both processes are time-consuming and yield only small amount of pure aza A. (Hien & Humme, 2000). There are other chromatographic and non-chromatographic methods such as multilayer counter current chromatography, supercritical fluid extraction that have been reported but they suffer the same disadvantages of being arduously time-consuming and giving a low yield (Dai *et al.*, 2000; Morgan, and Jarvis, 2001; Ambrosino e*t al*, 1999).

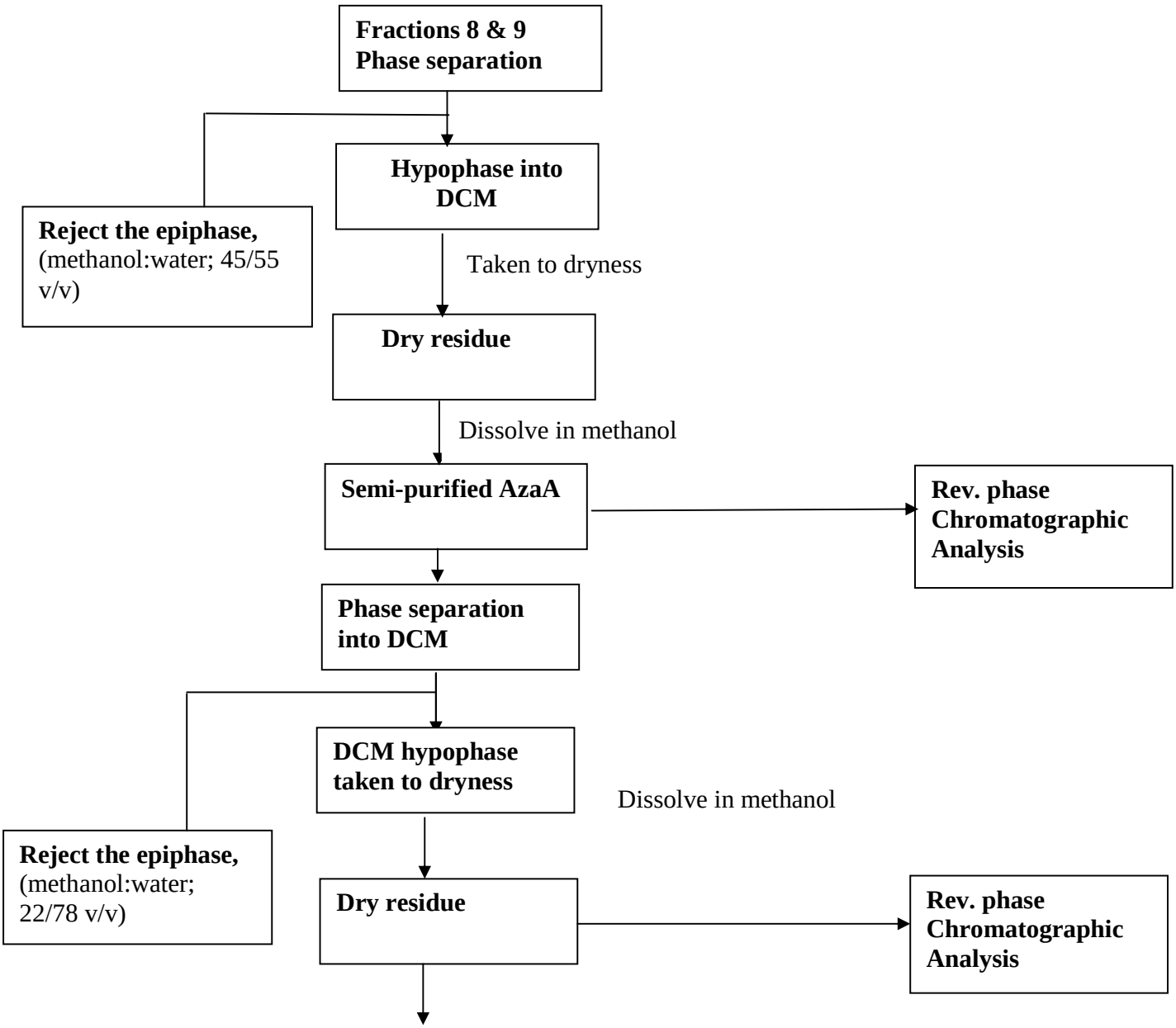
Figure 2.1: Flow Chart of Procedure for Pure Azadirachtin A Isolation.

The flow chart showing the outline of the method developed to purify aza A to > 95% purity from crude NSKE using flash column and preparative reverse-phased HPLC chromatography.

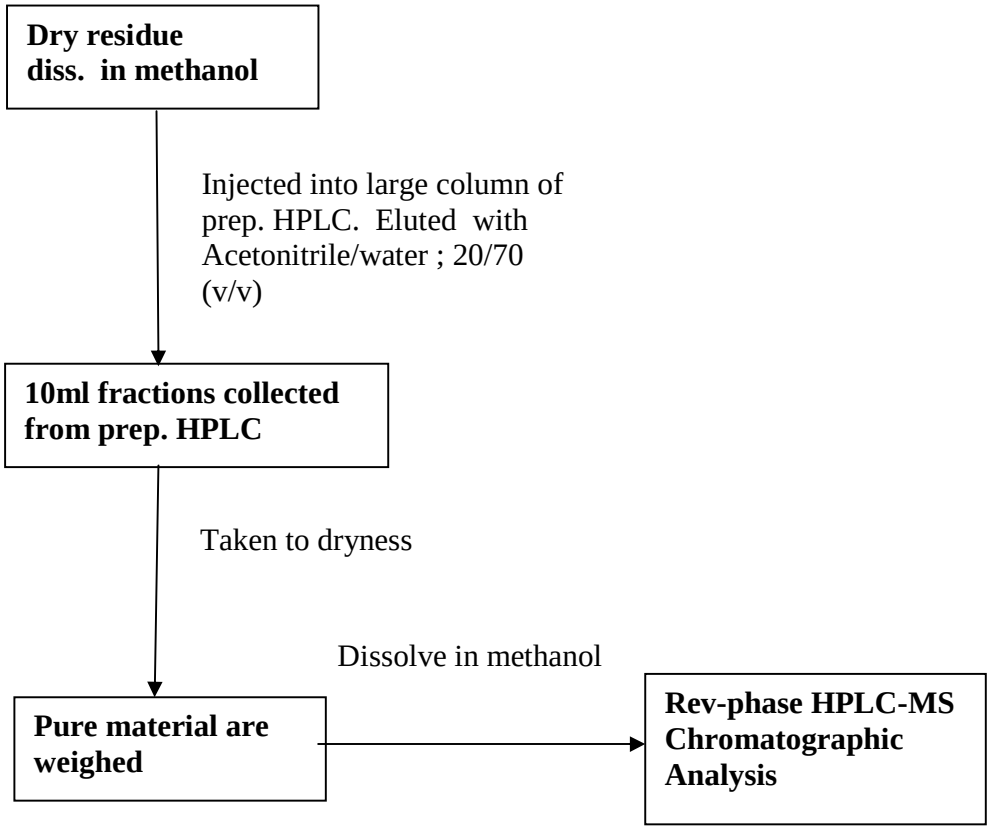


Stage two

Purification **Qualitative Analysis**



Stage three **Purification** **Qualitative Analysis**



2.1 Purification of Aza A.

2.1.1 Solvents.

All the organic solutions (Fisher Scientific UK) were either AR grade or HPLC grade as appropriate. Distilled water was filtered through a 0.2 µm filter before use. The process of purification of aza A is being separated in to three stages:

2.2 Stage One:

2.2.1 Flash chromatography.

a) NeemAzal®- Technical (NAT)

The starting material for preparation of aza A was NAT supplied by Trifolio GmbH, Lahnau, Germany. This yellow powder contained about 40% aza A by weight.

A quantity (4g) of this material was dissolved in 10ml of AR methanol by stirring with a magnetic stirrer at room temperature for 30 min. It was then filtered through a 0.2 µm filter (Sartorious, Sweden) before its injection on to a C18 reverse phase flash chromatography column (Biotage Ltd, Hertfordshire, UK. 150x40 mm, 35-70 micron particle size, average pore size of 60 Å)

The material was then eluted with methanol/water, 45/55 (v/v), under a pressure of 1793 mbar from a cylinder of nitrogen gas. This allowed a solvent flow of about 15ml.min^{-1}. 15 separate fractions of 200ml were collected. A sample (approx. 50µl) of each fraction was spotted onto aluminium-backed silica gel (see on 2.2.2) and stained for the presence of terpenoid compounds by means of the vanillin stain (see 2.2.2.1).

Those fractions showing the presence of high concentrations of material were analysed

by TLC.

2.2.2 Thin-layer chromatography.

Aluminium-backed, 0.02mm silica gel plates, 5x10cm (Macherey-Nagel GmbH, Dűren, Germany) were used for normal adsorption chromatography. Extracts were resolved in light petroleum/ethyl acetate (30/70, v/v) before drying and visualising the terpenoids by means of the vanillin stain.

2.2.2.1 Vanillin stain.

In order to monitor process of purification, vanillin stain, acidified methanol solution of vanillin, was used to give rough indication of quantification (Eweig and Shermer, 1972). The stain consisted of 3g of vanillin (4-hydroxy-3-methoxybenz-aldehyde), (Sigma-Aldrich, Pool, Dorset, UK) dissolved in 160ml of 95% ethanol, to which 40ml of 2M H_2S0_4 were carefully added. The stain was kept in a brown bottle. The aluminium-backed plates were dipped in the vanillin stain, and then heated by means of a hair-dryer, until the spots became visible. The pure standard of aza A developed a blue-purple colour, which helped identification of the compound in mixtures (Figure 2.2).

2.3 Stage Two:

2.3.1 Concentration by phase separation

The fractions from the flash chromatography described above found to contain most of the azadirachtin (Fractions 8 and 9 dissolved in methanol/water) were combined in a separating funnel, to which 100ml of dichloromethane (DCM) was added. An equal volume of water was then added and the funnel vigorously shaken, to drive the polar

terpenoids into the DCM. After the two layers had separated, the hypophase of DCM was retained and the epiphase of aqueous methanol re-extracted twice with DCM. The DCM extracts were combined, and the water removed by means of adding approximately 10g of anhydrous sodium sulphate.

The dry DCM solution was then taken to dryness in a rotary evaporator (Buchi Rotavapour) at reduced pressure (650 mbar) and lowered at a water-bath temperature of 40^{o}C. The yellow-white residue was dissolved in 5 ml of methanol, transferred to a 10 ml beaker, and the solvent allowed to evaporate at RT overnight. The dry residue was then weighed and further purified by preparative HPLC.

2.4 Stage Three:

2.4.1 Preparative HPLC.

Preparative HPLC was carried out with a large (21.7mm x 250mm) C-18 reverse-phase column (Phenomenex, model T5-430, Macclesfield, UK) maintained at a temperature of 40^{o}C to lower the solvent viscosity.

The mobile solvent was acetonitrile/water (35/65, v/v), and peak detection was by absorption at 217nm. The column eluate was collected in 10 ml samples by means of a fraction collector (Gilson FC 204 by Gilson Engineering Ltd., Newbury, UK).

The partially purified azadirachtin from the previous stage was dissolved in 20%v/v methanol (100mg in 100 ml of 20% methanol) This solution was further diluted in 1/10 in water, and 10 ml volumes injected onto the column. The rate of eluant flow was 5ml.min^{-1}. and the each run was 45 min in length. The column eluant was collected in 10ml samples by means of a fraction collector. This procedure was repeated until all the

partially purified material had been passed through the column.

2.4.2 Analytical High Performance Liquid Chromatography.

This was carried out in three locations (Davidson Bld., Graham-Kerr Bld. of Glasgow University and at NeemCo Ltd. Irvine), and on three different HPLC systems: Perkin-Elmer HPLC system, Surveyor HPLC system and Beckman HPLC system.

In each case the basic method was, however, the same. Analysis was carried out using a C-18 reverse phase column (4.6mm x 25 mm) and an eluant of 35/65 acetonitrile/water (v/v) with an isocratic elution. The terpenoids were located and quantified by their absorbance at 217 nm. These are the conditions which have been provisionally adopted, at the 49th council meeting (Utrecht, 2005), by Collaborative International Pesticides Analytical Council (CIPAC) CIPAC/4545 /P (CIPAC/4429, 2006) as the internationally agreed method for analysis of aza A.

2.4.3 Identity of azadirachtin by molecular weight.

This is was done by HPLC coupled to mass spectrometry with the kind assistance of Dr Bill Gemmell of the Division of Plant Sciences, Institute of Biomedical and Life Sciences. The solvent system (methanol/water) in this case routinely contained formic acid (10%) (Mr. 44), as it was used for analyses of a wide range of phytochemicals, including those with carboxyl and other acid groups.

The column (4.6mm x 25mm, Phenomenex, Macclesfield, UK) was maintained at 40^{o}C. The eluant was formic acid/ acetonitrile/ water 10/20/70 (v/v). The eluate was split after passage through the flow-cell of the diode array detector, and 0.3ml.min^{-1} was directed to an LCD DecaXP ion trap mass spectrometer fitted with and electro-spray interface

(Thermo Finnegan, San Jose, USA) the sample was analysed in a negative ion mode.

2.5 Quantitative Analysis of Aza A.

2.5.1 The Aim.

Once the standard curve has been achieved, few preliminary analyses of some commercial crude neem kernel extracts were done. Two methods were compared, HPLC and Vanillin Assay, in the quantification aza A in these extracts. The methods developed in these preliminary analyses were used in the rest of the project.

2.5.2 The Methods.

2.5.2.1 HPLC.

The main method of analysis that agreed by CIPAC, as already mentioned, employing reverse phase HPLC.

The HPLC used was a Perkin-Elmer HPLC system with a Perkin-Elmer series 200 pump, 785 UV/Vis detector and degasser. The data were collected and processed on a Perkin Elmer 1022 integrator. The column was a C18 reverse-phase analytical column (3 µm particle size). The eluants used were those set out in the CIPAC method:

a) Isocratic solvent: 35:65, (v/v) acetonitrile/water.
b) Gradient solvent: from 20:80 to 100:0 (v/v) acetonitrile/water.

(Only isocratic elution was used in the work reported here.)

Location and determination of aza A and other terpenoids was done by absorbance at 217nm. Flow rate was $1ml.min^{-1}$. The same volume (20µl) of sample was injected in each case.

In the purification process, the relative proportions of Aza A and the other azadirachtoids were quantified by a) estimating the area of the elution profile of the compounds and b) the peak height of each compound. The final purity of aza A obtained after preparative HPLC was also determined by MS analysis, comparing the peaks obtained with a standard supplied by Trifolio GmbH (Ruch, B, personal communication), and by TLC on silica gel, with repeated development of the chromatogram and staining by the vanillin stain, which if heated sufficiently, would be expected to show all carbon compounds present by charring.

2.5.2.2 Vanillin Assay.

The second method used for quantification of the limonoid was a fast colorimetric technique (Dai *et al*., 1999). This is a modification of the acidified methanol solutions of vanillin that has been used for the visualization of aza A presence in the limonoids by TLC (Allan, 1994). This Vanillin Assay was used to develop a colorimetric method for the quantification of aza A in the prilled NSKE pellets. The two methods were compared in terms of sensitivity.

2.5.2.2.1 Preparation of Standard Curve of Pure Aza A.

Aza A previously purified by flash and preparative HPLC to more than 95% purity, was dissolved in methanol and made up to 25ml using a volumetric flask. A sample of this solution (250 μgml^{-1}) was then serially diluted in methanol to give a range of concentrations down to 32 μgml^{-1}. These were used to construct a standard curve of aza A concentration against peak height and peak area.

2.5.3 Determination of aza A in crude neem extracts and pellets.

2.5.3.1 Commercial samples enriched for aza A

a) Crude Neem Seed Kernel Extracts (NSKEs).

Five commercially available samples of NSKE were compared. They were sourced as follows:

1) Sri Dhisha Biotech (Hydrabad, India).
2) Nickla Agricultural Industries(Mumbai, India)
3) Rym Exports (Mumbai, India)
4) Ascott (Mumbai, India)
5) NeemAzal® -Technical (Trifolio GmbH, Germany).

In each case, 30mg of the yellow powder was dissolved in 50ml of methanol using volumetric glassware. All were filtered through a 0.2 µm Sartorius Minisart single use syringe filter (Vivascience AG 30625, Hanover) before their application to the HPLC column.

b) Neem seed oil.

The aza A content in two neem seed oils, one from Trifolio GmbH., and the other from an unknown Ghanian source, were examined. 50 ml (1mg/10ml, v/v) of solution of each one was prepared; 5 ml of each neem seed oil was weighed into 50 ml volumetric flask and filled up with methanol.

c) Prilled NSKE Pellets.

A trial sample of pelleted material had been prepared by Germain's Technology Group (King's Lynn, UK), using NAT supplied by Trifolio GMbH. The amount of total

azadirachtoids and aza A in the pelleted materials were determined i) by reverse-phase HPLC and ii) Vanillin Assay.

i) reverse-phase HPLC.

A sample (0.5g) of the pellets were extracted into 5 ml methanol in 30 ml glass centrifuge tube using an Ultra Turrax bladed homogeniser. The homogenate was centrifuged at 2000g for 3 minutes, and the supernant poured off into a 50ml volumetric flask. The extraction process was twice repeated, and the supernants combined, and then made up to 50 ml, and well mixed, before filtration through 0.2µm Sartorius Minisart single use syringe filter by Vivascience, Hanover, Germany. Aliquots (20µl) were injected into the HPLC column for analysis. This was compared to standards according to their peak heights. Simultaneously this was authenticated by carrying out a vanillin assay as follows.

ii) Vanillin Assay.

A solution of 0.7 ml of methanol containing 1mg NAT with a known concentration of aza A, was prepared as a standard. Vanillin (0.02g/ml) in a solution of 0.2ml of methanol was added and shaken for 5 seconds. The mixture was left at room temperature for 2 minutes. A 0.1 ml of 20M sulphuric acid was, then shaken for 5 seconds. The solution was allowed to stand at room temperature for 5 minutes for the colour to stabilise. Finally the absorbance was measured at 574nm using a spectrophotometer equipped with tungsten lamp. Also, 0.5mg of pelleted material was prepared and read its absorbance as above standard. The test solutions were replaced by with an equal volume of methanol in above procedure. Quantification of pelleted materials was achieved by correlating its absorbance by that of a standard curve of NAT derived from a solution in methanol.

2.6 Results.

2.6.1 Thin-layer chromatography.

After collection of 14 fractions eluted from the Biotage 40 column, the fractions were tested for the presence of aza A by TLC on Aluminium-backed, 0.02mm silica gel plates, 5x10cm and visualised in the vanillin stain. A typical result is shown in Figure 2.2.

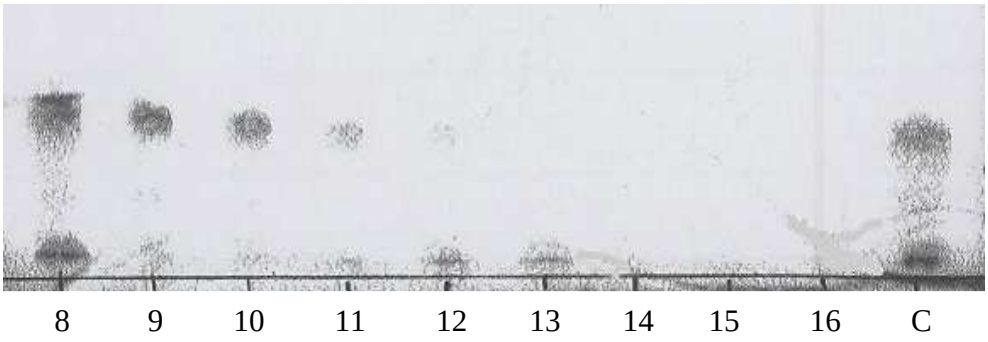


Figure 2.2: Identification of fractions from flash chromatography containing aza A. The result of TLC test to identify the fractions containing the highest concentration of aza A. in here fractions 8 and 9 contain the maximum amount of aza A with minimal impurities. The spot at the extreme right (C) is a standard of pure aza A.

2.6.2 Purification of aza A from the starting NSKE (NAT).

The process of purifying aza A to a high level is summarised in Table 2.1 and illustrated by the HPLC traces in Figures 2.4 a,b and c.

Neem material	Weight of material (mg)	Yield (%)	Aza A purity (%)
NAT	4000	100	42
Stage 1 (Flash chrom.)	300	7.5	75
Stage 2 (Prep. HPLC)	6.3	0.15	98

Table 2.1 Summary of purification of aza A.

The starting material was NeemAzal®-Technical (NAT)

The starting material for the purification, NAT, was already enriched in aza A, the purity of which was 42%. Choosing the aza A-rich fractions from the initial flash chromatography (Stage 1) raised this purity to 75%. The final stage of preparative HPLC (Stage 3) raised the purity of aza A to 98%, as determined by the methods described in section 2.5.2.1. The final yield of this pure material was, however, very low, at 0.15% of the weight of starting material.

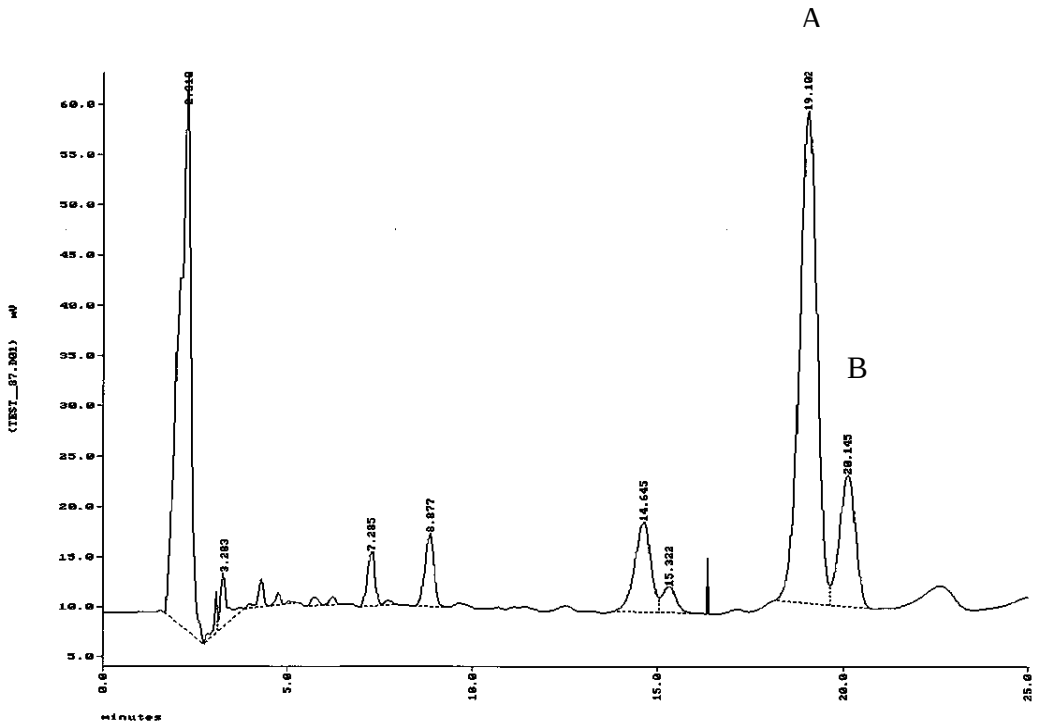


Figure 2.3a: HPLC analysis of the starting material, NeemAzal®-Technical (NAT). A sample (20µl at 1mg.ml^{-1}) was analysed by HPLC under the standard CIPAC conditions of reverse phase (C18) chromatography with an isocratic elution: acetonitrile/water; 35/65 v/v. Flow rate was 1ml.min^{-1}. Detection by light absorption at 217 nm. The large peak at 0 min is an injection artefact. A) aza A; B) aza B.

As the HPLC trace in Fig. 2.3a shows, the CIPAC method, now adopted as the internationally-agreed method of quantifying aza A, is very successful in separating the limonoids in the semi-pure mixture. Aza A which runs with an RT of 19.1 min. is adequately separated from aza B (RT: 20.1 min.). The minor peaks are presumed to be other azadirachtoids, but were not identified.

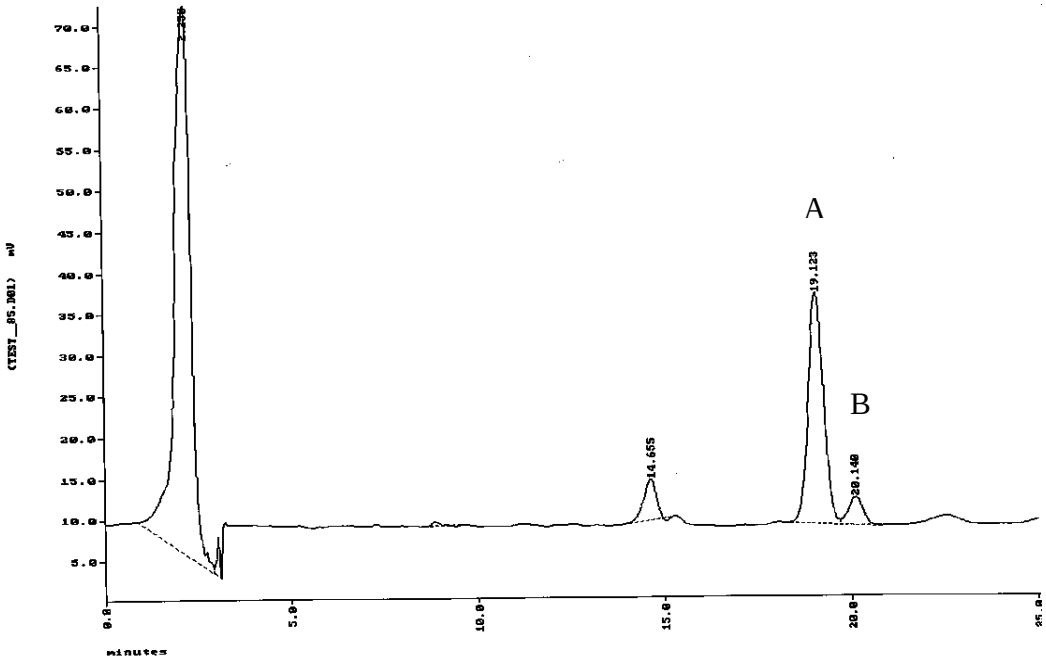


Figure 2.3b: HPLC analysis of material after Stage 1 (flash chromatography) of purification of aza A.
The material was that in Fractions 8 and 9: those containing most of the aza A. Conditions as in 2.3a. A) aza A; B) aza B.

2.6.3 Purified aza A

The aza A purified in a preparative HPLC. The result, as shown here, was a clear single peak of over 98% purity of aza A.

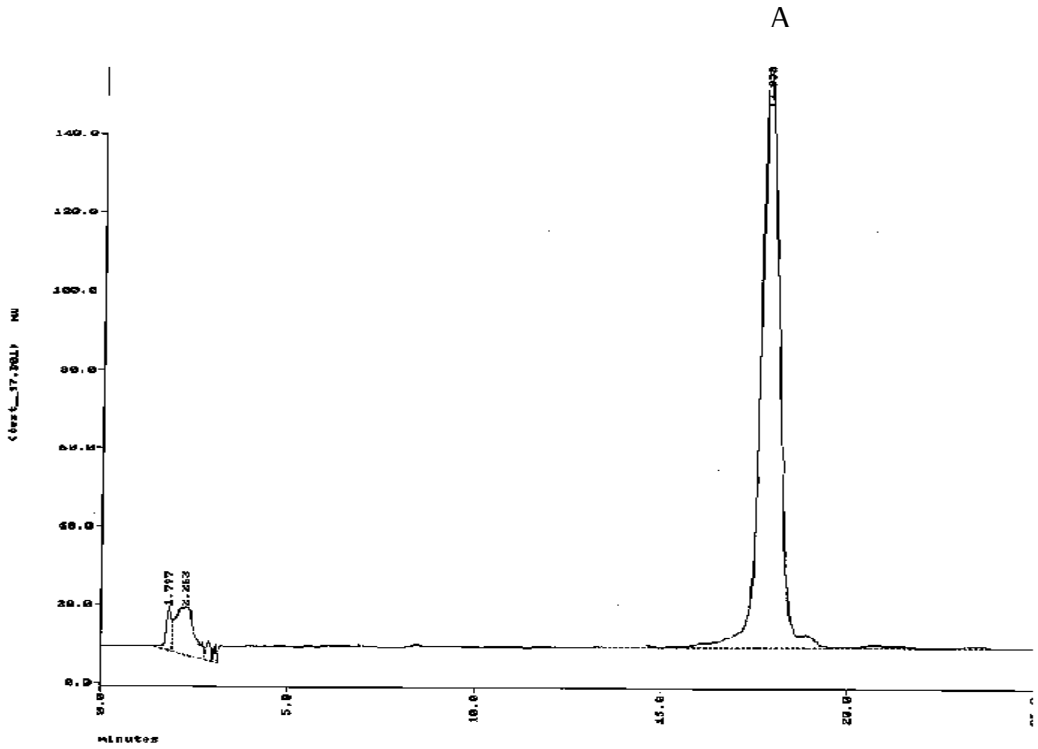


Figure 2.3c: HPLC analysis of material after Stage 3 (prep. HPLC) of purification of aza A.
Conditions as in Fig. 2.3a. A) aza A.

The HPLC trace in Fig. 2.3c shows that a high level of purity of aza A was achieved after two stages of reverse-phase chromatography. The final yield of about 6 mg of aza A was low, but sufficient to allow quantification of the limonoid in the rest of the project.

2.6.4 Confirmation of identity of aza A by MS.

The chromatogram (figure 2.3d) shown the suitability of the reverse- phase HPLC methods (HPLC and HPLC coupled to mass spectrometry) adopted here for the quantification of aza A in neem seed kernel extracts (NSKE's) and the purification to a single peak purity using flash and then preparative HPLC.

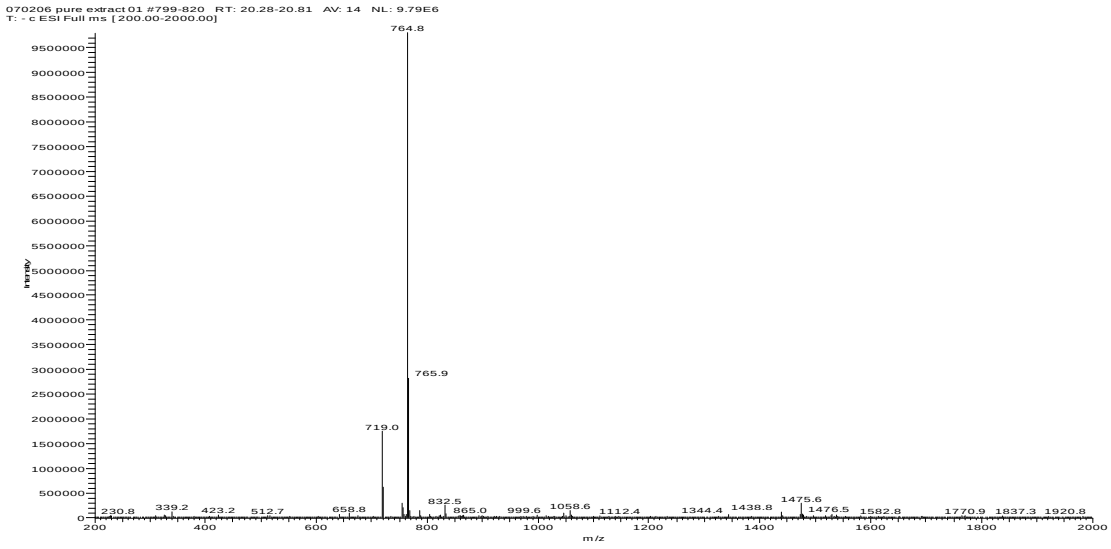


Figure 2.3d: HPLC-MS analysis of purified aza A.

The main peak is at 764.4. This represents an adduct of formic acid (Mr 44) and aza A. 764.2-44= 720.4: the Mr of aza A.

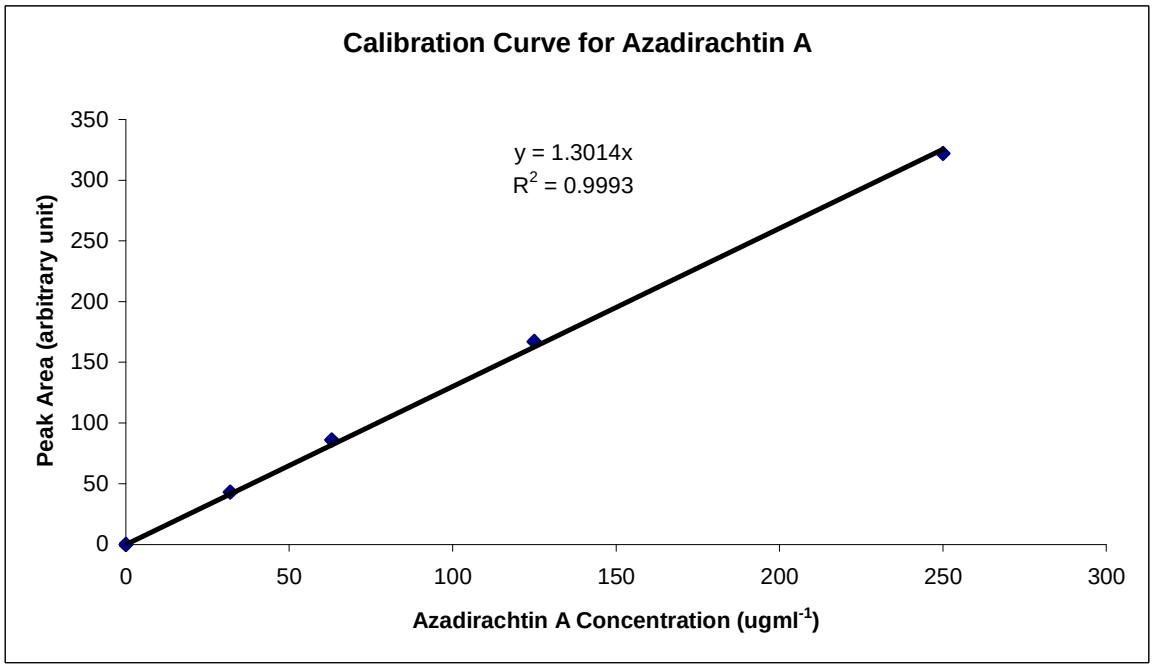
2.6.5 Standard curves of azadirachtin by peak areas and peak height.

2.6.5.1 The standard curve.

The standard curve, plotting the known concentrations of aza A against peak area (Figure 2.4a) and peak height (Figure 2.4b), show that there is an excellent correlation with the line passing through the origin. It was clear that both peak height and area can be used to quantify aza A by HPLC. These curves were therefore used to calculate the concentrations of the tetranortriterpinoid in various extracts throughout the remainder of the project.

The dilutions of purified aza A used to establish the standard curves, were retained, and run routinely in the quantification of aza A in plant and soil which will be described in Chapter 4. The solutions in methanol, stored at -20^{o}C, showed no deterioration over a period of a year, consistent with previous experience (R. Strang, personal communication).

From the results in Fig 2.4 were calculated the Limit of Detection (LOD) and Limit of Quantification (LOQ) for aza A. The LOD was found to be 7.5 $\mu g ml^{-1}$ and the LOQ was 25 $\mu g ml^{-1}$. Under the standard conditions of HPLC analysis used in determining aza A in the later parts of the study in 20µl samples, this meant that the minimum amount of aza A that could be determined with confidence was 6.0 $x10^{-8}$ g.



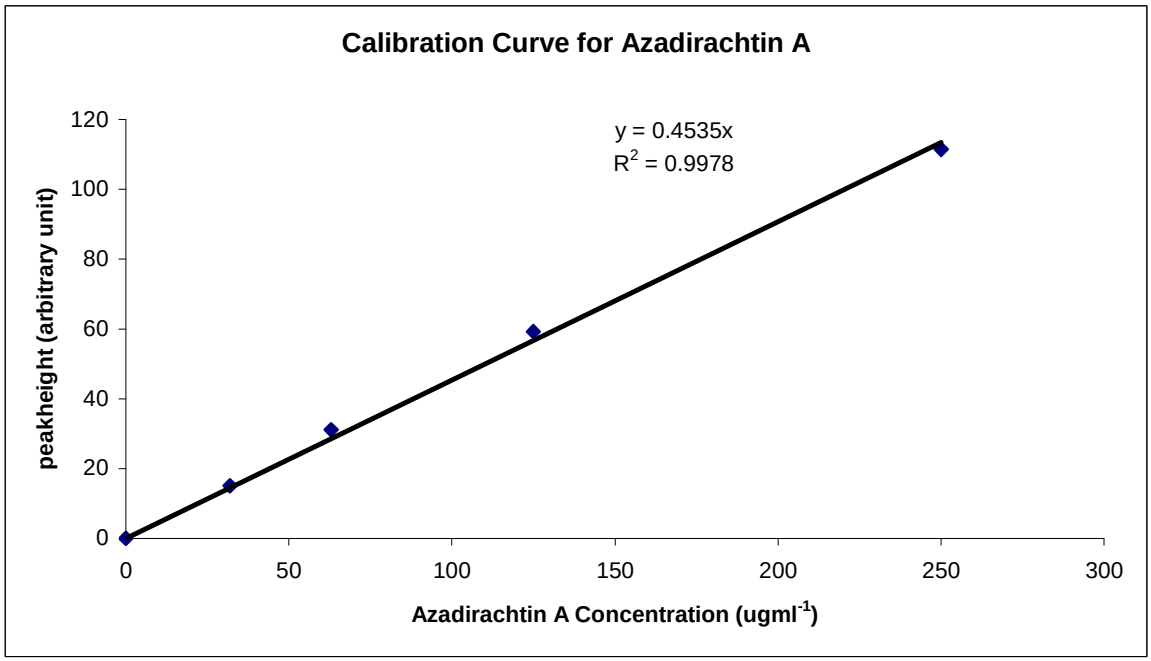


Figure 2.4 a and b: Aza A calibration curves. Standard curves of pure aza A showing excellent correlations (R^2) between the concentrations and the peak areas and height respectively with the line passing through the origin. A solution purified azadirachtin (98%) was made up to 25ml by dissolving in methanol. Samples of this solution (250µg.ml^{-1}) were then serially diluted down to 32µgml^{-1}. Samples of 20µl were run in duplicate and standard curve was drawn from the mean of duplicates.

2.6.6 Determination of aza A in commercial samples of NSKE, neem oil, and pellets.

The results of the analysis of the content of aza A in various commercial neem extracts and the pellets made for the project by Germains Technology Group are shown in Table 2.2.

Product	Source	Aza A Content (% w/w or w/v)
NSKE	Sri Disha Biotech	47
	Trifolio (A.I.D.Parry)	42
	Ascott	26
	Rym Exports	20
	Nickla Ag. Ind.	16
Neem oil	Ghana	0.43
	Trifolio	0.082
Pellets	Germains Tech. Gp.	14

Table 2.2 Aza A content of commercial neem extracts and pellets.

It was clear that the commercially-available NSKE all derived from various Indian manufacturers varied a lot in their content of the a.i. They range from almost 50% to 16% by weight. The Trifolio product NAT was shown to have a content of 42%. As expected, the two samples of neem oil had very little aza A.

The total azadirachtoid content of the pellets containing NAT was estimated by the vanillin method as well as by HPLC. The vanillin estimate was that the pellets contained 34±1.5 % total azadirachtoids. This is exactly consistent with the known amount added by Germains in making the pellets, and also with the 14% w/w aza A, which makes up 42% of the NAT (14/34=41%).

2.7 Discussion.

Several chromatographic methods have been reported for the isolation of aza A and its purification from neem seed kernels. The final separation are achieved by either high performance liquid chromatography (HPLC) (Schroeder and Nakanishi, 1987; Govindachari *et al*., 1992) and supercritical fluid chromatography (SCF) (Morgan and Johnson, 1997; Morgan and Jarvis, 2001; Morgan, 2008). Quantitative determination is usually by light absorption at a low UV wavelength, although there is also a colorimetric method based on vanillin assay in determination of azadirachtin- related limonoids in NSKE was reported (Daly, 2004; Dai *et al*., 1999).

For the isolation and quantitation of aza A by chromatographic methods, it is the polarity of the compound that is key to the process. Being highly polar compound and water soluble, aza A, in a normal absorptive chromatography with silica, is highly absorbed and eluted last. This made the isolation process slow and expensive in solvents. With the development of reverse-phased chromatography, however, the polar compounds such as the azadirachtoids are eluted quite rapidly.

The use of crude NSKE, in this case NAT with 42% aza A eliminated the problem of isolating from the kernels themselves. Schematic representation of the extraction of aza A from NSKE is shown in Figure 2.1. This speeded up the isolation of pure aza A, and allowed it to be achieved by two stages of reverse phase chromatography. Also, a mass spectrometer was used for purity and identification. Finally, the pure material obtained was used for the quantifications of number of commercially available semi-purified neem seed kernel extracts and prilled materials used for the application part of the project.

The initial 4g of NAT contained about 1680mg of aza A. The yield after the initial flash chromatography gave of yield of 300mg (21%) of material with a purity of aza A of 75%. Acidified vanillin dissolved in methanol was found effective method in visualising the presence of the terpenoid (Eweig and Shermer, 1972, Yamasaki *et al*., 1986; Allan *et al*., 1994) on aluminium-packed silica plates (Figure 2.2).

The purity of aza A at different stages of the isolation process was determined by the absorbance at 217 nm, by both peak height and the area under the peak produced by the HPLC method. On this basis the final product was estimated to be 98% pure. It can be argued that the purified aza A may be contaminated by compound(s) with no absorbance at 217 nm. However, the MS trace does not support the presence of any material other than aza A, and multiple development by TLC, staining with the non-specific vanillin stain also failed to show the presence of other compounds.

The yield from preparative reverse-phase HPLC was successful in isolating aza A to level of purity of 98%, but the yield was only 6.3mg (0.15%) of the estimated starting material. This contrasts with the results of Daly (2004) who developed the separation by flash chromatography, who reported a recovery of 8% aza A at a lower purity of 95%. The higher purity achieved here by the additional step of preparative HPLC, was at the cost of quantity of product. This explains the expensiveness of pure aza A commercially, as mentioned in the introduction to this chapter. It seems unlikely that aza A could ever be used as a PPP in pure form.

Determining the concentration of aza A depends on either light absorption in the low UV (usually 217nm) or the colorimetric method based on the vanillin stain used to monitor column fractions. Absorption at 217 nm is more sensitive than the second method. Used

here in conjunction with HPLC analysis, it was still accurate at a minimum concentration of $30\mu g.ml^{-1}$. The vanillin method was also found to be non-specific, and the colour developed unstable. Its only advantage would be the estimation of a large number of samples simultaneously.

The preliminary analysis of a range of commercial samples of NSKE indicated the variability of the extracts available on the market at the moment. The very low content of aza A in the two samples of neem oil was consistent with the fact that the polarity of aza A which makes it almost insoluble in the non-polar oil (Schmutterer, 2002). It also suggests that PPP based on the oil, although cheaper than NSKE, would have a low level of effectiveness against insects.

Analysis of the content of NAT in the pellets prepared for this project by Germains Technology Group indicated a concentration of aza A at 14% with a total azadirachtoid content of 34%, consistent with the specifications indicated by the manufacturer. The estimates made by the colorimetric and HPLC methods were also consistent with each other. Tests made throughout the project proved that the NAT in the pellets, stored at $4^{o}C$ remained constant over 2 years.

Chapter 3: Phytotoxicity of NeemAzal to seed germination and early growth.

3.1 Introduction.

Even though the systemic insecticides show greater retention in plant systems thus ability of protecting plants from insect attack, like residual herbicides they could affect the germination and following seedling development. Even though there are some other physical chemistry characteristics of respected pesticides like persistence in soil, leaching, solubility in soil, rate of evaporation and adsorption to soil particles that effect their influence, still some systemic pesticides like Cholropyrivous and Oxymal have shown some *in vitro* toxicity effect on germination and development of plants (Olofinboba, and Kozlowski, 1982).

As discussed in the Chapter 1, there is evidence from both *in vivo* and *in vitro* work that azadirachtin A inhibits the division of plant cells and the restricts the growth of whole plants. (Nisbet, 1991; Nisbet, *et al* 1993, 1996). Some herbicides act in this fashion. (Fedtke, 1982; Moreland, 1980; Gunning & Hardhan, 1982; Waldin *et al*., 1992; Binarova and Dolezel, 1993; Ellis *et al*, 1994; Mitrofanova *et al*, 2003; Morrissette *et al*., 2004) This might limit the amount of the terpepenoid which may be applied to plants either at the stage of germination or later. This might prevent the use of NeemAzal as a seed treatment.

Recent studies have shown that aza A behaves in insect cells as an antimitotic agent, acting in a similar fashion to colchicine by interfering with the polymerization of tubulin, and thus preventing cell division (Salehzadeh *et al*, 2003). As tubulin is found in all

eukaryotic cells, and is highly conserved, this would suggest that aza A should be able to prevent mitosis in all sorts of dividing cells, including plant cells.

The aim of this study was to test the phytotoxicity of NAT, of which aza A is the active ingredient, to germinating seeds, and their subsequent early growth. Experiments were set up *in vitro* to examine the effect of the terpenoid on the two plant species: cabbage (*Brassica oleracea, capitata,* Var. PrimoII), and sugar beet (*Beta vulgaris L.*, Var. Roberta). Two antimitotic herbicides, Trifluralin and Amiprophos-methyl, which are known to act as antimitotic agents, were used as comparators. (Fedtke, 1982; Moreland, 1980; Vaughan and Lehmen, 1991; Ellis *et al*, 1994; Tanaka *et al*,. 1999).

Both the germination and very early growth of both plant species seeds were looked at. A separate experiment examined the slightly later stages growth; measuring parameters such as plant fresh weight, whole plant growth and root growth.

3.2 Methods and Materials.

3.2.1 Preparation of Growth Medium.

Growth medium, (2.2L per batch), was made up by dissolving or 0.96g/120ml (0.8% w/v) micro-agarose (Duchefa Biochemie, Haarlem, The Netherlands). This was mixed with an equal volume of Murashige & Skoog (MS) basal salt (2.2g/L) (Murashige and Skoog, 1962). (Sigma-Aldrich Ltd., Poole, UK). They were mixed thoroughly with a magnetic stirrer while simultaneously adjusting pH to 6.8 with 0.1 M KOH solution, then autoclaved. The medium was allowed to cool, in 120ml aliquots, and kept sterile until required. Before pouring, it was reheated in microwave oven to liquefy the agar.

3.2.2 Stock solutions of NAT and herbicides.

The NSKE, NAT, and two herbicides, Trifluralin (Sigma-Aldrich, Laborchemikalien, GmbH, Seelze, Germany) and Amiprophos-methyl (Duchefa Biochemie, Haarlem, The Netherlands), were dissolved in dimethylsulphoxide (DMSO) (Sigma-Aldrich Cheme GmbH, steiheim, Germany) as they have a negligible water solubility. All materials were made initially to a concentration of 10^{-1}M and then each one further serially diluted in DMSO to give a range of concentrations from 10^{-1} M to 10^{-4} M. All were filter-sterilised in a flowhood. Volumes (1.2 ml) of each concentration were then added to 118.5 ml volumes of remelted basic medium to give a x100 dilution. This ensured that the final concentration of DMSO was 1% (v/v), a concentration which had been shown to be non-toxic to the plants, and giving a final range of concentrations of the active ingredients from 10^{-3} M to 10^{-6} M. Control plates contained only 1% (v/v) DMSO. Finally, 40ml

volumes of the various media were poured into three 140mm square Petri dishes, under sterile conditions, and allowed set, so that each test was done in triplicate.

3.2.3 Seed preparation for germination and growth bioassays.

3.2.3.1 Sterilisation

Cabbage (*Brassica oleracea, capitata,* Var. PrimoII,) and sugar beet (*Beta vulgaris L.*, Var. Roberta), seeds were sterilised by soaking them with 5% (v/v) commercial bleach for 10 minutes, occasionally mixing by gentle inversion. Then the liquid and the floating debris were decanted. Seeds were then serially washed five times with sterilised distilled water. By leaving the seeds in the final wash, they were allowed to imbibe for 48 hours at 4 ^{0}C.

3.2.3.2 Germination and Preliminary Development Assay.

36 sterilised seeds (four rows of nine seeds each) were placed in each Petri dish. Seeds of similar sizes were selected. Plates were then sealed with plastic film, to prevent moisture from escaping and avoid contamination, and left in the germination chamber to incubate at 21^{0}C in the dark. This process (each concentration of either NeemAzal, two herbicides or controls) was replicated three times. Germination, as judged by radical emergence and preliminary development (root and shoot) were evaluated in 8 days for cabbage and 14 days for sugar beet by counting number of geminated seeds, and measuring whole plant length.

3.2.3.3 Assay of Seedling Growth.

Filter paper (Anachem Ltd, Luton, UK) was cut to size to fit a 24 x 36cm plastic tray. The filter paper was put inside the tray and soaked with sterilised water. Seeds of either sugar beet or cabbage, sterilised and imbibed as above, were evenly set on the wet filter paper. The trays were sealed with plastic foil, to avoid water-loss. Also, trays were carefully wrapped with aluminium foil so that seeds were not exposed to light, thus mimicking germination mode of seeds in soil, and then left in the growth room at 21^{o}C to germinate. Seeds were examined twice daily for signs of germination. Newly germinated seeds were transferred in groups of 8 seeds to one Petri dish, onto all the media described above (3.1.2). As before, control plates contained only 1% DMSO. Seeds were placed in a row in the middle of the plates. Plates were then set in a vertical position and left to grow for a further 8 days for cabbage and 14 days for sugar beet. Temperature and humidity were kept at 20^{0}C and 60% respectively, and a light/dark cycle of 16 hours/ 8 hours. After 8 or 14 days plants were removed from the medium and measures were made of basic growth parameters:

a) plant fresh weight.
b) plant whole length.
c) root length.

3.2.3.4 Statistical Analysis.

Statistical comparisons were made on the basis of the averages (± SE) of the fresh weight (mg), whole plant length (cm) and root length (cm). One way and two ways (general linear model) analysis of variance (ANOVA) was used in analysing the collected data in Minitab statistical package.

3.3 Results.

3.3.1 The effect of two herbicides and NAT on germination and preliminary seedling growth of cabbage and sugar beet.

3.3.1.1 Cabbage.

a) Germination.

Cabbage seeds grown on control plates containing only 1% DMSO germinated before 8 days, and all seeds germinated. None of the possible inhibitors used: Trifluralin, Amiprophos-methyl (AMP) or NAT, showed any inhibitory effect on the emergence of the radical. (Results not shown)

The first growth occurring between radical emergence and 8 days, was, however, affected by the two herbicides. As is shown in Figure 3.1, both Trifluralin and AMP severely inhibited growth at concentrations above 10^{-6} M, with the former reducing the fresh weight by a maximum of 40% and the latter by a maximum of 55%. Both effects were statistically significant. The seedlings became swollen and stunted at those concentrations. In contrast, however, NAT, had no effect the germination and the ensuing radical elongation except at the highest concentration, 10^{-3} M.

An unexpected result was that the lowest concentration of the two herbicides seemed to slightly enhance the growth of the newly germinated plants, although this was not statistically significant.

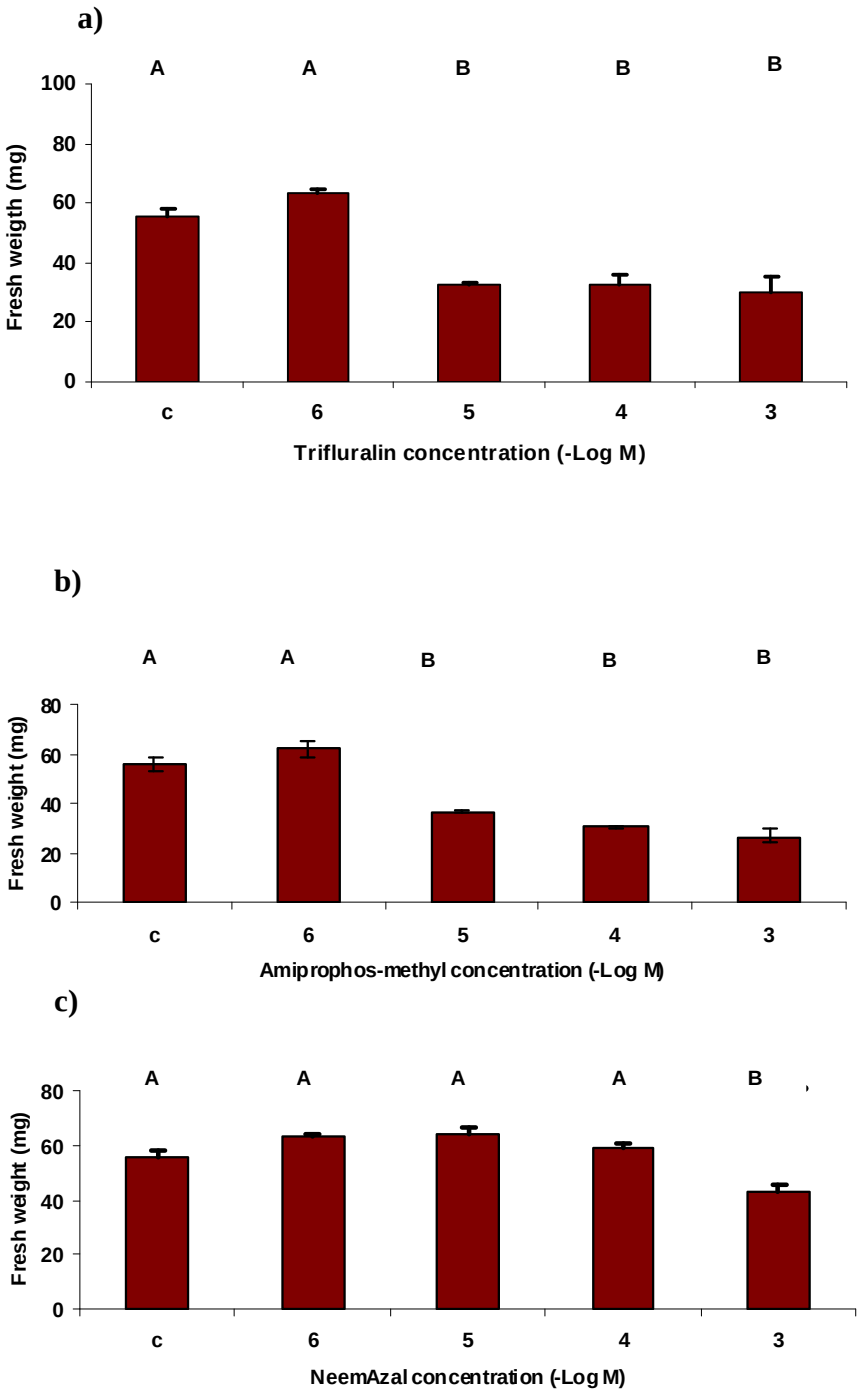


Figure 3.1: The effect of the herbicides and NAT on the early growth of cabbage seedlings. The results represent the fresh weights of the newly germinated plants by 8 days of incubation in a range of concentrations of test materials. Values are the averages ± SE of 3 plates each of 36 seeds. The compounds used were a) trifluralin; b) amiprophos-methyl; and c) NAT. Control plates (C) contained only 1% v/v DMSO. The letters indicate statistical significance: Those with different letters are significantly different at $p \leq 0.01$.

3.3.1.1.1. The effect of trifluralin on the growth of cabbage seedlings.

Fresh weight, total plantlet length and root length were measured at 12 days after first signs of germination of seedlings which were carefully time-matched to ensure that they were all at the same stage of development.

Trifluralin (Figure 3.2) reduced all three growth parameters, compared to controls, at all the concentrations used and in a concentration-dependent fashion, although this was only statistically significant at $p=0.01$ at concentrations of 10^{-5} M and greater. The effect of the compound was particularly marked on root and total plant length, reducing the former by a maximum of 80% at the highest concentration, and latter by 87%. The root growth which took place at concentrations of the herbicide greater than 10^{-5} M was stunted and abnormal.

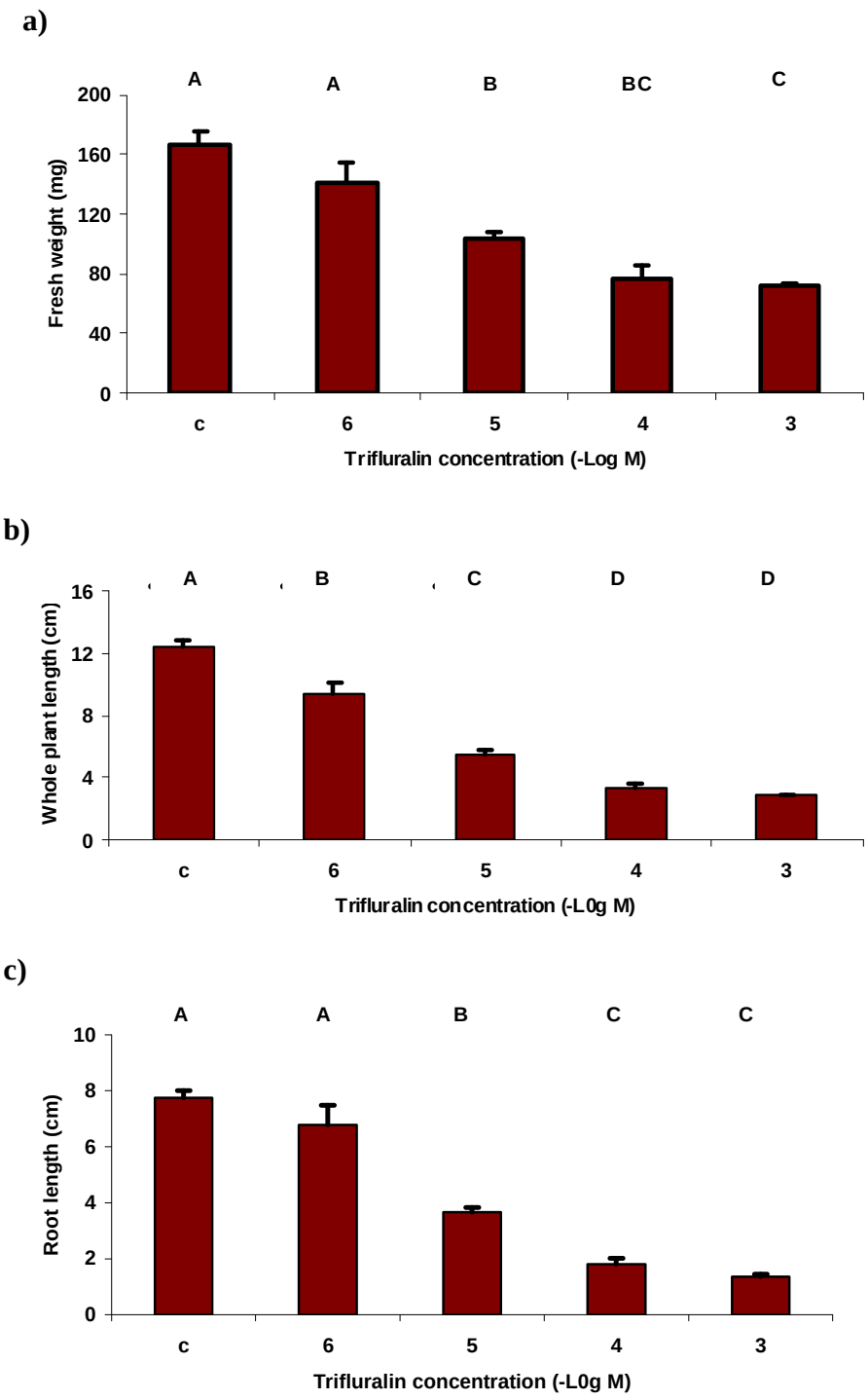


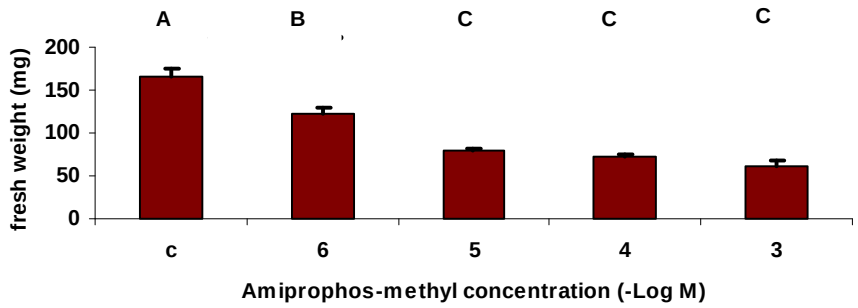
Figure 3.2: Effect of trifluralin on the growth of cabbage seedlings.
The results represent the averages ± SE of 3 replicate plates each of 8 germinated seeds after 12 days of growth on media containing different concentrations of the herbicide. The parameters measured were: a) fresh weight; b) total plant length; c) root length. Control (C) plates contained only 1% v/v DMSO. The letters indicate statistical significance: those with different letters are significantly different at $p \leq 0.01$.

3.3.1.1.2 The effect of amiprophos-methyl (AMP) on growth of cabbage seedlings.

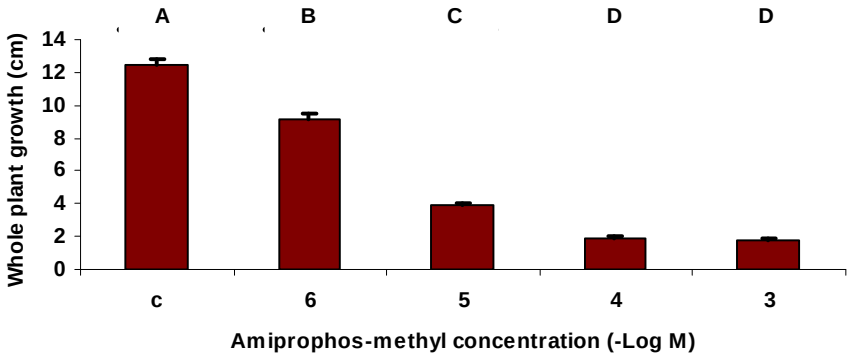
The effects of the herbicide on the growth of cabbage seedlings up to 16 days is almost identical to that of trifluralin (Figure 3.3). There was a concentration-related reduction in the three parameters of growth, which is statistically significant at either 10^{-6} M or 10^{-5} M. The most sensitive indicators, total plant length and root length, are both reduced by more than 80% compared to the controls, a result almost exactly the same as that found with trifluralin.

One anomalous result is that the herbicide appeared to increase root growth at 10^{-6} M.

a)



b)



c)

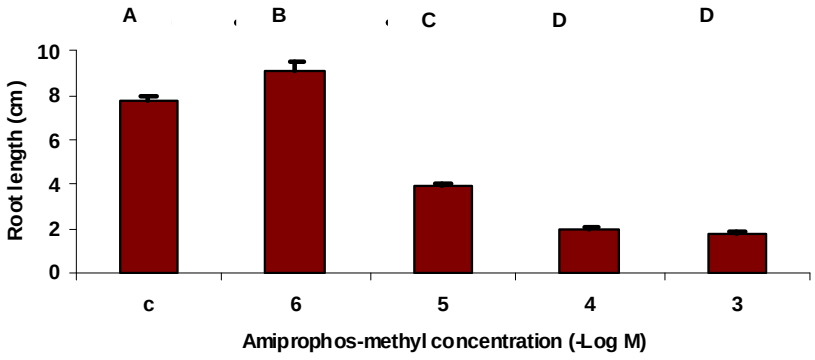
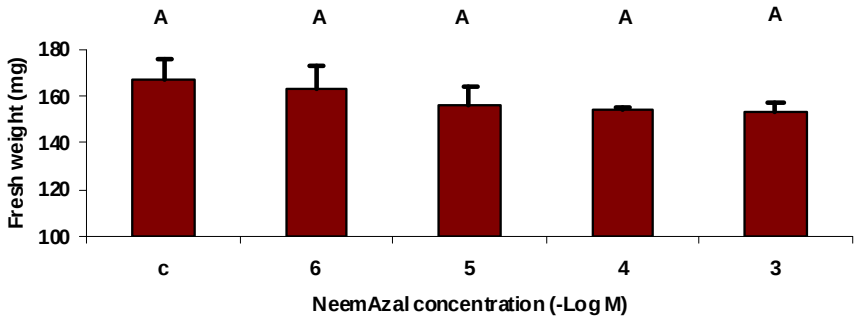


Figure. 3.3: The effect of AMP on the growth of cabbage seedlings.
The values are the averages ± SE of 3 replicate plates, each of 8 germinated seeds after 12 days on media containing different concentrations of the herbicide. The parameters measured were: a) fresh weight; b) total plant length; c) root length. Control (C) plates contained 1% v/v DMSO. The letters indicate statistical significance: those with different letters are significantly different at $p \leq 0.01$.

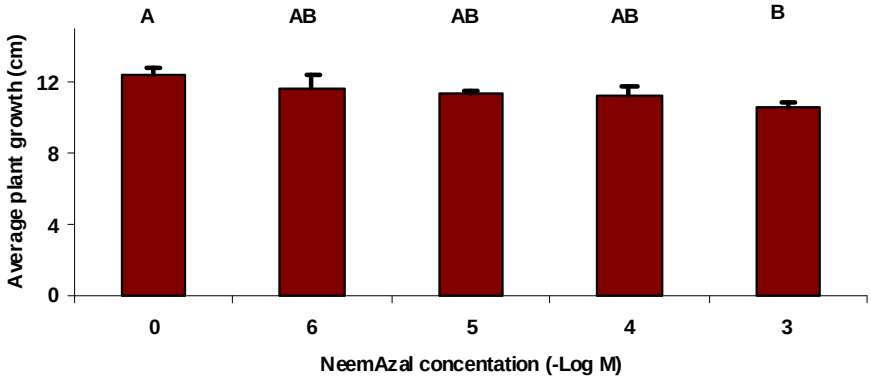
3.3.1.1.3 The effect of NAT on the growth of cabbage seedlings.

The effects on growth of a range of concentrations of NAT are shown in Figure 3.4. Although the averages of the parameters measured show a slight concentration-related decline, it is small compared to the antimitotic herbicides. At the maximum concentration of 10^{-3} M the reductions in the various measurements compared to the control are: fresh weight: 6%; total plant length: 19%; and root length: 17%. Only in the case of the total length is this reduction statistically significant ($p=0.01$).

a)



b)



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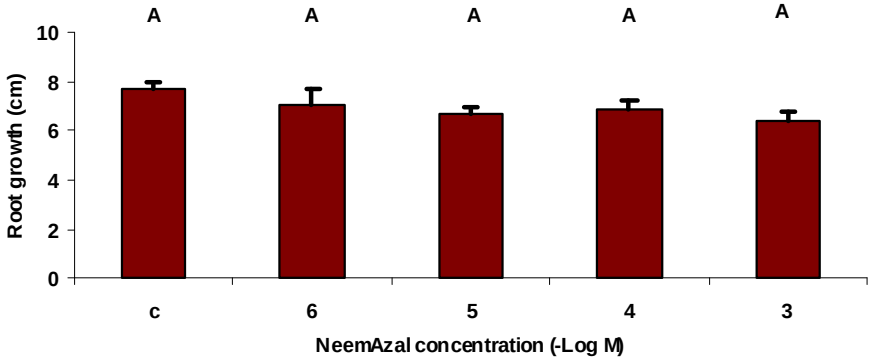


Figure 3.4: The effect of NAT on the growth of cabbage seedlings.
The results represent the averages ±SE of 3 replicate plates each of 8 germinated seeds after 12 days of growth on media containing different concentrations of the terpenoid extract. The parameters measured were: a) fresh weight; b) total plant length; c) root length. Control (C) plates contained only 1% DMSO. The letters indicate statistical significance: those with different letters are significantly different at $p \leq 0.01$.

3.3.1.2 Sugar Beet.

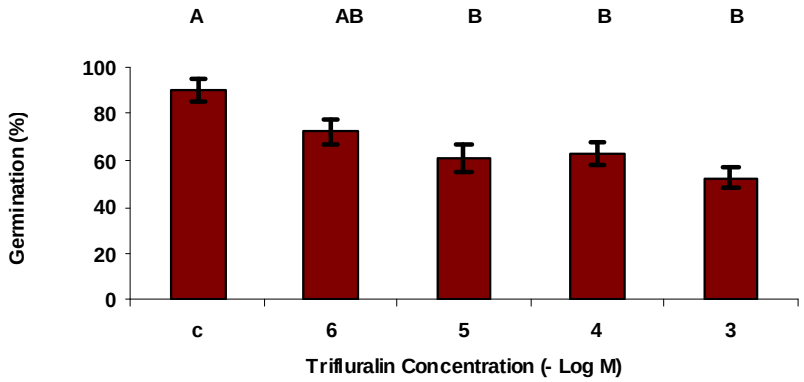
Sugar beet germination took longer than cabbage, and never reached 100% even on control plates, averaging 90% in the absence of the antimitotic compounds. A period of 8 days was required to achieve the maximum level of germination. Growth of the sugar beet seedlings was then much slower than the cabbage seedlings. The subsequent further period of early growth was thus set also at 8 days. Even after this longer period, the rate of growth as measured by fresh weight, total and root length, was generally less than half that of the cabbage seedlings.

3.3.1.2.1 The effect of Trifluralin on germination.

The sugar beet seeds were also much more sensitive to the presence of the antimitotic herbicides. As Figure 3.5 shows, both germination and early growth are inhibited by Trifluralin in a concentration-dependent manner. At the highest concentration of 10^{-3} M the herbicide reduced the rate of germination from the control level of 91% to 52%, a highly statistically significant reduction of 43% compared to the control value.

The effect of the herbicide was even more marked in restricting early growth. By 16 days after germination the fresh weight of the plants on medium containing 10^{-3} M was reduced by 70% compared to the controls.

a)



b)

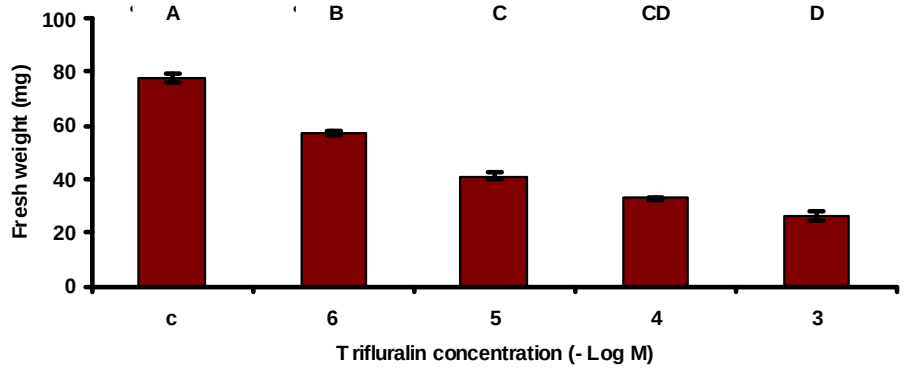
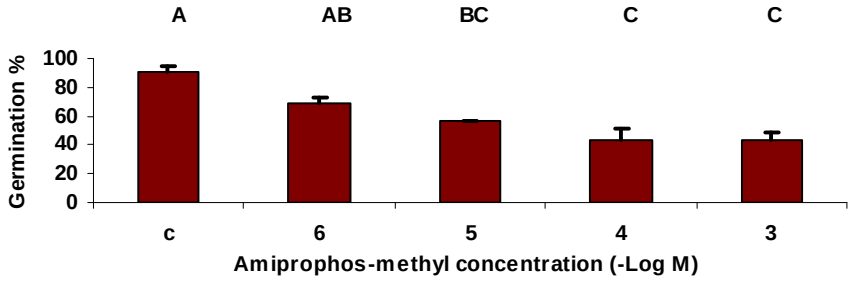


Figure 3.5: The effect of trifluralin on the germination and early growth of sugar beet. The results represent the averages ± SE of 3 plates, each containing 36 seeds on media containing a range of concentrations of the herbicide. The parameters measured were: a) germination (radical emergence) and b) growth as measured by fresh weight of seedlings. Control (C) plates contained only 1%v/v DMSO.
The letters indicate statistical significance: those with different letters are significantly different at $p \leq 0.01$.

3.3.1.2.2 The effects of AMP on germination of sugar beet.

The effects of the herbicide AMP on the germination and first growth of the sugar beet seeds (Figure 3.6) is almost exactly the same as that found with trifluralin. Both germination and early growth are severely disrupted in a concentration-dependent manner. At 10^{-3} M AMP, germination is reduced to 56% of the control (c), and early growth to 19%.

a)



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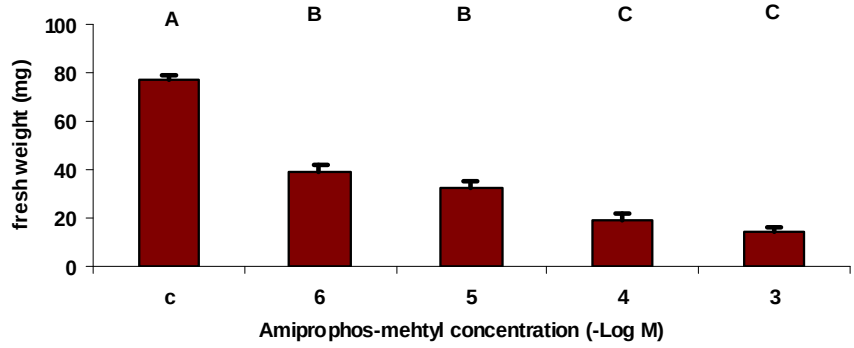
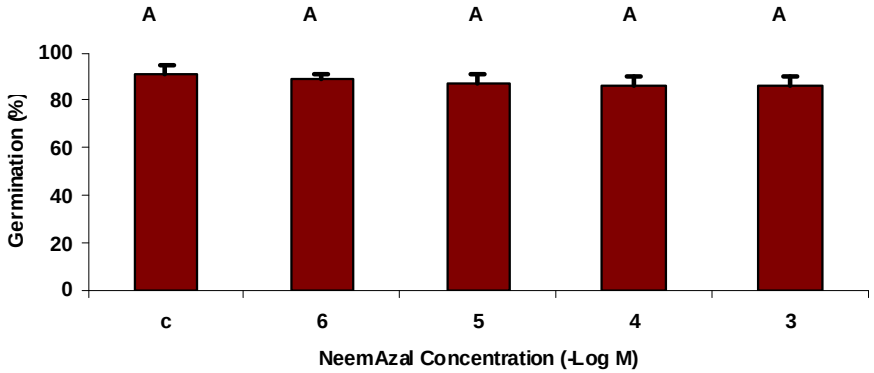


Figure 3.6: The effect of AMP on the germination and early growth of sugar beet.
The results represent the averages ± SE of 3 plates each of 36 seeds after 14 days of incubation, in the presence of a range of concentrations of the herbicide. The measured parameters were:
a) radical emergence, b) fresh weight of seedlings. Control plates (C) contained only DMSO. The letters indicate statistical significance: those with different letters are significantly different at $p \leq 0.01$.

3.3.1.2.3 The effect of NeemAzal on germination of sugar beet.

The results in Figure 3.7 show that NeemAzal had no negative effect on either the germination or early growth of the sugar beet.

a)



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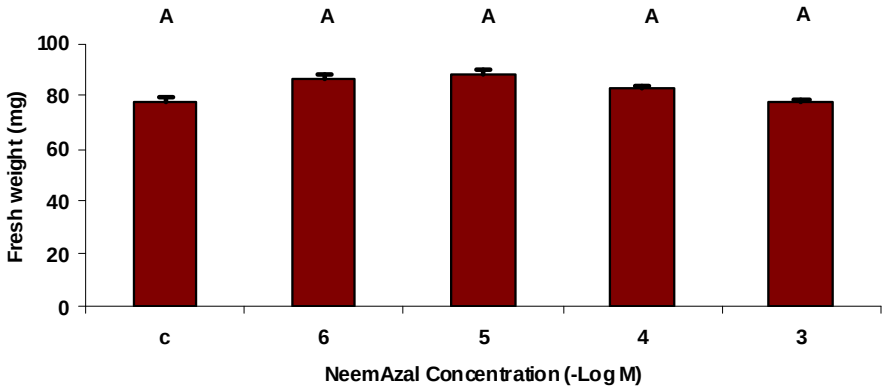


Figure 3.7: The effect of NAT on the germination and early growth of sugar beet. The results represent the averages ± SE of 3 plates each of 36 seeds 14 days after the start of incubation. The parameters measured were: a) radical emergence, b) fresh weight of seedlings. Control plates (C) contained only 1% v/v DMSO. The letters indicate statistical significance at $p \leq 0.01$: There are no significant differences between the different concentrations.

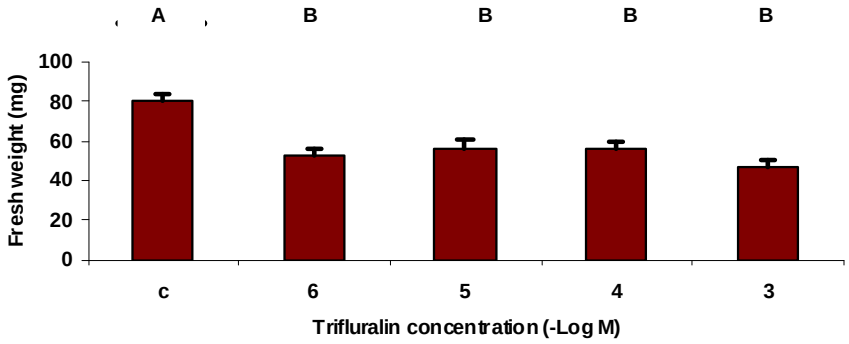
3.3.1.3 Sugar beet growth.

3.3.1.3.1 Effect of trifluralin on growth.

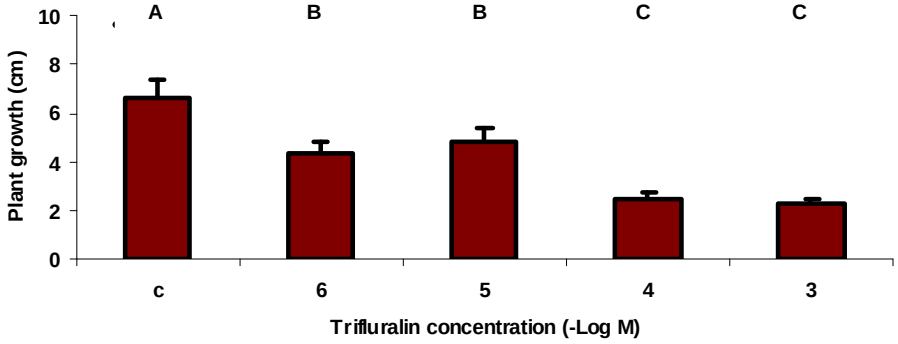
The effect of trifluralin was to inhibit the growth of the sugar beet seedlings between germination and 16 days (Figure 3.8). Total fresh weight of the plants was reduced by 36% compared to the controls. The amount of concentration dependence was not clear, as the effects of 10^{-6} M were not significantly different from 10^{-3} M.

The effects on the root and shoot length were more marked, being respectively reduced by 79% and 63% compared to the control values at the highest concentration of 10^{-3} M.

a)



b)



c)

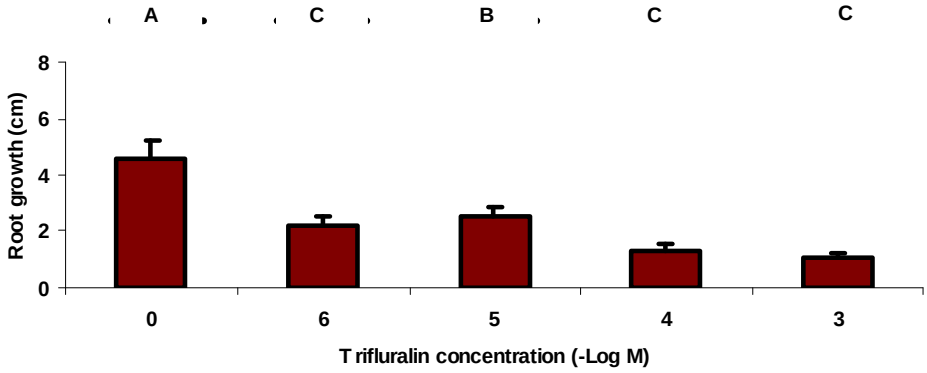
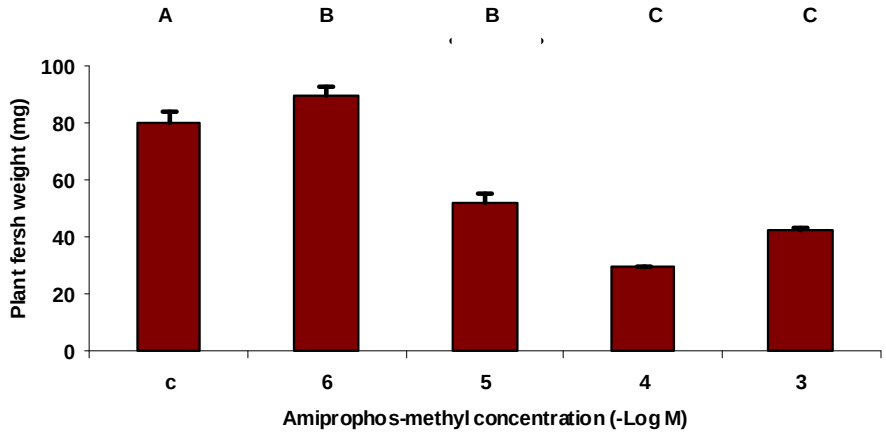


Figure 3.8: The effects of Trifluralin on the growth of sugar beet seedlings.
The results represent the averages ± SE of 3 replicate plates, each of 8 germinated seeds 16 days after radical emergence. The parameters measured are: a) fresh weight of seedlings; b) total plant length; c) root length. Control (C) plates contained only 1% v/v DMSO. The letters indicate statistical significance: those with different letters are significantly different at $p \leq 0.01$.

3.3.1.3.2 The effects of AMP on growth of sugar beet.

The effect of AMP on the growth of sugar beet seedlings is shown in Figure 3.9. As the case with trifluralin there is not a clear concentration dependence of the inhibition of growth. The lowest concentration of 10^{-6} M had no effect on the parameters measured, and higher concentrations all reduced growth to the same extent. This reduction was highly statistically significant, and was greatest with root length, which was only 33% of the control values.

a)



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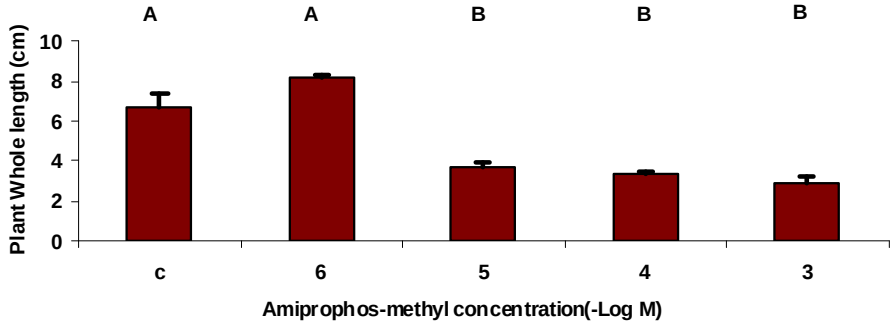

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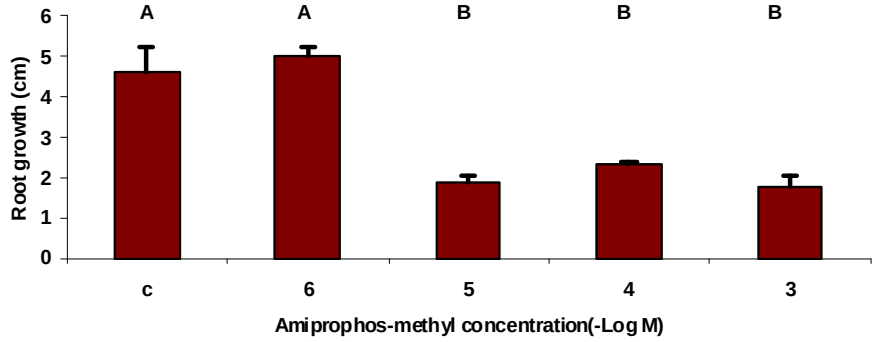


Figure 3.9: The effect of AMP on the growth of sugar beet seedlings. The results represent the averages ± SE of 3 replicate plates each of 8 germinated seeds 16 day after germination on growth media containing different concentrations of AMP. The control (C) plate contained only 1% v/v DMSO. The parameters measured were: a) fresh weight of seedlings; b) total plant length; c) root length. The letters indicate statistical significance: those with different letters are significantly different at $p \leq 0.01$.

3.3.1.3.3 The effects of NeemAzal on growth of sugar beet.

The effect of NeemAzal on the growth of sugar beet is shown in Figure 3.10. Throughout the range of concentrations used, the terpenoids had no effect on growth.

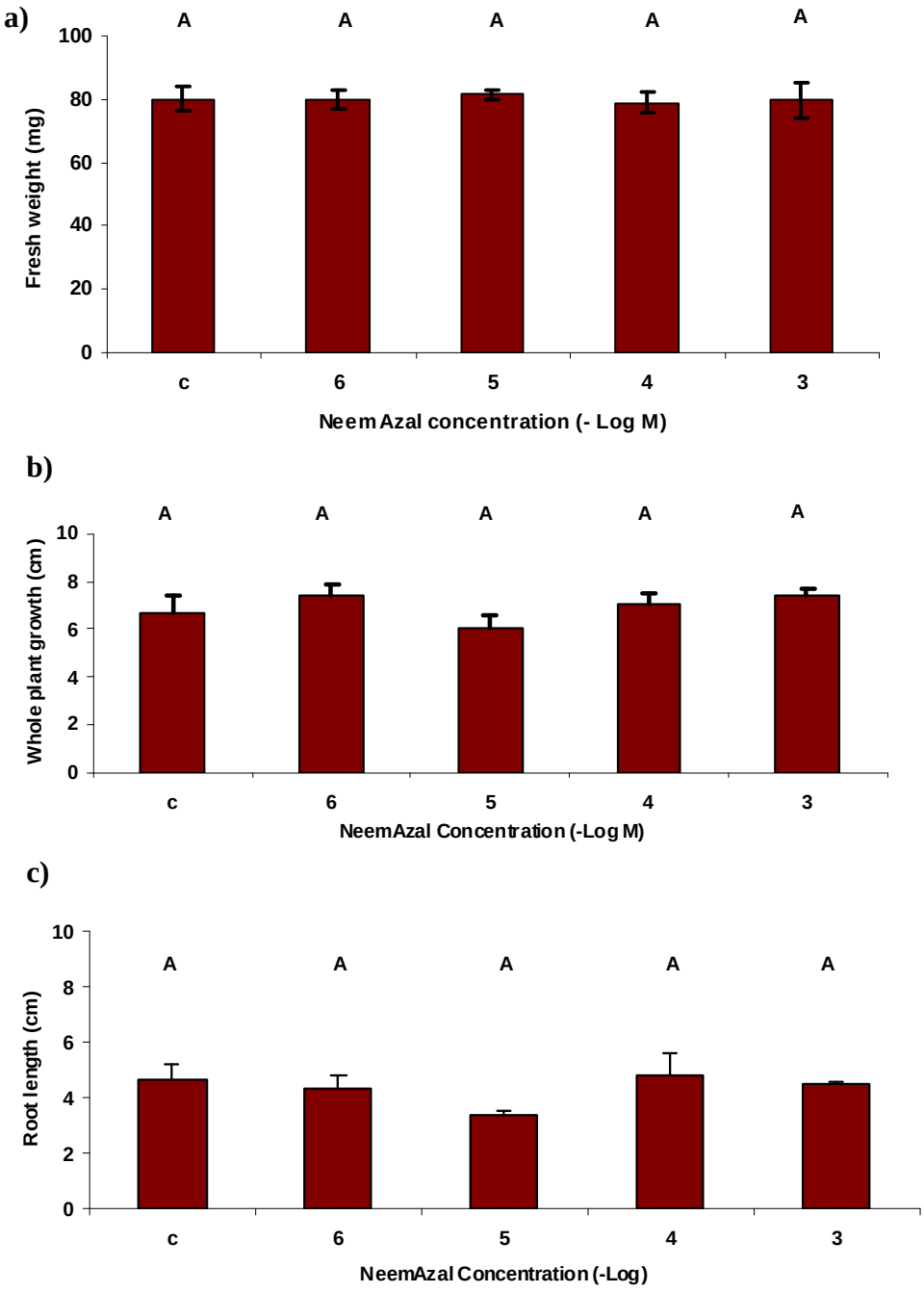


Figure 3.10: Effects of NAT on the growth of sugar beet seedlings. The results are the averages ±SE of 3 replicate plates each of 8 germinated seeds 16 days after germination on media containing different concentrations of the neem extract. Control (C) plates contained only 1% v/v DMSO. The parameters measured were: a) fresh weight; b) total plant length; c) root length. The letters indicate statistical significance at $p \leq 0.01$: There are no significant differences between the different concentrations.

3.4 The effect of aza A on Mitosis of Plant Cells.

There are several publications that have been done regarding aza A's effect on insect and mammalian cells (Elizabeth *et al*, 2003 ref. by Chapman, 2003; Rembold and Annadurai, 1993) at concentrations varying from 10^{-4} to 10^{-10} M. It was found that the limonoid had no affect on range of mammalian cells at concentrations up to 10^{-5} M, but Akundugu *et al* (2001) showed that azadirachtin affected the human glioma cells at these concentrations, and if that is true, it would cast doubt on its suitability as a safe PPP. Salehzadeh *et al* (2003) found that aza A has an antimitotic effect on insect cells, in the same way as the antimitotic plant metabolite, Colchicine, by interfering with the polymerization of tubulin, at a concentration of 5×10^{-6} M, and cells get stuck at G2/M phase of the cell cycle, but on that of mammalian cell it was found azadirachtin showed cytotoxic effects and inhibit proliferation, only in concentration over 10^{-4} M. This would suggest that azadirachtin might be able to prevent mitosis in all sorts of dividing cells, including plant cells (Nisbet, 1991; Nisbet, e*t al*, 1993 and 1996). This might be problematic if the terpenoid was applied to growing plants. A number of commercially available herbicides act by preventing plant-cell division. The following study was intended to establish if the terpenoid did have an effect on mitosis in plant cells.

Those findings raised the question of if aza A has negative effect on plants on its application in controlling insects, particularly when applied systemically. There is little or no work that has been done on Azadirachtin's cytotoxic effect on plant cells.

The literature on the application of neem-based plant protection products has reports of examples of phytotoxicity (Freiswinkel, 1989 ref. by Schmutterer, 2002), the basis of which is unknown. Earlier work in this project showed that the development of cabbage

and sugar beet seedlings was in fact inhibited by high concentrations of Azadirachtin A.

The effect of Azadirachtin A on cell division was studied using onion (*Allium cepa,* Bedford) root tips. Armburster *et al*., 1991; Singh *et al*., 2005) squash technique, with slight modifications, was used. After a few unsuccessful preliminary tests using cabbage and sugar beet roots, onion root tips were chosen as they have comparatively large cells and stain well.

3.4.1 The phytotoxic effect of Aza A due to anti-mitosis.

3.4.1.1 Materials and methods

a) Growth Medium and Stock solutions.

The Growth medium and stock solutions of azadirachtin A and herbicides was prepared as that of germination and growth bioassay of cabbage (Brassicae *oleracea, capitata*, Var. PrimoII), and sugar beet (*Beta vulgaris L*., Var. Roberta) (see 3.2.1 and 3.2.2). Onion (*Allium cepa,* Var: Bedford, home base, UK) seeds were germinated as reported for seedling growth assay, Schiff's reagent were used as a staining reagent, glacial acetic acid, 1N HCL, ethanol were used as a fixative reagents,

3.4.1.2 Methods.

Onion seeds were germinated as reported for seedling growth assay, and transferred into agarose growth medium (8 germinated seeds in each dish) containing two concentrations (10^{-3} M and 10^{-4} M) of Azadirachtin A. The antimitotic herbicide Trifluralin was used, at 10^{-4} M, as a comparison. Both compounds were added to the final medium in the solvent DMSO, to give a final concentration of the solvent of 1%. The control seedlings were

cultured on medium containing only 1% (v/v) DMSO. Root tips (5mm) were excised from onion seedlings at 24, 48 and 72 hours after treatment. They were fixed with acetic acid: HCL (1:3 v/v for 24 hours) and then rinsed with distilled water three times, then hydrolyzed with 1N HCL for 1 minute, and stained with Schiff's reagent (Sigma-Aldrich Ltd., England, UK) for 30 minutes. Finally the root tip was macerated in a drop of mounting agent, (Aquamount, Verebetered, BDH Laboratory, BH15 Ltd., England, UK) on slide, and covered with cover slip. They were examined microscopically at X40 under confocal microscope. The mitotic index (number of cells showing mitotic figures as a % of total cells in a sample of 150 cells), was determined in at least 4 microscopic fields for each treatment.

3.4.1.3 Results.

Mitotic indices of the onion root tips for three days after exposure to the phytotoxic compounds are shown in Figure 3.11. Control plants exposed only to DMSO, as expected, showed no change in mitotic index throughout the period of exposure. It remained steady at about 5%. The same was true of those exposed to 10^{-4} M Aza A. The plants exposed to 10^{-3} M aza A and 10^{-4} M trifluralin showed a dramatic, and highly statistically significant, ($p<0.01$), increase in mitotic index after 24 hours of exposure to the compounds, the former to 9.6% and the latter to 12%. After 48 hours exposure, both of these cultures showed a sharp fall in mitotic index to below the steady control level. This too was highly signicant ($p<0.01$) compared to the control value. This was accompanied by gross cell distortions and the appearance of multinucleate cells as normal cell replication ceased at 72 hours (Fig 3.13).

3.4.1.3.1 Effects of compounds on Mitotic figures of Onion Cells.

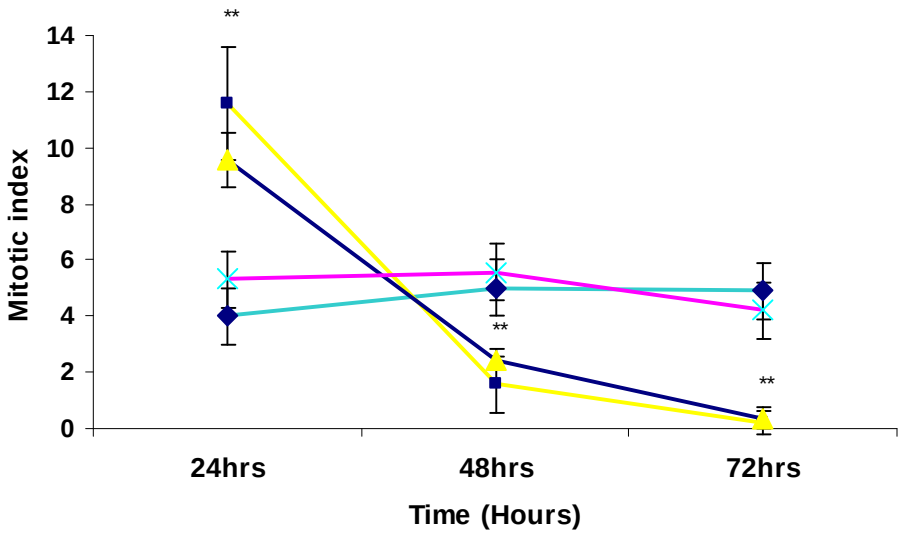


Figure 3.11: Effect of Aza A and Trifluralin on mitotic index on onion root tip. The seedlings were exposed to possible antimitotic compounds over a three day period as follows: ▲: Aza10^{-3}M; x: Aza10^{-4}M; ■:Trif. 10^{-4}M; ♦:DMSO (control). Values are the averages mitotic indices for at least 4 microscopic fields of 150. One-way analysis (unstacked) of variance (ANOVA) was carried in determining the statistical difference of mitotic index among the antimitotic compounds and control (♦DMSO). Those with different Asterisk (**) are significantly different from the control at $p \leq 0.05$. n=3.

3.4.1.3.2 The Average Mitotic Stages of Onion cells through 72 hours of exposure.

The average mitotic figures in squashed cells of controls, and those treated with aza A at the concentration of 10^{-4} showing in a steady state of dividing cells in different stages of mitotic stages on an average between 1 to 3. on the contrary, those treated with higher concentration of aza A and Trifluralin, at 10^{-3} M and 10^{-4} M respectively, the number of cells in prophase and metaphase stages increased, by over two-fold compared with the untreated for the first 24 hours, (Figure 3.12). After 48 hours those values remarkably decreased by lower than half of the original values, and after 72 hours there were hardly any cells in any stages of mitosis.

Fig. 3.14 shows the effect on the growing germinated plants after 7 days. While control seedlings and those growing in the presence of 10^{-4} M aza A have well-developed rooting systems, and have grown to an average of about 5 cm, those in the herbicide and 10^{-3} M aza A have failed to develop roots and show only stunted growth.

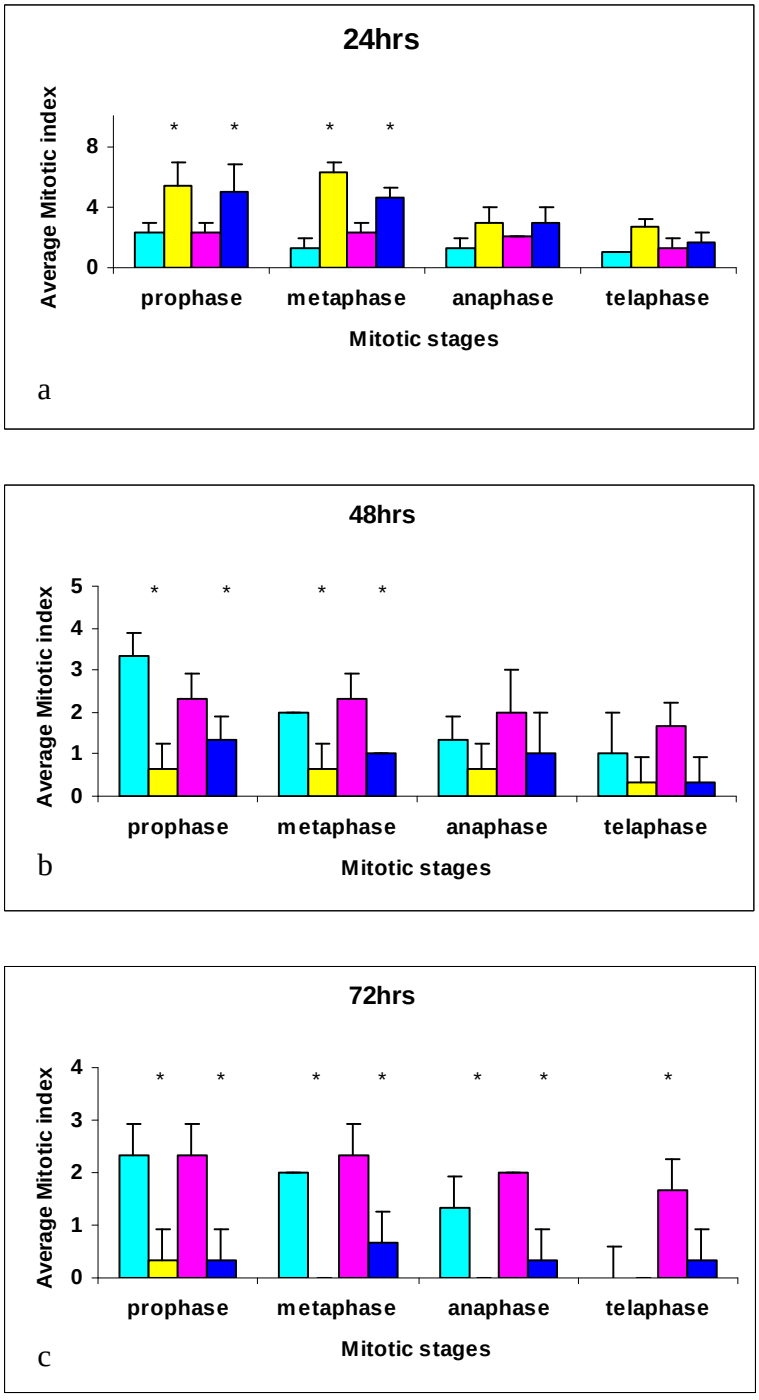


Figure 3.12: The average mitotic index of each mitotic stages onion rot tip cells. Each bar represents the mean ± SD of 3 replications of dividing cells of 150 cells per root tip a) 24 hours, b) 48 hrs, c) 72 hrs. ■ Control (DMSO), ■ Aza A 10^{-4}M, ■ Aza A 10^{-3}M ■ Trifluralin 10^{-3}M. Asterisks (*) indicate statistically significant differences ($p \leq 0.01$) of each mitotic stages of the treated cells compared to the control.

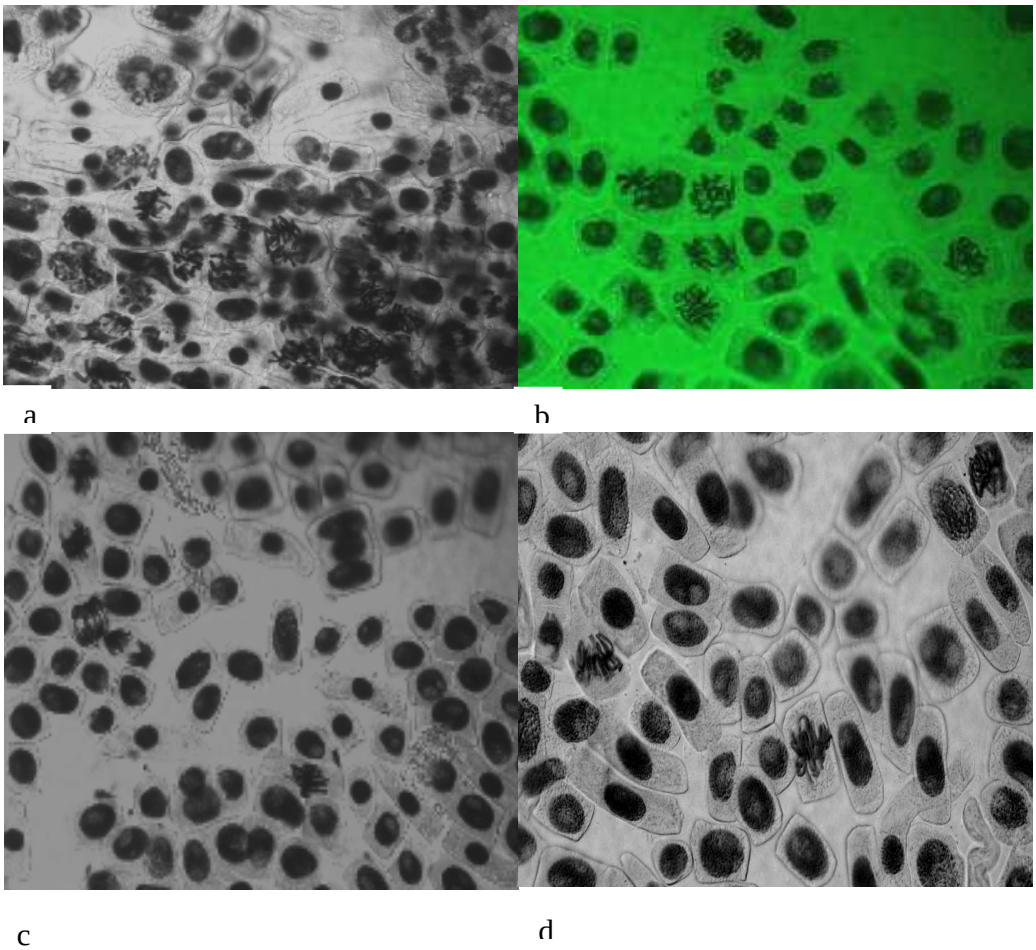
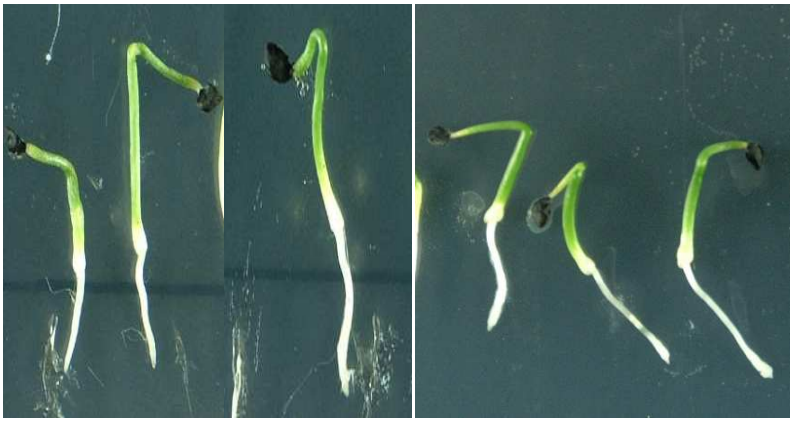


Figure 3.13: Photomicrography of root tip squashes. Root tips quashes at low magnification micrography (x400) of a) AzaA10^{-3}M the cells, b) Trifluralin 10^{-4}M with numerous arrested mitotic figures at metaphase and anaphase stages and c) aza A 10^{-4}M and d) DMSO with cells at different mitotic stages. (Scale: 1cm=25µm)



a) b)

c) d)

Figure 3.14: Onion seeds grown on agarose medium containing different concentrations of aza A, known antimitotic herbicide triflorulin and control for 7 days. a) 10^{-3}M concentration of aza A, b) 10^{-4} M concentration of Trifluralin. c) 10^{-4}M concentration of aza A and d) control (DMSO). To scale 1:1.

3.5 Discussion.

One of the advantages of using neem extracts as plant protection products, in which aza A is the active ingredient, is that they are said to be highly specific in their effects towards insects, while apparently having no effect on mammals, including Man (Salehzadeh *et al*., 2003).

There are, however, a number of reports that both crude neem-seed extracts and pure aza A have shown some phytotoxicity (Schmutterer, 2002). This is acknowledged by Trifolio GmbH in their "instructions for use" of formulated plant protection products based on NAT.

Nisbet (1991) found that azadirachtin at 500ppm (0.7×10^{-3} M) inhibited the early growth of tobacco (*Nicotiana sylvestris*) plants. Bittum *et al*., (2004) demonstrated that an inhibitory effect of azadirachtin A could be found using newly germinated plants of *Arabdopsis thaliana*. These authors showed that there was also an inhibitory effect on cell multiplication of the limonoid on *Arabdopsis* cells in liquid culture indicating that the effect was at the individual cells.

Aza A affects wide range of insects and its mode of action has been described in Chapter 1. Many of these may be attributed to the antimitotic effect on insect cells shown by (Salehzadeh *et al*., 2003). These authors showed that aza A bound to tubulin in the same way as the classical antimitotic colchicine, and apparently at the same site as colchicine, to prevent polymerization of the tubulin. This prevents the formation of the microtubules essential for cell division.

As tubulin is present in all eukaryotic cells, there is an obvious possibility that the phytotoxicity of aza A is due to the same sort of mechanism in plant cells as is found in insect cells.

As discussed in Chapter 1, a number of herbicides are known to act in this way. Two antimitotic herbicides, trifluralin and amiprophos-methyl were used as comparators. Trifluralin belongs to dinitroanalines: class of pre-emergency that cause severe morphological abnormalities root (meristematic) tips in plants (Morejohn *et al.*, 1987). AMP is organophosphorus, phosphorothioamidate herbicide. AMP , though different chemical class, act similarly and cause similar injury symptoms like that of dinitroanalins (Ellis, Taylor, and Hussey, 1994; Gunning and Hardham, 1982; Kiermayer and Fedtke, 1977; Morrissette *et al*., 2004). Both have been shown to have a clear antimitotic effect on plant cells.

The germination and early growth of plants, as was shown by the work reported here, are easily measured, and provide clear effects. This is likely to be the time when plants will be most sensitive to anti-mitotic effects. From a practical point of view, it will give an indication of how early a plant protection product can be applied.

Although there were some differences between the two plants used, (sugar beet seeds germinating later and less effectively than cabbage, for instance) both plants showed the same effects of the compounds used.

None affected germination of the seeds. As expected, the two antimitotic herbicides strongly inhibited root and shoot growth at low concentrations (10^{-5} M and higher). The

roots of affected plants became swollen and bulbous. At high concentrations, the leaves of the seedlings became curled and brittle. Root growth was severely inhibited. This is consistent with earlier findings (Kiermayer and Fedtke 1977; Gunning & Hardhan, 1982; Morejohn *et al*., 1987).

In contrast, although NAT did have an inhibitory effect on seedling growth, this was only found at the highest concentration used: 10^{-3} M. This concentration is in line with previous results which showed that azadirachtin A had a negative effect on growth of onion plant at a concentration of 10^{-3} M (Freiswinkel, 1989: Hilton and Nomura, 1964), and with Nisbet, 1992; Blake, 2002 and Betum *et al*., 2004).

The microscopic studies were carried out to see if the inhibitory effect of the azadirachtins was in fact due to an antimitotic effect. Such effects in plants are traditionally best seen in effects on the meristem of young plant roots, which are undergoing rapid cell division. Initial attempts to do this with cabbage and sugar beet roots were unsuccessful, and so the results here were with the "classical" tissue of onion roots. The results showed that the concentrations of tested compounds required for disruption of normal cell division processes coincided with the effects on the growing plants. At 10^{-3} M of aza A of NAT the root cells were abnormal and distorted. Many cells seemed to be stuck in anaphase and failed to reach prophase. No such effect was shown at 10^{-4} M. The same type of effect was shown by much lower concentrations of the herbicides used.

Several types of herbicides have their effect by antimitosis. The two used here, the

dinitroaniline trifluralin, and the phosphoric amide AMP, are known to prevent polymerisation of tubulin (Kiermayer and Fedtke, 1977; Morejohn *et al*., 1987; Morrissette *et al*., 2004). On the basis of the present results it is impossible to be sure that the phytotoxic effects shown by NAT are in fact due to the inhibition of polymerisation of tubulin, but the similarities with the herbicides, taken with the results of Salezadeh *et al*., (2002) on insect cells, strongly suggests that this is the case. Further work at the molecular level would be required to confirm that aza A inhibits plant microtubule polymerization by binding to individual tubulin monomers (α- or β-tubulin) as has been shown for insect cells.

In summary, the results presented in this chapter show that NAT with aza A as its main component is phytotoxic to newly germinated cabbage and sugar beet seedlings, but only at a concentration of 10^{-3} M, apparently by acting as an antimitotic agent.

What are the practical implications of these findings for the use of the crude extract as a plant protection product? In practice, the concentration of 10^{-3} M is unlikely to be ever achieved around plant roots, as it is represents the maximum solubility of aza A in water at normal temperatures. It would also be too expensive to be practical. It might however, prevent, the use of the neem extract in a seed pellet, although other pesticides also showing phytotoxic effects used in this way (B. Brown, personal communication).

Chapter 4: Persistence of aza A in soil and plants.

4.1 Introduction.

4.1.1 Use of appropriate pesticide formulations.

Since active ingredients (a.i.), are seldom used in pure form, pesticides come into the market in a wide range of different formulations: aerosols, dusts, baits, granules, ready-to-use, emulsifiable concentrates (EC), flowables (Rotteveel *et al*., 1997), wetable or soluble powders (WP or SP) and fumigants. Each one has its advantages and disadvantages which have already been outlined in Chapter 1. These formulations enable the a.i. to be easily handled as well as better delivery to its target (Devisetty, Chasin, and Berger, 1991; Matthews, 2008).

One of the important characteristics of aza A and its congeners (also called azadirachtoids) is that they are moderately water-soluble (Daly, 2004). This places aza A in a small group of plant protection products (See Table 4.1 in the discussion section for this chapter) which can move easily in soil water, and enter plants to act systemically against pests. The problem with such a.is. is that they may easily enter and pollute ground water, especially if they are used in excess, and have long half-lives in the soil (Pimentel *et al*., 1993; Pimentel *et al*., 1980; Wen and Pimentel, 1992).

In contrast, however, to the persistent synthetic pesticides, most botanical bio-pesticides, including the azadirachtins, disappear rapidly in the environment, and so they are considered less polluting than synthetic ones (Arnason, 1989). As aza A is the principal one of this group, it is the active ingredient in neem plant protection products, with multifaceted insecticidal effect on a wide range of insects (Isman, 1991; Saxena, 1986;

Schmutterer and Singh, 1995; Asher, 1993) and also discussed in Chapter 1. Several authors have suggested the use of aza A as a soil-applied systemic insecticide (Nisbet et al., 1993; Sundaram *et al.*, 1995; 1996; Thoeming and Poehling, 2006). Since azadirachtin, applied to plants or to soil, is likely to be rapidly destroyed by light, water and soil micro-organisms, and hence have a short half-life, an alternative way with better efficiency became desirable: development of a controlled release granular (pelleted) formulations of the terpenoids. This should make it possible for the pesticide to be delivered gradually to its target over a period of time thus reducing loss of pesticide in the soil, due to run off, leaching and biological breakdown. Also, unlike spraying of pesticides, this method of delivery should reduce the number of applications for the pesticide, and so increase its cost-effectiveness (Corbin *et al*., 2006, NAFTA report; Barlow, 1985; Collins *et al*.,1973 ref. by Daly, 2004).

4.1.2. Granular Formulations of Pesticides (GFP)

GFP is one of many options to deliver pesticides to target. Granules range in size between 200 μm to 2360 μm. Unlike liquid formulations which are usually sprayed directly on to the plant and pest, they are applied to the field as free flows and are delivered to the target indirectly by movement through soil and plant system, at a rate depending on the nature of the pellet material (Bowman, 1992; Matthews, 2008; Sawyer, 1983; Banks *et al*, 1990; Barlow, 1985).

Standard formulations of a typical granular are: carrier (70 to 98%), pesticide (2 to 30%), solvent or binder (0 to 10%), deactivator (0 to 7%) (Kalley *et al*., 1992; Goss *et al*., 1994). As already reported in Chapter 2, analysis of the granules used in this work found

34% w/w total azadirachtins, which puts the material at the top end of the range quoted above.

There are two main categories in granular carriers: mineral and organic. Most of those classed as organic are polymers, both synthetic and natural. Examples of synthetic ones are poly-ε-caprolactone, polyethylene and poly-phenyl chloride. The common natural ones include starch, alginate and lignins (Flores *et al*., 2007; Goss, Taylor, and Kallay, 1994); Boyston, 1992; Choudary *et al*., 1989; Solvey, 1998).

The use of granules opens the possibility of controlling and delaying the release of the a.i. so as to increase the effectiveness of the product. (Cryer and Laskowski, 1998; Kenaway, 1998; Kenaway, and Sakran, 1996). To achieve the desired controlled release, consideration of physical properties of the granular type such as mesh size, absorptive capacity of carrier, rate of breakdown, and hydrophilicity are important. The water-solubility and persistence of the a.i. are also important in determining the effectiveness of this method of delivery.

4.1.3. Previous work on release of aza A from granules

Daly (2004), whose work preceded this project, studied the basic physical parameters of aza A. He showed that it was moderately soluble in water (1900 mgL^{-1} at $22^{o}C$), and should therefore be readily mobile in soil, and systemic in plants. Using a tritiated tracer of azadirachtin A, ([22, 23 $^{3}H_2$] dihydroazadirachtin A) he was able to confirm that it was in fact mobile in both soil and the trial plant used: nasturtium (*Trepaeolum majus*). By using laboratory-made pellets loaded with the radio-active tracer, Daly (2004) was able also to show that rate of release of the terpenoid into an aqueous medium was dependent

on the nature of the pellets. Lack of time, however, prevented him applying these results in actual plant protection.

The work reported in this and the following chapter was undertaken to extend, and make more commercially relevant, the preceding work. The plants used were commercially important cabbage (*Brassica oleracea, capitata*) and sugar beet (*Beta vulgaris L.*), which are attacked by a wide range of pests encountered in Scotland, and which are described in the Chapters 1 and 5. Pellets, loaded with NAT (Trifolio GmbH) were prepared by Germains Technology Group, Kings Lynn, UK, who specialise in making pellets for application of plant protection products for agriculture. Daly's results with the tracer derivative meant that no estimates of the actual concentration of aza A were made either in soil or leaf material. For practical purposes, it was important to determine the concentration of the terpenoid in soil and plants, so that realistic estimates of usage could be determined.

4.1.4. The release of aza A from granules, and its persistence in soil and plants

The aim of this part of the project was to examine the behaviour of aza A in soil and plant environment by quantitative analysis of the terpenoid. It can be broken down into three parts:

The first part was to determine the limonoid's mobility and stability in soil and thus its half-life (DT_{50}).

The second part followed the release of aza A from the pellet formulations, with the aim of determining the degree of delayed release which could be achieved.

The third part looked at systemic plant uptake of the limonoid and its persistence inside the plant after its uptake from both a hydroponic medium and from soil.

4.2. Materials and Methods

4.2.1. Soil

The soil used throughout the work reported here was from a single batch of commercially-available "top soil" purchased from B and Q Ltd, UK. This is classed as a sandy loam type with a 3.2% carbon content and a pH of 7.0, the best of four main types of soil found in the UK. It's considered the best for broad range of plants as it has the advantages of sandy and clay soil and none of their disadvantages: the sandy part of it allows the plant root to easily penetrate into the soil, where the clay is beneficial for its nutrient and prevents quick loss of water (easy.net, 2004; gardeningdata.co.uk, 2003).

4.2.2. Maintaining a constant soil moisture content

Studies on the behaviour of aza A in soil depended on extracting the terpenoid from the soil water, as this represents the fraction available to the plant for uptake. As this work was done in pots, over many days, it was important to maintain a constant level of saturation of water in the soil. This was done by means of the system called "Osmogro Self-watering System®" (Aquagel Technologies Ltd, Scotland). The key component of this system is a semi-permeable membrane placed under the pot containing the soil, fed by a reservoir of water, which keeps the water content of the potted soil constant (Fig. 4.1).

After soaking with tap-water, the soil was transferred into a 12.7cm x 11.5cm flat-bottomed specially-designed pot of Osmogro Self-watering System®, the membranes put in place, the reservoir filled with water, and then the pots left for 2 days to equilibrate. Samples of soil were collected in triplicate from each pot, over a period of 3 days by

using a 1cm diameter coring tube, which removed a vertical column of soil from top to bottom of the pot.

The soil samples were placed in individual weighed 25ml Pyrex beakers, and dried in an oven at 100^{o}C, until constant weight (12 hours). The weight of water in the soil was determined by weighing before and after drying. Soil cores were taken as above from 4 pots, and the results averaged. A figure of 25 ± 1.2 % (w/w) for water in soil was obtained. The sampling over 3 days proved that the osmotic watering system could maintain this figure within 1% throughout the experiments.

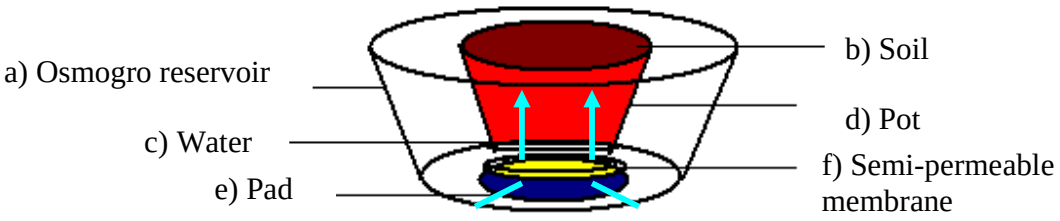


Figure 4.1: Osmogro Self-watering System®.
The figure shows in diagrammatic form the system used to maintain constant water content in soil. The water is drawn up into the soil through the osmotic membrane to maintain a constant osmolarity of the soil water, thus keeping its volume constant.

4.2.3. Persistence of aza A in soil

In order to investigate the basic behaviour of aza A in soil, NAT powder supplied by Triflolio-M GmbH, Germany, was used. This material contains 42% (w/w) aza A, as already indicated in Chapter 2. It was decided to add sufficient of the material to the soil in pots to achieve a theoretical maximum concentration of 10^{-4}M for aza A in the soil water. The results reported in Chapter 3 indicated that at this concentration, there should be no risk of phytotoxicity. To achieve this theoretical concentration the NeemAzal®-Technical was added to the prepared soil at a concentration of 43mg.kg^{-1}.

Equilibrated soil, in 1 kg batches, was thoroughly mixed with 43 mg of the crude terpenoid. Soil was then returned to the osmotic self-watering system and pots were left for 4 hours at room temperature (22±1^{o}C). Soil cores were collected as above, and each one extruded into a 10ml sterile plastic syringe, into which a 25mm glass-fibre filter (Whatman, UK), had been placed. At the tip of the syringe was fitted a Whatman's Spartan 3 syringe filter, pore size 0.2µm (Whatman, UK). The syringe was then suspended in a Corex 35ml centrifuge tube and centrifuged for 3 minutes at 3000rpm to remove and filter the water from the soil core (Fig. 4.2). About 200µl of water was collected consistently from each soil sample. An aliquot of this, (20-50µl), was injected into the reverse-phase HPLC for quantitative analysis of aza A in the soil by the standard CIPAC method as described in Chapter 2. (The normal aliquot volume for analysis was 20µl, but for very low concentrations, larger volumes were occasionally used to ensure that the amount of aza A was greater than the LOD, found to be 6x10^{-8} g (section 2.6.5.1) To ensure consistency, two standards of purified aza A from those used to establish the standard curves in Fig 2.4 were run with each batch of soil-water samples. These were

32µgml^{-1} and 250µgml^{-1}. Soil samples were taken every 24 hours for four days. (Beyond this time quantities of aza A had fallen below the LOD: 7.5x10^{-6} g.ml^{-1})

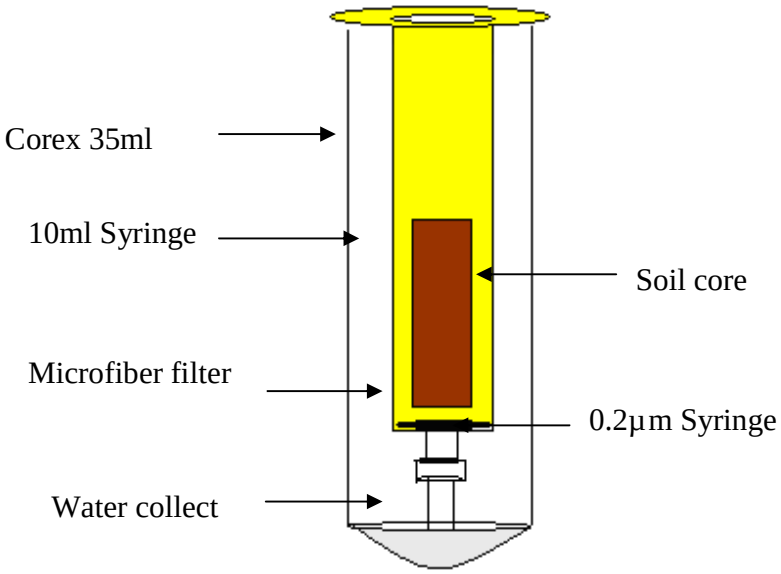


Figure 4.2: Recovery of water from soil samples.

The method used to extract and filter the water from a standard core of soil is shown diagrammatically. The water recovered after centrifugation was used to estimate the dissolved aza A.

4.2.4. Azadirachtin A release from the granules into the soil

4.2.4.1. The composition of the granules

Granules containing NAT were prepared for this project by Germains Technology Group, Kings Lynn, UK. Although some aspects of the nature of the pellets must remain confidential, they were composed of “wood flour” with carboxymethyl cellulose as

binder. As well as the basic pellet, which took up water, and disintegrated quite rapidly in the soil, a second type contained a hydrophobic polymer to delay release of the terpenoid. These will be respectively called “hydrophilic” (“Neem 1”) granules and “hydrophobic” (“Neem2”) granules.

4.2.4.2. Theoretical maximum concentration of azadirachtin A in soil

As stated previously in Chapter 2 the NAT content of both these types of pellet was shown to be 34±2% w/w. This meant, as aza A is 42% of the semi-purified extract, that the content of azadirachtin in the pellets was 140gkg^{-1} of pellets. To achieve the required theoretical maximum concentration of aza A of 10^{-4} M, 130 mg of the pellets were mixed with 1kg wet soil.

As described in 4.2.3 after the pellets had been incorporated into the soil in pots, they were left for 24 hours at room temperature, and the samples taken for estimates of aza A at daily intervals, until the concentration of azadirachtin had fallen below the level of detectability. Aza A in soil- and leaf-water was analysed by the CIPAC HPLC methods previously described. Concentrations were determined by peak height/area with regard to standards of known concentrations run at the same time.

4.2.5. Systemic uptake and persistence of aza A in plants

Systemic plant uptake and its persistence in the leaf of aza A was tested on plants which had been allowed to grow in soil for 21 days (4th leaf stage) before loading them hydroponically in growth solutions containing known concentrations of the terpenoids (Figure 4.3).

Cabbage and sugar beet (*Brassica oleraceaea* Var. PrimoII, *Beta vulgaris*) plants were

grown from seed as described in above section. After 3 weeks, plants were gently uprooted, the roots washed in tap water and then placed in half-strength Hoaglands solution, which contained the following nutrients: KNO_3, 6 mM; $Ca(NO_3)_2$, 4 mM; $NH_4H_2PO_4$, 2 mM; $MgSO_4$, 2 mM; H_3BO_3, 50 μM; $MnCl_2$, 10 μM; $ZnSO_4$, 0.77 μM; $CuSO_4$, 0.36 μM; Na_2MoO_4, 0.37 μM; Fe^{3+}–EDTA, 4.5 μM . The nutrient solution was put into 50x20cm porcelain troughs. The plants, supported by polystyrene sheets and cotton wool, were then placed with their roots immersed in the nutrient. The polystyrene sheets were wrapped in black polythene to exclude light from the medium. The system is shown in Figure 4.3. The troughs were put in the hydroponics growth room with a long day (light/dark, 16h/8h) set-up, to make them grow faster. They were maintained at 20 °C with humidity of 60%. The troughs were topped up every few days with water and the Hoagland's solution changed completely every week. The medium was constantly aerated by means of an air pump. Plants were let to grow on the aerated nutrients media for a week to recover from the shock. On the second week NAT as dissolved in the Hoaglands medium at two different concentrations of aza A, 10^{-3} M and 10^{-4} M, and the plant roots exposed to limonoids for 24 hours. After 24 hours the media was replaced by fresh Hoaglands, and the plants allowed to grow for a further 4 weeks. Throughout this time, individual leaves were taken for analysis of aza A.

a)

b)

Figure 4.3a and b: Plants growing in the hydroponic system.
The photograph shows a) cabbage and b) sugar beet plants growing in Hoaglands medium. They are supported by polystyrene sheets, covered with black plastic to mimic soil conditions and prevent algal growth.

4.2.5.1. Extraction of aza A from leaf tissue.

Harvested leaves were freeze-dried with LSL Secroio SR 1111 Aclens, Switzerland., at -59^{o}C for 24 hours. (Preliminary tests showed that they had reached constant weight by that time.) Then the dried leaves were weighed, and reduced to powder with a multispeed stirrer. Weighed samples of powder were suspended in methanol (1ml), shaken for 5 min. and centrifuged at 1000rp. for 3 minutes. The supernatants were collected. The process was repeated three times. The pooled supernatants were taken to dryness in stream of air and then redissolved in 1ml methanol. Aliquots of 50µl were analysed by the standard HPLC method (Chapter 2) to determine the concentration of aza A in the leaf tissue.

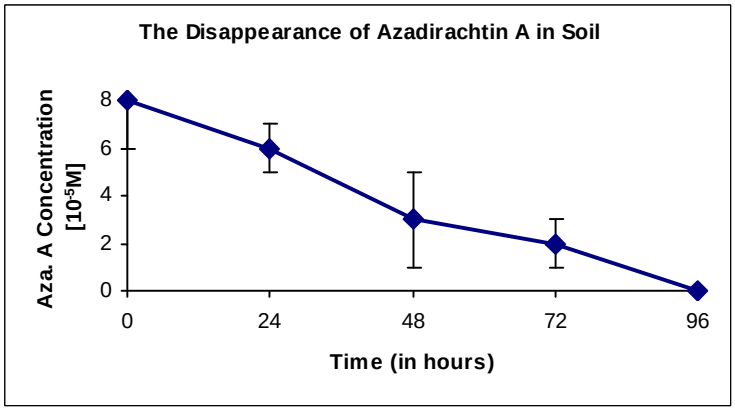
In order to calculate the concentration of aza A in the leaf water, preliminary experiments were done in which fresh leaves were freeze dried as above, weighing before and after to determine the water-content. It was found to be 88 ± 2% w/w. This figure was then used to calculate the concentration of azadirachtin in the leaf water.

4.3. Results

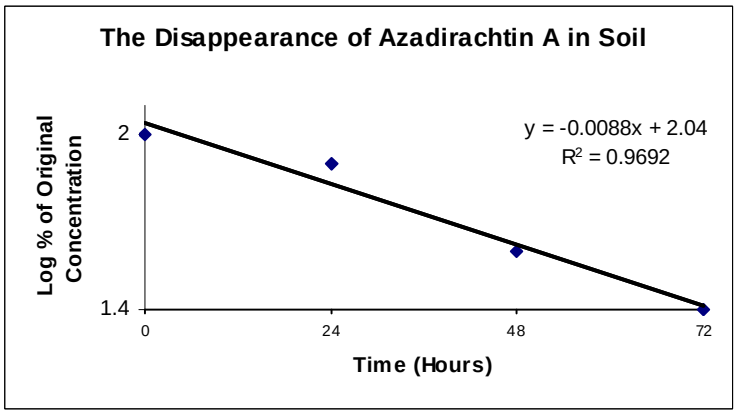
4.3.1. Mobility and disappearance

After the NAT had been mixed with soil to give a theoretical maximum concentration of 10^{-4} M aza A, the first sample of soil water, taken 4 hours later, gave a concentration of aza A of 8.11(±2.0) x 10^{-5} M. This mean value represents more than 80% of the expected maximum if all the aza A dissolved in the soil water. This suggests that azadirachtin is highly water-soluble, as expected from its known solubility.

The rate of disappearance of the terpenoid over the following 4 days is shown in Fig. 4.4. When the concentrations are presented in logarithmic form, it gives a half-life for aza A of 1.6 days.



a)



b)

Figure 4.4a and b Disappearance of aza A in soil.
Figures represent the concentration of aza A in the soil water after mixing NeemAzal®-Technical. a) The first sample (0 time) was taken at 4 hours. b) In the lower graph, the results are expressed in logarithmic form as a % of the first measurement. Each point is the mean ± SD of 3 samples from different pots.

4.3.2. Aza A release from the pellet into the soil.

a) Hydrophilic ("Neem 1") granules.

The release of aza A into the soil is shown in Fig. 4.5. No aza A could be estimated for the first 3 days of sampling. The concentration of the terpenoid reached its maximum after 5 days. The maximum concentration was found to be 4.2 (±0.18) $X10^{-5}$ M. This is 42% of the theoretical concentration which might have been achieved from the known terpenoid content of the granules. After 144 hours (6 days) the azadirachtin in the soil water declined steeply and was not measurable after 7 days.

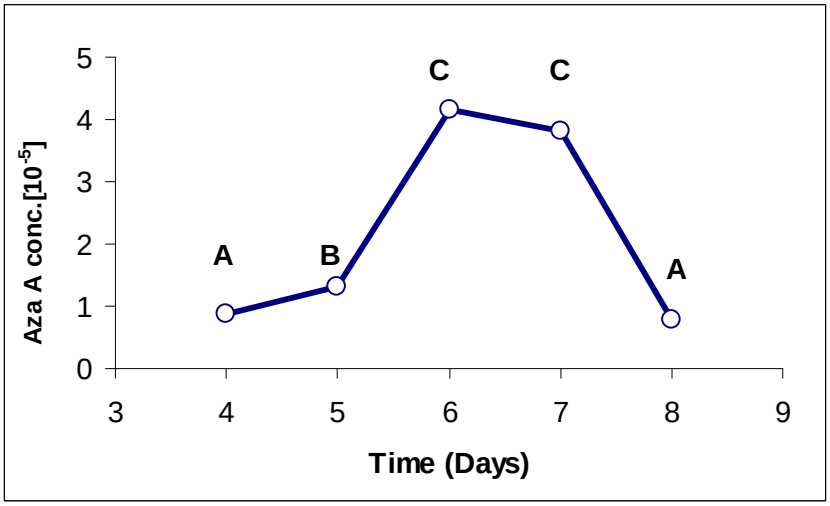


Figure 4.5: The release of aza A from hydrophilic granules.

Figures represent the mean concentration (n=6) of the terpenoid in soil water. The figures are the mean concentration (n=6) of aza A in soil water. Tukey's H.S.D 5% Analysis between the means was carried out and those with same letters are not significantly different at $p \leq 0.05$.

b) Hydrophobic ("Neem 2") granules

These granules contained an undisclosed hydrophobic polymer to delay their disintegration in water. The effects of this on the release of aza A are shown in Fig. 4.5. No terpenoid could be measured before the 9th day, and the concentration then rose slowly to its maximum on the 13th day. The maximum concentration was 2.5 (± 0.12) X10^{-5} M. This was 25% of the possible theoretical maximum expected. Then the azadirachtin declined slowly. After 17 days, it could no longer be estimated.

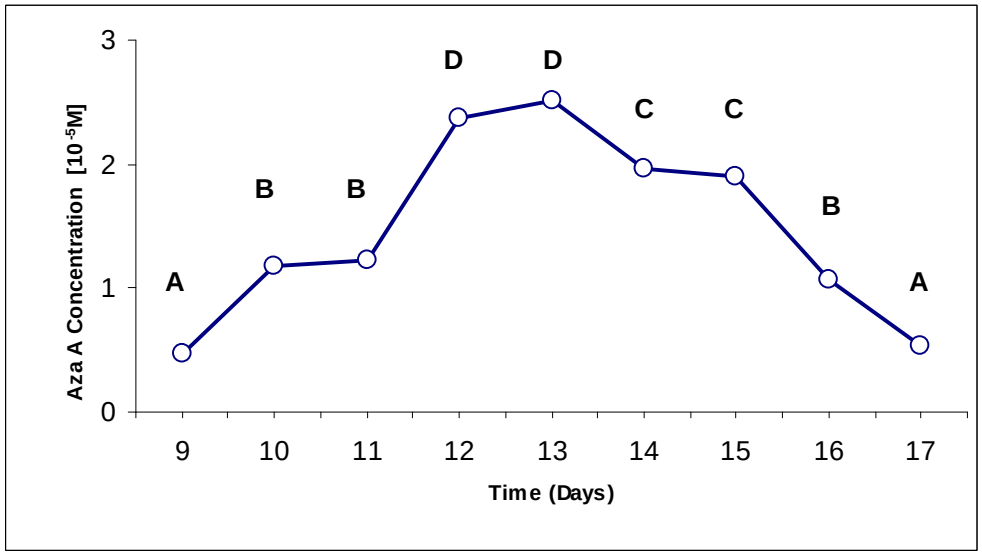


Figure 4.6: The release of aza A from the hydrophobic granules. The figures are the mean concentration (n=6) of aza A in soil water. Tukey's H.S.D 5% Analysis between the means was carried out and those with same letters are not significantly different at $p \leq 0.05$.

4.3.3 Systemic uptake and disappearance of aza A in plants

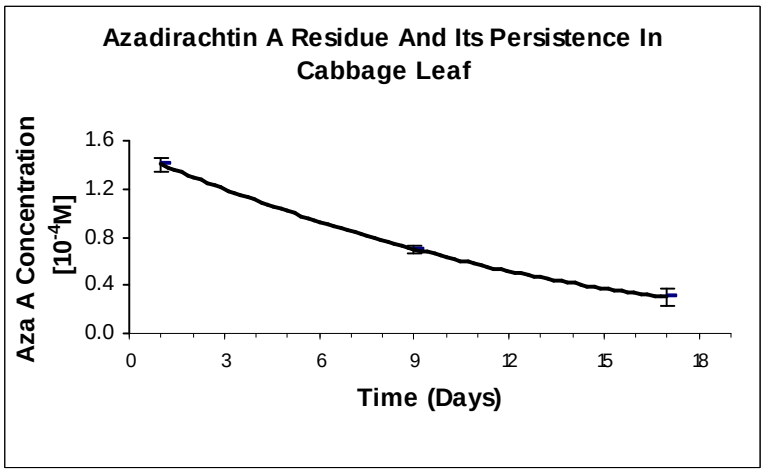
4.3.3.1 Hydroponic uptake

As described in the Materials and Methods section, both cabbage and sugar beet were loaded with aza A for 24 hours, from a medium containing either 10^{-3} M or 10^{-4} M, and the concentration of the limonoid measured in the leaves over 3 weeks.

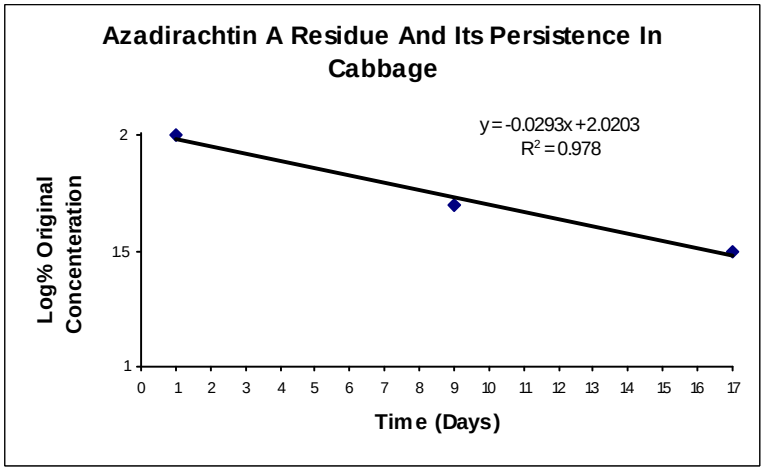
a) Cabbage

The concentration of aza A in leaf water measured at zero time (i.e. immediately after the 24 hour exposure to the terpenoid in the growth medium) was much lower than the concentration in the hydroponic medium. When the medium concentration was 10^{-3} M, the concentration in leaf water was 1.0 (± 0.67) x 10^{-4} M, and when it was 10^{-4} M, the leaf water concentration was 1.0(±0.5) x 10^{-5} M. In both cases, the leaf concentration was only 10% that in the bathing medium.

The disappearance of aza A from cabbage leaves is shown in Fig. 4.7 (a, b, c and d). The pattern was the same for the two concentrations of the terpenoid. The concentration of aza A declined exponentially and could not be measured after 17 days. The half-life of the terpenoid was 9 days.

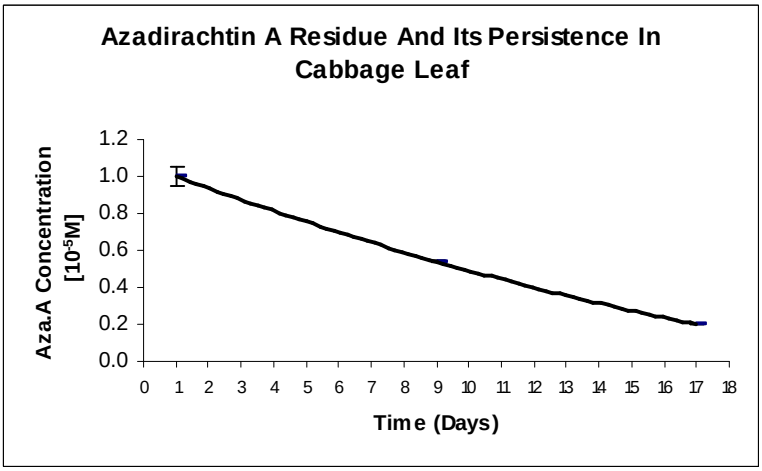


a)



b)

Figure 4.7a and b: Systemic uptake, and persistence of aza A inside cabbage plant. Plants were placed in hydroponic medium containing 10^{-3} M aza A for 24 hours, and then allowed to grow in medium without the terpenoid for 3 weeks, while leaves were taken in order to estimate the concentration of the terpenoid in leaf water. Points are the mean ± SD of 3 samples. Results in a) are the concentrations found and in b) a logarithmic representation based on % of the original concentration.



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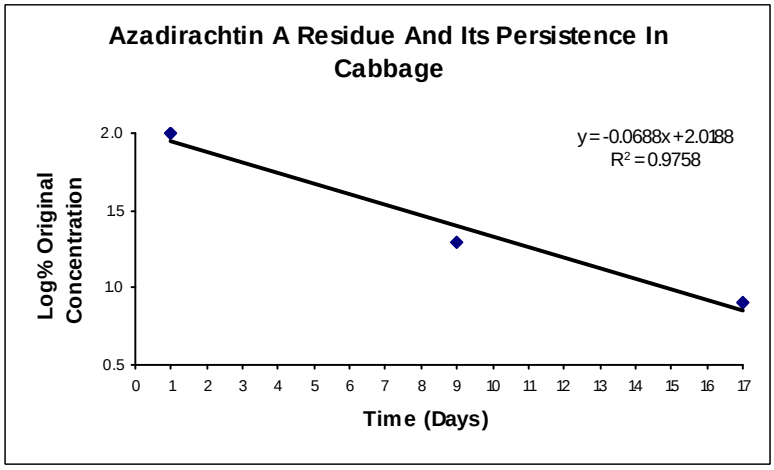
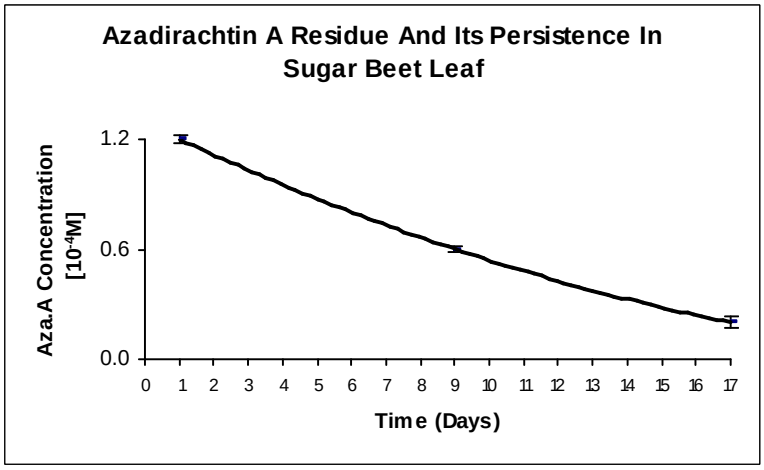


d)

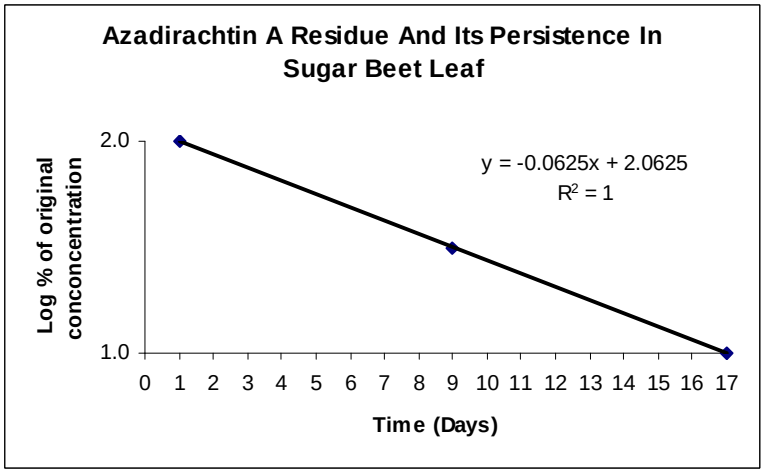
Figure 4.7c and d: **Systemic uptake, and persistence of aza A inside Cabbage plant over time**. c) The amount of aza A in the leaf water of cabbage at 10^{-4}M concentration after 1, 9 and 17 days respectively. Its shows aza A disappears exponential from leaf-water. Bars represent on standard errors of the mean values. d) log% of the original concentration as percentage of initial concentration plotted against time in days.

b) Sugar beet

The results for sugar beet were similar to those for cabbage. After loading the plants with the a.i. for 24 hours, the concentration in the leaf-water was found to be 1.2(±0.2) $x10^{-4}$ M, when the concentration in the hydroponic medium was 10^{-3} M, and 9.2(±2.0) x 10^{-5} M when the concentration in the medium was 10^{-4} M. The concentration of the a.i. then decreased in the plants in an exponential way, giving a half-life of 10 days.

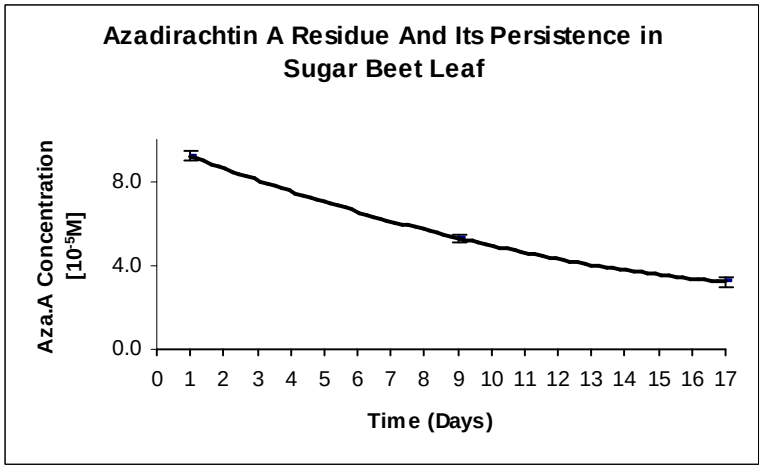


a)



b)

Figures 4.8a and b: Systemic uptake and persistence of aza A inside sugar beet plant over time. a) The amount of Aza A recovered from sugar Beet leaf water according to the original one (10^{-3}M) b) Log % of the original concentration.



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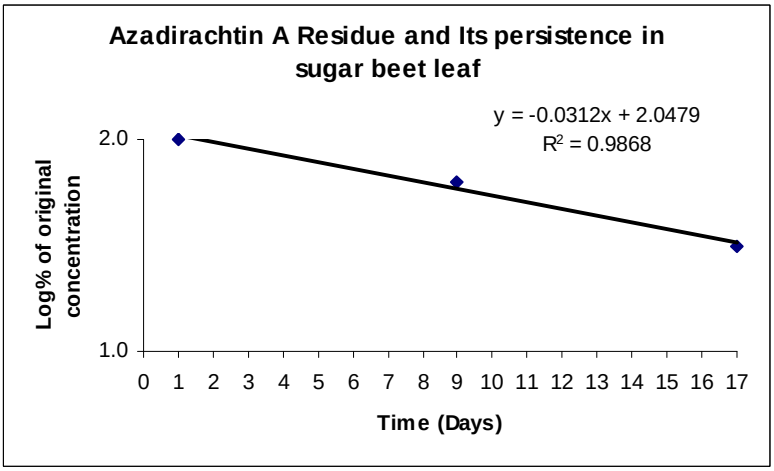


d)

Figures 4.8c and d: Systemic uptake and persistence of aza A inside sugar beet plant over time. c) The concentration of aza A in the leaf water of cabbage after 24 hours in relation to the original conc. Applied (10^{-4}M). d) Log % of the original concentration.

4.3.3.2. Aza A release from pellets and its subsequent uptake by plants

The results reported in section 4.3.2 a and b of this chapter showed that the delivery of the a.i. into the soil could be delayed by the use of pelleted material, and that the delay time could be modified by the inclusion of hydrophobic material. It was important to discover how this delay affected the appearance of aza A in the leaves of plants grown in soil treated with the pelleted material.

Both model plants were used: cabbage and sugar beet, and enough pelleted material included in the soil to give theoretical maximum concentrations of the a.i. of 10^{-3} M and 10^{-4} M in soil water.

After the addition of the pellets to the soil, the plants were allowed to grow for periods of up to a month. Leaves were removed, and the concentration of aza A in leaf water measured.

Due to lack of time and material, only the hydrophilic ("Neem 1") pellets could be used.

The results are shown in Figure 4.11. The results for the two types of plant were generally similar, but there were some differences between them in terms of the time course of the presence of aza A in the leaves. In the case of sugar beet the highest concentration of the a.i. was after 10 days, while it was not until 15 days in cabbage. Except for the cabbage exposed to the higher concentration of the terpenoid, no azadirachtin could be measured after three weeks.

In the case of both plants the concentration of the a.i. in the leaf water was lower than

might have been expected. The maximum concentration when the larger amount of pellets was used was 5.1(±0.3) x 10^{-5} M for cabbage and 8.0(±0.9) x 10^{-5} M for sugar beet. At the lower concentration of the a.i. the maxima were proportionately reduced.

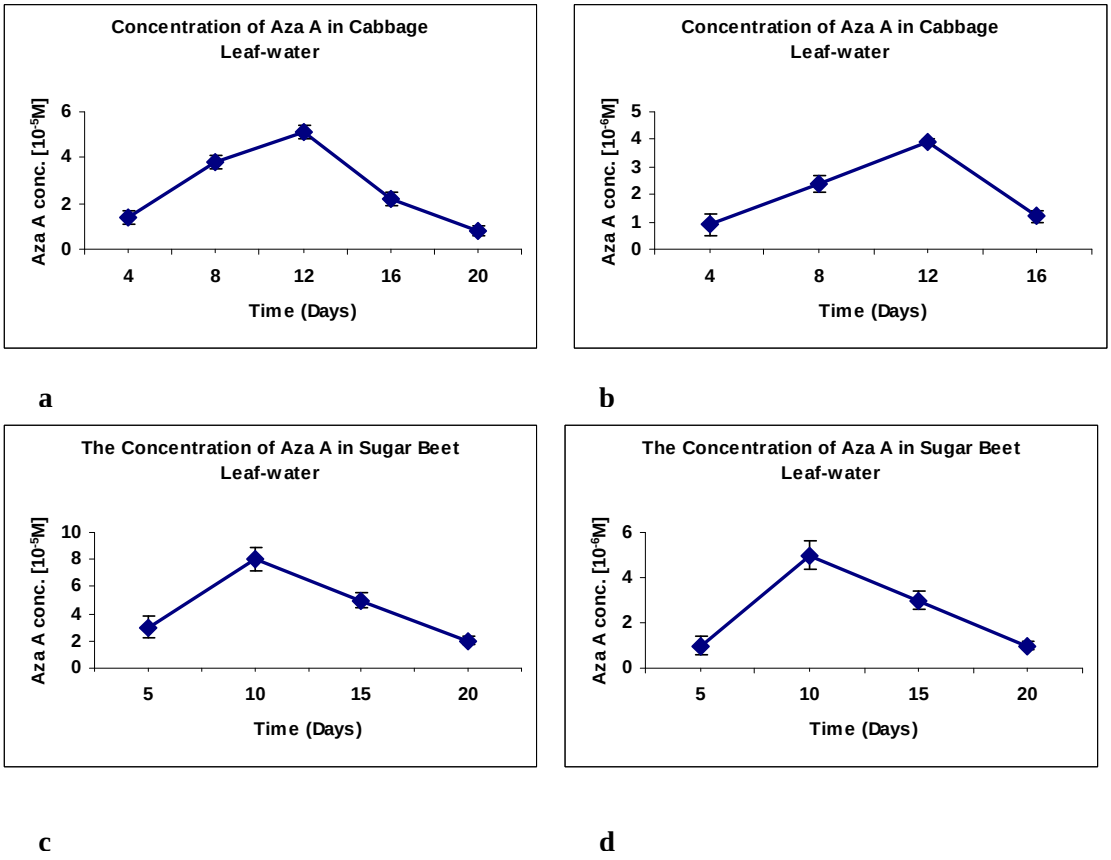


Figure 4.9a, b, c and d: Aza A concentration in leaves of cabbage and sugar beet. The figures represent the mean concentration of aza A (± SD; n=3) in the leaf-water of cabbage plants (a and b) and sugar beet (c and d), grown in soil treated with sufficient pelleted a.i. to give theoretical concentrations in soil water of a) and c): 10^{-3} M, and b) and d): 10^{-4} M.

4.4 Discussion .

It has been shown by previous studies (R.Strang, unpublished, 2000; Daly, 2004; Ruche, personal communication, 2005) that aza A breaks down in soil and artificial growth media in an exponential manner (1^{st} order kinetics). This is illustrated in Table 4.1. which records the results previously obtained in this laboratory (Daly, 2004). In order to minimize the number of analyses, fewer time points were used in the work reported here. This was probably a mistake, as it is not entirely clear from results presented in Figure 4.4 that the decay of the terpenoid is in fact exponential. If the rate of breakdown of aza A is a zero order reaction, it would suggest that the rate would be constant and thus independent of the concentration of the terpenoid. In the light of previous results, however, it seems reasonable to assume that disappearance of aza A is exponential, and to derive a DT_{50} of 1.6 days.

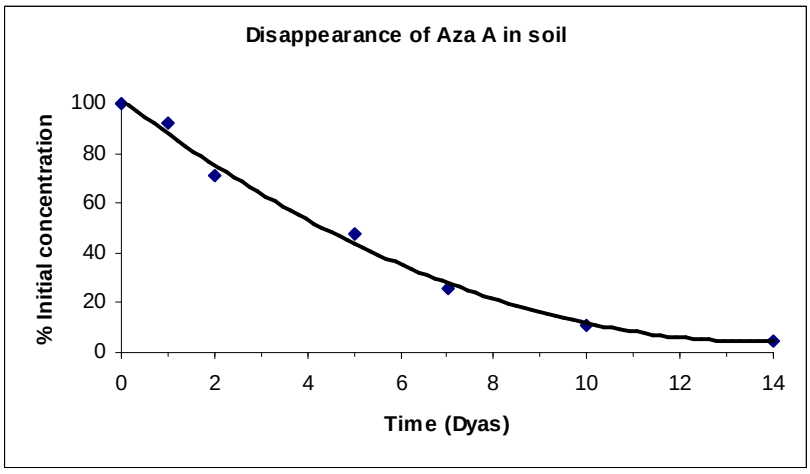


Table 4.1: Disappearance of Aza A in soil.

One of the important physical characteristics of aza A is that it is moderately water-soluble. Solubility at 22 ^{o} C has been previously determined as: 1.29g.L^{-1} (Daly, 2004), 2.9g.L^{-1} Kleeberg, H (personal communication, 2004). This means that a maximum concentration of more than 10^{-3} M should be possible, although, as shown in Chapter 3, at this concentration it might be phytotoxic.

This water-solubility means that the a.i. should be highly mobile in soil, and systemic in plants. The characteristics of aza A are compared to other systemic insecticides in Table 4.1. It also suggests that it should be well-suited for use in hydroponic systems of production, which are increasingly important for the production of high-value salad crops such as tomatoes and peppers (Abdul *et al*., 1989; Asher and Zur, 1993; Gill and Lewis, 1971; Osman and Port, 1990; Nisbet, 1991; Sundaram *et al*., 1995). Several authors have suggested that, although most neem plant protection products are foliar sprays, its effect on insects is due almost entirely to its systemic action (Ahmad and Basedow 2003; Pavela *et al*, 2004).

Pesticide	Log Kow	Water solubility (mgl^{-1})	Soil DT50 (days)	Soil applied	Systemic
Aldicarb	0.05	4930	2-9	Yes	Yes
Pirimicarb	1.70	3000	7-234	Yes	Yes
Ethiofencarb	2.04	1800	–	Yes	Yes
Carbofuran	1.50	320	30-60	Yes	Yes
Diazinon	3.30	60	–	Yes	No
Chlorpyrifos	4.70	1	60-120	Yes	No
Imidicloprid	0.57	610	-	Yes	Yes
Azadirachtin A	0.85	1300	2-4	Under trial	Yes

Table 4.2. Characteristics of some soil-applied/systemic pesticides (Daly, 2004; Tomlin, 2003).

One of the characteristics of a plant protection product which determines its mobility in soil is the log K_{ow}, representing the partition between oil and water. The log K_{ow} for aza A is 0.85 (Daly, 2004), reflecting its high water-solubility. Of the 500 pesticides whose physical characteristics have been reviewed recently (Wauchope *et al*., 2002; Tice, 2001) only 10% have log P_{ow} values less than 1.0. The terpenoid should be highly mobile in the soil water. The results reported in this chapter confirm this. 4 hours after mixing the crude terpenoid mixture in soil with 25% w/w water content, more than 80% of the total azadirachtin added was dissolved in the water which could be recovered from the soil. This suggests that in normal top-soil with a low organic carbon content (3-4 % is a

typical result (Daly, 2004), aza A should be rapidly available for uptake by plants. The other parameter which will determine the ease of movement of an a.i. in soil is the log Koc, the measure of affinity for organic carbon. In the case of aza A this is 1.5 (Kleeberg, H., unpublished results). Very few pesticides have such a low value.

The problem which arises from this high soil-mobility is that the a.i. may leach into ground-water. Any compound can be rated in this respect by the Groundwater Ubiquity Score (GUS), (Gustafson, 1989). This states:

$$GUS = \log DT_{50} \times (4 - \log K_{oc})$$

This indicates the importance of the rate of disappearance of the a.i. from the soil. The figure for the half-life of azadirachtin in soil from this work was 1.6 days. This is consistent with the previous findings of Daly (2004): 1.2 to 2.7 days, and (Kleeberg, H, personal communication): 1.9 to 3.8 days, depending on the soil type. If the figure of 1.6 is substituted in the above equation it gives a result of 1.2 for the GUS. Any pesticide with a GUS of less than 1.8 will pose no threat to ground-water despite its high mobility in soil (Pussemier, 1998).

Breakdown of azadirachtin in the soil is probably mainly due to its spontaneous hydrolysis in water, and to microbial action. As the half-life in pure water at slightly acid pH has been recorded as 19 days (Daly, 2004), it is likely that most of the breakdown is due to microbial activity. This means that the half-life will vary with temperature and nature of the soil. Proof of this is shown by the much longer DT_{50} figures found by Thoeming *et al*., 2006; and Sundaram *et al*., 1995, 1996). These authors reported DT_{50}

times of between 6-26 days. The key to these differences is the proportion of organic matter in the growing medium. It is clear that a high amount of organic matter increases the DT_{50}. This might be important in practice, as neem plant protection products applied to the to an artificial growth medium with a lot of peat etc. will remain available to the plant for a longer time.

The soil used in this project was a commercial soil, and was stored throughout the project in the plastic bag in which it was supplied. It is possible that this will have altered the level and type of microorganisms present in the soil, as the conditions may have become anaerobic, and possibly drier. However, the rate of breakdown of the active ingredient recorded here corresponds well with previous work done with soils which were taken freshly from the field or more carefully prepared (Daly, 2004, B. Ruch, (unpublished results)).

A practical problem which might result from the short half-life of azadirachtin applied to field soil with a low organic content is that it might require repeated application to achieve pest control. One of the potential advantages of a pellet version is that it may help to limit the number of applications by prolonging the effective presence in the soil (Darvari and Hasirci, 1996). Others have commented that the formulation of pesticides is important in determining their persistence (Bowman, 1992; Matthew, 2000, 2008). The main aim of using a pelleted formulation in this work was to prolong the life of the a.i. in the soil, and thus, hopefully, the plant.

For reasons which are not clear, the estimates of aza A in leaf water are much more

precise (i.e. low coefficient of variation, see below) than those which were obtained in soil-water, which are much higher. This makes statistical evaluation of the soil results difficult. This is particularly marked in following the release of a.i. from the hydrophobic ("Neem 2") granules, where the concentrations are very low. Despite this, however, all the recorded measurements were well above the LOQ, and the highest concentrations found were statistically significantly higher than the lowest, indicating that the general pattern of release was valid.

Bearing in mind the lack of precision of the individual estimates, the results confirm that the release of azadirachtin into the soil water was in fact delayed by encapsulating it in pellets. When NAT was simply added to soil, all trace of azadirachtin had gone by 4 days. In contrast, when the hydrophilic granules were used, the maximum concentration in the soil water was not achieved until 5 days, and the terpenoid could still be measured at 7 days. The addition of a hydrophobic polymer to the pellet medium resulted in a slow release of detectable azadirachtin over a period of up to 17 days, with a peak at 13 days. The pellet formulations also seem to protect the azadirachtin from the catabolic factors in the soil. The maximum concentration at 5 days of the a.i. is 42% of the maximum theoretical release when the hydrophilic granules were used and 25% at 13 days for the hydrophobic granules.

The behaviour of the terpenoid in the plant is more important than in the soil. Uptake from soil into both cabbage and sugar beet resulted in a concentration in the leaf water on about 10% that in the soil water (Table 1 in the Appendix). These figures are similar to those of the authors reported above, who also found that the concentration in the leaf was

less than 10% of that in the growing medium (Theoming *et al*, 2006; Sundaram *et al*, 1995, 1996). These results suggest that uptake into various plants is poor, even although there is evidence of systemic movement in plants. Daly (2004) in contrast, using the tracer 22,23-[$^{3}H_2$]dihydroazadirachtin A, in hydroponic conditions, found the same concentration of the compound in the leaves, as in the liquid medium. It is likely that uptake will vary with the plant used, but all the authors quoted were able to demonstrate systemic movement of aza A in the plants used: beans (Thoeming *et al.*, 2006), aspen (Sundaram *et al*., 1995,1996) and nasturtium (Daly, 2004).

Using radio-labelled dihydroaza A, Daly (2004) showed a clear exponential decline of the amount of the terpenoid, due to catabolism, after it had been taken up into leaf tissue. To lessen the number of analyses involved, only three times points were used in following the decay of aza A in the work presented here. The analyses showed a high degree of precision, with a low coefficient of variation (with an overall average of 4±1%). All the results presented in Figures 4.7 and 4.8, show the same pattern, suggesting high degree of accuracy of the analysis also. Thus there is no reason to doubt that the disappearance of aza A from the leaf tissue also follows 1st order kinetics, as would be expected.

The results presented here indicate that the aza A disappears more slowly once it is in the plant, than it does in the soil. The DT_{50} was 9 days. This is in general agreement with Thoeming *et al.*, 2006, who could find no measurable amount of the terpenoid after 14 days. Some work has reported a slower rate of disappearance from plant tissues. Sundaram (1996) found no decline of aza A in spruce over a three week period, and

Duthie-Holt *et al*., (1999) found biological activity in pine trees over a 6 week period. Some of these differences may be due to the different species used. At the moment there is no information available about the breakdown of azadirachtin in plant tissues. From a practical point of view, the persistence of the a.i. in the plant will affect the time of application before harvest.

Chapter 5: Control of Pests with Soil Applied NAT Pellets.

5.1 Introduction.

Controlling soil-borne pests such as nematodes and some insects, like cabbage root flies (*Delia radicum*), is more difficult than foliar pests, but was helped by the discovery of synthetic insecticides, mainly the organophosphates and carbamates. But effective control of such soil-borne pests with these synthetic pesticides required their application at high rates, which had an adverse effect on soil, ground water contamination and development of resistance by pests. This has prompted a renewed search for other biological plant protection products mainly from plants and other sources, with greater specificity towards insects. This has led to a growth in interest in the extracts of neem tree (*Azadirachtia indica*), ((Pavela, Barnet, and Kocourek, 2004; Nisbet *et al*., 1996; Lowery and Isman, 1994b; El-Wakeil and Saleh and, 2007; Frounier and Brodeur, 2000; Hummel & Kleeberg, 2003; Arpaia & Loon, 1993; Grisakova *et al*., 2006; Sayah, 2006; Mordue, 1996; Weintraub and Horowitz, 1997; Javed *et al*., 2007; Thoeming *et al*., 2003; Schulte *et al*., 2006; Lowery *et al*., 1997; Kumar and poehlingn, 2006; Thoeming and Poehling, 2rnet, 2006; Pavela and Barnet, 2005; Schmutterer, 2002)

Until recently, the only reported use of neem extracts applied to the soil has been for the control of plant-parasitic nematodes (Mojumder, 2000b; Mojumder and Mishara, 1997a, b); Akhtar, 2000; Javed et al., 2008). There are several reports that neem formulations cause 70 to 100% mortality on root-knot nematodes (Akhtar, 2000; Aziz *et al* ref by Javed *et al*., 2008). However, purified azadirachtin failed to control infestations, or to

show a nematicidal effect. This indicates the the effect of the crude materials is probably due either to secondary metabolites released during its decomposition or to other unknown terpenoids in the extracts (Blake, 2002; Javed *et al*., 2008), No claim is made by Trifolio-M GmbH is made about any nematocidal effect of NeemAzal®-T.

However, there have been several studies demonstrating that aza A can get into plant vascular system systemically (see Chapter 4) and have an effect in controlling phytophagous insects (Nisbet *et al* 1993; Blake, 2002; Thoeming *et al*., 2003; 2006; Daly, 2004; Pavela *et al*., 2004; Pavela and Bárnet, 2005; Grišakova *et al*., 2006). NAT/S was tested on 140 different insects and mites by spraying applications, and exhibited excellent control of feeding and sucking pests (Kleeberg and Hummel, 1999). Also Kleeberg and Hummel showed that NAT/S has an insecticidal effect in controlling black bean aphids (*Aphis fabae*) when applied hydroponically to the roots of bean plant *Phaseolus vulgaris*.

According to Guidelines to Good Plant Protection Practice (GPP) by the European and Mediterranean Plant Protection Organisation (EPPO, 1998), peach-potato aphid (*Myzus persicae* L.) and cabbage aphid (*Bervicoryne brassicae* L.) are very serious pests of brassicas such as cabbage, brussel sprouts, swedes and cauliflowers. Even small infestations of aphids can reduce the quality of the crop, while heavy infestations severely inhibit the growth of plants at younger stages, and so reduce the final yield. The most effective and prolonged control by insecticides was found to be granular application of aphicides to the soil. In some areas dual-component granular insecticides for control of aphids as well as cabbage root fly was reported (EPPO, 1998).

Lepidopteran pests of cabbage, such as diamondback moth and cabbage white butterfly,

are reported to have developed resistance to main-stream pesticides (Schmutterer, 2002), thus replacement insecticides become ever more important. Neem-based plant products have been reported to have antifeedant and growth disruption effect on *Plutella xylostella* and *Pieris brassicae* L. (Ruscoe, 1972 referenced by Osman and Port, 1990; Grisakova *et al*., 2006*)*. High concentrations (between 12.5g/L to 50g/L) of neem extracts, when sprayed weekly, showed good control of the moth (Dreyer, 1986; Schmutterer, 1990). All these reports are foliar spray application of NSKE, and the reports on specific systemic use of neem extracts by soil application are very sparse. Osman and Port, (1990) reported, however, that application of neem-seed powder to soil reduced damage due to *P. brassicae*.

5.1.2 Aim.

All the work reported in the previous chapters on the characteristics of release of aza A from the pellets, was a preparation for the work reported in this final chapter: the effect of the pellets applied to the soil around plants to control infestations of the major pests of brassicas. The overall aim was first to do the tests in pots in the glasshouse in the winter/spring, and then in a large field-test in the summer of 2008. For simplicity, the only plants used were cabbage (*Brassica oleracea, capitata*, Var: PrimoII).

The aim was to observe the effects of systemic aza A on the following insect pests of cabbage:

- Cabbage root fly (*Delia radicum* L.). Glasshouse and field-tested.
- Flea beetle (*Phyllotreta nemorum* L). Field-tested only.
- Cabbage white butterflies (*Pieris brassicae* L.). Glasshouse- and field-tested.

- Diamond Back Moth (*Plutella xylostella* L.). Field-tested only.
- Cabbage aphid (*Brevicoryne brassicae* L.). Field-tested only.
- Peach-potato aphid (*Myzus persicae* L). Glasshouse- and field-tested.
-

5.2 Materials and Methods

5.2.1 Experimental site

All experiments, both in glasshouse and field, were conducted at the Scottish Agricultural College (SAC) in Edinburgh.

5.2.2 Glass house Experiments

5.2.2.1 Insects and cultural conditions.

Peach-potato aphids (*Myzus persicae*) were obtained from the Scottish Crop Research Institute (SCRI) in Dundee, and cabbage root fly (CRF) (*Delia radicum* L.) were reared from eggs supplied by Warwick HRI, Wellesbourne. Cabbage white butterflies eggs were purchased from Blades Biological Ltd, Cowden, Edenbridge, Kent. Cultures of peach-potato aphid and CRF were maintained at a constant temperature of 20^{o}C with a 16 hour photoperiod. All insects were kept in plastic tanks approximately 40cm. square with a nylon netting front. Eggs of *Delia radicum* L. and Cabbage white were hatched and fed on cabbage before were used in the experiment.

5.2.2.2 Cabbage root fly (CRF).

The female CRF used for the experiment were 5 to 6 days old. (CRF mate between 4 to 7 days after hatching). Two concentrations of aza A (10^{-4} M and $5x10^{-5}$ M) were tested on

CRF. The amount aza A in milligrams was 19mg. and 9.5mgkg^{-1} soil in respective concentrations.

a) CRF Oviposition (Choice test).

Four pots, 2 pots with neem-treated soil, and 2 untreated (control) pots were placed per cage. The pots were evenly spaced without touching and treatments randomly arranged. 10 gravid females were released into each cage. The flies were provided with water-saturated cotton wool in a Petri dish, and a dish of 10% sucrose also soaked onto cotton wool. They also received a dish smeared with a thin layer of honey to provide the females with the protein and vitamins they require for oviposition.

After 3 days, one treated and one untreated pot were carefully removed to count eggs and the remaining two pots were left for further 7 days (10 days in total after introduction of insects). Throughout this time if the plants required watering this was done sparingly from below.

The laid eggs (hatched and un-hatched), were retrieved by removing the top 4 cm of the top soil and washed through a 2mm sieve in a Fenwick can and the residue caught in a 355 μm sieve. This was then washed onto filter paper, held in a funnel, using a saturated solution of $MgSO_4$. A drop of diluted detergent was added and excess liquid was allowed to drain. The filter paper was then pierced at the bottom to allow any liquid and detritus to escape. The filter paper was then removed from the funnel and placed on a metal disc. This was placed on turntable and the number of eggs counted using a stereo microscope.

For the plants that had been left 10 days, the roots of the plants were checked for larvae, and the numbers recorded and the level of root damage of CRF was compared between

the treated and untreated. Also the number of eggs (hatched and un-hatched) was counted, to determine total number of eggs laid.

b) CRF Oviposition (No-choice test).

Four pots of cabbage plants with soil of same the concentration of aza A (either 10^{-4}M or $5X10^{-5}$M: 19mg. and 9.5mgkg^{-1} respectively) and 4 untreated pots were prepared as for the oviposition experiment above. In this case, a cage contained only untreated or treated pots. As for the oviposition preference test, 2 pots were removed from each cage after 3 days. After a further 7 days (10 days altogether) eggs and larvae were checked.

5.2.2.3 Peach-potato aphids (*Myzus persicae* L.)

The aim was to investigate if the presence of systemic aza A in plants affects: a) number of nymphs produced by young adults, and b) survival of those nymphs over the following 5 days to ensure that the nymphs are the offsprings of the selected adult aphid. Also simultaneously the effect of aza A on aphids was examined.

5.2.2.3.1 Methods.

Cabbage plants at the 1 to 3 true-leaf stage were transplanted into soil containing neem granules with different concentrations (10^{-4}M, $5X10^{-5}$M or 10^{-5}M: 19mgkg^{-1}, 9.5mgkg^{-1} and 1.9mgkg^{-1} respectively) of aza A. The treated plants were left for 5 days or 10 days after the transplanting, before introducing the aphids. (as was shown in Chapter 4 the release of aza A into soil water reached its maximum after 5 days in the soil. The 5 day period was to coincide with this maximum, and the longer, 10 day period, was to see if the effect had started to decline.) By using a small brush, an adult *M. persicae* that had been removed from culture was transferred to a specially designed clip cage (Figure. 5.1). The clip cages, each with a single aphid, were attached to either upper or lower sides of the leaves of each test plant. There were at least 20 aphids in each treatment. After 5 days on the plants, survival and number of nymphs produced by adults were counted on control and treated plants.

Clip cages for insect rearing experiments.

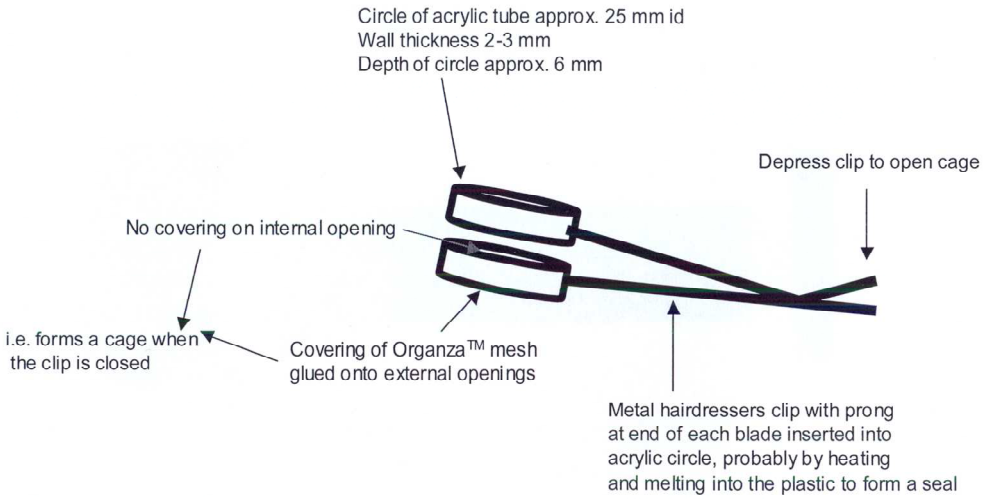


Figure 5. 1: Especially designed clip cage for tethering individual aphids on either top or bottom surface of the leaf.

5.2.2.4 Cabbage white Butterflies (*Pieris brassicae* L.).

a) Hatching and survival of 1st instar larvae.

The egg clutches (20 eggs per cage) of *P. brassicae* were placed onto cabbage plants grown on soil treated with granular formulations of NAT with aza A concentrations of 10^{-3} M, 10^{-4} M, 190mg. and 19mgkg^{-1} soil in respective concentrations, and untreated controls in a mesh cage. Each cage containing 5 plants at 3 to 4 true leaf-stage. The hatching rate, feeding and subsequent survival were observed.

b) Larval Survival and Development in later instars.

Egg clutches of *P. brassicae* were allowed to hatch onto an untreated cabbage in similar experimental conditions as that of hatching and survival. After hatching, the larvae were sorted according to their larval stages (2/3rd, 4th and 5th instars), removed, then transferred onto plants (6 plants in each cage) grown in soil treated with pellets, as that of the hatching experiment. The feeding behaviour, survival and development were observed over time.

5.2.3 Field experiment

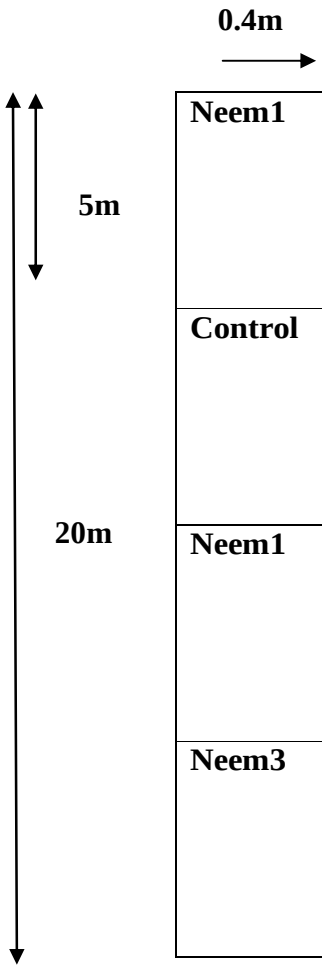
a) Study site.

A field trial was carried out at a field leased by SAC near Penicuik, Midlothian, during the growing season of 2008.

5. 2. 3.1 Experimental design.

The trial site was set out in a randomised block design (RBD) with evenly distributed treatments (Table 5.2) according to EPPO guidelines (1990). The field was prepared, ploughed, harrowed and laid out, before transplanting 2 to 3 leaf stage cabbage plants which had been grown in a glasshouse from seed. The pelleted neem materials were incorporated into soil using a tractor-mounted rotavator to a depth of approximately 5cm. 1936g (± 64g), which is equivalent of 10^{-4}M aza A (1pot of 1kg soil (77pots = 1m^2 area) contains 250ml H_2O, that is 18mg aza A/42mg of N/A/130mg pellet) in plot area of 2m^2 (0.4m x 5m). The total replication per treatment was: “Neem 1” (hydrophilic pellets): n=5; “Neem2” (hydrophobic pellets): n=1; “Neem3” (spray): n=5; “Control” (No

treatment): n=5. The total plot number was 16. There was a 1m gap between plots. There were 10 plants per row per plot, making 40 plants per plot. There was a 45cm gap between each plant in a row.



Neem1	**Control**	**Neem3**	**Control**
Control	**Neem1**	**Neem2**	**Neem3**
Neem1	**Control**	**Neem3**	**Neem1**
Neem3	**Neem1**	**Control**	**Neem3**

Table 5.1: The design of the field trial experimental site (not to scale).
The area per plot is $2m^2$ (0.4m x 5m) , and the whole block is $20m^2$. Treatments were as follows "Neem1": Hydrophilic pellets; "Neem 2": Hydrophobic pellets; "Neem3": Spray; "Control": untreated.

5. 2. 3.2 Sampling

Plants were left for a week before sampling, to allow the release of the azadirachtin from the pellets into the plant system. Ten plants per plot were randomly selected and marked with canes. These plants were assessed for the presence of pest and beneficial insects on a weekly basis. The overall growth stage of the crop was recorded on each visit.

5. 2.3.3 Pest Species

The target species were cabbage aphid (*Brevicoryne brassicae*) and peach potato aphid (*Myzus persicae*), cabbage white butterfly (*Pieris brassicae*), diamond back moth (*Plutella xylostella*), CRF (*Delia radicum*). The whole plant was checked for the presence of those pests, except for CRF. The number and species per plant was recorded on an appropriate data sheet (Appendix A). If large colonies were present where the individual aphids were difficult to count, then an estimation of colony size was made.

Identification was carried out in the field where possible. However, a sample was returned to the laboratory for identification when necessary. If this was the case, aphids were removed from the plants using a fine artist's brush dipped in aphid preserving fluid (2 parts 90% industrial methylated spirit to 1 part 75% v/v lactic acid). They were transferred to a phial of aphid-preserving fluid which was labelled with the study number, plot number, plant number and date.

Field sampling of CRF was based on the collection of eggs to estimate the activity in the field, based on the predicted second generation 50% egg laying peak, which was forecast on 6th July (Figure 5.11: CRF forecast egg-lying 2008 in Midlothian, Scotland). Using a

dessert spoon, soil was carefully removed from around the stem of the plants to a distance of 2.5 cm and to the same depth. The soil was placed in a plastic bag labelled with the study number and date sampled. Soil from between the drills was used to replace the soil removed around the stems. The soil sample was stored at 4°C on return to the laboratory before processing, as previously described in Section 5.2.2.2. One way and two ways (un-stacked) ANOVA and Chi square statistical tests were used in the analysis of the data.

5.2.3.4 Rates of application of pelleted NAT to soil in glass house and field experiments.

In the preliminary work reported in Chapter 2 and in the hydroponic experiments, it was possible to make up exact concentrations of aza A in the growing media. As a result of these experiments, the optimal concentrations of aza A in the medium around the plant roots was determined to be between 10^{-5} M and 10^{-4} M. In the preliminary experiments to define the characteristics of release from pelleted NAT, the quantities of pellet added to soil was based on a theoretical maximum concentration of aza A, if all the a.i. had been immediately released into the soil water. In fact, of course, release of a.i. is over a period of days, and could never reach this theoretical or nominal maximum. For consistency, however, the weight of pellets added to soil was based on these figures. (The actual figures for the concentrations of aza A throughout the project are summarised in Appendix A). As listed in this appendix the amounts of pellet added to soil were between 13mgkg^{-1} soil and 130mgkg^{-1} soil in pot experiments, and 10g.m^{2} in the field experiment, to give concentrations of aza A from 1.9mg. to 19mgkg^{-1} soil respectively.

5.3 Results.

5.3.1 Cabbage root fly

a) Oviposition and larval hatching (Choice experiment).

Although the numbers are relatively small, there was a statistically significant ($p < 0.01$) difference between the number of CRF eggs and larvae retrieved from the pots of treated soil compared to untreated soil, indicating that female CRF have ovipositional preference for soil without neem compounds (Table 5.1). At the lower concentration of the NSKE the number of eggs laid was half that of the controls, and was only 33% in the higher concentration. This effect of the presence of neem terpenoids was even more marked when larvae were counted at 10 days. In the control pots, almost 80% of larvae had survived, but where the soil had been treated, there were no surviving larvae at the higher concentration, and only 2 at the lower. Thus, over 91% of larvae were from the untreated soil.

Cage No.	Aza A conc. (mgkg-1 soil)	Total eggs laid	Total number of larvae	
			Living	Dead
1	19	5*	0*	1*
	0 (Control)	15	12	2
2	9.5	7*	2*	1*
	0 (Control)	13	10	2

Table 5.2: The effect of treatment with NAT pellets on the ovipositional preference of CRF females (Choice test).
The number of eggs and larvae (living and dead) retrieved from soil treated with pellets containing two concentrations of aza A, 19mgkg-1 and 9.5mgkg-1 soil, and that of untreated (controls) soil. Eggs were counted at 3 and again at 10 days, and the number of larvae were counted at 10 days. Chi square test was carried out to compare the neem treated and control. Results indicated (*) were highly significantly different from the control values at p=0.01.

b) Oviposition (No choice test).

One way analysis of variance (ANOVA) was carried out to establish the effect of aza A will have on the number of eggs laid and larvae after 10 days when there was no untreated soil available. The data of eggs collected from no-choice experiment showed a significant difference ($p<0.01$) between the number of eggs laid by female CRF of 5 to 6 days old, collected from the soil of pots treated with pellets containing two concentrations of aza A and (10^{-4}M & $5X10^{-5}$M, which is equivalent to 130mg (19mg aza A) and 65mg (9.5 aza A) of pellets respectively). (See explanation in Appendix B). The average

number of eggs retrieved from the untreated was 15 ± 1SE, that is, over 76% of all retrieved eggs (figure 5.3). While those from the treated ones were 5±1SE and 2±1SE for 19mg aza A and 9.5 aza A (10^{-4} & $5X10^{-5}$) respectively. Although the numbers for the lower concentration are unexpectedly lower, there was no significant difference found between the treated ones.

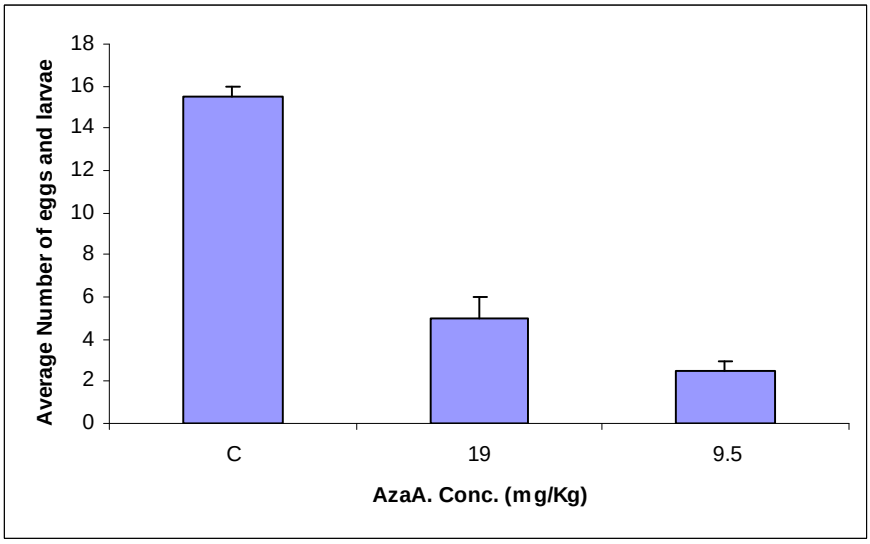


Figure 5.2: The effect of treatment with NAT pellets on oviposition and larval survival of CRF (No-choice Test).
Figures represent the averages of eggs and larvae retrieved from 4 of either treated or untreated (Control) pots. There is a significant different ($p<0.01$) between the control and the treatments, but no statistical significant difference between treatments.

a)

b)

Figure 5.3: Effect of NAT pellets NSKE on survival of cabbage plants infected with CRF.

Examples of plants treated with pellets containing aza A (a), and that of untreated (b). Root damage by CRF maggot resulting in plant death.

5.3.2 Aphids (Glasshouse experiment).

a) Fecundity of aphids on cabbages loaded systemically with NAT.

As described in the Methods section, the pellets loaded with NeemAzal®-T were introduced into the soil in the pots either 10 or 5 days before individual aphids were tethered in cages to either the upper or lower face of cabbage leaves. The insects were left for 5 days before counting the number of aphids in each cage. The results are shown in Figure 5.5.

Aphids on untreated plants showed an increase in numbers, which was the same for both experiments. In one case the individual females on the upper side of the leaf had increased to an average of 5.8 ± 0.3, and in the second experiment the number was 6.0 ± 0.5, i.e. the same level of population increase in both cases.

Where plants had grown in treated soil for 5 days, the rate of reproduction by parthenogenesis was reduced. At the lowest concentration of aza A used, the reduction was only 20% compared to the controls and was not statistically significantly different, but at the two higher treatments replication was almost completely prevented. Both were highly statistically significantly different from the control ($p > 0.01$). Only in 1.9 mg/kg was there any population growth at all. There was no increase in numbers at the highest level of treatment and only a small average increase of 0.5 aphids/cage at the intermediate concentration, which was not statistically different from the highest concentration.

When the plants had grown in soil for 10 days before the aphids had been introduced to the leaves, the results were almost identical (the lowest concentration was omitted). At

the highest concentration, there was no population increase at all, and only a doubling to 2.0 ± 0.2 aphids/cage in the intermediate concentration.

Another result of possible significance from the practical point of view is that no differences were found between aphids caged on the upper or lower sides of leaves.

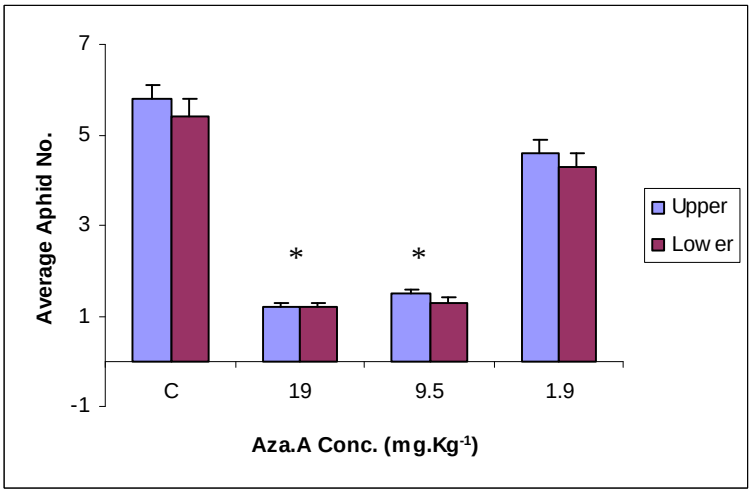


Figure 5.4a: The survival and fecundity of individual *Myzus persicae* on leaves of cabbage treated with NAT pellets.
The numbers represent the averages (± SE) per cage 5 days after individual aphids were placed on either the upper side ("Upper") or lower side ("Lower") side of a leaf. Pellets had been added to the soil 5 days before the introduction of the aphids. Control plants (C) were not treated with pelleted neem extract. The results marked (*) are significantly different from control values at p=0.01.

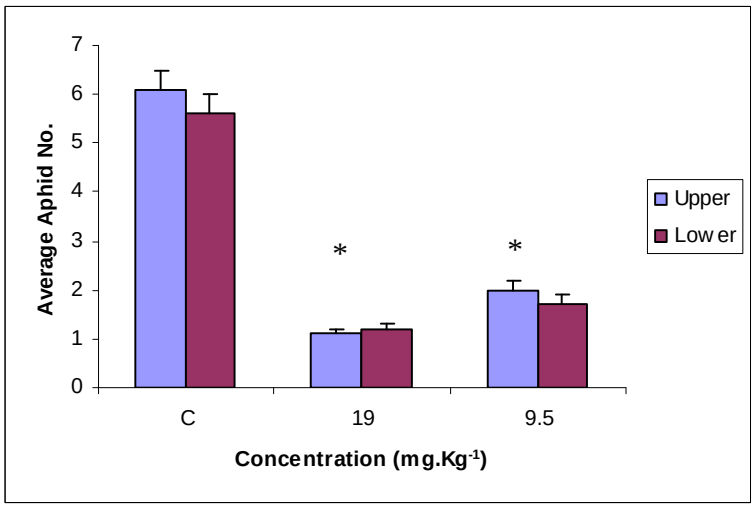


Figure 5.4b: The survival and fecundity of individual *Myzus persicae* on leaves of cabbage treated with NAT pellets.
The experimental conditions were almost the same as those in Fig. 5.5a, except that the pellets had been introduced into the soil 10 days before the aphids were placed in the cages. The lowest concentration of aza A (1.9 $mgkg^{-1}$ soil) was omitted as ineffective. The results indicated (*) were significantly different from the control values at p=0.01.

5.3.3- Cabbage white butterflies (*Pieris brassicae*).

5.3.3.1- Glasshouse

a) Hatching.

Cabbage white butterfly eggs placed onto plants in soil treated with neem pellets containing two different concentrations of aza A, 20 per cage, all hatched, but, as per table 5.2, those on treated cabbage failed to proceed to the second instar. After 4 days all those exposed to treated cabbages died. 14, (70%) survived in the untreated ones.

Aza A conc. ($mgkg^{-1}$ soil)	Time of exposure (Days)	Number of living larvae	Survival (%)
0 (Control)	3	20	100
	7	14	70
19	3	20	100
	7	0*	0*
190	3	20	100
	7	0*	0*

Table 5.3: The effect of treatment with NAT pellets on survival of newly hatched cabbage white caterpillars.
40 eggs, 20 per cage were fed on 5 cabbage plants with or without soil treated with pellets containing NAT pellets, and control (untreated). The results indicated (*) were significantly different from the control values.

b) Survival and development.

After hatching on an untreated (control) plant the larvae were allowed to feed on neem-free cabbage plants for 7 days. On the 7th day caterpillars were sorted into 2 groups according to their developmental stages – 2nd ,3rd, 4th instars. 22 of 4th in star were removed then transferred into plants (6 plants in each cage) grown into soil with neem pellets containing 9.5 mgkg^{-1} aza A (5X10^{-5}M) for 5 days as that of survival experiment. Second group (19) of 6 in 2nd and 13 in 3rd instar were transferred into cages with plants with neem pellets containing same amount of aza A concentration (9.5 mgkg^{-1}) (5X10^{-5}M). After 24 hours those in the 4th instars (figure 5.3, 18) moulted into 5th in star stage. 4 remained in the 4th in star. After 48 hours they stopped feeding and all of them all had fallen off on the floor. After 72 hours all died. For those of the smaller sizes, table 5.4, only 2 from 2nd in star group and 1 from 3rd in star stage remained in their previous stages. The rest moulted into next developmental stages. But after 72 hours all were dead.

Aza A conc. (mgkg^{-1} soil)	**Time of exposure (days)**	**Living larvae in instar: 2nd/3rd**	**4th**	**5th**	**pupae**	**Total survival rate (%)**
9.5	0	19	22	0	0	100
	1	3	16	18	0	86
	3	0	0	0	0	0
0 (Control)	0	12	8	0	0	100
	1	4	12	4	0	100
	3	0	6	12	0	90

Table 5.4: The effect of NAT pellets on the survival of large cabbage white *P. barassicae* L. caterpillars of 4th and 5th instar.

5.3.4 Field Experiment.

5.3.4.1 Cabbage Root Fly infestation.

The forecasts in the graph of egg lying forecast (Figure 5.11) shows late emerging flies of second generation in the spring peaked in the 1st week of July. That is when 50% have emerged/laid eggs. The plants were transferred into field in the second part of July to meet CRF at its peak emergence.

a) Field Experiment.

An extra plot was set up to estimate CRF migration into the trial plot. Soon after cabbage had been transplanted into the trials plots, CRF started to migrate in. The average number of CRF of soil sample collected from the site for 1st week was 2 (± SE). The number doubled for the 2nd week. From there it started to decline gradually.

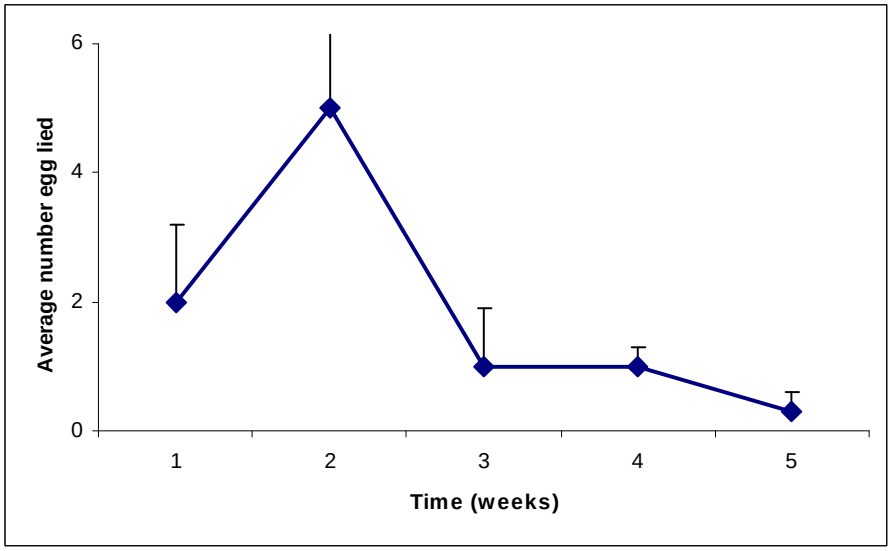


Figure 5.5: Oviposition by CRF. The figures are the means (± SE) recorded in untreated soil planted with cabbages at the test site, over the period of 23 July to 6th September.

5.3.4.2 Aphid Infestation.

After transplanting, the plants were allowed 7 days to become established, and to allow the release from the pellets of the a.i. By this time, aphid infestation was evident on cabbage plants. In sampling no preference was made on the species of aphids, though all of them were from either Cabbage aphid (*Brevicoryne brassicae* L.) or Peach potato aphid (*Myzus persicae* L).

In addition, due to continuous wet weather during the 5 weeks of the experiment, the number of aphid was lower than might have been. The average number of aphids between the treated plants and the untreated (controls) were compared employing one way un-stacked ANOVA. There was no significant difference between the treated plants and the control for the first three weeks of the experiment (Figure 5.7). From the 4^{th} week onwards the average numbers of aphid infestation in the untreated plants were significantly higher than the treated plants ($p<0.01$).

Aphid infestation on the cabbage over six weeks.

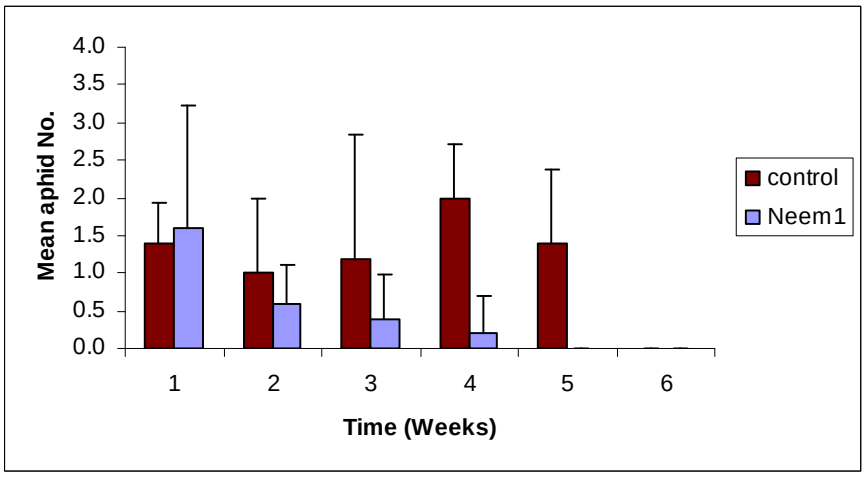


Figure 5.6: The effect of treatment with NAT pellets on aphid infestation of cabbage.
The results represent the average (±SE) number of aphids on individual plants "Neem 1" indicates treatment with the hydrophilic pellets at $10g.m^{2.}$ Control plants received no pellets.

5.3.4.3 Flea beetle level of damage.

The level of damage (Figure 5.8) by flea beetle (*Phyllotreta nemorum* L) on the treated plants was not different from the control on the 1st two weeks, but there was a significant different between the feeding behaviour, according to the leaf damage, of flea beetle specie on the treated and untreated cabbages from 3rd week onwards. The beetle damage on treated plants was statistically lower ($p<0.05$) than the untreated ones.

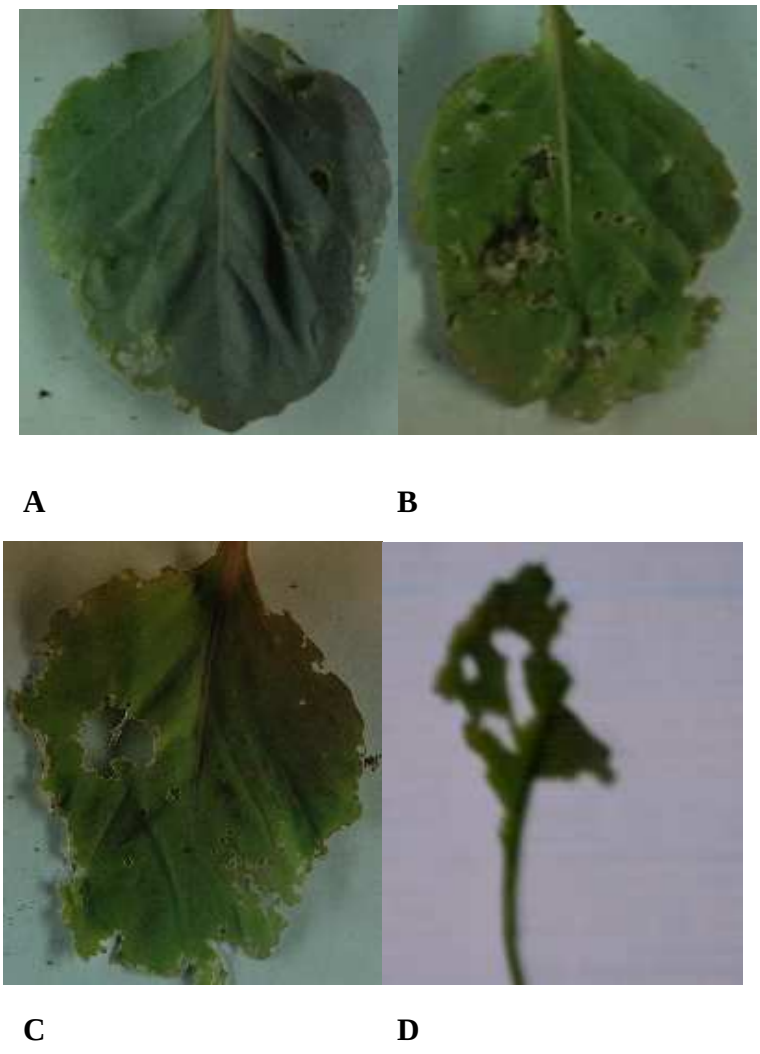


Figure 5.7: The Levels of Flea beetle damage
The images above were used as a reference in assigning the severity of leaf damage due to flea beetles: A=Level 1; B=Level 2; C=Level 3; D=Level 4.

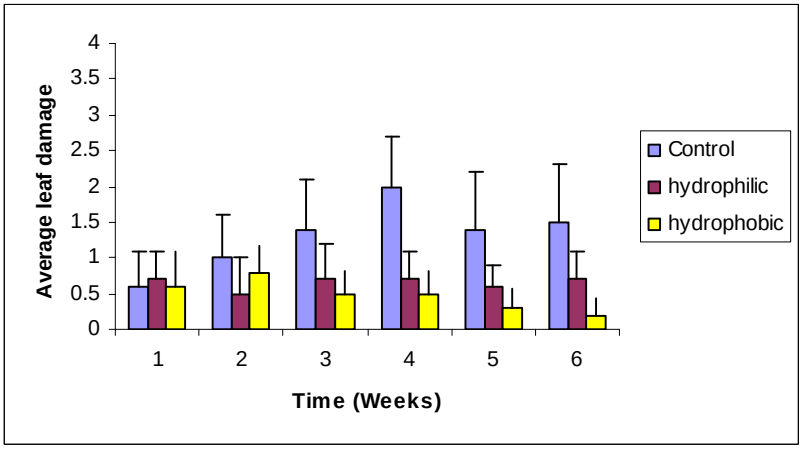


Figure 5.8: The effect of treatment with NAT pellets on the level of leaf-damage to cabbage by flea-beetle.
The level of damages were categorised into 1: low damage; 2: medium damage; 3: high damage; and 4: severe damage. (See Figure 5.8) The plants were growing in soil treated with either the hydrophilic pellets ("Neem1") or the hydrophobic pellets ("Neem 2"). Control plants were in soil which had received no pellets. The histogram gives exact means with ± SE.

5.3.4.4 Plant Growth.

The growth trend of cabbage plant was based on the increase in leaf number per plant over six weeks (Figure 5.10). There was no significant different between the growth of treated and untreated plants over the first four weeks. On the 5th week the average number of leaves per 10 plants per plot of treated plants was 11 while those of untreated was 9 (Figure 5.10), and after performing an unstacked one way ANOVA statistical analysis was significant at $p < 0.05$ value. On the 6th week the difference in leaf number was also statistically significant $p < 0.05$.

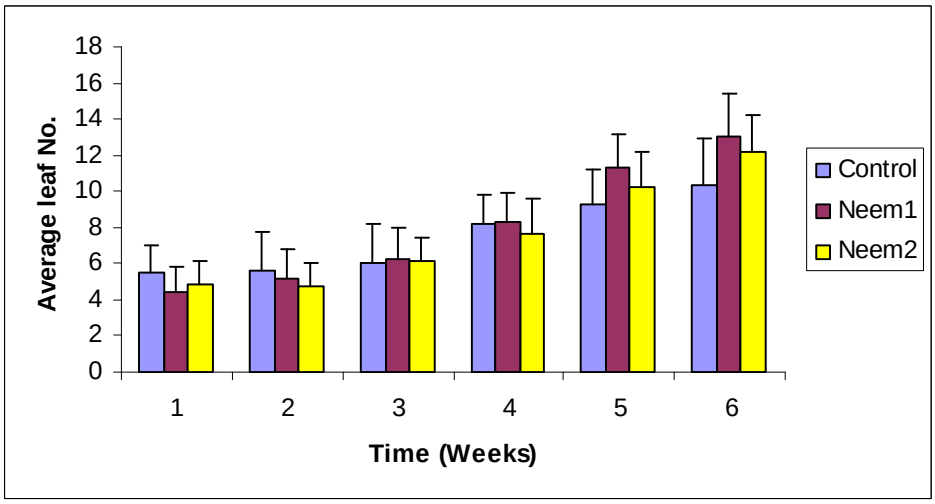


Figure 5.9: The effect of treatment with NAT pellets on the growth of cabbage plants
The figures are the average number of leaves (±SE) per plant (n=10). Plants were grown in soil treated with either hydrophilic ("Neem 1") or hydrophobic ("Neem 2") pellets. Control plants grew in untreated soil.

5.4 Discussion

As stated in the Introduction to this final chapter, the work reported was the final test of the effectiveness of the NeemAzal®-T delivered in pellet form to protect cabbage plants against both soil and foliar pests. The strategy was to make preliminary green-house tests against the pests which were available in culture in SAC Edinburgh, or which could be easily obtained from other sources. The final experiment was a large field trial to see if the results obtained in the greenhouse could be reproduced under realistic conditions.

The greenhouse trials were carried out and the results, as will be discussed below, were positive and showed a statistically-significant protective effect of aza A. Unfortunately, all field trials depend on the weather, and the weather in July and August 2008, in the east of Scotland was poor. As the figures in Table 5.5 show, the rainfall in July was 60% higher than the long-term average, and in August it was almost twice the 30-year average for that month. This meant that the expected level infestation of most pests did not occur. It also meant that collecting data in the waterlogged conditions was almost impossible. Only aphids and the flea beetles showed any increase in numbers. No results were collected for the lepidopteran pests, and it was not possible to assess the effect of CRF. Limitations of time, finances and material meant that it was not possible to repeat this large scale trial. The conclusions for individual pests discussed below are mainly based on the greenhouse studies.

Month	Sunshine(h)	Anomaly*(%)	Rainfall (mm)	Anomaly *(%)
July	140	89	104	140
August	90	60	162	194

Table 5.5: Rainfall and sunshine data for the East of Scotland for July and August 2008. *The data are expressed as a % of the 30 year average. (Data from Meteorological Office: *www.metoffice.gov.uk*).

5.4.1 Cabbage root fly.

The degree of deterrence of oviposition by the "choice" and "no choice" methods can be used to derive two coefficients of deterrence: "relative" (R) deterrence (choice), and "absolute" (A) deterrence (no choice). These are added together to give "total" (T) deterrence, (T=A+R) (Nawrot *et al*., 1982). According to these authors this gives an arbitrary classification of deterrence as follows: <50= weak; 51-100= moderate; 101-150=good; and 151-200=very good. Total oviposition deterrence when the concentration of a.i. was 9.5 mg.kg-1 was 98, and that at 19mg.kg-1 was 100, i.e. the same degree of deterrence for both concentrations. This places aza A at the top of the "moderate" category, and just short of "good". The fact that both concentrations produce the same level of deterrence suggests that there is an upper threshold for level of deterrence.

The choice and no-choice methods are most common laboratory based ways used to test the host preference. Papaj & Lewis (1993) showed that egg laying female of some phytophagous insect species gain experience when they lay their eggs for the first time. This might be true in CRF ovipositional preference. Kostal (1993) showed that combinations of physical and chemical stimuli greatly influence the oviposition of *Delia radicum* L. Also, when the period of depravation of natural (preferred) host is increased

heightens the chances of accepting available (presented) one: behavioral threshold (Barton-Browne and Withers, 2002). This is important point to consider in the CRF no-choice experiment and probably required longer period of exposure, because, as mentioned earlier a chemical stimuli might deter graved female of CRF to oviposit on to neem treated soil for the shorter period. There is no previous work reported on neem effect on oviposition of CRF, but Hellpap and Mercado (1996); Ayyangar and Rao (1989) reported that NSKE inhibited female tobacco cutworm, *S. litura*, from oviposition. The neem-seed terpenoids in NAT in a pelleted version have shown strong systemic ovipositional effect on CRF. The number of eggs laid in the treated soil was only half of that of the control in the choice test, and even fewer in the no-choice test. The proportion of live larvae after 10 days shows an even greater difference, with very few in the treated soil, compared to a survival of almost 100% in the control. It is not clear if this is due to failure to hatch or to larval death, but the overall effect is to give a high level of protection to the plants. Almost all the plants from untreated pots were killed due to destruction of the roots, while all of the treated plants were all growing normally. There are no previous reports on the effect of neem-seed extracts on CRF, but some other soil-based pests have been studied. The results presented here are consistent with the earlier findings of Meadows *et al*., 1999) on the effect of NAT on turnip root fly, (*Delia floralis* Fabr.) larval development. There are also several works on neem formulations applied as systemics on other plant insect pests (Hummel. E and Kleeberg, 2003; Kumar and Poehling, 2006; Pavela and BÃ¡rnet, 2005; Schulte, Martin, and Sauerborn, 2006; Sundaram *et al*., 1995; Thoeming *et al*., 2003; Thoeming and Poehling, 2006), and all strongly proved effective control potency. Thoeming, *et al* 2003 reported that NeemAzal-

U (a formulation of NAT specially designed for soil and hydroponic use), when applied at 10mg/kg aza A systemically into soil, reached the feeding site of larvae of western flower thrips, *Frankliniella occidentalis* on bean plants with a maximum mortality of over 50%. Thoeming *et al*., (2007) reported the same effect of 100% mortality on larvae of *Liriomyza sativa* Blanchard (Diptera: Agromyzidae) when N/A U containing17% aza A drenched in a potted soil. This suggestes that the 1st instar of CRF larvae is more susceptible in this stage.

In the "no choice" experiment, when CRF was left to lay eggs on either treated or untreated plants over 10 days, the effect of CRF maggots feeding on roots led to the control plants were almost all dead compared to the treated ones which were healthy and alive (Figure 5.4). This is an indication of severity of CRF feeding on young seedlings of 3 to 4 leaf stages of *Brassicae*, such as cabbage, plants (Bligaard, 1999). In addition, the damage sustained by untreated plants points to the high number of eggs laid, pupated and feeding on the plant root. There are some suggestions that ovipositional preference of CRF to untreated soil is to do with insect's gustatory system. In the process of locating its host, insects central and peripheral nervous system (CNS and PNS) may have a important role in CRF's behaviour in avoiding neem treated soil (Chapman, 1999).

On the experimental plot, the trend of egg laying by CRF over 5 weeks has been monitored and compared to that taken from other part of the Midlothian areas (Figure 5.11). Even though the number of eggs retrieved from soil sample at the experimental site is not as high as that of the Chapel Farm, Haddington CRF egg count, still this confirms

CRF migration to the experimental site. Both showed peaks of egg numbers during the 2nd week of July Nevertheless, unlike the glass house, no significant symptoms of CRF feeding damage has been physically observed on the field cabbage. This might be due to eggs laid in the field did not hatch as conditions such as temperature were not favourable.

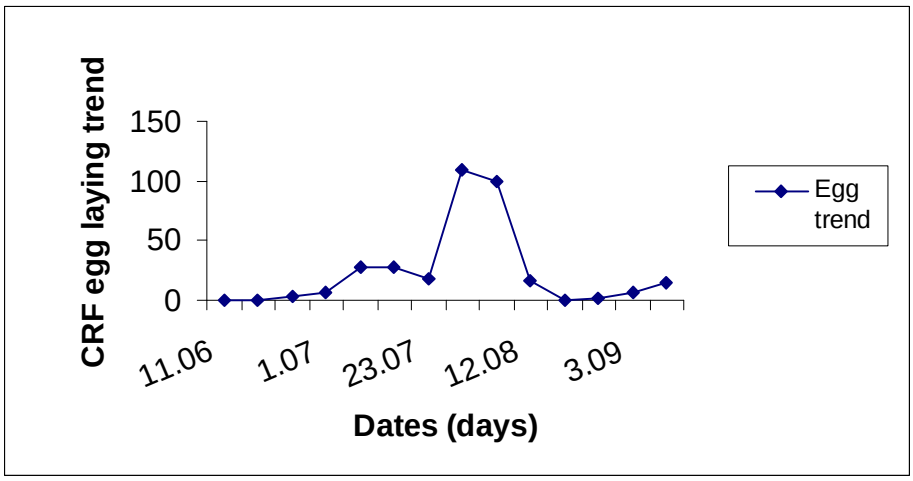


Figure 5.10: **Chapel Farm, Haddington CRF egg count**. From 11 Jun to 3 September 2008. (Data courtesy of A. Evans SAC, Edinburgh.).

5.4.2 Cabbage White Butterfly.

In glass-house experiments, the pellets deterred *P. brassicae* larvae of different stages of development (from 2nd to 4th instar) from feeding, retarded development, and caused first instar 100% mortality. The results found in this work agreed with that of (Gill and Lewis, 1971; Hummel and Kleeberg, 2003; Osman and Port, 1990; Seljasen and Meadow, 2006; Meadows *et al*., 1999). The work reported here suggests that neem components, of which aza A is the main active ingredient, applied in pelleted form to the soil, can be released and subsequently taken up by plant in an amount that acts as an antifeedant and growth

retardant five days after when first applied thus NAT was persistent in soil and resulted in 100% mortality to the caterpillar. The effect of NAT, of which aza A is the main active ingredient, on *P. brassicae* is confirmed in an earlier findings of Pierid pests crucifers (*P. rapae* and *P. brassicae*) susceptibility to neem formulations. Chiu (1989) reported single foliar application NSKE protected cabbage from *P. rapae* for 21 days. This ensured that young cabbage plants could be protected from *P. brassicae* larvae for at least 3 weeks. Some authors have noted phytotoxicity at higher concentrations (Nisbet, 1992; Karelina *et al*., 1992). It's important to note that cabbage white larvae fed on the treated cabbage seedlings for the first 12 hours caused some damage to the leaves, but plants recovered from this, and it did not affect their growth.

In field conditions, the severe weather conditions of continuous rain and low temperature made adult cabbage white inactive. No adults or caterpillars of *P. brassicae* were observed in the field area.

5.4.3 Aphids.

The clip cage has long been used to by entomologists and experimental ecologists to study plant-insect interactions, and to measure biological parameters such as development fecundity, fertility and mortality rates. It's a way to secure individual insects to specific plant leaf part (Crafts-Brandner et al., 1999; Muñiz and Nombela 2001; Moore *el al.*, 2003). It's important to make sure that the clip cage does not affect the insects', (here aphids'), life span and reproductive behavior (Muñiz and Nombela, 2001), and cages must be designed according to the size of individual insect pest under investigation.

However, there are some reports showing that the cage has confounding effect on the experimental results. Crafts-Brandner *et al*., 1999 reported that insect clip cages caused physiological effects, such as up-regulation of chlorophyll content of the leaf tissues due to the shading effect of the cages that caused the selected areas of sample leaf to show symptoms of senescence. In another finding, Moore *el al*., 2003, found that the mechanical pressure produced long-lasting effects on leaf growth. This suggests, when observing insects behaviour such as fecundity under different treatments only same type of clip cages has to be comparatively used in all treatments. No such effects of the cages on leaf growth or appearance were observed in the work reported here.

The systemic effect of azadirachtin on survival and reproduction behaviour of aphid was investigated, and in confirmation of previous reports, found to be effective in preventing infestation. Nisbet *et al*., (1993) found that azadirachtin was taken up systemically by the tobacco plant, and caused inhibition of feeding of *M. persicae*. They also found the confinement of the aphid in cage or tethered has little or no effect on the feeding behaviour of the insect, and found that initially insects imbibe phloem from treated plants, to produce a primary or secondary antifeedant effect (as explained on 1.6.1-C on page 33). Pavela and Barnet (2005) also found aza A in a concentration of from 0.5 to 5mg aza AL^{-1}, when applied systemically in soil can reduce the population of *Bevicoryne brassicae* up to 70%, and the effect was dose-dependent. The same result was found in the greenhouse experiment reported here, as aphids on treated plant leaves (upper or lower) failed to reproduce, and the majority of individuals were either dead or in dying at concentration of $5x10^{-5}$ M (9 mg aza Akg^{-1}) and above. Islam (2005) also reported, in an experiment that a fortmulation of NAT applied into soil systemically, led to high

mortality and significant population reduction of *A. fabae.* He also found the effect on the mortality rate of *A. fabae* was on younger nymphs more than older ones. The same is true in the work reported here, even though, during the selection period, aphids were not discriminated according to age. At the concentration of 1.9mg aza Akg^{-1} of soil, there was no effect found on the aphid fecundity and the rate of reproduction of *M. persicae* was almost same as that of untreated controls. Therefore, this suggests the application of concentration of active ingredient (aza A) that is 10^{-5} or below is not sufficient to control the aphid.

The bad weather in the field experiments meant that there was no great infestation of aphids. Numbers observed were low. Nevertheless, there was an indication that adult aphids found in the untreated plots were significantly higher that treated ones for the last three weeks of the experiment. So, despite the low numbers, the field results tend to confirm the greenhouse experiments and the results of previous authors. There are certain things that need more work in order to achieve better level of protection. As this work has been only one trial, more work is to be done in field situation due to lots of rain experienced during field trial period neem leached away from the granules. Also, different formulations of the granules and different rates of application must be tested in the field situation.

5.4.4 Flea beetle.

A systemic effect of NAT was also shown in the level of flea beetle damage assessment on the treated cabbage plants. The damage due to *P. nemorum* L. was indiscriminate for the first 2 weeks, but the beetle, like aphids, then avoided the leaves of treated plants.

Also, due to the fewer infestations of flea beetle and aphids treated cabbage plants grew better than untreated ones only in the last two weeks.

In deciding the rate of application of the pellets to soil in this Chapter, sufficient was added to give a purely nominal concentration in the soil water of between 10^{-5} M (1.9mgkg^{-1} soil) and 10^{-4} M aza A (19mgkg^{-1} soil). In fact the a.i. is slowly released and the actual concentrations measured in Chapter 3 were much lower, at their maximum about a factor of 10 lower. The concentration measured in the leaves was lower still, again by a factor of about 10. This means that the concentration of aza A in the leaves of plants growing in soil treated with the pellets is likely to be about 100 fold lower than might be expected, i.e. about 10^{-6} M. This still seems to give protection against insect attack.

In conclusion, aza A release from the pellet matrix into the soil and its subsequent plant uptake has show a promising insecticidal effect in controlled glass-house and in field situations. The result in the field experiment would have much clearer under good weather conditions. This paves the way for a much larger scale of pelleted version of neem- based plant protection products applications.

5.4 Push-pull strategy.

The work presented here has confirmed that aza A as formulated in NeemAzal®-T has antifeedant, repellent and anti-ovposition qualities on pest insects. This would make it a good candidate for use in "push-pull" strategies of insect control. Push-pull is a way of manipulating insect pests through repellant/ attractive stimuli simultaneously to minimize their infestation on the target crops or animals (Blackwell *et al*., 2004). Certainly azadirachtin has been suggested as a push component in such a strategy (Duraimurugan *et al*., 2005; Liu TX and Liu SS. 2006; Nisbet *et al*., 1992). Neem formulations are the most widely studied as an ovipositional deterrent among plant-derived products to reduce/prevent egg laying of species that cause damage through that or their maggots/larvae are pestiferous such as CRF, *Delia radicum* L., thus possible/important in push-pull strategy (Cook, *et al*., 2007).

Chapter 6

6.1 Final Discussion.

The work reported in this thesis was aimed at a commercial goal, and was intended to be part of a long-term project to develop a marketable product for plant protection in the UK and elsewhere. At the moment no neem plant protection product has been registered in the UK, but the company NeemCo., who have sponsored this work, intend to register neem-seed kernel extract in this country by the end of 2010. This project was part of the preparation for that registration, allowing experience and collaborations to be built up. Both of the other companies involved: Trifolio GmbH who supplied the NSKE, and Germains Technology Group, who did the pelleting of the NSKE have been very generous, and both will be involved in the long-term if the pelleted product is marketed in the UK.

Although no neem plant protection product has yet been registered in the UK, such extracts are available in many other parts of the world. The plant protection products are extracted from the seed kernels and are of two types: either based on the oil which makes up 40% of the weight of the seeds, or on polar solvent extracts which are enriched in the limonoid aza A. This is the active ingredient and is present in only low concentration in the oil, but makes up 20-50% in the latter extracts. (The preliminary analyses of various enriched extracts reported in Chapter 2 confirmed these figures, and showed that the commercial products have a range of proportions of the a.i.). The NSKE used most throughout Europe is that produced by the German company Trifolio-M GmbH and, as stated throughout the thesis, is called NeemAzal®-T (NAT). Analysis showed that it

contained more than 40% w/w aza A. This was used throughout the reported work, as it is this extract that will be registered in the UK, and will be used in any PPP sold here.

It is properties of aza A that decide the effectiveness of any PPP based on neem, and these have been investigated in this project. The analytical and purification work in Chapter 2 showed that it is not very easy to purify, although the development of reverse-phase flash and high performance chromatography make it easier than was the case when only conventional silica chromatography was available, as the polarity of terpenoid means that it comes quickly off a reverse-phase column. A high level of purity was obtained with an efficiency of 0.002%. It is unlikely that azadirachtin A will ever be used as a PPP except in a crude mixture. Another route to obtaining pure aza A is by synthesis, but as this involves about 70 steps, this can never be economical. Large companies do not like pesticides from natural sources, and so it may be that NSKE will remain a niche product produced by small companies.

One of the aims of this project was to obtain quantitative information about aza A at all stages in soil and leaves etc. Analysis of aza A is really only possible by reverse phase HPLC and this was used throughout the work, employing the recently accepted method by CIPAC, which worked well. No previous reports have been measured concentrations of the a.i. in soil and leaf water, so as to get a picture of the release and uptake of the compound.

One of the most important characteristics of aza A is that it is non-toxic to vertebrates and "beneficials" (organisms used for biologogical control of pests) so is generally thought of as being "good for the environment".

There is no doubt of the lack of toxicity towards mammals. Various neem PPPs have

been registered in the USA and many of the countries of the EU for almost 20 years without any adverse effects being reported. In the course of the rigorous registration procedures, the acual and chronic effects on living mammals was so slight as to place azadirachtin in the least toxic category (IV) of pesticides.

This *in vivo* work at the whole animal level has been more recently corroborated with *in vitro* work with cultured cells. Salehzadeh et al (2003) used mammalian cell lines derived from liver, lung and kidney and found that cell division was inhibited only when the concentration of aza A exceeded 10^{-4} M. A study using human glioma cell lines (Akudugu et al., 2001) found that cell replication was inhibited at $3\text{-}5x10^{-5}$ M. These findings contrast sharply with the observation that various insect-derived cell lines are inhibited at concentrations of aza A below 10^{-9} M (Salehzadeh et al, 2003). There seems no doubt that the large difference in sensitive to aza A between mammals and insects, lies at the cellular level.

There are no toxicity test information of commercially used neem materials on birds, but when 10% NSKE were incorporated with Japanese quail bird feed over 20 weeks, egg laying rate and its quality were not significantly affected (Elangovan *et al*, 2000). When Margosan-O was approved in USA as pesticide for food crops single oral dose of 16ml/kg body weight to mallard duck did not cause inducement of any adverse effect (Schmutterer , 2002). Also, when a feed containing 1000 to 7000mg/kg of Margosan-O of the diet to the same bird for 5 days has not shown any adverse effect (Johnson, 1996a, b).

As for the fish LC_{50} in rainbow trout was determined and 160mg/L of NAT/S for 96hrs, was considered low acute toxicity (Grunert. 1996). Although neem based formulations

have greater margin of safety to fish (young salmon), its toxicity is mainly due to carriers or emulsifiers (Wan *et al*, 1996).

In September, 2009, the EU Directive 91/414/EEC has become a Regulation (so far without an identifying number). In the Introduction, the Regulation states that *"substances should only be included in plant protection products where it has been demonstrated that they present a clear benefit for plant production, and they are not expected to have any harmful effect on human and animal health, or any unacceptable effects on the environment."* Aza A should fit these criteria very well.

One stated aim in the new Regulation is that as much as possible biological control and IPM should replace chemical control. Use of aza A in IPM depends on it having minimal toxicity towards beneficials. The results of semi-field and field trials have shown that neem products in which aza A is the a.i exhibit minimal side effect on non-targets including predatory insects and nematodes (Schmutterer, 1990). Saxena *et al*., (1981) showed that hymenopterous prasitoids are less sentitive to neem products than preditors. He also suggested azadirachtin application favours towards the parasitoid as it inhibits pupation in caterpillars. There is some evidence that oil formulations of neem product show stronger side effect to nontargets (Schmutterer, 2002) but this will not be relevant to semi-purified products such as NeemAzal®-T which contain high proportions of aza A.

In light of the many reports of the plight of the honey bee, and the suspicion that the systemic neonicotinoid Imidacloprid may be involved in the problem, it is important that any future systemic PPP be non-toxic to bees. Larson (1989); Leyman *et al*., (2000) Schmuterer and Holts, (1987) reported azadirachtin doesn't negatively effect the workers

of honey bee, *A. mellifera*, but repetitive applications of neem formulations may effect the nectar thus indirectly influence the honey they produce or cause some problem to the brood. This is an area which may require further study.

Overall, it can be concluded that neem formulations are suitable for use in IPM schemes, as their effect on non-targets and beneficials are minimal.

However, as indicated in Chapter 3 there is evidence that aza A is slightly phytotoxic. When it was shown that one of the targets of the limonoid is likely to be tubulin, which means that it acts as an antimitotic, it might mean that it affected plant tubulin also. The results reported in that chapter proved that it did have an antimitotic effect on newly germinated plants, although there is no proof that it is due to an effect on tubulin. The effect was only shown at a high concentration of aza A, $> 5X10^{-4}$, so in practice it may not be important. It might prevent the use of seed treatment with the NSKE, however. On the other hand, there is some evidence in Chapter 5 that over the growing season, treated plants grew slightly better than controls. Maybe lack of insect predation is more important than phytotoxicity. Imidacloprid, a neonicotinoid, also used systemically, inhibits germination and growth, but is widely used, presumably because its insecticidal effect outweighs this disadvantage.

As far as this project is concerned, the most important characteristic of aza A is its solubility in water. This allows it to move easily in soil water, and enter plants systemically. Very few insecticides are able to work in this way. The threat to pollution of ground-water is prevented by the short half-life in the soil. The trouble with this is that if the limonoid is just drenched onto soil, it will not have long to kill soil pests and enter plants. It was hoped that pellets might give a slow-release mechanism to give longer

effect. This was proved to be true. Although the work with the “hydrophobic” pellets was not completed, it was clear that the release of the terpenoid into the soil could be delayed by the composition of the pellets. Any product might have different types of pellet to give protection over the whole growing season, which would be an improvement on repeated spraying. Further improvement of stability of formulations in soil enviroments is necessary in order optimise the delivery of the a.i.. This could be achieved by modifying the formulations, such as product that contains high a.i, and granular carrier polymer to meet required performance such as optimum release rate and profile.

Although the terrible weather in the summer of 2008 meant that the results from the field trial were limited, the application of pellets to soil clearly gave protection to the cabbages from the pests. In the greenhouse trials, the NSKE was very successful against the CRF. This is the first time that such results have been reported against this soil-pest. It demonstrated that the pellet method can protect against both soil and foliar pests, and should be true of other soil pests also such as vine weevil.

As discussed earlier in the thesis, the main use of polar NSKE as PPP is by spraying an emulsifiable formulation of NAT in sesame oil, called NeemAzal-T/S. The work here and from other sources suggests that this may not be the most effective way to use the a.i. Added to the soil, it has the potential to protect the plant against soil-borne pests, as well as foliar. There are various ways of introducing the terpenoid to the soil, such as simple drenching, drip-irrigation etc. but the advantage of using pellets is that the presence of the active ingredient in the soil can be prolonged. The results here showed that with the hydrophobic pellets, the a.i. in soil peaked after 13 days, and was still 30% of the theoretical maximum concentration at this time. The field experiments, although limited

due to weather, indicated that azadirachtin A was still present in cabbage after 5 weeks.

Another possible systemic use of NSKE is in hydroponic cultivation, which is becoming very important for producing high-value crops such as peppers for the UK and other markets. The great advantage of hydroponics is that the concentration of a.i. can be determined exactly, as was done here, and monitored regularly.

At the moment, aza A is in the process of assessment for inclusion in Annex 1, which will allow its use for the foreseeable future. Assuming that it is included in the list of acceptable a.i. then it is likely to have a market in the UK similar to that in the countries where it is registered. The obvious market is the organic market. Currently this is in recession, but is likely to revive in the next few years.

6.2 Ideas for further research.

1. One obvious potential application of the pelleting technology is to use NeemAzal©-T in seed pellets to give the young plant immediate protection from insect attack. The problem with this is that the work in Chapter 3 indicated that the a.i. is phytotoxic at a high concentration. It is possible that there is a level of aza A which might be non-phytotoxic while still giving some protection to the plant. This is a development which might be further explored.
2. While the work in Chapter 3 suggested that aza A might have its antimitotic affect by preventing tubulin polymerisation, this can only be proven by studies at the molecular level, using, for instance, the methods employed by Salehzadeh *et al*. (2003). This work would help to prove that the main action of aza A is against tubulin, which is present in all eucaryotic cells. It would be of interest to find why

it is less effective than anti-mitotic herbicides, and if they bind at the same sites on the tubulin molecules.

3. There are obvious areas of study which would examine the possible role of aza A in specific IPM, and push-pull strategies in particular.
4. While aza A is generally thought not to affect nectar-gathering insects, including bees, this may not be true if the a.i. were to be employed as a systemic insecticide. This would be a very important study.
5. The field work using different sorts of pellet was only of limited success, due to the weather. Clearly this would be an area which could be repeated and extended in the future.

Appendix A. Summary of the concentrations of azadirachtin A applied in hydroponic media, and soil, and measured in soil and leaf water in the experiments reported in this project.

Type of Exp.	Formulation of Aza A	Aza A added to soil (mgkg-1 soil)	Pellets added to soil (mgkg-1 soil)	Theoretical max. conc.of Aza A in soil/leaf water	Actual max. conc. of Aza A in soil water Or hydroponic medium	b/a (%)	Actual max. Conc. in leaf water	c/a(%) or c/b*(%)
Hydro-	NeemAzal®-T	n.a.	n.a.	n.a.	$1.0x10^{-4}$		$1.0±1x10^{-5}$	10*
ponic	(powder)				$1.0x10^{-3}$		$1.0±1x10^{-4}$	10*
Pot	NeemAzal®-T	19.0	n.a.	$1x10^{-4}$	$8.0±1.0x10^{-5}$	80	n.a.	
	(powder)							
	Hydrophilic		130	$1x10^{-4}$	$4.2±1.0x10^{-5}$	42	n.a.	
	Pellets							
	("Neem 1")							
	Hydrophobic	19.0	130	$1x10^{-4}$	$3.0± 1.0x10^{-5}$	30	n.a.	
	Pellets							
	("Neem 2")							
	"Neem 1"	1.9	13	$1x10^{-5}$	n.a.		n.a.	
	Pellets	9.5	65	$5x10^{-5}$	n.a.		n.a.	
		19.0	130	$1x10^{-4}$	n.a.		$4.0±2x10^{-6}$	4
		190.0	650	$1x10^{-3}$	n.a.		$6.5±2x10^{-5}$	7
Field	"Neem 1"	**($g.m^2$)**	**($g.m^2$)**					
	Pellets	1.5	10	approx.$1x10^{-4}$	n.a.		$3.1±3x10^{-6}$	3

The concentrations of the a.i. azadirachtin A used in the work reported in Chapters 4 and 5 were based on the findings about the phytotoxicity of the limonoid in Chapter 3. These suggested that at 10-3M azadirachtin severely affected the growth of newly germinated cabbages and sugar beet.

Consequently, the concentrations used after that were almost all aimed to produce a lower concentration in the soil water. The exception to that was a hydroponic experiment when a concentration of 10^{-3}M was used to see if this concentration also affected plants at a later stage of growth. In soil-water most of the experiments aimed to produce a maximum theoretical concentration of 10^{-4}.

The calculations of maximum theoretical concentrations in soil water were based on the Know amount of aza A in the formulations used, and the known amounts of water in the Soil in pot experiments. When the pellets were used, this theoretical maximum could not be ac achieved, due to slow release and biological breakdown of the a.i. In fact, when the crude NSKE was simply added to the soil, and aza A measured after 24 hours, the concentration found was 80% of the possible, almost what might be expected with a rapid solution in the soil water and a half-life of 1.6 days.

Unsurprisingly, the two pelleted formulations resulted in lower maximum soil concentrations although these were still quite high at 42% and 30%.
The concentrations of the a.i. in the leaf-water are consistently only about a tenth or less of the concentration around the roots, and this is true for both hydroponic experiments and pot experiments.
In the experiments with insects the quantities of pellets added to the soil in pots and field were intended to produce a maximum concentration in the soil of 10^{-4}M or less. In fact, the soil concentration will have been at least a factor or two lower than the calculated maximum. When the concentration in the leaves was measured, there was good consistency between the field and pot experiments.

Appendix B **FIELD DATA SHEET** Title:- Week No:-	Date / /

No	**Green peach**			**Cabbage aphid**			**Cabbage white**			**Cabbage root fly**			**Flee beetle**
A	**Nymph**	**Adult**	**Winged**	**Nymph**	**Adult**	**Winged**	**Egg**	**Larvae**	**Adult**	**Egg**	**Larvae**	**Adult**	**1,2,3,4**
1													
2													
3													
4													
5													
6													
7													
8													
9													
10													

No	**Diamond back moth**			**Other insects**		**Slugs**	**Damage of leaves (extent)**				**Growth (No. leaves)**		
	Egg	Larvae	Adult			**1,2,3,4**							
1													
2													
3													
4													
5													
6													
7													
8													
9													
10													

Comments:-	Signature:- .

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