ALLELOCHEMICALS FROM *SORGHUM BICOLOR* THAT STIMULATE FEEDING BY THE LARVAE OF THE STEM BORER *CHILO PARTELLUS*

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by

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To Rita, O'Baka and Nii Sai,

who put up with so much.

DECLARATION

It is hereby declared that the following is the result of three years research undertaken by the author under supervision at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, and that it has neither wholly nor partly been presented elsewhere for another degree.

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" Much of life can be understood in rational terms if expressed in the language of chemistry. It is an international language, a language for all time, a language that explains where we came from, what we are, and where the physical world will allow us to go. Chemical language has great esthetic beauty and links the physical sciences to the biological sciences. Unfortunately, the full use of this language to understand life processes is hindered by a gulf that separates chemistry from biology. The gulf is not nearly so wide as the one between humanities and sciences. Yet, chemistry and biology are two distinct cultures and the rift between them is serious, generally unappreciated, and counterproductive....."

Nobel Laureate Arthur Kornberg

on Chemistry and Biology

CONTENTS

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

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ABSTRACT

Feeding bioassays with cellulose acetate discs impregnated with the hexane, ethyl acetate and methanol extracts of the leaf-whorls of field grown plants of sorghum cultivars IS 18363 (susceptible) and IS 2205 (resistant) showed that the methanol extracts were most stimulatory to the feeding of the third-instar larvae of *Chilo partellus*. Ethyl acetate extracts were intermediate in stimulatory activity whilst hexane extracts were the least stimulatory. Extracts of the more susceptible cultivar were more stimulatory than those of the more resistant cultivar and those of the whorls of the 3 week old plants were more stimulatory to larvae than those of the 6 week old plants.

The phagostimulatory compounds in the ethyl acetate extracts were phenolic, p-hydroxybenzaldehyde and p-hydroxybenzoic acid being the major components and ferulic and caffeic acids being in minor amounts. p-Coumaric acid was also present in minor amounts but was non-stimulatory at all the doses tested. p-Hydroxybenzaldehyde was a more potent feeding stimulant for the larvae relative to some of its possible theoretical biogenetic analogues. Limited structure-activity studies with some hydroxybenzoic acids and their corresponding cinnamic acids showed that the former were more stimulatory to the feeding of the larvae

than the latter and that oxygen substitution in the benzene ring was crucial for activity.

The phagostimulatory compounds in the methanol extracts were phenolic, identical to those in the ethyl acetate extracts, and sugars. The sugars which were identified in the extracts comprised sucrose, fructose, glucose and xylose. The feeding response of larvae to these sugars followed the order sucrose >> glucose ≈ fructose; xylose was non-stimulatory. Comparison of the activities of sucrose with mixtures of glucose and fructose showed that the high activity of the disaccharide is due to its total structure and not to a summation of its monosaccharide moieties.

Sugars synergised with phenolics to give enhanced feeding response of the third-instar larvae.

Chromatographic analyses of the extracts showed that stimulatory and non-stimulatory components in the extracts differed quantitatively rather than qualitatively in the whorls of the two cultivars at the two growth stages. This may have implication in resistance screening and breeding programmes.

INTRODUCTION

Sorghum bicolor (L.) Moench (*Andropogeneae* : *Graminaeae*) is a native of Africa and Asia (Brounk, 1975). The plant varies in height from 0.5 to 6 m depending on the cultivar. It is an annual with a single stem, usually erect, dry or juicy, with a diameter ranging from 0.5-3 cm. It is similar to maize, but with only one type of inflorescence which is a panicle consisting of spikelets with bisexual flowers. The plant is adapted to a wide range of ecological conditions, growing in hot and dry conditions and in areas with high rainfall in which waterlogging occurs. The optimum temperature for growth is 30 ^{o}C (Purseglove, 1975).

Sorghum is ranked as the fourth most important world cereal, following wheat, rice and maize and it is the staple food in the drier parts of tropical Africa, India and China (Purseglove, 1975). The grain is used in preparing a variety of foods. It is ground into a wholemeal flour and made into a thin porridge or a dough by boiling in water. The pealed grain is sometimes cooked like rice or ground into flour for making biscuits or bread. In certain parts of Central, Eastern and Southern Africa, some varieties of the grain are used for brewing beer. In the developed

countries like the United States, the grain and plant are used as food for livestock while syrup is manufactured from the varieties called sorgos (Purseglove, 1975). These have large juicy stems which have been found to contain up to 10% sucrose. It is estimated that 2-3 million gallons of syrup are produced per year from sorghum in the United States.

Sorghum is attacked by a number of insect pests. The worst species are the stalk-borers. In Eastern Africa and India, *Chilo partellus* (Swinhoe) has been found to cause the severest damage to the plant (Teetes *et al.*, 1983). In addition *Busseola fusca* (Fuller), *Locusta migratoria* L., *Eldana saccharina* (Walker) and *Sesamia calamistis* Hmps. are important stemborers of the plant, and in some locations one of this is found to be its predominant pest (Teetes *et al.*, 1983; Seshu Reddy, 1985). It is also attacked by the sorghum shootfly *Atherigona soccata* (Rondani) and a variety of aphids (Teetes *et al.*, 1983).

The work reported in this study relates to *C. partellus*. In the field, it attacks all parts of the plant except the roots. The first indication that the plant is infested by the larvae of this insect is the appearance of shot-holes and lesions in the young whorl leaves (Dabrowski and Kidiavai, 1983; Teetes *et al.*, 1983). A dead heart and a ragged appearance of the leaves indicate severe attack on the plant. Larvae also bore into the stem causing extensive

tunnelling, which affects the growth and survival of the plant.

The bases of sorghum resistance to its insect pests has attracted the attention of a number of researchers all over the world. At the International Centre of Insect Physiology and Ecology (ICIPE), the focus is on resistance and susceptibility manisfestations of different cultivars at different stages in the colonisation of the plant by *C. partellus*, *B. fusca*, and *E. saccharina*. At the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), similar studies have been reported for the migratory locust *L. migratoria* (Bernays and Chapman, 1978; Woodhead and Bernays, 1978; Woodhead, 1982 and 1983). The greenbug aphid *Schizaphis graminum* is the insect of interest at the United States Department of Agriculture (USDA) (Dreyer *et al.*, 1981).

Saxena (1985a) described two broad categories of factors which govern the interaction between plants and their insect pests. These include responses of the insect to the plant leading to its establishment, and the characters of the plant which control these responses. The insect's main responses which include orientation (attraction or repulsion), oviposition and feeding are determined by the plant's physical and chemical characters. It is believed that these plant characters if identified can

be used in various pest management strategies such as behaviour manipulation with active host chemicals and the use of these characters as a basis for screening and breeding for resistance.

As part of this program of the ICIPE, this study was undertaken to identify the feeding stimulants in sorghum for *C. partellus* larvae. The report on this study is divided into six chapters. Chapter 1 gives an overview on insect phagostimulants and antifeedants. Chapter 2 gives a summary of the previous allelochemical work on sorghum and the detailed objectives of this study. Preliminary investigation involving the development of feeding bioassays for the third-instar larvae of *C. partellus* and the development of chromatographic separation conditions for sorghum extracts are described in Chapter 3. Chapter 4 describes the experimental details and results of the investigations carried out in this study, and Chapter 5 discusses these results. Some suggestions for future studies as a follow up of the present work are given in Chapter 6. This is finally followed by a glossary of special terms and a list of references.

CHAPTER 1

INSECT PHAGOSTIMULANTS AND ANTIFEEDANTS: AN OVERVIEW

Insects are neccesary to plants for their pollination and thus for their reproduction (Edwards and Wratten, 1980). Plants have therefore evolved features like alluring colours and odours to attract insects for fertilization, and in return they provide pollen and nectar to the insects. However, research has shown that not all insects are useful to the plant (Marini-Bettolo, 1983; Boppre, 1986). Some are harmful to it, and these include phytophagous insects which solely depend on plants for their food and oviposition (egg-laying) sites (Edwards and Wratten, 1980). The mechanisms by which plants escape from the damaging effects of these insects and the adaptation of the insects, in turn, to their food supplies have been studied by Erhlich and Raven (1964), Jermy (1976) and Feeny (1983). They postulated that plants have evolved a great variety of defensive mechanisms, mainly production of toxic chemicals to protect themselves from these insects. In turn, phytophagous insects have also evolved mechanisms enabling them to avoid or detoxify these toxins in their food, and in some cases have been able to use the chemicals characteristic of certain species of plants as cues for the recognition and selection of their hosts. These workers described this phenomenon between plants and phytophagous insects as coevolution.

Before the theory of coevolution was promulgated, studies on the role of plant chemicals on the behaviour (feeding and oviposition) of phytophagous insects were already in progress. The earliest experimental evidence of the importance of these chemicals as cues for food selection by phytophagous insects was provided by Verschaeffelt (1910) working with cruciferous feeding insects. His work showed that members of the plant family *Cruciferae* owed their interaction with *Pieris* species to a single set of chemicals, the mustard oils and their glycosides. Though this discovery set the direction of research for several decades, attention was mainly focussed on plant attractants and stimulants with little emphasis on repellents and deterrents (Dethier, 1982). However, recent years have witnessed intense studies in the establishment of the influence of the various classes of plant chemicals on the feeding and oviposition of several insect pests, and noteworthy reviews on the subject have been provided by Dethier (1966), Chapman (1974), Hedin *et al.*, (1977), Schoonhoven (1982) and Staedler (1986).

In this chapter an attempt has been made to summarise the literature on some of the salient features on the chemical basis of feeding by phytophagous insects. The focus is on studies on well-characterised feeding stimulants and deterrents. Tables 1.1 and 1.2 list these compounds, their plant sources and the insects they have been

demonstrated to affect (see pages 17-23 and 30-43). Compounds which play opposite roles, acting as stimulants to certain insect species but deterrent to others, are also included. Also included in this chapter is a summary of the various bioassay methods which have been used in testing for phagostimulation and deterrency in insects.

1.1 Allelochemics

Chemicals that are produced by individuals of one species and which affect the physiology or behaviour of individuals of another species are called allelochemics (Whittaker and Feeny, 1971).

In insect-plant relationships, two broad categories of allelochemics are recognized: allomones and kairomones. Allomones are chemicals used by the plant to protect itself from insect attack. They may act as repellents, deterrents or antibiotics which interfere metabolically with normal growth and development of the insect. Kairomones are chemicals used by the insect for host location and recognition, and food detection. They may act as attractants, arrestants or stimulants. Reese (1979) found that allelochemic effect is both concentration-dependent and situation-dependent. Thus a chemical classified as a stimulant for one insect species may act as a deterrent to

the same insect when present in a high concentration.

Although plant chemicals are important cues for food selection by phytophagous insects, studies have shown that they seldom alone determine insect behaviour. The other cues used by the insect in discriminating between various potential foods include biophysical factors of the plant such as shape, colour, texture, water content and ultrastructure (Dethier, 1982; Staedler, 1983)

1.2 Insect Feeding Behaviour

Insect feeding involves various behavioural responses which can be divided into four distinct steps (Dethier, 1966; Munakata,1977; Schoonhoven, 1982; Miller and Strickler, 1984): (1) host plant recognition and orientation, comprising locomotion which brings the insect to its food and cessation of locomotion on arrival, also termed arrest; (2) initiation of feeding (biting, probing or sucking); (3) continuation of feeding; and (4) termination of feeding as a result of satiation.

Feeding has been studied with a variety of assays reflecting the different behaviours of the insects described. From these assays, it has been suggested that the acceptance or rejection of a plant as food by an insect is determined by a number of factors (Dethier, 1982). These

include the intensity of olfactory and gustatory feeding stimulants, the intensity of repellents and deterrents, the metabolic state of the insect (i.e. the degree of deprivation, specific dietary deficiencies, malaise etc.) and finally learning acquired as a result of previous feeding experience.

Evidence to support some of these suggestions has been provided by electrophysiological studies at the sensory level (Schoonhoven and Jermy, 1977; Dethier, 1980; Staedler, 1982). These have shown that chemoreceptors are the means by which the insect detects plant chemicals and is able to differentiate between a stimulant and a deterrent compound. Thus insects are said to respond to a summation of inputs 'gestalt' from various chemoreceptors which perceive complex mixtures of compounds present in their foods.

1.3 Insect Feeding Stimulants

A feeding stimulant enhances the feeding of an insect and it can be classified as nutritional or non-nutritional (Dethier, 1966). The nutritional compounds are essential for the proper development of the insect, and they include sugars, amino-acids, inorganic salts, vitamins and phospholipids. The non-nutritional chemicals, also described as token stimuli (Fraenkel, 1959), are said to only guide the insect in discriminating its food, and they

comprise secondary plant compounds (Dethier, 1966). Some insects are stimulated to feed by a single dominant compound while others require several different chemicals in combination (Dethier, 1982; Miller and Stricker, 1984; Staedler, 1986).

Of the nutritional compounds, sugars, especially sucrose, have been recognized as a feeding stimulant to the majority of insects (Thorsteinson, 1960; Dethier, 1966; Schoonhoven, 1968; Sutherland, 1977; Staedler, 1983). According to Beck (1956), the choice by the European corn borer *Ostrinia nubilalis* (Hubner) of sites on the corn plant is based on the highest sugar concentration and is not influenced by local attractants. The importance of sucrose as a phagostimulant has been demonstrated for a number of lepidopteran and coleopteran insects (Dethier, 1966; Hsiao and Fraenkel, 1968; Hsiao, 1969; Peacock and Fisk, 1970 ; Sutherland, 1971; 1977; Doss and Shanks, 1984; Ladd, 1986 Shanks and Doss, 1987). Sucrose is also known to synergise with other compounds to give enhanced feeding stimulation. Sitosterol in combination with sucrose was found to stimulate more feeding by obscure root weevil *Sciopithes obscurus* Horn than sucrose alone (Doss, 1983). A similar synergism was found by Shanks and Doss (1987) with the same mixture with black vine weevil *Otiorhynchus sulcatus* (F.).

Other related sugars have been found to possess phagostimulatory activities. These include raffinose for the red cotton bug *Dsydercus koengii* (F.) (Hedin *et al.*, 1977), fructose for the eastern spruce budworm *Choristoneura fumiferana* Clem. (Albert *et al.*, 1982) and the onion maggot *Delia (=Hylemya) antiqua* (Meigen) (Mochizuki *et al.*, 1985). The eastern spruce budworm is stimulated to feed on cellulose discs by glucose and inositol (Albert *et al.*, 1982), and Numata *et al.*, (1979) found that pinitol, fructose and myo-inositol stimulated feeding of the larvae of the yellow butterfly *Eurema hecabe mandarim*. Ladd (1986) has reported that several *Scarabaeidae* species are stimulated to feed by a vareity of sugars including sucrose, maltose, fructose and glucose. However, arabinose, xylose and raffinose were rather weak feeding stimulants for these species of insects.

Tests with amino-acids have shown that they are generally weak feeding stimulants (Dethier, 1966). However, for the brown rice planthopper *Nilaparvata lugens* (Stal), asparigine is the major phagostimulant (Sogawa, 1982). Amino-acids have been shown in some studies as chemicals which act in combination with other compounds, especially sugars, to give enhanced feeding stimulation in a variety of insects. An example of this is demonstrated by the study of Beck and Hanec (1958). They found synergistic effects between serine and glucose for the European corn borer.

leucine and methionine each have been shown to synergise with sucrose to enhance the feeding of the green peach aphid *Myzus persicae* (Sulzer) (Mittler and Dadd, 1964). In a recent study, Albert and Jerrett (1981) found that feeding by the eastern spruce budworm is enhanced by a two component mixture of sucrose with a variety of amino-acids. These amino-acids include L-proline, L-hydroxyproline, L-glutamic acid and L-arginine. Protein deprived females of the housefly *Musca domestica* (L.) are stimulated to feed by a mixture of L-leucine and sodium phosphate buffer solution (Browne and Kerr, 1985).

In addition to sugars and amino-acids, a number of other nutrient components have been reported as insect feeding stimulants. Examples of these include betaine, ascorbic acid and thiamine for *Chorthippus curtipennis* (Harris) (Dethier, 1966), adenosine for the sweetclover weevil *Sitona cylindricolis* Fahraeus phospholipids for the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Hedin *et al.*, 1977) and sitosterol for the weevils, *Otiorhynchus sulcatus* (F.), *Sciopithes obscurus* (Horn) and *Hypera postica* (Gyllenhal) (Shanks and Doss, 1987).

Extensive studies have been carried out on the role played by secondary plant (non-nutritional) chemicals in the selection of food by phytophagous insects. Table 1.1 shows that these compounds range from high molecular weight

compounds such as glycosides to low molecular weight ones such as simple phenols.

The well investigated glycosides include mustard oils as well as phenolic, cyanogenic and iridoid glycosides. Mustard oil chemicals are associated with cruciferous plants. Of these sinigrin, glucapparin and glucoiberin have been found to trigger on the feeding of the following insects: the diamondback moth *Plutella maculipennis* (L.), the mustard beetle *Phaedon cochleariae* F., *Pieris brassicae* L. and the flea beetles *Phyllotreta cruciferae* (Goeze) and *Phyllotreta striolata* (F.) (Whittaker and Feeny, 1971). According to Wensler (1962), this class of compounds also accounts for the feeding by the cabbage aphid *Brevicoryne brassicae* (L.) on cruciferous plants. Iridoid and cyanogenic glycosides are not common insect feeding stimulants. However, for the Mexican bean beetle *E. varivestis* feeding is promoted by the cyanogenic glycosides phaseolunatin, lotaustrin and linamarin (Hedin *et al.*, 1977). Bowers (1983 and 1984) in a systematic search isolated and identified two iridoid glycosides, catalpol and aucubin from the plant *Plantago lanceolata* which stimulated feeding by the checkerspot butterfly *Euphydryus challedona* (Doubleday) and the buckeye butterfly *Junonia coenia* both of which are pests of the plant.

Phenolic and flavonoid compounds are well known feeding stimulants for certain coleopteran insects. The smaller European elm bark beetle *Scolytus multistriatus* (Marsham) is strongly stimulated to feed on pith discs treated with *p*-hydroxybenzaldehyde or some of its analogues (Baker and Norris, 1968), (+)-catechin 5-α-D-xylo-pyranoside or its aglycone (+)-catechin from *Ulmus americana* (L.) (Norris, 1977). The horse-raddish beetle *Phyllotreta armoraciae* (Koch) is stimulated to feed by glucosinolates, kaempferol 3-O-xylosylgalactoside and quercetin 3-O-xylosylgalactoside (Nielsen *et al.*, 1979). Coleopteran insects have also been used in feeding bioassays for tests involving three phenolic glycosides: salicin, populin and luteolin-7-glucoside. Significant feeding stimulation were found for various concentrations of salicin for the willow beetle *Gonioctena vitallinae* (L.) (Hedin *et al.*, 1977) and the salicaceous feeding beetle *Phyllodecta vitellinae* (Hutchinson, 1931). When presented on filter paper discs, populin and luteolin-7-glucoside promoted the feeding of *Chrysomelia vigontipunctata costella* (Marseul), *Plagiodera versicolora distincta* Baly and *Lochmaea capreae cribata* Solosky (Matsuda and Matsuo, 1985). Other active phenolic and flavonoid compounds reported to be active insect feeding stimulants include chlorogenic acid and cyanidin-3-glucoside (Hedin, *et al.*, 1977).

Some phytosterols have been reported as potent feeding stimulants for certain insect species. An example of this is β-sitosterol which is a feeding and biting stimulant for silkworm *Bombyx mori* (L.) (Hamamura *et al.*, 1962; Ito *et al.*, 1964), Colorado potato beetle *Leptinotarsa decemlineata* (Hsiao and Fraenkel, 1968), black vine weevil *Otiorhynchus sulcatus* (F.) (Doss and Shanks, 1984) and alfalfa weevil *Hypera postica* (Gyllenhal) (Shanks and Doss, 1987). The obscure root weevil *Sciopithes obscurus* Horn is stimulated to feed by sitosterol and stigmasterol (Doss *et al.*, 1982 ; Doss, 1983).

Synergistic effects between secondary plant compounds have also been demonstrated. Chromatographic fractions were generally found to be less stimulatory than the mother extracts, and full activity is often not regenerated by recombining the test mixtures (Hedin *et al.*, 1977). The major cause of this is the breakdown of some compounds during isolation and purification. However, these studies go to confirm the suggestions of some workers that adequate feeding response from some phytophagous insects is obtained from a complex profile of chemicals.

Studies have also been conducted on a number of insects which feed on only a few plant species. Such insects often use specific secondary plant metabolites as cues for feeding. Smith (1966) found that the change of feeding

sites on broom *Sarothamus scoparius* by adults of the aphid *Acyrthosiphon spartii* is associated with the movement of sparteine between the various parts of the plant. A similar example was reported by Kogan (1976) that several species of *Diabrotica* and *Acalymona* show considerable preference for certain species of *Cucurbitaceae* with high cucurbitacin concentrations. Similar examples have been found for the insect *Calpe excavata* which feeds on *Cocculus trilobus* in response to the alkaloid isoboldine (Wada and Munakata, 1968) and the monophagous beetle *Chrysolina brunsvicensis* which lives on *Hypericum* and requires hypericin in its food (Rees, 1969). Kogan (1976) reported that three solanaceous feeding insects, *Lema trilineata daturaphila* Kogan and Goeden, *L. decemlineata* and *Manduca* sexta (L.), show preferences for certain species of solanaceous plants characterised by the presence of certain classes of alkaloids. Solanum plants characterised by the presence of tropane alkaloids are accepted by *L. trilineata* and those characterised by steroidal alkaloids such as solanine and chaconine by *L. decemlineata* but *Manduca sexta* shows broad spectrum tolerance for the various classes of alkaloids.

Table 1.1 INSECT FEEDING STIMULANTS.

Feeding stimulant	Plant source	Insect name	Reference
ADENOSINE	sweet clover	*Sitona cylindricolis* F.	Hedin *et al.*,(1977)
ALLYL SULFIDE	onion	*Hylemya antiqua* (Meigen)	Miller *et al.*,(1984)
ANISIC ACID	general	*M. bivittatus* (Say)	Hedin *et al.*,(1977)
ANISIC ALDEHYDE	anise,citrus	*P. polyxenes* Stoll	Hedin *et al.*,(1977)
ARGININE	pine	*C. fumiferana* Clem.	Albert *et al.*,(1981)
ASCORBIC ACID	general	*Chorthippus curtipennis* (Harris)	Dethier (1966)
ASPARIGINE	rice	*Nilaparvata lugens* (Stal)	Sogawa(1982)
AUCUBIN	*P. lanceolata*	*Junonia coenia*	Bowers(1984)
BENZOIC ACID	general	*M. bivittatus* (Say)	Hedin *et al.*,(1977)

BETAINE	general	*C. curtipennis* (Harris)	Dethier(1966)
CARLINOSIDE	rice	*N. lugens* (Stal)	Kim *et al.*,(1985)
CARVONE	*Umbelliferae*	*P. polyxenes* Stoll	Dethier(1966)
CATALPOL	*P. laceolata*	*Euphydryus challedona* (Doubleday)	Bowers(1983)
(+)-CATECHIN-T-α-XYLO-PYRANOSIDE	elm	*Scolytus multistratus* (Marsham)	Norris(1977)
CHACOLINE	potato leaves	*Leptinotarsa decemlineata*(Say)	Kogan(1976)
CHLOROGENIC ACID	potato leaves	*L. decemlineata* (Say)	Hedin *et al.*,(1977)
CITRONELLOL	cotton	*Spodoptera litoralis* (Boisd)	Hedin *et al.*,(1977)
COUMARIN	sweet clover	*S. cylindricolis* F.	Hedin *et al.*,(1977)
CUCURBITACINS	*Cucurbitaceae* *Cruciferae*	*Diabrotica undecimpunctata* (Barber), *Aulacophora foveicollis*	Hedin *et al.*,(1977)
FORMIC ACID	cotton	*A.grandis* Boheman	Hedin *et al.*,(1977)
FRUCTOSE	onion	*Hylemya antiqua* (Meigen)	Mochizuki *et al.*,(1985)
	pine	*C. fumiferana* Clem.	Albert *et al.*,(1981)

GLUCOCAPPARIN	*Cruciferae*	*Pieris brassicae* (L.)	Whittaker *et al.*,(1971)
GLUCOHEIROLIN	*Brassica* spp.	*P. brassicae* (L.)	
	Capparidaceae	*Plutella* maculipennis (L.)	Whittaker *et al.*,(1971)
GLUCOIBERIN	*Cruciferae*	*P. brassicae* (L.)	Whitakker *et al.*,(1971)
GLUCOSE	cottonseed	*Dsydercus koenigii* (F.)	Hedin *et al.*,(1977)
		C. fumiferana Clem.	Albert *et al.*,(1982)
GLUCOSINALBIN	*Brassica spp.*	*P. brassicae* (L.)	Hedin *et al.*,(1977)
GLUCOTRICIN	rice	*N. lugens* (Stal)	Sogawa(1982)
GLUTAMIC ACID	pine	*C. fumiferana* Clem.	Albert & Jerrett(1981)
GOSSYPOL	cotton	*A. grandis* Boheman	Hedin *et al.*,(1977)
HOMOINETIN	rice	*N. lugens* (Stal)	Sogawa(1982)
o-HYDROXYBENZOIC ACID	rice	*N. lugens* (Stal)	Sogawa(1982)
o-HYDROXYBENZYL ALCOHOL	elm	*S. multistratus* (Marsham)	Baker *et al.*,(1968)
p-HYDROXYBENZALDEHYDE	elm	*S. multistriatus* (Marsham)	Baker *et al.*,(1968)

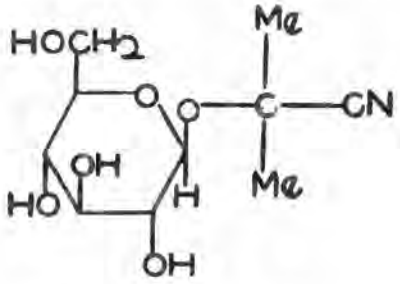
HYPERICIN	*Hypericium hirsutum*	*Chrysolina brunsvicensis*	Rees(1969)
INOSITOL		*C. fumiferana* Clem.	Albert *et al.*,(1982)
ISOBOLDINE	*Cocculus trilobus*	*Calpe excavata*	Wada & Munakata(1968)
α-KETOGLUTARIC ACID	cotton	*A. grandis* Boheman	Hedin *et al.*,(1977)
LACTIC ACID	cotton	*A. grandis* Boheman	Hedin *et al.*,(1977)
LECITHIN	corn, soybean	*M. bivittatus* (Say), *Camnula pellucida* (Scudder)	Dethier (1966)
LINAMARIN	*Phaseolus spp.*	*Epilachna varivestis* Mulsant	Hedin *et al.*,(1977)
LOTAUSTRIN	*Phaseolus spp.*	*E. varivestis* Mulsant	Hedin *et al.*,(1977)
LUTEOLIN-7-GLUCOSIDE	*Salicaceous spp*	*Chrysomelia vigintipunctata costella* (Marseul) *Plagiodera versicolora distincta* Baly.	Matsuda & Matsuo(1985)

MALONIC ACID	cotton	A. *grandis* Boheman	Hedin *et al.*,(1977)
MONOSODIUM GLUTAMATE		*C. curtipennis* (Harris)	Dethier(1966)
NEOCARLINOSIDE	rice	*N. lugens* (Stal)	Kim *et al.*,(1985)
NEOSCHAFTOSIDE	rice	*N. lugens* (Stal)	Kim *et al.*,(1985)
ORIZATIN	rice	*N. lugens* (Stal)	Sogawa(1982)
ORYZANONE	rice	*Chilo suppressalis* (Walker)	Hedin *et al.*,(1977)
PHASEOLUNATIN	*Phaseolus spp.*	*E. varivestis* Mulsant	Hedin *et al.*,(1977)
POPULIN	*Salicaceous spp.*	*C. vigintipunctata costella* (Marseul), *P. versicolora distincta* Baly, *Lochmaea capreae cribata* Solosky	Matsuda & Matsuo(1985)
PROLINE	pine	*C. fumiferana* Clem.	Albert & Jerrett(1981)
QUERCETIN	cotton	*A. grandis* Boheman	Hedin *et al.*,(1977)
QUERCETIN-3'-GLUCOSIDE	cotton	*A. grandis* Boheman	,,

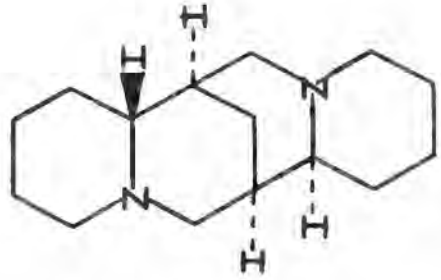
QUERCETIN-3-O-XYLOSYL-GALACTOSIDE	cotton	*A. grandis* Boheman	,,
RAFFINOSE	cottonseed	*D. koenigii* (F.)	Hedin *et al.*,(1977)
SALICIN	*Salicaceous* spp.	*C. vigintipunctata*	Matsuda & Matsuo(1985)
		costella (Marseul),	
		P. versicolora distincta Baly, L.	Matsuda & Matsuo(1985)
		Capreae cribata Solosky	Matsuda & Matsuo(1985)
SCHAFTOSIDE	rice	*N. lugens* (Stal)	Kim *et al.*,(1985)
SINALBIN	*Cruciferae*	*Plutella maculipenis* (L.)	Whitakker *et al.*,(1971)
SINIGRIN	*Cruciferae*	*P. rapae* (L.),	Vershaeffelt (1910)
		P. brassicae (L.)	
β-SITOSTEROL	cotton,	*A. grandis* Boheman	Hedin *et al.*,(1977)
		B. mori (L.)	Ito *et al.*,(1964)
		Hypera postica (Gyllenhal)	Shanks and Doss(1987)
SOLANINE	potato leaves	*L. decemlineata* (Say)	Kogan(1976)

SPARTEINE	*Sarothamus scoparius*	*Acyrthosiphon spartii*	Smith(1966)
STIGMASTEROL		*Sciopithes obscurus* Horn	Doss(1983)
SUCCINIC ACID	cotton	*A. grandis* Boheman	Hedin *et al.*,(1977)
SUCROSE		*Ostrinia nubilalis* (Hubner)	Beck(1956)
		Oulema melanopus (L.),	Hedin *et al.*,(1977)
		E. varisvestis Mulsant,	
		D. Koenigii (F.)	
		C. fumiferana (Clem)	Albert & Jerrett(1981)
		Hylemya antiqua (Meigen)	Mochizuki *et al.*,(1985)
		Scarabaeidae spp.	Ladd(1986)
TERPINEOL	cotton	*S. litoralis* (Boisd)	Hedin *et al.*,(1977)
THIAMINE		*C. curtipennis* (Harris)	Dethier(1966)
TRICIN-5-GLUCOSIDE	rice	*N. lugens* (Stal)	Sogawa(1982)

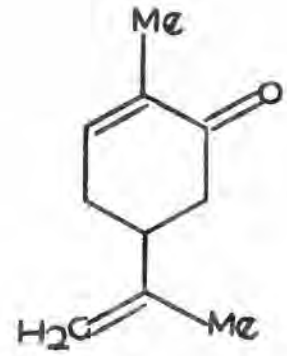
Structures of some selected feeding stimulants.



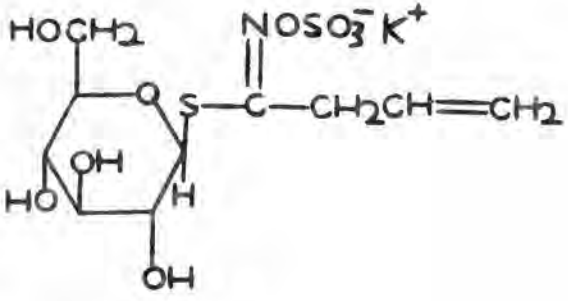
Phaseolunatin



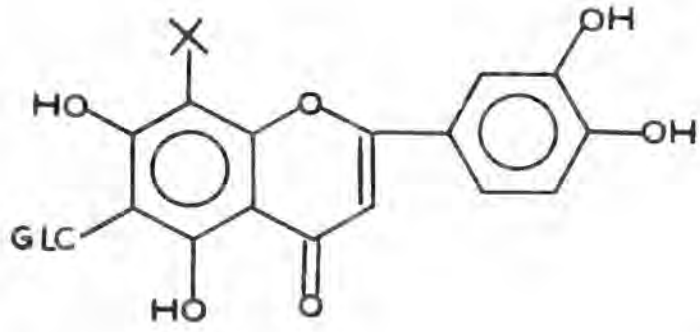
Sparteine



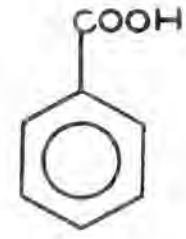
(-)-(R)-carvone



Sinigrin



Carlinoside
X = α-L-arabinopyranosyl



Benzoic acid

1.4 Insect Feeding Deterrents

Plant chemicals which possess feeding deterrent activities against insects are known as antifeedants (Dethier *et al.*, 1960). They prevent or decrease the feeding of the insect without killing it, with the insect remaining near the treated plant and dying from starvation (Dethier, 1960; Munakata, 1977). It has been suggested that an antifeedant is concerned with deterring the initiation of feeding therefore may act as a resistant factor protecting plants against insect attack (Munakata, 1977). However, it is plausible that in some cases it is the insect that has evolved the means of detecting and avoiding a potentially deleterious compound. Many deterrents are also toxic to insects.

Table 1.2 shows that plant deterrent chemicals are derived from virtually all classes of natural products, but studies to date suggest that the most potent insect antifeedants belong to the terpenoid and alkaloid classes (Schoonhoven, 1982). Some of the more prominent examples from these two classes will be discussed in this section.

Among the terpenes, the tetranortriterpenoid azadirachtin isolated from the neem tree *Azadirachta indica* Meliaceae (Butterworth *et al.*, 1972) is an antifeedant for a host of different lepidopteran, coleopteran and orthopteran

insects. Other potent related tetranortri-terpenoid compounds from *A. indica* include salanin (Warthen, 1979), 3-desacetylsalanin, nimbandiol and meliantriol (Warthen, 1983). Obacunone, harrisonin, pedonin from *Harrisonia abyssinica* and related tetranortriterpenoid compounds, have shown antifeedant activities against the African armyworm *Spodoptera exempta*, the cowpea podborer *Maruca testulalis* (Geyer) and the sugarcane stalk borer *Eldana saccharina* (Hassanali *et al.*, 1986; 1987).

Several diterpenoid compounds function as phago-deterrents for a number of insects. Among the well known ones are the clerodane diterpenes from *Ajuga* and *Verbenaceae* plants (Munakata, 1977; Belles *et al.*, 1985). The potent ones in this class include clerodendrin-A and B, clerodin, caryoptin and some derivatives of caryoptin. Other reported antifeedant diterpenes are the grayanoid diterpenes grayanotoxin III and kalmitoxin I and II which inhibit feeding of the polyphagous gypsy moth *Lymantria dispar*, inflexin and isodomedin from *Isodon* species which are effective antifeedants for *S. exempta* (Norris, 1986). Hydroxygrindelic acid, also a diterpene is, an antifeedant for the greenbug aphid *Schizaphis graminum* (Rondani) (Rose *et al.*, 1981).

Antifeedant sesquiterpenoids have been reported for a variety of insects. Warbuganal, muzigadial are both effective antifeedants and cytotoxins to *S. exempta* (Norris, 1986). Polygodial and ugandensidial which are both related sesquiterpenoids of warbuganal were found to be less active than the latter when presented to *S. exempta* (Kubo *et al.*, 1976). Studies on the feeding of some species of *Neodipron* sawflies on juvenile and mature needles of the pine tree *Pinus banksiana*, have revealed that larvae do not feed on the juvenile ones because they contain relatively high concentrations of the sesquiterpene antifeedant, 13-keto-8(14)-podocarpen-18-oic acid (Norris, 1986). Other potent sesquiterpenes include the germacranes shiromodiol di- and mono- acetates isolated from the leaves of *Parabenzoin trilobium* (Munakata, 1977), and glaucolide-A (Norris, 1986), all of which deter the feeding of a number of lepidopteran species.

Monoterpenes with insect antifeedant properties have been tested in most cases against the migratory locust. Examples of this include 1,4-cineole, carvone, *p*-cymene, citral, geraniol and some derivatives of these compounds (Warthen, 1983).

Among the alkaloids, the steroidal alkaloids in the *Solanaceae* have proved to be the most effective antifeedants against *L. decemlineata*, the two-striped grasshopper *Melanoplus bivittatus* (Say) and *C. fumiferana* (Harley and Thorsteinson, 1967; Chapman, 1974; Bentley *et al.*, 1984a). The potent ones include tomatine, demissine, tomatidine, α-solanine, α-chaconine and solanidine. *C. fumiferana* is also inhibited to feed by pyrrolizidine and lupine alkaloids (Bentley *et al.*, 1984b and 1984c), and for *Dysdercus* species drinking is inhibited by strychnine, caffeine and nicotine (Schoonhoven and Derksen-Koppers, 1973). Broom and lupine plants have been shown to be resistant to the aphids, *Aphis cytisoris* (Hartig) and *Acyrthosiphon pisum* (Harris) respectively due to the alkaloids sparteine in broom and lupanine in lupine (Wink *et al.*, 1982; Wink and Witte, 1984). Verma *et al.*, (1986) isolated tylophorine, tylophorinine and pergularinine from *Tylophora asthmatica* and bioassays of these compounds showed that they deterred feeding of *S. litura*. Other potent insect antifeedant alkaloids include castanospermine and related compounds from *Castanospermum australe* (Dreyer *et al.*, 1985), cocculodine and isoboldine from *Cocculus trilobus* (DC) (Wada and Munakata, 1968) and several 9-acridone alkaloids isolated from *Teclea trichocarpa* (Hassanali *et al.*, 1983).

Apart from terpenoid and alkaloid classes of compounds some furanocoumarins, steroids and acetogenins have been found to deter insect feeding. Examples of the furanocoumarins include isopimpelline, xanthotoxin, imperatorin and xanthoxyletin all of which are derived from *Rutaceae* and *Umbelliferae* plants (Yajima *et al.*, 1979; Berenbaum, 1978; Gebreyesus and Chapya, 1983). Steroids are generally not well established potent antifeedants, but a few like the ergostane-type steroids from *Solanaceae*, *Physalis*, *Withania* and *Nicandria* species deter the feeding of *E. varivestis* (Ascher *et al.*, 1980). The acetogenous aglycone, 6-methoxybenzoxazolinone derived from the glycoside, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) in maize, is an antifeedant for the larvae of *O. nubilalis*, and tests with various analogues of this compound have shown similar effects (Beck, 1960).

Broad spectrum antifeedants are also known. Apart from azadirachtin, the mustard glycoside sinigrin, the carbohydrate phlorizin, the flavonoid glycoside rutin, several quinones and napthoquinones and some ellagitannins have been found to be broad spectrum insect antifeedants (Schoonhoven, 1972; Bernays and Chapman, 1977; Hedin *et al.*, 1977; Norris, 1986; Jones and Klocke, 1987).

Table 1.2 INSECT FEEDING DETERRENTS.

Feeding deterrent	Plant source	Insect name	Reference
ARBUTIN		*Locusta migratoria* (L.)	Adams and Bernays(1978)
ATROPINE		*M. bivittatus* (Say)	Hedin *et al.*,(1977)
		P. brassicae (L)	
AUCUBIN		*L. migratoria* (L.)	Bernays and Chapman(1977)
AZADIRACHTIN	*Azadirachta*	*Acalymma vittatum* (F.)	Kubo and Nakanishi(1977)
	indica	*D. u. howardii* Barber	Norris(1986)
	Melia azedarach	*Earias insulana* (Boisd)	
		Galleria mellonella (L.)	
		Heliothis virescens (F.)	
		Heliothis zea (Boddie)	
		Hypsipyla grandella (Zeller)	
		L. decemlineata (Say)	

		L. migratoria (L.)	
		P. xylostella (L.)	
		P. japonica Newman	
		S. gregaria Forsk	
		S. frugiperda (J. E. Smith)	
		S. littoralis (Boisd)	
4-BENZOQUINONE		*S. multistriatus* (Marsham)	Norris(1977)
		O. nubilalis (Hubner)	
BENZYL ALCOHOL	small grains	*S. graminum* (Rondani)	Hedin *et al.*,(1977)
BERBERINE		*B. mori* (L.), *P. brassicae* (L.)	Levinson(1976)
BETAINE		*Danaus plexippus* (L.)	
BRUCINE	*Srychnos spp.*	*B. mori* (L.), *D. plexippus* (L.)	Levinson(1976)
		P. brassicae (L.)	
CAFFEIC ACID	*Sorghum bicolor* (L.) Moench	*L. migratoria* (L.)	Adams and Bernays(1978)

CAFFEINE		*D. plexippus* (L.)	Schoonhoven *et al.*,(1973)
		M. sexta (L.)	
CARYOPHYLLENE		*L. m. migratoriodes*	
		(Reiche &. Fairmaire)	
CASTANOSPERMINE	*Castanospermum*	*Acyrthosiphon pisum* (Harris)	Dreyer *et al.*,(1985)
	australe		
CHLORDIMEFORM		*Calopilos miranda* (Butler)	Hirata and Sogawa(1976)
		Hyadaphis erysimi (Kaltenback)	
		Laodelphax striatellus (Fallen)	
		Myzus persicae (Sulzer)	
		Nephotettix cincticeps (Uhler)	
		N. lugens (Stal)	
		Pryeria sinica (F.)	
		S. litura (F.)	
CHLOROGENIC ACID	Salicaceous spp	*Lochmaeae capreae cribata*	Matsuda and Senbo(1986)
CHOLESTEROL ACETATE	corn	*Diatraea grandiosella* (Dyar)	

CLERODEDRIN	*Clerodendrum*	*C. miranda* (Butler)	Munakata(1977)
	crytophyllum	*Euprotis subflava* (Bremer)	
	Clerodendrum	*O. nubilalis* (Hubner)	
	trichotomum	*S. litura* (F.)	
COCCULOLIDINE	*Cocculus*	*Calospilos miranda* (Butler)	Wada and Munakata(1968)
	trilobus	*Spodoptera littoralis* (Boisd.)	
o-COUMARIC ACID	*Sorghum bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
		S. multistriatus (Marsham)	
p-COUMARIC ACID	*Sorghum bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
		S. multistriatus (Marsham)	
(*z*)-o-COUMARIC ACID	*Melilotus*	*Epicauta fabricii* (Leconte),	Hedin *et al.*,(1977)
GLUCOSIDE	*infesta*	*Epicauta pestifera* Werner,	
		Epicauta vittata(F.)	
4-HYDROXYCOUMARIN		*A. pisum* (Harris)	Dreyer *et al.*,(1987)
CUCURBITACINS	*Iberis*	*Phyllotreta nemorum* (L.)	Norris(1986)
	amara	*Apis melliferea* L.	

		Phaedon cochleariae F.	
		Phyllotreta undulata Kutschelina	
		Epilachna tredecimnotata (Latreille)	
p-CYMENE	*Amorpha frcticosa*	*L. decemlineata* (Say)	
		L. m. migratoroides (Reiche & Fairmaire)	
		P. brassicae (L.)	
DEMISSINE	*Solanum demissum*	*L. decemlineata* (Say)	Hedin *et al.*,(1977)
DHURRIN	*Sorghum bicolor*	*L. migratoria* (L.)	Woodhead *et al.*,(1977)
DIGITONIN	*Digitalis purpurea*	*M. bivittatus* (Say)	Hedin *et al.*,(1977)

2,4-DIHYDROXY-7-METHOXY-2H-1,4-BENZOXAZIN-3(4H)-ONE (DIMBOA)	corn	*O. nubilalis* (Hubner)	Chapman (1974)
ECDYSTERONE		*P. brassicae* (L.)	Chapman (1974)
FERULIC ACID	*Sorghum bicolor*	*L. migratoria* (L.)	Adams and Bernays (1978)
FRIDELIN	*Acokanthera oblongifolia*	S. *littoralis* (Boisd.)	
GENTISIC ACID	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays (1978)
GLAUCOLIDE-A	*Vernonia gigantea*	*Diacrisia virginica* (F.)	Norris (1986)
		Sibine stimulea (Clemens)	
	Vernonia glauca	*S.* eridania (Cramer)	
		S. frugiperda (J. E. Smith),	
		S. ornithogalli (Guenee)	
		Trichoplusia ni (Hubner)	

GOSSYPOL	cotton	*Boarmia* (Ascotis) *selenaria* (Dennis & Schiffermuller)	Norris(1986)
		L. m. migratorioides (Reiche & Fairmaire)	Bernays and Chapman(1977)
		Heliothis spp.	
		S. littoralis (Boisd)	
HARRISONIN	*Harrisonia abyssinica*	*S. exempta* (Walker)	Hassanali *et al.*,(1986)
HILDECARPIN	*Tephrosia hildebrandtii*	*Maruca testulalis* (Geyer)	Lwande *et al.*,(1986)
p-HYDROXYBENZALDEHYDE	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
m-HYDROXYBENZOIC ACID	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
P-HYDROXYBENZOIC ACID	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
IMPERATONIN	*Clausena anisata*	*S. exempta* (Walker)	Gebreysus *et al.*,(1983)
INFLEXIN	*Isodon inflexus*	*S. exempta* (Walker)	Kubo and Nakanishi(1977)

ISOBOLDINE	*C. trilobus*	*S. litura* (F.)	
ISOFERULIC ACID	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
ISOMEDIN	*Isodon shikokianus*	*S. exempta* (Walker)	Kubo and Nakanishi(1977)
ISOPIMPINELLIN	*Orixa japonica*	*Blattella germanica* (L.)	Yajima and Munakata(1979)
		Neostylopyga rhombifolia (Stoll)	
		Periplaneta americana (L.),	
		S. litura (F.)	
JUGLONE	*Carya ovata*	*P. americana* (L.)	Norris(1977)
		S. multistriatus (Marsham)	Norris(1977)
KAEMPFEROL	*Robinia pseudoacacia*	*S. multistriatus* (Marsham)	Norris(1977)
LIMONENE	*Pinus silvestris*	*Dendrolimus pini* (L.)	
		L. m. migratorioides (Reiche & Fairmaire)	Bernays and Chapman(1977)

LINAMARIN		*L. m. migratorioides* (Reiche & Fairmaire)	Bernays and Chapman(1977)
MELIANTRIOL	*A. indica*	*S. gregaria* Forsk	Warthen(1983)
6-METHOXY-2-BENZOX-AZOLINONE	*Zea mays*	*Heliothis zea* (Boddie)	Hedin *et al.*,(1977)
		O. nubilalis (Hubner)	
2-METHYL-1,4-NAPHTHO-QUINONE		*Acalymma vittatum* (F.)	
		P. americana (L.)	Norris(1977)
		S. multistriatus (Marsham)	Norris(1977)
MONOCROTALINE	*Senecio spp.*	*A. pisum* (Harris)	Dreyer *et al.*,(1985)
MORPHINE		*P. brassicae* (L.)	Hedin *et al.*,(1977)
MUZIGADIAL	*Warbugia stuhlmanii*	*S. exempta* (Walker)	Warthen(1983)
1,4-NAPHTHOQUINONE		*A. vittatum* (F.)	Chapman(1974)
		Neodipron rugifrons Middleton	
		Neodipron swainei Middleton	
		P. americana (L.)	Norris(1977)
		S. multistriatus (Marsham)	

NICOTINE	*Nicotiana*	*Dysdercus* spp.	Chapman(1974)
	tabacum	*B. mori*	Levinson(1976)
		P. brassicae (L.)	
NIMBANDIOL	*A. indica*	*E. varivestis* Mulsant	
OBACUNONE	*Harrisonia*	*M. testulalis* (Geyer)	Hassanali *et al.*,(1986)
	abyssinica	*Eldana saccharina*	
PEDONIN	*H. abyssinica*	*M. testulalis* (Geyer)	Hassanali *et al.*,(1987)
		E. saccharina	
PHASEOLUNATIN		Coleopteran sp.	Warthen(1983)
PHLORIZIN	*Malus sp*	*Amphophora agathonica* Hottes	Chapman(1974)
		Aphis pomi De Geer	
		L. m. migratorioides	
		(Reiche & Fairmaire)	
		M. persicae (Sulzer)	

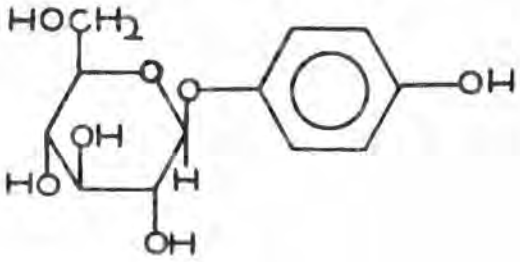
PHYTOL	*Clerodendron fragrans*	*S. litura* (F.)	
PLUMBAGIN	*Plumbago auriculata*	*S. exempta* (Walker) *S. littoralis* (Boisd.)	
POLYGODIAL	*Warburgia stuhlmannii*	*S. exempta* (Walker) *S. littoralis* (Boisd.)	Kubo *et al.*,(1976)
PROTOCATECHUIC ACID	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
QUININE	*Cinchona officinalis*	*B. mori* (L.) *L. m. migratorioides* (Reiche & Fairmaire) *P. brassicae* (L.)	
RIDELLINE	*Senecio*	*A. pisum* (Harris)	Dreyer *et al.*,(1985)
SALANNIN	*A. indica*	*A. vittatum* (F.) *Aonidiella aurantii* (Maskell) *D. u. howardii* Barber *Earias insulana* (Boisd.)	

		Locusta spp.	
		M. domestica L.	
SALICIN		*B. mori* (L.)	
		L. m. migratorioides	Bernays and Chapman(1977)
		(Reiche & Fairmaire)	
		M. sexta (L.)	
SHIROMODIOL DIACETATE	*Parabenzoin*	*Calospilos miranda* (Butler)	Norris(1986)
	trilobum	*S. litura* (F.)	
SINIGRIN	*Cruciferae*	*L. migratoria* (L.)	Adams and Bernays(1977)
		Aphis fabae Scop	Chapman(1974)
		A. pisum (Harris)	
		P. p. asterius Stoll	Hedin *et al.*,(1977)
SOLANINE	*Solanum*	*P. brassicae* (L.)	Levinson(1976)
	punae	*C. fumiferana* Clem.	Bentley *et al.*,(1984)
SPARTEINE			

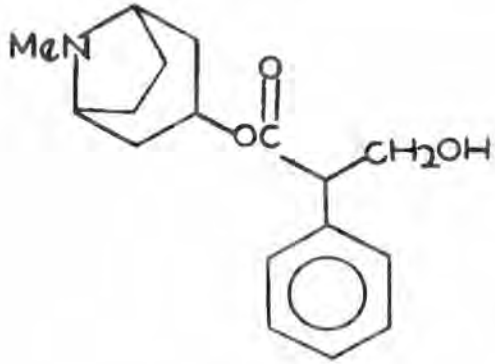
SWAINSONINE	*Astragalus lentiginosus*	*A. pisum* (Harris)	Dreyer *et al.*,(1985)
STRYCHNINE	*Strychnos spp.*	*Dysdercus* spp	Chapman(1974)
		P. brassicae (L.)	
SYRINGIC ACID	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
α-TERPINEOL	*Amorpha fructicosa*	*Dendrolimus pini* (L.)	
		L. decemlineata (Say)	
		L. m. migratorioides (Reiche & Fairmaire)	
		P. brassicae (L.)	
TOMATINE	*Nepeta cataria*	*P. brassicae* (L.)	Chapman(1974)
		L. decemlineata (Say)	Levinson(1976)
		C. fumiferana Clem.	Bentley *et al.*,(1984a)
		M. sexta (L.)	
		M. bivittatus (Say)	
TYLOPHORINE		S.litura (F.)	Verma *et al.*,(1986)

UGANDENSIDIAL	*W. stuhlmanii*	*S. exempta* (Walker)	Kubo and Nakanishi(1979)
		S. littoralis (Boisd.)	
VANILLIC ACID	*S. bicolor*	*L. migratoria* (L.)	Adams and Bernays(1978)
WARBURGANAL	*W. stuhlmanii*	*S. exempta* (Walker)	Kubo and Nakanishi(1977)
		S. littoralis (Boisd.)	
XANTHOTOXIN	*Orixa japonica*	*Blattella germanica* (L.)	Yajima and Munakata(1979)
		Neostylopyga rhombifolia (Stoll)	
		P. americana (L.)	
		S. litura (F.)	
XANTHOXYLETIN		*S. exempta* (Walker)	Gebreysus *et al.*,(1983)
XYLOMOLLIN	*Xylocarpus*	*S. exempta* (Walker)	Kubo *et al.*,(1976)
	molluccensis		
ZANTHOPHYLLINE	*Zanthoxylum*	*Hemileuca oliviae* Cockerell	Warthen(1983)
	monophyllum	*Hypera postica* (Gyllenhal)	
		Melanoplus sanguinipes (F.)	

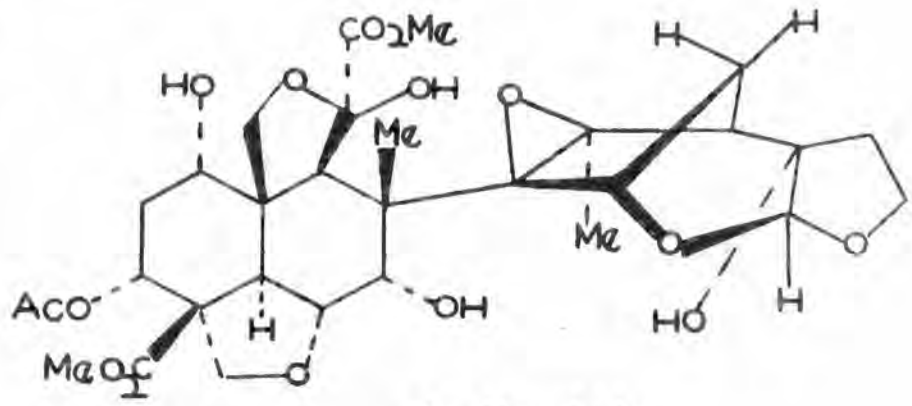
Structures of some selected feeding deterrents.



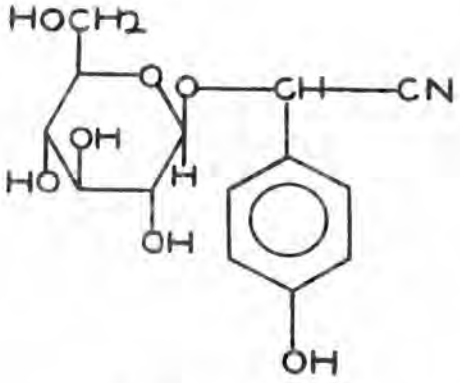
Arbutin



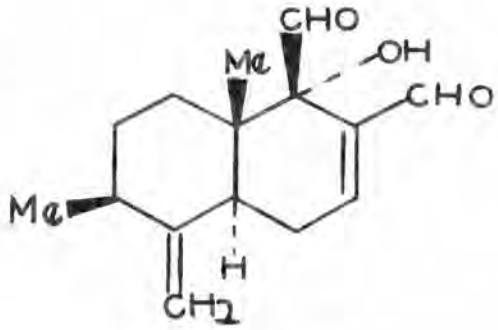
Atropine



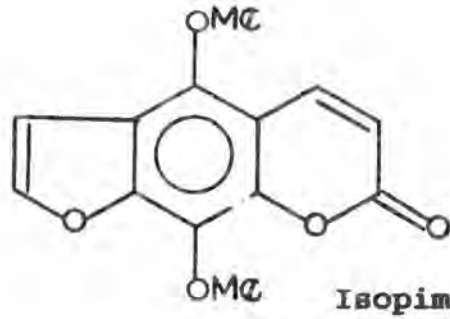
Azadirachtin



Dhurrin



Muzigadial



Isopimpinellin

1.5 Compounds which act both as stimulants and deterrents

A comparison of the list of chemicals in Tables 1.1 and 1.2 shows that a number of compounds function as anti-feedants for certain insect species and as phagostimulants for others. Gossypol, a dimeric sesquiterpenoid found in cottonseed pigment glands, is stimulatory to the bollweevil *Anthomonus grandis* but deters feeding of *Heliothis* species, *L. migratoria* and *S. littoralis* (Hedin *et al.*, 1977). Sinigrin which is a broad spectrum phagostimulant for a number of lepidopteran and coleopteran insects (Whittaker and Feeny, 1971) is an antifeedant for *L. migratoria*, *Myzus persicae* and *Papilio polyxenes* (Chapman, 1974). The isoquilonine alkaloid, isoboldine inhibits the feeding of *Calospilos miranda*, *S. litura* and *Prodenia litura* (Levinson, 1976) but enhances food intake of *Calpe excavata* (Wada and Munakata, 1968). Salicin, a phenolic glycoside, is stimulatory to some salicaceous feeding beetles (Matsuda and Matsuo, 1985) but shows antifeedant activity against *B. mori*, *L. migratoria* and *M. sexta* (Chapman, 1974). p-Hydroxybenzaldehyde, a lignin degradative product is a feeding stimulant for *S. multistriatus* (Baker and Norris, 1968) but deterrent to *L. migratoria* (Woodhead, 1982). Chlorogenic acid is a feeding stimulant for the Colorado potato beetle *L. decemlineata* (Hsiao and Fraenkel, 1968) but deterrent to the salicaceous feeding beetle *Lochmaea capreae cribata* (Matsuda and Sembo, 1986). Coleopteran insects

feeding on *Cucurbitaceae* species do not all accept cucurbitacin in their food (Kogan, 1976).

1.6 Bioassay Methods

Phagostimulation or phagodeterrency of chemicals for phytophagous insects have been tested by several methods. Studies have shown that the ideal bioassay is different for each species of insect (Cook, 1976). However, in all testing procedures the insect may be exposed to the test substances only (no choice situation) or may be offered a choice between food with and without the test substance (choice situation).

The main procedure for testing for phagostimulation involves the presentation of the test material on an inert substrate. Where discs have been used, the test substance is impregnated by soaking the discs in a solution of the substance or by pipetting a known volume of the test solution unto individual discs (Cook, 1976). Where agar/cellulose has been used as the substrate, the test substance is added to the agar/cellulose before cooling (Hsiao and Fraenkel, 1968). The following inert substrates have been used in the form of discs: ordinary filter paper (Daad, 1960; Wensler and Dadzinski, 1972), elder pith (Heron, 1965; Norris and Baker, 1967), styropor (Meisner and Ascher, 1968), glass-fibre filter paper (Woodhead and

Bernays, 1978) and membrane filters (Doss and Shanks, 1986). In some assays, tests substances have been applied directly in solution to the mouthparts of the insect (Harley and Thorsteinson, 1967) and for drinking insects, the test substance has been applied in a polythene film (Kim *et al.*, 1985).

Procedures for testing for antifeedants also vary. They include adding it to an artificial substrate such as agar cellulose which has been made palatable usually with sucrose (Schoonhoven, 1972), or mixing it with dried food e.g. wheat flour wafers (Bernays and Chapman, 1977), spraying of the test chemical on the natural food which could be a leaf disc (Kubo and Nakanishi, 1979), or cellulosic filter paper or glass fibre discs (Schoonhoven, 1982). Parafilm sachets have been used for sucking insects (Schoonhoven and Derksen-Koppers, 1976; Rose *et al.*, 1981) and insects drinking from free water sources have been tested by applying antifeedants in their drinking water (Schoonhoven and Derksen-Koppers, 1973).

CHAPTER 2

PREVIOUS FEEDING ALLELOCHEMICAL WORK ON SORGHUM AND OBJECTIVES OF THE PRESENT STUDY.

Both physical and chemical characteristics of the sorghum plant have been shown to affect feeding and oviposition behaviour of some of its insect pests (Blum, 1968; Chadha and Roome, 1980; Maiti *et al.*, 1980; Jotwani, 1981; Reddy *et al.*, 1981; Bernays *et al.*, 1983; 1985). Insects affected by physical features of the sorghum plant include *Chilo partellus* and the sorghum shootfly *Atherigona soccata* (Rondani). These physical characteristics include trichomes, dryness, turgidity, morphology, anatomical complexity and colour of the leaves. In addition to these, wax on the surface of the leaves and the extent of lignification of cells round the vascular bundles and silica bodies in the epidermis of the leaves have also been found to affect the behaviour of these insects. Chemical constituents of the plant have also been shown to be crucial in host selection before feeding and oviposition by some of the pest insects of sorghum. Insects on which allelochemical studies have been conducted include the migratory locust *Locusta migratoria*, the greenbug aphid *Schizaphis graminum* and the spotted stem borer *Chilo partellus*. The insect behaviour which has been the main

concern of most researchers in sorghum allelochemical studies is feeding. This chapter gives an overview of these studies and summarises the objectives of the present study.

2.1 *Locusta migratoria* L. (Acrididae : Orthoptera)

Nymphs of the migratory locust feed on a wide variety of grasses, but it has been reported that seedlings of sorghum are more distasteful to the insect than most grasses (Woodhead and Bernays, 1978). This distastefulness has been attributed to the presence of a variety of deterrent chemicals which override the effects of stimulatory compounds in the plant (Bernays and Chapman, 1978; Woodhead and Bernays, 1978). These workers identified sugars, amino acids and phospholipids as feeding stimulants for the nymphs, and different surface wax and tissue chemicals of sorghum leaves as playing either deterrent or stimulatory roles.

The surface wax components include n-alkanes, esters free fatty acids, alcohols and aldehydes (Atkin and Hamilton, 1982; Woodhead, 1982 and 1983). In some cultivars, *p*-hydroxybenzaldehyde is a component in the surface wax (Woodhead, 1982; Haskins and Gorz, 1985) and can be present at levels deterrent to the migratory locust. Woodhead (1983) found that n-alkanes with 19, 21 and 23 carbon atoms were deterrent, while other n-alkanes between

C-18 and C-28 had no effect on feeding. Atkin and Hamilton (1982) reported that the major alkanes in the surface waxes of some other sorghum cultivars were C-27, C-29 and C-31. These did not deter feeding, unlike the shorter chain alkanes identified by Woodhead. Woodhead (1983) found that a C-32 alkane on the other hand was stimulatory. None of the free fatty acids, alcohols, or aldehydes present in the wax had any effect on larval feeding. However, an ester containing a C-12 fatty acid was deterrent, while that with C-22 acid was not. It was concluded from these bioassays that differences in surface waxes may contribute to cultivar differences in resistance to attack by grasshoppers.

Deterrent compounds which were identified in the tissues of the leaves were mainly phenolic acids, and these include *p*-hydroxybenzoic, vanillic, o-coumaric, *p*-coumaric, gentisic, ferulic and caffeic acids. These phenolic acids occur in the leaves of the plant mainly as their esterified/glycosidic forms, but following insect attack they are released in the free form by enzymes present in the plant (Woodhead and Cooper-Driver, 1979). Adams and Bernays (1978) have shown that the individual phenolic acids at the concentrations occurring in the plant have no effect on the feeding of *L. migratoria*, but in combination they are deterrent.

Another tissue component is HCN (Woodhead and Bernays, 1978; Woodhead, 1983), and it is also similarly released into the tissues of the leaves from its cyanogenic glucoside, dhurrin (Akazawa *et al.*, 1960). Changes in HCN and phenolic content with age of the sorghum plant and the effects of the changes on the feeding of the migratory locust has been studied (Woodhead, 1980). HCN and phenolic content of sorghum were found to decrease steadily as plants got older but this change varied with the cultivar (Woodhead, 1980). It was established that cultivars with high phenolic content suffered little damage from the insect. Woodhead (1981) also compared the phenolic contents of field and laboratory grown plants and found that the phenolic content was higher in the field than the laboratory grown plants. Woodhead attributed this difference to factors such as light intensity and attack by insects and pathogens on the field grown plants.

2.2 *Schizaphis graminum* (Rondani) (Aphididae : Homoptera)

p-Hydroxybenzaldehyde, dhurrin and procyanidin were identified as the major greenbug feeding deterrent isolates from sorghum seedlings (Dreyer *et al.*, 1981). Dreyer and coworkers carried out tests for synergism between two component mixtures of these compounds and found no effect for combinations of dhurrin, luteolin-7-glucoside, p-hydroxybenzaldehyde and p-hydroxybenzoic acid.

2.3 *Chilo partellus* (Swinhoe) (Pyralidae : Lepidoptera)

At the ICIPE, Mbita field station in Western Kenya and the ICRISAT, in India, studies have been conducted both in the field and the laboratory on the feeding behaviour of *C. partellus* larvae on sorghum (Roome and Padgham, 1978; Dabrowski and Kidiavai, 1983; Alghali, 1985; Saxena, 1985b). It was established that young larvae (first and second instars) fed in the whorl and older larvae (fourth and fifth instars) in the stem of the plant. The intermediate larval stage (third instar) fed in the whorl and gradually moved into the stem of the plant. In addition, feeding varied with the cultivar and the age of the plant with larvae preferentially feeding on the young plant rather than the older ones (Alghali, 1985).

Roome and Padgham (1978) performed bioassay experiments which revealed that the feeding responses of *Chilo partellus* larvae to surface wax extracts, steam distillates and homogenates of the whorl leaves of sorghum varied with the age of the plant. Larvae preferred artificial diets containing leaf extracts of young sorghum to leaf extracts of the older plants. However, no detailed chemical investigations were performed by these workers to elucidate the basis of these observations. In another experiment, Roome and Padgham (1978) showed that *C. partellus* larvae fed on artificial diets containing phenolic acids up to three

times the concentration normally occurring in sorghum. A combined mixture of the phenolic acids which had previously been reported to deter feeding by *Locusta* (Adams and Bernays, 1978) did not similarly affect the feeding of *C. partellus* larvae.

The production of HCN in the whorls of sorghum has been shown to be inversely correlated with damage caused by the first-instar larvae (Woodhead et al., 1981). However damage by larvae did not correlate with phenolic acid production in the whorl.

OBJECTIVES OF THE PRESENT STUDY

The review of the feeding allelochemical work on the spotted stem borer *C. partellus* shows that no detailed and methodical study has been carried out to identify the chemicals from sorghum which affect the feeding of the larvae of this insect. Secondly, the literature available on this subject suggests that no work has been undertaken to demonstrate whether or not there are any allelochemical bases for feeding preferences shown by larvae to different sorghum cultivars.

To fill this gap, this study was undertaken with the following objectives:-

(i) to develop a bioassay to monitor the search for chemicals in sorghum that affect the feeding of third-instar *C. partellus* larvae;
(ii) to isolate and characterise the active chemicals which affect *C. partellus* larval feeding;
(iii) to undertake detailed dose-response studies of these chemicals and a few selected analogues;
(iv) to develop chromatographic methods to quantify the amounts of the active chemicals in various sorghum cultivars and at different plant growth stages.

Details of the study are described in the chapters that follow.

CHAPTER 3

PRELIMINARY INVESTIGATIONS

The work described in this study was conducted in two stages:

1. preliminary investigation of the whorls of greenhouse grown plants of sorghum cultivars IS 18363 (susceptible) and IS 2205 (resistant) (Saxena, 1985b) with the purpose of (a) developing a suitable feeding bioassay, (b) comparing the extractive efficiencies of different solvents and (c) comparing the chromatographic profiles and relative stimulatory activities of different extracts of the two cultivars 3 and 6 weeks after plant emergence;

2. detailed investigation on the extractives of the whorls of field grown plants of these two cultivars with the purpose of identifying the active allelochemics.

Only the details of the preliminary investigation will be described in this chapter. Details of the second part of the study are described in the next chapter.

3.1 MATERIALS AND METHODS

3.1.1 Plant material

Sorghum cultivars IS 18363 (susceptible) and IS 2205 (resistant) were grown in plastic pots in a greenhouse at 28 ± 2 °C at a day length of 9 h at the ICIPE, Chiromo Campus in November, 1985.

3.1.2 Extraction

After 3 and 6 weeks of growth, about 250-500 leaf whorls of each cultivar were cut off and extracted successively with hexane, ethyl acetate and methanol by immersion for 30 min in each case. All the extracts were concentrated to dryness in vacuo at 40 °C and the residues weighed. The methanol extracts were lyophilized after removal of the organic solvent by rotary evaporation.

3.1.3 Development of a feeding bioassay

The following factors were considered in the development of a feeding bioassay: size and type of petri-dish, larval instar, feeding substrate, moisture retention within the dish and the number of larvae in each feeding run. The factors were evaluated purely on the basis of qualitative observations.

Petri-dish: Two types and sizes of petri-dishes , plastic and glass pyrex, 60 x 70 mm and 90 x 100 mm, were used to determine which would be easier to handle and which would allow greater contact between two 12 mm discs used as feeding substrates and the test larvae. The 60 x 70 mm petri-dish was found to allow greater contact between the larvae and the discs. The glass pyrex dish was more suitable for the study because it was easy to wash and dry.

Larval instar: Since the first three stages feed on the leaf whorl, feeding tests were conducted using first, second and third instar larvae to determine the larval stage most convenient for use within a short assay period. A high mortality was observed for the first instars, but although this factor was low for the second instars, they were found to be relatively slow feeders. Third instar larvae were found to feed better, and accordingly were used in subsequent bioassays.

Feeding substrate: Feeding tests on cellulose acetate, cellulose nitrate and cellulose discs loaded with whorl extracts of sorghum showed that cellulose was the least preferred of the three substrates for larval feeding. Cellulose acetate and cellulose nitrate were equally preferred, but in subsequent assays, cellulose acetate was used. Unlike cellulose nitrate, it is insoluble in

methanol, the medium used for the transfer of extracts, and it is presumed that on hydrolysis in the gut of the larvae, acetic acid would be produced which is a common metabolic product in the biochemical system of an insect.

Moisture retention: To prevent larvae from becoming dehydrated or drowning, tests were carried out to determine the best way of maintaining moisture in the petri-dish for an assay period of 24 h. Cellulose and cotton-wool were tested for moisture retention but cellulose was found to be more suitable. With cotton-wool, larvae got entangled within the strands. Filter-paper, 55 mm diameter soaked with 600 µl of distilled water was found to keep the petri-dish moist for more than 24 h. A fine wire-gauze (Fig. 3.1) was used to stop the larvae from contacting the moist paper which was found to immobilise the larvae.

Number of larvae: Three third-instar larvae were found to give measurable feeding rates on a 12 mm disc for a period of 24 h without any apparent overcrowding.

3.1.4 Feeding Tests

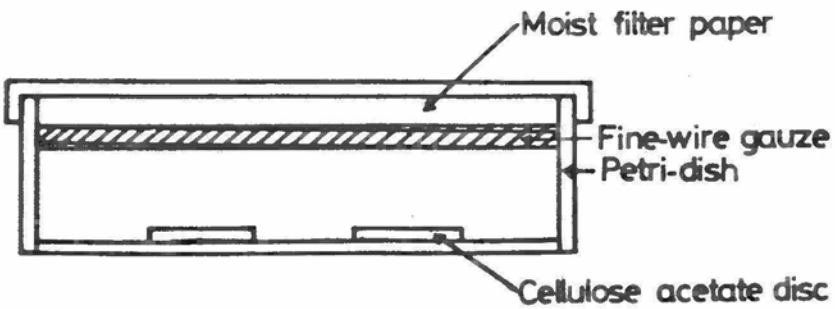
3.1.4a Choice situation

Three third instar larvae were given a choice between a treated disc and a control disc which were separated by a

distance of almost 5 mm (Fig. 3.1). Each treated disc was loaded with 500 μg of extract, 250 μg on each side of the disc. Control discs were treated with test solvent only. The control and treated discs were air-dried and weighed several times to a constant weight on a Cahn 21 milligram balance to ± 0.01 mg before and after the assay. All the tests were carried out in the dark in an environmentally controlled room (RH = 78 ± 2%, temperature = 29 ± 2 ^{o}C), and each feeding run was 24 h. Larvae used for the tests were starved for 24 h prior to the assay.

Feeding tests were carried out on the hexane, ethyl acetate and methanol extracts of the whorls of the two cultivars. Each test was replicated 9 times.

Feeding data were treated in two ways. First, the feeding response in each test was expressed as percentage preference calculated from the formula (1 - Xo/Xt) x 100 where Xt is the mean weight of treated disc consumed and Xo the mean weight of control disc consumed. A second parameter expressing total percentage of substrate consumed from the food available in the dish was derived from the formula 100 x (Xt + Xo)/(Xt_i + Xo_i), where Xt_i and Xo_i are the initial weights of treated and untreated discs respectively.



Apparatus for choice feeding bioassay.

Fig.3.1

3.1.4b No choice situation

The same apparatus in Fig. 3.1 was used for feeding tests in the no choice situation. However, in this experiment, only one test disc (12 mm diameter) loaded with the extract was offered in each feeding run to three third instar larvae. Control discs were similarly tested, and all the tests were maintained for 24 h as previously described in the choice situation. Each test was replicated 25 times.

A dose response test was carried out on all the crude extracts and the feeding response for each dose was expressed in logarithms as the Relative Feeding Response (RFR) and was calculated from the formula, RFR = Xt/Xo.

3.1.5 Chromatography

The main techniques used in the fractionation of extracts in this study were high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC), and the factors considered in the development of chromatographic conditions for the separation of the extracts were as follows : type of column, solvent system (HPLC), type of detector, flow rate and temperature depending on the type of chromatographic system used for separation of the extracts.

3.1.5a High performance liquid chromatography (HPLC)

Type of column: For analytical work, a DuPont C-18 reverse phase column, Zorbax ODS, 25 cm x 4.6 mm was found to be suitable for the separation of the ethyl acetate and methanol extracts, and for preparative separation of these extracts, a Varian MCH-10 C-18 reverse phase column 50 cm x 8 mm was used.

Solvent system: The separation of the ethyl acetate and methanol extracts was achieved by using a 20% aqueous methanol solvent system.

Flow rate: For analytical studies, a flow rate of 1.0 ml/min was found to give the best separation of the ethyl acetate and methanol extracts. A flow rate of 3.0 ml/min was used for preparative separation and this maintained the separation profiles of the extracts.

Type of detector: Two types of detectors were used, a UV and a RI detector. The UV detector was sensitive to the chromophoric components whilst the refractive index (RI) detector was sensitive to the non-chromophoric components. Components in the ethyl acetate and methanol extracts were easily detected at 240 and at 254 nm.

Temperature: Fractionation of the two extracts was performed at 25 ^{o}C.

3.1.5b Gas liquid chromatography

Type of column: A packed column 3 m x 2 mm i.d. of 5% silicone OV-17 on chromosorb WHP 80-100 mesh was used as the adsorbent for the separation of the hexane extracts and this was fitted into a Packard gas chromatograph, model 428.

Detector: A flame ionisation detector (FID) was used to detect the components in the hexane extracts.

Temperature: A temperature programme of 220-300 ^{o}C at 3 ^{o}C/min gave the best separation of the components in the hexane extracts.

Flow rate: The carrier gas was nitrogen at 20 ml/min.

3.2 RESULTS

3.2.1 Yields of extracts from sorghum whorls

Table 3.1 shows the yields of extracts for sorghum cultivars IS 18363 (susceptible) and IS 2205 (resistant) at two growth stages about 3 and 6 weeks after plant emergence.

Table 3.1 Yields of extracts from sorghum whorls(mg/g whorl)

Cultivar	plant age(wks)	wt (g) whorls	wt (mg) extract hexane	EtAc	MeOH	% total yield
IS 18363	3	135	123.1	341.7	279.9	1.1
IS 2205	3	79	45.8	332.0	236.4	1.8
IS 18363	6	550	357.5	817.7	2723.2	1.8
IS 2205	6	485	412.3	742.7	4816.4	4.9

For both cultivars, the yield of extract from the whorls of the 6 week old plants was higher than that from the 3 week old plants. At the two growth stages, the resistant cultivar IS 2205 yielded more extract than the susceptible cultivar IS 18363.

3.2.2 Feeding Assays

3.2.2a Choice situation

The feeding responses of third-instar larvae of *C. partellus* to crude extracts of the whorls of sorghum cultivars IS 18363 (susceptible) and IS 2205 (resistant) at the two growth stages are shown in Tables 3.2 and 3.3.

Table 3.2 Feeding responses of third instar larvae of *C. partellus* to cellulose acetate discs treated with 500 µg of leaf whorl extracts of sorghum plants (3 weeks old) in a choice bioassay.

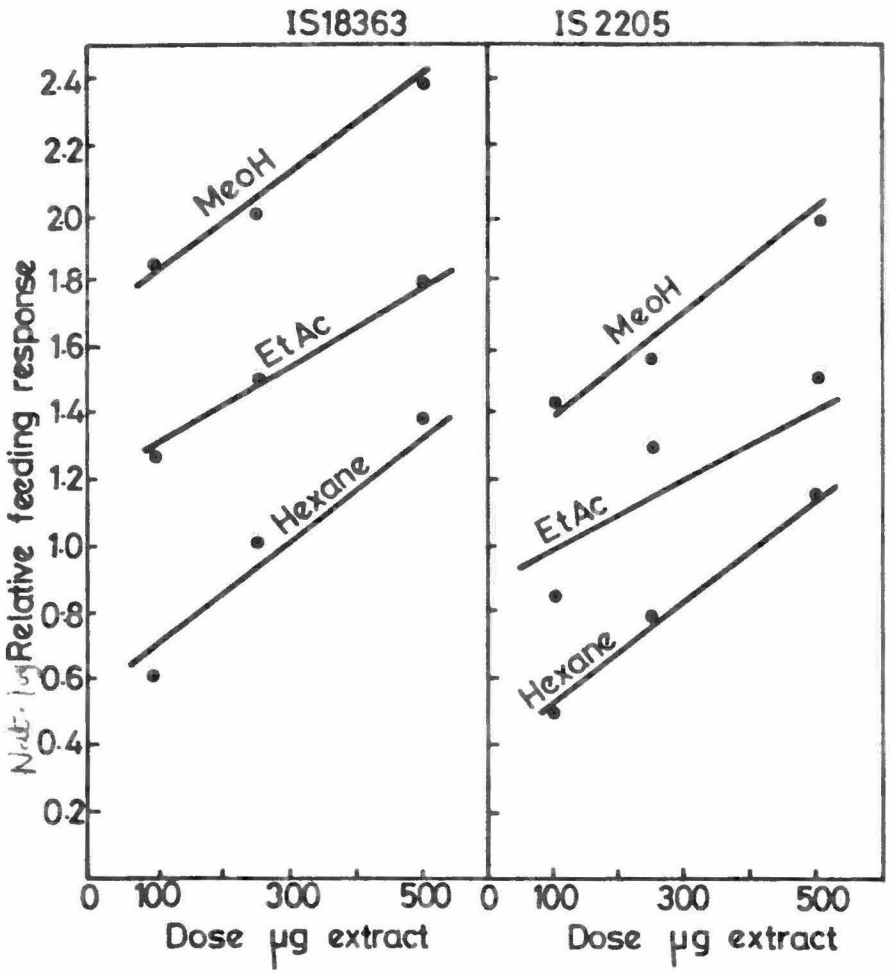
	IS 18363			IS 2205		
Feeding response	Hexane	EtAc	MeOH	Hexane	EtAc	MeOH
X_T ± S.D (mg)	0.41 ± 0.32	2.14 ± 1.63	3.00 ± 1.54	0.50 ± 0.19	0.78 ± 0.57	3.35 ± 1.55
X_0 ± S.D (mg)	0.12 ± 0.18	0.11 ± 0.08	0.03 ± 0.03	0.04 ± 0.03	0.04 ± 0.06	0.12 ± 0.25
Preference $X_T - X_0$	0.29	2.03	2.97	0.46	0.74	3.23
% Preference	72	95	99	92	96	96
Total feeding	0.47	2.06	2.74	0.49	0.74	3.13
*Relative feeding	1	4.4	5.8	1	1.5	6.4

* Feeding response relative to hexane

Table 3.3 Feeding responses of third instar larvae of *C. partellus* to cellulose acetate discs treated with 500 µg of leaf whorl extracts of sorghum plants (6 weeks old) in a choice bioassay.

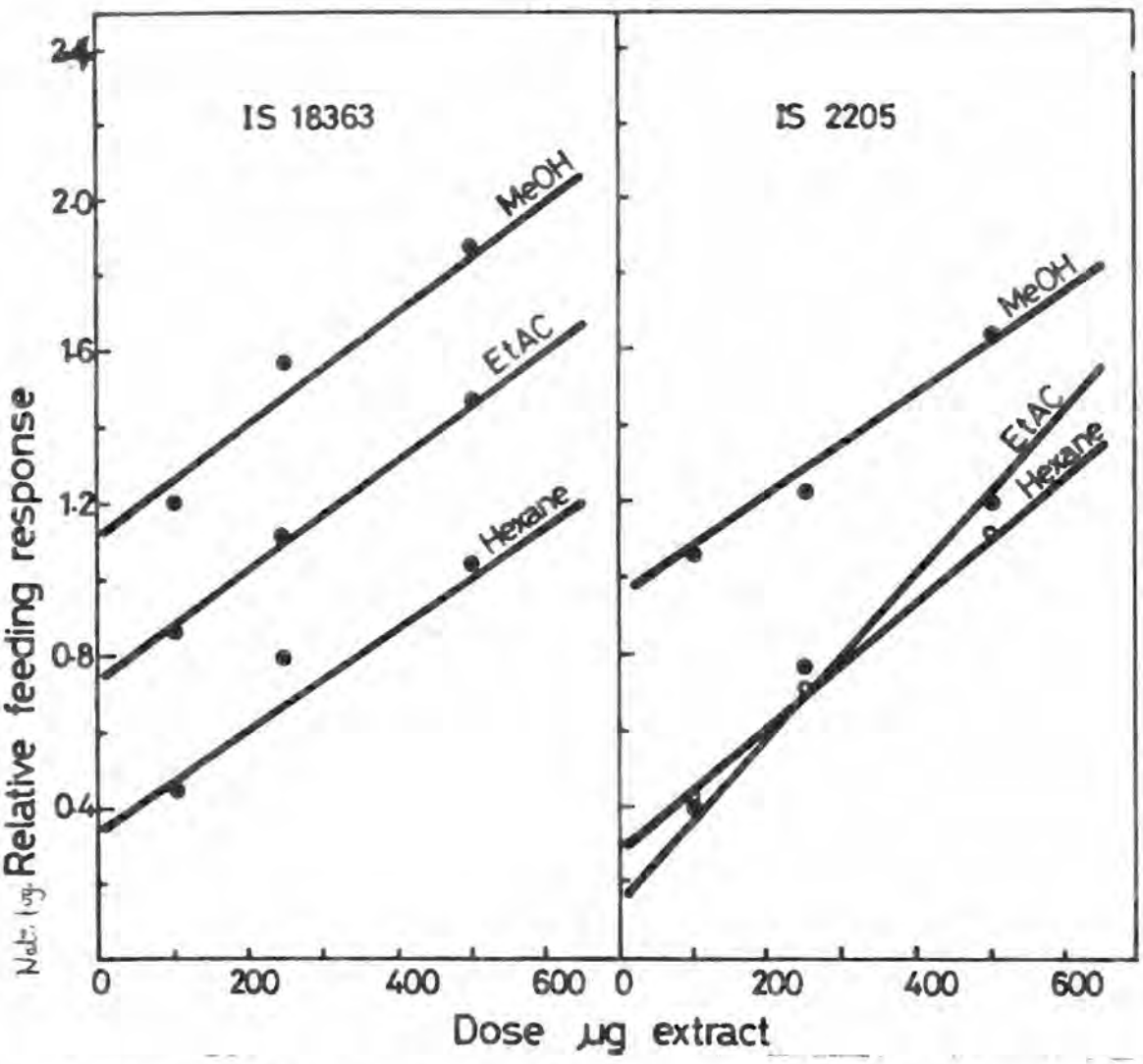
	IS 18363			IS 2205		
Feeding response	Hexane	EtAc	MeOH	Hexane	EtAc	MeOH
$X_T \pm$ S.D (mg)	0.80 ± 0.80	1.12 ± 0.31	4.16 ± 1.15	0.72 ± 0.59	1.70 ± 1.08	3.34 ± 1.57
$X_0 \pm$ S.D (mg)	0.10 ± 0.05	0.08 ± 0.13	0.01 ± 0.01	0.38 ± 0.47	0.22 ± 0.41	0.05 ± 0.12
Preference $X_T - X_0$	0.70	1.04	4.15	0.34	1.48	3.29
% Preference	87	92	99	48	87	98
Total feeding	0.77	1.08	3.76	0.99	1.72	3.18
*Relative feeding	1	1.4	4.9	1	1.74	3.2

* Feeding response relative to hexane



Dose response curves for the crude extracts of the whorls of the 3 week old plants of IS 18363 and IS 2205.

Fig. 3.2



Dose response curves for the crude extracts of the whorls of the 6 week old plants of IS 18363 and IS 2205.

Fig. 3.3

3.2.2b No Choice situation

Dose response curves for the extracts are shown in Figs. 3.2 and 3.3. The mean feeding on the control disc was 0.008 ± 0.004 mg

3.3 DISCUSSION

In the choice situation, treated discs were more preferred by larvae to untreated discs (Tables 3.2 and 3.3) indicating that all the extracts contained compounds that were stimulatory to the feeding of the larvae. Calculation of the degree of preference as defined on page 5, gave values 72, 95 and 99 for the hexane, ethyl acetate and methanol extracts respectively of the whorls of the 3 week old plants of IS 18363 and 92, 96 and 96 for those of IS 2205. Values obtained for similar extracts of the whorls of the 6 week old plants were 87, 92 and 99 for IS 18363 and 48, 87 and 98 for IS 2205. These values clearly fail to differentiate between the stimulatory efficacies of the three extracts and between the two different cultivars at the two growth stages. Comparison of the total feeding on discs treated with the different extracts clearly showed the difference. Indeed the methanol extracts of the 3-week-old whorls of the two cultivars were about 6-fold more stimulatory than the corresponding hexane extracts. The ethyl acetate extracts were about 1.5 to 4-fold more

stimulatory than the hexane extracts.

The data on the relative feeding response of larvae to treated discs in the no choice situation confirmed that all the crude extracts stimulated larval feeding (Figs. 3.2 and 3.3). The feeding response increased with increasing dose of extract, and also with the polarity of the extract. Thus of the three sets of extracts, hexane , ethyl acetate and methanol of the whorls of the two cultivars tested for feeding response, the methanol extracts were most stimulatory followed by those of ethyl acetate and hexane. It was also clear from the dose response curves that the feeding pattern was similar in the two cultivars. However, it appeared that larvae responded more to extracts of the susceptible cultivar IS 18363 than to extracts of the resistant cultivar IS 2205. The feeding pattern did not change when extracts of the whorls of the 6 week old plants were tested (Fig. 3.3). However, the results suggested that there were less stimulatory compounds in the 6 week old plants.

An additional advantage of this method over the choice situation is that it simulates more closely the feeding of the insect in the natural environment. An improvement of the no choice bioassay involved the use of a smaller container which allowed greater contact between larvae and the test disc. Details of this are described in Chapter 4.

Preliminary high performance liquid chromatographic analysis (HPLC) of the ethyl acetate and methanol extracts of the two cultivars suggested that differences between the two cultivars were quantitative rather than qualitative. A similar observation was made for the hexane extracts of the two cultivars which were analysed by gas chromatography. Details of each analysis are described in Chapter 4.

CHAPTER 4

EXPERIMENTAL DETAILS AND RESULTS

4.1 Plant materials

Whorls of sorghum cultivars IS 18363 (susceptible) and IS 2205 (resistant) were obtained from field grown plants at the Mbita Point Field Station, ICIPE in Western Kenya. Only undamaged (as a result of insect feeding or mutilation) whorls were used so as to exclude inducible defensive chemicals which are likely to be produced in the plants as a result of herbivore feeding (Haukioja, 1982).

4.2 Insects

Freshly moulted third-instar larvae of *C. partellus* were obtained for tests from stock cultures maintained on an artificial diet described by Ochieng *et al.*, (1985) at the ICIPE insectary.

4.3 Extraction

500 (3.5 kg) and 800 (3.4 kg) pieces of freshly cut whorls of 3 week old plants of sorghum cultivars IS 18363 and IS 2205 respectively were extracted successively in

batches, with hexane and ethyl acetate by immersion for 15 min in each case to extract surface components. After ethyl acetate extraction the plant materials were soaked in methanol for 24 h to extract tissue components. All the extracts were filtered to remove plant debris and then concentrated to dryness in vacuo. The methanol extracts were lyophilized after solvent removal by rotary evaporation. In another experiment, 80 pieces (3.5 kg and 2.9 kg) of the whorls of 6 week old plants of each of the cultivars IS 18363 and IS 2205 respectively, were similarly extracted and the resulting extracts concentrated to dryness.

In view of the different extraction procedures, yields of extracts (g/kg whorl) were calculated in two different ways. Percentage yield of surface extractives from only the hexane and ethyl acetate extracts and percentage total yield from the three extracts hexane, ethyl acetate and methanol of the whorls of the two cultivars at the two growth stages. The results from these calculations are shown in Table 4.1.

Table 4.1. Yields of extracts from sorghum whorls (g/kg whorl)

Cultivar	Plant age(wks)	wt (kg)	wt(g) of extract			% yield	
			Hexane	EtAc	MeOH	surface	Total
IS 18363	3	3.5	1.7	1.1	46.3	0.16	2.8
IS 2205	3	3.4	1.4	1.7	46.3	0.11	1.8
IS 18363	6	3.5	1.4	0.8	29.0	0.78	11.1
IS 2205	6	2.9	0.8	1.3	34.9	0.91	15.9

For both cultivars the yield of extract increased with increasing age of plant reflecting an increase in size of the whorls.

4.4 Chromatography

Thin layer chromatography (tlc) was performed on MN polygram precoated silica gel/UV_{254} 40 x 80 mm plates (0.25 mm thickness), and preparative tlc on similar plates of 20 x 20 cm. High performance liquid chromatography (HPLC) was performed on a Varian 5000 LC model, equipped with a DuPont Zorbax ODS C-18 reverse phase column, 25 cm x 4.6 mm and a UV detector at 254 nm. The column was eluted with 20% aqueous methanol at a pump pressure of 210 atm and at a flow rate of 1.0 ml/min. For preparative separation, a Varian MCH-10 C-18 reverse phase column, 50 cm x 8 mm and a UV

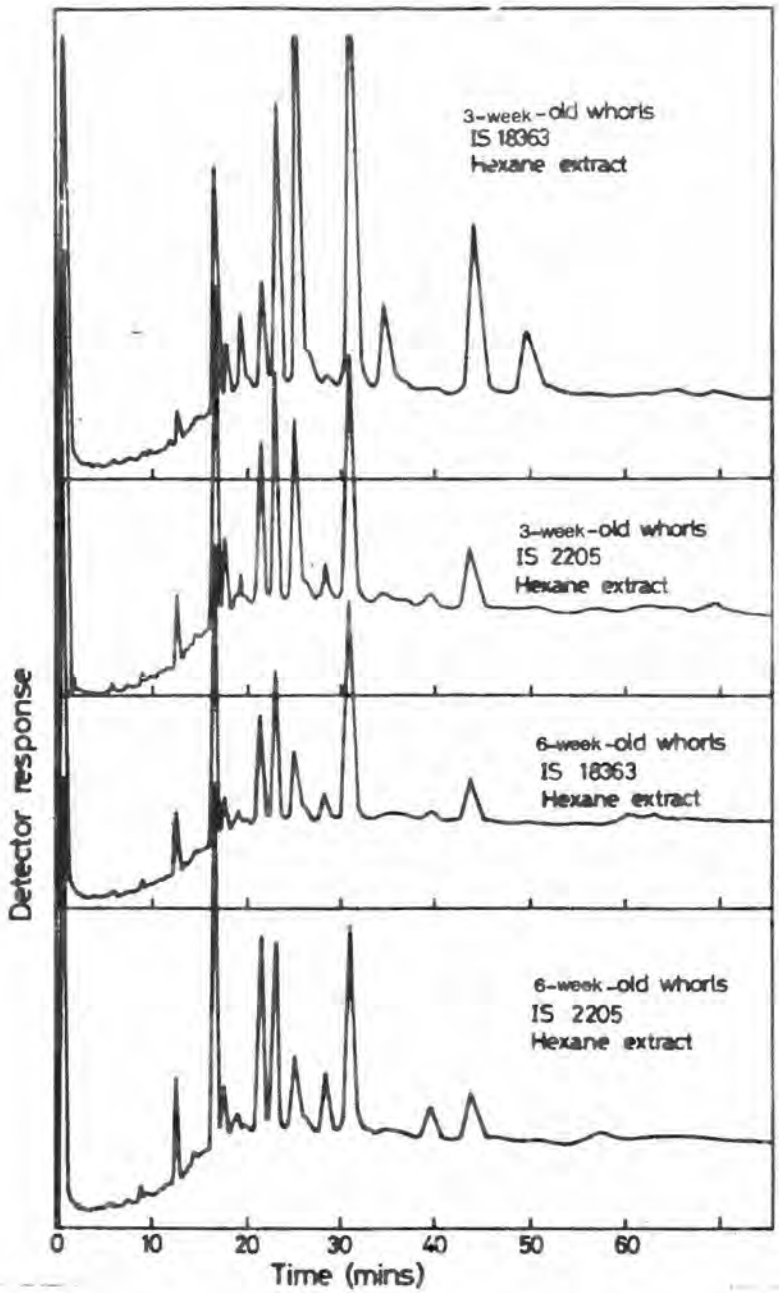
detector at 240 nm were used; the column was eluted with 20% aqueous methanol at 3.0 ml/min at 25 ^{o}C.

Gas liquid chromatography (GLC) was performed on a Packard 428, equipped with a flame ionisation detector, utilising a 3 m x 2 mm i.d. column of 5% silicone OV-17 on chromosorb WHP 80-100 mesh. The column temperature was programmed from 250-300 ^{o}C at 3 ^{o}/min. Carrier gas was nitrogen at 20 ml/min.

For profile studies, 5 μg/μl each of the ethyl acetate and methanol extracts were prepared in methanol, filtered through a wad of cotton wool plugged in a Pasteur pipette and 5 μl aliquot of each preparation were analysed by HPLC on the DuPont Zorbax ODS C-18 reverse phase column. All solvents used in the analysis were HPLC grade (Aldrich Chem. Co. Ltd.).

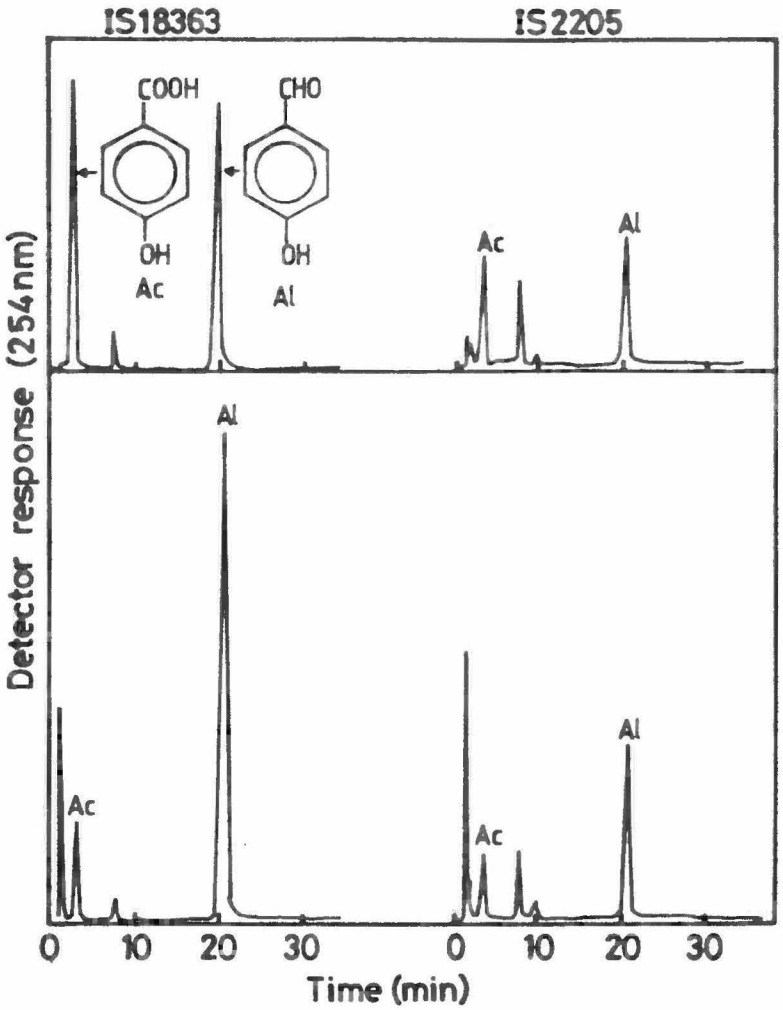
Hexane extracts were analysed by GC. 1 μl of solutions containing 20 μg/μl of the extracts were used for analysis.

Figures 4.1a, 4.1b and 4.1c show the chromatographic profiles of the hexane, ethyl acetate and methanol extracts of the two cultivars, and the most striking feature in the chromatograms is quantitative differences between identical peaks.



Gas chromatograms of sorghum leaf-whorl extracts.

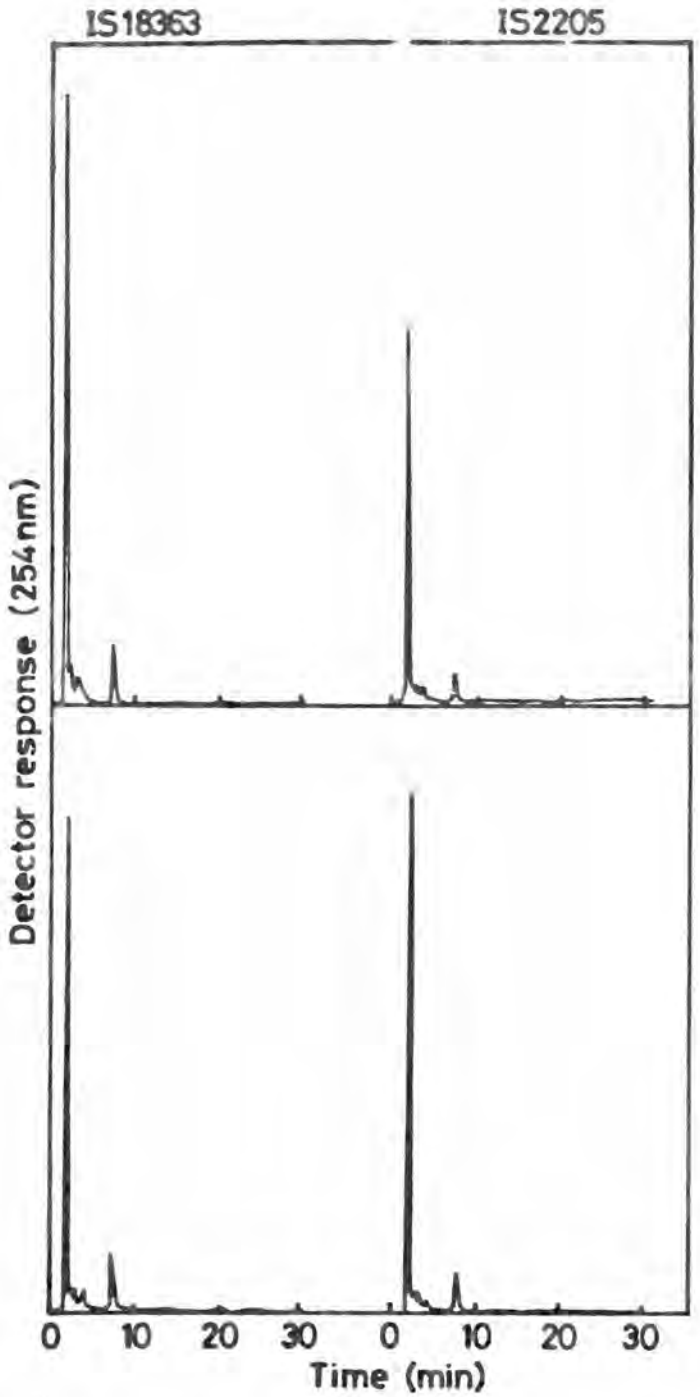
Fig. 4·1a



HPLC profiles of the ethyl acetate extracts of sorghum whorls.

The upper profiles represent extracts of the whorls of the week old plants and the lower profiles, extracts of the whorls of the 6 week old plants of IS 18363 and IS 2205.

Fig. 4.1b



HPLC profiles of the methanol extracts of sorghum whorls

The upper profiles represent extracts of the whorls of t week old plants and the lower profiles, extracts of the whorls of the 6 week old plants of IS 18363 and IS 2205.

Fig. 4.1c

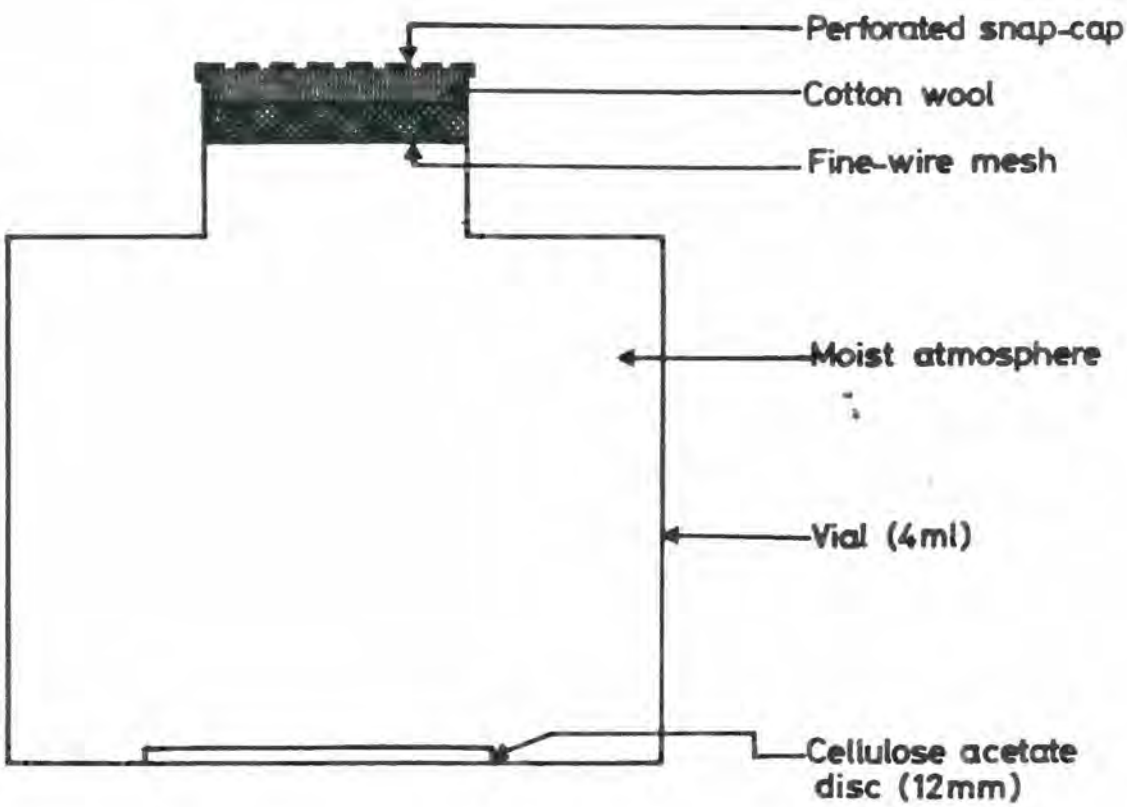
4.5 Spectral analysis

GC/MS analysis of compounds was carried out on a VG Masslab (Chesire, UK) mass spectrometer, model VG12-250 interfaced with a HP5790 gas chromatograph. Mass spectral data were obtained by electron impact at 70 eV and are reported as m/e vs relative intensity. Carrier gas for GC was helium.

4.6 Bioassays For Feeding Activity

Feeding tests were conducted in a no choice situation in small glass vials, 23 x 27 mm (4 ml). The vials had tight fitting plastic snap-on caps which had holes punched with a dissecting pin. Moisture was maintained in each vial by soaking a wad of cotton-wool (30 $\pm$ 3 mg) placed inside the cap with 200 μl of double distilled water. The cotton-wool was covered with a fine-wire mesh which fitted tightly inside the cap (Fig. 4.1d).

Test samples in solvents were applied topically unto both sides of cellulose acetate discs. One test disc (12 mm in diameter), dried in a stream of warm air, was placed at the bottom of the vial containing three third-instar larvae of *C. partellus* which had been starved for 24 h. All the feeding tests were maintained for 24 h in the dark in an



Apparatus for no choice feeding bioassay

Fig. 4·1d

environmentally controlled room (RH = 78 ± 2%, temperature, 29 ± 2 ^{O}C)

4.7 Solubilisation of extracts and solvent effects on feeding

Crude extracts of hexane were prepared in hexane, ethyl acetate extracts dissolved best in methanol and methanol extracts in water. Other medium polarity solvents like chloroform, acetone and ethyl acetate were found to dissolve the feeding substrate and therefore were unsuitable.

Preliminary tests showed that methanol slightly distorted the feeding substrate. Feeding assays were therefore carried out on the feeding substrates after they had been dipped into the solvents used for solubilisation of the extracts. There were two treatments, no water and water treatments. In the no water treatment, discs were only dipped in the test solvents and air dried. In the water treatment, discs dipped into solvent and air dried were further loaded with 15 µl of double distilled water. Each test was replicated 8 times.

The data collected on discs consumed for the different solvents were subjected to analysis of variance and the means separated by the Duncan's multiple range test.

Table 4.2 shows the feeding responses of third-instar larvae of *C. partellus* to cellulose acetate discs treated with different solvents.

Table 4.2. Effect of solvent on the feeding response of third-instar larvae of *C. partellus* in a no choice bioassay.

Test performed	Solvent	Mean feeding (mg)	Anova model F-ratio
No water added	no solvent	0.02850 ab	
	hexane	0.02625 b	
	methanol	0.00413 c	
	water	0.03213 a	
			27.02***
Water added (15 µl)	no solvent	0.02600 b	
	hexane	0.02988 ab	
	methanol	0.02712 ab	
	water	0.03025 ab	

*** Pr>F 0.0001

Means followed by the same letter are not significantly different ($P < 0.05$; Duncan's multiple range test).

There was large variability in the mean weights of discs consumed by larvae in the no water treatment, particularly in the methanol case, but the addition of 15 µl of water to discs already dipped into solvent eliminated this variability.

4.8 Bioassays of the crude extracts

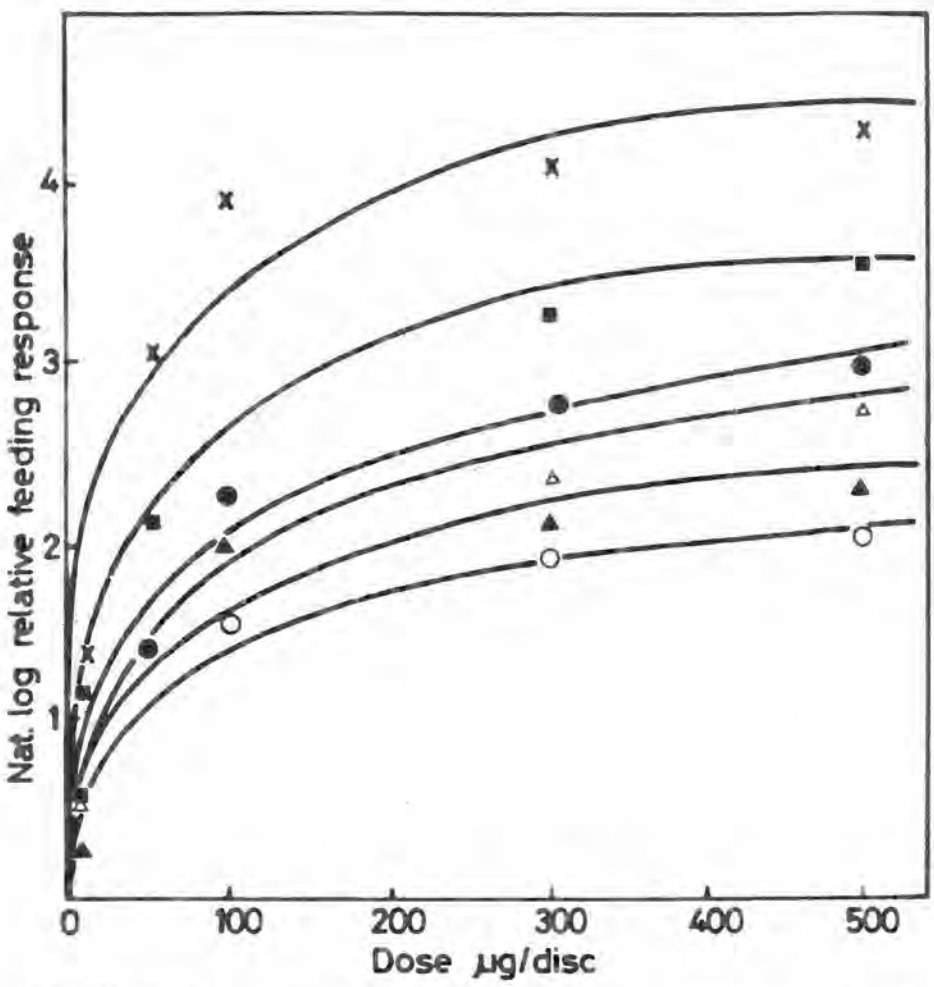
Dose response tests were carried out on hexane, ethyl acetate and methanol extracts of the whorls of the two cultivars. The crude extracts were each tested at 10, 50, 100, 300 and 500 µg. Chromatographic fractions of crude extracts were tested singly and in combination. Each test was replicated 15 times.

Each test disc was dried thoroughly in a stream of warm air and then weighed several times to a constant weight on a Cahn 21 milligram balance to ± 0.001 mg before and after the assay. The feeding response on each disc was expressed as the Relative Feeding Response (RFR) calculated from the formula RFR = Xt/Xo where Xt and Xo are the mean weights of treated and control discs consumed respectively. Natural logarithms of RFR were plotted against different doses to depict the dose-response relationships.

These relationships which are shown in Figures 4.2a and 4.2b were expressed in the regression equation ln y = ln a + b ln x where y = relative feeding response and x = dose in µg/disc. The intercepts (a) and slopes (b) are compared in Tables 4.3a and 4.3b.

Table 4.3a. Constants for the regressions of the crude extracts of the whorls of the 3 week old plants.

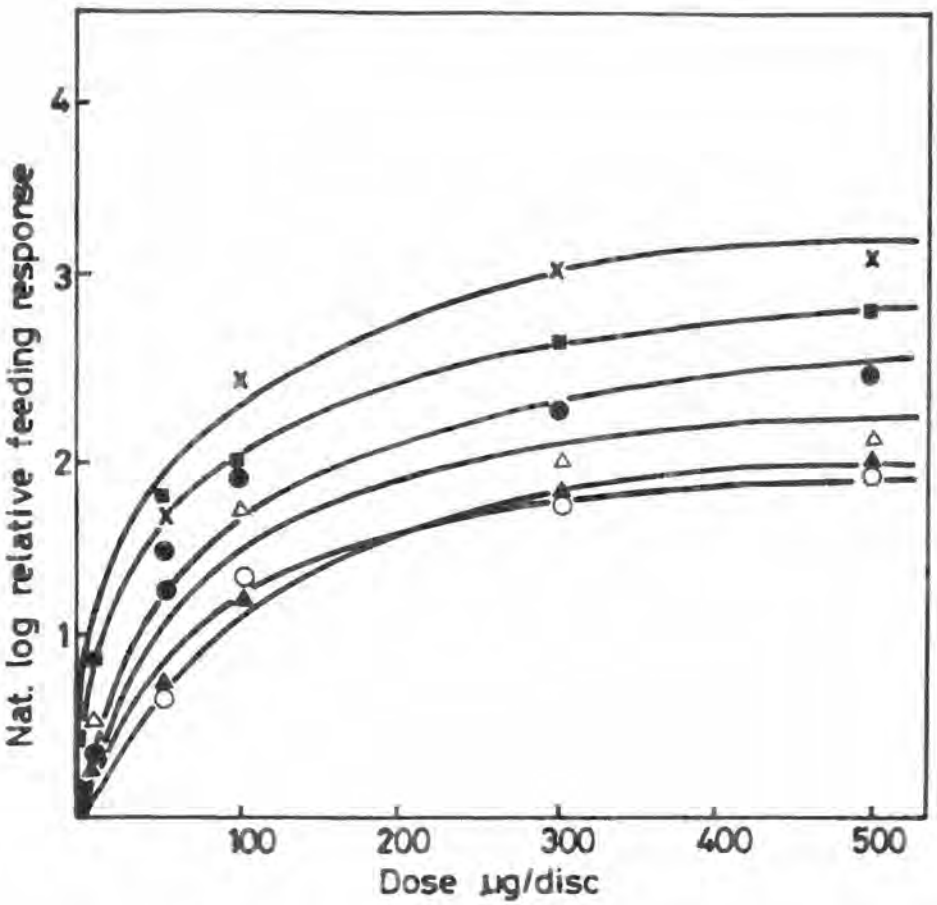
	IS 18363		IS 2205	
Extract	ln a	b	ln a	b
hexane	-0.8352	0.5221	-0.7056	0.4427
ethyl acetate	-0.9582	0.6426	-0.5387	0.5272
methanol	-0.0741	0.7486	-0.2656	0.6331



Dose response curves for the crude extracts of the whorls of the 3 week old plants of IS 18363 and IS 2205.

IS 18363: x methanol, ● ethyl acetate, ▲ hexane
IS 2205: ■ methanol, △ ethyl acetate, O hexane

Fig. 4.2a



Dose response curves for the crude extracts of the whorls of the 6 week old plants of IS 18363 and IS 2205.

IS 18363: x methanol, ● ethyl acetate, ▲ hexane
IS 2205: ■ methanol, Δ ethyl acetate, O hexane

Fig. 4.2b

Table 4.3b. Constants for the regressions of the crude extracts of the whorls of the 6 week old plants.

	IS 18363		IS 2205	
Extract	ln a	b	ln a	b
hexane	-1.0000	0.4709	-0.8131	0.4371
ethyl acetate	-1.0073	0.5728	-0.1925	0.3778
methanol	-0.4885	0.6024	-0.3330	0.5124

A comparison of the data on the feeding responses of the third-instar larvae to the extracts of the two cultivars showed that they followed the same pattern. Polar extracts were more stimulatory to larvae than the nonpolar ones. However, the b values indicate that larvae responded more to extracts of the more susceptible cultivar, IS 18363 than to extracts of cultivar IS 2205. The response was also dependent on the age of the plant, with larvae feeding more on extracts of the 3 week old whorls than those of the 6 week old whorls.

4.9 Fractionation of extracts

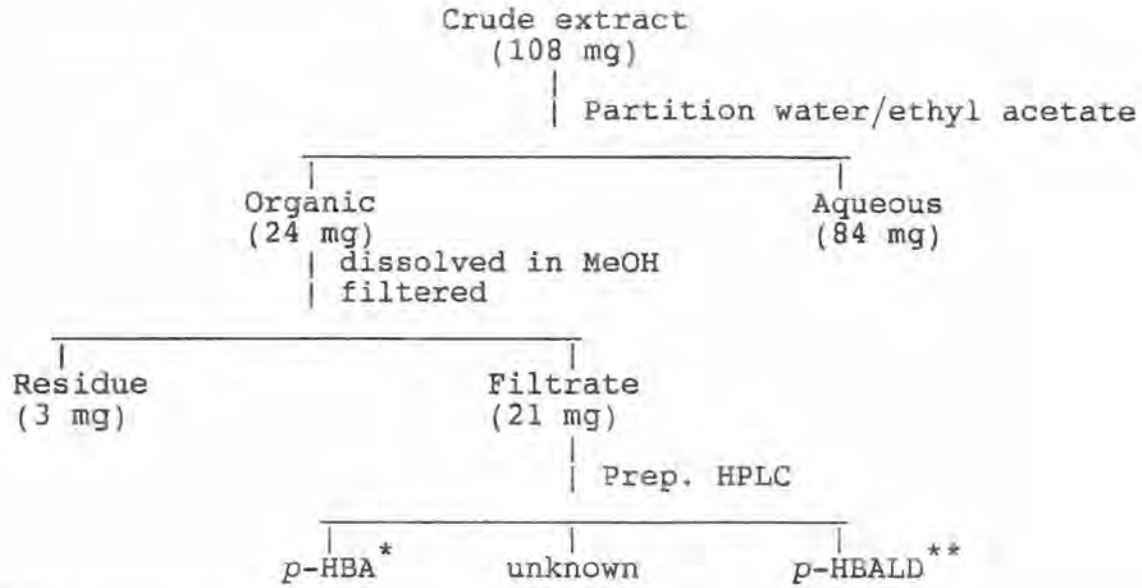
As a priority the more stimulatory methanol and ethyl acetate extracts were fractionated with the purpose of identifying the active compounds. Unfortunately no similar study could be undertaken on the hexane extract in this investigation.

4.10 Fractionation of ethyl acetate extract and acetylation of organic fraction

There were four different ethyl acetate extracts comprising those of the whorls of 3 and 6 week old plants of sorghum cultivars IS 18363 and IS 2205. From the preliminary study, it was found that differences between the extracts were quantitative rather than qualitative. Hence detailed fractionation studies were undertaken only on one extract (3 week old plants). Other extracts could then be analysed quantitatively by comparison of their chromatographic profiles.

Fractionation of the crude ethyl acetate extract was undertaken as shown in Scheme 4.1.

Scheme 4.1 Flow diagram for the isolation of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde from ethyl acetate extract of 3 week old plants.

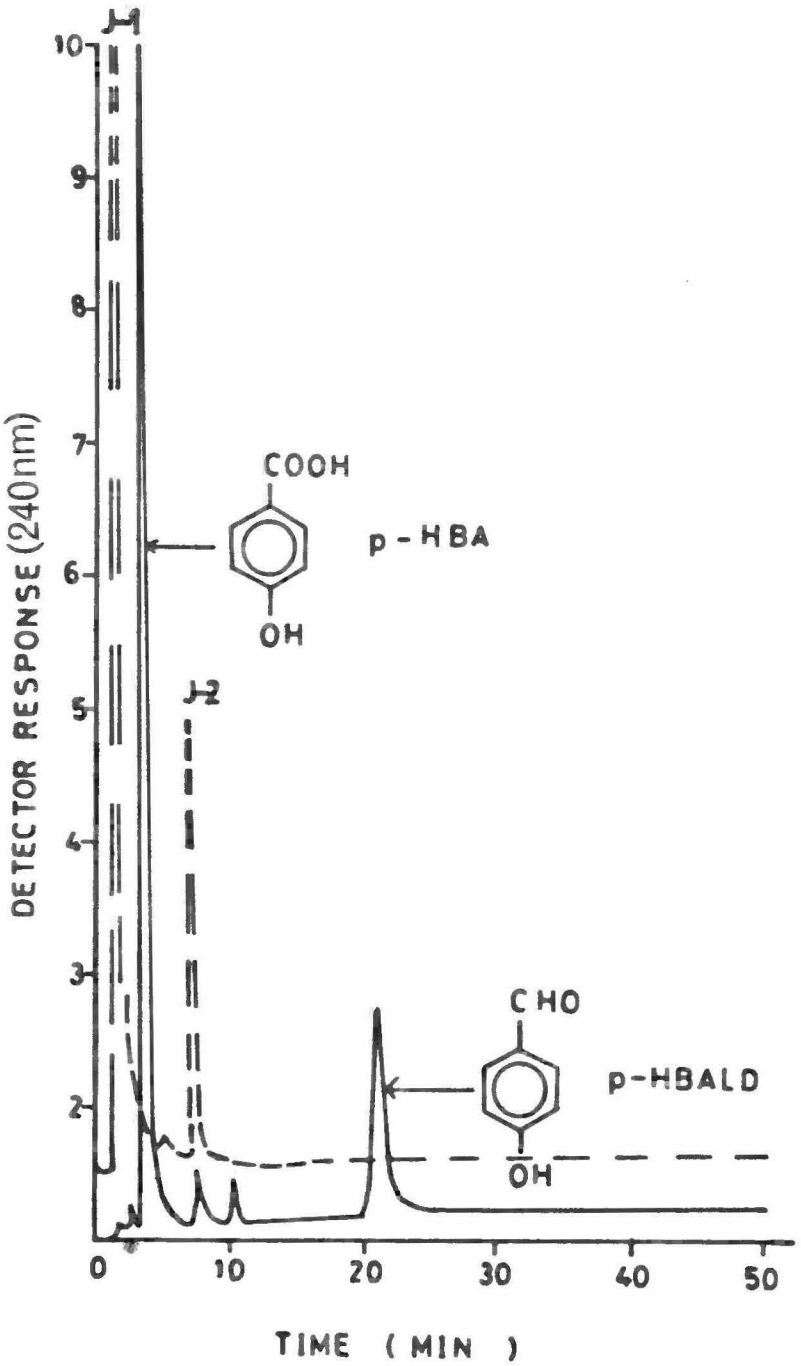


* *p*-hydroxybenzoic acid

** *p*-hydroxybenzaldehyde

The extract was partitioned between water and ethyl acetate. The aqueous fraction constituted about 80% of the extract and the organic fraction about 20%.

Preparative HPLC of the organic phase gave *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde as the major components (Fig. 4.3a). The two compounds were identified by mass spectrometry and coinjection with authentic samples on an HPLC reverse phase column. The mass spectrum of *p*-hydroxybenzaldehyde showed peaks at m/e (%), M^+-122 (100),



HPLC profiles of the partition phases of the ethyl acetate extract of the whorls of the 3 week old plants of IS 18363

(——) organic phase, (- - -) aqueous phase.

Fig·4·3a

93 (48), 65 (48), 39 (58) and for *p*-hydroxybenzoic acid, M^+-138 (70), 121 (100), 93 (30), 65 (28), 39 (34). The retention time of *p*-hydroxybenzaldehyde on the reverse phase column was R^+=20 min and for *p*-hydroxybenzoic acid Rt=5 min. The identities of the two compounds were further established by preparing their methylated derivatives with diazomethane in peroxide free ether and characterising them by GC-MS and GC-coinjection with authentic samples. The mass spectral data for the methoxy methyl ester of the acid were m/e (%) M^+-166 (30), 135 (100), 107 (12), 92 (18), 77 (28), 64 (12) and for the methoxybenzaldehyde M^+-136 (100), 107 (24), 92 (28), 77 (46), (18), 63 (16), 51 (14), 39 (18).

Acetylation of the organic fraction with acetic anhydride in dry pyridine at 0 ^oC for 24 h gave a mixture of phenolic acetyl derivatives. In addition to the acetyl derivatives of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid, GC/MS analysis of the mixture revealed the presence of the acetyl derivatives of caffeic, *p*-coumaric and ferulic acids. The mass spectrum of the acetyl derivative of *p*-coumaric acid showed peaks at M^+-206 (4), 163 (6), 119 (42), 105 (8), 93 (22), 70 (38), 61 (56) and 45 (100); that of caffeic acid at M^+-264 (4), 221 (100), 178 (8), 143 (60), 128 (30), 103 (24), 91 (64), 77 (12), 61 (14) and 43 (64); and that of ferulic acid at M^+-236 (28), 221 (35), 193 (12), 178 (8), 165 (8), 143 (88), 128 (38), 105 (48), 91 (100), 77 (45), 65 (15), 51 (28) and 41 (34). These spectra were in

agreement with those of authentic samples of these compounds. The presence of these cinnamic acids in the organic fraction was confirmed by coinjection with authentic samples on an HPLC reverse phase column at detector wavelength of 280 nm (Fig. 4.3b). The retention times (Rt) of caffeic acid, *p*-coumaric acid, and ferulic acid were 6, 10 and 13 min respectively.

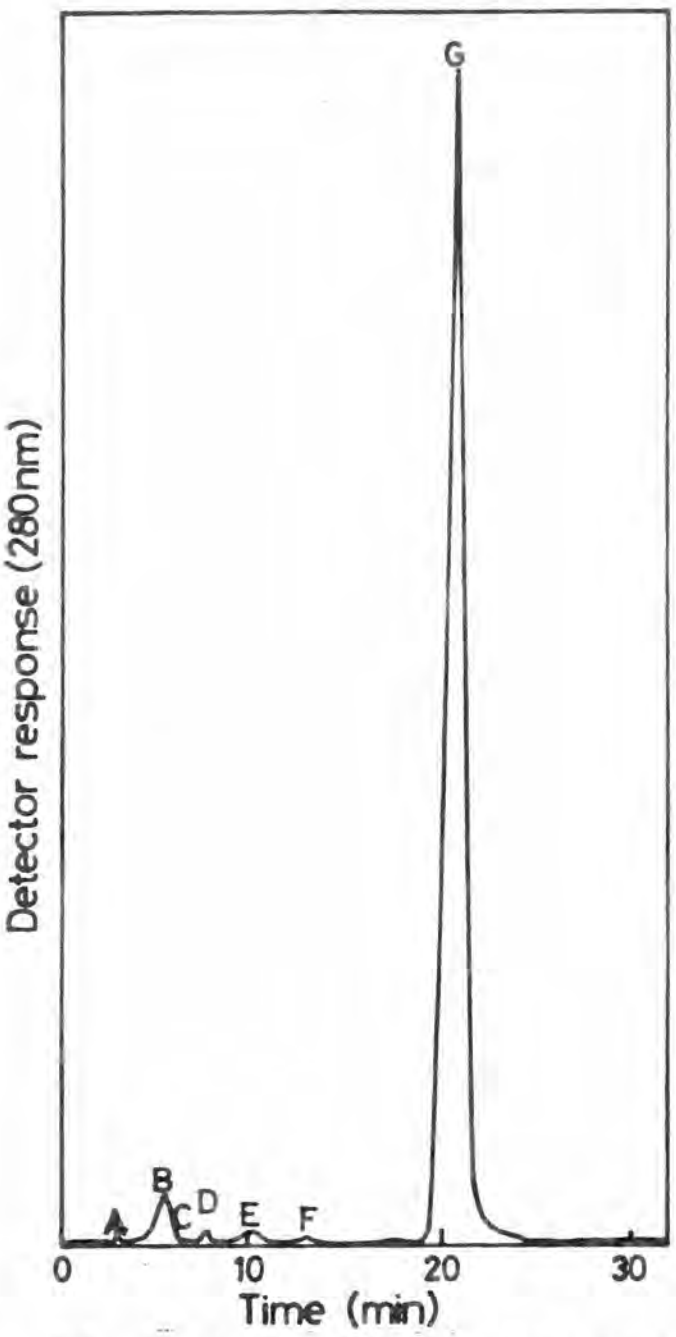
No further separation was carried out on the aqueous fraction since its HPLC profile was superimposable on that of the crude methanol extract of the whorls. Fractionation of the latter is described on page 102.

4.11 Feeding bioassays of fractions of the ethyl acetate extract, phenolics identified and their analogues

The following fractions and phenolics were bioassayed:

(a) the organic and aqueous fractions of the ethyl acetate extract individually and as blend in the proportion they were fractionated from the original extract;

(b) purified synthetic samples of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde and a mixture of the two in the proportion occurring in the crude extract.



HPLC profile of the ethyl acetate extract of the whorls o
the 3 week old plants of IS 18363 (280 nm).

A-J-1, B-p-hydroxybenzoic acid, C-caffeic acid, D-J-2,
E-p-coumaric acid, F-ferulic acid, G-p-hydroxybenzaldeh

Fig. 4.3b

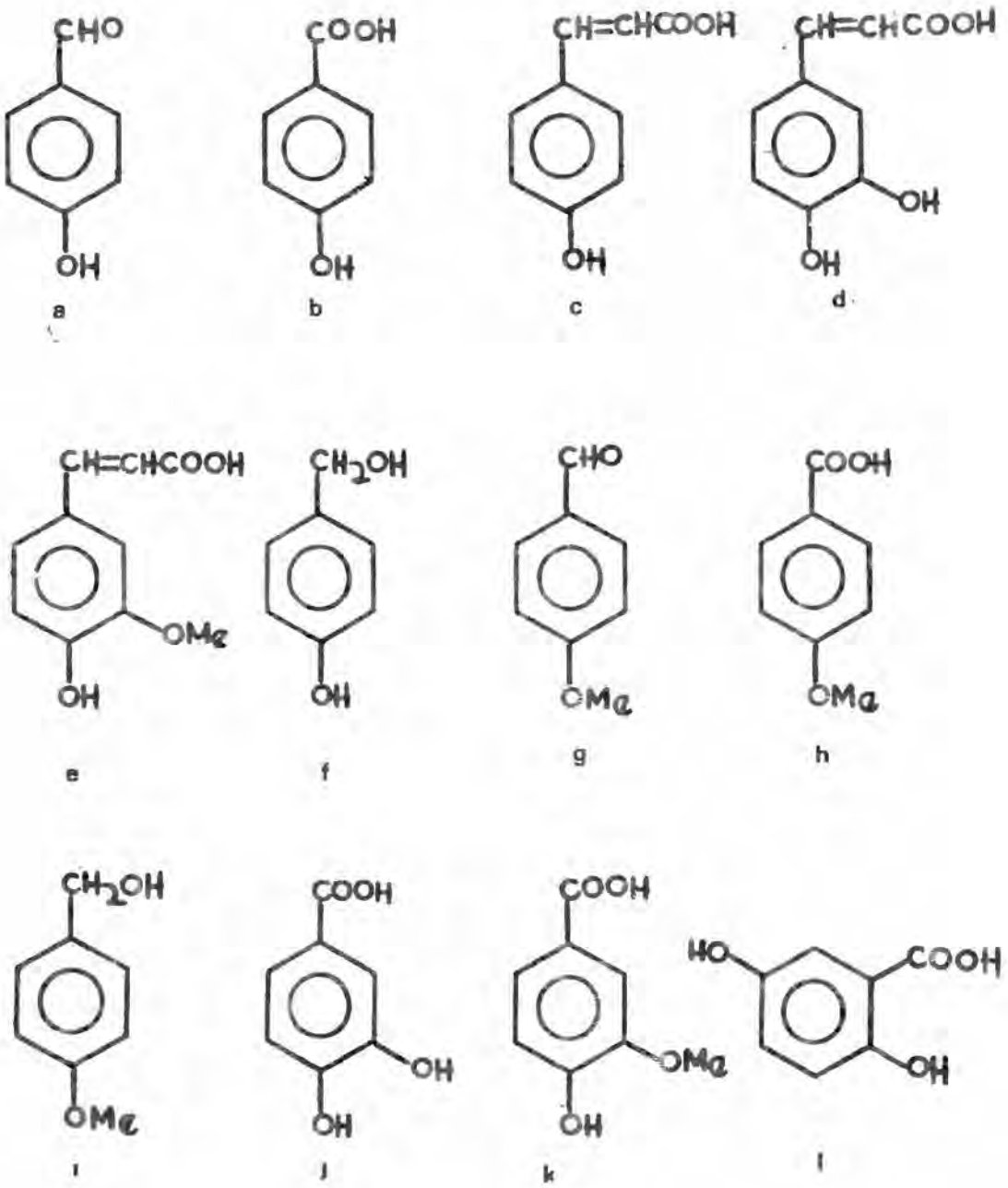
(c) some selected analogues of the above phenols comprising *p*-hydroxybenzyl alcohol, *p*-methoxybenzaldehyde, *p*-methoxybenzoic acid and *p*-methoxybenzyl alcohol.

(d) purified synthetic samples of *p*-coumaric acid, caffeic acid, ferulic acid, vanillic acid, protocatechuic acid, gentisic acid and chlorogenic acid a common constituent of plants (Sondheimer, 1964) and quinic acid, a degradative product of chlorogenic acid.

All the phenolic compounds (Fig. 4.4) were obtained from Aldrich Chem. Co. Ltd. and the purity of each chemical was checked by HPLC and/or GLC. Each phenolic compound was tested at 10, 25, 50, 150, 250, 350 and 450 μg.

The dose response curves for the fractions of the ethyl acetate extract (Fig. 4.5) were also expressed in the regression equation $\ln y = \ln a + b \ln x$ where y = relative feeding response and x = dose μg/disc. The equations were as follows: organic, $\ln y = -0.9662 + 0.4226 \ln x$; aqueous, $\ln y = -0.6881 + 0.5322 \ln x$; organic+aqueous, $\ln y = -0.9918 + 0.6209 \ln x$ and for the crude extract, $\ln y = -0.9582 + 0.6426 \ln x$.

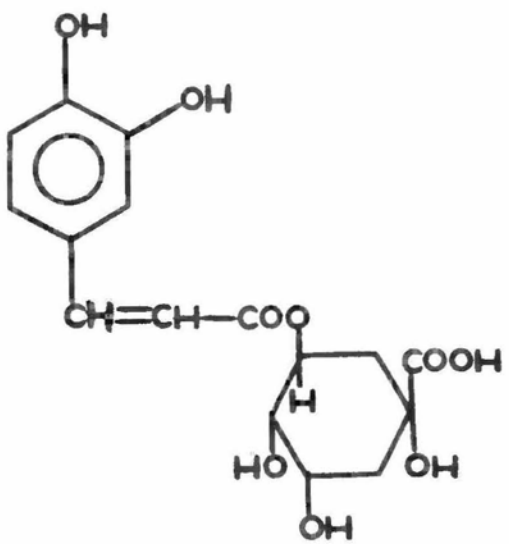
A comparison of the regressions showed that fractionation lowered the feeding response of the larvae, and that the polar fraction retained a higher



Phenolic compounds tested for larval feeding response.

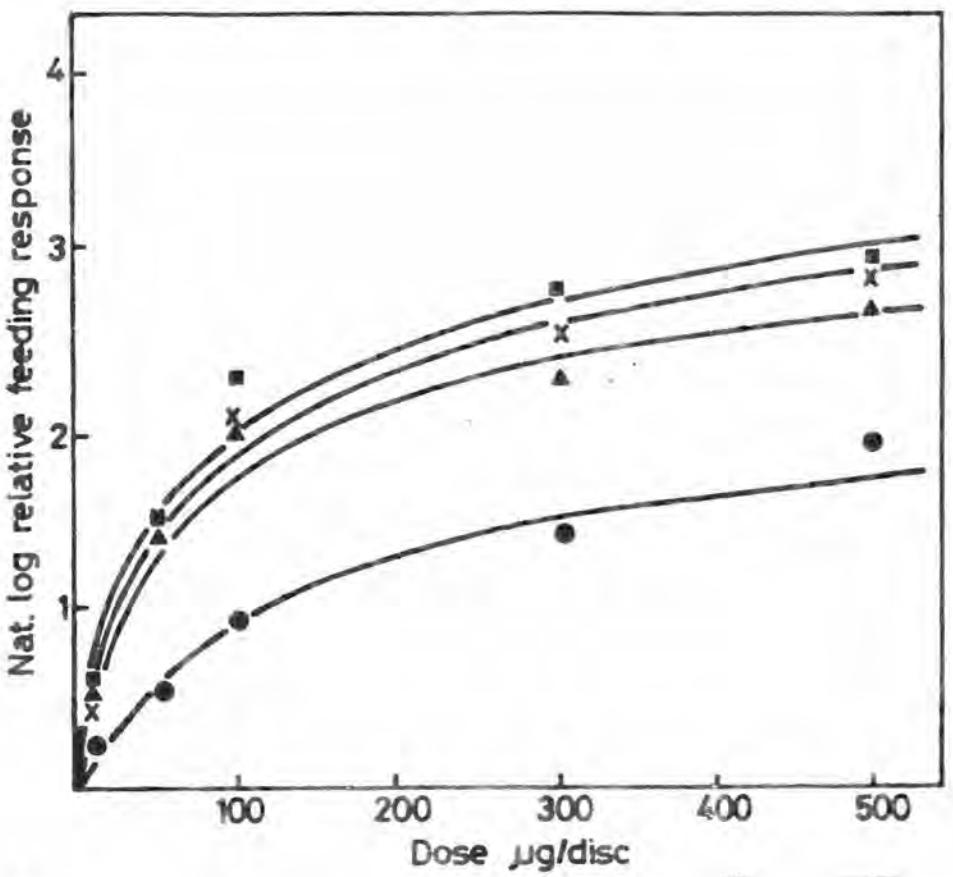
(a) p-hydroxybenzaldehyde, (b) p-hydroxybenzoic acid, (c) p-coumaric acid, (d) caffeic acid, (e) ferulic acid, (f) p-hydroxybenzyl alcohol, (g) p-methoxybenzaldehyde, (h) p-methoxybenzoic acid, (i) p-methoxybenzyl alcohol, (j) protocatechuic acid, (k) vanillic acid, (l) gentisic acid.

Fig. 4 4



chlorogenic acid

Fig. 4.4



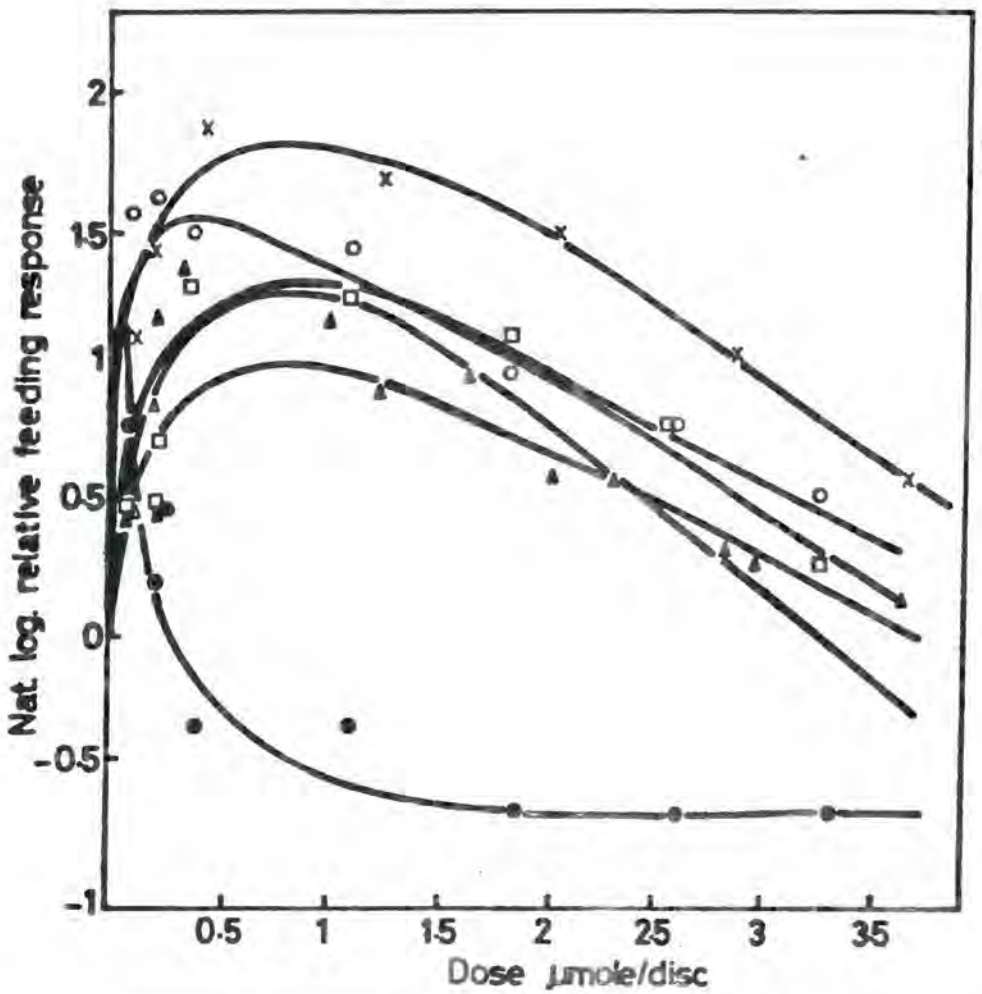
Dose response curves for the partition phases of the ethyl acetate extract of the whorls of the 3 week old plants of IS 18363.

■ crude, x aqueous+organic, ▲ aqueous, ● organic.

Fig. 4.5

activity than the less polar one (Fig. 4.5). The blend from the fractions which was constituted in the proportion obtained from the fractionation of the extract, aqueous : organic (4:1), was almost as active (b = 0.6209) as the crude extract (b = 0.6426). This suggested that chemicals in the aqueous and organic fractions acted synergistically to give an enhanced larval feeding response.

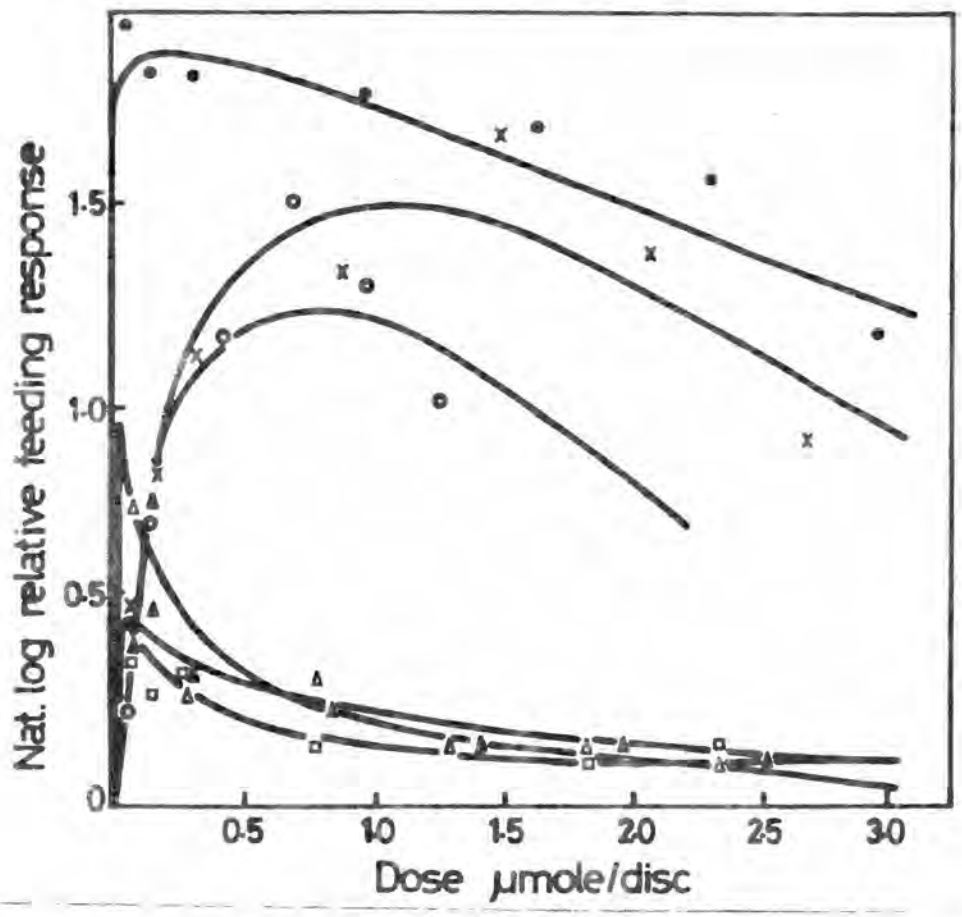
The dose response curves for the phenolic compounds (Figs. 4.6 and 4.7) were expressed in the regression equation $\ln y = \ln a + b \ln x + x \ln c$ where y = relative feeding response, x = dose, μmole/disc ; a, b, and c are constants. The a, b and c values are listed in Table 4.4.



Dose response curves for p-hydroxybenzaldehyde, p-hydroxybenzoic acid and some synthetic analogues of the two compounds.

x p-hydroxybenzaldehyde, O p-methoxybenzyl alcohol,
□ p-hydroxybenzoic acid, ▲ p-methoxybenzoic acid,
△ p-hydroxybenzyl alcohol, ● p-methoxybenzaldehyde.

Fig. 4.6



Dose response curves for some hydroxybenzoic and cinnamic acid compounds.

● protocatechuic acid, x vanillic acid, O chlorogenic acid, Δ quinic acid, ▲ caffeic acid, □ ferulic acid.

Fig. 4.7

Table 4.4 Regression constants for phenolics and some derivatives.

Compound	ln a	b	ln c
p-hydroxybenzaldehyde	-0.1343	0.5575	-0.0060
p-hydroxybenzoic acid	-1.0196	0.5199	-0.0055
p-hydroxybenzyl alcohol	-0.1879	0.3533	-0.0043
p-methoxybenzaldehyde	1.9694	-0.5601	0.0018
p-methoxybenzoic acid	-0.6328	0.5043	-0.0048
p-methoxybenzyl alcohol	1.6133	0.0017	-0.0024
p-HBALD + *p*-HBA*	-1.2784	0.8347	-0.0051
vanillic acid	2.1145	0.5945	-0.6074
protocatechuic acid	2.0369	0.0446	-0.2869
caffeic acid	0.2679	-0.0624	-0.0524
ferulic acid	0.1456	-0.0748	0.0029
chlorogenic acid	2.0330	0.5559	-0.8040
quinic acid	0.1167	-0.3239	0.0711
p-coumaric acid	not stimulatory		
gentisic acid	not stimulatory		

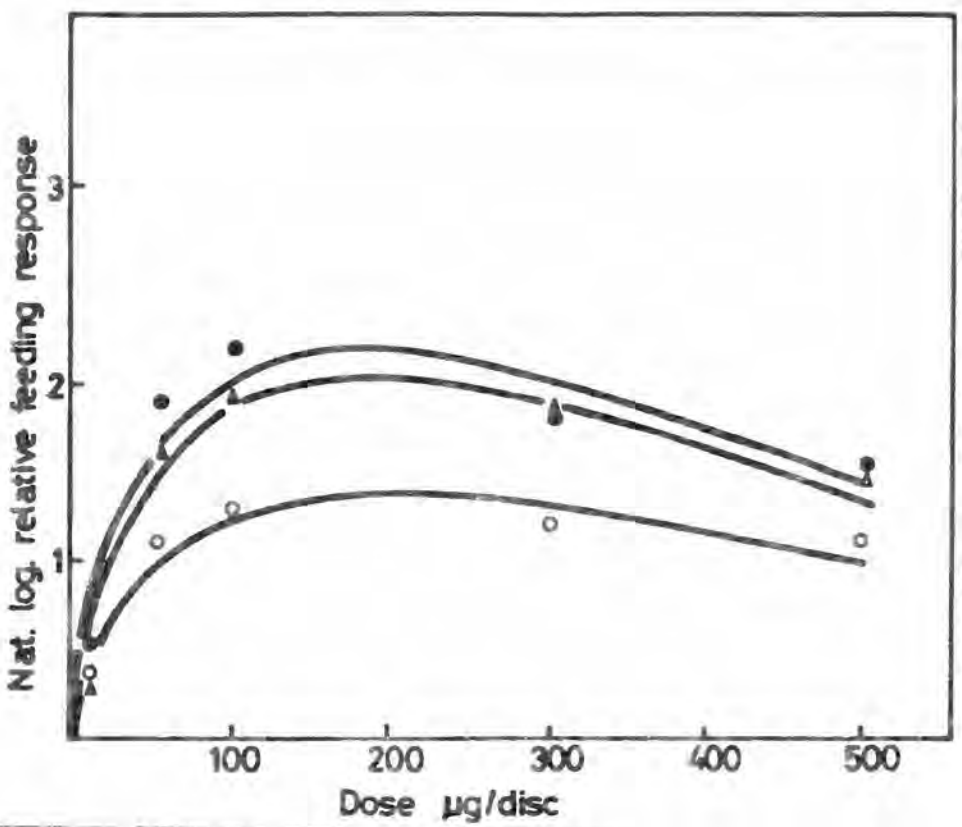
* *p*-hydroxybenzaldehyde+*p*-hydroxybenzoic acid

The data on the phenolics showed that larval feeding response varied with the nature of the compound. All the phenolic compounds except *p*-coumaric acid and gentisic acid stimulated larval feeding, but became less stimulatory at

higher doses. *p*-Methoxybenzaldehyde was stimulatory at very low doses but deterrent at higher doses. The feeding response of larvae to a blend of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid as occurring in the crude extract of the whorls of the 3 week old plants of cultivar IS 18363 showed no synergism between the two compounds (Fig. 4.8).

4.12 Extent of oxidation of p-hydroxybenzaldehyde during bioassays

Since aldehydes are rapidly oxidised on exposure to air, an attempt was made to determine the extent of oxidation of *p*-hydroxybenzaldehyde during the assay period. A methanol solution of *p*-hydroxybenzaldehyde (10 μg/μl) was prepared. 10 cellulose acetate discs were each loaded with 200 μg of *p*-hydroxybenzaldehyde followed by 15 μl of double distilled water (to correct any distortion of the discs by the methanol) and then air dried in a stream of warm air. 5 out of these discs were immediately extracted with 1000 μl of methanol, and 10 μl of each extract were analysed on an HPLC reverse phase column. The remaining discs were set under the bioassay conditions previously described, but without larvae. After 24 h the discs were similarly extracted with methanol and analysed. The mean peak areas corresponding to *p*-hydroxybenzaldehyde after 0 and 24 h of bioassay were calculated and compared. Peaks representing



Dose response curves for p-hydroxybenzaldehyde, p-hydroxy benzoic acid and a blend of the two compounds.

● p-hydroxybenzaldehyde+p-hydroxybenzoic acid (3:2),
△ p-hydroxybenzaldehyde, O p-hydroxybenzoic acid.

Fig.4.8

the absorptions of *p*-hydroxybenzoic acid were also checked for in the analysis.

The results showed that there was no significant difference between peak areas of *p*-hydroxybenzaldehyde after 0 h (2.30 ± 0.05 cm^2) and 24 h (2.30 ± 0.07 cm^2) of bioassay indicating that very little oxidation of *p*-hydroxybenzaldehyde occurs under the bioassay conditions.

4.13 Phenolic levels in the ethyl acetate extracts

Free phenolics in the extracts were *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, *p*-coumaric, ferulic and caffeic acids. Preliminary HPLC analysis of the three extracts hexane, ethyl acetate and methanol showed that there was very little of these compounds in the hexane and methanol extracts indicating that the bulk of them were in the ethyl acetate extracts. The HPLC data of the ethyl acetate extracts showed that ferulic, caffeic, and *p*-coumaric acids were present in trace amounts whilst p-hydroxybenzoic acid and p-hydroxybenzaldehyde were in relatively large proportions (Fig. 4.3b). Analysis of the free phenolic levels in the ethyl acetate extracts was therefore determined from the amounts of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde from a calibration of a standard solution containing a mixture of the two. The

concentrations of *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde in the standard solution were 0.18 μg/μl and 0.24 μg/μl respectively. 1, 2, 5, 10 and 15 μl aliquots of the standard mixture were analysed on a HPLC reverse phase column and the effluent detected by UV at 254 nm. 5 μg/μl methanol solution of each of these ethyl acetate extracts was prepared and 5 μl each of the extracts were similarly analysed. For each volume analysed the peak areas for the absorptions of the the two compounds were calculated and converted into their corresponding amounts in micrograms. From these the amounts in the whorls of the two cultivars at the two growth stages were calculated. These data are summarised in Tables 4.5 and 4.6.

Table 4.5 Phenolic levels in 25 μg of each of the ethyl acetate extracts

Extract	Plant age (wks)	*p*-hydroxy benzaldehyde (μg)	*p*-hydroxy benzoic acid (μg)	Total phenolic (μg)	Ratio of the two
IS 18363	3	1.8	1.2	3.0	1.5
IS 2205	3	0.9	0.5	1.4	1.8
IS 18363	6	3.3	0.4	3.7	8.3
IS 2205	6	1.2	0.3	1.5	4.0

Table 4.6 Phenolic levels in the whorls % (μg/kg whorl)

Cultivar	Plant age(wks)	wt (kg)	No of whorls	% phenolic in extract	% phenolic in whorl
IS 18363	3	3.5	500	30	0.17
IS 2205	3	3.4	800	14	0.05
IS 18363	6	3.5	80	37	1.32
IS 2205	6	2.9	80	15	0.65

Several noteworthy results emerge from these data:-

(a) the extracts of the whorls of the resistant cultivar IS 2205 contains less *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid than the susceptible one IS 18363 for both the 3 and 6 week old plants. Similarly, the total phenolic level was less for the extracts and whorls of the resistant cultivar at the two growth stages of the plant.

(b) the total phenolic levels were not significantly different for the extracts of the whorls of the 3 and 6 week old plants. The whorls of the 6 week old plants contained a higher phenolic level. At the two growth stages, the susceptible cultivar IS 18363 contained a higher phenolic level than the resistant one IS 2205.

resistant one IS 2205.

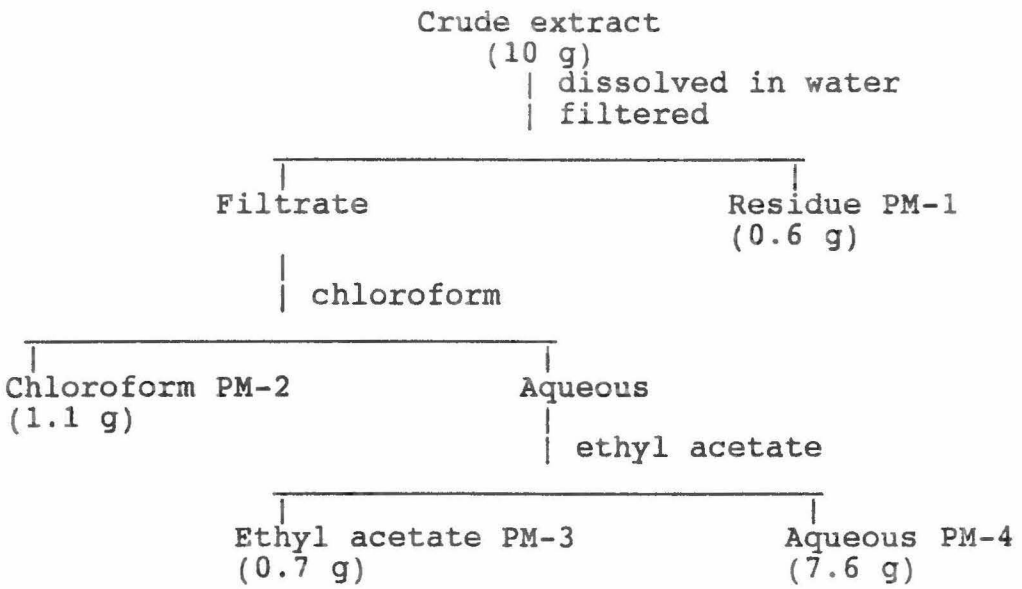
(c) the level of *p*-hydroxybenzaldehyde was higher in the extracts of the whorls of the 6 week old plants than the 3 week old ones, whereas the situation for *p*-hydroxybenzoic acid was reverse.

4.14 Fractionation of methanol extract

Only the extract of the whorls of the 3 week old plants of sorghum cultivar IS 18363 was fractionated for detailed study. The composition of the other extracts was determined by comparisons of their chromatographic profiles.

Fractionation of the crude methanol extract was undertaken as shown in Scheme 4.2.

Scheme 4.2 Flow diagram for the fractionation of the methanol extract of the whorls of the 3 week old plants



The extract (10 g) was dissolved in water (300 ml) and filtered by suction. This removed the insoluble materials (PM-1, 0.6 g). The resulting filtrate was extracted with chloroform (5 X 50 ml). The chloroform extracts were combined, dried over anhydrous sodium sulphate and then evaporated to dryness in vacuo to give a dark green oily mass (PM-2, 1.1 g). The above process was repeated with ethyl acetate. The combined ethyl acetate extracts yielded a yellowish-green solid after solvent removal in vacuo (PM-3 0.7 g). The aqueous phase after ethyl acetate extraction was concentrated first on a rotary evaporator and then on a freeze-drier to give a dark brown solid (PM-4, 7.6 g).

Fraction PM-1 constituted 6%, fraction PM-2, 11%, fraction PM-3, 7%, and fraction PM-4, 76% of the crude extract showing that about three-quarters of the components in the crude methanol extract of the 3 week old whorls of cultivar IS 18363 were water soluble.

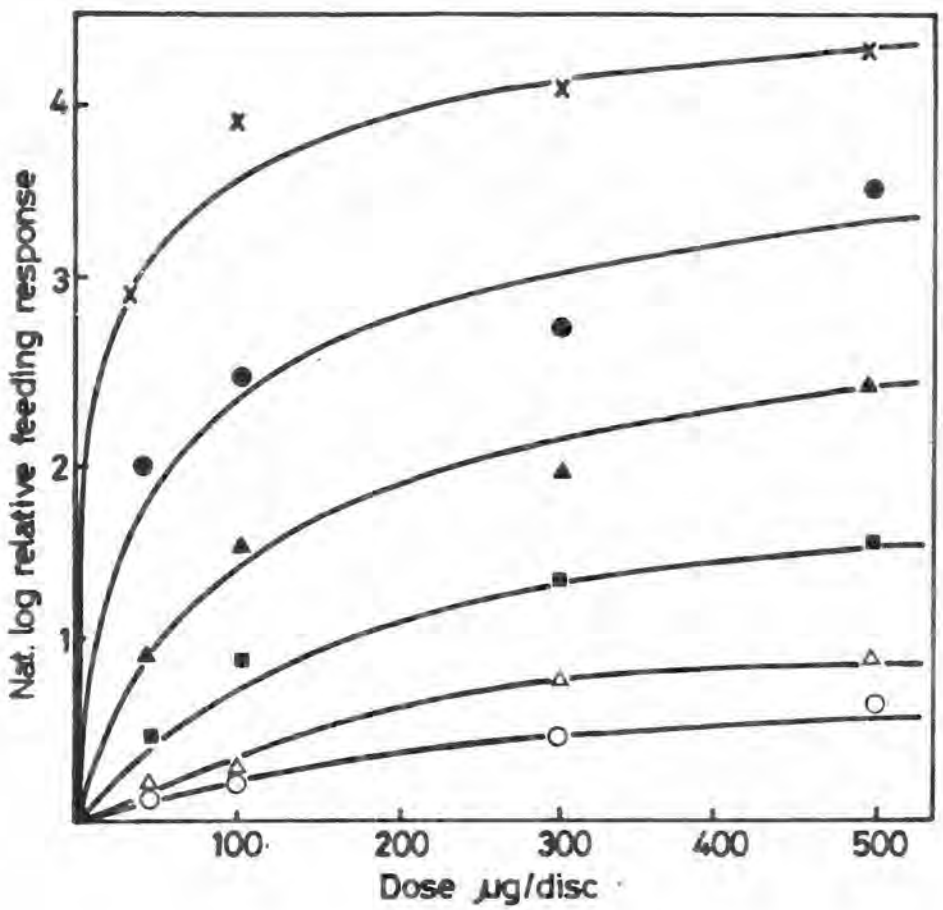
4.15 Bioassays of the fractions

The fractions PM-1-PM-4 were each bioassayed for effects on feeding of *C. partellus* third-instar larvae. Dose response tests were performed for each fraction and for a combined mixture of all the four fractions in the proportion in which they were obtained from the crude

the mixture was tested by elimination. The results of these tests are shown in Fig. 4.9 and Table 4.7.

Fig. 4.9 shows the dose response curves for the different fractions expressed in the regression equation $\ln y = \ln a + b \ln x$ where y and x are relative feeding response and dose in µg/disc respectively. These equations were as follows: PM-1, $\ln y = -0.8456 + 0.2234 \ln x$; PM-2, $\ln y = -1.1611 + 0.4156 \ln x$; PM-3, $\ln y = -0.7366 + 0.6613 \ln x$; PM-4, $\ln y = -1.2695 + 0.5751 \ln x$ and for the crude extract $\ln y = -0.0741 + 0.7486 \ln x$.

A comparison of the b values showed that fractionation lowered the feeding response of the larvae, with the most active fraction being the most polar fraction, PM-4. Fractions PM-2 and PM-3 were intermediate while the least stimulatory was PM-1. A blend of the fractions reconstituted in the ratio in which they were fractionated was more stimulatory than any of the individual fractions but less active than the crude methanol extract.



Dose response curves for the partition phases of the methanol extract of the whorls of the 3 week old plants of IS 18363.

x crude, ● PM-1+PM-2+PM-3+PM-4, ▲ aqueous (PM-4) △ chloroform (PM-2), ■ ethyl acetate (PM-3), ○ residue (PM-1).

Fig. 4.9

Table 4.7 Feeding responses of *C. partellus* larvae to cellulose acetate discs treated with 300 µg of various combinations of the fractions of the methanol extract in a no choice bioassay.

	Fraction Combinations	Mean disc consumed	Anova model F-value
I.	Crude extract	1.6874 a	
II.	PM1+PM2+PM3+PM4	1.1986 b	
III.	PM2+PM3+PM4	0.9353 c	
IV.	PM3+PM4	0.6729 d	
V.	PM1+PM2+PM4	0.5838 d	
VI.	PM1+PM3+PM4	0.4184 e	156***
VII.	PM1+PM4	0.4176 e	
VIII.	PM2+PM4	0.4034 e	
IX.	PM4	0.2634 f	
X.	PM1+PM2+PM3	0.1206 g	
XI.	Control	0.0283 g	

***Pr>F 0.0001

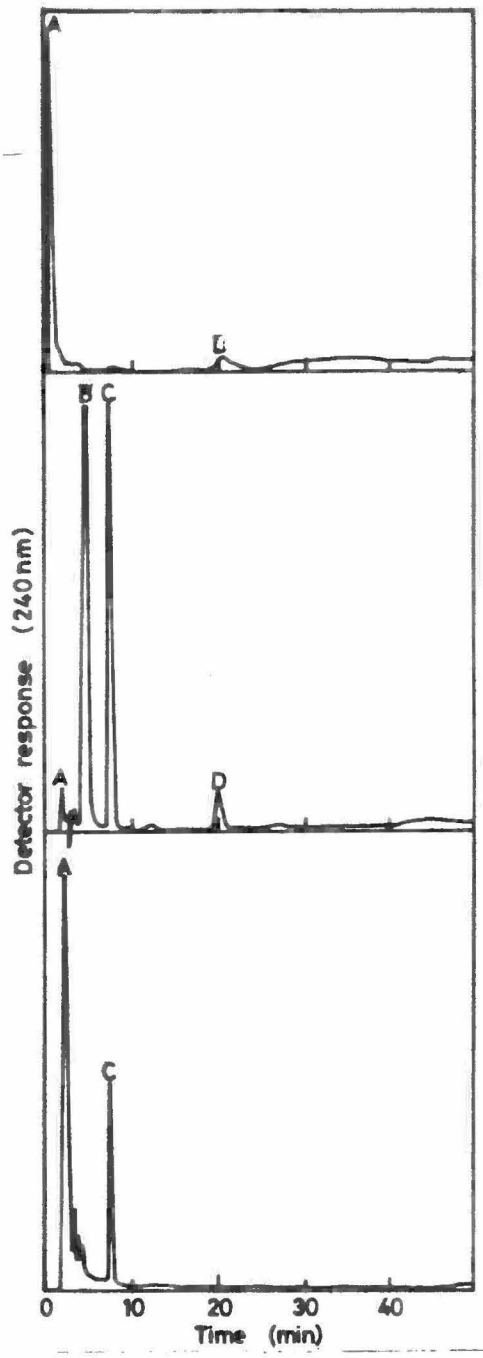
Means with the same letter are not significantly different (P<0.05; Duncan's multiple range test)

The results show synergism between components in the water soluble fraction PM-4 and components in the organic fractions PM-1, PM-2 and PM-3 as evident from I to IX in the

table. The combination containing all the fractions was most stimulatory but significantly less active than the crude extract suggesting that some of the components were lost or deactivated during fractionation. The response of larvae to the mixture containing the less polar fractions was not significantly different from control showing that they play a synergistic role in the mixture. Fraction PM-4, which forms about 76 percent of the total extract when tested alone was significantly more stimulatory than control.

4.16 Chromatographic analysis of the methanolic fractions

The four methanolic fractions were examined by HPLC on a reverse phase analytical column under conditions identical to those used for the fractions of the ethyl acetate extract using a UV detector at 240 nm. Fraction PM-1 did not show any well-defined components. Fraction PM-2 contained only two major components, one of which corresponded to *p*-hydroxybenzaldehyde and a second highly polar component (R_t = 2 min) (J-1) which was also present in a larger proportion in fraction PM-4 (Fig. 4.10). PM-3 and PM-4 contained a common component (J-2) in addition to a series of polar and less polar compounds, one of which was *p*-hydroxybenzoic acid (Fig. 4.10). Since the chromatographic data of the crude methanol extract (Fig. 4.1c) did not show the presence of *p*-hydroxybenzaldehyde and



HPLC profiles of the partition phases of the methanol extract of the whorls of the 3 week old plants of IS 18363.

from top to bottom. PM-2, PM-3 and PM-4.

A-J-1, B-p-hydroxybenzoic acid, C-J-2, D-p-hydroxy-benzaldehyde.

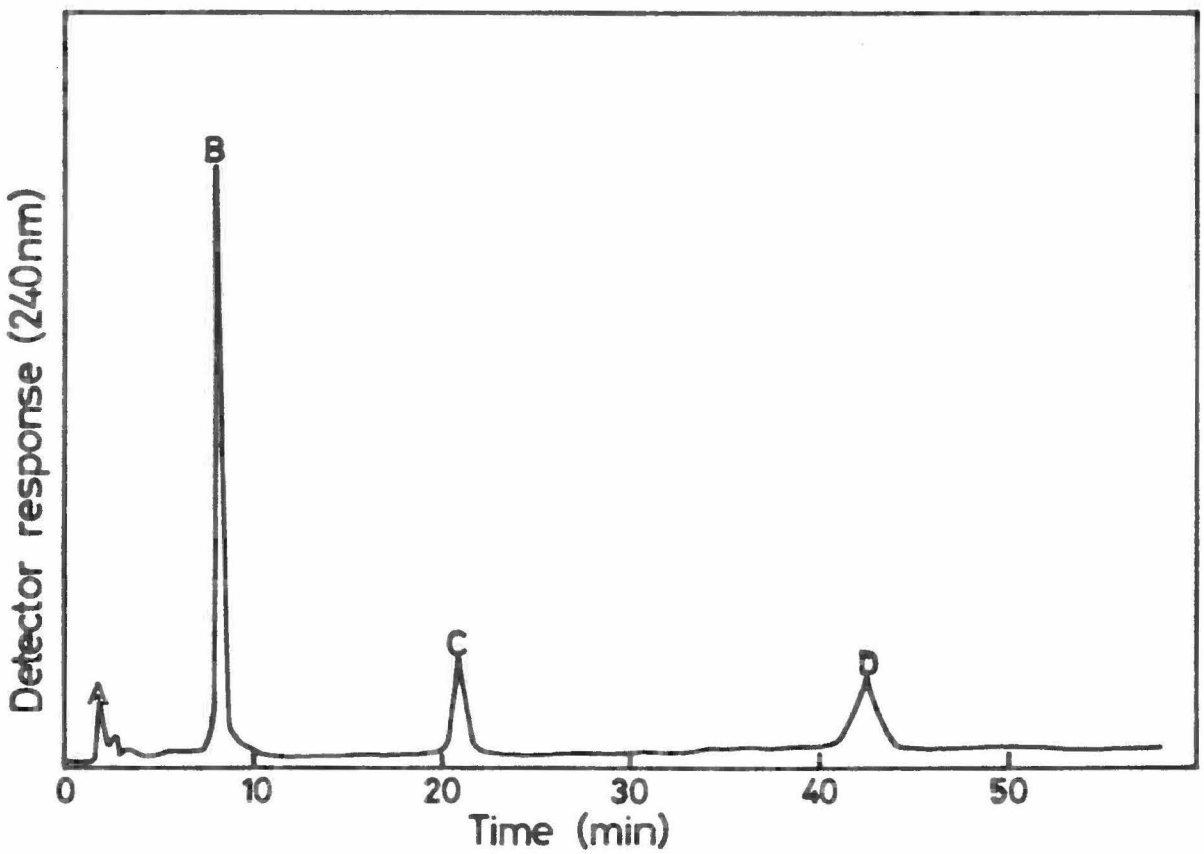
Fig. 4.10

p-hydroxybenzoic acid in the extract, it appears that these two components might be degradative products of some other components in the extract. Since PM-3 and PM-4 between them contained all the unidentified compounds they were subjected to further analysis as described below.

(a) Micropreparative HPLC analysis of PM-3 and TLC of PM-4.

These were carried out to isolate pure samples of J-2. A sample of PM-3 (20 mg) was chromatographed on a DuPont Zorbax ODS 25 cm x 4.6 mm column. Five fractions were collected (PM-3-I to PM-3-V). Of these PM-3-II and PM-3-V corresponded to *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde respectively. PM-3-III which was obtained as a dark yellow solid corresponded to the unidentified compound J-2. No significant quantities of materials were obtained from fractions PM-3-I and PM-3-IV.

Further chromatographic analysis of this sample of J-2 on an HPLC reverse phase column showed that a proportion of it had decomposed into two relatively less polar components J-3 and J-4, among others (Fig. 4.11). These results confirm the earlier suggestion of the presence of breakdown components in some of the fractions of the methanol extract. J-3 and J-4 were isolated as a mixture from micropreparative HPLC of J-2 on a reverse phase column. GC-MS analysis of this mixture showed that J-3 was a major breakdown component



HPLC profile of J-2 showing its breakdown components.

A-unknown, B-J-2, C-p-hydroxybenzaldehyde and D-p-methoxybenzyl alcohol.

Fig·4·11

of J-2, and this was identified as *p*-hydroxybenzaldehyde. The minor component J-4 was identified as *p*-methoxybenzyl alcohol. Both components were identified on the basis of their mass spectral data and GC coinjection with authentic samples. The mass spectrum of *p*-methoxybenzyl alcohol showed peaks at M^+-138 (70), 121 (42), 109 (96), 94 (52), 77 (100), 65 (35), 51 (52) and 39 (80). The identities of the two compounds were further established by HPLC coinjection on a reverse phase column with authentic samples (*p*-hydroxybenzaldehyde R_t=20 min, *p*-methoxybenzyl alcohol R_t=43 min). These results suggested that component J-2 had the characteristics of a ketal, with *p*-hydroxybenzaldehyde and *p*-methoxybenzyl alcohol moieties of the molecule.

Since a pure sample of J-2 could not easily be isolated by micropreparative HPLC of PM-3 (Fig. 4.10), an attempt was made to isolate it from PM-4 by conventional thin layer chromatography. Analysis of PM-4 on silica gel/UV_{254} (0.25 mm thickness) in solvent system methanol/hexane 3:1 showed two spots, both of which fluoresced blue in UV light. A sample of PM-4 (0.44 g) was dissolved in water and separated by preparative tlc in the same solvent system. The two fractions were extracted with methanol, the resulting extract filtered and concentrated to dryness in vacuo. The leading fraction gave a highly hygroscopic pale yellow solid (0.17 g) which was identified as J-2 and the trailing fraction a dirty white solid (0.03 g) identified as J-1 by

comparison of their HPLC profiles with that of a sample of PM-4.

J-1 was recrystallized from methanol to give a white solid mp > 250 ^{o}C. The mass spectral data for this compound were m/e (%) M^{+}-502 (100), 347 (60), 303 (14), 229 (18), 191 (18), 60 (14) and 44 (90). The peaks at m/e 60 and 44 were suggestive of fragments of a carboxylic acid. Thus the physical data of J-1 were suggestive of a high molecular weight carboxylic acid.

Further purification of J-2 was achieved by chromatography on silica gel in the same solvent system. The mass spectrum of this compound showed that it was a mixture of six related compounds (J-2-1 to J-2-6) with a common fragmentation pattern. The m/e (%) values for peaks corresponding to these components in the mixture were as follows:

J-2-1, 236 (8), 193 (8), 177 (18), 165 (100), 151 (18), 101 (8), 73 (8), 59 (26), 55 (12), 43 (100) and 39 (30);

J-2-2, 193 (6), 177 (6), 163 (12), 150 (12), 101 (2), 73 (6), 59 (12), 55 (12), 43 (100), 39 (42);

J-2-3, 193 (6), 177 (12), 163 (12), 151 (10), 101 (4), 73 (12), 69 (6), 60 (18), 55 (16), 43 (100), 39 (18);

J-2-4, 206 (2), 193 (4), 177 (10), 164 (8), 153 (10), 97 (10), 73 (8), 69 (12), 60 (12), 55 (12), 43 (100), 39 (40);

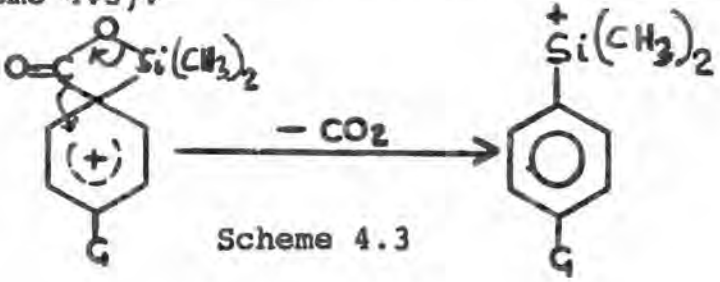
J-2-5 206 (2), 193 (6), 177 (18), 165 (16), 152 (10), 97 (10), 83 (8), 73 (6), 69 (10), 60 (14), 55 (20), 43 (100), 39 (30);
J-2-6 206 (2), 177 (8), 165 (8), 153 (8), 97 (10), 83 (6), 73 (4), 69 (12), 60 (10), 55 (12), 43 (100), 39 (36).

Peaks which were common to these components were at the following m/e values: 193, 177, 165, 151, 101, 73, 60, 43 and 39. The peaks at m/e 193, 177 and 165 were also present in the mass spectrum of the acetyl derivative of ferulic acid. The peak at m/e 60 was suggestive of a carboxylic acid moiety in the molecule. These results suggested that components J-2-1 to J-2-6 may be phenolic in nature and may be related to ferulic acid or some other phenolic compounds in the plant. To confirm the presence of these components in J-2, the latter was converted into a more volatile derivative and the product analysed by GC-MS. Details of this are described below.

(b) GC-MS analysis of trimethylsilyl derivatives of J-2.

1 mg of a sample of J-2 in 100 μl of dry pyridine was derivatized in a 1 ml sample vial with 100 μl of a solution of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS). The mixture was capped and heated to 60 ^{o}C for 16 hr. GC-MS (Fig. 4.11b) analysis revealed, as expected, the trimethylsilyl

derivatives of a mixture of components which appeared to be related. Peaks common to these compounds were at the following m/e values; 44, 57, 73, 103, 117, 129 or 133, 147, 205, 217. As suggested by Budzikiewicz et al., (1967), the peak at m/e 73 is due to the trimethylsilyl cation $(CH_3)_3Si^+$ but that at m/e 147 is an artefact due to the ion $(CH_3)_3Si=\overset{+}{O}-Si(CH_3)_2$ which is formed by expulsion of a methyl group from hexamethyl disiloxane $(CH_3)_3Si-O-Si(CH_3)_3$, a condensation product of trimethylsilanol $(CH_3)_3SiOH$. Of particularly interest was the peak at m/e 44 which corresponds to the expulsion of CO_2 from a trimethylsilyl benzoate (Scheme 4.3).



Scheme 4.3

An intense M-CH_3 peak which can be used for molecular weight determination of trimethylsilyl ethers (Budzikiewicz et al., 1967) appeared to be present in the spectra of all the derivatives in the mixture. These peaks were at m/e 243, 365, 379 and 437 and were found for more than one component in the spectra suggesting the presence of isomeric compounds in the mixture. The spectra were compared to those of the TMS derivatives of 8 standard phenolic compounds which include *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, syringic acid, *p*-hydroxybenzyl

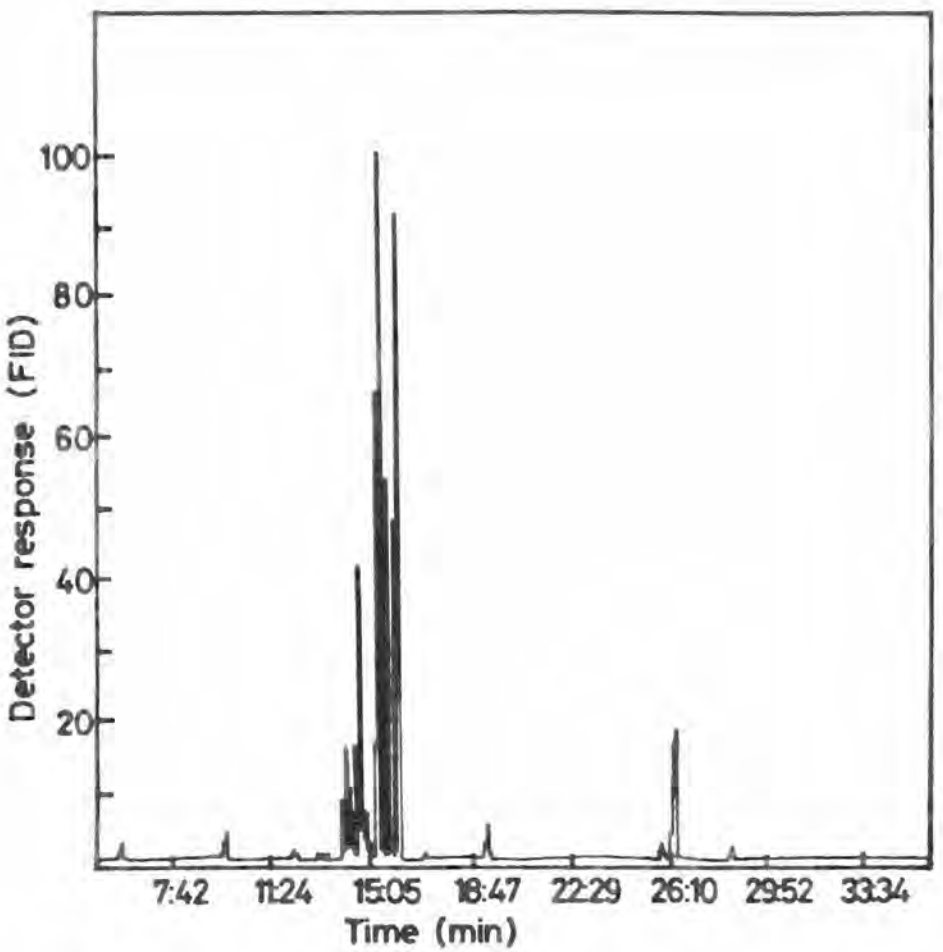
alcohol, gentisic acid and caffeic acid but none was identical to any of the components in J-2. However, the general fragmentation pattern of the TMS derivatives of these compounds was similar to that of the components in J-2 confirming that components in the latter were phenolic in nature. The precise structural identities of the components in J-2 were not clear so no further work was carried out on it.

(c) GC-MS analysis of acetylated sample of PM-3.

Since in addition to J-2, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid, PM-3 also contained minor quantities of polar and less polar components, an attempt was made to identify these by GC-MS of their acetylated derivatives. Acetylation of PM-3 was carried out in acetic anhydride in dry pyridine at 0 ^{o}C for 24 h. GC-MS (Fig. 4.12) revealed the presence of the acetyl derivatives of *p*-coumaric, caffeic, and ferulic acids and other high molecular weight compounds. Some of these showed fragmentation patterns charateristic of acetyl derivatives of higher phenols (P1, P2 and P3). The rest were characteristic of hydrocarbon compounds. The peaks for P1, P2 and P3 were at the following m/e values:

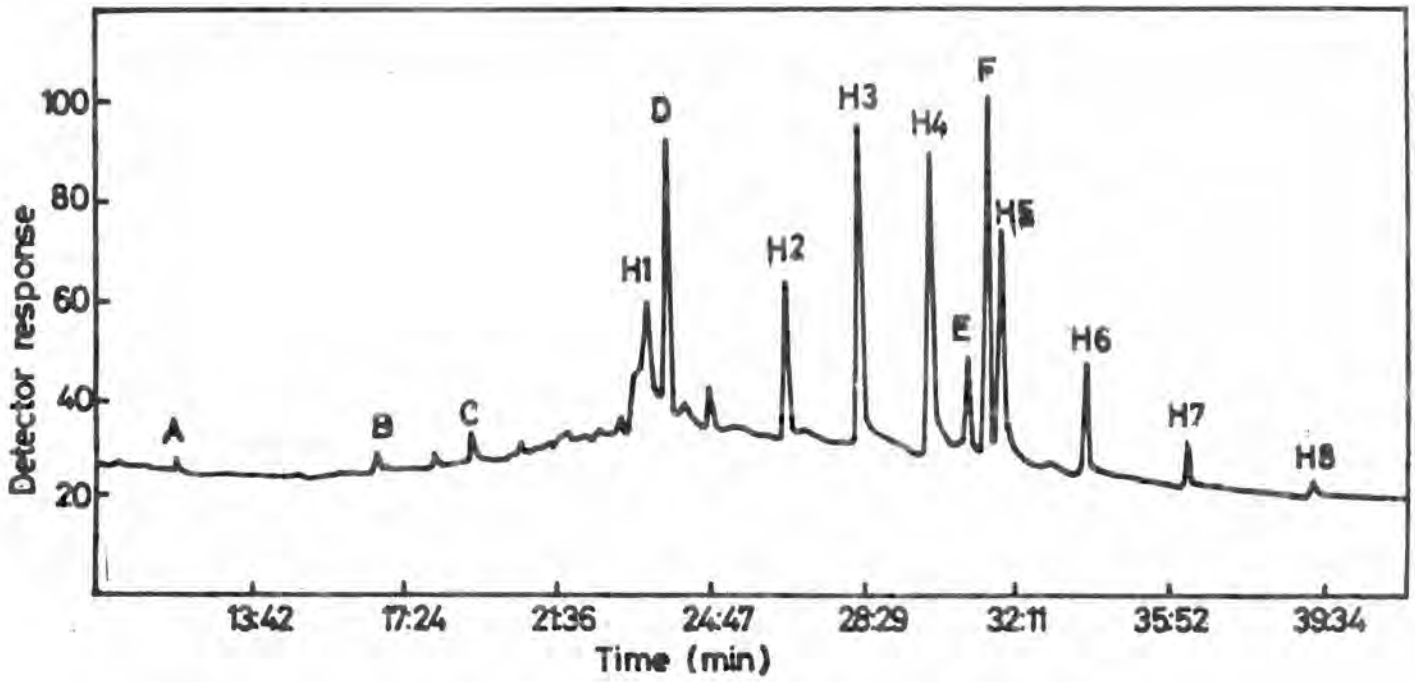
P1, M^{+}-324 (20), 309 (100), 147 (10), 133 (15), 119 (36), 103 (18), 91 (48), 77 (12), 57 (80) and 41 (72).

P2, M^{+}-330 (35), 315 (100), 253 (8), 237 (22), 178 (8), 165



Gas chromatogram of the trimethylsilyl (TMS) derivatives of the components in J-2.

Fig. 4.11b



Gas chromatogram of the acetylated product of PM-3.

A,B and C are peaks representing the acetyl derivatives of p-coumaric, ferulic and caffeic acids. D,E and F represent P1, P2 and P3 described in the text. H1-H8 are peaks representing hydrocarbon compounds in the product.

Fig.4.12

(62) and 41 (94).

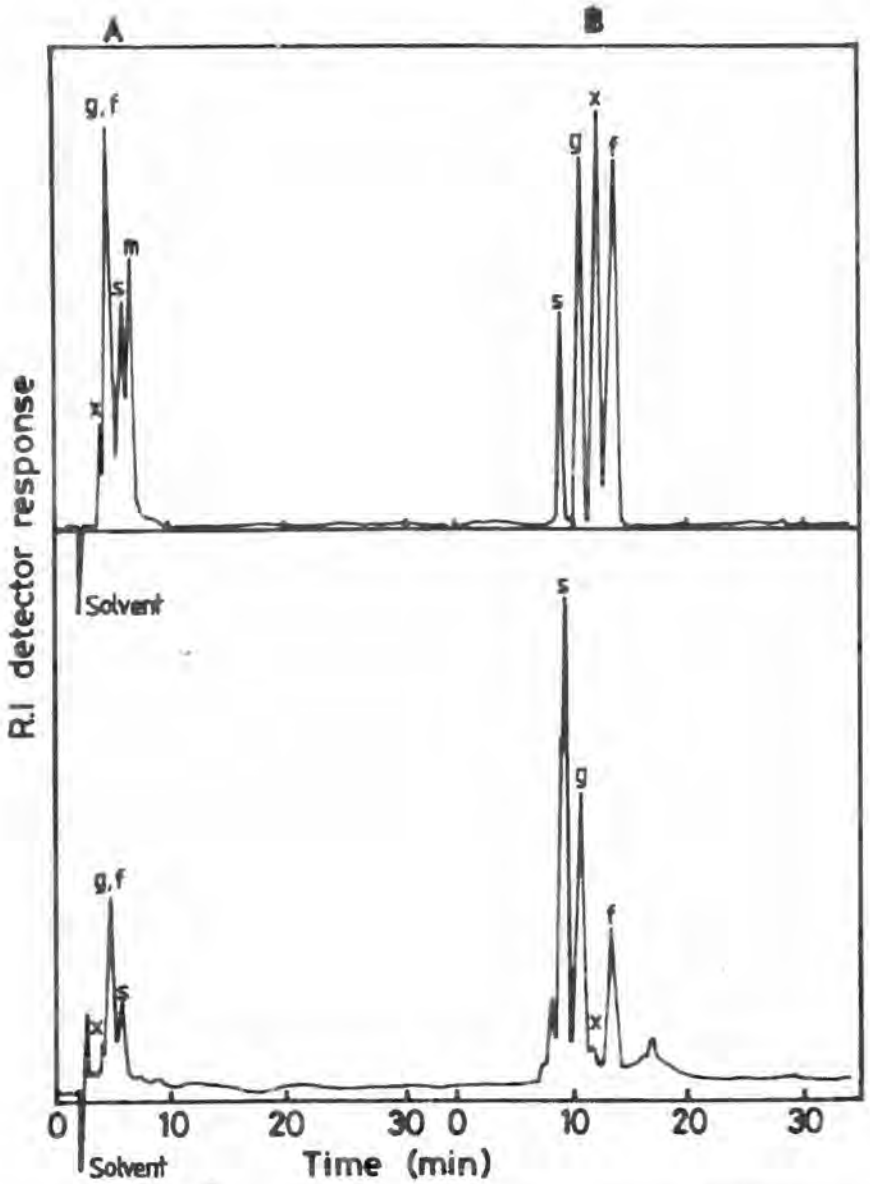
P3, M^+-386 (28), 371 (62), 293 (28), 178 (22), 165 (6), 139 (8), 119 (68), 103 (38), 91 (100), 77 (22), 57 (80) and 41 (85).

(d) Chromatographic analysis of sugars in PM-4

PM-4 was found to be water soluble, containing both chromophoric J-1 and J-2 (UV detection at 240 nm) and non-chromophoric compounds (RI detection). Attempts were made to separate all the components of this fraction by various chromatographic methods. These methods included column chromatography on Pharmacia Sephadex G-10 and Sephadex LH-20 with a UV monitor at 254 nm and reverse phase thin layer chromatography on Merck RP-18 F_{254S} plates. However, these methods did not give very satisfactory separation of the components in fraction PM-4. A sample of the crude fraction was analysed by PMR in D_2O on a Varian XL 200 MHz. The PMR spectrum showed a concentration of signals between 3 and 4 ppm which are signals characteristic of the protons of a carbohydrate (Lemieux and Stevens, 1966). Fraction PM-4 was therefore subjected to carbohydrate analysis on two different columns. (a) a Biorad carbohydrate column, Aminex HPX-87C, 30 cm x 7.8 mm and (b) a Varian normal phase column, Micropak NH_2-10, 30 cm x 4 mm. The effluents from these columns were monitored by a refractive index detector (Varian RI-3).

An aqeuous solution of PM-4 (0.3 mg/μl) was prepared and 40 μl aliquot of the solution were analysed separately on the two columns. The solvent system for the analysis on the Biorad column was degassed double distilled water and this was maintained at a flow rate of 0.6 ml/min at 80 ^{o}C. For the normal phase analysis, the Micropak NH_2-10 column was eluted with 70% aqueous acetonitrile at a flow rate of 1.4 ml/min at 25 ^{o}C. Standard sugars which included sucrose, glucose, fructose, galactose, mannose and xylose (Sigma Chem., Co.) were similarly analysed both individually and in a mixture. The identities of the sugars in fraction PM-4 were established by comparison of the chromatograms of the standards from the two analytical columns with those of PM-4.

Fig. 4.13 shows the HPLC profiles of the mixture of standard sugars and fraction PM-4. The upper and lower chromatograms represent the standard sugar mixture and fraction PM-4 respectively analysed on (a) Micropak NH_2-10 and (b) Biorad Aminex HPX-87C columns. The sugars identified by comparison of the chromatograms were sucrose, fructose, glucose and xylose. The identities of these sugars in fraction PM-4 were confirmed by coinjection with authentic samples on the two HPLC carbohydrate columns.



HPLC profiles of a mixture of standard sugars (upper profile) and a sample of the aqueous fraction (lower profile) of the methanol extract of the whorls of the 3 week old plants of IS 18363 analysed on two different columns, A-Micropak NH_2 and B-carbohydrate Biorad Aminex HPX-87C.

s-sucrose, g-glucose, x-xylose, f-fructose, m-maltose

Fig. 4.13

4.17 Bioassays of components from methanolic extract

Feeding bioassays were carried out on all the sugar and nonsugar components in fraction PM-4. Each component was tested at 10, 25, 50, 150 and 250 µg.

(a) Nonsugars J-1 and J-2 (chromophoric components).

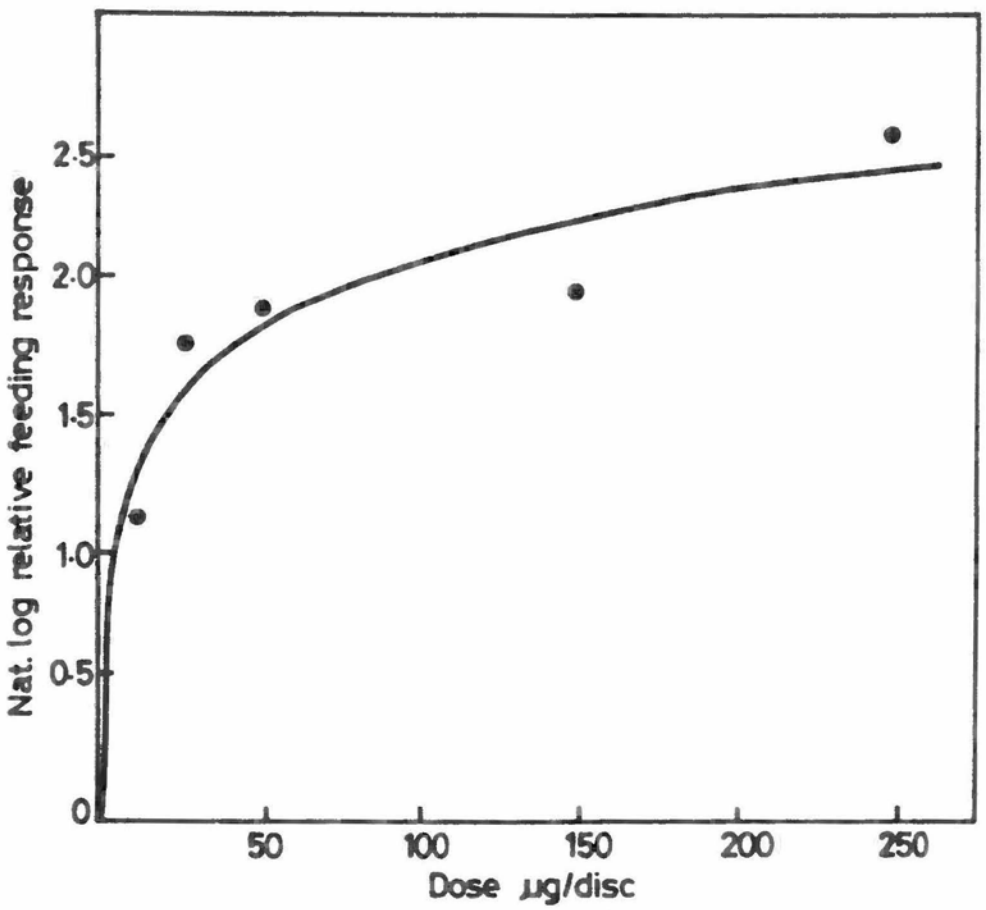
Component J-1 was non-stimulatory at all the doses tested.

Fig. 4.14 shows the dose response curve for component J-2 expressed in the regression equation $\ln y = 0.4435 + 0.3542 \ln x$ where y = relative feeding response and x = dose, µg/disc.

The dose response curve showed that J-2 stimulated larval feeding at all the doses tested.

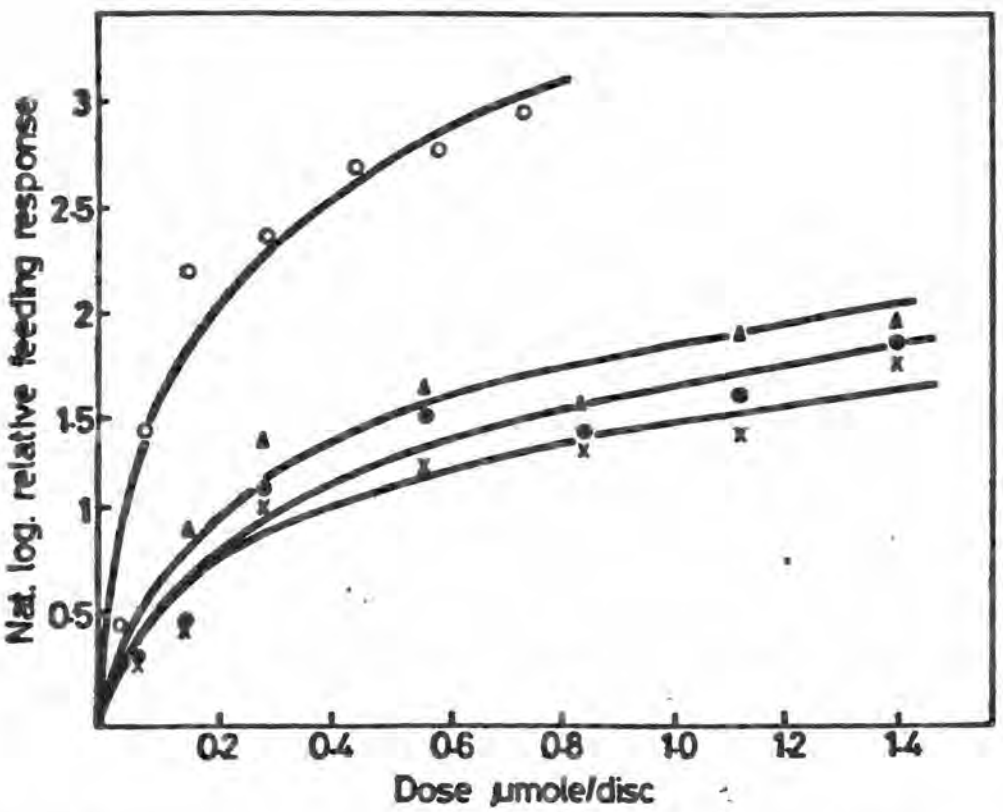
(b) Sugars (non chromophoric components)

Fig. 4.15 shows the dose response curves for the different sugars which were expressed in the regression equation $\ln y = \ln a + b \ln x$ where y = relative feeding response and x = dose, µmole/disc. These equations were as follows: sucrose, $\ln y = 3.2902 + 0.7539 \ln x$; glucose,



Dose response curve for the total mixture of components i
J-2.

Fig·4·14



Dose response curves for the sugar components in the methanolic extract of the whorls of the 3 week old plants of IS 18363.

O sucrose, Δ glucose+fructose, ● glucose, × fructose.

Fig·4·15

ln y = 1.6158 + 0.5108 ln x; fructose, ln y = 1.4632 + 0.4364 ln x; glucose + fructose, ln y = 1.8618 + 0.4918 ln x.

The dose response curves showed that sucrose was most stimulatory to the larvae followed by glucose and fructose, both of which were similar in their effect on the feeding of the larvae. Larvae did not respond to xylose at any of the doses tested. A mixture of glucose and fructose in a 1:1 ratio was more stimulatory to larvae than the individual sugars tested alone, but less stimulatory than sucrose.

(c) A mixture of *p*-hydroxybenzaldehyde and sucrose

Tests were performed for synergism between sucrose and *p*-hydroxybenzaldehyde. Cellulose acetate discs were loaded with 10 µg of sucrose and air dried. In a previous assay, *p*-hydroxybenzaldehyde was tested at 10, 25, 50, 150, 250, 350 and 450 µg. In this experiment, the sucrose treated discs were loaded with the same doses of *p*-hydroxybenzaldehyde and bioassayed for third-instar larval feeding response.

The dose response curves for *p*-hydroxybenzaldehyde with or without sucrose are shown in Fig. 4.16. These were

expressed in the regression equation ln y = ln a + b ln x where y = relative feeding response, x = dose of *p*-hydroxybenzaldehyde in μ mole/disc; a,b and c are constants. The regression equations were: *p*-hydroxybenzaldehyde, ln y = 2.5288 - 0.7251x + 0.5462 ln x; *p*-hydroxybenzaldehyde + sucrose, ln y = 3.8739 - 0.9215x + 0.9023 ln

.

The dose response curves showed that *p*-hydroxybenzaldehyde alone stimulated feeding at all the doses tested in the above experiment but the effect of it decreased with increasing dose. Addition of 0.03 μ mole (10 μg) sucrose to *p*-hydroxybenzaldehyde at the doses tested increased larval feeding response by an increment which was significantly greater than a simple summation of the activities of the two compounds would suggest. This suggested that *p*-hydroxybenzaldehyde and sucrose acted synergistically to give enhanced feeding response.

4.18 Sugar and nonsugar levels in the methanol extracts

The relative amounts of the individual sugars identified in the aqueous fractions of the methanol extracts of the two cultivars at the two growth stages were determined from a calibration of a standard solution containing a mixture of the sugars. These sugars were sucrose, glucose, fructose and xylose, and the

respectively. 5, 10, 15, 20, and 30 µl aliquots of these standard solutions were analysed on the Biorad carbohydrate column, Aminex HPX-87C and the effluent monitored by a RI detector. The methanol extracts of the whorls of the 3 and 6 week old plants of IS 18363 and IS 2205 were fractionated as previously described (page 102) and 40 µl of each of the resulting aqueous fractions similarly analysed. The peak areas corresponding to the different sugars in the extracts were calculated and in amounts in micrograms. The total amount of nonsugar components in each of the fractions was calculated by difference. The total amount of sugar in the whorls of the two cultivars at the two growth stages were also calculated. The data on these calculations are shown in Tables 4.8 and 4.9.

Table 4.8 Sugar and nonsugar levels in 12 mg of each of the aqueous fractions of the methanol extracts.

Aqueous fraction	Plant age (wks)	Suc (mg)	Glu (mg)	Fru (mg)	Xyl (mg)	Total sugar (mg)	Total nonsugar (mg)
IS 18363	3	5.3	2.5	1.9	0.6	10.3	1.7
IS 2205	3	4.5	2.5	1.6	0.5	9.1	2.9
IS 18363	6	4.8	1.8	2.1	0.2	8.9	3.1
IS 2205	6	5.4	2.4	2.3	0.2	10.3	1.7

Table 4.9 Sugar levels in the whorls % (mg/kg whorl)

Cultivar	Plant age(wks)	No of whorls	wt (kg)	Total sugar (mg)	% sugar
IS 18363	3	500	3.5	10.3	0.6
IS 2205	3	800	3.4	9.1	0.3
IS 18363	6	80	3.5	8.9	3.2
IS 2205	6	80	2.9	10.3	4.4

The findings from these data were as follows:

(a) sugars formed between 65 to 85 percent of the mixture in the aqueous fractions of the methanol extracts of the whorls of the two cultivars at the two growth stages.

(b) the amounts of the identified sugars in the fractions were in the order sucrose > glucose $\simeq$ fructose > xylose.

(c) there was more sugar in the whorls of the 3 week old plants of the susceptible cultivar IS 18363 than those of the resistant cultivar IS 2205. The sugar levels in the whorls of the 6 week old plants of the two cultivars were approximately the same, but were higher than the levels found for the 3 week old plants.

CHAPTER 5

DISCUSSION

Previous studies on the effect of plant chemicals on the feeding of phytophagous insects have involved bioassays employing different feeding substrates such as agar/cellulose (Hsiao and Fraenkel, 1968), ordinary filter paper (Daad, 1960; Wensler and Dadzinski, 1972), styropor (Meisner and Ascher, 1968) and glass-fibre filter paper (Staedler and Hanson, 1976; Woodhead and Bernays, 1978). In addition, membrane filter discs, which are available in sizes within a narrow weight range, have been used with advantage (Bristow *et al.*, 1979; Doss *et al.*, 1980; Doss *et al.*, 1982; Albert *et al.*, 1982 Capinera *et al.*, 1983; Doss and Shanks, 1984; Doss and Shanks, 1986). For bioassays in this study, cellulose acetate membrane filter discs were tested and found to be well suited for monitoring the feeding responses of the third-instar larvae to sorghum extracts. However, contact with methanol caused slight distortion of the discs and were poorly fed on by the larvae as shown by the results in Table 4.2. Similar observations were made by Doss and Shanks (1986) who found that contact with organic solvents caused membrane filters either to dissolve or to wrinkle. However, our experimentation showed that addition of water to solvent-treated discs restored

their palatabilty to the larvae since no significant differences were found between the feeding responses of larvae to these discs and to control, hexane, and water treated discs (Table 4.2). The procedure of adding water was thereafter adopted for all discs in the bioassays.

In no choice tests, third-instar larvae of *C. partellus* were found to exhibit a dose-dependent response to crude extracts of sorghum cultivars IS 18363 and IS 2205 applied to cellulose acetate discs, confirming the effectiveness of the bioassay procedure. The results of the bioassays of the crude extracts of hexane, ethyl acetate and methanol showed that none of them was deterrent to the feeding of the larvae although this did not rule out the presence of deterrent components in these extracts. The methanol extracts were most stimulatory and showed a more linear dose/response relationship than the other crude extracts which were tested (Figs. 4.2a and 4.2b; Tables 4.3a and 4.3b). Ethyl acetate extracts were intermediate in stimulatory activity and these were followed by the hexane extracts. The stimulatory activities of the hexane extracts were consistent with previous results (Roome and Padgham, 1978) in that larvae of this insect showed little preference for a lipid extract of the whorls of sorghum plants.

Extracts of the whorls of the 3 week old plants were more stimulatory to larvae than those of the 6 week old

plants. These results were consistent with earlier findings (Alghali, 1985) in that leaf feeding and overall plant damage by the larvae of *C. partellus* was more acute at the younger than the older vegetative stages.

The bioassay tests showed that at both growth stages (ie. 3 and 6 weeks old plants), larvae responded more to extracts of the susceptible cultivar IS 18363 than to those of the resistant cultivar IS 2205 (Figs. 4.2a and 4.2b). These results are in agreement with observations made earlier where larval feeding was found to be high on the leaves of IS 18363 but medium on IS 2205 (Saxena, 1985b). HPLC analyses showed quantitative rather than qualitative differences between the crude extracts of these two cultivars (Figs. 4.1a, 4.1b and 4.1c). The chromatographic data suggested that the methanol and ethyl acetate extracts contained some common components (Figs. 4.1b and 4.1c) but the bioassay results suggested that the more potent phagostimulatory compounds were in the methanol extract. Unfortunately, no detailed analytical studies could be carried out on the least stimulatory hexane extracts which are likely to contain wax components shown in earlier studies to comprise n-alkanes, aldehydes, fatty acids and esters (Woodhead, 1983; Avato *et al.*, 1984).

When fractions of the ethyl acetate and methanol extracts obtained by partitioning between organic and

aqueous phases were tested, it became apparent that these fractions were less stimulatory than their mother extracts (Fig. 4.5 and 4.9). For both extracts, the more potent fractions were the aqueous ones. When the fractions were recombined the activities of the crude extracts were restored suggesting that several groups of compounds combined additively or synergistically to give the enhanced feeding activity of the mother extracts.

Phagostimulatory compounds identified in the ethyl acetate extracts of the whorls of sorghum cultivars IS 18363 and IS 2205 for the third-instar larvae of *C. partellus* were all phenolic. The major ones were *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid previously reported to occur in the surface wax of sorghum cultivars studied by Woodhead et al. (1982) and Haskins and Gorz (1985). In addition, ferulic, caffeic and *p*-coumaric acids were present as minor components. Since hydroxyaromatic acids rarely occur in the free form in plants (Harborne, 1964) but occur as soluble esters or O-glycosides (Harborne and Corner, 1961), or as insoluble esters bound to the cell wall (Hartley and Jones, 1977), the relatively large proportion of *p*-hydroxybenzoic acid found in the extracts may have been due to formation by oxidation of *p*-hydroxybenzaldehyde.

Phagostimulatory compounds identified in the methanol extracts of the whorls of the two cultivars included the

phenolics mentioned earlier and sugars. These sugars comprised sucrose, glucose, fructose and xylose, identified by comparison of their HPLC retention times and coinjection with authentic samples on two columns: (a) carbohydrate Biorad Aminex HPX 87C and (b) normal phase Micropak NH_2-10. Sucrose accounted for the largest proportion by weight of the sugars in the methanol extracts followed by either glucose or fructose and then xylose (Table 4.8). Components J-1 and J-2 were isolated as nonsugar components from the aqueous fraction of the methanol extract. J-1 was non-stimulatory at all the doses which were tested whilst J-2 stimulated larval feeding (Fig. 4.14). The latter compound was found to be a mixture of related phenolic derivatives (Fig. 4.10b). The fragmentation patterns of the components in this mixture were suggestive of glycosides of some of the phenolic compounds identified in the extracts including those of *p*-hydroxybenzaldehyde, ferulic and caffeic acids, all of which are known constituents of sorghum seedlings (Saunders *et al.*, 1978; Woodhead and Cooper-Driver, 1979). It was apparent from its chromatographic performance that J-2 was thermally labile, and partially decomposed to components two of which were identified as *p*-hydroxy-benzaldehyde and *p*-methoxybenzyl alcohol (Fig. 4.4). It appears that J-2 is not the cyanogenic glucoside dhurrin present in high concentration in younger than older sorghum leaves, and whose hydrolysis products are HCN and p-hydroxy-benzaldehyde (Woodhead and Bernays, 1978). The exact

structural identities of J-2 components are not clear. However, it is possible that they act as reservoirs for *p*-hydroxybenzaldehyde and other phenolic compounds in the plant. Identification of these compounds, and investigation of their biological roles in relation to sorghum pests are clearly warranted.

Phenolics have previously been isolated from the leaves of sorghum and have been shown to be responsible for resistance against *L. migratoria* and *S. graminum* feeding (Woodhead and Bernays, 1978; Dreyer *et al.*, 1981; Woodhead, 1982). On the other hand, *C. partellus* larvae have been found to feed on artificial diets containing these phenolics at levels up to three times the quantities that are normally found occurring in sorghum (Roome and Padgham, 1978). A similar observation was made by Fisk (1980) working with the homopteran insect *Peregrinus maidis* (Ashun) on a phenolic extract from sorghum. Baker et al. (1968) found *p*-hydroxybenzaldehyde and some of its derivatives strong feeding stimulants for the elm bark beetle *Scolytus multistriatus*. In this study, third-instar larvae of *C. partellus* were stimulated to feed by *p*-hydroxybenzoic acid and *p*-hydroxybenzaldehyde and some derivatives of these compounds (Fig. 4.6). The feeding responses of larvae to increasing doses of these phenolic compounds followed broadly the same pattern, reaching an optimum and then dropping. However, they varied with the nature of

substitution of the phenolic compound suggesting that the different functionalities on the benzene ring may play a significant role in the food discriminative behaviour of the larvae. A comparison of the hydroxy compounds (Fig. 4.6) showed that *p*-hydroxybenzaldehyde was most stimulatory followed by *p*-hydroxybenzoic acid and *p*-hydroxybenzyl alcohol in that order. It may be pointed out that under the bioassay conditions of this study, very little of *p*-hydroxybenzaldehyde is oxidised so the activity observed is essentially due to the aldehyde. These results follow closely that obtained by Baker *et al.*, (1968) for the elm bark beetle where *p*-hydroxybenzaldehyde elicited the strongest feeding response from the insect. A comparison of the activities of the methoxy derivatives of these compounds, showed that larvae responded best to *p*-methoxybenzyl alcohol and poorest to *p*-methoxybenzaldehyde (Fig. 4.6), a complete reversal of the trend observed for the hydroxy compounds. Comparing the feeding responses of the larvae to *p*-hydroxybenzaldehyde and to its biogenetic analogues tested in this study showed that *p*-hydroxybenzaldehyde was the most potent feeding stimulant whilst its methoxy derivative was the weakest. These findings suggest that *p*-hydroxybenzaldehyde probably plays a prominent role in larval discrimination between potential foods. Bioassays of the cinnamic acids present in the ethyl acetate and methanol extracts showed that *p*-coumaric acid was non-stimulatory at all the doses tested, whilst ferulic

and caffeic acids gave similar feeding rates (Fig. 4.7), both being effective at very low doses. Caffeic and ferulic acids differ from *p*-coumaric acid in having an extra oxygen function at the 3-position in the benzene ring which may thus be important for the activities of this class of phenolic compounds.

Comparison of hydroxybenzoic acid and its analogues with cinnamic acids showed that the former were generally more stimulatory than the latter. Thus *p*-hydroxybenzoic acid (4-hydroxybenzoic acid) was more stimulatory to larvae than *p*-coumaric acid (4-hydroxycinnamic acid); vanillic acid (4-hydroxy-3-methoxybenzoic acid) was more stimulatory than ferulic acid (4-hydroxy-3-methoxycinnamic acid); and protocatechuic acid (3,4-dihydroxybenzoic acid) was also more stimulatory than caffeic acid (3,4-dihydroxy-cinnamic acid). Of particular interest was the complete lack of response to gentisic acid (2,5-dihydroxybenzoic acid), a known phenolic constituent of sorghum seedlings (Woodhead and Cooper-Driver, 1979). This shows that the positions of the functional moieties in the benzene ring are crucial for the activity of the compound.

Bioassays were also carried out on chlorogenic acid, a common plant constituent (Sondheimer, 1964). Its role in host plant selection by some insects has been reported. Kato and Yamada (1966) reported that it served as a growth

factor for silkworm larvae, and Hsiao and Fraenkel (1968) reported that it was a feeding stimulant in potato leaves for the Colorado potato beetle *L. decemlineata*. Chawla *et al.*, (1974) found that incorporation of chlorogenic acid in an artificial diet improved the growth of the potato aphid *Macrosiphum euphorbiae*. Matsuda and Senbo (1986) reported that it deterred feeding by *Lochmaeae capreae cribata* but was stimulatory to *Gastrophysa atrocyanea*, both of which feed on salicaceous plants. In the present study, the compound stimulated larval feeding but became less stimulatory at higher doses (Fig. 4.7). It was a better stimulant than its degradative products, quinic and caffeic acids (Fig. 4.7) both of which elicited weak stimulatory responses from the larvae at very low doses, although quinic acid was a better stimulant than caffeic acid.

Although only a few phenolic compounds were investigated in this study, some tentative inferences can be made from the results. The results suggest that there are chemoreceptors in the mouthparts of the third-instar larvae of *C. partellus* which perceive different classes of phenolic compounds and their derivatives. This may suggest the involvement of generalised receptors. However, the different structural requirements for the activities of benzoic and cinnamic compounds suggest that different groups of receptors may be present. Detailed sensory physiological studies may help to shed some light on the question.

Sugars have been demonstrated as strong phagostimulants in a large number of insect species (Thorsteinson, 1960; Dethier, 1966; Schoonhoven, 1968; Sutherland, 1977; Staedler, 1983). In this study, sucrose, fructose and glucose stimulated the feeding of the third-instar larvae of *C. partellus*. Sutherland (1977) reported that these sugars were ubiquitous nutrient chemicals which function as phagostimulants for general plant feeders. The data for *C. partellus* (Fig. 4.5) showed that sucrose was most stimulatory and it exhibited a more linear dose/response relationship than the other sugars which were tested. These results are in agreement with previous reports on the strong stimulatory activity of sucrose to a wide variety of insects feeding on a diverse range of plants (Dethier, 1966; Hsiao and Fraenkel, 1968; Hsiao, 1969; Peacock and Fisk, 1970; Sutherland, 1971; 1977; Doss and Shanks, 1984; Ladd, 1986; Shanks and Doss, 1987). Glucose and fructose which are hexose sugars gave similar feeding rates in our study; but xylose, which is a pentose sugar was non-stimulatory at all the doses tested. In a recent study, Ladd (1986) found that xylose, which is generally unknown as an insect feeding stimulant (Chippendale, 1978), elicited a weak stimulatory feeding response from the Japanese beetle, *Popillia japonica* Newman, but noted that his results were not conclusive. Our results are in agreement with previous reports on the

activities of pentose and hexose sugars (Chippendale, 1978; Ladd, 1986) in that hexose sugars are more stimulatory to phytophagous insects than pentose sugars. In summation, apart from phenolic receptors, it appears that the larvae have receptors which perceive sugars, but they seem to be more receptive to sucrose than to the other sugars which were identified in the extracts.

The feeding tests on blends of some of these phagostimulatory compounds revealed interesting results. The stimulatory activity of a blend of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in a proportion occurring in the crude ethyl acetate extract was virtually due to the activity of *p*-hydroxybenzaldehyde suggesting no synergism between the two compounds (Fig. 4.8). It appears that the absolute amount of *p*-hydroxybenzaldehyde in the plant may play a significant role in the feeding of the larvae. It would be interesting to assay a blend of all the phenolic components in the proportion they are found occurring in the plant to elucidate more precisely the relative importance of phenolics in the feeding of the larvae of this insect.

Bioassays of a blend of glucose and fructose in equal ratio (Fig. 4.15) gave a response which appeared to be a simple addition of the activities of the two hexoses as there was no evidence of synergistic effect. The stimulatory activity of this blend was also significantly

less than that of sucrose showing clearly that the activity of the dissacharide is due to its total structure and not to a summation of its component moieties. The results of the bioassays of a blend of *p*-hydroxybenzaldehyde and sucrose, on the other hand, indicated synergism between the two compounds since the dose/response plot of a summation of the activities of the two compounds had a distinctly lower slope than that of the experimental one (Fig. 4.16). It would be interesting to extend this investigation to studying the effect of blending all the phenols and sugars identified in sorghum extracts to determine the relative importance of each constituent in the total blend. Nevertheless, the results obtained so far support the view by Dethier (1982) that host acceptance by phytophagous insects is controlled by both non-nutritional secondary plant compounds (token stimuli), and nutritional chemicals.

Chromatographic analyses of the levels of the major phenolic and sugar components in the extracts of the whorls of the two cultivars showed, as expected, that their amounts were dependent on the nature of the cultivar. The results of these analyses showed that there was less *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in the resistant cultivar IS 2205 than the susceptible cultivar IS 18363 for both the 3 and 6 week old plants (Tables 4.5 and 4.6) and these findings are consistent with the greater palatability of the latter cultivar. However, for both cultivars the

total amounts of the phenols in the older whorls were significantly higher than those in the younger ones. Analyses of the sugar components showed that sucrose formed about 37-44% of the sugar fraction whilst glucose, fructose and xylose formed between 15-21%, 13-19%, and 1-5% respectively (Table 4.8). The total nonsugar level in these fractions ranged between 14-24%. Comparing the total sugar levels in the whorls of the 3 week old plants of the two cultivars showed , as expected, that there was less sugar in the resistant than the susceptible cultivar (Table 4.9). However, the sugar levels in the whorls of 6 week old plants of the two cultivars were approximately the same, and, again, significantly higher than the levels found for the 3 week old plants.

The higher levels of both phenolics and sugars in the older sorghum plants of both IS 18363 and IS 2205 were quite unexpected and appeared inconsistent with their lower preference by the larvae in the field (Alghali, 1985; Saxena, 1985b). Since our crude extracts of 3 week old seedlings were also more stimulatory than those of 6 week old seedlings of both cultivars, any biophysical differences between the younger and older seedlings cannot fully account for the difference in preference for the two groups and point toward a chemically mediated difference. We propose the presence of deterrent compound(s) which increase in proportion with the age of the seedlings. The most likely

candidate which fits this proposition is the "non-stimulatory" highly polar component J-1 present in both the ethyl acetate and methanol extracts. The exact roles of this component and other non-stimulatory components identified in the extracts in the plant need to be reinvestigated further with a modified feeding bioassay. It is suggested that these components should be applied to sucrose treated discs and assayed for any reduction in larval feeding response in both choice and no choice situations. It is noteworthy to mention that in this study, the complete lack of response by larvae to some of the constituents of the sorghum plant support the view by Dethier (1982) that host acceptance by phytophagous insects is mediated by positive and negative stimuli from plants and by the insect's physiological condition.

CHAPTER 6

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

It is apparent from this study that feeding behaviour of the third-instar larvae of *C. partellus* is mediated by a complex blend of chemicals. These include phenolics, sugars, hexane extractables and components which may be present in the plant as deterrent compounds. The major phenolic compounds were *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. Ferulic, *p*-coumaric, and caffeic acids were present in minor quantities. All, but *p*-coumaric acid stimulated larval feeding. The major sugar components were sucrose, glucose and fructose, xylose being in minor amounts. All but xylose were stimulatory to the feeding of the larvae. Some components in the extracts could only be characterised partially. These include J-1, a highly polar high molecular weight carboxylic acid and J-2, a mixture composed of related phenolic derivatives. J-1 might be the deterrent component implicated in the present study. J-2 appeared to stimulate larval feeding and broke down to give p-hydroxybenzaldehyde, among other products. Further study to investigate the nature, biological activities and degradative studies of the J-2 components would prove useful

to the understanding of the feeding behaviour of the larvae of this insect.

It became apparent in this study that the bioassays using cellulose acetate were more effective for monitoring phagostimulation rather than phagodeterrency of sorghum constituents. Thus, it would be interesting to investigate further, with a modified bioassay the precise roles of J-1, p-coumaric acid, xylose and constituents in the least stimulatory hexane extracts (when identified) on the feeding of the larvae. This may provide information on the nature of deterrency in the plant, and additional insight into the bases of food selection by the larvae. However, it is hoped that the present work has laid down the groundwork for a detailed study of the complex mechanisms underlying the chemical basis of food discriminative behaviour of the larvae of this insect.

Blend effects could not be fully investigated in this study. However, the study demonstrated that there was an additive or synergistic effect between some constituents of the plant as a result of reblending of fractions obtained from crude extracts. No synergism was found for a blend of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. On the other hand, bioassays of a blend of *p*-hydroxybenzaldehyde and sucrose clearly showed synergism between the two compounds. A blend of glucose and fructose in equal ratio

showed no synergism but an effect which appeared to be additive. For a thorough understanding of blend effects on the feeding of the larvae the following combinations need to be assayed: p-hydroxybenzaldehyde with the other phenolic constituents of the plant; sucrose with the other sugar components; phenolics and sugars; phagostimulatory components in the hexane extract (when identified) combined with phenolics and sugars; and lastly all the stimulant components with any deterrent compounds identified.

The difference between the two cultivars studied IS 18363 and IS 2205 appears to be quantitative rather than qualitative, with the more potent blends being present in the more susceptible cultivar IS 18363. Larval preference for extracts of the whorls of IS 18363 corresponded with the preference for this cultivar in the field and the preferred age for feeding. The levels of phagostimulants per fresh weight of whorl increased with plant age but this was not directly correlated with larval feeding response to extracts containing these phagostimulants. Thus, a study relating the levels of the stimulants (phenolics and sugars) to the surface areas and dry weights of the plants at the two different growth stages which were studied will throw more light on this question. In addition, it would be interesting to investigate the relationship, if any, between phagodeterrency and the age of the sorghum plant, and to

extend full allelochemical studies to other sorghum cultivars.

The process of host selection by a phytophagous insect is complex and involves physical and morphological factors and complex biochemical interactions between the plant and the insect. Therefore it will be interesting and enlightening to be able to match this work with detailed behavioural and electrophysiological studies to determine the precise roles played by the different stimulant components in the feeding behaviour pattern of the larvae of the insect. In addition, it will also be interesting to investigate whether the different levels of allelochemicals which appear to account for the different levels of susceptibility of these two cultivars in the field are correlated with those physical characteristics of the plants which are associated with pest performance and avoidance.

The larva of this insect goes through five larval instars before pupation. Therefore it will be useful to investigate whether these phagostimulatory compounds affect the feeding of the first, second, fourth and fifth instar larvae of the insect, and particularly to investigate if there are any chemical basis for the shift in the feeding of the later instars from the leaves to the stem.

It has been shown in previous studies that some plant chemicals which stimulate larval feeding also stimulate egg-laying by the adult gravid female of the insect (Hedin *et al.*, 1977) Therefore investigations to determine whether these stimulants affect oviposition of the adult gravid female of this insect need to be undertaken. This will provide additional knowledge for the understanding of the colonisation of the plant by the insect.

Although further investigations are needed to understand fully the ecochemistry of this insect, several potential applications of these phagostimulants in the management of this pest may be mentioned. Firstly, they may be used as markers in selective screening and breeding programmes. Secondly, they may be combined with other semiochemicals of the pest in the development of selective control technologies. For example, host plant attractants might initially be used to attract adult gravid females of the insect into traps containing an artificial substrate impregnated with oviposition stimulants to stimulate egg-laying by the female. When the eggs hatch, the larvae can be reared on an artificial diet impregnated with these feeding stimulants mixed with suitable insect growth regulator (IGR) to disturb the normal growth and development of the insect. By use of suitable IGR it may be possible to have a system which continously produces sterile males and females. Adults which emerge from these traps may be

released back into the environment where they would mate with normal members. The system could thus provide a basis for controlling the population of the insect.

Larvae feeding on disc loaded with sorghum extract in a choice bioassay (right). Control disc (left) with only shot-holes.

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Glossary of Special Terms

Allelochemical:	A chemical released by one species which affects another species.
Allomone:	A molecule, ion or free radical which is operative as an interspecific messenger in chemical ecology, and is adaptively advantageous to the emitter (releaser) of the substance.
Antifeedant activities:	Treatment effects which reduce or prevent feeding.
Antifeedant:	A substance which prevents, or reduces, feeding.
Assay substrate:	The material on which, or in which, the test substance is presented in the bioassay.
Chemical profile:	Species of chemicals contained.

Choice test:	Bioassay in which the insect is given a choice between two or more treatments.
Cultivar:	Variety of plant.
Deterrent:	A chemical which discourages (deters) a given organismal behaviour (e.g., feeding).
Drinking:	Ingestion of a liquid.
Feeding response:	Increase or decrease in feeding in a bioassay, attributable to treatment.
Feeding:	The act of eating.
Herbivore:	An animal that eats plant tissue.
Host range:	Grouping of species or varieties which an insect will use for food or shelter.
Host:	A live organism that serves as a food substance or shelter.

Inducible chemical: A chemical produced in an organism (e.g., plant) when it is placed under environmental stress.

Inhibition: Reduction of behavioural activity level (e.g. feeding).

Instar: Stage which larva assumes between moults.

Kairomone: A molecule, ion or free radical which is operative as an interspecific messenger in chemical ecology, and is adaptively advantageous to the perceiving organism.

Monophagy: Feeding on one species, or a very few species, in one genus.

Neutral substrate: Substrate which does not influence feeding in a bioassay.

No-choice test: Bioassay in which the insect is exposed to only one candidate substrate for feeding.

Non-host:	A live organism that is not serving as a food substrate or shelter for the insect of interest.
Oligophagy:	Feeding on several species in a few genera in one or a very few families.
Perception:	Direct acquaintance (recognition) with anything through the senses.
Receptor:	Macromolecular entity which binds (complexes with) a chemical, ion or free radical ligand (messenger).
Repellent:	A volatile substance which elicits an avoidance behaviour by an organism (e.g., insect).
Semiochemical:	Chemicals involved in the interaction between living organisms.
Specialist species:	A species that practices monophagy.

Stimulant:	A chemical which alters the behaviour of an organism through its nervous system.
Substrate:	Any substance ingested in conjunction with the act of feeding.