The Effects of Azadirachtin on the Feeding Behaviour and Virus Transmission of the Green Peach Aphid, *Myzus persicae* (Sulzer)

by

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Abstract

Azadirachtin was isolated from neem seeds, by flash column chromatography, with a purity of >95%. Yields from Ghanaian seeds were higher than those from Pakistani seeds (0.04% w/w and <0.0015% w/w respectively). An unsuccessful attempt was made to produce ^{14}C-labelled azadirachtin for use in systemic movement studies. Qualitative and preliminary quantitative chromatographic analyses of the leaf extracts of tobacco seedlings (*Nicotiana clevelandii*) whose roots had been immersed in azadirachtin solutions showed systemic movement of the compound from the roots to the aerial parts.

The electrical penetration graph (EPG) method was used to analyse the feeding behaviour of apterous, adult *Myzus persicae* (Homoptera : Aphididae) on *N. clevelandii* seedlings, treated systemically with azadirachtin. The percentage of the 9h recording period devoted to non-penetration activities and to stylet pathway patterns, the number of probes initiated and the numbers of sieve tube penetrations all increased with increasing azadirachtin concentration. Azadirachtin treatment significantly reduced the percentage of probes that reached sieve elements and increased non-penetration activity before and after the first period of ingestion from the sieve elements. The percentage of the recording period spent in the EPG pattern associated with sieve tube penetration was significantly reduced by an azadirachtin concentration of 300ppm, and the duration of each individual sieve tube penetration was significantly reduced by an azadirachtin concentration of 100ppm.

A novel quantitative method, based on honeydew production, was used to measure aphid feeding on artificial liquid diets containing various concentrations of azadirachtin. During a 52h period on diets containing azadirachtin at concentrations of 100 and 300ppm, adult apterous *M. persicae* produced 4-5 times less honeydew than those feeding on control diets and aphids on diets containing 500 and 1000ppm azadirachtin produced no more honeydew than aphids which were starved for the same period. During this period the rate of nymph production by aphids on all treated diets fell to less than half that of aphids on control diet. When the aphids were subsequently transferred to untreated diets for 44h, a large proportion of the nymphs produced by aphids which had been on diets treated with 100-500ppm azadirachtin were born dead with undeveloped. appendages.

After 26h on diets containing azadirachtin at lower concentrations (25-100ppm), the rate of nymph production by adult apterous *M. persicae* on treated diets fell to less than half that of aphids on control diets. After 50h on the treated diets nymph production had virtually ceased and all nymphs produced by adults that had fed on azadirachtin-treated diets were dead. During the first 26h period, honeydew production was unaffected on azadirachtin-treated diets but was approximately 3 times less than on control diets during the subsequent 24h period.

Adult apterous *M. persicae* acquired PLRV and PVY less readily from infected *N. clevelandii* seedlings whose roots were immersed in 500ppm azadirachtin solutions than from seedlings with their roots immersed in control solution. However, this treatment did not cause any difference in the number of azadirachtin-treated or untreated seedlings which became infected with either virus when exposed to viruliferous aphids.

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Declaration

I hereby declare that the thesis which follows is my own composition, that it is a record of work done by myself and that it has not been presented in any previous application for a Higher Degree.

Alasdair J. Nisbet

Chapter 1. Introduction

1.1 Aphids as crop pests

Infestation of crops by aphids (Homoptera: Aphididae) can result in heavy losses by:

a) direct damage to the plants;

b) the transfer of plant viruses during feeding;

c) the injection of toxic material in saliva;

d) reducing photosynthetic activity; and,

e) providing a medium for fungal growth on their excreta (Schepers, 1989).

The peach-potato aphid, *Myzus persicae* (Sulzer), is a prolific vector of plant viruses in the temperate zone, though it rarely reaches high enough populations on single plants to cause direct damage through feeding. The efficiency of *M. persicae* as a virus vector is related to: a) its ability to overwinter as the apterous, virginoparous form and build up large populations early in the growing season, and b) the tendency of the aphids to disperse from their host plant even at low population densities (Hodgson, 1991).

The viruses transmitted by aphids have been classified into three categories based on their retention periods in the vector ():

a) non-persistently transmitted, e.g. potato virus Y (PVY): These can be transmitted to healthy plants shortly after the insect has fed on an infected plant. These viruses need only a short acquisition probe, usually less than one

minute, to render the insect infective and only a short probe to be transmitted to another plant. Aphids do not require to feed in the phloem sap to acquire these viruses and the ability to infect further plants is short lived.

b) semi-persistently transmitted, e.g. beet yellows virus (BYV): These are acquired after several minutes of feeding and the vectors may remain infective for some days, but not through a moult.

c) persistently transmitted e.g. potato leafroll virus (PLRV) and barley yellow dwarf virus (BYDV) : These viruses cannot be acquired from an infected plant by brief probes but only after periods of feeding, usually from the phloem, lasting 24-48 h. Once virus is acquired the insect may remain infective for several days. The persistently transmitted viruses are mostly phloem borne.

1.2 Control of *Myzus persicae* and the spread of aphid-transmitted potato viruses

The increase in the use of insecticides in Scottish seed potato production is typical of the current methods used to control virus spread by *M. persicae*. Until the mid-1970's the use of insecticides in the production of Scottish seed potato was virtually unknown (Woodford *et al.*, 1977; Turl, 1981). In 1974 there was no recorded use of a granular insecticide treatment in the seed potato crop and only 6% of the entire potato crop was treated with organophosphorous (OP) insecticide sprays (Chapman *et al.*, 1977). By 1977, after epidemics of PLRV in the Scottish seed potato crop,

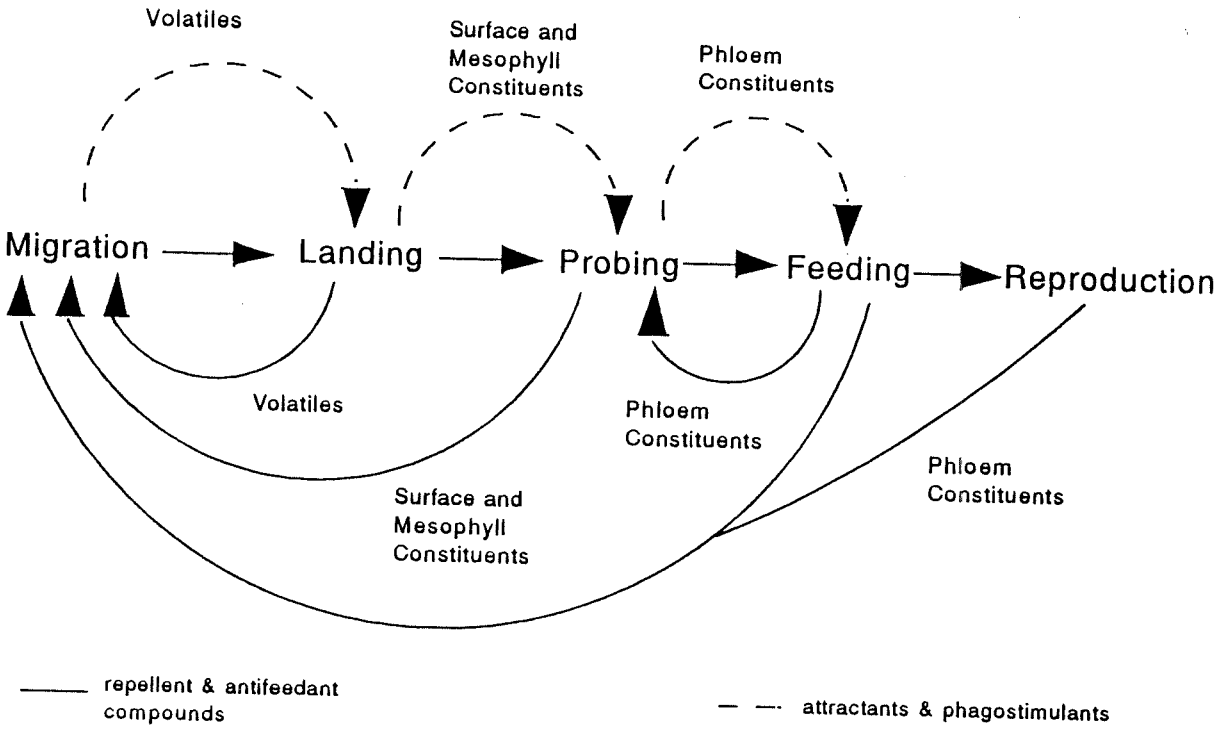
36% of the seed crop was treated with granular OP's at sowing and 80% of this crop was sprayed with OP or carbamate insecticide (Steed *et al.*, 1979). The use of multiple sprays of insecticide to control *M. persicae* has led to a rapid selection for resistant variants (Foster, 1986; ffrench-Constant *et al.*, 1987) and in 1976 the presence of OP and carbamate resistant field populations were confirmed in west and central Scotland (Devonshire *et al.*, 1977). While OP and carbamate aphicides may restrict spread of semi-persistently transmitted and persistently transmitted viruses from sources within a crop (secondary spread), they seldom kill aphids fast enough to control secondary spread of non-persistently transmitted viruses, or the introduction of persistently transmitted and non-persistently transmitted viruses to the crop from outside sources (primary spread) (Hille Ris Lambers, 1980). The potential of synthetic pyrethroid insecticides to control the acquisition and transmission of non-persistently and semi-persistently transmitted viruses through fast knockdown and by prolonged incapacitation (Gibson *et al.*, 1982a,b), as well as repellant properties, was short lived because of cross resistance in the aphid population. Rice *et al.* (1983) found that apterae of the more resistant of the common *M. persicae* variants, (R_2), were not so readily repelled from deltamethrin-treated potato leaves as susceptible (S) and less resistant (R_1), variants. Whereas deltamethrin treatment reduced transmission of PVY by both S and R_1 variants, there was no significant difference in PVY transmission by R_2 apterae from plants treated with water

compared with plants treated with deltamethrin. Pyrethroids and pyrethroid/ OP mixtures provide poor control of *M. persicae* in the field and select for resistance more rapidly than either OP or carbamate insecticides (ffrench-Constant *et al.*, 1987, Dewar *et al.*, 1992). There are now seven different levels of resistance, based on E4-esterase levels, in *M. persicae* populations with each successive variant displaying approximately double the titre of the enzyme responsible for resistance. Within each variant, resistance is least to carbamates, greater to organophosphates and greatest to pyrethroids (Devonshire, 1989). Currently, pirimicarb and demeton-S-methyl are the most widely used aphicides to control *M. persicae*. However recent studies have shown that demeton-S-methyl applied to sugar beet crops can rapidly select for resistance, giving poor control (Dewar *et al.*, 1992). There is therefore an urgent requirement for new methods of controlling the aphid vectors of plant viruses.

1.3 Plant-derived behaviour-altering chemicals as a means of virus vector control.

The use of non-insecticidal behaviour-altering chemicals (semiochemicals) to interfere with host selection and the feeding behaviour of aphids has received particular attention in the past decade. Host plant selection by aphids involves a sequence of behavioural events which are influenced to some extent by chemical cues as shown in Figure 1 (Niemeyer, 1991). Chemicals on the plant surface,

Figure 1. Host selection by aphids: Influence of plant chemistry (after Niemeyer, 1991).



e.g. components of the epicuticular layer or trichomes, are perceived by antennal chemoreceptors (Bromley *et al.*, 1979) and by receptors on the tibiae and tarsi (Anderson & Bromley, 1987). Probing involves penetration of the cuticle and the epidermis of the leaf by the stylets (Klingauf, 1987a). Probing is thought to involve tasting of the plant's internal fluids by the gustatory papillae of the epipharyngeal organ which is positioned on the dorsal side of the pharyngeal duct within the aphid's head at the anterior end of the food canal (Ponsen, 1987).

If the aphid receives positive stimulation from the probing behaviour it may then initiate a deeper probe into the leaf and imbibe the vascular fluids, usually the phloem sap. The host selection procedure is therefore a sequence of events which may be influenced at each stage by a balance between positive and negative stimuli. At each step the selection procedure may be negatively influenced by repellant or antifeedant compounds. A **repellant** compound causes insects to make a directed movement away from its source (Dethier *et al.*, 1960) and may act before feeding is attempted. An **antifeedant** compound prevents feeding on the substrate upon which it is present (Munakata, 1975) and acts when feeding is attempted. An antifeedant may cause the insect to die of starvation if it remains on the substrate attempting to feed or it may cause the insect to move away from that food source. When these compounds are present the overall palatability of a food source is determined by the combination of positive external sensory inputs (e.g. from phagostimulants and arrestants), negative external sensory

inputs, (e.g. from repellants and antifeedants) and factors arising within the insect, (e.g. degree of satiation). Only when the overall input is positive can feeding begin and be sustained (Dethier, 1982).

Many plant-derived compounds from several chemical classes have been found to possess antifeedant and/or repellant activity against aphids. Schoonhoven & Derksen-Koppers (1976) incorporated twenty-four individual plant-derived compounds into artificial diets to test settling and survival of *M. persicae* in choice and no-choice bioassays respectively. None of the compounds that they tested enhanced diet acceptability in choice tests and in no-choice tests eight of the compounds had a strong negative effect on aphid survival equivalent to that found when aphids were starved during the same period. Rose *et al.* (1981) isolated a series of diterpenoid acids from members of the Compositae (*Chrysothamnus nauseosus* and *Grindelia humilis*), which prevented settling by greenbugs (*Schizaphis graminum*) when incorporated into artificial diet at low concentrations. The lipophilic amides capsaicin and piperine deterred aphid settling in a bioassay in which half of the surface of a leaf from a host plant was treated with the chemical, whilst the other half acted as the control (Griffiths *et al.*, 1989). Sesquiterpene compounds in the drimane series e.g. warburganal, derived from the east African tree *Warburgia ugandensis* and (-)-polygodial from the water pepper (*Polygonum hydropiper*) also deterred aphid settling in the bioassay described above. Polygodial treatment of virus-infected plants reduced the acquisition of PVY and BYV by *M.*

persicae in laboratory tests (Gibson *et al.*, 1982b), though the mechanism of this inhibition is not fully understood, as polygodial does not affect the initial settling behaviour of the aphid, other than to increase the durations of individual penetrations of the leaf surface (Powell *et al.*, 1992a). In field experiments, polygodial treatment of winter barley reduced the numbers of the cereal aphids *Rhopalosiphum padi* and *Sitobion avenae* infesting the crop and significantly reduced the incidence of BYDV within the crop, resulting in an improved yield compared with an untreated control plot. Application of extracts of *Ajuga remota* (Labiatae) which contain the clerodane compound ajugarin 1 also reduced the incidence of BYDV in winter barley in field experiments, but not as effectively as polygodial (Dawson *et al.*, 1986).

Extracts and compounds from the neem tree *Azadirachta indica* A. Juss (Meliaceae) have received particular attention from entomologists in the last thirty years. The neem tree is native to India and is now widespread in Africa and Asia. Extracts of neem seeds and leaves have been used for centuries in traditional Indian agriculture to protect crops and stored produce from insect attack (Saxena, 1989).

1.4 Azadirachtin

Recent interest in neem extracts as crop protectants dates back to the work of Pradhan *et al.* (1962) who reported that dilute aqueous seed extracts completely prevented feeding by the desert locust *Schistocerca gregaria*.

In 1968 the most active antifeedant principle was isolated from neem seeds and named azadirachtin (Butterworth & Morgan, 1968). Azadirachtin possesses a wide range of activities against insects including: deterrent, anti-ovipositional, antifeedant, growth retarding, insecticidal and fitness- and fecundity-reducing properties (Schmutterer, 1990). Recently azadirachtin has also been shown to depress the immune system of *Rhodnius prolixus* (De Azambuja *et al.*, 1991). The chemical, antifeedant, growth-disrupting and fecundity-reducing properties of azadirachtin are discussed below.

1.4.1 Chemical properties of azadirachtin

Azadirachtin is found at its highest concentrations in the seeds of the neem tree. Neem oil, which may comprise up to 40% of the seed weight (Jacobson, 1986), contains the less polar triterpenoids e.g. nimbin and salannin (Figure 2 a & b) and sulphur-containing compounds which give the oil an unpleasant garlicky odour. Although azadirachtin occurs at concentrations of 0.02-0.35% (w/w) in the seed (van Beek & de Groot, 1986), neem oil pressed from the seed (expeller grade oil) usually contains the material at relatively low but variable concentrations e.g. 0.005%-0.4% (w/vol) (Isman *et al.*, 1990).

All of the well-characterised compounds identified from neem are triterpenoids and all of the known triterpenoids are derived from the parent tetracyclic triterpenoid tirucallol (Fig. 2 c) (Jones *et al.*, 1988). Azadirachtin

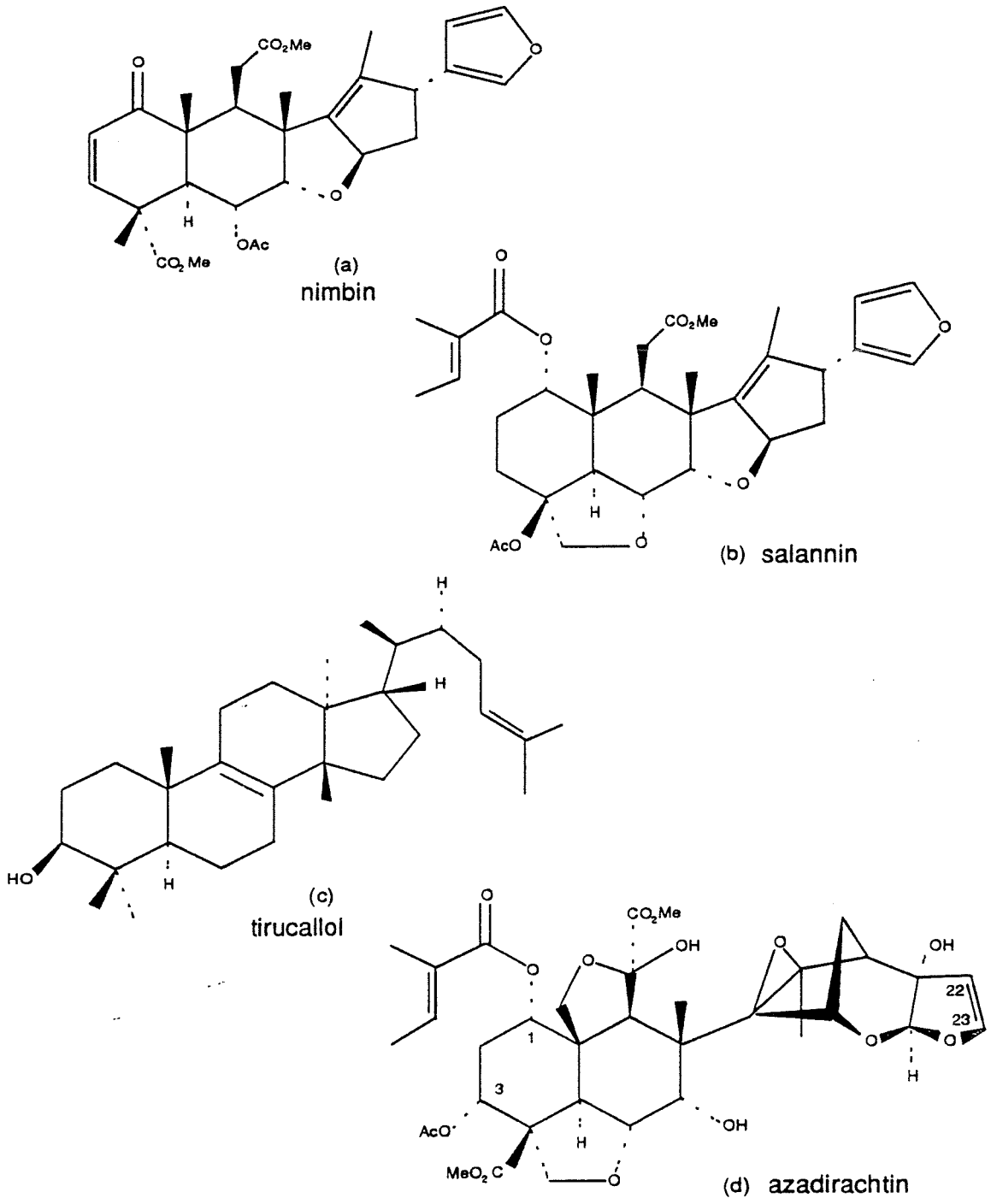


Figure 2. Limonoids from neem seeds and their precursor, tirucallol.

(Fig. 2 d) is a highly oxidised triterpenoid with molecular formula $C_{35}H_{44}O_{16}$ (molecular mass 720). It is freely soluble in polar organic solvents and sparingly soluble in water (Butterworth & Morgan, 1971). The correct structure of azadirachtin was elucidated only recently through X-ray crystallography of a detigloylated dihydro derivative of the molecule (Broughton *et al.*, 1986). Azadirachtin has only been obtained as a white microcrystalline powder with a melting point of 154-158°C. It has been suggested that the compound does not form discrete crystals because azadirachtin is a mixture of at least seven distinct isomers named azadirachtins A-G (Rembold, 1988). Azadirachtins A (Fig. 2 d) and B are the predominant isomers comprising more than 80% and 15% of the mixture respectively. Azadirachtin B differs from azadirachtin A by having a tigloyl group at position 3, a free 1-hydroxy group and reduction to the de-oxy moiety at position 11. Each of the remaining isomers comprise less than 0.2% of the mixture. The predominant azadirachtins do not differ markedly in their biological activity (Rembold, 1988). Azadirachtins H and I have recently been isolated from neem seeds in small yields (4.0% and 1.2% of the total azadirachtin content respectively) by Govindachari *et al.* (1992) but the biological activity of these molecules has not yet been described.

Azadirachtin is stable in neutral solution but decomposes rapidly in alkaline media (E.D. Morgan pers. comm., 1988). The compound is also susceptible to U.V. degradation and decomposes exponentially over time with a half life of 25h when exposed to constant U.V. irradiation. After 80%

decomposition the resulting complex mixture of compounds retained the original growth regulatory properties of azadirachtin (Barnby *et al.*, 1989).

1.4.2 Antifeedant properties of azadirachtin

Butterworth & Morgan (1971) demonstrated that azadirachtin completely inhibited feeding by *S. gregaria* when it was incorporated into sucrose-impregnated filter papers at concentrations as low as 0.04ppm despite the lack of a repellant effect. Since this original study azadirachtin has been shown to inhibit feeding in many species of insects from diverse orders. The reviews of Jacobson (1986) and Warthen (1989) cite reports of the antifeedant activity of neem extracts against seventy-one species of insects from seven orders.

It has been proposed that two types of antifeedant activity evoked by azadirachtin should be distinguished: Primary (gustatory) antifeedant activity, represented by a regulation of food intake resulting from contact between the antifeedant and the sensory organs of the mouthparts; and Secondary (non-gustatory) antifeedant activity observed after ingestion, application or injection of the antifeedant, resulting from the disturbance of hormonal and/or other physiological systems (see section 1.4.3) (Schmutterer, 1985).

The mechanism of action of the primary antifeedant response has been investigated in Lepidopteran larvae and in locusts. Perception of the antifeedant at the sensory level

may involve different mechanisms. Azadirachtin may stimulate specific deterrent receptors as in larval *Pieris brassicae* (Schoonhoven, 1982) or the molecule may modify the activity of receptors which are specific for other compounds e.g. phagostimulants. The medial sensilla styloconica on the maxillae of the oligophagous larvae of *Spodoptera exempta* and *Mamestra brassicae* have distinct receptors which are responsive to azadirachtin or to sucrose, but there is no peripheral interaction between the receptors. In several polyphagous species, e.g. *Spodoptera littoralis*, interaction occurs between these receptors resulting in a modified neural input from the sucrose receptor if the azadirachtin-sensitive receptor is stimulated (Simmonds & Blaney, 1984).

Gill & Lewis (1971) demonstrated the systemic movement of azadirachtin from the roots of bean plants by showing that desert locusts were deterred from feeding on the aerial parts of plants growing in treated compost. Translocation of azadirachtin from sprayed bean plant foliage to untreated foliage has also been reported, apparently via the phloem sap (Radwanski, 1977). This finding was confirmed (Larew, 1988), by the demonstration of the movement of a "neem seed extract toxin" from a sprayed bean leaf to an unsprayed leaf on the opposite side of the plant. This movement was also shown to be associated with a significant dilution of the active principle. Systemic movement in the plant is important for two reasons: Firstly, unless the antifeedant effect can be expressed in new foliage and growth, the new growth will be selectively attacked by the insect while the treated parts are avoided and secondly, systemic uptake may

reduce the exposure of the chemical to environmental factors such as ultraviolet radiation, thus prolonging the active life of the compound.

1.4.3 Growth-disrupting effects of azadirachtin

Ruscoe (1972) first described the growth-disrupting effects of azadirachtin in *P. brassicae* larvae feeding on cabbage leaves treated with the compound at concentrations which were not antifeedant. Final instar larvae which had fed on leaves treated with 50ppm azadirachtin were unable to complete their pupal moult. When the larvae had fed on leaves treated with 12.5ppm azadirachtin some were able to complete the pupal moult but the pupae showed malformations such as larval heads and distended wing pads. These pupae died prior to the imaginal moult. According to the reviews of Jacobson (1986) and Warthen (1989) the growth-disrupting effects of neem seed extracts have been reported for forty-one species of insects covering almost every economically important order. Lepidopteran insects appear to be the most susceptible to the growth-disrupting effects and comprise nearly 40% of the species for which these effects have been described.

The growth-disrupting effects of azadirachtin on larvae of the Mexican bean beetle *Epilachna varivestis* are typical of many other species and are as follows (Rembold, 1988) : When larvae feed on treated substrates they may remain in the larval state without progressing to the pupal moult. Those

larvae which reach the pre-pupal stage may die from desiccation. If metamorphosis to a normal pupa occurs, the resulting adult may be unable to shed the pupal integument. If the adult emerges it usually has deformed wings or mouthparts and dies within a few hours.

The mechanism of action of azadirachtin on the metamorphic processes of insects is only partially understood. Koul *et al.* (1987) demonstrated that injection of azadirachtin into final instar *Bombyx mori* larvae caused severe developmental defects but azadirachtin did not affect the production of ecdysone by prothoracic glands stimulated with prothoracicotropic hormone (PTTH) *in vitro*. This finding was supported by further studies using different insect species (e.g. Barnby & Klocke, 1990) and it appears that azadirachtin has no direct effect on prothoracic gland function, nor does it interfere with the action of PTTH. It has been suggested that azadirachtin may disrupt the storage and secretion of PTTH or PTTH-like neurohormones (Barnby & Klocke, 1990).

The first description of the effects of azadirachtin on ecdysteroid titres came from Sieber & Rembold (1983) when they demonstrated that injection of the compound into *Locusta migratoria* larvae produced a marked delay in the ecdysteroid peak associated with moulting. They also demonstrated that, although the overall ecdysteroid titre in azadirachtin-treated larvae was not reduced, the contribution of 20-hydroxyecdysone (20-HE) to the peak was diminished. Marco *et al.* (1990) have shown that the peak ecdysteroid level in *Tenebrio molitor* pupae injected with

azadirachtin was reduced by more than 50%. This reduction was also attributed to diminished 20-HE levels rather than ecdysone levels which were similar to those found in untreated pupae.

In the blue blowfly *Calliphora vicina* injection of mature larvae with azadirachtin produced a slight delay in the ecdysteroid peak associated with the early pupal stage and the mid-pupal peak was diminished and delayed for several days. Azadirachtin did not inhibit the synthesis of ecdysone but the release of the hormone from the ring gland was prevented. Furthermore azadirachtin could neutralise the effects of atropine and picrotoxin which stimulate the release of ecdysone from ring glands *in vitro*. Azadirachtin also extended the persistence of ecdysone by interfering with the hydroxylation of the hormone to 20-HE (Bidmon *et al.*, 1987).

Juvenile hormone (JH) synthesis is also affected by azadirachtin. Malczewska *et al.* (1988) demonstrated that treatment of final instar *Galleria mellonella* larvae with azadirachtin caused a reduction in cold-induced elevation of JH levels, preventing the super-numerary moults observed in untreated larvae.

Inhibition of ecdysis in azadirachtin-treated insects is therefore probably caused by interference with the hormonal control of moulting which may also result in lowered weight gain through a secondary antifeedant effect. For example Kraus *et al.* (1987) found that *E. varivestis* larvae which had fed on a diet containing azadirachtin, at concentrations which did not produce primary antifeedant effects, rapidly

reduced their feeding rate even after transfer to untreated diets resulting in mortality within one week. Schluter *et al.* (1985) observed that final instar *Manduca sexta* larvae experienced a dose-dependant reduction in weight gain after injection with azadirachtin. Larvae treated with the highest concentrations of azadirachtin moulted to supernumerary larvae, an effect which can also be produced by starvation. Similarly, Koul *et al.* (1987) found that final instar *B. mori* larvae injected with azadirachtin experienced a dose-dependant reduction in weight gain resulting in a failure to pupate in those larvae which did not reach a threshold weight of 2.5g. In *L. migratoria* azadirachtin reduced the frequency of gut contractions *in vitro* and slowed the passage of a meal along the alimentary tract when injected into fifth instar nymphs. These factors may be responsible for the reductions in food intake and weight gain observed in azadirachtin-treated locusts and it has also been suggested that the effects on gut motility may be partly responsible for the disruption in endocrine events (Mordue (Luntz) *et al.*, 1985).

1.4.4 Fecundity-reducing effects of azadirachtin

Inhibition of reproduction through azadirachtin treatment has been demonstrated for insects in most orders and is thought to be caused by hormonal imbalances in the adult. In the Heteroptera topical application of methanolic neem seed extracts to final instar *Dysdercus fasciatus* nymphs resulted in adult females which produced only 59% of the eggs

produced by untreated females (Ochse, 1981, cited by Schmutterer, 1990). Topical application of azadirachtin to *Oncopeltus fasciatus* females produced complete sterility (Dorn *et al.*, 1986). As in larval insects, adults may suffer weight loss or reduced weight gain after azadirachtin treatment. For example, injection of azadirachtin into female *L. migratoria* 2-13 days after the adult moult resulted in a 50% weight reduction compared to untreated females (Rembold, 1988).

The mechanism by which fecundity is affected has been studied in detail in *L. migratoria*. In this insect juvenile hormone III (JH III) controls the synthesis of vitellogenin by the fat body and so controls egg maturation in the ovary. In untreated females the titre of JH III increases around eight days after the imaginal moult, inducing synthesis of vitellogenin while in azadirachtin-treated females JH III synthesis is prevented and consequently vitellogenin synthesis and egg production are abolished (Rembold *et al.*, 1984). In untreated locusts ecdysteroids are produced by the ovaries and reach peak titres towards the end of vitellogenesis. Injection of azadirachtin after the end of oogenesis results in a depletion of ovarian ecdysteroids (Rembold, 1988). Overall the reduction in fecundity seen in azadirachtin treated *L. migratoria* females is thought to be the result of delayed peaks of JH III, ecdysteroid and vitellogenin.

1.5. Effects of Neem Extracts on Aphids

The reported effects of neem extracts on aphids can be placed into three categories: direct toxicity, effects on population build-up and repellant and antifeedant effects.

i) Direct toxicity: Goyal *et al.* (1971) tested the toxicity of eleven extracts and isolates from crushed neem seed against the water-nut aphid *Rhopalosiphum nymphaeae*. They found that a crude methanolic extract of the seed was more insecticidal than any of the purified extracts or isolates, while Devakumar *et al.* (1986) found that a mixture of the limonoids associated with neem oil were highly insecticidal to the safflower aphid (*Uroleucon carthami*).

ii) Effects on population build-up: Schauer (1984) found that a methyl-tertiary-butyl-ether (MTB) extract of neem seeds, formulated with sesame oil and a lecithin, caused high mortality in first and third instar pea aphids (*Acyrthosiphon pisum*) and, used at lower concentrations, reduced the fecundity and longevity of adults raised from first instar nymphs on treated plants. The nymph mortality was attributed to moulting disturbances as no contact toxicity or repellancy was noted.

Kirpal-Singh *et al.* (1986) reported that aqueous suspensions of neem seed kernel extract, leaf extract and neem oil caused significant reductions in the build-up of cabbage aphid (*Brevicoryne brassicae*) populations on cauliflower and cabbage under field conditions.

Safflower aphids were controlled equally well by an aqueous suspension of ground neem seeds and the OP

insecticide phosphamidon, but neither preparation was as effective as dimethoate or endosulphan (Singh *et al.*, 1988).

The commercially available neem-based formulation Margosan-O has been used effectively to control infestations of the crapemyrtle aphid *(Tinocallis kahawaluokanali)* in field experiments (Booth *et al.*, 1990). However reports on the effects of the formulation on *M. persicae* are contradictory. Lagnaoui *et al.* (1990) reported that in a field experiment to study the effects of Margosan-O on an infestation of Colorado potato beetle (*Leptinotarsa decemlineata*), the populations of *M. persicae* in treated plots were not significantly reduced, while Meisner *et al.* (1990) (citing a personal communication from Larson), stated that Margosan-O gave excellent control of *M. persicae*.

Griffiths *et al.* (1978) studied the effects of a crude methanolic neem seed extract on the survival and fecundity of adult *M. persicae* on systemically and topically treated kale plants. They found that, when applied to the roots, the extract caused reductions in survival and larviposition only at concentrations which were phytotoxic. Similarly, a population of aphids building up on tobacco was influenced by an azadirachtin-enriched neem extract only at high concentrations (Schmutterer, 1985).

iii) Repellant and antifeedant effects: When Griffiths *et al.* (1978) incorporated a crude methanolic neem seed extract into artificial diet at a range of concentrations in a choice test, *M. persicae* showed a dose-dependant reduction in settling behaviour. Azadirachtin, incorporated into an artificial diet in a choice test, prevented *M. persicae* from

settling on the treated diet and caused the aphids to show a strong preference for untreated diet. When the aphids were presented with azadirachtin treated diet in a no-choice test, they suffered the same mortality rate as aphids which had been starved during the same period (Schoonhoven & Derksen-Koppers, 1976). West & Mordue (Luntz) (1992) reported that when the leaves of barley plants were painted with different concentrations of azadirachtin and presented to cereal aphids (*Rhopalosiphum padi* and *Sitobion avenae*) in a choice test, there was a strong bias towards settling on untreated leaves and leaves treated with low concentrations of the compound. Topical and systemic treatment of the seedlings resulted in a reduction in the number of occasions on which aphids were observed to be penetrating the plant surface.

1.6 Effects of neem extracts on virus transmission

The majority of studies on the effects of neem extracts on plant virus transmission have involved rice viruses. Tungro is a disease of rice caused by the rice tungro bacilliform virus (RTBV) which is dependent on the rice tungro spherical virus (RTSV) for successful transmission by the leafhopper *Nephotettix virescens*. Both viruses are phloem-borne and transmitted in a semi-persistent manner. Application of expeller grade neem oil to rice plants reduced the incidence of tungro by more than 50% after exposure of the plants to viruliferous leafhoppers (Mariappan & Saxena, 1983). The lowered rate of tungro transmission can be attributed to an

overall reduction in feeding by *N. virescens* on treated plants and reduced phloem sap ingestion in particular (Saxena & Khan, 1985). The feeding behaviour of *N. virescens* can be altered in this way without physical contact between the insect and neem oil, as neem oil odour can produce similar effects (Saxena & Khan, 1986). Rice plants which were grown in neem cake-treated soil were also protected from tungro infection on exposure to viruliferous leafhoppers as a result of reduced phloem sap ingestion by the vectors (Saxena *et al.*, 1987).

There are some reports of the effects of neem products on virus transmission by aphids e.g. Srivastava *et al.* (1986) found that transmission of a non-persistently transmitted virus (cucumber mosaic virus) by *Aphis gossypii* was inhibited if the aphid came into contact with neem oil before and during acquisition, between acquisition and inoculation or at the time of inoculation. This effect was related to the reluctance of aphids to probe on treated surfaces and a reduction in the duration of initial probes when settling occurred. Recently, Hunter & Ullman (1992) reported the effects of a commercial formulation (RD-Repellin) on the settling behaviour of *A. pisum* and on the aphids' ability to transmit zucchini yellow mosaic virus (a non-persistently transmitted virus of curcurbits). RD-Repellin is a combination of extracts from the seeds of neem, karanjia, castor, mahua and gingelly. The formulation had repellant properties, affecting pre-probing behaviour and increasing locomotory activity even in those aphids which did not contact it. However in a no-choice test, RD-

Repellin did not prevent viruliferous aphids from infecting treated plants although symptom expression was delayed. This latter observation may have been an effect of the anti-viral properties of some of the compounds found in neem oil (Verma, 1974).

1.7 Plan of Research

From the above it can be seen that aphid antifeedants may offer an alternative to the use of synthetic insecticides in the control of plant virus spread. Azadirachtin is a potent antifeedant against many species of insects but in the majority of studies with aphids, only crude or enriched neem extracts have been used rather than the pure compounds which they contain. In addition, the above studies and the few in which pure azadirachtin was used have assessed effects that cannot be attributed directly to an antifeedant mode of action. This reflects the difficulties involved in measuring aphid feeding quantitatively as most aphids imbibe fluids while their stylets are located within sieve elements.

The aims of this thesis are:

i) To examine the effects of azadirachtin on the peach-potato aphid;

ii) To establish the nature of the relationship between azadirachtin concentration and the response of aphids to the compound and to explain this response in terms of primary and secondary effects:

iii) To investigate the possibility of reducing the acquisition and transmission of non-persistently and

persistently transmitted plant viruses through the use of azadirachtin against aphids.

The thesis is divided into three sections:

i) Isolation, purity analysis and systemic movement of azadirachtin (Chapter 2);

ii) Assessment of antifeedant effects of azadirachtin on *M. persicae* (Chapters 3 and 4);

iii) Virus acquisition and transmission studies (Chapters 5 and 6).

These sections are followed by a general discussion of the conclusions reached and the implications of the results for the use of azadirachtin as an aphid antifeedant (Chapter 7).

Chapter 2. The Purification and Systemic Movement of Azadirachtin.

2.1 Purification of azadirachtin from neem seeds.

2.1.1 Introduction

Azadirachtin was extracted using a modification of the procedures described by Schroeder & Nakanishi (1987) and Yamasaki *et al.* (1986), with final purification of the compound by flash column chromatography and preparative thin layer chromatography (TLC).

Azadirachtin was extracted from neem seeds on five occasions. Seeds from Ghana were used in extractions 1-3 and seeds from Pakistan were used in extractions 4 and 5. Extraction 1 is described in detail below, and subsequent modifications to this procedure are described for extractions 2-5.

2.1.2 Materials and Methods: Extraction

General: All solvents used in the initial stages of extractions were "Bulk" grade. Solvents used for flash column chromatography and thin layer chromatography were "AnalaR" grade. Partially purified fractions were stored in sealed containers at -20^{o}C after the complete removal of solvents by rotary evaporation and high pressure evacuation using an oil pump.

Flash column chromatography: Two methods of flash column chromatography were employed. The original method of Still *et al.* (1978) was modified to use a vacuum-driven system with "wide" (7cm x 15cm) or "narrow" (4cm x 15cm) columns according to the amount of material to be purified. The "dry-column" technique described by Harwood (1985) was also employed in some of the purifications using P40 (5cm length) flash columns. Silica gel, grade 60, 230-400 mesh 60 Å (Merck) was used for all flash chromatographic separations.

Analytical TLC: Fractions eluting from the columns were monitored for the presence of azadirachtin and other limonoids by performing analytical TLC on 20cm x 20cm polyester-backed silica gel plates (250µm thickness, 2-25µm particle size). Identification of azadirachtin and salannin was by co-chromatography with authenticated standards kindly provided by Dr. A.J. Mordue, University of Aberdeen and Dr. J.D. Connolly, University of Glasgow. Analytical TLC plates were run in 70% ethyl acetate : 30% petroleum ether 60^{o}-80^{o} and developed using one of two methods. Initially the plates were sprayed with ceric solution (1% $CeSO_4$ in 10% H_2SO_4) and then heated. This method was used until the monitoring of the chromatographic separation of fraction C in extraction 1, (see below). Thereafter, plates were developed in an atmosphere of iodine then sprayed with 25% H_2SO_4 and heated. This latter method gave a more permanent visualisation of the compounds present.

Preparative TLC: Preparative TLC was performed on 20cm x 20cm glass-backed plates spread with Kieselgel GF_{254} (Merck) to a depth of 0.5mm. A maximum of 30mg of impure material was applied as a single line to each plate. Plates were run three times in 60% ethyl acetate : 40% petroleum ether 60^{o}-80^{o}. After final drying, a strip of silica approximately 1.5cm wide on the vertical edge of each plate was developed using ceric solution. The unstained section of the azadirachtin-containing band was scraped off and washed four times with warm chloroform.

Crystallisation: Crystallisation of azadirachtin was originally performed by dissolving the material in a minimum amount of chloroform, then adding carbon tetrachloride until the mixture became cloudy. The mixture was stored in a freezer at $-20^{o}C$ for three hours then filtered under suction to remove solids. This method was used until the re-crystallisation of fractions A and B in extraction 1, (see below). Thereafter, the azadirachtin was dissolved directly into carbon tetrachloride by gentle heating and then immediately filtered before storage in the freezer and harvesting as described above (Schroeder & Nakanishi, 1987).

Semi-preparative high performance liquid chromatography (HPLC): Semi-preparative HPLC of fractions B1 and B2 in extraction 5 (see below) was performed on a C18 reverse phase column using the system described below;

pump: Perkin-Elmer Series 400 liquid chromatograph;

settings: 3.0 ml/min flow rate, 30 min run time, 20µl loop;

column: Hichrom S10 ODS2 25005;

detection: Perkin-Elmer LC90 spectrophotometer

settings: 214nm, 0.1 range;

solvent system: methanol:distilled water, (1:1 vol/vol) filtered to 0.2 µm and de-gassed under vacuum.

Identification and quantification of azadirachtin was by co-chromatography with an authenticated sample kindly provided by Dr. J. Anderson, Imperial College, London.

Extraction 1.

1.39kg of neem seed were finely ground and immediately transferred to a percolator. Fat extraction of the ground seed was then implemented using petroleum ether 60^{o}-80^{o}C (approximately 2.5l) for 3h, followed by a further extraction with hexane (approximately 2.5l) until no further yellow-coloured oil could be extracted by the solvent. The extracted oils were discarded and extraction of the remaining de-oiled seed (marc) by percolation with methanol was attempted. The flow of methanol through the marc was extremely slow, probably because exhaustive oil extraction had left the marc finely fragmented and compacted. Overnight percolation with methanol yielded little material, so cold extraction with agitation was employed. For this procedure, the marc was filtered using

Buchner apparatus, then divided into two approximately equal quantities and placed in 3l capacity conical flasks. Sufficient methanol was added to cover the seed and the mixtures were shaken by hand for 10min and then for 5min at 15min intervals during a 1h period. The marc was then filtered and the methanol was removed by rotary evaporation. This procedure was repeated three more times and was followed by an overnight extraction with mechanical stirring instead of shaking. After removal of the methanol from the combined filtrates, the residue - a brown tar, was re-dissolved in methanol and partitioned with equal volumes of hexane to remove any remaining lipids. The methanolic fraction was evaporated to dryness, then dissolved in ethyl acetate and partitioned twice with deionized water to remove water-soluble proteins and sugars. The ethyl acetate-soluble fraction was relieved of solvent by rotary evaporation. The subsequent purification procedure is shown schematically in Figure 3, and is described in detail below:

The ethyl acetate-soluble fraction was dissolved in 100ml of chloroform, to which 30g of silica gel was added. The resulting mixture was evaporated to dryness and added to the top of a wide flash chromatography column. The column was eluted using the solvent system shown in Table 1. Eighteen fractions were collected and monitored for azadirachtin content by analytical TLC. Azadirachtin was present in impure form in the last four fractions eluted by 100% ethyl acetate (Figure 4).

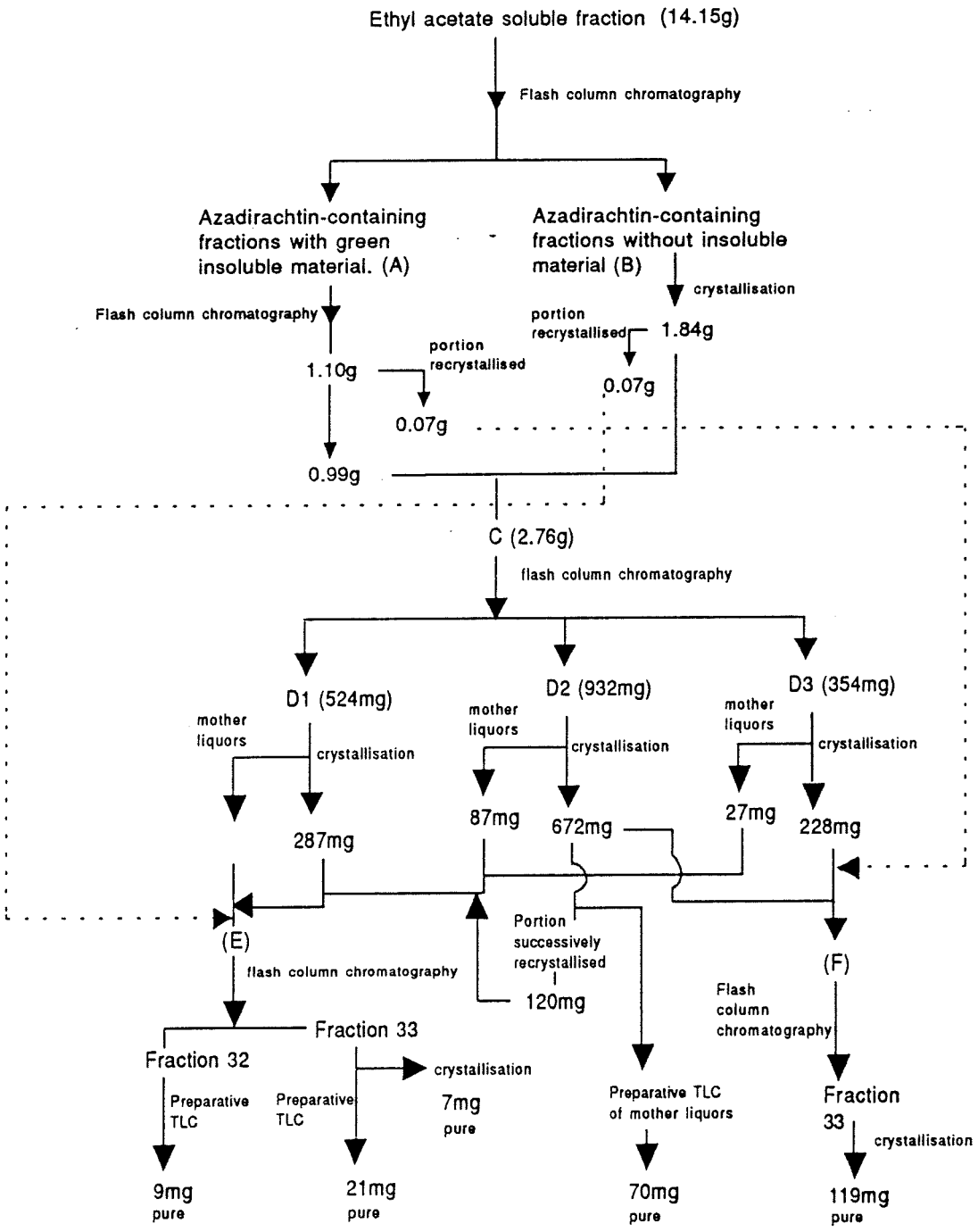
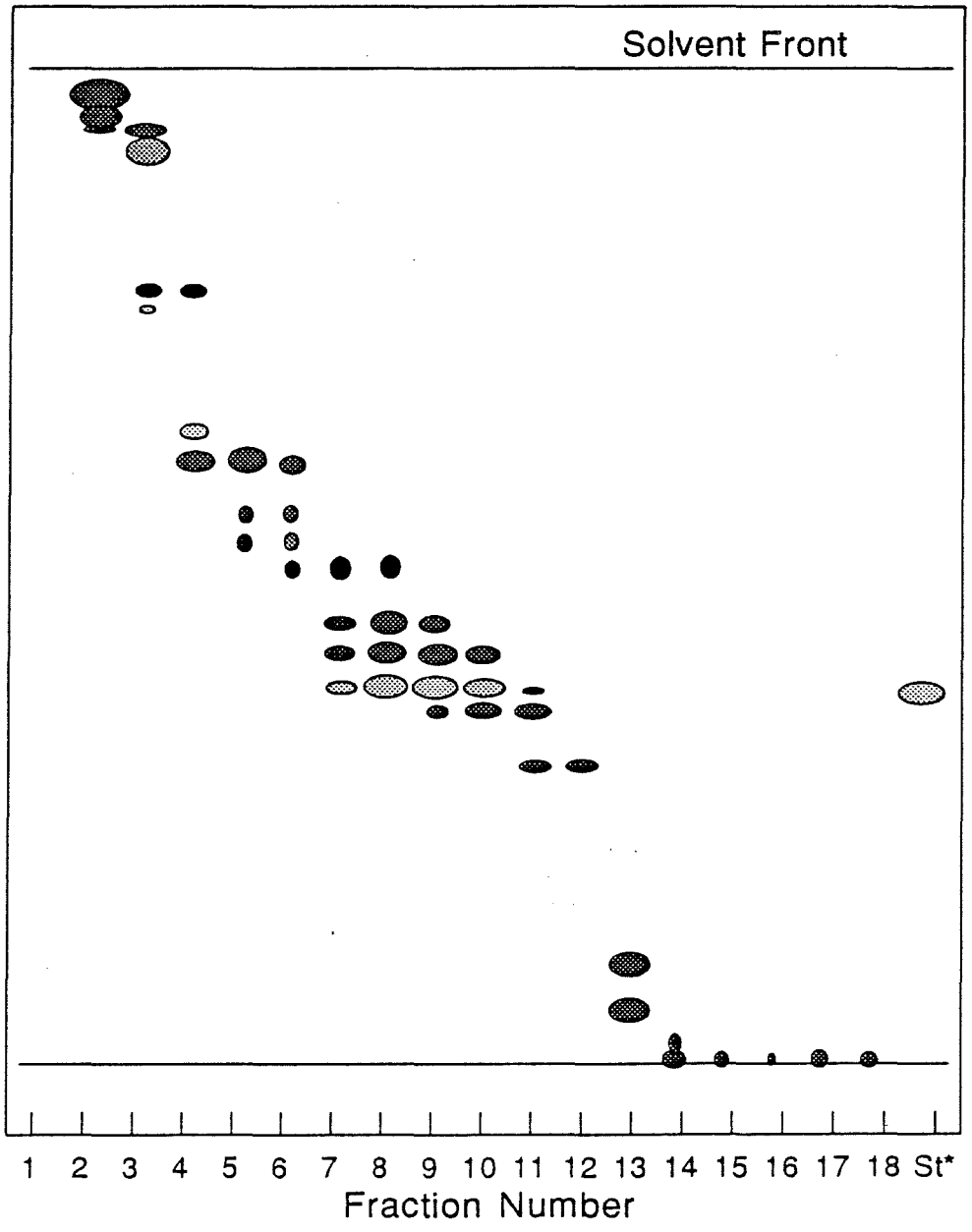


Figure 3. Purification of Azadirachtin from Ethyl Acetate-soluble fraction in Extraction 1.

Figure 4. Thin Layer Chromatogram of the fractions eluted from the flash column chromatographic separation of the ethyl acetate-soluble extract in Extraction 1.



* St = azadirachtin standard, Rf value 0.38, running solvent 70:30 ethyl acetate:petroleum ether.

Table 1. The solvent system used in the first flash column chromatography purification of azadirachtin.

Solvent System			Number of Fractions Taken*
Petroleum Ether 60-80	: Ethyl Acetate :	Methanol	
50	: 50 :	0	5 (1-5)
0	: 100 :	0	5 (6-10) a**
0	: 50 :	50	4 (11-14)
0	: 0 :	100	4 (15-18)

* Fraction size was approximately 30ml. Fraction numbers are in parenthesis. Azadirachtin-containing fractions are denoted by the letter "a".

** Fractions A & B in Figure 3.

Crystallisation of these fractions was attempted but was hindered by the presence of excess impurities. In each case, a green-coloured, insoluble, material was found floating on the surface of the mother liquors after attempted crystallisation. This material was collected and pooled with fraction 7 to give fraction 'A' (Figure 3). The remaining mother liquors from the crystallisation steps were pooled to give fraction 'B' which was re-crystallised.

Fraction A was further purified by flash column chromatography using a narrow column and the solvent system shown in Table 2. The azadirachtin-containing fractions were pooled and crystallised. Portions of this pooled fraction and the re-crystallised fraction B were then subjected to further re-crystallisation in an attempt to purify them.

Table 2. The solvent system used in the second flash column chromatography purification of azadirachtin.

Solvent System			Number of Fractions Taken*
Petroleum Ether 60-80	:	Ethyl Acetate	
50	:	50	4 (1-4)
30	:	70	5 (5-9)
15	:	85	3 (10-12) a**
0	:	100	4 (13-16)

* Fraction size was approximately 30ml. Fraction numbers are in parenthesis. Azadirachtin-containing fractions are denoted by the letter "a".

** Re-crystallised or added to Fraction C in Figure 3.

The purity of the resulting off-white amorphous material was determined by ^{1}H NMR and analytical TLC. Both procedures showed the material to be impure. The remaining semi-purified material was pooled giving fraction 'C' which was subjected to flash column chromatography using a narrow column. A more precise polarity gradient of eluting solvents was used and smaller fractions were taken (Table 3) to aid purification.

The azadirachtin-containing fractions were pooled according to their purity, hence fraction D1 contained two less-polar impurities, fraction D2 contained one less-polar impurity, and D3 contained traces of a more-polar impurity. These fractions were crystallised and the mother liquors of the crystallised material were pooled.

Table 3. The solvent system used in the third flash column chromatography purification of azadirachtin in extraction 1.

Solvent System			Number of Fractions Taken*	
Petroleum Ether 60-80	:	Ethyl Acetate		
60	:	40	4 (1-4)	
50	:	50	10 (5-14)	
40	:	60	10 (15-24)	
30	:	70	10 (25-34)	a^{1}**
20	:	80	2 (35-36)	a^{2}
0	:	100	2 (37-38)	

* Fraction size was approximately 10-15ml. Fraction numbers are in parenthesis. Azadirachtin-containing fractions are denoted by the letter "a".

**a^{1} = Fraction D1, a2 = Fractions D2,D3 (Figure 3).

A 600mg portion of fraction D2 was successively re-crystallised yielding approximately 120mg of impure azadirachtin after four attempts. The mother liquors from these crystallisations were evaporated to dryness and the residue was purified by preparative TLC, which yielded 70mg azadirachtin. The product of the successive re-crystallisation of D2 was pooled with D1, the mother liquors from the original crystallisations of D1,D2 and D3 and the re-crystallised portion of fraction B to form fraction E. The portion of fraction D2 which had not been used for successive re-crystallisation was pooled with fraction D3 and the re-crystallised portion of fraction A to form fraction 'F'. Fractions E and F were purified independently by flash column chromatography on narrow columns, eluting with the solvent gradient shown in Table 4.

Table 4. The solvent system used in the fourth flash column chromatography purification of azadirachtin.

Solvent System			Number of Fractions Taken*	
Petroleum Ether 60-80	:	Ethyl Acetate		
60	:	40	5	(1-5)
50	:	50	5	(6-10)
40	:	60	8	(11-18)
30	:	70	10	(19-28)
20	:	80	5	(29-34) a
0	:	100	2	(35-36)

* Fraction size was approximately 10-15ml. Fraction numbers are in parenthesis. Azadirachtin-containing fractions are denoted by the letter "a".

Azadirachtin was eluted from fraction E by 80% ethyl acetate : 20% petroleum ether 60^{o}-80^{o} with one, less-polar contaminant in fraction 32, and in a pure state in fraction 33. Fraction 32 was relieved of its contaminant by preparative TLC, yielding 9mg of pure azadirachtin on crystallisation. Fraction 33 yielded 7mg of pure azadirachtin on crystallisation. When the mother liquors of both fractions 32 and 33 were combined and purified by preparative TLC, crystallisation of the resulting purified material yielded a further 21mg of azadirachtin. Flash column chromatography of fraction F produced pure azadirachtin in a single fraction (fraction 33) which yielded 119mg of the compound on crystallisation.

Extraction 2.

All remaining extractions followed the initial protocol of extraction 1 until the chromatographic purification of the ethyl acetate soluble fraction after partitioning. One alteration was made to the initial stages; to improve

the efficiency of the fat extraction steps, the ground seed was extracted simultaneously in three equal amounts in separate percolators.

For extraction 2, the purification of azadirachtin from the ethyl acetate-soluble fraction is shown schematically in Figure 5. Initial flash column chromatographic separation of the ethyl acetate-soluble fraction was on a wide flash chromatography column eluting with the gradient shown in Table 5.

Table 5. The solvent system used in the first flash column chromatography purification of azadirachtin in extraction 2.

Solvent System			Number of Fractions Taken*	
Petroleum Ether 60-80	:	Ethyl Acetate		
60	:	40	2 (1-2)	
50	:	50	6 (3-8)	
40	:	60	10 (9-18)	
30	:	70	10 (19-28)	s
20	:	80	4 (29-32)	a^1**
0	:	100	4 (33-36)	a^2

* Fraction size was approximately 20-25ml. Fraction numbers are in parenthesis.

** Salannin and azadirachtin-containing fractions are denoted by the letters "s" and "a" respectively. a^1 = Fraction A and a^2 = Fractions B or C in Figure 5.

Salannin was detected in fractions 19-22 and azadirachtin eluted in fractions 30-35. Fractions 30-33 contained less-polar contaminants and were pooled to give fraction A. Further flash column chromatography was performed on this fraction using a narrow column (20cm

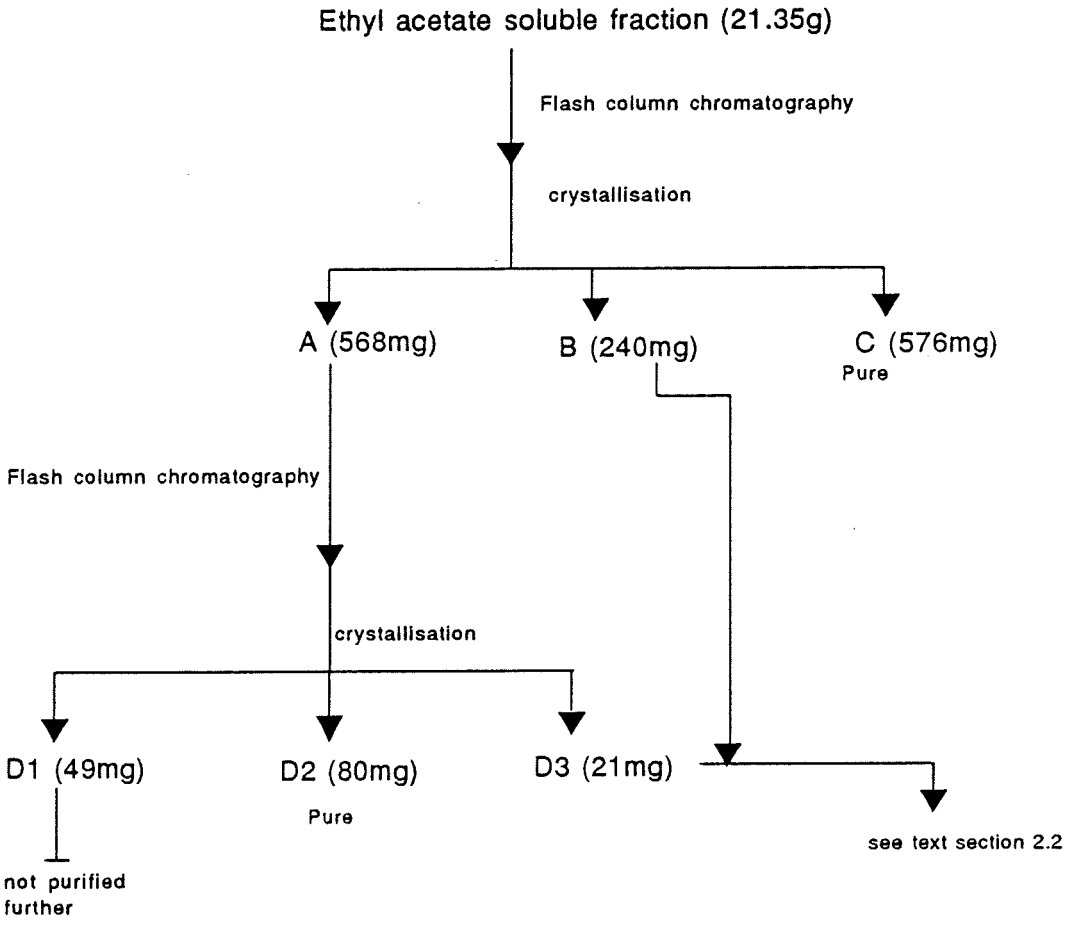


Figure 5. Purification of Azadirachtin from the Ethyl Acetate-soluble fraction in Extraction 2.

length) and eluting with the solvent system shown in Table 6.

Table 6. The solvent system used in the second flash column chromatography purification of azadirachtin in extraction 2.

Solvent System			Number of Fractions Taken*	
Petroleum Ether 60-80	:	Ethyl Acetate		
40	:	60	5 (1-5)	
30	:	70	8 (6-13)	
20	:	80	8 (14-21)	
10	:	90	5 (22-26)	a**
0	:	100	2 (27-28)	

* Fraction size was approximately 10-15ml. Fraction numbers are in parenthesis.

** Azadirachtin-containing fractions are denoted by the letter "a" (= Fraction D2 in Figure 5).

Azadirachtin was eluted with one less-polar contaminant (D1), pure (D2) and with one more-polar contaminant (D3). Fraction D2 was crystallised, yielding 80mg azadirachtin.

Extraction 3.

Extraction 3 is shown schematically in Figure 6. Initial flash column chromatography of the ethyl acetate-soluble fraction was performed on a wide column, eluting with the solvent system shown in Table 5 (above). Fractions were pooled according to their relative purity and further flash column chromatography was performed using dry-column flash chromatography. Fractions A1 and A2 were

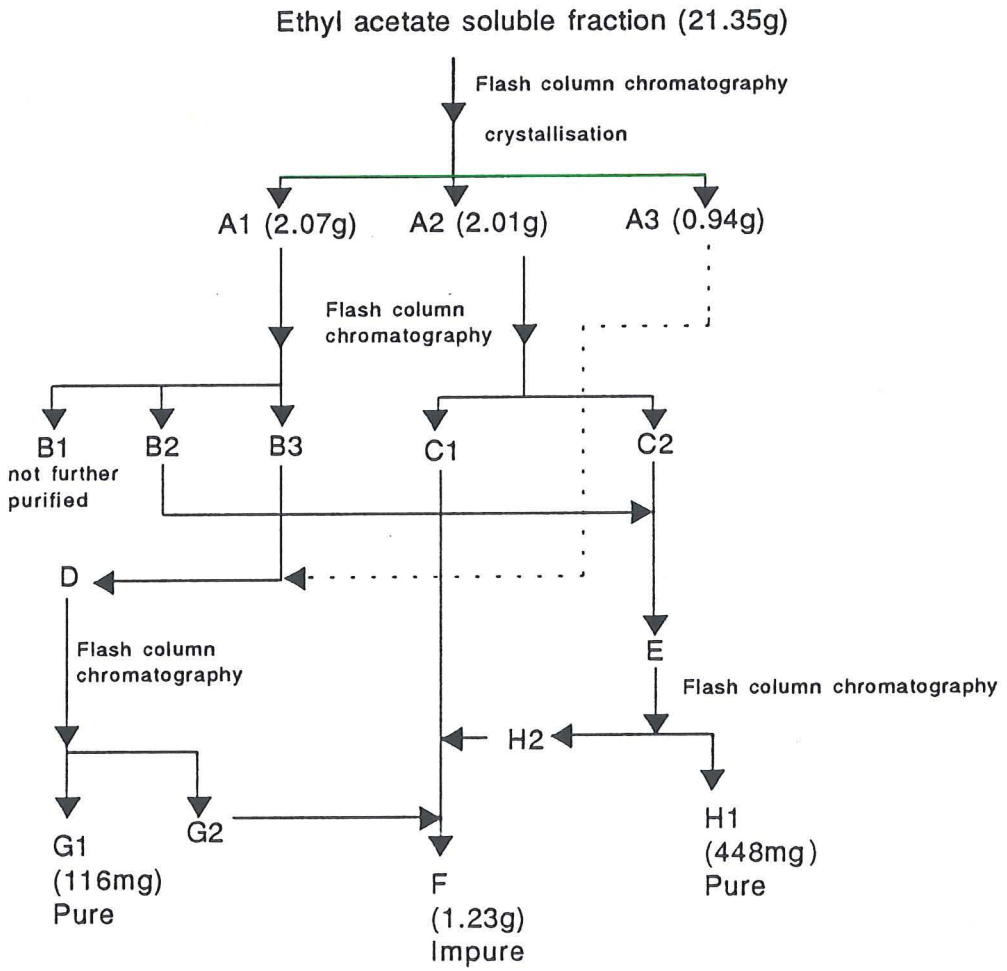


Figure 6. Purification of Azadirachtin from the Ethyl Acetate-soluble fraction in Extraction 3.

purified further using isocratic elution with 50% ethyl acetate: 50% petroleum ether 60^{o}-80^{o}, taking fraction sizes of approximately 25ml. Fraction E was purified further using an elution gradient as shown in Table 7:

Table 7. The solvent system used in the chromatographic separation of azadirachtin from fraction E in the third extraction.

Solvent System			Number of Fractions Taken*	
Petroleum Ether 60-80	:	Ethyl Acetate		
70	:	30	15 (1-15)	
60	:	40	5 (16-20)	
50	:	50	27 (21-47)	a**

* Fraction size was approximately 10-15ml. Fraction numbers are in parenthesis.

** Azadirachtin-containing fractions are denoted by the letter "a" (= H1 in Figure 6).

Fraction F was retained in its impure state as a reserve supply of azadirachtin for purification if the need arose.

Extraction 4.

Extraction 4 is shown schematically in Figure 7. Initial flash column chromatography of the ethyl acetate-soluble fraction was performed on a wide column using the solvent system shown in Table 5. After the initial attempted separation, all azadirachtin-containing fractions contained the same impurities and were therefore pooled for further chromatographic separation using a narrow

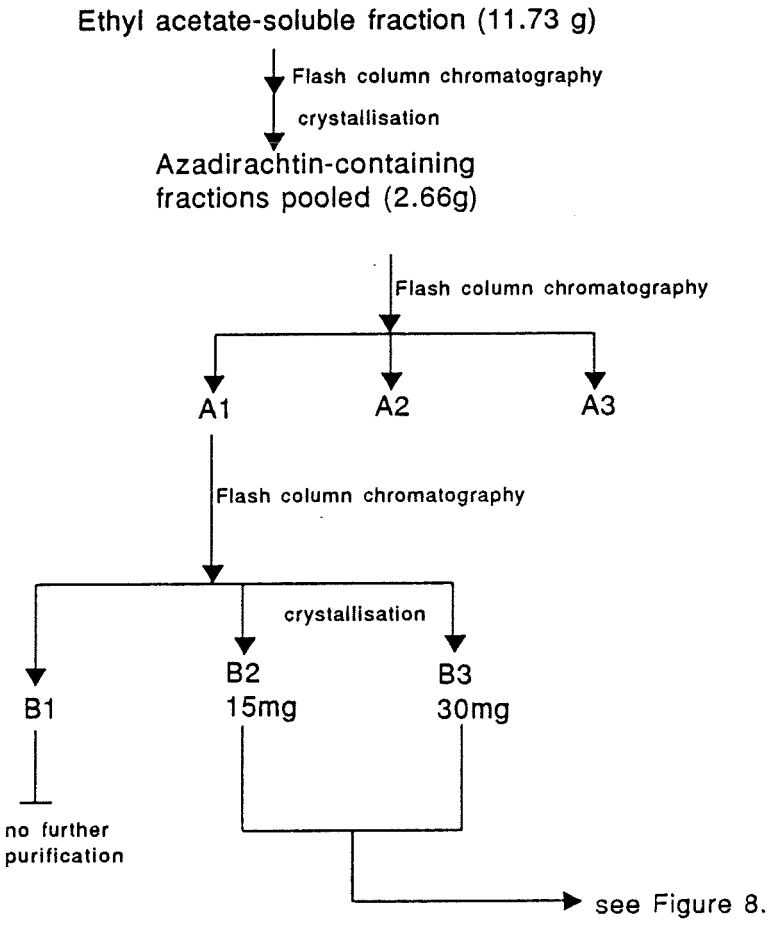


Figure 7. Purification of Azadirachtin from the Ethyl Acetate-soluble fraction in Extraction 4.

column and eluting with the solvent system shown in Table 8 below:

Table 8. The solvent system used in the chromatographic separation of the pooled azadirachtin-containing fractions in extraction 4.

Solvent System			Number of Fractions Taken*	
Petroleum Ether 60-80	:	Ethyl Acetate		
60	:	40	2 (1-2)	
50	:	50	3 (3-5)	
40	:	60	8 (6-13)	
30	:	70	10 (14-23)	a^{1}**
20	:	80	10 (24-33)	a^{2}

* Fraction size was approximately 10-15ml. Fraction numbers are in parenthesis.

** Azadirachtin-containing fractions are denoted by the letter "a". a^{1} = Fraction A1 and a^{2} = Fractions A2 and A3 in Figure 7.

Fractions were pooled according to their relative purity. Further purification of fractions A2 and A3 was not attempted because of the small sizes of the fractions. Purification of fraction A1 by flash column chromatography was performed on a narrow column eluting with a more gentle polarity gradient (Table 9) in an attempt to remove a less-polar contaminant that chromatographed very close to azadirachtin on the analytical TLC.

Fractions were again pooled according to their relative purity and, because of the small sizes of the fractions, no further purification was attempted at this stage.

Table 9. The solvent system used in the chromatographic separation of the fraction A1 in extraction 4.

Solvent System			Number of Fractions Taken*	
Petroleum Ether 60-80	:	Ethyl Acetate		
50	:	50	3	(1-3)
45	:	55	5	(4-8)
40	:	60	4	(9-12)
35	:	65	5	(13-17)
30	:	70	11	(18-28)
20	:	80	6	(29-34) a**

* Fraction size was approximately 10-15 ml. Fraction numbers are in parenthesis.

** Azadirachtin-containing fractions are denoted by the letter "a".

Extraction 5.

Extraction 5 is shown schematically in Figure 8. After initial flash column chromatography using a wide column and eluting with the solvent system shown in Table 5 (above), all azadirachtin-containing fractions were pooled because of the low weight of these fractions. Fractions B2 and B3 from extraction 4 were added to the pooled material, and the resulting fraction (A) was further purified by flash column chromatography using a dry column and eluting isocratically with 50% ethyl acetate : 50% petroleum ether 60^{o}-80^{o}. Azadirachtin-containing fractions were pooled according to their relative purity and after an initial attempt to purify these fractions by semi-preparative HPLC, no further purification was attempted.

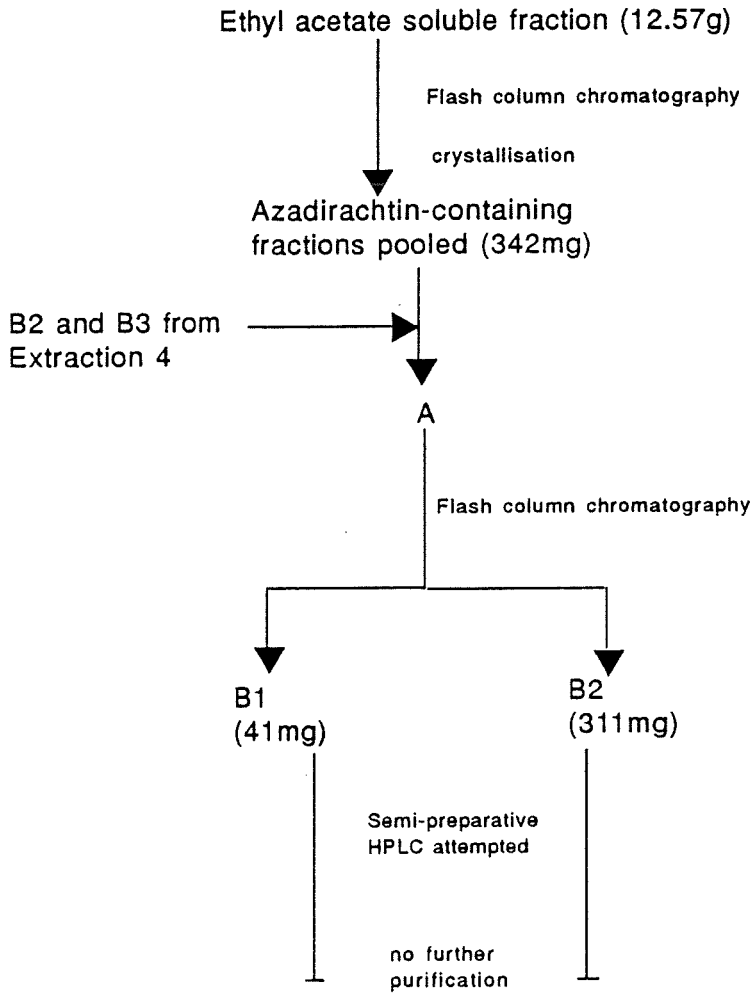


Figure 8. Purification of Azadirachtin from the Ethyl Acetate-soluble fraction in Extraction 5.

2.1.3 Purity analysis

General: The purity of each extract was assessed using at least two of the following methods:

i) Analytical TLC (as described in section 2.1.2.).

ii) Analytical HPLC was performed using a 25cm x 4.6cm C18 reverse phase column (Spherisorb ODS2, 5µm particle size) on the following system;

Pump: Gilson 303, flow rate 1ml/min

Detector: Gibson 'Holochrome' spectrophotometer

settings: 220nm, 0.5 range

Solvent: methanol: water (HiPerSolv grade), (1:3 vol/vol) or

methanol:water (1:1 vol/vol) de-gassed and filtered as in section 2.1.2.

iii) 200MHz ^{1}H nuclear magnetic resonance (nmr) spectroscopy was performed on a Bruker WP 200 SY instrument. Shifts in ppm are relative to $CHCl_3$ at 7.25ppm.

iv) melting point analysis.

2.2 Attempted radiolabelling of azadirachtin

2.2.1 Introduction

A radiolabelled derivative of azadirachtin has previously been produced by hydrogenation of the 22,23 enol ether bond with tritium to produce [22,23-

$^{3}H_2$]dihydroazadirachtin (Rembold *et al.*, 1989). An attempt was made to produce azadirachtin labelled with ^{14}C on the acetate group at position 3 (see Figure 2) to study the systemic movement and mobility of the molecule in phloem sap. Using azadirachtin with an isotope incorporated into the original structure is preferable to using [22,23-$^{3}H_2$]dihydroazadirachtin to study uptake as the tritiated molecule may have different mobility from the compound itself.

2.2.2 Materials and methods

i) *Deacetylation of azadirachtin.* Azadirachtin was deacetylated on three occasions using a modification of the procedure published by Butterworth *et al.* (1972). On two occasions pure azadirachtin was used as a starting material and on one occasion the combined impure fraction (B+D3) from extraction 2 was used. A 5% (w/w) solution of azadirachtin in methanol was stirred with 1% potassium hydroxide for 3.5h at room temperature. After this period, the mixture was made just acidic by adding dilute hydrochloric acid. The mixture was then extracted with ethyl acetate four times, leaving a crude, acidic product on evaporation of the solvent.

ii) *Esterification of the acidic product.* The acidic product was dissolved in chloroform and freshly prepared diazomethane in diethyl ether was added in excess. This mixture was stirred for 3 days at room temperature and

then evaporated to dryness prior to purification by preparative TLC. Preparative TLC was performed as described in section 2.1.2 but the running solvent was changed to 80% ethyl acetate : 20% petroleum ether 60°-80° to accommodate the increased polarity of deacetylazadirachtin compared to the original compound. Purity of the product was confirmed by co-chromatography with an authenticated standard kindly provided by Dr. A.A. Kadir, University of Glasgow.

iii) *Re-acetylation using ^{14}C-acetic anhydride.* Two attempts were made to re-acetylate azadirachtin with ^{14}C-acetic anhydride. In the second attempt, an acylation catalyst, 4-dimethylaminopyridine (DMAP), was used in an attempt to overcome steric hindrance (Holfe & Steglich 1972).

a) *First attempted re-acetylation*: 1 mCi of ^{14}C-acetic anhydride was purchased from Amersham International plc. The material was supplied as a film of liquid lining the interior of a break-seal tube. The tube was suspended, upright, overnight in a bath of acetone and dry ice to condense the acetic anhydride and let it collect in the bottom of the vessel. When the seal was broken 100µl of unlabelled, re-distilled acetic anhydride was mixed with the contents. 3.2mg of deacetylazadirachtin dissolved in 80µl re-distilled pyridine were then added to the acetic anhydride, and a further 80µl of pyridine were washed down the inside walls of the vessel. The vessel was then sealed and removed from the acetone/dry ice bath. The

contents were gently shaken and left at room temperature overnight before excess methanol was added to stop the reaction. The resulting material was washed into a flask with chloroform and the solvent was removed under high vacuum. The residue (product 1A) was analysed for the presence of azadirachtin using analytical TLC combined with radioscanning (see below). The reaction had not gone to completion, so product 1A was dissolved in 160µl re-distilled pyridine and 200µl acetic anhydride were added. The mixture was again left overnight at room temperature followed by removal of the solvent as above. The product (1B) was analysed for the presence of ^{14}C-labelled azadirachtin, and preparative TLC on 5cm x 20cm plates (solvent system as in section 2.1.2) was used to purify the product. Analytical TLC and radioscanning were performed on the purified product (1C).

b) *Second attempted re-acetylation*: 3.5mg deacetylazadirachtin and 0.2mg DMAP in 200µl re-distilled pyridine were added to 500µCi ^{14}C-acetic anhydride (purchased from Amersham International plc.) which had been condensed by immersion of the container in liquid nitrogen. The container was removed from the liquid nitrogen and left at room temperature for 24h. 200µl unlabelled acetic anhydride were then added and the mixture was left at room temperature for a further 6h before the reaction was stopped with methanol. Following solvent removal, the residue was washed twice with saturated $CuSO_4$ solution and then distilled water to

remove any residues of the acylation catalyst. Analytical TLC of the product and radioscanning of the TLC plate suggested that a radiolabelled product (2A) was present. Product 2A was purified by preparative TLC as above giving product 2B. Analytical TLC of product 2B suggested that it contained mostly deacetylated material but with some radioactivity present. Product 2B was then re-acetylated using unlabelled acetic anhydride as described above. Preparative TLC revealed two distinct bands. The upper band was removed (product 2C) and analysed for purity and activity as described below.

iv) *Determining purity and activity of labelled compounds*. Purity and activity of fractions were monitored throughout both attempted re-acetylations by analytical TLC accompanied by radioscanning of the plates using a Panax TLC radioscanner (range 100-300 c.p.s., time constant 30s, Geiger dead time 200 μs, scanning and recording at 1cm/min). In addition, the activity and purity of product 2C were determined using the following methods:

a) Counting using a Geiger-Muller counter suspended over a measured quantity of the material on a watchglass. Twenty counts were made, each of one minute in length, and a mean figure was calculated for activity in counts per minute (c.p.m.).

b) HPLC combined with liquid scintillation counting. HPLC was performed as described in section 2.1.3, collecting fractions of 1ml as they eluted. Liquid

scintillation counting was performed on 200µl of each fraction in 10ml Ecoscint using an Intertechnique SC30 liquid scintillation spectrometer. The activity of each fraction was measured in c.p.m. (mean of 10, 1min counts per sample). 100µl of an internal standard (quoted activity $2.43x10^3$ d.p.m./g, specific gravity 0.8) was then added to each fraction and activity was corrected for the efficiency of the counter.

c) Two dimensional TLC with autoradiography. Four, 10cm x 10cm analytical TLC plates were cut from a polyester-backed 20cm x 20cm plate described above. Two dimensional analytical TLC was then performed on product 2C as follows: in the first direction the running solvent was ethyl acetate 80% : petroleum ether (60-80) 20%. Following a clockwise rotation of the plate round 90° and the application of a second standard, the running solvent was changed to diethyl ether 98% : methanol 2%. After drying, visualisation of the plates was achieved using iodine vapour. Two plates were then placed face down on to photographic film and stored in darkness at room temperature. One was developed after 4days, and the other after 7days.

2.3 Determination of systemic movement of azadirachtin in *Nicotiana clevelandii*.

The movement of azadirachtin from the roots to the aerial parts of *Nicotiana clevelandii* was determined qualitatively by analytical TLC. A preliminary

quantitative measure of systemic movement was also performed using HPLC.

2.3.1 Qualitative determination of systemic movement by analytical TLC.

i) *General*

Treatments: Azadirachtin solutions were prepared by dissolving the microcrystalline solid in dried, AnalaR grade ethanol, and diluting the solution with distilled water to give an ethanol concentration of 2%. Tween 20 was then added to the solution to a concentration of 0.02%. Control solutions were 2% ethanol, 0.02% Tween 20 in distilled water. Solutions were freshly prepared in the morning of the day that they were used.

Plants: Tobacco (*Nicotiana clevelandii* Gray) seedlings, germinated in gibberellic acid (300ppm), were transferred to seed trays for 10days, kept at 23°C with a daylength regime of 18h light: 6h dark (L18:D6), and then planted in 4" pots with general purpose, peat-based compost, and kept at 21°C, L18:D6 until use at 21-25days after germination. The roots of each plant were washed with distilled water, and immersed in 1.5ml azadirachtin or control solution in a plastic vial. The plants, with their roots immersed up to the distal portion of the hypocotyl, were kept at 17-18°C, 70% relative humidity (rh) L18:D6 for 36h. A barrier of Nescofilm prevented contact between the azadirachtin solution and the leaves.

ii) *Preliminary analysis*: Leaves from four seedlings which had been treated with 300ppm azadirachtin solution were extracted with 2ml methanol for 2h. The resulting material was centrifuged at 14000rpm for 3min and the supernatant was evaporated to dryness. The residue was dissolved in 5ml ethyl acetate and partitioned twice with equal volumes of water. The water-soluble fraction was discarded and the remaining material was concentrated to 1ml. 5µl of this concentrate were analysed by analytical TLC (as in section 2.1.2) using 80% ethyl acetate: 20% petroleum ether 60^{o}-80^{o} as the running solvent.

iii) *Standardisation of technique*: 0.501g (fresh weight) of leaf tissue from *N. clevelandii* seedlings which had received no treatments were extracted as above. The ethyl acetate-soluble portion was evaporated to dryness after partitioning with water, and was then re-dissolved in ethyl acetate to give concentrations of 0.59%, 1.18%, 2.36% and 4.72% (w/vol). Analytical TLC was performed on these extracts as above, using 80% ethyl acetate : 20% petroleum ether 60^{o}-80^{o} as a running solvent.

iv) *Definitive qualitative analysis of systemic movement*. The leaves of *N. clevelandii* seedlings which had been treated with a range of concentrations of azadirachtin or control solutions, were extracted as above and prepared as 1% (w/vol) solutions in ethyl acetate. Analytical TLC was performed on these extracts, as above, alongside two standards; a) azadirachtin in ethyl acetate, and b)

azadirachtin dissolved in a portion of the extract of plants from the untreated group (spiked standard).

2.3.2 Preliminary quantification of systemic movement of azadirachtin by HPLC of extracts.

Leaf tissue from treated *N. clevelandii* seedlings was extracted as above (section 2.3.1) and the ethyl acetate-soluble portions were dissolved in methanol which was then diluted to 10% (vol/vol) with water. Two azadirachtin concentrations were used as treatments; 300ppm and 500ppm. The extracts were freed of pigments and, in the case of the extract from 300ppm treated plants were concentrated, by passage through disposable, suction-driven extraction columns (Bond Elut C18 columns, 1ml capacity, Analytichem International, USA). The extraction columns were eluted first with 10% methanol to remove the most polar contaminants, and then with 100% methanol to elute azadirachtin. The azadirachtin-containing fraction was eluted in 200µl methanol in the case of the 300ppm treated plants, and in two fractions of 300µl and 100µl in the case of the 500ppm-treated plants. These fractions were analysed by HPLC using the system described in section 2.1.3. A standard curve of absorbance at 220nm against azadirachtin concentration was constructed using known concentrations of azadirachtin, and the quantity of the compound in the extracts was calculated from this standard graph. The moisture content of the leaves of untreated *N.*

clevelandii was calculated by drying the leaves to a constant weight in an oven at 60°C. Using this figure, the original concentration of azadirachtin in the leaf tissue was calculated.

2.4 Results

2.4.1 Purification of azadirachtin from neem seeds

In total 1.44g pure azadirachtin was extracted from 4.29kg of Ghanaian neem seeds, (Table 10). No pure azadirachtin was obtained from the Pakistani neem seeds and the results from the initial semi-preparative HPLC steps suggested that less than 40mg of pure material was present in the most pure extracts from more than 2.5kg of seed.

Analytical TLC of the extracts gave accurate indications of purity. Purity was confirmed by HPLC, melting point analysis and ^{1}H nmr spectroscopy, (see Table 11 and Figure 11)). ^{1}H nmr spectroscopy was used to establish the purity of the major purified fractions (>30mg) only. The ^{1}H spectra of an impure sample (recrystallised portion of fraction B, extraction 1) and a pure sample (fraction D2, extraction 2) are shown in Figures 9 and 10 respectively. The nmr data and assignments of fraction D2 (extraction 2) are given in Table 12.

Table 10. Yields of azadirachtin and azadirachtin-containing fractions in each extraction.

Extraction number	Weight of crushed seed (kg)	Weight of methanolic extract (g)	Weight of ethyl acetate-soluble fraction (g)	Total pure azadirachtin (mg)	% yield
1	1.39	165.71	14.15	226	0.016
2	1.48	209.73	28.31	>656*	>0.044
3	1.41	186.11	21.35	>564*	>0.040
4	1.58	121.99	11.73	<40	<0.0015
5	1.13	77.15	12.57		

* In extractions 2 and 3 not all the material was purified so the quoted figure is for pure material only.

Table 11. Purity analysis of the most pure fractions from each extraction

Extraction number	Fraction	Quantity (mg)	Method of purity assessment: TLC	m.p.	nmr	HPLC (%)
1	32 (E)	9	s*			
	33 (E)	28	s			
	prepTLC	70	s		p	93
	33 (F)	118	s		p	98.9
2	C	576	s		p	98.8
	D2	80	s		p	
3	H1	448	s		p	96.6
	G1	116	s	155-157 oC		
4	B2	15	1mc			
	B3	30	1mc (tr)			
5	B1	41	1lc+1mc			20.6
	B2	311	1mc			10

*s= single spot purity, mc= more-polar contaminant, lc= less polar contaminant, tr= trace, p= pure by nmr spectroscopy.

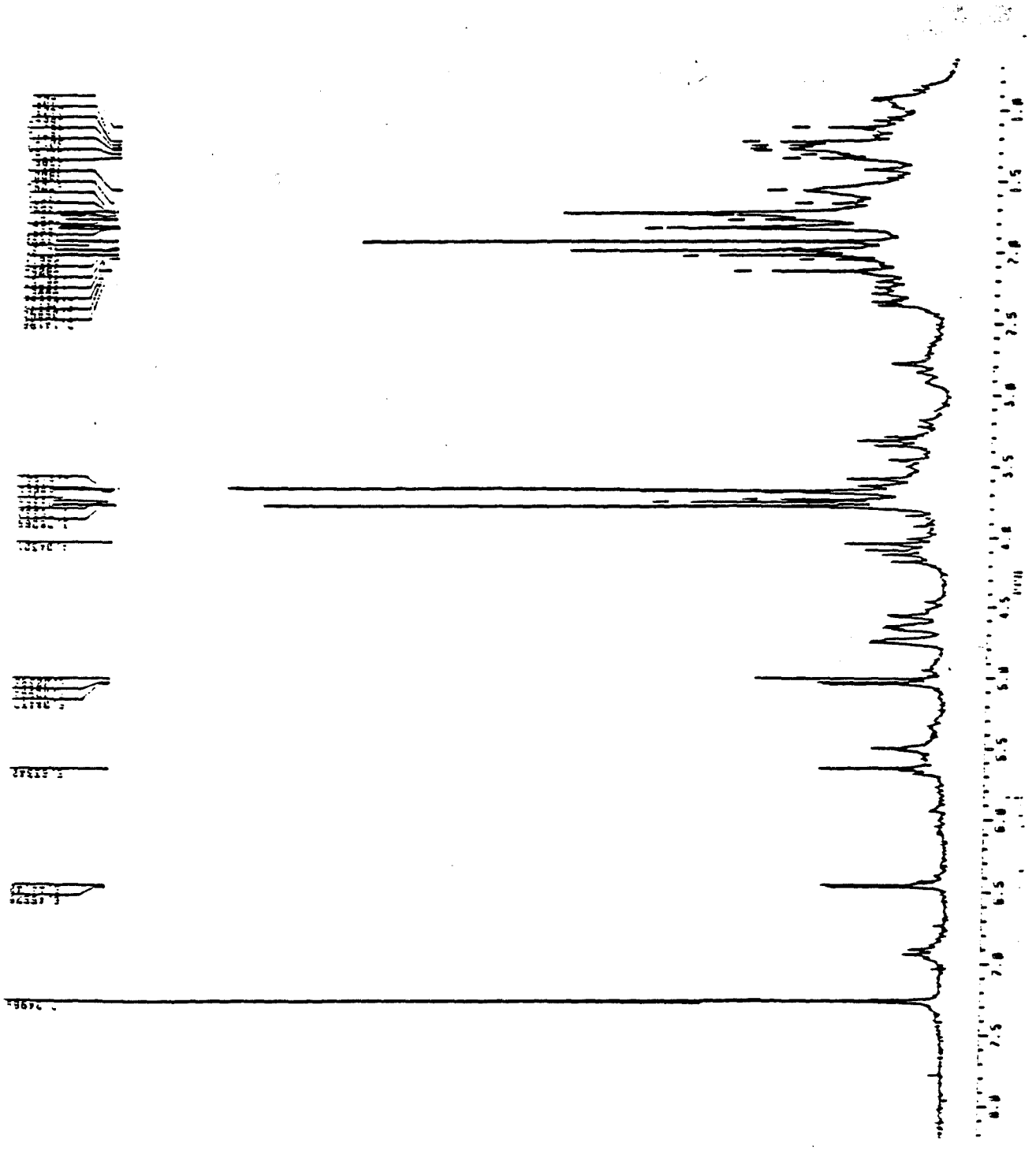


Figure 9. The proton nmr spectrum of impure azadirachtin (fraction B recrystallised, Extraction 2)

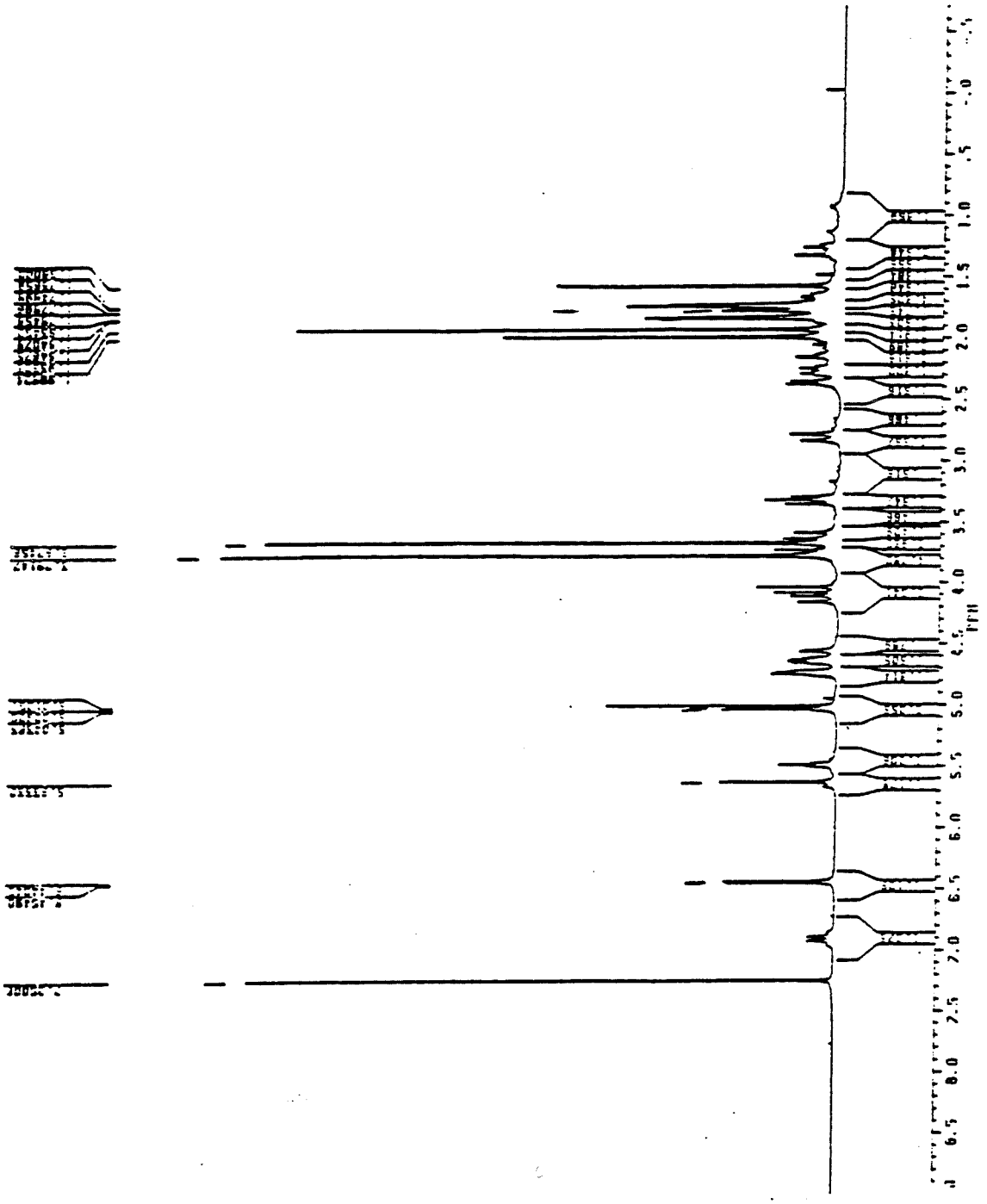


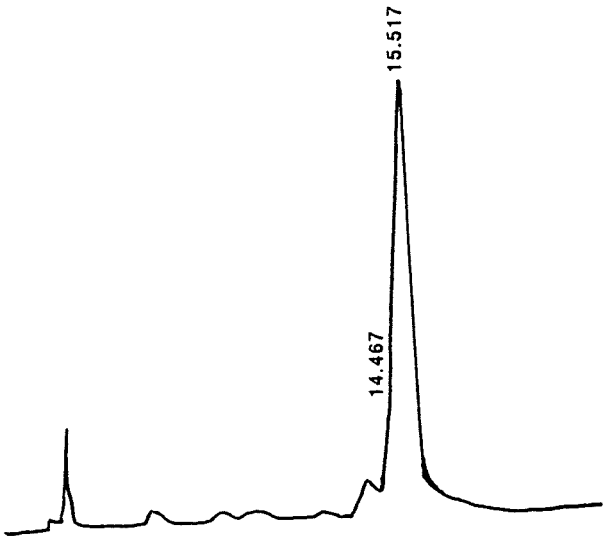
Figure 10 The proton nmr spectrum of pure azadirachtin (fraction B3, Extraction 2).

Table 12 . Proton nmr shifts and assignments of fraction D2 (extraction 2) in comparison to previously published data for azadirachtin (Kraus et al. 1987).

	Chemical shifts (ppm) and multiplicity*	
Assignment	D2 (extr. 2)	Published
1-H	4.75 (t,3.0)	4.75 (dd,2.9,3.1)
2-Ha	2.30 (dt,16.0,3.0)	2.34 (ddd,16.7,2.9,2.7)
2-Hb	2.15 (dt,16.0,3.5)	2.13 (ddd,16.7,3.1,2.9)
3-H	5.49 (t,2.60)	5.50 (dd,2.7,2.9)
5-H	3.33 (d,12.5)	3.35 (d,12.5)
6-H	4.60 (dd,12.5,2.7)	4.60 (dd, 12.5,2.7)
7-H	4.75 (d,2.7)	4.75 (d,2.7)
9-H	3.33 (s)	3.34 (s)
15-H	4.66 (d,3.4)	4.67 (d,3.4)
16-Ha	1.72 (ddd,13.0,3.3,5.1)	1.73 (ddd,13.0,3.4,5.1)
16-Hb	1.31 (d,13.0)	1.31 (d,13.0)
17-H	2.37 (d,5.2)	2.38 (d,5.1)
18-H	2.01 (s)	2.01 (s)
19-Ha	3.62 (d,9.6)	3.63 (d,9.6)
19-Hb	4.15 (d,9.6)	4.15 (d,9.6)
21-H	5.63 (s)	5.65 (s)
22-H	5.04 (d,2.9)	5.05 (d,2.9)
23-H	6.45 (d,2.9)	6.46 (d,2.9)
28-Ha	4.07 (d,8.9)	4.08 (d,9.0)
28-Hb	3.75 (d,8.9)	3.76 (d,9.0)
30-H	1.74 (s)	1.74 (s)
7-OH	2.79 (s)	2.89 (s)
11-OH	5.01 (s)	5.05 (s)
20-OH	2.84 (s)	2.92 (s)
12-OCH_3	3.68 (s)	3.68 (s)
29-OCH_3	3.78 (s)	3.76 (s)
CH_3COO	1.94 (s)	1.95 (s)
Tigloyl		
3'H	6.91 (qq,7.1,1.4)	6.93 (qq,7.0,1.5)
4'H	1.77 (dq,7.1,1.2)	1.78 (dq,7.0,1.1)
5'H	1.84 (quintet,1.2)	1.85 (dq,1.5,1.1)

* s = singlet, d = doublet, t = triplet, q = quartet

a)



Column conditions in both analyses, as described in text section 2.1.3, solvent; methanol: water (1:1 vol/vol).

b)

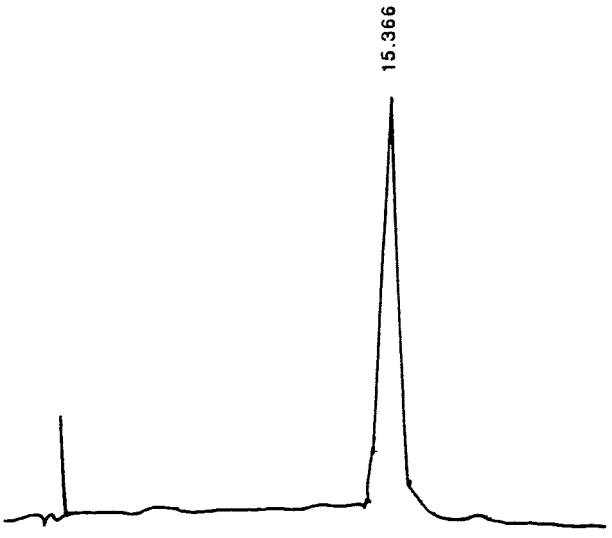


Figure 11. HPLC traces of a) azadirachtin standard provided by Dr. J. Anderson, Imperial college, b) fraction C, Extraction 2.

2.4.2 Attempted radiolabelling of azadirachtin

i) *Deacetylation.* In each preparation yields of deacetylazadirachtin were poor, particularly when the starting material was not pure (Table 13).

Table 13. Yields of deacetylazadirachtin from each preparation.

Preparation	weight of starting material (mg)	weight of acidic product (mg)	yield of deacetyl-azadirachtin (mg)	% yield (w/w)
1	127	45	2	1.4
2	261*	187	2	0.8
3	156	89	12	7.7

*Impure starting material (Fractions B+D3, extraction 2)

ii) *Re-acetylation using ^{14}C-acetic anhydride.*

First attempt: Radioscanning of the TLC of product 1A showed a major peak of activity at *Rf* 0.09 and minor peaks with *Rf* values from 0.84-0.91. Visualisation of the plate revealed the presence of azadirachtin and deacetylazadirachtin with *Rf* values of 0.47 and 0.35 respectively. Analytical TLC of product 1B showed that most of the deacetylazadirachtin had been re-acetylated. Further purification to product 1C yielded 1.8mg of azadirachtin with no associated radioactivity.

Second attempt: Radioscanning of the TLC of product 2A showed a major peak of activity with an *Rf* value of 0.47. Visualisation of the plate revealed azadirachtin at *Rf* 0.45. Similar analysis of product 2B, however, showed that the major peak of activity was associated with deacetylazadirachtin. Analysis of product 2C showed that the major peak of activity was now associated with azadirachtin.

Analysis of purity and activity:

a) Counting using a Geiger Muller tube;

Mean activity of 0.06mg of sample	6.075 cps
Mean background activity	0.495 cps
Activity of sample	5.580 cps
Total activity of product 2C	93.0 cps/mg
	= 5580 cpm/mg

b) HPLC combined with liquid scintillation counting; Product 2C co-chromatographed with pure azadirachtin (retention time 20.1min) but contained some, more-polar impurities which appeared as a shoulder running before the main peak. The major peak of radioactivity (calculated as 0.186μCi/mg) was associated with the peak of U.V. absorbance, (Figure 12), with several minor peaks of radioactivity occurring in the fractions eluting before the major peak. The minor peaks were not associated with any strongly U.V. absorbing compounds eluted from the column.

c) Two-dimensional TLC combined with autoradiography. After development of the TLC plates in iodine vapour, the samples all appeared as single spots with *Rf* values in

close agreement with those of the standards. When the autoradiographs of these plates were developed, all of the radioactivity was also shown to be associated with a single spot on each plate, but not with the same *Rf* values as the spots visualised using iodine vapour (Table 14 and Figure 13).

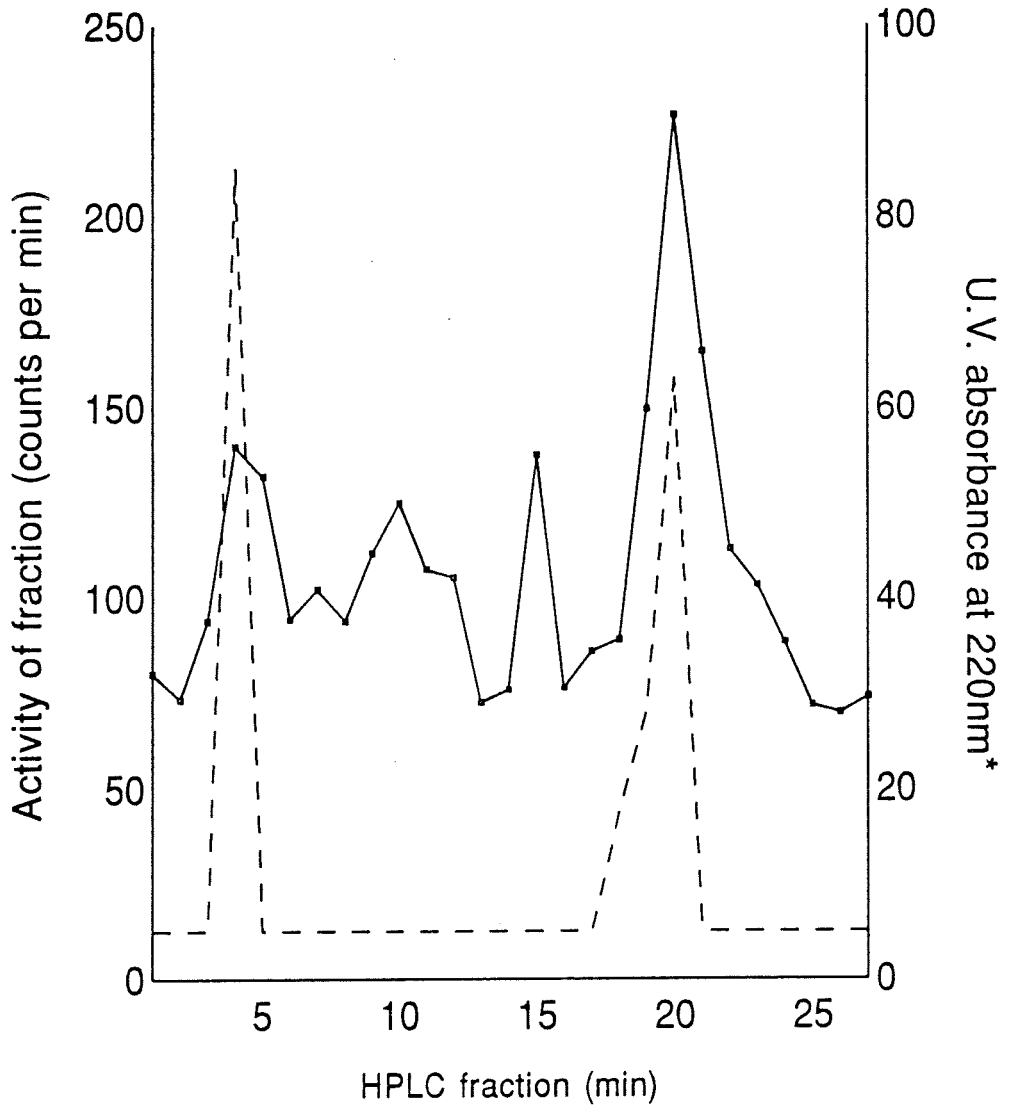
Table 14. Typical *Rf* values from the two-dimensional TLC of product 2C, visualised with iodine vapour and autoradiography (4 day exposure period).

	Method of visualisation	
Direction of running	Iodine vapour	Autoradiography for 4 days
1	0.63 (0.64)*	0.41
2	0.35 (0.37)	0.15

* figures in parentheses are *Rf* values of the standard preparations.

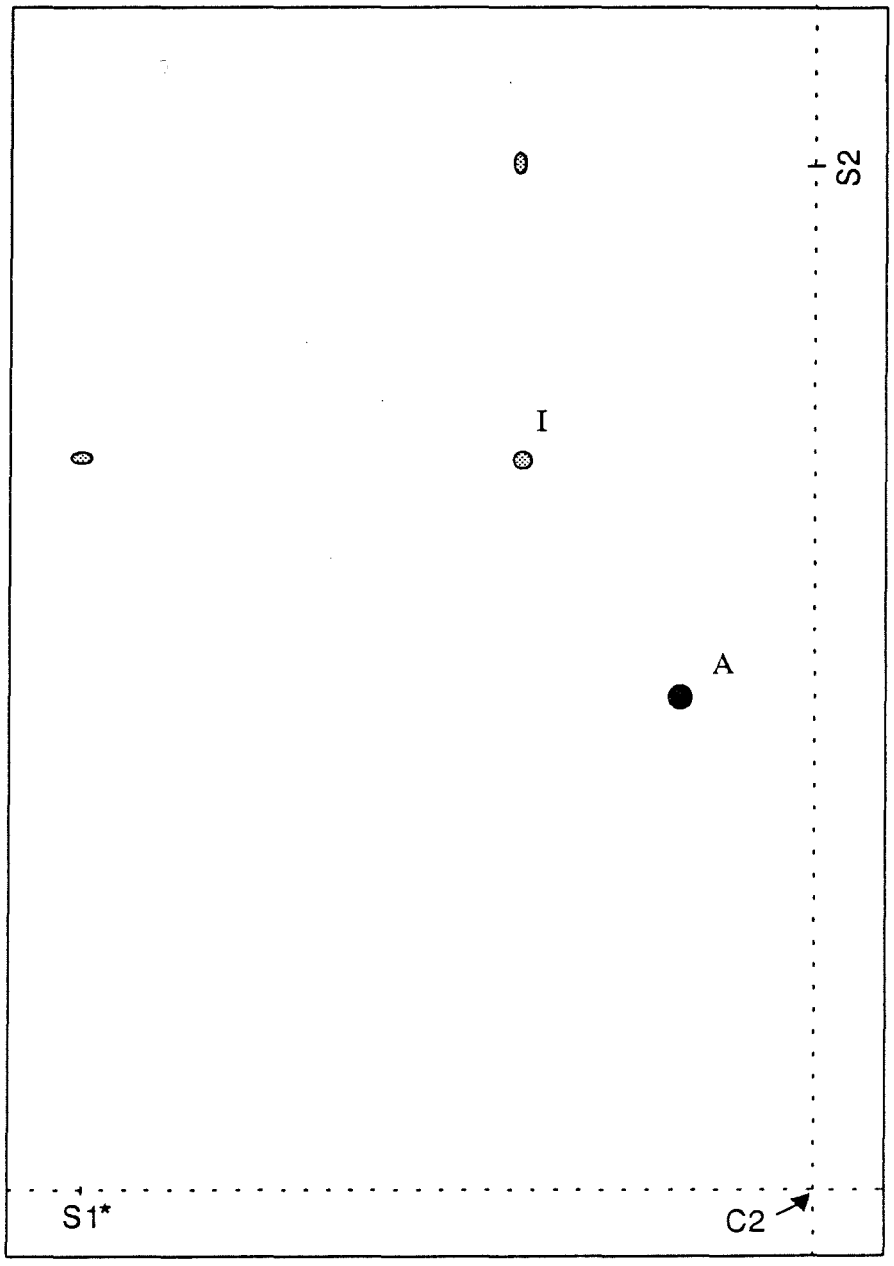
The results were similar for plates developed after 4 or 7days. It appeared that, although azadirachtin had successfully been reformed, the unknown radioactive material detected by autoradiography was more polar than azadirachtin and possessed much higher activity than any radiolabelled azadirachtin which may have been present.

Figure 12. The radioactivity associated with each fraction eluted from an HPLC separation of Product 2C



* U.V. absorbance is on an arbitrary scale

Figure 13. Two-dimensional thin layer chromatogram and autoradiograph of product 2C.



* S1,S2 and C2 are the points of origin of the first and second standards and fraction C2 respectively.

I and A are the spots developed using Iodine vapour and autoradiography respectively.

2.4.3 Qualitative estimation of systemic movement of azadirachtin.

i) *Preliminary analysis*: After development of the TLC plate, six spots were seen. A faint spot with an *Rf* value of 0.38 was seen which co-chromatographed with an azadirachtin standard.

ii) *Standardisation of technique*: As the concentrations of the leaf extract increased, more compounds were detectable by TLC (see Table 15). No compounds were detected with similar *Rf* values to azadirachtin.

Table 15. Number of compounds observed and their relative positions on a thin layer chromatogram of the leaf extracts of *Nicotiana clevelandii*.

Concentration of extract (%) (w/vol)	Number of spots	*Rf* values of spots	*Rf* value of azadirachtin standard
0.59	3	0.05,0.65,0.77	0.29
1.18	5	0.05,0.59,0.62, 0.72,0.78	0.32
2.36	5	0.05,0.59,0.62, 0.73,0.78	0.32
4.72	6	0.06,0.43,0.54, 0.62,0.73,0.78	0.31

Extract concentrations of above 1.18% caused smearing and definition of individual compounds was difficult.

iii) *Definitive qualitative analysis of systemic movement of azadirachtin.*

A purple spot which co-chromatographed with azadirachtin could clearly be seen in the leaf extracts of plants which had previously had their roots immersed in solutions of azadirachtin ranging from 100 to 1000ppm (Figure 14). There were no compounds present with similar *Rf* values to azadirachtin in the untreated plant extract. All compounds that were present in the extract from untreated plants were also present in those from treated plants.

2.4.4 Preliminary quantitative measurement of systemic movement of azadirachtin

A single compound, co-chromatographing with azadirachtin, was detected in all three of the fractions analysed and was quantified using the standard graphs of azadirachtin concentration plotted against absorbance at 220nm. The moisture content of fresh *N. clevelandii* leaves was estimated as 90% (w/w) and, using this figure, the putative azadirachtin concentration in fresh leaf tissue was calculated.

e.g. in the case of the extract from plants which had been treated with 300ppm azadirachtin for 36h:

Quantity of azadirachtin in

sample used for HPLC.....4.48µg/ 20µl

Quantity eluted from Bond Elut...

i.e. in 200µl 4.48 x 10 = 44.8µg

Weight of leaf tissue extracted.....71.1mg

Volume of tissue fluid in leaf...0.9 x 71.1 = 64.0mg

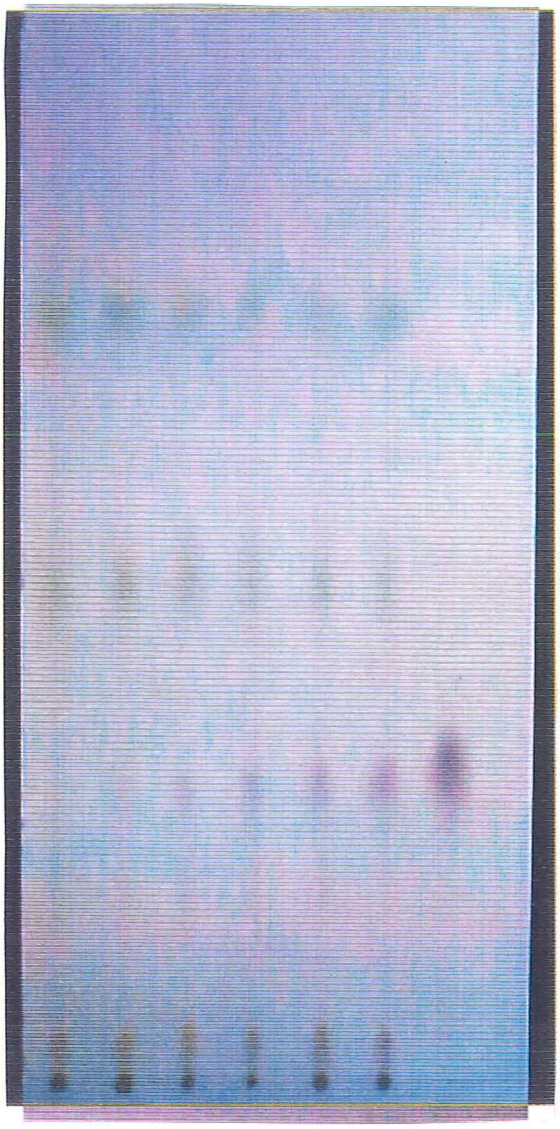
= 0.064 ml

Concentration of azadirachtin in leaf tissue

= 44.8µg in 0.064 ml

= 700ppm.

The total quantity of azadirachtin present in the fractions obtained from plants treated with 500ppm azadirachtin solutions was 151µg, representing a tissue concentration of 1675ppm.



C 1 3 5 10 ss as

C= Control
1,3,5,10= extracts from plants treated with 100,300,500 and 1000ppm azadirachtin solutuions for 36h.
ss= spiked standard
as= azadirachtin standard

Figure 14. Thin layer chromatogram of leaf extracts from N. clevelandii seedlings which had been systemically treated with azadirachtin solutions.

Chapter 3. Assessment of the systemic antifeedant effects of azadirachtin on *Myzus persicae*

3.1 Introduction

It is difficult to quantify the ingestion of plant sap by sucking insects because the stylets are positioned within the plant tissue and are therefore hidden from view. Electrical monitoring of aphid feeding was introduced by McLean & Kinsey (1964) as a method of determining the position of stylets during plant penetration. The original system made use of an AC power source and was developed for use with a DC power source by Schaefers (1966). The DC system was further developed by Tjallingii (1978a, 1985a) and had the advantage over the AC system that signals generated by fluctuations in electromotive force (emf) which arise from potential sources within the system could be distinguished from signals generated by changes in resistance, while those signals generated by the AC system could only be distinguished in terms of resistance fluctuations, (Tjallingii, 1985a). The principle of electrical monitoring is to make the aphid and its feeding substrate (plant or diet) components of an electrical circuit. A small, adjustable voltage is applied to the feeding substrate and the aphid is connected to an amplifier by a fine, flexible wire. When the aphid's stylets penetrate the feeding substrate and the food canal fills with fluid the circuit is complete and, when using the DC system, a signal known as the electrical penetration graph (EPG) (Tjallingii, 1985a) is generated. The EPG can be differentiated into several

waveform patterns characterised by four components; amplitude, frequency, voltage level and electrical origin (Tjallingii, 1988). To date six waveform patterns have been distinguished on the basis of the above criteria. These patterns have been correlated with the location of the aphid's stylets within the feeding substrate and, in some cases, with specific feeding activities (see Table 16).

The patterns A,B and C are seen in succession during the passage of the stylet tips from the plant surface through the epidermal and mesophyll cells. Tjallingii (1978a) has shown that the quantity of ^{32}P-labelled aphid saliva secreted into an artificial diet during feeding was closely correlated with the duration of A,B and C patterns and that a close correlation also exists between pattern B and the formation of the salivary sheath. Pattern C is characterised by the incidence of brief, repeated potential drops (-100 to -180mV) which are associated with stylet puncture of the protoplasts of epidermal, mesophyll or vascular bundle cells (Tjallingii, 1985b). These brief penetrations of the protoplasts may be involved in sap-sampling by the aphids during the stylet pathway to the vascular tissue (Kimmins & Tjallingii, 1985) as there is evidence for the uptake of small quantities of fluid during patterns A,B and C (Tjallingii, 1978a).

Table 16. Summary of main features and correlations of EPG patterns. Amplitude is given relative to pattern A (100%), repetition rate is in Hertz, voltage level is extracellular (e) or intracellular (i) and main electrical origin is either resistance fluctuation (R) or electromotive force (emf). (After Tjallingii, 1990).

EPG pattern[1]	Feature characteristics				correlations	
	rel. amplit.	rep. rate	volt. level	el. orig.	plant tissue	aphid activity
A	100	5-10	e	R	epidermis	electrical on/off stylet contacts
B	75	0.2-0.3	e	R	epidermis/ mesophyll	sheath salivation
C	30	mixed	e	R	all tissues	activities during stylet pathway
pd	.	0.02	i	emf	all living cells	stylet tip puncture
E1(c)	.	2-4	e	emf	unknown	unknown
E1(pd)[2]		2-4	i	emf	sieve elements	unknown
E2(pd)[3]p	5	0.5-4	i	R	sieve elements	(passive) ingestion
w	.	4-9	i	emf	sieve elements	(watery?) salivation
F	5	11-19	e	R/ emf	all tissues	mechanical stylet work
G w	.	4-9	e	emf	xylem	active ingestion
p	0-60	4-9	e	R	xylem	unknown

[1] p = peaks, w = waves, [2]described herein as "unsustained E(pd)", [3]described herein as "sustained E(pd)".

Pattern E occurs on two potential levels representing extracellular or intracellular positioning of the stylet tips. Transmission electron microscopic studies have shown that pattern E is correlated with the positioning

of the maxillary stylet tips within phloem sieve tubes when it is recorded at the lower potential level (E(pd)) (Kimmins & Tjallingii, 1985). When stylets are amputated using high frequency radio microcautery during pattern E(pd) the amputated stylets may yield phloem sap (Mentink *et al.*, 1984) but only if pattern E(pd) has been observed for more than 8min (Kimmins & Tjallingii, 1985). Further evidence of a correlation between pattern E and sap ingestion comes from the observations that, during EPG recordings, honeydew production has only been recorded during pattern E(pd) (Tjallingii, 1990) and pattern E(pd) is the only pattern that has been recorded continuously for more than eight hours. The E(pd) pattern is composed of peaks (0.5-4Hz) and waves (4-9Hz). Using electromyograms from the muscles of the salivary and cibarial pumps, Tjallingii (1978a) demonstrated that the peaks coincided with activity of the salivary pump but not the cibarial pump. The uptake of ^{32}P-labelled diet increased with an increase in the frequency of the waves seen in pattern E suggesting that the waves may represent cibarial pump activity (Tjallingii, 1987). During the initial stages of pattern E(pd), there is a period when "non-typical peaks" occur (Tjallingii, 1990). These peaks differ from the "typical" peaks in sign, shape, amplitude constancy and electrical origin. Non-typical peaks are also seen throughout the extracellular E pattern (E(c)) and for this reason pattern E(c) and the non-typical peaks of the initial stages of pattern E(pd) have been re-classified as E1 while the remainder of pattern E(pd)

has been classified as E2 (Tjallingii, 1990). The functional correlation of pattern E1 has yet to be determined.

During pattern F the stylets may be located in the phloem tissue, but not within the sieve tubes (Mentink *et al.*, 1984). Further electron microscopic studies have shown that the stylet tips are positioned in cell walls during pattern F (Tjallingii, 1987). When pattern F was displayed by aphids feeding on sections of stem from *Vicia faba* labelled with ^{32}P-labelled solution, the uptake of the isotope by the insects was ten times higher than during patterns A,B and C but not as high as in pattern E(pd) (Tjallingii, 1987). Electron microscopy has also revealed that during the recording of pattern G, the aphid's stylets were located within xylem vessels (Tjallingii, 1987) and active ingestion at approximately double the rate recorded during E(pd) patterns was seen.

In the following work the EPG method has been used to examine the feeding behaviour of *M. persicae* on the foliage of tobacco seedlings which had their roots immersed in azadirachtin solutions and on seedlings which had been sprayed with the compound.

3.2 Materials and Methods

3.2.1 Preliminary experiments.

Two preliminary experiments (*a* and *b*) were conducted to find the optimum pre-treatment which would minimise the variability of the feeding behaviour of aphids used in

EPG recording. An experiment (*c*) was also conducted to assess the behavioural effects of the restricted movement imposed by tethering the aphids to the thin gold wire of the EPG setup.

a) *Optimisation of pre-treatments 1.*

Plants: *N. clevelandii* seedlings were raised as described in section 2.3.1. Plants were removed from the potting compost 2-3h prior to the experiment. Their roots were washed with distilled water, then immersed in approximately 1.5ml distilled water in plastic vials. A barrier of Nescofilm prevented contact between the leaves and the root-soaking solution.

Aphids: Adult apterous *M. persicae* were collected from a clonal culture maintained on turnip (*Brassica campestris*) at 17-18^{o}C, 70% rh, L16:D8. All manipulations of aphids were performed using a damp, camel hair paintbrush.

The aphids were placed in a Petri dish for 1, 2 or 3h before they were prepared for EPG recordings by attaching them, with a water-based conducting silver paint (DEMETRON, Gesellschaft fur Electronik-werkstoffe m.b.H., Hanau, Germany) to a 3-4cm length of thin (25µm diameter) gold wire which was attached at its distal end to the amplifier. When paint had dried (approximately 5min) the aphids were placed on the mid vein, in the centre of the underside of the second true leaf of each plant. EPG recordings were made from each aphid for 1h after the

aphid had been placed onto the plant. One EPG was recorded from each of five aphids per starvation period.

EPG recording: EPG recordings were made using a single EPG amplifier (Murphy Developments, Hilversum, The Netherlands) arranged in a Faraday cage. The amplifier gain of this equipment was x50, and the input resistance was $10^9\Omega$. Amplifier output was exported from the Faraday cage by a shielded lead connected directly to one channel of a twin channel chart recorder (Washington Oscillograph 400 MD/2, Palmer Bioscience, Sheerness, Kent, U.K). A flexible fibre optic cable provided light in the Faraday cage from an external cold light source.

EPG Analysis: The EPGs were interpreted using the characteristics of each pattern shown in Table 16. The time taken prior to the initiation of the first probe (A pattern) and the number of probes initiated during the recording period were recorded as measures of the ability of the aphids to settle. The proportion of aphids which initiated pattern E(pd) was taken as a measure of the ability of the aphids to feed normally once settled.

b) Optimisation of pre-treatments 2.

Plants: As above.

Aphids: *M. persicae* were collected from a culture as above, but were not starved. The aphids were immediately

attached to thin gold wires (3-4cm) which were connected to the amplifier. After allowing for the paint to dry, the aphids were placed onto potted 3wk old *N. clevelandii* plants for 1, 2 or 3h to allow them to acclimatise to the restricted movement imposed by the tethering wire and any effects of the conducting paint (see below). The aphids were then removed from the untreated plant, and placed in a Petri dish for 30min to allow the repositioning of the stylets in the labial groove, in case they had been displaced during removal from the leaf.

EPG recording: EPGs were recorded for 90min using the equipment described previously.

EPG analysis: The ability of the aphids to settle was assessed by the number of probes initiated. The ability to feed normally was determined by the proportion of aphids initiating E(pd) patterns and by the time taken for the aphids to initiate this pattern.

c) *Tethering effects*: One of the constraints of the EPG method for studying aphid feeding behaviour is the restriction on locomotion caused by tethering the aphid to the input probe of the amplifier (Tjallingii, 1986). An experiment was performed to assess the effects of tethering using a modification of previously published methods (Tjallingii, 1986; Spiller, 1988; Montllor & Tjallingii, 1989). Aphids were attached to a short length (approximately 0.5cm) of fine gold wire with silver

conducting paint. The aphids were then given 3h access to untreated *N. clevelandii* seedlings. After this pre-treatment, single aphids were placed on to the same leaf positions on treated or control *N. clevelandii* plants (see section 3.2.2) as those used for EPG recording. The plants were illuminated in the same manner as that used for EPG experiments. The aphids were examined at 15min intervals for 3h using a binocular microscope. Those that remained stationary with the rostrum held at right angles to, and touching, the leaf surface continually for a 10s observation period were recorded as being in a 'probing position'. For each observation, the number of aphids recorded in this position was compared with the number of tethered aphids from the same treatment that were involved in any feeding activity during the first 3h of the subsequent EPG recordings (see section 3.2.2). Ten replicates were performed of each aphid/concentration combination. The mean number of probing aphids at each observation in the tethered and non-tethered groups was compared using Fisher's exact test.

3.2.2 Quantitative assessment of the systemic antifeedant effects of azadirachtin on *M. persicae* using the EPG method.

Plants: *N. clevelandii* seedlings were raised and treated with azadirachtin or control solutions as described in

section 2.3.1, with the exception that the plants were treated with the test solutions for 27h instead of 36h. Azadirachtin, at concentrations of 100,300,500 or 1000ppm, was applied to ten seedlings per treatment.

Aphids: Aphids were collected and given 3h of the pre-treatment as described above (section 3.2 *b*).

EPG recording: Simultaneous EPG recordings from two aphids were made using two identical EPG amplifiers. Two flexible fibre optic cables provided light in the Faraday cage from an external cold light source.

Seedlings treated with azadirachtin or control seedlings were placed, in a Faraday cage, on mountings which could be rotated and tilted for ease of aphid observation. Light from the flexible fibre optic cables was trained onto the upper surface of the leaves of the plants.

An EPG was recorded from each aphid for 9h starting when the stylets first penetrated the leaf. Ten replicates, each of one aphid on one plant, were recorded for each concentration of azadirachtin, and from aphids on control plants. The EPG records were examined and the wave forms categorised as described above.

EPG Analysis: The duration and frequency of occurrence of the following categories of feeding behaviour were analysed on treated and untreated plants:

1. Pathway activities in all tissues, comprising patterns A,B and C .

2. Penetration of phloem sieve elements, as indicated by "sustained E(pd)", i.e. pattern E at a low potential level for more than 8 minutes, and "unsustained E(pd)" patterns, resembling the above, but lasting less than 8 minutes. (Kimmins & Tjallingii, 1985).

3. Non-penetration activity; no electrical contact between the aphid stylets and the plant.

The proportions of time that aphids in each treatment group devoted to the various categories of feeding behaviour were analysed over the 9h recording period.

Effects of azadirachtin on behaviour prior to sieve element contact: The following parameters were analysed:

1. The number and duration of individual periods of non-penetration prior to the first E(pd);

2. The total time taken to reach a sieve element from the start of the recording;

3. The time taken to reach a sieve element during the first "successful probe" i.e. that probe in which E(pd) pattern was first recorded;

4. The proportion of all probes which were successful;

5. The proportion of time that aphids devoted to non-penetration, pathway patterns and sieve element penetration during the first hour of recording.

Effects of azadirachtin on behaviour after sieve element contact: The effects on aphids of imbibition of phloem

sap from azadirachtin-treated plants were examined by analysing:

1. The duration of the first period of non-penetration after the first sustained E(pd);
2. The time taken to reach the sieve elements during the first successful probe after the first sustained E(pd);
3. The proportions of time during each of the three successive 3h time intervals that aphids in each treatment group devoted to non-penetration, pathway patterns and sieve element penetration.

The percentages of the EPG recording periods devoted to each pattern and the mean percentages of probes initiated which reached phloem tissue were angularly transformed prior to analysis of variance. A square root transformation was used for: i) the number of probes initiated during the recording period; ii) the number of periods of non-penetrating activity prior to the first sieve element contact, and iii) the numbers of sustained and unsustained E(pd) patterns. Linear regressions were made using azadirachtin concentration as the independent explanatory variable and the square root-transformed values as the dependant response variables. Untransformed values of i) the duration of sustained E(pd) patterns; ii) the duration of periods of non-penetration prior to the first sieve element contact and; iii) the times taken to reach the sieve elements were examined by regression analysis or analysis of variance. The durations of the first periods of non-penetration after the first

sustained E(pd)s were transformed to $\log_{10}$ and examined by analysis of variance.

3.2.3. Quantitative assessment of the feeding behaviour of *M. persicae* on tobacco seedlings sprayed with azadirachtin.

Aphids; As in section 3.2.2.

Plants; N. clevelandii seedlings were raised as described in section 2.3.1. The plants were removed from their potting compost, and their roots were washed and then immersed in distilled water immediately prior to the application of treatments.

Solutions of azadirachtin (500ppm concentration) were prepared in 2% ethanol, 0.02% Tween 20 in distilled water. Control solutions were prepared by omitting the azadirachtin. A fine spray of the solutions was applied to the plants using an airbrush kit ("Aerograph sprite", Letraset U.K. Ltd., London). The treatments and controls were sprayed on to both upper and lower surfaces of the leaves until run off. The plants were then kept for 24h at 17-18 ^{o}C, 70% rh, L18:D6, prior to their use.

EPG recording; Recordings were made simultaneously from aphids on treated and control plants for 9h. Five replicates were performed.

EPG Analysis; The proportions of the recording period devoted to non-penetration, pathway patterns (ABC) and sieve tube penetration (E(pd)) were compared using parametric (paired comparison t-tests) and non-parametric (sign test) analyses on the untransformed data. The number of probes initiated was square root-transformed prior to analysis by a paired comparison t-test. The results were also compared with those obtained using systemic applications in experiment 3.2.2, though, as the experiments were entirely separate, no statistical comparisons could be performed.

3.3 Results

3.3.1 Preliminary experiments

a) Optimisation of pre-treatments 1: Starvation of the aphids prior to attachment to the input probe did not enhance their ability to probe during a 1h EPG recording period (Table 17). Aphids usually terminated probes before E(pd) patterns were initiated. An increased number of probes was assumed to indicate increased restlessness and the inability to locate or penetrate phloem sieve tubes.

Table 17. The effects of starvation prior to electrode attachment on aphid probing behaviour during a 1h EPG recording period.

Probing Behaviour	Starvation period (h) 1	2	3**
Time to first probe (min)	4.2 (1.1)*	2.1 (0.8)	12.3 (7.8)
Number of probes initiated	5.6 (1.1)	7.6 (1.7)	4.7 (1.3)
Proportion of aphids initiating pattern E(pd)	0.4	0.2	0.3

* Figures in parenthesis are standard errors

** Values in this column are means based on three replicates.

b) Optimisation of pre-treatments 2: Aphids which had been connected to the input probe and allowed access to an untreated plant for 3h prior to EPG recording showed a consistent ability to settle and to locate and penetrate phloem sieve tubes a short time after EPG recording was commenced (see Table 18).

Table 18. The effects on aphid settling and probing behaviour of wiring and plant access prior to 90min EPG recordings.

Probing behaviour	Access period after attachment (h) 1	2	3
Number of probes initiated	7.4 (2.8)*	6.8 (1.9)	3.8 (1.2)
Proportion of aphids initiating E(pd)	0.6	0.6	0.8
Time to start of first E(pd) (min)	52.6 (12.6)	55.5 (18.7)	20.5 (3.7)

* Figures in parenthesis are standard errors.

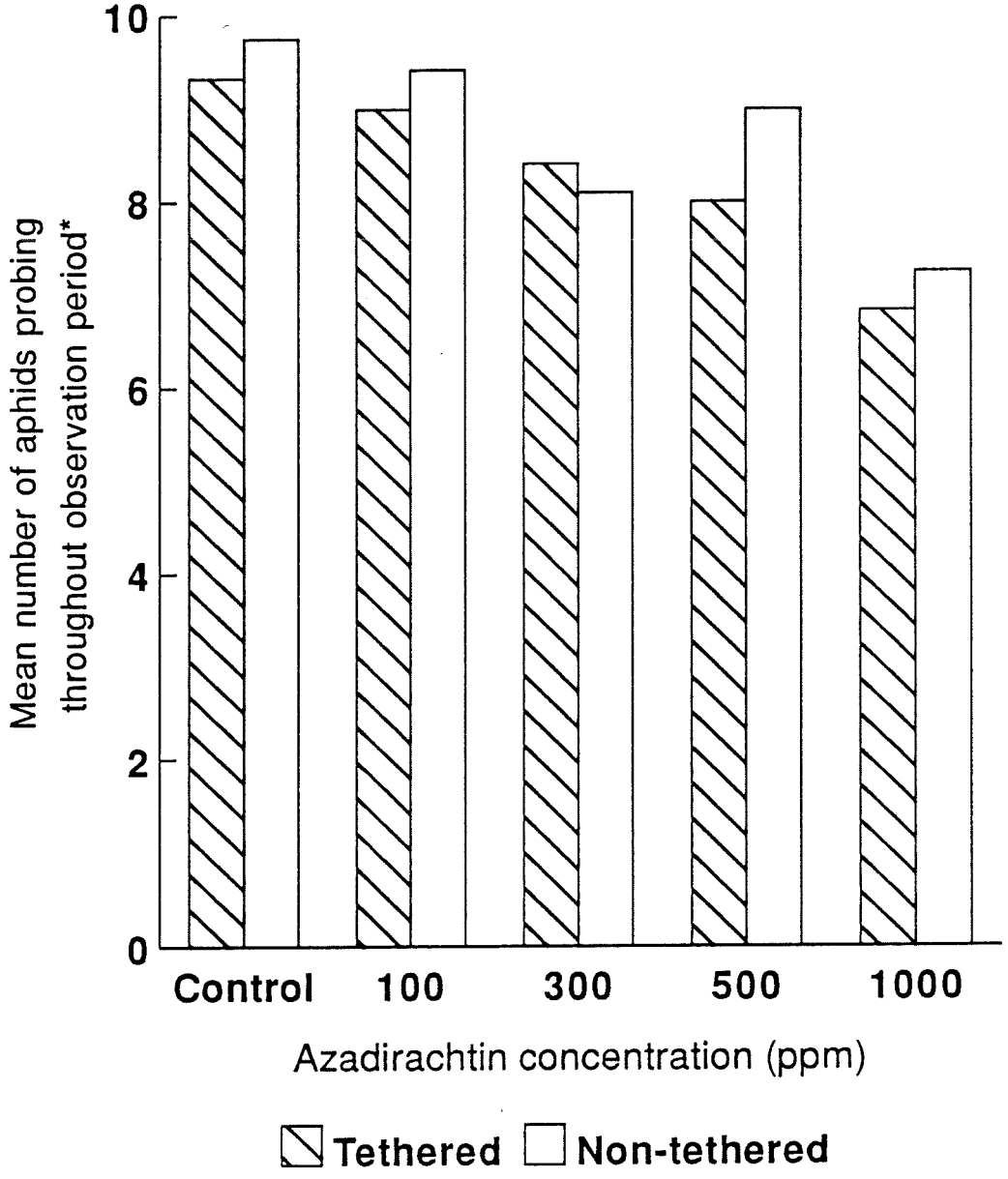
c) *Tethering effects:* The probing behaviour of tethered and non-tethered aphids was very similar within each treatment. This is shown in Figure 15 where the average numbers of aphids probing on treated and untreated plants were obtained by dividing the sum of the number of aphids seen in the probing position (or recorded as probing from the EPG) at the various observations for all plants of a treatment by the product of the number of plants and the number of observations (Bindra & Sylvester, 1961).

Significant differences between the numbers of tethered and non-tethered aphids that were probing were observed in only three of the sixty comparisons (5 treatments X 12 observations). These occurred first at 15min when significantly more non-tethered than tethered aphids were probing on plants treated with azadirachtin at a concentration of 1000ppm. At 30min, significantly more tethered than non-tethered aphids were probing on plants treated with azadirachtin at a concentration of 500ppm. However, at 45min significantly more non-tethered than tethered aphids were probing on these plants.

3.3.2 Quantitative assessment of the systemic antifeedant effects of azadirachtin on *M. persicae* using the EPG method.

EPG analysis: 9h period; Patterns F and G rarely occurred in the EPGs, and were not analysed. Non-penetration behaviour, i.e. the time elapsed between the

Figure 15. The effects of tethering on the probing behaviour of M. persicae on N. clevelandii



$$\text{* Mean number} = \frac{\text{No. probing at each observation}}{\text{No. of plants x No. of observations}}$$

withdrawal of the stylets from the leaf tissue until the re-establishment of electrical contact at the start of the next probe, increased as the concentration of azadirachtin in the root soaking medium was increased (Table 19).

As the azadirachtin concentration was increased, the percentage of the total recording period devoted to A,B, and C patterns increased also.

The number of probes initiated, analysed after square root transformation, increased significantly ($P<0.001$) as the concentration of azadirachtin increased, (Figure 16). Increasing the concentration of azadirachtin significantly increased ($P<0.001$) the numbers of sustained and unsustained E(pd)s (Figure 17), but the percentage of the total recording period devoted to E(pd) decreased with increasing azadirachtin concentration (Table 19) because the mean duration of each sustained E(pd) decreased with increasing azadirachtin concentration (Figure 18). Short, sustained E(pd)s displayed by aphids on treated plants were often interrupted by short (1-3min) periods of C pattern, usually containing several potential drops or, less often, were terminated and followed by periods of non-penetration activity.

Effects of azadirachtin on behaviour prior to sieve element contact: Azadirachtin had no effect on the number of periods of non-penetration prior to the first E(pd), but the duration of individual patterns of non-penetration on 1000ppm-treated plants was significantly

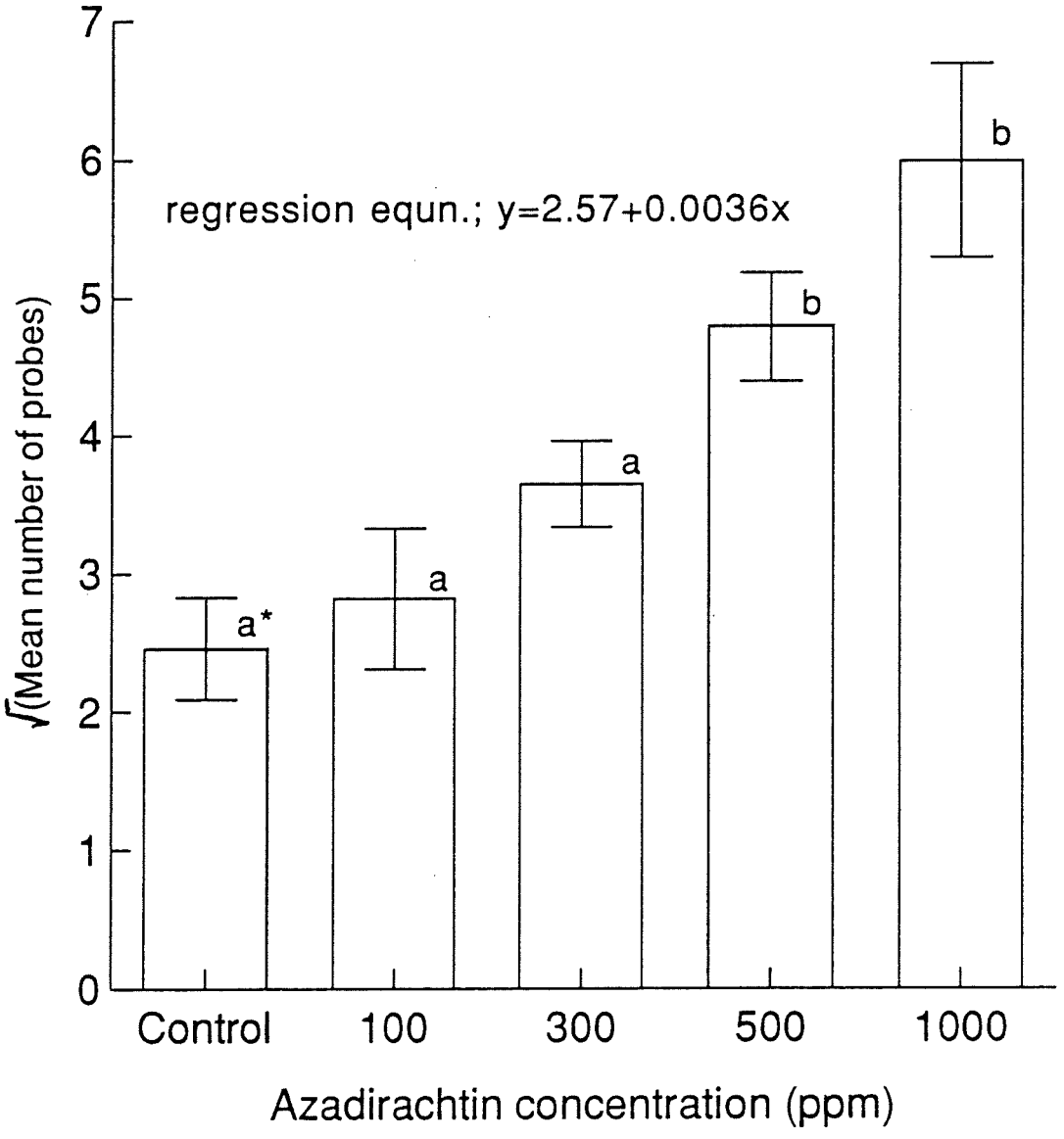
Table 19. Percentage of total recording time devoted to each probing category

Recorded pattern	Azadirachtin Concentration in Treatment					
	Control	100ppm	300ppm	500ppm	1000ppm	s.e.d.
non-penetration	6.9 a* (2.3)**	11.4 a (5.1)	21.4 b (15.8)	37.7 c (39.5)	34.7 c (33.7)	4.75
A,B and C	17.3 a (10.1)	18.1 a (11.7)	24.4 a 18.2)	33.3 b (30.5)	34.2 b (32.3)	3.66
E(pd)	68.5 a (84.4)	63.4 ab (77.1)	51.4 b (59.6)	30.2 c (28.3)	31.4 c (28.9)	6.43

* Values followed by the same letter across rows are not significantly different, (P=0.05)

** Values in parenthesis are detransformed percentages

Figure 16. The influence of azadirachtin concentration on the number of probes initiated by aphids on treated plants.



* Values annotated with the same letter are not significantly different, (P=0.05). Vertical bars indicate 2x Standard Error of Mean.

Figure 17. The influence of azadirachtin concentration on the number of sustained and unsustained E(pd) patterns.

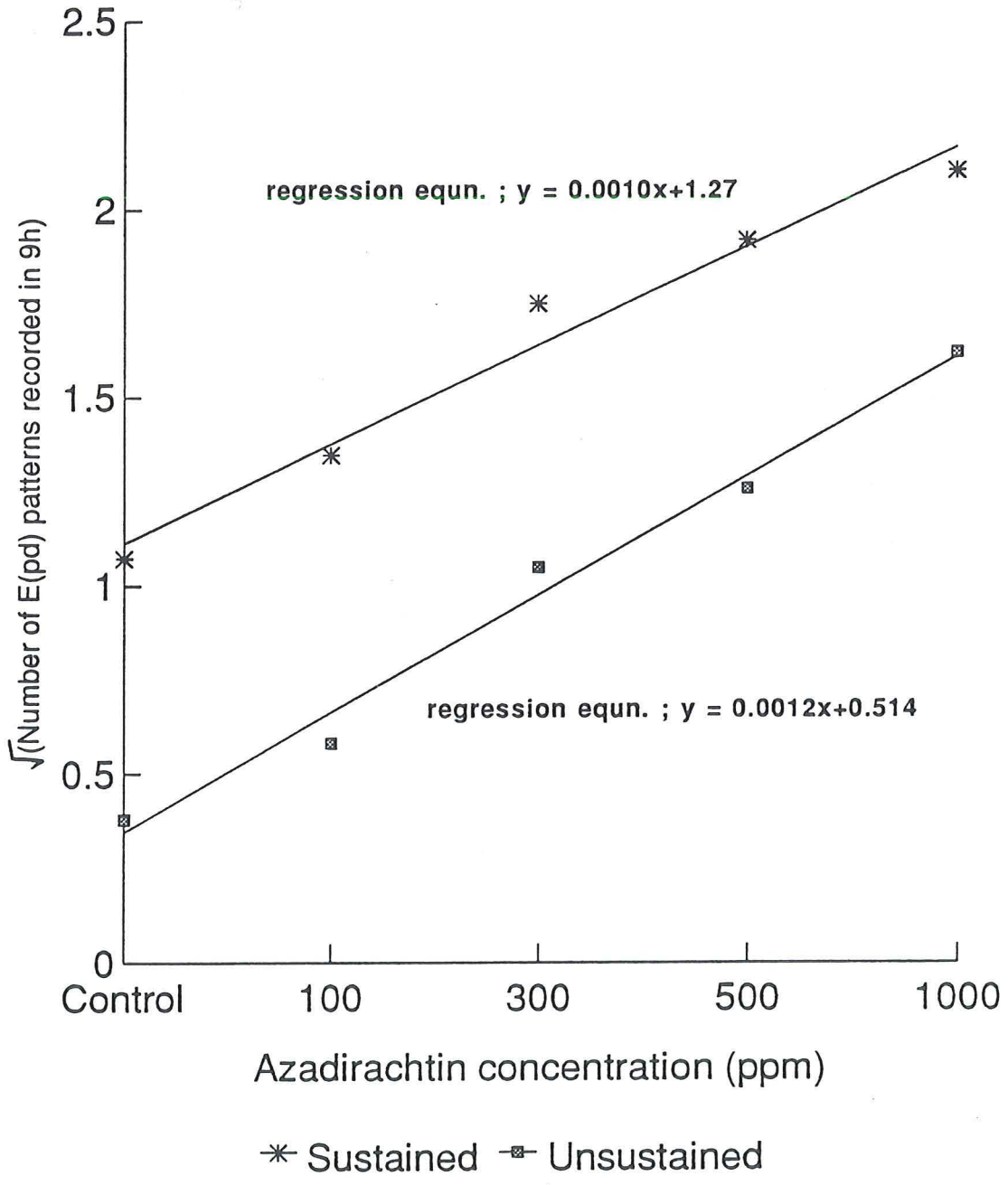
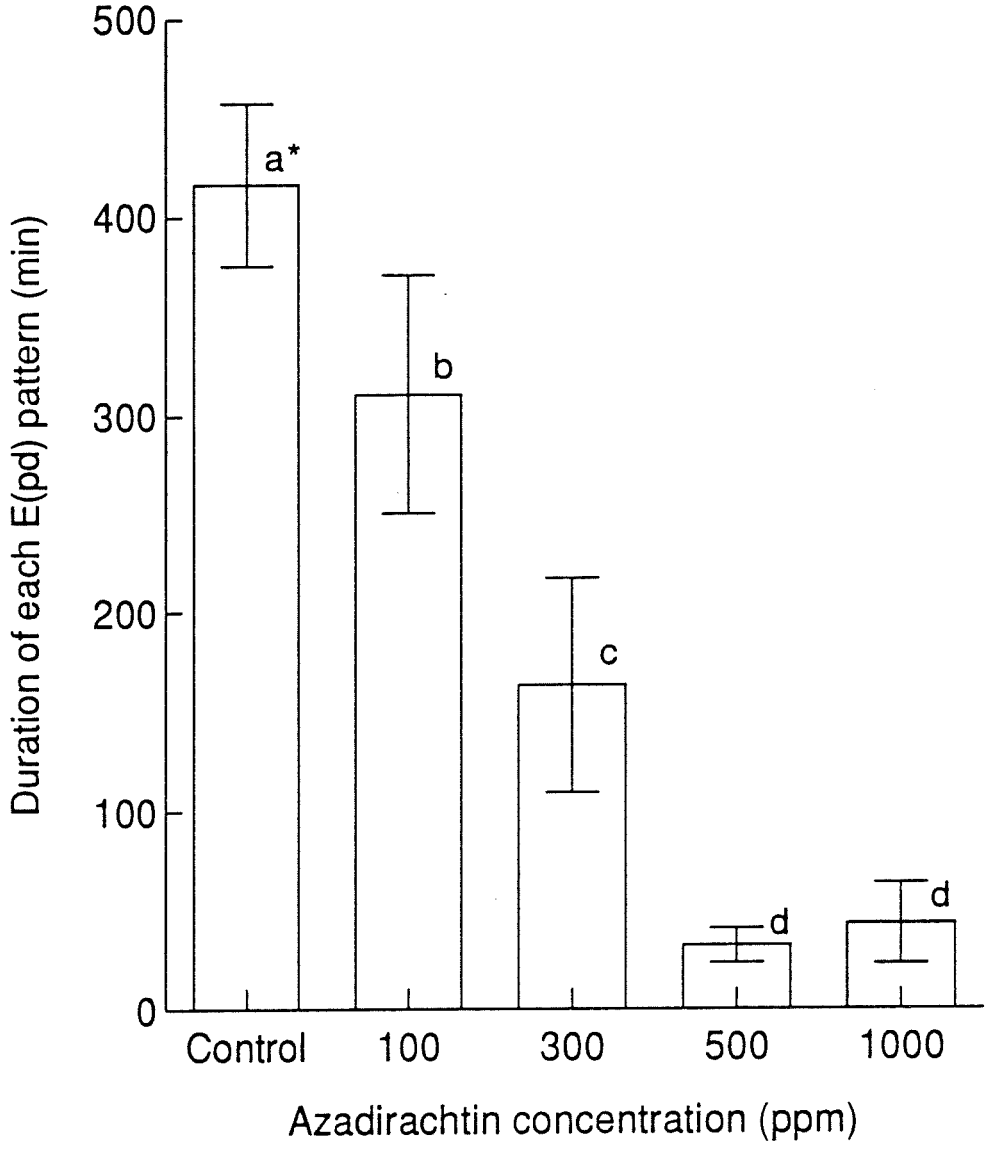


Figure 18. The influence of azadirachtin concentration on the duration of each sustained E(pd) pattern.



* Values annotated with the same letter are not significantly different (P=0.05). Vertical bars indicate 2x Standard Error of Mean.

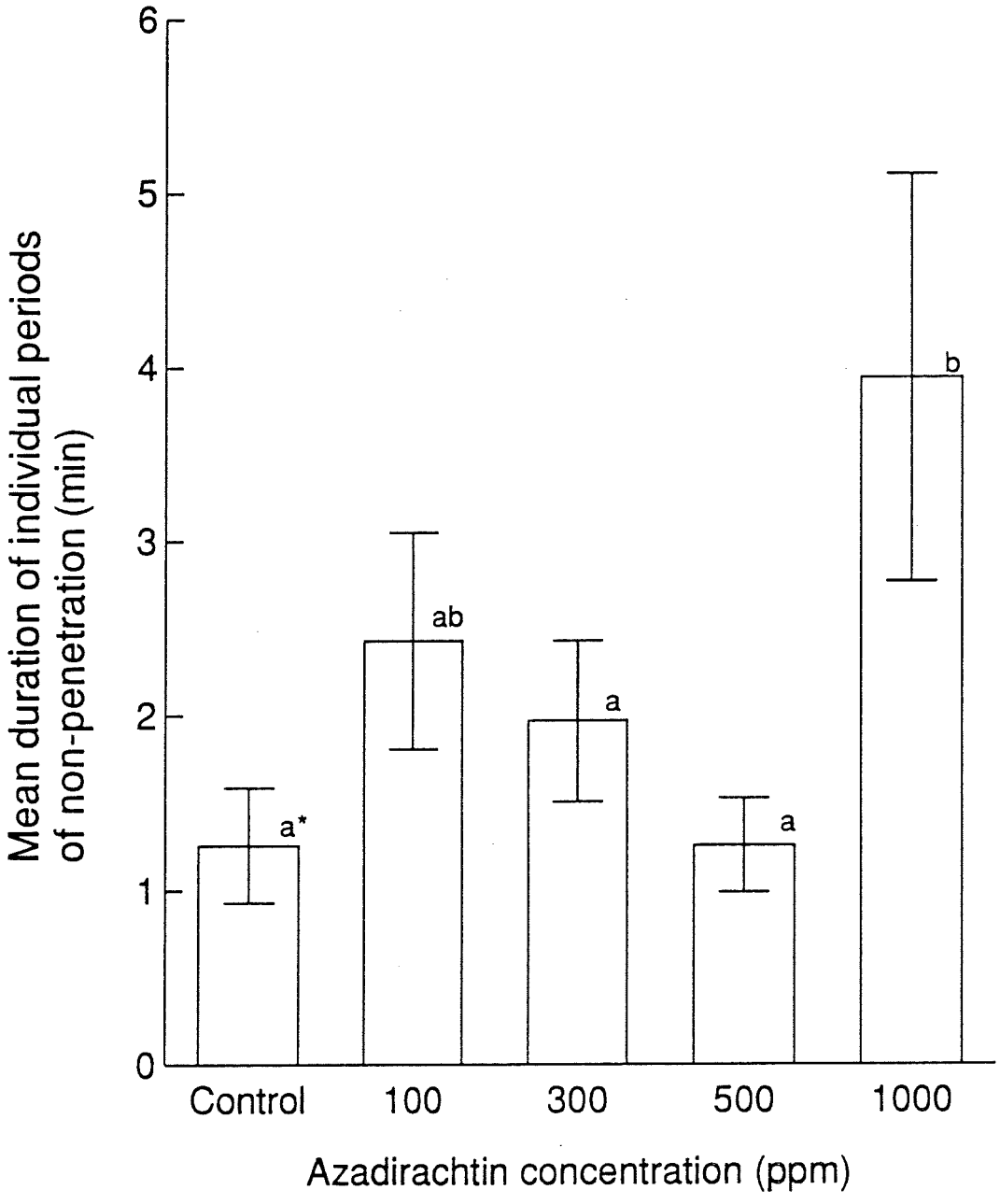
($P<0.05$) longer than that on the control plants (Figure 19). At azadirachtin concentrations above 300ppm a significantly lower ($P<0.05$) percentage of probes ended in pattern E(pd) than in controls (Figure 20). However, azadirachtin had no significant effect on either the time elapsed from the first probe to the first E(pd) pattern or on the duration of pathway patterns prior to sieve element contact in the first successful probe.

During the first hour of recording there was a significant positive relationship between azadirachtin concentration and the proportion of time devoted to non-penetration activity ($P<0.001$). This was accompanied by a significant negative relationship ($P<0.05$) between the concentration of the compound and the proportion of time devoted to pattern E(pd) (Figure 21). The proportion of the first hour devoted to patterns A,B and C was not altered by azadirachtin treatment.

Effects of azadirachtin on behaviour after sieve element contact: On plants treated with azadirachtin concentrations above 300ppm the mean durations of the first non-penetration periods after initial sieve element contact were significantly higher than on control plants or plants treated with 100ppm azadirachtin (Figure 22). However, the duration of pathway patterns in the next successful probe was unaffected.

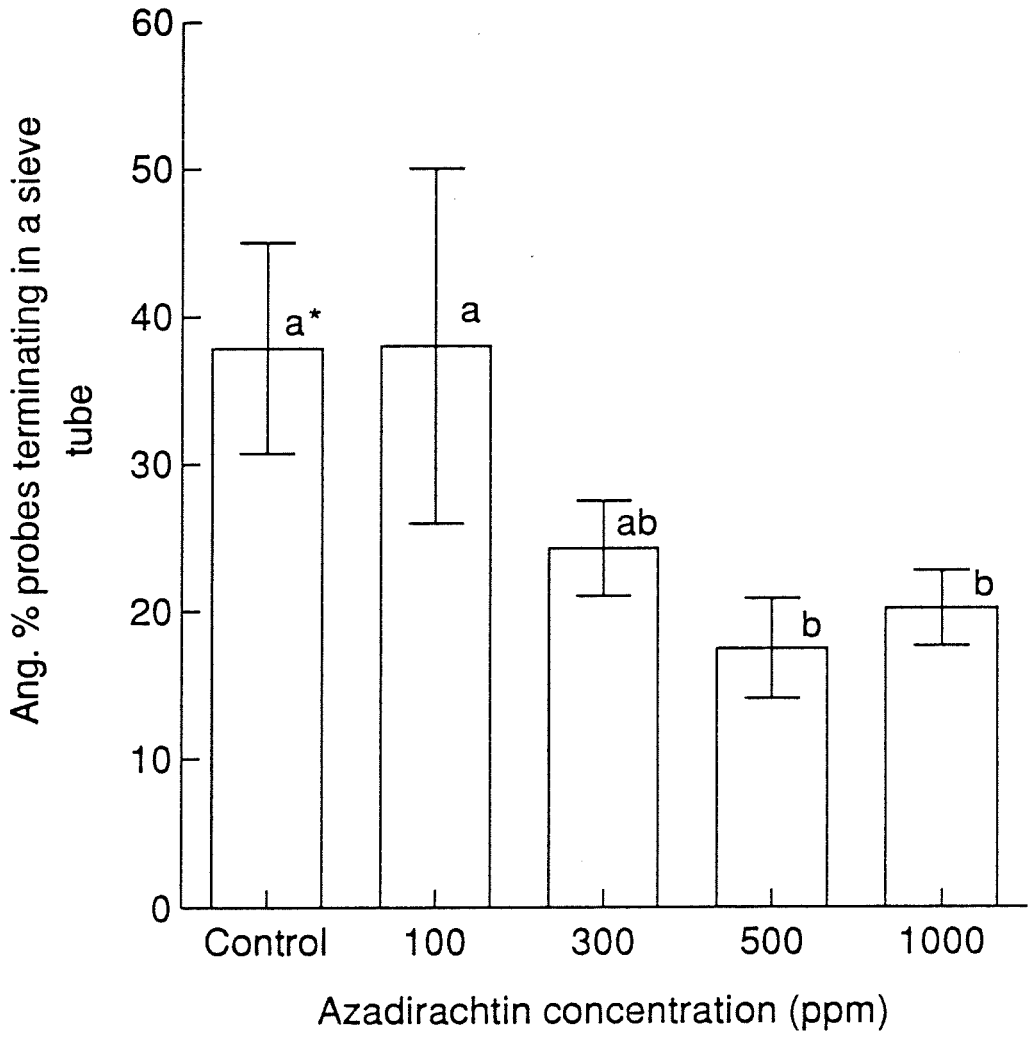
There were significant interactions between the proportion of each 3h time interval devoted to each feeding pattern category and azadirachtin concentrations (Table 20).

Figure 19. The influence of azadirachtin concentration on the duration of periods of non-penetration prior to the first sieve element contact.



* Values annotated with the same letter are not significantly different (P=0.05). Vertical bars indicate 2x Standard Error of Mean.

Figure 20. The influence of azadirachtin concentration on the proportion of probes initiated which penetrated a sieve tube.



* Values annotated with the same letter are not significantly different (P=0.05). Vertical bars indicate 2x Standard Error of Mean

Figure 21. The influence of azadirachtin on the proportion of the first hour of EPG recording devoted to non-penetration and pattern E(pd).

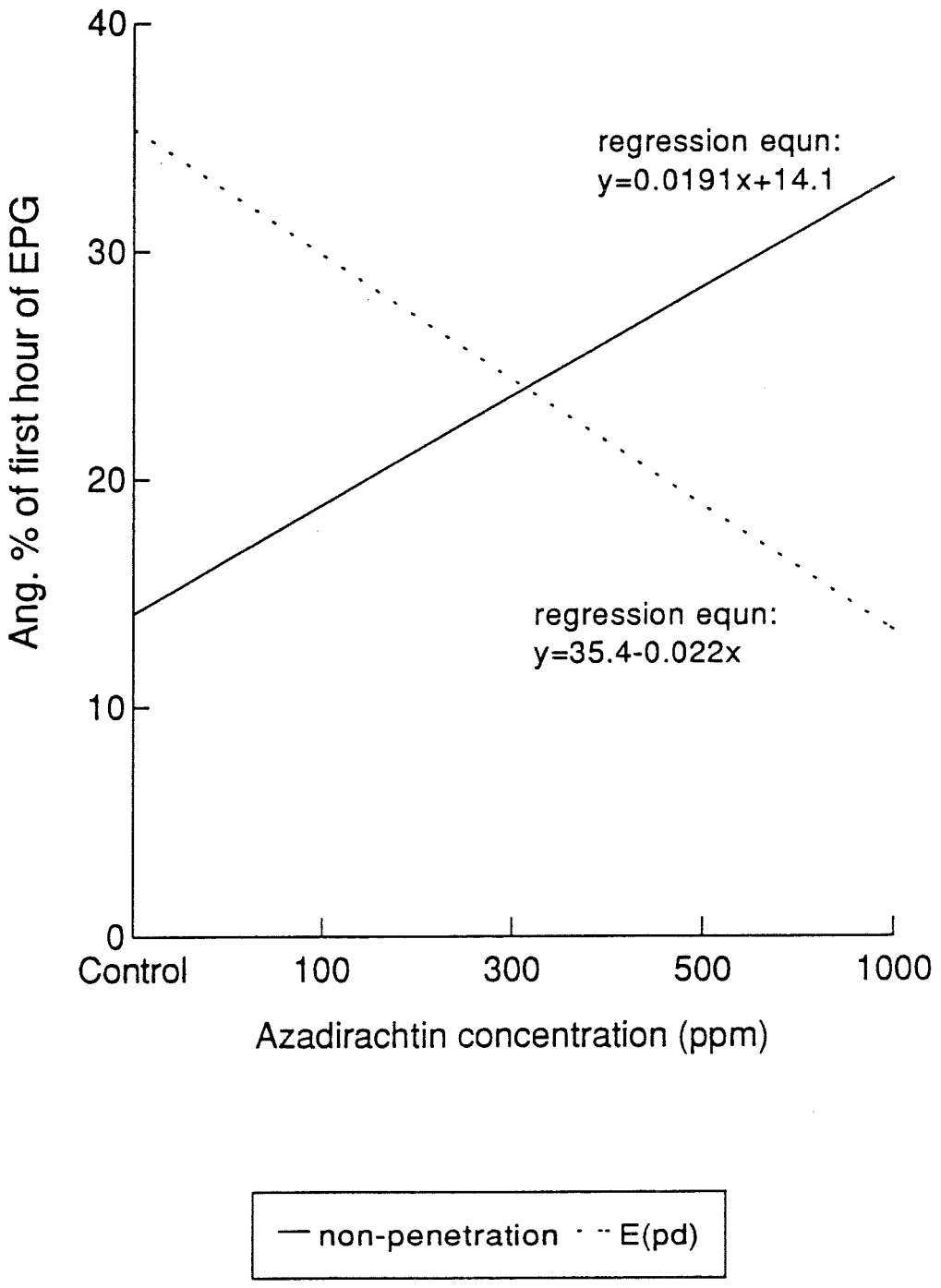


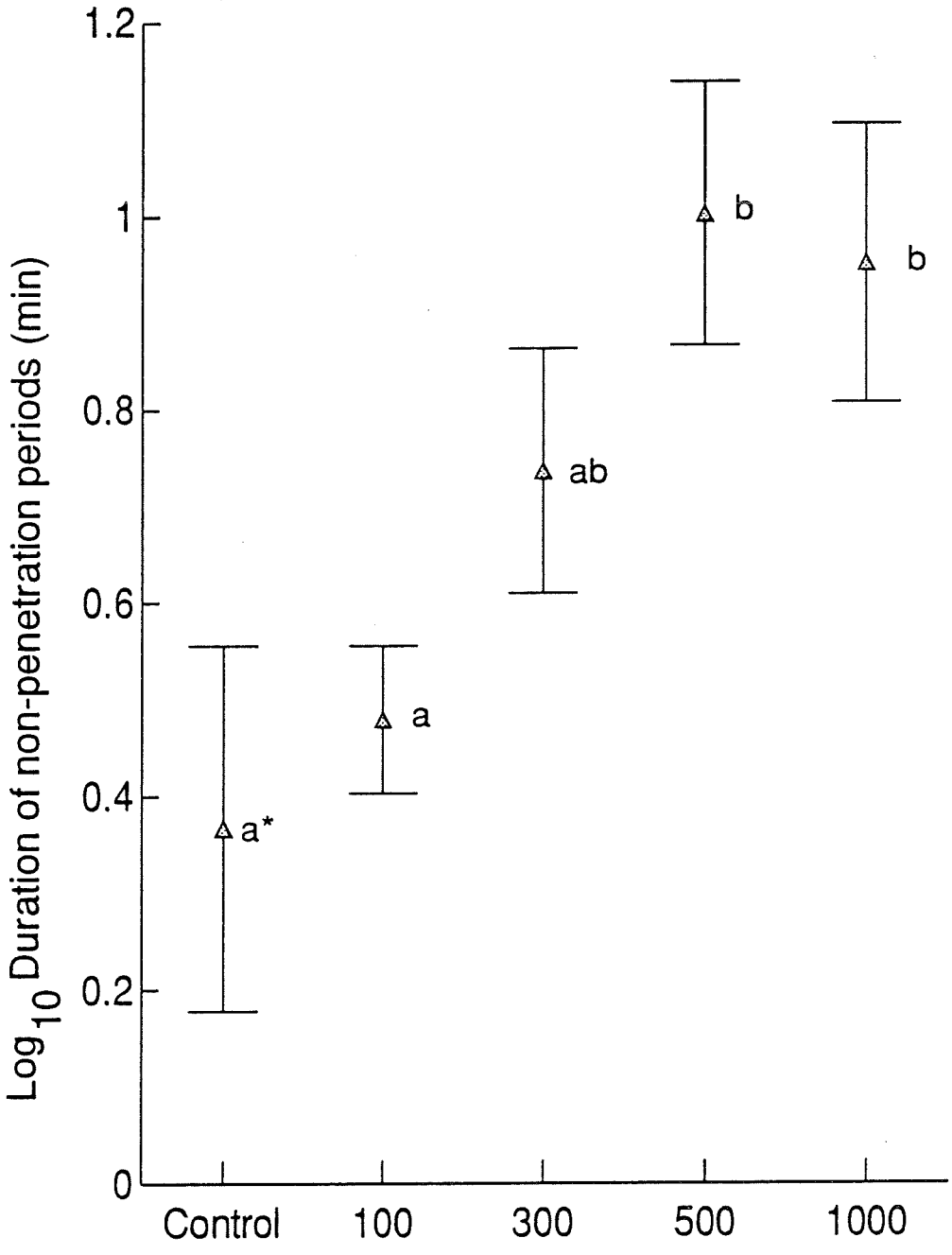
Table 20. Percentage of each successive 3 hour period devoted to each probing pattern category

Recorded pattern	Time period**	Azadirachtin concentration in treatment: Control	100ppm	300ppm	500ppm	1000ppm
non-penetration	1	11.4 a*	15.8 a	21.8 a	22.3 a	29.0 a
	2	0 b	5.1 a	12.1 a	37.1 b	38.7 a
	3	4.7 ab	4.8 a	27.4 b	54.0 c	35.1 a
A,B and C	1	28.2 a	25.0 a	27.9 a	40.1 a	37.6 a
	2	2.3 b	8.1 b	17.2 b	33.8 a	28.2 a
	3	6.5 b	9.9 b	23.5 ab	22.0 b	35.7 a
E(pd)	1	56.1 a	50.6 a	37.9 a	37.0 a	34.1 a
	2	87.7 b	74.0 b	65.2 b	26.8 ab	27.2 a
	3	77.3 b	77.4 b	55.8 ab	16.6 b	28.3 a

*Transformed values followed by the same letter within columns are not significantly different, (P=0.05)

** Time period 1 refers to the first 3 hours of recording, time period 2 to the second three hours, and time period 3 to the final three hours.

Figure 22. The influence of azadirachtin on the duration of the first period of non-penetration after the first sustained E(pd).



* Values annotated with the same letter are not significantly different (P=0.05). Vertical bars indicate 2x Standard Error of Mean.

i) Non-penetration: The proportion of time during which aphids on control and 100ppm-treated plants were involved in non-penetration activities was less during the second and third 3h periods than in the first. On plants treated with azadirachtin at a concentration of 300ppm non-penetration also decreased after the first 3h period but increased during the final period. In contrast, aphids on plants treated with 500 or 1000ppm azadirachtin spent longer proportions of time engaging in non-penetration activity in the second and third periods than in the first.

ii) A,B and C patterns: For most treatments the percentage time devoted to patterns A,B and C decreased in the second 3h period, then increased slightly in the final period. However, in the 500ppm treatment group, the percentage time devoted to these patterns decreased throughout the recording periods.

iii) E(pd) pattern: Aphids on 500ppm and 1000ppm treated plants devoted a greater percentage of time to E(pd) pattern during the first 3h of recording than in the subsequent periods, whereas on control plants and plants treated with lower concentrations of azadirachtin less time was spent in this pattern during the first 3h of recording than in the subsequent periods.

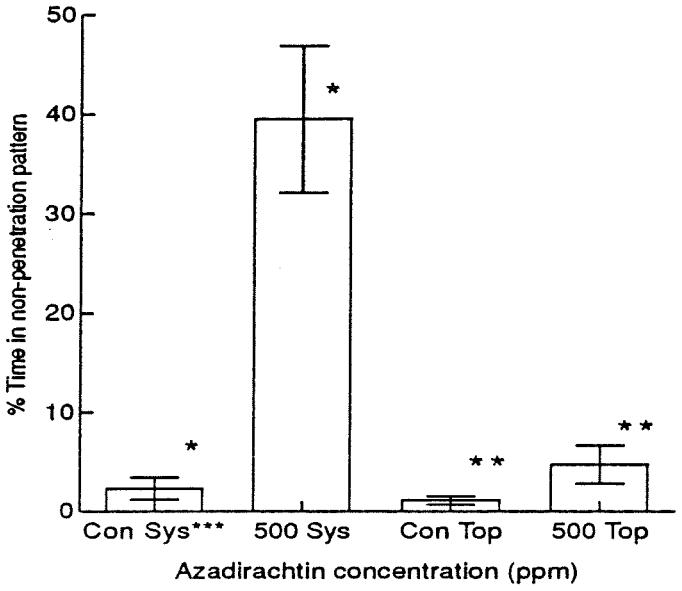
3.3.3 Quantitative assessment of the feeding behaviour of *M. persicae* on tobacco seedlings sprayed with azadirachtin.

The effects of topical applications of 500ppm azadirachtin are compared with the systemic effects of root immersion in azadirachtin at the same concentration in Figures 23-26 overleaf. The values relating to systemic effects are taken from the previous experiment. Standard error bars have been fitted to the data. It can be seen from these figures that azadirachtin does not have the same effects on aphid feeding when applied to the foliage, rather than systemically. The following points are evident when considering the effects of topically-applied azadirachtin on the three main categories of recorded aphid feeding patterns:

i) The percentage of the total 9h recording period devoted to non-penetration activities was similar for both the control group and the treated group with means of 1.1% and 4.7% respectively. When the same concentration of azadirachtin was applied via the roots, the treated group spent on average 39.5% of the 9h period engaging in non-penetration activity, while the value recorded for control group was 2.3%.

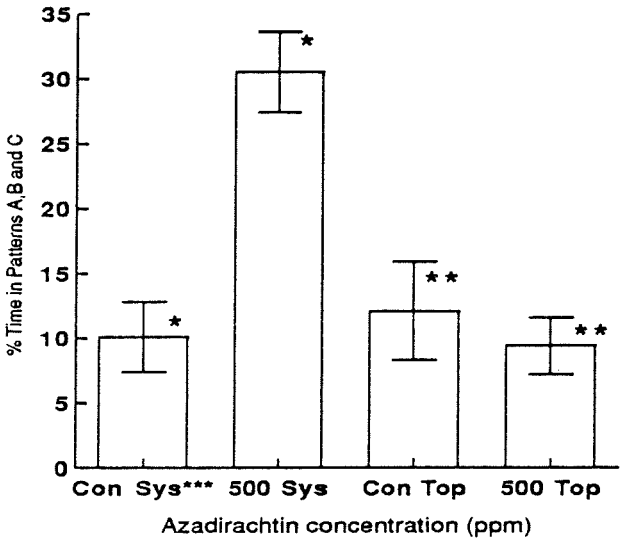
ii) The percentage of the recording period devoted to the patterns A,B and C (taken together as pattern ABC) on sprayed plants was again similar for both the control and treated groups, with means of 12.1% and 9.4% respectively. The values recorded from the comparable

Figure 23. Effect of systemic and topical azadirachtin treatments on the percentage of the recording period devoted to non-penetration.



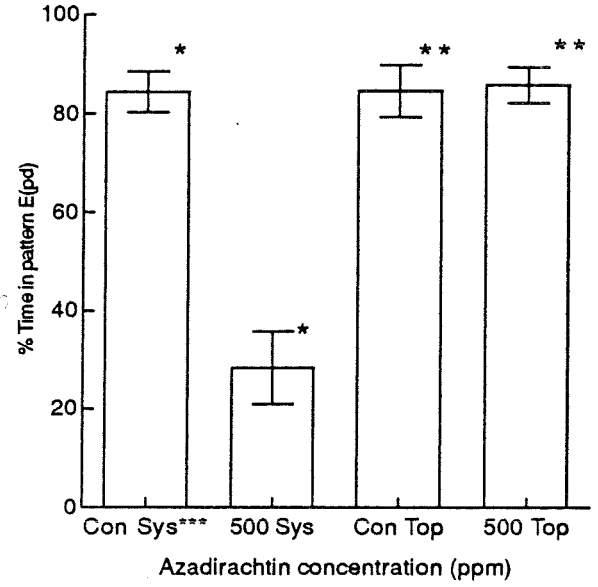
* Mean based on ten replicates
** Mean based on five replicates
*** Sys = systemically and Top = topically applied.

Figure 24. The effect of systemic and topical azadirachtin treatments on the percentage of the recording period devoted to patterns A,B and C.



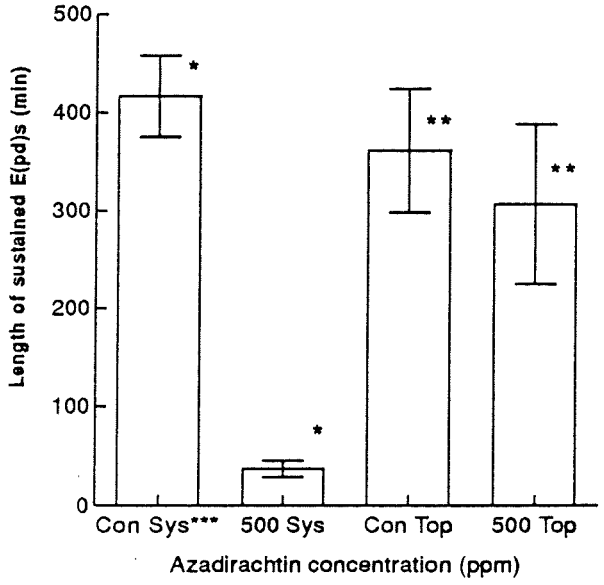
* Mean based on ten replicates
** Mean based on five replicates
*** Sys = systemically and Top = topically applied

Figure 25. Effects of systemic and topical azadirachtin treatments on the percentage of the recording period devoted to pattern E(pd)



* Mean based on ten replicates
** Mean based on five replicates
*** Sys = systemically and Top = topically applied

Figure 26. Effects of systemic and topical azadirachtin treatment on the length of each sustained E(pd) pattern.



* Mean based on ten replicates
** Mean based on five replicates
*** Sys = systemically and Top = topically applied

systemic treatments were; 10.1% in the control group, and 30.5% in the treated group.

iii) The percentage of the recording period spent in pattern E(pd) was also very similar for the control and treated groups with mean values of 84.6% and 85.8% respectively. The values recorded from the comparable systemic treatments were; 84.4% for the control group, and 28.3% for the treated group.

iv) The mean lengths of each individual E(pd) pattern were also compared. Only E(pd) patterns which were sustained for more than 16min were used for the comparisons. Previously, the value of 8min was used. However, as the lengths of a number of the unsustained feeds in the present experiment fell between 8 and 16min, and none between 16 and 39min, it was decided to use 16 min as a cut-off point.

The mean length of sustained E(pd) patterns in the topically-treated groups were 361.3min in the control group and 306.7min in the azadirachtin-treated group. These values were similar and both had large standard errors associated with them. In contrast, when the treatments were applied systemically, azadirachtin treatment caused a substantial reduction in the mean length of individual sustained feeds, from 416.3min in the controls to 37.0min in the treated group.

Topical application of azadirachtin did not affect the number of probes initiated during the 9h recording period. After square root transformation of the number of probes initiated the mean counts (± S.E.) were 2.46 (±

0.32) and 2.26 (± 0.33) for treated plants and control plants respectively.

Chapter 4: The primary and secondary effects of azadirachtin on *Myzus persicae*.

4.1 Introduction

The use of techniques in which aphids feed on chemically-defined artificial diets behind membranes has allowed detailed study of many aspects of aphid feeding behaviour including the effects of plant secondary compounds, systemic insecticides and growth regulators on probing, salivation, uptake, longevity and fecundity (for review see Mittler, 1988). Several quantitative methods employing radioactive isotopes have been used to determine uptake of artificial diet solutions (e.g. Arn & Cleeve, 1971). However, the usefulness of these methods is limited because, after ingestion, the isotope may be excreted in the honeydew, transpired, secreted in the saliva or incorporated into the cuticle which is subsequently shed at ecdysis. While it is possible to measure diet uptake accurately in short term experiments by using radioactive isotopes (e.g. ^{3}H-inulin) that are difficult to metabolise (Wright *et al.*, 1985), information on the uptake of a diet over periods of a day or longer is necessary to determine its overall acceptability (Mittler, 1988). In some cases dyes have been used to determine the uptake of diet, though the dye itself may have deleterious effects on the aphids (Mittler & Dadd, 1963a,b).

Banks & Macaulay (1964) calculated that the total quantity of food ingested by an adult *Aphis fabae* during its lifetime was approximately equal to the total weight of its excreta plus the weight of its progeny at birth. Because the former measure was responsible for approximately 90% of the total, the weight of excreta produced and the rate of excretion (volume in a given period) were considered to be accurate indices of the total food ingested and the rate of feeding respectively.

The use of honeydew production as a measure of food uptake is particularly appropriate for *M. persicae* because:

i) This species excretes only while feeding.

ii) Feeding and excretion occur continuously in light and dark.

iii) The adults do not stop excreting while producing young (Broadbent, 1951).

In the present work, settling and honeydew production were used as measures of aphid feeding on artificial diets which had been treated with azadirachtin at a range of concentrations. The aim of this work was to estimate the theoretical concentration of azadirachtin which would have an antifeedant effect on aphids feeding on phloem sap.

A new quantitative method of measuring honeydew production is described, based on techniques used to quantify amino acids in chromatographic separations (Polson *et al.*, 1947, Thompson *et al.*, 1951, Atfield & Morris, 1961). It was developed from a method used to

quantify the amino acid content of individual honeydew droplets (Auclair, 1958). The honeydew produced by aphids feeding on sachets of artificial diet was collected and reacted with ninhydrin solution, which produces a purple coloured complex with amino acids and amides. The honeydew excreted by *M. persicae* while feeding on artificial diets is known to produce a ninhydrin reaction of much greater intensity than that of honeydew collected from the aphid while feeding on some host plants (Dadd & Krieger, 1968).

Mittler, cited in Auclair (1958), stated that any quantitative measurement of honeydew production must take into account the **frequency** (number of droplets in a given time) and the **rate** of excretion. In the method described herein both criteria are met simultaneously by collecting all the honeydew produced by a defined number of aphids during a given period. In addition, detection of amino acids in the honeydew of aphids placed on sachets of artificial diet demonstrates that the aphids have been feeding on the diets because *M. persicae* does not excrete non-dietary amino acids, (Mittler & Dadd, 1962, Bragdon & Mittler, 1963).

Several recent studies have shown that azadirachtin can affect the digestion and utilisation of proteins in caterpillars which had been treated with the compound (Ayyangar & Rao, 1989, Sridhar & Chetty, 1989, Timmins & Reynolds, 1992). If this were also true for aphids which had ingested azadirachtin then quantitative measurements of feeding, based on the amino acid content of the

honeydew produced by aphids feeding on azadirachtin-treated diets, may give erroneous estimations of the quantity of diet consumed when compared with the honeydew of aphids which had fed on untreated diets. For this reason, preliminary experiments were conducted, first to establish the nature of the relationship between the quantity of diet consumed by aphids and estimates, based on amino acid content, of the amount of honeydew that they produced and secondly to establish whether this relationship also applied to aphids feeding on diets which contained azadirachtin.

4.2 Materials and methods

General:

Aphids: Adult apterous aphids of similar age (11 days ± 24 h), raised from first instar nymphs produced over a 24h period on turnip (*Brassica campestris*) plants, were used in all experiments. Only adults which had begun larviposition were used. The aphids were starved for 6h prior to each experiment. Aphids were raised, and all experiments were performed, in constant conditions as described in section 3.2.1.

Diets: A holidic diet (a chemically defined synthetic diet) was used rather than a meridic or oligidic diet (diets prepared from ingredients of undefined composition) as the latter may not have been accepted so readily when first presented to aphids (Mittler, 1988).

The "Base 30" diet described by Griffiths *et al.* (1975) was used in all experiments and the diet described by Dadd & Mittler (1966) was also used in one initial experiment (see section 4.2.1). The diets were prepared aseptically by the methods of Dadd *et al.* (1967) and Adams & van Emden (1972) and were stored in 20ml aliquots at -20°C until immediately before use. Azadirachtin was dissolved in ethanol (AnalaR grade) and incorporated into the diet just before use. The final concentration of ethanol in the azadirachtin-treated and control diets was 0.5%.

Test chambers: $4cm^2$ sections of Nescofilm were surface-sterilised for 5min 18cm beneath an ultraviolet sterilising lamp (Philips HPW, 125W). All subsequent procedures were performed under aseptic conditions in a laminar flow cabinet at room temperature. A square of Nescofilm was stretched, with the sterilised surface facing up, across one end of a clear acrylic cylinder (internal diameter: 24mm, external diameter: 30mm, height: 22mm) which had been washed in 10% sodium hypochlorite solution and rinsed in distilled water. Suction was applied to the lower surface to form a well which was then filled with 400µl of diet solution. A second Nescofilm square was then stretched across the top of the well, sterilised surface down, to form a sealed sachet. On release of the suction the seal was temporarily broken to exclude air from the sachet which was then re-sealed. The walls and floor of each chamber were lined with chromatography paper (Whatman, No. 1) and

a square of yellow, colour-compensating filter (Kodak CC20Y) was placed above each diet sachet to encourage the aphids to probe (Mittler & Dadd, 1965) (Figure 27).

Measurement of uptake: i) In some of the initial experiments uptake was measured by the weight of diet consumed (weight of test chamber before aphid access minus weight of test chamber after access). In all of these experiments a test chamber to which no aphids were allowed access was included to monitor for any weight changes that may have been induced by environmental factors (e.g. ambient relative humidity) rather than aphid feeding. When weight changes were found in the empty chambers, each of the estimates of uptake was corrected by the appropriate amount. In some cases substantial gains or losses in weight (>5mg) were recorded in these chambers and the reliability of uptake estimates based on weight of diet consumed was considered to be questionable. In cases where the weight gain or loss from these chambers exceeded 1mg the results from those chambers to which aphids had had access were discarded.

ii) Settling: Counts of the number of aphids settled on each diet sachet were made hourly during defined observation periods. Aphids were recorded as "settled" if they remained stationary on the lower surface of the diet sachet for 10s.

iii) Honeydew production: The chromatography papers which lined the walls and floor of the test chambers were removed at intervals and dipped in a freshly prepared

solution of 0.1g cadmium acetate, 1.0g ninhydrin in 10ml distilled water, 2ml glacial acetic acid, 100ml acetone (Atfield & Morris, 1961).

These papers were then suspended in darkness at room temperature for 19h. Ammonia was excluded from the atmosphere in which the papers were held by placing a dish of conc. H_2SO_4 in their immediate vicinity. After this period the purple spots which appeared (Figure 28) were cut from the papers and the reacted honeydew was eluted into a defined volume of 90% methanol (AnalaR) for 1h at 4°C. 1ml of this solution was then centrifuged at 8000 rpm for 2min prior to spectrophotometric analysis using a Shimadzu UV-visible recording spectrophotometer, model UV-260. Absorbance was read at 500nm against a 90% methanol standard. All absorbances were in arbitrary units and were multiplied by 1000 to make the data more manageable.

Additional measurements: The number of adult aphids surviving in each chamber and the numbers of live and dead nymphs produced were counted at intervals throughout the experiments. It was not possible to use these records as indicators of the effects of azadirachtin on feeding because death of adults and reduced fecundity may result from direct toxic effects as well as a reduction in food intake (Schoonhoven & Derksen-Koppers, 1976).

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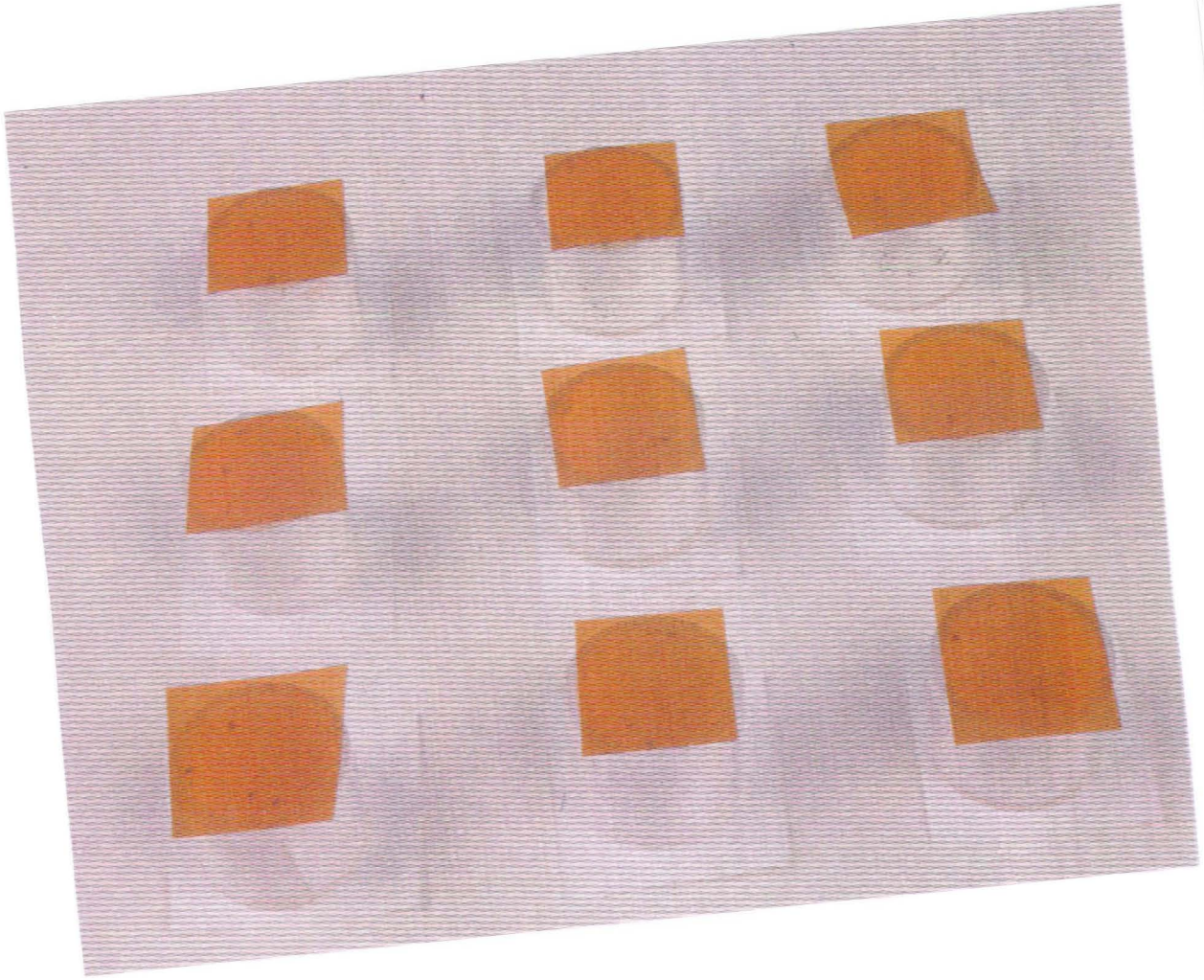


Figure 27. Test chambers used in artificial diet experiments.

Original in colour

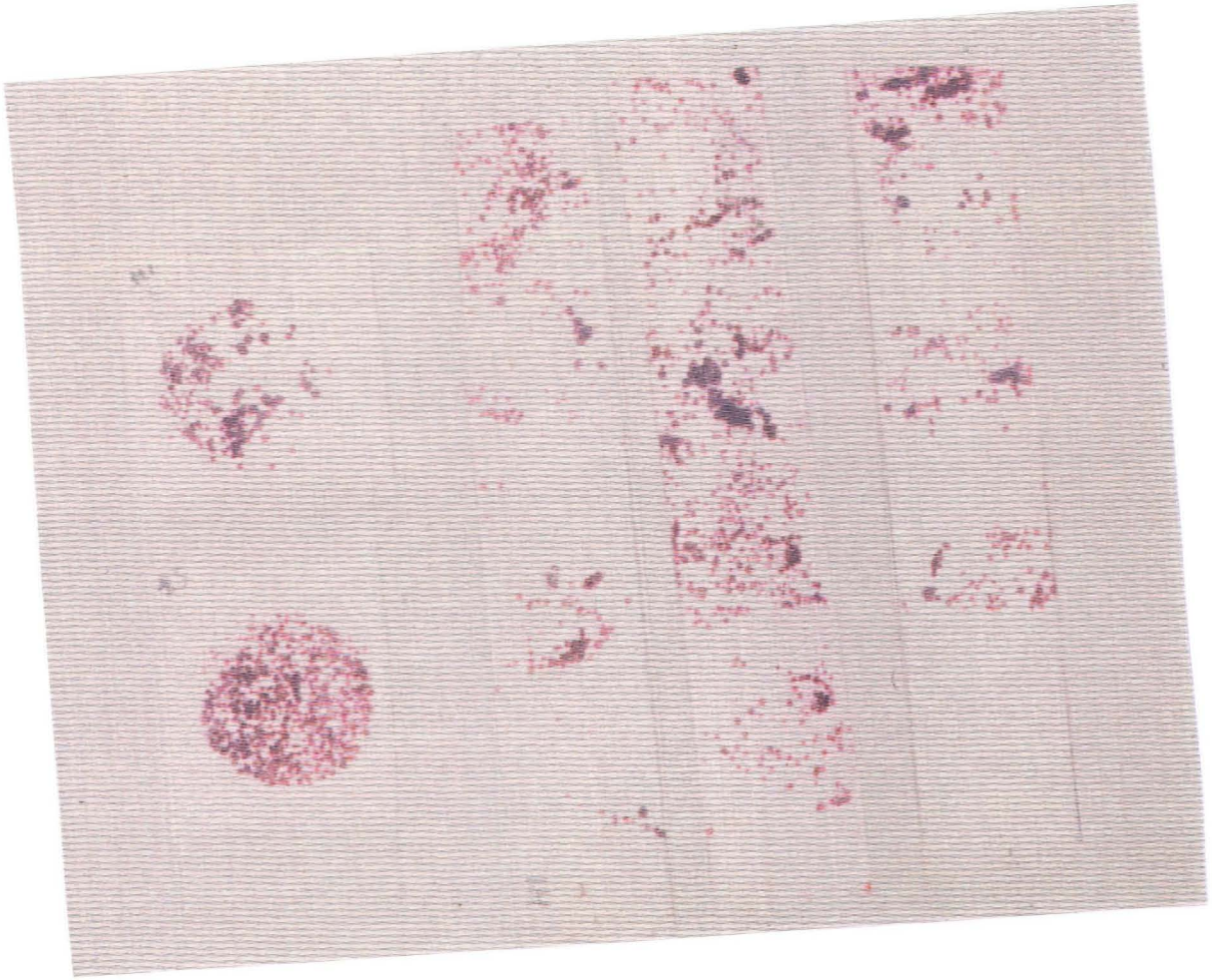


Figure 28. Sections of chromatography paper used to collect aphid honeydew in experiment 4.2.4 (after staining with ninhydrin).

4.2.1. Comparison of the acceptability of two holidic diets.

Test chambers were prepared with sachets of either Base 30 (diet 1) or the Dadd & Mittler (1966) diet (diet 2).

The diets were not treated with azadirachtin or ethanol. The chambers were weighed and lined with chromatography paper. Five aphids were placed for 48h in five replicates for each treatment.

After 18h, settling was recorded every hour for the next eight hours. At the end of the 48h access period all of the stained honeydew was extracted into 4ml of 90% methanol prior to spectrophotometric analysis. The weight of diet consumed and the number of nymphs produced was also measured. Diet 1 was used in all subsequent experiments.

4.2.2 Relationship between amino acid content of honeydew and the weight of diet consumed.

Forty eight test chambers were prepared with sachets of untreated diet, weighed and then lined with chromatography paper. Eight replicates containing 0,1,2,3,4 or 5 aphids per chamber were prepared. Nymphs were carefully removed with a damp camel hair paintbrush after 24h. After a further 24h the aphids and their progeny were removed and the chromatography papers were stained. The stained honeydew was extracted into 4ml of

90% methanol for spectrophotometric analysis and the final weight of the test chambers was determined. Regression analysis was performed on the relationship between absorbance of honeydew produced in each chamber and the weight of diet consumed in that chamber. Any chambers containing sachets of diet that were visibly contaminated with fungus, or in which no adults survived the 48h period were excluded from the analysis.

4.2.3. Relationship between amino acid content of honeydew and weight of diet consumed by aphids feeding on azadirachtin-treated diets

Forty eight test chambers were prepared with sachets of diet containing azadirachtin at concentrations of 0,50,100 or 200ppm. The chambers were weighed and then lined with chromatography paper. Three replicates containing 0,2,3 or 4 aphids were prepared for each azadirachtin concentration. Nymphs were removed after 24h and all aphids were removed after 48h.The final weights of the chambers were measured and the honeydew was stained with ninhydrin then extracted into 2ml of 90% methanol for spectrophotometric analysis. Multiple linear regression was performed on absorbance of reacted honeydew against weight of diet consumed using azadirachtin concentration as a qualitative variable. Exclusions from the analysis were as described above (section 4.2.2).

4.2.4 Initial investigation into aphid feeding behaviour on diets containing azadirachtin.

Azadirachtin was incorporated into the diet at concentrations of 100, 300, 500 and 1000ppm. Five aphids were placed into each weighed, paper-lined chamber for 48h. This protocol was followed for five repetitions of the experiment, with one replicate per azadirachtin concentration in each repetition. After 48h access to azadirachtin-treated or control diets, or starvation for the same period, the aphids in four of the repetitions were transferred onto untreated diet sachets for a further 48h. After each access period the honeydew on the papers was stained with ninhydrin and then extracted into 4ml of 90% methanol. The weight of diet consumed and absorbance of stained honeydew per surviving adult were calculated for the periods of feeding on treated and untreated diets and were analysed by analysis of variance. The number of nymphs produced per surviving adult during both feeding periods (first and second 48h) was also calculated and transformed by square root transformation prior to analysis of variance. In three of the repetitions the percentage of those nymphs that were dead at birth was calculated and angularly transformed prior to analysis of variance.

4.2.5 Effects of feeding by nymphs on weight of diet consumed and honeydew produced.

It was apparent from the results of experiment 4.2.4 that nymph production was strongly affected by azadirachtin in the diets. It was suspected that differences between the numbers of nymphs amplified the differences in the weight of food consumed and the quantity of honeydew produced between controls and treatments. The following experiments were undertaken to investigate this:

i) Effect on honeydew production

Aphids ; First instar nymphs were produced on sachets of untreated artificial diet by adult aphids which had been starved for 24h. The nymphs were produced over the subsequent 24h period.

Method; Three groups, containing 10,30 and 50 individual first instar nymphs, were placed, without prior starvation, onto sachets of diet. The honeydew produced during the subsequent 48h period was collected and quantified by staining with ninhydrin followed by extraction into 2ml of 90% methanol and spectrophotometric analysis. Only one replicate was performed for each group of aphids.

ii) The experiment was repeated measuring the weight of diet consumed in 48h instead of the amount of honeydew produced.

In both of the above experiments, the number of nymphs surviving after 48h and the number that had moulted to the second instar were also measured.

4.2.6 Aphid feeding behaviour on diets containing azadirachtin using amended protocol to exclude nymphs.

The design of the artificial diet experiment using an amended protocol was similar to that in which the original protocol was used. Azadirachtin was incorporated into the diet at concentrations of 100, 300, 500 and 1000ppm in 0.5% ethanol. Controls had either 0.5% ethanol incorporated into the diet or were starved. Four replicates of each of the six treatments were prepared and arranged in a randomised block design. After 52h access to treated or control diets, or starvation, the surviving aphids were transferred, still in 24 discrete groups, to chambers with sachets of untreated diet for a further 44h.

Method; The protocol was amended to exclude data which clearly included nymph feeding. No method could be found which could distinguish between the weight of diet consumed by adults or by nymphs. The only quantitative measure of feeding recorded was honeydew production. However, settling was also taken as a measure of diet acceptability.

Settling observations; The protocol for settling assessment is outlined schematically in Figure 29.

The settling data from each separate observation period were analysed using a generalised linear model. This analysed the mean number of aphids settled as a proportion of the total alive at the start of each observation period, using a binomial distribution. Where no aphids had survived, this was treated as a missing value.

Nymph removal; Nymphs produced by the feeding aphids usually stayed in close proximity to the adult, and their frequent removal would have caused a good deal of disturbance. For this reason nymphs were removed, using a damp camel hair paintbrush, from the chambers once every 12h while the adults were feeding on treated diets and every 24h after transfer to untreated diet. Nymphs were removed 1h before the first settling observation and immediately after the last while on treated diets, and 12-13h prior to the start of observations on untreated diets. The rates of nymph production (number of nymphs produced per surviving adult per hour) of aphids on each diet were compared using analysis of variance after a square root transformation of the data. The data were analysed for each time period separately, and were also compared across time periods for the same diet treatment.

Honeydew recording; The chromatography paper which lined the chambers in which the aphids were feeding was replaced after the first 28h, and then every 24h throughout the duration of the experiment. After staining with ninhydrin the honeydew produced by adults was easily distinguished from that produced by nymphs by the

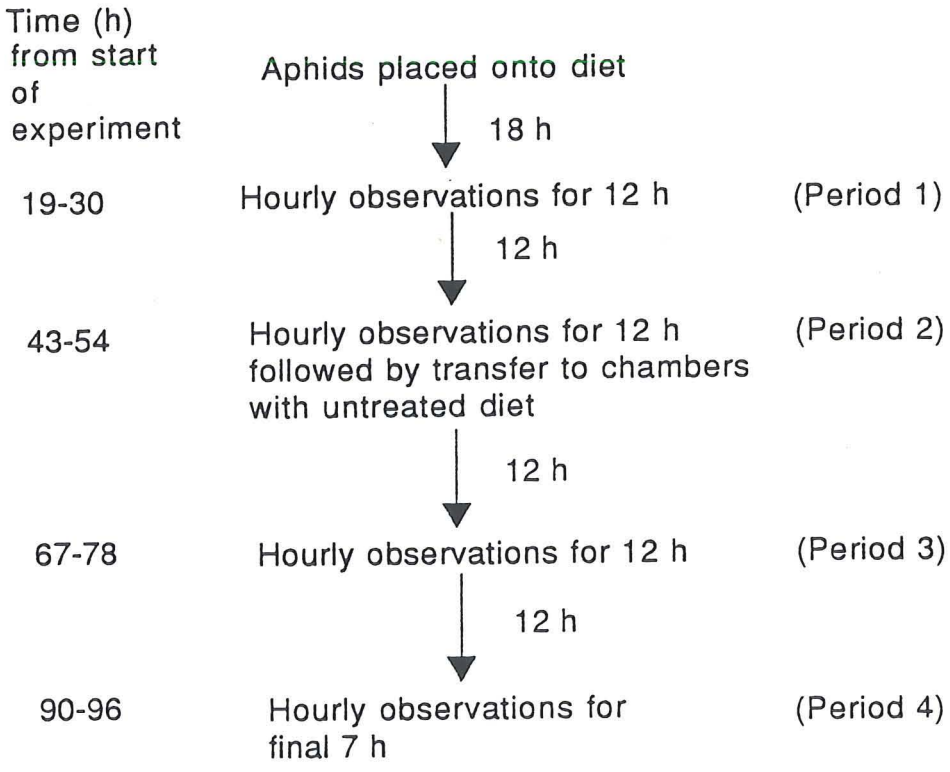


Figure 29. Protocol for observing settling in experiment 4.2.5

diameter of the droplets. Consequently, the nymph honeydew was effectively excluded from absorbance measurements by discarding the sections of chromatography paper containing the smaller droplets after removing the adult droplets for analysis. The stained honeydew was eluted into 1.5ml of 90% methanol for spectrophotometric analysis. Analysis of variance was performed on the absorbance of honeydew produced per surviving adult in separate time periods.

4.2.7 Aphid feeding behaviour on diets containing lower concentrations of azadirachtin.

Aphids: To enhance acceptance of the diet during the first 24h of these experiments, aphids were removed from the turnip culture plants at 9 days old (± 24h) and placed onto sachets of untreated diet for 48h. After this period the aphids were removed and starved for 6h before access to the treated diets.

Diets: Azadirachtin was incorporated into the diet at 25, 50, 75 and 100ppm. Controls were as previously described (section 4.2.6).

The experimental protocol was similar to the amended protocol described above (section 4.2.6) with the following exceptions:

i) The observations on settling lasted for 8h instead of 12.

ii) The aphids were transferred to untreated diet after 50h instead of 52 and spent 48h on untreated diets instead of 44.

iii) The first honeydew recording period was 26h instead of 28.

4.3 Results

4.3.1 Comparison of the acceptability of two holidic diets.

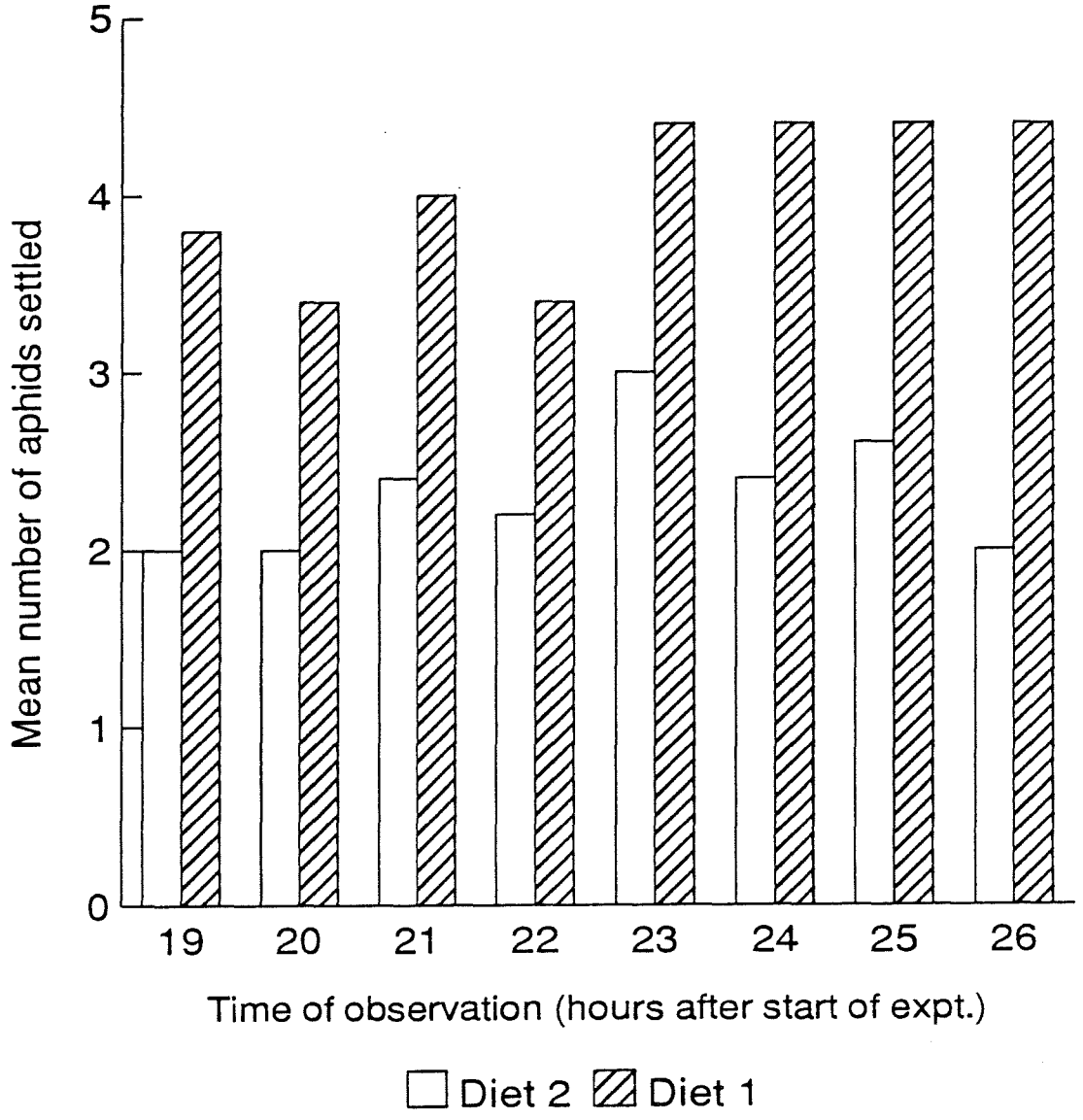
Fewer aphids were settled on diet 2 than on diet 1 at every time interval (Figure 30). The values presented in the graph are means taken from the five replicates. The results of the other measurements are presented in Table 21.

Table 21. The effects of two different holidic diets on the feeding and larviposition of *M. persicae*.

Diet	Number of nymphs produced (± SE)	Weight of diet consumed (mg ± SE)	Absorbance of ninhydrin-treated honeydew at 500nm ($x10^{-3}$)
Diet 1	31.0 ± 2.7	8.58 ± 0.73	226.4 ± 35.9
Diet 2	17.4 ± 4.6	3.42 ± 1.06	147.5 ± 44.9

Diet 1 was superior to diet 2 in all the criteria measured. Aphid responses were also less variable on diet

Figure 30. Comparison of the mean number of aphids settled at hourly intervals, 19 to 26h after placement on two different holidic diets.



1. Diet 1 was therefore used in all later experiments involving artificial diets.

4.3.2 Relationship between the amino acid content of honeydew produced and the weight of diet consumed in 48h.

A linear relationship existed between the weight of diet consumed and the absorbance of ninhydrin-treated honeydew ($r = 0.888$, 35 df $P<0.001$). The relationship was described by the equation $y = 14.87x + 72.47$ (Figure 31).

4.3.3. Relationship between amino acid content of honeydew produced and weight of diet consumed by aphids feeding on azadirachtin-treated diets

Multiple linear regression of absorbance of stained honeydew against weight of diet consumed, using azadirachtin concentration as a qualitative variable showed that the relationships were linear for each treatment (Figure 32). There were no significant differences between the relationships for each of the treatments in either the elevation or the gradient of the individually fitted regression lines (Table 22). The relationship between honeydew production and weight of diet consumed could therefore be described by a single regression line with equation $y = 52.76x - 4.6$ for all treatments (Figure 32).

Figure 31. The relationship between weight of diet consumed and the absorbance of ninhydrin-stained honeydew after development.

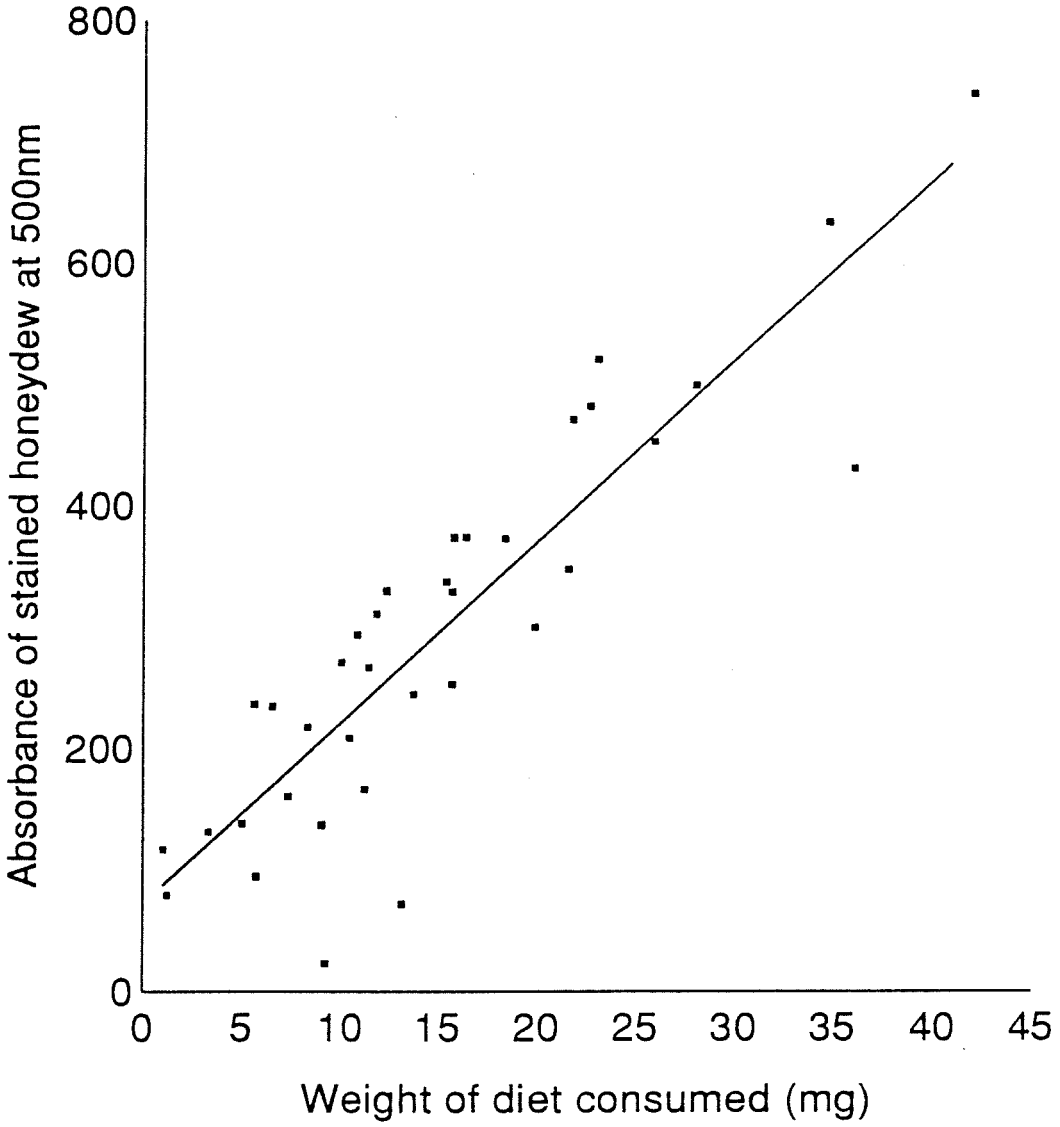
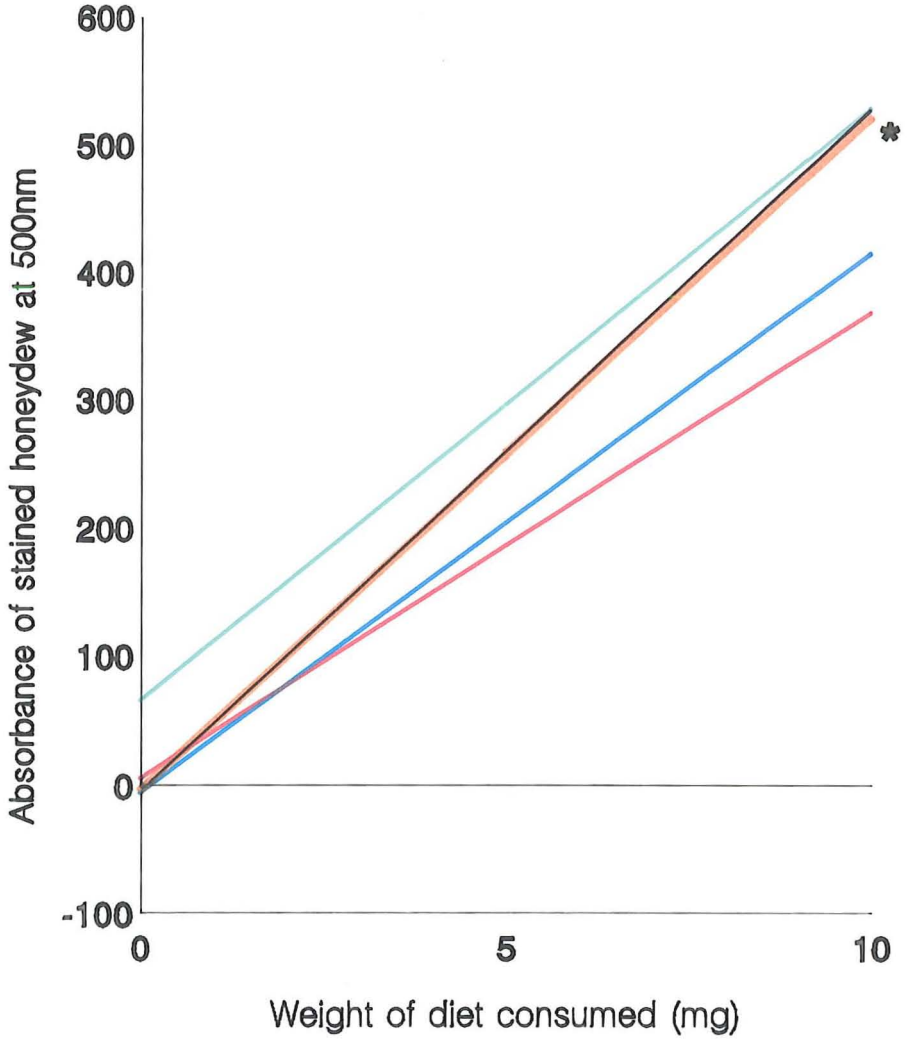


Figure 32. Comparison of the relationship between honeydew production and weight of diet consumed on azadirachtin-treated or untreated diets.



* Regression lines of "Control" and "Overall" are superimposed

Table 22. Summary of comparison of regressions describing the relationship between weight of diet eaten and amino acid content of honeydew for different azadirachtin concentrations.

Source of Variation	degrees of freedom	Sum of Squares	Mean Square	Variance Ratio
overall regression	1	1137679	1137679	163.34
Intercept	3	34332	11414	1.64
Slopes	3	8286	2762	0.40
Residual	28	195019	6965	

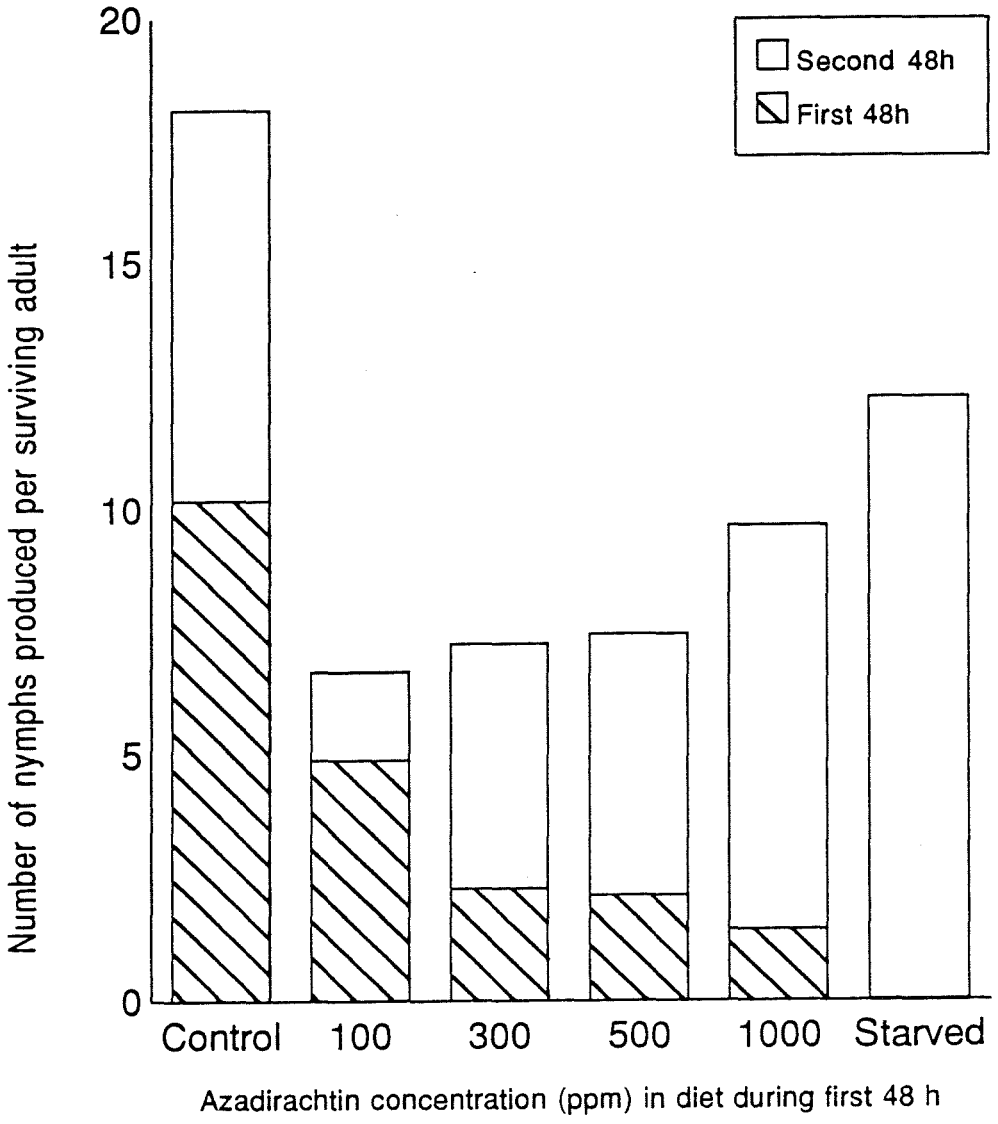
4.3.4 Initial investigation into aphid feeding behaviour on diets containing azadirachtin.

Nymph production: During the first 48h period the aphids on control diets produced significantly more nymphs than those on diets treated with 100ppm azadirachtin, which in turn produced more than those on any other diet containing azadirachtin. After transfer to untreated diet sachets, aphids which had previously fed on 100ppm treated diets produced significantly fewer nymphs than those which had fed on control diets or diets containing 1000ppm azadirachtin, or aphids which had been starved. The aphids which had fed on 300 and 500ppm treated diets also produced fewer nymphs than those which had fed on 1000ppm treated diets (Figure 33 and Table 23).

After the adults had been transferred to untreated diets, a proportion of the nymphs that they produced were born dead with undeveloped appendages (Figure 34).

Adults which had previously fed on diets treated with 100ppm azadirachtin produced the highest proportion of

Figure 33. Nymph production by adult M. persicae feeding on azadirachtin-treated diets - primary and secondary effects.



The figures shown are mean counts for four replicates and have not been transformed.

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a)

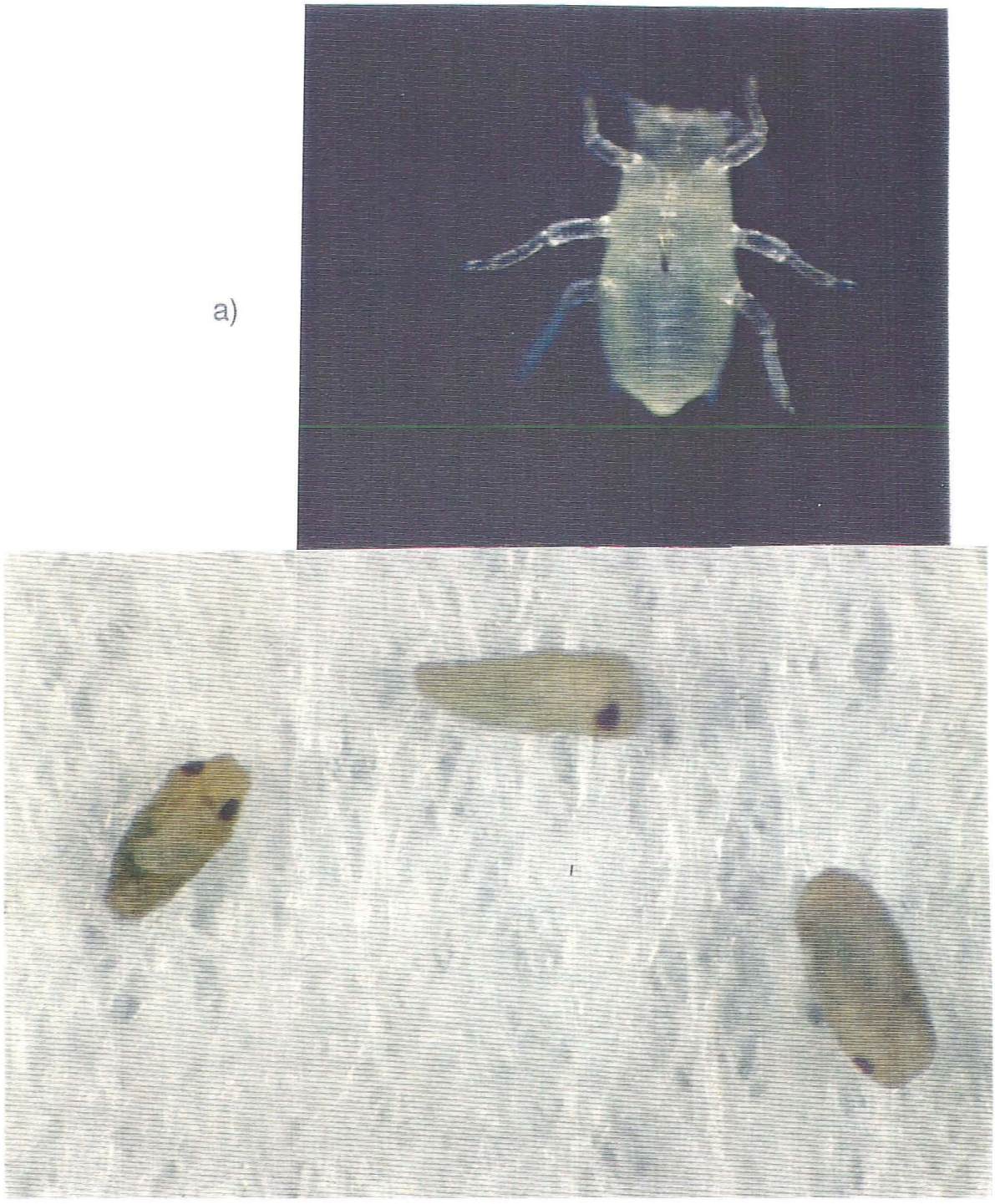
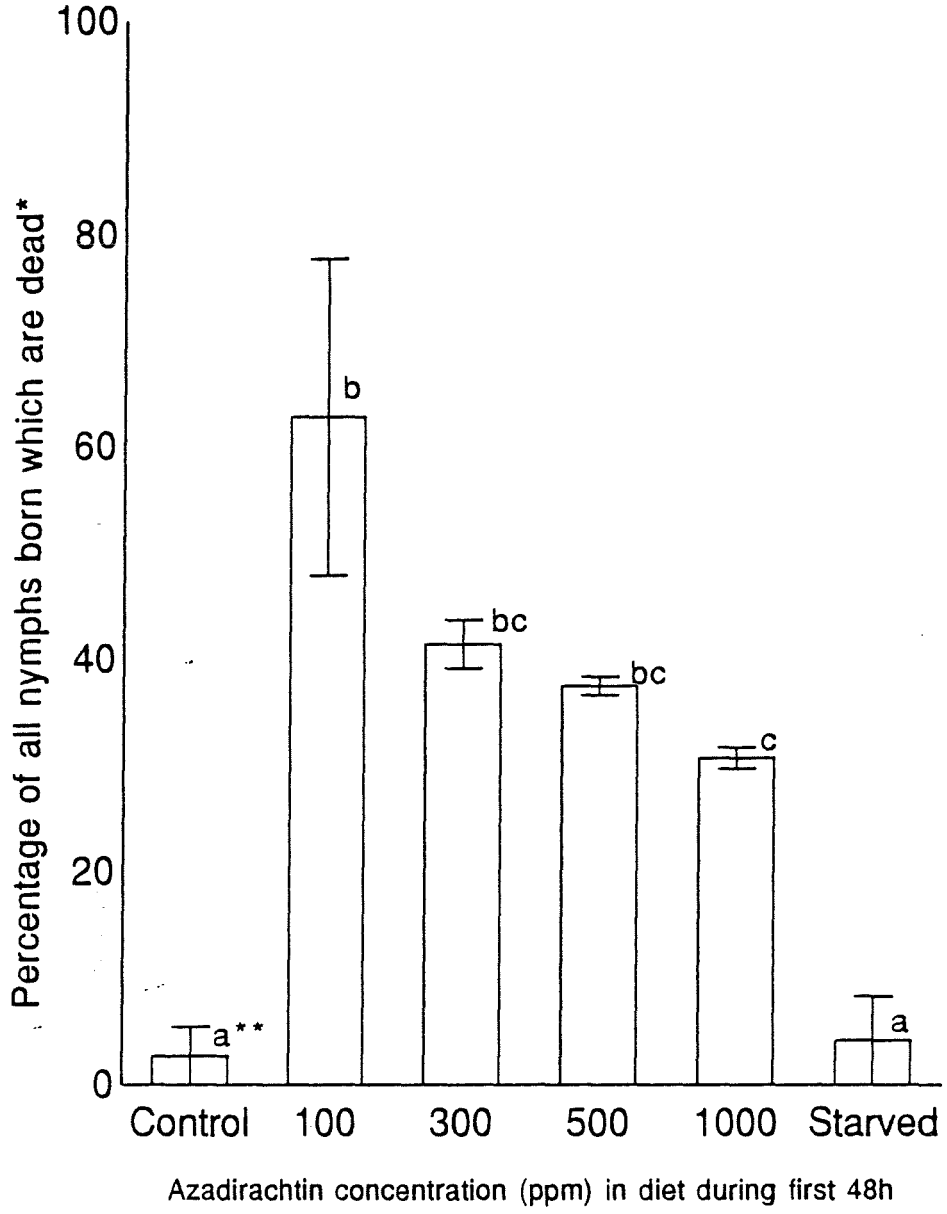


Figure 34. a) Normal, 1st instar M. persicae nymph produced by adults feeding on control diet in experiment 4.2.4.
b) Nymphs born dead without developed appendages, produced by adults which had fed for 48h on diet treated with 100ppm azadirachtin.

Figure 35. The effect of azadirachtin on the percentage of dead nymphs produced by adults after transfer to untreated diets.



* Percentages have been angularly transformed
** Same letter denotes no significant difference (P=0.05)
Vertical bars indicate 2x Standard Error of Mean

dead nymphs and the proportion of dead nymphs produced fell as the azadirachtin concentration increased (Figure 35).

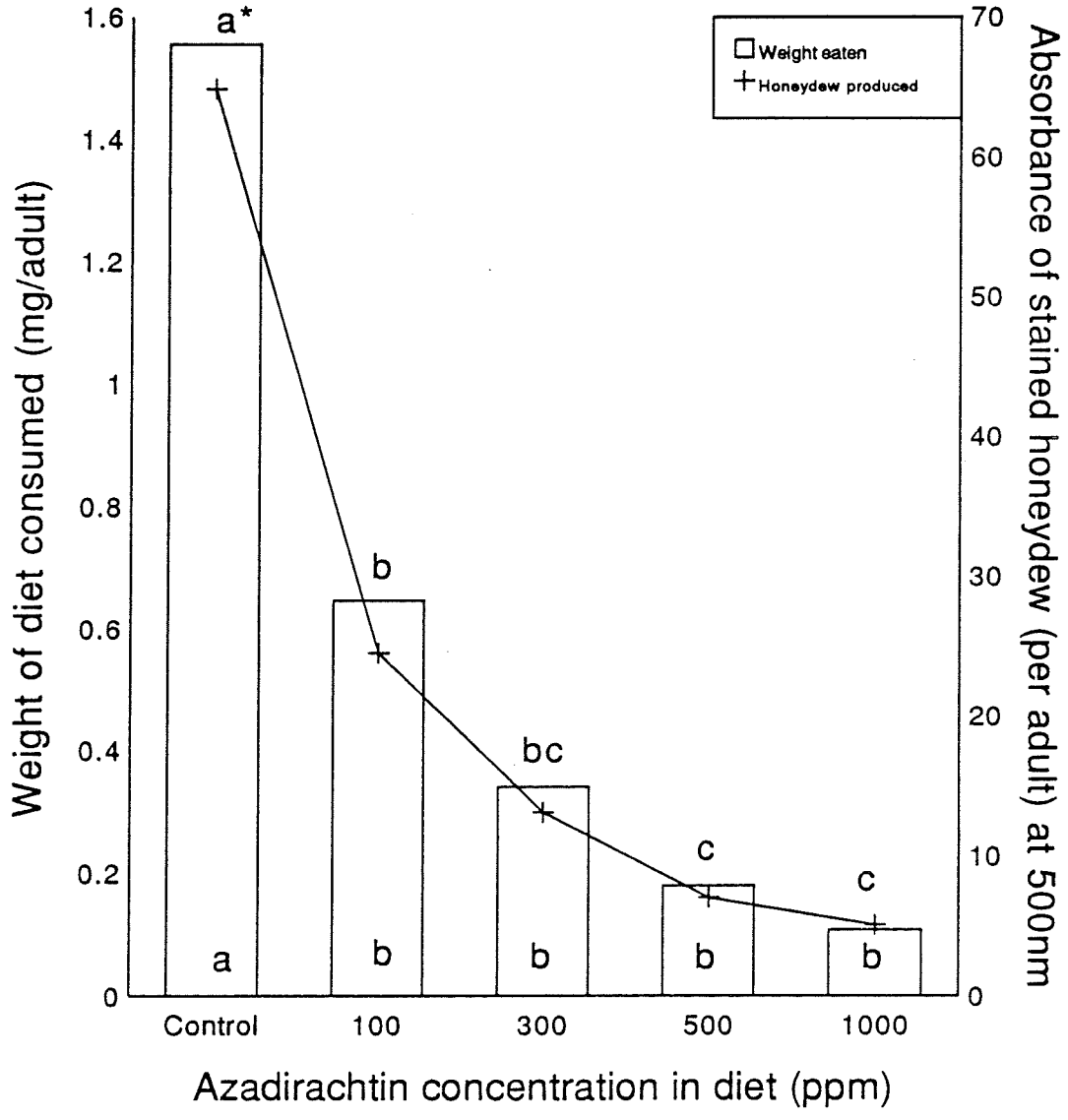
Table 23. Nymph production by adult *M. persicae* while on azadirachtin-treated diets and after transfer to untreated diets.

Treatment of diet in first 48h	Number of nymphs (square root transformed ±SE) produced per adult in two 48h periods	
	First 48h	Second 48 h
Control	$3.19 \pm 0.11^{a*}$	2.81 ± 0.12^{ac}
100ppm	2.19 ± 0.16^{b}	1.20 ± 0.34^{b}
300ppm	1.45 ± 0.24^{bc}	2.12 ± 0.39^{ab}
500ppm	1.44 ± 0.13^{bc}	2.22 ± 0.35^{ab}
1000ppm	1.00 ± 0.39^{c}	2.83 ± 0.23^{ac}
Starved	0^{d}	3.49 ± 0.23^{c}

* Values followed by the same letter are not significantly different (P=0.05) within columns.

Weight of diet consumed and quantity of honeydew produced: During the first 48h period, aphids on the control diet sachets consumed more than twice as much diet and produced more than twice as much honeydew as aphids on any of the treated diets (Figure 36). The quantity of diet consumed and the amount of honeydew produced by aphids feeding on azadirachtin-treated diets decreased with increasing azadirachtin concentration. After transfer to sachets of untreated diet, aphids which had previously fed on control diet consumed more than

Figure 36. The quantity of diet consumed and the honeydew produced by aphids feeding for 48h on diets treated with azadirachtin.



*Values annotated with the same letter in a series are not significantly different. Upper letters refer to honeydew produced, lower values refer to weight consumed.

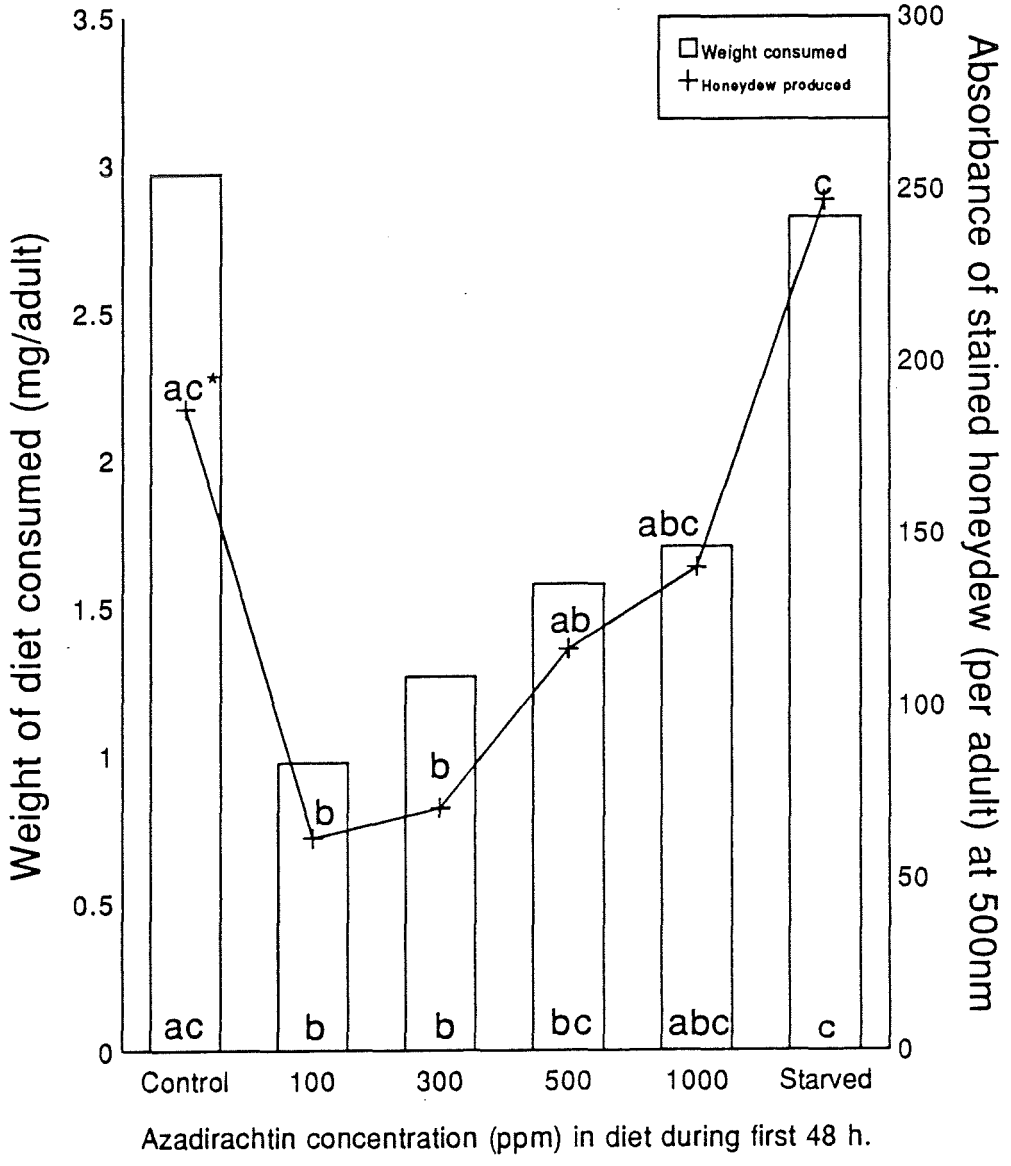
three times as much diet and produced more than three times as much honeydew as those which had previously fed on diet containing 100ppm azadirachtin (Figure 37). The quantity of diet consumed and the production of honeydew increased as the concentration of azadirachtin in the initial diets increased. Aphids which had been starved during the initial 48h period consumed as much diet as those which had previously fed on control diet and produced a similar amount of honeydew (Figure 37).

4.3.5 Contribution of nymphs to weight of diet consumed and honeydew produced.

i) *Honeydew production*: The relationship between the number of nymphs present on a diet sachet for 48h and the absorbance of the stained honeydew collected from that sachet appeared to be linear (Figure 38). The mean absorbance of stained honeydew produced per surviving nymph (± S.E.) was 40.5 ± 1.9 and 86.1 % of the nymphs had moulted to the second instar.

ii) *Weight of diet consumed*: The relationship between the number of nymphs present on a diet sachet and the weight of diet consumed from that sachet also appeared linear (Figure 39). The mean weight of diet consumed per surviving aphid was 0.27 ± 0.02 mg and 90.4% of the nymphs had moulted to the second instar.

Figure 37. The quantity of diet consumed and honeydew produced by aphids in 48h after transfer from azadirachtin-treated diets to untreated diet.



*Values annotated with the same letter are not significantly different.
Upper letters refer to honeydew, lower letters to weight consumed.

Figure 38. The relationship between the number of nymphs present on a diet sachet and the amount of honeydew produced.

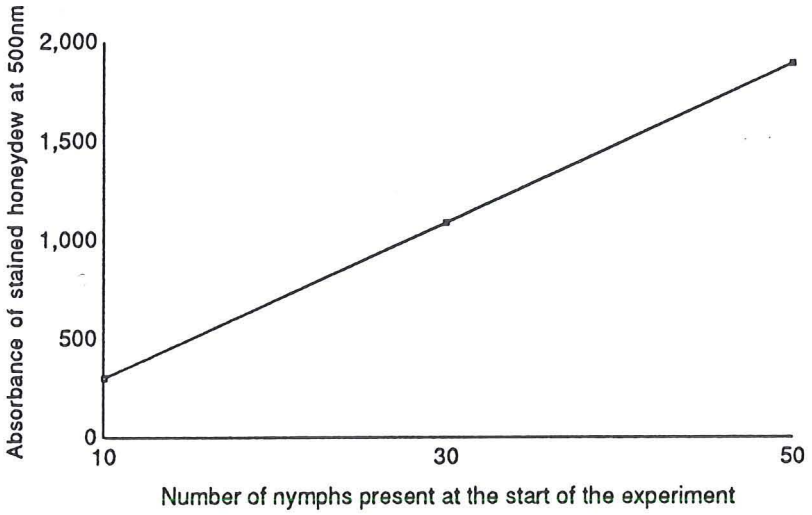
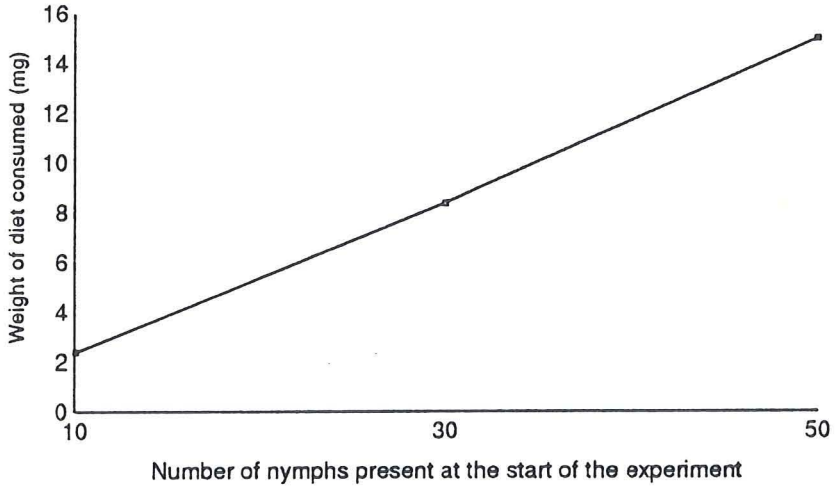


Figure 39. The relationship between the number of nymphs present on a diet sachet and the quantity of diet that they consume.



4.3.6 Aphid feeding behaviour on diets containing azadirachtin using amended protocol to exclude nymphs.

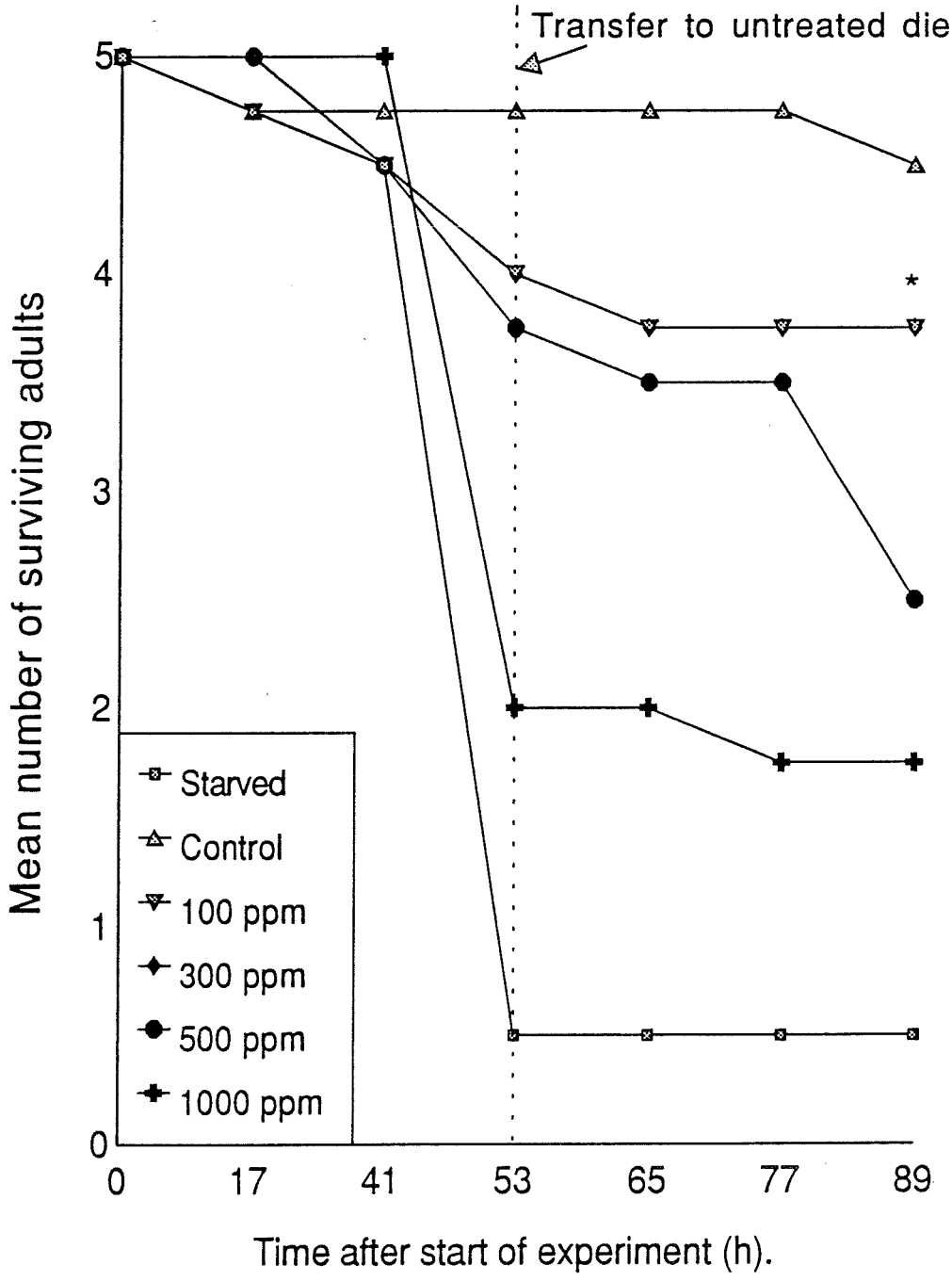
i) *Mortality*; Mortality was higher in groups of aphids on treated diet than on control diet, but was highest in aphids which had been starved for the first 52h period (Figure 40). For this reason, settling, nymph production and honeydew production were expressed as proportions per surviving aphid.

ii) *Settling*;

a) First observation period (treated diet, 19h-30h); There were significant differences ($P<0.05$) between the diets despite poor settling (<50%) even in the control group. Figure 41 shows the proportions of surviving aphids settled as predicted from the generalised linear model. The starved aphids were significantly ($P<0.01$) less settled than all other groups with the exception of the aphids on 1000ppm treated diet. The aphids on 1000ppm treated diet were significantly less settled than those on the control, 100 or 300ppm treated diets, ($P<0.05$). There was no significant difference between the settling of aphids on control diet and that of aphids on diets treated with up to 500ppm azadirachtin.

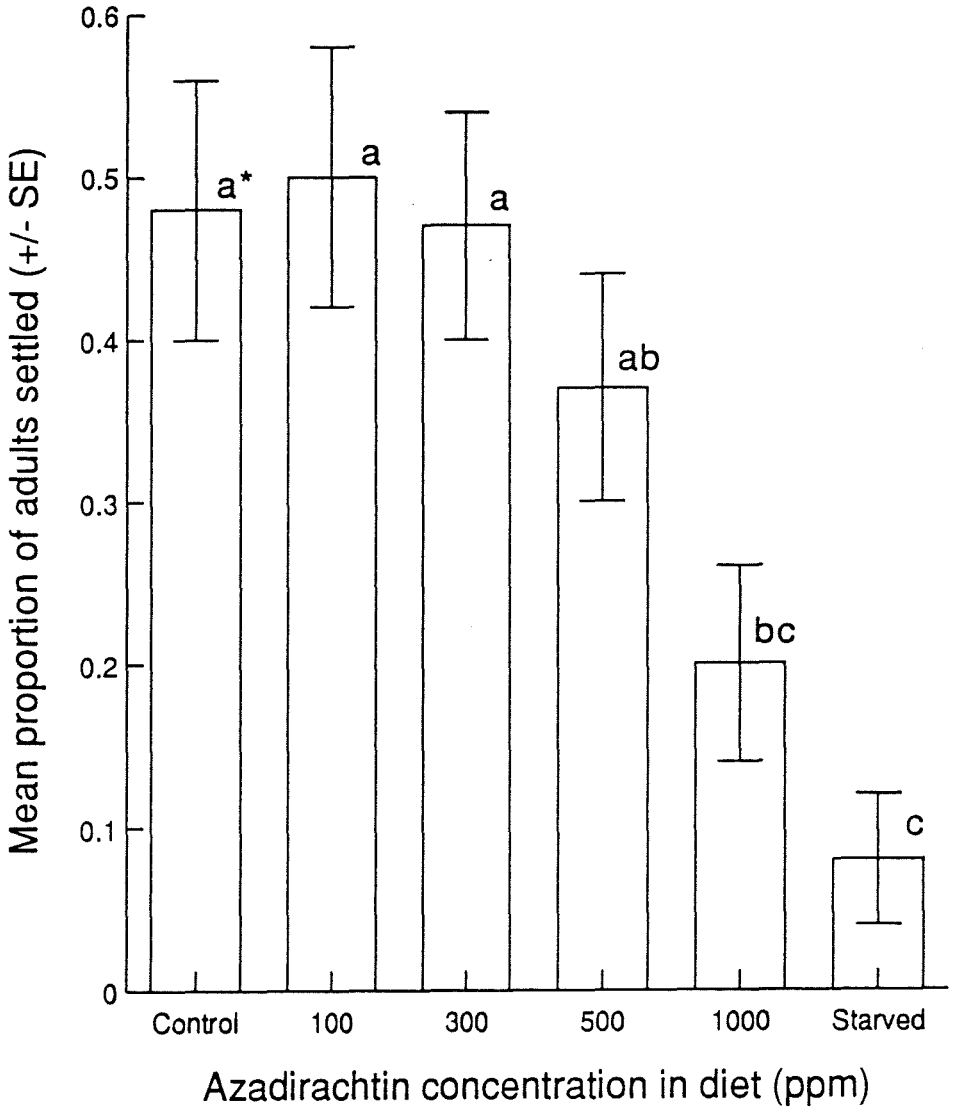
b) Second observation period (treated diet, 43h-54h); During this period there were significant differences between the treatments, ($P<0.001$). The starved aphids were the least settled, significantly less than all other groups ($P<0.01$). The aphids on all treated diets were

Figure 40. The influence of azadirachtin concentration in the diet on survival of M. persicae adults.



*data for 100 and 300ppm are superimposed

Figure 41. Settling behaviour of adult M. persicae on artificial diets treated with azadirachtin during the first observation period (19-30 h).



*Values annotated with the same letter are not significantly different (P=0=05).

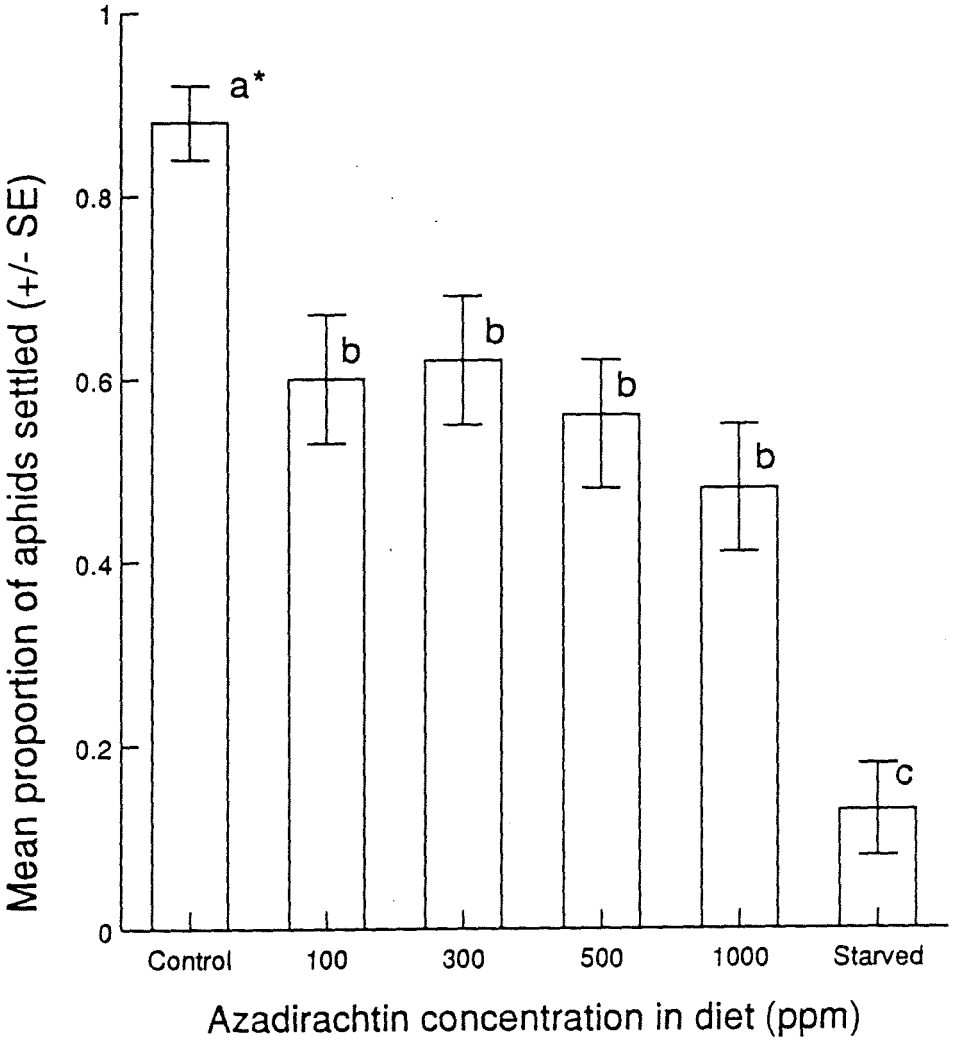
significantly less settled than those on the control diet, ($P<0.01$) (Figure 42). During this period the aphids in the control group were more settled than in the first period, with nearly 90% settling.

c) After transfer to untreated diet (Third and fourth observation periods, 67h-78h and 90h-96h) ; There were no significant differences in the settling of aphids from treated or control groups. Almost all aphids were settled during this period.

iii) *Nymph production;* The number of adults alive at the start of each period of nymph counting was used to calculate the mean number of nymphs produced per adult per hour (Table 24). Overall, there were significant differences between the diets in the rate of nymph production, ($P<0.01$).

While the aphids were feeding on azadirachtin-treated diets (time periods 1-3), nymph production decreased with increasing azadirachtin concentration. This relationship continued into the initial period of feeding on untreated diet (period 4) but during period 5 the opposite relationship was found, i.e. nymph production rates increased directly with the concentration of azadirachtin in the diet to which the aphids had previously been given access. This relationship continued into period 6 with the exception that aphids which had previously been given access to diet containing 500ppm azadirachtin had virtually stopped producing nymphs. In period 7 aphids which had previously been given access to diets

Figure 42. The settling behaviour of adult M. persicae on azadirachtin-treated diets during the second observation period (43-54 h).



*Values annotated with the same letter are not significantly different (P=0.05)

Table 24. Numbers of *M. persicae* nymphs per adult per hour (square root transformed) produced on azadirachtin-treated diets and after transfer to untreated diets.

	Time period						
	Treated diets			Transfer to untreated diets			
Treatment	1 (0-16h)	2 (16-40h)	3 (40-52h)	4 (52-64h)	5 (64-76h)	6 (76-88h)	7 (88-95h)
Control	0.39 ab*	0.40 a	0.69 a	0.57 a	0.36 a	0.27 ac	0.33 a
100 ppm	0.41 b	0.33 ab	0.32 b	0.24 b	0.04 b	0 b	0 b
300 ppm	0.28 ac	0.30 bc	0.06 c	0.16 bc	0.12 cb	0.13 ab	0.17 c
500 ppm	0.26 c	0.21 cd	0.23 bd	0.12 bc	0.18 cb	0.05 b	0 b
1000 ppm	0.18 c	0.18 d	0.21 bd	0.04 c	0.25 c	0.24 ac	0.07 bc
Starved	0.23 c	0.14 d	0.15 cd	0 c	0.54 d	0.39 c	0.64 d
S.E.D.	0.06	0.05	0.07	0.09	0.08	0.09	0.07

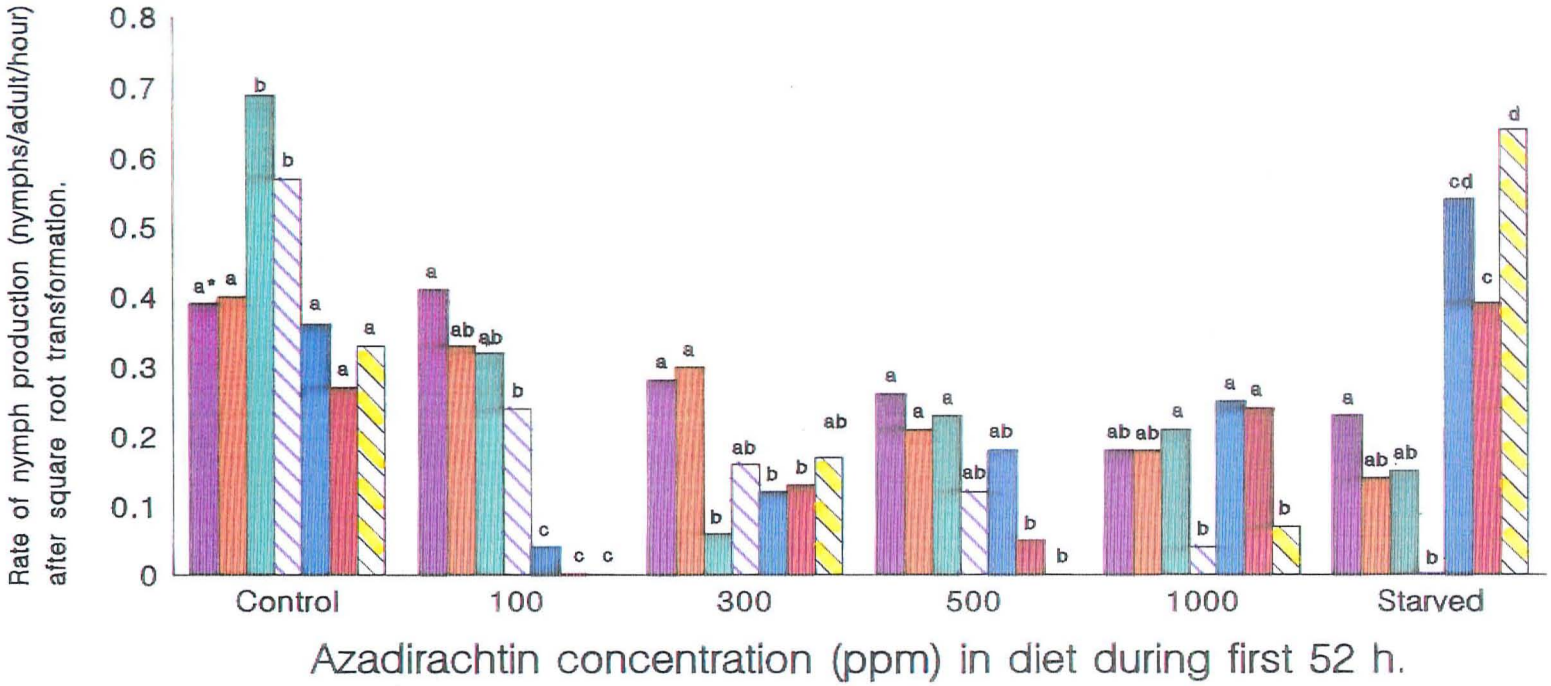
* Values followed by the same letter within columns are not significantly different (P=0.05),

containing 100,500 or 1000ppm azadirachtin had either stopped producing nymphs or were producing them at a very slow rate, while those that had previously been given access to diets containing 300ppm azadirachtin continued to produce nymphs at about half the rate of those which had fed on control diets.

The rate of nymph production by aphids on each treatment was also compared for individual time periods (Figure 43). Aphids which had fed on control diets reached a peak rate of nymph production while on the original diets and this peak continued into the initial period of access to untreated diet (period 4). No dead nymphs were produced by aphids which had fed on the control diets. Nymph production by aphids which had been given access to diets containing 100 and 500ppm azadirachtin declined throughout the experiment to zero in the latter periods on untreated diets. During the final periods in which the aphids in these groups produced nymphs (period 4 and period 5 respectively) all of the nymphs were dead (Table 25).

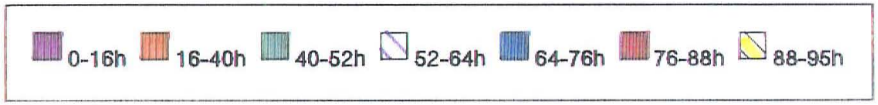
The rate of nymph production by aphids which had been given access to diets containing 300 and 1000ppm reached very low levels during the final period on treated diet and the initial period on untreated diet respectively, before recovering to some degree. The rate of nymph production by aphids which had been starved for the first 52h of the experiment declined throughout the first period reaching zero during the initial period on

Figure 43. The effect of azadirachtin treatment on the rate of nymph production by M. persicae while on treated diets and after transfer to untreated diet.



Original in colour

* Values annotated with the same letter are not significantly different (P=0.05) within treatments.



untreated diet, but recovered rapidly to high levels in the remaining three periods on untreated diet.

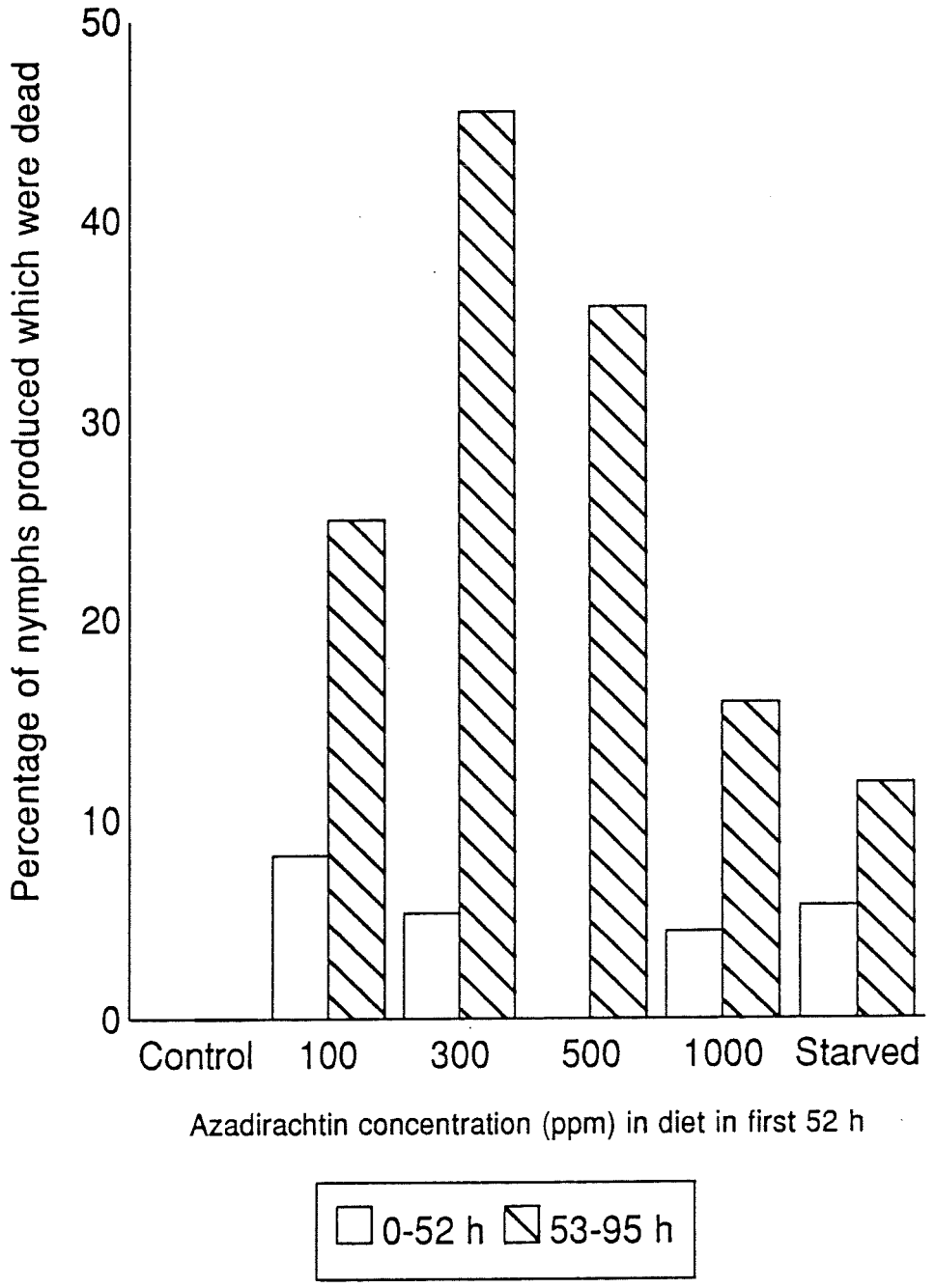
Table 25. Percentage of dead nymphs produced in each time period.

	Azadirachtin concentration in diet (ppm)					
Time period	Control	100	300	500	1000	Starved
1	0	0	0	0	0	0
2	0	0	2.3	0	0	0
3	0	44	75	0	18.2	28.3
4	0	20	14.3	0	0	0
5	0	100	67	50	9	0
6	0	-*	40	100	33.3	50
7	0	-	75	-	0	0

-* No nymphs were produced in these periods.

High mortality of the adult aphids in some of the treatment groups made statistical analysis of the data on dead nymphs produced in each time period impossible. However, the percentages of nymphs born dead during the period when adult aphids had access to treated diets (0-52h) and the subsequent period when they had access to untreated diets (53-95h) are shown in Figure 44.

Figure 44. The percentage of dead nymphs produced by aphids feeding on azadirachtin-treated diets (0-52h) and after transfer to untreated diets (53-95h).



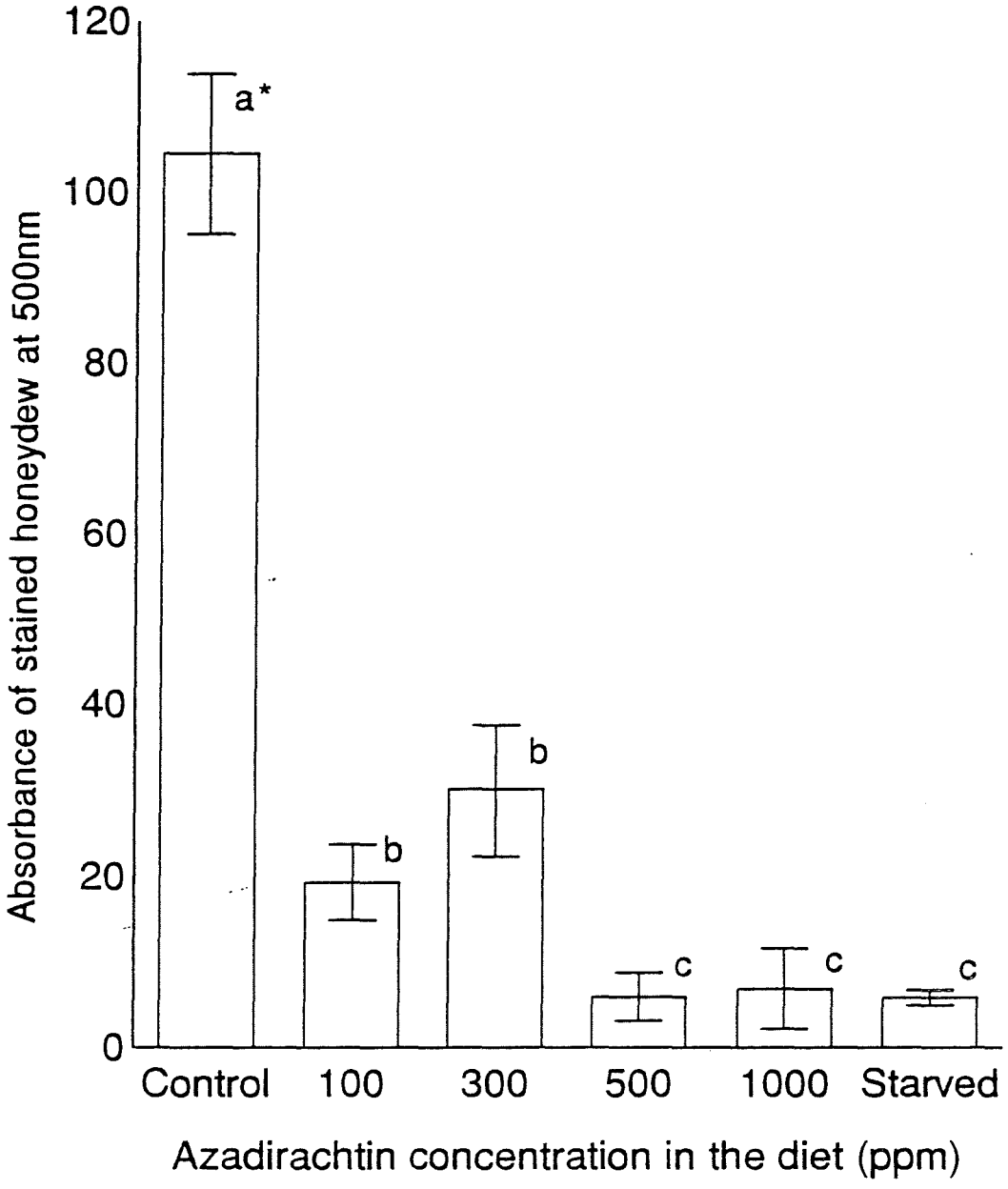
4) *Honeydew production*: Honeydew production was expressed as absorbance per mean surviving adult. The parameter 'mean surviving adult' was calculated as;

$$\frac{\text{No. of adults alive at start of period} + \text{No. of adults alive at end of period}}{2}$$

i) Honeydew recording period 1 (Treated diets, 28-52h); During this period there were significant differences between the absorbance of honeydew produced by the aphids on different diets, ($P<0.001$), (Figure 45). Aphids feeding on control diets produced approximately five times more honeydew than those on diet treated with 100ppm azadirachtin. The amount of honeydew produced by aphids on diets treated with 500ppm azadirachtin and 1000ppm azadirachtin did not differ from the amount produced by starved aphids.

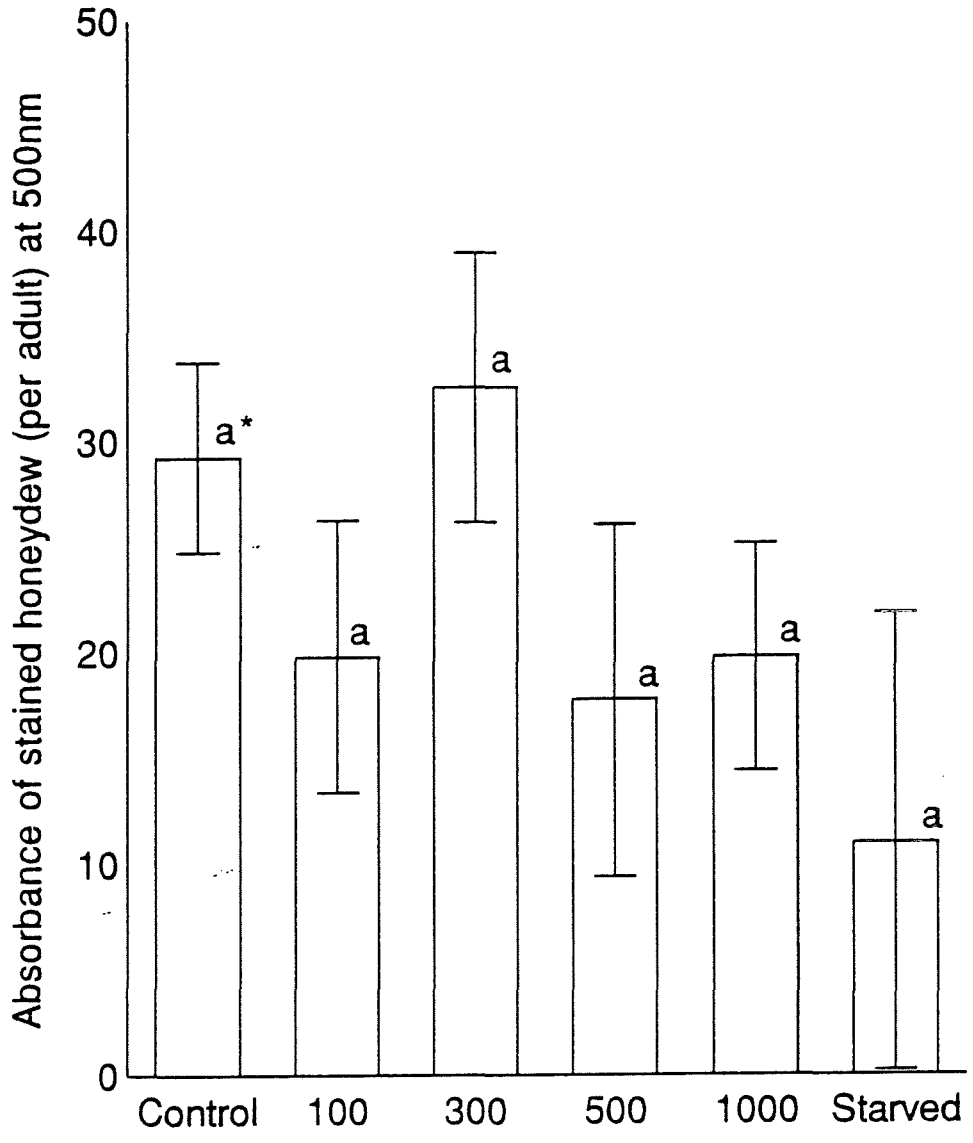
ii) Honeydew recording periods 2 and 3 (Untreated diets 53-77h, and 78-96h): Although there appeared to be large differences in the mean values presented (Figures 46 and 47), there were also large standard errors associated with these means. Analysis of variance showed no significant differences in the honeydew production of aphids from different treatment groups after transfer to untreated diets. Although accurate comparisons of honeydew production between the different time periods cannot be made (because the reproducibility of the method of honeydew estimation has not been proven) honeydew

Figure 45. Honeydew production by M. persicae feeding on azadirachtin-treated diets in honeydew recording period 1 (28-52 h).



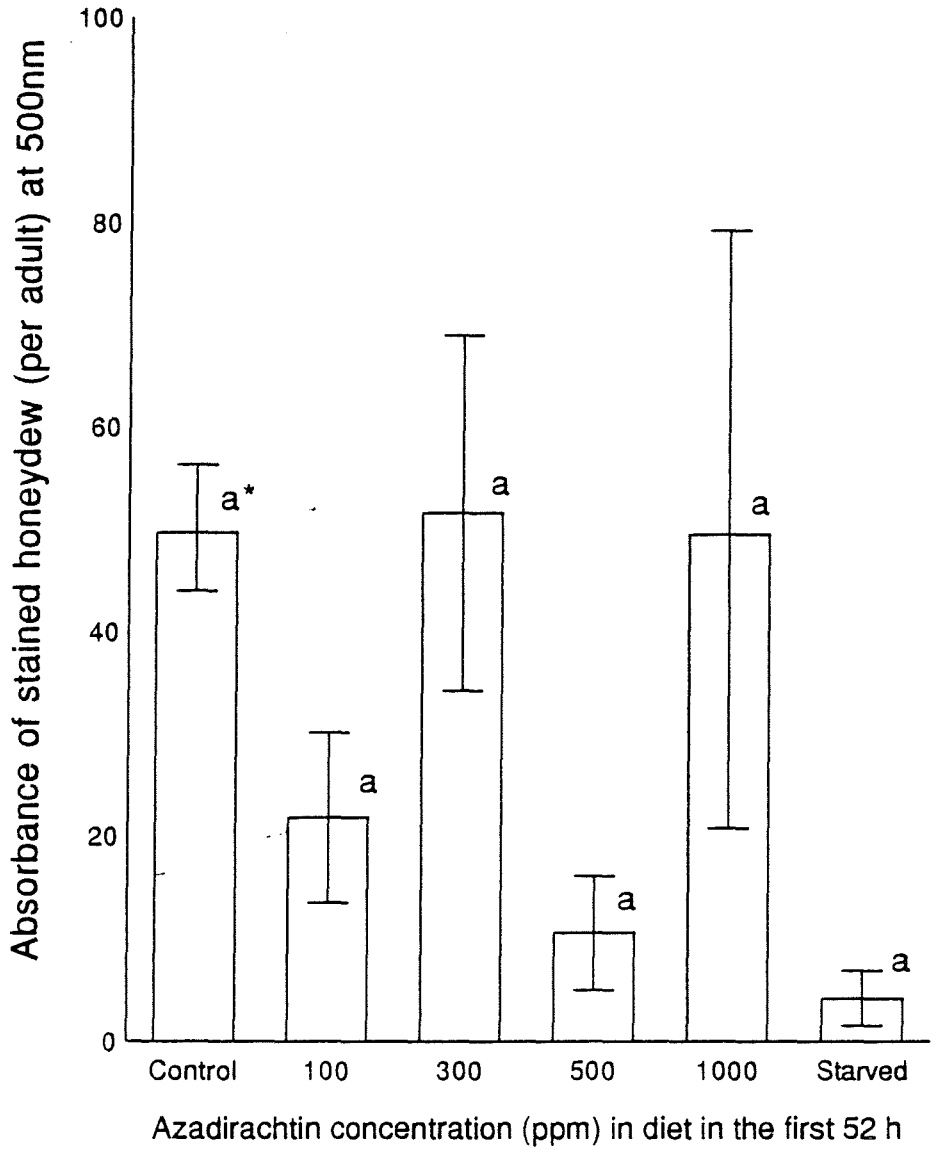
* Values annotated with the same letter are not significantly different (P=0.05)
Vertical bars indicate 2x Standard Error of Mean

Figure 46. Honeydew production by M. persicae in the first period after transfer from azadirachtin-treated diets to untreated diet (53-77 h).



*Values annotated with the same letter are not significantly different (P=0.05)
Vertical bars indicate 2x Standard Error of Mean

Figure 47. Honeydew production by M. persicae in the second period after transfer from azadirachtin-treated diets to untreated diet (78-95h)



*Values annotated with the same letter are not significantly different (P=0.05)
Vertical bars indicate 2x Standard Error of Mean

production by aphids in the control group appears to have fallen substantially after transfer to untreated diets while that produced by aphids on azadirachtin-treated diets remained fairly constant. This may have been caused by a reduced feeding rate after an initially high diet uptake on control diets. It is notable that the lowest honeydew production during periods 2 and 3 was seen in the groups of aphids which ceased nymph production after transfer to untreated diets (100ppm and 500ppm treated groups), while those which continued to produce nymphs also produced the largest quantities of honeydew. The exception to this was the starved group which, despite high nymph production rates after transfer to untreated diets, produced small quantities of honeydew. This result implies that the measures to exclude nymph honeydew from the analysis had been successful. Honeydew produced by nymphs was therefore not responsible for the differences in honeydew production between the groups of aphids after transfer to untreated diet.

4.3.7 Aphid feeding behaviour on diets containing lower concentrations of azadirachtin.

1) *Mortality*: Survival rates were high in all treatment and control groups (Figure 48) but aphids which were starved during the first 50h suffered higher mortality.
2) *Settling*: In all time periods 80-100% of the aphids were settled in all treatment and control groups. The aphids which were starved during the first 50h were significantly less settled ($P<0.001$) than the aphids in all other groups during this period but all of these aphids were settled after transfer to untreated diet (Figure 49).
3) *Nymph production*: During the first period on treated or control diets (0-26 h) nymph production rates decreased as azadirachtin concentration increased but were all significantly higher than the rate of nymph production of starved aphids (Table 26).

During the second period in which the aphids had access to treated or control diets (27-50 h) nymph production rates of aphids on all azadirachtin-treated diets were lower than on control diets and were not significantly different from the production rate observed for starved aphids. After transfer to untreated diet (51-98 h) the adults which had previously been given access to all treated diets stopped producing nymphs, or produced them at very low rates while aphids which had been starved produced nymphs at more than twice the rate of aphids which had been given access to control diets.

Figure 48. Mortality of adult M. persicae feeding on diets treated with lowered concentrations of azadirachtin.

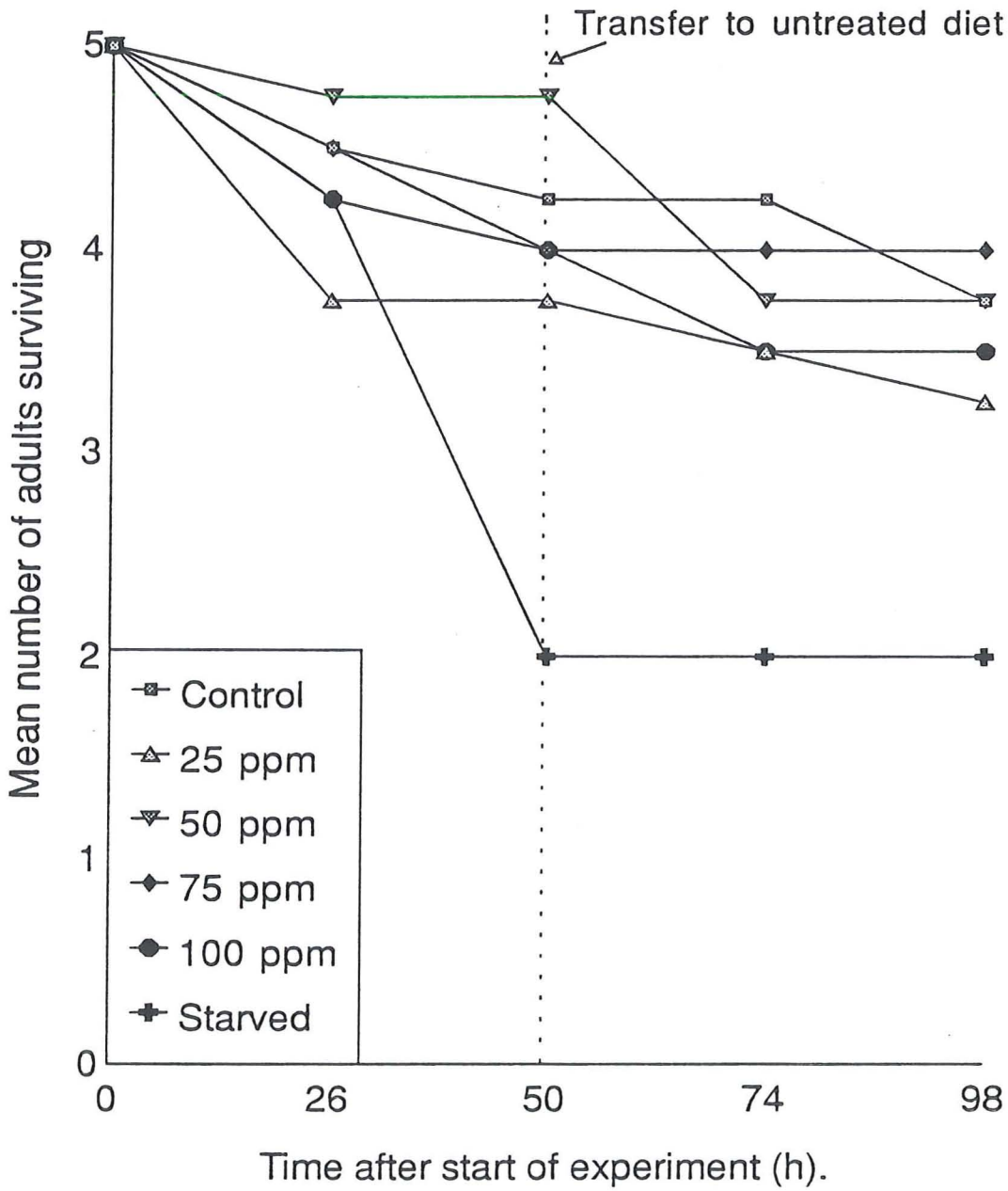
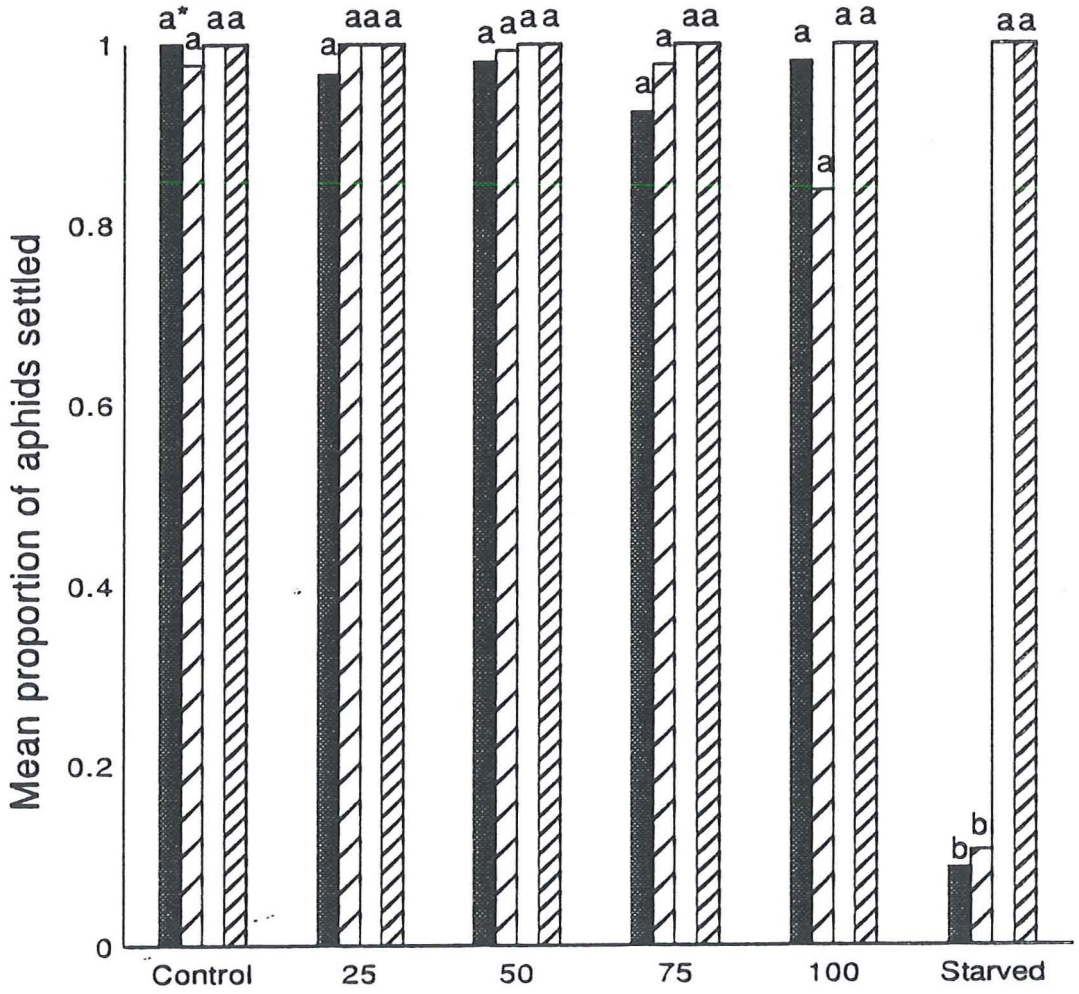


Figure 49. Settling behaviour of adult M.persicae on artificial diets treated with lowered concentrations of azadirachtin during each observation period.



*Values annotated with the same letter are not significantly different (P=0.05) within treatments.

Table 26. The rate of nymph production by adult *M. persicae* feeding on artificial diet treated with azadirachtin at lowered concentrations and after transfer to untreated diets in consecutive time periods.

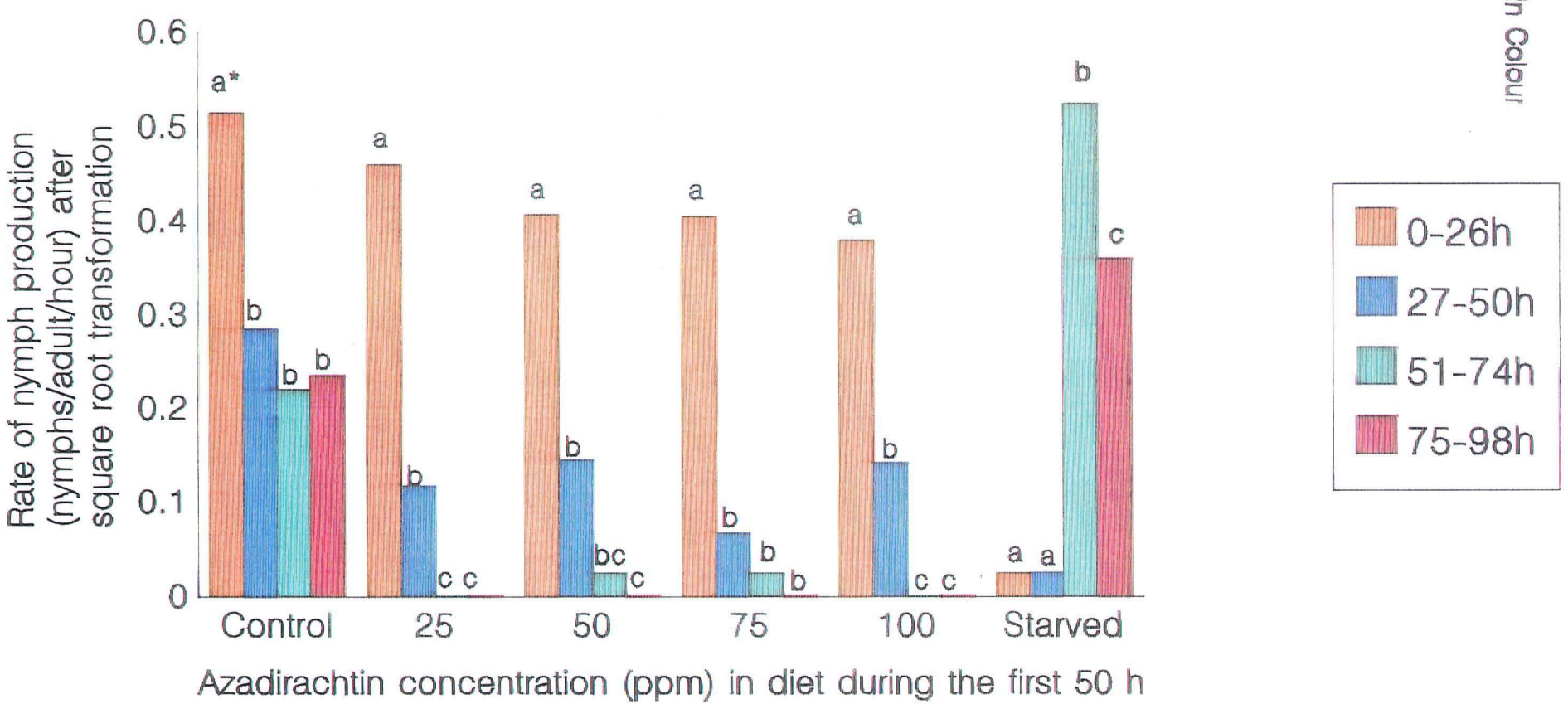
	Time period			
	Treated Diets		Untreated Diets	
Treatment	1	2	3	4
	(0-26h)	(27-50h)	(51-74h)	(75-98h)
Control	0.51 a*	0.28 a	0.22 a	0.23 a
25ppm	0.46 ab	0.12 b	0 b	0 b
50ppm	0.41 b	0.14 ab	0.02 b	0 b
75ppm	0.41 b	0.06 b	0.02 b	0 b
100ppm	0.38 b	0.14 ab	0 b	0 b
Starved	0.02 c	0.02 b	0.52 c	0.36 c

* Values shown are means of the square root transformations of the number of nymphs produced per adult per hour. Values followed by the same letter are not significantly different (P=0.05) within columns.

Aphids which had access to the control diet reached a peak of nymph production during the first period (0-26 h) (Figure 50). The rate of nymph production by these aphids fell by approximately 50% thereafter and remained at a steady rate during the subsequent periods. The rate of nymph production by aphids on all treated diets declined to zero during the course of the experiment. In the first 50h of the experiment the aphids which were starved produced nymphs at a low rate. After transfer to untreated diet nymph production peaked during the initial period and then fell to a lower rate.

None of the nymphs produced by aphids feeding on the control diets was dead but the percentage of nymphs born

Figure 50. The rates of nymph production by M. persicae while on diets treated with lowered concentrations of azadirachtin and after transfer to untreated diet.



Original in Colour

* Values annotated with the same letter are not significantly different within treatments.

dead from adults which had been given access to azadirachtin-treated diets generally increased during the second time period (Table 27). By the third time period aphids which had fed on diets containing azadirachtin at concentrations of 25 and 100ppm had stopped producing nymphs and all the nymphs produced by aphids which had fed on diets containing 50 and 75ppm azadirachtin were born dead. A small percentage of the nymphs born to aphids which had been starved for the first 50h of the experiment were born dead in periods 3 and 4.

Table 27. Percentage of all nymphs born to aphids which had fed on diets containing lowered concentrations of azadirachtin which were dead at birth.

	Azadirachtin concentration in diet (ppm)					
Time period (days)	Control	25	50	75	100	Starved
1	0	4.9	6.0	10.2	4.6	0
2	0	22.2	38.5	0	25.0	0
3	0	-*	100	100	-	1.9
4	0	-	-	-	-	4.0

* No nymphs were produced in these periods.

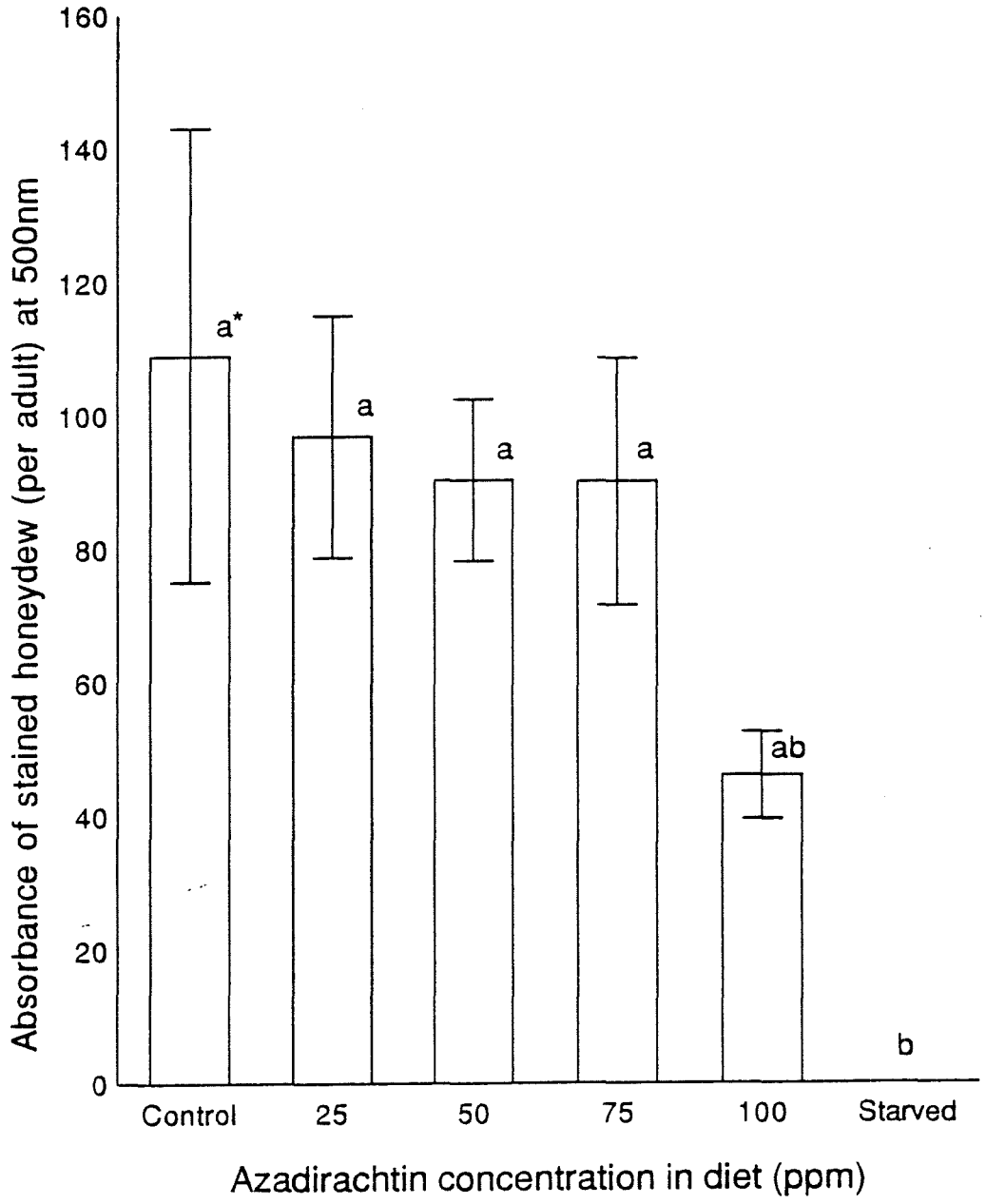
4) *Honeydew production*:

i) Honeydew recording period 1, (0-26 h). Aphids which had access to control diets and diets containing azadirachtin at concentrations of 25-75ppm produced approximately twice as much honeydew as those with access to diets containing 100ppm azadirachtin (Figure 51).

ii) Honeydew recording period 2 (27-50 h). During this period, aphids with access to control diets produced more than three times the amount of honeydew produced by aphids on any of the treated diets (Figure 52). There was no significant difference between the quantity of honeydew produced by aphids feeding on any of the treated diets and that produced by aphids which were starved.

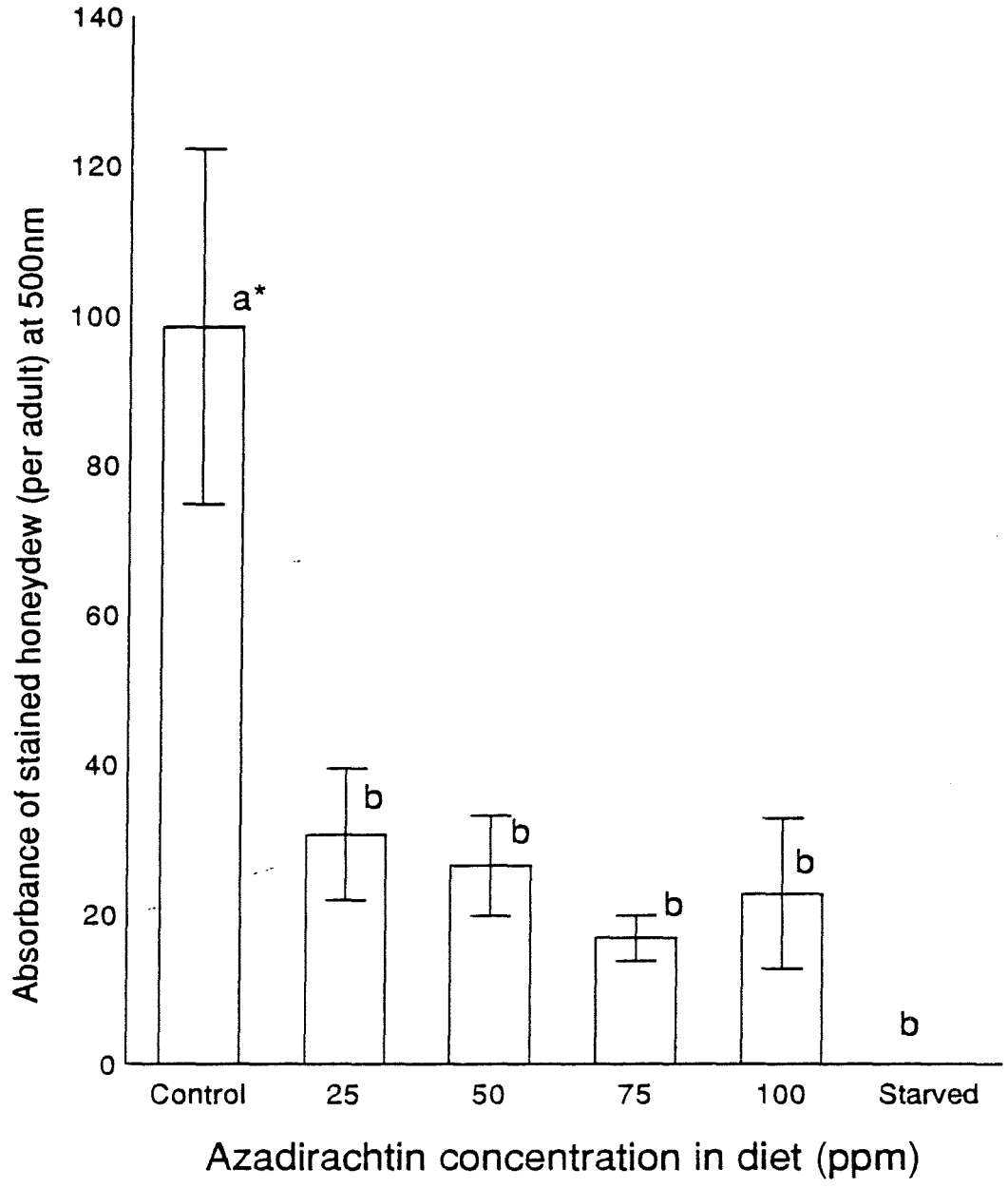
iii) Honeydew recording periods 3 and 4, (51-74h and 75-98 h). Aphids which had previously been given access to azadirachtin-treated diet produced less honeydew than those which had previously had access to control diets, but the only statistically significant reduction was seen during the final 24h period in the 100ppm treated group (Figures 53 and 54).

Figure 51. Honeydew production by M. persicae feeding on artificial diets treated with azadirachtin at lowered concentrations during honeydew recording period 1 (0-26h).



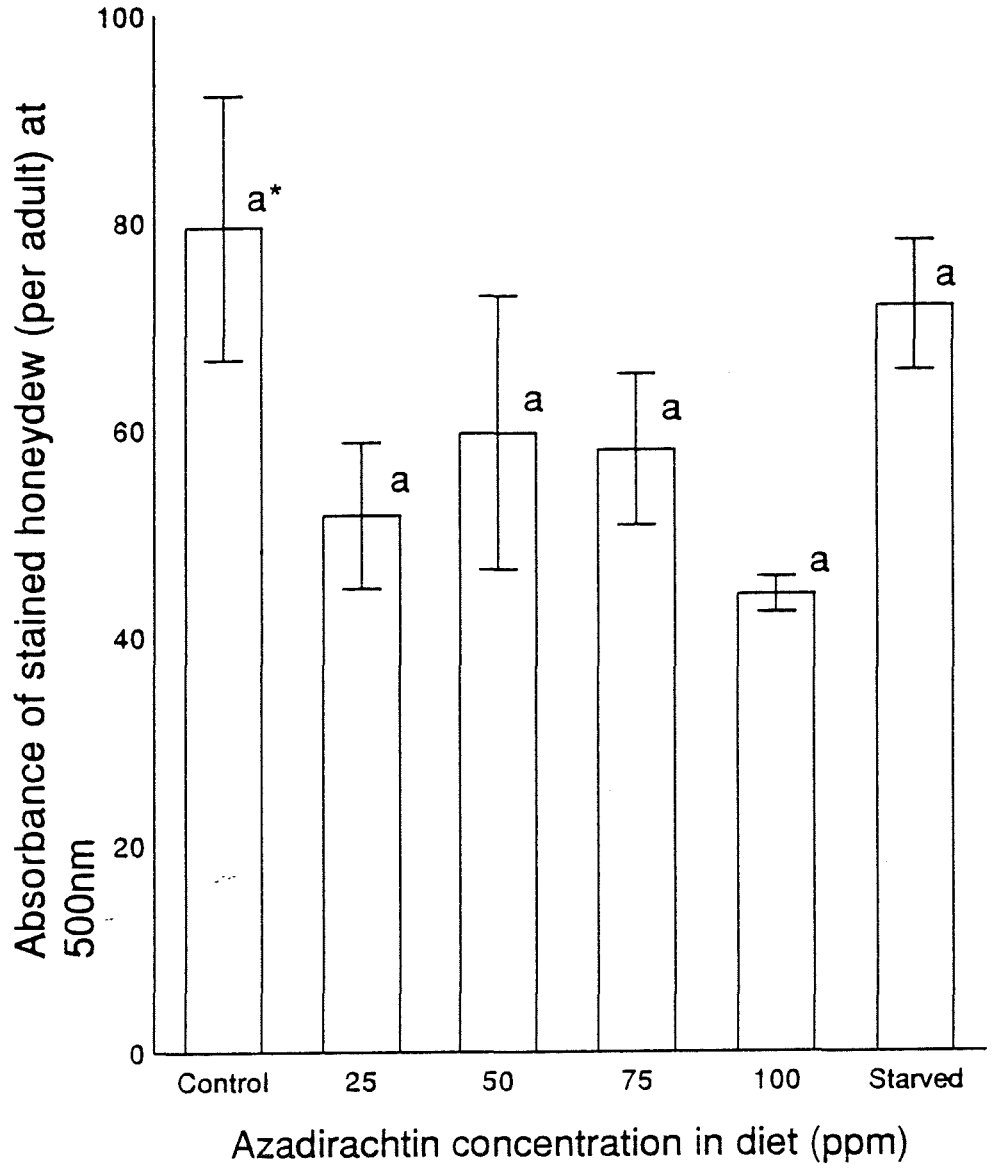
* Values annotated with the same letter are not significantly different (P=0.05).
Vertical bars indicate 2x Standard Error of Mean

Figure 52. Honeydew production by M. persicae feeding on artificial diets treated with azadirachtin at lowered concentrations during honeydew recording period 2 (27-50 h).



* Values annotated with the same letter are not significantly different (P=0.05).
Vertical bars indicate 2x Standard Error of Mean

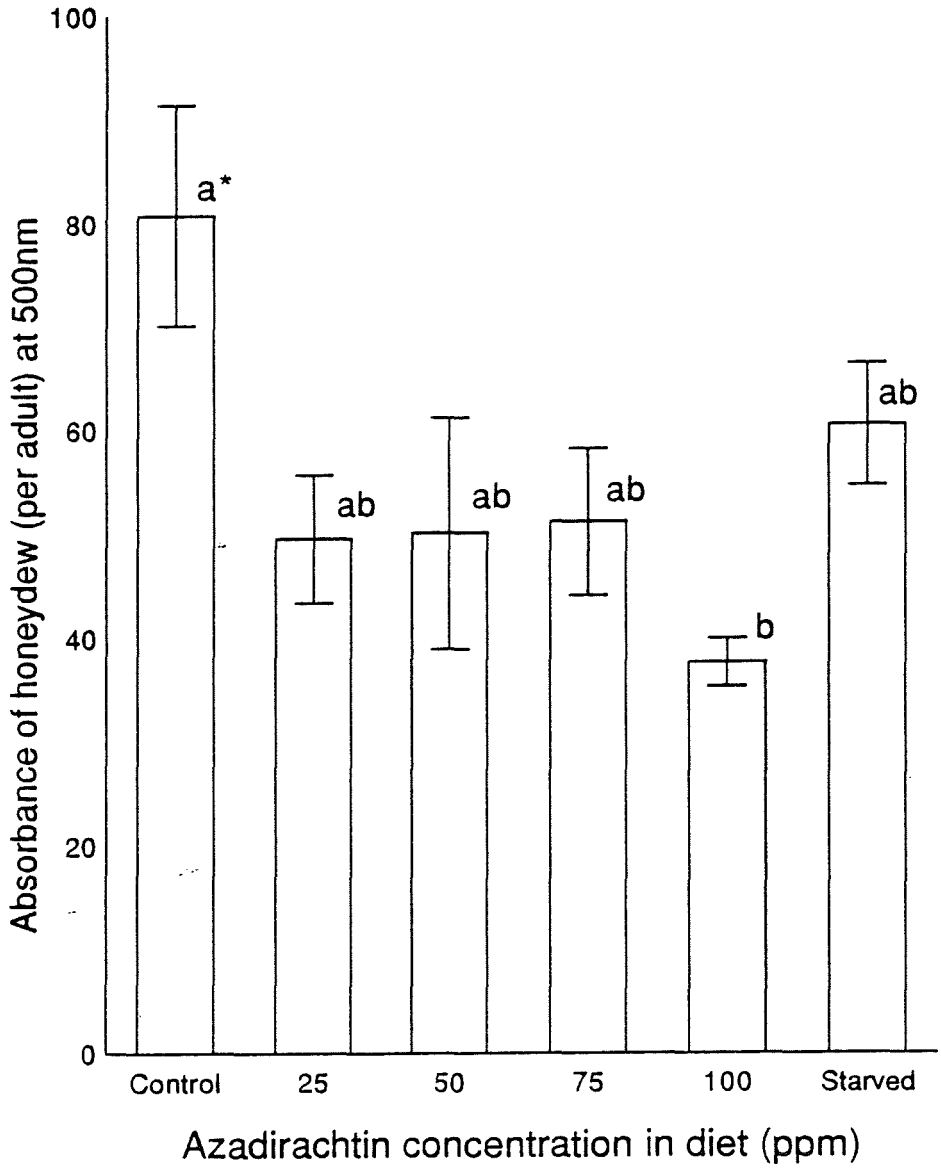
Figure 53. Honeydew production by M. persicae feeding on artificial diets treated with azadirachtin at lowered concentrations during honeydew recording period 3 (51-74 h).



* Values annotated with the same letter are not significantly different (P=0.05).

Vertical bars indicate 2x Standard Error of Mean

Figure 54. Honeydew production by M. persicae feeding on artificial diets treated with azadirachtin at lowered concentrations during honeydew recording period 4 (75-98 h).



* Values annotated with the same letter are not significantly different (P=0.05).
Vertical bars indicate 2x Standard Error of Mean

Chapter 5. The effects of azadirachtin on the acquisition and inoculation of potato leafroll virus by *Myzus persicae*.

5.1 Introduction

Potato leafroll virus (PLRV) is a member of the luteovirus group which replicates in the phloem tissue of about 20, mostly solanaceous, host plants (Harrison, 1984). After infection, virus particles are initially observed in sieve elements and in the plasmodesmata between sieve elements and companion cells (Shepardson *et al.*, 1980). PLRV may cause necrosis of the sieve elements and companion cells in susceptible genotypes. The necrosis is characterised by the death and collapse of cells with no concomitant hyperplastic growth, a process known as necrotic obliteration (Esau, 1957).

PLRV symptoms: The symptoms of primary PLRV infection (infection of a previously healthy plant) in potato are dependent on the resistance of the variety to PLRV multiplication (e.g. Barker & Harrison, 1985) and can be detected soon after the infection becomes systemic. The leaf margins may turn purple as a result of anthocyanin accumulation which is indicative of disturbed sugar metabolism. Ultimately the leaves become thickened, leathery and brittle and the plant is severely stunted. The tips of the leaves may also curl upwards at the edges (Bos, 1978). In secondarily infected plants (plants grown from infected tubers) large amounts of starch and other

carbohydrates are stored in the leaves and there is excessive formation of callose in the phloem tissue followed by phloem necrosis. The plants may be stunted and the leaflets of the lower leaves roll upwards (Harrison, 1984).

PLRV transmission: PLRV is transmitted in a persistent manner by a limited range of aphid vectors of which *M. persicae* is considered to be the most efficient (Sylvester, 1980). Virus particles, ingested by a small number of aphid species while feeding on the phloem sap, pass along the gut lumen to the hindgut where they may be either excreted in the honeydew or transported through the epithelium of the hindgut into the haemocoel (Sylvester, 1980, Gildow, 1987). When the virus particles, suspended in the haemolymph, come into contact with the accessory salivary glands they are transported in coated vesicles from the basal lamellae of the accessory salivary gland cells, through the cells and are released into the lumen of the salivary canal. The virus particles pass out of the salivary duct during feeding to complete the cycle of infection (Gildow, 1982).

Acquisition: The ability of *M. persicae* to acquire PLRV from an infected plant increases as the period of feeding on the plant (the acquisition access period, AAP) increases. Tamada & Harrison (1981) demonstrated that the quantity of PLRV retained by *M. persicae* feeding on infected *Physalis floridana* increased linearly with increasing AAP until 5 days after the initial access. After 7 days the virus titre of the aphids remained at a

high, steady level. Acquisition efficiency is also affected by several other factors as follows;

i) Feeding rate during the AAP: van den Heuvel & Peters (1990) demonstrated that nymphs which transmitted PLRV from infected *P. floridana* plants to indicator plants produced significantly more honeydew during the AAP than those which did not transmit virus after the same length of AAP.

ii) Virus titre of source: Barker & Harrison (1986) demonstrated that, in the PLRV-resistant potato variety Pentland Crown, the maximum concentration of virus measured in the leaves was less than 10% of that observed in the leaves of the PLRV-susceptible variety Maris Piper. As a result *M. persicae* adults feeding on Pentland Crown acquired approximately 23 times less virus than those feeding on Maris Piper and transmitted PLRV with an efficiency of 7% compared with 85% efficiency for those aphids which had acquired virus from Maris Piper. When PLRV was incorporated into artificial diets at different concentrations, *M. persicae* acquired the virus more readily from those diets which contained higher concentrations (van den Heuvel, 1991).

iii) Age of infected source material: PLRV is acquired less efficiently from lower, older leaves than upper, younger leaves of secondarily infected plants despite the occurrence of higher virus titres and symptom expression in the former (Tamada & Harrison, 1981; van den Heuvel & Peters, 1990). This is thought to be caused by reduced availability or retention of virus by aphids from older

infected tissue as a result of changes in the coat protein at the surface of the virus capsid (van den Heuvel, 1991).

iv) Developmental stage of vector: Robert (1971) demonstrated that nymphal apterae were more efficient vectors of PLRV than nymphal alatae. This was also true for adult apterous and alate virginoparae. First instar nymphs acquire and transmit PLRV more efficiently than older nymphs independent of the virus titre in the source (van den Heuvel, 1991).

v) Temperature and relative humidity: Singh *et al.* (1988) found that exposure of PLRV-infected *P. floridana* to combinations of 20 or 25°C with 80% rh for 48h prior to the AAP resulted in optimum acquisition of the virus by *M. persicae*. Temperatures of above 20°C during the AAP have resulted in reduced acquisition of the virus by aphids (Tamada & Harrison, 1981).

Inoculation: The maximum efficiency of inoculation of plant tissue with PLRV by aphids is usually achieved in a shorter period (inoculation access period, IAP) than that required for maximum efficiency of acquisition (Sylvester, 1980). Tamada & Harrison (1981) found maximum numbers of infective insects 4 days after the beginning of the AAP. Kennedy *et al.* (1962), cited by Sylvester (1980), reported an optimum IAP of less than 1h for PLRV. Inoculation is thought to be more a function of salivary sheath formation than of sustained feeding and may occur in the non-vascular as well as the vascular tissue (Sylvester, 1980). The efficiency of inoculation depends

on successful acquisition followed by the completion of a latent period. The length of the latent period is strongly affected by the temperature at which the aphids are kept (Tamada & Harrison, 1981). Other factors which affect inoculation efficiency include;

i) Settling and feeding during the IAP: Bindra & Sylvester (1961) found that in instances where the inoculation efficiency of PLRV was lower than expected, the percentage of aphids found settled on the indicator plants at the end of the IAP was considerably lower than the percentage found settled on plants which developed infections at the expected rate.

ii) The number and virus titre of aphids used to inoculate: Empirical evidence supports the theory that inoculations by individual aphids within a group are separate and independent events and that virus inoculation efficiency will follow that predicted by the binomial theorem (Bindra & Sylvester, 1961). A group of aphids will therefore transmit virus to a plant if any one of the group would have transmitted virus singly. Similarly the group will not transmit virus if none of the aphids would have transmitted singly.

iii) Temperature and relative humidity. Tamada and Harrison (1981) reported that inoculation of PLRV by *M. persicae* is more efficient at temperatures above those which are optimal for acquisition. In addition Singh *et al.* (1988) found that the number of plants developing symptoms of PLRV could be increased by keeping the plants

at temperatures of 25 or 30°C with 80 or 90% rh for 48h after the IAP.

In the present work, preliminary experiments were performed to optimise the conditions for acquisition and inoculation of PLRV from and to tobacco seedlings. These were followed by definitive experiments to determine any effects of azadirachtin treatment on virus acquisition and inoculation and to examine the nature of these effects.

5.2 Acquisition Experiments: Materials and Methods

General

Virus source potato plants; Potato tubers (cv. Maris piper), harvested from plants which had been infected with PLRV in the previous season, were planted in individual pots and placed in a glasshouse at approximately 20°C until emergence. In experiment 5.2.1 the plants were then placed in a growth cabinet at 10°C, 16h daylength for 11 days to slow growth, as they were growing very vigorously in the glasshouse. In all other experiments the plants remained in the glasshouse at approximately 20°C in insect-proof polythene cages.

Virus source tobacco plants and indicator plants; All virus source tobacco plants and indicator plants were *N. clevelandii* seedlings raised as described in section 2.3.1. In experiment 5.2.1 the indicator plants were 21 days old at the time of inoculation and in all subsequent experiments the source and indicator tobacco plants were

10-11 days old at the time of inoculation unless otherwise stated.

Quantitative virus determination; Quantitative analyses of virus presence were performed using the double antibody sandwich (DAS) enzyme linked immunosorbent assay (ELISA) technique (Clark & Adams, 1977).

Buffers; coating buffer, phosphate buffered saline (PBS) and PBS Tween were prepared as described in Clark & Adams (1977) with the exception that sodium azide was omitted from the solutions which were preserved by refrigeration. Extraction buffer was 2% (w/vol) polyvinylpyrrolidone (PVP) in PBS Tween.

Antisera were kindly provided by Dr. H Barker, S.C.R.I., Invergowrie. All antisera used were polyclonal and produced in rabbits immunised with preparations of purified virus particles. In experiment 5.2.1 the plates were prepared in duplicate, and two different gamma globulin conjugates were used for comparison;

a) alkaline phosphatase conjugate,

b) penicillinase conjugate.

The substrates used for these conjugates were 0.06% 4-nitrophenylphosphate in 10% diethanolamine (pH 9.8) and potassium penicillin G in 0.01% bromothymol blue (pH 7.2) (Singh & Barker, 1991) respectively.

All virus tests were performed using polystyrene microtitre plates, (NUNC Immunological 1, NUNC, Denmark), in which the outer wells were unused. Two replicate wells were used for each sample. Plates were coated with gamma globulin at a concentration of 1µg/ml in coating buffer

for 3h at 37°C. Plant tissue was disrupted by pestle and mortar or in a Pohlane roller press and was then diluted to 4% (w/vol) with extraction buffer in experiments 5.2.1 to 5.2.6 or 10% (w/vol) in all subsequent experiments. Dilution series of PLRV standards from 800ng/ml to 3.125ng/ml were prepared in disrupted leaf tissue from healthy *N. clevelandii*, diluted to 4% (w/vol) with extraction buffer in experiments 5.2.1 to 5.2.5 and 10% (w/vol) thereafter. The plates were then thoroughly washed with PBS Tween, and 200µl of sample or standard was added to each well before incubation at 4°C for 18h. After thorough washing with PBS Tween 200µl of the appropriate conjugate, at a concentration of 0.1% (w/vol), was added to the wells in conjugate buffer. The conjugate buffer, in the case of the alkaline phosphatase conjugate, contained disrupted leaf tissue from healthy *N. clevelandii*, diluted as previously. After 3h incubation at 37°C, and thorough washing with PBS Tween, the appropriate substrates were added, and the plates were incubated at room temperature for 4h and then at 4°C for a further 14h. The absorbance of the contents of each well was determined, using a Titertek Multiskan photometer (Flow Laboratories, Irvine, Scotland), hourly for 4h and after 18h at 405nm or 625nm for the alkaline phosphatase and the penicillinase reactions respectively. When the alkaline phosphatase reaction was used, samples with an absorbance of less than double that of healthy controls were regarded as being free from virus. When using the penicillinase reaction, samples with an

absorbance of more than half that of healthy controls were considered to be free from virus. The reason for the difference between the techniques is as follows: Alkaline phosphatase reacts with its substrate to produce an intense yellow coloured product and the increase in the intensity of this colour reflects the quantity of virus antigen present in each well. Penicillinase reacts with its substrate to form an acidic product. The associated reduction in pH turns the indicator (bromothymol blue) from a deep blue colour to a bright yellow/orange. Reading for absorbance at 625nm, the reduction in intensity of the blue colour reflects the quantity of virus antigen present.

Virus titres in infected samples were calculated from the standard curve, fitted using a computer programme, (Curvefit, BBC Master).

5.2.1. Optimisation of the number of aphids used for inoculation and time from inoculation to detection of virus in plant tissue.

Adult *M. persicae* (10 days old ± 24h) were raised from first instar nymphs on turnip plants in the conditions described in section 3.2.1. The aphids were starved for 22h before transfer to the virus source potato plants (50 per plant) which had been moved from the 10^{o}C growth cabinet to a glasshouse maintained at approximately

ambient temperature and relative humidity. The aphids were reared on these source plants for an AAP of 7 days. On their removal from the plants it was noted that the aphids had suffered high mortality and only 46% had survived. The surviving aphids were starved for 6h and transferred to *N. clevelandii* indicator plants. Thirty-two indicator plants were infested with one, two, three or four aphids, to give eight replicates of each number of aphids. The aphids were enclosed in an area around each plant by a cylinder of clear plastic, approximately 4cm in diameter, sealed at one end with bolting nylon, and sunk into the compost to a depth of approximately 1cm. The plants were held in a glasshouse at approximately 20°C. After an IAP of 4 days the aphids and any nymphs that they had produced were removed using a fine paintbrush and the plants were fumigated with nicotine shreds. After a further 10 day period, eight of the indicator plants, comprising two representatives of each aphid number group, were tested for PLRV infection by ELISA. Because of the small size of the indicator plants at this stage the whole plants were used for virus testing. Approximately 1g of leaf tissue was removed 4 days later from each of the remaining replicates and stored at 4°C for 2 days before virus testing. Eighteen days after the end of the IAP sixteen of the replicates comprising four representatives from each aphid number group, were tested for virus presence by ELISA and after a further 4 days eight replicates, two from each aphid number group, were similarly tested. At each 4 day period

during the experiment, the plants were visually assessed for presence and severity of PLRV symptom expression, i.e. leaf shortening, and plant stunting.

5.2.2 Attempted comparison of PLRV acquisition from azadirachtin treated and control plants.

Inoculation of virus source tobacco plants; 60-70 adult aphids, raised as described in section 5.2.1, were placed onto each of six, 7 week old virus source potato plants in polythene cages in a glasshouse at approximately 20^{o}C for an AAP of 6 days. The regulation of temperature helped to reduce aphid mortality during the AAP. The remainder of the experimental protocol is shown diagramatically in Figure 55.

For 10-12h before the AAP and during the AAP itself, the virus source tobacco plants were treated with azadirachtin or control solutions in plastic cages ("systemic movement cages") designed to enclose aphids (see Figure 56). The honeydew produced by the feeding aphids was collected on collars of filter paper from 15 randomly selected replicates of both treated and control plants during this virus acquisition period. The honeydew was stained using the ninhydrin reagent described in section 4.2. The resulting stained material was eluted into 1.5ml of 90% methanol and the absorbance was read at 500nm as previously described. After the AAP the aphids were counted and removed and the virus source plants were given a phytotoxicity score of 0-5 as follows:

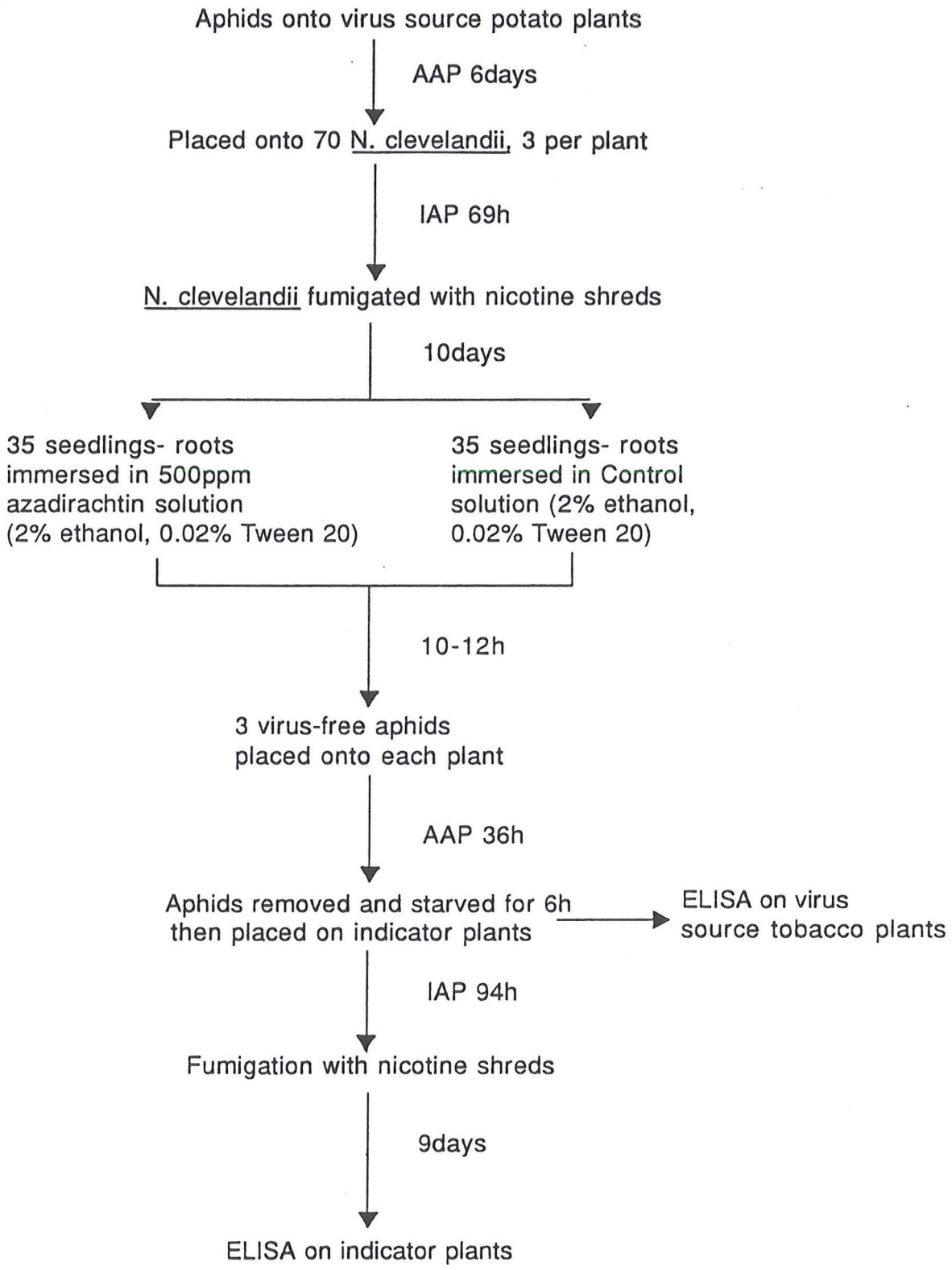


Figure 55. Experimental protocol for experiment 5.2.2; Attempted comparison of PLRV acquisition from azadirachtin-treated or control plants.

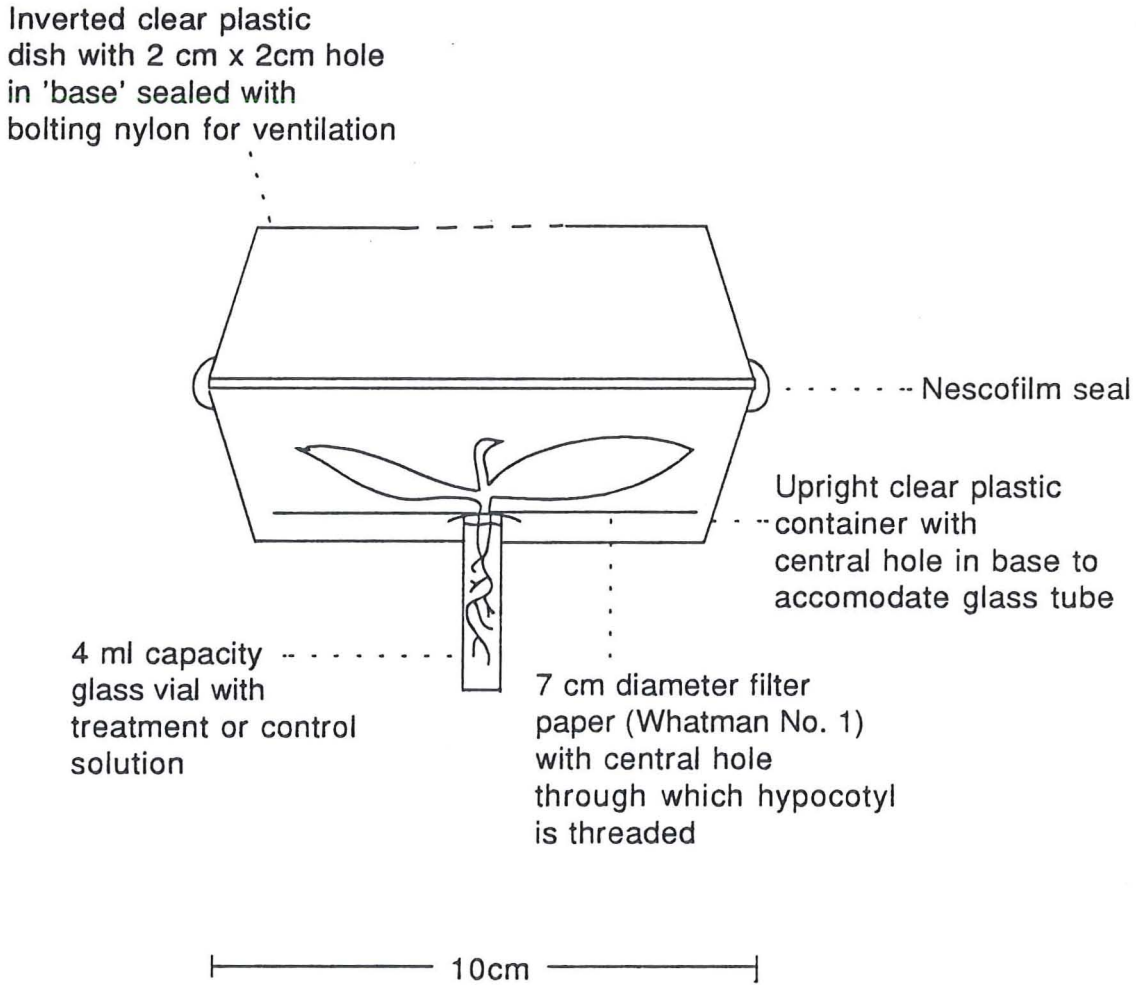


Figure 56. The cages used to enclose aphids on virus source tobacco plants during the AAP in experiments 5.2.1-5.2.3.

0= no phytotoxicity

1= 1-25% of leaf area wilted

2= 25-50% leaf area wilted

3= 50-75% leaf area wilted

4= terminal bud alive, 75-100% leaf area wilted

5= plant dead

Each source plant was weighed and assayed for virus titre using alkaline phosphatase-based ELISA.

Nymph production during the IAP on indicator plants was recorded for 15 replicates of both treated and control plants.

Analysis of results: The proportions of aphids surviving on azadirachtin-treated and control virus source tobacco plants after the AAP were compared using a generalised linear model with a binomial distribution. The titres of PLRV in the virus source tobacco plants, phytotoxicity and honeydew and nymph production per surviving adult were all compared using Student's t-tests. The data on nymph production were square root transformed prior to analysis.

5.2.3 Optimisation of AAP for *M. persicae* adults feeding on PLRV-infected *N. clevelandii* and determination of the feasibility of maintaining the seedlings in solution for this period.

Inoculation of source tobacco plants; N. clevelandii seedlings were each infested with 3 *M. persicae* nymphs (1st-4th instar) which had been produced by adult aphids

on PLRV source potato plants. The subsequent experimental protocol is shown diagramatically in Figure 57.

After the AAP on treated or control virus source tobacco plants, adult mortality and phytotoxicity were measured as in section 5.2.2.

Analysis of results: The PLRV titres of the virus source tobacco plants were analysed as described in section 5.2.2. The effects of azadirachtin treatment, access periods and the interactions of these factors on aphid survival were analysed using a generalised linear model with a binomial distribution. Similarly the effects of these factors on phytotoxicity were analysed using ANOVA.

5.2.4 Attempt to reduce mortality during the AAP and increase the transmission of PLRV to indicator plants.

It was suspected that removal of the virus source tobacco plants from the potting compost may adversely affect the survival of the aphids feeding on these plants. The aim of the following experiment was to remove all factors that may have been responsible for the observed low survival of aphids and to attempt to increase the acquisition of PLRV from the virus source tobacco plants by manipulating the environment in which the plants were kept prior to and during the AAP.

Method: 56 virus source tobacco plants were produced as in section 5.2.3 with the exception that aphids were removed by hand at the end of the IAP instead of by nicotine fumigation to exclude the possibility of

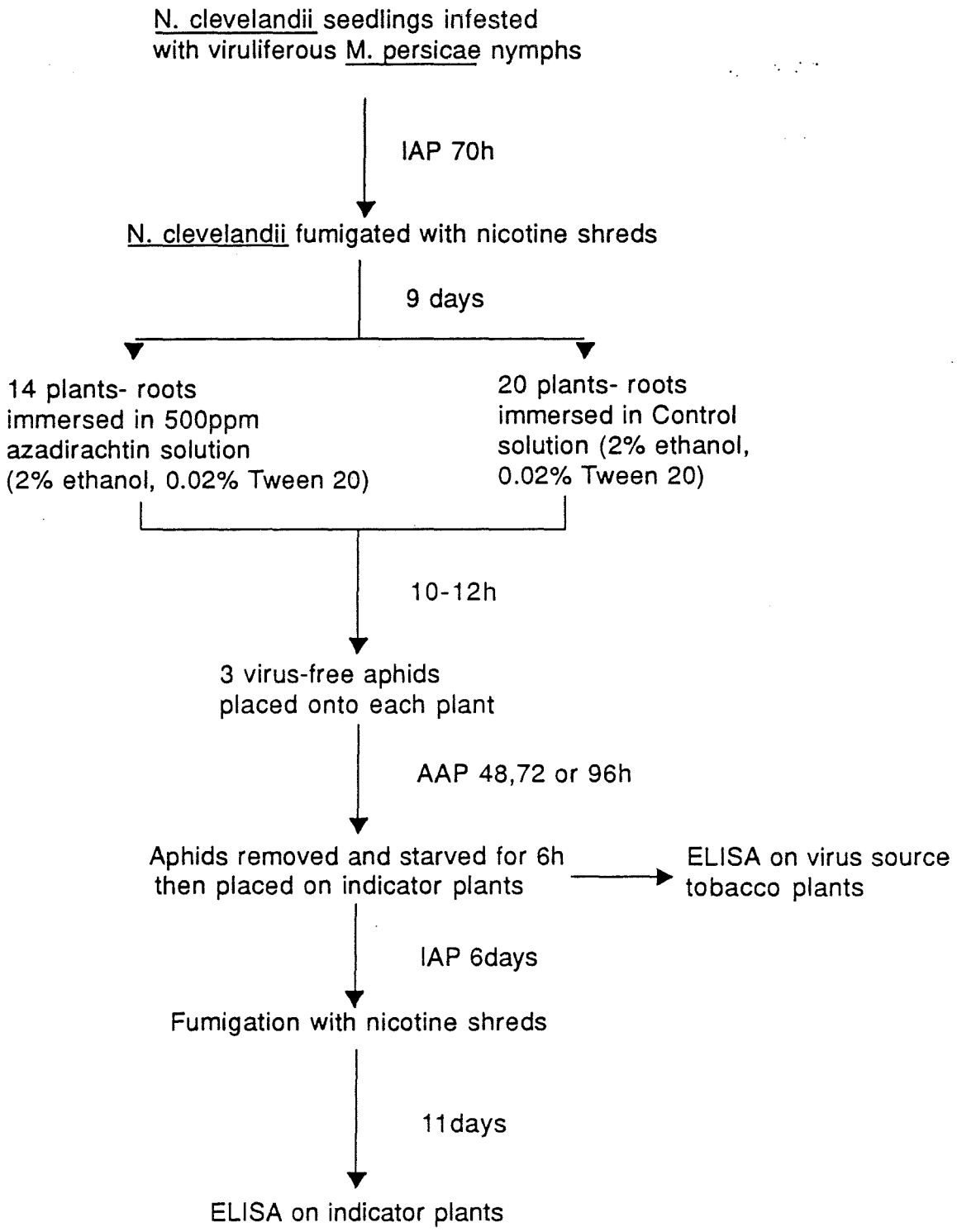


Figure 57. Experimental protocol for experiment 5.2.3; Optimisation of AAP for M. persicae adults feeding on PLRV-infected N. clevelandii and determination of the feasibility of maintaining the seedlings in solution for this period.

insecticidal residues. After a further 10 day period the plants were divided into four groups and were either removed from the compost or remained potted prior to confinement in constant environment rooms at 18°C, 70-80% rh or 25°C, 80-90% rh for 16h as shown in Table 28.

Table 28. The groups (A-D) in which the virus source tobacco plants were placed according to whether or not they were removed from the potting compost and the temperature at which they were held for 16h prior to the AAP. The figures in parenthesis indicate the number of plants in each group.

		Removed	Potted
Temperature	18°C	A (13)	B (15)
	25°C	C (13)	D (15)

The systemic movement cages were modified to reduce any build-up of toxic volatiles by dispensing with the inverted plastic dishes that formed the cage lids. Aphids were prevented from escaping by a 1cm deep film of Fluon (polytetrafluoroethylene) around the inner rim of the upright dish (see Figures 59 and 60). The subsequent experimental protocol is shown in Figure 58.

After the AAP, the number of aphids surviving on treated or untreated virus source tobacco plants was counted and each of these plants was scored for phytotoxicity before being tested for PLRV presence by ELISA.

Analysis of results: The PLRV titres of the virus source tobacco plants were analysed as described in section

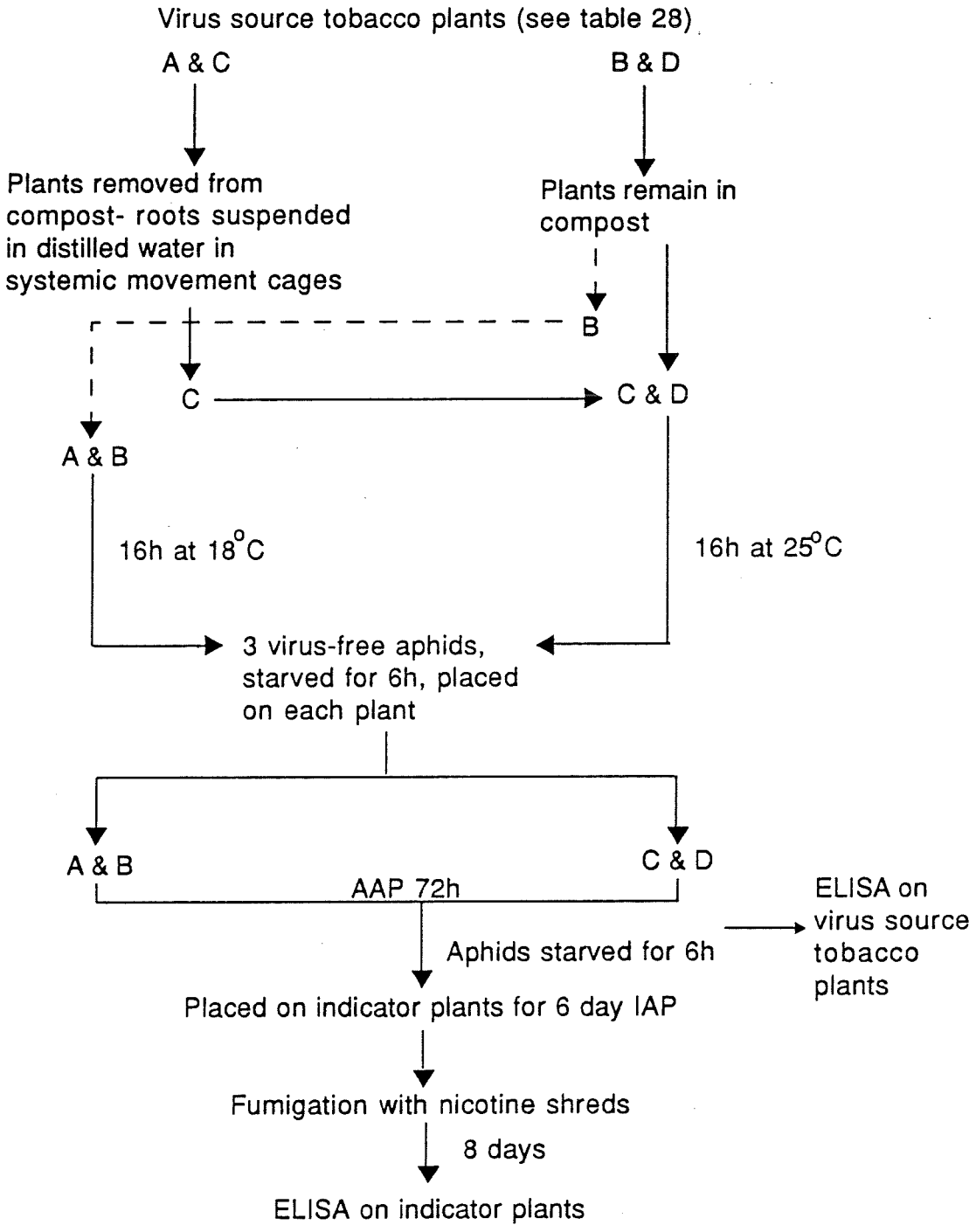


Figure 58. Experimental protocol for experiment 5.2.4; Attempt to reduce mortality during the AAP and to increase the transmission of PLRV to indicator plants.

Original in colour

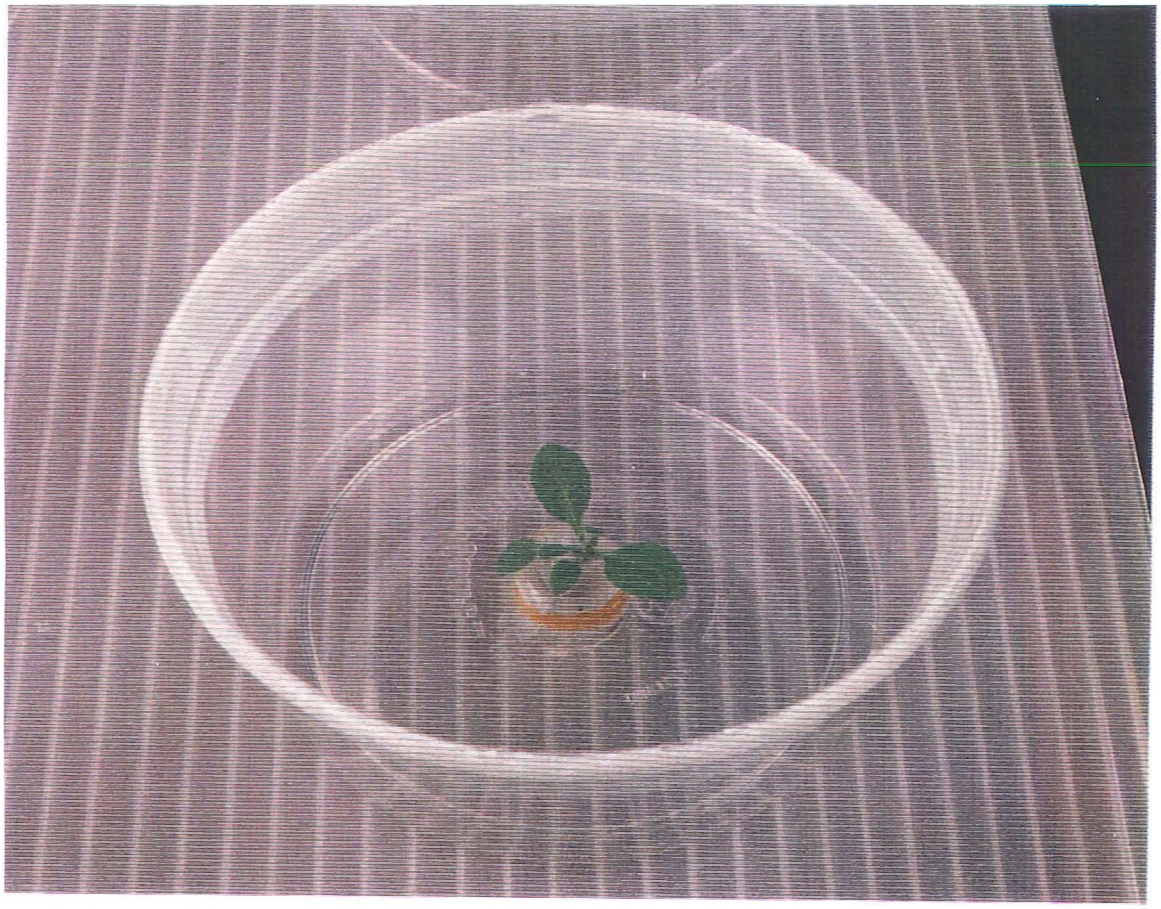


Figure 59. Modified systemic movement cage used from experiment 5.2.4 onwards.

Original in colour

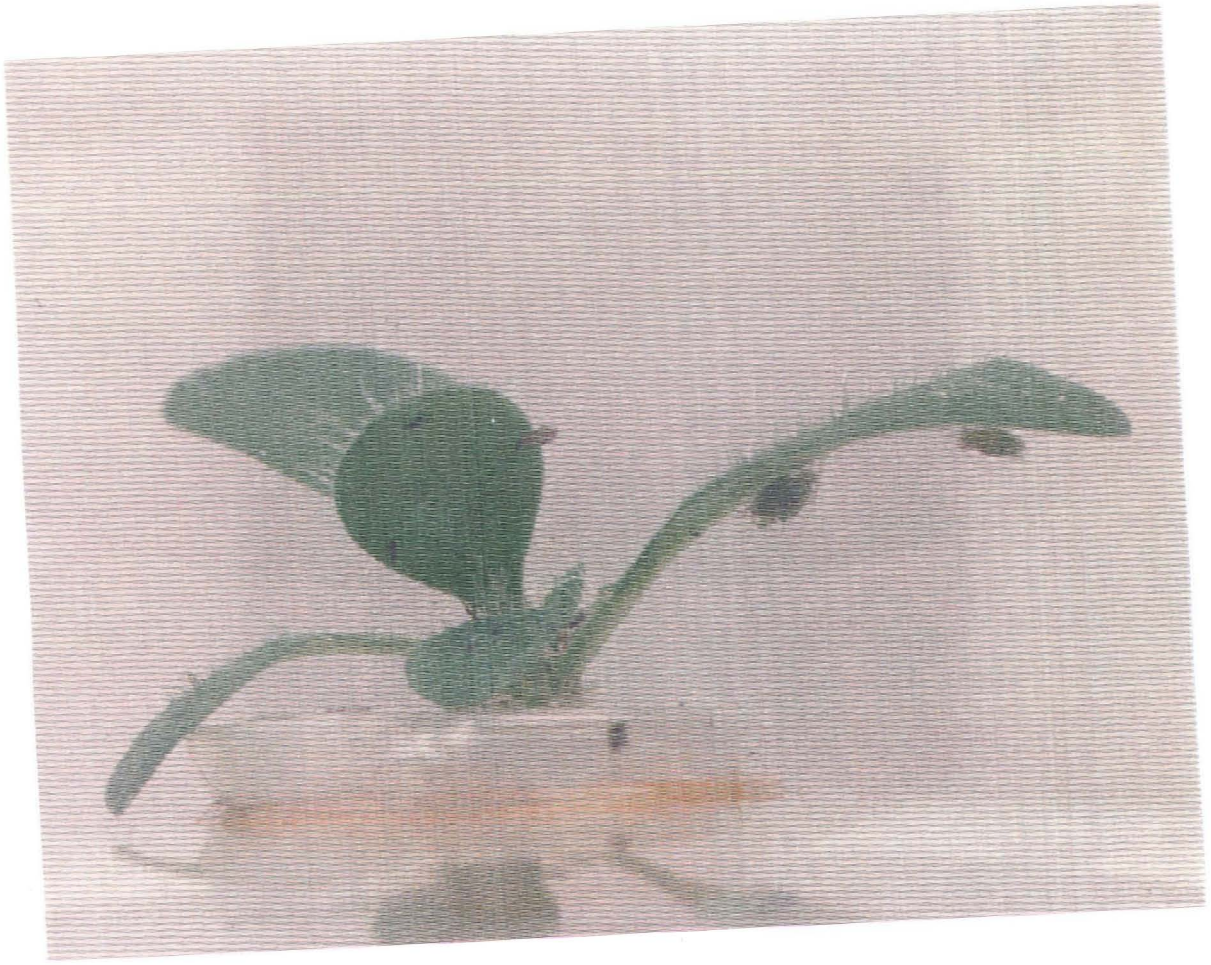


Figure 60. Adult M. persicae feeding on leaves of N. clevelandii seedling in modified systemic movement cage.

5.2.2. The effects of environmental conditions, removal from compost and the interactions of these factors on both aphid survival and the proportion of indicator plants infected with PLRV were analysed using a generalised linear model with a binomial distribution. The effects of these factors and their interactions on phytotoxicity were analysed using ANOVA.

5.2.5 Quantitative detection of PLRV by ELISA in single adult *M. persicae*.

As a result of the low numbers of infected indicator plants in all of the above experiments a method was developed to augment or replace the use of indicator plants to determine the ability of *M. persicae* to acquire PLRV from azadirachtin-treated plants.

Aphids; i) Viruliferous: Adult apterous *M. persicae* raised from birth on PLRV infected potato plants in a glasshouse at 20°C.

ii) Non-viruliferous aphids: as in section 3.2.1.

Method; The method was based on a penicillinase double antibody sandwich ELISA technique used for the detection of luteoviruses in whiteflies (P. McGrath, pers.comm., 1991) and was similar to that previously described for the plant assay, with the following exceptions;

i) Extraction buffer; as above with 5% Marvel dried milk.

ii) Extraction was performed using a minidrill in a plastic vial.

iii) Volumes of coating buffer, sample (in extraction buffer), conjugate buffer and substrate buffer were all 100µl.

iv) Plant sap from healthy *N. clevelandii* was not added to any preparation.

48 individual aphids were assayed in total, (24 viruliferous, 24 non-viruliferous). Microtitre plates were prepared in duplicate and both the alkaline phosphatase and the penicillinase-based techniques were used to compare background interference. A dilution series of PLRV was prepared as previously, and three wells on each plate were left with no sample added to compare with non-viruliferous samples.

Detection factors (DF) were calculated for each of the reactions as described below (after Singh & Barker, 1991):

a) for alkaline phosphatase-based reaction;

$$DF = \frac{\text{Mean absorbance of virus-containing samples}}{\text{Mean absorbance of healthy samples}}$$

b) for penicillinase-based reaction;

$$DF = \frac{\text{Mean absorbance of healthy samples}}{\text{Mean absorbance of virus-containing samples}}$$

5.2.6 Further attempt to reduce aphid mortality during the AAP.

Aphid mortality during the AAP in experiment 5.2.4 was high, even in the groups of aphids which spent this period on plants which remained potted. This indicated that the mortality may not have been caused by the removal of the plants from the compost and the subsequent phytotoxicity observed, but may have been caused by an inability of aphids raised on different host plants to sustain feeding on tobacco seedlings. To attempt to overcome this possible problem aphids which were used to acquire PLRV from virus source tobacco plants were given access to uninfected *N. clevelandii* seedlings prior to the AAP and only aphids which survived this pre-treatment were used for acquisition experiments. The results of experiment 5.2.4 also indicated that removal of the plants from the compost may have some effect on aphid survival. This may have been caused by changes in the phloem chemistry and/or nutrient availability induced by root damage. To attempt to reduce these effects, a proportion of the virus source tobacco plants were removed from potting compost and grown in nutrient solution for several days prior to their use in acquisition studies.

Aphids; 11 day old (± 24h) apterous *M. persicae*, raised as described in section 3.2.1, were transferred, 6 per plant, to 21 day old *N. clevelandii* and were kept for 66h in a glasshouse at approximately 20°C.

Method; Virus source tobacco plants, which had been infected with PLRV as described in section 5.2.4, were divided into two groups; those to remain in compost (8 plants) and those to have their roots immersed in nutrient solution (14 plants). The plants which were to be treated with nutrient solution were removed from the compost and, after thorough washing, the roots of each plant were suspended in 1.5ml of the inorganic nutrient solution described by Smith *et al.* (1983). All of the virus source tobacco plants were then placed in a glasshouse at 20°C for 3 days. The plants were then removed from the compost or the nutrient medium and, after thorough washing, their roots were immersed in 2% ethanol, 0.02% Tween 20 solution in the modified systemic movement cages described in section 5.2.4 in a constant environment of 18°C, 70-80% rh, for 16h. After this period two aphids, which had been removed from uninfected *N. clevelandii* seedlings and starved for 16h, were placed on each virus source tobacco plant for an AAP of 66h. After the AAP, the surviving aphids were counted and removed, phytotoxicity was scored, and the virus source tobacco plants were tested for PLRV using ELISA. The groups of putative infective aphids were starved for 6h and then transferred to indicator plants. Following an IAP of 90 h, the aphids were removed. The indicator plants were grown for a further 14 days before they were tested for virus presence using ELISA.

Analysis of results: The effect of removing the virus source tobacco plants from compost on aphid survival and

the proportions of indicator plants infected were analysed using a generalised linear model with a binomial distribution. The PLRV titres of the virus source tobacco plants and the effects of the different root treatments on phytotoxicity were analysed using Student's t-tests.

5.2.7 Comparison of PLRV acquisition by *M. persicae* on individual azadirachtin-treated or untreated tobacco seedlings.

The results of experiment 5.2.6 suggested that transfer of the virus source tobacco plants to nutrient solution prior to the AAP did not aid virus acquisition and subsequent inoculation to indicator plants. Mortality of aphids was however reduced by using aphids that had already survived for a prolonged period on *N. clevelandii* seedlings and this practice was employed in the following experiment.

Virus source tobacco plants; PLRV infected *N. clevelandii* plants were produced as in section 5.2.4 and were removed from potting compost, washed and treated with a solution of azadirachtin (23 plants) or a control solution (22 plants) as described in section 5.2.2 in modified systemic movement cages for 12h before the introduction of aphids.

Aphids; Adult apterous *M. persicae* (9 days old ± 24h) were raised from 1st instar nymphs on turnip plants at 18°C, 70-80% rh, 18L:6D and were then transferred, 6 per

plant, to 21 day old, virus-free *N. clevelandii* for 90h. After this period the aphids were removed and starved for 6h. Three aphids were then placed on each virus source tobacco plant for an AAP of 72h. The surviving aphids were then counted and removed, phytotoxicity was scored, and the virus source tobacco plants were tested for PLRV using ELISA. The groups of putative infective aphids were transferred to indicator plants without prior starvation. Following the 90h IAP, the aphids were removed and tested for virus presence using penicillinase-based ELISA described in section 5.2.5. After a further 14 day period alkaline phosphatase-based ELISA was performed on the indicator plants.

Analysis of results: The effects of azadirachtin treatment on aphid survival during the AAP and the proportions of aphids and indicator plants infected with PLRV were analysed using a generalised linear model with a binomial distribution. The PLRV titres of the virus source tobacco plants, those indicator plants and aphids that were infected and the phytotoxicity scores of the virus source tobacco plants were analysed using Student's t-tests.

5.2.8 Comparison of PLRV acquisition by *M. persicae* on groups of azadirachtin-treated or untreated tobacco seedlings.

It was suspected that the differential survival observed in groups of aphids feeding on azadirachtin-treated and

control virus source tobacco plants strongly affected the ability of these groups to transmit the virus to indicator plants in the previous experiment (see section 5.4.7). The following experiment was designed to remove the differences in transmission that resulted from differential mortality within groups.

Virus source tobacco plants; PLRV infected *N. clevelandii* plants were produced as in section 5.2.4. They were removed from potting compost, washed and their roots were immersed in solutions of 500ppm azadirachtin (40 plants) or a control solution (40 plants) as described in section 5.2.2 in 1.5ml capacity plastic vials. The plants were then transferred, in eight groups of ten, to 500ml capacity white plastic food containers. The plastic vials were approximately equally spaced in the containers and were secured to the base with Blu-tack (Bostik Ltd.). A 1cm deep band of Fluon was deposited around the inner rim of the container to prevent aphid escape. The containers were then placed in a constant environment room at 18°C, 70-80% rh and 18L:6D for 12h prior to the introduction of aphids.

Aphids; Apterous adult *M. persicae* were raised from first instar nymphs deposited over a 24h period on 6 week old *N. clevelandii* seedlings. When the aphids were 11 days old (± 24h) they were removed from the seedlings and starved for 6h prior to use.

After the starvation period and when the virus source tobacco plants had been in the constant environment room for 12h, 60 aphids were introduced into each container

for an AAP of 72h. After the access period the aphids were removed from the plants and were starved for 6h. The virus source tobacco plants were tested for PLRV presence by ELISA. Following the starvation period 3 putative viruliferous aphids were placed on each of 80 indicator plants for an IAP of 96h. Fourteen days later virus presence was measured in the indicator plants by ELISA.

Analysis of results: Analysis of the PLRV titres of virus source tobacco plants and the effects of azadirachtin treatment on the number of indicator plants infected were performed as described in section 5.2.7.

5.3 Inoculation experiments: Materials and Methods

General:

Aphids; 11 day old (± 24h) adult apterous *M. persicae* were reared from first instar nymphs on virus source potato plants in a glasshouse at approximately 20°C. The aphids were removed from the plants and were starved for 6h before the IAP on indicator plants.

Indicator plants; 20 day old *N. clevelandii* plants were used as indicator plants in both experiments. The plants were removed from the potting compost and after thorough washing the roots of each plant were immersed in azadirachtin solution or in a control solution as described in section 5.2.2 in 1.5ml capacity plastic vials. Both experiments (5.3.1 and 5.3.2) were performed in constant conditions of 18°C, 70-80% rh, 18D:6L.

5.3.1 Comparison of the ability of *M. persicae* to inoculate azadirachtin-treated or untreated tobacco seedlings with PLRV in a "no-choice" experiment.

Each *N. clevelandii* indicator plant was kept in isolation from the others in the modified systemic movement cages described in section 5.2.4. The plants were treated with azadirachtin or control solutions as described above for 12h. After this period, three of the aphids which had been raised on the virus source potato plants were placed onto each plant, without prior starvation, for an IAP of 72h. The number of aphids surviving at the end of this period was noted and plants were then removed from the systemic movement cages and re-potted in compost. The re-potted plants were fumigated with nicotine shreds and grown for 16 days in an aphid-free glasshouse at 22^{o}C. At the end of this period each plant was weighed and tested for virus presence by ELISA.

Analysis of results: The effects of azadirachtin treatment on aphid survival and the proportion of indicator plants infected were analysed using a generalised linear model with a binomial distribution. The weights of control and azadirachtin-treated indicator plants and their PLRV titres were compared using Student's t-tests.

5.3.2 Comparison of the ability of *M. persicae* to inoculate azadirachtin-treated or untreated tobacco seedlings with PLRV in a "choice" experiment.

All aphids and plants used were raised and treated with azadirachtin or control solutions as in experiment 5.3.1 with the exception that one azadirachtin-treated plant and one control plant were placed in each of 40 choice chambers (Figure 61). The choice chambers were arranged in a randomised block design with each alternate row having the opposite orientation of azadirachtin-treated and control plants. 12h after the roots were immersed, 2,4,6 or 8 aphids (10 replicates of each) were placed in the centre of each choice chamber. The numbers of the surviving aphids which were in a probing position (see section 3.2.1 for definition) on treated or control plants were counted after 24, 48 and 72h. After the final count the aphids were removed and the plants were re-potted and fumigated with nicotine. 16 days later the indicator plants were tested for virus presence by ELISA.

Analysis of results: The settling data were analysed using a generalised linear model with a binomial distribution to predict the probability of the aphids selecting treated plants. These data were analysed separately for each observation period and for each aphid group size. The effects of azadirachtin treatment, the number of aphids used to inoculate and the interaction of these factors on the proportion of indicator plants

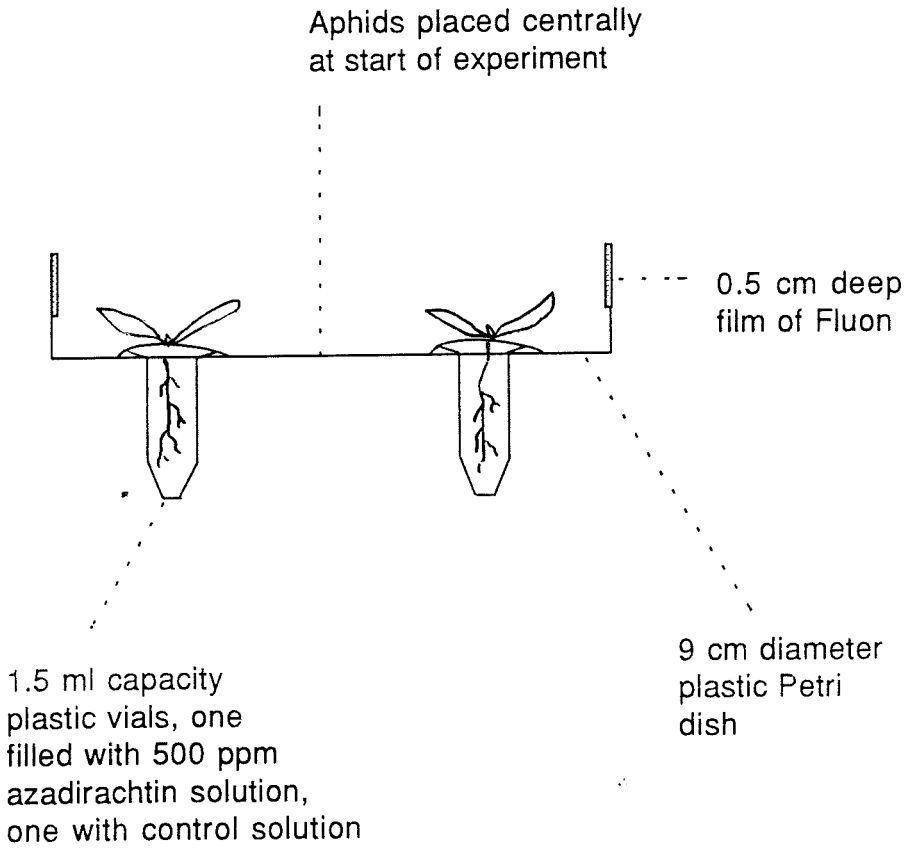


Figure 61. Choice chambers used in experiment 5.3.2.

infected were also analysed using a generalised linear model with a binomial distribution. The PLRV titres of the virus source tobacco plants and of the infected indicator plants were analysed using a Student's t-test and an ANOVA respectively.

5.4 Results

5.4.1 Optimisation of the number of aphids used for inoculation and time from inoculation to detection of virus in plant tissue.

Of 32 indicator plants only 9 were shown, by ELISA, to be infected with PLRV. Successful inoculation was achieved using 2,3 or 4 aphids (see Table 29).

All plants which were shown to be infected on days 18 and 22 had previously been shown to be infected on day 14. By day 22 after infection, virus titres were, for all plants tested, too high to be quantified.

Table 29. The number of successful inoculations of PLRV using different numbers of aphids.

No. of aphids used	No. of successful infections
1	$^{0}/_{8}$
2	$^{3}/_{8}$
3	$^{2}/_{8}$
4	$^{4}/_{8}$

Symptom expression as a means of determining infection was unsafe, although in every case where the combination of strong leaf symptom expression and severe stunting occurred, virus presence was confirmed using ELISA.

No symptoms were apparent in one plant after 14 days although PLRV was detected by ELISA at this juncture. Symptoms became visible in this plant 18 days after inoculation.

Alkaline phosphatase and penicillinase-based ELISA techniques were both able to detect PLRV presence in plant tissue, though the conversion of substrate and accompanying colour change took longer using the penicillinase-based technique. In addition the relationship between absorbance and virus titre was linear over a wider range of virus concentrations when the alkaline phosphatase-based technique was used and determination of virus titre from the standard curve was therefore more accurate (see Figure 62). For this reason, the alkaline phosphatase-based technique was used to determine PLRV presence and titre in plant tissue in all subsequent experiments.

5.4.2 Attempted comparison of PLRV acquisition from azadirachtin-treated and control plants.

i) PLRV titres of the virus source tobacco plants: The concentrations of PLRV in the virus source tobacco plants which had been treated with azadirachtin solution or control solution were not significantly different (see

Figure 62. Standard curves of PLRV titre against absorbance for alkaline phosphatase and penicillinase reactions.

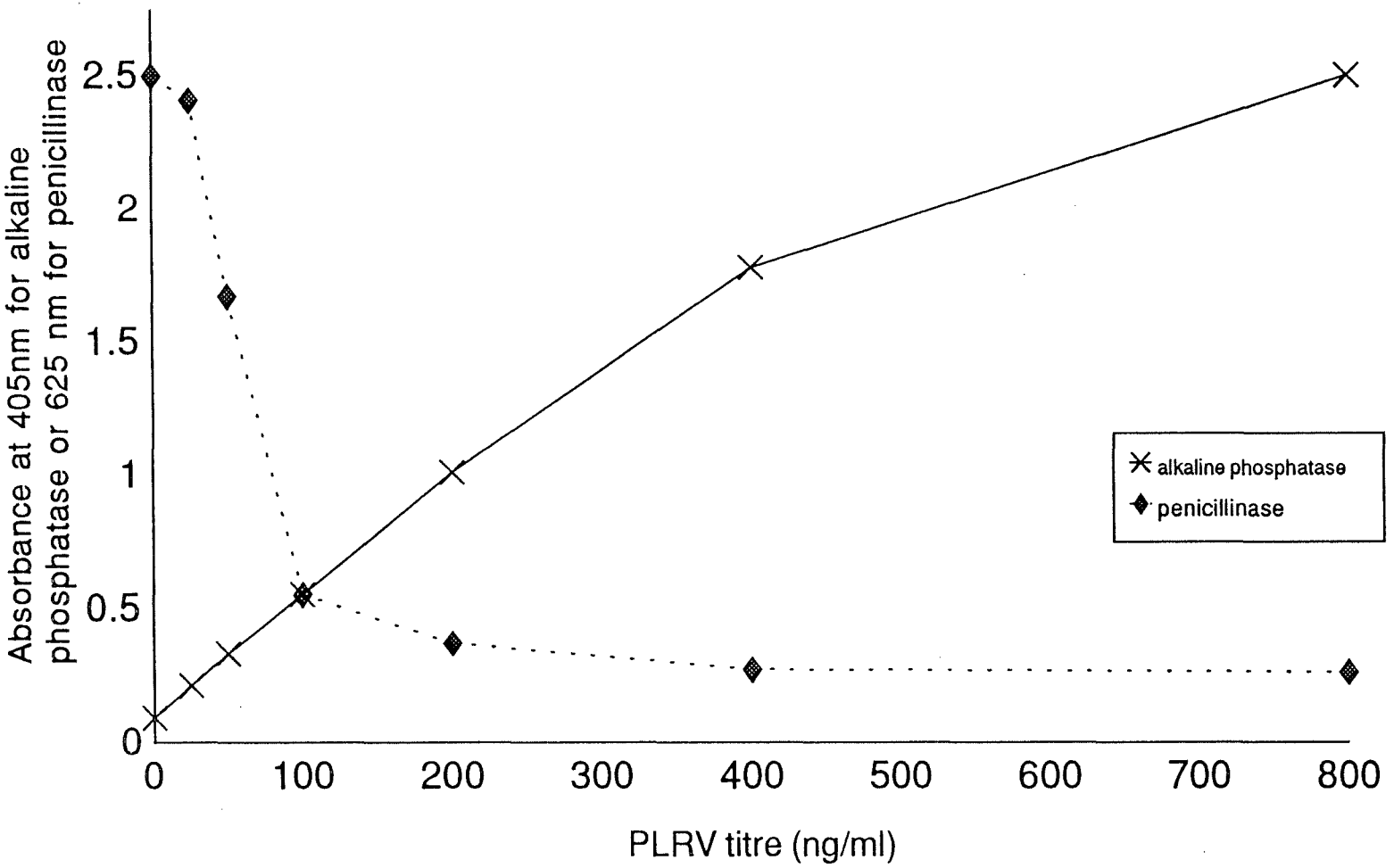


Table 30) and it was concluded that azadirachtin did not possess antiviral properties against PLRV.

ii) Survival of adults during AAP: The proportions of adults surviving the AAP on azadirachtin-treated plants was significantly less than on control plants (see Table 30).

iii) Honeydew production during the AAP: As a result of the large amount of variation in the amount of honeydew produced by aphids feeding on control plants, the mean absorbance of honeydew produced by aphids on azadirachtin treated plants was not significantly less than that produced by aphids on untreated plants (see Table 30).

iv) Nymph production during the IAP: The total number of nymphs produced on 15 indicator plants by aphids which had previously spent the AAP on azadirachtin-treated plants was 5 compared with a total of 365 produced on the same number of indicator plants by aphids which had spent the AAP on control plants. Aphids which had spent the AAP on azadirachtin-treated plants produced significantly fewer nymphs per surviving adult during the IAP than aphids which had spent the AAP on control plants (see Table 30). When the adult aphids which had spent the AAP on azadirachtin-treated plants were removed from the indicator plants, dark oval shapes were seen in their abdomens. These shapes were never seen in aphids from control plants. On dissection of the adults, the dark objects proved to be fully-formed melanised nymphs.

v) Phytotoxicity: Plants which had been treated with azadirachtin suffered significantly higher phytotoxicity scores than untreated plants (see Table 30).

Table 30.Summary of results from experiment 5.4.2: Attempted comparison of PLRV acquisition from azadirachtin-treated and control plants.

	Treatment of virus source tobacco plants during the AAP			
	Azadirachtin	Control	n	Significance
PLRV titre of source tobacco plants (ng/g fresh weight)	447 (34)*	418 (21)	32	n.s.
% of adult aphids surviving the AAP	90.5	98.1	70	P<0.01
Absorbance of honeydew produced in AAP	9.6 (2.9)	55.3 (24.9)	30	n.s (P=0.08)
¶no. of nymphs produced per surviving adult during IAP	0.16 (0.1)	3.1 (0.1)	30	P<0.001
Phytotoxicity score	1.29 (0.15)	0.69 (0.11)	70	P<0.01

* Values in parenthesis are standard errors of means.

vi) Infection of indicator plants: None of the indicator plants was infected with PLRV. It was suspected that this was because the AAP of aphids on the virus source tobacco plants (36h) was too short.

5.4.3 Optimisation of AAP for *M. persicae* adults feeding on PLRV-infected *N. clevelandii* and determination of the feasibility of maintaining the seedlings in solution for this period.

i) PLRV titres of the virus source tobacco plants: The PLRV titre of the virus source tobacco plants at the end of the AAP was unaffected by azadirachtin treatment or the duration of the AAP. The mean PLRV titres of azadirachtin-treated and control plants (± SE) were 1.4 (± 0.2) and 1.1 (± 0.1) µg/g fresh weight respectively.

ii) Survival of adults during the AAP: Survival was poor in all experimental groups (see Table 31) and was not significantly influenced by azadirachtin treatment, the duration of the AAP or the interaction between these factors.

Table 31. The influence of the duration of the AAP and azadirachtin treatment of the virus source tobacco plants on the proportion of *M. persicae* adults surviving the AAP.

	Duration of AAP (h)		
	48	72	96
Azadirachtin-treated	0.53*	0.60	0.33
Control	0.57	0.28	0.28

* These values are fitted proportions predicted from the generalised linear model.

iii) Phytotoxicity: The mean phytotoxicity scores were not significantly affected by azadirachtin treatment

alone but were significantly influenced by the duration of the AAP ($F=3.69$, 2,27 d.f. $P<0.05$) and the interaction between the duration of the AAP and azadirachtin treatment. The phytotoxicity scores for azadirachtin-treated plants were generally higher than those for control plants and, in the case of azadirachtin-treated plants, the scores became lower as the AAP increased in length (see Table 32).

iv) Infection of indicator plants with PLRV: None of the indicator plants were infected with PLRV. It was suspected that this may have been caused by a reluctance of the aphids to feed on plants that had been removed from the compost resulting in the low survival observed and very few indicator plants being infested with enough viruliferous aphids to become infected.

Table 32. The influence of azadirachtin treatment and the duration of the AAP on the phytotoxicity scores of the virus source tobacco plants.

	Duration of AAP (h)		
	48	72	96
Azadirachtin-treated	2.40 (0.41)*	2.20 (0.41)	1.33 (0.53)
Control	2.29 (0.35)	0.71 (0.35)	2.33 (0.38)

* Figures in parentheses are standard errors of means.

5.4.4 Attempt to reduce mortality during the AAP and increase the transmission of PLRV to indicator plants.

i) PLRV titres of the virus source tobacco plants: The mean PLRV titres of virus source tobacco plants were not significantly affected by the environmental conditions that the plants were held in immediately prior to and during the AAP or by removing the plants from their potting compost. The mean PLRV titre (± SE) of plants which remained in the compost was 779 (± 74)ng/g fresh weight and that of the plants that were removed from compost was 810 (± 82) ng/g fresh weight.

ii) Survival of adults during AAP: Aphid survival during the AAP was slightly reduced on plants which had been removed from the potting compost (see Table 33) but was not significantly affected by this operation, different environmental conditions or the interaction of these factors.

Table 33. The influence of environmental conditions and removal of the virus source tobacco plants from compost on the proportion of *M. persicae* adults surviving the AAP.

	Environmental conditions	
	18°C/70-80% rh	20-25°C/80-90% rh
potted	0.62	0.58
removed from compost	0.44	0.44

* These values are fitted proportions predicted from the generalised linear model.

iii) Phytotoxicity: Pre-treatment of the virus source tobacco plants at the higher temperature and humidity resulted in increased phytotoxicity (see Table 34), but this effect was not statistically significant ($F=3.17$, 1,52 d.f., $P=0.081$). Removal of the plants from compost significantly increased the phytotoxicity score ($F=187.94$, 1,52 d.f. $P<0.001$), but there was no significant interaction between environmental conditions and removal from compost.

Table 34. The influence of environmental conditions and removal from compost on the phytotoxicity scores of the virus source tobacco plants.

	Environmental conditions	
	18°C/70-80% rh	20-25°C/80-90% rh
potted	0.07	0.27
removed from compost	3.00	3.62

iv) Infection of indicator plants with PLRV: The proportions of indicator plants which were infected with PLRV were not statistically significantly influenced by environmental conditions, removal from compost or the interaction of these factors. The proportion of indicator plants which became infected after inoculation by aphids which had spent the AAP on plants held at the higher temperature and relative humidity was higher despite lower aphid survival and higher phytotoxicity (cf. Tables 33-35).

Table 35. The influence of environmental conditions and removal of the virus source tobacco plants from compost on the proportion of indicator plants infected with PLRV.

	Environmental conditions	
	18°C/70-80% rh	20-25°C/80-90% rh
potted	0.60*	0.73
removed from compost	0.40	0.73

* These values are fitted proportions predicted from the generalised linear model.

The titres of PLRV in the indicator plants were approximately 4 times less than the titres measured in the virus source tobacco plants, with mean titres of 241.8 and 233.9ng/g fresh weight for plants which remained potted or were removed from the compost respectively. The environmental conditions in which the virus source plants had been kept and the removal of the plants from compost had no statistically significant effects on the PLRV titres of the indicator plants.

5.4.5 Quantitative detection of PLRV in single adult *M. persicae* by ELISA.

PLRV was detected in all aphids which had been raised on virus source potato plants but not in any of the aphids which had been raised on turnip. The average virus titre of the aphids was determined as 4280 (± 560)pg/g by alkaline phosphatase-based ELISA and 5035 (± 675)pg/g by penicillinase-based ELISA. The background reaction measured in the wells containing extracts of non-

viruliferous aphids was more intense in the alkaline phosphatase-based technique where the detection factor was 12.9 compared with a detection factor of 39.5 for the penicillinase-based technique. For this reason the latter technique was used to determine PLRV titres in aphids in subsequent experiments.

5.4.6 Further attempt to reduce aphid mortality during the AAP.

i) PLRV titres of the virus source tobacco plants: The mean (± SE) PLRV titres of the virus source tobacco plants which had been treated with nutrient solution or had remained potted were not significantly different and measured 1.2 (± 0.2) and 0.9 (± 0.2) µg/g fresh weight respectively.

ii) Survival of adults during AAP: Survival of the aphids on plants that remained potted and on plants in nutrient solution was high: 81.2% and 71.4% respectively. These values were not significantly different.

iii) Phytotoxicity: Mean phytotoxicity scores for plants that remained potted and on plants in nutrient solution were not significantly different and measured 0.50 (± 0.27) and 0.25 (± 0.12) respectively.

iv) Infection of indicator plants with PLRV: There were no significant differences in the proportions of indicator plants which became infected with PLRV or the virus titre of these plants after inoculation by aphids

which had spent the AAP on plants in nutrient solution or plants which remained potted (see Table 36).

Table 36. The influence of root treatment with nutrient solution or remaining in compost on the proportion and PLRV titre of indicator plants which became infected.

	Proportion of indicators infected	Virus titre of infected indicators (ng/g fresh weight)
Potted	0.37*	515 (± 122)
In nutrient solution	0.36	523 (± 133)

* These values are fitted proportions predicted from the generalised linear model.

5.4.7 Comparison of PLRV acquisition by *M. persicae* on individual azadirachtin-treated or untreated tobacco seedlings.

i) PLRV titres of the virus source tobacco plants: PLRV titres of the virus source tobacco plants were not significantly altered by azadirachtin treatment (see Table 37).

ii) Survival of adults during AAP: Significantly fewer aphids survived the AAP on azadirachtin-treated plants than on control plants (deviance ratio: 20.77, 1,43 d.f., $P<0.001$) (see Table 37).

iii) Phytotoxicity: Virus source tobacco plants which were treated with azadirachtin suffered higher phytotoxicity than control plants (see Table 37), but the

Table 37. The effects of azadirachtin treatment of virus source tobacco plants on their PLRV titre and phytotoxicity score, the amount of virus acquired by aphids during the AAP and the proportion and virus titre of indicator plants subsequently infected.

	Proportion of adults surviving the AAP	PLRV titre of virus source tobacco plants (µg/g)	Phytotoxicity score	PLRV titre of aphids (µg/g)	Proportion of indicator plants infected	PLRV titre of indicator plants (ng/g)
Azadirachtin -treated	0.45 a*	1.1 a (± 0.06)**	0.87 a (± 0.25)	17.9 a (± 2.6)	0.17 a	325.8 a (± 39.0)
Control	0.86 b	1.1 a (± 0.08)	0.54 a (± 0.17)	89.3 a (±26.0)	0.59 b	437.0 a (± 77.0)

* Values followed by the same letter are not significantly different (P=0.05) within columns.

** Figures in parenthesis are standard errors of the means.

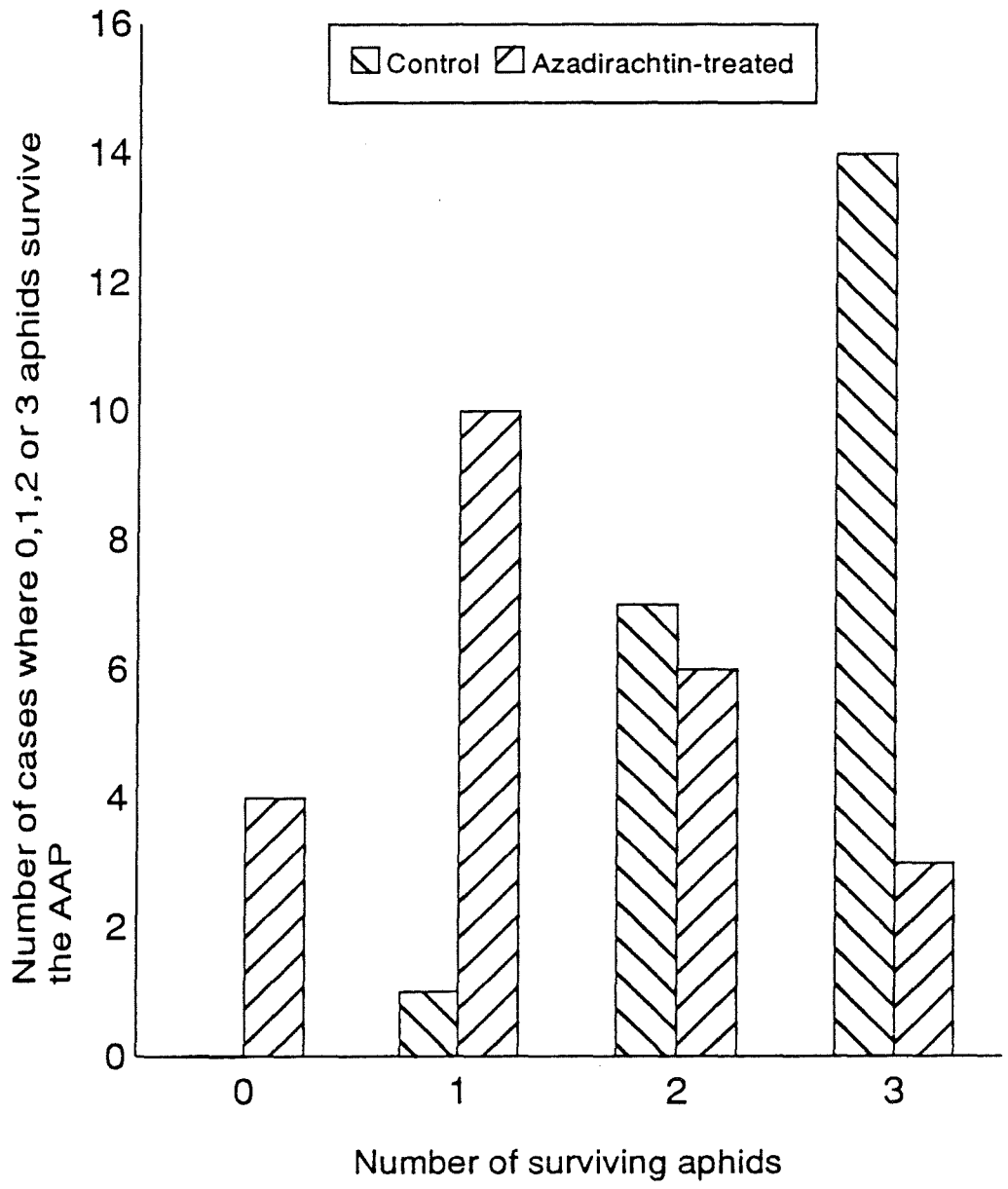
difference in the mean scores was not statistically significant.

iv) PLRV titre of aphids: PLRV was found in only 11 aphids (7 from control plants and 4 from azadirachtin-treated plants) after the IAP on indicator plants. PLRV titres in aphids from control plants were generally higher than those in aphids from azadirachtin-treated plants, but were variable, and the differences between the means were not statistically significant (see Table 37).

v) Proportion and PLRV titre of infected indicator plants: 13 of the 22 indicator plants that had been exposed to aphids from untreated virus source tobacco plants were infected with PLRV, while only 4 of the 23 exposed to aphids from azadirachtin-treated plants were infected (see Table 37). This difference was statistically significant (deviance ratio: 7.29, 1,43 d.f., $P<0.05$). The PLRV titres of the indicator plants were not significantly different.

vi) Relationship between proportion of aphids surviving and proportion of indicator plants infected: Successful inoculation of indicator plants could only occur when at least one aphid survived the AAP on virus source tobacco plants. The number of cases in which 0, 1, 2 or 3 aphids survived the AAP is shown in Figure 63. In 14 of the 22 replicates where the aphids had spent the AAP on control plants, all the aphids survived compared with only 3 of the 23 replicates where the aphids had spent the AAP on azadirachtin-treated plants. Because successful

Figure 63. The number of cases where 0, 1, 2 or 3 aphids survive the AAP on azadirachtin-treated and control virus source tobacco plants



transmission is more likely with increasing numbers of viruliferous aphids, the lowered rate of transmission observed in this experiment may have been caused by differential mortality. Further evidence of this is shown in Table 38 where the percentage of successful transmissions by groups of aphids of different sizes is examined. When the optimum number of aphids for successful transmission survived, the percentage of successful transmissions was similar, independent of where the aphids had spent the AAP.

Table 38. The influence of group size on the percentage of successful transmissions of PLRV to indicator plants from azadirachtin-treated and control virus source tobacco plants.

	Number of surviving aphids		
	1	2	3
Azadirachtin -treated	10*	16.6	66.7
Control	0	42.8	64.3

*Calculated as;

$$\frac{\text{number of actual transmissions}}{\text{number of possible transmissions}} \times 100$$

5.4.8 Comparison of PLRV acquisition by *M. persicae* on groups of azadirachtin-treated or untreated tobacco seedlings.

The results of this experiment are summarised in Table 39. There was no significant effect of azadirachtin treatment on the PLRV titre of the virus source tobacco

plants. The proportion of indicator plants infected with PLRV was significantly reduced by azadirachtin treatment (deviance ratio: 6.24, 1,78 d.f. $P<0.05$), but the titre of PLRV in those plants that were infected was unaffected.

Table 39. The effects of azadirachtin on the PLRV titre of virus source tobacco plants and the proportion and titre of infected indicator plants.

	PLRV titre of virus source plants (µg/g)	Proportion of indicator plants infected	PLRV titre of indicator plants (µg/g)
Azadirachtin -treated	1.76 (± 0.01)*	0.23**	1.03 (± 0.06)
Control	1.78 (± 0.01)	0.53	1.18 (± 0.05)

* Figures in parentheses are standard errors of means.
** These values are fitted proportions predicted from the generalised linear model.

5.5.1 Comparison of the ability of *M. persicae* to inoculate azadirachtin-treated or untreated tobacco seedlings with PLRV in a "no-choice" experiment.

The results of this experiment are summarised in Table 40. Aphid survival during the IAP and the proportion of indicator plants infected with PLRV were unaffected by azadirachtin treatment. However, the PLRV titre of the infected indicator plants was significantly reduced in

Table 40. The effects of azadirachtin treatment of indicator plants on the proportions of aphids surviving the IAP, phytotoxicity and infection of the indicators with PLRV.

	Proportion of aphids surviving at end of IAP**	Proportion of indicator plants infected**	PLRV titre of indicator plants (ng/g)	Weight of indicator plants (g)
Azadirachtin -treated	0.89 a*	0.45 a	670 a	0.16 a
			(± 72)***	(± 0.02)
Control	0.92 a	0.30 a	1114 b	0.74 b
			(± 71)	(± 0.04)

* Values followed by the same letter are not significantly different (P=0.05)

** These values are fitted proportions predicted from the generalised linear model.

*** Values in parenthesis are standard errors of the means.

plants which had been treated with azadirachtin (t=4.20, 28 d.f., P<0.001). The growth of indicator plants was abnormal after treatment with azadirachtin. The leaves were deformed and the plants were often stunted. This occurred whether the plants were infected with PLRV or not (see Figures 64-67). When the plants were weighed prior to PLRV determination by ELISA the azadirachtin-treated plants were significantly lighter than those which had spent the IAP in control solutions (t=11.92, 78 d.f. P<0.001).

5.5.2 Comparison of the ability of *M. persicae* to inoculate azadirachtin-treated or untreated tobacco seedlings with PLRV in a "choice" experiment.

i) Settling during IAP: In nearly every case fewer than 50% of the aphids were settled on azadirachtin-treated plants (see Table 41).

The number of aphids used in the choice tests had no significant effect on the proportions of aphids choosing to settle on the azadirachtin-treated plants.

Original in colour

Figure 64. PLRV-infected N. clevelandii indicator plant 14 days after treatment with 500ppm azadirachtin.

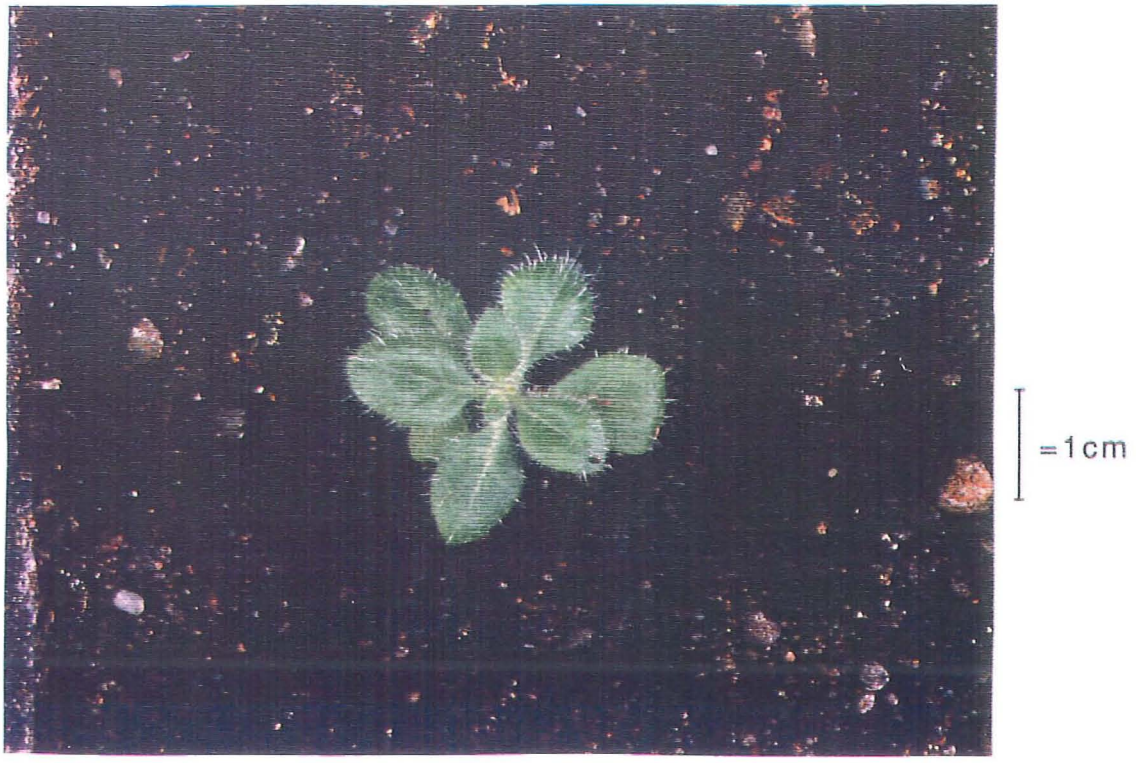


Figure 65. PLRV-infected N. clevelandii indicator plant 14 days after treatment with control solution.

original in colour

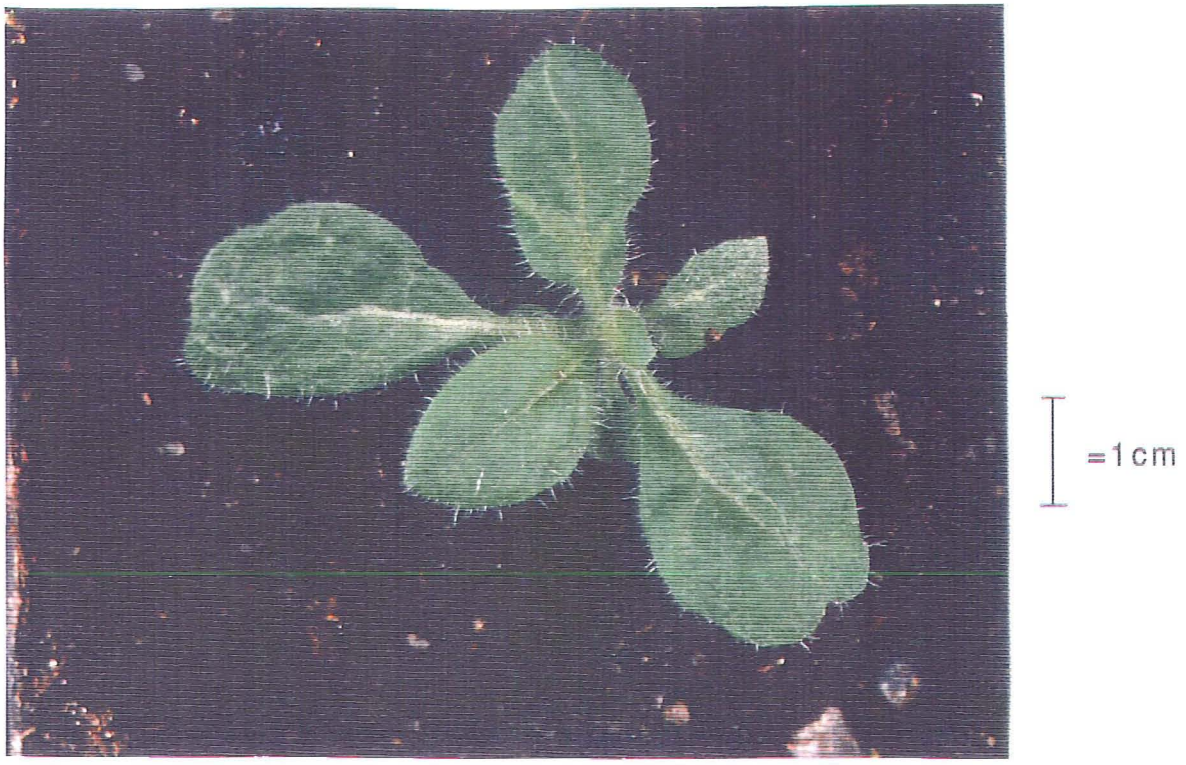


Figure 66. Uninfected N. clevelandii indicator plant 14 days after treatment with 500ppm azadirachtin.

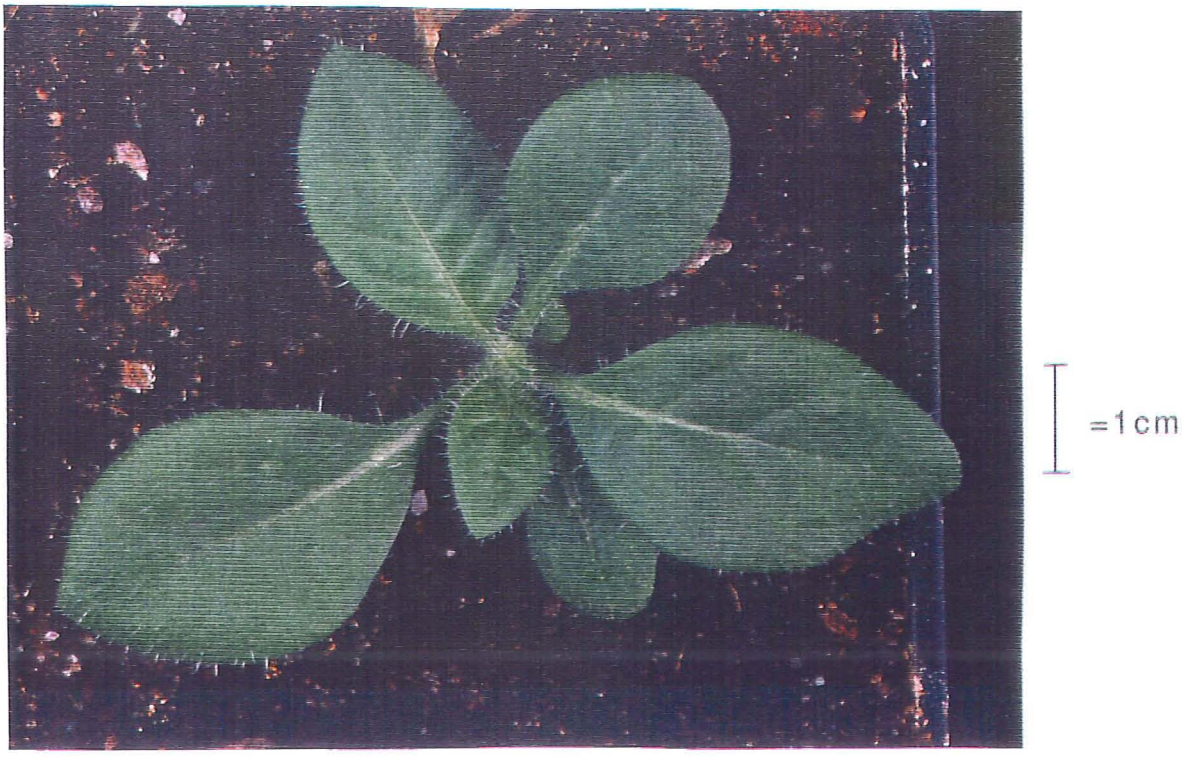


Figure 67. Uninfected N. clevelandii indicator plant 14 days after treatment with control solution.

Table 41. The proportions of the aphids in choice tests which settled on azadirachtin-treated plants.

Time of observation	Number of aphids present: 2	4	6	8
24 h	0.50$^{\Phi}$	0.26*	0.41	0.44
48 h	0.47	0.43	0.28*	0.36*
72 h	0.40	0.27*	0.25*	0.31*

$^{\Phi}$ These values are fitted proportions predicted from the generalised linear model.

* Values followed by an asterisk are significantly different from the null hypothesis of a 50:50 distribution

ii) Proportion of indicator plants infected with PLRV:

The number of aphids used in the choice tests had a significant influence on the proportion of indicator plants infected with PLRV (see Table 42) (deviance ratio: 3.72, 3,36 d.f., $P<0.05$).

Table 42. The influence of azadirachtin treatment and the number of viruliferous aphids used in choice tests on the proportion of indicator plants infected.

	Number of aphids used: 2	4	6	8
Azadirachtin -treated	0.40*	0.40	0.30	0.80
Control	0.30	0.60	0.60	1.00

* These values are fitted proportions predicted from the generalised linear model.

The proportion of azadirachtin-treated indicator plants infected with PLRV was, in most cases, less than that of untreated indicator plants. However, there was no statistically significant effect of azadirachtin treatment or any significant interaction between azadirachtin treatment and the number of aphids used.

Chapter 6. The effects of azadirachtin on the acquisition and inoculation of potato virus Y by *Myzus persicae*.

6.1 Introduction

Potato virus Y (PVY) is the type member of the potyvirus group (de Bokx, 1981). Potyviruses are flexous rods (11 x 680-900nm) of single stranded RNA which typically replicate in the cytoplasm of non-vascular plant tissues and usually have aphids as natural vectors. When a leaf is inoculated with PVY the virus remains in the cells of the inoculated leaf and multiplies there before moving short distances to adjacent cells. When high titres are reached virus particles enter the phloem sap and are rapidly translocated to new tissue (Beemster, 1972).

PVY strains: Groups of strains of PVY can be distinguished according to the severity of symptoms that they cause in host plants. The three main groups of strains are PVY^O, PVY^N and PVY^C. PVY^O is comprised of the common strains of the virus which produce more severe symptoms in potato than PVY^N. However, the latter group spreads more rapidly in the field (Beemster & Rozendaal, 1972). PVY^N is the group of "tobacco veinal necrosis" strains which cause severe, often lethal necrosis in tobacco plants, whereas in some potato cultivars the infection can remain symptomless (Weidemann, 1988). PVY^C causes severe stipple/streak necrosis in potato but some strains in this group are very difficult for aphids to transmit (de Bokx, 1981).

PVY symptoms in potato: The symptoms of primary PVY infection (i.e. infection of a previously healthy plant) depends on the variety of potato or species of indicator plant used (e.g. Barker & Harrison, 1984). In potato varieties which are most susceptible to PVY, necrosis occurs primarily in the collenchyma of the aerial parts of the plant and sometimes extends to other tissues of the cortex, but not the vascular bundles. In petioles the parenchyma between the vascular bundles may also become necrotic resulting in visible streaks. Necrosis of the petiole in some susceptible varieties results in "leaf-dropping"; the leaves of infected plants wilt and wither (Bos, 1978).

Plants which are grown from PVY-infected tubers (secondarily infected plants) may be stunted with ruffled upper leaves, scattered yellow spots and necrosis of the lower leaves (de Bokx, 1981).

PVY transmission: PVY is transmitted in a non-persistent manner by several aphid species with a range of vector efficiencies. *M. persicae* is an effective vector of PVY and is often responsible for the majority of virus spread in potato crops (Beemster & Rosendaal, 1972). PVY is acquired during brief epidermal penetrations (probes) on infected leaves and the aphid can infect a healthy plant by probing immediately after acquisition. Powell (1991), using *M. persicae* and PVY^N-infected tobacco plants, recorded acquisition and transmission thresholds of 7.6 and 3.6 seconds respectively, with optimum acquisition

and transmission periods of 10-15 seconds. Longer and deeper probes result in decreased acquisition of PVY. Using the EPG technique Powell, (1991) and Powell *et al.* (1992b) also demonstrated that, in the majority of cases where PVY was successfully transmitted from virus source tobacco plants to indicator plants, the vector aphids penetrated the cell membrane of an epidermal cell of the source plant at least once during the acquisition period. For an aphid to successfully acquire PVY it must also acquire a "helper component" either before or during virus acquisition (Govier & Kassanis, 1974a,b). The helper component is a non-structural protein, serologically unrelated to PVY particle protein or inclusion body protein and is expressed only in tissue which is infected with PVY (Govier *et al.*, 1977).

The site of retention of the virus in the vector has not yet been fully determined. Some authors believe that non-persistently transmitted viruses are "stylet borne" (Kennedy *et al.*, 1962) and are retained on the distal portion of the stylets. Harris (1977) has proposed the "ingestion-egestion transmission hypothesis" which explains non-persistent transmission as contamination of the anterior part of the alimentary canal as far as the foregut with virus-laden sap during brief probes followed by inoculation when a portion of the sap is egested during subsequent probes on uninfected tissue.

The maximum period for which an aphid can retain infectivity with non-persistent viruses is also a matter of some debate. It is generally accepted that maximum

retention periods are several hours and that this period is rapidly reduced by feeding and increased temperatures. Recently, several authors (e.g. Zeyen & Berger, 1990) have questioned these maximum retention periods on the basis that aphids can transmit non-persistent viruses after periods of several days if they are not allowed to probe solid surfaces.

In the present work experiments were performed to determine effects of systemic azadirachtin treatment on the ability of aphids to acquire and inoculate PVY.

6.2 Materials and Methods

General

Virus source potato plants; Potato tubers (cv. Craig's Snow White), harvested from plants which had been infected with a field-maintained strain of PVY in the previous season, were planted in individual pots and placed in insect-proof cages in a glasshouse at approximately 20^{o}C. Leaves from these plants were used as virus sources 63 days after sowing in the case of experiment 6.2.1 and 55 days after sowing in experiment 6.2.2.

Virus source tobacco plants and indicator plants; All virus source tobacco plants and indicator plants were *N. clevelandii* seedlings raised as described in section 2.3.1.

Virus determination; Determination of PVY presence in plant tissue was qualitative only as there was no

purified PVY available for standard series. The technique used was similar to that described in section 5.2 but, because monoclonal antibodies were used, there were some differences, as shown in the protocol described in Figure 68. All antisera were kindly provided by Dr. H. Barker, S.C.R.I., Invergowrie.

Aphids; In both experiments aphids were raised on *N. clevelandii* as described in section 5.2.8 with the exception that aphids were starved for 2h before each experiment instead of 6h.

6.2.1 Comparison of the ability of *M. persicae* to acquire PVY from azadirachtin-treated or untreated tobacco seedlings.

Thirty 21 day old *N. clevelandii* seedlings were infected with PVY as follows: One leaf was detached from the middle stem section of a virus source potato plant and was used in all inoculations. Individual aphids were placed on the leaf for an AAP of 90s. Probes were not individually timed but aphids which did not probe were not used for inoculation. Aphids were immediately transferred and caged, five per plant, on the *N. clevelandii* seedlings overnight. The aphids and their progeny were then removed manually and the plants were allowed to grow in the glasshouse at approximately 20^{o}C for 15 days. After this period the plants were assayed for PVY presence by symptom expression and ELISA of leaf tissue. Infected plants showed strong symptoms *viz.*

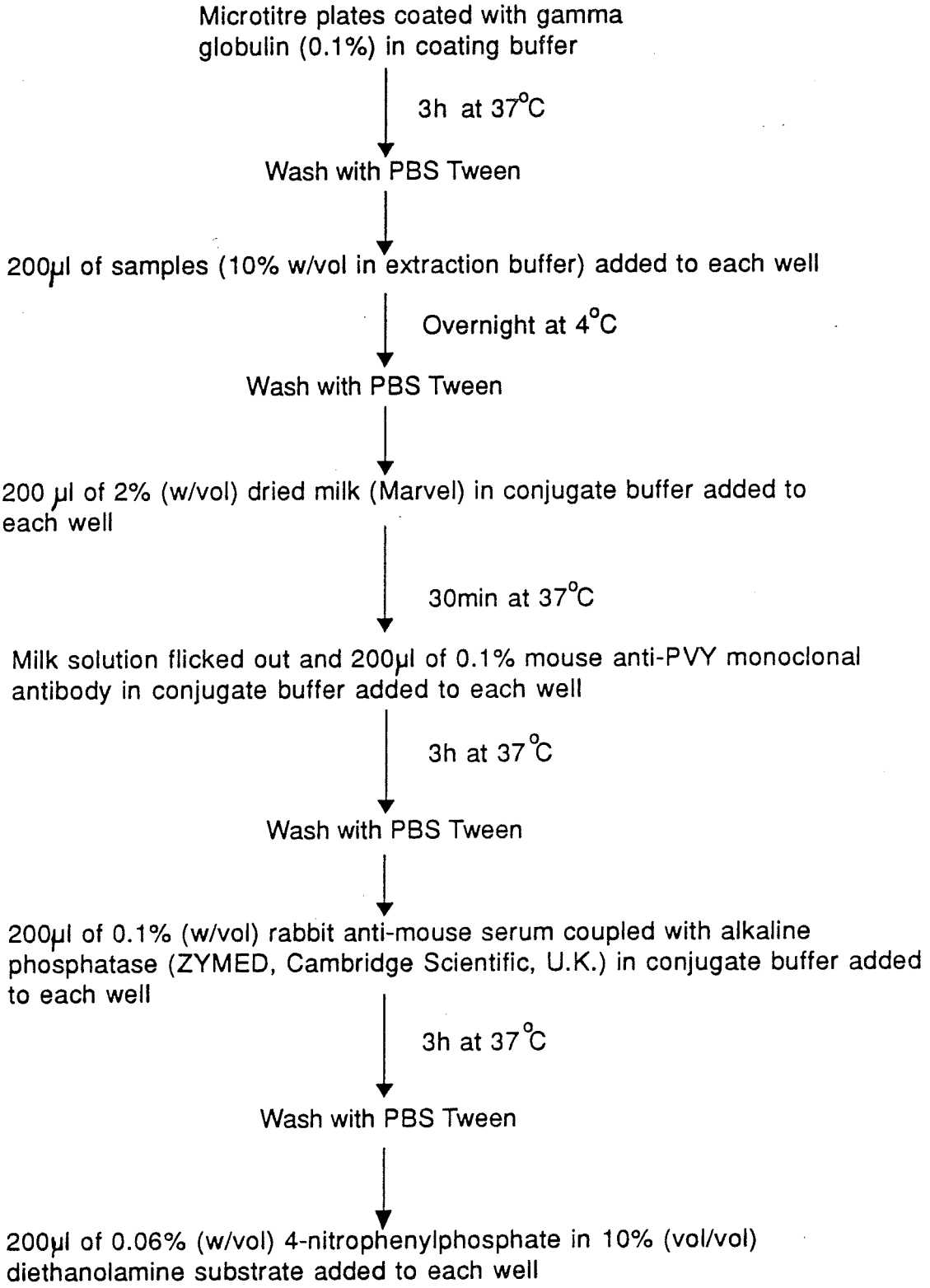


Figure 68. ELISA protocol for using monoclonal antibodies to detect PVY in experiments 6.2.1 and 6.2.2

chlorosis of large areas of leaf tissue and commonly death of the hypocotyls. PVY infection was confirmed in 29 of the plants. However, 5 of these displayed such strong symptoms that they would probably not have survived the subsequent experimental procedure and were excluded. The remaining plants were divided into two groups of 12 and treated with 500ppm azadirachtin solution or a control solution as described in section 3.2.2. After 27h, a single aphid was placed onto each plant for an AAP of 90s and was then immediately transferred to a 21 day old *N. clevelandii* indicator plant overnight. Each of the 24 virus source tobacco plants was used for 10 such acquisitions. The 240 indicator plants were fumigated with nicotine and then grown in an aphid-free glasshouse for 17 days before virus determination by ELISA.

Analysis of results; The results for each virus source tobacco plant were expressed as a proportion of indicator plants infected as follows:

$$\frac{\text{Number of surviving indicator plants infected with PVY}}{\text{Number of surviving indicator plants}}$$

The proportions of indicator plants infected with PVY after acquisition from azadirachtin-treated or control plants were then compared using a generalised linear model with a binomial distribution.

6.2.2 Comparison of the ability of *M. persicae* to inoculate PVY to azadirachtin-treated or untreated tobacco seedlings.

Two hundred *N. clevelandii* seedlings were split into two equal groups and treated with 500 ppm azadirachtin solution or control solution. After 27h, single aphids, which had previously been given an AAP of 90s on a leaf of the virus source potato plant, were placed onto each seedling overnight. After this period the aphids and their progeny were removed manually and the seedlings were re-potted in compost and fumigated with nicotine. The seedlings were then grown in a glasshouse at approximately 20^{o}C for 9 days. At this point it was noted that some of the plants had developed severe symptoms and some had already died. The plants which had developed severe symptoms were removed and frozen at -20^{o}C until ELISA was performed along with the remaining plants 5 days later.

Analysis of results; The number of infected indicator plants was expressed as a proportion of the surviving indicator plants. The proportions of azadirachtin-treated and untreated indicator plants which were infected were compared using a generalised linear model with a binomial distribution.

6.3 Results

6.3.1 Comparison of the ability of *M. persicae* to acquire PVY from azadirachtin-treated or untreated tobacco seedlings.

Aphids which had spent the AAP on untreated plants infected a significantly higher proportion of indicator plants than aphids which had spent the AAP on azadirachtin-treated plants (deviance ratio: 12.04, 1,23 d.f. $P<0.01$). 38.4% ($^{43}/_{112}$) of the indicator plants which had received aphids from azadirachtin-treated virus source tobacco plants were infected with PVY compared with 53.5% ($^{61}/_{114}$) of the indicator plants which had received aphids from untreated plants.

6.3.2 Comparison of the ability of *M. persicae* to inoculate azadirachtin-treated or untreated tobacco seedlings with PVY.

Aphids inoculated azadirachtin-treated indicator plants and untreated indicator plants with almost exactly the same efficiency. 52.1% ($^{49}/_{94}$) of azadirachtin-treated indicator plants and 52.7% ($^{48}/_{91}$) of untreated indicator plants were infected with PVY. There was no statistically significant difference. Azadirachtin-treated indicator plants showed similar phytotoxicity to that described in section 5.5.1.

Chapter 7. Discussion

7.1 Purification and Systemic movement of Azadirachtin:

As indicated in section 1.6, few studies on the effects of neem extracts on aphids have employed pure compounds. Crude or enriched extracts are of more interest from a practical point of view for pest control, but it is difficult to quantify the specific effects of these extracts on insects because of possible synergism between the various compounds that they contain and particularly because of their variable content of active ingredients. Purification of specific compounds is both difficult and time consuming but if azadirachtin is to be used as a model compound for the synthesis of antifeedants and/or insect growth regulators it is important that it is isolated from other compounds in neem extracts which may also possess these properties. The extraction techniques used in Chapter 2 were based on an existing technique described by Schroeder & Nakanishi (1987) and were adapted to make use of the available equipment. Extraction 1 yielded less material than the other extractions of Ghanaian seed, for the following possible reasons:

i) Initial exhaustive extraction of lipids is known to be advantageous to the efficient purification of azadirachtin from neem seed (Schroeder & Nakanishi, 1987). In extraction 1 the preliminary lipid removal was inefficient because of compaction of the ground seed in

the percolator. When the seed was then extracted by agitation with a fixed volume of methanol the solvent rapidly became saturated with lipids and low polarity compounds making the extraction of higher polarity compounds less efficient.

ii) The initial flash column chromatographic separation of the ethyl acetate-soluble fraction used a small range of eluting solvents which increased in polarity abruptly and fraction size was fairly large. As a result the separation was imprecise and the azadirachtin-containing fractions were very impure. The purification of these fractions involved four further flash column chromatographic separations. Assuming a 95% recovery rate from each separation (Still *et al.*, 1978), almost 20% of the azadirachtin was lost during these stages. In subsequent extractions it was found to be extremely advantageous to use shallow elution gradients throughout the purification procedure.

iii) While final purification of azadirachtin in extraction 1 was achieved using flash column chromatography (*c.* 55% of total product) and preparative TLC (*c.* 45% of total product), successive re-crystallisation from CCl_4 was a wasteful and unsuccessful method of purification.

In the subsequent extractions the efficiency of lipid removal was improved by extracting the ground seed in three separate portions using percolation with petroleum ether 60^{o}-80^{o} and then hexane before extraction with methanol while stirring. Extraction of de-fatted, ground

neem seed with methanol is less efficient using stirring than by percolation because the latter uses a constant flow of distilled methanol at just below boiling point through the seed, whereas with the stirring method a constant volume of cold methanol is used, which rapidly becomes saturated (Schneider & Ermel, 1987). It is possible that the de-oiled marc would possibly have been less compacted if the seed had been ground less finely.

Azadirachtin isolated from the Ghanaian seed was of high purity (>95% in most cases). Higher purity can be achieved by preparative HPLC techniques. The procedure described by Yamasaki *et al.* (1986) used flash column chromatography followed by preparative HPLC for the purification of azadirachtin from neem seed extract (see Table 43). This produced azadirachtin with a purity of more than 99%, but in a yield of only 0.0056% (w/w).

Table 43. The isolation procedure and purity of azadirachtin from neem seed extract (described by Yamasaki *et al.*, 1986).

Step	Isolation procedure	Azadirachtin purity after each step
1	Flash column chromatography on silica	7%
2	Flash column chromatography on ODS	26%
3	Preparative HPLC on silica	70%
4	Preparative HPLC on phenyl column	>99%

Yields of azadirachtin from Ghanaian seed were low (approximately 0.04% w/w) but better than those from

Pakistani seed. Seeds of different ecotypes of neem tree contain different quantities of the compound. Ermel *et al.* (1984) found a fourfold difference in the azadirachtin content of neem seed from different geographical locations. The highest yields of azadirachtin are not restricted to seed from any specific country, but come from individual trees of different geographical origin.

Benge (1988) lists genetic and environmental elements and maturity as factors which affect the azadirachtin content of seeds from individual trees. The ripeness of the seed at harvest is also important because, as ripening proceeds, the products formed are successively more highly oxidised. For this reason the major triterpenoid present varies from nimbin to salannin to azadirachtin as the seed ripens (Jones *et al.*, 1988). The conditions and duration of storage of the seed after harvest can also have a profound effect on azadirachtin content. Ermel *et al.* (1987) reported that the azadirachtin content of neem seeds fell to 10% of its original value when the seeds had been stored at 100% rh, 60^{o}C for 3 weeks. Schmutterer & Zebitz (1984) found that seeds which had been in storage for 18 months contained less methanol-extractable material than those which had been stored for 4-6 months, though it was not clear which components of the seed were lost or reduced over time. The seeds used in extractions 1-3 had been in storage, at room temperature, for approximately 2 years. The storage

conditions of the seeds used in extractions 4 and 5 are unknown.

It is possible that any or all of the above factors were responsible for the differences in yield found in the present work.

7.1.1 Attempted synthesis of radiolabelled azadirachtin:

Rembold (1989) stated that most structural modifications of azadirachtin result in low yields because of the lability of the molecule and that only hydrogenation/tritiation of the 22,23 enol ether bond can be achieved with reasonable success (e.g. 42.1% yield, Rembold, 1988). Despite an attempt to increase yields of deacetylazadirachtin by adapting the method described by Butterworth *et al.* (1972) (azadirachtin was stirred in 1% potassium hydroxide for 3.5h instead of 2.5% potassium hydroxide for 1h), yields were still low; 0.8-7.7% compared with 12.5% published (Butterworth *et al.*, 1972). Labelling deacetylazadirachtin with ^{14}C-acetic anhydride was unsuccessful, though in both attempts unlabelled azadirachtin was re-formed. Initially it was thought that this lack of success may have been because of steric hindrance at the 3-hydroxy position (H. Rembold pers comm, 1990) however no ^{14}C-labelled azadirachtin was produced even when an acylation catalyst was employed. Very small volumes of labelled acetic anhydride (<1µl) were used in both attempted re-acetylations and it is possible that the reactants may have become contaminated

with water despite the use of re-distilled solvents. Because acetic anhydride decomposes to form acetic acid on contact with water, all of the radiolabelled material could be decomposed by a very small quantity of the contaminant. A radiolabelled product with low specific activity was formed by the second attempted re-acetylation. Two dimensional thin layer chromatography coupled with autoradiography revealed that this product was more polar than azadirachtin, though it was neither visible on the TLC plate after staining with iodine vapour nor evident on the HPLC trace in experiment 2.2.2b. This compound was not identified but, if water contamination had resulted in the decomposition of acetic anhydride to acetic acid, it is possible that ^{14}C-α and/or β acetoxyazadirachtin were formed by reaction of acetic acid with the enol ether bond (Figure 69).

7.1.2 Systemic movement of azadirachtin:

It had been intended to produce ^{14}C-labelled azadirachtin to follow and quantify systemic movement of the compound throughout the vascular system of tobacco plants. The systemic movement of azadirachtin from plant roots to aerial parts has been demonstrated in several species of plants (Radwanski, 1977) but the quantitative movement of the compound within the vascular tissues and its location and concentration within other tissues has never been determined. The production of ^{14}C-labelled azadirachtin

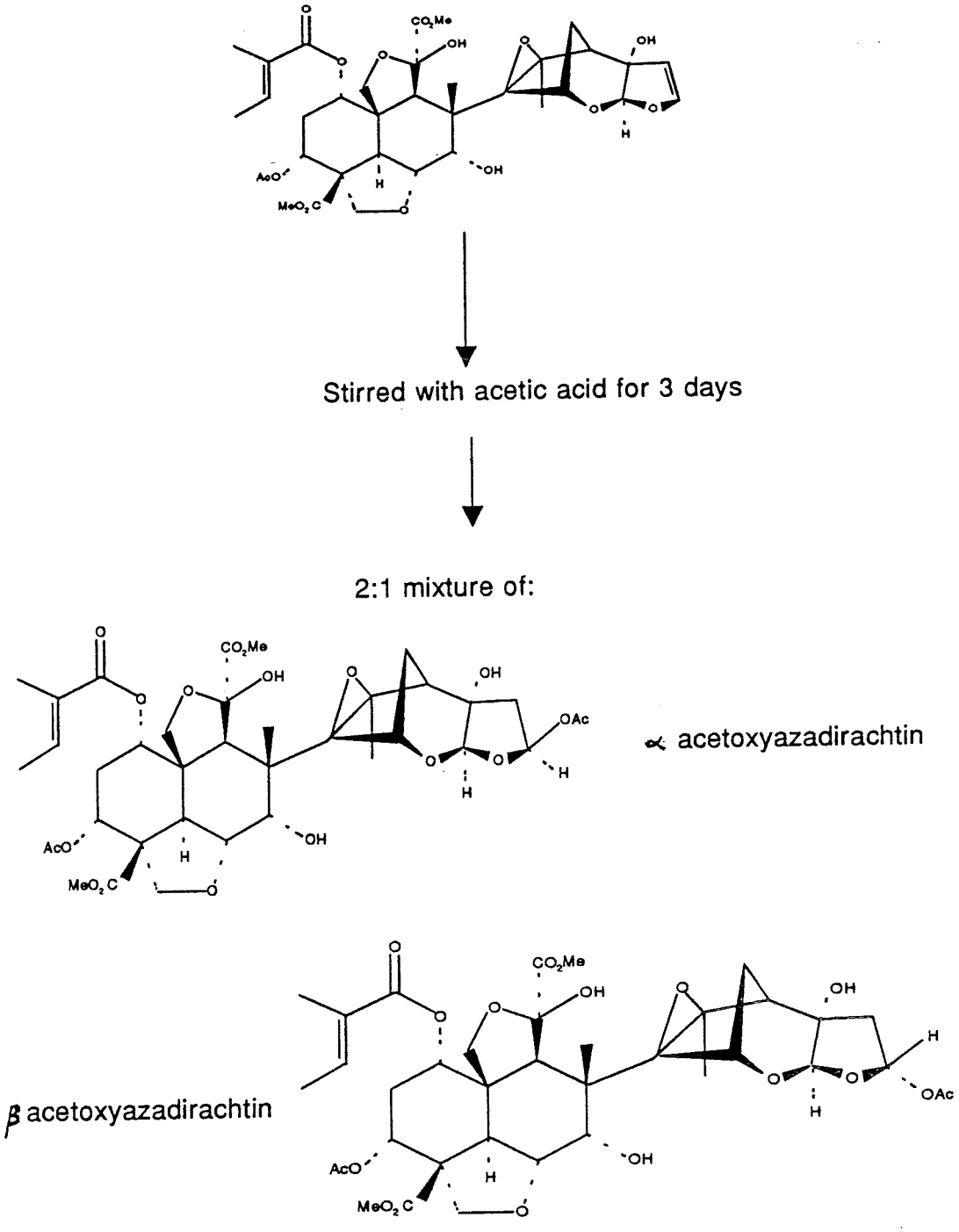


Figure 69. The reaction of azadirachtin with acetic acid (described by Ley et al., 1988)

by the method described in Chapter 2 does not seem practical and, unless ^{14}C can be incorporated into the skeleton of the molecule e.g. during a total synthesis, such studies will probably rely on the use of the tritiated compound. If this approach is used, the results will require verification using the unaltered molecule to examine changes in chemical behaviour resulting from tritiation of the 22,23 bond. To determine, and also partially quantify the phloem mobility of azadirachtin, an assay could be designed utilising the honeydew produced by phloem-feeding insects. It should be possible to detect the compound or its breakdown products (cf. Molyneux *et al.*, 1990) in the honeydew of phloem-feeding insects on plants which had been treated with low concentrations of azadirachtin. Quantification of the azadirachtin present in the honeydew could be achieved by analytical HPLC or TLC accompanied by fluorescence scanning (Ermel *et al.*, 1984).

Another approach to determining the concentrations of compounds in various plant tissues is through the use of immunoassays (e.g. Kolossa *et al.*, 1987; Weiler, 1989). Attempts are currently being made to link azadirachtin to a protein molecule to produce an antigenic compound from which antisera to azadirachtin could be raised (H. Rembold pers. comm., 1991).

The results presented in sections 2.4.3 and 2.4.4 demonstrated that a compound, possibly azadirachtin, was detectable in the leaves of tobacco seedlings after their roots had been immersed in a range of concentrations of

the compound. The results of a preliminary study, presented in section 2.4.4, suggested that the concentration of azadirachtin in leaf tissue was 2-3 times higher than in the solution in which the roots were immersed. This observation, if ratified by further testing, could partially explain the persistence of azadirachtin in the leaf tissue of systemically treated plants. Meisner *et al.* (1987) reported that neem extracts, applied to the soil in which corn seedlings were growing, had stronger effects on the growth and development of European corn borer (*Ostrinia nubilalis*) when the larvae were placed onto plants 4 days after treatment instead of 2 days after treatment. The same authors also reported that the effects of the extract on development of the serpentine leafminer (*Liriomyza trifolii*) were more pronounced if larvae were deposited on the leaves of bean seedlings 5 days after application of the extract to the soil than after 3 days or 1 day. These observations, and the long-term phytotoxicity described in the present work (sections 5.3 and 6.3.2), might be explained by an accumulation of azadirachtin in the leaf tissue over time. To test this hypothesis, extracts of leaf tissue could be made from plants which had had their roots immersed in azadirachtin solutions for different time intervals e.g. 6,12,18,24 and 30h. The concentrations of azadirachtin in the leaf extracts could then be quantified by HPLC as described in Chapter 2. The uptake and accumulation of tritiated azadirachtin could be examined and compared with that of the unaltered

molecule using the same experimental system. If both compounds had similar behaviour, the experiments could be repeated using substantially lower concentrations of tritiated azadirachtin to examine accumulation more accurately by scintillation counting. Accompanied by microautoradiography of leaf and stem sections to examine the relative concentrations of the compound in different tissues, this would give a comprehensive examination of the systemic movement of azadirachtin in plants.

7.2 The systemic antifeedant effects of azadirachtin against *M. persicae*:

Although it has been suggested that systemicity is not a prerequisite for an antifeedant against aphids, (Griffiths *et al*, 1989), it may, nevertheless, be a considerable advantage in the light of evidence that many species of aphid prefer the parts of host plants which contain low concentrations of semiochemicals (Miles, 1978, Leszczynski & Dixon, 1990). Aphids feeding on plant growth that emerged after treatment of the plant surface with a non-systemic compound would avoid the antifeedant, whereas a systemic compound would reach these parts through the plant vascular system.

The EPG method was used to examine the systemic antifeedant effects of azadirachtin on *M. persicae* (Chapter 3). An important prerequisite to the use of this method is to ensure that the feeding behaviour of aphids is not detrimentally affected by tethering. Initial host

selection by aphids typically involves brief probes followed by limited movement before "settling" (Wensler, 1962). Tethered aphids have less opportunity to move than free aphids but there were no consistent differences between the behaviour of tethered or free *M. persicae* in experiment 3.2.1 (Figure 15). Similar evidence was presented for the reaction to tethering of *M. persicae* on resistant and susceptible lettuce, (Montllor & Tjallingii, 1989). Any effects on aphid behaviour in the present work may have been minimised by the 3h pre-treatment given to the aphids after attachment, as tethering effects diminish as the EPG experiment progresses (Tjallingii, 1986).

Aphids probed more frequently on treated than untreated plants (see Figure 16). This may have been a result of tethering as well as antifeedant effects since it has been shown that free cereal aphids probed less frequently on azadirachtin-treated plants than on untreated plants (West & Mordue (Luntz), 1992). Tjallingii (1986) found that tethered cabbage aphids (*B. brassicae*) placed on non-host plants (*V. faba*) maintained initial settling behaviour, i.e. brief probes followed by limited movement (Wensler, 1962), throughout the course of 5h EPG recordings while non-tethered aphids left the plants after 2-3h.

The results presented in section 3.3.2 suggest that azadirachtin was taken up systemically by the plant, and that concentrations in the phloem sap caused premature termination of sustained phloem feeding by aphids, even

at the lowest concentration tested (100ppm), thereby resulting in an overall reduction in the time devoted to E(pd) activity. Aphid mouthparts are devoid of external chemoreceptors (Tjallingii, 1978b). Gustation of plant sap by the epipharyngeal organ may only occur after plant penetration (Kimmins & Tjallingii, 1985).

It remains to be shown whether azadirachtin also affects the **rate** of phloem sap ingestion. E(pd) pattern indicates the positioning of the stylets in sieve elements, but the rate of uptake of phloem sap is regulated by the muscles of the aphid's anterior alimentary canal (Ponsen, 1972 cited in Ponsen, 1987). There may be correlations between the recently described E pattern waves (Tjallingii, 1990) and the rate of imbibition. However, E(pd) waves were not separately identified in the present work and no attempt was made to distinguish E1 and E2 peaks, (Tjallingii, 1990).

Aphids on plants treated with azadirachtin concentrations of 1000ppm spent longer individual periods in non-penetration activity before they ingested phloem sap than those on plants treated with lower concentrations of the compound (see Figure 19). This, along with the evidence for an increased number of probes and a lower proportion of probes reaching the sieve elements on plants treated with high concentrations of azadirachtin, may indicate that they detected azadirachtin or an antifeedant metabolite in the epidermal and mesophyll tissues prior to phloem sap ingestion. The detection of an antifeedant in such

tissues could be responsible for an increase in the frequency of exploratory probes and locomotory activity. Azadirachtin treatment did not increase the proportion of time devoted to pathway patterns during the first hour of recording or the time required to reach the sieve elements in successful probes. This suggests that the presence of the compound in non-vascular and vascular tissues did not affect the perception of phagostimulatory cues e.g. pH, which guide the stylets to the sieve elements, either before or after ingestion. A successful probe on treated plants implies that the aphid imbibed phloem sap before responding to the antifeedant. When exploratory probes were initiated on treated plants, the aphid may have detected the antifeedant in non-vascular tissues during sap-sampling. These exploratory, or "unsuccessful" probes could have been triggered by a "primary" antifeedant effect (caused by contact between the chemical and chemoreceptors, Schmutterer, 1985) while changes in feeding behaviour after a successful probe could have resulted from either primary or "secondary" effects (toxic effects after ingestion of azadirachtin).

The imbibition of sap by aphids in the 500ppm treatment group during the first 3h was followed by a reduction in the duration of all other probing activities in the final time period (Table 20). The same was not, however, true for aphids feeding on 1000ppm treated plants. It is possible that high concentrations of azadirachtin evoked a high primary antifeedant response and that aphids feeding on these plants imbibed a smaller quantity of

azadirachtin and were thus not subjected to possible secondary effects such as intoxication.

According to Schauer (1984) and Schmutterer (1985), neem seed extracts have no contact effects on aphids and, for aphid control, the concentration of active ingredients in neem seed extracts should be not less than 1000ppm. Lower concentrations in foliar applications are thought to result in insufficient uptake and translocation of the compounds to control aphids. Although substantially lower concentrations of azadirachtin were found to influence feeding behaviour when applied systemically in the present work, they are still, nevertheless, very much greater than the concentrations of azadirachtin reported to produce antifeedant effects on other species of insect. For example, Gill & Lewis (1971) demonstrated that azadirachtin had systemic antifeedant effects on *S. gregaria* when applied to the roots of *P. vulgaris* seedlings at concentrations as low as 0.2ppm. Several factors may account for the high concentrations required to affect aphid feeding behaviour:

i) As aphids feed mainly from the sieve elements of their host plants, active concentrations of antifeedant are required to be present in the phloem sap. Absorption of azadirachtin in solution through the plant root may be facilitated by apoplastic movement, whereby the compound moves inwards from the root epidermis to the vascular tissue without crossing cell membranes until it encounters the endodermis. The compound must then

traverse the cell membrane of this tissue before entering the apoplast of the xylem. It is unlikely that azadirachtin, as a foreign molecule, is actively loaded from the apoplast into the symplastic phloem tissue via the companion cell complex. The compound must therefore diffuse accross the phospholipid bilayer of the plasma membrane of the companion cell complex, cross this membrane via a passive carrier, or diffuse directly into the sieve element. The phloem concentration of azadirachtin therefore depends on its ability to cross cell membranes unhindered.

ii) It is possible that azadirachtin is compartmentalised within the tissues of the plant, i.e. it is present in some tissues at high concentrations and at low concentrations in others. For example it is the compartmentalisation of nicotine into non-vascular tissues of tobacco plants which allows *M. persicae* to thrive on tobacco plants without sustaining mortality (Klingauf, 1987b). Reese *et al.* (1987) suggest that aphids have a "window of sensitivity" to the pH of plant tissues while locating the sieve tubes and that when the aphid is committed to phloem sap ingestion it becomes insensitive to pH. In an extreme example of this, presented by Reese *et al.* (1987), groups of *S. graminum* which were feeding on sachets of artificial diet with a pH of 8.5 continued to feed when the diet was replaced with 40% formic acid (pH 1.8). If aphids avoid detection of azadirachtin while locating the sieve tubes then they may also be insensitive to the antifeedant once they

begin phloem sap ingestion, particularly if it is present in low concentrations. Tjallingii (1990) reported that, during electrically-monitored feeding of aphids on artificial diet, pattern E was maintained even after the introduction of strong antifeedants to the diet. If azadirachtin had the same effect, a primary antifeedant response would be observed only when very high concentrations were used. With lower concentrations, aphids would ingest phloem sap until internal stimuli, e.g. resulting from toxicity, forced them to stop.

iii) The pH of phloem sap is slightly alkaline (pH 7.2-8.5), and azadirachtin decomposes in alkaline media (E.D. Morgan, pers. comm. 1988).

iv) Azadirachtin may operate in two ways to produce an antifeedant response by; a) blocking sugar receptors, and, b) exciting deterrent receptors (Schoonhoven, 1982). The blocking of sugar receptors is known to be reversible and concentration-dependant, (Dethier, 1982). The presence of a high concentration of a strong phagostimulant such as sucrose may be sufficient to offset the effects of azadirachtin.

This hypothesis could be tested by examining the antifeedant effects of azadirachtin on *M. persicae* when it was present at a constant concentration in diets with varying concentrations of sucrose and/or phagostimulatory amino acids e.g. methionine, isoleucine, leucine, phenylalanine, tryptophan or asparagine (Auclair, 1969).

If specific deterrent receptors exist in *M. persicae*, they may be insensitive to azadirachtin. A polyphagous

species such as *M. persicae* is less likely to be deterred from feeding by specific compounds than a monophagous or oligophagous species because host specialisation is related to increased sensitivity to specific compounds or the presence of specific chemoreceptors (Schoonhoven, 1982).

v) Azadirachtin may be broken down by the enzymes and/or non-enzymic reducing material (Miles & Harrewijn, 1991) in the saliva produced by aphids while feeding in plant tissue.

There are several similarities between the results presented in Chapter 3 and those obtained using electrical techniques to monitor aphid feeding behaviour on resistant plants. Campbell *et al* (1982) noted that greenbugs (*Schizaphis graminum*) made numerous phloem contacts of short duration on resistant varieties of sorghum. They suggested that this behaviour may have arisen from a difference in the phloem chemistry of the resistant and susceptible varieties. In the present work, increases were observed in the number of both sustained and unsustained E(pd)s. Unsustained E(pd)s are usually represented by E1, the non-typical E(pd) pattern composed of slowly increasing peaks without accompanying waves (Tjallingii, 1990). They are not thought to involve ingestion from sieve elements (Kimmins & Tjallingii, 1985) though they may be indicative of sieve element penetration (Hogen Esch & Tjallingii, 1992). Electrical penetration graphs showed that *Nasonovia ribisnigri* was not prevented from reaching sieve elements on resistant

lettuce plants, but that the overall duration of pattern E(pd) was very much less than on susceptible plants (Mentink *et al*, 1984). A "phloem factor" was thought to be responsible for the modified feeding behaviour of *N. ribisnigri* on resistant lettuce plants, (Montllor & Tjallingii, 1989). They found that the E(pd) patterns recorded from *N. ribisnigri* on resistant lettuce plants were shorter (none lasted more than 8 min) than those on susceptible lettuce plants and that the number of probes, the total time devoted to non-penetration activities, and the length of time spent in patterns A,B and C all increased on resistant lettuce plants. They attributed the increase in the duration patterns A,B and C to an atypical C pattern. In the present work, the increase in durations of these patterns with increasing azadirachtin concentration is the result of increased initiation of probes and the presence of typical C pattern separating terminated E(pd) patterns.

Dethier (1982) stated that when an insect is deprived of food, the ratio of positive to negative gustatory stimuli from a marginally acceptable plant is biased in the positive direction and the plant is accepted as a food source. The duration of the meal is short because, as partial satiety approaches, internal negative stimulation causes the positive/negative ratio to become smaller. Brief, repeated feeds on plants treated with antifeedants also compensate for water loss in insects in tropical conditions according to Schmutterer (1990). This may also be a very important factor to aphids which, by

virtue of their small size, have a large surface area to volume ratio.

7.2.1 Comparison of systemic and spray applications:

Application of azadirachtin (500ppm) to the roots of *N. clevelandii* seedlings had strong effects on the ability of *M. persicae* to sustain feeding from the phloem sap whereas application of the same concentration as a spray onto the leaves did not (cf. Figures 23-26). It is possible that topical application of azadirachtin to the leaves resulted in poor uptake into the leaf tissue itself and that the compound was therefore not detected by probing aphids. West & Mordue (Luntz) (1992) found that probing behaviour by *R. padi* and *S. avenae* was less frequently observed on winter barley seedlings which had been treated with 250 and 500ppm azadirachtin solutions applied systemically or as foliar treatments. The same authors found that, in a choice experiment, winter barley leaves painted with solutions of azadirachtin at concentrations as low as 50ppm were rejected in favour of untreated leaves or leaves painted with lower concentrations of the antifeedant. Compounds applied to the leaf surface may have repellent effects so decreases in aphid settling cannot be attributed solely to antifeedant effects. However, cereal aphids may also be more susceptible to the primary antifeedant effects of azadirachtin than *M. persicae*. Different species of aphid are known to respond very differently to neem extracts

e.g. Schauer (1984) demonstrated that *Aphis fabae* was less susceptible to the effects of a neem seed extract than *Acyrthosiphon pisum*. The respective LC_{50}'s for the adult insects were 850.9ppm and 111.4ppm.

7.3 Determination of active concentrations of azadirachtin in artificial diets:

The response of *M. persicae* to the presence of azadirachtin in artificial diets can be used as a model for its response to the antifeedant in phloem sap. It is recognised that concentrations that may be antifeedant when presented in artificial diet studies cannot be translated exactly to phloem sap concentrations because leaf topography and chemistry may alter an aphid's response to the compound (Griffiths *et al.*, 1978). As demonstrated in Chapter 4, the choice of diet can strongly influence acceptability. The diet described by Griffiths *et al.* (1975) was more readily accepted than the one described by Dadd & Mittler (1966). The latter diet (Diet 2) contained L-tryptophan, L-arginine monohydrochloride, L-glutamic acid, L-histidine, L-valine, L-ascorbic acid, aneurine hydrochloride, riboflavin, nicotinic acid and choline chloride, which were all absent from the more acceptable diet (Diet 1). Each of these compounds has been shown to variously deter settling and feeding, reduce survival and fecundity, impair development or have no advantageous effect if included in a complex aphid diet (Mittler, 1967, 1970;

Mittler & Kleinjan, 1970; Griffiths *et al.*, 1975; Boisvert & Auclair, 1981). L-leucine, L-alanine, L-serine, folic acid, pyridoxine and calcium pantothenate were all present in Diet 1 at double the concentration used in Diet 2. These compounds enhance settling, survival or nymph production when incorporated at the higher concentration (Griffiths *et al.*, 1975). Because the experiments with artificial diets involved testing for short term effects of azadirachtin, the more acceptable diet was used rather than the one which was more nutritionally complete.

7.3.1 Development of a technique to quantify aphid feeding on artificial diet:

The acceptability of aphid diets has, in the past, been based on measurements of:

i) Settling (Dreyer *et al.*, 1981; Rose *et al.*, 1981);

ii) Nymph production ("larviposition") (Greenway *et al.*, 1978; Griffiths *et al.*, 1975,1978);

iii) Nymph growth and development (Mittler & Dadd, 1963b);

iv) Survival (Mittler & Dadd, 1962,1963b; Schoonhoven & Derksen-Koppers, 1976);

v) Uptake of dyes (Mittler & Dadd, 1963a)

vi) Weighing a) groups of aphids before and after feeding (Dadd & Kreiger, 1968) and b) sachets of diet before and after feeding (Auclair, 1965; Mittler, 1967).

vii) Measurement of honeydew production by a) counting excretory spots and determining the volume of such spots (Griffiths *et al.*, 1975; Mittler & Meikle, 1991) or b) the excretion of rabiolabelled compounds (Arn & Cleeve, 1971; Wright *et al.*, 1985).

Settling was not used as the definitive measure of antifeedant activity in the present work because it can be strongly influenced by aggregation behaviour and is therefore unreliable. Nymph production, mortality and the uptake of dyes could not be used to measure the antifeedant effect of azadirachtin for reasons discussed in Chapter 4. Weighing of diet sachets proved to be unreliable and, where different numbers of nymphs are produced on diets with varying concentrations of azadirachtin, it is impossible to determine to what extent the nymphs have influenced the total weight of diet consumed.

In the technique described in section 4.2 (iii), the amino acids present in aphid honeydew were stained with ninhydrin to give a quantitative measure of excretion and therefore feeding. Nitrogenous material ingested by aphids feeding on plants or artificial diets is almost entirely composed of amides and amino acids (Auclair, 1963; Srivastava, 1987). These compounds are usually present in the ingested material in quantities in excess of the aphids' requirements and, although in some species differential retention of amino acids occurs (Bragdon & Mittler, 1963), all of the amino acids present in

honeydew are also detectable in the food source (Mittler, 1953). The number and quantity of amino acids present in honeydew therefore depends on the number and quantity present in the food source. In the present work, the artificial diet was very rich in these compounds (approximately 2% w/vol) and measurement of the amino acid content of honeydew was therefore an appropriate method of estimating food intake. The amino acid : carbohydrate ratio of honeydew increases exponentially with dietary amino acid level (Mittler, 1988), so this method may only be appropriate for use with diets which contain high concentrations of the nitrogenous compounds. In cases where the dietary amino acid content is low (e.g. 0.003-0.4% w/vol in phloem sap (Zimmerman, 1960)) measurement of the carbohydrate content of honeydew using a similar method (e.g. an adaptation of the method described by Mittler & Meikle, 1991) may be more appropriate.

In the present work, there was a strong positive linear relationship between the weight of diet consumed and the absorbance of stained honeydew. There was a slight scattering of individual values around the line which described the relationship, probably resulting mainly from inaccuracies in the measurements of weight of diet consumed rather than the amount of honeydew produced. The method of honeydew measurement described in the present work is less complicated than those previously described because frequency and volume of droplets need not be measured independently. In contrast, the methods

described by Banks & Macaulay (1964), Mittler & Meikle (1991) and others require firstly the collection of honeydew onto paper, which is stained, to determine the frequency of excretion and secondly, the independent collection of honeydew into mineral oil to determine the volume and thus rate of excretion.

A further advantage of the method described in the present work over previously published methods is that no special procedures for handling radioactive markers are required and there is no loss of accuracy as a result of metabolism of radiolabelled material.

In the method described above, the stained honeydew was eluted into a specified volume of solvent. The volumes of solvent used were in excess of 1ml to permit colorimetric analysis. Where small volumes of honeydew are to be quantified e.g. those collected from individual nymphs or from adults over short time periods, a more accurate approach may be considered. Fluorimetric quantification of amino acids is approximately one hundred times more sensitive than colorimetry (Roth, 1971) but does have the following disadvantages;

i) proline and cysteine do not fluoresce under the conditions described by Roth (1971);

ii) the fluorescence of the products of the reaction of *o*-phthalaldehyde and 2-mercaptoethanol with amino acids is not constant but reaches a peak intensity after approximately 5 min. Fluorescence therefore must either be measured at a set time interval or over the course of a set period to obtain comparable results. This may pose

logistical problems when handling large numbers of samples.

Azadirachtin effects on amino acid content of honeydew: The relationship between the quantity of food consumed and the quantity or composition of honeydew produced did not appear to be affected by the presence of azadirachtin in the diet at concentrations that produced primary and secondary antifeedant effects. However, if ingestion of azadirachtin were to cause the rate of honeydew production to fall, e.g. because of inhibition of proctolin-induced gut contractions (Mordue (Luntz) & Plane, 1988) but the amino acid concentration of the honeydew to increase, because of decreased uptake or metabolism (e.g. Ayyangar & Rao, 1989; Sridhar & Chetty, 1989; Timmins & Reynolds, 1992), the absorbance of each stained honeydew droplet would be higher than that of droplets produced by aphids feeding on control diets. This would result in an over-estimation of food intake by aphids on azadirachtin-treated diets. If feeding were only slightly depressed, the antifeedant effect would then be obscured. An initial experiment (4.2.4) did not support this hypothesis. In this experiment both the weight of diet consumed and the quantity of honeydew produced were used as measurements of feeding on azadirachtin-treated diets. It demonstrated that the relationship between the two measurements was consistent for azadirachtin-treated and control diets and that any over-estimation of food intake on treated diets was slight.

The measurements recorded in experiment 4.2.4 could not be used to determine the antifeedant effect of azadirachtin on adult *M. persicae* because the values were probably strongly influenced by nymph production. The quantity of honeydew produced and weight of diet consumed in 48h by first and second instar nymphs was high. First and second instar nymphs of the pea aphid (*A. pisum*), feeding on plants, produced 6.3% and 18.9% of the volume of honeydew produced by adults (Auclair, 1958), whereas first and second instar *M. persicae* nymphs (48-72h old) feeding on untreated diets produced 19.5% of the quantity of honeydew produced by adults. It is doubtful whether the nymphs that were born during experiment 4.2.4 contributed as much honeydew as this to the recorded total because they were less than 48h old at the end of the recording period. Nevertheless, if the nymphs produced 10% as much honeydew as adults and if each adult produced 10 nymphs in a 48h period on control diets then the actual quantity of honeydew produced by each adult would be only half the total amount produced by adults and nymphs.

7.3.2 Antifeedant effects of azadirachtin presented to aphids in artificial diet:

The experimental protocol described in section 4.2.6 made it possible to exclude the effects of nymph feeding from the overall measurements of diet consumption.

Concentrations of azadirachtin as low as 100ppm produced a strong primary antifeedant effect against adult *M. persicae* (Figure 45). Concentrations below 100ppm failed to produce primary antifeedant responses but did induce strong secondary effects on feeding after an initial 24h period of exposure to diets treated with azadirachtin at concentrations as low as 25ppm (cf. Figures 51-54). In most cases aphids which had previously fed on azadirachtin-treated diets produced less honeydew after transfer to untreated diet than aphids which had previously fed on control diets. This may have been partly an effect of starvation/dehydration, but the results were variable and difficult to interpret.

The initial settling behaviour of aphids on azadirachtin-treated diets was influenced only by the highest concentration tested (1000ppm), but as the experiment progressed, concentrations as low as 100ppm disrupted settling (see Figures 41 and 42). In experiment 4.2.7, when aphids had fed for a short period on untreated diets before the start of the experiment, initial settling was high and azadirachtin, incorporated into the diet at concentrations of 25-100ppm, exerted no effect on the number of aphids settling in any time period (Figure 49). In all periods in experiments 4.2.6 and 4.2.7, but especially during the second 24h period on diets treated with low concentrations of azadirachtin, settling was less strongly affected than honeydew production. This indicates that the rate of feeding/honeydew excretion was strongly reduced even when

the aphids appeared to be feeding normally. Schoonhoven (1982) stated that contact with a feeding deterrent may not only interrupt food intake but may also result in increased locomotory activity of insects. Experiments 4.2.6 and 4.2.7 indicated that the locomotory activity of aphids on diets containing high concentrations of azadirachtin was increased, but lower concentrations, which strongly affected ingestion, had no initial effect on locomotory activity. This may account for the observations by Griffiths *et al.* (1978) that only high concentrations of neem extracts produced visible effects on aphid settling behaviour. It also demonstrates the value of using several measures of feeding behaviour to investigate the effect of an antifeedant on aphids.

7.3.3 Effects of azadirachtin on aphid mortality:

Mortality of aphids was associated with the period in which they were exposed to azadirachtin-treated diets rather than after transfer to untreated diets. Increased mortality was therefore attributed to starvation effects rather than secondary toxic effects after uptake of the compound. The highest rates of mortality, which were recorded for starved aphids and aphids placed on diets that contained 1000ppm azadirachtin, occurred after 41-53h. This period agrees with previously published estimates of the longevity of starved aphids (Mittler & Dadd, 1963b).

7.3.4 Effect of azadirachtin ingestion on nymph production:

A decrease in nymph production was one of the most noticeable effects that azadirachtin had on adult *M. persicae* feeding on treated diets. Higher concentrations of the antifeedant, which strongly decreased ingestion, had the smallest effects on nymph production 9cf. Figures 36 and Table 23). Aphids were not deterred from feeding during first 26h period on diets containing 25-75ppm azadirachtin (Figure 51) but their rates of nymph production were lower than those of aphids that had fed on control diets (Table 26). After a further 24h period, nymph production on diets treated with 25-100ppm azadirachtin had virtually ceased and any nymphs that were born had been prematurely extruded. These were not the effects of starvation as aphids which had been starved started to produce viable nymphs at high rates as soon as they had access to untreated diets.

The effect of neem extracts on the fecundity of aphids has been described previously. Dimetry & Schmidt (1992) found that when adult *A. fabae* were placed on bean plants which had been sprayed with the commercial neem formulations "Neem-Azal S" or "Margosan-O" at concentrations which contained 0.875ppm and 0.75ppm azadirachtin respectively, fecundity and longevity were decreased. This may, however, have been an effect of repellency. Pea aphids (*A. pisum*), reared from first instar nymphs on bean plants which had been sprayed with

a methyl-tertiary-butyl-ether neem seed extract at a concentration of 20ppm, produced less than 10% of the number of nymphs produced by adults on untreated plants (Schauer, 1984). Many workers have found that the effects of azadirachtin on the growth, development and fecundity of insects are produced by concentrations lower than the thresholds for antifeedant activity. For example, Kraus *et al.* (1987) found that larval *Epilachna varivestis* fed normally at first on leaves which had been treated with 10-25ppm azadirachtin but rapidly reduced their feeding rate after one day and, after transfer to untreated leaves, the larvae died within one week. Larvae which had been offered leaves treated with azadirachtin at a concentration of 500ppm did not feed and suffered no secondary effects after transfer to untreated leaves. Simmonds & Blaney (1984) also noted that increasing the concentration of azadirachtin in diets decreased the food consumption and increased the starvation-related mortality of oligophagous species of Lepidopteran larvae whereas the highest mortality of polyphagous species was associated with low concentrations of azadirachtin and high food intake.

Garcia *et al.* (1991) compared the concentrations of azadirachtin that inhibited feeding and moulting in *Rhodnius prolixus*. They found that the ED_{50} for moult inhibition was 625-fold lower than the ED_{50} for feeding inhibition. In the present work 25ppm azadirachtin, the lowest concentration tested, decreased the fecundity of

aphids and it is possible that much lower concentrations could be effective.

The effects of azadirachtin on the fecundity of *M. persicae* may result from disturbances in the endocrine mechanisms which control embryo development and/or parturition. These effects have been studied more closely in other Hemipterans. For example, Dorn *et al.* (1986) demonstrated that fifth instar milkweed bugs (*Oncopeltus fasciatus*), which had been injected with azadirachtin, failed to moult to the adult stage. Nevertheless, a high proportion of these "permanent larvae" developed ovaries which contained chorionated eggs. These ovaries were very much smaller than those of normal adults and contained fewer eggs. Oocytes were arrested at an early stage of vitellogenesis. Garcia *et al.* (1991) found that treatment of adult female *R. prolixus* with azadirachtin also reduced oocyte growth and diminished egg production. These effects were correlated with reduced titres of vitellogenin, vitellin and ecdysteroids. Koul (1984) demonstrated that vitellogenesis was also prevented in adult *Dysdercus koenigii* which had been injected with azadirachtin. Topical application of azadirachtin to *D. koenigii* inhibited or delayed embryogenesis with many of the embryos remaining at a late stage of development. In aphids, juvenile hormone (JH) stimulates and controls embryogenesis and the rate of embryo development (Hardie, 1987; Hardie *et al.*, 1990). If azadirachtin has the same effects on JH titre in aphids as it does in other insects

(Rembold, 1988) this may partly explain the effects of the compound on the fecundity of *M. persicae*.

7.4 Effects of azadirachtin on the acquisition and transmission of PLRV and PVY:

EPG and artificial diet studies indicated that azadirachtin could have a primary antifeedant effect against adult *M. persicae* on plants if the phloem sap contained high concentrations of the compound. The diet studies also demonstrated that lower concentrations of azadirachtin have secondary antifeedant effects and a profound effect on fecundity after ingestion. Since feeding rate and duration are closely related to the ability of *M. persicae* to transmit PLRV (van den Heuvel & Peters, 1990), any effect of azadirachtin in decreasing the feeding activity of aphid vectors could be expected to decrease their transmission of PLRV.

7.4.1 Effects of azadirachtin on the acquisition of PLRV:

In PLRV acquisition tests where the vector aphids had previously fed on uninfected *N. clevelandii* plants infected with PLRV (experiment 5.2.7), about 85% survived the AAP on untreated plants and subsequently transmitted the virus to approximately 60% of the indicator plants. Less than 50% of the aphids survived the AAP on azadirachtin-treated plants and these aphids transmitted PLRV to less than 20% of the indicator plants. In a

subsequent PLRV acquisition test (experiment 5.2.8) the numbers of putative viruliferous aphids transferred to indicator plants from treated and untreated virus source plants were equal. Fewer indicator plants became infected by aphids that had spent the AAP on azadirachtin-treated virus source plants than by aphids from untreated virus source plants. The higher mortality during the AAP amongst groups of aphids on azadirachtin-treated virus source tobacco seedlings was therefore not solely responsible for the inability of groups of these aphids to transmit PLRV. The evidence from EPG studies, honeydew collection from aphids feeding on azadirachtin-treated plants and the mortality of aphids during the AAP on these plants strongly suggests that the reduction in PLRV acquisition resulted from reduced phloem sap imbibition as an antifeedant response to azadirachtin. Aphids which survived the AAP on azadirachtin-treated plants must have fed to some extent as they could not have survived starvation for this period (72h). Further evidence of feeding during the AAP is provided by the observations that aphids did produce a small amount of honeydew on azadirachtin-treated plants during this period and that, on transfer to indicator plants, aphids which had survived the AAP on azadirachtin-treated plants produced nymphs at very low rates. It is therefore possible that a secondary antifeedant effect caused by the imbibition of azadirachtin was partly responsible for the reduced transmission of PLRV to indicator plants. Such an effect could have operated at the acquisition or inoculation

stage, or both. Some indication that the antifeedant affects mainly the acquisition of PLRV is given by the titres of PLRV in aphids which had survived the AAP on azadirachtin-treated or untreated plants. The titres of PLRV in aphids which had spent the AAP on untreated plants were higher than those caged on azadirachtin-treated virus source plants (experiment 5.2.7). However, the results of this experiment must be viewed with caution because very few of the aphids tested gave a positive result for virus presence. This is probably because the tests were performed after the aphids had fed on indicator plants for 90h. Tamada & Harrison (1981) demonstrated that the PLRV titre of aphids which were allowed to feed on uninfected plants fell rapidly during the first 48h after plant access and reached a low steady level thereafter.

Determination of virus presence in aphids: Published methods of determining virus titres in aphids have relied on alkaline-phosphatase-based DAS ELISA and complex cocktail-ELISA techniques. For example, Tamada & Harrison (1981) used an alkaline phosphatase-based technique to determine the PLRV titres of adult *M. persicae*. They found high background absorbance from uninfected samples. Van den Heuvel (1991) compared an alkaline phosphatase-based DAS ELISA technique with an amplified cocktail ELISA to detect PLRV in *M. persicae* and found that the latter was substantially more sensitive and gave much lower background absorbance. The major drawbacks of the amplified cocktail ELISA technique are the relative

complexity and expense of the method and the requirement for NADP, *o*-nitrophenylphosphate, alcohol dehydrogenase, lipoamide dehydrogenase and *p*-iodonitrotetrazolium violet.

The penicillinase-based technique described in section 5.2.5 is simple, inexpensive and safer than techniques which use 4-nitrophenylphosphate as a substrate. The product of the reaction of penicillin with penicillinase is penicilloic acid which is non-toxic, whereas the product of the reaction of alkaline phosphatase with 4-nitrophenylphosphate is the highly toxic 2,4-dinitrophenol, (Singh & Barker, 1991).

The quantity of intrinsic penicillinase in aphids seems to be predictably low compared with intrinsic phosphatase levels. Thus the background reaction is reduced when penicillinase-based DAS ELISA is used. However, estimates of PLRV titre obtained using penicillinase-based techniques also have some disadvantages. They are probably less accurate than those obtained from amplified cocktail ELISA techniques because the standard curve of virus titre against absorbance with the penicillinase based technique was linear only over a small range of concentrations. The conversion of substrate in penicillinase-based DAS ELISA also takes considerably longer than in amplified cocktail ELISA so the detection of PLRV is not as rapid. For quantitative studies, the amplified cocktail method is therefore probably more appropriate than the penicillinase-based method but where

qualitative or semi-quantitative results are required the penicillinase-based method is cheaper and more simple.

Other factors affecting PLRV acquisition: The acquisition of PLRV by vector aphids from virus source tobacco seedlings was greatly enhanced by manually removing the original inoculating aphids instead of using nicotine fumigation. Nicotine residues which appear to have been present on the leaves even 9-10 days after fumigation probably affected the acquisition process. Fumigation of the virus source tobacco seedlings with nicotine had more of an effect on virus acquisition than either environmental conditions or removal of the seedlings from compost. Nicotine residues hampered the acquisition of PLRV but did not adversely affect survival of vector aphids.

Both the survival of vector aphids during the AAP and, to some extent, the ability to acquire virus from the virus source tobacco plants were strongly affected by the immediate feeding history of the vector aphid. Aphids which had been reared on, or had last fed on, PLRV-infected potato plants transmitted PLRV efficiently to tobacco plants whereas aphids which had been reared on turnip plants did not survive or feed for long enough periods on the virus source tobacco plants to efficiently transmit PLRV to indicator plants. Survival of vector aphids on infected tobacco seedlings was improved by previous exposure to uninfected tobacco plants. The effect of the feeding history of *M. persicae* on its ability to thrive on different plants has previously been

described by Lowe (1973). He found that there were wide variations in the ability of clones of *M. persicae* to colonise sugar beet depending on whether they had been raised on Chinese cabbage, sugar beet or broad bean. Aphids which had previously been reared on broad bean settled and colonised sugar beet most readily, but aphids which had previously been reared on Chinese cabbage were least able to settle and colonise the new host. In addition, Klingauf (1987b) reported that when *M. persicae* were transferred from *Brassica n. napus* to *Nicotiana tabacum*, only 5% of the aphids survived while aphids which had been reared on tobacco exhibited higher survival rates when transferred to swede. Therefore, it is concluded that, to accurately measure the true effects of an antifeedant on the acquisition of persistently-transmitted viruses, it is necessary to exclude all other factors that may influence the acquisition process e.g. differential mortality, residues of other chemicals and the influence of feeding history.

7.4.2 Effects of azadirachtin on the acquisition of PVY:

Aphids acquired PVY less well from azadirachtin-treated plants than from untreated plants. This was contrary to expectations based on the EPG experiments which indicated that aphids probed more frequently on azadirachtin-treated plants than on untreated plants. It is possible that the interaction between the severe symptoms of PVY and the short-term phytotoxic effects of azadirachtin

(i.e. wilting) resulted in a reduction in the number of intact cells from which PVY could be acquired during probes. The short-term phytotoxicity is unlikely in itself to have prevented normal settling and feeding and may even have encouraged such behaviour (e.g. Klingauf, 1987b; Polonsky *et al.*, 1989). However, *M. persicae* usually has to penetrate a viable infected cell to acquire a non-persistently transmitted virus (Powell, 1991; Powell *et al.*, 1992b).

7.4.3 Effects of azadirachtin on the inoculation of PLRV and PVY:

Azadirachtin did not affect the ability of *M. persicae* to inoculate indicator plants with PLRV in choice and no-choice experiments, although the number of aphids settling on treated plants was reduced in choice experiments. Inoculation of plant tissue with PLRV is thought to be more a function of salivary secretion and non-vascular feeding activities than ingestion from the vascular tissue (Sylvester, 1980). Evidence from the EPG studies demonstrated that treatment of *N. clevelandii* with azadirachtin did not affect the ability to produce a salivary sheath during penetration.

Choice tests were performed to examine the possibility that aphids fed for short periods on azadirachtin-treated indicator plants in no-choice tests. Starvation might have overcome the antifeedant effect of azadirachtin in no-choice tests. While no-choice tests provide a more

accurate measure of how aphids behave in the field, choice tests are more sensitive indicators of antifeedant activity, especially for polyphagous species (Schoonhoven, 1982). For example, salannin is an effective antifeedant against *S. littoralis* in choice tests but is ineffective in no-choice tests (Blaney *et al.*, 1990). However, Blaney *et al.* (1990) suggest that, no-choice bioassays are probably the best method of determining the strength of an antifeedant.

Treatment of indicator plants with azadirachtin resulted in severe long-term phytotoxicity characterised by leaf distortion and stunting. This malformation of the leaf tissue may have contributed to the low PLRV titres observed in infected plants that had been treated with azadirachtin previously. Despite inducing short-term and long-term phytotoxicity, azadirachtin did not affect the ability of viruliferous *M. persicae* to infect treated plants with either PLRV or PVY.

Partial wilting of *Physalis floridana* did not affect the ability of *M. persicae* to infect these plants with PLRV (Bindra & Sylvester, 1961). The same is probably true for *N. clevelandii* and the short-term phytotoxicity observed in azadirachtin-treated plants is unlikely to have affected the probability of infection with PVY because the aphids had an improved chance of infecting an intact epidermal or mesophyll cell during the long IAP.

7.5 Prospects for the practical use of azadirachtin to control virus spread by *M. persicae*:

Van Beek & de Groot (1986) listed 14 characteristics of an ideal antifeedant:

i) non-phytotoxic

ii) non-toxic to man, animals, beneficial insects and other organisms

iii) inhibits feeding by as many pest species as possible

iv) preferably also toxic against the same insects

v) active at very low concentrations

vi) reasonably persistent when applied to a crop

vii) yields no toxic or bad-tasting metabolites

viii) absorbable by the plant and translocated through the plant

ix) easily applied

x) low costs and constant supply

xi) compatible with other forms of pest management

xii) it should not affect the taste, smell or appearance of the produce

xiii) no habituation or resistance should occur

xiv) stable during storage.

In the present work high concentrations of azadirachtin did not prevent the inoculation of PLRV or PVY, but they did cause mild short-term phytotoxicity and severe long-term effects on plant morphology. While most of the other requirements for an ideal antifeedant are met by azadirachtin, it is the inability to control inoculation

which precludes its use as a means of preventing the introduction of virus to potato crops.

Miller & Cowles (1990) stated that damage by insect pests could be described by the expression;

$$\text{Damage} = D.A.S.T$$

where D = pest density per unit crop

A = plant acceptability

S = plant suitability for the insect

T = time or duration of interaction

Antifeedants influence D,A and S (S only if the compound has secondary effects after ingestion) but the authors believe that such compounds, used alone, do not have a large enough impact on any of the four factors to control damage effectively. This conclusion was explained by the theory of dynamic acceptance thresholds. Thus when an insect contacts a plant which has been treated with an antifeedant it remains hungry and in the vicinity of the food plant. The effect of the antifeedant diminishes as increasing external and internal stimuli lower acceptance thresholds over time as discussed in section 1.3. Miller & Cowles (1990) suggested that the effectiveness of feeding deterrents might not be overcome if insects that were experiencing mounting food deprivation could intermittently feed on a highly acceptable source - a concept which they term "stimulo-deterrent diversion strategy (SDDS)" and which is more succinctly described

elsewhere as "push-pull strategy" (Griffiths, 1990; Pickett, 1991). Griffiths *et al.*, (1991) described a system where antifeedant extracts of *Ajuga* spp. were used to protect the upper areas of foliage on mustard plants and force mustard beetle larvae (*Phaedon cochleariae*) onto the lower foliage which had been treated with triflurobenzuron (an acylurea IGR) where they were effectively controlled. Many authors (e.g. Schmutterer, 1988, 1990; Jermy, 1991) consider the effects of azadirachtin on fecundity, development and growth to be more important for the practical control of insects than the antifeedant effects. The work presented in this thesis supports this conclusion for *M. persicae*. The fecundity-reducing property of azadirachtin could be its most useful effect for the control of virus spread in potato crops. Most PLRV spread in Scottish potato crops results from aphid population growth and movement within crops and often occurs early in the season (Woodford *et al.*, 1983; Barker, 1990). Using existing technology, it would be possible to apply azadirachtin-enriched granules with the seed tubers at planting. These granules are already commercially available in India under the name "Neemark" (Saxena, 1989). This treatment could be supplemented by a powerful aphid antifeedant or repellent (e.g. (E)-ß-farnesene derivatives (Griffiths *et al.*, 1989) or polygodial (Gibson *et al.*, 1982b) applied to the upper foliage to control the spread of PVY when aphid migration was forecast. The aim would be to force immigrant aphids away from the crop or onto the lower

leaves of the plants. This would have two advantages for the control of virus spread. First, PLRV particles from older tissue are less readily acquired by aphids than those from younger leaves (Tamada & Harrison, 1981; van den Heuvel & Peters, 1990). Secondly, there is some evidence that azadirachtin concentrations in lower leaves are higher than in upper leaves after uptake from the roots (Rovesti & Deseo, 1991). Provided that azadirachtin concentrations in the phloem sap of treated plants were insufficient to produce antifeedant effects but were high enough to have profound effects on fecundity, populations of aphids would be prevented from establishing on treated plants. Apterous *M. persicae* leave their host plants in response to a deterioration in the plants' condition which is caused by increased population pressure (Hodgson 1991). The majority of aphids leaving the host plants are adults and final instar nymphs. If the fecundity-reducing effects of azadirachtin were also combined with developmental effects against nymphs, as reported for other aphid species (e.g. *A. pisum* and *A. fabae*, Schauer 1984), the stages most likely to emigrate would not be produced and virus spread within the crop could be controlled effectively. Sylvester (1989) listed the four major approaches to the control of plant virus spread as: i) the elimination of virus sources; ii) isolation of crop from virus sources; iii) manipulation of crop, and iv) reduction in numbers of active vectors. The reduction in numbers of active vectors through the use of the fecundity-reducing effects of azadirachtin is compatible

with the elimination of and isolation from virus sources (by roguing and spatial separation from ware crops). The cultivation of PLRV and PVY resistant varieties (Barker & Harrison, 1984,1985,1986) could further reduce virus transmission.

Azadirachtin, used as an insect growth regulator (IGR) against *M. persicae*, appears to have an advantage over synthetic IGR compounds because it can sterilise adult aphids shortly after uptake. In contrast, Bauernfeind & Chapman (1984) found that even kinoprene, the most effective synthetic IGR tested against *M. persicae*, sterilised the adult only if it had been applied to the insect before the fourth instar. In the present work, fecundity-reducing effects were noted after only 24h when adult aphids had fed on artificial diets containing azadirachtin. On plants the rate of sap uptake is thought to be at least double that observed on artificial diets (Mittler, 1988) so the effects of azadirachtin may be even more rapid on plants.

The persistence in the field of topically-applied azadirachtin is reported to be only a few days (Larson, 1987; Schmutterer, 1988). However, the residual effect is prolonged by systemic uptake (von der Heyde 1984). Gill & Lewis (1971) reported persistence of antifeedant activity of azadirachtin against *S. gregaria* for up to 15 days when a 10ppm solution was applied to the soil. In addition, Barnby *et al.* (1989) found that even after 80% degradation of azadirachtin by UV irradiation, the IGR effects persisted in the resulting mixture of compounds.

The commercially available neem product Margosan-O is registered in the USA for use on non-food crops. It has therefore undergone the rigorous toxicological tests of the Environmental Protection Agency (EPA). The product has an acute oral toxicity in rats of >8500mg/kg and no dermal toxicity. The avian LC_{50} is also >7000ppm (Larson 1987). Jacobson (1986,1988) has reviewed the toxicological activity of azadirachtin and neem products and it seems certain from the scientific and anecdotal evidence that azadirachtin poses no risk to warm-blooded animals.

Saxena *et al* (1989) found that systemic and spray applications of neem seed extracts to rice fields had no adverse effects on the populations of predatory mirids or spiders. Importantly for aphid control, predaceous coccinellids were not affected by the application of a neem formulation, and the spraying of aphid mummies containing the larvae or pupae of braconid wasps did not prevent the successful emergence of these parasitoids (Schauer, 1985; Srivastava & Parmar, 1985 (cited by Schmutterer, 1990)).

Aphid resistance to insecticides is one of the main reasons for the search for alternative means to control the spread of aphid-transmitted viruses. Given the multiple modes of action of azadirachtin on the behaviour and physiology of insects, it is unlikely that resistance will develop. Habituation to the antifeedant effect of azadirachtin is possible, e.g. *S. gregaria* does not respond to antifeedant concentrations of the compound

after it has encountered them for four days continuously (Schoonhoven, 1982), but habituation to the antifeedant response is likely to be followed by IGR or fecundity-reducing effects after ingestion. Larvae of the diamondback moth, *Plutella xylostella*, treated continuously with neem seed extract for 35 generations did not develop resistance to the antifeedant and fecundity-reducing effects, and there were no increases in the activity of esterases or multifunctional oxidase. (Vollinger, 1987).

In spite of the increasing area of neem cultivation to supply the botanical insecticide industry, Taylor (1987) expresses the opinion that it is unlikely that enough azadirachtin will be readily available for widespread use and that the most hopeful prospect is for the development of simple synthetic analogues. Synthetic analogues which retain the IGR and fecundity-reducing properties of the original molecule while minimising the antifeedant properties are probably of most practical use. Some progress has been made towards this end, e.g. Simmonds *et al* (1990) tested the IGR activity of a series of natural and synthetic azadirachtin derivatives against four species of lepidopteran larvae. None of the derivatives tested was more active than azadirachtin but evidence was presented that the nature of substitutes at the C-1 and C-3 positions of the decalin ring were important.

Although the prospects of controlling potato virus spread using azadirachtin as an aphid antifeedant are poor, this work has demonstrated that its profound

effects on the fecundity of *M. persicae* could provide the basis for a novel and lasting method of controlling aphid populations and virus spread.

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