Endogenous resistance to insect pests in alfalfa: engineering for enhanced resistance

Mazahery-Laghab, Hojjatollah

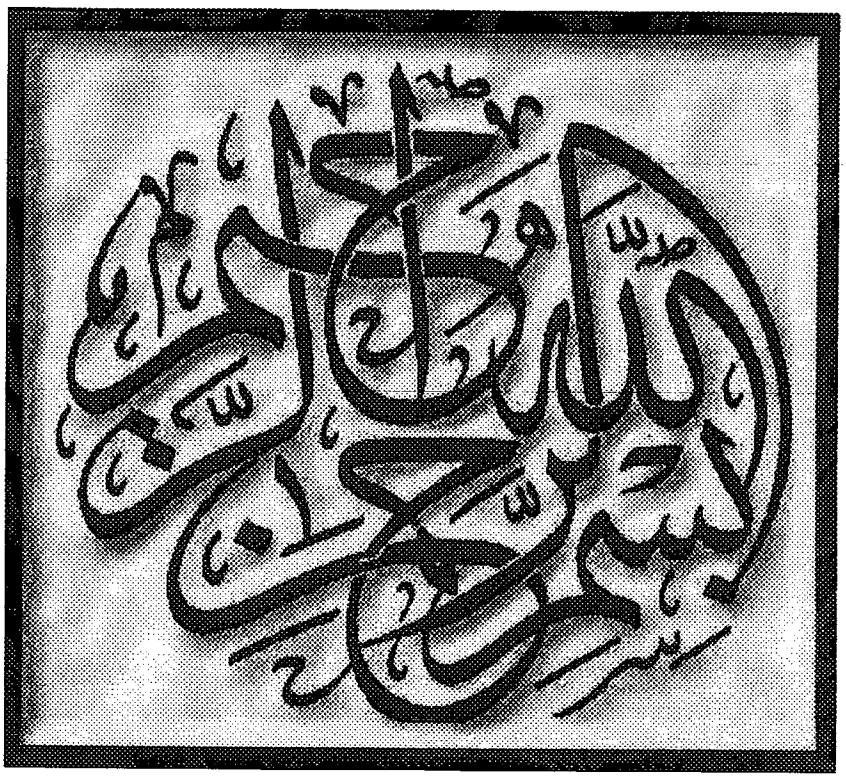
Endogenous Resistance to Insect Pests in Alfalfa; Engineering for Enhanced Resistance

A Thesis Submitted by Hojjatollah Mazahery-Laghab, M. Sc.

In Accordance with the Requirements of the University of Durham for the Degree of Doctor of Philosophy.

Department of Biological Sciences

August 1997



ABSTRACT

Alfalfa (*Medicago sativa*) is a valuable forage crop grown throughout the World. While the crop is resistant to attack by many insect pests, it is subject to potentially severe losses through the action of several specific pests, which are adapted to alfalfa as a host. The most economically damaging of these pests is the alfalfa weevil, *Hypera postica*. This thesis investigates the endogenous defences of alfalfa against insects, which are responsible for its resistance to non-pest species, and develops a strategy for increasing the resistance of alfalfa towards pest species, specifically alfalfa weevil.

The role of saponins in the resistance of alfalfa towards non-pest species has been investigated by using successive insect bioassays, carried out with extracts, mixtures of compounds, and purified compounds, to identify which compounds present in alfalfa tissues are responsible for toxicity towards insects. Crude saponin extracts, in 80% methanol, from alfalfa seedling tissues were bioassayed against the cowpea seed weevil, *Callosobruchus maculatus*. Both extracts from shoot and root tissues caused larval mortality and delayed development when incorporated into an artificial diet at levels comparable to those found in alfalfa, but lower levels of root saponin extracts showed probiotic effects, whereas lower levels of shoot saponins were still toxic. Hydrolysis of the saponins present in these extracts decreased their toxicity.

Purified saponin mixtures were prepared by butanol partition and ether precipitation, and were bioassayed against potato aphid (*Aulacorthum solani*) in a liquid artificial diet, which allowed quantitative effects to be accurately assayed. Shoot saponins showed a concentration-dependent toxic effect, decreasing survival over an initial 5 day period, decreasing growth, and inhibiting fecundity (measured by nymph production) in these insects. Alfalfa root saponins showed no deleterious effects below a threshold level, but caused complete mortality above this level. The alfalfa saponin mixtures were separated into fractions by chromatography on a reverse phase column. Bioassays showed that the toxicity towards potato was associated only with fractions containing saponins, and that fractions containing a component identified as soyasaponin I were more toxic to the aphids than others. Finally, two saponins purified from alfalfa, soyasaponin I and medicoside A, were assayed. These assays showed that soyasaponin was consistently more toxic in effects on mortality, growth and fecundity. It was concluded that alfalfa saponins, and in particular soyasaponin I, were major factors in the resistance of alfalfa towards potato aphid, and other insects. A saponin mixture from another species, sugar beet (*Beta vulgaris*) was also toxic to aphids, supporting the view that saponins have a general role in resistance to insects.

Inhibition of insect digestive proteolysis by expression of a foreign protein protease inhibitor was selected as a strategy to protect transgenic alfalfa against alfalfa weevil. The major protease activity in larvae of this insect was shown to be due to cysteine proteases, which could be inhibited by cystatins. Rice cystatin was produced in large quantity using a recombinant protein expression system in *E. coli* for use in a "proving" experiment. Incorporation of the rice cystatin into an alfalfa weevil larvae artificial diet decreased survival, showing that this approach was feasible.

Contents

Abbreviations

AMC----------7-Amido-4-methylcoumarin

Ara----------L-Arabinose

BApNA----------α-N-Benzoyl-DL-arginine-p-nitroanilide

BSA----------Bovine Serum Albumin

BuOH----------Butanol

(c)DNA----------(Complementary) Deoxyribonucleic acid

d.H_2O----------Distilled water

DMSO----------Dimethyl Sulphoxide

DTT----------Dithiothreitol

E-64----------L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane

EDTA----------Ethylenediaminatetra-acetic-acid

EtOH---------- Ethanol

Et_2O---------- Diethyl ether

Gal----------D-Galactose

Glc----------Glucose

GlcUA----------D-Glucuronic acid

HPLC----------High Performance Liquid Chromatography

IPTG----------Isopropyl-β-D-thiogalactoside

LC_{50}----------50% Lethal Concentration

Man----------D-Mannose

MeOH----------Methanol

MeSH----------2-Mercaptoethanol

NMR----------Nucleic Magnetic Resonance

O.D.----------Optical Density

Ozc----------Oryzacystatin

PAGE----------Polyacrylamide Gel Electrophoresis

PCMB----------p-Chloromercuribenzoic Acid

PMSF----------Phenylmethylsulphonyl fluoride

PVP----------Polyvinyl Pyrrolidone

r.h.----------Relative humidity

Rha----------L-Rhamnose

RNase----------Ribonuclease

Rozc----------Recombinant oryzacystatin

SBTI----------Soybean Trypsin Inhibitor

SDS----------Sodium dodecyl sulphate (or lauryl sulphate)

TEMED----------NNN'N'tetramethylethylenediamine

TLC----------Thin Layer Chromatography

Tris----------Tris(hydroxymethyl)aminomethane

Xgal----------5-bromo-4chloro-3-indolyl-β-D-galactoside

Xyl----------D-Xylose

Acknowledgements

In The Name of ALLAH, who says if somebody is going to thank me, he/she must first thank my created people that has/have helped him/her. So, first of all, I should gratefully thank my supervisor although all people in the world, all moving on the earth, all birds in the sky and all creatures that move in the seas become authors, all water in the seas and oceans become ink and all trees on the earth become pencils, it is impossible to make a complete acknowledgement to the teacher.

However, I would like to thank Dr. J. A. Gatehouse as my sympathetic supervisor for all his worthy and helpful directions and advices, Dr. A.M. R. Gatehouse for her helpful guidances in bioassays, enzyme, and inhibitor assays, Dr. David Bown for his helpful practical guidances, Dr. R. Edwards for providing some required materials and using facilities in Lab. 2, Dr. B. A. Whitton for using spectrofluorimeter in Lab. 9 and Dr. Tony Fordham-Skelton and Dr. M. Bharathi for proof reading the manuscript. John Gilroy should be thanked for all sample concentrating and lyophilising. Other respected people in the Lab. 1 and Insectory section and also, others in the department are acknowledged.

I would also like to thank Matthew Squires for providing results on sugar beet saponins, which are described in this thesis.

I also gratefully acknowledge the financial support provided by the Ministry of Culture and Higher Education of the Islamic Republic of Iran. Also, many thanks to the Department of Biological Sciences, Durham University for the use of facilities.

I may thank also to Drs. G. Massiot and C. Lavaud from Reims University, France, for providing saponin references and doing NMR for saponin identification, Dr. W. Oleszek from Institute of Soil Science and Plant Cultivation, Pulawy, Poland, for shipping the saponin medicoside A as a reference compound, Dr. B. Yazdi-Samadi from Tehran University, Agriculture College for providing resistant and susceptible alfalfa seed cultivars. Drs. T. Elden from Agriculture Research Services, Beltsville, Maryland, USA and Drs. S. Blodgett from Montana State University for shipping alfalfa weevil eggs. Also, many thanks to Dr. R. Ratcliffe from USDA ARS, Insect and Weed Pest Control Research Unit, USA and Dr. H. Hsiao from Utah University due to their looking for any alfalfa weevil source

Finally, Many thanks to my parents for their financial and moral support during my study and to my wife Vajih and my sons Hamzeh and Hamed for their patience and encouraging for working hard.

Chapter 1

Introduction

1.1. General Introduction

As one of the basic requirements, food has a vitally important role in human life. The supply of most of Man's foodstuffs is dependent on crop production, either to provide food directly, or to produce forage crops to feed to animals. Increasing population, and a limited capacity of the plant to produce food, make crop losses due to factors such as plant diseases caused by viral, bacterial, fungal, and other pathogens, and due to attack by pests, a major economic and social problem world wide.

Crop damage caused by insects is a major economic factor in agriculture in tropical and temperate regions of the world. Globally, expenses for insect control using pesticides were estimated at about $7,655 millions in 1990 (Gatehouse *et al.*, 1992), but 13% of total crop production worldwide was still lost to insect pests. Failure to prevent insect attack can have devastating consequences, both for plants in the field and for stored plant products. For instance, untreated stored cowpea seeds are attacked by the seed weevil *Callosobruchus maculatus* Fab., and as much as 100% of seed can be destroyed in 5 months (Gatehouse 1991; Gatehouse and Boulter 1983). Large concentrations of a single crop species have long been recognized as favouring dramatic increases in pest species, with the numbers of herbivorous insects increasing according to the area occupied by the crop. Modern agricultural practices, which lead to insect species becoming serious pests, have been discussed by Chrispeels and Savada (1994). Although many species attack one plant species only (monophagous), or a limited range of host species (oligophagous), many serious agricultural pests are polyphagous.

1.2. Alfalfa (*Medicago sativa* L.)

Alfalfa is one of the major forage crops throughout the world. The high content of protein in alfalfa (*Medicago sativa)* plants offers a source of nutrients, which can help meet the protein requirements for humans as well as animals. This

plant is a valuable source of protein for temperate climates, and aerial parts of the plant may be used as forage crop or extracted to provide leaf protein concentrate (Oleszek *et al.*, 1994). Also, the plant can be grown in a wide range of climatic conditions such as cold, hot, dry, and semidry regions, but not in wet regions. The plant is rich in protein, calcium, vitamins, is highly palatable, has high yield, and contains reduced cellulose compared to other forage crops (Hanson *et al.*, 1988; Karimi 1990). Alfalfa cultivation is also important in improving the agronomic qualities of soil when used as a fertilizer plant; besides the nitrogen and organic matter added to soils by ploughing in alfalfa, the effects of land ventilation, crop rotation, and drainage are also beneficial. For all these reasons, alfalfa as a forage crop has been known as " green gold " from an economic point of view (Karimi 1990).

Alfalfa forage is extensively used as an animal foodstuff, although its utilization as a protein source is limited by the antinutritional properties of the saponins and other compounds present. Much work has been devoted to the factors limiting the value of alfalfa for non-ruminants such as poultry and swine (Oleszek *et al.*, 1994). Although saponins have been suggested to act as an antinutritional component, beneficial effects of these compounds have also been claimed (Price *et al.*, 1987). Intake of alfalfa by man is generally very low, but increasing consumption of alfalfa sprouts as green salad, or in the form of tablets or juice, has been suggested in diets for hypocholesterolaemic control (Oleszek *et al.*, 1994). This has been useful in the control of cardiovascular disease and may significantly increase saponin intake.

The yield of alfalfa in agriculture is limited by many factors, but important among these are insect pests such as alfalfa weevil (*Hypera postica* Gyll.). So, pest management is essential for agricultural production. One of the ways of pest management is by using pesticides. Unfortunately most of the pesticides also cause environmental pollution and contaminate the ecosystem. They can destroy other useful insects, which can be used as the predators for insect pests. There are efficient mechanical and agrotechnical ways to control insect pests but unfortunately they are temporary. Plant resistance to insect pests has not only an effective role in the pest management, but using this method can give protection in the long term (Mazahery-Laghab 1991).

Alfalfa contains biologically active substances like saponins, toxic amino acids, phenolics and quaternary amino bases, some of which are recognised as allelochemicals (Gorski *et al.*, 1991). In addition, some of these compounds have been suggested to have insecticidal properties, including saponins. The purpose of this research was to study the insecticidal properties of alfalfa saponins, and to develop a strategy for protecting alfalfa against its major insect pest, and thus to increase productivity of this crop.

1.3. Major Insect Pests of Plants

Among different orders of insects, Lepidoptera, Coleoptera, Homoptera, Diptera, and Orthoptera are the most significant plant pests. In the Lepidoptera such as moths and butterflies, it is normally caterpillars (the larval stage) that cause damage, usually to the green aerial parts of the plant. Coleoptera such as bruchid beetles also have a larval stage that is usually the major cause of damage to plants; in this case, almost any part of the plant can be attacked, with many species specialising on a particular organ, and/or a particular host species. Homoptera such as aphids do not pass through a larval stage, and both immature and adult forms damage plants by sucking sap from leaves, stem and shoots. In Diptera such as flies the larval stage is the familiar maggot. These pests can attack fruits, bulbs, roots, and leaves. Orthoptera such as grasshoppers and locusts are polyphagous in their habits, and both adults and immature nymphs cause damage (Gatehouse 1991).

Work in this thesis will concentrate on three insect species, all of which are crop pests. Two are coleopterans; the seed weevil *Callosobruchus maculatus* Fab., which is a polyphagous pest of stored seeds, and alfalfa weevil, *Hypera postica* Gyll., an oligophagous pest of alfalfa. In addition, a Homopteran species, the oligophagous glasshouse potato aphid, *Aulacorthum solani*, is used extensively as a subject for bioassays.

1.3.1. Alfalfa Weevil (*Hypera postica* Gyllenhal)

More than 100 insect species damage alfalfa, *Medicago sativa* (L.), but of these alfalfa weevil causes the greatest insect damage to the crop. The Animal and

Plant Health Inspection Service of the Department of Agriculture, United States considered alfalfa weevil as the most serious alfalfa pest in the United States (USDA 1991), costing farmers from $400 million to $1 billion a year by lowering both alfalfa yields and the nutritional value of the hay. Sixty percent of the pest damage to alfalfa has been assigned to this insect (Behdad 1989), which is a serious early-season pest of alfalfa, causing the loss of most of the first cutting yield of forage (Mazahery-Laghab and Yazdi-Samadi 1994, Mazahery-Laghab 1991; Behdad 1989). It is found throughout the United States, Canada, and most Asian and European countries. Heavily infested fields can experience substantial yield reductions and lower forage quality (Roda *et al.*, 1996). *Hypera postica* Gyll. is oligophagous, feeding almost exclusively on plants of the genus *Medicago,* only occasionally using a few species of the related genera, *Melilotus*, *Trifolium*, and *Trigonella* (Hsiao 1969; Hsiao and Hsiao 1974a).

In most areas, alfalfa weevil has one generation per year and over-winters as an adult. The eggs are deposited inside the stems of alfalfa plants. In early spring when stimulated by increasing temperature, the larvae emerge from the eggs, then from the stem, and move to the top of the plant to feed on the buds while hiding between sprouts (Manglitz and Ratcliffe 1988). *Hypera postica* gyll. feeds on the alfalfa shoots in all of the larval stages, but newly emerged adults can also cause considerable feeding damage (Barnes *et al.*, 1969). However, the majority of the injury is caused by the third and fourth instars. More than 50 larvae in different ages per plant can destroy nearly all of the leaves. The most important consequence of alfalfa weevil larval injury is consumption of leaf tissue and subsequenced first growth yield reductions. 17.1% yield reduction (losses) over the total growing season have been observed (Hanson *et al.*, 1988). Wilson *et al.* (1979) found that seasonal yield loss was 6398 kg ha^{-1} when alfalfa was infested with 7.5 larvae per stem.

1.3.2. Cowpea Seed Weevil (*Callosobruchus maculatus Fab.* Fab.)

Cowpea seed weevil (*Callosobruchus maculatus Fab.: Bruchidae*) is a major insect pest of the cowpea, *Vigna unguiculata* (the main host of the beetle), mungbean, *Vigna radiata* (L.), pigeon pea, *Cajanus cajan* (L.), and other pulses, which are important world-wide as grain legumes. It is found in Africa and Asia both on field

crops and in stores. *C. maculatus Fab.* is the most serious pest attacking food legume seeds during storage, reducing seed quality and affecting germination. (Hill 1983; Peng, 1990; Visarathononth *et al.*, 1990; Singh and Singh 1990). The adult is a small brownish beetle, which usually lays eggs onto the seed shell, or onto the outside of desiccating pods; each female lays up to 90 eggs. The hatched larvae bore into the pea or bean, where they spend the entire larval period (approx. 20 days). Pupation takes place in a chamber just under the testa of the seed, this being known as the 'window' stage; pupation takes about seven days to complete (Hill 1983). The adult then pushes its way out of the pupation chamber to complete the life cycle. Infestations of cowpea seed weevil usually originate from farm stores, but the adult beetles can fly for up to about half a mile so that infestation can be transferred from store to field. If the infested pods are then harvested and taken into the farm stores further infestation takes place.

Bruchids such as *C. maculatus Fab.* have obvious economic importance because they develop on grain legumes and cause serious post-harvest damage. However, they are a typical of the family as a whole, where the insect pests are usually very restricted in their host range, or monophagous. Many potential host legume species have evolved to produce antimetabolites such as alkaloids, non-protein amino acids, and saponins in their seeds and have thus acquired resistance to insect attack. Specific bruchid species have evolved to be able to overcome the plant defences, and thus there is a close relationship between the host, which is able to defend itself against most insect predators, and a specific bruchid species, which has evolved to overcome the specific defence mechanisms used by the host (Gatehouse 1991; Kitamura *et al.*, 1990). Such interactions are typical of "wild" plant species; selection of species for crops has generally eliminated those plants, which contain toxic compounds.

1.3.3. Glasshouse Potato Aphid (*Aulacorthum solani* Kalt.):

The main host for this oligophagous pest is potato and other *Solanaceae*, alternatives are sugar beet, beans, etc. Its primary host is foxglove (*Digitalis sp.*). The distribution of potato aphid includes Europe, Japan, New Zealand, Kenya, Peru, USA, and Canada. Similarly to most aphids, glasshouse potato aphid feeds on phloem

sap, removing water and nutrients. In addition, aphids acquire viruses from infected plants and pass them on, thus acting as virus vectors. Yield losses due to virus diseases are far more serious than those caused directly by the feeding of the aphids, although even these losses may be substantial. The glasshouse potato aphid is a medium-sized aphid, shiny, yellow-green or brownish and occurs on a wide variety of hosts in glasshouses throughout the year; it can also overwinter outdoors (Gratwick 1992). It is often found on potatoes in the field but is rarely numerous. It also lives on chitting potatoes during the winter. The aphid breeds on the sprouts of seed potatoes in store and weakens the shoots. It also acts to spread virus to healthy tubers, which are very susceptible to infection at this stage. Aphids may infest the tubers before they are brought into store or when stores are left open during the autumn. Very heavy infestations of aphids can seriously damage the haulm and reduce the yield of main crop potatoes.

1.4. Mechanisms of Plant Resistance to Pests and Pathogens

Plant resistance towards insect predators may be based on physical defences, such as thorns, stinging hairs, and tough, leathery leaves, on the production of a high yield of foliage to compensate for the damage caused by pests, or, most commonly, on the presence of specific chemicals associated with defence against pest and pathogens (Taiz and Zeiger 1991). These chemical have been considered to be "secondary metabolites", but are now often referred to as defensive chemicals. They can be found as either constitutive components in various plant tissues or are synthesised in response to attacking pests or pathogens. They include such complex substances as antibiotics, alkaloids, and terpenes, as well as "primary metabolites" such as enzymes, enzyme inhibitors, and lectins (Ryan 1990). For instance, biologically active substances such as saponins, toxic amino acids, phenolics and quaternary amino bases have been reported in alfalfa (Gorski *et al.*, 1991). Defensive chemicals can operate by a number of mechanisms; they can be toxins, antifeedants, or can prevent the insect from recognising the plant tissue as a suitable food source or substrate for oviposition (Taiz and Zeiger 1991; Gatehouse 1991).

Many plants intended for human consumption (including legumes) contain secondary metabolites; however, the protective effects of these compounds against

pests and diseases are rarely sufficient to allow their growth under agricultural conditions without additional protection (usually exogenous). In contrast, some herbaceous weeds produce secondary metabolites, which offer extremely effective protection to the plant (Dawson *et al.*, 1989). Plants also have the capacity to synthesise and accumulate biologically active, low molecular weight compounds (phytoalexins) when exposed to micro-organisms, nematodes or insects (Fenwick *et al.*, 1991). Consequently, in many cases plant protection is a result of the presence of both pre-formed secondary metabolites and phytoalexins. Thus, such compounds are present in the harvest of plant materials for human and animal consumption.

Secondary metabolites are often the products of complex biosynthetic pathways, and possess chemical structures quite different from the primary metabolites from which they are produced. Secondary products are produced by only some species of a genus, and are not distributed evenly throughout the plant, either quantitatively or qualitatively, in space and time. The production of secondary metabolites can also be affected by environmental factors, and in general the levels of these compounds increase when plants are under environmental stress (Swain 1997; Wyman-Simpson *et al.*, 1991).

1.5. Saponins

Saponins, which derive their name from their ability to form soap-like foams in aqueous solutions, are a diverse and chemically complex group of compounds which occur naturally in plants, and to a lesser extent in marine animals like starfish, sea-cucumber and snake venom (Fenwick *et al.*, 1991; Ireland 1987). They are glycosylated steroidal or triterpenoid compounds, and are common plant secondary metabolites, occurring in over 100 families. They are frequently found in leguminous species such as alfalfa. Many saponins have pronounced antifungal properties, and it is possible that they act as preformed determinants of resistance to attack by fungi (Bowyer *et al.*, 1995). Saponins generally occur as complex mixtures, varying in nature and also in amount (Fenwick *et al.*, 1991; Bowyer *et al.*, 1995). In cultivated crops, the triterpenoid saponin group is predominant, although steroidal saponins are commonly found in plants used as herbs or for their health-giving properties

The main crops containing saponins are listed in Table 1 (FAO 1985; Fenwick

et al., 1991). This table includes individual saponin-containing crops in 1984 taken from the production yearbook of the FAO. First, consideration is given to plants used as human foods; second, to those species cultivated as animal feeding-stuffs or, to plants which may be consumed by animals; and third, to plants which presently find favour as herbs, health foods, tonics, etc. The aglycones are described by Fenwick *et al.* (1991).

Table 1.1 Saponins in plants used as human foods

Plant used as:	**Plant source of saponins**	**Type of aglycones**
Human foods	*Glycine max*	Triterpenoid
	Phaseolus sp.	Triterpenoid
	Other peas and beans	Triterpenoid
	Avena sativa	Steroid
	Solanum species	Steroid
	Lycopersicon esculentum (seeds)	Steroid
	Allium species	Triterpenoid
	Asparagus officinalis	Steroid
	Camellia sinensis	Triterpenoid
	Arachis hypogaea	Triterpenoid
	Spinacia oleracea	Triterpenoid
	Cucurbita sp.	Steroid
	Beta vulgaris	Triterpenoid
	Dioscorea sp.	Steroid

Table 1.2 Saponins in plants used as animal feeding stuffs

Plant used as:	**Plant source of saponins**	**Type of aglycones**
Animal feeding stuffs	*Medicago sativa*	Triterpenoid
	Forage and cover crops	Triterpenoid
	Helianthus annuus	Triterpenoid
	Aesculus hippocastanum	Triterpenoid
	Cyamopsis tetragonolobus	Triterpenoid
	Lupinus sp.	Triterpenoid

Table 1.3 Saponins in plants used as flavouring, health, tonics, etc

Plant used as:	**Plant source of saponins**	**Type of aglycones**
Flavouring, health foods, tonics, etc	*Trigonella foenum-graecum*	Steroid
	Glycyrrhiza glabra	Triterpenoid
	Myristica fragrans	Triterpenoid
	Quillagia	Triterpenoid
	Saponaria sp.	Triterpenoid
	Yucca sp.	Steroid
	Gypsophila sp.	Triterpenoid
	Edible seeds	Triterpenoid
	Panax sp.	Triterpenoid

1.5.1. Biological Properties of Saponins

Saponins have been found to have a variety of biological properties. These include anti-flammatory, hypocholesterolemic, contraceptive, foaming, haemolysis of red blood cells, chick growth inhibition, depression of egg production, seed germination inhibition, insecticidal, antifungal, and allelopathic activity (Gestetner *et al.*, 1970; Jain and Tripathi, 1991; Small *et al.*, 1990). A further special effect acts on grazing animals, causing distension by retained gas (bloat); this causes considerable pain and even some times death of the affected animals. The reduction of transmural potential difference in mammalian small intestine has also been reported (Burnouf-Radosevich 1986; Livingston *et al.*., 1977; Gorski *et al.*, 1991; Oleszek *et al.*, 1994). These properties are useful in medicine, agriculture, and industry. Biologically active saponins are made up of an aglycone (known as a sapogenin) linked to one or more oligosaccharide moieties to form a glycoside.

Since saponins may have a deleterious physiological influence on agriculturally important animals, it has been considered beneficial to develop low saponin-containing varieties of alfalfa. From a pharmaceutical point of view, however, it is conceivable that a high saponin producing variety might be desirable, illustrating the general point that secondary metabolites like saponins can have both deleterious and beneficial effects in crop plants. On one hand they can be harmful for ruminants or because they have an allelopathic effect on other crops; on the other hand they can be useful because of their effects against some insect pests or diseases.

In alfalfa, selection for particular secondary metabolites has been carried out with regard to particular targets. Successful selection for low saponins has led to the release of new varieties with reduced saponin content coupled with high dry matter yield (Fenwick *et al.*, 1991; Price *et al.*, 1987). Successful selection programs for alfalfa lines, high or low in saponins, will depend on the development of a rapid method of screening by determining saponin content of individual plant selections (Zimmer *et al.*, 1967). Selection for low saponin genotypes has been conducted by applying various biological tests, such as the inhibition of the growth of the fungus *Trichoderma viride*, the insect *Tenebrio molitor*, the fish *Lebistes reticulatus* or through the haemolytic test. By using such biological tests, new cultivars with good agronomic traits, coupled to high quality requirements for animal feeding have been

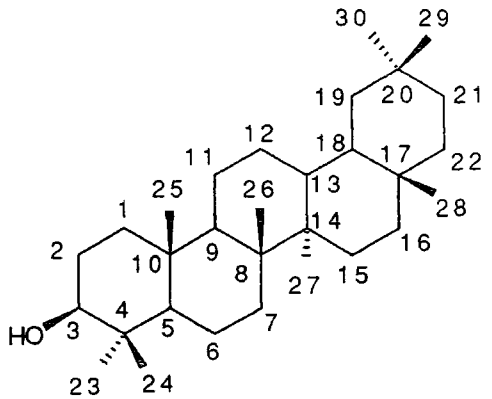
realized (Zimmer *et al.*, 1967; Pedersen *et al.*, 1976). The reduced content of saponins in the new varieties is usually ascribed to a reduction of biologically active fraction of total saponins (Dawson *et al.*, 1989).

Alfalfa breeders are justified in breeding for low saponin content in alfalfa cultivars without undue concern about detrimental effects on pest resistance. However, since saponins have a capacity to affect the biological activity of other organisms, breeders will need to continually check their low saponin selections for resistance to a broad array of pests. This will be a safeguard to avoid the unintentional development of cultivars with extreme susceptibility to the same pest (Pedersen *et al.*, 1976).

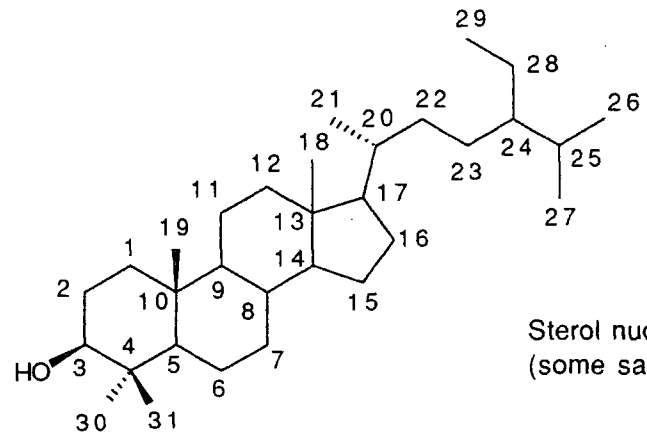
1.5.2. Chemical Properties of Saponins

Saponins are glycosides which consist of a polycyclic ring system, either steroid with 27 carbons or triterpenoid with 30 carbons, termed the aglycone or sapogenin, which is linked to one or more carbohydrate moieties (Fig. 1.1) (Ireland 1987). Aglycones are generally linked to D-galactose (gal), L-arabinose (ara), L-rhamnose (rha), D-glucose (glc), D-xylose (xyl), D-mannose (man) and D-glucuronic acid (glcUA), some of which may be acetylated (Fig. 1.2). Chain lengths of 2-5 saccharide units are most frequent. The oligosaccharide chains are generally linear, although the branching is possible (Fenwick *et al.*, 1991). In cultivated crops, the triterpenoid saponin group is predominant, although steroidal saponins are commonly found in plants used as herbs or for their health-giving properties (see Tables 1-1, 1-2, and 1-3).

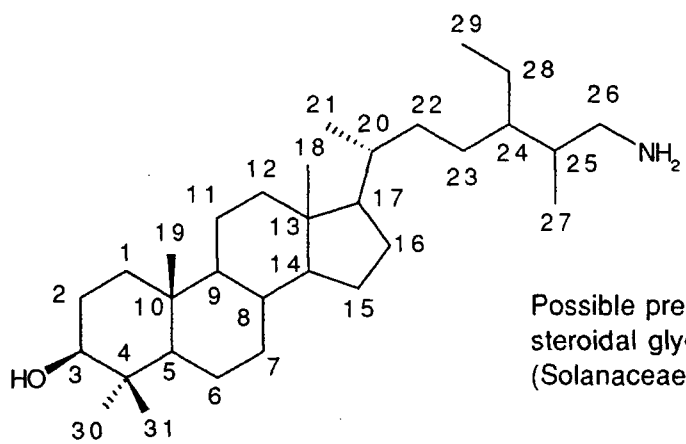
Saponins possess amphipathic properties, which are derived from their two-part compositions, the hydrophobic aglycone and hydrophilic saccharide. For this reason they have surfactant properties, such as their ability to lower surface tension and form foams, and also their ability to lyse red blood cells (which provides a useful way to identifying and locating saponins). The linkage between an aglycone and a single oligosaccharide group makes saponins, which are more amphipathic than saponins with a linkage between an aglycone and two or more oligosaccharide groups. Therefore the former saponins tend to have higher haemolytic and other biological activities (Small *et al.*, 1990). Other than this property, the biological



C-30 triterpenoid skeleton
(most saponins)



Sterol nucleus
(some saponins)



Possible precursor of
steroidal glycoalkaloids
(Solanaceae)

Fig. 1.1 Structures of the main carbon skeletons (sapogenins) present in the two major types of plant saponins, and in the closely related steroidal alkaloid glycosides. All three types also contain one or more carbohydrate chains; the hydroxyl group on C-3 is usually glycosylated.

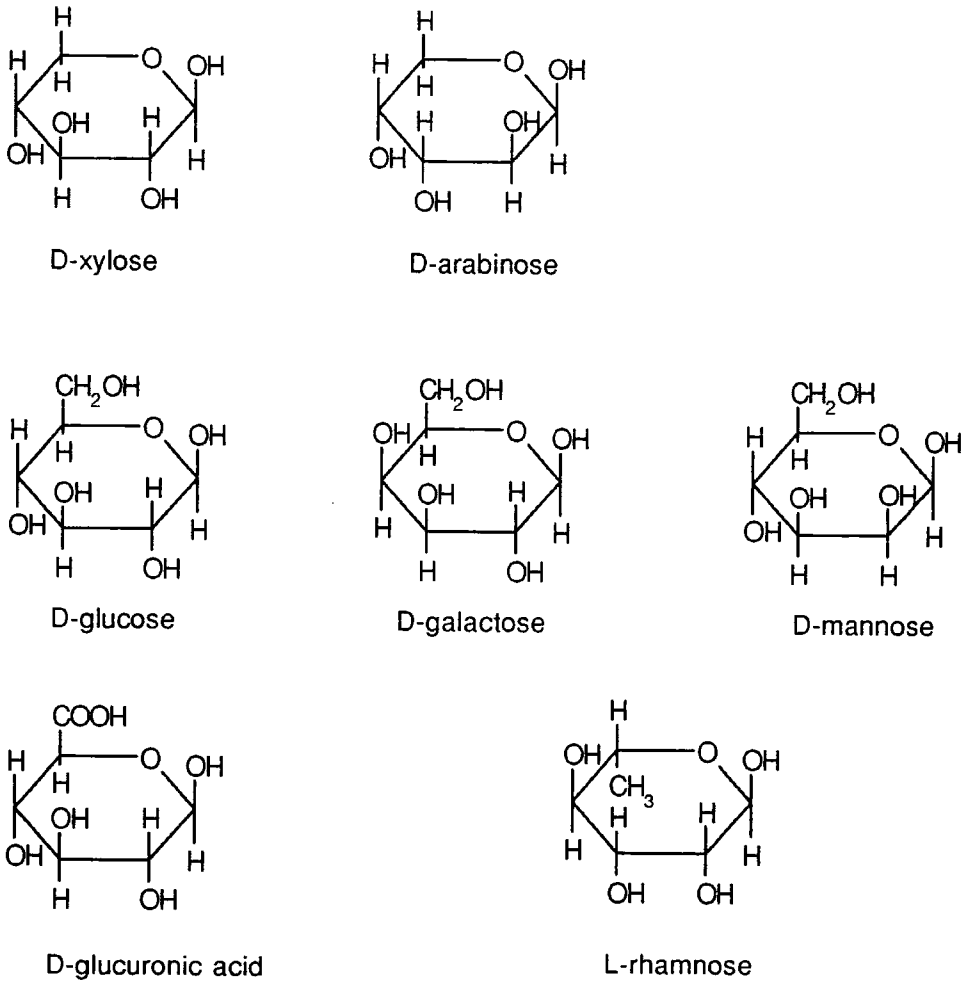


Fig. 1.2 Monosaccharide sugars found in saponins. β-pyranose forms are shown.

activity of saponins is not only dependent on the structure of lipophilic aglycone, but also on the sugar composition. The spatial conformation is also an important feature (Oleszek *et al.*, 1994).

Triterpenoid saponins are divided into two types, according to the attachment of sugar at the specific carbons in triterpene ring system. Those glycosides which have a single chain attached at C-3 of the aglycone are called monodesmosidic glycosides. These types have shown biological activity. When a second sugar chain is attached at C-28, the glycoside is called a bisdesmoside. These saponins have not shown any biological activity (Domon *et al.*, 1984). Some of the saponins have more than two glycosidic side chains. For example, a type of zanhic acid glycoside which has three sugar moieties and no free carboxyl group is called tridesmosidic glycoside. Most of the works on saponins have shown that monodesmosidic glycosides have more biological activity than bisdesmosides or tridesmosides, e.g. against noctuid larvae (*Spilosoma obliqua*) (Jain and Tripathi 1991).

1.5.3. Biosynthesis of Saponins

The synthesis of secondary plant materials is catalysed by a series of enzymes, which are products of numerous genes (Dawson *et al.*, 1989). Little data on the biosynthesis of pentacyclic triterpene glycosides are available. The aglycone moiety is the first synthesized, then glycosylated at C-3 (Peri *et al.*, 1979). These compounds are characterized by a relatively complex structure containing in some cases more than 10 sugar residues, often in the form of branched chains (Wojciechowski 1975). Triterpenoids and steroids are built up of six isoprene units (originating from mevalonic acid) and have a common biosynthetic origin in that they are all derived from squalene, presumably via ring opening of squalene-2,3-epoxide (oxidosqualene), followed by a concerted cyclization. The corresponding cyclases have been characterized (Abe *et al.*, 1993). While the true triterpenes have 30 carbon atoms, the steroids have only 27 carbons by virtue of the oxidative cleavage of three methyl groups from a C_{30} intermediate (Hostettmann and Marston 1995).

The biosynthesis of sapogenins as the aglycones, especially medicagenic acid from [2-^{14}C] acetate was investigated in germinating seeds of alfalfa (*Medicago sativa*) by Nowacki *et al.* (1976). During the first 10 days, the highest radioactivity

was found in medicagenic acid and not in the other sapogenins. It was suggested that medicagenic acid is the first synthesized sapogenin in germinating seeds, and the other sapogenins are formed from medicagenic acid.

The biosynthesis of sapogenins from [2-^{14}C] mevalonic acid was investigated in alfalfa and soya by Peri *et al.* (1979). In order to follow the biosynthesis of saponins *in vivo,* alfalfa and soybean seeds were incubated with radioactive mevalonic acid. Labelled saponins and respective sapogenin could be detected by chromatography, scanning for labelled sapogenins, and autoradiography. In the soya plant the radioactive mevalonic acid was readily incorporated into the sapogenin fraction of 5 and 10 day old soya seedlings. The formation of labelled sapogenins again supports the validity of scheme whereby mevalonic acid serves as the source of five carbon atoms to form the isoprenoid units and, via squalene, the sterol or triterpenoid structure.

Certain plants are capable of incorporating a nitrogen atom from arginine into the side chain of the steroid moiety, resulting in the nucleus of steroidal alkaloids. It is possible that this involves direct replacement of the hydroxyl group in 26-hydroxylcholesterol by an amino function but this has not been confirmed (Hostettmann and Marston 1995). The glycoalkaloids of the Solanaceae are thus closely related to saponins in structures and functional properties. Glycosylation of solanidine has been found to be catalysed by crude enzyme preparations from potato sprouts and tubers, while tomatine synthesis and accumulation in the tomato occurs mainly in the leaves and roots

1.5.4. Inheritance of saponins

Crosses of alfalfa lines were made in a genetical study in Bolivia, where the lines included bitter, saponin-rich and sweet, saponin-free plants. A 3:1 ratio of bitter to sweet character was found in the F_2 generation, implying that bitterness (and hence, high saponin content) was determined by a single dominant gene (Galwey *et al.*, 1990). The occurrence of semi-sweet varieties of quinoa however, suggests that the amount of saponin is polygenically controlled in some species (Fenwick *et al.*, 1991). Heritability for saponin content in alfalfa is rather high (Stuteville and Skinner 1987).

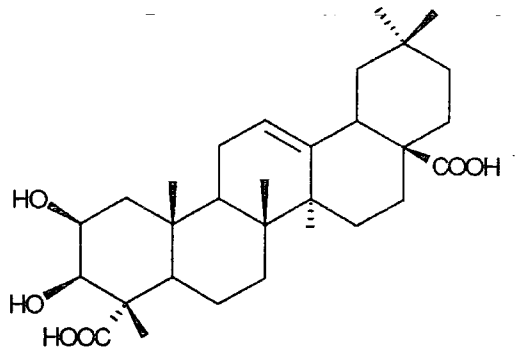
1.6. Alfalfa saponins

Alfalfa contains a complex mixture of saponins. About 30 different saponins have been found in tissues of alfalfa including root, shoot, flowers, seeds and callus and also, in cell suspension cultures. Chemical fractionation and TLC procedures revealed 33 saponins in cv. Du Puits and 27 in Lahontan. (Gestetner *et al.*, 1970; Price *et al.*, 1987; Tava *et al.*, 1993). A great deal of attention has been paid to the saponins in alfalfa, primarily because of their anti-nutritional and other effects on the physiology of higher animals. Their detrimental effects on ruminants, poultry, mice, rats, insects, fungi, and germinating seeds have been reported (Gestetner *et al.*, 1965; Su *et al.*, 1972; Pedersen *et al.*, 1976).

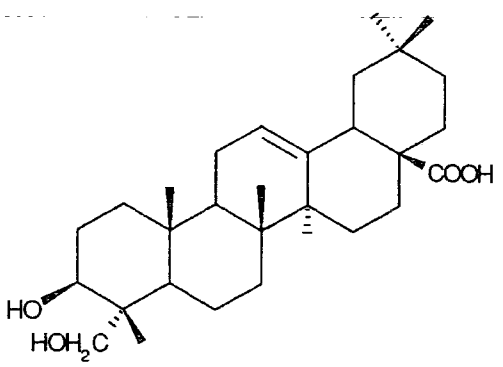
In chemical terms, alfalfa saponins are triterpenes linked to one, two or three sugar groups, and occur as a mixture of glycosides of medicagenic acid, zanhic acid, hederagenin, and soyasapogenin. Characterisation of these compounds took place following the isolation of a novel sapogenol from dehydrated alfalfa meals in 1957. This compound was identified as 2,3 dihydroxyolean-12-en-23,28-dioic acid (medicagenic acid). A further product, lucernic acid, isolated in same manner, does not appear to have been investigated further. It was later found that the toxicity of alfalfa was associated with a saponin fraction precipitated by sterols, including cholesterol; hydrolysis of these saponins revealed the presence of medicagenic acid and a related compound, identified as 3,23-dihydroxyolean-12-en-28-oic acid, known as hederagenin (Price *et al.*, 1987). Further investigation of alfalfa sapogenol fractions by MS revealed 8 aglycones, including soyasapogenols A and B, medicagenic acid,

---

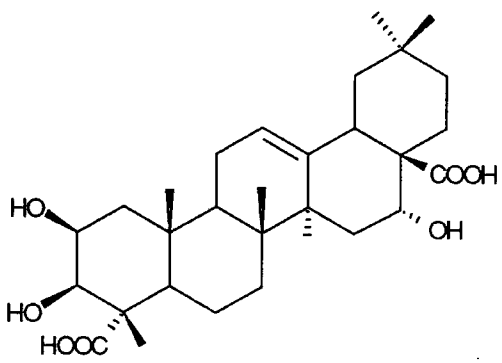
Fig. 1.3. Sapogenins of alfalfa. Codes in brackets indicate tissues in which these sapogenins are present; l = leaf; r = root; s = seed. Adapted from Massiot *et al.* (1988a).



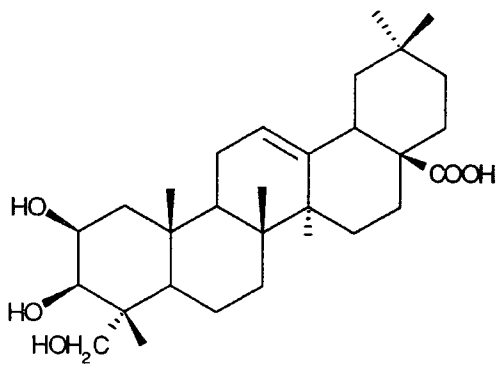
medicagenic acid (r,l)



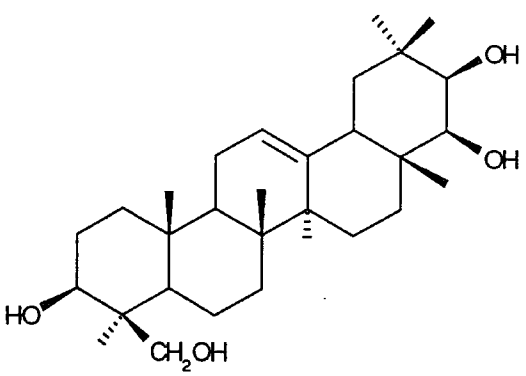
hederagenin (r,l)



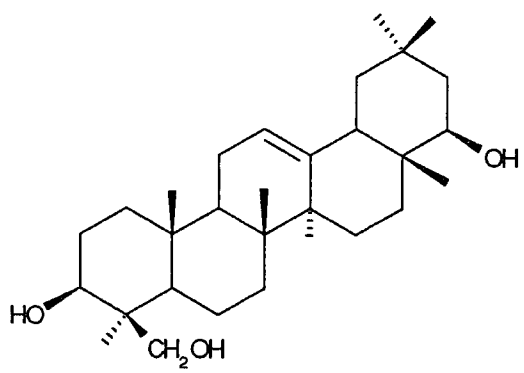
zanhic acid (l)



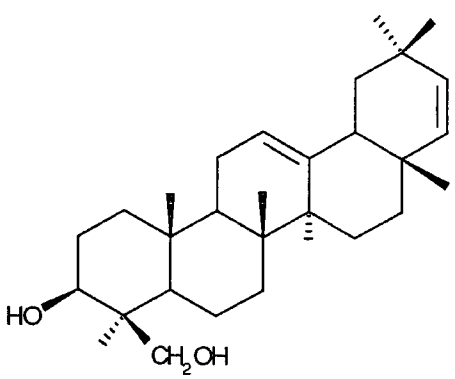
bayogenin (r,l)



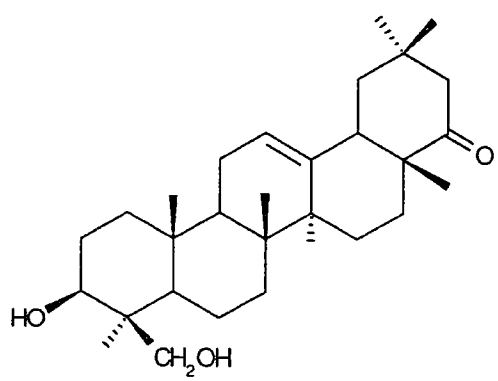
soyasapogenol A (r,l)



soyasapogenol B (l,s)



soyasapogenol C (r,s)



soyasapogenol E (r)

hederagenin, and lucernic acid (Berrang *et al.*, 1974); these sapogenins are shown in Fig. 1.3, with the exception of lucernic acid, which is probably a hydrolysis artefact (Massiot *et al..*, 1988a). Timbekova and Abubakirov (1984) have detected 13 sapogenins in alfalfa possessing the pentacyclic triterpene aglycone.

Medicagenic acid is the most abundant sapogenin in alfalfa, and its glycosides are present in leaves and roots. Hederagenin glycosides accumulate predominantly in alfalfa root. These saponins have shown a limited biological activity against some micro-organisms, insect pests and diseases.(Massiot *et al.*, 1988a; Oleszek *et al.*, 1994). Soyasapogenol glycosides occur both in the root and shoot tissues. Again, these saponins are not biologically very active; their biological activity *in vitro* is at least 10-fold less than the most active saponins. Zanhic acid has been found predominantly in the aerial parts of alfalfa as a tridesmosidic glycoside with a low biological activity (Nowacka and Oleszek 1992).

1.6.1. Insecticidal Activities of Alfalfa Saponins

Alfalfa saponins have been shown to be toxic compounds to many insects. The toxic effects of saponins are attributed to their ability to form complexes with membrane sterols, resulting in loss of membrane integrity (Bowyer *et al.*, 1995). Investigations of the interactions between saponin-containing plants and insects, fungi, and micro-organisms has led to the conclusion that alfalfa saponins are more active toxins than soybean saponins (Applebaum and Birk 1979, Birk and Peri 1980, Fenwick *et al.*, 1991). It has been concluded that the saponins in soybeans act as a defence against the bruchid beetle *Callosobruchus chinensis* L., because larvae were not able to hydrolyse saponins (Applebaum 1965). Soya bean saponins were found to be highly toxic to the rice weevil, *Sitophilus oryza;* wheat, dusted with 300 ppm of soybean saponins was protected from damage by this pest (Pedersen *et al.*, 1976). The saponins in alfalfa were also able to inhibit or reduce damage by insects, because of the presence of medicagenic acid glycosides, which are found both in the root and shoot, or soyasapogenol, and hederagenin glycosides found in the root; alfalfa root saponins, which are rich in medicagenic acid derivatives were markedly toxic to the flour beetle (*Tribolium castaneum*) (Fenwick *et al.*, 1991). It has been shown that alfalfa root saponins are stronger haemolytic agents and are more toxic to *Tribolium*

castaneum larvae than foliage saponins (Gestetner *et al.*, 1970).

Selection for high or low saponin concentration in alfalfa using the fungal pathogen *Trichoderma* also affects resistance to insect pests. Selection for high saponin content in alfalfa increased resistance to the pea aphid (*Acyrthosiphus pisum* Harris) while selection for low saponins content decreased resistance. Resistance to spotted alfalfa aphid (*Trioaphis maculata*) was not affected by these selections (Pedersen *et al.*, 1976). Massiot *et al.* (1991) compared two cultivars of alfalfa, Resis as the saponin-rich variety and Lahontan as the saponin-poor variety, and showed that their acceptance when fed to the yellow mealworm *Tenebrio molitor* was inversely proportional to the saponin and medicagenic acid levels . Also, Pederson *et al.* reported that the resistance of alfalfa to the aphid *Macropysum pisi* was dependent on the high concentration of saponins in resistant cultivars (Singh 1986).

It is unclear whether the adverse effect of alfalfa saponins on insect growth and survival is due to a toxic effect as antibiosis, or simply a result of saponin acting as feeding deterrents, hence leading to growth inhibition and death by starvation. Indeed, the role of saponins in prevention of the insect attack may differ from insect to insect, and it has been suggested that alfalfa saponins may play a more crucial role in preventing attack by polyphagous insects compared to oligophagous insects, which have found a means of overcoming any toxic effect.

1.6.2. Allelopathy of Alfalfa Saponins

Alfalfa and other saponin-containing plant also show allelopathic activity towards other crops or weeds, and inhibit their growth or the germination of seeds. Saponins leach into the soil from the epidermal cells of root and cause a major reduction in the yield of the crops. The allelopathic effects of *Medicago lupulina, M. media* and red clover were investigated, and it was concluded that alfalfa has the greatest allelopathic effects, which was attributed to the differences in saponin contents (Levy *et al.*, 1989a and 1989b). Alfalfa saponins are known to inhibit the germination of cottonseeds, exerting their action by interfering with oxygen uptake through the seed coat and seed membrane. The residues of alfalfa saponins have been found in soil upon which alfalfa has previously been grown, and the soil inhibited the germination of cottonseeds. These effects can be attributed to the surface activity of

saponins (Shany *et al.*, 1970; Ireland 1987).

Some work has been carried out on the allelopathic activities of individual saponins. The allelopathic activity of alfalfa saponins can be attributed mostly to the presence of medicagenic acid glucoside. This saponin was used in petri dish tests to determine the allelopathic potential towards wheat. Root growth was inhibited by 50% when medicagenic acid glucoside was present in the growth medium at a concentration of 100 ppm. Wheat shoots exposed to this concentration of saponin also suffered some retardation, however to a much lesser degree than roots. A higher concentration of medicagenic acid glucoside also inhibited wheat germination severely (Oleszek *et al.*, 1992).

1.6.3. Variation of Saponin Content in Alfalfa

Analysis of saponins can be carried out by a variety of methods; thin layer chromatography (TLC) is a good method for qualitative analysis, but is difficult to use quantitatively. High Performance Liquid Chromatography (HPLC) has been used by some researchers for analysis of biologically active compounds in alfalfa (Oleszek and Jurzysta 1990). This method has also been employed for the separation and quantitation of the both aglycones and intact saponins (Fenwick *et al.*, 1991).

A number of factors have been shown to affect saponin content in alfalfa. In addition to the plant species, the genetic origin, part of the plant examined, its physiological age and state are all important factors in determining saponin content, as are the environmental and agronomic factors associated with growth of the plant, and post harvest treatments, including storage and processing. Cooking and silage for fermentation process have been shown to reduce saponin levels in soybean and alfalfa. Evidence in variation in saponin content has also found in lentils, mothbean, alfalfa, and quinoa (Fenwick *et al.*, 1991 and Tava *et al.*, 1993). The quantity of saponins is different in various cultivars. For example, cultivar Resis is described as a saponin-rich variety, whereas cultivar Lahontan is saponin-poor; the amounts of saponins for the two cultivars were 14 and 21 g/kg in Lahontan and Resis respectively (Massiot *et al.*, 1991). It has been found that different parts of the plant contain different amount of saponins. For example, the saponin content of alfalfa seed was 1.3 g/kg dry matter, the average for the leaf protein concentrates was 2 g/kg and the content in the root was 30 g/kg (Massiot *et al.*, 1988a). Fractionation of alfalfa

saponins has shown a different yield of saponins in the root and shoot (Gestetner *et al.*, 1970). Two cultivars can have different amounts of saponins in specific tissues. As reported by Tava *et al.* (1993), saponin accumulation in the Equipe cultivar was less than Dupuits, but the amounts of saponins were approximately the same in their roots. The two cultivars were significantly different in the amount of saponins in the aerial parts. Saponins and sapogenins were quantified in the two different cultivars as shown in Table 1-4.

A classification of alfalfa varieties can be made, based on levels of saponins. Alfalfa varieties with saponin levels less than 0.3% are termed sweet varieties, whereas lines with saponin levels more than 1.5% are known as bitter, or high-saponin lines.

Saponin content also varies with plant development. Massiot *et al.* (1988a and b) indicated that the yields of saponins are highly dependent on the time of collection. For instance, different cuttings of alfalfa have different amounts of saponins. The first cutting on average exhibited a lower saponin content than the second or third. Over three successive harvests, saponin content was positively correlated with protein, ash, fat and nitrogen-free extract and negatively correlated with crude fibre and hay yield. The average saponin contents of leaves were found to be twice those of stems and a significant reduction occurred in the saponin contents of older plants (Fenwick *et al.*,

Table 1-4 Saponin and sapogenin content in the Shoot and Root from alfalfa cv Equipe and DuPuits

Cultivars	Equipe		Dupuits	
Plant parts	Shoot	Root	Shoot	Root
Saponins (% dry Matter)	1.64±0.09	3.75±0.27	2.37±0.15	3.67±0.12
Hederagenin (mg/g dry matter)	0.03	1.32	0.03	0.82
Medicagenic acid (mg/g dry matter)	0.55	5.22	1.25	4.43
Soyasapogenol B (mg/g dry matter)	0.01	0.06	0.01	0.02
Soyasapogenols C, D, E & F (mg/g dry matter)	0.14	1.43	0.26	0.72

1991). Groski *et al.* (1991) found that during the first eight days, saponin concentration rose from zero in the alfalfa seeds to 8.7% in the roots and 1.8% in the shoots, and then decreased slowly to 7.6% in root and 0.8% in shoots present on the 24th day.

Particular saponins may be found in the different parts of plant. As Tani *et al.* (1985) reported, the majority of soyasaponins in soybean seeds are located in the plumule, hypocotyl and radicle, those parts that develop into the mature plant. Aerial parts were richer in saponins than seed hypocotyl, which was in turn richer than stem, branch and petiole. In the underground parts the soyasaponin B level decreased in the order root hair > lateral > main root (Shimoyamada *et al.*, 1990). Massiot *et al.* (1988a) studied saponins and prosapogenins from alfalfa and reported that soyasapogenol A, hederagenin, bayogenin, and medicagenic acid were found both in the roots and shoots. Lucernic acid and zanhic acid were reported to be in the leaves, but soyasapogenol C and E were found in roots.

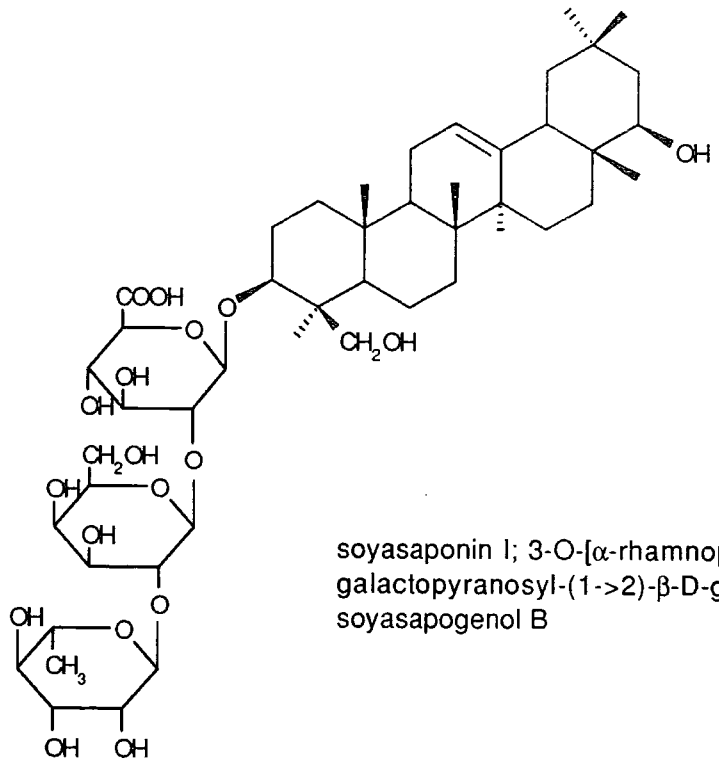
1.7. Properties of Individual Saponins

Two specific saponin types were found to be of particular significance for the work described in this thesis, and thus their properties are described in more detail.

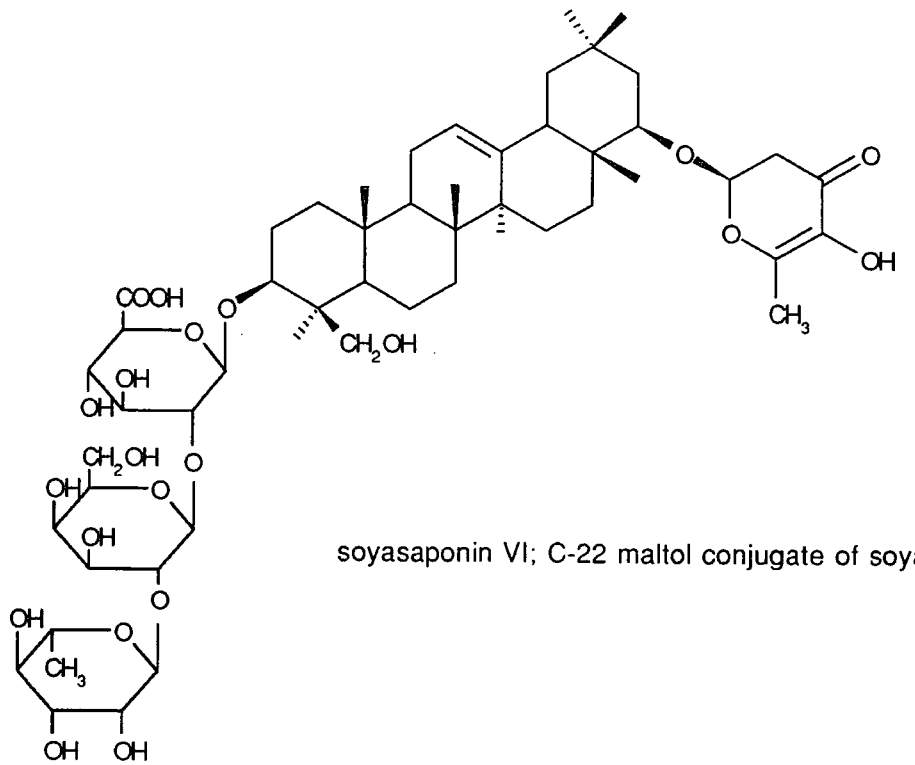
1.7.1. Soyasaponins

Soybean seeds (*Glycine max*) contain about 2% of glycosides, in the form of saponins and isoflavonoid glycosides. The aglycones present in soybean saponins are the four soyasapogenols A, B, C, D and E, which are pentacyclic triterpenoids substituted with -OH groups, differing from each other in the number and positions of substituents. These sapogenins correspond to a series of soyasaponins. Medicagenic acid is not present in soya bean, and was not detected in labelling experiments (Peri *et al.*, 1979). Unfortunately, different authors differ in their descriptions of the soyasaponins. A consensus of current opinion suggests that soyasapogenols A, B, and E are true aglycones and corresponding saponins are "group A", "group B", and "group E" saponins, while soyasapogenols C, D, and F are artefacts (Price *et al.*, 1987; Hostettmann and Marston 1995). Soyasaponins are also present in alfalfa. Massiot *et al.* (1988a) concluded that soyasapogenol B was present in both seeds and leaves of alfalfa but not in the root whereas Oleszek and Jurzysta (1986) isolated soyasapogenol B from alfalfa root tissues.

Soyasaponin I (Fig. 1.4) is a major saponin in soybean. This saponin possesses soyasapogenol B as sapogenin, linked to an oligosaccharide at C3, and was also isolated from alfalfa shoot and probably root tissues (Kitagawa *et al.*, 1976). Tsurumi *et al.* (1992) found a new triterpenoid saponin containing soyasaponin I conjugated to γ-pyrone. Soyasaponin I, known as phytochrome inhibitor, is released from this saponin during the extraction of pea seedlings. Massiot *et al.* (1992) extracted the seeds of alfalfa and isolated soyasaponin I conjugated to maltol, for which the name soyasaponin VI was proposed. This compound may be the natural precursor of soyasaponin I. Soyasaponin VI was first isolated from alfalfa sprouts, and it was thought that this substance was produced during germination. The only natural product related to soyasaponin VI previously isolated was γ-pyrone hoslundin, which is a C-maltol derivative (Ngadjui *et al.*, 1991).



soyasaponin I; 3-O-[α-rhamnopyranosyl-(1->2)-β-D-galactopyranosyl-(1->2)-β-D-glucuronopyranosyl] soyasapogenol B



soyasaponin VI; C-22 maltol conjugate of soyasaponin I

Fig. 1.4. Structures of soyasaponin I and soyasaponin VI

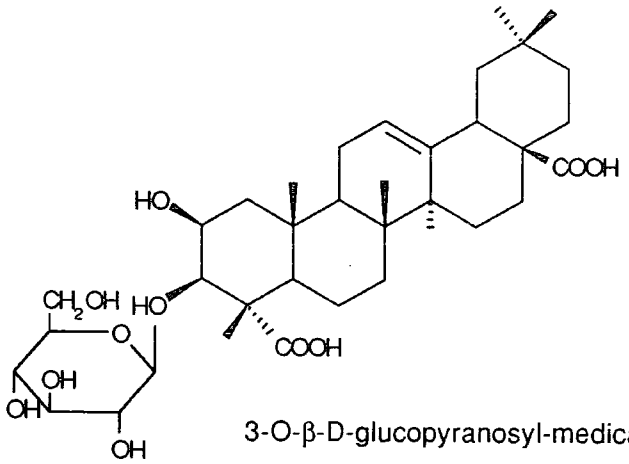
Soyasaponin I has been found in numerous legumes, such as kidney bean (*Phaseolus vulgaris*), runner bean (*P. aureus*), butter bean (*P. lunatus*), scarlet runner bean (*P. coccineus*), field bean (*Vicia faba*), lentil (*Lens culinaris*), chick pea (*Cicer arietinum*), and pea (*Pisum sativum*) (Hostettmann and Marston 1995; Gestetner 1971).

The study of biological activity of saponins in soybean demonstrated that a crude saponin extract partially inhibited the proteolytic activity of the larval midgut of *Tribolium castaneum*, and significantly inhibited trypsin and α-chymotrypsin (Baker 1978).

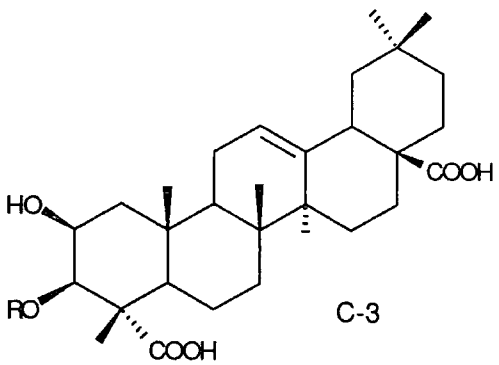
1.7.2. Medicagenic acid glycosides

The compound most commonly identified as the major saponin in alfalfa is the 3-O-glucopyranoside of medicagenic acid (Fig. 1.5), which has been separated by several groups (Morris *et al.*, 1961; Timbekova and Abubakirov 1984; Levy *et al.*, 1986; Price *et al.*, 1987; Oleszek and Jurzysta1990). Medicagenic acid and its glycosides have been implicated as the major biologically active component of alfalfa saponins. Medicagenic acid 3-O-β-D-glucopyranoside was first isolated from alfalfa root tissue, and its structure was determined by Morris *et al.* (1961). Medicagenic acid glucoside was identified as the most active antimycotic compound (towards the fungal pathogens *Trichoderma viride* and *Sclerotium rolfsii*) in alfalfa root extracts. An improved method for the isolation of this compound and the elucidation of its structure by chemical, physical, and enzymatic techniques has been reported (Levy *et al.*, 1986). Nowacki *et al.* (1976) investigated the biosynthesis of medicagenic acid from ^{14}C-acetate in germinating seeds of alfalfa and showed that when sapogenins were analysed the highest radioactivity was present in medicagenic acid, suggesting that this compound is the first saponin to be synthesised in germinating seeds. Medicagenic acid was detected and increased-level after the first 10 days of germination of seeds.

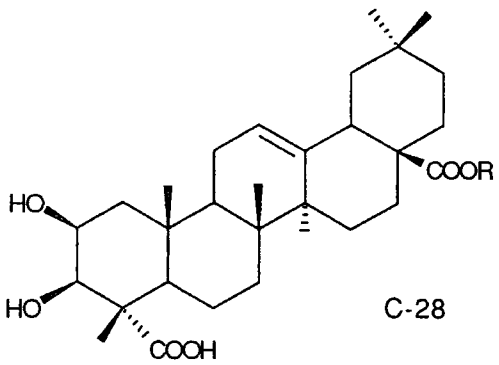
Besides the glycoside, other glycosides of medicagenic acid are present in alfalfa (Fig. 1.5). Flowers contain medicagenic acid linked to the trisaccharide Rha-GlcUA-Glc at the 3-O position (Oleszek *et al.*, 1992). Medicagenic acid was also isolated as the 3-O-glucopyranoside-(1,6)-glucopyranoside-(1,3)-glucopyranoside



3-O-β-D-glucopyranosyl-medicagenic acid

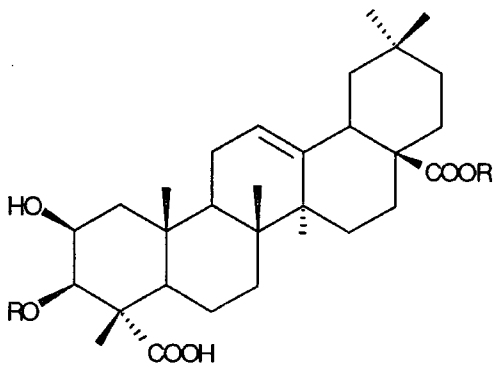


C-3



C-28

Monodesmosides of medicagenic acid; R = glycosyl-



Bisdesmosides of medicagenic acid; R = glycosyl-

Fig. 1.5. Structures of saponins based on medicagenic acid

from alfalfa tops by Gestetner (1971). Monodesmosides where the glycoside is attached by an ester linkage at the carboxylic acid groups at C-28 are also known (Massiot *et al.*., 1988b), and a series of bisdesmosides with glycosides linked to both C-3 and C-28 have also been characterised.

Glycosides of medicagenic acid have been found to possess a high level of biological activity, and to be the most toxic of the alfalfa saponins (Shany *et al.*, 1970). The mono and bisdesmosides of medicagenic acid have also shown high levels of toxicity using haemolytic, antifungal, and allelopathic bioassays, with concentrations of the compounds necessary to cause 50% inhibition, or mortality, several times lower than those of other saponins. The highest toxicity was found for the 3-O-β-D-glucopyranoside of medicagenic acid, and fungal growth was completely inhibited at a very low concentration (0.1%) of this compound (Shany *et al.*, 1970). Bisdesmosides of medicagenic acid showed lower activities, which did not fully correlate to the number of sugars attached to the aglycone (Oleszek *et al.*, 1990). When crude saponins of alfalfa were separated into cholesterol-precipitable and non-precipitable fractions, the precipitable fraction (6% of root dry matter) consisted of medicagenic acid glycosides; Glc, Ara, Xyl, and Rha were found as their sugar chain components and the mixture showed a high biological activity towards the fungal pathogen *Trichoderma viride* (Oleszek and Jurzysta 1986).

1.8. Saponin Degradative Enzymes of Insects, Fungi and Plants

The toxicity of many plant secondary compounds is dependent on whether they are glycosylated or not. For example, cyanogenic glycosides are only toxic after hydrolysis (when they release hydrogen cyanide), and plants containing these compounds also contain a specific enzyme which hydrolyses the cyanogenic glycoside when tissues are damaged (Oxtoby *et al.*, 1991). The enzyme and its substrate are normally separated in different cellular compartments. In the case of saponins, deglycosylation is thought to make these compounds less toxic, since it decreases the amphipathic nature of the molecule, and thereby prevents it from disrupting membranes (Roddick and Drysdale 1984; Osbourn 1996). Consequently, an attacking pathogen or pest can attempt to overcome the plant's biochemical defence by producing an enzyme which will hydrolyse saponins. Such detoxifying

enzymes have been identified in pathogens. Although some pathogenic fungi have intrinsic resistance to the membraneolytic action of saponins because of their membrane composition, others produce enzymes that specifically detoxify particular plant saponins (Bowyer *et al.*, 1995). Tolerant fungi are able to bring about enzymatic deglycosylation of saponins. For example, *Fusarium avenaceum*, the isolates of fungi pathogenic to oats contain an α-glucosidase active against the oat saponin avenacin, hydrolysing it to the aglycone (Crombie *et al.*, 1986).

Whereas fungal glycosidase enzymes are intended to detoxify saponins, there is some evidence that plants contain similar saponin hydrolases which are intended to make saponins more toxic. The oat saponin avenacoside is stored as the bisdesmoside, which has a relatively low membrane-disrupting capacity, and so does not pose a threat to plant cell membrane integrity. The presence of hydrolysing enzymes in plant tissues can remove one of the sugar chains and produce a monodesmosidic compound, which is probably more toxic than bisdesmosidic one (Nisius 1988; Osbourn 1996). As with cyanogenic glycosides, the hydrolase and its saponin substrate are probably separated in different cellular compartments until tissue damage occurs. The isolation of genuine saponins thus sometimes necessitates pre-treatment of plant material before subjecting it to the usual extraction and isolation procedures. For example, the saponins momordin I and momordin II have been isolated from the root of *Momordica cochinchinensis*. However, only momordin II, a bisdesmoside, is present in the living plant; it is converted to the monodesmoside momordin I by intracellular esterases present in the root during the drying process (Mahato and Nandy 1991).

1.9. Plant Protein Protease Inhibitors

Besides the use of secondary metabolites as defensive compounds in plants, a series of types of proteins are also intimately involved in protecting plants against attack by pests and pathogens. Like secondary metabolites, these proteins may either be present constitutively, or may be induced by damage to plant tissues on attack (wounding response). One of the best characterised types of plant defensive proteins are inhibitors of digestive proteases.

The presence of proteins in plants which inhibit mammalian digestive

enzymes has been known for many years, and was first confirmed after the isolation of a trypsin inhibitor from soybean, *Glycine max* (Edmonds 1994). Since then, it has become clear that protein protease inhibitors are widespread in plants (Richardson, 1977). Because of the importance of serine proteases in the mammalian digestive system, and the consequential medical and nutritional significance of their inhibitors, the focus of much of the early research was the characterisation of various plant-derived serine protease inhibitors. More recently, the effect of these inhibitors on insect proteases has been investigated, and the lack of apparent serine protease activity in some cases and the lack of inhibition in others, has led to investigation of the other type of proteases employed within the digestive system of insects and to a search for inhibitory proteins from plants.

While the activity of plant protein protease inhibitors is clear, the detailed mechanism by which these proteins are toxic to insects is not clear. They may work by direct inhibition of enzymes, reducing the breakdown of dietary protein and causing a lack of free essential amino acids; alternatively, inhibition may induce hyperproduction of proteases to compensate for the inhibited enzymes, and this enzyme synthesis, itself, depletes the available supply of essential, sulphur-containing amino-acids and leads to problems with metabolism. Either way, supplementation of the diet with methionine alone, or with other free amino acids, has been shown to reduce some of the detrimental effects of protease inihbitors in some cases (Gatehouse and Boulter, 1983; Broadway and Duffey 1986; Hines *et al.*, 1991). Broadway and Duffey (1986) speculated that high endogenous levels of methionine in plant tissues or seeds might increase their susceptibility to predatory insects.

1.9.1. Insect digestive proteases

Proteases, the enzymes that catalyse protein digestion are divided into four groups on the basis of their catalytic mechanism. Serine proteases contain an essential serine residue which participates as a nucleophile in peptide bond cleavage; similarly, cysteine proteases use an essential cysteine residue for peptide bond cleavage, aspartic or acid proteases contain an essential acidic amino acid residue in their active site centres, and metalloproteases contain an essential metal ion involved in the catalytic mechanism (Storey and Wagner 1986; Edmonds 1994; Wolfson and Murdock 1987). In higher animals, the major digestive endoproteases are serine

proteases (trypsin, chymotrypsin, elastase), and it was assumed that insect contained similar enzymes in their guts. Evidence from assays of proteolytic activity in bees and Lepidopteran larvae supported this conclusion, although little direct evidence for the nature of insect digestive proteases was available, and the digestive proteinases of relatively few insect species had been characterized (Wolfson and Murdock 1987). More recently, however, it has been realised that in many insects, especially Coleopteran species, cysteine proteinases play crucial roles in digestion (Matsumoto *et al..*, 1995; Irie *et al.*, 1996). In general, lepidopteran larvae contain serine rather than cysteine proteases; for example, Christeller *et al.* (1992) reported no occurrence of cysteine protease activity in the gut of lepidopteran larvae since inhibitors specific for cysteine proteases (E-64, cystatin and N-ethyl maleimide) were ineffective both at their usual optimal pH (pH 5.9) and at alkaline pH at normal inhibitory concentrations. On the other hand, Coleopteran larvae clearly do contain cysteine proteases. For instance, in a study of protein digestion in larvae of the bruchid beetle, *Callosobrochus maculatus Fab.*, Gatehouse *et al.* (1985a) obtained evidence that the midgut in this species contains a proteinase whose activity was not blocked by the usual serine proteinase inhibitors, but was powerfully inhibited by specific cysteine proteinase inhibitors. This work was confirmed and extended by Murdock *et al.* (1987), who showed that the enzyme had a pH optimum of 5.0, and that the cysteine protease chymopapain inhibitor from potatoes was also an effective inhibitor. Colorado potato beetle (*Lepinotarsa decemlineata*) also showed susceptibility to the inhibitor E-64, which strongly inhibited the cysteine proteinase activity in the midgut of the insect (Wolfson and Murdock 1987), whereas other protease inhibitors had no significant effect on this insect. Similarly, the protease activities in extracts from the midgut of rice weevil, *Sitophilus oryzae*, and the red flour beetle, *Tribolium castaneum* were inhibited by E-64, PCMB, antipapain, and oryzacystatin from rice seeds (Liang *et al.*, 1991). Cysteine protease activity has also been reported in Colorado potato beetle (Michaud *et al.*, 1993), in bean insect pests, such as *Callosobruchus chinensis* (Coleoptera) and *Riptortus clavatus* (Hemiptera), (Kuroda *et al..* 1996) in blood sucking bugs (Houseman 1978; Houseman and Downe 1982; Garcia *et al..*, 1978), and in two species of corn rootworms (*Diabrotica virgifera virgifera* LeConte and *D. undecimpuncata howardii* Barber) (Edmonds 1994).

In confirmation of the biochemical data showing the presence of cysteine

digestive proteases in many insects, a gene encoding a putative digestive cysteine proteinase from *Drosophila melanogaster* was isolated. The mature enzyme from the encoded protein, termed *Drosophila* cysteine proteinase-1, consisted of 218 amino acid residues. This enzyme showed significant similarity to cysteine proteinases of higher animals (cathepsin H and L) and of plant origin (rice oryzains) and was predominantly expressed in the midgut of the insect (Matsumoto *et al.*, 1995). In the work considered in the present thesis, the alfalfa pest insect used, alfalfa weevil, is a coleopteran, and thus is also likely to rely on cysteine digestive proteases.

In support of this conclusion, Elden (1995) stated that alfalfa weevil larvae use cysteine proteinases as major digestive enzymes, although other proteinases may be present as well. The nature of the alfalfa weevil digestive proteases was deduced from observations of inhibition of the development of the larvae, pupae, and adults of alfalfa weevil (*Hypera postica*) by inhibitors of cysteine proteinases like E-64, pHMB, and leupeptin, which were incorporated into a 6.5% gelatin solution and applied to the upper and lower leaf surfaces of alfalfa leaves (Elden 1995). In this study it was also reported that soybean trypsin inhibitors (serine protease inhibitors) with higher molecular weight than the cysteine protease inhibitors (E-64 and pHMB) had little or no effect towards the weevil. Chicken egg white cystatin, an inhibitor of lysosomal cysteine proteinases (Anastasi *et al.*, 1983) was the only cysteine protease inhibitor that had no deleterious effect on alfalfa weevil growth or development (Elden 1995).

1.9.2. Plant Cystatin Inhibitors

Cystatins are reversibly-binding protein inhibitors of cysteine proteases (Bode *et al.*, 1988), and are widely distributed throughout the plant and animal kingdoms. Cystatins have been shown to form equimolar complexes with cysteine proteases like papain, leaving the active side of enzyme inaccessible to substrates (Anastasi *et al.*, 1983; Lindahl *et al.*, 1988). The cystatin binds to the enzyme in a simple manner, different from the binding of the substrate, and in marked contrast to the more complicated interactions of serine protease inhibitors with their target enzymes (Bjork and Ylinenjarvi, 1990; Lindahl *et al.*, 1991).

The most extensively studied of the plant cystatins are those from rice. Two

distinct inhibitors have been isolated (Konodo *et al.*, 1990); the first, termed oryzacystatin (later oryzacystatin-I) (Abe *et al..*, 1987) is a potent inhibitor of papain and of the endogenous cysteine protease responsible for the hydrolysis of the reserve glutelin during seed germination. Although the amino acid sequence of the second inhibitor, oryzacystatin-II shows marked similarities to the sequence of oryzacystatin-I (approximately 55% identity) the two inhibitors were remarkably distinct in their enzyme specificities and the developmental patterns of expression of their mRNAs in developing seeds (Kondo *et al.* 1990). Oryzacystatin-I was found to inhibit papain (K_i 3.0 x 10^{-8} M) more effectively than cathepsin H (K_i 0.79 x 10^{-6} M), while oryzacystatin-II was more effective against Cathepsin B or L (Michaud *et al.*, 1993). Also, while the mRNA for oryzacystatin-I was expressed at a maximum level 2 weeks after flowering and could not be detected in mature seeds, the mRNA for oryzacystatin-II was expressed constantly throughout the maturation stages and was clearly detectable in mature seeds. Both inhibitors show significant homology to several of the animal cystatins (Abe *et al.*, 1987; Kondo *et al.*, 1991)

Liang *et al..* (1991) demonstrated that nearly all the proteolytic activity (demonstrated against casein) in larval midgut extracts of two stored grain pests, the rice weevil (*Sitophylus oryza*), and the red flour beetle (*Tribolium castaneum*), could be inhibited by oryzacystatin-I. If this inhibition were exerted *in vivo* as well as *in vitro*, this would then affirm the role of cystatins in protection, as well as the endogenous regulatory role previously demonstrated for both soybean cystatin and oryzacystatin (Hines *et al.*, 1991 and Kuroda *et al.*, 1996).

Recently the x-ray crystal structures of two members of the cystatin superfamily, chicken egg-white cystatin (Bode *et al.*, 1988) and stefin B in complex with an S-carboxymethylated papain (Stubbs *et al.*, 1990) have been elucidated. Both structures are composed of a long central α-helix wrapped in a 5-stranded, antiparallel β-related sheet, with a subsidary trunk of helix or strand. At one end of molecule is an exposed hairpin loop, comprising the QVVAG amino acid sequence highly conserved in the cystatins, and flanked on their side by a second hairpin-loop and the projecting amino terminal "trunk". In the enzyme:inhibitor complex, both of these exposed loops interact with subsites adjacent to the catalytic residues of the enzyme, while the trunk segment loops over the catalytic cysteine residue of the enzyme and binds to a subsite nearby, preventing the access of substrates to the

enzyme's catalytic site through pure steric hindrance (Bode and Huber 1992). This mechanism is quite different to that employed by the serine inhibitors and the potato carboxypeptidase inhibitor.

1.9.3. A strategy for Increasing the Resistance of Alfalfa towards Alfalfa Weevil

Plant proteins such as proteinase or α-amylase inhibitors, lectins, and lipoxygenases can act as defenses against herbivorous insects (Gatehouse *et al.*, 1992; Elden, 1995), and form part of the multiple mechanisms of resistance which plants exploit to defend themselves against attack by pests. However, successful insect pest species will be able to overcome these defences in order to be able to utilise the plant as foodstuff, and thus endogenous resistance in crop plants is often difficult to produce by conventional techniques.

Different conventional breeding techniques have been attempted to protect alfalfa from attack by alfalfa weevil, and relatively weevil-resistant cultivars have been produced. Phenotypic selection in the random mating populations has been effective in development of various degree of resistance to the alfalfa weevil and other alfalfa pests (Barnes *et al.*, 1969 and 1970). Resistant alfalfa cultivars, when available, provide protection from damage when temperature keeps parasites and predators inactive, or prevents the application of insecticides. They contribute to establishment and maintenance of stands and to forage yields and quality. When large acreages are involved, even the slightest resistance expressed as reduced damage or increased tolerance leads to significant savings. However, resistant cultivars are not a panacea for all pest problems. Conventional breeding approaches for introducing resistance to some insect pests such as alfalfa weevil and Lygus bug into cultivated varieties have been unsuccessful since alfalfa germplasm with a high levels of resistance to these insects has not been identified (Sorensen *et al.*, 1988). Backcross breeding takes a long time, and hybridization between two cultivars of alfalfa is not easy. Moreover, methods of plant breeding for insect resistance such as mutation breeding or protoplasm fusion have not been used to produce a successful insect-resistant crop (Gatehouse 1991). Under these circumstances, the possibility of using transgenic plants, and being able to specifically exploit a gene pool outside the limits of that available within alfalfa for insect resistance genes, becomes a desirable option. Transgenic plants, which have the necessary basic biosynthetic capacity to produce

and express the product of the transformed gene, have been used to enhance the resistance of a number of model plant species and crop plants to insects (Gatehouse 1991). Although the most common strategy to engineer insect resistance is to express the insecticidal toxins from *Bacillus thuringiensis* in transgenic plants (Perlak *et al.*, 1990), genes encoding plant defensive proteins have also been used for this purpose.

The strategy which is proposed in this thesis for control of alfalfa weevil is to produce transgenic plants expressing protease inhibitors effective against alfalfa weevil gut proteases, and thereby hinder digestion, inhibit development, and cause mortality in the larvae. A similar strategy was pioneered in experiments aiming to protect transgenic tobacco against lepidopteran larvae using inhibitors of serine proteases (Hilder *et al.*, 1987). Over-expression of several inhibitors from constitutive promoters has been shown to afford protection in transgenic tobacco plants against attack by lepidopteran larvae (Gatehouse *et al.*, 1993). Transformation of a single gene responsible for the production of a protein protease inhibitor using existing genetic engineering technology is relatively straightforward. Since alfalfa weevil seems to use mainly cysteine digestive proteases (section 1.9.1), it is most likely that the most effective inhibitor for control of this pest would be a plant cystatin

Irie *et al.* (1996) introduced a gene construct encoding corn cystatin under control of the CaMV (cauliflower mosaic virus) 35S promoter into rice, and produced transgenic plants for insect bioassays. Corn cystatin prepared from transgenic rice plants showed potent inhibitory activity against proteinases that occur in the gut of the insect pest, *Sitophilus zeamais*.

Other potential approaches for producing insect-resistant transgenic alfalfa have been considered. Thomas *et al.* (1994) expressed the anti-elastase insect protease inhibitor in transgenic alfalfa for use as an insecticide. They described the transformation and regeneration as a simple and efficient technique, which was also used for insertion of genes encoding herbicide resistance and virus resistance into alfalfa. However, the same authors stated that the use of *Bacillus thuringiensis* toxin genes for producing insect resistant alfalfa was not possible, since no toxins with significant activity against the alfalfa weevil or Lygus bug have been reported.

1.10. Aims and Objectives

The programme described in this thesis had the following aims and objectives:

(i) To investigate the insecticidal properties of saponins, specifically those from alfalfa.

(ii) To purify individual saponins from alfalfa and determine which saponins are most insecticidal.

(iii) To investigate how insects are able to overcome toxic effects of saponins.

(iv) To consider the role of saponins in protecting plants against insect predators.

(v) To investigate the digestive biochemistry of the alfalfa weevil, the major insect pest of the crop.

(vi) To investigate the feasibility of protecting alfalfa against alfalfa weevil through the use of transgenic plants expressing proteins toxic to the insect.

Chapter 2

Materials

2.1. Plant Materials

Seeds of two alfalfa *(Medicago sativa)* cultivars, Europe and Euver, supplied by Elsoms Ltd, were sown on moist vermiculite in 38 x 22 x 6 cm plastic seed trays and were covered with a thin layer of vermiculite. The trays were placed in a growth room at 25°C with 16 hours of illumination per day. Alfalfa seedlings were harvested after 2 weeks and were split into the root and shoot tissues, which were quickly frozen using liquid nitrogen. Frozen tissues were stored in a cold room at -20°C until required.

Another cultivar of alfalfa (Ahar), which was susceptible to alfalfa weevil attack in a field experiment dispatched kindly by Dr. Yazdi-Samadi from Tehran University, was grown up as above. The seedlings were transferred to plastic pots containing John Innes compost no.2 to mature, and used then for either rearing alfalfa weevil larvae or incorporating as powder in artificial diet for insect (alfalfa weevil) bioassay.

Potato tubers of variety Desiree were planted in pots containing John Innes compost no.2. The potato plants were grown to maturity under the same conditions as alfalfa. Individual potato leaves were used for rearing potato aphids needed for insect bioassays, or for saponin extraction.

Sugar beet seeds were provided by Broom's Bank Research Station, IACR Broom's Barn, Bury St. Edmunds, Suffolk, Norfolk; plants were grown under similar conditions to those described for alfalfa.

2.2. Insect Materials

2.2.1. Alfalfa Weevil (*Hypera postica* Gyll.)

The eggs of this pest were provided by Dr.Blodgett from Montana State University and Dr. Elden Agriculture Research Services, Beltsville, Maryland, U.S.A. The eggs were shipped at 48C on a filter paper in medium size covered petri dishes, which had been fitted with moist sponges. Eggs were stored at 48C until required.

2.2.2. Glasshouse Potato Aphids (*Aulacorthum solani* Kalt.)

Potato aphids were from a laboratory stock, reared on potato leaves which had been placed in Blackman boxes in an incubator at 22.58C with an illumination of 16/8hrs light/dark.

2.2.3. African Migratory Locusts (*Locusta migratoria migratorides* R. & F.)

The locusts were reared in an insectory in a photoperiod time of 14/10 (L/D) at 30°C and uncontrolled relative humidity.

2.2.4. Cowpea Seed Weevil (*Callosobruchus maculatus* Fab.)

The beetles were reared in culture jars containing either chickpea or cowpea seeds. The bottles were lidded with porous tissue lids and were placed on the top of a 10cm-depth tray containing water to make a humid microclimate.

2.3. Reference Saponins

For identification of saponins from alfalfa, six purified alfalfa saponins extracted from alfalfa root tissues were kindly presented by Dr. Massiot (Faculte' de Pharmacie, URA CNRS 492, 51, Reims-Cedex, France). A sample of medicagenic acid glucoside was also kindly donated by Dr. Oleszek (Department of Biochemistry, Institute of Soil Science and Plant Cultivation, Pulawy, Poland).

2.4. Materials for Enzyme and Inhibitor Assays

2.4.1. Enzymes

β-D-glucosidase from almond and papain from *Papaya latex* were obtained from Sigma Chemical Company (Poole, Dorset, UK)

2.4.2. Inhibitors

pCMB (p-chloromercuribenzoic acid), E64 (L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane), cystatin from hen egg white, soybean Kunitz trypsin inhibitor) and PMSF (phenylmethylsulfonyl fluoride) were purchased from

Sigma Chemical Company.

2.4.3. Substrate

Z-PheArg-AMC.HCl was supplied by Novabiochem (UK) Ltd, Nottingham. p-Nitrophenyl β-D-glucoside was purchased from Sigma Chemical Co.

2.5. Artificial Diet Compounds

L-amino acids, vitamins, trace metal salts, sucrose, phenylalanine, potassium orthophosphate and other ingredients for aphid diets were "standard" grade, purchased from Sigma Chemical Co., as were casein, vitamin C, methylparabenzoate, and agar. Aureomycin was purchased from Cyanamid. Chick pea seeds were purchased from a local Health Food supplier.

2.6. Reagents for Electrophoresis

Acrylamide stock solution (30 g acrylamide, 0.8 g bisacrylamide 100 ml^{-1}) was obtained from Appligene. Tris was obtained as Trizma base from Sigma, as were protein size markers (SDS-7: bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine erythrocyte carbonic anhydrase, 29 kDa; trypsinogen (PMSF-treated), 24 kDa; soybean trypsin inhibitor, 20 kDa; alpha-lactalbumin, 14 kDa). Other reagents were from BDH, Poole, Dorset, UK.

2.7. Reagents for Bacterial Culture and Molecular Biology

Trypticase peptone (from pancreatic digest of casein) was supplied by BBL Ltd. Yeast extract (Oxoid) was supplied by Unipath Ltd., Basingstoke. Bacto-tryptone was provided from Becton Dickinson, Cowley, Oxon., and bacto-agar was purchased from Difco. DNA size markers and IPTG (isopropylthio-β-D-galactoside) were purchased from Northumbria Biologicals Ltd., Cramlington, Northumberland, UK, as were restriction enzymes, with the exception of SacI, which was supplied by Boehringer Mannhem. Agarose was supplied by Gibco. BRL, Life Technologies, Ltd., Paisley, Scotland.

2.8. Other Solvents and Reagents

2.8.1. Protein Assays

Bradford reagent was obtained from Bio-Rad, and the standard protein BSA (bovine serum albumin) was supplied by Sigma.

2.8.2. HPLC and Other Solvents

Diethyl ether, methanol, petroleum ether, ethyl acetate, n-butanol, and iso-propanol were supplied by BDH, and were analytical or equivalent grade.

2.8.3. Miscellaneous Reagents

Sulphuric acid, acetic acid, acetic anhydride, Fast orange G, EDTA, chloroacetic acid, sodium chloride, ammonium persulphate and 2-mercaptoethanol were supplied by BDH Ltd., Poole, England, UK. Kenacid Blue R, Coomassie Brilliant Blue, PVP (polyvinylpyrrolidone), DTT (dithiothreitol), Brij-35, ethidium bromide, ampicillin, sorbic acid and SDS were purchased from Sigma Chemical Co. Ltd., UK. DMSO (dimethylsulphoxide) was supplied by Koch-Light Laboratories Ltd., England.

2.9. HPLC and TLC

Thin layer chromatography was carried out on Alltech and Merck Silica Gel 60 F254 20 x 20 cm plates. These plates were used as 20 x 20 cm, or as cut plates in 10 x 10 or 10 x 20cm sizes. Milipore Sep-Pak (C18) cartridges (1 ml volume) were obtained from Water Associates, Milford, MA, U.S.A. A LOBAR pre-packed preparative chromatography column size A (240-10), RP-18 (40-63 μm) reverse phase packing material for liquid chromatography was supplied by Merck Company, via BDH.

2.10. Miscellaneous Items

Cellulose acetate membrane filters (0.22 μm pore size 47 μm diameter) were supplied by Oxoid NuFlow. Dialysis tubing (Visking size 2, 18/32" diameter) was

purchased from Medicell International Ltd., London, UK. It was prepared by boiling the tubing in 10 mM EDTA containing ammonium bicarbonate for 20 min and then re-boiled in d.H_2O for further 20 min.

Chapter 3

Methods

3.1. Analytical Methods

3.1.1. Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out on silica gel coated glass TLC plates (with fluorescent indicator), using different plate sizes (20 x 20 cm, 20 x 10 cm and 10 x 10 cm) based on the number of samples, and the purpose of the analysis. Solutions of samples for TLC were centrifuged at 12000 g for 2-3 min, and the supernatants (10-20 µl per spot) were spotted onto the plates. An appropriate amount of purified saponin mixture was used as a reference and control on the TLC plate. The spotted samples were dried with a hair drier.

TLC plates were run in tanks lined with filter paper and pre-equilibrated with solvents. Two solvent systems were used.

For saponin separations: ethyl acetate:distilled water:acetic acid 7:2:2 (v/v/v)

For sapogenin separations: petroleum ether:chloroform:acetic acid 7:2:2 (v/v/v)

Plates were removed from the tank when the solvent front had reached approx. 1 cm from the top of the TLC plate, and were allowed to dry in air. TLC plates were then sprayed with a reagent for saponins, containing MeOH:acetic anhydride:sulphuric acid in the ratio of 10:1:1 (v/v/v), freshly made. After spraying with this reagent, plates were transferred to a drying oven at 104°C for 15 min or 180°C for 2.5 min. Sprayed plates were observed under normal light and under UV light at 300 nm, using a transilluminator (UVP Chromato-VUE). Each TLC plate was then photographed and R*f* values of the samples were measured and recorded. Selected TLC plates were recorded using an Agfa Studio Scan desktop flat bed scanner connected to an Apple Macintosh computer, on which the digitised images were stored.

3.1.2. SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide minigels were prepared according to Hames and Rickwood (1981). Fifteen percent acrylamide gels were prepared and used. Solutions

used are detailed below. For alternative acrylamide concentrations, adjustments were made to this basic recipe according to those suggested by Hames and Rickwood (1981). Samples, in 1xSDS Sample Buffer (0.1 M Tris pH 6.8, 10% Glycerol, 1% SDS, 0.001% Bromophenol Blue), were boiled for 3 min, and 2-25 µl loaded per well along with 1 µl β-mercaptoethanol.

Ingredients	Resolving Gel	Stacking Gel
Acrylamide	6.25 ml	1.25 ml
3.0 M Tris-HCl pH 8.8	1.875 ml	-
0.4 M Tris-HCl pH 6.8	-	2.50 ml
d.H_2O	6.165 ml	5.65 ml
De-gas for 5 min		
10% (w/v) SDS	0.15 ml	0.10 ml
2% (w/v) Ammonium persulphate	0.56 ml	0.50 ml
TEMED	7.5 µl	7.5 µl

Electrophoresis was carried out in an ATTO electrophoresis tank, following the supplier's instructions. After electrophoresis, gels were stained with a solution of 0.05% Kenacid Blue in 40% MeOH, 7% acetic acid, and destained briefly in MeOH/acetic acid solution.

3.1.3. Gel Drying

Gel drying was carried out in a Bio-RAD Gel Drying Frame in accordance with the supplier's instructions, taking care that no air bubbles became trapped between the gel and cellophane sheets. The assembled frame was then placed in a drying oven at 60°C.

3.1.4. Restriction of Plasmid DNA

Restriction of plasmid DNA was performed at 37°C for 3 hrs using buffer supplied with the restriction enzymes. Final dilution of restriction enzymes was at least 20-fold.

For a total volume of 20 µl assay, 6 µl of mini-prep Plasmid DNA was mixed with 6-13 µl d.H_2O and 2 µl of MBI Buffer Y. Then 1 µl each of of EcoRI and SacI (Ecl 136I) were added to the plasmid DNA. The mixture was incubated on a Grant heating block at 378C for 3 hrs. After heating, 4 µl of orange colour loading buffer (2 µl per 10 µl volume) was added to each sample, which was loaded onto a 1% agarose gel. pUC18 DNA (1 µl) was used as the control (negative).

3.1.5 Agarose Gel Electrophoresis

Electrophoresis of DNA was carried out in a Pharmacia GNA-100 minigel apparatus. 45 ml distilled H_2O was added to 0.5 g agarose in a 100 ml conical flask. The suspension was microwaved for 1 min to dissolve the agarose, and then 5 ml of 10x TAE buffer (400 mM Tris-acetate pH 7.7, 100 mM EDTA) containing 5 µl ethidium bromide (from 10 mg ml^{-1} stock) was added. The mixture was shaken well and poured into a gel-casting mold. The gel was placed in a gel tank into which 400 ml of 1x TAE buffer (40 mM Tris-acetate, pH 7.7, 10 mM EDTA; diluted from 10x stock) was poured. The comb was taken out gently and samples were loaded into the wells. Prior to loading, 0.2-0.5 volumes of dye mixture (10 mM Tris-HCl , 10 mM EDTA, pH 8.0, 1mg ml^{-1} fast orange G, 30% glycerol) were added to the samples before loading. λPstI, a commercially prepared restriction digest of lambda DNA, was run as a size marker. Electrophoresis was carried out at 2-10 volts cm^{-1} for approximately 2 hrs and gels were photographed on a UV transilluminator.

3.2. Insect Cultures and Diets

3.2.1. Aphid Maintenance

Blackman boxes were used to rear aphids on excised leaves of potato. Small sponges were placed inside the lower small section from which water is absorbed by the leaf in the upper and larger chamber. The leaves were taken from plants grown in a growth room at 25°C and 60% r.h. with an illumination of 16/8 L/D. Blackman boxes were placed in a plastic tray containing water to a height of about 5 mm and then placed in an incubator at 22.5°C and 16/8 L/D illumination.

Glasshouse potato aphids (*Aulacorthum solani*) were placed on the potato leaf

to feed. Adults were used for the production of nymphs for bioassay. Five adult aphids were removed from host plant leaf and placed on another potato leaf in a Blackman box. The newly produced nymphs, which were less than 24 hrs old, were used for insect bioassay.

3.2.2. Preparation of Aphid Diet

A liquid diet based on that reported by Febvay et al (1988) was used. This diet was improved by altering the levels of aromatic amino acids. The diet formulation is described below.

Vitamin Solution;

Eleven vitamins (see below) were weighed out and mixed together in a 200 ml autoclaved beaker. One hundred and fifty ml d.H_2O was then added to the mixture which was continually stirred for 30 minutes, or until all components had dissolved. The volume of the solution was adjusted to 200 ml and then ten 20-ml aliquots were placed in 25-ml sterilised universal tubes and stored at -20°C.

Vitamins	Per 200ml (mg)	Vitamins	Per 200ml (mg)
Amino benzoic acid	20.00	Folic acid	2.00
Ascorbic acid	200.00	i-Inositol (anhydrous)	84.00
Biotin	0.20	Nicotine acid	20.00
Calcium pantothenate	10.00	Pyridoxine (HCl)	5.00
(D-form)		Riboflavin	1.00
Choline chloride	100.00	Thiamin (HCl)	5.00

Amino Acid Solution;

The following L-amino acids, trace metals and other diet components were weighed out into a sterilised 200-ml beaker:

L-Amino acids	Per 200 ml (mg)	L-Amino acids	Per 200 ml(mg)
Alanine	357.42	Leucine	463.12
β-Alanine	12.44	Lysine (HCl)	702.18
Arginine	489.80	Methionine	144.70
Asparagine (H_2O)	597.10	Ornithine (HCl)	18.82
Aspartic acid	176.50	Phenylalanine	463.86
Cysteine	59.18	Proline	258.66
Glutamic acid	298.72	Serine	248.56
Glutamine	891.22	Threonine (allo free)	254.32
Glycine	333.12	Tryptophan	85.50
Histidine ($HCl.H_2O$)	272.04	Tyrosine	77.26
Isoleucine (allo free)	329.50	Valine	381.70

Other components:	*Per 200ml*
β-Alanyltyrosine	218.30 mg (or Phenylalanine 122.2 mg)
Sucrose	40.00 g

Trace metals	*Per 200 ml (mg)*
$CuSO_4.5H_2O$	0.94
$FeCl_3.6H_2O$	8.90
$MnCl_2.4H_2O$	1.30
NaCl	5.08
$ZnCl_2$	1.66

Other ingredients	*Per 200 ml (mg)*
Calcium citrate	20.00
Cholesterol benzoate	5.00
$MgSO_4.7H_2O$	484.0
KH_2PO_4	500.0

These components, except KH_2PO_4, were dissolved in 80 ml $d.H_2O$, to which was added 20 ml of vitamin stock solution. Insoluble amino acids were dissolved separately by addition of small amounts of 1M HCl. The pH of the solution was

adjusted to 7.00 with 1 M KOH and then 500 mg potassium dihydrogen orthophosphate was added. The solution was then placed on a stirrer to dissolve all compounds. Finally the volume of solution was adjusted to 200 ml and the pH of the solution was adjusted to 7.5. Twenty 10-ml aliquots of diets were passed through 0.2 µm Millipore sterilised filters, collected into sterilised universal tubes, and stored at -20°C till required.

3.2.3. Rearing Alfalfa Weevil (*Hypera postica* Gyll.)

Petri dishes containing alfalfa weevil eggs were placed in an insectory room at 25°C and 70% relative humidity, with a 9/15 L/D illumination (Hsiao and Hsiao 1974a and 1974b). Newly hatched first instar larva were then transferred into 10 x 15 x 10 cm plastic containers with a piece of wet tissue at the bottom of the container. Three 70 day-old branches of alfalfa cultivar "Ahar" were placed on the tissue and larvae were transferred onto the shoots using a fine camel hair brush. The top of the containers was perforated to provide ventilation. The cultivar "Ahar" had been shown to be the most susceptible to alfalfa weevil attack among 138 cultivars of alfalfa varieties (Mazahery-Laghab 1991). Every day wet tissue was inspected and replaced if the previous one had dried out. First instar larvae were used for insect bioassay, and third and fourth instar larvae were collected for extraction of their gut for enzyme assays.

3.2.4. Preparation of Alfalfa Weevil Diet

The artificial diet for alfalfa weevil larvae was made up as described below, based on a potato leaf diet . Oryzacystatin (Rozc) protein at 2% and 5% of the total protein in the diet was added to the diet in small petridishes. The diet was based on freeze dried alfalfa leaf of cultivar "Ahar" in agar supplemented with vitamin free casein and vitamins.

Mixtures	Diet Components	Control diet	Artificial diets 2% Rozc	5% Rozc
1	Ground freeze dried Alfalfa-shoot tissues	0.2525 g	0.2525 g	0.2525 g
	Casein	0.101 g	0.101 g	0.101 g
	Casein	3.02 mg	---------	--------
	Ozc Protein	----------	3.02 mg	7.55 mg
	distilled H_2O	2.0 ml	2.0 ml	2.0 ml
2	L-Ascorbic acid (Vitamin C)	0.0101 g	0.0101 g	0.0101 g
	Methylparabenzoate	0.005 g	0.005 g	0.005 g
	Aureomycin	8.8 mg	8.8 mg	8.8 mg
3	Agar	187.5 mg	187.5 mg	187.5 mg
	distilled H_2O	3.0 ml	3.0 ml	3.0 ml

The agar was microwaved in a portion of the water, and cooled to 60°C before addition of the remaining diet components. The levels of Rozc (recombinant oryzacyststin) used in the 2% Rozc experimental diet was supplemented in control diets with additional casein, thus ensuring that experimental and control diets contained the same protein levels. Prepared diets were transferred into small petridishes, which were then covered with the lids, and sealed with a strip of para-film, these were then stored in a fridge until required. It was important not to store the diet for longer than a week as the diet would then start to deteriorate.

3.3. Extraction of Saponins from Plant Tissues

3.3.1. Extraction of Saponins from Alfalfa.

Frozen alfalfa root or shoot tissues were weighed out, and placed in a cooled mortar. The tissue was ground finely using liquid nitrogen to prevent any enzymatic degradation, and to make the tissue brittle enough to grind. The powdered tissue was then transferred to a conical flask and 80% MeOH (5 ml g^{-1} tissue) was added to the

powder and saponins were extracted at 4°C, by stirring overnight. The solution was filtered through a fine glass sinter. The residue was washed with a small volume of distilled water and the filtrate was evaporated under vacuum in a rotary evaporator at 40°C. 1.5 ml g^{-1} of $d.H_2O$ was added to the residue after evaporation and the sample was stored at -20°C until required.

3.3.2. Extraction of Saponins from Potato and Sugar Beet Leaves

Potato or sugar beet leaves were frozen in liquid nitrogen, and then lyophilised. Dried tissue was weighed out, transferred into a mortar, and ground finely. The powdered tissue was transferred into a glass universal tube. Crude saponins were extracted with 25 ml 80% MeOH (5 ml per g. fresh tissue) at 4°C by stirring overnight. The solution was centrifuged at 3600 rpm (3000 g). The supernatant was taken and evaporated at 40°C. The residue was taken up in 1.5 ml MeOH per gram tissue, and stored at -208C.

3.3.3. Acid Hydrolysis of Saponins

In order to hydrolyse saponins to sapogenins, 1ml of either alfalfa shoot or root extract containing crude saponins from cultivars Euver and Europe were mixed with 5 ml 2N HCl. The solution was then refluxed at 100°C for three hours and then evaporated to dryness. Five ml $d.H_2O$ (equal to 2N HCl volume) was added to the residue, and the mixture was transferred into an appropriate vial and shaken with 5 ml ethyl acetate. Two layers were formed. The upper ethyl acetate layer was removed and stored, the aqueous layer was re-extracted twice with ethyl acetate as above. The combined ethyl acetate layers were evaporated at 40°C under nitrogen. The residue was then dissolved in 0.5 ml MeOH. A similar procedure was used to hydrolyse soyasaponin I from shoot tissue after purification by HPLC

3.4. Purification of Saponins

3.4.1. Extraction with *n*-Butanol

Sixty gram of plant tissues was extracted in 80% MeOH, evaporated in vacuum at 40°C and the residue was taken up in 50 ml $d.H_2O$. The crude saponin

extract was transferred into a 250-ml separating funnel and was mixed with 25 ml water saturated *n*-BuOH. Then the funnel containing the mixture was shaken and allowed to stand. Two distinguishable phases, an upper BuOH layer, and a lower aqueous layer formed. Sometimes an interface layer was also present, depending on the sample being partitioned. The upper BuOH layer was first drawn off and stored. The inter-phase layer was transferred into a centrifuge tube and centrifuged at >3600 rpm at 4°C. The precipitate was removed and aqueous supernatant was added to the lower H_2O layer. The combined aqueous layers were re-extracted twice with 25 ml BuOH as described above. The three BuOH layers were combined, evaporated in vacuum at 55°C and the residue was dissolved in MeOH. This method was scaled down for smaller amounts of starting material.

3.4.2. Ether Precipitation

The saponin solution after extraction with butanol was filtered through a glass micro-fibre filter CF/G. 10 ml MeOH was used to wash the residue. The filtrate was mixed with 5 volumes of diethyl ether. The suspension, in a beaker covered by parafilm, was shaken, then left until a precipitate of saponins had formed. The supernatant was drawn off carefully and the mixture of precipitate and remaining supernatant layer was centrifuged at 4°C and 3600 rpm for 10 min. 30 ml Et_2O was added. The precipitate mixed, divided into two equal portions and centrifuged as before. The supernatant layer was again drawn off and the precipitate mixed with another 15 ml Et_2O in each tube, and centrifuged. Washing was continued until the ether wash solution was colourless after centrifugation. The residue was then allowed to dry in air over silica gel in a desiccator, giving a purified saponin mixture.

3.4.3. Reverse-Phase Chromatography Using Sep-Pak Cartridge

Purification of saponins using Sep-Pak cartridges was carried out essentially as described previously (Nowacka *et al.*, 1994; Oleszek 1988; Nowacka and Oleszek 1992; Oleszek and Jurzysta 1990). Crude saponin extracts from the root and shoot tissues of alfalfa seedlings were purified by reverse-phase chromatography at their natural pH. Saponin extracts were loaded onto a 1-ml Millipore Sep - Pak (C18) cartridge, which had been equilibrated with 5 ml of MeOH followed by 5ml d.H_2O.

A 5-ml aliquot of crude saponin extract was passed through the cartridge. The cartridge was then eluted with a step gradient of 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% v/v of MeOH in water, using 5 column volumes of solvent at each step. The procedure was repeated five times and eluted material was collected from each solvent step. Then the five eluates for corresponding gradient steps were combined and evaporated in vacuum at 40°C to dryness. The residues were each taken up in 1 ml distilled water.

3.4.4. High Performance Liquid Chromatography (HPLC) of Saponin Mixtures.

Saponin mixtures after butanol extraction and ether precipitation were further purified by reverse-phase HPLC on a L0BAR RP-18 (40-63 μm) C-18 preparative column (240 mm length, 10 mm dia, 18.8 ml volume), applying the sample in water, and using a gradient of isopropanol in water to elute bound components. All solvents were filtered using 0.22 μm cellulose acetate membranes. The column was first conditioned with $d.H_2O$ and then with MeOH each for 24 hrs at 0.5 ml min^{-1} flow rate, and was then subjected to a complete HPLC gradient run with no sample loaded. Chromatography was carried out using a Gilson HPLC System with detection at 216 nm, at a flow rate of 0.5 ml min^{-1}. The gradient profile was varied slightly for different runs. For purifying fractions from alfalfa shoot and root saponins (**Sa-Sf, Ra-Rf**) the total run time was 200 min. A two-stage gradient of 0 to 70% propanol over 140 min and 70-100% propanol over 10 min was employed. A 100% plateau of iso-propanol was used to elute low polarity components. After 170 min, the concentration of propanol was decreased to 0% within 2 min and the column was washed with distilled water for 28 min to elute any remaining compounds in the column and prepare the apparatus for the next chromatography. Eluate fractions (5 min) were collected after saponin samples were injected onto the column using a Pharmacia fraction collector. 20 μl of each fraction was analysed by TLC. On the basis of TLC results, fractions were pooled and evaporated using a Howe Gyrovap GT rotary vacuum concentrator. Residues were dissolved in a small amount of MeOH and precipitated with 5 volumes of Et_2O. The precipitates were collected by centrifugation, dried and stored in a desiccator.

HPLC carried out for re-purification of bioactive components used a modified protocol, with 180 min total run time, and 3 min fraction collection time. Samples

were loaded onto the HPLC column and eluted with a gradient of propanol from 0% to 45% for 30 min following the elution with 45% to 55% for 70 min and 70%-100% for 10 min. Elution with 100% propanol for 20 min allowed the elution of low polarity compounds, and then column was washed with a 100%-0% propanol gradient over 5 min, followed by washing with water for 45 min.

3.5. Analysis of Saponins

3.5.1. Identification of Saponins Using Reference Compounds

Either 1 mg of reference saponins or 10 mg of ether precipitated purified saponin mixtures from alfalfa shoot and root tissues were dissolved in 1ml of MeOH, micro-centrifuged, and 20 µl of solution of reference compounds (0.1%) and 8.5 µl of purified saponin mixture (10%) solutions were spotted on glass TLC plate. The plate was developed in a TLC tank containing the ethyl acetate solvent system. Different saponins in purified saponin mixture were compared with references (both under normal and uv light) after staining of TLC plate with saponin (sulphuric acid-acetic anhydride) reagent.

3.5.2 Characterisation of Soyasaponin by NMR

A re-purified sample of saponin fraction Sf was despatched to Drs Massiot and Lavaud, University of Reims, France for determination of structure by ^{13}C NMR.

3.5.3. Examination of Root and Shoot Saponins in the Different Stages of Alfalfa Growth

Five gram of root and shoot tissues from different ages of alfalfa cultivar Euver (14, 40, and 70 days old), grown at 258C under a photo-period of 16/8 L/D, were collected, weighed, frozen in liquid nitrogen and lyophilised. Dried tissues were ground in a mortar and crude saponins extracted with 80% MeOH by stirring at 4°C overnight, following the procedure decribed above. Crude extracts were partitioned between *n*-BuOH and d.H_2O and precipitated with Et_2O. Residue pellets containing a mixture of saponins were dissolved in MeOH. TLC was carried out using 20-µl samples and resultant spots on TLC plate were analysed.

3.6. Extraction and Purification of Recombinant Oryzacystatin (Rozc) from *E.coli* (*Escherichia coli*)

3.6.1. Preparation of YT-amp Media

Materials for YT-medium (8 g Trypticase, 5 g Yeast Extract, 5 g NaCl per litre of medium) were mixed and taken up in 900 ml of $d.H_2O$, stirred, and adjusted to 1L with $d.H_2O$. Ten-ml aliquots of solution were dispensed into universal tubes and autoclaved. After autoclaving ampicillin (50 $\mu g\ ml^{-1}$) was added. For preparation of ampicillin stock, 10 mg ampicillin was first dissolved either in 1 ml $d.H_2O$ followed by filtration through a 0.22 μm membrane filter and stored in aliquots as stock at -20°C, or was dissolved in 1ml of 70% EtOH and stored at room temperature. 10 ml YT-amp aliquots were stored at 4°C until required.

Agar plates were made using YT-medium, autoclaved with 15 $g\ l^{-1}$ $^{-1}$ bacto-agar. For YT-amp Xgal plates, ampicillin (50 $\mu g\ ml^{-1}$) was added to the autoclaved agar and YT-amp medium prior to pouring into petri dishes to prepare agar plates. The plates were then placed in a sterile air flow hood and sealed with parafilm and stored at 4°C. Prior to use, plates were warmed and equilibrated to room temperature.

3.6.2. Plasmid DNA Minipreparation

Single colonies from agar plates, or aliquots taken using a wire loop from glycerol preserved cells, were inoculated into 100 ml aliquots YT-amp (YT medium with 50 $\mu g\ ml^{-1}$ ampicillin) and incubated overnight at 37°C on a rotating wheel. Plasmid DNA was prepared from cultures using a scaled up version of the method of Edmonds (1994).

Culture samples (1.5-ml) were transferred into Eppendorf tubes, spun at full speed using a micro-centrifuge for 1.5 min and the supernatant discarded. Another 1.5 ml of culture was added to the tube and spun as before and the supernatant again discarded. 200 µl of ice-cold solution I composed of 2 mg lysozyme, 9 mg glucose (50 mM), 50 µl 0.2 M EDTA (10 mM), 25 µl 1 M Tris-HCl pH8.0 (25 mM), and 925 µl $d.H_2O$, was added and cells were re-suspended by pipetting. 200 µl of solution II (room temperature) composed of 200 µl 1 N NaOH (0.2 N), 50 µl 20% SDS (1%)

and 750 µl d.H_2O, was added followed by gentle inversion. 200 µl ice-cold solution III (3 M Na-acetate pH4.8, adjusted with glacial acetic acid) was then added and mixed by inversion. The solution was spun in a micro-centrifuge for 5 min to pellet the precipitate. Supernatant was transferred to a clean Eppendorf while taking care to avoided pipetting any of the precipitate and spun for 1 min. Two volumes of EtOH stored at -20°C (1 ml) was added to the supernatant and incubated on ice for at least 15 min until DNA was precipitated. Mixture was spun at full speed in a microfuge for 15 min. EtOH was poured off and replaced with 1 ml of 70% EtOH at -20°C, spun for 3 min, and the EtOH was again poured off and spun for a few seconds to bring any drops of EtOH to the bottom of the tube, and the EtOH was removed with a tissue. DNA samples were allowed to dry for a few seconds. DNA pellets were re-suspended in 30 µl of T.E (containing RNAase, optional). Small volumes of samples were run on agarose gels with DNA size marker.

3.6.3. Batch Culture for Expression of Rozc

Transformant PHEV1-6 bacterial cells containing the DNA insert were grown overnight in 5 ml YTamp media at 37°C. These cells were then added to 1 L of YT-amp containing of 0.5 mM IPTG. The suspension was incubated at 37°C for 15 hrs with shaking at 85 rpm on a rotary shaker (INFORS AG). Bacterial cells were harvested by centrifugation at 3600 rpm at 4°C for 15 min.

3.6.4. Purification of Rozc

Harvested cells carrying the recombinant plasmid were re-suspended in 10 mM Tris pH 8.0, 2 mM EDTA and were disrupted by sonication using MSE Soniprep 150 sonicator with 3 times 30 second bursts with 15 second intervals. Where possible samples were kept on ice to prevent heat gain during sonication. Cell debris was removed by centrifugation at 15900 g. The supernatant was subjected to heat treatment at 82°C in a water bath for 15 min., allowed to cool to 4°C, and then centrifuged at 15900 g for 15min. The pellet was discarded. The supernatant was fractionated by ammonium sulphate precipitation. Protein was collected after 0-60% saturation with ammonium sulphate (6.60 g in 16.15 ml supernatant) according to Harris and Angal (1995). The suspension was then centrifuged at 3600 rpm at 4°C for

5 min. and the precipitate was re-suspended in 4 ml of 20 mM Tris pH8.0. The re-suspended protein solution (see 3.3.7) was transferred to the dialysis tubing and dialysed against 2 litres of 20 mM Tris buffer pH 8.0 at 4°C. Several buffer changes were carried out so as to ensure complete desalting of the protein solution. The protein solution was transferred into a plastic centrifuge tube and "Shell" frozen in liquid nitrogen and finally lyophilised using a Flexi-dry µp, FTS system freeze dryer. SDS-PAGE was carried out using 15% gels to analyse the extracted Rozc protein

3.7. Preparation of Enzyme Extracts

3.7.1. Enzyme Extraction of Alfalfa Shoot Tissues

Crude enzymes of alfalfa were extracted as described by Parry and Edwards (1994). Shoot tissue (18 g) from alfalfa seedlings was frozen in liquid nitrogen and homogenised using a Moulinex blender in 4 volumes of ice-cold 0.2 M Tris-HCl, pH 7.5 containing 14 mM MeSH, 1 mM Phenyl methyl sulfunyl fluoride (PMSF) and 2 mM EDTA. The mixture was centrifuged at 14000 g for 20 min. The supernatant (150 ml) was stirred with 10% Sigma insoluble PVP for 20 min at 4°C. Protamine sulphate (1.4% w/v stock) was added to 134 ml of supernatant (10%), and after 30 min stirring, the precipitated nucleic acids were removed by centrifugation. The supernatant was fractionated with solid ammonium sulphate and the protein fraction, which precipitated at 90% saturation after 1.5 hr incubation, was collected by centrifugation at 14000 g at 4°C. It was stored at -20°C, where no significant loss of activity was observed.

3.7.2. Gut Enzyme Extraction from Alfalfa Weevil

Third and fourth instar alfalfa weevil larvae were transferred from the rearing plastic vial and immobilised by placing in an ice-cold beaker. The larvae were then transferred into a glass petri dish on ice and heads and tails, 1 mm from the body end, were cut off using scissors. Forceps were also used to facilitate cutting off the ends and removing the gut from the larval body. Removed gut was transferred to the tip of a teflon pestle and placed into a glass homogenizer containing 1 mM DTT in distilled water (40 larvae in 1 ml buffer) and the guts were squeezed while the content was placed on ice. Further homogenisation was carried out in the glass homogenizer using

a CABLAB electric homogenizer at 4800 rpm. 50 µl aliquots of homogenised gut extract containing digestive enzymes were pipetted into 1.5-ml Eppendorf tubes, frozen in liquid nitrogen and stored at -20°C.

3.7.3. Gut Enzyme Extraction from Locust

The tail of African migratory locust (*Locoust migratoria migratorides*) was cut off 5 mm from the caudal end using forceps and scissors. The head was twisted off and the gut removed in one piece. Gut contents were squeezed onto a flat petri dish on ice and then transferred onto the tip of a teflon pestle followed by placing into a glass homogenizing tube. One ml distilled water containing 2-β-mercaptoethanol (1µl 200 ml^{-1}) was added to the gut contents and homogenised using a TR1-R STIR-R electric homogenizer at 4800 rpm. All homogenised gut contents were divided into 50-µl aliquots in Eppendorf tubes and to each portion an equal volume of chloroform was added to remove lipids from the gut contents by shaking on a whirlimixer and centrifugation at 1000 g for 2 min. The upper aqueous layer was transferred into another Eppendorf tube and was quickly frozen in liquid nitrogen and stored at -20°C until required

3.7.4. Crude Enzyme Extract from Potato Aphid

Adult aphids, reared on potato leaves, were transferred from the leaves into a glass homogenizer contain 1 ml of 1 mM DTT. The aphids were then squashed using a fine teflon pestle and homogenised as described previously. Lipids were removed by chloroform extraction as described previously (3.7.3). The aqueous phase was carefully removed and then dispensed into 100 µl aliquots, frozen in liquid nitrogen, and stored at -20°C until required.

3.7.5. Gut Enzyme Extraction from Cowpea Seed Weevil

The extraction of cowpea seed weevil gut enzymes was carried out as described by Gatehouse et al (1985b). Cowpea seeds infested with *Callosobruchus maculatus* larvae were dissected and late instar larvae were taken out and placed in a glass petri dish on ice. The head of the larvae was held with thin forceps and the bottom was nicked by dissector scissors and subsequent decapitation was also carried

out on ice. The alimentary tracts were pushed out gently through the bottom of the gut. Gut extract was placed on the tip of a teflon pestle and transferred immediately into a glass homogenizer containing 250 µl 1 mM DTT per 20 larvae. Homogenization was carried out on ice using a TRI-R STIR-R electric homogenizer at 4800 rpm. The homogenized extract was then extracted with chloroform as described previously. The aqueous layer was aliquoted (125 µl), frozen with liquid nitrogen and stored at -20°C until required.

3.8. Enzyme Assays

3.8.1. Standard Glucosidase Assay

β-D-glucosidase activities were assayed using p-Nitrophenyl-β-D-glucoside as a chromogenic substrate. Enzyme assays were performed with a mixture of 1 ml of 10 mM p-nitrophenyl substrate in acetate buffer pH 5.0 and 10 µl of enzyme extract from either alfalfa tissue or insect gut, or with 1 µl purified almond β-D-glucosidase. Assays were monitored on a SPB-15 UV/VIS spectrophotometer at room temperature at 410 nm for 25 min. Absorbance changes were measured on a chart recorder.

3.8.2. Assay for saponin hydrolysis

A method adapted from that of Gestetner (1971) was used. 10µl of β-D-glucosidase from almond or 20 µl of alfalfa or insect enzyme extracts were mixed with 90 µl of a 0.1% purified saponin mixture from the root and shoot tissues of alfalfa seedlings. 390 µl acetate buffer, pH 5.0, was added and extracts were incubated at 37°C for 20 hrs with continuous agitation throughout the incubation period. After incubation, the reaction was stopped by addition of 1 ml water saturated BuOH and saponins were extracted 3 times with *n*-BuOH. BuOH layers were combined, evaporated, and residue was taken up in 50 µl MeOH. The resuting saponins were analysed by TLC, spotting 20 µl of solution on the TLC plate for each sample. Saponins were analysed after staining the plate with sulphuric acid reagent and visualising under both UV and normal light.

3.8.3. Assay for Alfalfa Weevil Gut Protease Activities

The fluorescent substrate Z-Phe-Arg-AMC was used to investigate the activity of alfalfa weevil proteases. All assays were carried out in a total volume of 2060 µl in duplicate.

0-10 µl of insect gut enzyme (10x diluted with d.H_2O) of alfalfa weevil were mixed with a 250 µl of buffer containing 340 mM Na acetate, 60 mM acetic acid, 40 mM Na_2EDTA and 8 mM DTT, pH 5.5. The mixtures were then diluted to 760 µl with 0.1% Brij35. The assay mixture was pre-incubated at 30°C for 2 min. 50 µl of 20 µM Z-Phe-Arg-AMC was then added, and the assay was incubated at 30°C for 10 min. The reaction was stopped using 1250 µl stopping reagent containing 100 mM chloroacetic acid, 30 mM Na acetate, and 70 mM acetic acid pH 3.0. Released product (AMC) was measured on a Perkin-Elmer LS-3B spectrofluorimeter at emission and excitation wavelengths of 450 nm and 358 nm respectively. A similar procedure was followed when the effects of inhibitors on the alfalfa weevil protease activity were assayed, except that inhibitors (2-10 µl) were added to the enzyme prior to pre-incubation.

The assay was calibrated for moles of product released by repeating the above procedure omitting the enzyme solution, and replacing it with known amounts of AMC (from a stock solution of 2.5 µM AMC in DMSO). A calibration graph was drawn (Fig. 3.1), which showed a linear response of fluorescence with amount of product.

3.8.4. Bradford Protein Assay for Alfalfa Weevil Gut Enzymes

To estimate the total protein content in alfalfa weevil gut extract, a dye-binding protein assay was carried out in a microtitre plate with flat-bottomed wells. 5 µl of the stock solution of BSA (Bovin Serum albumin as standard protein) equal to 50 µg BSA was diluted to 50 times with d.H_2O and used to construct a standard curve. 10 µl of gut extract (diluted x10) was used for protein assay. Duplicate assays were carried out for each sample. A total volume of 300 µl was used for each assay containing either weevil gut extract or BSA. A range of appropriate dilutions of BSA from 0 µl to 20 µl was added to 300-280 µl Bradford reagent. The plate was incubated for 2 min while shaking well on a shaker to prevent any settlement of protein before reading at 570 nm using a Dynatech MR 5000 plate reader. A standard

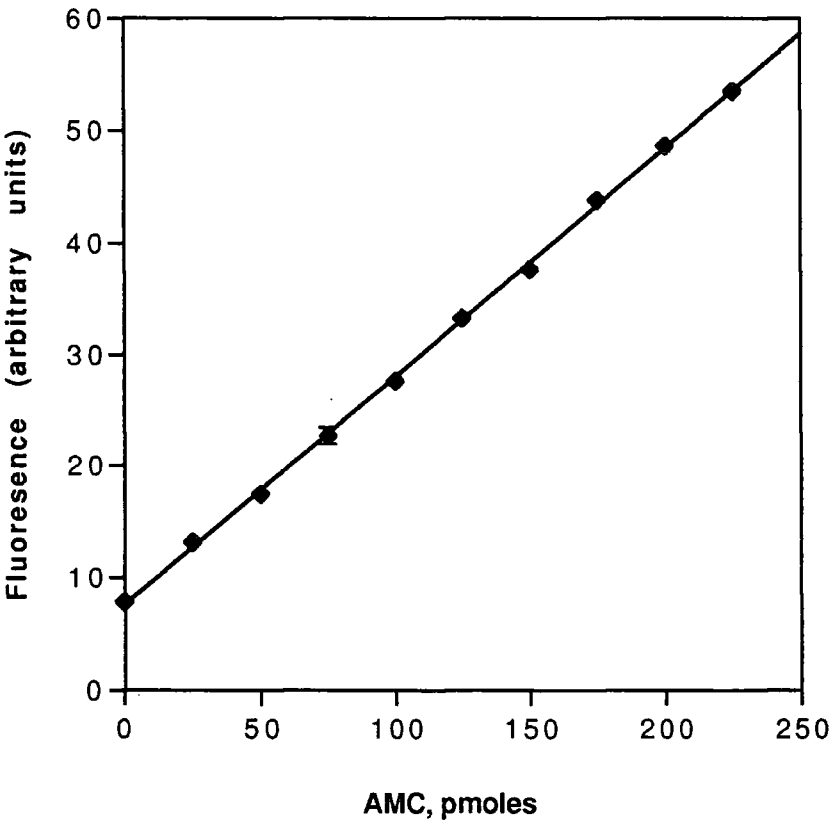


Fig. 3.1 Calibration curve for fluorescence of aminomethyl coumarin (AMC) under standard assay conditions for the protease assay using Z-arg-phe-AMC as substrate. Each point is the mean of duplicates; range is indicated by vertical error bars.

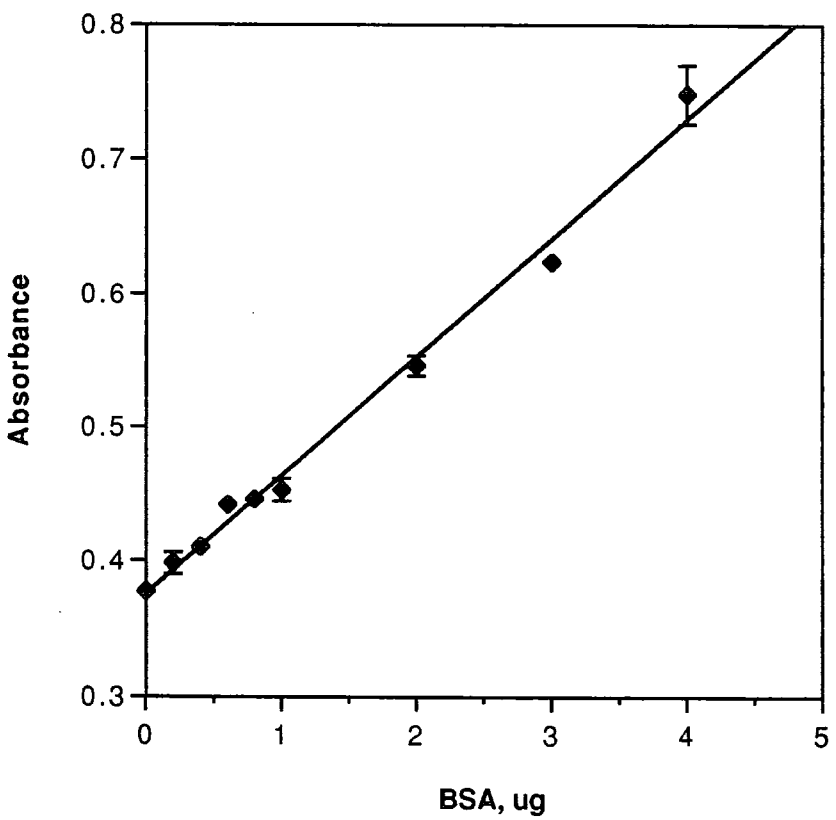


Fig. 3.2 Calibration curve for protein estimation using BSA as standard. Each point is the mean of duplicates; ranges are indicated by vertical error bars.

curve (Fig. 3.2) was used for calibration.

3.8.5 Assay for Papain Activity

One µl papain (0.1% in acetate buffer) was added to 250 µl pre-warmed acetate buffer containing 8 mM DTT. 500 µl 0.1% Brij35 was added and the solution was preincubated at 30°C for 2 min. 50 µl substrate (Z-Phe-Arg-AMC.HCl) was then added to initiate the reaction at 30°C for 10 min incubation. After stopping the reaction with 1250 µl stop solution as above, fluorescence was read at 450 nm emission and 358 nm excitation. The effects of inhibitors were determined by adding inhibitor to enzyme prior to pre-incubation.

3.9. Insect Bioassays

3.9.1. Bioassays with Cowpea Seed Weevil (*Callosobrochus maculatus*) Larvae

The insecticidal effects of saponins from alfalfa roots and shoots were investigated by allowing cowpea seed weevil (*Callosobruchus maculatus*) larvae to develop in diet pellets containing the saponins. Artificial diet pellets based on ground chickpea meal were prepared using the method of Gatehouse and Boulter (1983).

2.5 g portions of chickpea meal were suspended either in an excess volume of d.H_2O, or in solutions of crude extracts containing known amounts of saponin. The different treatments contained 3.75, 0.375 or .038 ml crude saponin extract from both root or shoot tissues to give amounts of saponin in the diet pellets equivalent to 100%, 10% and 1% of those present in tissues. These suspensions were then frozen in liquid nitrogen and lyophilised. Pellets were prepared by adding 1ml of d.H_2O to each of the 2.5 g portions and stirring to form a uniform thick paste; 5 pellets of similar size were prepared for each treatment. Five replicas each of 5 pellets were prepared. The pellets were dried down over silica gel for 24 hrs, equilibrated for seven days at 27°C, 70% r.h. and then covered with Snappies cling-film/PVC sheeting. Pellets were then placed in culture jars containing infested cowpea seeds and females were allowed to oviposit. Uniform egg lay on all pellets was assumed (mass infestation technique). After 2 days, the pellets were placed in individual 5 x 2.5 cm diameter glass specimen vials. The time to first appearance of windows on the pellets, and the

first day of adult emergence, was recorded for all pellets in all treatments. The numbers of adults emerging were recorded daily so that the mean development period for each treatment could be calculated. The bioassay was run for a total of 41 days and on termination, the number of non-emerged adults, pupae and larvae produced from each pellet were measured by dissection of the pellets. Statistical analysis of data was carried out using unpaired t-tests.

The above bioassay method was also used to evaluate the effects of crude alfalfa shoot and root sapogenins on cowpea seed weevil. In this case, 1.87, 0.187 and 0.019 ml sapogenin solution in methanol were added to the chickpea meal suspension to give amounts of sapogenin equivalent to 100%, 10% and 1% of the saponin level present in tissue. Bioassays and subsequent analyses were carried out as described above.

3.9.2. Aphid Bioassays

Five neonate nymphs (maximum 24 hrs old), were removed from the host plant, using a fine paintbrush to minimise physical damage, and placed in a plastic feeding chamber (small petri dishes). A single layer of stretched parafilm membrane was placed over the chamber and 200 µl of artificial diet with or without saponin (weighed as dry solid and dissolved in diet) was pipetted onto the parafilm. The diet was then covered with a second layer of stretched parafilm so as to form a feeding sachet through which the nymphs could feed. The feeding chambers were then housed in an incubator at 22.5°C with an illumination of 16/8 L/D. Diets and feeding sachets were renewed every other day to avoid diet deterioration and microbial contamination. The number of nymphs surviving was recorded daily. Trials were carried out for 20 days unless stated otherwise; this allowed nymph production from aphids which developed to maturity (after 10-11 days) to also be monitored daily.

For each set of bioassays, two controls were also run; these were basal diet only, and no diet. Unless otherwise stated, 5 replicates each of 5 nymphs, were carried out.

In order to determine which fractions from a hplc separation of root and shoot saponin mixtures were toxic to aphids, a slightly different procedure was followed. Fractions from the hplc run were evaporated, and the residues were taken up in 600 µl artificial diet, and tested against potato aphid in bioassay as previously described.

Survival was recorded over a period of 6-7 days.

In some trials, aphid growth was monitored by measuring the size prior to maturity (usually after 8 days). Aphids were viewed through a low-power microscope, and the image was also monitored using a Nikon video camera attached to the microscope. Images were digitised and captured using the on-board video system on an Apple Power Macintosh 7100/66AV computer, and stored on the computer's hard disk. Captured images were analysed for aphid length (head to tail) and width (body immediately behind the first pair of legs) using NIH Image software. Sizes were calibrated using a captured image of a standard microscope calibration slide.

3.9.3. Alfalfa Weevil Bioassay

This bioassay was carried out to study the effects of Rozc upon the development of alfalfa weevil larvae. Two experimental treatments, 2% or 5% Rozc, and a control were set up. For each treatment at least 10 replicates each of 5 first instar larvae were set up. All of the larvae in each replicate were transferred into an Eppendorf tube containing the diet. The tubes had been made with 12 holes (pores) and one hole in the bottom. Compressed pieces of wetted tissues were placed inside the Eppendorf tube lid to increase the relative humidity inside the tube and prevent drying of the artificial diet.

Survival was measured daily, and diets were changed every other day. The tubes along with the tissues were changed if necessary. Another "no diet" control with neither artificial diet nor Rozc was used to evaluate the potential of larvae to remain alive without any food.

3.10. Statistical Methods

Statistical analysis was carried out using Statview software (v. 4.5; Abacus Concepts, Ca. USA) on Apple Macintosh computers. Most differences in parameters between controls and treatments were evaluated using unpaired t-tests, but differences in survival were evaluated using a non-parametric Mann-Whitney U-test. The criterion for rejecting the null hypothesis was $p < 0.05$.

Chapter 4

Experimental Procedures and Results

SECTION 1:

4.1. Purification and Characterisation of Saponins from Alfalfa and Other Plants

4.1.1. Extraction of Saponins from Alfalfa Tissues

The distribution of saponins in alfalfa root and shoot tissues was studied in two cultivars, Europe (Erp) and Euver (Evr). Roots and shoots from alfalfa seedlings were collected, frozen immediately in liquid nitrogen, ground while frozen, and extracted in 80% MeOH at room temperature. These conditions were intended to minimise any post-harvest changes to the saponins (e.g. hydrolysis). After evaporation, the resulting extracts were analysed by thin layer chromatography (TLC) on silica gel plates, using ethyl acetate-distilled water-acetic acid (7:2:2) as solvent. Saponins were visualised after separation by spraying plates with MeOH-acetic anhydride-sulphuric acid reagent.

Multiple spots were visualised in all extracts by this method. The colours of the spots, both under normal and UV light were used to indicate the nature of the compounds present. Spots which were coloured brown, purple, green or combinations of these colours under normal light, and yellow, green or dark under UV light were considered to be due to the saponins. Spots, which were fluorescent blue under UV light, and were not visible under normal light, were considered to be due to phenolic compounds.

- When the spots produced by extracts from roots and shoots were compared, certain spots were common to both extracts, whereas others were different. Spots produced are shown in Fig. 4.1, and are summarised in Tables 4.1 and 4.2. Roots contained comparatively little detectable material after TLC, and most of the spots had high Rf values, in the region 0.4 - 1. Three main components, with approximate Rf values 0.43 (major component; UV yellow spot), 0.49 (UV yellow

spot) and 0.67 (UV green spot) were present. The most mobile of these spots was present in higher amounts in cultivar Erp than in cultivar Evr. Fluorescent blue spots were also present in these root extracts. Shoot extracts contained high amounts of detectable material after TLC, with most of the detected spots having low Rf values, in the range 0 - 0.5. A brown (visible) / yellow (UV) spot at approx. Rf 0.43, apparently the same as the major spot in the root extract, was present in shoot extract, and formed the most intense spot with $Rf > 0.2$. However, shoot extracts also contained large amounts of components with $Rf < 0.2$, which gave dark green-yellow spots on TLC. Variable amounts of material running near to the solvent front were also present in the shoot extracts. Cultivar Erp contained an extra dark spot running near the solvent front, and an extra spot at low Rf (approx.0.07) compared to cultivar Evr; a minor yellow spot at Rf approx. 0.53 was also more intense in cultivar Erp than in cultivar Evr. The two spots in shoot extract which fluoresce strongly blue ($Rf = 0.49$ and 0.91) are present in greater amount in cultivar Erp than in cultivar Evr. Otherwise, the pattern of spots in the two cultivars was similar. Many of the spots visualised on TLC of the crude root and shoot extracts were considered to be due to components other than saponins; however, certain spots were subsequently tentatively identified by comparison with the standard saponin samples (see section 4.1.6).

The extract of alfalfa tissue in 80% MeOH, after evaporation and re-dissolving in water, was designated "crude saponin extract" in subsequent bioassays.

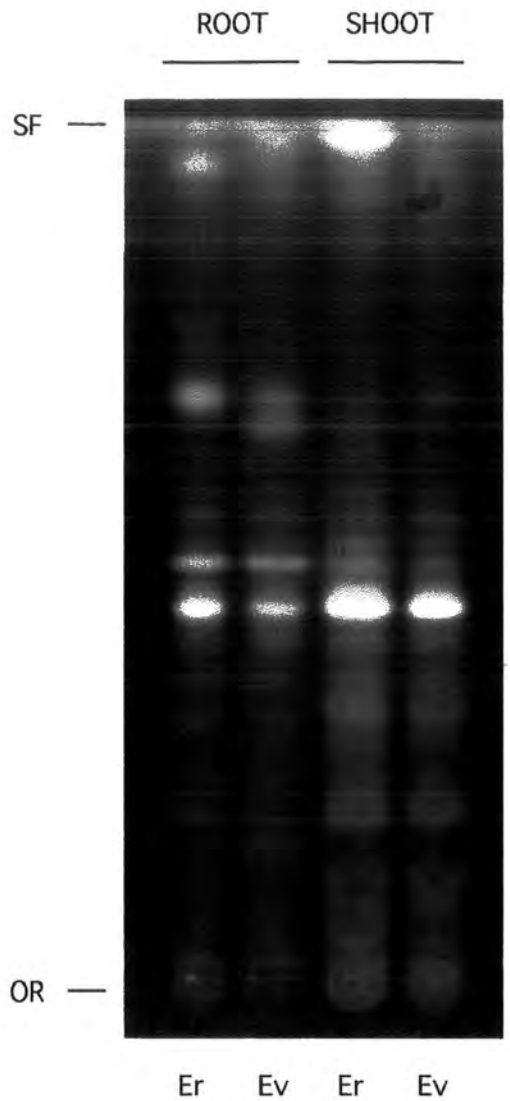


Fig. 4.1 Tlc analysis of crude saponin extracts from root and shoot tissues of alfalfa seedlings of cultivars Europe (Er) and Euver (Ev). Tlc plate visualised and photographed under uv light.
OR = origin; SF = solvent front.

Rf	Erp	Evr	Intensity	Fluorescence Colour
0.04	*	*	++	bl-gr
0.07	*	*	++	bl-gr
0.12	*	*	+	bl
0.15	*	*	+++	gr
0.19	*	*	+	bl
0.32	*	*	+	
0.40	*	*	+	
0.43	*	*	+++	yl
0.49	*	*	+++	bl
0.51	*	*	++	bl
0.53	*	-	+++	
0.53	-	*	++	
0.54	*	-	+	
0.63	*	-	+	
0.67	*	-	+	
0.91	*	-	+++	bl
0.91	-	*	+	bl
0.94	*	*	+	yl
0.97	*	-	+++	yl
1.00	*	*	++	yl

Table 4.1. R*f* values of spots detected in crude saponin extract from alfalfa shoot tissue after TLC.

Explanation of symbols:

Erp = Cultivar Europe; Evr = Cultivar Euver

* = Presence of spot; - = Absence of spot

bl = blue; gr = green; yl = yellow

(colour of fluorescence; spot dark if no colour noted)

+++ = High intensity; ++ = Medium intensity; + = Low intensity

Rf	Erp	Evr	Intensity	Fluorescence Colour
0.16	*	*	+	
0.19	*	*	+	
0.31	*	*	+	
0.33	*	*	+	
0.39	*	*	+	
0.43	*	*	+++	yl
0.49	*	*	+++	yl
0.54	*	*	+	bl
0.64	-	*	+++	bl
0.67	*	-	+++	gr
0.67	-	*	++	gr
0.96	*	-	+++	bl
1.00	*	*	+	

Table 4.2. R*f* values of spots detected in crude saponin extract from alfalfa root tissue after TLC.

Explanation of symbols:

Erp = Cultivar Europe; Evr = Cultivar Euver

* = Presence of spot; - = Absence of spot

bl = blue; gr = green; yl = yellow

(colour of fluorescence; spot dark if no colour noted)

+++ = High intensity; ++ = Medium intensity; + = Low intensity

4.1.2. Hydrolysis of Alfalfa Saponins

Crude saponin extracts from the root and shoot tissues of alfalfa seedlings were acid hydrolysed. Sapogenins were extracted into ethyl acetate and analysed by TLC as above. This showed that hydrolysis had taken place, as the spots previously observed had disappeared, and instead spots running near the solvent front were present (Fig. 4.2A). TLC on silica plates in petroleum Et_2O -chloroform-acetic acid (7:2:2) was used to separate sapogenins; spots were visualised as previously. Both root sapogenin samples showed a single major yellow spot at R*f* 0.30. Yellow spots of lower intensity at higher R*f* values were also present. The shoot sapogenin samples contained a very intense dark green spot at R*f* 0.11; the next most intense spot was at R*f* 0.61 (yellow). Minor spots at higher R*f* values were also present. Many spots at R*f* > 0.6 in both extracts (root and shoot) appeared to have the same R*f* values. No differences were apparent in the patterns of spots given by the two cultivars. These results are shown in Fig. 4.2B and summarised in Tables 4.3 and 4.4.

The major sapogenins in root and shoot tissues were considered to be represented by the spots at R*f* 0.30 and 0.61 respectively.

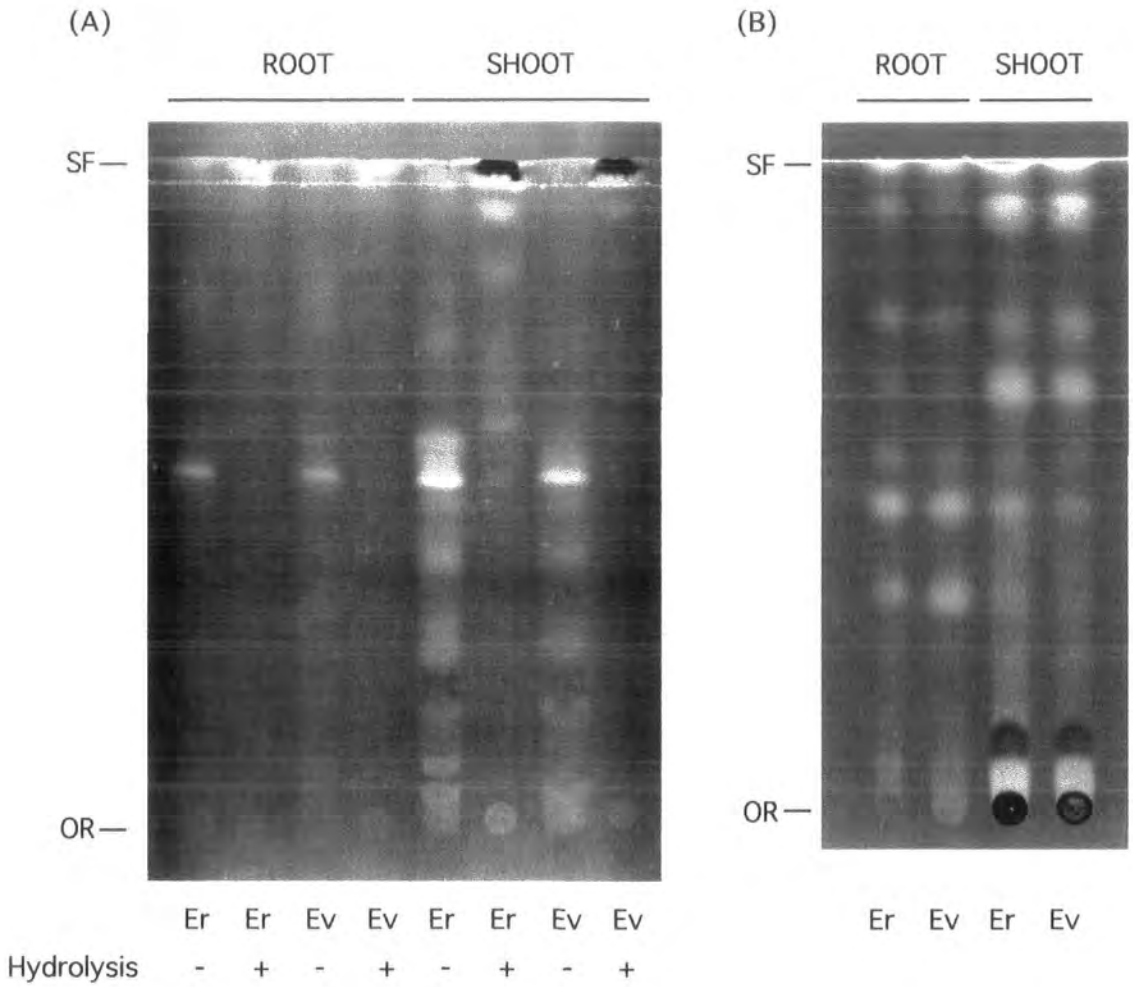


Fig. 4.2 Hydrolysis of compounds in crude saponin extracts from root and shoot tissues of alfalfa seedlings of cultivars Europe (Er) and Euver (Ev). Tlc plate visualised and photographed under uv light.
OR = origin; SF = solvent front.
(A) Analysis of extracts before (-) and after (+) hydrolysis under tlc conditions for saponins, showing material running at the solvent front after hydrolysis.
(B) Analysis of extracts after hydrolysis under tlc conditions for sapogenins (ether-chloroform-acetic acid solvent).

Rf	Erp	Evr	Intensity	Fluorescence Colour
0.05	*	*	+++	bl
0.11	*	*	+++	gr
0.22	*	*	+	
0.30	*	*	+	
0.44	*	*	++	bl
0.52	*	*	+	
0.61	*	*	+++	yl
0.71	*	*	++	yl
0.84	*	*	++	yl
0.87	*	*	++	yl
0.92	*	*	++	yl
1.00	*	*	++	yl

Table 4.3. R*f* values of putative sapogenin spots detected in hydrolysed saponin extract from alfalfa shoot tissue after TLC.

Explanation of symbols:

Erp = Cultivar Europe; Evr = Cultivar Euver

* = Presence of spot; - = Absence of spot

bl = blue; gr = green; yl = yellow

(colour of fluorescence; spot dark if no colour noted)

+++ = High intensity; ++ = Medium intensity; + = Low intensity

Rf	Erp	Evr	Intensity	Fluorescence Colour
0.05	*	*	++	bl
0.22	*	*	+	
0.30	*	*	++	yl
0.44	*	*	++	bl
0.52	*	*	+	yl
0.61	*	*	+	yl
0.71	*	*	++	yl
0.84	*	*	++	yl
0.87	*	*	++	yl
0.92	*	*	++	yl
1.00	*	*	++	yl

Table 4.4. *Rf* values of putative sapogenin spots detected in hydrolysed saponin extract from alfalfa root tissue after TLC.

Explanation of symbols:

Erp = Cultivar Europe; Evr = Cultivar Euver

* = Presence of spot; - = Absence of spot

bl = blue; gr = green; yl = yellow

(colour of fluorescence; spot dark if no colour noted)

+++ = High intensity; ++ = Medium intensity; + = Low intensity

4.1.3. Purification of Alfalfa Saponins from Crude Extract by Reverse-Phase Chromatography

Chromatography of methanolic extracts of alfalfa tissue on disposable reverse-phase columns has been suggested in the literature (Nowacka *et al.*, 1994; Oleszek 1988; Nowacka and Oleszek 1992; Oleszek and Jurzysta 1990) as a one-step method for saponin purification. Aliquots of crude extract (after evaporation and re-dissolving in water) were loaded onto a C-18 column (Sep-Pak cartridge) that had been equilibrated with water. The column was washed with water, and eluted with a step gradient in MeOH : d.H_2O mixtures. Eluted material was collected from each solvent step, evaporated to dryness, and analysed by TLC (ethyl acetate-distilled water-acetic acid solvent).

When the shoot extract was separated by this technique, components giving dark (under usual light) and green (under UV light) coloured spots with Rf < 0.2 were eluted at low MeOH concentrations (up to 20%). Components giving fluorescent blue spots with higher Rf values (> 0.6) also eluted at low MeOH concentrations (up to 30%). The major yellow (UV) spot at Rf approx. 0.4 eluted in the range of MeOH concentrations 30-70%, with most of the material eluting in the 40-60% MeOH elution steps. No detectable material eluted at MeOH concentrations above 70%. These results are shown in Fig. 4.3.

Root extract showed a similar separation of components on Sep-Pak chromatography. Fluorescent blue or green spots with high Rf values (> 0.6) eluted at low MeOH concentrations (up to 50%) whereas the two yellow spots at Rf 0.43 and 0.49 eluted in the MeOH concentration range 40% to 70%, with most of the material eluting in the 50% and 60% MeOH washes. Results are shown in Fig. 4.4.

This technique gave good separations of the different components present in the extracts, but could only be used on a small scale, as the column was overloaded by moderate amounts of extract. A large-scale separation technique for the saponins and other components in the extracts was thus investigated.

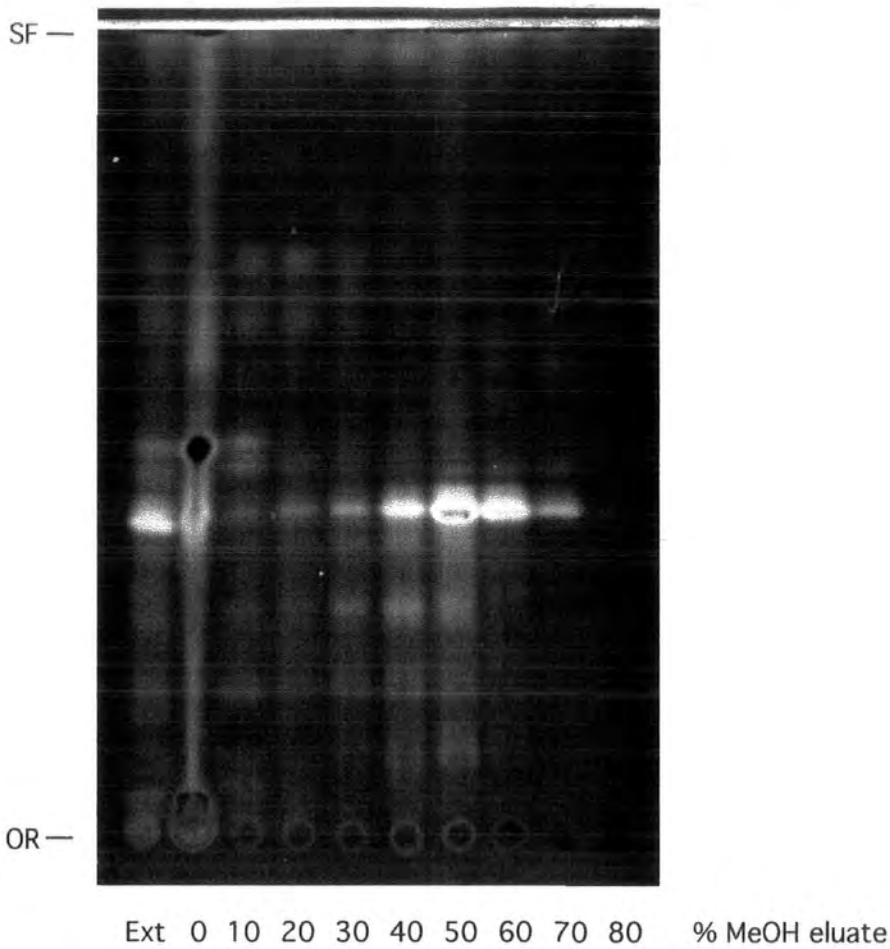


Fig. 4.3 Separation of crude saponin extract from alfalfa seedling shoots (cv. Euver) on Sep-Pak reverse phase cartridge. Tlc analysis visualised and photographed under uv. light.
Ext = crude saponin extract
OR = origin; SF = solvent front

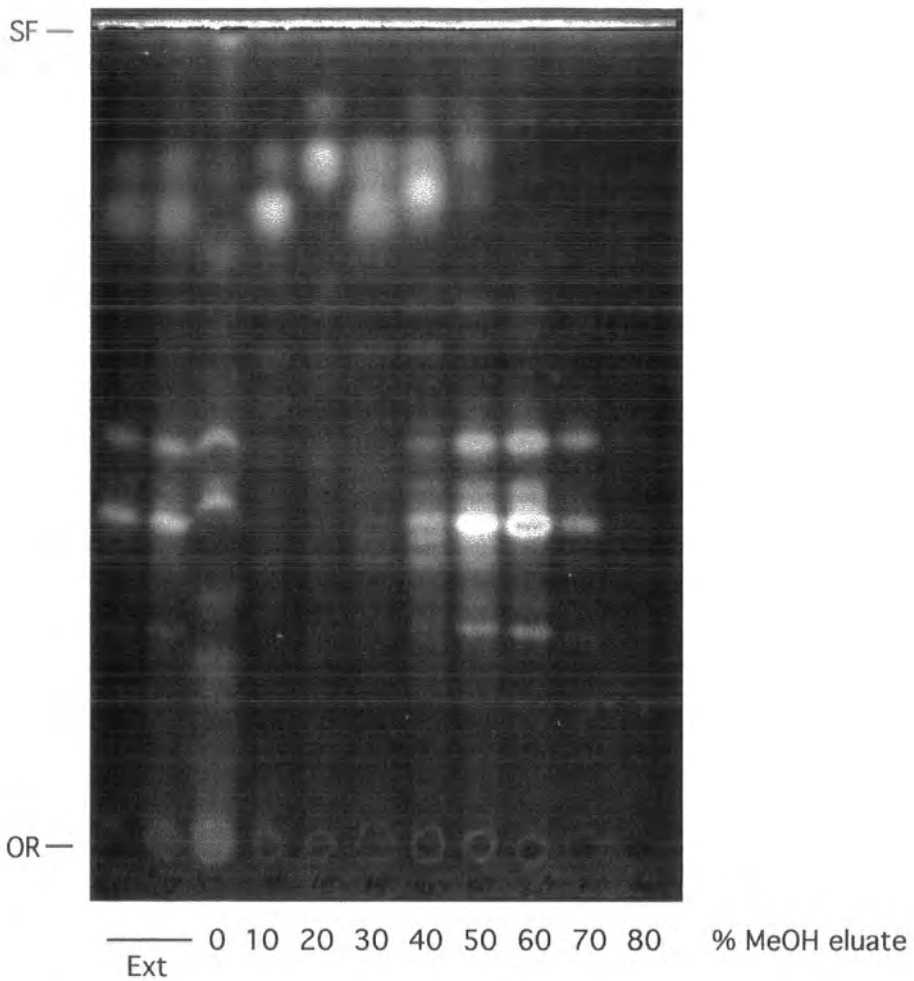


Fig. 4.4 Separation of crude saponin extract from alfalfa seedling roots (cv. Euver) on Sep-Pak reverse phase cartridge. Tlc analysis visualised and photographed under uv light.
Ext = crude saponin extract
OR = origin; SF = solvent front

4.1.4. Solvent Fractionation as a Purification Method for Alfalfa Saponins

The crude extracts from alfalfa tissues were purified by partition between *n*-BuOH and water, followed by precipitation with $_{Et2O}$. Both these techniques have been employed in purification schemes for saponins previously (Wyman-Simpson 1991; Massiot *et al.*, 1994); all saponins, except the most polar tridesmosides of zanhic acid, partition from the aqueous layer into the *n*-BuOH layer during the first step, as they are relatively non-polar, and thus this step removes polar small molecules. Precipitation of saponins by addition of Et_2O to MeOH solution is based on their relatively high polarity, due to the attached glycoside residues, compared to soluble non-polar small molecules. A series of trial experiments was carried out to ensure that saponins were not being lost during either of these two steps.

The 80% MeOH tissue extracts were evaporated to dryness at 40°C and re-dissolved in water prior to extraction 3 times with water-saturated *n*-BuOH. The resulting aqueous and *n*-BuOH phases were pooled, concentrated by evaporation, and analysed by TLC. In both root and shoot extracts, this technique retained almost all of the components tentatively identified as saponins in the *n*-BuOH layer, with no qualitative change in the saponin spots being observed. However, spots provisionally identified as belonging to phenolic compounds, and certain leaf pigments, were removed into the aqueous layer (results not presented).

For Et_2O precipitation, the *n*-BuOH layer was evaporated and re-dissolved in MeOH. Precipitation with Et_2O retained the saponin components in the precipitate, which, if washed carefully, could be isolated as a cream powder. Dark green leaf pigments were not precipitated by this technique, and only small amounts of putative saponins were left in the supernatant when analysed by TLC.

The combination of *n*-BuOH extraction and $_{Et2O}$ precipitation removed most of the non-saponin contaminants which could be visualised on TLC plates after spraying with the saponin reagent, and did not appear to cause any qualitative differences in the pattern of spots of putative saponins on TLC. However, it is possible that some minor components, particularly more polar saponins, could have been lost at the *n*-BuOH

Fig. 4.5 Purification of saponins from alfalfa seedling root and shoot tissue. Analysis by tlc, visualised and scanned under normal light (0 = origin, 1 = solvent front).

Tracks A,B: saponins from shoot tissue.

Tracks C,D: saponins from root tissue.

Tracks A,D: saponin spots from "crude saponin extracts"

Tracks B,C: saponin spots from "saponin mixtures" after butanol extraction and ether precipitation.

extraction stage. There was also carry over of non-saponin compounds into the $_{Et2O}$ precipitate; it still contained some sugars, and appeared to contain polar lipids. The precipitate from Et_2O was designated "saponin mixture", and was used for subsequent bioassays. TLC analyses of the crude and purified saponins are shown in Fig. 4.5.

4.1.5. Purification of Alfalfa Saponins by High Performance Liquid Chromatography (HPLC)

To purify individual saponins from the mixture produced after n-BuOH extraction and $_{Et2O}$ precipitation, chromatography on a preparative scale reverse phase column (C-18) was employed. Saponin mixtures were applied in aqueous solution to this column, and were eluted with a gradient of iso-propanol. Eluted fractions were then analysed by TLC.

Results for separation of components in the saponin mixture from alfalfa seedling shoots are shown in Fig. 4.6, and for the separation of components present in the saponin mixture from alfalfa seedling roots in Fig. 4.7. The profile of absorbance at 216 nm shows that most of the UV-absorbing material elutes early on the gradient, but that this peak of absorbance does not include the major saponin components. The nature of these components was not investigated. The saponins elute later on the gradient (35-70% iso-propanol). Material was left on the column after the separation, and could be eluted with 100% iso-propanol; this appeared to be a polar lipid mixture. The HPLC step thus not only separated the saponins, but also removed contaminants not detected on TLC.

On the basis of the saponin spots shown by TLC, two series of fractions from root and shoot saponins were designated. Fractions were labelled "S" and "R" for shoot and root respectively, and were lettered a-g in order of elution from the column. Subsequent preparative scale separations were used to prepare amounts of these fractions sufficient for bioassays against potato aphids, to assess the toxicities of the different saponin components. These fractions are described in more detail below.

The shoot saponin fractions are shown in Fig. 4.8. The saponin mixture

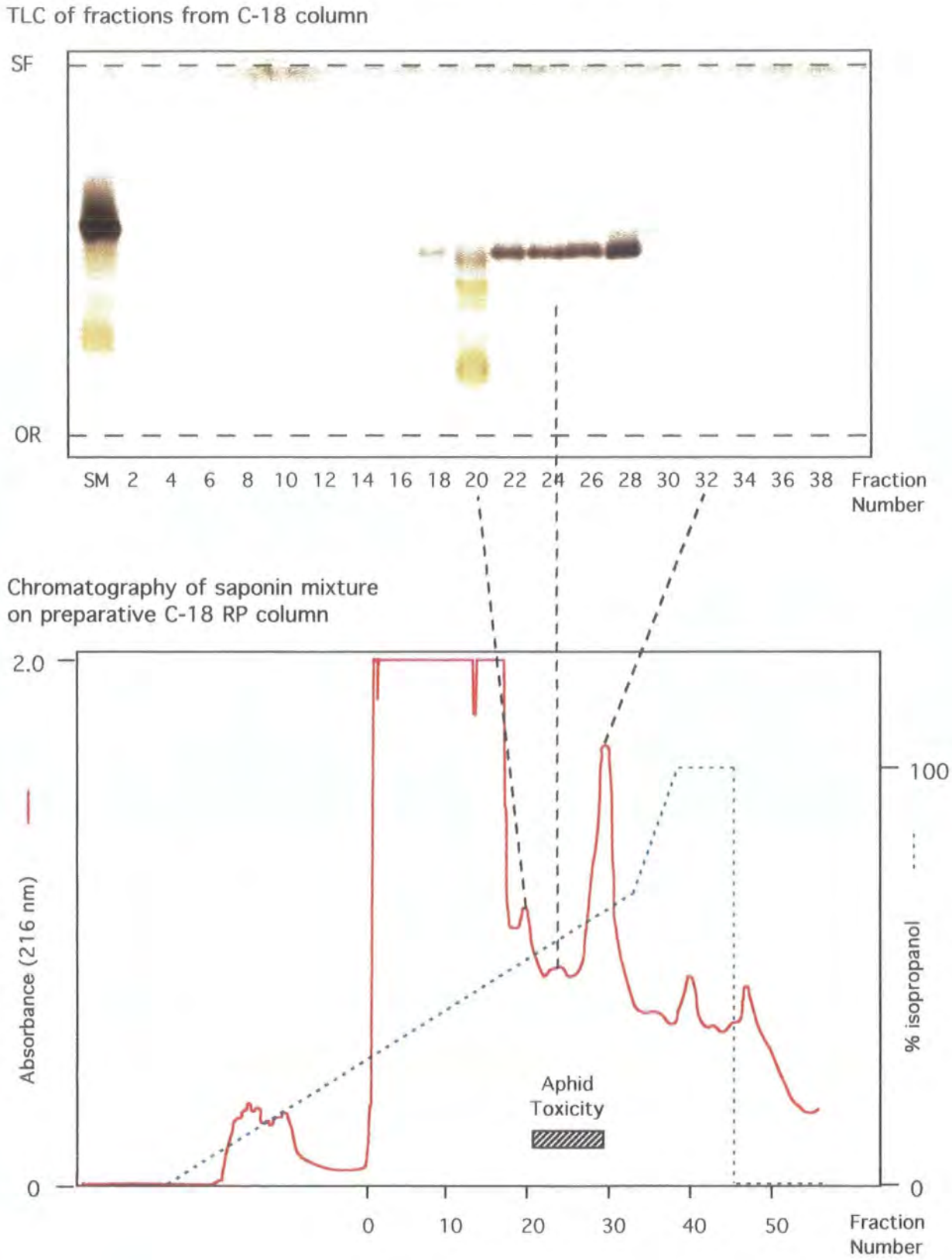


Fig. 4.6 Separation of shoot saponin mixture by chromatography on C-18 reverse phase column. The horizontal diagonally striped bar in the elution profile indicates fractions from the whole separation (not just those numbered above) shown in a separate experiment to contain compounds toxic to aphids.
SM = Saponin mixture (starting material); OR = origin; SF = solvent front.

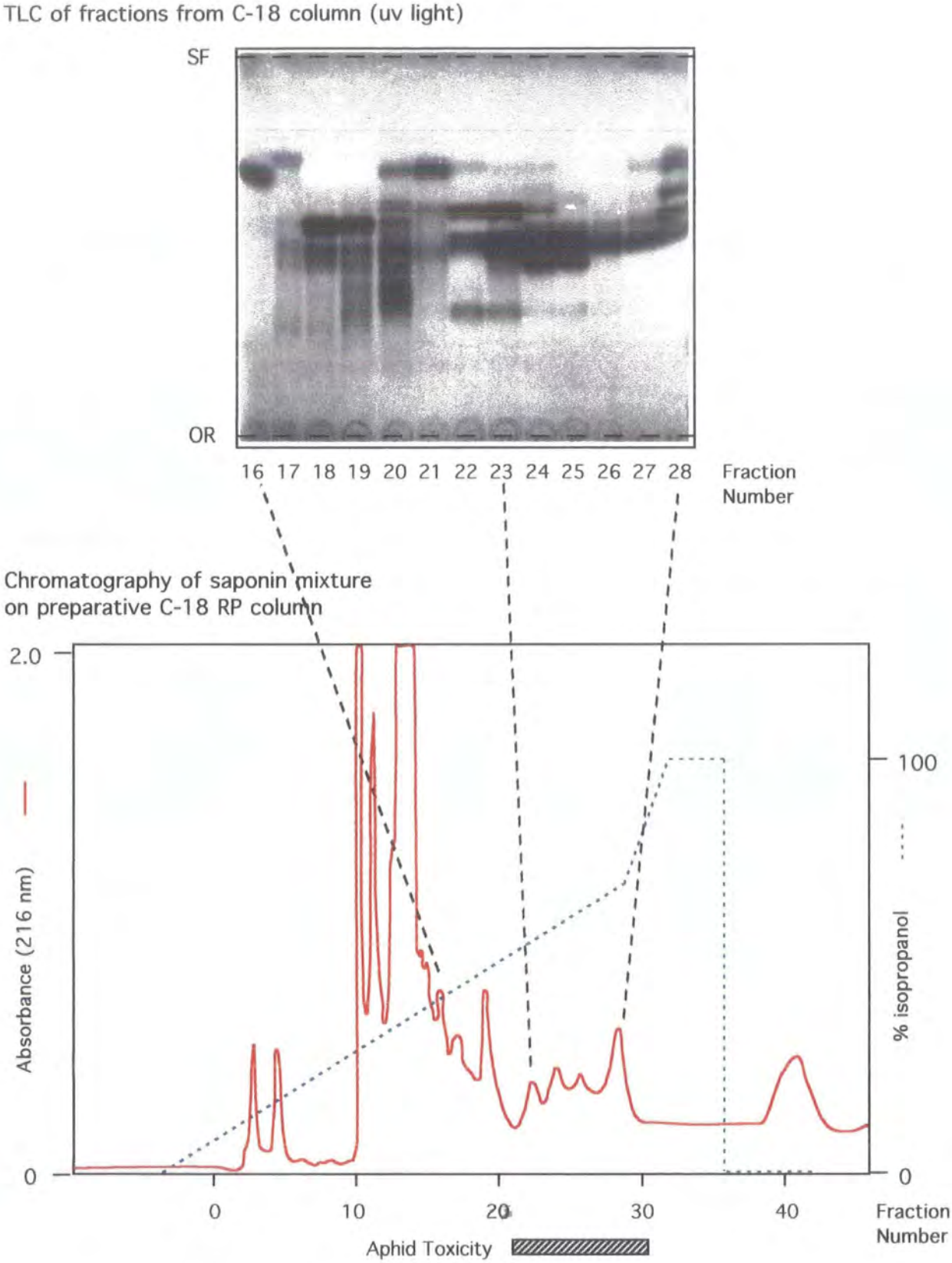


Fig. 4.7 Separation of root saponin mixture by chromatography on C-18 reverse phase column. The horizontal diagonally striped bar in the elution profile indicates fractions from the separation shown in a separate experiment to contain compounds toxic to aphids. The tlc plate in this separation is viewed under uv light; note fluorescent compounds (phenolics?) in fractions 18 and 19.
OR = origin; SF = solvent front.

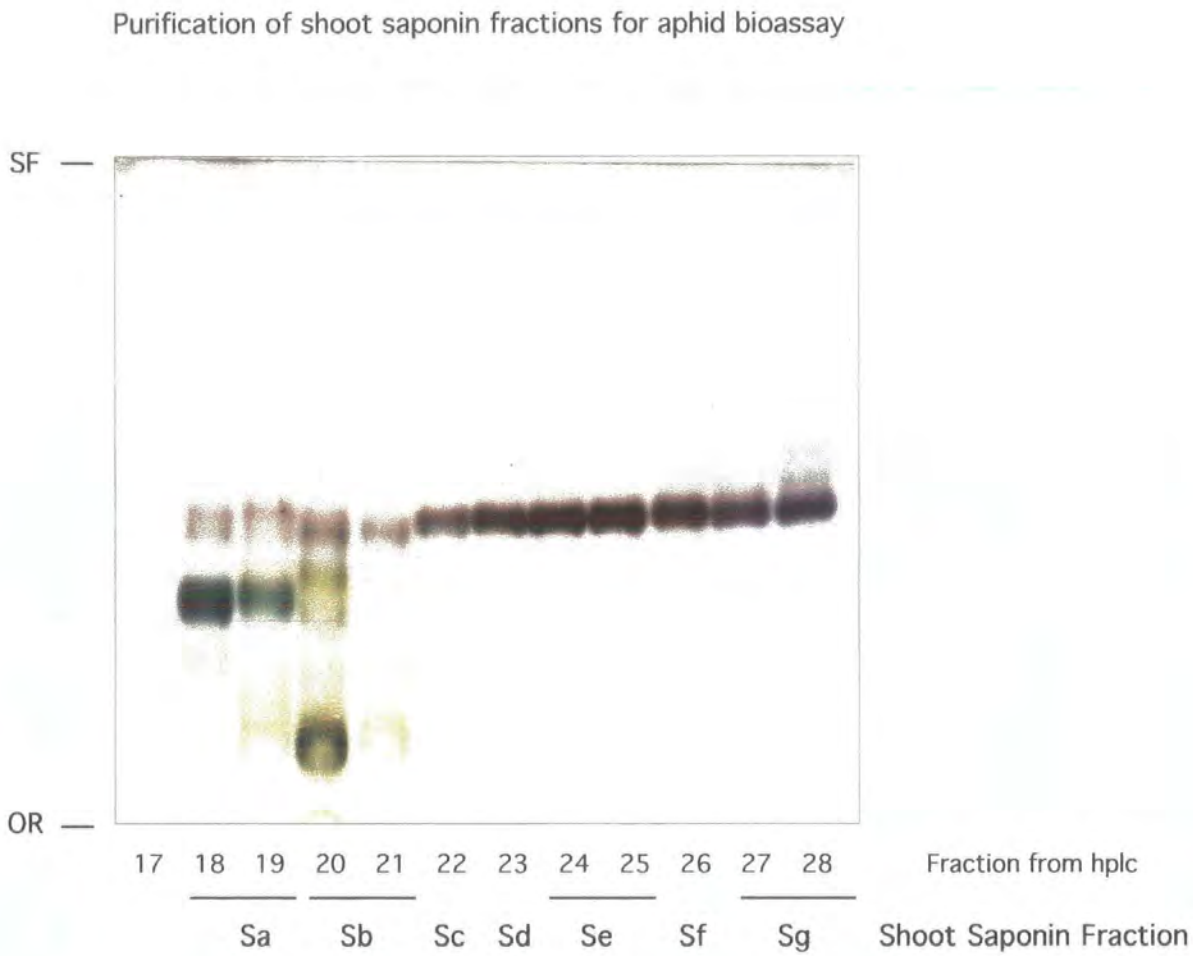


Fig. 4.8 Purification of shoot saponin fractions by reverse-phase chromatography for bioassay against potato aphids. Tlc analysis of fractions (5ml) from C-18 column is shown; fractions pooled for bioassay fractions **Sa-Sg** are indicated.
SF = solvent front; OR = origin.

contained a fairly simple pattern of spots, and several fractions resulting from the separation appeared to contain only a single component.

Sa; two spots on TLC, at R*f* 0.35 (green-blue) and 0.50 (brown-green)

Sb; green spots on TLC, at R*f* 0.14 (major), 0.33, 0.37 (weak); brown-green spot at R*f* 0.50 (weak)

Sc; brown-green spot at R*f* 0.50

Sd; brown spot at R*f* 0.50

Se; red-brown spot at R*f* 0.50

Sf; purple-brown spot at R*f* 0.50;

Sg; purple spot at R*f* 0.56 with fainter purple spots at lower and higher R*f* of these fractions, Sa, Sb, Sf and Sg clearly contained more than one saponin component, although Sf contained only small amounts of the second component. Sg was isolated only in small amounts. Fractions Sc, Sd and Se appeared to contain only one component, although the colour of the spot at R*f* 0.50 changed slightly across the fractions. This band corresponded to the major saponin band observed in crude and partially purified shoot saponins (the slightly different R*f* previously observed, 0.43, was found to be due to differing conditions on TLC).

The root saponin fractions are shown in Fig. 4.9. In this case, a more complex pattern of spots was observed, suggesting that a more diverse mixture of saponins was present in root tissue. The green spots at R*f* < 0.5 were not present; instead a series of purple spots at R*f* > 0.5 were observed. It was not found possible to obtain any fractions containing only a single saponin component by HPLC. A typical separation of saponins from roots is shown in Fig. 4.7.

Ra; purple spots at R*f* 0.56 (major); brown spot at R*f* 0.50 (faint);

Rb; purple spot at R*f* 0.58 (major), 0.38 (faint); brown spot at R*f* 0.50;

Rc; brown spot at R*f* 0.50; purple spot at R*f* 0.47;

Rd; purple-brown spot at R*f* 0.50 (major); purple spots at R*f* 0.55, 0.63, 0.68 (faint)

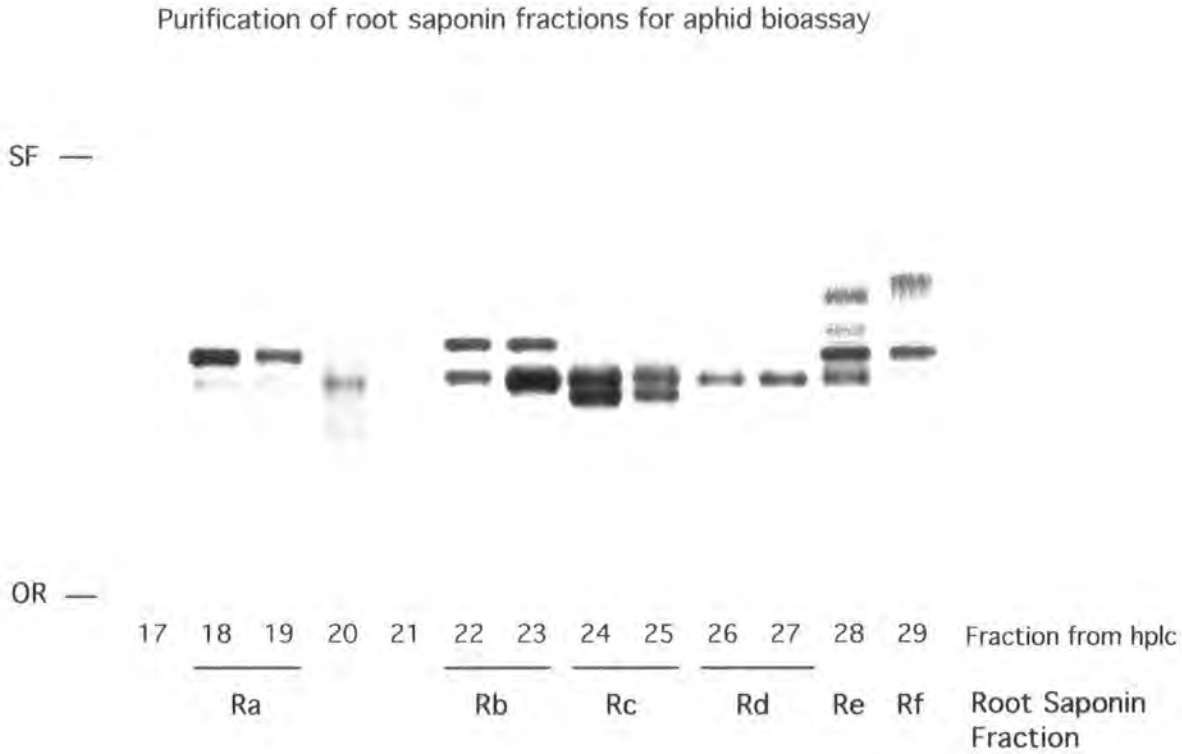


Fig. 4.9 Purification of root saponin fractions by reverse-phase chromatography for bioassay against potato aphids. Tlc analysis of fractions (5ml) from C-18 column is shown; fractions pooled for bioassay fractions **Ra-Rf** are indicated. SF = solvent front; OR = origin.

Re; purple spots at R*f* 0.54 (major), 0.68, 0.60 (last faint); brown spot at R*f* 0.50

Rf; purple spots at R*f* 0.54 (major), 0.71, 0.68

In these fractions, the spots at R*f* 0.5 in **Rb-Re** corresponded to the spots at R*f* 0.50 in shoot fractions **Sc-Sf**, respectively.

The saponin fractions collected after HPLC were evaporated to dryness, and stored as solids.

4.1.6. Identification of Alfalfa Saponins using Reference Compounds

A series of standard saponins of known structure were obtained, and were used to tentatively identify saponin spots on TLC, using similar R*f* and similar spot colour after spraying with the acetic anhydride-sulphuric acid reagent as criteria for identification. Results are presented in Fig. 4.10.

Two monodesmoside saponins were provided as standards, and both were present in the alfalfa saponin mixtures. 3-O-β-D-glucopyranosyl medicagenic acid (medicoside A; compound 16, Massiot *et al.*, 1988a) gave a purple spot at R*f* 0.59. This compound was present in the root saponin mixture as a significant but not major component, and purified into fractions **Re and Rf** (eluted with 60-68% iso-propanol). It was not detectable in the shoot saponin mixture. The second monodesmoside contained soyasapogenol B as the aglycone; 3-O-α-L-rhamnopyranosyl (1->2)-β-D-galactopyranosyl (1->2)-β-D-glucuronopyranosyl soyasapogenol B (soyasaponin I; Massiot *et al.*, 1988a) gave a red-brown spot at R*f* 0.33 (under these conditions). Comparison with the root and shoot saponin mixtures showed that this compound appears to be the major saponin present in both tissues, previously described as having R*f* 0.43. This compound was purified in fractions **Sc-Sf**, and **Rb-Rd** (eluted with 45-56% iso-propanol).

The bisdesmoside saponin 3-O-β-D-glucopyranosyl (1->2)- β-D-glucopyranosyl]-28-O-[β-D-xylopyranosyl(1->4)- α-L-rhamnopyranosyl(1->2)-α-L-

Identification of saponins in alfalfa root and shoot extracts using reference compounds

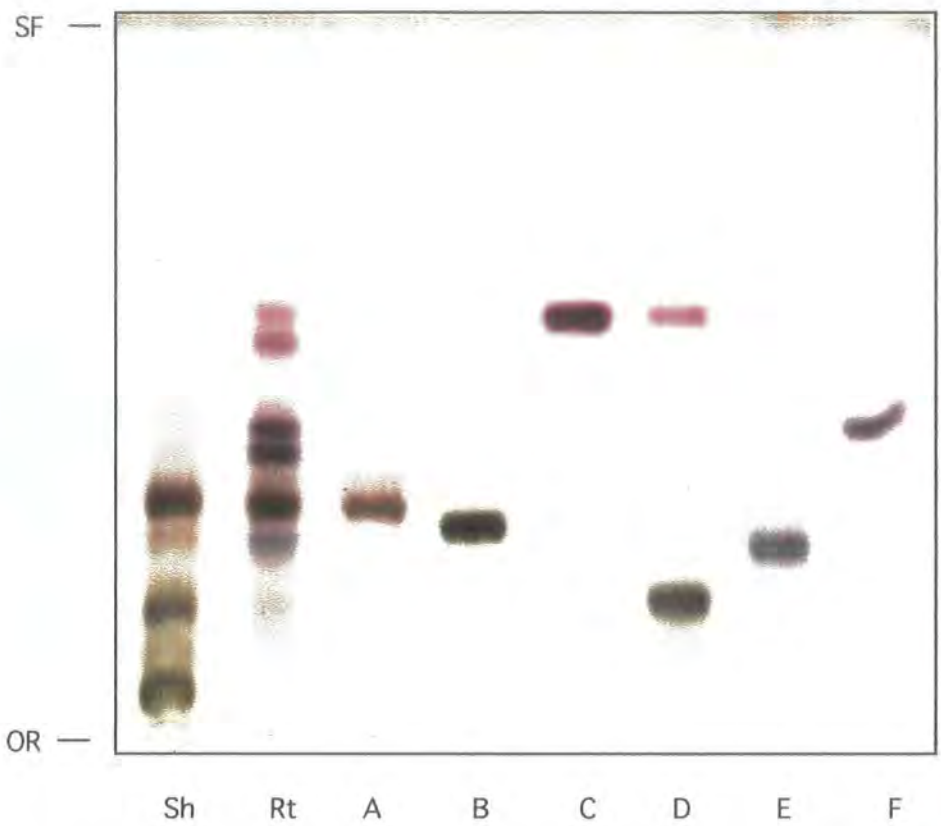


Fig. 4.10 Identification of saponins present in alfalfa seedling roots and shoots by tlc using reference compounds. OR = origin; SF = solvent front.

Sh = Shoot saponin mixture

Rt = Root saponin mixture

A = Soyasaponin I (3-O-α-L-rhamnopyranosyl (1->2)-β-D-galactopyranosyl (1->2)-β-D-glucuronopyranosyl soyasapogenol B)

B = Medicoside J (3-O-β-D-glucopyranosyl-28-O-[β-D-glucopyranosyl (1->4)- α-L-rhamnopyranosyl (1->2)-α-L-arabinopyranosyl] medicagenic acid)

C = Medicoside A (3-O-β-D-glucopyranosyl medicagenic acid)

D = 3-O-[β-D-glucopyranosyl (1->2)- β-D-glucopyranosyl]-28-O-[β-D-xylopyranosyl(1->4)- α-L-rhamnopyranosyl(1->2)-α-L-arabinopyranosyl] medicagenic acid

E = Medicoside I (3-O-[α-L-arabinopyranosyl (1->2)-β-D-glucopyranosyl (1->2)-α-L-arabinopyranosyl]-28-O-β-D-glucopyranosyl medicagenic acid)

F = Medicoside G (3-O-β-D-glucopyranosyl-28-O-β-D-glucopyranosyl medicagenic acid)

arabinopyranosyl] medicagenic acid (compound 7 of Massiot *et al.*, 1988b) produced an olive green spot at R*f* 0.19 (under TLC conditions used). An additional, fainter purple spot at R*f* 0.60 was also present in this sample, which could be identified as a 3-O-β-D-glucopyranosyl medicagenic acid by comparison with the other reference compounds. The additional spot had probably resulted from partial hydrolysis of the bisdesmoside. The shoot saponin mixture contained this compound as a major component; it is present in fraction **Sb** (eluted with 44% iso-propanol).

A second bisdesmoside component, structurally identified as 3-O-[α-L-arabinopyranosyl (1->2)-β-D-glucopyranosyl (1->2)-α-L-arabinopyranosyl]-28-O-β-D-glucopyranosyl medicagenic acid (medicoside I; compound 4, Massiot *et al.*, 1988b), was detected as a blue-grey spot at R*f* 0.26. This compound appeared to be a minor component of the root saponin mixture, present in fraction **Rc** (eluted with 52-56% iso-propanol). A further bisdesmoside, 3-O-β-D-glucopyranosyl-28-O-β-D-glucopyranosyl medicagenic acid (medicoside G; compound 1, Massiot *et al.*, 1988b) was detected as a purple spot at R*f* 0.43. This spot is probably present in fractions **Re** anf **Rf** (eluted with 60-68% iso-propanol); although spots of similar mobility are present in fractions **Ra** and **Rb**, the relatively low polarity of medicoside G (which only contains a single sugar residue, and is more mobile on TLC) would suggest that it is eluted from the column at higher concentrations of iso-propanol. The bisdesmoside 3-O-β-D-glucopyranosyl-28-O-[β-D-glucopyranosyl (1->4)- α-L-rhamnopyranosyl (1->2)-α-L-arabinopyranosyl] medicagenic acid (medicoside J; compound 6, Massiot *et al.*, 1998b) gave a dark olive-coloured spot at R*f* 0.29, but did not appear to be present in the root or shoot saponin mixtures.

Although the standards allowed the tentative identification of some of the root and shoot saponins, some spots, notably two more mobile purple spots in the root saponin mixture and present in fractions **Ra** and **Rb**, remain unidentified.

4.1.7. Confirmation of Structures of Alfalfa Saponins by NMR

Fractions **Sf** and **Rd** were re-purified on the HPLC system described above, using a narrow range gradient of 45-55% iso-propanol, to produce material for structural analysis. Whereas re-chromatography of **Sf** resulted in a peak, which gave a single spot on TLC analysis, **Rd** could not be purified to a single spot by this technique, and thus was not characterised further. The repurification of fraction **Sf** to give a single component is shown in Fig. 4.11.

The structure of compound **Sf**, the major saponin component of alfalfa seedling roots and shoots, identified by comparison with standards as soyasaponin I, was confirmed by NMR. ^{13}C NMR was carried out on fraction **Sf** in Durham, but the spectrum was too weak to analyse further. A sample of **Sf** was also analysed by Dr. Massiot, and the NMR spectrum of the compound was consistent with the identification as soyasaponin I.

4.1.8. Qualitative Comparison of Root and Shoot Saponins in Alfalfa Plants of Different Ages

Crude saponin extracts were prepared from root and shoot tissue of alfalfa plants (cultivar Europe) at three different stages of plant development; 14 days after germination (seedling), 40 days after germination (immature) and 70 days after germination (mature). The extracts were subsequently analysed by TLC; results are shown in Fig. 4.12.

The spot pattern given by root saponins did not change greatly with the age of the plant except for two spots of low mobility, neither of which could be identified by comparison with standards. An olive-coloured spot at R*f* 0.37 became less intense as the plants aged, whereas another olive spot at R... 0.23 became more intense, so that it became a major component in the mature plants, whereas in the seedlings it had only been a minor component. Two purple spots of higher mobility were also present as minor components in seedlings and increased in relative amount as development progressed.

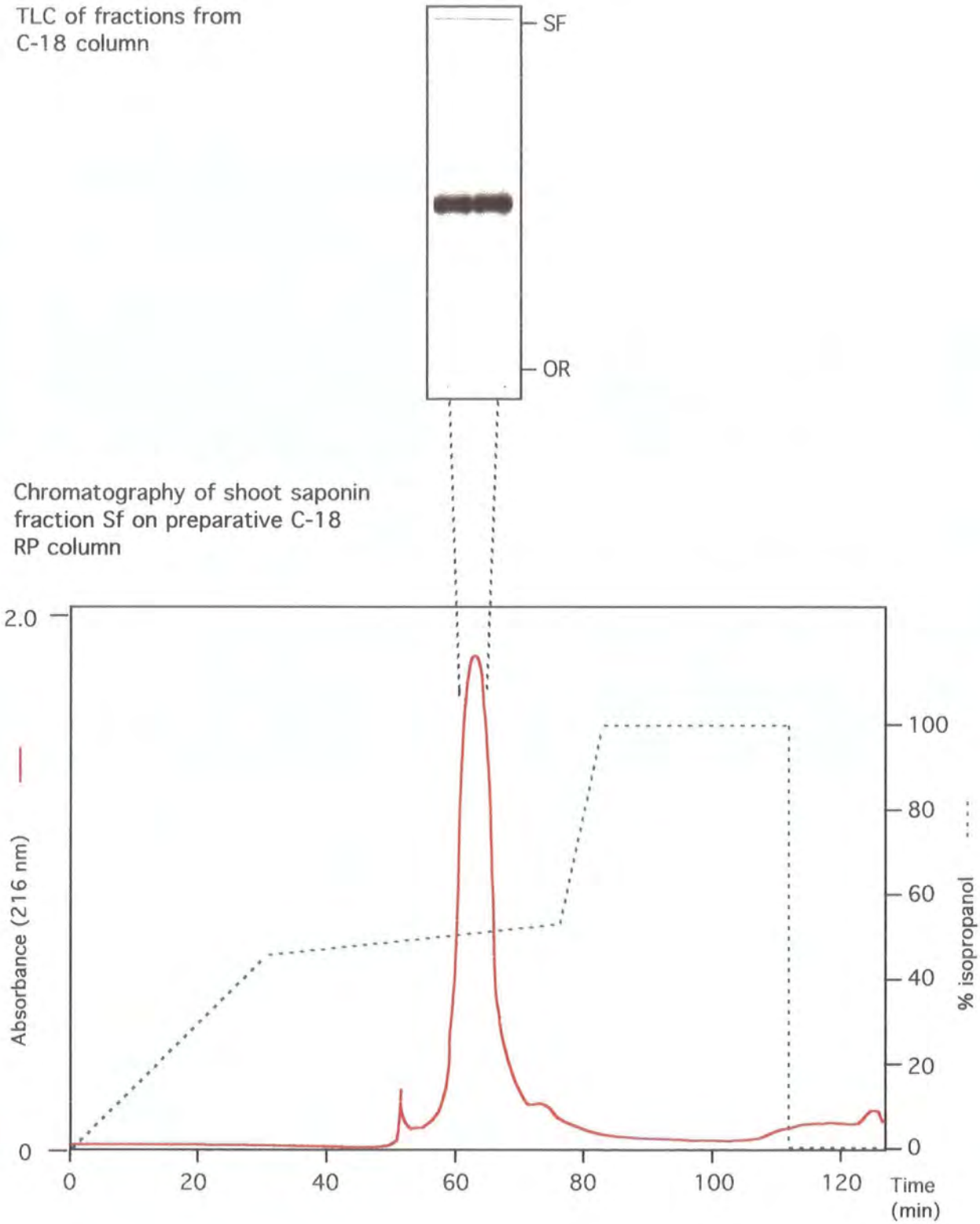


Fig. 4.11 . Chromatography of shoot saponin fraction Sf on C-18 reverse phase column. Fractions from the peak were pooled to give the sample used for nmr.

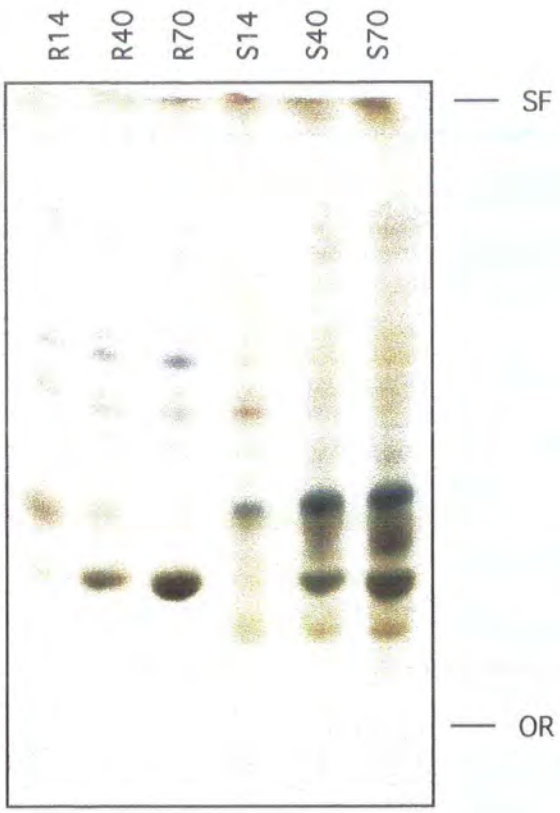


Fig. 4.12 Crude saponin extracts from roots and shoots (aerial parts) of plants of alfalfa var. Euver at different ages after germination. Saponins analysed by tlc, viewed under normal light.
R = root tissues; S = shoot tissues
14 = 14 day old plants (seedlings); 40 = 40 day old plants; 70 = 70 day old plants.
OR = origin; SF = solvent front.

The spot pattern given by shoot tissues showed more significant changes with development. As in roots, an olive spot at R*f* 0.23 became a more significant component as development proceeded (present only at trace levels in seedlings). Purple bands of high mobility, one of which was medicoside A, were not detectable in seedlings, but became more significant with development. The brown band identified as soyasaponin I, which was the major shoot saponin in seedlings, declined with development, so that only small amounts of this compound were present in mature plants.

4.1.9. Purification of Saponins from Sugar Beet and Potato

Saponin mixtures were purified from leaves of mature potato and sugar beet plants by extraction, partition into *n*-BuOH, and ether precipitation as described above. In both cases the saponins were recovered as cream-coloured powders, which were analysed by TLC.

Sugar beet leaves gave multiple coloured spots on analysis of crude extracts by TLC, indicating that saponins were present. After purification, saponin mixtures from sugar beet gave two closely spaced spots at R*f* approx. 0.80, 0.82; the less mobile spot was purple-red in colour, the more mobile grey-green (Fig. 4.13). These two spots may be tentatively identified as the two major saponins reported to be present in sugar beet leaves, beta vulgarosides 5 and 9 (Yoshikaum *et al.*, 1996), although confirmation of this identification was not carried out.

Crude extracts of potato leaves also contained numerous coloured spots after TLC. Most of these spots were removed by purification, and the saponin mixture obtained contained two components, of approximately equal amount; a green-brown spot at R*f* 0.33, and a second similar spot at R*f* 0.50 (Fig. 4.14). These spots are probably not saponins. Glycosylated steroidal alkaloids are known to be major secondary compounds in potato; two compounds have been described, α-chaconine and α-solanine, both of which are based on the aglycone solanidine (Price *et al.*, 1987). These compounds will co-purify with saponins, as their hydrophilic/hydrophobic

properties are very similar.

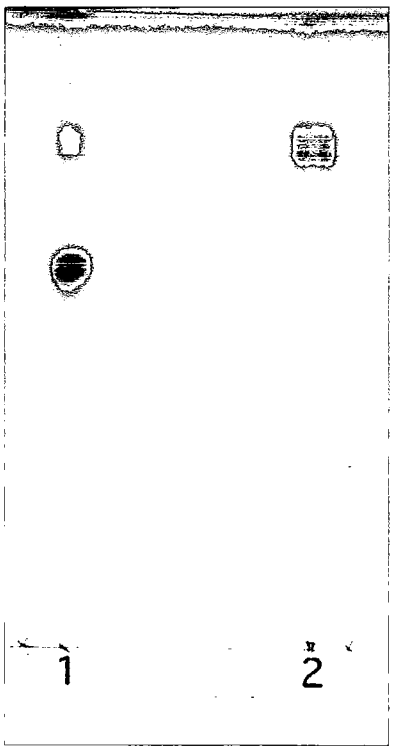


Fig. 4.13. Saponins extracted from sugar beet leaves after separation by tlc.
Track 1; crude saponin extract
Track 2; saponin mixture after butanol extraction and ether precipitation
OR = origin; SF = solvent front.

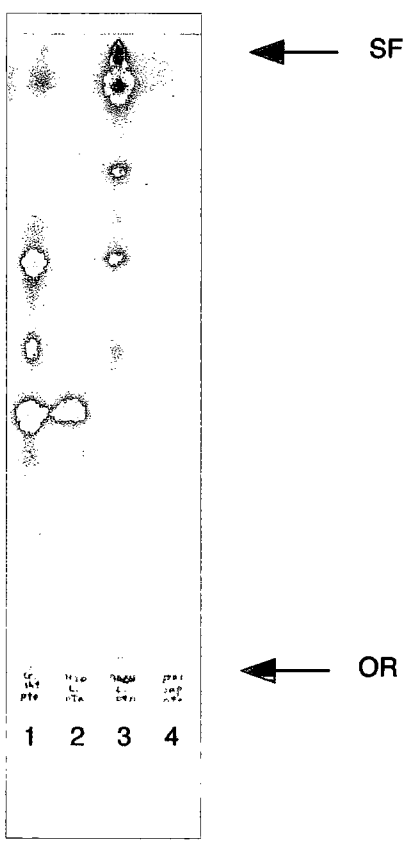


Fig. 4.14. Saponins extracted from potato leaves after separation by tlc.
Track 1; crude saponin extract
Track 2; aqueous layer from butanol partition
Track 3; butanol layer from butanol partition
Track 4; saponin mixture after butanol extraction and ether precipitation
OR = origin; SF = solvent front.

SECTION 2:

4.2. Effects of Saponins from Alfalfa and other Plants on Insects

4.2.1. Effects of Alfalfa Crude Saponin Extract on Larvae of *Callosobruchus maculatus* (Cowpea Seed Weevil)

Crude saponin extracts were prepared from alfalfa root and shoot tissues of cultivar Euver using 80% MeOH as extractant, as described above. The extracts were evaporated to dryness at 40?C and the residue was dissolved in water. The crude saponins were then incorporated into artificial diet pellets for *Callosobruchus maculatus* (based on ground chickpea seed flour). Saponin concentrations were adjusted so that the pellets contained a level equivalent to the alfalfa tissue (i.e. 1 g of artificial diet contained the amount of saponin extracted from 1 g of alfalfa tissue), 10% of that present in alfalfa tissue, and 1% of that present in alfalfa tissue.

Artificial diet pellets were infested with adult weevils, and after eggs had been laid on the pellets, they were separated into individual containers, and larval development of the insect was monitored. The appearance time of the first windows (holes) on pellets was noted and recorded. Numbers of adults and pupae produced from each pellet were measured, and the time to first emergence of adults was also noted. After 41 days the pellets were dissected and all surviving insects were assessed. Results are presented in Fig. 4.15 and Table 4.5.

Control diet pellets supported good development of the seed weevil, with 6.44 ± 0.78 (mean ± SE) surviving insects produced per pellet. Crude saponin extract from root tissues of alfalfa had no deleterious effects on *C. maculatus* development; rather, insect development was enhanced, with numbers of surviving insects increased by approx. 40% by saponin at 100% level and 10% level, and by approx. 100% by saponin at 1% level. In contrast, the crude saponin extract from alfalfa shoots was insecticidal; no insects survived when shoot saponin was incorporated into the diet at 100% level, and only approx. 40% of control insect survival was produced by saponin extract at 10% level. At 1% level of incorporation, the shoot saponin extract enhanced

insect survival by approx. 60%.

The time of adult emergence after initial oviposition was measured on each diet pellet and the mean time of first emergence was calculated for each treatment. The first emergence time on the control diet pellet in saponin bioassay was 30.8 ± 0.6. Among the root saponin treatments only saponin at 100% incorporation level had a significant effect in increasing the first time of adult emergence compared to the control diet pellets to 38.00 ± 1.00 days; the time of first adult emergence in the treatments containing root saponins at 1% and 10% incorporation levels were not significantly different from the control. Chickpea diet pellets incorporating shoot saponins at the 10% and 1% were also found to give an emergence later than control diet pellets (1.5 - 1.8 days later), although the differences were not significant; the effect of the 100% treatment on adult emergence could not be assessed as no insects survived. The results of the first emergence time of adults are summarised in Table 4.5. The time taken to first appearance of pupation "windows" was also increased for root saponins at 100% (by 4 days) and shoot saponins at 10% (by 2.7 days); other treatments either had no effect, or in the case of shoot saponins at 100%, no pupation windows were observed.

The insecticidal effects produced by the shoot saponin extracts were tested for significance using the unpaired t-test, comparing the mean surviving insects produced. The effects were significant at $p < 0.001$ for saponin incorporated at 100% level, and at $p < 0.05$ for saponin incorporated at the 10% level. Similarly, the enhancement of survival was significant for both root and shoot saponin extracts at $p < 0.001$ and $p < 0.05$ respectively. In this assay, whereas root saponin extracts have no insecticidal activity, shoot extracts at levels resembling those found in alfalfa tissues are insecticidal.

The enhancement of survival observed in some treatments in these assays is likely to be due to the presence of simple sugars in the crude saponin extracts. Most sugars are soluble in 80% MeOH. Development of *C. maculatus* larvae is limited by starch digestion, since inhibiting α-amylase activity in this insect by incorporation of α-

Bioassay of *Callosobruchus maculatus* against alfalfa root and shoot saponin preparations (crude saponin extract).

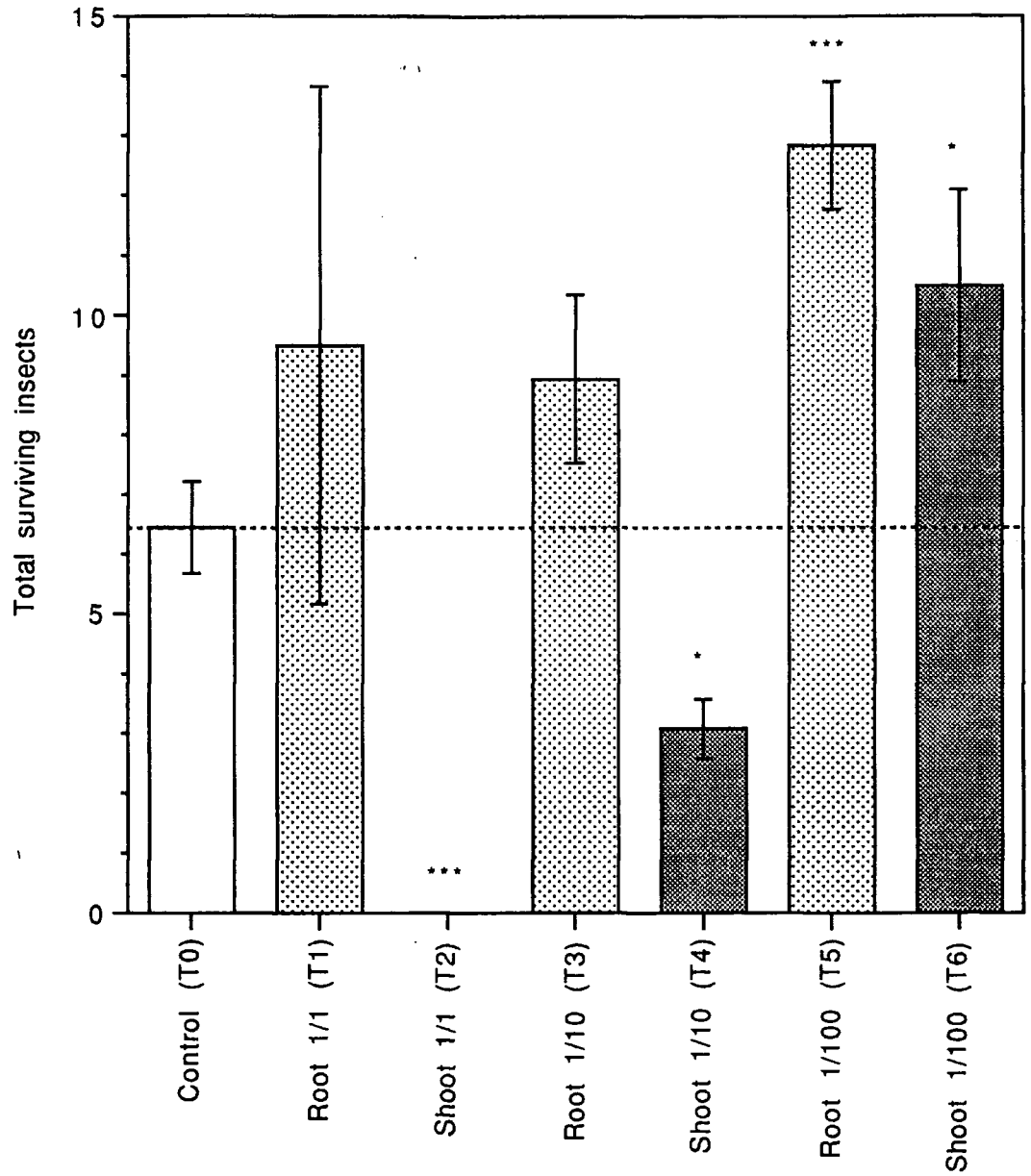


Fig. 4.15. Effects of crude saponin extracts from alfalfa seedling roots and shoots on larvae of *C. maculatus*. Figures 1/1, 1/10, 1/100 indicate estimated dilution of crude saponin extract in diet pellets. Error bars show mean ± S.E. Columns marked with asterisks are significantly different to control at $p<0.05$ (*), $p<0.001$ (***)

amylase inhibitors into artificial diets (Gatehouse *et al.*, 1986), or by producing transgenic plants which express α-amylase inhibitors in the seeds (Gatehouse *et al.*, 1993; Ishimoto *et al.*, 1996) causes mortality of the larvae. Therefore, the addition of the crude saponin extracts effectively supplemented the diet with sugars, and enhanced the survival of larvae by circumventing the limitation on starch digestion. In these assays, therefore, the observed results not only reflect the insecticidal effects of compounds in the extracts, but also beneficial effects.

4.2.2. Effects of Alfalafa Sapogenins on Larvae of *Callosobruchus maculatus* (Cowpea Seed Weevil)

Crude saponin extracts from root and shoot tissues of alfalfa cultivar Euver were hydrolysed as described above (section 3.3.3). The crude sapogenins were then

Treatment	Control	100% shoot saponin	10% shoot saponin	1% shoot saponin	100% root saponin	10% root saponin	1% root saponin
Mean time to appearance of pupation "windows"	23.0	No windows	25.7	22.5	27.0	21.7	21.4
Mean time to first emergence of adults ± SE	30.8 ±0.6	No emergence	32.3 ±1.0	32.6 ±1.3	38.0** ±1.0	30.2 ±1.0	30.8 ±0.9

Table 4.5. Mean times to emergence and pupation of *C. maculatus* in artificial diet pellet bioassay of crude alfalfa saponins. Each treatment contained 25 replicate pellets. Emergence time marked ** is different from control at $p < 0.01$. Other differences are not significant at $p < 0.05$.

Treatment	Control	100% shoot sapo-genin	10% shoot sapo-genin	1% shoot sapo-genin	100% root sapo-genin	10% root sapo-genin	1% root sapo-genin
Mean time to appearance of pupation "windows"	19.0	No windows	23.6	23.1	No windows	22.0	20.6
Mean time to first emergence of adults ± SE	28.4 ±0.4	No emergence	33.8 ±0.6	33.1 ±0.5	No emergence	31.0 ±0.4	30.7 ±0.3

Table 4.6. Mean times to emergence and pupation of *C. maculatus* in artificial diet pellet bioassay of crude alfalfa sapogenins. Each treatment contained 25 replicate pellets.

incorporated into artificial diet pellets as described in section 4.2.1, with incorporation levels of 100%, 10% and 1% compared to the original alfalfa tissue as before. Bioassay against *C. maculatus* was carried out as described; summarized results are presented in Fig. 4.16. In this assay, insect development on control pellets was high, with 14.6 ± 1.2 (mean ± SE) insects surviving per pellet. Results for root and shoot sapogenins were similar, in contrast to results with unhydrolysed extracts. Both extracts were insecticidal when incorporated at the 100% level; no insects survived at all. At the 10% incorporation level, the root sapogenin extract decreased survival by 16% (not significant), and the shoot extract by 21% (significant at $p < 0.05$). At 1% incorporation level, root extract enhanced survival by 15% (not significant) whereas shoot extract decreased survival by 9% (not significant).

The development of *C. maculatus* larvae was estimated by times to pupation and emergence of adults. Sapogenin treatments delayed the appearance of pupation windows by up to 4.6 days, and the inhibition of development caused by shoot sapogenins was greater than that caused by root sapogenins. The time of the adult emergence after oviposition was also measured on different treatments. Results for the different treatments are summarised in Table 4.6. Again, the sapogenin treatments delayed adult emergence, by up to 5.4 days, and shoot sapogenins (delay 4.7 - 5.4 days) were more effective than root saponins (delay 2.2 - 2.6 days).

Hydrolysis of the saponins in the extracts from shoot tissue decreased their insecticidal activity in this assay. Results from the pellets containing sapogenins incorporated at the 100% level may appear anomalous, but the complete mortality of larvae observed for both root and shoot saponin 100% treatments was probably not due to sapogenin toxicity. Artificial diet pellets prepared with this level of crude sapogenin incorporation were very hard, and the physical characteristics of the pellets were not suitable for larval tunnelling. In fact, no evidence of larval tunnelling could be seen anywhere on these pellets, although the eggs had hatched, and the larvae had penetrated the clingfilm layer around the diet pellet. The results from the 100% saponin treatments were therefore discounted.

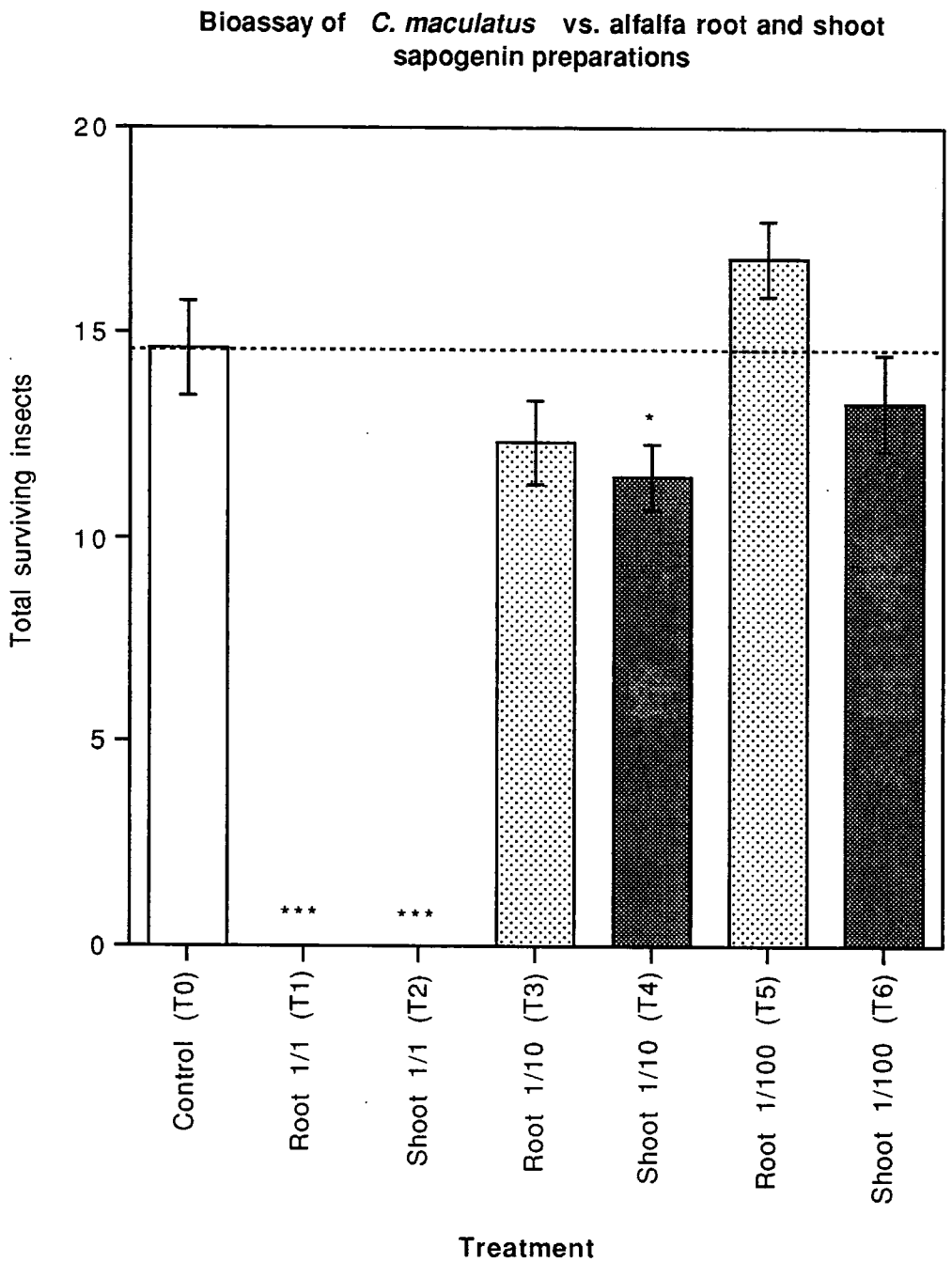


Fig. 4.16. Effects of crude sapogenins from alfalfa seedling roots and shoots on larvae of *C. maculatus*. Figures 1/1, 1/10, 1/100 indicate estimated dilution of crude sapogenins in diet pellets. Error bars indicate mean ± S.E. Columns marked with asterisks are significantly different to control at $p<0.05$ (*), $p<0.001$ (***)

4.2.3. Bioassay of Toxic Effects of Saponin Mixtures Purified from Root and Shoot Tissues of Alfalfa Seedlings on Potato Aphid

The assays with *C. maculatus* indicated that the alfalfa saponins were insecticidal, but the assay method had severe shortcomings. The bioassays using artificial seed pellets were labour-intensive and required large amounts of material to set up a sufficient number of replicates. It was therefore decided to use a more sensitive insect bioassay, which could be carried out with smaller amounts of material, in order to assay purified saponins. Aphids provided an ideal bioassay subject, in that they are small, have a short life cycle, reproduce parthenogenetically (allowing the entire life cycle to be studied), and feed on liquid diet, in which compounds can be incorporated at known concentrations. An initial bioassay was carried out with crude saponin extract to confirm that the alfalfa saponins were toxic to the aphid used.

The alfalfa saponin mixtures obtained from alfalfa root and shoot seedling tissues after extraction, solvent partition with *n*-BuOH and Et_2O precipitation were assayed for effects on the survival and fecundity of potato aphids by incorporation into a liquid artificial diet (Febvay *et al.*, 1988). This diet, which is described in the methods section, contains sucrose, a full set of amino acids and vitamins, and allows normal growth of the aphid from a newly-hatched nymph to adulthood, including the parthenogenetic production of second generation nymphs. In these assays, both the survival of the aphids, and production of nymphs, were monitored on a daily basis. Both root and shoot saponin mixtures were incorporated into the diet at three different concentrations; 0.1 mg/ml (0.01% w/v), 0.3 mg/ml (0.03% w/v) and 1.0 mg/ml (0.1% w/v).

Results for the saponin mixtures from roots and shoots are shown in Figs. 4.17 and 4.18. Two controls are used for these assays. The "no diet" control is used to show whether feeding by aphids has taken place. In the absence of diet, every time this control was carried out, all aphids were dead by day 3 of the assay; these data are not presented in subsequent figures. A diet control is also carried out, to give a measure

of performance for the aphids used; since these data did vary from assay to assay, due to variability in the aphids used, the "control" data are presented in each figure subsequently. Significance of differences between treatments was evaluated by means of survival analyses, or by Mann-Whitney U-test of data from the individual replicates.

The root saponin mixture at 1.0 mg/ml was highly toxic to potato aphids, with a survival curve similar to the "no diet" control (Fig. 4.17), so that all aphids were dead by day 4 of the assay on this treatment. In contrast, survival of the aphids on lower concentrations of the root saponin mixture did not differ significantly from aphids on control diet until after day 10. Up to this time survival on the control and two treatments was < 80%. After day 10 survival declined to >5% on day 20, coinciding with the production of nymphs from day 11 to day 20. The decline in survival was less on the two treatments than on the control during days 10-15, and during this period nymph production was also less, mainly due to a lag of approx. 1 day in starting nymph production. These data suggest that the lower concentrations of root saponin mixture are causing a slight inhibition of aphid development, which delays both nymph production and the decline in survival associated with nymph production.

Results with the shoot saponin mixture showed a more consistent trend, as presented in Fig. 4.18. All saponin treatments caused a sharp decrease in survival over days 0-4 of the assay, and this mortality correlated with the dose of saponin mixture (20% for 0.1 mg/ml, 29% for 0.3 mg/ml and 78% for 1.0 mg/ml). The survival curve for the diet control was similar to that described above, with survival declining from >90% on day 9 to <5% on day 20. Survival also declined slowly to <5% by day 20 in the 1.0 mg/ml saponin diet, but in both the lower doses of saponin, the decline in survival was slower after the initial period, so that both treatments showed higher survival than controls after day 10, and had only declined to approx. 15% by day 20. Production of nymphs in the control diet aphids started on day 11, and increased up to day 20, as survival declined. The shoot saponin treatments all showed significant decreases in nymph production, with the decrease being dose-dependent, so that by day 20 the numbers of nymphs were approx. 50% of control at 0.1 mg/ml saponins,

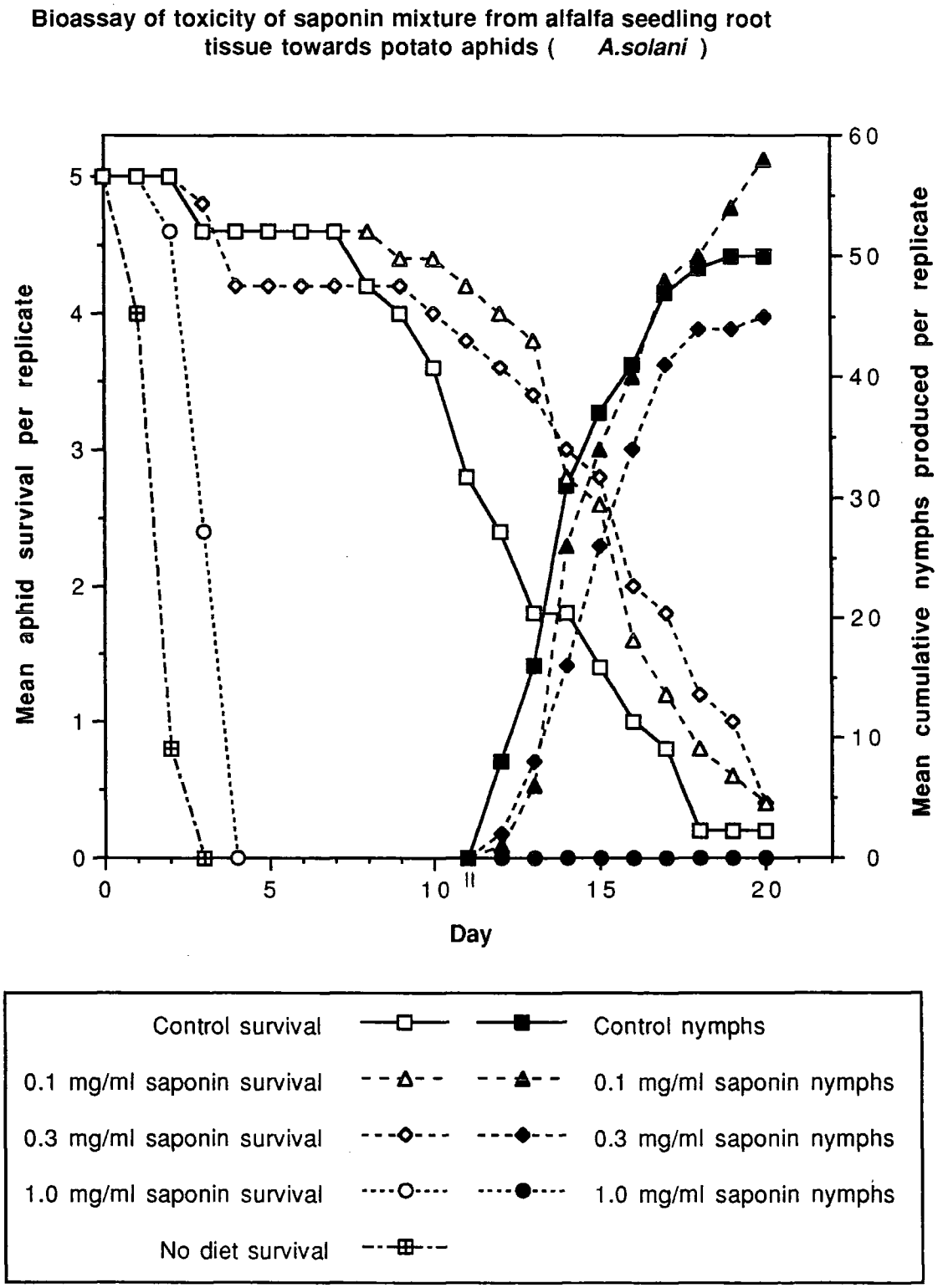


Fig. 4.17. Bioassay of saponin mixture from alfalfa seedling roots at different concentration levels in artificial diet against potato aphid(*solani*).

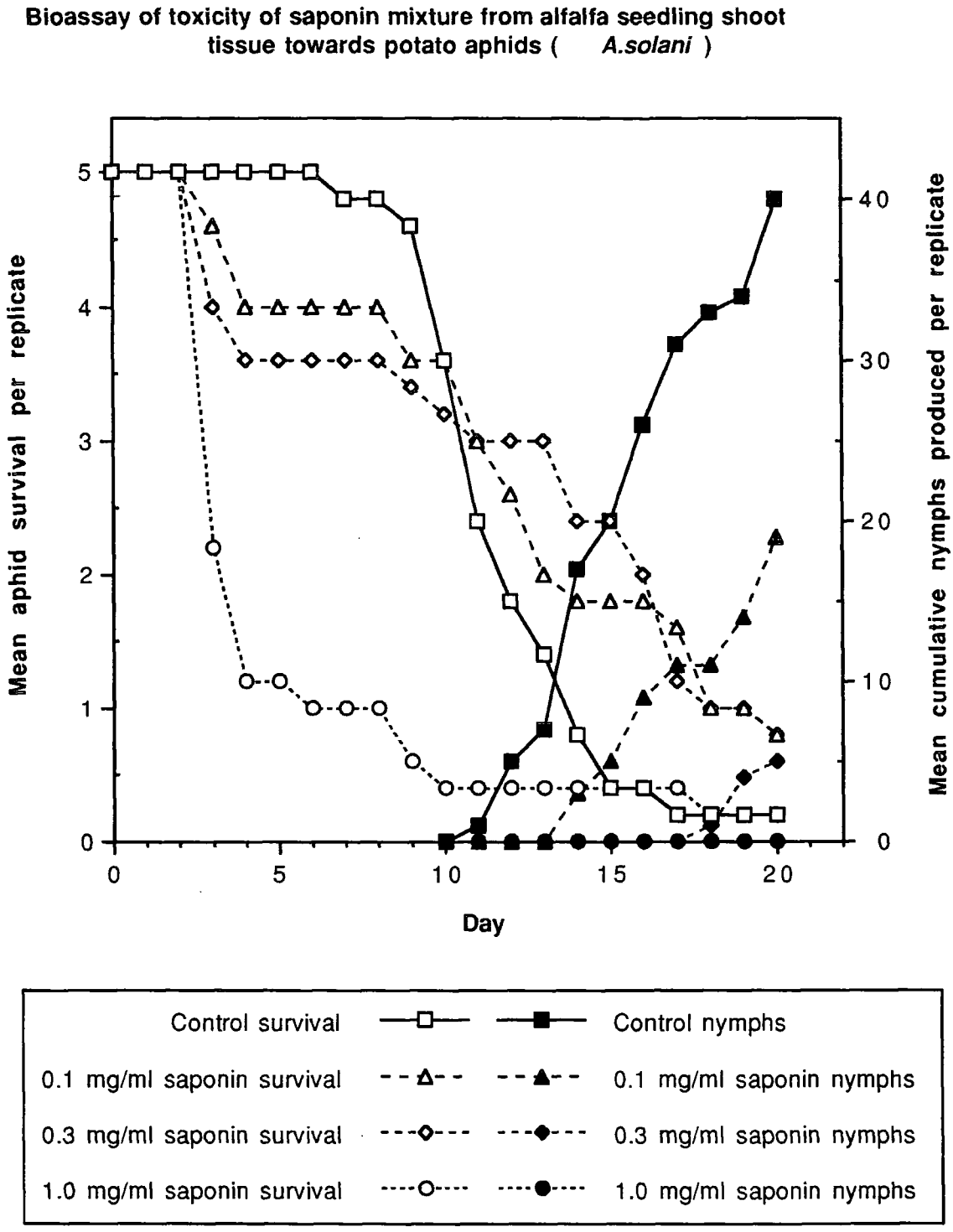


Fig. 4.18 Bioassay of saponin mixture from alfalfa seedling shoots at different concentrations in artificial diet against potato aphid (*A. solani*).

12% of control at 0.3 mg/ml saponins, and 0 at 1.0 mg/ml saponins. 0.1 mg/ml saponins caused a 3 day delay in nymph production, whereas 0.3 mg/ml saponins caused a 7 day delay, and 1.0 mg/ml saponins prevented nymph production completely. Similar results were obtained in repeats of this assay carried out with different shoot saponin preparations and with different aphid populations (results not presented).

Since both saponin mixtures used in these assays may have contained other compounds as well as saponins (see section 4.1.1 above), interpretation of the results must be subject to caution. However, it is clear that the saponin mixtures have significant toxic effects on aphids, and that for the shoot saponin mixture at least, these effects are progressive and dose-dependent. Results with the root saponin mixture, on the other hand, suggest that a component in the mixture is exhibiting a "threshold" effect, where toxicity is only seen at dose levels above the threshold. The enhanced survival observed for lower concentrations of both root and shoot saponin mixtures during nymph production may be a result of probiotic effects of components (not necessarily saponins) in these mixtures, but can also result from the reductions observed in fecundity, since delays in nymph production will enhance the survival of adult aphids.

The effects of the shoot saponin mixture on aphid development were further assessed by measuring the length and width of the aphids, before they had reached maturity, using a video image capture system attached to a low-power microscope. Decreases in the size of aphids imply a slower growth rate, and thus a retardation of development. Shoot saponins at three concentrations as above were used; results for survival and sizes at day 8 are shown in Fig. 4.19. Survival at day 8 was enhanced (although not significantly) by saponins at 0.1 or 0.3 mg/ml in this assay, but was significantly decreased (by approx. 60%) by saponin at 1.0 mg/ml. However, all saponin treatments significantly ($p < 0.005$) decreased both length and width of the aphids, and the decrease observed was progressive and dose-dependent. Thus saponins at 0.1, 0.3 and 1.0 mg/ml decreased length by 17%, 21% and 47% respectively, and width by 9%, 11% and 32% respectively. These results agree with

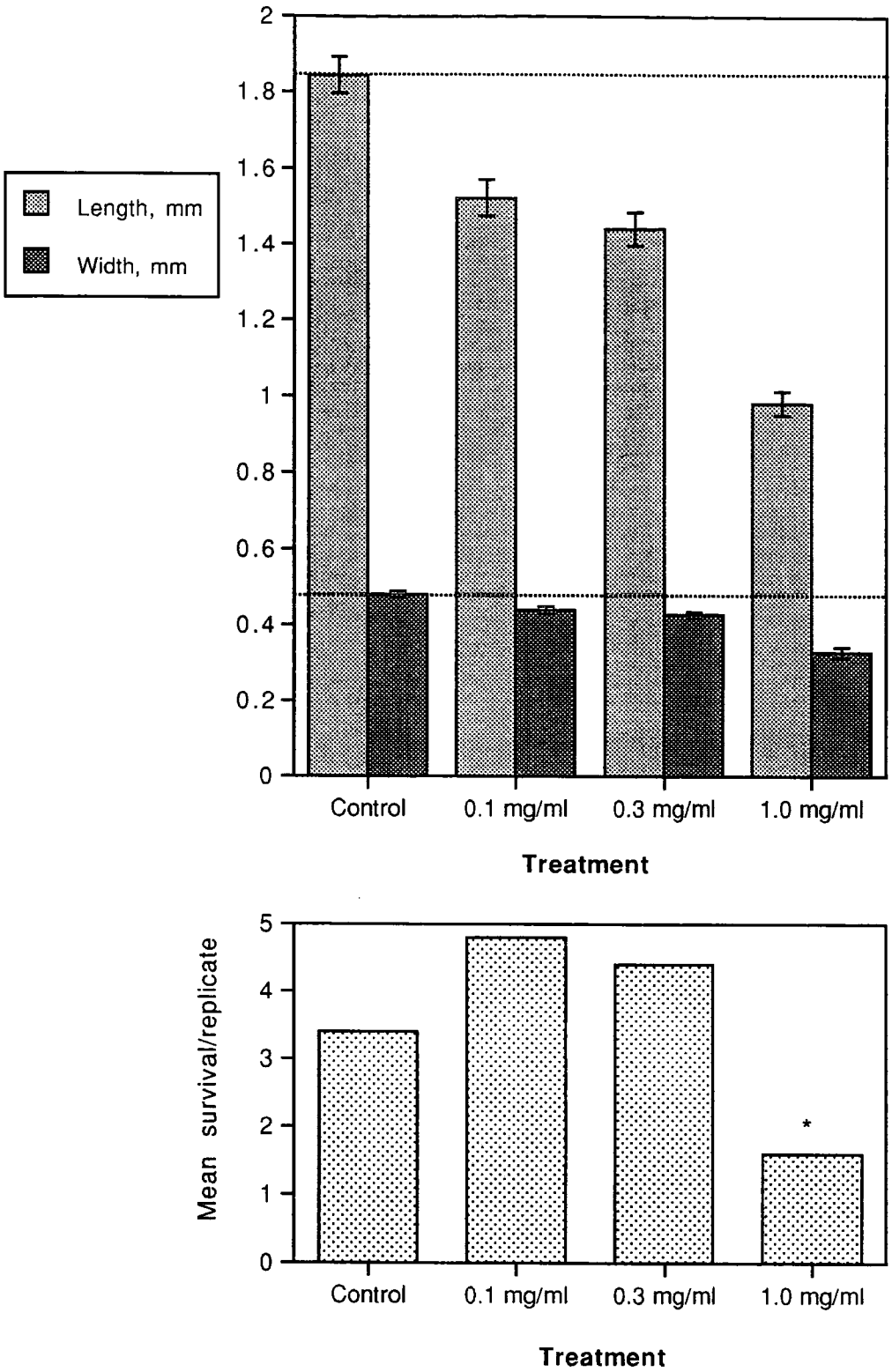


Fig. 4.19 Aphid sizes and survivals (day 8) after feeding on diets containing different concentrations of alfalfa shoot saponin mixture. Error bars show means ± S.E. All saponin treatments significantly ($p < 0.005$) decrease size compared to control. * = survival significantly different to control ($p < 0.05$).

the effects of the shoot saponin mixture on development deduced from the decreases in nymph production.

4.2.4. Identification of Possible Insecticidal Compounds after HPLC Separation of Alfalfa Saponins

In order to identify which compounds in the saponin extracts were responsible for aphid toxicity, experiments were set up where both root and shoot saponin extracts were separated by reverse-phase HPLC (see section 3.4.4), and all fractions (whether containing saponins or not) were analysed for insecticidal activity.

Crude saponin extracts were applied to the HPLC column in aqueous solution, and fractions were collected over the whole gradient of iso-propanol, evaporated to dryness, and subsequently tested by dissolving the residue in aphid diet (3 mg ml^{-1}). Survival of aphids feeding on the diet was monitored over a 6 day period.

For both root and shoot saponin mixtures, the only fractions which produced significant mortality in the aphids were those containing saponins; the initial peak of UV-absorbing material (see Figs. 4.6 and 4.7) eluted from the column was not toxic. Mortality was confined to fractions containing the major saponin component of root and shoot tissue, which gave a brown band on TLC at Rf approx. 0.4.

These assays demonstrated that the alfalfa saponins did indeed show insecticidal activity, and suggested that the major saponin component was the most toxic.

4.2.5. Effects of Purified Alfalfa Shoot Saponin Fractions on the Potato Aphid

Saponin fractions from alfalfa shoots were prepared as described in section 3.4.4. These fractions were incorporated into artificial diet at known concentration (0.3 mg/ml), and their effects on the survival and development of potato aphids was assayed. In these assays aphid survival was measured over an 11-day period (from neonates to maturity on control diets), and development was assessed by measurements of the length and width of the aphids. Results are presented in Fig.

4.20.

Aphids on control diet showed a survival of approx. 75% over the duration of this assay, whereas survival in the aphids on different treatments ranged from 10% to 60%. Whereas survival was only decreased by approx. 15% by fraction **Sa**, and by approx. 30% by fraction **Sb**, fractions **Sc**, **Sd** and **Sf** all decreased survival by >50%. Fractions **Se** and **Sg** had an intermediate effect on survival, with decreases of approx. 45% and 40% respectively. Due to the relatively small numbers of aphids in these assays, differences between individual fractions are not significant, but differences between survival in treatments containing fractions **Sb-Sf** and the control are significant at $p < 0.05$ (Mann-Whitney U-test). Effects on development largely paralleled effects of survival, with fraction **Sa** causing a small and non-significant effect on length and width, and fractions from **Sb** to **Sf** causing a progressively more severe depression of both length and width, from 12% and 3% (**Sb**) to 35% and 16% (**Sf**). Fraction **Sg** was less deleterious to development than **Sf**, being similar to **Sb** and **Sc** in its effects. All differences in length between treatments and control were significant at $p < 0.01$, except fraction **Sa**; decreases in width were significant at $p < 0.01$ for fractions **Sd**, **Se** and **Sf**. **Sf** was the most toxic of all the fractions tested, causing the greatest mortality, and the greatest decreases in both length and width of the aphids.

The results given by the different saponin fractions in this bioassay can be compared with their composition, as shown by TLC analyses. It is apparent that the major saponin component of shoots, giving a brown spot on TLC identified as soyasaponin I, appears to correlate with increased toxicity, although it is clear that the other saponins present also have toxic effects.

4.2.6. Effects of Purified Alfalfa Root Saponin Fractions on the Potato Aphid

Saponin fractions prepared from alfalfa roots were tested for insecticidal activity in a similar assay (0.3 mg/ml incorporation in diet) to that described for shoot saponin fractions above. Results are presented in Figs. 4.21 and 4.22; in this assay, nymph production was also measured by allowing the assay to continue to day 20.

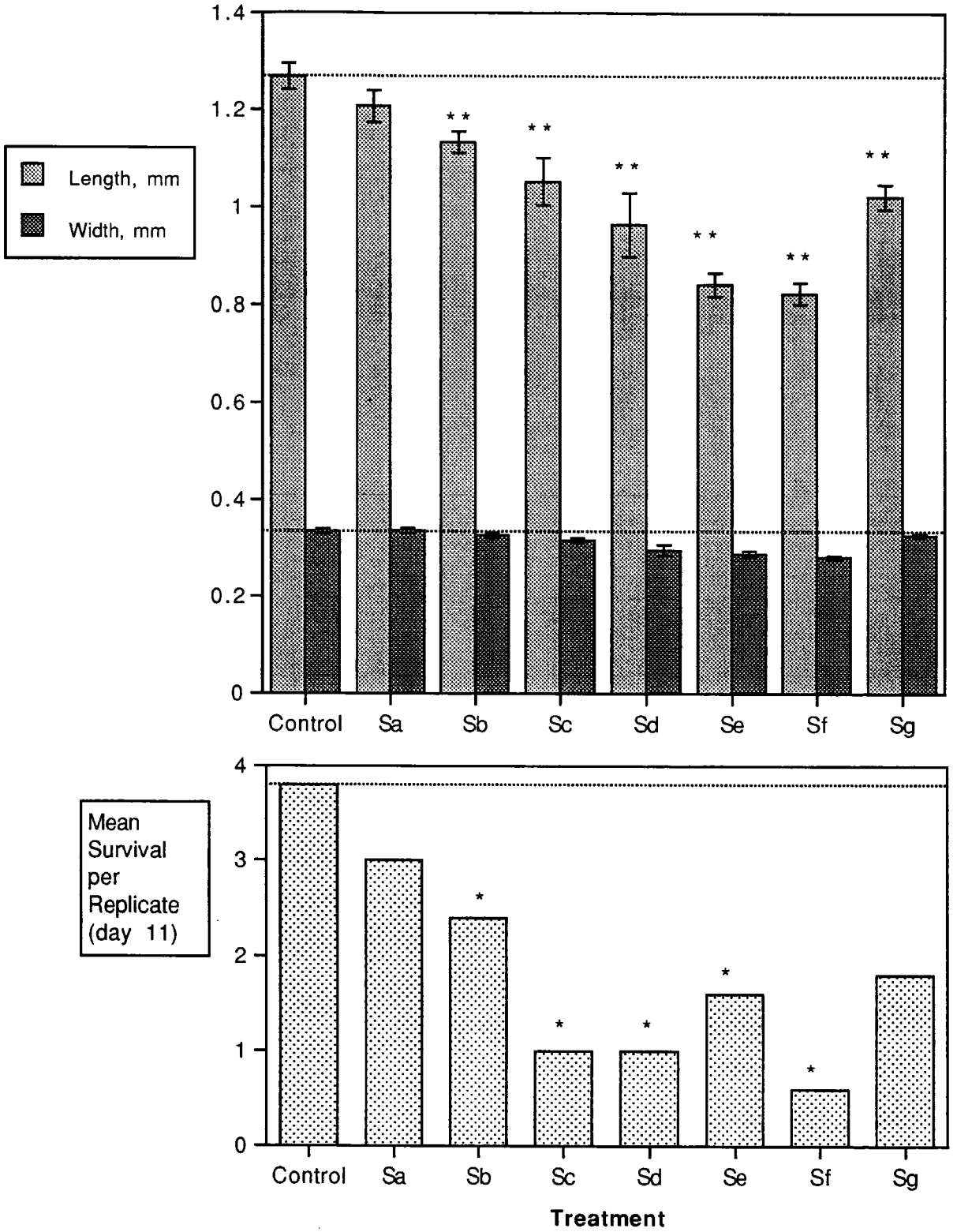


Fig. 4.20 Aphid sizes and survival (day 11) after exposure to different fractions from alfalfa shoot saponins. Error bars show means ± S.E.
** = length significantly different to control ($p < 0.01$); * = survival significantly different to control ($p < 0.05$).

Fraction **Rf** was not purified in great enough amount to be used in this bioassay.

Aphids in this assay showed 100% survival over the 11 day assay period, and all the saponin fractions except **Ra** caused significant decreases in survival, ranging from approx. 10% (Rc) to approx. 50% (Rd). **Rd** and **Rb** were the most toxic fractions, causing the greatest decreases in survival, with **Re** showing intermediate toxicity, and **Ra** and **Rc** low, or no toxicity, with minimal effects on survival. Effects of the saponin fractions on development were also apparent, with aphid sizes at day 11 showing decreases in length ranging from approx. 7% to 30%, and in width ranging from approx. 1.5% to 17% when compared to controls. However, the decrease in length was not significant ($p > 0.05$) for fraction **Rd**, and decreases in width were not significant for fractions **Rd** and **Re**. Fraction **Rb** caused the greatest decreases in size, with **Ra** and **Rc** giving intermediate effects, and **Rd** and **Re** little effect. As would be expected on the basis of effects on size, fractions **Ra** and **Rb** caused large decreases in cumulative nymph production at day 20 (90% and 98% respectively, and long delays in the time to first nymph production (8 days and 10 days respectively). Fraction **Rc** only decreased nymph production by 30% and delayed it by 3 days; fraction **Re** caused a similar delay but had more effect in decreasing nymph production (by 72%). Surprisingly, fraction **Rd** had the greatest effect on nymph production; no nymphs at all were produced on diets containing this fraction, and all the aphids were dead by day 17. The small effects on growth shown by this fraction are therefore not an indication of its toxicity.

As in the assay of shoot saponin fractions, the greatest effects in this assay are produced by fractions containing high proportions of the saponin giving a red-brown spot identified as soyasaponin I. The relatively low toxicity of fraction **Rc** in this assay may be due to the other saponin component in this fraction; as described in section 4.1.5, this component (identified as medicoside I, 3-O-[α-L-arabinopyranosyl (1->2)-β-D-glucopyranosyl (1->2)-α-L-arabinopyranosyl]-28-O-β-D-glucopyranosyl medicagenic acid) is readily hydrolysed, and thus may be ineffective as a toxin.

A second bioassay to determine whether fraction **Rf** was highly toxic towards

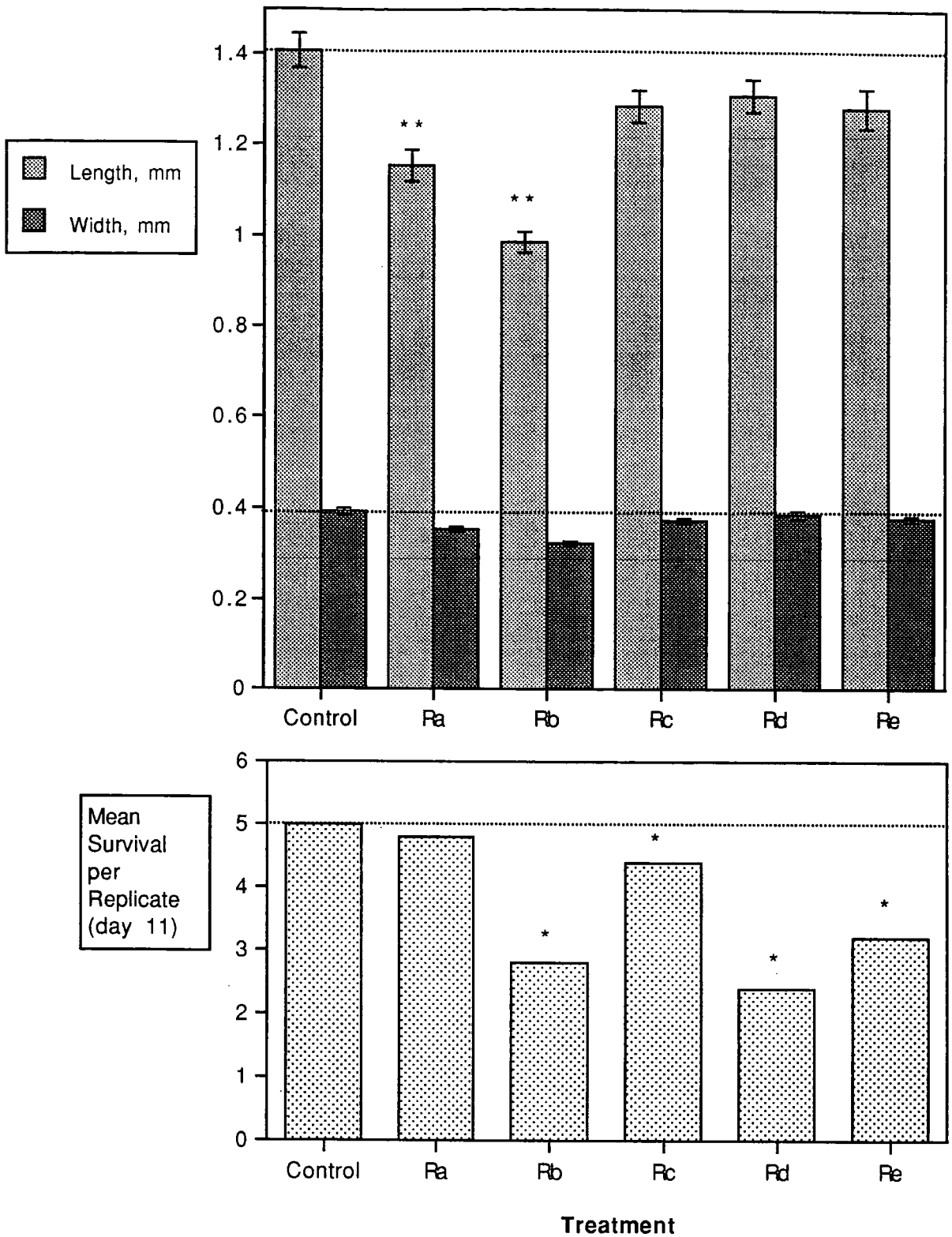


Fig. 4.21 Aphid sizes and survival (day 11) after exposure to different fractions from alfalfa root saponins. Error bars show means ± S.E.
** = length significantly different to control ($p < 0.01$); * = survival significantly different to control ($p < 0.05$).

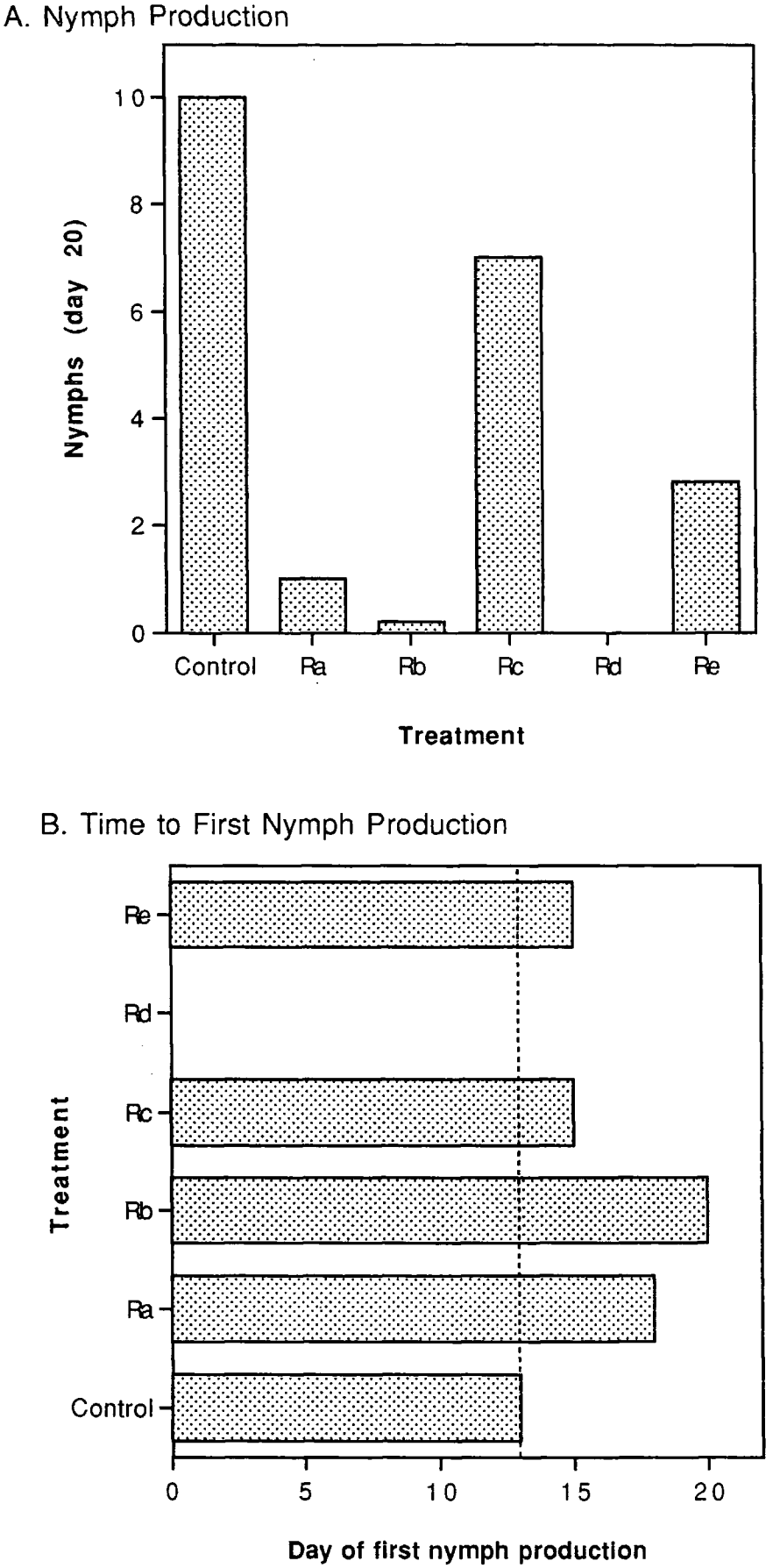


Fig. 4.22. Nymph production by aphids used in the assay shown in fig. 4.21.

the potato aphid was carried out. This bioassay was carried out for 9 days, due to limited amounts of material available. Over this period, fraction **Rf** had no effect at all on aphid survival, but did decrease aphid length (by 16%) and width (by 10%) when compared to controls. These results are summarised in Fig. 4.23. This bioassay shows that fraction **Rf**, which does not contain soyasaponin I, but which does contain medicoside A (medicagenic acid glycoside) is of lower toxicity towards the aphid than fractions **Rb** and **Rd**, in agreement with the earlier bioassay.

4.2.7. Effects of Characterised Alfalfa Saponins on the Potato Aphid

Assays were carried out with medicoside A (3-O-β-D-glucopyranosyl medicagenic acid), as supplied by two independent laboratories (those of Dr. Oleszek and Dr. Massiot) and soyasaponin I (3-O-α-L-rhamnopyranosyl (1->2)-β-D-galactopyranosyl (1->2)-β-D-glucuronopyranosyl soyasapogenol B), prepared by re-purification by HPLC of fraction **Sf** (see section 3.4.4). Compounds were incorporated at 0.3 mg/ml in diet.

The first assay compared medicoside A to control diet. In this assay, survival and nymph production were determined over 20 days, and sizes of the aphids were measured on day 9. Results are presented in Figs. 4.24 and 4.25. Medicoside A increased aphid survival over days 9-11 (similar to fraction **Rf**, which contains this saponin). However, it did decrease both length and width of the aphids (by 23% and 14% respectively), delayed the onset of nymph production by 2 days, and decreased cumulative nymph production at day 20 by 60%. These results are similar to those obtained with root saponin fraction **Rf**, which was assessed to show low toxicity, although it is clear from the decrease in nymph production that there is a significant effect on development.

The second assay compared medicoside A and soyasaponin I. Parameters were measured as for the previous assay, except that sizes were measured on day 10 of the assay. Results are presented in Figs. 4.26 and 4.27. Medicoside A performed very similarly in this assay as in the previous one, with no effect on survival, but decreases in size at day 10 (length decreased by 18%, width by 8%). There was no delay in the onset of nymph production, but the cumulative nymphs were decreased by 60% at day

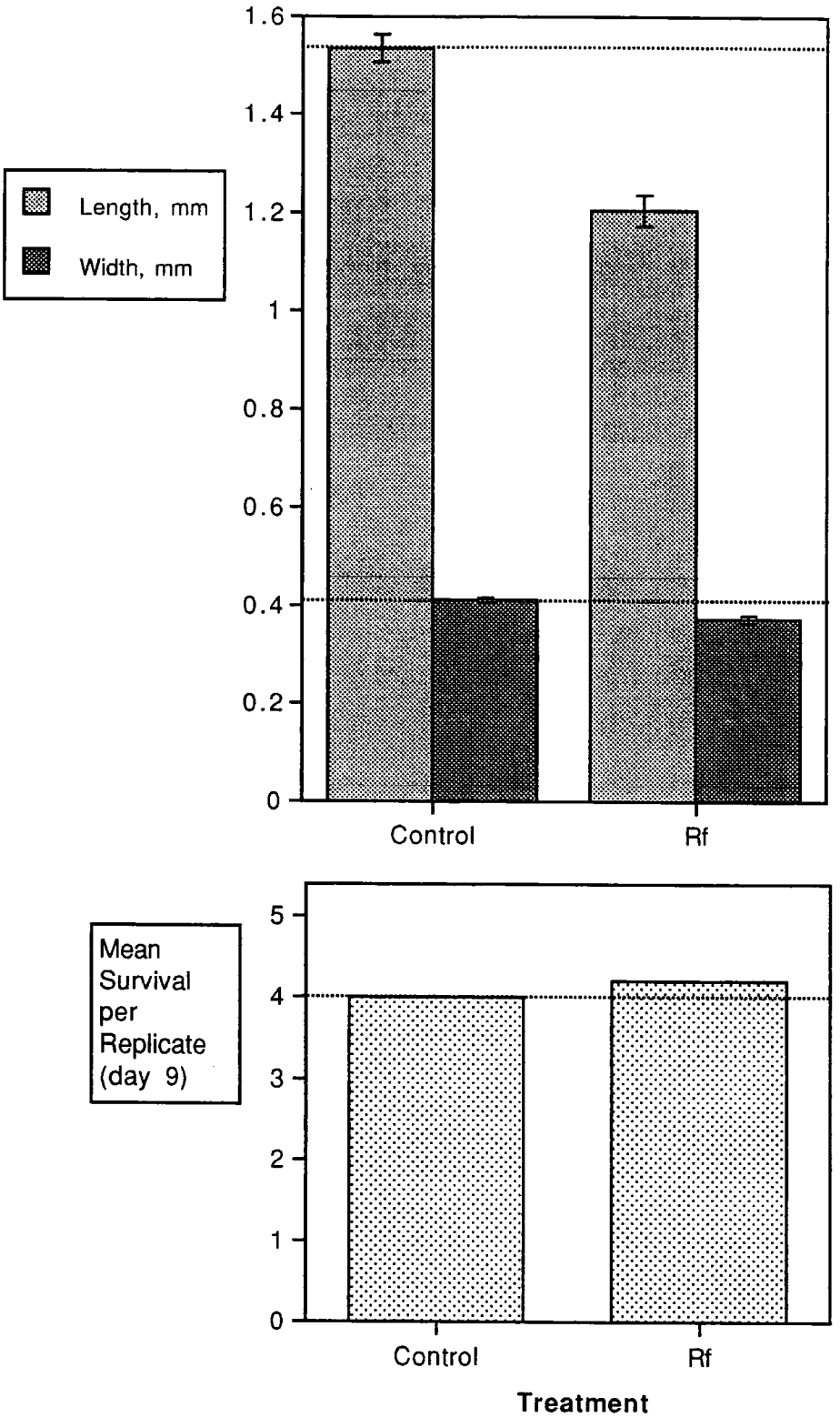


Fig. 4.23 Aphid sizes and survival (day 9) after exposure to fraction Rf from alfalfa root saponins. Error bars show means ± S.E.

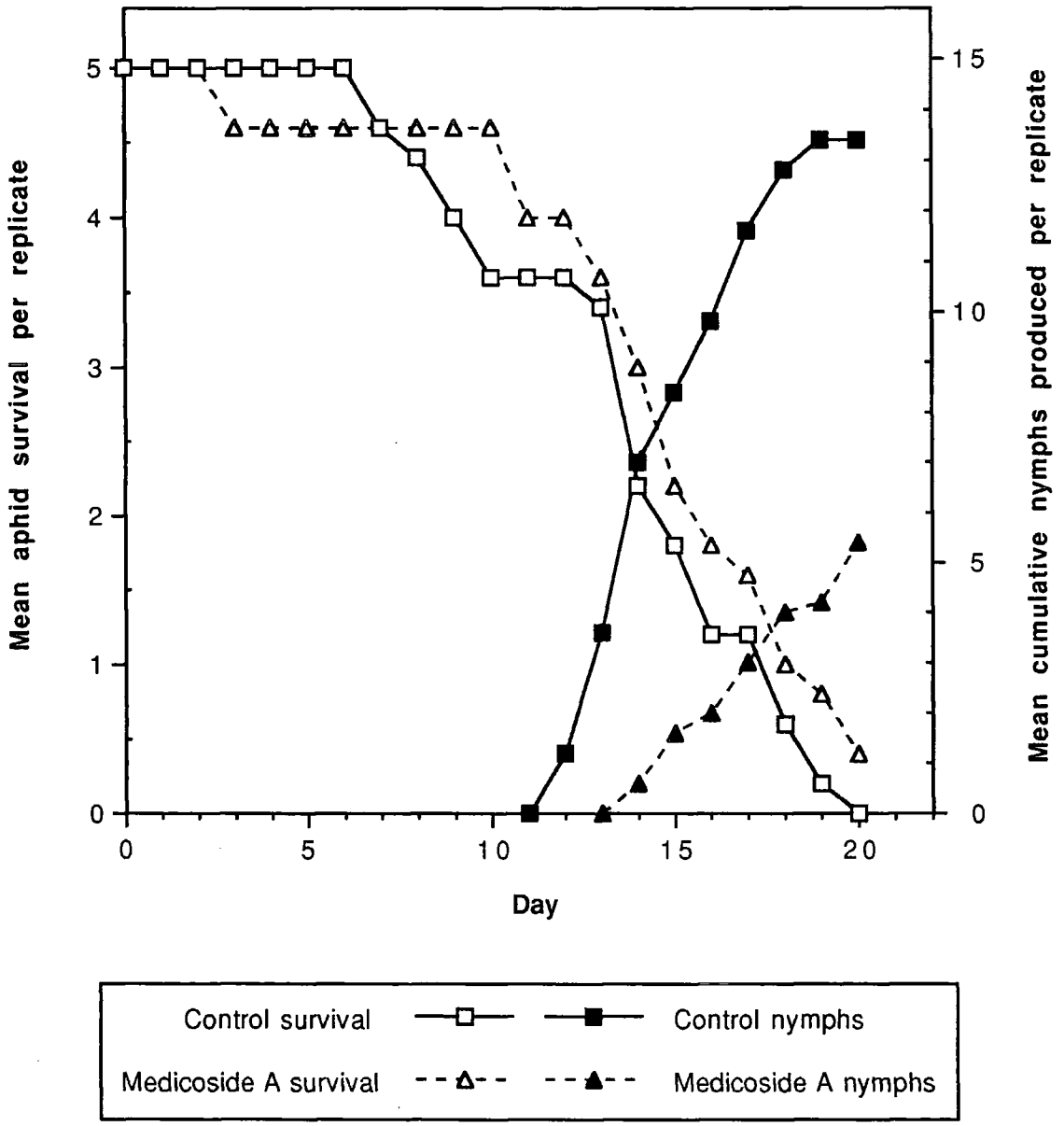


Fig. 4.24 Bioassay of toxicity of medicoside A (medicagenic acid glucoside) towards potato aphids

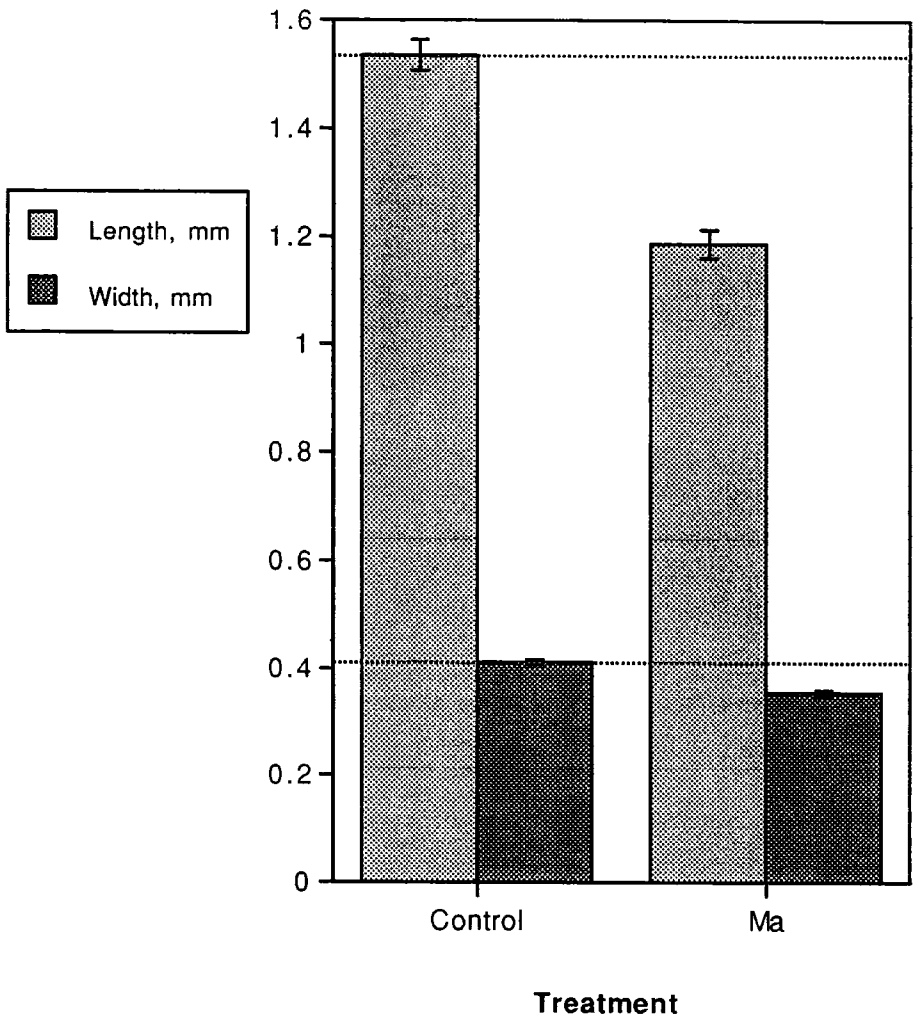


Fig. 4.25 Aphid sizes (day 9) after exposure to medicoside A (Ma)
Error bars show means ± S.E.

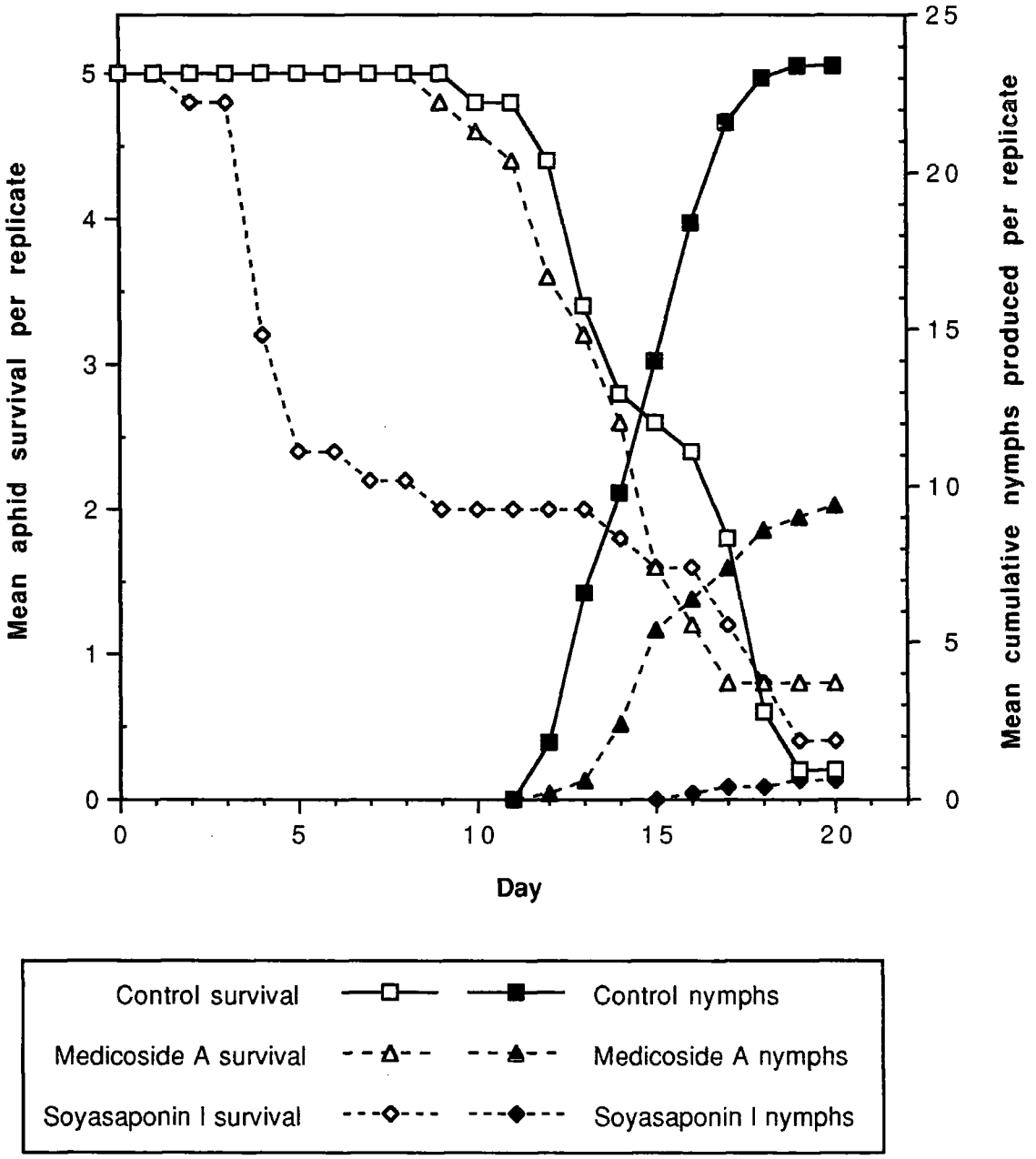


Fig. 4.26 Bioassay of toxicity of medicoside A and soysaponin I towards potato aphids.

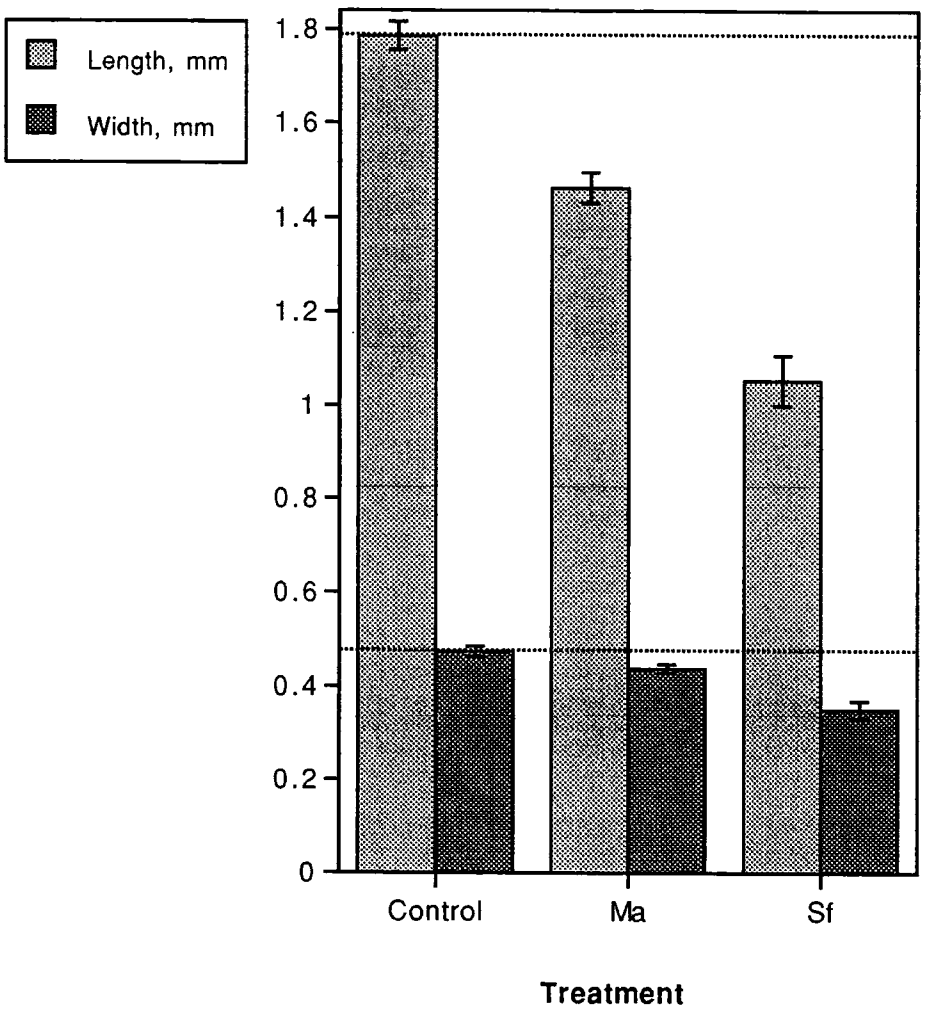


Fig. 4.27 Aphid sizes (day 10) after exposure to medicoside A (Ma) and soyasaponin I (Sf). Error bars show means ± S.E.

20. In contrast, soyasaponin I caused 60% mortality compared to control over days 5-11, delayed the onset of nymph production by 5 days, and almost eliminated the production of nymphs, with cumulative nymph production after 20 days being only 3% of the control. Soyasaponin also had significantly ($p < 0.0001$) greater effects on aphid size on day 10 than medicoside A; reductions in length and width were 41% and 26% respectively for soyasaponin I when compared to controls. These data demonstrate that soyasaponin I is toxic towards potato aphids, having a significant effect on aphid survival, as well as strongly affecting development, and that its toxic effects are much greater than medicoside A. The toxic effects of soyasaponin I are comparable to those obtained with total shoot saponins (section 4.2.3), and with fraction **Sf** from shoot saponins (section 4.2.5), showing that soyasaponin I is the most active component of the alfalfa saponins in producing insecticidal effects.

A third assay was carried out to assess the dependence of the toxicity of soyasaponin I on its concentration in the diet. Concentrations of 0.1, 0.3 and 1.0 mg/ml of the saponin in artificial diet were used. In this assay the highest concentration of soyasaponin I caused complete mortality in 6 days (control survival 80%), with both lower concentrations giving mortality >50% over the interval 5-13 days. Nymphs were only produced by the control aphids and aphids feeding on the lowest saponin concentration; this reduced cumulative nymph production by 80%. Results are shown in Fig. 4.28. These data show that the concentration of soyasaponin I which is necessary to reduce survival of aphids from nymph to adulthood by 50% (LC_{50}) on artificial diet is of the order of 0.1 mg/ml.

4.2.8. Effects of Saponin Mixtures from Sugar Beet on the Potato Aphid

A saponin mixture from sugar beet leaves was prepared as described in section 3.3.2. Assays similar to those described for the alfalfa shoot saponin mixture, where the purified sugar beet saponin mixture was incorporated into artificial diet at three different concentrations, were set up. Results are presented in Figs. 4.29 and 4.30.

All three concentrations of the sugar beet saponin had an effect on aphid survival in the first four days of the bioassay, reducing survival to 55-60% on day 4 compared to 83% on the control diet. After this initial rapid decline in survival, the saponins at 0.1 mg/ml caused no significant further mortality until nymph production

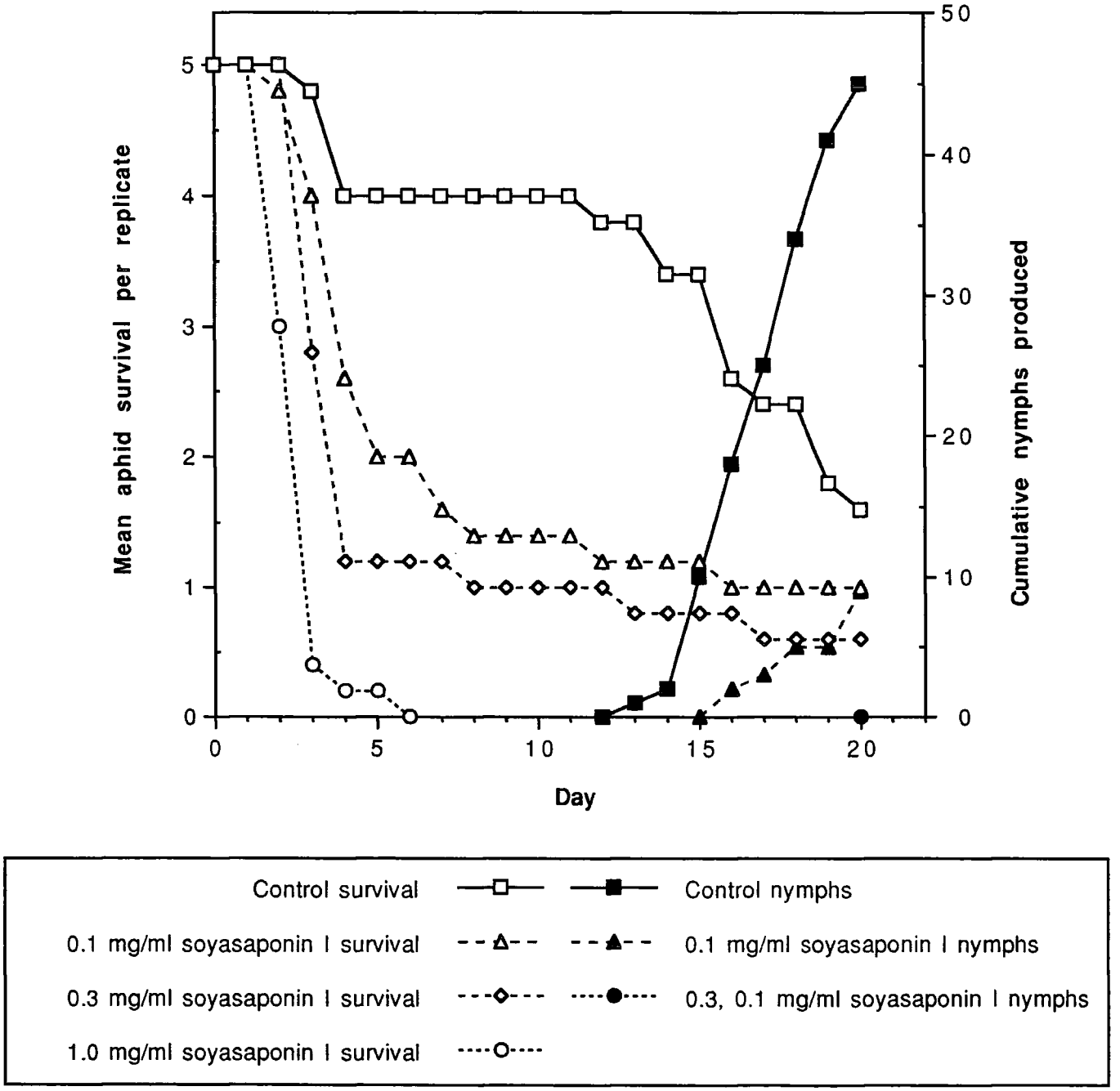


Fig. 4.28 Bioassay of toxicity of soyasaponin I at different concentrations towards potato aphids

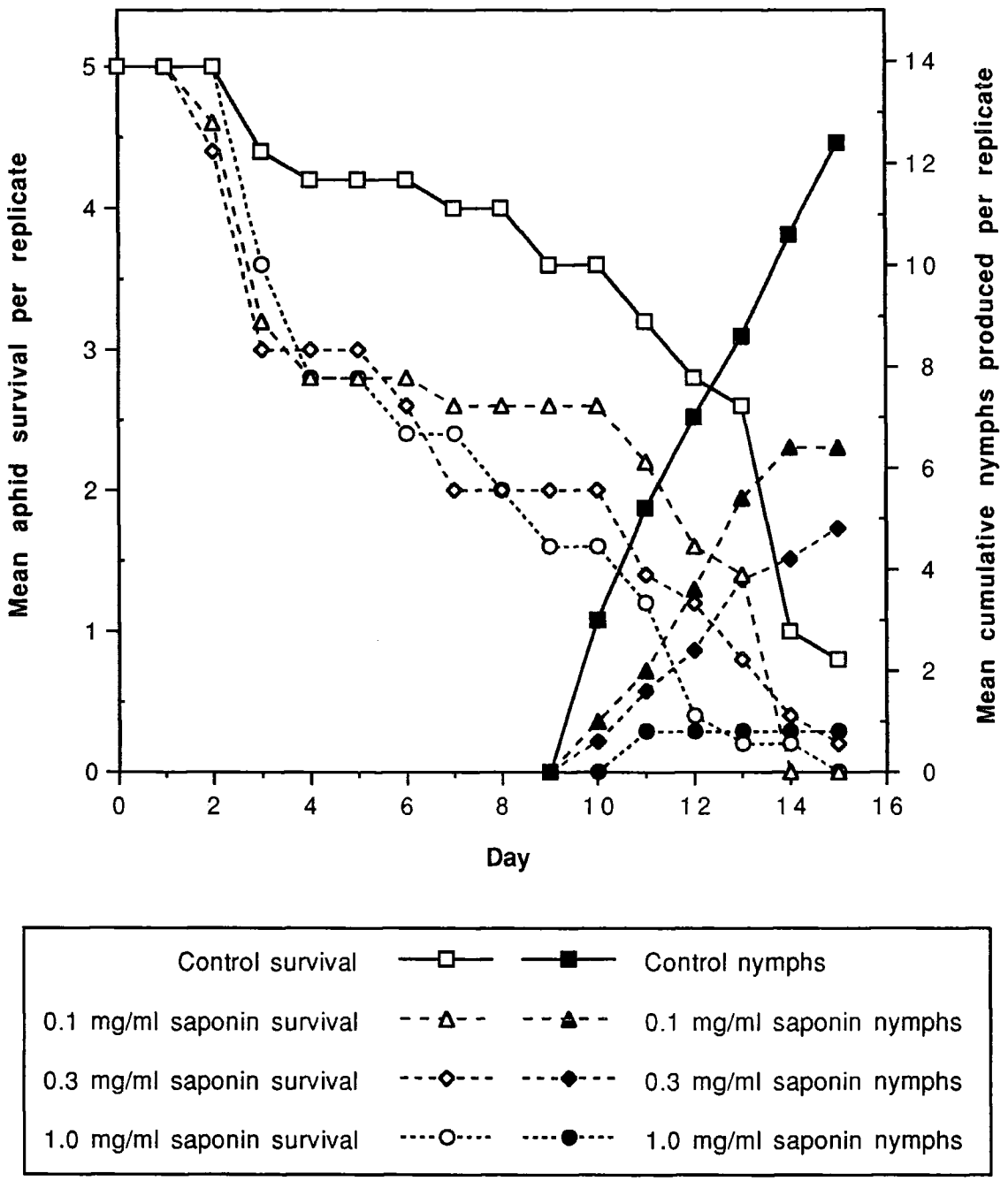


Fig. 4.29 Bioassay of toxicity of saponin mixture from sugar beet leaves towards potato aphids.

commenced, whereas the highest dose of saponin did cause further mortality, until survival was less than 5% by day 13. The 0.3 mg/ml level of saponin had intermediate effects. Effects on nymph production were also progressive. Only the highest concentration of saponin, 1.0 mg/ml, caused any delay in the onset of nymph production, and then only by 1 day (day 11 vs. day 10). However, the rate of nymph production was significantly and progressively decreased by increasing dose of saponins, so that at day 15, cumulative nymph production was decreased by 48%, 61% and 94% by 0.1, 0.3 and 1.0 mg/ml saponins respectively. However, on a per surviving aphid basis, the decreases in nymph production compared to control were small except for the highest saponin dose.

Measurements of the sizes of aphids on day 8 of the bioassay described above confirmed that the sugar beet saponins had only limited effects on aphid development, as suggested by the failure to affect the onset of nymph production at concentrations below 1.0 mg/ml. The length and width of the aphids fed on diet containing 0.1 and 0.3 mg/ml saponins were not significantly different to controls (Fig. 4.30). However, aphids fed sugar beet saponins at 1.0 mg/ml were significantly smaller than controls (length was decreased by 35% and width by 16%). The results suggest that the "threshold level" at which the sugar beet saponins start to show toxic effects against potato aphids is between 0.3 and 1.0 mg/ml, if the hypothesis that the initial mortality is due to an antifeedant effect is accepted.

4.2.9. Hydrolysis of Alfalfa Saponins by Enzymes from Insects and Plants

Hydrolysis of saponins to the corresponding sapogenins (and a mono- or oligosaccharide) by glycosidases has been suggested as a method used by fungi for detoxifying these compounds (Osbourn *et al.*, 1991 and 1995). However, plants themselves contain glycosidases, which are capable of hydrolysing a wide range of substrates, and these enzymes, especially β-glucosidases, have been implicated in plant defence through the release of toxic compounds from glycosides (Falk and Rask 1995, Nisius 1988, Levy *et al.*, 1989a). The β-glucosidase from almond is stated to be able to hydrolyse medicoside A (3-O-β-D-glucopyranosyl medicagenic acid (Levy *et al.*, 1986). It was therefore of interest to see whether selected insect species were able to hydrolyse the alfalfa saponins, and whether the plant itself contained glycosidases

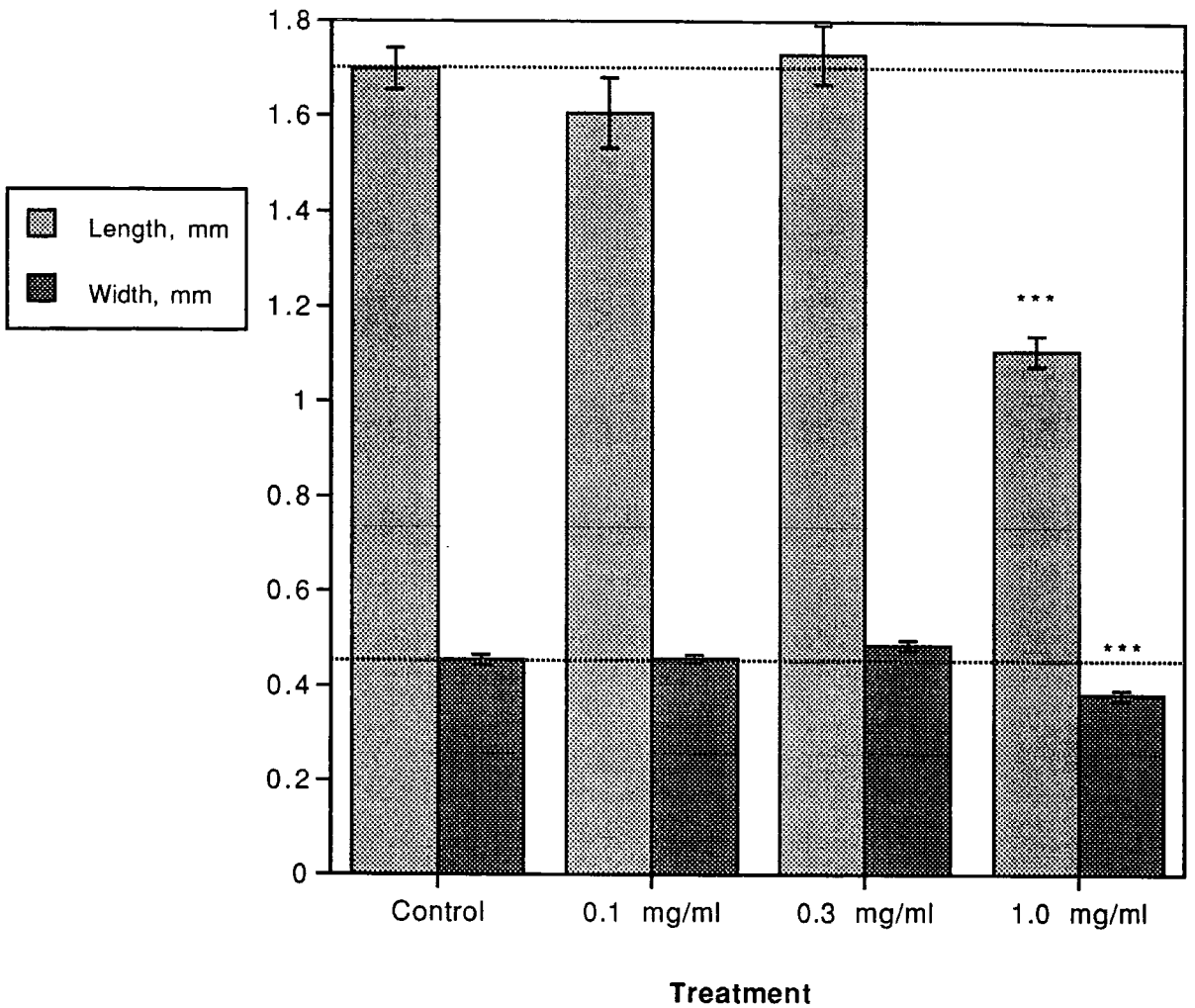


Fig. 4.30 Aphid sizes (day 8) after feeding on diets containing different concentrations of sugar beet saponin mixture. Error bars show means ± S.E. Bars marked *** are significantly different to control at $p < 0.001$.

capable of hydrolysing its own saponins.

Saponin hydrolysis was determined by incubation of purified saponin mixtures, or fractions after HPLC, with enzyme extracts of gut tissues from insects, or extracts from plant tissue, or purified enzymes. Reactions were quenched, and saponins were extracted from the reaction mixtures by *n*-BuOH partition. Changes to the saponins were assayed by running samples on TLC with appropriate controls.

(1) Hydrolysis by Almond β-glucosidase

Almond β-glucosidase had little effect on purified alfalfa root and shoot saponin mixtures when incubated at a ratio of 25 units of enzyme per 120 μg saponin at pH 5.0, 37?C for 20 hr. Results are shown in Fig. 4.31. Examination of the TLC plate under UV light showed that some hydrolysis had occurred, as dark spots with high R*f* values (approx. 0.93) were produced when saponins were incubated in the presence of enzyme, but not alone. This putative sapogenin spot appeared to result from partial hydrolysis of medicoside A in root saponins (purple spot, R*f* 0.59), and an unidentified component in shoot saponins (yellowish spot, R*f* 0.50). Most of the saponin spots were unchanged, showing a general resistance to hydrolysis by this enzyme. Hydrolysis of medicoside A in root saponins was confirmed in a second experiment, using 5 units of enzyme per 100 μg root saponin mixture; results are shown in Fig. 4.32. Again, partial hydrolysis of medicoside A was observed. Analysis of the extracted saponins by TLC using a solvent system for sapogenin separation (petroleum $_{Et2O}$) showed the presence of a sapogenin spot of low R*f* liberated by hydrolysis (Fig. 4.32), which was assumed to represent medicagenic acid. Purified root and shoot saponin fractions (**Rc** and **Sf**) were unaffected by this enzyme.

(2) Hydrolysis by Alfalfa Extracts

An enzyme extract from alfalfa seedling shoots showed little hydrolytic activity towards both root and shoot saponins under assay conditions similar to those above (data not presented). Slight changes to minor saponin components were observed, but

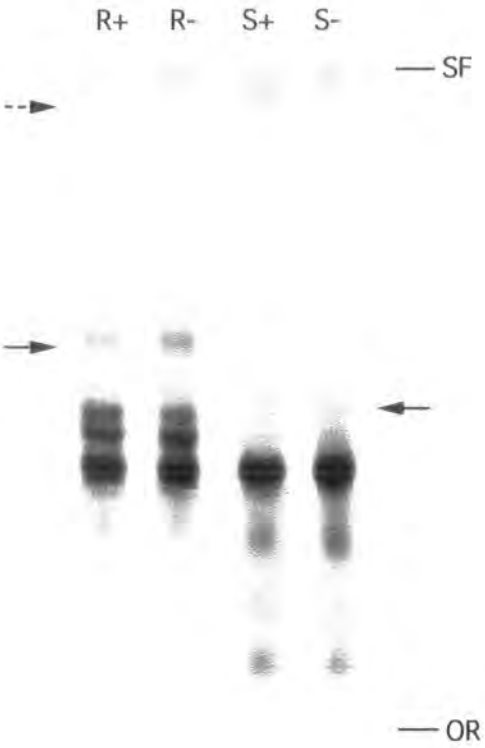


Fig. 4.31 Effect of almond β-glucosidase on saponin mixtures from roots and shoots of alfalfa seedlings.
R = roots; S = shoots
+ = incubated with enzyme; - = controls
Solid arrows indicate components partially hydrolysed by β-glucosidase; dashed arrow indicates product.
OR = origin; SF = solvent front.

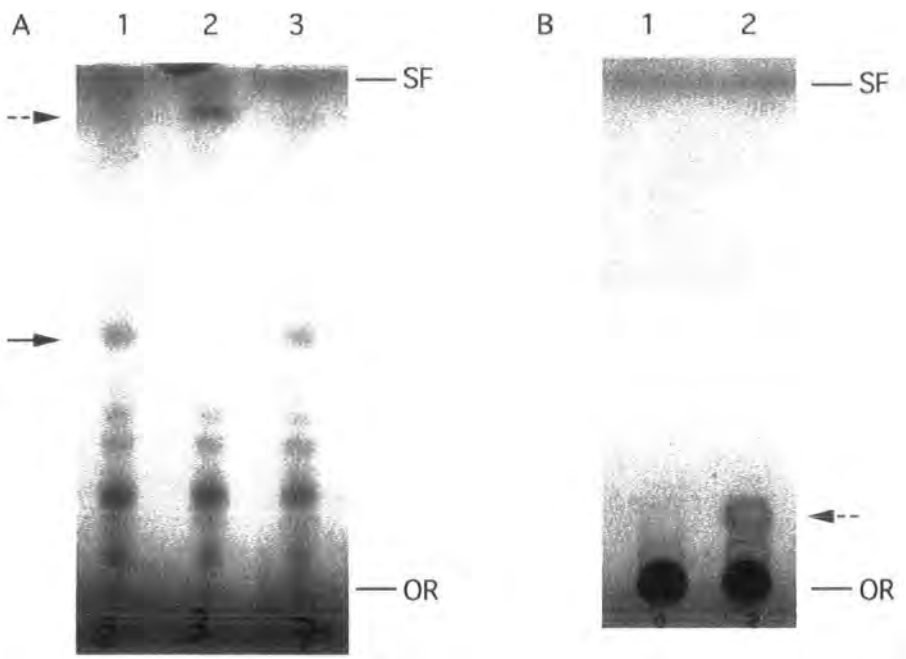


Fig. 4.32 Effect of almond β-glucosidase on saponin mixtures from roots of alfalfa seedlings. Tlc analyses visualised with uv light.
A: partial hydrolysis of saponins (normal tlc solvent system)
B: production of sapogenin (petroleum ether solvent system)
1 = control incubation (no enzyme); 2 = incubation with enzyme; 3 = zero incubation time control.
Solid arrows indicate components partially hydrolysed by β-glucosidase; dashed arrow indicates product. OR = origin; SF = solvent front.

these could not be characterised. However, when assayed with purified saponins as substrates, the alfalfa extract showed some hydrolytic activity towards the medicoside I component in **Rc**, but no activity at all towards soyasaponin I (Fig. 4.33). The hydrolysis product of medicoside I was similar to that described for hydrolysis by insect enzymes (q.v.)

(3) Hydrolysis by Insect Gut Enzyme Extracts

Insect gut extracts were prepared as described in the Methods chapter, and were used to determine whether hydrolytic activity towards alfalfa saponins was present in the insect gut. Three insect species were used; locust (*Locusta migratoria*: Orthoptera), a polyphagous insect which utilises plant tissues containing saponins, alfalfa weevil (*Hypera postica*: Coleoptera) larvae, an oligophagous insect specific to alfalfa, which therefore utilises tissues containing alfalfa saponins, and cowpea seed weevil (*Callosobruchus maculatus*: Coleoptera) larvae, an oligophagous insect which utilises plant tissues with low, or no, saponin content. Preliminary assays using p-nitrophenyl-β-D-glucoside showed that the locust gut extract contained detectable β-glucosidase activity, but the other two extracts did not (results not presented).

The insect gut extracts were incubated with purified root and shoot saponin fractions (**Rc** and **Sf**); results are shown in Fig. 4.34. No hydrolysis of soyasaponin I was observed in any case, but the medicoside I component in fraction **Rc** was hydrolysed by extracts from locust and alfalfa weevil, but not by the extract from cowpea seed weevil. The purple spot of medicoside I (3-O-[α-L-arabinopyranosyl (1->2)-β-D-glucopyranosyl (1->2)-α-L-arabinopyranosyl]-28-O-β-D-glucopyranosyl medicagenic acid) was hydrolysed to a more mobile purple spot (R*f* approx. 0.4 under TLC conditions used) by both locust and alfalfa weevil extracts; this spot was also produced on hydrolysis by alfalfa enzymes (see above). The hydrolysis product is similar in colour and mobility on TLC to medicoside G (3-O-β-D-glucopyranosyl-28-O-β-D-glucopyranosyl medicagenic acid). The locust enzyme also produced a further, more mobile purple spot (R*f* approx. 0.55), which is similar in colour and mobility to

Fig. 4.33 Hydrolysis of saponin components by enzyme extract from alfalfa.
1 = root saponin fraction Rc
2 = alfalfa enzyme extract
3 = incubation of Rc with enzyme extract
Solid arrow indicates hydrolysed component, dashed arrow indicates product.
OR = origin, SF = solvent front.

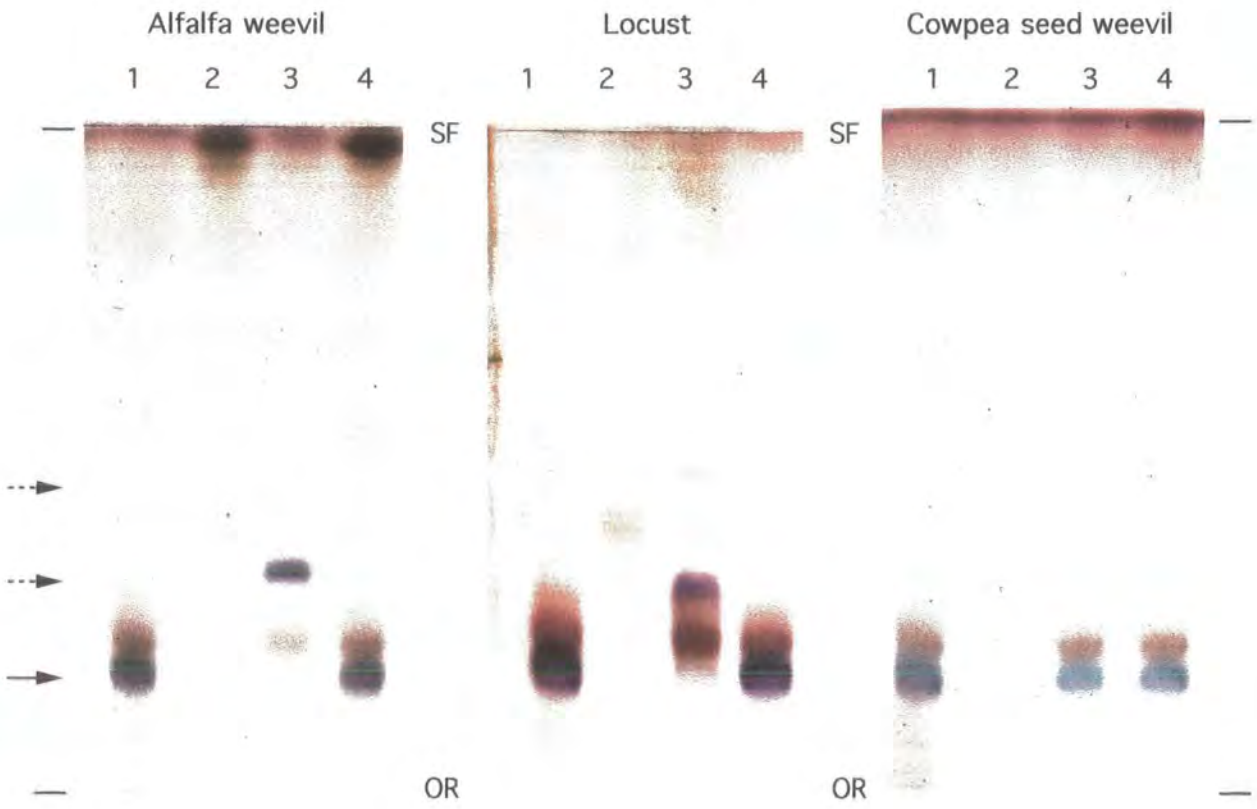


Fig. 4.34 Hydrolysis of saponin components by enzyme extracts from guts of alfalfa weevil larvae, locusts and cowpea seed weevil larvae
1 = root saponin fraction Rc; 2 =insect enzyme extract; 3 = incubation of Rc with enzyme extract; 4 = zero time control for incubation of Rc with enzyme extract
Solid arrow indicates hydrolysed component, dashed arrow indicates product(s).
OR = origin, SF = solvent front.

medicoside A (3-O-β-D-glucopyranosyl medicagenic acid).

SECTION 3:

4.3. Development of a Strategy to Control Alfalfa Weevil (*Hypera postica*) through Inhibition of Digestive Proteases

4.3.1. Characterisation of Digestive Proteases in Alfalfa Weevil

Gut enzyme extracts were prepared from third and fourth instar larvae of alfalfa weevil by removing and homogenizing the entire gut (and contents) in 25 µl 10 mM DTT per gut. Protease activity was assayed in these extracts by using the substrate Z-phe-arg-AMC, which is hydrolysed by trypsin-like and papain-like proteases to yield a fluorescent product, 7-amino-4-methyl coumarin (AMC). Alfalfa weevil larval gut extracts showed a high level of activity towards this substrate, estimated as 14 pmoles product liberated min^{-1} per larva (approx. 2.7 pmoles product min^{-1} per µg protein).

The protease activity present was characterised by including inhibitors specific for different classes of proteases in the assay. Results are shown in Table 3.1.

Table 3.1:

Inhibitor	Concentration (µg/ml)	% Inhibition
E-64	1.23	91
PMSF	61.7	0
SKTI	12.3	0
Cystatin (hen)	12.3	85

Inhibition of alfalfa weevil gut protease activity (substrate; Z-phe-arg-AMC) by various protease inhibitors. Each assay contained the equivalent of 0.04 larval guts in a toal volume of 810 µl. Assay results are the means of duplicate determinations; ranges were <10%.

In these assays, inhibitors of serine proteases, both chemical (phenylmethylsulphonyl fluoride, PMSF) and protein (soybean Kunitz trypsin inhibitor, SKTI) caused no inhibition of the proteolytic activity detected by Z-phe-arg-AMC. On the other hand, both chemical (L-trans-epoxysuccinyl-L-leucylamido (4-guanido)

butane; E-64) and protein (hen egg white cystatin) inhibitors of cysteine proteases inhibited approx. 90% of the detectable activity when present in excess. It was concluded that almost all the proteolytic activity detected in alfalfa weevil larvae is due to cysteine proteases.

The inhibition assays were repeated with smaller amounts of both E-64 and cystatin to assess the sensitivity of the gut enzyme to inhibition. Results are shown in Fig. 4.35. Neither inhibitor gave a linear graph when amount of inhibitor added was plotted against enzyme activity, suggesting that more than one cysteine protease was present, and that the different proteases differed in their sensitivity to the inhibitors. Although cystatin is significantly less effective as an inhibitor than E-64 on a weight-for-weight basis, when the two inhibitors are compared on a mole-for-mole basis, the alfalfa gut protease is of similar sensitivity to both inhibitors. Since E-64 is an irreversible covalent inhibitor of cysteine proteases, this suggests that the binding of cystatin to the alfalfa weevil gut proteases is characterised by a very low dissociation constant; i.e. inhibition is very effective.

4.3.2. Production of Recombinant Oryzacystatin (R-ozc)

In order to be able to test the effects of a cystatin protein protease inhibitor *in vivo*, at least 10 mg of protein were needed. Also, if cystatins were to be used in transgenic plants to protect against alfalfa weevil, a cystatin of plant origin might prove easier to express than egg white cystatin. It was therefore decided to use the cystatin from rice, oryzacystatin; while this is present in small quantities (<1 mg/kg) in rice seed, an expression construct to allow the protein to be produced in larger quantities in the bacterium *E. coli* was available.

The expression construct pHEV1(ozc) was described by Edmonds (1994); a plasmid map is presented in Fig. 4.36. In this construct the coding sequence for rice oryzacystatin is expressed using the bacterial *lacZ* promoter present in the cloning vector pUC8; expression can be induced by the presence of IPTG (isopropylthiogalactoside) in the medium. As a result of the way in which the recombinant plasmid was constructed, the coding sequence of oryzacystatin is present as a translational fusion. The protein expressed in bacteria contains an extra 14 amino

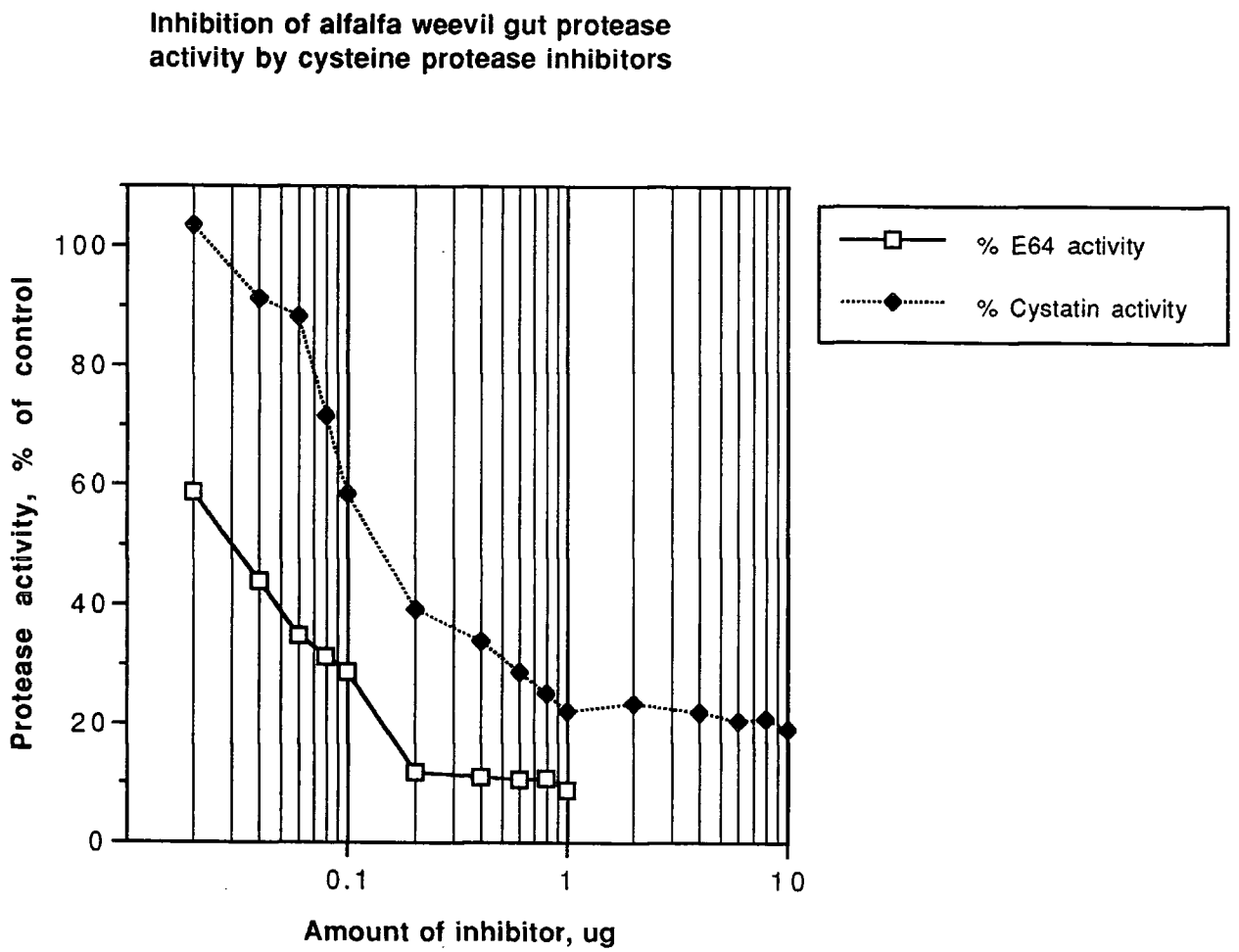


Fig. 4.35. Inhibition of alfalfa weevil larval gut protease activity (substrate Z-phe-arg-AMC) by specific inhibitors of cysteine proteases. Each point is the mean of two assays. Each assay contained the equivalent of 0.04 larval guts in a total volume of 810ul.

Rozc expression construct

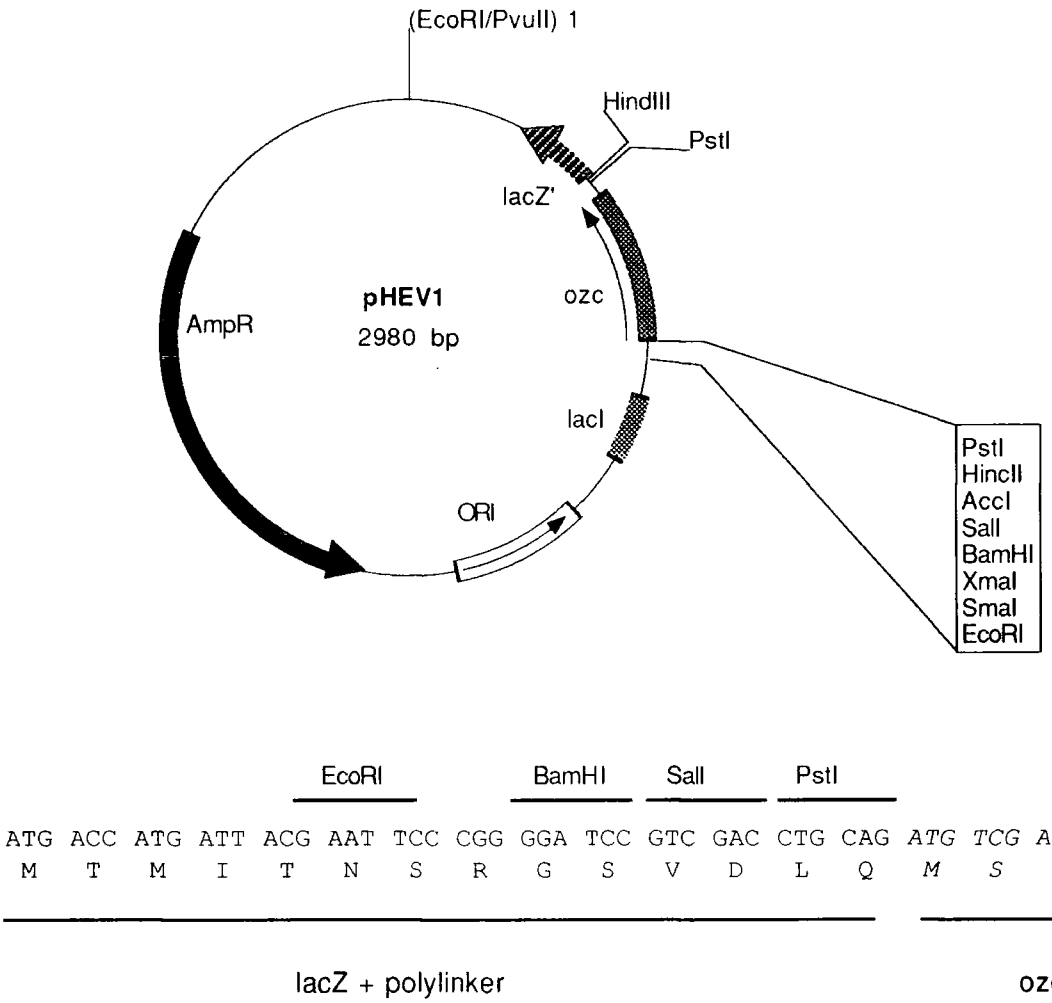


Fig. 4. 36 Map of plasmid pHEV1 containing the expression construct for oryzacystatin (ozc). The ozc coding sequence is expressed in frame with the lacZ polypeptide (shown in sequence below map), and terminates with its own stop codon. The resulting ozc polypeptide contains an extra 14 amino acids at the N-terminus, derived from lacZ and the polylinker in pUC8. Adapted from Edmonds (1994).

acids at the N-terminus, derived from the first few amino acids of the lacZ gene product, and amino acids encoded by the polylinker (see Fig. 4.36). Assays reported in Edmonds (1994) have shown that the extra N-terminal amino acids do not affect the inhibitory activity of the recombinant oryzacystatin when compared to the "native" protein. Bacteria from a stock of the clone were plated out for single colonies, and DNA was prepared from selected colonies by a standard "miniprep" protocol. Plasmid DNA was digested with EcoRI and SacI to confirm the presence of the oryzacystatin coding sequence insert in the plasmid. Results are shown in Fig. 4.37. Clone 6 was selected for further work.

A large scale culture of bacteria containing pHEV1(ozc) was set up, and allowed to grow for 15 hrs at 378C in the presence of inducer. Cells were harvested and disrupted by sonication as described in "Methods", and oryzacystatin was purified from the cell supernatant by heat treatment (828C, 15 min) and ammonium sulphate precipitation (65% saturation). After dialysis, the protein was lyophilised. Analysis of this protein by SDS-polyacrylamide gel electrophoresis showed that the oryzacystatin was substantially pure, although small amounts of *E. coli* proteins were still present (Fig. 4.38). The protein was considered of sufficient purity for bioassays.

To confirm that the oryzacystatin produced in *E. coli* was functionally active, inhibition of papain was determined *in vitro* using Z-phe-arg-AMC as substrate. 10 µg of inhibitor caused >80% inhibition of 1 µg of papain when tested in a standard enzyme assay (as in section 4.3.1). In a preliminary assay against alfalfa weevil larval gut enzyme, 50 µg inhibitor caused >60% inhibition of protease activity in 0.04 larval guts. further assays using different quantities of inhibitor showed that inhibition did not show a linear response with amount of inhibitor, as was observed for egg white cystatin, and that up to 70% inhibition of the larval gut protease activity could be achieved. Results are shown in Fig. 4.39. Oryzacystatin was not found to be as effective an inhibitor of the alfalfa weevil gut enzyme on a weight-for-weight basis as egg white cystatin (c.f. Fig. 4.35 and Fig. 4.39); however, further assays with oryzacystatin that had been purified to homogeneity would be necessary to confirm this conclusion.

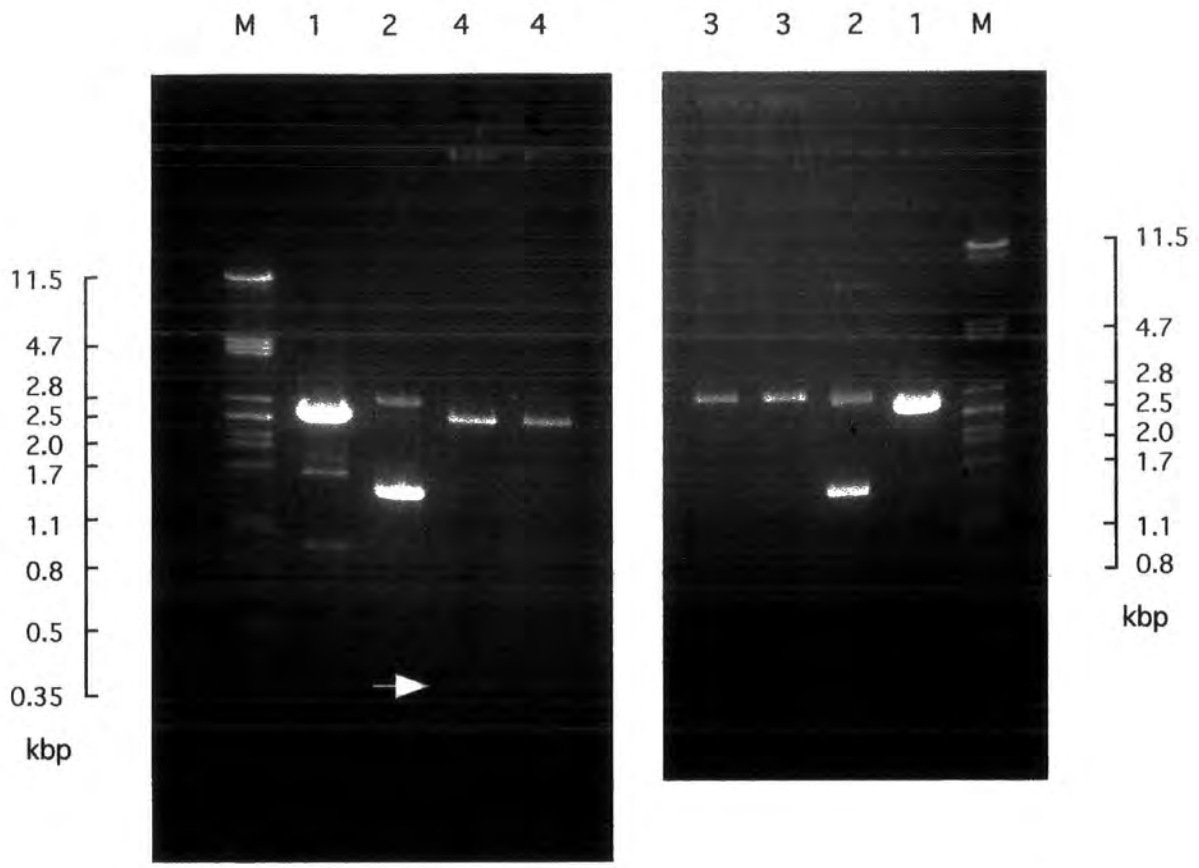


Fig. 4.37 Analysis of DNA from recombinant plasmid pHEV1, containing the oryzacystatin coding sequence.

M = molecular weight marker (λ DNA digested with Pst I)

1 = pUC19 DNA digested with EcoR I (right) or EcoR I and Sac I (left)

2 = pUC19 DNA undigested

3 = pHEV1 digested with EcoR I

4 = pHEV1 digested with EcoR I and Sac I; arrow indicates insert in plasmid.

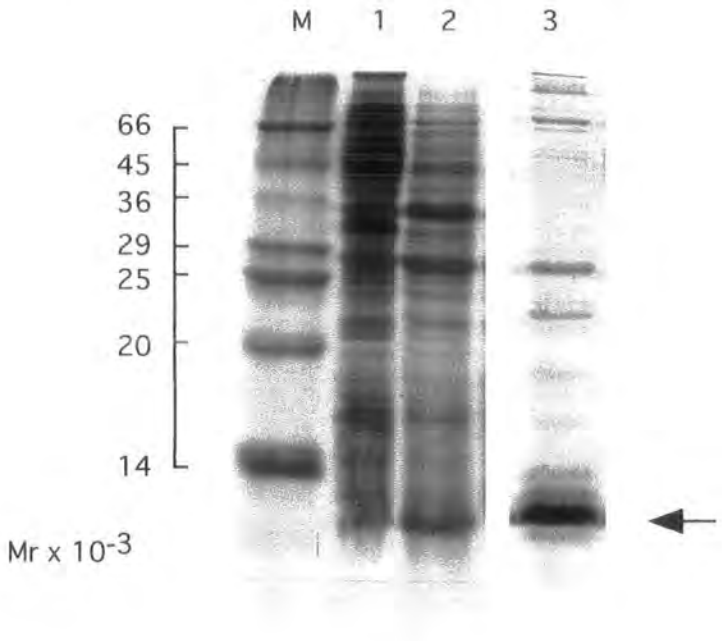


Fig. 4.38 Expression of recombinant oryzacystatin in *E. coli*.
M = molecular weight marker
1 = total protein extract from *E. coli* cells containing pUC19
2 = total protein extract from *E.coli* containing pHEV1
3 = purified recombinant oryzacystatin after heat treatment, ammonium sulphate precipitation, dialysis and freeze-drying.
Arrow indicates oryzacystatin polypeptide.

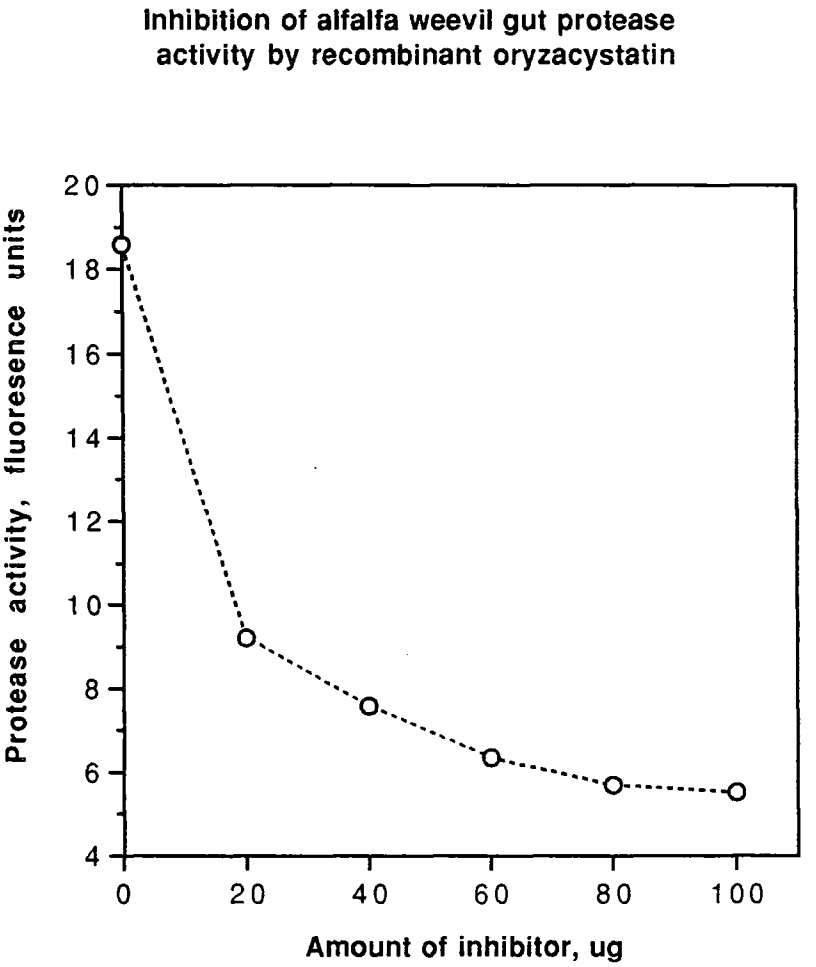


Fig. 4.39 Inhibition of alfalfa weevil larval gut protease activity (substrate Z-phe-arg-AMC) by recombinant oryzacystatin. Each point is the mean of two assays. Each assay contained the equivalent of 0.04 larval guts in a total volume of 810ul.

4.3.3. Artificial Diet Bioassay of Effects of Oryzacystatin on Alfalfa Weevil Larvae

First instar larvae of alfalfa weevil were transferred onto artificial diet based on alfalfa leaf powder (see Methods section). Larvae were divided into batches of 5, and each batch was placed either on diet containing no inhibitor (control), or incorporating oryzacystatin at 2% or 5% of the estimated total protein content to form a single assay replicate. Survival of larvae in each replicate was estimated daily over a 2 week assay period. Results are shown in Fig. 4.40.

Survival on the control diet was 100% to day 2 of the assay, but then declined steadily to approx. 35% by day 7. The decline in survival then levelled off, so that survival on day 14 was approx. 20%. All larvae offered moisture but no diet were dead by day 4. Therefore, although the artificial diet is clearly significantly sub-optimal, it is good enough to allow the effects of added compounds to be assessed. Incorporation of oryzacystatin at 2% of total protein decreased larval survival throughout the assay, although the shape of the survival curve was similar to that on control diet. Survival on 2% oryzacystatin-containing diet was approx. 18% at 7 days and approx. 3% at day 14. Differences between survival on control diet and 2% oryzacystatin diet were significant at $p < 0.05$ for days 2-14 inclusive (Mann-Whitney U-test), and at $p < 0.01$ for days 6, 9-11. Unexpectedly, survival on diet containing 5% oryzacystatin was intermediate between the 2% and control diets. Up to day 5, the decline in survival was similar to that observed on 2% oryzacystatin (and significant at $p < 0.05$), but after this time survival declined less rapidly; survival at day 7 was approx. 26%, and at day 14 approx. 6%, with the difference between control and 5% oryzacystatin diet only significant at day 10. Both overall survival curves for 2% and 5% oryzacystatin are significantly different to that for the control group ($p < 0.05$).

The artificial diet bioassay has thus shown that rice oryzacystatin has a significant effect on survival of larvae of alfalfa weevil, and suggests that expression of the protein in transgenic plants would have a protective effect against this pest.

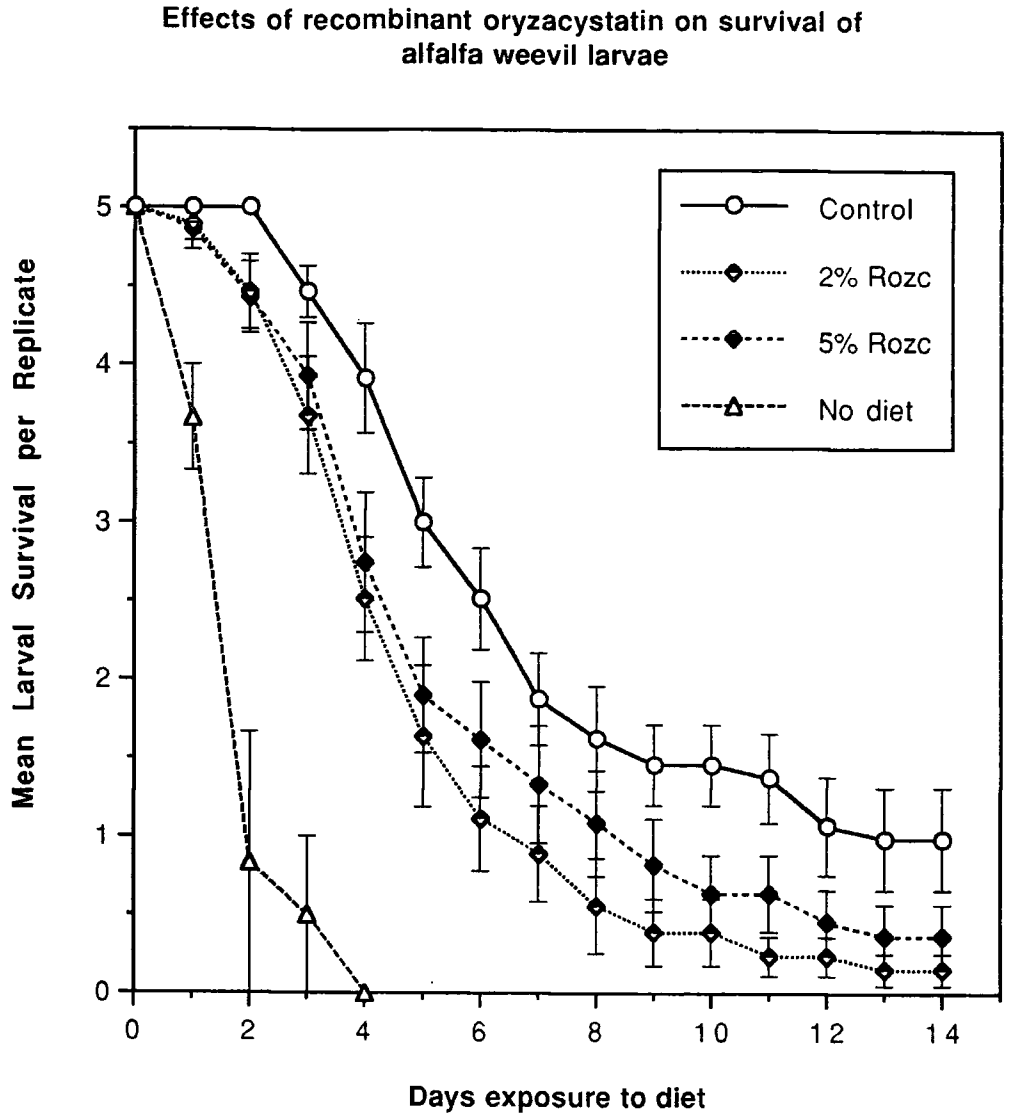


Fig. 4.40 Survival of alfalfa weevil larvae (first instar at day 0) on artificial diet containing recombinant oryzacystatin (Rozc). Error bars are ± SE for the means of at least 10 replicates of 5 larvae.

Chapter: 5

DISCUSSION

5.1. Analysis and Purification of Saponins

5.1.1. Analytical Technique for Saponin Compounds

Although many different procedures have been used for saponin analysis, such as foam production, haemolytic activity, inhibition of fungal growth, insecticidal or piscicidal activity, gravimetery, spectrophotometery, TLC, gas chromatography, HPLC, etc. (Price *et al.*, 1987; Hostettmann and Marston 1995), TLC was used as the principal analytical technique in the present study. While this technique has a number of drawbacks, in that it is semi-quantitative at best, and Rf values of specific components are not routinely reproducible between different TLC plates, these drawbacks are outweighed by its advantages. Separations obtained on TLC plates are very reproducible, and the technique can handle both pure saponins and crude extracts. It is also very sensitive (detection limits in the present study were approx. 1 µg for specific compounds), and has a high resolving power.

Centrifugation was used in the preparation of samples to remove insoluble material, which would distort the spot pattern. It was found that desalted samples gave a good chromatogram of spots, in agreement with Plummer (1978), who stated that before chromatography, biological samples should be desalted using electrolysis or electro-dialysis. The presence of excess salts in the chromatography medium causes spreading of spots on the plate and changes in their Rf values. To obtain reproducible results, it is also necessary to ensure a constant atmosphere in the solvent container. For this reason, during the development of the chromatogram, not only should any exhausting of the evaporated solvent from the tank be prevented, but also any importing of air from outside to the tank. The tank should also be lined with filter paper, dipping in the solvent; this paper will keep the container saturated with

the vapour of the solvent and will aid the ascent of the solvent front (Oleszek *et al.* 1990).

Despite the above precautions, comparison between R*f* values obtained for a particular sample between different TLC plates, or with values stated in the literature, was difficult, and R*f* values for specific saponins varied significantly on different TLC plates in this research (including examples presented in this thesis). However, the separations achieved were qualitatively highly reproducible, and the band patterns obtained allowed components to be readily identified and compared between different plates.

The solvent system used to develop the silica TLC plates used in the present study (ethyl acetate: water: acetic acid, 7: 2: 2) is typical of those reported in the literature for saponin separations. The stationary phase here is relatively polar, while the mobile phase is non-polar and acidic (and thus ensures that saponins containing carboxylic acid groups are uncharged). Saponins should thus separate on the basis of polarity; the less polar the saponin, the further it should migrate. Since the sugar residues attached to the sapogenin are more polar than the sapogenin nucleus, in general the less glycosylated the saponin, the more mobile it will be on TLC under the conditions used. This is broadly borne out by the results presented; particularly, on partial hydrolysis of saponins by insect and plant extracts (section 4.2.9), less mobile spots on TLC disappear, and more mobile spots are produced. Similar solvent systems for saponin analysis were used by Dubois *et al.* (1988) e.g. a) $CHCl_3$: MeOH : H_2O (64 : 40 : 8); b) $CHCl_3$: MeOH : AcOH : H_2O (60 : 32 : 12 : 8); c) EtOAc : AcOH : HCOOH : H_2O (100 : 11 : 11 : 26).

The relatively non-polar sapogenins migrate virtually with the solvent front in the system used for saponins, and a less polar solvent system, which increases polar interactions between solutes and stationary phase, must be used for sapogenin separation and analysis. Medicagenic acid, hederagenin, lucernic acid, and soyasapogenol B were identified to be more mobile than their intact saponins by TLC (Wyman-Simpson *et al.* (1991). Petroleum ether : chloroform : acetic acid (7 : 2 : 1

v/v/v) was used as a solvent for sapogenin separation in the present study; other comparable systems are quoted in the literature, e.g. toluene-acetone (4: 1) (Dubois *et al.*; 1988); benzene: MeOH (92: 8) (Wyman-Simpson *et al.*, 1991).

Saponin spots were visualized on the thin-layer chromatograms by spraying with MeOH: Acetic Anhydride: Sulphuric Acid (10 : 1 : 1 v/v/v) as reagent, and subsequently drying the plate in an oven at 104°C for 15 min or at 180°C for 2.5 min (cf. literature values of 120°C for 2 min (Oleszek 1988; Nowaka and Oleszek 1992; Oleszek *et al.* 1990; Oleszek & Jurzysta 1986), or 120°C for 5 min Ireland (1987)). The coloured spots which are produced can be viewed under normal or UV light; the latter has the advantage that phenolic compounds give fluorescent spots, and thus can be distinguished from saponins. Alternative staining reagents for saponins have been described (Price *et al.*, 1987); for example, Dubois *et al.* (1988) detected saponins with Komarowsky's reagent (a mixture (5: 1) of p-hydroxybenzaldehyde (2% in MeOH) and 50% H_2SO_4), Godin's reagent (vanillin-sulphuric acid) and Nile blue reagent; Wyman-Simpson *et al.* (1991) sprayed TLC plates with Libermann-Burchard spray reagent and visualized coloured and fluorescent spots under long wave UV light (366 nm).

5.1.2. Extraction Procedures

Source Material:

The extraction of saponins from root and shoot tissues of alfalfa was carried out routinely on material that had been frozen immediately after harvesting, using liquid nitrogen. This procedure will minimise artefacts due to any possible enzymatic or chemical alterations to saponins. The tissues were ground while frozen and immediately extracted in 80% MeOH; this procedure will again prevent enzymatic changes to saponins, and the use of a low temperature (4°C) for the MeOH extraction will also minimise any chemical hydrolysis or other alterations. Many previous workers who have studied saponins in alfalfa have used dried plant material, which has been extracted under relatively harsh conditions; for example, Morris *et al.*

(1961) used alfalfa root tissue, collected as ploughed in the alfalfa stands, and dried in a forced-draft oven until the roots became brittle. Saponins were then extracted with 95% ethanol at 80°C for 6 hrs. While such harsh treatments are not deleterious if the primary aim is to investigate sapogenins, for extraction of saponins with intact carbohydrate side chains and other substituents, it is important to prevent any unwanted enzymatic activity after harvesting tissue. Enzymes have been found to be active when ground tissue is left in aqueous solution. Although no changes were detected in the saponin quality during drying (results of preliminary experiments not shown), the use of dried alfalfa tissue was avoided, since saponin degradation has been shown to occur in other species during tissue drying. For example, momordin II, a major bisdesmoside saponin isolated from the root of *Momordica cochinchinensis*, was converted to the monodesmoside momordin I by intracellular esterases present in the root during the drying process (Kawamura *et al.*, 1988). Alfalfa contains enzymes capable of modifying or degrading saponins, probably by a hydrolytic process, and most methods for extracting saponins from alfalfa have used an initial drying stage, so this may have introduced artefacts into experiments due to saponin hydrolysis.

Saponin hydrolysis on drying alfalfa foodstuffs may be an important factor in increasing the nutritional value of the crop. When ruminant animals are fed with green or fresh alfalfa, or grazed on alfalfa in the field, they develop bloat in their rumen stomach. In contrast, if they feed on the same alfalfa after drying not only they will not have any problem with bloat, but also the alfalfa is a good source of nutrients. Although it has been stated that alfalfa saponins are degraded by ruminal micro-organisms and that they have little effect on the animal (Lu and Jorgensen 1987), it is likely that they are involved in causing bloat due to their foam-forming properties.

Extraction Conditions:

Saponins are generally extracted with mixtures of miscible organic solvents with water, in a rather non-specific manner; methods described in the literature for

extraction of alfalfa saponins rely on the solubility of saponins in water and alcohol such as MeOH or butan-1-ol (BuOH) (Massiot *et al.*, 1988a and 1992). 80% MeOH was the preferred solvent for saponin extraction in this study. Extraction was routinely carried out at 4ºC , for reasons mentioned above; in a comparative experiment, extraction was carried out in a boiling water bath. In this case, extra bands were observed on the TLC analysis, which could have been produced by hydrolysis of saponins at 100ºC. Extraction with water at 100ºC, followed by partition into *n*-BuOH was used by Massiot *et al.* (1988a and 1992) to study saponins and their sapogenins in alfalfa; however, these authors suggested that extraction in boiling water may have hydrolysed the saponin content of the tissues to sapogenin. Nowaka and Oleszek (1992) concluded that the saponins in alfalfa are present in a number of glycosidic forms, each having a different polarity, thus in order to obtain the highest recovery possible, the extraction solvent must be carefully selected. The extraction method used follows that of Oleszek (1988). However, other methods are found in the literature; for example, although mono- and bisdesmosidic saponins can be separated after extraction in aqueous MeOH, extraction by partitioning between BuOH and water mixture has been recommended (Fenwick *et al.* 1991).

The crude saponin extract obtained by the procedure described in this thesis contains many other types of compounds besides saponins; fluorescent spots after TLC analysis indicated the presence of phenolic compounds, and simple sugars were also present. Nevertheless, the pattern of spots obtained on TLC was reproducible, and could be considered to represent the entire spectrum of saponins present in the source tissue.

5.1.3. Hydrolysis of Alfalfa Saponins

The reaction conditions of hydrolysis have been investigated to determine the best conversion of saponins to sapogenins. Tava *et al.* (1993) suggested eight hours as the best time for refluxing alfalfa root saponins with 2M HCl in 50% MeOH to purify sapogenins, but prolonged times give reduced medicagenic acid yield, probably

owing to decomposition. Rao and Bories (1987) stated the best period for hydrolysis of saponins to be 12 hrs. Acid hydrolysis of alfalfa saponin extracts with 2N HCl was carried out with 3 hrs refluxing to produce sapogenin extracts in the present thesis. Acid hydrolysis with 2N H_2SO_4 is also possible; for example, Oleszek and Jurzysta (1986) hydrolysed saponins with 2N H_2SO_4 in 50% MeOH for 4 hrs, and Gestetner (1971) used 0.4N H_2SO_4 for 2.5 hrs in an autoclave at 125°C.

Analysis of the sapogenin mixtures produced by hydrolysis by TLC showed three major sapogenin components, represented by a spot at R*f* 0.31 (yellow) in roots, and spots of R*f* 0.11 (intense dark green) and 0.61 (yellow) in shoots (colours under UV light). These results should be directly comparable to those of Waller *et al.* (1993), who used the same solvent system as in this investigation to analyse hydrolysed alfalfa saponins. R*f* values of 0.06 (green spot), 0.22 (brick/cherry red) and 0.16 (peach-rose; colours under normal light)) were estimated for medicagenic acid, soyasapogenol and hederagenin, respectively. Characterisation of the sapogenin spots on TLC plates in the present work is difficult because of the lack of any standard sapogenin, and none of the spots obtained can be clearly identified. Since standard saponins became available to aid the identification of saponin spots on TLC, it was not considered worth pursuing identification of sapogenins, although hydrolysis of characterised saponins under the conditions used, followed by analysis by TLC, would enable the sapogenin spots to be readily identified.

5.1.4. Purification of Alfalfa Saponins

The complexity of alfalfa saponins, as described earlier, and observed in the present investigations, is a significant problem if single components are to be isolated and investigated. However, the availability of chromatographic techniques for saponin separation offers a means to achieve high resolution in separations of preparative amounts of materials.

Preliminary Experiments on Micro-Scale Reverse Phase Columns:

Separation of the crude saponin extract on 1-ml disposable Sep-Pak reverse phase (C18) cartridges (intended for sample clean-up prior to HPLC) was used to show the utility of reverse-phase chromatography in separating alfalfa saponins. Aqueous MeOH was used to elute samples at their "natural" pH (acidic), to protect the silica in cartridge against any damage (Oleszek 1988). The use of unbuffered eluants can cause problems with non-reproducibility, since the selectivity of the C-18 sorbent to retain saponins is strongly dependent on the pH of the sample loaded (Oleszek 1988). In reversed-phase chromatography, the stationary phase is non-polar, and mobile phase such as water or MeOH is relatively polar, so solvent mixtures with different polarity can elute compounds of different polarity, or, in this context, different saponins. The most polar component will elute at the lowest MeOH concentration (Skoog and Leary 1992). Elution of the Sep-Pak cartridge with a stepped gradient allowed a series of fractions to be collected; analysis of these fractions by TLC revealed the compounds present.

According to Oleszek (1988), saponins could not be eluted from C18 Sep-Pak cartridges with solutions containing less than 40% v/v MeOH in water, but washing the cartridge with water removed carbohydrates, which may affect saponin isolation, and other unwanted polar components. The saponin extract must be presented to the column as an aqueous solution instead of in MeOH, since MeOH causes removal of the sugars, phenolics, glycosides, oligosaccharides and flavonoids in addition to the saponins from Sep-Pak (C-18) cartridges (Fenwick *et al.* 1991).

In general, analysis of fractions from separations of alfalfa crude saponin extracts showed that blue fluorescent spots on TLC, considered diagnostic for phenolic compounds, were present in fractions eluted in the concentration range 10-50% MeOH, whereas coloured spots diagnostic for saponins eluted principally in the range 40-70% MeOH. Separation of different saponin components in the different fractions was apparent. No saponin spots were observed in the range 80-100%

MeOH, although other authors have stated that hederagenin and soyasapogenol glycosides are present in the eluates of 70-100% MeOH (Oleszek 1988; Fenwick *et al.* 1991). In shoot extracts, the 10% and 20% MeOH fractions gave two green spots, R*f* 0.14 and 0.20. Oleszek (1988) has suggested that these spots may be zanhic acid tridesmosides; the presence of three glycosides makes this saponin relatively polar, leading to early elution from the reverse-phase column, and a low R*f* value on TLC (Fig. 4.4).

Chromatography on the Sep-Pak cartridge was not a practical technique for preparative purposes. The cartridge overloaded with relatively small quantities of extract, and separations under these circumstances were poor. No further development of the technique was feasible after the preliminary experiments described.

Solvent fractionation:

Although preparative-scale HPLC on a C-18 reverse phase column was suggested as the purification method of choice for alfalfa saponins, based on the preliminary experiments, loading crude saponin extracts directly onto HPLC columns was not successful. It was observed that columns became partially blocked by crude saponin extracts, leading to high back pressures, even after filtration of the samples that were applied. Removal of non-saponin components was thus necessary, and was effected by solvent fractionation. Although BuOH partition did not give any significant purification of saponins as judged by the spots detected after TLC, it was effective in removing polar non-saponin components that caused column blocking, which separated into the water layer. There may have been some loss of zanhic acid tridesmosides in this step, but these are a minor component in the total alfalfa saponins, and are not in themselves responsible for the toxicity of alfalfa saponins towards insects, since the saponin mixture after BuOH extraction and ether precipitation is comparable in toxicity to the crude saponin extract (q.v.).

BuOH extraction has been employed in other purification schemes for

saponins; for example, Shao *et al.* (1995) purified medicagenic acid glycoside from *Aster batangensis* roots after extraction with petrol, ethyl acetate, and *n*-BuOH successively. The *n*-BuOH soluble fraction contained the saponin, which was purified by chromatography. Similarly, Shashida *et al.* (1992) re-extracted methanolic extracts of steroidal saponins from the roots of *Smilax piparia* with *n*-BuOH and chromatographed *n*-BuOH –soluble phase to purify saponins.

Saponins present as a solution in water-saturated *n*-BuOH can be recovered by evaporation, dissolution in MeOH, and precipitation by the addition of ether. The saponins are relatively polar, and addition of non-polar ether causes them to come out of solution. This technique was used routinely to recover saponins; after centrifugation and drying a brown- or cream-coloured powder which could be stored successfully was generated. Evaporation of saponin solutions left a sticky residue, which could not be removed from flasks, weighed, or quantitated in any way. Ether precipitation may also be useful for removing impurities not detectable on TLC analysis. The combination of extraction in 80% MeOH, partition with *n*-BuOH and ether precipitation was used as a standard method for preparing saponin mixtures. However, such mixtures still contain other components, as is apparent when chromatography on reverse-phase columns is carried out.

Saponin purification by the use of cholesterol, ammonium, or sodium salts to fractionate saponins into two classes was once much used but tends now to be superseded by chemical and chromatographic procedures (Fenwick *et al.*, 1991).

High-Performance Liquid Chromatography (HPLC):

Saponin mixtures were further purified by HPLC using a preparative scale reverse-phase column (C18). Despite of the complexity of saponin mixtures in the root and shoot tissues of alfalfa seedlings, this method was found to be an effective technique of separation and purification of saponins. Saponin mixtures were applied in aqueous solution to the column, and then eluted with a gradient of aqueous iso-propanol. Iso-propanol was used instead of MeOH (used on Sep-Pak cartridges) as

the non-polar eluant, both to improve separation and to decrease the concentration of non-polar component required for saponin elution. Although the capacity of this column was still limiting, up to 100 mg of saponin mixture could be loaded on per chromatographic run, from which individual saponin fractions were recovered in 1-10 mg amounts.

Similar systems have been used by other investigators for saponin separations. Domon *et al.* (1984) developed an HPLC method to separate the olean-12-ene triterpenoid glycosides, to investigate the bioactivity of the intact oleanane saponins. They isolated both mono- and bis-desmosidic saponins. Triterpenoid saponins were successfully chromatographed on a reversed-phase column and polar bonded supports such as DIOL. The solvent used was a system composed of either MeOH and H_2O or acetonitrile and H_2O. In contrast, Kitagawa *et al.* (1976) used silica gel column chromatography in chloroform: MeOH: H_2O solvent mixtures to isolate soyasaponin I, II and III from soya, while soyasaponin bisdesmosides A1 and A2 were separated and purified by centrifugal liquid chromatography and acidic ion-resin treatment (Price *et al.*, 1987). Fenwick *et al.* (1991) reported that a combination of preparative HPLC on silica gel and reversed-phase columns could be used to effect separations by complementary techniques.

Detection of saponins after chromatographic separations poses problems due to a lack of suitable chromophoric groups, which will absorb UV light and allow detection by standard UV-flow cell photometers. The presence of a double bond in the sapogenin nucleus, or a carboxyl group, allows the use of low wavelength UV detection (200-216 nm), but use of these wavelengths is subject to interference by solvents, and by other compounds which absorb much more strongly at these wavelengths. As a consequence, the elution profiles reported in this thesis for purifications of saponin mixtures do not show saponins as major peaks in the elution profiles, whereas TLC analysis of the fractions from chromatographic separations shows saponins as major components. Coloured impurities are present in the saponin mixtures, which absorb strongly at low UV wavelengths. For this reason, absorption

could not be used to identify saponin-containing fractions, and analysis of fractions by TLC was routinely necessary.

Most other workers have used low wavelength UV light as a detection method for saponins; for example, oat avenacoside saponins at 200 nm (Ireland 1987), 216 nm for the cortex saponins from *Quillaja saponaria.* (Kensil *et al.* 1991), 203 nm for ginsenosides (Fenwick *et al.* 1991), and 206 nm for potato glycoalkaloid saponins (Price *et al.* 1987). If a suitable chromophore is present, the use of a higher wavelength is advantageous, as in the case of glycyrhizin in liquorice roots and extracts, which can be detected using UV light at 254 nm. The use of a refractive index detector for saponin identification has also been reported (Fenwick *et al.*, 1991), but this method is also subject to severe interference by other compounds present in extracts.

Fractions collected from chromatographic separations were pooled and evaporated. The high proportion of water in pooled fractions makes evaporation slow; this was identified as a problem in the chromatography of soyasaponins (Ireland 1987). After evaporation, the sticky residue was again converted to a powder by ether precipitation after dissolving in MeOH; the resulting cream powders could be stored successfully if kept desiccated.

Resolution of individual saponins:

Purification of the alfalfa saponins on the preparative C18 reverse phase column, while a good method, was not entirely ideal, as only some saponins could be purified to single components by this method. In particular, the more complex nature of the saponin mixture from roots made purification of individual saponins from roots impossible. Oleszek (1992) has stated that leaves of mature alfalfa plants contain about 30 individual saponin components, the polarity of which is very similar, which makes separation extremely difficult. In the present work, a second chromatographic step would be desirable to effect a complete separation of the different components; however, this was not the primary aim of the research, and was not feasible if

sufficient material for bioassays was required. Fortunately, the separation into saponin fractions was sufficient to allow relative toxicities of the different components to be investigated. Although the presence of non-saponin components in these fractions was not specifically analysed, no other components were detected either by TLC or by NMR on saponin fractions which had been repassaged through the reverse phase column.

5.1.5. Characterization and identification of saponins

Identification of saponins using TLC:

Although tentative identification of saponins can be made on the basis of R*f* values on TLC, and values given in the literature, it is not valid to identify saponins on the basis of R*f* values only, since mobility of components on TLC plates is subject to a high level of random variation between different TLC runs. However, the use of standard saponins of known structures, which can be run on the same TLC plate as unknowns, allows specific components to be identified. This identification is still not conclusive, since correspondence of spots on TLC does not prove identity, and single spots may be composed of more than a single saponin or aglycone, depending on the sample and the TLC system, but is sufficient as a working definition if other evidence is taken into account. In agreement with this conclusion, Tava *et al.* (1993) stated that on the basis of TLC analysis, it was possible to identify major components of the crude saponin extracts by comparison with authentic compounds. Nevertheless, the identification of components in the root and shoot saponins using standard saponins supplied by other workers described in section 4.1.6 cannot be regarded as conclusive except in the case of soyasaponin I, where structural studies were carried out. The relative polarity of the compounds, their elution from the reverse phase column (% range of elution with isopropanol increases with decreasing polarity, so that monodesmosides elute after bisdesmosides, and sapogenins containing carboxyl groups, such as medicagenic acid, elute after those that do not, such as soyasapogenol), and their mobility on TLC (mobility increases with decreasing

polarity, so that monodesmosides are more mobile than bisdesmosides, and sapogenins with carboxyl groups are more mobile than those without) are all consistent with the assignments given. The identification of medicoside A (3-O-β-D-glucopyranosyl medicagenic acid) is valid by Tava's critieria, but some of the other identifications must be regarded as tentative.

As indicated in the results section, identification of the sugar beet and potato saponins cannot be regarded as other than preliminary.

Identification of the Structure of Soyasaponin I using NMR:

Further identification to confirm the structure of the major sapoinin purified from alfalfa seedling shoots was carried out by NMR, after repurification of the compound by HPLC. ^{13}C NMR spectroscopy is widely used to examine the structure of aglycones of saponins, and ^{1}H NMR for determination or confirmation of the glycosidic species (Price *et al.*, 1987). Assignments of the signals of various carbons of the saponins are generally made by comparison with the ^{13}C NMR data of the aglycone and methyl sugar using known chemical shift rules, and glycosylation shifts (Mahato *et al.*, 1988). The nature of the aglycone-sugar linkage is normally determined enzymatically or by inspection of ^{1}H or ^{13}C NMR spectra (Fenwick *et al.*, 1991). NMR is the favoured technique for structure determinations of saponins; in many cases complete structure determination of saponins is possible by NMR spectra alone in approx. 6 hrs of instrument time (Mahato and Nandy 1991). ^{13}C and ^{1}H NMR have been used to characterise the *Solanum* glycoalkaloids, and the ^{13}C NMR spectra of the aglycones, demissidine, solanidine, tomatidine, solasodine and soladulosidine have been reported by Radeglia *et al.* (1977).

The structure of soyasaponin I, (3-O-α-L-rhamnopyrosyl (1->2)-β-D-galactopyranosyl(1->2)-β-D-glucoronopyranosyl soyasapogenol B) was fully elucidated by Tsurumi *et al.* (1992), using both NMR and FD-mass spectrometry. This saponin had been extracted and purified from soybean (*Glycine max*) previously, and identified by Kitagawa *et al.* (1976, 1982). Soyasaponin I is also found in many

other species besides soya and alfalfa. For example, Ireland (1987) extracted this compound from haricot bean and red kidney bean. However, Massiot *et al.* (1991) concluded that soyasaponin I is not uniform in all species, and that some species (including alfalfa) differ from the "original" soyasaponin I by having a glucose instead of a galactose residue in the trisaccharide on C-3. The possible presence of a maltol conjugate of soyasaponin I in alfalfa has also been noted previously. The wide range of elution from the reverse phase column shown by soyasaponin I from alfalfa seedling roots and shoots in the present work suggests that some undetected heterogeneity in the substituents on the sapogenin nucleus may be present, but this has not been investigated further.

A further factor to be considered in the identification of soyasaponin I is the possible presence of substituents on the hydroxyl group at C_{22}. A γ-pyronyl substituent has been reported in soyasaponin isolated from germinating pea seedlings, and a maltol substituent in is present in a soyasaponin isolated from alfalfa sprouts, as described earlier (section 1.7.1). These, or similar, substituents may be present on the soyasaponin isolated from alfalfa seedling tissues. C_{22} hydroxyl substituents might not be detected on TLC, because of their lability in the solvent system used, which is strongly acidic (18% acetic acid); on the other hand, the extraction and purification steps do not involve conditions likely to cause hydrolysis. Consequently, these substituents could account for the wide range of elution of soyasaponin I from the reverse phase column; bands that are identified as soysaponin I on TLC could in fact represent hydrolysis products of soyasaponin I derivatives containing labile substituents on the C_{22} hydroxyl. Further work would be required to investigate the presence of these labile substituents.

Soyasaponin I has been reported to have poor solubility in water (Oleszek and Jurzysta 1990), and this was confirmed in the present work. To dissolve this saponin from purified solid in water, suspensions were treated to a freeze-thaw process (three cycles of freezing and melting). Interestingly, when this saponin was present in the purified saponin mixture prior to HPLC, the mixture as a whole was freely soluble in

water. Possibly other components in the mixture were providing counter-ions for the glucuronic acid residues present on the carbohydrate side chain of soyasaponin I, and thus increasing its solubility.

5.1.6. Changes in Saponin Content with Alfalfa Development

The analysis of alfalfa saponins at different ages of the plant reported in this thesis, and shown in Fig. 4.12, demonstrates that significant changes to major saponin components take place as the plant develops. Although the analysis shown was carried out on crude saponin extracts, so that some of the spots shown may not be due to saponins, analysis of purified saponin mixtures (after BuOH partition and ehter precipitation) gave essentially similar results (data not presented). Although many of the spots cannot be identified, it is clear that soyasaponin declines in relative amount as plant development proceeds, while components of lower mobility, which may be glycosides of medicagenic acid, increase. Although other authors have investigated the presence or absence of different saponins in roots, leaves and seeds of alfalfa (see introduction), changes with plant age have not been systematically investigated. Although outside the scope of this thesis, these developmental changes in saponin content would merit further investigation.

5.2. Toxic Effects of Saponins on Insects

5.2.1. Bioassays of Saponin and Sapogenin Extracts against Cowpea Seed Weevil (*Callosobruchus maculatus*)

Rationale for the use of Callosobruchus maculatus:

Cowpea seed weevils are easy to culture and maintain, and the larvae have been used by many workers (e.g. Applebaum 1965) as a subject for bioassay of potentially toxic compounds. The bioassay technique using artificial diet pellets into which compounds could be incorporated was developed in Durham (Gatehouse *et al.* 1979), and has been used to show that proteins such as enzyme inhibitors and lectins

are toxic to developing larvae of this insect. C. *maculatus* larvae are oligophagous, and will tolerate a limited range of host species, but are still sensitive to many toxic compounds, and thus are a good subject for bioassays. Survival of larvae can be measured as one parameter of toxicity, and time taken for adult emergence can be used to give a measure of whether added compounds retard development of the insect.

Saponin Bioassay:

For the study of insecticidal properties of alfalfa saponins, the crude saponin extracts from alfalfa root and shoot tissues were used to assay the effects of the compounds present against larvae of the cowpea seed weevil (*Callosobruchus maculatus*). The artificial diet system used was based on chickpea seed meal, and since chickpea is an appropriate host for cowpea seed weevil, so control diet pellets supported a good development of the seed weevil. The saponin extract was incorporated into the diet at concentrations equivalent to that in alfalfa seedlings (based on the fresh weight of tissue extracted), 10% of the level, and 1% of the level. Since the water content of chickpea seed meal is <10% that of seedling tissue, on a dry weight basis the levels of saponins used would be approx. o.1x those on a fresh weight basis (i.e 10%, 1% and 0.1% the "natural" level).

In the bioassays reported here, saponin extracts from alfalfa roots showed no insecticidal activity. In fact there was a positive effect from the root extract on *C. maculatus* development (Fig. 4.15). This result is surprising, since most of the bioassays of the effects of alfalfa saponins on insects and fungi have been done with material extracted from roots, and have shown a negative biological activity. For example, Shany *et al.* (1970) concluded that alfalfa root saponins are stronger haemolytic agents and are more toxic to *Tribolium castaneum* larvae than alfalfa shoot saponins. The toxicity of the root saponins is commonly ascribed to high levels of medicagenic acid glycosides, especially medicoside A, the 3-O-glucoside (Massiot *et al.* 1988b; Gestetner *et al.* 1970; Shany *et al.* 1970; Oleszek and Jurzysta 1986; Wyman-Simpson *et al.* 1991; Livingston *et al.* 1979; Tava *et al.* 1993). The absence

of toxicity in root saponins in the present assay may be due to the source of the saponins, which were extracted from alfalfa seedlings (2 weeks old). As shown in Fig. 4.12, the level of medicoside A found in root tissues of alfalfa seedlings is low, and increases as plants develop. On the other hand, seedlings contain higher levels of soyasaponin I, which may have low toxicity towards *C. maculatus*. The percentage saponin content of chickpea has been reported as about 0.23% of dry matter (Fenwick *et al.* 1991), which is close to the average amongst the peas and beans. Ireland (1987) analysed chickpea and reported soyasapogenol B as the sole aglycone, and the saponin preparation chromatogram indicated the presence of two components, including soyasaponin I. It is therefore possible that *C. maculatus* has adapted to a low level of saponins in its diet, and is specifically adapted to soyasaponin I (as a common saponin component of different legume species). However, if this is the case, it is difficult to account for the results for shoot saponins in this assay, which showed a significant toxic effect, and thus suggest that soyasaponin I is showing toxic effects, since this is the major component of the shoot saponin extract.

Problems in interpreting the results from this assay system from serious shortcomings in the experimental scheme. The crude saponin extracts contain many other compounds besides saponins; the presence of free sugars has been considered in the results section (4.2.1), but there are also phenolics and polar lipids present. The effects observed are thus not ascribable to any one compound, but are a combination of probiotic and antibiotic effects caused by different components. For example, the presence of individual sugars in an artificial diet, shortened the duration of development of *C. maculatus* compared to the development of weevils on control diet (Applebaum *et al.*, 1965). For this reason, the assays have been regarded only as indicative that saponins from alfalfa tissues can show insecticidal effects, and that broadly shoot saponins appear more effective than root saponins.

A further factor affecting these assays is alterations in the physical structure of the diet pellets caused by the compounds added to them. This will be considered further subsequently, but may also have played a part in these assays. Evidence that

changes had taken place in the physical structure of the chickpea diet pellets was provided by the pellets containing shoot saponins at "10%" concentration, where many larvae emerged from the pellets and passed their pupal stage outside the diet, whereas normally pupation occurred inside the pellets. Where insects passed their pupal life stage outside the diet pellets, the pupal stage was shorter than normal (Table 4.5).

Sapogenin Bioassay:

This experiment explored the hypothesis that hydrolysis of saponins made them less toxic to insects. It has been claimed that some sapogenins are not biologically active; for example, although medicagenic acid did have a toxic effect, the soyasapogenols have no detrimental effect at all on growth of the *Tribulium castaneum* larvae. The crude sapogenin residue, after hydrolysis, was dissolved in MeOH in preparation of the diet pellets, in contrast to the saponin residue, which was dissolved in water. Sapogenins are insoluble in water because of the cleavage of the linkage between triterpene and sugars. Although MeOH is known to be toxic to insects, freeze drying the diet pellets was a good way to remove alcohol and to remove the unfavourable effects in the insects.

The results of sapogenin bioassay showed that the treatments with highest concentration of sapogenins (at 100% incorporation level) had a most significant effect on the cowpea seed weevil. These treatments did not allow the emergence of any adults from the diet pellets containing either root or shoot sapogenins. The results are almost certainly due to physical factors, as described in the results section (4.2.2), and are not considered further.

The bioassays with lower concentrations of sapogenins showed no significant enhancements of adult emergence, but little toxic effect either; there was some evidence that the shoot sapogenin extract had some deleterious effect on larval survival, and some delay in adult emergence caused by both extracts. Thus, broadly, the results support the hypothesis that hydrolysis of saponins decreases their

biological activity, although due to the drawbacks in these assays noted above, it is not possible to draw firm conclusions from the results. Bioassays with pure compounds would be necessary to demonstrate that the effects observed are actually due to specific saponins or sapogenins, and to assess their relative toxicities towards the cowpea seed weevil.

5.2.2. Bioassays of Purified Saponin Mixtures against Potato Aphid (*Aulacorthum solani*)

Rationale for use of Aphids:

The practical reasons for switching to the use of aphids for further bioassays of saponin toxicity against insects have been considered in the results chapter (section 4.2.3). Potato aphids (*Aulacorthum solani*) were used as they were available in the laboratory, and are oligophagous rather than polyphagous, and thus were likely to show sensitivity to insecticidal compounds. Highly polyphagous aphid species such as *Myzus persicae* (peach-potato aphid), which were also available, are more likely to have the capacity to tolerate, or to detoxify, insecticidal compounds from a wide range of plant species.

The use of aphids allows survival, development and fecundity of insects to be measured. Survival of aphids on control diet was routinely high (> 90%) over the period of the bioassay until nymph production commenced, showing that the diet supports good development in this species. Nutritional requirements of many aphid species have been studied by rearing them on chemically defined liquid diets (Minks and Harrewijn 1987). The decline in survival observed when nymph production is in progress is also observed in aphids reared on plants. The only indication that the artificial diet is not optimal for these aphids is in the lower production of nymphs per aphid observed on diet compared to *in planta* (decreased to approx. 25-50%), and the failure of "second generation" aphids to produce nymphs themselves. The quantity and quality of the diet influences fecundity in many phytophagous insects (Heinrichs, 1988), and fecundity is thus a sensitive indicator of antibiotic effects. The diet

system was used as a good model for assessing toxic effects of added compounds.

Data produced by the different measurements possible in this bioassay system can give results that may appear inconsistent. In considering survival, the use of a corrected mortality parameter (Abbott 1925) was not found to be useful in the aphid bioassays; aphids on the recommended "no diet" control survived for only a short time (3-4 days), so that corrected mortality values were often low, even for saponins that subsequently showed significant growth depressive effects. The short lifespan of aphids which are not feeding can result in a fall in survival over the initial period of a bioassay if a compound had a feeding deterrent effect, since some aphids die before they are able to overcome the deterrent effect; however, in those aphids that do survive, effects on subsequent development and fecundity are small. Conversely, a compound may not act as an anti-feedant, and thus have little effect on initial survival, but can be significantly toxic, causing survival to decline after the initial 3-4 day period, and/or a retardation of development and reduced fecundity. Both these effects can be observed in different bioassays reported in this thesis. In assessing the relative toxicities of different saponin preparations and fractions, both these effects must be considered.

Despite potential complications in interpreting data from aphid bioassays, this system proved to be sensitive and reproducible in measuring effects of added saponins. However, further experiments would be necessary to determine the extent to which conclusions drawn on the basis of assays with this aphid species can be extended to other aphids, or other insect pests. Susceptibility to allelochemical differs from insect to insect, and with other pest invertebrates; for example, a high level of saponins in plant species was correlated with resistance to pea aphids (*Thosiphon pisum*) but had no effect on chalcids (*Brchophagus roddi*), clover root curculio (*Sitano hispidulus*), spotted alfalfa aphid (*Therioaphis maculata*), alfalfa weevil (*Hypera postica*), northern root-knot nematode (*Meloidogyne hapla*) or stem nematode (*Ditylenchus dipsaci*) (Pedersen *et al.*, 1976).

Assays of purified saponin mixtures:

Crude saponin extracts could not be used in aphid bioassays due to severe discolouration of the diet, presumably due to chemical reactions between diet components and compounds in the extracts. Thus, in order to demonstrate that alfalfa saponins were toxic to potato aphids, it was necessary to use the saponin mixtures purified from seedling shoot and root tissues by BuOH partition and ether precipitation. The purified saponin mixtures could be dissolved in diets at known concentrations, allowing dose-response effects to be assayed. The dose-dependence of toxic effects is an important criterion for ascribing resistance to a particular component; for example, increased mortality in leafhopper and pea aphid nymphs was related to increased saponin concentration rather than saponin type and source, although these were also important factors (Horber *et al.*, 1974).

The results of these bioassays have been discussed in section 4.2.3. Whereas the shoot saponin mixture showed dose-dependent progressive effects on aphid development and fecundity, the effects of the root saponin mixture suggest a relatively small toxic effect, since nymph production was not significantly decreased by the lower concentrations of root saponin mixture used. However, the root saponin mixture at the highest concentration used produced complete mortality over the inital 4 day period. This result might lead to the conclusion that the root saponin mixture is more toxic to aphids than the shoot saponin mixture; however, the results of the assays using different doses of saponins clearly demonstrate that the shoot saponin mixture is in fact more toxic. The most likely explanation for this apparent contradiction has been discussed above; the root saponin mixture appears to have a strong antifeedant effect, whereas the shoot saponin mixture has a lower (though still significant) antifeedant effect, but the shoot saponin mixture appears to be more toxic than the root saponin mixture.

The insect bioassay results obtained with the purified saponin mixtures on potato aphid are similar in some respects to those of crude saponin extracts on larvae of *C. maculatus*. In both cases, the shoot saponin material showed a consistent and

dose-dependent toxic effect, and the root saponin material was less toxic, or in the case of *C. maculatus*, non-toxic. The probiotic effects observed in the earlier bioassay were not, however, reproduced in the assay with potato aphids, which suggests that these were largely due to components other than saponins in the crude extracts. The slower decline in survival observed in aphids during the later stages of the assay with the shoot saponin mixture does not represent a probiotic effect, but antibiosis, since the aphids are failing to produce nymphs. Normally, aphid survival declines once nymph production has commenced. The antibiotic effect of the shoot saponin mixture is also seen in the effects on aphid growth, as determined by the aphid sizes measured during the growing phase (prior to nymph production), and in the delay of onset of nymph production.

These assays showed that the purified saponin mixtures showed toxic effects towards potato aphids, establishing that the insecticidal effects observed in crude saponin extracts were not due to components removed during the purification.

5.2.3. Identification of Insecticidal Components in Saponin Mixtures

In order to establish that the toxicity of crude saponin extracts, and saponin mixtures, was actually due to the saponins present, and not other components, separation of the components on reverse phase HPLC was followed by bioassay of individual fractions collected from the separations against potato aphid. These assays were limited by technical considerations, and were effectively determinations of survival over a 6 day period, for a single replicate, with a variable amount of added component in different fractions, corresponding to the amount of each compound present and separated into the different fractions. Nevertheless, the assays gave very useful indications of which fractions contained compounds toxic (in the short term) to potato aphids.

These assays gave a consistent and clear result in identifying fractions containing saponins as the only components eluted from the HPLC column which affected the survival of the aphids, and thus provide strong evidence that the saponins

are the major insecticidal components extracted from alfalfa tissues by 80% MeOH. This does not exclude the possibilities that alfalfa contains insecticidal compounds which would not be extracted under these conditions, such as proteins, or other insecticidal compounds in the 80% methanolic extract whose effects have not been detected by this assay. The assays also consistently associated saponin toxicity with the spot on TLC which was subsequently identified as soyasaponin I, although toxicity was also present in saponins eluted slightly later on the gradient.

The identification of saponins as the major insecticidal components in both root and shoot extracts from alfalfa seedlings allowed a more detailed study of the effects of different saponin fractions purified from the HPLC column, and a comparison of the effects of different saponins. It is significant that other workers have reported that when purified individual saponins, such as avenacosides A, B, C, and D have been investigated, they have often been found to elicit quite different biological responses compared to the activities of crude saponins (Ireland, 1987).

5.2.4. Effects of Saponin Fractions from Alfalfa Roots and Shoots Separated by HPLC on the Potato Aphid

These assays allowed a true comparison between different saponin fractions to be made, based on the incorporation of equal concentrations of purified material into artifical diets. Since a complete purification of individual saponins was not achieved by a single chromatographic separation on the reverse phase column, saponin fractions of defined composition were used for these assays; most fractions contained at least two individual components, but some (such as **Sd**) contained only a single component. Repeated purification runs on the reverse phase column were used to generate these fractions, and the separations were completely reproducible from run to run.

The shoot saponin fractions gave results that were readily interpretable in these assays. They show clearly that although all saponins present in the shoot saponin mixture decrease aphid survival, and retard development (as measured by

aphid size), the greatest effects are consistently associated with fractions containing the highest proportions of soyasaponin I. The saponins that are less mobile on TLC (putatively glycosides of medicagenic acid) are consistently less toxic. The assays thus allowed a tentative identification of soyasaponin I as the major insecticidal component present in the shoot saponin mixture, and thus as a major factor in insect resistance in alfalfa.

Results from the root saponin fractions are less easy to interpret, and require that effects on survival, aphid development (as estimated by size) and fecundity (nymph production) are all taken into account, as discussed above. A similar treatment of saponin toxicity was made by Applebaum *et al.* (1965), who, in bioassays of saponin fractions against *C. maculatus* , found that some saponin fractions decreased the sizes of insects, whereas others retarded development. The different effects observed with the alfalfa root saponin fractions are presented in Table 5.1 below.

Effect on: / **Fraction:**	**Survival**	**Aphid size**	**Nymph Production**	**Time to First Nymph Production**
Ra	ns	++	++	++
Rb	+++	+++	+++	+++
Rc	+	+	+	+
Rd	+++	+	++++	++++
Re	++	+	++	+
Rf	ns	++	ND	ND

Table 5.1 Effects of different fractions from alfalfa root saponins on parameters measured in potato aphid bioassay. ND = not determined.

Survival: ns = no significant effect; + = 0-20% decrease; ++ = 20-40% decrease; +++ = 40-60% decrease

Aphid size: + = 0-20% length decrease; ++ = 20-30% length decrease; +++ = >30% length decrease.

Nymph production: + = 0-50% decrease; ++ = 50-90% decrease; +++ = 90-99% decrease; ++++ = no nymphs

Time to first nymph production: + = 2 days delay; ++ = 5 days; +++ = 7 days; ++++ = no nymphs

Soyasaponin I is present as a significant component in fractions **Rb-Re** in these assays, and all of these fractions decreased survival, retarded development, and decreased nymph production. However, fraction **Ra**, which contained no soyasaponin I, also affected development and nymph production, although it did not have an effect on aphid survival. This fraction is clearly toxic, but was not investigated further, as fractions **Rb** and **Rd** were more toxic in these assays. Fraction **Rf** is also clearly less toxic, as is fraction **Re**, which contains a lower proportion of soyasaponin. The apparent inconsistency in this assay is shown by fraction **Rc**, which should have a toxicity similar to fractions **Rb** and **Rd**, but instead is markedly less toxic than either. This result can only be explained by assuming that the other saponin component in this fraction, a purple spot slightly less mobile on TLC than soyasaponin I (see Fig. 4.9) is having a probiotic effect on the aphids. Evidence for this assumption is that this component is readily hydrolysed by insect and plant enzymes (see section 4.2.9); this is a route of saponin detoxification in fungi (Osbourn, 1996), but requires the further assumption in this case that the products of hydrolysis are utilised by the insect, or decrease toxicity of the unhydrolysed soyasaponin I. Jain and Tripathi (1991) have suggested that sapogenins may show feeding stimulatory effects, which could account for the putative probiotic effect observed by the unidentified component present in fraction **Rc**. Further work to characterise this component, and investigate its hydrolysis in aphids, would allow this possibility to be investigated.

With the proviso about fraction **Rc** taken into account, the results with root saponin fractions again support the conclusion that soyasaponin I is the major toxic component in the root saponin mixture, although other saponins have toxic effects also.

5.2.5. Effects of Purified Alfalfa Saponins on the Potato Aphid

Results presented in this thesis (section 4.2.7) show that the purified alfalfa saponins soyasaponin I and medicoside A both show toxic effects towards potato

aphids, but by all criteria that were assessed, soyasaponin I is significantly more toxic. The main difference in their effects is that soyasaponin I causes significant aphid mortality, whereas medicoside A has little effect on survival. Although both compounds decrease aphid sizes and nymph production, soyasaponin I has greater effects at the same concentration. This conclusion is entirely consistent with all the previous bioassays on saponin fractions from root and shoot tissues.

This result is surprising, given that most reports in the literature report that glycosides of medicagenic acid are responsible for saponin toxicity in alfalfa, and that insect bioassays using larvae of the flour beetle, *Tribolium castaneum*, identified medicoside A as the most insecticidal alfalfa saponin (Oleszek and Jurzysta 1986). The possibility of different saponins having different levels of toxicity towards different insects must be considered in this context. It is possible that soyasaponin I is used as a general defensive compound in a number of plant species, since it has a relatively wide distribution, whereas saponins based on medicagenic acid are restricted to alfalfa and are specifically directed against pests of alfalfa which have overcome the insecticidal effects of soyasaponin. The result reported here has some support from earlier literature; an unidentified sapogenin chemically related to medicagenic acid inhibited fungal growth significantly, but did not affect leafhopper or pea aphid when tested in bioassays (Horber *et al.*, 1974). On the other hand, Krzymanska and Danuta (1985) concluded that the inhibition of development of green pea aphid (*Acyrthosiphon pisum*) by alfalfa saponins they observed was associated with medicagenic acid. Both soyasaponin I and medicoside A are monodesmosides, thus the extent of glycosylation is not a factor in their relative toxicity, although soyasaponin carries a trisaccharide substituent, whereas medicoside A only carries a single glucose residue.

5.2.6. Effects of Saponins from other Plant Species on Aphids and Other Insects

In general, although many authors have stated that saponins are insecticidal, little detailed work to support this assertion has been presented, and very few studies

have attempted to compare the toxicities of different saponins and sapogenins. An investigation of the effects of some saponin glycosides and sterols isolated from *Balanites roxburghii*, *Agave cantala*, and *Phaseolus vulgaris* on larvae of *Spilosoma abliqua* using a leaf disc method (Jain and Tripathi, 1991), concentrated on antifeedant activity shown by the compounds considered. Sapogenins showed little feeding deterrent effect in this study. For example, β-amarin, diosgenin, gitogenin and soyasapogenin B reduced consumption by less than 20%, or showed stimulatory properties. However, the saponins tested exhibited anti-feeding potential, reducing consumption by 20-75%. Differences between saponins showed correlation with the number of sugars, with monodesmosides being more active as anti-feedants than bisdesmosides; similarly, bisdesmosidic saponins were less active than the monodesmosides as molluscicides.

The work with sugar beet saponins reported in this thesis supports the statement that saponins are generally toxic to insects, since the sugar beet leaf saponins are toxic to potato aphids in the artificial diet bioassay, although the levels of toxicity are clearly lower than alfalfa shoot saponins (c.f. Figs. 4.29 and 4.30 with Figs. 4.18 and 4.19). The effects of the sugar beet saponins are more limited than those of alfalfa saponins; the main effect is a reduction in survival over an initial 4 day period, and there is little effect on aphid size, except at the highest concentration used, and a correspondingly small effect on nymph production per surviving aphid. Sugar beet saponins are based on a triterpenoid sapogenin, but the glycosides are not fully characterised, and thus further conclusions cannot be drawn.

An evaluation of the role played by saponins in the resistance of particular plant species to phytophagous insects is not likely to be able to identify single saponins as being responsible for resistance. Not only is there the complication of multiple saponins normally being isolated from a single source tissue, but the contributions of other classes of secondary metabolites, and other compounds, must be considered also, since the observed resistance is generally a product of the interaction of a number of different resistance mechanisms which the plant is able to

deploy. Nevertheless, saponins clearly do play a significant role in the plant's defensive strategy, although the importance of the contribution made by saponins will vary from saponin to saponin, insect to insect, and plant species to plant species.

5.3. Detoxification of Saponins by Insects

The hydrolysis of avenacosides A-D, the saponins found in oats, by fungal pathogens, has been found to be a determinant in the resistance or susceptibility of the plant towards the pathogens; fungi which are successful pathogens are able to hydrolyse the saponins, whereas fungi unable to attack oats do not hydrolyse the saponins (Osbourn *et al.*, 1991; and 1995). The reports that sapogenins are less toxic to insects than saponins, both in this thesis and elsewhere, support the view that a similar detoxification mechanism may operate in insects. However, the results of attempted hydrolyses of alfalfa saponins by enzymes from a variety of sources show that these compounds are generally resistant to hydrolysis. Even highly active enzyme preparations from locusts, which are a truly polyphagous species, or enzyme preparations from alfalfa weevil, which is a pest of alfalfa, did not cause hydrolysis of the major components of root or shoot saponin mixtures, and thus it seems unlikely that insects rely on hydrolysis to detoxify these compounds. In agreement with the results presented, no hydrolytic enzyme activity towards soyasaponin I was detected in homogenates of *C. maculatus* larvae by Applebaum *et al.* (1965).

One component of the saponin mixtures is, however, readily hydrolysed, both by enzyme extracts from alfalfa itself, and by insect enzymes; this is the purple spot present in fraction **Rc**. The identification of this component as medicoside I must be regarded as tentative, but is supported by the locust enzyme extracts partially hydrolysing this compound to what appears to be medicoside A. The nature of the hydrolysis reactions occurring cannot be fully elucidated without chemical characterisation of the substrate and products, which was not possible in this investigation. Characterisation of the first product of hydrolysis, which is similar in mobility on TLC to medicoside G, would be particularly useful. However, it seems

likely that the ester linkage at the C-28 carboxylic acid group in medicagenic acid is involved, since this glycosidic bond will be more labile than the ester linkage at C-3, and insects contain non-specific esterases. Possibly hydrolysis of glycosidic bonds between carbohydrate units in the oligosaccharide side chains of the substrate is also involved.

5.4. A Strategy to Protect Alfalfa against its Major Insect Pest, Alfalfa Weevil

Although saponins have been shown to play a major role in protecting alfalfa against non-pest insect species, alfalfa weevil must be able to overcome any toxic effect of these compounds, since it is a successful pest. Conventional plant breeding strategies for insect resistance in this crop, which rely on endogenous compounds to give resistance have therefore largely been unsuccessful. Although varieties with enhanced resistance have been identified, the basis of resistance has not been identified, and resistance is still only partial. Sorensen *et al.* (1988) stated that introducing resistance to insects such as alfalfa weevil into cultivated varieties has been unsuccessful because alfalfa germplasm with high levels of resistance to these insects has not been identified. Even if such germplasm were identified, it is likely that alfalfa weevil would be able to adapt to new resistant varieties produced from it. Elden (1995) concluded that chemical factors responsible for alfalfa weevil resistance were not present in the plant and resistance is limited to low level of tolerance to weevil damage. The use of genetic engineering technology to introduce novel insecticidal compounds has been discussed in the Introduction, and is developed in this thesis.

The use of genes encoding protease inhibitors to enhance the resistance of transgenic plants towards insect pests has been discussed in a number of reviews (e.g. Ryan, 1990; Gatehouse *et al.*, 1993). In order to apply this strategy it was first necessary to characterise the proteases present in alfalfa weevil larvae.

5.4.1. Characterization of Digestive Proteases in Alfalfa Weevil

Protease activity in gut extracts of alfalfa weevil larvae was assayed using the fluorimetric substrate Z-phe-arg-AMC. This substrate is very sensitive, and allowed proteolytic activity to be detected readily. However, Z-phe-arg-AMC is specific for trypsin-like and papain- (or cathepsin-) like proteases, and proteases with specificity directed towards other amino acids than lys/arg C-terminal to the point of cleavage (such as chymotrypsin) will not be detected. Assays in which non-specific proteolysis is detected, such as those using azoalbumin, or gelatin, as substrates, would be required to confirm that this substrate is detecting all the proteolytic activity present. The assumption that the substrate used is detecting a major proteolytic activity, is, however, reasonable.

The inhibition of the activity of the alfalfa weevil proteases using the chemical cysteine proteinase inhibitor E-64 showed that almost all of the proteolytic activity detected by Z-phe-arg-AMC in the gut is due to the cysteine proteases. E-64 is a small peptide isolated from *Aspergillus japonicus* (strain TRP-64) and is a specific and irreversible inhibitor of cysteine proteinases (Wolfson and Murdock 1987). Several families of Hemiptera and Coleoptera are found to have cysteine proteinases in their midgut where they appear to play important roles in the digestion of food proteins. These particular insects characteristically have mildy acidic pHs in their midguts near the pH optima of cysteine proteases (pH ≅ 5.0) (Ryan, 1990). Isolation of the midgut proteinases of the larvae of the cowpea seed weevil and from the bruchid beetle, *Zabrotes subfasciatus*, confirmed that proteinases of the cysteine mechanistic class were present (Ryan, 1990). Other pests are known to use cysteine proteases as the major proteolytic enzymes in the alimentary tracts (Kuroda *et al.*, 1996). These include *Leptinotarsa decemlineata* (Wolfson and Murdock, 1987), *C. maculatus* (Gatehouse *et al.*, 1985), *Acanthoscelides obtectus* (Kuroda *et al.*, 1996), and *T. castaneum* (Chen *et al.*, 1992). Cysteine proteases are common in animals, eucaryotic micro-organisms and bacteria as well as in plants. In animals, they are normally sequestered in lysosomal compartments or in the cytoplasm, where they are

thought to be involved in intracellular protein turnover, and thus their use as digestive proteases in Coleoptera is surprising. The non-linear graph of inhibition against amount of inhibitor produced for E-64 (and for cystatin), with most of the inhibition detected at low concentrations of inhibitor, indicates that more than one cysteine protease was present in the gut extract, and that the enzymes differed in their sensitivities to the inhibitors.

The protease activity detected in alfalfa weevil gut extracts was also inhibited by the protein protease inhibitor hen egg white cystatin, which was of similar effectiveness to E-64 on a mole-for-mole basis. Cystatins are found in both animals and plants, and have their inhibitory effects on both endogenous and exogenous cysteine proteinases. This means that in plants cystatins could play roles in protection against crop insect pests that contain cysteine proteinases in their guts (Misaka *et al.*, 1996), although they are generally present in plant tissues at very low levels. Cystatins have been found in, and isolated from plant species including pineapple, potato, corn, rice, cowpea, mungbean, tomato, wheat, barley and millet (Ryan, 1990).

5.4.2. Expression of Oryzacystatin In *E. Coli*

Oryzacystatin, the cystatin present in rice seeds (at a very low concentration), has been suggested as an anti-metabolic protein towards insects, which use cysteine proteases as digestive enzymes, by a number of authors, e.g. Edmonds (1993). Liang *et al.* (1991) partially purified and characterised the major proteinase extracted from midguts of the rice weevil, *Sitophilus oryza*, and red flour beetle, *Tribolium castaneum*.; oryzacystatin from rice seeds and other inhibitors of cysteine proteases were effective inhibitors. Oryzacystatin consists of two molecular species, ozc I and ozc II, which occur in rice seeds, are water soluble, and are resistant to heating at >100°C in d.H_2O (Aoki *et al.*, 1995; Abe *et al.*, 1987). As a plant protein, its encoding gene should be easy to express in transgenic plants, and thus it is a good candidate for plant protection via genetic engineering. Since cystatin from hen egg white inhibited alfalfa weevil gut extract proteolysis, it was considered likely that oryzacystatin would do so also. However, relatively large amounts of protein are required for bioassays and characterization of inhibitory specificities towards insect

enzymes, and purification of oryzacystatin from rice seeds does not lead to isolation of sufficient material. Production of this protein in *E. coli* using an expression system would allow both enzyme assays and insect bioassays with this protein to be carried out.

A standard *E. coli* expression system was used to produce this protein, which is produced in the bacterium in active form. The construct encoded a sample fusion protein with 14 amino acid residues N-terminal, originating from the polylinker of pUC8 and 102 residues of the full-length ozcI encoding sequence; the extra amino acids were not found to have any effect on the inhibitory properties of the protein (Edmonds, 1993). After induction and production of the recombinant protein, cells were disrupted by sonication, and oryzacystatin was purified by heat treatment (80°C), ammonium sulphate precipitation, dialysis, and lyophilization. The final product also contained small amounts of *E.coli* protein (Fig. 4.38); this could have been removed by ion-exchange chromatography (Edmonds 1993), but the oryzacystatin preparation was considered sufficiently pure for insect bioassay. Inhibition of papain with the oryzacystatin protein produced in *E.coli* confirmed that the inhibitory activity of this protein was present.

Abe *et al.* (1988) have also reported expression of ozcI in *E.coli*. A cDNA clone composed of 598 base pairs for a cysteine proteinase inhibitor of rice immature seeds was isolated by Abe *et al.* (1987) via screening with synthesized oligonucleotide probes based on partial amino acid sequences of ozc. An expression plasmid containing a full length ozc cDNA at the multi-cloning site of pUC18 was constructed and produced a lacZ - oryzacystatin fusion protein in *E.coli* (Abe *et al.*, 1988). They reported an efficient expression of ozc and truncated fragments in *E.coli* and found that the inhibitory activity of the fusion protein lacking the N-terminal 21 amino acid residue from ozc was unaffected and that the NH_2-terminal residues, and the COOH-terminal are not essential for inhibition of papain.

5.4.3. Effects of Oryzacystatin on Alfalfa Weevil Larvae

The oryzacystatin produced in *E. coli* was effective as an inhibitor against alfalfa weevil gut proteases, but compared to egg white cystatin, ten-fold higher amounts of this inhibitor were needed to produce similar levels of inhibition, and the

maximum inhibition obtained was significantly lower (approx. 70% vs. approx. 90%). This result may be due to impurities in the oryzacystatin preparation, but could also indicate that this inhibitor is not as effective towards the alfalfa weevil enzymes. However, it was considered worthwhile to continue to use this material in an insect bioassay. Inhibitor activities *in vitro* are always not consistent with *in vivo* results. For instance, although the Bowman-Birk soybean trypsin inhibitor (BBTI) was a strong inhibitor of midgut proteolytic activity of honey-bee *in vitro* and was responsible for causing a decrease in trypsin activity *in vivo*, it did not exhibit acute toxicity (Burgess and Gatehouse, 1997).

Previous reports have shown that inhibitors of digestive cysteine proteases can have significantly deleterious effects on survival and development of insects which rely on these enzymes. For example, the effects of E-64 on life-history parameters of the Mexican been beetle, *Epilachna varieties*, was assessed in a bioassay by Wolfson and Murdock (1987). The results showed that time to oviposition and number of days to complete larval development were increased, and fewer eggs were produced. Similarly, potato multicystatin, a unique plant cystatin isolated from potato tubers, was shown to have growth depressing effects on the corn rootworm, *Diabrotica* (Waldron *et al.*, 1993). Also, Kuroda *et al.* (1996) reported that oryzacystatin was effective for growth inhibition and increased mortality of bean insect pests, not only Coleoptera (*C. chinensis*), but also Hemiptera (*Riptortus clavatus*), and Michaud *et al.* (1993) reported a potential growth depression of the Colorado potato beetle, an economically important potato pest, by oryzacystatin. In the latter case it was shown that both the ozc I and ozc II inhibitors specifically inhibit Cathepsin H, which represents an important fraction of the Colorado potato beetle proteinases.

While the artificial diet system used for the bioassay of alfalfa weevil larvae was far from ideal, as survival and development on the control diet was not particularly good, the results obtained are better than any other artificial diet bioassay system reported in the literature for this insect. Previous workers have attempted to bioassay compounds against alfalfa weevil larvae by painting solutions of the

compounds onto alfalfa leaves; while this method can give an assessment of toxicity, it is non-quantitative, and does not duplicate the situation *in vivo* when a product is being expressed in the plant tissue. The bioassay system was adequate to allow the effects of oryzacystatin to be estimated, and showed that the protein did affect larval survival. The experimental results suggested that the lower concentration of oryzacystatin (2% of total protein) caused higher mortality of alfalfa weevil larvae than the higher concentration (5% of total protein). This result suggests that the inhibitor is having complex effects on digestion in this insect.

5.4.4. Conclusion

The experiments carried out with alfalfa weevil have shown that inhibitors of cysteine proteases inhibit proteolysis in this insect, and can decrease the survival of larvae. Consequently, genes encoding cystatins would be worthwhile candidates to consider for inclusion into transgenic alfalfa to give protection against this insect pest. However, the results suggest that protection may only be partial if oryzacystatin were to be used, and that investigation of both the alfalfa weevil proteases and their interactions with different cystatins would be valuable in enabling a more effective inhibitor to be selected. From a study of inhibitory effects on alfalfa weevil growth and development, Elden (1995) concluded that low molecular weight protease inhibitors such as E-64, p-hydroxymercuribenzoate and leupeptin were more effective than protein protease inhibitors, but it would not be practical to produce these compounds in transgenic plants.

The only comparable experiments in this area is a report of the use of an anti-elastase insect protease inhibitor in transgenic alfalfa for use as an insecticide (Thomas *et al.*, 1994); no further information has since been published on these experiments.

REFERENCES

Abbott, W.S. (1925) A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **18,** 265-267.

Abe, I., Rohmer, M. and Prestwich, G.D. (1993) Enzymatic Cyclization of Squalene and Oxidosqualene to Sterols and Triterpenes. *Chem. Rev.* **93,** 2189-2206

Abe, K., Emori, Y., Kondo, H., Arai, S. and Suzuki, K. (1988) The NH_2-terminal amino acid Residues are not Essential for the Papain-Inhibitor Activity of Oryzacystatin, a Member of the Cystatin Superfamily. *J. Biol. Chem.* **263,** 7655-7659

Abe, K., Emori, Y., Kondo, H., Suzuki, K. and Arai, S. (1987) Molecular Cloning of A Cysteine Proteinase Inhibitor of Rice (Oryzacystatin); Homology With Animal Cystatins and Transient Expression in the Ripening Process of Rice Seeds. *J. Biol. Chem.* **262,** 16793-16797

Anastasi, A., Brown, M. A., Kembhavi, A. A., Nicklin, M. J. H., Sayers, C.A., Sunter, D.C. and Barrett, J. (1983) Cystatin, a Protein Inhibitor of Cysteine Proteinases. *Biochem. J.* **211,** 129-138

Aoki, H., Akaike, T., Abe, K., Kuroda, M., Arai, S., Okamura, R., Negi, A. and Maeda, H. (1995) Antiviral Effect of Oryzacystatin, a proteinase inhibitor in Rice, against Herpes Simplex Virus Type 1 In Vitro and In Vivo. *Antimicrob. Agents Chemother.* **April 1995,** 846-849

Applebaum, S. W., Gestetner, B. and Birk, Y. (1965) Physiological Aspects of Host Specificity in the Bruchidae- IV. Developmental Incompatibility of Soybeans for *Callosobruchus. J. Insect Physiol.* **11,** 611-616

Applebaum, S.W. and Birk, Y. (1979) Saponins In *Herbivores, Their Interaction with Secondary plant Metabolites* (Rosenthal, G.A. and Janzen, D.H. eds.), pp. 539-566, *Academic Press, Inc.*, London

Baker, A. M. R. (1978) "Protease Inhibitors and the Biochemical Basis of Insect Resistance in *Vigna unguiculata*". *PhD thesis*, Durham University, U.K. (138)

Barnes, D. K., Hanson, C. H., Ratcliffe, R. H., Busbice, T. H., Schillinger, J. A., Buss, G. R., Campbell, W. V., Hemken, R. W. and Blickenstaff, C. C. (1970) The Development and Performance of Team Alfalfa: A Multiple Pest Resistant alfalfa with Moderate Resistance to the Alfalfa Weevil. USDA- ARS 34-115. U.S. Government Printing Office, Washington, DC.

Barnes, D. K., Norwood, B. L., Hanson, C. H., Ratcliffe, R. H. and Blickenstaff, C. C. (1969) A Mass screening procedure for Isolating Alfalfa Seedlings with Resistance to the Alfalfa Weevil. *J. Econ. Entomol.* **62**, 66-69

Behdad, E. (1989) Pests of Field Crops in Iran. *Sepehr*, Tehran

Berrang, B., Davis, K. H., Jr., Wall, M. E. and Hanson, C. H. (1974) Saponins of Two Alfalfa Cultivars. *Phytochem.* **13**, 2253-2260

Birk, Y. and Peri, I. (1980) Saponins In *Toxic Constituents of Plant Foodstuffs* (Liener, I. E. ed.), pp. 161-182, *Academic Press*, London

Bjork, I. And Ylinenjarvi, K. (1990) Interaction Between Chicken Cystatin and the Cystatin Proteinase Actinidine, Chymopapain A, and Ficin. *Biochemistry* **29**, 1770-1776

Bode, W. and Huber, R (1992) Natural Protein Proteinase Inhibitors and Their Interaction with Proteinases (Review). *Eur. J. Biochem.* **204**, 433-451

Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J. and Turk, V. (1988) The 2.0 X-ray Crystal Structure of Chicken egg White Cystatin and its Possible Mode of Interaction with Cysteine Proteinases. *The EMBO j.* **7**, 2593-2599

Bowyer, P., Clarke, B. R., Lunness, P., Daniels, M.J. and Osbourn, A. E. (1995) Host Range of a Plant Pathogenic Fungus Determined by a Saponin Detoxifying Enzyme. *Science* **267**, 371-374

Broadway, R. M. and Duffey, S. S. (1986) The Effects of Dietary protein on the Growth and Digestive Physiology of Larval *Heliothis zea* and *Spodoptera Exi*gua. *J. Insect physiol.* **32**, 673-680

Burgess, E. P. J. and Gatehouse, A. M. R. (1997) Engineering for Insect Pest Resistance in Biotechnology and the Improvement of Forage Legumes (McKeresie, B. D. and Brown, D. C. W. eds.), pp. 229-258, *CAB International*, New York, USA

Burnouf-Radosevich, M. and Delfel, N. E. (1986) High-performance Liquid Chromatography of Triterpene Saponins. *J. Chromatogr.* **368.** 433-438

Chen, M., Feng, G., Zen, K. C., Richardson, M., Valdes-Rodriguez S., Reeck, G. R. and Kramer, K. J. (1992) α-Amylases from Three Species of Stored Grain Coleoptera and Their Inhibition by Wheat and Corn Proteinaceous Inhibitors. *Insect Biochem. Mol. Biol.* **22**, 261-268

Chrispeels, M. J. and Savada, D. E., (1994) Plant Genes and Agriculture. *Jones and Bartlett*, Boston, MA, USA

Christeller J. T., Laing, W.A., Markwick, N. P. and Burgess, E.P.J. (1992) Midgut Protease Activities in 12 Phytophagous Lepidopteran Larvae: Dietary and Protease Inhibitor Interactions. *Insect Biochem. Mol.* **22**, 735-746

Crombie,W. M. L., Crombie, L., Green, J. B. and Lucas, J. A. (1986) Pathogenicity of "Take-All" Fungus to Oats: Its Relationship to the Concentration and Detoxification of the Four Avenacins. *Phytochem.* **25**, 2075-2083

Dawson, G. W., Hallahan, D. L., Mudd, A., Patel, M. M., Pickett, J. A., Wadhams, L. J. and Wallsgrove, R. M. (1989) Secondary Plant Metabolites as Targets for Genetic Modification of Crop Plants for Pest Resistance. *Pestic. Sci.* **27**, 191-201

Dixon, A, F. G. (1987) The way of Life of Aphids: host Specificity, Speciation and Distribution In; Aphids, Their Biology, Natural Enemies, and Control, Volume A (Minks, A. K. and Harwijn, P. eds.) pp. 197-207, *Elsevier*, Oxford

Domon, B., Dorsaz, A. and Hostettmann, K. (1984) High Performance Liquid Chromatography of Oleanane Saponins. *J. Chromatogr.* **315**, 441-446

Dubois, M., Bauer, R., Cagiotti, M. R. and Wagner, H. (1988) Foetidissimoside A, A New 3, 28-Bidesmosidic Triterpenoid saponin, and Cucurbitacins from *Cucurbita foetidissima*. *Phytochem.* **27**, 881-885

Edmonds, H. S. (1994) "Antimetabolic Proteins from Plants and Their Potential use in Conferring Resistance Against Corn Rootworms *(Diabrotica sp.)*". *PhD thesis*, Durham University, U.K.

Elden, T.C. (1995) Selected Proteinase Inhibitor Effects on Alfalfa Weevil (*Coloptera: Curculionidae*) Growth and Development. *J. Econ. Entomol.* **88**, 1586-1590

Falk, A. and Rask, L. (1995) Expression of A Zeatin-O-Glucoside-Degrading β-Glucosidase in *Brassica napus*. *Plant Physiol.* **108**, 1369-1377

FAO (1985) Production Year Book *Food and Agriculture Organization of the United Nations* **38**, Rome,

Febvay, G., Delobel, B. and Rahbe, Y. (1988) Influence of the Amino Acid Balance on the Improvement of an Artificial Diet for A Biotype of *Acrythosiphon pisum* (Homoptera: Aphididae). *Can. J. Zool.* **66**, 2449-2453

Fenwick, G. R., Price, K. R., Tsukamoto, C. and Okubo, K. (1991) Saponins In *Toxic Substances in Crop Plants* (FelixD'Mello, J. P., Duffus C. M. and Duffus, J.H., eds.), pp. 285-327, *Royal Society of Chemistry*, Cambridge

Galwey, N. W., Leakey, C. L. A., Price, K. R. and Fenwick, G. R. (1990) Chemical Composition and Nutritional Characteristic of quinoa (*Chenopodium quinoa* Yilld.). *Hum. Nutr. , Fd. Sci., Nutr.*, **42F**, 245-261

Garcia, E. S., Guimaraes, J. A., and Prado, J. L. (1978) Purification and Characterization of a Sulfhydryl-Dependent Protease from *Rhodinus prolixus* Midgut. *Arch. Biochem. Biophys.* **188**, 315-322

Gatehouse, A. M. R. and Boulter, D. (1983) Assessment of Antimetabolic Effects on Trypsin Inhibitors from Cowpea *(Vigna unguiculata)* and other Legumes on Development of the Bruchid Beetle *Callosobruchus maculatus*. *J. Sci. Food Agric.* **34**, 345-350

Gatehouse, A. M. R., Butler, K. J., Fenton, A. and Gatehouse, J. A. (1985a) Presence and Partial Characterization of a Major Proteolytic Enzyme in the Larval Gut of *Callosobruchus maculatus*. *Entomol. Exp. Appl.* **39**, 279-286

Gatehouse, A. M. R., Fenton, K. A. and Anstee, J. H. (1985b) Carbohydrase and Esterase Activity in the Gut of Larval of *Callosobruchus maculatus*. *Experientia* **41**, pp. 1202-1205

Gatehouse, A. M. R., Fenton, K. A., Jepson, I. And Pavey D. J. (1986) The Effects of α-amylase Inhibitors on Insect Storage Pests: Inhibition of α-amylase in vitro and effects on Development in vivo. *J. Sci. Fd. Agric.* **37**, 727-734

Gatehouse, A. M. R., Hilder, V. A., Powell, K., Boulter, D. and Gatehouse J. A. (1992) Potential of Plant-Derived Genes in the Genetic Manipulation of Crops for Insect Resistance In *Proc. 8th Int. Symp. Insect-Plant Relationshiops, Dordrecht*: (Menken, S.B.J., Visser, J.H. and Harrewijn, P. eds.), pp. 221-234 *Kluwer Acad. Publ.*

Gatehouse, A. M. R. (nee Baker), Gatehouse, J. A., Dobie, P., Kilminster, A. M. and Boulter, D. (1979) Biochemical basis of Insect Resistance in *Vigna unguiculata*. *J. Sci. Food Agric.* **30**, 948-958

Gatehouse, A. M. R., Shi, Y., Powell, K. S., Brough, C., Hilder, V. A., Hamilton, W. D. O., Newell, C. A., Merryweather, A., Boulter, D. and Gatehouse J. A. (1993) Approaches to Insect Resistance Using Transgenic Plants. *Phil. Trans. Roy. Soc. Lond.* B, **342**, 279-286

Gatehouse, J. A. (1991) Breeding for Resistance to Insects; in Advanced Methods in Plant Breeding and Biotechnology (Murray, D. R. ed.). Biotechnology in Agriculture, No.4, *Red Wood Press Ltd.*, Melksham

Gestetner, B. (1971) Short Communication: Structure of A Saponin from Lucerne (*Medicago sativa*). *Phytochem.* **10**, 2221-2223

Gestetner, B., Birk, Y. and Bondi, A. (1965) The Enzymatic Breakdown of Soybean Saponins In the Digestive Tract of Chicks and Mice. *Israel J. Chem.* **3**, 88p

Gestetner, B., Shany, S., Tencer, Y., Birk, Y. and Bondi, A. (1970) LUCERNE SAPONINS: II. - Purification and Fractionation of Saponins from Lucerne Tops and Roots and Characterisation of the Isolated Fractions. *J. Sci. Food Agric.* **21**, 502-507

Gorski, P. M., Miersch, J. and Ploszynski, M. (1991) Production and Biological Activity of Saponins and Canavanine in Alfalfa Seedling. *J. Chem. Ecol.* **17**, 1135-1143

Gratwick, M. (1992) Crop Pests in the U.K. Collected Edition of MAFF Leaflets. *Chapman and Hall,* London

Hames, B. D. and Rickwood, D. (1981) Gel Electrophoresis of Proteins: A practical Approach. *IRL Press Ltd*, England

Hanson, A. A., Barnes, D. K., Hill, R. R. and Jr (1988) Alfalfa and Alfalfa Improvement.No.29 in the series Agronomy, *Madison,* Wisconsin, USA

Harris, E. L. V. and Angal, S. (1995) Protein Purification Methods, A Practical Approach. *Oxford University Press*, Oxford

Heinrichs, E. A. (1988) Plant Stress- Insect Interactions. *John Wiley and Sons*, Canada

Hilder, V. A., Gatehouse, A. M. R., Sheerman, S. E., Barker, R. F. and Boulter, D. (1987) A Novel Mechanism of Insect Resistance Engineered into Tobacco. *Nature* **300**, 160-163

Hill, D. S. (1983) Agricultural Insect Pests of the Tropics and Their Control. *Cambridge University press*, Cambridge

Hines, M. E., Osuala, C. I. and Nielsen, S. S. (1991) Isolation and Partial Characterization of a Soybean Cystatin Cysteine Proteinase Inhibitor of Coleopteran Digestive Proteolytic Activity. *J. Agric. Food Chem.* **39**, 1515-1520

Horber, E., Leath, K. T., Berrang, B., Marcarian, V. and Hanson, C.H. (1974) Biological Activities of Saponin Components from DuPuits and Lahontan Alfalfa. *Entomol. Exp. Appl.* **17**, 410-424

Hostettmann, K. and Marston, A. (1995) Saponins. *Cambridge University Press*, Cambridge

Houseman, J. (1978) A Thiol-Activated Digestive Proteinase from Adults of *Rhodnius prolixus* Stal (Hemiptera: Reduviidae). *Can. J. Zool.* 56, 1140-1143

Houseman, J. G. and Down, A. E. R. (1982) Identification and Partial Characterization of Digestive Proteinases from Two species of Bedbug (Hemiptera: Cimicidae). *Can. J. Zool.* **60**, 1837-1840

Hsiao, T. H. (1969) Adenine and Related Substances as Potent Feeding stimulants for the Alfalfa Weevil, *Hypera postica. J. Insect Physiol.* **15**, 1785-1790

Hsiao, T. H. and Hsiao, C. (1974a) Feeding Requirements and Artificial Diets for the Alfalfa Weevil, *Entomol. Exp. Appl.* 17, 83-91

Hsiao, T. H. and Hsiao, C. (1974b) A practical Artificial Diet for Laboratory Rearing of the Alfalfa weevil (*Hypera postica* Gyll.) *Ann. Entomol. Soci. Am.* **67**, 149-150

Ireland, P.A. (1987) "Analysis of Saponins." *Ph.D. thesis*, Reading University, U.K.

Ishimoto, M. and Chrispeels, M. J. (1996) Protective Mechanism of the Mexican Bean Weevil Against High levels of α-Amylase Inhibitor in the Common Bean. *Plant Physiol.* **111**, 393-401

Jain, D. C. and Tripathi, A. K. (1991) Insect Feeding - Deterrent Activity of Some Saponin Glycosides. *Phytotherapy Res.* **5**, 139-141

Karimi, H. (1990) Alfalfa. *University Publishing Centre*, Tehran

Kawamura, N., Watanabe, H. and Oshio, H. (1988) Saponins from Roots of *Momordica cochinchinensis. Phytochem.* **27**, 3585-3591

Kensil, C. R., Patel, U., Lennick, M. and Marciani D. (1991) Separation and Characterization of Saponins with Adjuvat Activity from *Quillaja saponaria* Molina Cortex. *J. Immun.* **146**, 431-437

Kitagawa, I., Yoshikawa, M. and Yosioka, I. (1976) Saponin and Sapogenol. XIII. Structures of three Soybean Saponins. Soyasaponin I, Soyasaponin II, and Soyasaponin III. *Chem. Pharm. Bull.* **24**, pp.121

Kitagawa, I., Yoshikawa, M., Wang, H. K., Saito, M., Tosirisuk, V., Fugiwara, T. and Tomita, K. (1982) Revised Structures of Soyasapogenols A, B and E, Oleanene-Sapogenols from Soybean. Structure of Soyasaponins I, II and III. *Chem. Pharm. Bull.* **30**, 2294-2297

Kitamura, K., Ishimoto, M. and Ishii, S. (1990) Bruchid Resistance Factors in *phaseolus* and *Vigna* Legumes In *Bruchids and Legumes: Economic, Ecology, and Coevolution* (Fujii, K., Gatehouse A. M. R., Johnson, C. D., Mitchel, R. and Yoshida eds), 229-240, Proceeding of Second International Symposium Of Bruchids and Legumes, *Klumer Academic Publishers*, Okayama, Japan

Kondo, H., Abe, K., Emori, Y. and Arai, S. (1991) Gene Organization of Oryzacystatin-II, a New Cystatin Superfamily Member of Plant Origin, is Closely Related to That of Oryzacystatin-I but Different from Those of Animal Cystatins. *FEBS*. **278**, 87-90

Kondo, H., Abe, K., Nishimura I., Watanabe, H., Emori, Y. and Arai, S. (1990) Two Distinct Cystatin Species in Rice Seeds With Different Specificities Against Cysteine proteinases. *J. Biol. Chem.* **265**, 15832-15837

Krzymanska, J. and Danuta, W. (1985) Significance of Saponins in Resistance of Alfalfa Against Green Pea Aphid (*Acyrthosiphon pisum* Harris). *Chemical Abstr.* **102**, 128998y

Kuroda, M., Ishimoto, M., Suzuki, K., Kondo, H., Abe, K., Kitamura, K. and Arai, S. (1996) Oryzacystatins Exhibit Growth-Inhibitory and Lethal Effects on Different Species of Bean Insect Pests, *Callosobruchus chinensis* (Coleoptera) and *Riptortus clavatus* (Hemiptera). *Biosci. Biotech. Biochem.* **60**, 209-212

Levy, M., Zehavi, U. and Naim, M. and Polacheck, I. (1989a) Isolation, Structure Determination, Synthesis and Antifungal Activity of a New Native Alfalfa-Root Saponin. *Carbohyd. Res.* **193**, 115-123

Levy, M., Zehavi, U., Naim, M. and Polacheck, I. (1986) An Improved Procedure for the Isolation of Medicagenic Acid 3-O-β-D-Glucopyranoside from Alfalfa Root and Its Antifungal Activity on Plant Pathogens. *J. Agric. Food Chem.* **34**, 960-963

Levy, M., Zehavi, U., Naim, M., Polacheck, I. And Evron, R. (1989b) Structure-Biological Activity Relationship in Alfalfa Antimycotic Saponins: the Relative Activity of Medicagenic Acid and Synthetic Derivatives Thereof Against Plant Pathogenic Fungi. *J. Phytopathol.* **125**, 209-216

Liang, C., Brookhart, G., Feng, G. H., Reeck, G. R. and Kramer, K. J. (1991) Inhibition of Digestive Proteinases of Stored Grain Coleoptera by Oryzacystatin, a Cysteine Proteinase Inhibitor From Rice Seed. *Eur. J. Biochem.* **278**, 139-142

Lindahl, P., Raub-Segall, E., Olson, S. T. and Bjork, I. (1991) Papain Labelled with Fluorescent Thiol-Specific Reagents as A Probe for Characterization of Interactions Between Cysteine Proteinases and Their Protein Inhibitors by Competitive Titrations. *Biochem. J.* **276**, 387-394

Livingston, A. L., Knuckles, B. E., Edwards, R. H., deFremery, D., Miller, R. E. and Kohler, G. O. (1979) Distribution of Saponin in Alfalfa Protein Recovery Systems. *J. Agric. Food Chem.* **27**, 362-365

Livingston, A. L., Linda, A. L., Whitehand, L. C., and Kohler, G. O. (1977) Microbiological Assay for Saponin in Alfalfa Products. *J. AOAC* **60**, 957-960

Mahato S. B., Nandy, A. K. (1991) Review Article Number 60; Triterpenoid Saponins Discovered Between 1987-1989. *Phytochem.* **30**, 1357-1390

Mahato S. B., Sarkar, S. K. and poddar, G. (1988) Review Article Number 38; Triterpenoid Saponins. *Phytochem.* **27**, 3037-3067

Manglitz, G. R. and Ratcliffe, R. H. (1988) Insects and Mites in *Alfalfa and Alfalfa Improvement* (Hanson, A. A., Barnes, D. K., Hill, R. R. and Jr, R. R. eds.), pp. 671-704, *American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc.*, Madison, Wisconsin, U.S.A

Massiot, G., Dijoux, M., Lavaud, C., Men-Oliver, L. L., Connolly, J. D. and Sheeley, D. M. (1994) Seco-Glycosides of Oleanolic Acid from *Beta vulgaris*. *Phytochem.* **37**, 1667-1670

Massiot, G., Lavaud, C. and Men-Oliver, L. L., Binst, G. V., Miller, S. P. F. and Fales, H. M. (1988b) Structural Elucidation of Alfalfa Root Saponins by Mass Spectrometry and Nuclear magnetic Resonance Analysis. *Chem. Soc. Perkin Trans* **I**, 3071-3079

Massiot, G., Lavaud, C., Benkhaled, M. and Men-Oliver, L. L. (1992) Soyasaponin, A

New Maltol Conjugate from Alfalfa and Soybean. *J. Nat. Prod.* **55**, 1339-1342

Massiot, G., Lavaud, C., Besson, V., Men-Oliver, L. L. and Binst, G. V. (1991) Saponins from Aerial Parts of Alfalfa (*Medicago sativa*). *J. Agric. Food Chem.* **39**, 78-82

Massiot, G., Lavaud, C., Guillaume, D. and Men-Oliver, L. L. (1988a) Reinvestigation of the Sapogenins and Prosapogenins from Alfalfa *(Medicago sativa). J. Agric. Food Chem.* **36**, 902-909

Matsumoto, I., Watanabe, H., Abe, K., Arai, S. and Emori, Y. (1995) A Putative Digestive Cysteine Proteinase from *Drosophila melanogaster* is Predominantly expressed in the Embryonic and Larval Midgut. *Eur. J. Biochem.* **227**, 582-587

Mazahery-Laghab, H. (1991) "Study of the Resistance of Different Alfalfa Cultivars to Alfalfa Weevil *(Hypera postica Gyll.)." M.Sc. thesis*, Tehran University, Iran

Mazahery-Laghab, H. and Yazdi-Samadi, B. (1994) Resistance of Alfalfa Varieties to Alfalfa Weevil (*Hypera postica* Gyll.). *Iranian J. Agric. Sci.* **25**, 12-17

Michaud, D., Nguyen-Quoc, B. and Yelle, S. (1993) Selective Inhibition of Colorado Potato Beetle Cathepsin H by Oryzacystatin I and II *FEBS*, **331**, 173-176

Miller, R. W., Kleiman, R., Powell, R. G. and Putnam, A. R. (1988) Germination and Growth Inhibitors of Alfalfa. *J. Nat. Prod.* **51**, 328-330

Minks, A. K. and Harrewijn, P. (1987) Aphids, Their Biology, Natural Enemies and Control, Volume A, World Crop Pests, 2A, *Elsevier*, Oxford

Misaka, T., Kuroda, M., Iwabuchi, K., Abe, K. and Arai, S. (1996) Soyacystatin, a Novel Cysteine Proteinase Inhibitor in Soybean, is Distinct in Protein Structure and Gene Organization from other Cystatins of Animal and Plant Origin. *Eur. J. Biochem.* **240**, 609-614

Morris, R. J., Dye, W. B. and Gisler, P. S. (1961) Isolation, Purification, and Structural Identity of an Alfalfa Root saponin. *J. Org. Chem.* **26**, 1241-1243

Murdock, L. L., Brookhart, G., Dunn, P. E., Foard, D. E., Kelley, S., kitch, L., Shade, R. E., Shukle, R. H. and Wolfson, J. L. (1987) Cysteine Digestive Proteinases in Coleoptera. *Comp. Biochem. Physiol.* **87 B**, 783-787

Ngadjui, B. T., Ayafor, J. F., Sondengam, B. L., Connolly, J. D. and Rycroft, D. S.(1991) Hoslundin, Hoslundal, and Hoslundiol: Three New Flavonoids from the Twigs of *Hoslundia Opposita* (Lamiaceae). *Tetrahedron*, **47**, 3555-3564

Nisius, A. (1988) The Stromacentre in Avena Plastids: An Aggregation of β-Glucosidase Responsible for the Activation of Oat-Leaf Saponins. *Planta* **173**, 474-481

Nowacka, J. and Oleszek, W. (1992) High Performance Liquid Chromatography of Zanhic Acid Glycoside in Alfalfa *(Medicago sativa). Phytochemical Analysis* **3**, 227-230

Nowacka, J. and Oleszek, W. (1994) Determination of Alfalfa *(Medicago sativa)* Saponins by High –Performance Liquid Chromatography. *J. Agric. Food chem.* **42**, 727-730

Nowacki, E., Jurzysta, M. and Dietrych-Sz¤stak, D. (1976) Biosynthesis of Medicagenic Acid in Germinating Alfalfa. *Biochem. Physiol. Pflanz.* **156**, 183-186

Oleszek, W. (1988) Solid - Phase Extraction-Fractionation of Alfalfa Saponins. *J. Sci. Food Agric.* **44**, 43-49

Oleszek, W. and Jurzysta, M. (1986) Isolation, Chemical Characterization and Biological Activity of Alfalfa *(Medicago media* Pers.*)* Root Saponins. *ACTA Soc. Bot. Pol.* **55**, 23-33

Oleszek, W. and Jurzysta, M. (1990) High - Performance Liquid Chromatography of Alfalfa Root Saponins. *J. Chromat.* **519**, 109-116

Oleszek, W., Nowacka, J., Gee, J. M., Wortley, G. M. and Johnson, I. T. (1994) Effects of Some Purified Alfalfa (*Medicago sativa*) Saponins on Transmural Potential Difference in Mammalian Small Intestine. *J. Sci. Food Agric.* **65**, 35-39

Oleszek, W., Jurzysta M. and G¤rski, M. (1992) Alfalfa Saponins- the Allelopathic Agents; in Allelpathy Basic and Applied Aspects (Rizvi, S. J. H. and Rizvi, V. eds.), pp. 151-167, *Chapman and Hall*, London

Oleszek, W., Price, K.R., Colquhoun, I. J., Jurzysta, M., Ploszynski, M. and Fenwick, G. R. (1990) Isolation and Identification of Alfalfa (*Medicago sativa* L.) Root Saponins: Their Activity in Relation to a Fungal Bioassay. *J. Agric. Food Chem.* **38**, 1810-1817

Osbourn, A. (1996) Saponins and Plant Defence a Soap Story. *Trends Plant Sci.* **1**, 4-9

Osbourn, A. E., Clarke, B. R., Dow, J. M. and Daniels M. J. (1991) Partial Characterization of Avenacinase from *Gaeumanomyces graminis* Var. *avenae*. *Physiol. Mol. Plant Pathol.* **38**, 301-312

Osbourn, A., Bowyer, P., Lunness, P. Clarke, B. and Daniels, M. (1995) Fungal Pathogens of Oat Roots and Tomato Leaves Employ Closely Related Enzymes to Detoxify Different Host Plant Saponins. *Mol. Plant-Microb Int.* **8**, 971-978

Oxtoby, E., Dunn, A., Pancoro, A. and Hughes, A. (1991). Nucleotide and derived amino acid sequence of cyanogenic β-glycoside (linamarase) from white clover (*Trifolium repens* L.). *Plant Mol. Biol.* **17**, 209-219

Parry, A. D. and Edwards, R. (1994) Characterization of O-Glucosyltransferases with Activities Toward Phenolic Substrates in Alfalfa. *Phytochem.* **37**, 655-661

Pedersen, M. W. (1975) Relative Quantity and Biological Activity of Saponin in Germinating Seeds, Roots and Foliage of Alfalfa. *Crop Science* **15**, 541-543

Pedersen, M. W., Barnes, D. K., Sorensen, E. L., Griffin, G. D., Nielson, M. W., Hill, R. R., Jr., Frosheiser, F. I., Sonoda, R. M., Hanson, C. H., Hunt, O. J., Peaden, R. N., Elgin, J. H., Jr., Davine, T. E., Anderson, M. J., Goplen, B. P., Elling, L. J. and Howarth R. E. (1976) Effects of Low and High Saponin Selection in Alfalfa on Agronomic and Pest Resistance Traits and the Interrelationship of these Traits. *Crop Sci.* **16**, 193-199

Peng, W. K. (1990) Carbon dioxide as a Control Agent for *C. maculatus* (Fab.) In *Stored Adzuki Bean; in Bruchids and Legumes: Economics, Ecology and Coevolution* (Fujii, K., Gatehouse, A. M. R., Johnson, C. D., Mitchel, R. and Yoshida, T. eds.), **pp.** 75-80, Proceeding of Second International Symposium Of Bruchids and Legumes, *Klumer Academic Publishers*, Okayama, Japan

Peri, I., Mor, U., Heftmann, E., Bondi, A. and Tencer, Y. (1979) Biosynthesis of Triterpenoid Sapogenols in Soybean and Alfalfa Seedlings. *Phytochem.* **18,** 1671-1674

Perlak, F. J., deaton, R. W., Armestrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T. and Fishoff, D. A. (1990) Insect Resistant Cotton Plants. *Bio/Tech.* 8, 939-943

Plummer, D. T. (1978) An Introduction to Practical Biochemistry; pp. 61-98. *McGraw-Hill*, U.K.

Polacheck, I., Levy, M., Guizie, M., Zehavi, U., Naim, M. and Evron, R. (1991) Mode of Action of the Antimycotic Agent G2 Isolated from Alfalfa Roots. *Zbl. Bakt.* **275,** 504-512

Price, K. R., Johnson, I. T. and Fenwick, G. R. (1987) The Chemistry and Biological Significance of Saponins in Foods and Feeding Stuffs: in *CRC Critical Reviews in Food Science and Nutrition* (Furia, T. E. ed.), **26,** pp.27-135

Radeglia, R., Adam, G. and Ripperger, H. (1977) ^{13}C NMR Spectroscopy of *Solanum* Steroid Alkaloids. *Tetrahedron Lett.* No. 11, 903-906

Rao, D. and Bories, G. (1987) Simple Gas Chromatographic Method for the Determination of Medicagenic Acid in Alfalfa (*Medicago sativa*). *J. Chromatogr.* **410,** 169-175

Rao, G. V., Rao, P. S., Tomimori, T. and Kizu, H. (1985) Soyasaponin I from *Rothia trifoliata*. *J. Nat. Prod.* **48,** 135-138

Roda, A. L., Landis, D. A., Coggins, M. L., Spandl, E. and Hesterman, O. B. (1996) Forage Grasses Decrease Alfalfa Weevil Damage and Larval Numbers in Alfalfa Grass Intercrops. *J. Econ. Entomol.* **89,** 743-750

Roddick, J. G. and Drysdale, R. B. (1984) Destabilitization of Liposome Membranes by the Steroidal Glycoalkaloid α-Tomatine. *Phytochem.* **23,** 543-547

Ryan, C. A. (1990) Protease Inhibitors in Plants: Genes for Improving Defenses Against Insects and Pathogens. *Annu. Rev. Phytopathol.* **28,** 425-449

Shany, S., Gestetner, B., Birk, Y. and Bondi, A. (1970) LUCERNE SAPONINS: III.- Effect of Lucerne Saponins on Larval Growth and their Detoxification by Various Sterols. *J. Sci. Food Agric.* **21**, 508-510

Shao, Y., Zhou, B., Ma, K., Wu, H., Lin, L. and Cordell, G. A. (1995) Medicagenic Acid Saponins from *Aster batangensis*. *Phytochem.* 39, 875-881

Shashida, Y., Kubo, S., Mimaki, Y., Nikaido, T. and Ohmoto, T. (1992) Steroidal Saponins from Smilax riparia and S. china. *Phytochem.* **31**, 2439-2443

Shewry, p. (1991) Plant Proteins that Confer Resistance to Pests and Pathogens. *J. Exp. Bot.* **42**, 3

Shimoyamada, M., Hudo, S., Okubo, K., Yamauchi, F. and Harada, K. (1990) Distributions of Saponin Constituents in Some Varieties of Soybean Plant. *Agric. Biol. Chem.* **54**, 77-81

Singh, B. B. and Singh, S. R. (1990) Breeding for Bruchid Resistance in Cowpea In *Bruchids and Legumes: Economics, Ecology and Coevolution* (Fujii, K., Gatehouse, A. M. R., Johnson, C. D., Mitchel, R. and Yoshida, T. eds.), pp. 219-228 Proceeding of Second International Symposium Of Bruchids and Legumes, *Klumer Academic Publishers*, Okayama, Japan

Singh, D. P. (1986) Breeding for Resistance to Diseases and Insect Pests. *Springer - Verlag*, New York

Small, E., Jurzysta, M. and Nozzolillo, C. (1990) The Evolution of Haemolytic Saponin Content in Wild and Cultivated Alfalfa (*Medicago sativa*, Fabaceae). *Econ. Bot.* **44**, 226-235

Sorensen, E. L., Byers, R. A. and Horber, E. K. (1988) Breeding for Insect Resistance in *Alfalfa and Alfalfa Improvement* (Hanson, A. A., Barnes, D. K., Hill, R. R. and Jr, R. R. eds.), pp. 859-902, *American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc.*, Madison, Wisconsin, U.S.A

Storey, R. D. and Wagner, F. W. (1986) Review Article Number 21, Plant Proteinases: A Need for Uniformity. *Phytochem.* **25**, 2701-2709

Stubbs, M. T., Laber, B., Bode, W., Huber, P. and Jerala, R., Lenarcic, B. and Turk, V. (1990) The Refined 2.4 A X-Ray Crystal Structure of Recombinant human Stefin B in Complex with the Cysteine Proteinase Inhibitor Papain: A Novel Type of Proteinase Inhibitor Interaction. *EMBO J.* **9**, 1939-1947

Stuteville, D. L. and Skinner, D. Z. (1987) Effects of Selecting for Downy Mildew Resistance in Alfalfa Saponin Content. *Crop Sci.* **27**, 906-908

Su, H. C. F., Speirs, R. D. and Mahany, P. G. (1972) Toxic Effects of Soybean Saponin and Its Calcium Salt on the Rice Weevil. *J. Econ Entomol.* **65**, 844-847

Stubbs, M. T., Laber, B., Bode, W., Huber, R. and Jerala, R., Lenarcic, B. and Turk, V. (1990) The Refined 2.4 a X-Ray Crystal Structure of Recombinant human stefin B in Complex with the Cysteine Proteinase Inhibitor Papain: A Novel Type of Proteinase Inhibitor Interaction. *EMBO J.* **9**, 1939-1947

Swain, T. (1977) Secondary Compounds as Protective Agents. *Ann. Rev. Plant Physiol.* **28**, 497-501

Taiz, L. and Zeiger, E. (1991) Plant Physiology. *The Benjamin/Cummings Publishing Company, Inc.* New York

Tani, T., Katsuki, T., Kubo, M., Arichi, M. and Kitagawa, I (1985) Histochemistry. V. Soyasaponins in Soybeans (*Glycin max*) Seeds. *Chenm. Pharm. Bull.* **33**, 3829

Thomas, J. C., Wasmann, C. C., Echt, C., Dunn, R. L., Bohnert, H. J. and McCoy, T. J. (1994) Introduction and Expression of an Insect Proteinase Inhibitor in Alfalfa (*Medicago sativa* L.). *plant Cell Rep.* **14**, 31-36

Timbekova, A. E. and Abubakirov, N. K. (1984) Triterpene Glycosides of Alfalfa. I. Medicoside G., A novel Bisdesmoside from *Medicago sativa. Khim. Prir. Soedin.* pp.451

Tsurumi, S., Takagi, T. and Hashimoto, T. (1992) A γ-Pyronyl-Triterpenoid Saponin from *Pisum sativum. Phytochem.* **31**, 2435-2438

Turner, E. M. C.(1961) An Enzymic Basis for Pathogenic Specificity in *Ophiobolus graminis. J. Exp. Bot.* **12**, 169-175

USDA (1991) Biological Control of the Alfalfa Weevil. Animal and Plant Health Inspection Service, National Biological Control Instute, *Program Aid*, No. **1321**, 11 pages

Visarathanonth, P., Khumlekasing, M. and Sukprakarn, C. (1990) Insecticidal Control of Cowpea Weevil, *Callosobruchus maculatus* Fab., A Pest of Mungbean In *Bruchids and Legumes: Economic, Ecology, and Coevolution* (Fujii, K., Gatehouse A. M. R., Johnson, C. D., Mitchel, R. and Yoshida, T. eds.), 101-104, Proceeding of Second International Symposium of Bruchids and Legumes, *Klumer Academic Publishers*, Okayama, Japan

Waldron, C., Wegrich, L. M., Merlo, P. A. O. and Walsh, T. A. (1993) Characterization of a Genomic Sequence Coding for Potato Multicystatin, an Eight-Domain Cysteine Proteinase Inhibitor. *Plant Mol. Biol.* **23**, 801-812

Waller, G. R., Jurzysta, M. and Thorne, R. L. Z. (1993) Allelopathic Activity of Root Saponins from Alfalfa *(Medicago sativa L.)* on Weeds and Wheat. *Botan. B. S. A.* **34**, 1-11

Wilkomirski, B. and Kasprzyk, Z. (1979) Free and Ester-Bound Triterpenes Alcohols and Sterols in Cellular Subfractions of *Calendula officinalis* Flowers. *Phytochem.* **18**, 253-255

Wilson, M. C., Stewart, J. K. and Vail, H. D. (1979) Full Season Impact of the Alfalfa Weevil, Meadow Spittlebug, and Potato Leafhopper in an Alfalfa yield. *J. Econ. Entomol.* **72**, 830-834

Wojciechowski, Z. A. (1975) Biosynthesis of Oleanolic Acid Glycosides by Subcellular Fractions of *Calendula Officinalis* Seedlings. *Phytochem.* **14**, 1749-1753

Wolfson, J. L. and Murdock, L. L. (1987) Suppression of Larval Colorado Potato Beetle Growth and Development by Digestive Proteinase Inhibitors. *Entomol. Exp. Appl.* **44**, 235-240

Wyman-Simpson, C. L., Waller, G. R., Jurzysta, M., McPherson, J. K. and Young, C.C. (1991) Biological Activity and Chemical Isolation of Root Saponins of Six Cultivars of Alfalfa *(Medicago sativa L.). Plant and Soil* **135**, 83-94

Yoshikawa, M., Murakami, T., Kadoya, M., Muraoka, O., Yamahara, J. and Murakami, N. (1996) Medicinal Foodstuffs. III. Sugar Beet. (1): Hypoglycemic Oleanolic Acid Oligoglycosides, Beta vulgarosides I, II, III, and IV, from the Root of *Beta vulgaris* L. (Chenopodiaceae) *Chem. Pharm. Bull.* **44**, 1212-1217

Zimmer, D. E., Pedersen, M. W. and McGuire, C. F. (1967) A Bioassay for Alfalfa Saponins using the Fungus, *Trichoderma viride*. *Crop Sci.* 7, 223-224