Components of resistance to sweetpotato weevils *Cylas puncticollis* (Boheman) and *Cylas brunneus* (Fabricius) (Coleoptera: Apionidae) in Ugandan sweetpotato germplasm

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DECLARATION

I, MUYINZA HARRIET hereby declare that this thesis is from my original research and has not been submitted for the award of a degree in any University.

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To my beloved mother Christine Namakula Mukuye and to the memory my beloved Dad Disan Famison Mukuye for their dedication and commitment to educate a girl child.

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ABSTRACT

Sweetpotato, *Ipomoea batatas (L.)* Lam. Family Convulvulaceae, is an important staple food, feed and raw material for both industrial and non-industrial products. Production in Africa is estimated at over 13.4 million hectares and is second only to China in the world. Sweetpotato production is however constrained by the presence of biotic and abiotic stresses. *Cylas puncticollis* (Boheman) and *Cylas brunneus* (Fabricius) are the main field pests of sweetpotato in Africa causing yield losses estimated at between 60-100% during the dry season. The management of these pests therefore, calls for the development of interventions that can be replicated and used across Sub-Saharan Africa and elsewhere in the world. Host plant resistance is one of the valid options especially at small-scale resource poor farmer level, where an affordable and effective weevil management is urgently needed. Previous research indicated variation in levels of resistance among Ugandan varieties to *Cylas* spp. It was hypothesised previously that bio-chemical content of sweetpotato may influence this variation and provide insight into the mechanisms of resistance. The amount and kinds of these bio-chemicals in sweetpotato varieties with differences in susceptibility to *Cylas* spp. and their relationships to weevil resistance was unknown. The objectives of this study therefore were to: evaluate levels of susceptibility in farmer-selected sweetpotato varieties (improved and landraces) and sweetpotato breeding lines in Uganda to *C. brunneus* and *C. puncticollis* in field trials; determine and validate the modes of resistance to *C. brunneus* and *C. puncticollis* observed in the field through laboratory feeding and oviposition bioassays; identify and quantify plant chemicals in sweetpotato varieties that may confer resistance to *C. brunneus* and *C. puncticollis;* and determine the effect of selected sweetpotato phytochemicals on *C. brunneus* and *C. puncticollis* feeding, oviposition and development.

Field evaluation of 136 Ugandan sweetpotato varieties was done over two seasons and at two sites (Namulonge and Serere) in Uganda. Using Principal Component Analysis (PCA) of percentage root damage data and external and internal stem base damage rankings among others, sweetpotato varieties that were consistently less damaged across sites and seasons were identified. These were further evaluated in the laboratory using oviposition and feeding bio-assays and analysed for biochemical content and composition using Liquid Chromatography-Mass spectrometry (LCMS) and High Performance Liquid Chromatography (HPLC). Root surface, latex and whole root content of the varieties

were analysed. Artificially synthesized root compounds were incorporated into weevil diets and their effect on the development of 1st instar larvae of both species investigated. Additionally the artificially synthesized compounds were smeared on root surfaces to investigate their effect on adult female weevil biology.

Laboratory experiments confirmed resistance of seven resistant varieties including New Kawogo, Anamoyito, Dimbuka2, Ororengo 2, Kyebagambire, ARA228 and APA356. These varieties showed protracted development time and reduced adult weevil eclosion compared to the susceptible control varieties (NASPOT 1 and Tanzania). From bio-chemical analyses, hydroxy cinnamic acids including hexadecyl *p*-coumaric acid, hexadecyl caffeic acid and octadecyl caffeic acids were identified in sweetpotato latex flesh and surfaces of sweetpotato varieties. The compounds such as hexadecyl caffeic acids and hexadecyl-*p*-coumaric acids were found to occur in significantly higher amounts in the latex, root surfaces and flesh of the roots of resistant varieties such as New Kawogo compared to Tanzania and NASPOT 1 varieties. There were significantly ($P \leq 0.05$ df= 12) more emergent *C. puncticollis* adults from susceptible varieties compared to New Kawogo in incubation bio-assays and significant reduction ($P \leq 0.05$) in the mean oviposition and feeding by *C. brunneus* and *C. puncticollis* on this variety compared to susceptible NASPOT 1. Field resistant varieties when screened in the laboratory gave prolonged adult weevil development time for both *Cylas* spp. and low susceptibility indices while the reverse was true on susceptible varieties. Diet incorporated bio-assays with hexadecylcaffeic acid and hexadecyl-*p*-coumaric acids significantly ($P \leq 0.05$) reduced larval survival weights and mean survival to pupation in the treated periderms compared to controls; and this response was dose dependent for both compounds on both weevil spp.

This study clearly showed that weevil resistance in sweetpotato must be more than simply escape, but is quantifiable and manageable and thus capable of being used in the development of resistant varieties through breeding. It recommends that the recent advances in identifying the sweetpotato genome and success in transforming sweetpotato and incorporating weevil resistant *Bt* toxins by other researchers involved in transgenic research, could be complemented with the identification of quantitative trait loci responsible for variations in phytochemical composition and thus develop ecologically more stable and sustainable resistant varieties, for sweetpotato resistance to *Cylas* species in Uganda and elsewhere in the world.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Economic importance of sweetpotato

Sweetpotato (*Ipomoea batatas (L.) Lam.* Family Convulvulaceae) is the 7th most important food crop globally with an estimated production of 135 million metric tones (FAO STAT, 2007; CIP, 2009). In sub-Saharan Africa, the crop is the staple food for a large proportion of the population. Sweetpotato is easy to propagate and is highly adaptable, tolerant to high temperatures, low fertility soils and is drought tolerant. Because of its hardy nature and broad adaptability, sweetpotato is easy to propagate and highly adaptable to temperature extremes, low soil fertility and drought (Kapinga and Carey, 2003); sweetpotato is widely grown than any other root crop in the world (CIP, 1999). It is grown in diverse agro-ecological conditions, ranging from semi-arid to high-altitude and temperate climates (Woolfe, 1992).

Globally sweetpotato is grown principally for its storage roots which are used diversely, as a vegetable, snack food, animal feed, and a commodity for industrial starch extraction and in fermented form as brew (Lin *et al.*, 1985; Jannson and Raman, 1991). The roots provide over 90% of the nutrients per calorie required for a well balanced human diet (Watt and Merril, 1975; Islam *et al.*, 2002). It is an important source of carbohydrates, fibre, iron, potassium, protein and vitamins. Sweetpotato is particularly known to contain vitamin A (retinol) precursors and thus, provides one of the cheapest means of fighting vitamin A deficiency, which is a common health problem in the developing world (Andrade *et al.*, 2009, Woolfe, 1992). Other vitamins found in sweetpotato roots, include riboflavin (B_2), pantothenic acid (B_5), pyridoxine and its derivative (B_6), niacin, folic

acid, thiamine, vitamin C and Tocopherol (vitamin E) (Woolfe, 1992). In addition, sweetpotato leaves constitute an important and abundant vegetable used by numerous communities in the world (Islam *et al.*, 2002) and the vines constitute a major livestock feed component (Andrade *et al.*, 2009).

Sweetpotato is a staple food for a large proportion of the population in many African countries (Bouwkamp, 1985), with acreage in sub-Saharan Africa estimated at 3.2 million hectares with an estimated production of 13.4 million tonnes of roots in 2005 (FAOSTAT, 2007). In North Africa, Nigeria is the largest sweetpotato producer with over 2.7 million metric tones, produced for food, as a source of industrial rawmaterial and animal feed (FAOSTAT 2008). It is one of three widely grown root crops in SSA, and assumes greatest importance in East Africa where it forms a major staple in the food systems of Uganda, Rwanda, Burundi and Eastern Congo (Stathers *et al.*, 2003b). In this region, sweetpotato is an ideal crop for mitigating against famine and ensuring food security. Compared to many other crops, sweetpotato requires few inputs and is relatively less labor intensive, making it particularly suitable for households threatened by migration, civil disorder or diseases such as HIV and AIDS (Padmaja, 2009).

It is harvested either piecemeal (small lots at a time whenever there is need) and used in steamed and or boiled form, and constitutes many local diets (Kapinga *et al.*, 1995). In some drier areas of the region, especially in Tanzania and Uganda, the fresh sweetpotato roots are sliced, dried and stored for use during seasons of low food supply contributing significantly to household food security (Kapinga and Carey, 2003; Agona, 1998). Sweetpotato is suitable for industrial processing including extruded products, chips, starch and juice, and in making of composite flours used in desserts and in confectionery

industries (CIP, 1999). The demand for these products is increasing within the region, providing an opportunity for sweetpotato to become an important raw material in food processing industries and, thus, a major source of income for the smallholder peasant farmers (Bashaasha *et al.*, 1995).

Uganda, with an annual production of 2.8 million metric tonnes, is the second largest producer of sweetpotato in the world after China (FAOSTAT, 2009). The per capita sweetpotato consumption in Uganda is 110 Kg, making it the third major source of carbohydrates after banana and cassava (Smit and Odongo, 1997). It is regarded highly as a food security crop because of its high yields per unit area and time (Woolfe, 1992) and low input requirements for its production (Bashaasha *et al.*, 1995; Aritua and Gibson, 2002). Sweetpotato is grown in almost all parts of Uganda, mainly by small scale female farmers on plots that rarely exceed 0.5 ha (Kapinga and Carey, 2003), although some larger-scale production exists (Smit, 1997). The crop is grown in broadly two types of farming systems: the cereal-based farming, where sweetpotato is grown as a food-security crop, such as in eastern, north-eastern and northern Uganda; and in the non-cereal crop-based farming system, in the central, western, south-western Uganda where it is a major part of the staple food system (Zandstra and Gregory, 1998).

Ugandan farmers regard sweetpotato as a food security crop because of high yields and low input requirements for its production (Bashaasha *et al.*, 1995; Aritua and Gibson, 2002). The rural poor grow it near their homes to feed their families and also provide income from the sale of the excess produce (Hakiza *et al.*, 2000; Mwanga *et al.*, 2001b). The crop is mainly harvested piecemeal for providing fresh daily food for

the family almost throughout the year (Bashaasha *et al.*, 1995). However, during the long dry season in north-eastern Uganda, harvested roots are sun-dried to products such as amukeke (dry-sliced form) or inginyo (dry-chunk form) for storage (CIP, 1998; Owori and Hagenimana, 2000). It is also widely used as an animal feed and compares favourably with alfalfa as forage (Woolfe, 1992; Jansson and Raman, 1991).

Uganda has a long tradition of sweetpotato cultivation and there are many local landraces/cultivars that are grown. Additionally, many improved varieties have been introduced to boost production (Mwanga *et al.*, 2003). However, the yield potential of the new varieties as observed at research stations has not been expressed in farmer fields (CIP, 2009). Besides, the on-farm sweetpotato productivity at 4-5 t/ha in SSA is well below expected average of 15 t/ha experienced in countries such as China (Padmaja, 2009).

The low sweetpotato yields in the region are a result of a combination of factors, including human population pressure, socio-economic considerations (such as farm labour and competing activities like rural to urban migration), declining soil fertility and frequent droughts (CIP, 2009; Kapinga and Carey, 2003). However, pests and diseases are by far the most important production constraint to sweetpotato production in Uganda and worldwide (Low *et al.*, 2009; Ebregt *et al.*, 2004).

1.2 Constraints to sweetpotato production

Much as sweetpotato is a very important dietary and income earner globally, it succumbs to pests and diseases prior to harvest and during storage (Sutherland, 1986; Zhang *et al.*,

1995). Losses are usually high depending on the area of cultivation, prevailing environmental conditions, variety and method of harvesting (Smit, 1997; Chalfant, 1990).

Among diseases, sweetpotato virus disease (SPVD) is due to dual infection by Sweetpotato feathery mottle virus (SPFMV), a *Potyvirus* and Sweetpotato chlorotic stunt virus (SPCSV), a *Crinivirus*, which can reduce yields by 59-98% (Gibson *et al.*, 1998; 2004), while the fungal disease caused by *Alternaria* spp. causes yield losses of over 70% (Osiru *et al.*, 2008). The prevalent insect pests include stem borers of family Sesiidae, leaf defoliators of family Nymphalidae (Order Lepidoptera) (Hill, 1984) and storage pests of dried sweetpotato which include *Rhyzopertha dominica*, *Dinoderus minutus* and *Araecerus fasciculatus* (Hall *et al.*, 1998; Agona, 1998).

However, by far, the most important production constraint on sweetpotato in East Africa and across the world, is damage by the sweetpotato weevil *Cylas* spp. (Coleoptera: Apionidae) (Zhang *et al.*, 1995; Stathers *et al.*, 2003b). The weevils belonging to Order Coleoptera: family Apionidae include *Cylas brunneus (Fabricius)*, *C. puncticollis (Boheman)* and *C. formicarius (Fabricius)*. In Asia and America, damage by the weevils is predominantly due to *Cylas formicarius elengatulus* well as in Africa, this particular species has not been detected, with *C. brunneus* (Fabricius) and *C. puncticollis* (Boheman) being the most prevalent pests in the field and storage (Jannson and Raman, 1991). Losses due to *Cylas* spp. often reach 60- 100% under subsistence farming systems worldwide (Chalfant, 1990; Nottingham and Kays 2002), leading to significantly lower income and food security for the resource poor farmers (Smit, 1997; Magira, 2003).

1.3 The Sweetpotato weevils (*Cylas spp.*): Ecology, Biology Economic importance and Management

1.3.1 Ecology of sweetpotato weevils

The sweetpotato weevils, *C. puncticollis, C. brunneus* and *C. formicarius elegantulus* (Fab.) are pests of economic importance in different areas of the world where sweetpotato is grown (Jannson and Raman, 1991; Downham *et al.*, 2001) C*ylas brunneus* and *C. puncticollis* are restricted to Africa (Smit and Van Huis, 1998) while *C. formicarius elegantulus* is a pest on sweetpotato in Asia, the United States and the Caribbean (Stathers *et al.*, 2003a and b).

The two African sweetpotato weevil species have some differences in their biology, although their ecological interactions in the field are similar (Smit, 1997). Adult female weevils usually feed on the epidermis of vines, scraping oval patches off young vines and petioles. They also feed on external surfaces of storage roots resulting in round feeding punctures. Adult *Cylas* spp. weevils are not usually seen on the crop, the soil surface under the vines or in the soil around the base of plant, apart from early in the morning before the sun heats the plant surface, when adult weevils may be seen feeding below the leaf surfaces in heavily infested fields (Smit and Van Huis, 1998).

1.3.2 Biology of *Cylas* weevils

Eggs are usually laid on root surfaces and stem bases, singly in an egg hole made by the adult females. Following egg deposition the egg hole is covered with a greyish mass which hardens to form a protective cap over the developing egg. Egg incubation period ranges from 4 – 8 days depending on temperature (Ekobu *et al.*, 2010). The emergent

larvae feed internally within the root or vine for 12 – 150 days during which they complete three larval instars. The developing larvae are legless, curved and white, extensively tunnel when feeding in the vines and storage roots, and deposit frass within the tunnels (Fig 1.1, 1.2, 1.3 and 1.4) (Smit, 1997). In response to the damage, storage roots produce terpenelike chemicals which render the root inedible (due to bad odour and taste) even at low levels of insect damage (Jannson and Raman, 1991) (Fig 1.2).

Pupation takes place within the roots or stems; the pupal period lasting 4 - 8 days. Soon after emergence, the adults mate but oviposition does not occur for a minimum of 5 - 8 days. Mean generation duration from egg to egg may be up to 85 days. However, the mean development of the weevil from egg to adult takes an average of 32 days (Smit, 1997; CIP, 2009).

Adult sweetpotato weevil survival varies greatly from 63 - 120 days under field conditions, and fecundity also varies greatly; a single female can lay up to 90 - 340 eggs, depending upon the number of matings and crowding (Smit 1997). Adult *C. brunneus* is brown and smaller than the larger black *C. puncticollis*, while *C. formicarius* is as small as *C. brunneus* but has a blue-black abdomen and a red thorax. Adult female and male adult sweetpotato weevils have distinctive differences in shapes of their antennae. The antennae of the males are straight while those of the female are round and club shaped (CIP, 2009). Infestation is determined by uprooting the storage roots and cutting them open to expose galleries or tunnels containing different stages of the weevil (Stathers *et al.*, 2003a & b).

Fig 1.1. *Cylas* pupa developing inside stem

Fig1.2. Sliced damaged sweetpotato root

Fig 1.3. Sliced undamaged root

Fig 1.4. Surface of a weevil damaged root

1.3.3 Economic importance of *Cylas* spp.

Crop losses from *Cylas* weevil damage range from 5 to 80%, with weevil damage increasing the longer the crop remains unharvested (Jannson and Raman, 1991; Mwanga *et al.*, 2007a). Weevil damage increases sharply between 24 and 30 weeks after planting and is usually accentuated in dry conditions, with both weevil species co-existing in sweetpotato fields (Fig 1.2). Weevil feeding on the inside of the vines causes malformation, thickening and cracking of the vine, and paling of leaves. Severe weevil

infestation affects the growth and overall vigour of the plant, resulting in reduced yields (CIP, 1999).

Damage due to the weevil is one the biggest constraint on sweetpotato production in East Africa (Sutherland, 1986). Damaged roots are of low quality and the quantities of roots available for food and marketing are significantly lowered. Even small weevil populations reduce root quality (Proshold, 1983). This is because the damaged storage roots produces bitter tasting and toxic sesquiterpenes, which render the damaged roots unfit for human consumption (Akazawa *et al.*, 1960) (Fig 1.2). As a result of this damage, the small scale resource poor farmers lose significant portions of their sweetpotato harvest, resulting in loss in household food and income security. Since damage by the pests is accentuated during the dry season, losses will increasingly become even more serious as climate change predictions for SSA indicate an expanding dry season.

Thus developing technologies that manage sweetpotato weevils is paramount as this would boost and sustain production dramatically, and have a positive impact on the livelihoods of millions of poor farmers across sub-Saharan Africa (SSA). These technologies should be appropriate to small-scale sweetpotato production circumstances; that is, non-costly, user-friendly and sustainable (Smit, 1997a).

1.3.4 Management of *Cylas* spp.

Conventional integrated pest management procedures for weevil management are not easily adopted by small holders in SSA. This is because it is extremely difficult to control field sanitation in small-scale subsistence production systems, where sweetpotato is grown year around (CIP, 2009). This is especially true since in-ground storage, piecemeal

and strip harvesting are common practices. These practices guarantee that fresh roots are available for consumption during most of the year, but it also means that sweetpotato crops are exposed to weevils throughout the year (Mwanga *et al.*, 2007a; Stathers *et al.*, 2003a).

1.3.4.1 Chemical control

Chemical control of the *Cylas* weevil is an option which has been adopted in some countries such as India. This involves dipping planting materials into a synthetic pesticide including Chloropyrifos (Dursban) granules at 2.25 Kg a.i/ha; Monochrotophos 36% SL, phoimidaclopid 17.8% SL and profenophos 50% EC. Treatment, has been found to result in delayed pest infestation for several months however, the cryptic nature of the adult and feeding by the root tunnelling weevil larvae reduce the reliance on insecticides, since their effect on these stages is minimal (Tarafdar and Sarkar, 2006). Also the resulting environmental contamination such as effects on non-target organisms and health hazards to man and animals, and high cost of the pesticides, makes this option unsustainable, especially at small scale farmer level (CIP, 2009; Smit, 1997) Thus chemical use for weevil management has become only being feasible for large scale commercial root production or vine-multiplication nurseries (CIP 2009).

1.3.4.2 Use of pheromone traps

Pheromone traps have been developed for the management and monitoring of weevil populations. Heath *et al.*, (1986) identified the female –produced sex pheromone of C. formicarius. Development of lures and traps for this species has been carried out by Jansson *et al.*, (1991). Then pheromones of *C. puncticollis* and *C. brunneus* were

identified and confirmed as decyl- and dodecyl (E) -2-butenoate respectively (Downham *et al.,* 1999). Development of traps for *C. puncticollis* and *C. brunneus* were then done (Smit and Odongo, 1997). Subsequently, traps impregnated with *Cylas* sex pheromones, have been produced in a laboratory and applied to small rubber capsules that are placed in traps in the field. The rubber capsules are placed above the foliage and covered to protect them from rain and sunlight. A container of soapy water is usually placed under the capsule. Male adults that are attracted by the sex pheromone fall into the pail of water and can easily be collected and removed from the field (CIP 2009). Mass trapping of male weevils has thus been done and has shown suppression of populations of *C. formicarius* males in some countries but this has not always resulted in significant reductions in infestations rate and or increases in yield (Braun and Van Den Fliert, 1999). Similarly in Uganda, mass trapping of both African sweetpotato species reduced numbers of males without any benefits on yield or infestation rates (Laboke *et al.,* 1997) although some reports of pest reduction have been reported in India and Taiwan (Hwang, 2010). Thus mass trapping using sex pheromone traps did not lead to a reduction in weevil damage to roots and had minimal adoption for *Cylas* weevil management.

1.3.4.3 Cultural control measures

One of the most practiced prevalent management strategies involves the use of cultural management practices. These have included the implementation of cultivation practices aimed at preventing infestation, including: Sanitation of the field involving the removal and destruction (through burning or feeding to livestock) of infested vine and root residues (Jannson and Raman, 1991). However, if vines are left in the field to help increase soil fertility, care should be taken to ensure they are dead and not able to sprout. Where piecemeal harvesting of the crop is practiced, care is taken to remove

and destroy any infested roots that are found. Removal of volunteer plants and alternative hosts is also done, and results in reduction in losses due to the pest (Smit, 1997).

Crop rotation with other crops that are not hosts to sweetpotato weevils for 2-3 seasons, has been recommended to break the pest cycle. This however only works where residual crop material are removed from the garden to ensure that no residual weevil populations are left. This however, is usually not done making this practice of minimal use in pest management at small holder level (Tarafdar and Sarkar, 2006). Other cultural management measures included use of clean (uninfested) planting materials, timely planting and prompt harvesting to avoid the dry period is recommended. Other practices against the pest include; avoiding planting in weevil-infested fields, and/or using barrier crop such as cassava, maize, bananas or sorghum planted around the perimeter in strips of at least 3-5 m in width between fields to restrict sweetpotato weevil migration the soil (CIP, 1999).

Hilling up of soil around the base of plants to prevent or fill soil cracks is another practice which has been recommended for pest management (Stathers *et al.*, 2003a). Soil is usually dug around the sweetpotato mound to reduce cracks in the soil by bulging roots which expose them to damage by *Cylas* weevils. Mulching is also recommended to keep the soil moist and prevent cracks, and provide a more favourable place for natural enemies. It is recommended that care should be taken not to use a mulching material that weevils can feed or develop on. These methods however, have not been much adopted as control measures by small holder farmers against *Cylas* spp. This is because most farmers regard these practices as mundane

daily routine operations, with not much significance in weevil management. As a result, their adoption as methods of pest management especially in situations when the crop is under intense weevil pressure, have remained minimal.

Another cultural practice involves piecemeal harvesting to remove the largest storage roots most at risk from weevil attack and subsequently hilling up the soil around the remaining roots to prevent sweetpotato weevils from being able to access the roots through cracks in the soil (Smit, 1997a). This hilling up has been recommended for pest management. This practice however could also mean that farmers may carelessly leave pieces of sweetpotato in the garden, which attracts more weevils to the field resulting in enhanced damage to the remaining unharvested crop. However, although this can be an important component of pest management system the potential to individually cause reduction in weevil populations is minimal (Jannson and Raman, 1991).

1.3.4.4 Biological control

Biological control methods have not been much used for weevil management. However, recent advances have seen the identification of a fungus (*Beauveria bassiana*) as a fungal pathogen. This however, though commercially available in some countries, has not yet been taken up for wider use and thus has little application in pest management (CIP 1999). The fungus is applied on the soil surface beneath the sex pheromone trap or sprayed on the foliage around the trap. Weevils attracted to the sex pheromone will be infected by the fungus and killed after several days (CIP, 2009). Others reported the identification of entomopathogenic nematodes of Family Steinernematidae and Heterorhabditidae which were isolated in *C. formicarius* (Jannson and Raman, 1991).

Other nematodes including *Rhabditis* sp., *Aphelenchus avenae*, *Acrobeloises* sp. and *Steinernema* were reportedly isolated from *C. formicarius* in the U.S.A., with *Steinernema* was reportedly particularly pathogenic to *C. formicarius* in the laboratory (Jansson, 1991, Jannson *et al.*, 1990b). However, several constraints such as soil moisture, solar radiation and temperature may limit the effectiveness of entomapathogenic nematodes as biological control agents of insects (Kaya, 1985). In addition, none of these has been found effective on the African weevil spp. and in the field against *C. formicarius*. Thus their potential as components of the integrated management of *Cylas* spp. has not yet been attained.

1.3.4.5 Host plant resistance

Host plant resistance, is a viable strategy, since smallholder farmers as using resistant varieties do not require additional skill or investment (Wanda and Humberto, 1991). Further the approach is compatible with other pest management practices and thus forms an important component of an integrated management strategy. However, progress in breeding sweetpotato varieties with resistance to *C. puncticollis* and *C. brunneus* has been slow (CIP, 2009; Ekobu *et al.*, 2010) largely due to the scarcity of varieties with significant levels of resistance. Breeders have spent many years trying to develop varieties that are resistant to the weevil. So far they have not been very successful. However, varieties that form roots relatively deep in the soil are less attacked because the weevils cannot easily reach the roots to lay eggs. Other varieties escape weevil damage because their storage roots mature quickly and can be harvested early (Stathers *et al.*, 2003a & b). Thus among the available germplasm, there exists a wide variation in levels of susceptibility, with some varieties such as New Kawogo reported by farmers as resistant (Yada, 2008), well as others are highly

susceptible. There however, exists a gap in knowledge in the mechanisms of resistance to *Cylas* spp. and this had resulted in slow progress in research geared towards developing of resistant varieties.

Where cultivar differences in susceptibility to *Cylas* spp. were observed, the mechanism was reportedly escape, specifically through the depth of sweetpotato storage roots and the tendency of shallow swollen roots to crack dry soil and so provide access for the insects (Stathers *et al.*, 2003a). Early maturing cultivars enabled farmers to harvest roots before the onset of the dry season and subsequent increase in *Cylas* populations (Smit 1997). However, there is increasing evidence for the existence of active resistance to sweetpotato weevils, associated with factors mediating processes of oviposition (preference) and feeding (antibiosis) (Stathers *et al.*, 2003b). For example, increased sweetpotato weevils and damage have been associated with increase in bergatomone (surface oviposition stimulant) on the surface of some sweetpotato roots (Nottingham *et al.*, 1989). Others have reported differences in sweetpotato chemical composition, including presence of caffeic acids and other latex compounds, with possible effect on weevil resistance (Data *et al.*, 1996; Son *et al.*, 2003a) Additionally, phenolic compounds in roots was also associated with preference for feeding by *C. formicarius elangantulus* (Nottinghan *et al*, 1989). More recently, Wang and Kays, (2002) showed quantitative and qualitative differences in the profiles of hexadecyl and coumaric acid esters on sweetpotato surfaces and latex of a weevil resistant variety whose effect on weevil biology is yet to be elucidated. Thus calling for more research, into gaining a deeper understanding of the mechanisms of resistance to the weevil, to guide the breeding effort.

Other breeding efforts have involved the development of transgenic sweetpotato varieties resistant to sweetpotato weevils have been attempted through isolation of *Bacillus thurigiensis* proteins toxic to the weevil for enhanced weevil resistance. Research has identified *Bt Cry* proteins which are being targeted through sweetpotato genetic transformation for enhanced resistance to sweetpotato weevil damage in the field (Ekobu *et al.*, 2010). These efforts however, are still in their early stages, and are yet to come up with transformed commercial varieties especially for resistance to African sweetpotato weevils and still need to be complemented with plant based resistance mechanisms to ensure lasting and stable basis for resistance to the *Cylas* spp.

1.4 Statement of the problem

Resistance to sweetpotato weevil is not known in Ugandan germplasm, but there are varieties with varying levels of susceptibility to the weevils. This calls for screening wide selection of materials to identify representative genotypes with consistent responses to weevil infestation. Although some characters such as deep rooting have been identified and associated with differing levels of infestation and thus moved forward the understanding of host plant weevil interactions to some extent, considerably no progress in this respect has been made in the determination of factors responsible for resistance in African varieties against the African weevil species, *C. puncticollis* and *C. brunneus.* Where the latex of sweet potato has been reported to contain phenolic compounds that may influence levels of resistance as shown in studies on *C. formicarius*, it is possible but was never shown that these components were responsible for the biological activity of the latex, and thus affect resistance on African weevil species. Thus understanding the underlying factors that may explain

the variation, for example identification of inherent genotype characteristics (biochemical and physical) that confer the observed variation, and determining how these factors affect weevil biology and facilitate breeding for weevil resistance in sweetpotato is not yet available.

1.5 The conceptual framework

The existing cultivar variation in levels of resistance to *Cylas* species could be due to 'escape' where some varieties could escape being damaged by the weevil due to thick vegetative cover which on one hand will enhance soil moisture conservation. As a result of this there will be reduced cracking of the soil by bulging roots thus resulting in reduced exposure to damage by *Cylas* spp. Alternatively deep rooting varieties may be difficult to access by the adult weevils resulting in reduced exposure of roots and thus low oviposition and feeding thus resulting in resistance. On the other hand deep rooted varieties will mean that soil temperature around storage roots will be very low resulting in reduced weevil development and damage thus susceptibility to *Cylas* spp. On the other hand, the mechanisms of resistance could be active. In this case, active resistance could be due to the presence of inherent biochemical compounds in sweetpotato roots, which could have an effect on weevil biology, thus lowering sweetpotato susceptibility to *Cylas* spp. damage. Of these different theoretical framework approaches, this study targeted the investigation of the role of active inherent biochemical sweetpotato compounds on levels of resistance to *Cylas* spp. This was because there was growing evidence that resistance in sweetpotato could be inherent and capable of use for breeding against *Cylas* spp. This approach also could provide a basis on which quantitative trait loci for resistance could be identified and used for enhanced breeding (Fig 1.5).

there exists variation in damage by *Cylas* spp. among sweetpotato varieties, the mechanisms of these varietal differences are not known. Understanding these mechanisms and the possibility of their genetic inheritance would facilitate targeted breeding to develop resistant varieties. Amongst the factors that may confer this variation is the type, quantity and quality of root chemicals that may influence *Cylas* preference of the specific species for oviposition, feeding and their effect on weevil development.

1.7 Objectives of study

The overall objective of this study was to understand sweetpotato resistance mechanisms to *Cyla*s spp. and the possibility of their genetic inheritance in order to facilitate targeted breeding to develop resistant varieties.

The specific objectives were;

1. to evaluate levels of susceptibility in farmer-selected sweetpotato varieties (improved and landraces) and sweetpotato breeding lines in Uganda to *C. brunneus* and *C. puncticollis* in field trials
2. to determine and validate the modes of resistance to *C. brunneus* and *C. puncticollis* observed in the field through laboratory feeding and oviposition bioassays
3. to identify and quantify plant chemicals in sweetpotato varieties that may confer resistance to *C. brunneus* and *C. puncticollis,*

and,

4. to determine the effect of sweetpotato phytochemicals on *C. brunneus* and *C. puncticollis* biology (feeding, oviposition and development).

1.8 Study hypotheses

1. There are valid and observable differences in the level of susceptibility to *Cylas* spp. weevils among the local landraces and improved sweetpotato germplasm grown in the various agro-ecological zones of Uganda and in breeding lines of the National Sweetpotato Breeding Programme.

2. The observed differences in the level of susceptibility to *Cylas* spp. weevil among the local landraces and improved sweetpotato germplasm in Uganda is due to presence of different phytochemicals which vary in quality and/or quantity among the different lines, landraces and varieties.

3. The phytochemicals present in the roots of the different lines, landraces and varieties affect sweetpotato weevil biology: attraction to roots, feeding, oviposition and development.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Weevil culturing and sexing

Both *C. puncticollis* and *C. brunneus* weevils were reared on sweetpotato storage roots in cages made of metal and cloth meshes which allowed free air circulation. They were kept at room temperature (25 to 28° C) and relative humidity of 55 to 75%. Weevils were allowed to feed and oviposit on sweetpotato roots for about three days before they were transferred to fresh storage roots. The roots were then incubated until a new generation of weevils emerged after approximately four to five weeks. Weevils were collected as they emerged and held for two to three weeks to ensure optimal fecundity before females were used in the bio-assays (Wilson *et al.*, 1990). The weevils were separated into male and female based on distinct antennal sexual dimorphism characteristics of both species (Smit, 1997). Females of the two species possess club shaped antenna tips, whereas the antennae of males are straight and un-clubbed.

2.2 Larval extraction for use in bio-assays

Larvae used in bio-assays were obtained from roots that had been artificially infested with adult weevils to lay eggs. After withdrawal of the adults, the roots were incubated for 3-4 days before peeling the surface to expose the larvae. The larvae were gently brushed onto the bio-assay trays using an entomological soft brush.

2.3 Composition of the insect diet bio-assay

A diet developed by Shimoji and Kohama (1996), and modified by Ekobu *et al.*, (2010), was adopted and used in this study to rear larval stages of *C. puncticollis* and C. *brunneus* to adulthood and to investigate biological activity of the compounds. The ingredients used to make the diet included; Chemical agar ; distilled water; dried sweetpotato powder; casein (protein); cellulose; sucrose; yeast; salt mixture; ascorbic acid; vitamin B-mixture for insects; choline chloride; inositol; cholesterol; potassium sorbate; methyl hydroxyl benzoate and antibiotic (tetracycline).

The diet was prepared as follows;

1. The agar (40g) was dissolved in distilled water (900 ml) and heated on a heated magnetic stirrer at 100°C and then allowed to cool to 55°C and then the remaining ingredients were added and blended using a common kitchen blender. The smaller ingredients (including vitamin B-mixture for insects, choline chloride, inositol, cholesterol, potassium sorbate, methyl hydroxyl benzoate and antibiotic) were dissolved in 20 ml of ethanol prior to being added to the diet to ensure equal distribution. Then the test compounds were added to 90 g of diet as solutions in ethanol. Control diets were treated with the same volume of ethanol only, to demonstrate effect of solvent on the development of the larvae.

2. For diet bio-assays, to 90 g of diet, the tested root compounds including hexadecyl-*p*- coumaric acid, hexadecyl caffeic acid and chlorogenic acid were added at concentrations of 0.1, 0.01, 0.001 and 0.0001% w/w as serial concentrations (as described further in Chapter 5 of this thesis) and mixed using a magnetic stirrer.

The diets were poured when still warm into sterile plastic petri dish replicates. After the diet had cooled and set, five small burrows were excavated in the diet surface using a plastic spatula by scooping out a small piece of diet (Fig 2.1). Larvae were then placed in the burrow and the displaced diet was gently replaced on top to reduce the risk of desiccation.

Fig 2.1. The diet bio-assay set up showing the way larvae were inoculated into the diet

2.4 Separation and identification of phytochemicals

The separation and purification of plant compounds was achieved using a combination of different chromatographic techniques, such as flash column chromatography, thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). The choice of the techniques depended largely on the solubility and volatility of the compounds being separated (Bidlingmeyer, 1992).

2.4.1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is an analytical technique that evolved from preparative flash column chromatography. The principle is the same, except that a pump is used to move the solvent (mobile phase) under high pressure (up to 4000-5000 psi) through the stationary phase of small particle size (2 to 10 mm) which is contained in a stainless steel column to withstand the high pressure. The solvent may remain constant (isocratic separation) or might change continuously in its proportion during separation (gradient elution). Solvents require degassing to avoid the formation of air bubbles in the column, which could cause fluctuations in pressure and hence affect resolution and reproducibility. A detector, measuring absorbance in the ultraviolet/visible range (photodiode array detector, PDA), monitors the compounds eluting from the column and a signal processor captures and processes the data. HPLC is mainly used for compounds that are non-volatile such as large terpenoids, phenolics, alkaloids, lipids and sugars and when detecting using a PDA is best for compounds that have a chromophore and can thus be detected in the ultraviolet and visible regions of the spectrum (Harborne, 1988; Bidlingmeyer, 1992).

In this study HPLC was performed on a Waters system with 600E quaternary pump, 717 autosampler and 996 diodarray detector. Chromatographic separation was performed on 150 mm x 4.6 mm internal diameter (5 nm particle size) Zorbax Eclipse C18 column using a linear mobile phase gradient of 0.94 ml/min flow rate with 5% acetic acid (A): MeOH (B): 18:1:1 MeOH: water: acetic acid (C). Initial conditions were 80% A, 0% B and 20% C and 80% B: 20% C at t = 20 min and 100% B at 21 min and maintained until

41 minutes. Injection volume was 5 µl and data analysis was performed using Millennium software.

2.4.2 Liquid Chromatography Mass Spectrometry (LC-MS)

In this study, LC-MS was carried out on a Thermo-Finnigan LC/MS/MS system consisting of a 'Surveyor' autosampling LC system interfaced to a LCQ Classic quadrupole ion trap mass spectrometer. The ion trap MS was fitted with an Atmospheric Pressure Chemical Ionisation (APCI) source operated under standard conditions; i.e. vaporiser temperature 450°C, needle current 5 mA, heated capillary temperature 150 °C, sheath and auxiliary nitrogen gas pressure 80 and 20 psi, and the source voltages tuned for the optimal transmission of protonated rutin. The ion trap was set to monitor ions from m/z 125-1200 with collision energy of 45%. Chromatographic separation was performed on a 150 mm x 4.6 mm internal diameter (5 mm) Phenomenex Luna C18 column using a linear mobile phase gradient of 1 ml/min flow rate, water: MeOH:5% HOAC in MeOH 80:0:20 at T=0 to 0:80:20 at T=20 and to T=25). Injection volumes were 10 µl and data analysis was performed using Xcalibur 1.2 software. LC-MS was carried out at Royal Botanical Gardens, Kew, United Kingdom.

The following was how extracts were prepared for analysis;

2.4.3 Root chemical extraction

2.4.3.1 Methanol extraction

A weighed amount of fresh root material was placed in a covered glass beaker and methanol was added to cover the root completely. Extraction was carried out for 24 hours in laboratory. Methanol was chosen as the solvent because it extracts a wide spectrum of

plant chemicals of differing polarity (Kestenholz, 2002), and due to its low boiling point *in vacuo* (30 - 40°C) allows rapid evaporation and minimises the risk of overheating of compounds present in the plant extract. The extract was filtered through a filter paper (Whatman 1PS) using a vacuum pump (KNF Neurnberg, Germany, type N820.3FT18, vacuum < 100 mbar, flow rate 20 l min^{-1}). The combined extract was filtered, and then evaporated *in vacuo* on a rotary evaporator (R110, Büchi, Switzerland), as further described in Chapter 5. The concentrated crude extract was subsequently re-dissolved in a few ml of methanol to remove it from the round bottom flask and stored in sealed glass vials at 2 °C.

2.4.3.2 Hexane extraction

Roots were also extracted in hexane. This solvent was used to extract non-polar compounds that would otherwise not be extracted with methanol and which might be associated with biological activity in the plant materials. The same protocol was used as described above for methanol except that hexane extraction of surface root compounds was done for 1 minute to prevent deeper layers of the root from being extracted. Hexane extraction was performed first to extract the non-polar compounds prior to extraction of the less polar compounds with methanol. After this, the extract was evaporated *in vacuo* as described previously. Concentrated extracts were re-dissolved in about 5 ml of methanol and stored at 2°C. It was from this extract that samples were obtained and loaded into the HPLC and LCMS system for analysis.

2.4.3.3 Sweetpotato root surface area: weight evaluation

Additionally, the surface area relative to the weights of the sweetpotato roots was estimated. To attain this, at least 10 roots were used for area assessment. These were covered with calibrated graph paper and the surface area of each of the roots was then evaluated (as described further in section 5.2.2). The resulting data was pooled and used to plot a graph of surface area weight relationship. The gradient of this graph gave the ratio at which the weight of the roots related to their surface area and this value was used to estimate the amount of compound obtained per unit area of surface of the roots for different varieties.

Also quantification of the compounds from the different root extracts was done by comparing the area below the retention peaks of chromatograms with that of a known chemical standard. In this way the amount of compound per unit volume of extract was obtained; it was then used to obtain the total compound per unit area and/ or weight of roots extracted as described further in sub-section 5.2.5.

2.5 Data analyses

Most of the data analysis was done using SAS statistical software. Effects of treatments were analysed and when significant differences were observed, means were separated using error mean square values obtained after one way ANOVA. The values were used to obtain the standard error difference between test means. This was then used to compare the means. Two means were declared to be significantly different if the difference between them was greater than twice the standard error difference (SED) (Agona, 1998). Choice experiment data was analysed using Students T-test for paired samples. Also multivariate analysis was done for varietal categorization using principal component analysis (SAS 2001).

CHAPTER THREE

FIELD SCREENING OF SELECTED UGANDAN VARIETIES FOR LEVELS OF *Cylas* WEEVIL INFESTATION

3.1 Introduction

Host-plant resistance (HPR) is an important component in the development of integrated pest management strategies in many crops (Teetes, 2009). In the case of sweetpotato weevils, for which conventional pest management is difficult to implement because of its occluded behaviour (Smit and Odongo, 1997), HPR offers a potential long-term and sustainable crop protection for subsistence sweetpotato farmers (Taleker, 1987). In recent years, there has been increased interest in searching for resistance within existing sweetpotato germplasm in many countries; for example in Asia (Katwale *et al.*, 1989), South America (Taleker, 1987) and East Africa (Stathers *et al.*, 1999; 2003a; 2003b; Magira, 2003). In most of these studies only a small number of cultivars ranging between 10-30, usually differing from country to country, were used in screening for *Cylas* spp. resistance. Within this diverse germplasm, there was no germplasm with high resistance to *Cylas* spp. but differences in susceptibility were indicated. This suggests that wider sweetpotato variety collections needed to be further made and a robust sweetpotato weevil resistance screening done in consultation with farmers so that promising genotypes can be released immediately and/or refined into elite genotypes on-station based on farmers' selection criteria and preferences.

Thus the objectives of this part of the study were (i) to screen a representative sample of Ugandan sweetpotato germplasm for levels of sweetpotato weevil infestations in order to identify sources of resistance for use in breeding programmes; and (ii) to identify weevil damage parameters which can be used to consistently evaluate this response. A total of

136 local and improved sweetpotato varieties were selected, that were initially characterized by farmers as having some levels of resistance to sweetpotato weevils and that made up a sub-set of the sweetpotato germplasm collected from 12 different agro-ecological zones and major sweetpotato producing districts of Uganda; (Yada, 2008) were used in this study.

3.2 Materials and Methods

3.2.1 Experiment sites

The experiments were conducted in fields at the National Crops Resources Research Institute (NaCCRI) Namulonge and the National Semi-Arid Resources Research Institute (NaSARRI) Serere. Namulonge is located in central Uganda (0' 32" N, 32' 35" E at an elevation of 1128 m above sea level) with mean annual rainfall of 1200 mm divided into two peaks: a broad peak in March to May and a narrow peak in October to November of each year. The daily maximum temperatures vary from 26°C in July to 28.5°C in January and daily minimum temperatures vary from 15.5°C in July/August to 17.4°C in April (Lusembo *et al.*, 2010). Serere is located in north-eastern Uganda (35° 27'E, 0°32'N, at elevation of 1128 metres above sea level), with mean annual rainfall of 1280 mm also divided into 2 peaks, a broad peak in April to May, and a narrow peak in August to September of each year. Average daily temperatures of 25-30°C are experienced (Mulindwa *et al.*, 2006). These two sites are representative of sweetpotato growing areas of Uganda (Bashaasha *et al.*, 1995) and also represent areas with low and high sweetpotato weevil pressure, respectively (Smit, 1997a).

3.2.2 Experimental layout

The 136 varieties were initially planted in September 2005 at NaSARRI so that sweetpotato root availability was synchronized with the dry season peak. This normally occurs in December – February of each year, thereby exposing the selected varieties to high weevil infestation pressure and eliminating the extremely susceptible varieties from subsequent evaluations. Each of the varieties was planted in a plot made of three 1.5 m-long and 15 cm-wide hand-hoe-made ridges laid 1 m apart (from the middle points of the ridge) using 30 cm-long apical vine pieces that were spaced 30 cm apart within the ridge. Care was taken to select weevil and virus free materials at planting. Where there were duplicate entries of varieties, both were planted and named 'a' or 'b', respectively. Where identities of the varieties were not known at their collection points but were part of this evaluation, they were given an identification code name of the area in which they were collected and a reference number to enable future tracking of their sources. Two known (Magira, 2003) weevil-susceptible varieties, Tanzania and NASPOT 1, were included in the trial as susceptible checks. The varieties were arranged in a completely randomised design and replicated three times. Each ridge plot was artificially infested with 9 adult (1 - 2 weeks old) *Cylas* spp. weevils in the ratio of 2 *C. brunneus* to 1 *C. puncticollis* observing a (1:1 sex ratio of the species) at 9 and 13 weeks after planting (WAP). This ratio was based on earlier observations of adult weevil emergence from naturally infested left–over sweetpotato roots collected in gardens around the experimental sites. The weevils used in the trials were obtained from a culture maintained on sweetpotato storage roots in cages at NaCRRI and NaSARRI, respectively. Hilling up of the ridges and weeding was done three times with a hand hoe at 4, 8 and 12 weeks after planting (WAP).

3.2.3 Trial assessment

3.2.3.1 Assessing weevil damage in the sweetpotato vines

At crop maturity (20 WAP), the number of surviving plants in each plot was recorded and from each plot five vine (one vine per plant if all the 5 plants in a plot survived) were randomly selected for weevil damage assessment. A basal segment (15 cm from the crown) of each vine, was cut off, visually assessed and scored for external weevil damage using a scale of 1 – 5; where 1 = 0 – 20% of the basal segment damaged; 2 = 21 – 40% ; 3 = 41 – 60% ; 4 = 61 – 80% and 5 = 81 – 100% (Stathers *et al.*, 2003a; Magira, 2003). Thereafter, each piece was split lengthwise using a knife to reveal internal weevil damage which was also scored using the above scale (Fig 3.1). After scoring for weevil damage, all the vines on each ridge were collected and weighed.

3.2.3.2 Assessing weevil damage on sweetpotato roots

Roots from surviving plants in each plot were carefully dug up, collected and weighed to obtain the overall root weight per plot. The harvested roots were then sorted into weevil damaged and un-damaged roots. A root was considered damaged if it had characteristic dark scarred spots on the root surface, which are typical symptoms of weevil penetration and feeding (Stathers *et al.*, 2003b), while those without any surface damage were considered clean. The weevil-damaged roots were further categorised into slightly and completely damaged. Each of the roots with slight damage was peeled to remove all the damaged portions which were combined with the roots that were completely damaged and weighed together to obtain the weight of the damaged roots. This weight was, thereafter, expressed as a percentage of the overall weight of all the roots (clean and

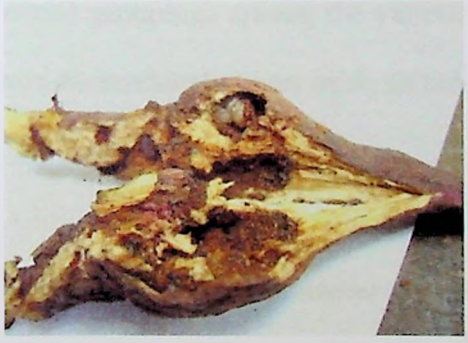
damaged) per plot. Additionally, a ratio between overall root weight and vine weight per plot was calculated per plot as the root-vine weight index.

1 (0-20%)

2 (21-40%)

3 (41-60%)

4 (61-80%)



5 (80-100%)

Fig 3.1. Internal stem damage severity (1= 0 - 20%; 2 = 21 - 40%; 3 = 41 - 60%; 4 = 61 - 80% and 5 = 81 - 100% damage)

3.3 Data Analyses

Simple (Pearson) correlations among the number of surviving plants, external stem damage index, internal stem damage index, percentage damaged roots and root-vine weight index, were calculated to explore the respective interrelationships. Principal component analysis (PCA) based on these correlation coefficients was run using PRINCOMP procedures of SAS (SAS, 2001) to determine the most important sweetpotato weevil damage parameters. Based on these parameters, 30 with low damage, 6 intermediate, and 6 very susceptible sweetpotato varieties together with NASPOT 1 and Tanzania as susceptible checks, were selected for further field evaluation at NACCRI and NaSARRI. The selected varieties were planted in the field during May 2006 and assessed in November 2006 and again planted during October 2006 and assessed in April 2007. The data collected on the 42 varieties was analysed as above followed by variance maximization rotation (Varimax) procedure to obtain a clear pattern of factor loadings for each variety (Everitt *et al.*, 2001). Bi-plots of the varietal factor loadings were drawn to reveal groupings among the varieties. The groupings were further elucidated by running non-hierarchical cluster analysis using FASTCLUS procedure in SAS.

Additionally, the data collected was transformed to "normalize" its distribution before analysis of variance; number of surviving plants was *log* (x+1) transformed, percentage damaged roots was angular transformed ($y' = 100 \times \text{Asin}((\text{Sqrt}(y+0.5) / 100) \times \pi/4)$), and, internal and external stem damage indices were square-root transformed (Gomez and Gomez, 1984; Jerrold, 1984) prior to Analysis of variance which was performed using Generalized Linear Model (GLM) procedure of SAS (SAS institute, 2001). Where there were significant differences among varieties, varietal means were separated using the Least Square Means (LSMEANS) procedure of SAS (SAS Institute, 2001).

CHAPTER FOUR

HOST PLANT PREFERENCE AND THE INFLUENCE OF DIFFERENT SWEETPOTATO CULTIVARS ON OVIPOSITION, FEEDING AND DEVELOPMENT OF *Cylas* SPP.

4.1 Introduction

Insect infestation of a host plant is a result of the (i) ability of the respective host to attract the insect and the insect subsequently accepting it for feeding and/or oviposition; and (ii) the suitability of the host plant for the growth and development of the insect eggs and larval stages (Wilson *et al.*, 1990). Understanding the processes by which insect pests select host plants and preferred sites within plants is of considerable interest in the biology of the pests, their population dynamics and management of insect pests (Stathers *et al.*, 1999).

It has been shown that varieties may have differences in their suitability as hosts for *Cylas* spp. and this could be the reason for considerable differences in levels of field damage by the weevils. There was however, little information on the link between the observed varietal differences in susceptibility and weevil preference for feeding, oviposition and development. This part of the study investigated preferences by *Cylas* spp. for the sweetpotato plant, varieties and sites for its feeding and oviposition; and the effect of the host choice on *Cylas* spp. larval development. This study was designed to provide some insight into the possible mechanisms of resistance to *Cylas* spp. in sweetpotato.

4.2 *Cylas* spp. recognition and selective preference of sweetpotato plant and specific plant parts

This part of the study investigated whether *Cylas* spp. could recognise and select sweetpotato when presented with alternative substrates; and whether *Cylas* spp. selected sweetpotato roots on leaves.

4.2.1. Materials and Methods

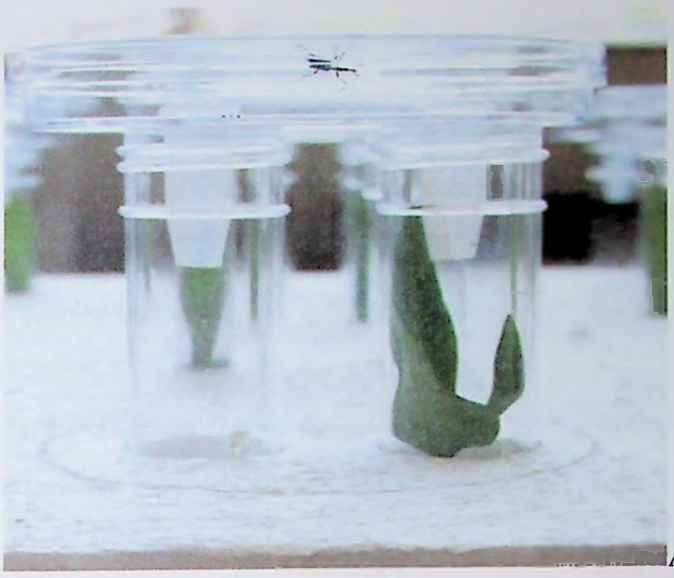
Bioassays were performed to investigate whether adult female sweetpotato weevils could differentiate between sweetpotato and *Ipomoea cairica* (Morning glory) and *Dioscorea* spp. (yam), which are reported to be alternative hosts of *Cylas* spp. (Austin, 1988)- as alternative substrate to sweetpotato were done. Also an investigation to find out whether adult female sweetpotato weevils showed any preference for roots or leaves of sweetpotato was conducted. The test apparatus for the bioassays was made of a 10 cm wide (diameter) plastic petri-dish that was perforated at 2 places where plastic eppendorf tubes, with the lid and bottom end cut off, were inserted. A 2 cm wide (diameter) glass vial was then fixed at each of perforations, surrounding each eppendoff tube (Fig. 4.1).

The test substrate (sweetpotato root or leaf) of NASPOT 1 and Tanzania varieties which were found susceptible in field evaluation, were placed in one of the plastic vials and the alternative substrate in the other vial. For the control, the alternate vial was left empty. One adult (0 – 1 week old) *Cylas puncticollis* weevil, obtained from cultures reared as described in section 2.1 of Chapter 2 and deprived of food for 24 hours, was placed in the petri-dish and the lid replaced. The experiment was replicated ten times and the tubes firmly fixed onto a single polystyrene tray (Fig. 4.1c) with closely fitting holes to hold the vials in place. The lower outer end of the eppendorf tube was smeared with fluon to

prevent insects from moving out of the vial once they made a choice. The set-up was left at ambient conditions (25-28°C) on a laboratory bench for 16 – 18 hours. Thereafter, the numbers of weevils that had oriented to either treatment were counted and their means compared using Students' T-test at $P \leq 0.05$.

4.2.2 Results and discussion

Cylas weevils were able to detect both sweetpotato roots and leaves. The number of adults in tubes containing these substrates was significantly higher than in tubes containing no substrate. Significantly ($P < 0.01$ and $P < 0.05$) more adult weevils preferred sweetpotato substrates when presented with either *Dioscorea* substrates or control (Table 4.1). There was no significant difference in the number of adult weevils in each tube when *Ipomoea cairica* leaf was presented as the alternative substrate to sweetpotato leaf and similarly, there was no difference in the number of adult weevils in each tube when sweetpotato roots and leaves of NASPOT 1 were compared, whereas significantly more weevils oriented towards roots than leaves of Tanzania.



A

B

C

Fig 4.1. Choice bioassay apparatus set-up for an individual choice (A); bio-assay set up from above (B); tray for the choice–bio-assay (C).

Table 4.1. *C. puncticollis* preference for sweetpotato plant and plant parts in laboratory choice bioassays

Treatment pairing	No. of weevils choosing sweetpotato (mean ± SE)	T-value (df = 18 two-tail)	P-value (t > T)
NASPOT 1 leaf Vs Control	8.0 ± 0.6	4.34	0.012
NASPOT 1 leaf Vs *I. cairica* leaf	6.0 ± 1.1	1.62	0.18
NASPOT 1 root Vs Control	8.4 ± 0.5	5.21	0.006
NASPOT 1 root Vs NASPOT 1 leaf	6.4 ± 0.9	7.06	0.205
NASPOT 1 root Vs *Dioscorea* root	8.4 ± 0.6	6.67	0.003
Tanzania leaf Vs Control	8.4 ± 0.4	6.53	0.003
Tanzania leaf Vs *I. cairica* leaf	6.6 ± 0.9	1.79	0.147
Tanzania root Vs Control	7.8 ± 0.6	5.49	0.005
Tanzania root Vs Tanzania leaf	8.6 ± 0.5	7.06	0.002
Tanzania root Vs *Dioscorea* root	8.4 ± 0.5	6.67	0.003

The ability of *Cylas* spp. to perceive sweetpotato and orient towards it was demonstrated by more adult weevils preferring sweetpotato substrates when presented with either *Dioscorea* substrates or empty vials (control) (Table 4.1). The ability of *Ipomoea cairica* to attract *Cylas* spp. to similar extents as sweetpotato indicates that *I. cairica* could have similar chemical constituents to sweetpotato, although it lacks swollen roots and may attract *Cylas* spp. in the wild. However, due to lack of swollen roots, it may not be an important alternative host to the pest, although further studies could be done to confirm this. However, prior to this study, little information on *I. cairica* and its possible attraction to *Cylas* spp. in was not available. The results also showed that leaves and roots of the some sweetpotato varieties such as NASPOT 1, were equally attractive to *Cylas* spp. indicating that sweetpotato leaves and roots may be perceived by the weevil as possessing similar odours, suggesting that chemical composition may be an important

factor for attracting weevils to the host. From this part of the study, it can be postulated that attractiveness of *Cylas* spp. to the host is mediated by chemical cues from the genus *Ipomoea* and that host selection by the weevils is an active process.

4.3 *Cylas* spp. recognition and preference of a susceptible sweetpotato variety

4.3.1 Materials and methods

The test apparatus for the bioassay were two glass jars interconnected by a transparent glass tube. A freshly harvested storage root of either New Kawogo a resistant variety or Tanzania a susceptible variety was placed in a respective jar. In the mid-point of the interconnecting glass tube, 20 two-week old adult weevils of either *C. brunneus* or *C. puncticollis,(*that were initially deprived of food for 24 hours), were released and allowed to select and settle on either of the two roots over a period of 14 days. The number of insects on either roots was counted after 7 and 14 days and the data were analysed using a two-tailed Students T-test for paired means at $P \leq 0.05$ level of significance. Each experiment was replicated 3 times and repeated 4 times (d.f. =22; N=12)

4.3.2 Results and discussions

Results of the weevil preference between New Kawogo and Tanzania varieties are presented in Table 4.2. Significantly ($P < 0.05$ and $P < 0.01$) more adult weevils preferred Tanzania when presented with roots of the two varieties at both 7 and 14 days for each of the two species. On both New Kawogo and Tanzania varieties, the number of adult weevils of both species appeared to decrease and increase respectively with time, and these values were statistically significant.

The preference of a susceptible sweetpotato variety by the weevil as indicated by the significantly higher number of weevils on Tanzania roots relative to New Kawogo indicates that Tanzania may possess more attractive substances (or less repellent substances) on the root surface compared to New Kawogo (Stathers *et al.*, 1999; Nottingham and Kays, 2002; Wang and Kays, 2002).

Table 4.2. Differences in weevil preference between Tanzania and New Kawogo varieties

Weevil species	Variety	Mean insects infesting root when incubated for	
		7 days	14 days
Cylas brunneus	New Kawogo	7.3 ± 0.8	5.5± 0.6
	Tanzania	12.8± 0.8	14.5 ±0.6
P (t > T) two-tail, df (12)		0.035	0.006
Cylas puncticollis	New Kawogo	7.8 ± 1.4	6.3 ± 0.3
	Tanzania	12.3 ± 1.4	14.3 ±0.6
P (t >T) two-tail, df (12)		0.037	0.002

4.4 The influence of different sweetpotato cultivars on *Cylas* spp. feeding and oviposition

This part of the study consists of bioassays that demonstrated the suitability of sweetpotato for feeding and oviposition by *Cylas* spp.

4.4.1 Materials and Methods

4.4.1.1 No choice bioassay

This involved bio-assays where sweetpotato materials of the same variety were presented to the weevil for feeding and oviposition. They were then compared to similarly treated varieties, to get an evaluation of weevil preferences for feeding and oviposition on the

resistant and susceptible varieties under laboratory conditions. This could then provide an understanding of whether variations in the field were due to escape or due to host suitability

In no-choice bioassay, plugs of root periderm obtained using a 24 mm diameter cork borer from a freshly harvested sweetpotato tuber, were fitted snugly into a 12-well-plate such that insects introduced to the area would only be exposed to the root periderm and not the inner cortex. Onto each periderm plug one adult (2 weeks old) female of either *C. puncticollis* or *C. brunneus* was placed before covering the well-plate with the lid. The lid was raised to about 1 cm above the wells to allow for free movement of the weevils between 12 periderms using clay into which tiny holes were made to allow air circulation (Fig. 4.2). Five sweetpotato varieties were selected for this part of the study based on their observed field-level damage by *Cylas* spp. (sub-section 3.4) to represent resistant (New Kawogo, Dimbuka 2 and ARA228 and susceptible (NASPOT 1 and Tanzania) varietal categories. Each variety was put in its respective well-plate and replicated 50 times.

The bio-assay was left to stand for 24 hours, following which the number of oviposited eggs, feeding punctures and droppings on each of the periderms was evaluated. Data was analysed using Add –In software in Excel. Means were different if the difference between them was more than twice standard error difference.

A

B

Fig 4.2. Bioassay set up to investigate selective preference of sweetpotato varieties for feeding and oviposition; set up of bio-assay seen from sides (A); set up when seen from above (B)

4.4.1.2 Choice bioassay

In a choice bioassay, six periderm plugs of the standard susceptible variety Tanzania were fitted snugly into half of the 12-well-plate, and the other half fitted with either of the test resistant varieties New Kawogo, Dimbuka 2 and ARA228. Onto each periderm plug one adult (2 weeks old) female of either *C. puncticollis* or *C. brunneus* were introduced as described above. The experiment was of a completely randomised design. Each experimental pairing was replicated 10 times. The experiment was left at ambient conditions (25-28℃) for 24 hours. Thereafter, the weevils were removed and the numbers of feeding holes, eggs laid and droppings on each periderm plug were counted. The number of droppings was recorded to give an indication of the amount of feeding by each adult. The data collected was normalized using square-root transformation before analysis of variance and mean separation using least significant difference values.

4.4.2 Results and Discussion

In the no-choice bio-assay, and for both species, there were significantly ($P \leq 0.05$) more eggs oviposited, droppings left and feeding punctures on susceptible varieties Tanzania and NASPOT1, than on the resistant varieties New Kawogo and Dimbuka 2. However oviposited eggs by *C. brunneus* on NASPOT 1 and ARA228 were not significantly different (Table 4.3).

It is possible that *C. brunneus* may be generally less aggressive than *C. puncticollis* even on the more susceptible Tanzania and NASPOT 1 varieties (Table 4.3 and 4.4). Lower oviposited eggs on the resistant varieties for both weevil species also provided more support to previous findings that reported oviposition as an indicator of plant resistance to

Cylas weevils since it is a factor in host selection process by the weevil that is quantifiable (Wilson *et al.*, 1990).

Table 4.3. Variations in mean oviposition, feeding holes and droppings on periderms in no-choice bioassays with different varieties

Variety	Oviposited eggs (mean ± se)	Droppings (mean ± se)	Feeding holes (mean ± se)
C. puncticollis (‡)			
New Kawogo	1.7 ± 0.1 (1.8)	2.3 ± 0.5 (5.0)	2.5 ± 0.1 (5.5)
Dimbuka 2	2.1 ± 0.1 (3.3)	2.2 ± 0.7 (4.0)	2.2 ± 0.1 (3.7)
ARA228	2.1 ± 0.1 (3.5)	1.4 ± 0.2 (1.0)	1.4 ± 0.2 (1.0)
NASPOT1	2.5 ± 0.0 (5.3)	4.3 ± 0.4 (17.5)	5.5 ± 0.3 (30)
Tanzania	2.6 ± 0.1 (6.0)	4.7 ± 0.0 (20.7)	9.3 ± 0.1 (86)
SED (19 d.f.)	0.1	0.4	0.2
CV (%)	4%	5	14.1
C. brunneus (‡‡)			
New Kawogo	1.1 ± 0.0 (0.2)	1.1 ± 0.0 (0.1)	1.1 ± 0.0 (0.1)
Dimbuka 2	1.1 ± 0.1 (0.3)	1.5 ± 0.1 (1.2)	1.9 ± 0.1 (2.6)
ARA228	1.3 ± 0.7 (0.8)	1.2 ± 0.0 (0.4)	1.5 ± 0.2 (1.3)
NASPOT1	1.3 ± 0.1 (0.7)	2.0 ± 0.3 (3.8)	2.9 ± 0.4 (7.1)
Tanzania	1.6 ± 0.0 (1.3)	1.6 ± 0.0 (1.7)	2.8 ± 0.1 (6.9)
SED	0.06	0.2	0.13
CV (%)	14.2	7.5	6.1

Means ± standard error; SED= Standard error difference; two treatment means are significantly different when the difference between them is greater than twice the SED value for each column at $P \leq 0.05$ per species; ‡ means are from data of 12 periderms; ‡‡ means are for 1 peridem; All data was normalised using $\sqrt{(x+1)}$ transformation and actual means are in parenthesis.

Similarly, the results showed that the susceptible variety Tanzania had more ($P < 0.05$) number of eggs oviposited and feeding hole punctures than New Kawogo, Dimbuka 2 and ARA228 varieties for both weevil species (Table 4.4). However, the number of faecal droppings, on Tanzania compared to the three resistant varieties, were not significantly different ($P > 0.05$) for both weevil species.

Table 4.4. Variation in numbers of oviposited eggs, feeding punctures and faecal droppings on periderms of sweetpotato varieties in choice bioassays with Tanzania variety

Varietal comparisons	Eggs (Mean ± se)	Feeding holes (Mean ± se)	Droppings (Mean ± se)
C. puncticollis			
Tanzania	36 ± 0.6	27 ± 1.3	50 ±.01
Dimbuka2	18 ± 0.0	6.0 ± 0.02	18 ± 0.0
P(t > T)	0.001	0.002	0.12
Tanzania	3.3 ± 0.2	26.5 ± 0.3	20.5 ± 0.8
New Kawogo	1.2 ± 0.1	10.0 ± 0.7	19.5± 0.7
P(t > T)	0.008	0.007	0.41
Tanzania	16 ± 0.1	75 ± 3.0	38.0 ± 10.4
ARA228	6.0 ± 0.7	8.0 ± 0.7	11.0 ± 2.3
P(t > T)	0.01	0.01	0.16
C. brunneus			
Tanzania	1.3 ± 0.2	4.2 ± 0.1	1.7 ± 0.0
Dimbuka2	0.0 ± 0.0	0.8 ± 0.2	0.2 ± 0.1
P (t > T)	0.04	0.003	0.006
Tanzania	1.50 ± 0.0	4.7 ± 0.4	5.5 ± 0.3
New Kawogo	0.00 ± 0.0	2.2 ± 0.0	0.3 ± 0.0
P(t > T)	0.02	0.04	0.02
Tanzania	1.60 ± 00	83 ± 5.6	20.3 ± 3.4
ARA228	0.0 ± 0.0	16.0 ± 3.3	4.5 ± 0.5
P(t > T)	0.06	0.03	0.073

d.f.= 36

4.5 Influence of sweetpotato varieties differing in susceptibility to *Cylas* spp. on insect growth and development

4.5.1 Materials and methods

Freshly harvested roots of susceptible (Tanzania) and resistant (Dimbuka2 and New Kawogo) varieties harvested at 120 DAP, weighing 200 - 250 g were washed, and dried

in the sun for a few hours to remove excess moisture on the root surface and then placed in separate one-litre plastic jars that were lined with paper napkins. To each jar, 10 mated 2-week-old female *C. puncticollis* for one set of the jars and *C. brunneus* weevils for another, were introduced, the jar mouth covered with thin polyester cloth and then a lid, into which circular holes of 1 cm diameter had been made, placed over the jar and tightened. The weevils were left to oviposit on the root for 2 days before removing them. Thereafter, the experimental jars were covered with sugar-paper so as to reduce illumination which would otherwise cause root-sprouting. Each varietal treatment was replicated 20 times for each weevil species and the experiments repeated 3 times.

The experiment was left on a laboratory bench at ambient conditions (25-28°C and relative humidity between 46-80%) until adult weevil emergence. The emerging weevils from each jar were removed and counted daily until emergence stopped. Data on emergent adults was categorized into 10 day periods from day 25 to final day of weevil emergence, i.e. 0-25, 26-35, 36-45, and 46-55 days.

Data was pooled for analysis using One way Analysis of Variance and to determine susceptibility index (SI); where SI= Log e N/T (where Log e is the Natural Logarithm, N is the mean total emergent adults and T is the time taken from the mid-oviposition time to the last day of emergence of F1 generation (Dobie, 1984; Muyinza, 1998). The generation time, (which is the mean time taken for all F1 weevils to emerge) was evaluated.

4.5.2 Results and discussion

For each weevil species, there was significant difference in mean emergent adult weevils from incubated roots among varieties (Table 4.5), with significantly more adults emerging from Tanzania ($P \leq 0.05$) than Dimbuka 2 and New Kawogo.

Table 4.5. Variation in total mean emergent adults among varieties

	‡Mean emergent adults (mean ± se)	
Variety	*C. puncticollis*	*C. brunneus*
Dimbuka 2	20.8 ± 5.3	13.4 ± 2.2
Tanzania	33.9 ± 1.7	34.5 ± 9.0
New Kawogo	3.00 ± 1.2	1.90 ± 0.8
sed (d.f.=8)	4.6	7.4
CV %	23	5

‡ se= standard error; Two treatment means are significantly different when differences between them are greater than the standard error difference (sed)

There was variation in percentage of weevils emerging at different times from roots of the resistant and susceptible varieties (Table 4.6). Both Dimbuka 2 and New Kawogo had the highest percentage emergent adults at between 36 - 45 days and New Kawogo had all weevil emergence delayed until this time. This was in contrast to Tanzania variety where about 71.9% and 89% of *C. puncticollis* and *C. brunneus* weevils, respectively, emerged by 35 days of incubation. No weevils emerged from New Kawogo during this period, instead, weevils from both Dimbuka 2 and New Kawogo had protracted development, at as many as 54 days from mid- oviposition for *C. puncticollis,* on both varieties and 54 and 46 days respectively on Dimbuka 2 and New Kawogo varieties respectively, for *C. brunneus* weevils (Table 4.7).

The generation time of *Cylas* spp. was less in the more susceptible variety Tanzania than in Dimbuka2 and New Kawogo varieties. It was noted, however, that the development of both *Cylas* spp. were more protracted in New Kawogo and Dimbuka 2 and this was

demonstrated by the majority of adults emerging between 36-45 days, unlike in Tanzania where the majority emerged between 25 and 35 days (Fig. 4.3).

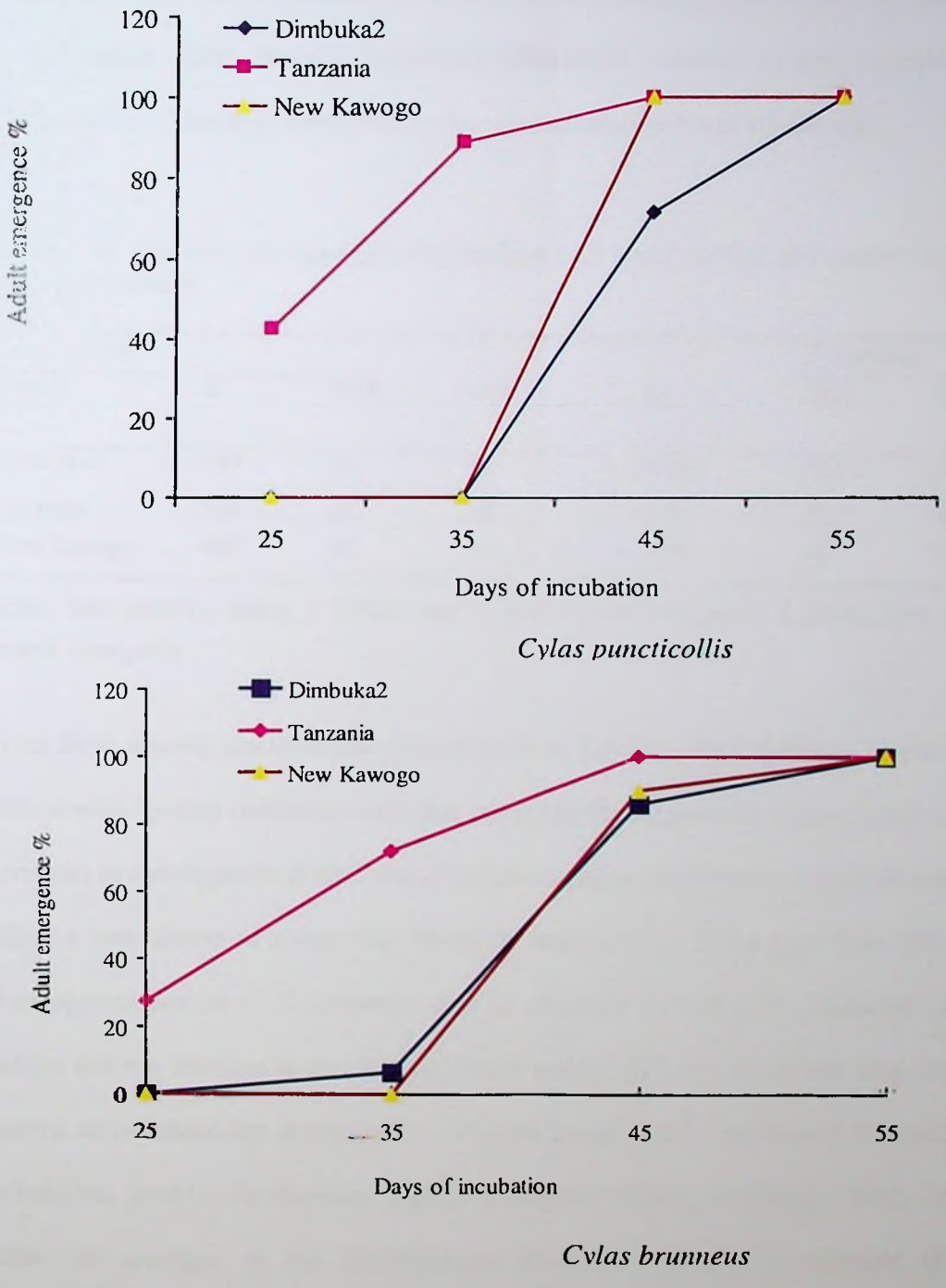


Fig.4.3. Adult *C. puncticollis* and *C. brunneus* emerging from previously infested roots of different varieties incubated in the laboratory for up to 60 days

The median of the generation time for both weevil species was also shorter for Tanzania variety compared to both Dimbuka2 and New Kawogo. From the susceptibility index New Kawogo was found to be relatively resistant, followed by Dimbuka2 whereas Tanzania was the most susceptible to both weevil species (Table 4.6). This corroborates earlier results which showed significant differences in levels of host acceptability for oviposition on Tanzania compared to the two resistant varieties (Table 4.6).

Table 4.6. Variation in susceptibility indices and adult weevil emergence days on different varieties

C. puncticollis				*C. brunneus*		
Variety	SI	DFE	DLE	SI	DFE	DLE
Dimbuka2	0.07	35	54	0.06	38.5	54
Tanzania	0.09	25	45	0.11	23	46
New Kawogo	0.03	41	54	0.03	38	46

‡‡SI= Susceptibility Index; ‡ DFE= Day of first weevil emergence ‡ DLE= Day of last weevil emergence

From these results, susceptibility indices of New Kawogo and Dimbuka 2 were lower for both weevil species compared with that recorded for Tanzania variety (Table 4.6). Also, variation in development period was consistent among the different varieties and by itself offers a new means of comparing levels of resistance to *Cylas* spp. The differences in development period of *C. brunneus* and *C. puncticollis* could be attributed to intrinsic factors that are inherent in the different sweet potato varieties. Resistant crop varieties are known to influence the development of pests by providing unfavourable conditions for infestation, growth, development and or emergence (Wang and Kays, 2002). The insects either die outright or the development of their offspring is retarded (Hahn and Leuschener, 1982; Dobie, 1984). It is possible that such similar effects in the less susceptible sweetpotato varieties could have delayed the development of *C. brunneus* and

C. puncticollis. The growth period of developing larvae could have been delayed and the number of emergent adults significantly affected.

Among the varieties evaluated, New Kawogo and Dimbuka 2 had low emergent adults and exhibited protracted development. These varieties may be possessing antibiotic and antixenotic factors which increased developmental time, reduced number of emergent adult weevils that confer some levels of host resistance (Son *et al.,* 1991b). These could be a result of variation in root chemical composition which may be variable in resistant and susceptible varieties. Farmers could benefit by growing these less susceptible varieties to reduce field pest infestations. However, before any of these varieties is recommended for adoption, other factors including resistance to diseases, taste and marketability need to be considered. Further, it is important to pinpoint the mechanisms of resistance and focus on stable heritable characters for breeding against *Cylas* spp. Once these mechanisms are identified, then this information could be used to focus breeding for these characters and thus foster the development of resistant varieties against *Cylas* spp.

4.6 Conclusion

Results in this section of the study provide strong evidence that the variation in levels of resistance in sweetpotato varieties to *C. brunneus* and *C. puncticollis* is active and affects weevil feeding and oviposition and larval development. The mechanisms of differential resistance, seems to be linked to root surface and internal chemical factors but this needs further confirmatory studies.

CHAPTER FIVE

COMPOSITION OF CHEMICAL EXTRACTS FROM LATEX, CORTEX AND SURFACES OF ROOTS OF SWEETPOTATO VARIETIES DIFFERRING IN SUSCEPTIBILITY TO *Cylas spp.*

5.1 Introduction

Insect deterrent or attractant chemicals and stimulants are complex sets of compounds that mediate behaviour of insect pests. Understanding plant chemistry and specific compounds that modulate behaviour between insects and their hosts (Son *et al.,* 1990; Stevenson *et al.,* 2009), is essential for a precise parent progeny selection in plant breeding (Kays and Horvat, 1983). Thus identifying pragramatic use of differences in the plant chemistry is a realistic strategy in developing resistant lines against pests (Son *et al,* 1990).

Recent years have seen advances aimed at identifying plant chemical mediation in host resistance to several insect pests; for example against mango fruit flies (Nankinga *et al.,* 2010), cabbage root fly, *Delia brassicae* and against *Heliocoverpa zea* (Summers and Felton, 1994). However, phytochemical contents of sweetpotatoes and the variation thereof, in resistance to *Cylas* spp. among sweetpotato varieties, is still largely unknown. This knowledge would guide research and development of resistant lines against these pests.

Chemical differences in vine and root latex were reported to occur in sweetpotato varieties and were reported as affecting levels of acceptability of sweetpotato varieties by *C. formicarius* (Snook *et al.*, 1994; Wilson *et al.*, 1990). Existence of surface chemicals

such as bergamotene in sweetpotato with attractant effects on *C. formicarius* (Wang and Kays, 2002), hexadecyl, octadecyl and eicosylcoumaric acid esters in sweetpotato latex and sesquiterpenes such as α-gurjunene, α-humulene, and ylangene in root tissues, have been reported (Wilson *et al.*, 1990; Data *et al.*, 1996). These studies however, did not quantify the amounts of these compounds in the various sweetpotato varieties examined and didn't link these compounds to differences in varietal resistance to *Cylas* spp. It is however, highly possible that these and other yet unidentified components occur in variable amounts in sweetpotato and may account for differences in field susceptibility of sweetpotato to *Cylas* spp. The exact identity, location and quantities of chemicals that may mediate suitability for feeding, oviposition and development of *Cylas* spp. remained largely unknown.

In chapter three and four of this thesis it was shown that varietal differences in susceptibility to damage by sweetpotato weevils amongst local and improved varieties exist. However, there was need to identify the basis for this variation so as to gain more understanding of the underlying mechanisms conferring these varietal differences. This Chapter presents studies aimed at identifying and quantifying chemicals in the root latex, cortex and root periderm in sweetpotatoes from germplasm collected in Uganda. These studies elucidated whether the observed levels of field susceptibility to *Cylas* spp. are linked to differences in chemical root composition in different varieties.

5.2 Materials and methods

5.2.1 Identification of chemicals in root latex, surfaces and whole roots of sweetpotato

5.2.1.1 Root latex extraction

Whole sweetpotato roots of two susceptible (NASPOT 1 and Tanzania), two moderately susceptible (Kyebigambire and APA356) and six resistant varieties, OrurengoA, Andinyaku, Anamoyito, Dimbuka 2, ARA228 and New Kawogo sweetpotato varieties were harvested at full maturity (6 months after planting). From each variety, storage 3-5 roots were broken in two by hand to optimize the latex flow (compared to cutting using a knife). The latex was collected from the exposed broken root surface with a capillary tube. An aliquot of the latex was transferred to a vial, weighed, dissolved in methanol, and stored at 3 ± 3°C prior to being used for High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) analyses.

5.2.1.2 Root surface extraction with methanol

Medium weight (80-120 g) and freshly harvested roots of the 10 varieties used in sub-section 5.2.1 were selected and weighed prior to extracting chemicals from their surfaces. Selected root surface area was estimated by wrapping a graph paper around each of the roots of known weight and marking out the paper which covered the root. Ten roots of each variety were used and the data of root weight and surface area plotted. The gradient of the graph gave the ratio of weight to surface area of roots for each of the varieties. This ratio was used to calculate the amount of compounds obtained per root.

Each root was then placed in a glass beaker into which ordinary grade methanol (95%) was poured and left to stand for 24 hours in a fume-hood chamber in the laboratory. Methanol was chosen as the solvent because it extracts chemicals with a wide spectrum of polarity and its low boiling point *in vacuo* (30-40°C) allows rapid evaporation and minimises the risk of overheating of compounds present in the plant extract (Harborne, 1988). After 24 hours, the extract was filtered through a filter paper (Whatman 1PS) using a vacuum pump (KNF Neurnberg, Germany, type N820.3FT18, vacuum < -100 mbar, flow rate 20 l min-1) (Stevenson *et al.*, 2009). The extract was filtered and then evaporated *in vacuo* on a rotary evaporator (R110, Büchi, Switzerland). The concentrated crude extract was subsequently re-dissolved in HPLC grade methanol to remove it from the round bottom flask and stored in sealed glass vials at 2°C.

5.2.2 Root surface extraction with hexane

The root samples of each of the varieties used in sub-section 5.2.1 were extracted in HPLC grade hexane. Hexane was used to extract non-polar compounds that would otherwise not be extracted with methanol and which might be associated with biological activity in sweetpotato. The same protocol was used as described above for methanol on the same lot of roots, except that for hexane, the extraction for surface root compounds was done for 1 minute to prevent deeper layers of the root from being extracted. After this, the extract was evaporated *in vacuo* as described previously in 5.2.2. The concentrated extracts were re-dissolved in a few (2-2.5) ml of methanol and stored at 2°C.

5.2.3 Whole root extraction

Following surface extraction, the roots of each of the varieties were blended with a kitchen solid blender. The crushed root was then mixed with ordinary grade (95%)

methanol of known volume. Then the extract was allowed to settle over a period of 24 hours and filtered off. Filtration was achieved with aid of a filter pump by passing the extract through a filter paper. The filtrate from each of the varieties was evaporated *in vacuo* on a rotary evaporator. The fraction left in the round bottomed flask was then dissolved in ordinary grade methanol of a known volume and these were the samples from which 0.5 µl samples were obtained for analysis using High performance liquid chromatography (HPLC) technique (Bidlingmeyer, 1992).

5.2.4 High-Performance Liquid chromatography (HPLC)

The filtrates from methanol extraction of the different sweetpotato varieties were compared using high-performance liquid chromatography. HPLC was performed on a Waters system with 600E quaternary pump, 717 auto sampler and 996 diode ray detector. Chromatographic separation was performed on 150 mm x 4.6 mm internal diameter (5 nm particle size) Zorbax Eclipse C18 column using a linear mobile phase gradient of 0.94 ml/min flow rate with 5% acetic acid (A): MeOH (B): 18:1:1 MeOH: water: acetic acid (C). Initial conditions were 80% A, 0% B and 20% C and 80% B:20% C at t= 20 minutes and 100% B at 21 minutes and maintained until 41 minutes. Injection volume was 5 µl and the chromatogram of each of the extracted materials and data analysis were performed using Waters Millennium software (Kestenholz, 2002; Stevenson *et al.*, 2009). The integrals from peaks in resulting chromatograms (peak areas) were compared to those of synthetic standards (Stevenson *et al.*, 2009) which were used to calculate the amounts of compound per unit surface per unit weight of storage roots.

The quantities of compounds from each variety represented by characteristic chromatograms, was obtained by calculating the area below the peaks at specific points of the chromatograms and comparing this to a standard which was run at the same wavelength and under similar conditions. The standards included hexadecylcaffeic acid and haxadecyl-*p*-coumaric acid at 325 nm. The variation in areas of retention of the different compounds from the varietal extracts were thus evaluated using values obtained for the known chemical standards which were run at the time of the analysis (Stevenson *et al.,* 2009). These areas were used to calculate the amounts of compounds per unit µl of extracted per unit weight and / or surface of the roots, as described in sub-section 2.4.3.3.

The obtained compound concentrations on the surface of roots were correlated using Spearmans' correlation coefficient with numbers of feeding holes and oviposited eggs obtained, for the varieties presented earlier in Table 4.3. The oviposition levels per unit area of periderm (about $1cm^3$ surface area) as obtained in sub-section 4.4.2 was correlated with amounts of compound per unit area of root (cm^3) .

5.2.5 Liquid chromatography –Mass Spectrometry

Further analysis of the chemical components of the latex was carried out using LC-MS on a Thermo-Finnigan LC/MS/MS system consisting of a 'Surveyor' autosampling LC system interfaced to a LCQ Classic quadrupole ion trap mass spectrometer. This was fitted with an Atmospheric Pressure Chemical Ionisation (APCI) source operated under standard conditions; injection volumes applied were of 10 µl and data analyses were performed using Xcalibur 1.2 software, and adopted from Stevenson *et al.,* (2009).

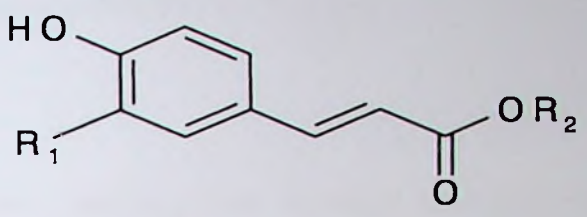
5.3 Results

5.3.1 Nature of the compounds identified in latex extracts of varieties

The compounds identified and quantified in the latex of the different sweetpotato varieties were mainly phenylpropanoid esters characterised by a long chain alcohol (Table 5.1). These compounds varied in nature and amount among the different varieties. They included hexadecyl-*p*-coumaric acid with molecular weight of 388, heptadecyl-*p*-coumaric acid with molecular weight of 402, octadecyl –*p*-coumaric acid with molecular weight of 416, and hexa-, hepta-, octa- , nona-decylcaffeic acids with molecular weights of 404, 418, 432 and 446, respectively. Others included and hexa- and octadecyl ferulic acids, with molecular weights of 418 and 446, respectively. These compounds vary in structure, based on substitution patterns on the aromatic ring and the length of the long chain alcohol and can be generally represented by the structural formula shown in Table 5.1 below.

Typical fragmentation in Pressure Chemical Ionisation (APCI) source (positive mode) of these compounds was characterised by the occurrence of a protonated molecule representative of the whole compound with specific fragments. For example for hexadecyl-*p*-coumaric acid protonated molecule was recoded at *m/z* 389 $[M + H]^+$ with protonated fragments at m/z 165 and m/z 147 representative of coumaric acid and coumaroyl ions consistent with the loss of $-C_{16}H_{33}$ and $[O\text{-}C_{16}H_{33}]^{\cdot}$ respectively, corresponding to the mass associated with the hexadecyl alcohol, which led to the positive identification of the compound. Hexadecyl caffeic acid was identified by a similar process and by comparison of the MS spectrum and retention time with standards.

Table 5.1. Phenylpropanoid esters identified in root latex of sweetpotato varieties with varying susceptibility to *Cylas* spp.



Compound	Molecular weight	$^1R_1$	$^1R_2$	Retention time (minutes) (*Z* and *E*)
Hexadecyl-*p*-coumaric acid*	388	H	$-(CH_2)_{15}CH_3$	24.8& 25.1
Heptadecyl-*p*-coumaric acid	402	H	$-(CH_2)_{16}CH_3$	25.6
Octadecyl-p-coumaric acid*	416	H	$-(CH_2)_{17}CH_3$	26.4& 27.7
Hexadecylcaffeic acid	404	OH	$-(CH_2)_{15}CH_3$	24.6& 23.9
Heptadecylcaffeic acid*	418	OH	$-(CH_2)_{16}CH_3$	24.4& 25.0
Octadecylcaffeic acid*	432	OH	$-(CH_2)_{17}CH_3$	25.4& 25.1
Nonadecylcaffeic acid*	446	OH	$-(CH_2)_{18}CH_3$	26.3& 26.6

Compounds **1** and **2** are the *Z* and *E* isomers of hexadecyl-*p*-coumaric acid and hexadecyl caffeic acid and are here illustrated showing the structural difference between the two isomers. All other compounds where *Z* and *E* forms were identified are indicated by *.

Table 5.2. Compound type by variety in the root latex of sweetpotato varieties

Compound	Varieties**									
	TZ	NP	KY	AN	DB	NK	OR	AR	AP	AND
Hexadecyl-*p*-coumaric acid* (388)	+	+	+	+	+	+	+	+	+	+
Heptadecyl-*p*-coumaric acid (402)	+	+	+	+	+	+	+	+	+	+
Octadecyl-*p*-coumaric acid*(416) Z	-	-	+	-	+	+	+	+	+	+
Octadecyl-*p*-coumaric acid*(416) E	+	+	+	+	+	+	+	+	+	+
Hexadecylcaffeic acid (404)	+	+	+	+	+	+	+	+	+	+
Heptadecylcaffeic acid* (418)	-	-	-	+	+	+	+	+	+	-
Octadecylcaffeic acid* (432)	-	-	-	-	+	+	+	+	+	-
Nonadecylcaffeic acid* (446)	-	-	-	-	-	+	-	-	-	-

Figures in parenthesis are: *Molecular weights; TZ: Tanzania; NP: Naspot1; KY: Kyebagambire; AN: Anamoyito; DB: Dimbuka; NK: New Kawogo; OR: OrurengoA; UNK4: AR: ARA228; AP: APA356; AND: Andinyaku; ** (+) means present and (–) means absent in the root latex

The chemical composition of the root latex varied among sweetpotato varieties occurring in resistant and some constituents were absent in some varieties including in susceptible ones such as Tanzania and NASPOT 1 (Table 5.2).

The resistant varieties including New Kawogo, Dimbuka, OrurengoA (Orurengo), ARA228 and APA356 had most of the compounds, while heptadecyl, octadecyl-and nonadecyl caffeic acids were not detected in the susceptible varieties, Tanzania and NASPOT 1. Four main compounds were identified in the latex of sweetpotato varieties evaluated:

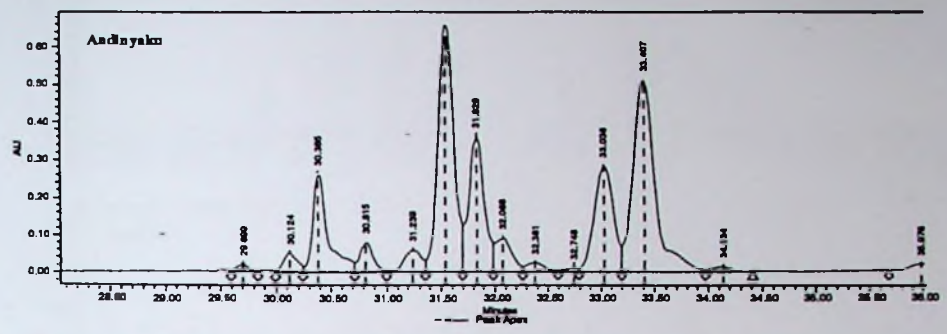
1. Hexadecylcaffeic acid

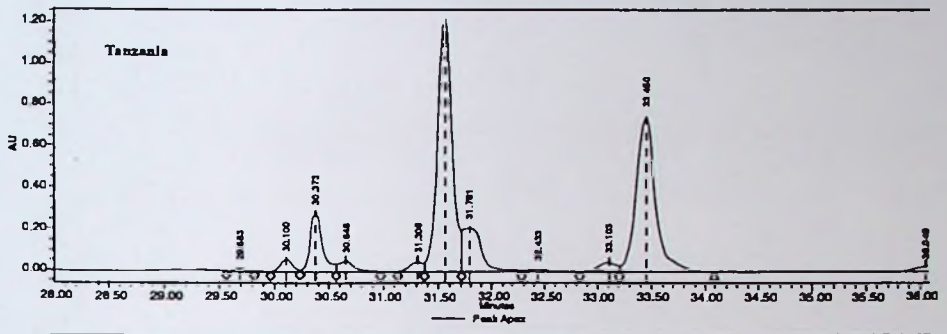
2. Heptadecyl-*p*-coumaric acid

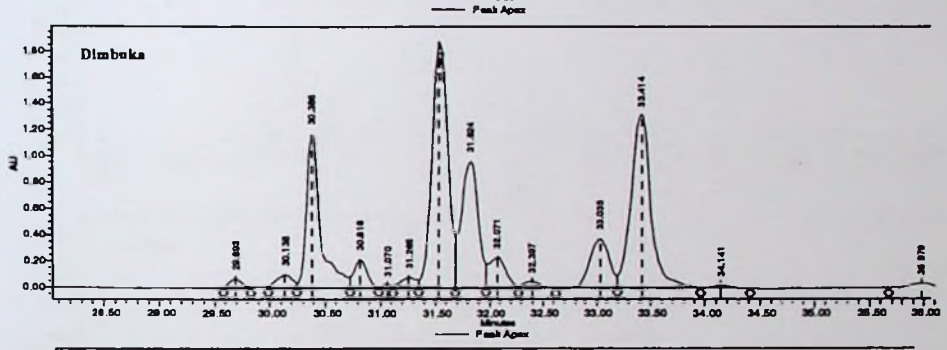
3. Hexadecyl-*p*-coumaric acid

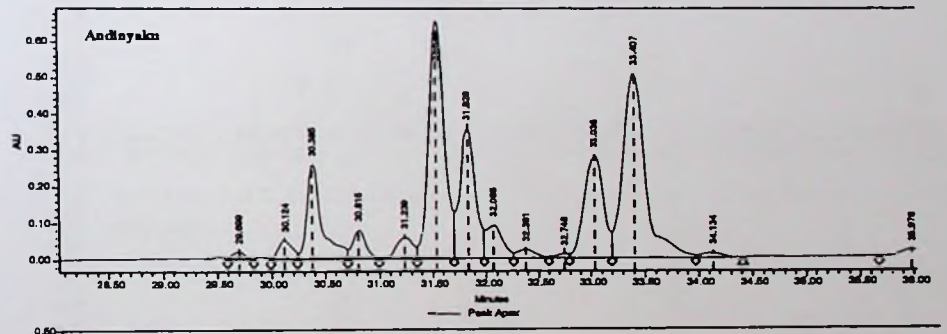
4. Octadecyl-*p*-coumaric acid

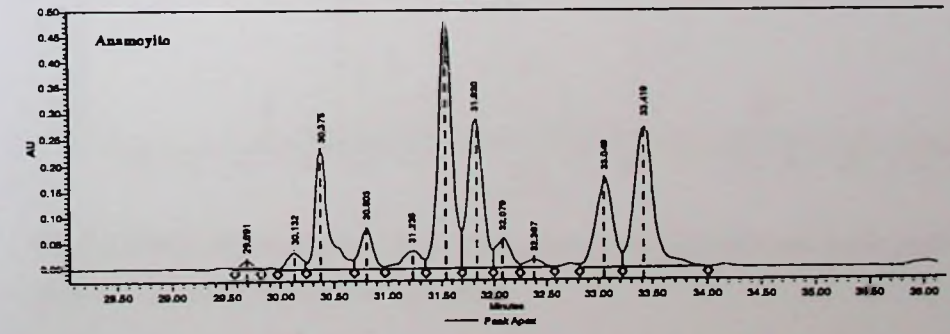
Nonadecylcaffeic acid was only detected in New Kawogo variety. The variations in the peaks in the chromatograms obtained from the root latex of all the varieties are presented in Fig. 5.1. The peaks show the maximum retention times for the compounds and sizes represent characteristic amounts of these compounds among the evaluated varieties; the fewer the peaks the less number of compounds found in the extract.


Andinyaku
AU
Minutes
Peak Apex


Tanzania
AU
Minutes
Peak Apex


Dimbuka
AU
Minutes
Peak Apex


Andinyaku
AU
Minutes
Peak Apex


Anamoyito
AU
Minutes
Peak Apex

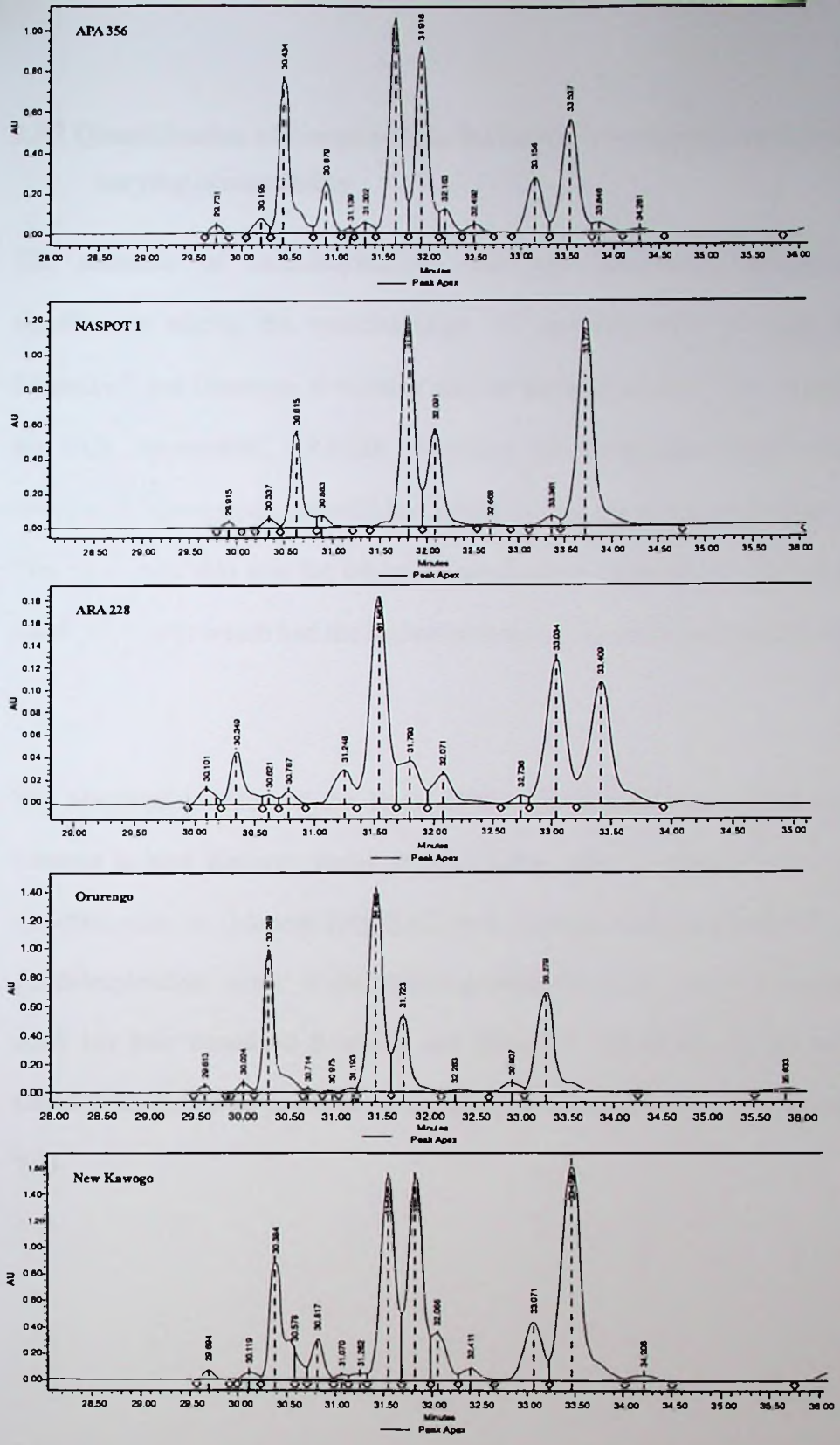


Fig.5.1 HPLC chromatograms at 325 nm showing the phenolic acid ester peaks in the non-polar region of the latex from all the varieties

5.3.2 Quantification of compounds in the latex of sweetpotato varieties of varying susceptibility

The amounts of hexadecylcaffeic acid and hexadecyl-*p*-coumaric acid varied significantly among the varieties (Figs. 5.2 and 5.3). New Kawogo, Kyebagambire, Dimbuka 2 and Orurengo A varieties had the highest amounts of hexadecylcaffeic acid in the latex. Meanwhile, ARA228, Andiyaku and Anamoyito which were observed as resistant to sweetpotato weevils had relatively low quantities of hexadecyl caffeic acid. This trend was also true for hexadecyl-*p*-coumaric acids among the varieties; except in ARA228 variety which had the highest amount of hexadecy-*p*-coumaric acids (Fig. 5.2).

The chemical profiles of the latex of the less susceptible varieties especially those inherent in New Kawogo varied in composition when compared to those of susceptible varieties such as Tanzania (Fig 5.4). New Kawogo contained peaks for compounds **1** (hexadecylcaffeic acid), **2** (hexadecyl-*p*-coumaric acid) and **4** (octadecyl-*p*-coumaric acid) but also contained **3** which was identified tentatively as octadecylcaffeic acid. Octadecylcaffeic acid occurred at negligible levels in the latex of Tanzania variety (Fig. 5.4).

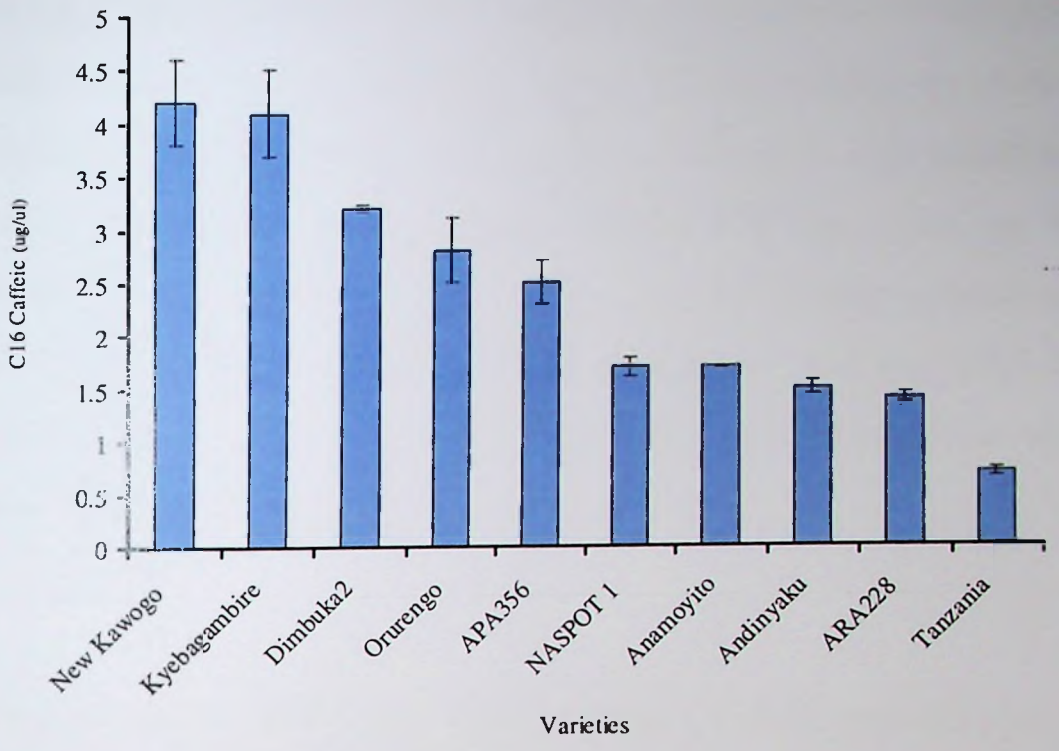


Fig.5.2. Concentration of hexadecyl-*p*- caffeic acids in latex of roots of sweetpotato varieties differing in susceptibility to *Cylas spp.*

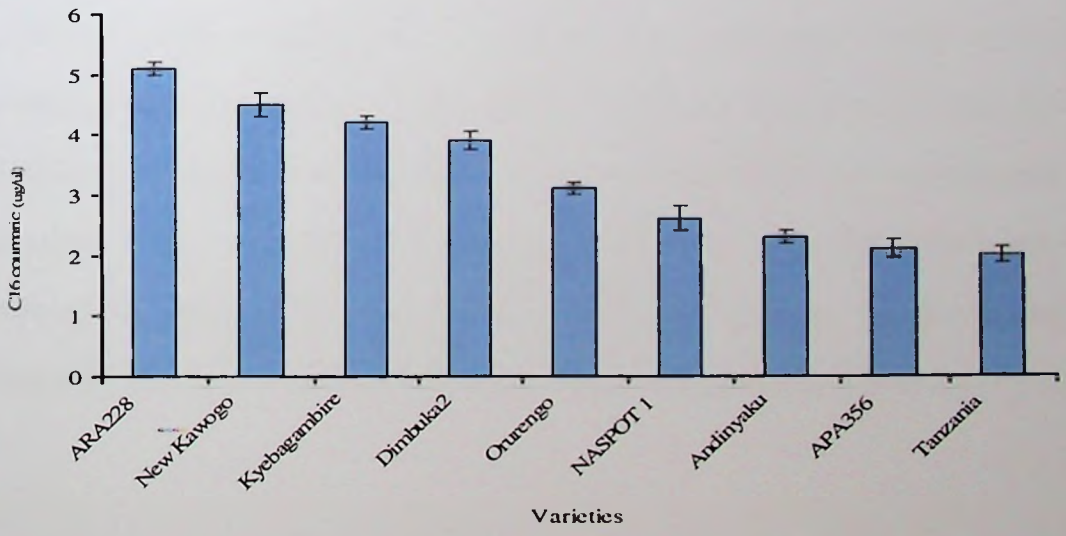


Fig.5.3.Concentration of hexadecyl- *p*-coumaric acid in µg/ml of latex among varieties differing in susceptibility to *Cylas* spp.

Hexadecylcaffeic acid and octadecylcaffeic acid identified in these varieties, were previously unknown as constituents of sweetpotato and thus discovered for the fist time during this study. Thus this study provided for the first time, evidence of a relationship between the field and laboratory varietal resitance to *Cylas* species, which enabled the pin pointing of these compounds as possible sources of resistance to *Cylas* spp. When hexadecyl-*p*-coumaric acid (**2**) was left in solution for 3 days at room temperature and in the presence of light, the compounds converted partially to the Z form which eluted approximately 30 seconds earlier on the LC-MS and was characterized by a mass and UV spectra consistent with the *Z*-form of coumaric acid (Fig. 5.4).

It is important to note that only hexadecylcaffeic acid, hexadecyl-*p*-coumaric acid, heptadecyl-*p*-coumaric acid and octadecyl-*p*-coumaric acid (E-form) were identified in the root latex of the susceptible variety Tanzania with hexadecyl-*p*-coumaric acid being the major component (Fig.5.4 and Table 5.1). Conversely, all of the compounds occurred in the latex of the resistant variety New Kawogo. Their abundance relative to the most abundant peak in each analysis indicated that for the two susceptible varieties, the most abundant compounds were hexadecylcaffeic acid and hexadecyl-p-coumaric acid with complete absence of compound 3 (octadecylcaffeic acid) in Tanzania variety and lower amounts of octadecyl-*p*-coumaric acid (E-form) and absence of the Z-form this compound (octadecyl-*p*-coumaric acid) in the latex of Tanzania (Fig. 5.4).

There was variation in amounts of these four compounds in the latex of the rest of the varieties (Fig. 5.6). The resistant varieties Dimbuka 2, Andinyaku, Kyebagambire,

Anamoyito, ARA228, APA356 and Ororengo possessed higher amounts (larger peak areas) of hexadecylcaffeic acid whereas Tanzania and NASPOT 1 had very low amounts of these compounds. Thus the amount of hexadecylcaffeic acid in the resistant varieties followed a pattern similar to New Kawogo and susceptible varieties NASPOT 1 and Tanzania correspondingly with lower amounts of these compounds.

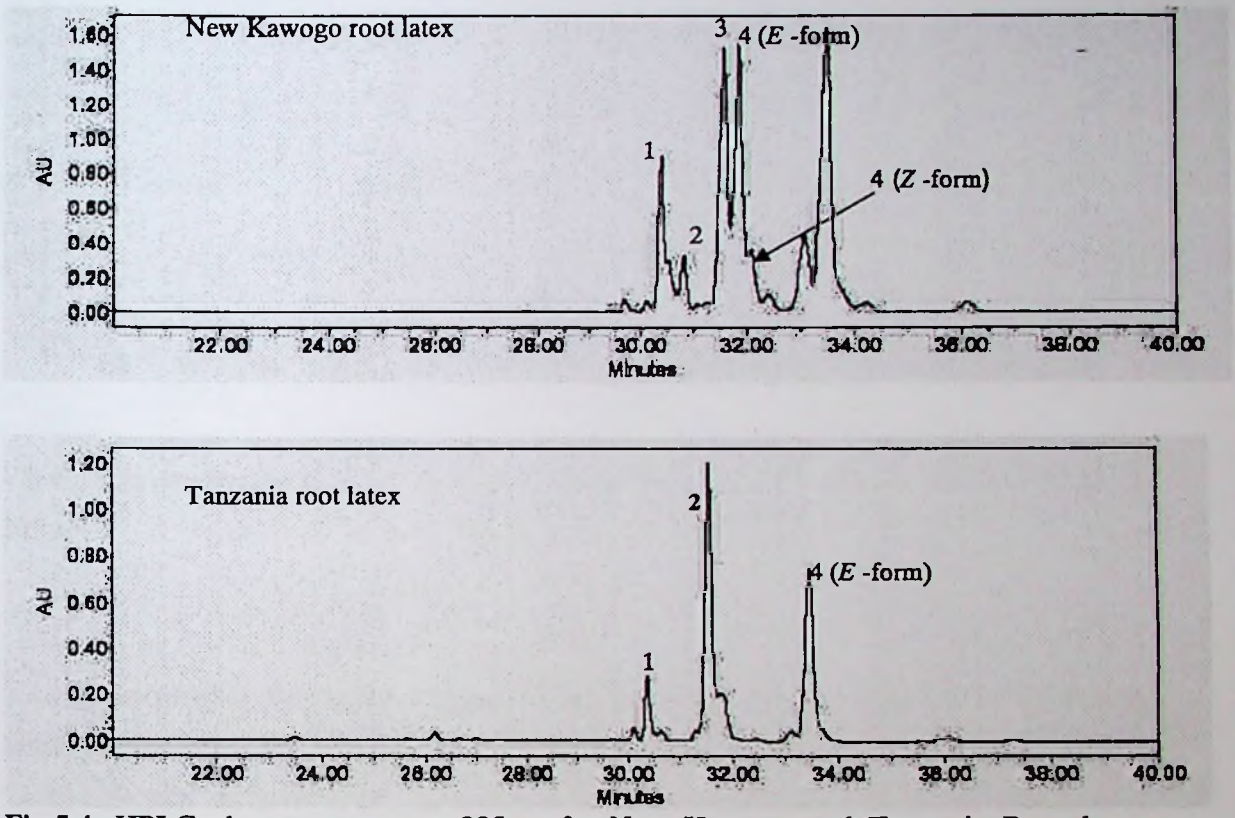


Fig.5.4. HPLC chromatograms at 325nm for New Kawogo and Tanzania Root latex (40 mg latex/ml) illustrating differences in chemical profile (1= hexadecylcaffeic acid, 2= hexadecyl-*p*-coumaric acid 3= octadecylcaffeic acid) and 4= octadecyl-*p*-coumaric acid)

The identified compounds had different in their UV spectra. Hexadecyl caffeic acid showed band maxima at 245.7nm and 328 nm (Fig 5.5) while hexadecyl-*p*-coumaric acid had band maxima at 238 and 314nm, respectively.

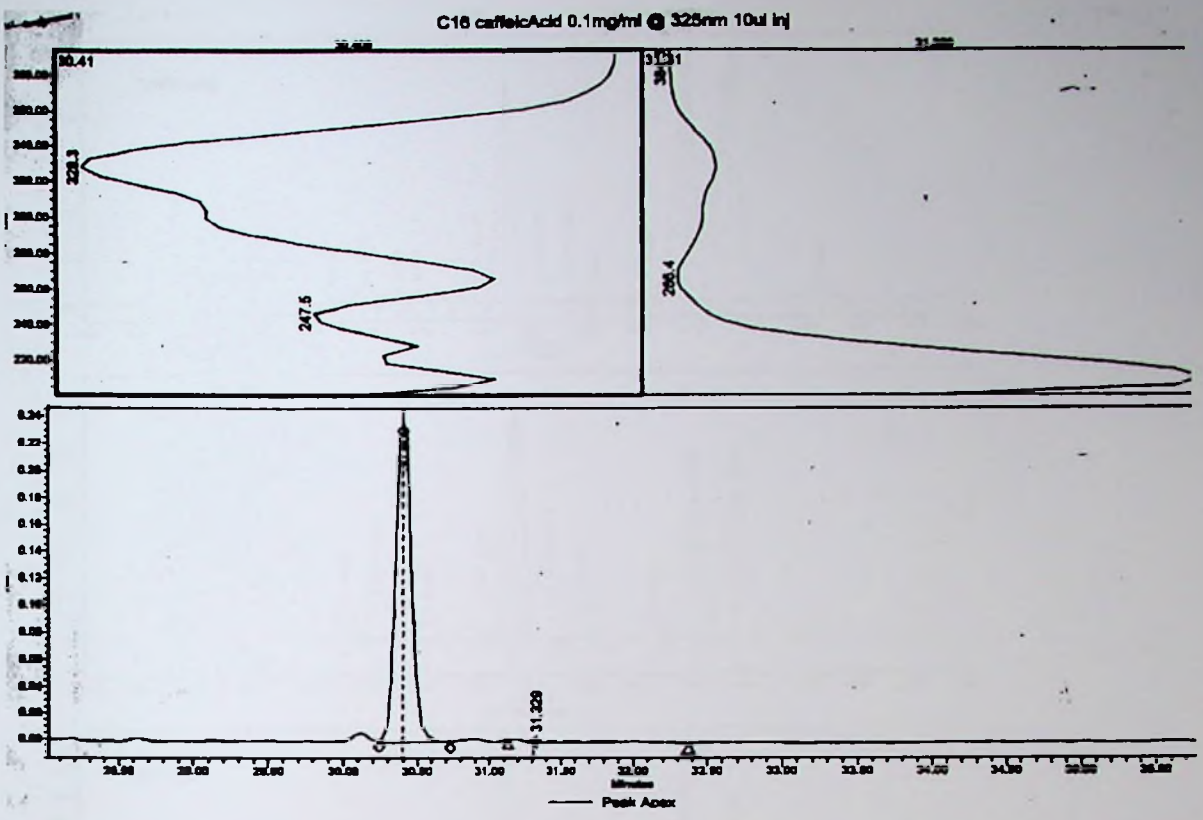
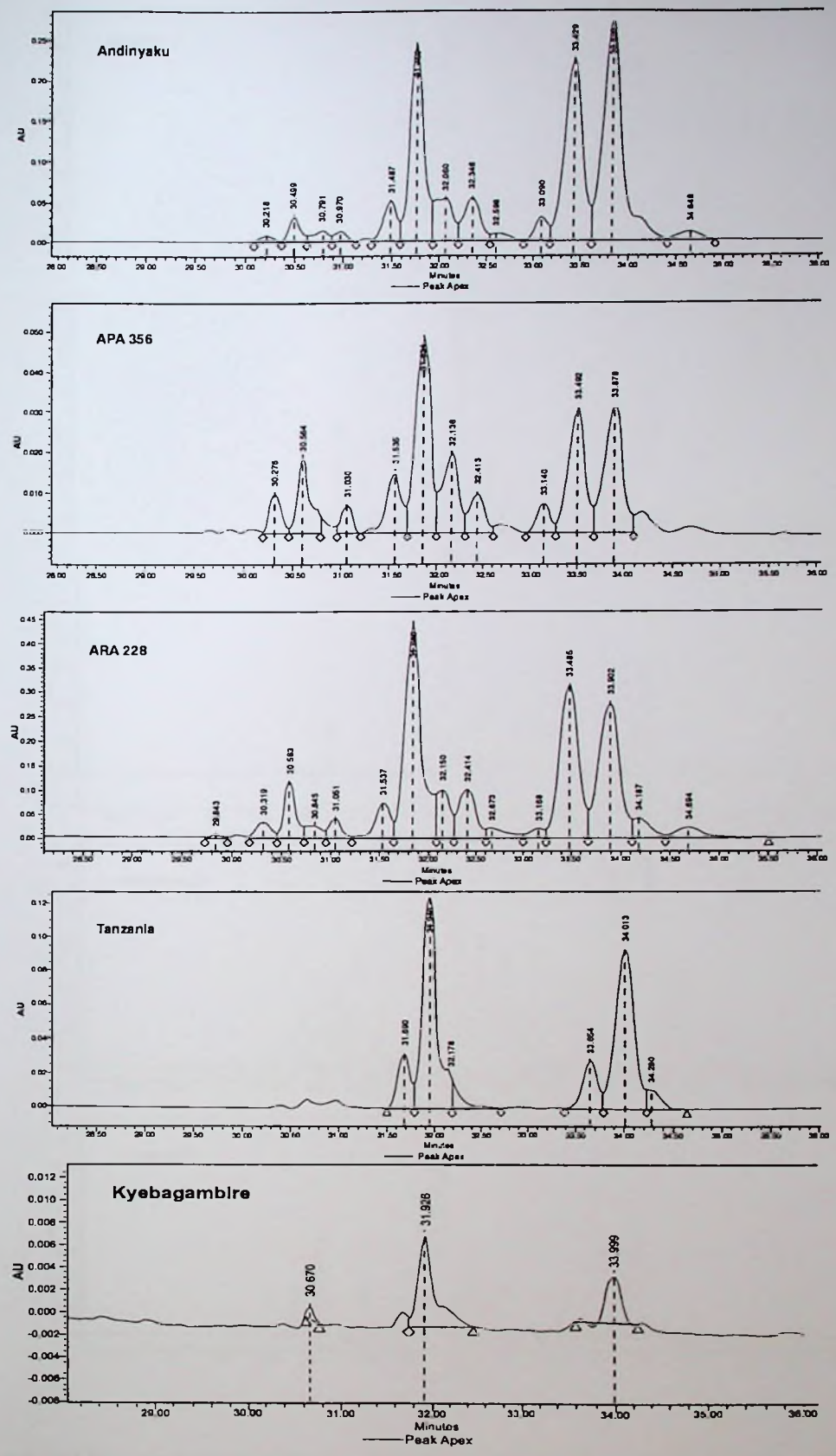


Fig 5.5. Chromatogram and UV spectrum of one of hexadecyl caffeic acid (standard) at 325nm

5.3.3 Sweetpotato root surface chemical composition among varieties with varying levels of resistance to *Cylas* spp.

The qualitative and quantitative variations in the constituents of the surfaces of resistant and susceptible varieties is shown in Table 5.3 below.

Surfaces of susceptible varieties such as Tanzania and NASPOT 1 had no hexa-, hepta-, octa- and nona-decyl caffeic acids, whereas they occurred on all the resistant varieties. Intermediate varieties represented by Kyebagambire, had only hexa- and heptadecyl caffeic acids and lacked the rest of the caffeic acid esters. All the varieties had coumaric acid esters (Table 5.3; Fig. 5.6).


Andinyaku
AU
Minutes
Peak Apex
APA 356
AU
Minutes
Peak Apex
ARA 228
AU
Minutes
Peak Apex
Tanzania
AU
Minutes
Peak Apex
Kyebagambire
AU
30.670
31.928
33.999
Minutes
Peak Apex

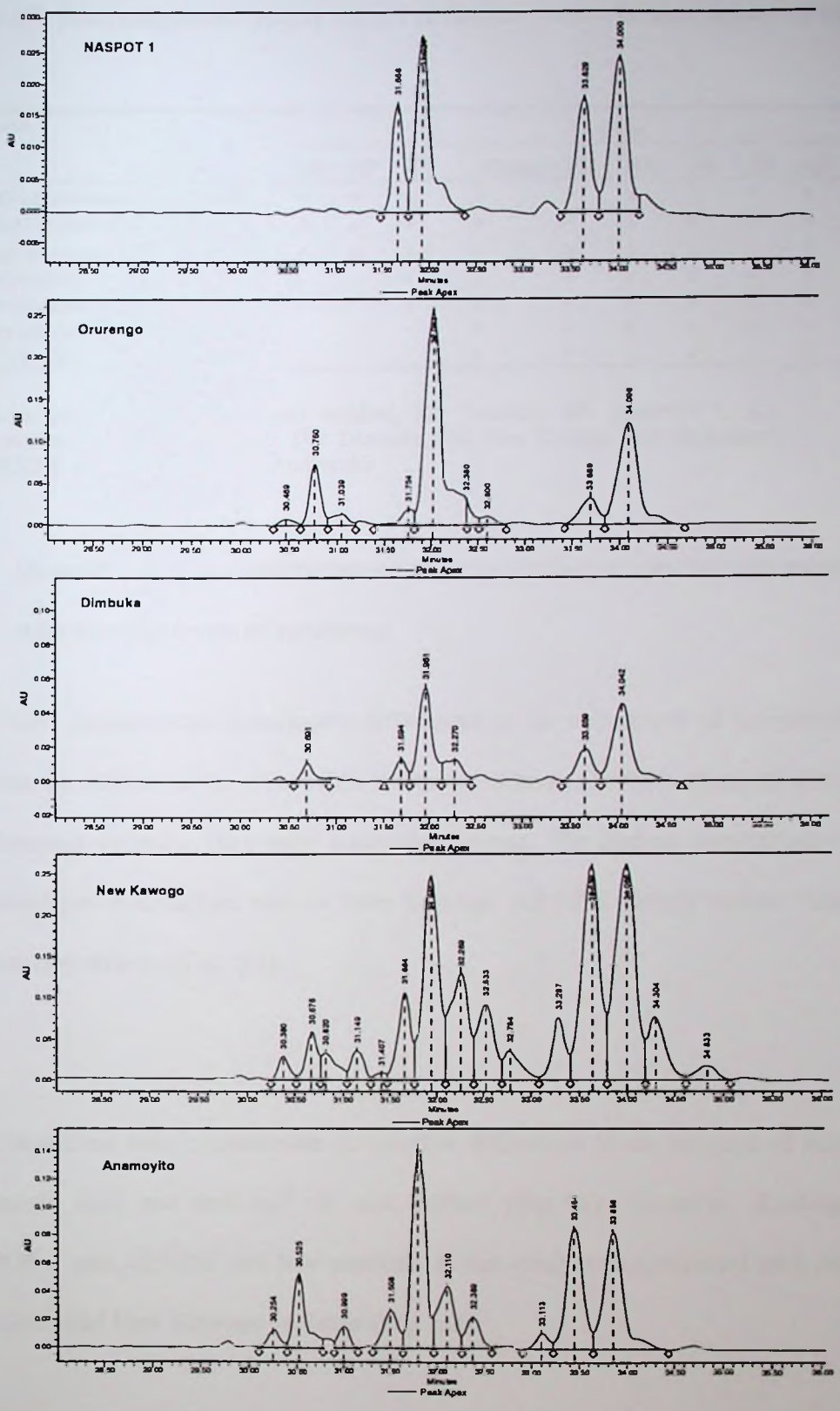


Fig 5.6. HPLC chromatograms at 325 nm showing the phenolic acid ester peaks in the non-polar region of the surface extracts of all the varieties

Table 5.3. Root compound type by variety in the root surface of sweetpotato varieties

Compound	Varieties									
	TZ	NP	KY	ANAM	DB	NK	OR	AR	AP	AND
Hexadecyl-*p*-coumaric acid* *(388)*	+	+	+	+	+	+	+	+	+	+
Heptadecyl-*p*-coumaric acid (402)	+	+	+	+	+	+	+	+	+	+
Octadecyl-p-coumaric acid* (416)	+	+	+	+	+	+	+	+	+	+
Hexadecylcaffeic acid (404)	-	-	+	+	+	+	+	+	+	+
Heptadecylcaffeic acid* (418)	-	-	+	+	+	+	+	+	+	+
Octadecylcaffeic acid* (432)	-	-	-	+	+	+	+	+	+	+
Nonadecylcaffeic acid* (446)	-	-	-	+	+	+	+	-	+	+

Figures in parenthesis are Molecular weights; TZ: Tanzania; NP: NASPOT 1; KY: Kyebagambire; ANAM: Anamoyito; DB: Dimbuka; NK: New Kawogo; OR: OrurengoA; AR: ARA228; AP: APA356; AND: Andinyaku

5.3.4 Quantification of constituents on the root surface of sweetpotato varieties with varying levels of resistance

There were considerable quantitative differences in the occurrence of hexadecylcaffeic esters on the surface of the roots of the different varieties. On the surfaces of NASPOT 1 and Tanzania varieties, they were completely absent. The highest concentration of the compound per unit surface was on New Kawogo, ARA228 variety and on Anamoyito varieties respectively (Fig. 5.7).

In addition there were considerable quantitative differences in the amounts of hexadecyl-*p*-coumaric acid per unit cm^2 of root surface (Fig.5.8). Tanzania, Kyebagambire, NASPOT 1 and APA356 had low amounts of this compound compared with ARA228, OrurengoA and New Kawogo varieties (Fig. 5.8).

5.3.5 Variation in root chemical content

Total amounts of the compounds per unit area of root surface followed the general trend recorded above, with the resistant varieties ARA228, OrurengoA and New Kawogo having higher quantities of total hexadecyl esters than the susceptible NASPOT 1 and Tanzania (Fig.5.9). Kyebagambire and APA356 varieties had relatively low amounts of the compounds per unit of surface, which may be related to their intermediate resistance status.

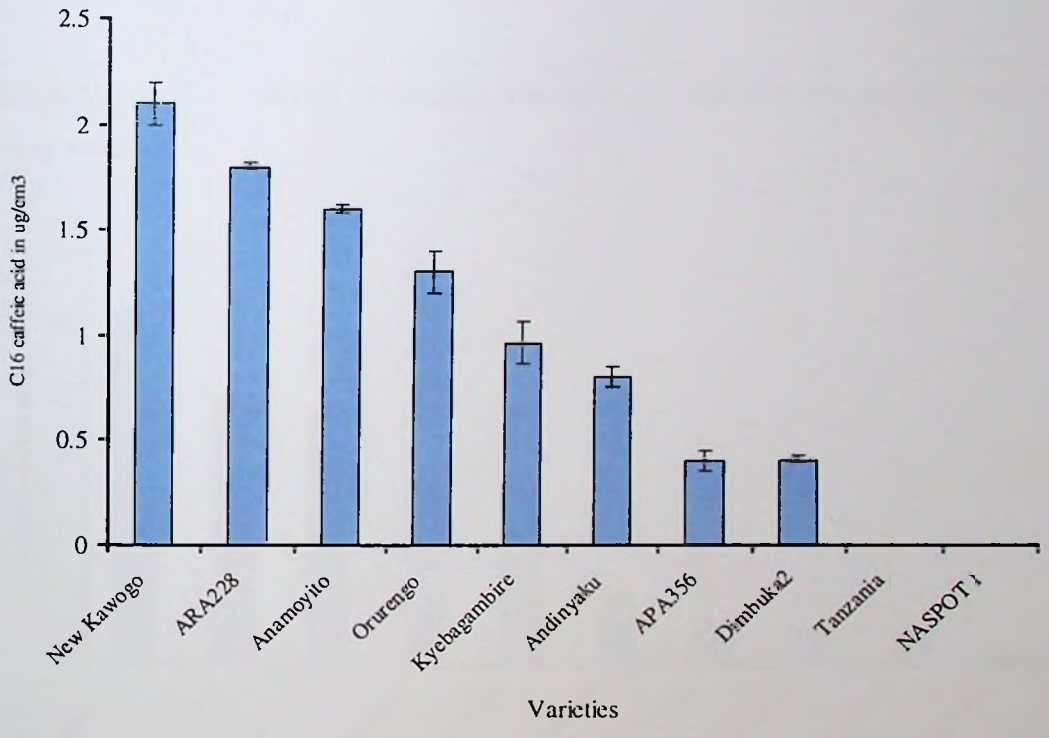


Fig.5.7 Variation in amount of hexadecylcaffeic acids in $\mu g/cm^3$ of root surface among varieties

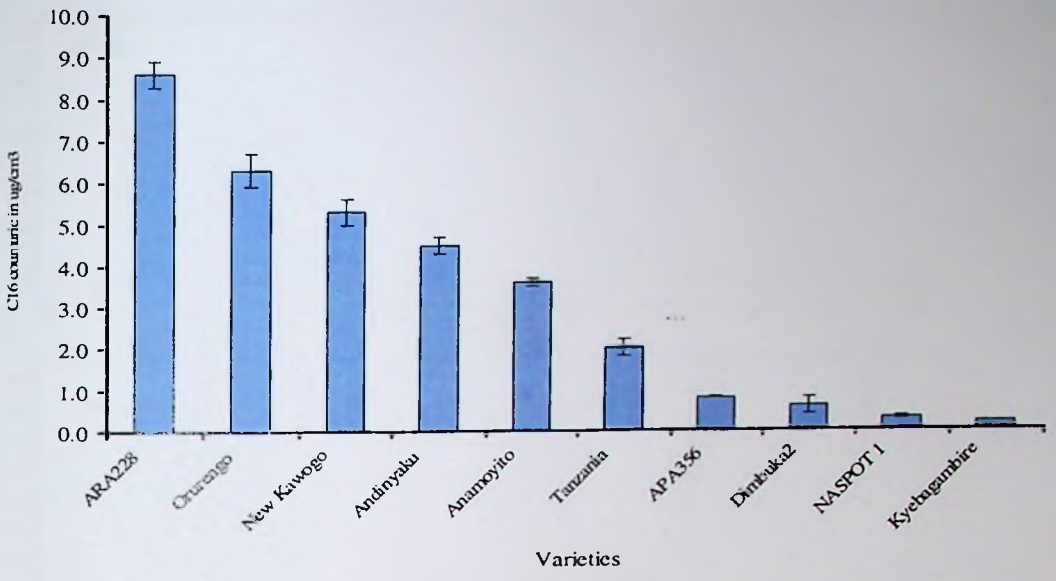


Fig.5.8. Variation in amount of hexadecyl-*p*- coumaric acid per unit cm^2 of root surface among varieties

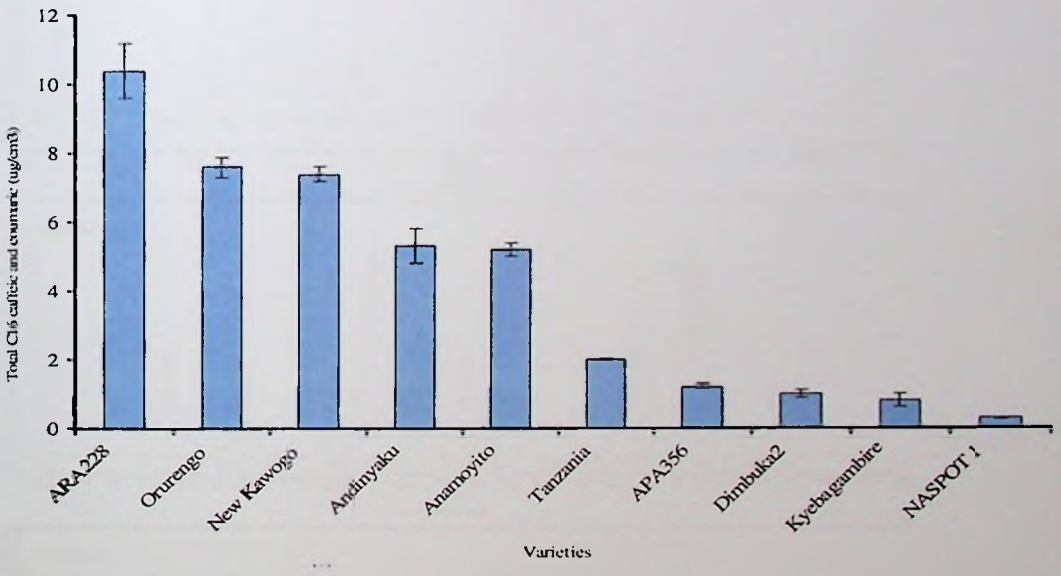
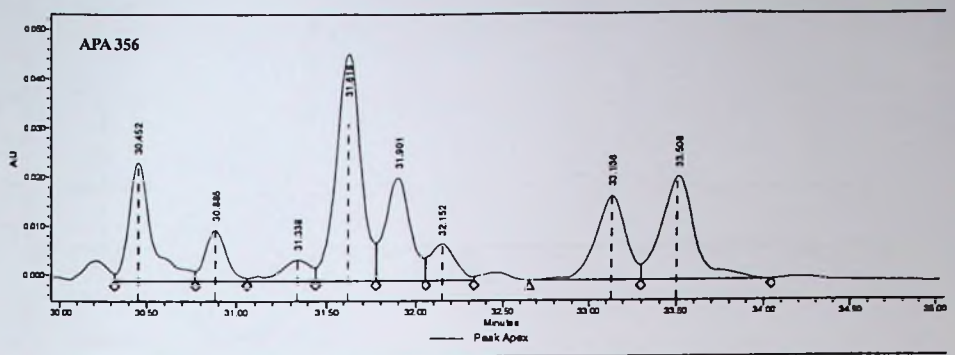
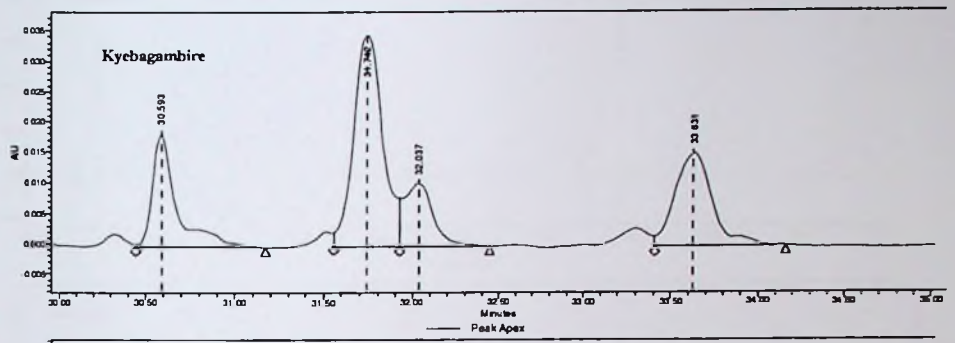
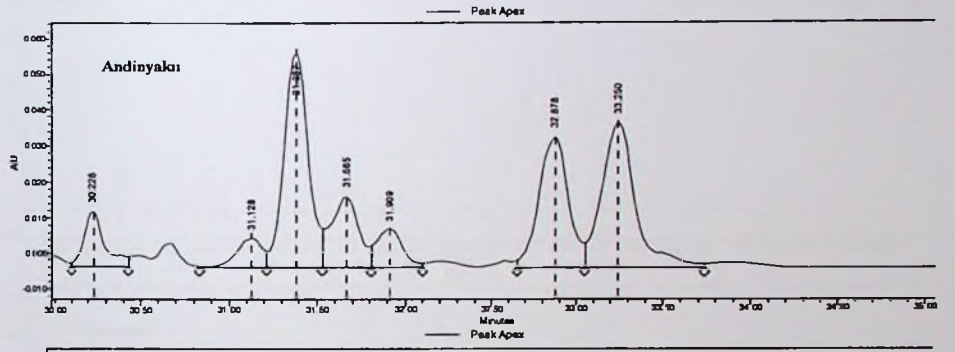


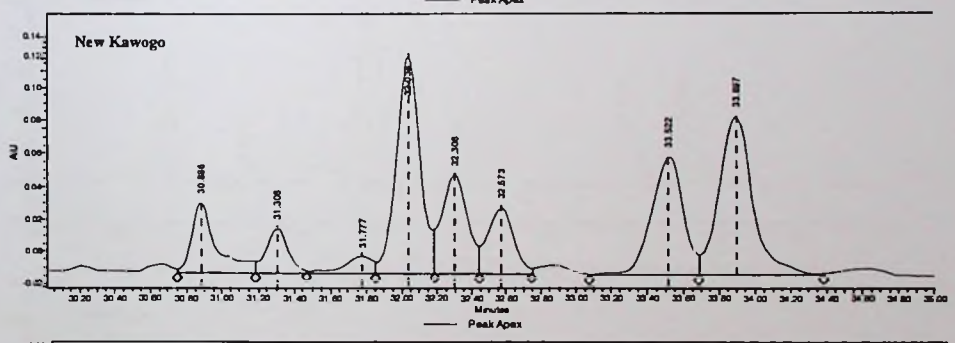
Fig.5.9. Variation in total hexadecylcaffeic acid and hexadecyl-*p*- coumaric acid in $\mu g/cm^2$ of root surfaces of varieties

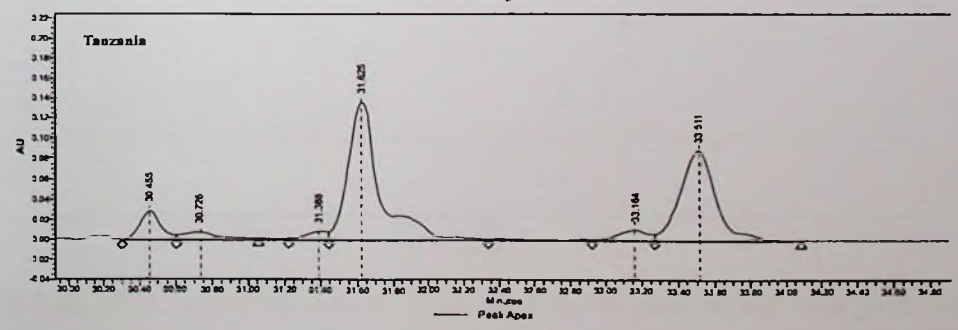
HPLC chromatograms at 325nm of the whole root extracts of the different varieties are presented in Fig 5.10. There were clear variations in the qualitative composition and content of the chemicals in whole roots of varieties.


APA 356
AU
30.452
30.886
31.338
31.611
31.901
32.152
33.104
33.508
Minutes
Peak Apex


Kyebagambire
AU
30.593
32.037
33.631
Minutes
Peak Apex


Andinyakı
AU
30.226
31.128
31.665
31.909
32.878
33.250
Minutes
Peak Apex


New Kawogo
AU
30.884
31.306
31.777
32.306
32.573
33.522
33.887
Minutes
Peak Apex


Tanzania
AU
30.455
30.726
31.625
33.164
33.511
Minutes
Peak Apex

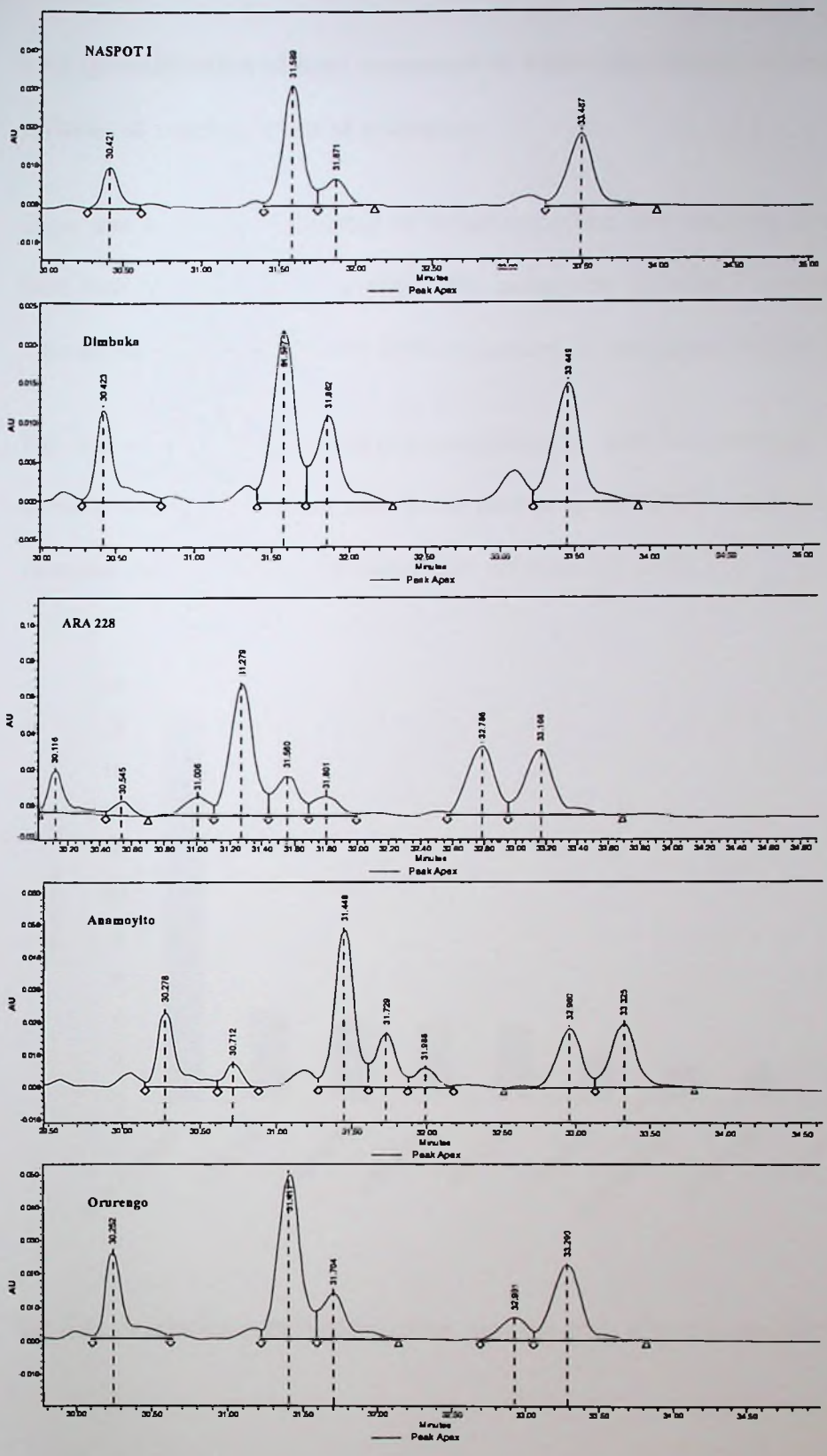


Fig 5.10. HPLC chromatograms at 325 nm showing the phenolic acid ester peaks in the non-polar region of whole root extracts of the varieties

5.3.6 Quantification of total compound in whole root extracts of sweetpotato varieties of varying levels of resistance

There was higher concentration of hexadecylcaffeic acid esters in New Kawogo whole roots than in all the other varieties. The susceptible varieties Tanzania and NASPOT 1 varieties had very low amounts of the compound per unit gram of roots (Fig 5.11).

This was also true for hexadecyl-*p*-coumaric acid, with New Kawogo having very high levels of the compound and none being present on NASPOT 1 and very low amounts in Tanzania, Andinyaku and Kyebagambire varieties (Fig 5.12).

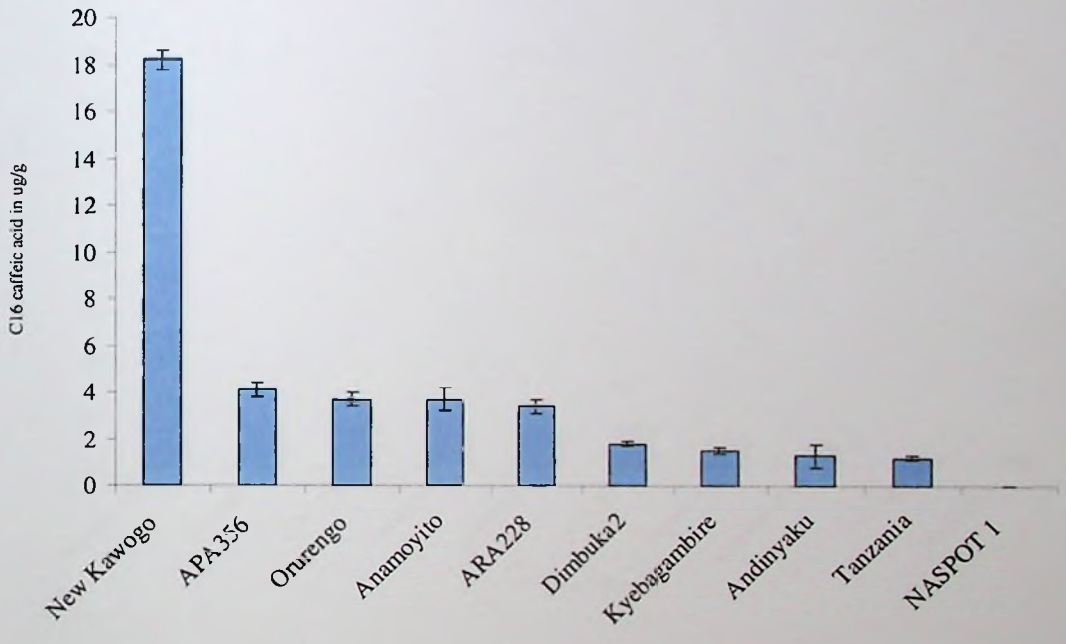


Fig 5.11. Variation in hexadecyl caffeic acid per gram of root in varieties.

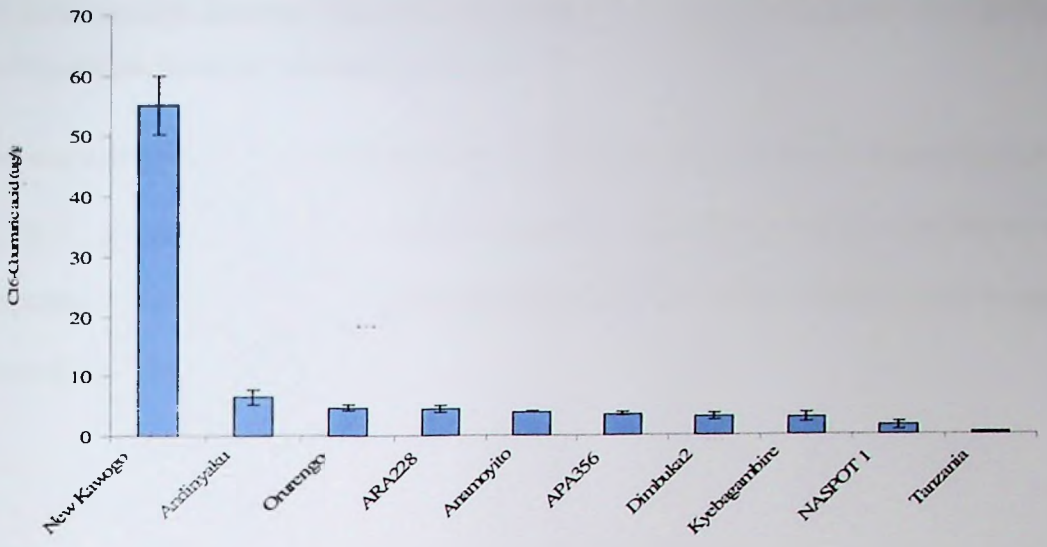


Fig 5.12 Variation in hexadecyl-*p*-coumaric acid per gram of roots among varieties

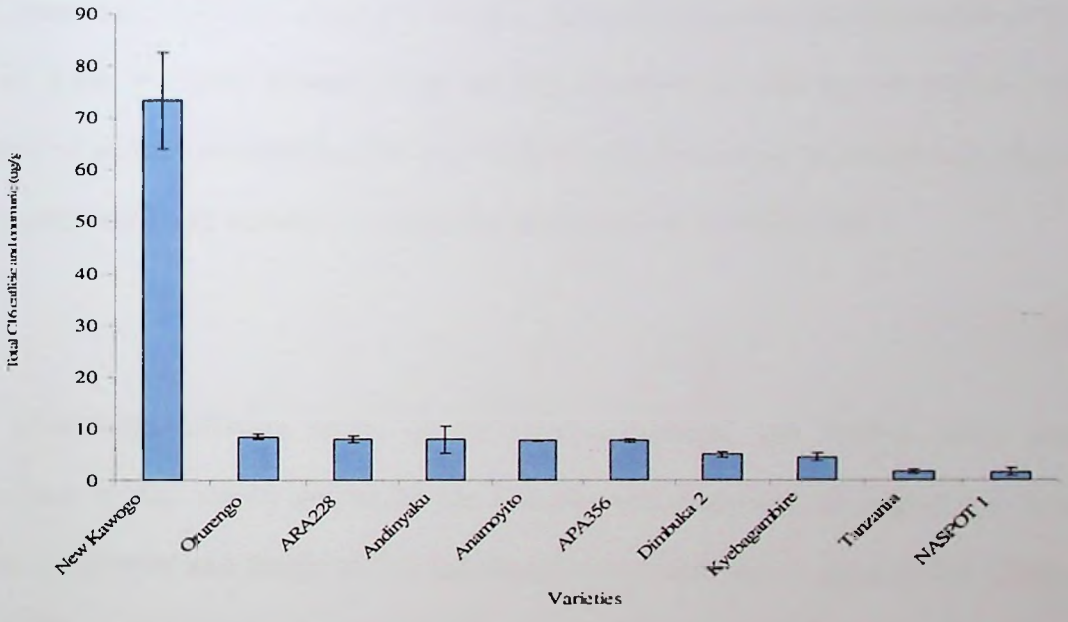


Fig.5.13. Variation in total hexadecyl-*p*-coumaric and hexadecylcaffeic acid per gram of root among varieties

Investigation of the total amount of hexadecylcaffeic and hexadecyl-*p*-coumaric acids in the root extracts of all varieties revealed that the highest concentration per unit gram was found in New Kawogo and the lowest in NASPOT 1 and Tanzania varieties (Fig 5.13).

5.3.7 Relationship between amounts of surface root chemical content and feeding and oviposition levels of selected varieties

There was a negative relationship (r=-0.58; -0.39 and P< 0.05) between mean oviposited eggs by *C. puncticollis* and *C. brunneus* weevils, respectively, on root periderms of sweetpotato varieties (Table 4.3) and total hexadecylcaffeic and *p*-coumaric acid in their surfaces (Table 5.4).

The relationship between feeding holes was also strongly negatively correlated (r= -0.74 P< 0.05) to feeding by *C. brunneus* and slightly (r=-0.47) negatively correlated to feeding by *C. puncticollis* weevils. There was a highly significant negative correlation (R= -0.85 and r= -0.64; P< 0.05) between mean feeding punctures of both weevil species and amounts of surface hexadecylcaffeic acid (Table 5.4) and between levels of oviposition by *C. puncticollis* and amounts of hexadeclycaffeic acid (r= -0.84; P< 0.05).

The relationship between levels of hexadecyl-*p*-coumaric and caffeic acids and oviposition by the weevil species on the varieties was negatively correlated for both species of weevils and highly significant (r=-0.84 P< 0.001) for *C. puncticollis* (Table 5.4). This meant that occurrence of these compounds was strongly associated with reduced oviposition and feeding punctures.

Table 5.4. The relationship between surface root chemical composition and oviposition and feeding of *C. brunneus* and *C. puncticollis* on the varieties

Surface chemical	r –values			
	Oviposition		Feeding	
	C.brunneus	*C .puncticollis*	*C. brunneus*	*C .puncticollis*
Total surface hexadecyl coumaric + caffeic acids	-0.39	-0.58*	-0.74*	-0.47
Surface hexadecyl coumaric acid	-0.35	-0.49	-0.69*	-0.4
Surface hexadecylcaffeic acid	-0.52	-0.84**	-0.85**	-0.64*

Df= 9; * Significant at P< 0.05; ** Significant at P< 0.001

5.4 Discussion

There were marked differences in the chemistry of the extracts from root surfaces, latex and whole root of the different sweetpotato varieties. The identified components were mainly phenylpropanoids esterified to long chain alcohols. The findings in this study have contributed to new previously unreported information on the chemistry of sweetpotato varieties. This confirms earlier reports which suggested possible existence of plant chemicals such as complex phenolic compounds in sweetpotato (Snook *et al.,* 1994; Wilson, 1990; Muyinza *et al.,* 2007) and provides the identity of compounds and their location in different parts of sweetpotato roots.

Root surface extracts of resistant varieties like New Kawogo and ARA228 had higher concentration of hexadecyl-*p*-coumaric and caffeic acids per unit gram of root. The root surface content of these constituents were also relatively high. Susceptible varieties NASPOT 1 and Tanzania, for example, unlike in less susceptible varieties, had no

octadecyl caffeic and nonadecylcaffeic acids (both Z and E forms). The absence of these compounds in the latex and on root surfaces of susceptible varieties such as Tanzania and NASPOT 1 and their presence in resistant varieties like New Kawogo may indicate their possible mediation of different varieties by *Cylas* species. The observed absence of the compounds in NASPOT 1 internal root tissues, may explain the lower oviposition levels on the surfaces of varieties like New Kawogo and higher ones on susceptible varieties.

Further, the total hexadecylcaffeic and hexadecyl-*p*-coumaric acids per unit weight of the roots were higher in less susceptible varieties than in the more susceptible ones. This may be related to longer development time and lower numbers of emergent adults from resistant varieties such as New Kawogo (sub-sections 4.4 and 4.5). Earlier reports suggested the possibility of mediation of root chemical constituents in sweetpotato resistance to *Cylas spp.* and differences in root chemistry was implicated in differences in varietal resistance to these pests (Son *et al.*, 1990; Stevenson *et al.*, 2009; Muyinza *et al.*, 2007). The absence of heptadecyl, octadecyl and nonadecylcaffeic acids in susceptible varieties and extremely low levels of hexadecylcaffeic acids on the more susceptible varieties such Tanzania provides support to this hypothesis.

The high content of surface compounds was positively associated with reduced oviposition and feeding by *C. puncticollis* and *C. brunneus* on resistant varieties such as New Kawogo. The results provided for the first time a link between field levels of resistance and sweetpotato chemistry. In a study conducted by Stevenson *et al.*, (2009), there was a significant reduction in number of feeding punctures and lower oviposition by *Cylas* spp. when synthesized root latex compounds were surface treated on to the roots

of susceptible sweet potato varieties (sub-section 6.4.1). This strongly suggested that latex chemicals may have deterrent effects on functions such as oviposition, feeding and larval development. The resulting antixenosis and /or antibiosis effects probably accounts for the differences in levels of resistance to *Cylas* spp. among varieties. There is, however, need for further validation using bio-assays to determine the effect of key compounds identified in sweetpotato on the biology of *Cylas* spp.

5.5 Conclusion

The findings in this study clearly showed the existence of variations in the chemical composition of the root latex, periderm and flesh of the sweetpotato varieties examined. The results of this study suggest a possible link beween *Cylas* weevil infestation and sweet potato root chemical composition, which may account for the resistance of different sweetpotato varieties.

Further studies on the specific effects of the compounds on oviposition, feeding and larval development were needed to shed more light on their roles in conferring resistance to sweetpotato.

CHAPTER SIX

THE EFFECT OF SWEETPOTATO ROOT CHEMISTRY ON *Cylas brunneus* AND *Cylas puncticollis* BIOLOGY

6.1 Introduction

Phenolic hydroxycinnamic esters, such as hexadecyl and octadecyl-*p*–coumaric caffeic acids, were found on the root surface of resistant varieties like New Kawogo in greater quantities than the on susceptible varieties such as Tanzania and NASPOT 1. Previous research on the effect of phenolic compounds, including cinnamic acids like coumaric acid, showed their inhibitory effects on the biology of herbivorous insects such as *Helicoverpa zea* (Summers and Felton, 1994), and stimulatory activity on the oviposition of *Spodoptera littoralis* (Boisd.). The pattern of qualitative and quantitative differences in sweetpotato varieties obtained in the present study in different sweetpotato varieties, suggested an active role by plant phenolic compounds in influencing the biology of herbivores, including that of *Cylas* spp. In addition, other researchers have reported existence of considerable quantities of chlorogenic and isochlorogenic acids in sweetpotato (Islam *et al*, 2003a). The effects of all these compounds, however, on the biology of the pests, was largely unknown; thus, underlining the need for a deeper understanding of the link between chemical root composition and mechanisms of resistance to the pests.

Thus, the purpose of this part of the study was: to investigate the effect of selected sweetpotato root compounds on oviposition, feeding and development of *C. puncticollis* and *C. brunneus* and identify candidate compounds that could be used for sweetpotato breeding for *Cylas* weevil resistance.

6.2 Materials and methods

6.2.1 Effect of hexadecyl caffeic and hexadecyl-*p*-coumaric acids on oviposition and feeding of female *C. brunneus* and *C. puncticollis* on surface treated periderms of susceptible varieties

Freshly harvested roots of NASPOT 1 variety were washed gently with water to remove soil and, using a cork borer, root periderms of each of these varieties were placed in 12 tray bio-assay for choice and no-choice bio-assays (similar to description in sub-section 4.4.1).

Hexadecylcaffeic acid and hexadecyl-*p*-coumaric acids were artificially synthesized in the laboratory at the Natural Resources Research Institute (Dr. Hall). They were then mixed with acetone to make 1, 0.1, 0.01 and 0.001 mg/ml solutions for use in bio-assays. A choice bio-assay tray that involved untreated NASPOT 1 and treated NASPOT 1 periderms were set up as described previously (sub-section 4.4.1). On to each treated periderm, 50µl of the test solution was applied using an Eppendorf pipette (the amount of compound per unit periderm at the different concentrations was comparable to the quantities expressed naturally on root surfaces of sweetpotato varieties where they occur). On the control periderms similar amounts of acetone was applied. Twelve mated adult 7-14 day old female *C. brunneus* or *C. puncticollis* weevils were introduced into separate trays, which were covered to prevent escape of the weevils but allowed movement between wells, and left to stand for 24 hours. Each of the treatments was replicated 20 times in sets of 5 tray regimes. The mean numbers of eggs, feeding holes and droppings on periderms of each variety were recorded.

6.2.2 Effect of hexadecyl-*p*-coumaric, hexadecylcaffeic acid and chlorogenic acid on larval development in diet bio-assays

Hexadecylcaffeic and hexadecyl-*p*-coumaric acids were incorporated as solutions in ethanol into an artificial diet that could rear larval stages of *C. puncticollis* and *C. brunneus* to adulthood (Ekobu *et al.*, 2006)

Different concentrations of the respective compounds (hexadecylcaffeic acid, hexadecyl-*p*-coumaric acid and chlorogenic acid) were made up by adding varying amounts of the compounds to 90 g of diet as follows;

i) Concentration 1: 0.1% w/w- 100 mg of the test compound was dissolved in 2 ml of ethanol. From this 1.8 ml of the solution containing 90 mg of compound, was added to 90 g of diet to make 0.1 w/w dosage treatment.

ii) Concentration 2: 0.01 % w/w- 10 mg of test compound was dissolved in 2 ml of ethanol, from which 1.8 ml containing 9 mg of compound was added to 90 g of the diet to make 0.01 w/w dosage treatment.

iii) Concentration 3: 0.001 % w/w –A solution containing 1 mg of test compound in 2 ml of ethanol was obtained. From this 1.8 ml containing 0.9 mg of compound was added to 90 g of the diet to make 0.001 w/w dosage.

iv) Concentration 4: 0.0001 w/w- A solution containing 0.1 mg of test compound in 2 ml of ethanol was made. From this 1.8 ml containing 0.09 mg of compound was added to 90 g of the diet to make 0.0001 w/w dosage.

Then diets above were mixed using a magnetic stirrer and poured when still hot into sterile plastic petri dish replicates. After the diet had cooled and set, 5 small pieces of diet were

scooped out with a plastic spatula leaving burrows on the diet surface. Early second instar larvae (noticed to be at this development stage, basing on their head capsule width) (Ekobu, 2006) for both species, were obtained from inoculated sweetpotato roots, which had been previously infested with adult *Cylas* weevils of either species. The roots were first sterilised by washing them with 100% ethanol to reduce diet contamination during larval extraction. Small layers of the periderms were cut with a sharp blade to expose and collect larvae. The larvae were then gently brushed into the test diets using a soft entomological brush. They were then placed in the burrows and the displaced diet was gently replaced on top to reduce the risk of desiccation (Fig 2.1 Sub-section 2.3). The experiment was then left at ambient temperature and relative humidity until 50% pupation was recorded in the controls. Mean larval mortality, larval weight and mean number of pupating larvae was recorded for all treatments.

6.3 Data analyses

Data generated in all these experiments was analysed using descriptive statistics in Microsoft Excel and STATICA statistical package. Students t-test was used to compare means from choice bio-assay data at $P \leq 0.05$.

6.4 Results

6.4.1 Effect of cinnamic esters on adult *Cylas* weevil biology

Surface treatment of sweetpotato with hexadecylcaffeic acid and hexadecyl-*p*-coumaric acid affected the biology of both *C. puncticollis* and *C. brunneus* weevils. The feeding frequency, number of oviposited eggs and frass produced on the root surfaces of NASPOT 1, a weevil susceptible sweetpotato variety, treated with hexadecylcaffeic acid or hexadecyl-*p*-coumaric acid, were significantly lower than on untreated root surfaces.

Similarly, there were significant variation in mean feeding frequency on periderms treated with hexadecyl-*p*-coumaric acid or hexadecylcaffeic acid compared to untreated NASPOT 1 controls, specifically at 1, 0.1 w/w for both weevil spp. (Table 6.1). Treated periderms generally had significantly ($P \leq 0.05$) fewer feeding punctures than the controls. Weevil feeding generally increased with decrease in applied dosages for *C. puncticollis* (Table 6.1), although this trend was not distinct with *C. brunneus.*

Table 6.1. Variation in feeding of *C. puncticollis* and *C. brunneus* with treatment in a choice bio-assay between NASPOT 1 treated with compound versus untreated controls

	Concentration	Hexadecylcaffeic acid			Hexadecyl-*p*- coumaric acid		
Treatment	(mg/ml)	feeding holes	T-value	P*	feeding holes	T-value	P*
C. puncticollis							
Treated	1.0	0.80 ± 0.5	2.45	*0.03**	2.5 ± 2.2	2.50	*0.024**
Control		8.25 ± 0.5	(df=6)		6.8 ± 2.4	(df=6)	
Treated	0.1	3.0 ± 0.0	0.02	*0.01**	3.7 ± 0.7	1.81	*0.00**
Control		12.5± 2.1	(df=4)		9.8 ± 1.3	(df=10)	
Treated	0.01	3.5 ± 0.6	3.18	*0.06*	23.7 ± 4.1	1.56	0.09
Control		11.0 ± 2.4	(df=3)		32.3 ± 3.8	(df=4)	
C. brunneus							
Treated	1.0	3.0 ± 0.6	2.77	*0.01**	0.5 ± 0.3	2.45	*0.04**
Control		10 ± 0.0	(df=4)		1.0 ± 0.4	(df=6)	
Treated	0.1	4.6 ± 2.1	0.04	*0.04**	2.8 ± 1.2	2.52	*0.01**
Control		23.7 ±10	(df=10)		12.6± 1.7	(df=8)	
Treated	0.01	0.0 ± 0.0	0.12	0.116	1.0 ± 0.0	2.77	0.29
Control		2.0 ± 0	(df=4)		2.0 ± 0.0	(df=4)	

*P values are means significantly different at $P \leq 0.05$

The mean numbers of droppings were generally lower on treated periderms compared to the untreated ones although they were not significantly different ($P > 0.05$) (Table 6.2).

This could mean that the reduction in feeding seen on treated periderms may not necessarily correspond to numbers of weevil droppings, although other factors such as possibility that the compounds induce more droppings in insects may not be completely ruled out. From these findings, it is possible to conclude that feeding punctures and oviposited eggs may be good measures of *Cylas* weevil resistance in chemical screening bio-assays.

Table 6.2. Variation in number of faecal droppings of *C. puncticollis* and *C. brunneus* on treated and untreated periderms with hexadecylcaffeic and hexadecyl-*p*-coumaric acids

	Concentration	Hexadecylcaffeic acid			Hexadecyl-*p*- coumaric acid		
Treatment	(mg/ml)	droppings	T-value	P*	droppings	T-value	P*
C. puncticollis							
Treated	1	0.0 ± 0.0	4.3	*0.18*	0.0 ± 0.0	2.78	*0.37*
Control		2.0 ± 1.0	(df= 2)		0.3 ± 0.3	(df= 4)	
Treated	0.1	1.0 ± 0.4	2.45	*0.02**	2.8 ± 0.8	2.23	*0.08*
Control		3.5 ± 0.6	(df= 6)		7.5 ± 2.3	(df= 10)	
Treated	0.01	1.0 ± 1.0	2.45	0.56	27.3 ± 2.2	2.78	0.46
Control		2.5 ± 2.1	(df= 6)		20.7 ± 8.0	(df= 4)	
C. brunneus							
Treated	1	3.0 ± 0.6	2.78	0.1	1.5 ± 0.5	4.30	1.00
Control		8.0 ± 2.3	(df=4)		1.5 ± 1.5	(df= 2)	
Treated	0.1	6.0 ± 3.2	2.31	*0.06*	2.8 ± 0.9	2.78	*0.11*
Control		21.6 ± 6.3	(df= 8)		12.6 ± 4.6	(df= 4)	
Treated	0.01	1.0 ± 0.0	2.78	0.37	2.8 ± 0.8	2.23	0.15
Control		3.7 ± 2.7	(df= 4)		6.7 ± 2.3	(df= 10)	

*P values are means significantly different at $P \leq 0.05$

The cinnamic acid treated periderms were less preferred for oviposition than the controls for both *Cylas* species (Table 6.3). No eggs were oviposited on periderms by *C. puncticollis* on periderms treated with hexadecylcaffeic acid or hexadecyl-*p*-coumaric acid at the three dosage rates and with hexadecyl –*p*-coumaric acid for *C. brunneus* at 1 and at 0.1w/w of compound (Table 6.3). The effect on oviposition was profound probably implying that small differences in the content of these compounds at the root surface can easily affect weevil oviposition rates and thus affect levels of resistance to the pests.

Table 6.3. Effect of Hexadecylcaffeic acid and Hexadecyl-*p*-coumaric on oviposition of *C. puncticollis* and *C. brunneus* in compound treated versus untreated NASPOT 1 periderms

	Concentration	Hexadecylcaffeic acid			Hexadecyl-*p*- coumaric acid		
Treatment	(mg/ml)	eggs	T-value	P*	eggs	T-value	P*
C. puncticollis							
Treated	1	0.0 ± 0.0	4.3	0*	0.0 ± 0.0	2.78	0.16
Control		0.0 ± 0.0	(df=2)		1.0 ± 0.6	(df= 4)	
Treated	0.1	0.0 ± 0.0	2.45	*0.01**	0.3 ± 0.3	2.57	0.41
Control		2.5 ± 0.7	(df=6)		0.8 ± 0.3	(df=5)	
Treated	0.01	0.0 ± 0.0	0.09	*0.05**	1.0 ± 1.0	2.78	0.37
Control		1.3 ± 0.6	(df=4)		0.0 ± 0.0	(df=4)	
C. brunneus							
Treated	1	0.3 ± 0.3	2.78	0.37	0.0 ± 0.0	4.30	0.42
Control		1.0 ± 0.6	(df=4)		0.5 ± 0.5	(df=2)	
Treated	0.1	0.0 ± 0.0	2.31	0.35	0.0 ± 0.0	2.78	0.37
Control		2.5 ± 0.7	(df=8)		0.3 ± 0.3	(df=4)	
Treated	0.01	0.3 ± 0.3	2.78	0.72	0.3 ± 0.3	2.23	0.29
Control		0.5 ± 0.3	(df=4)		0.83 ± 0.3	(df= 10)	

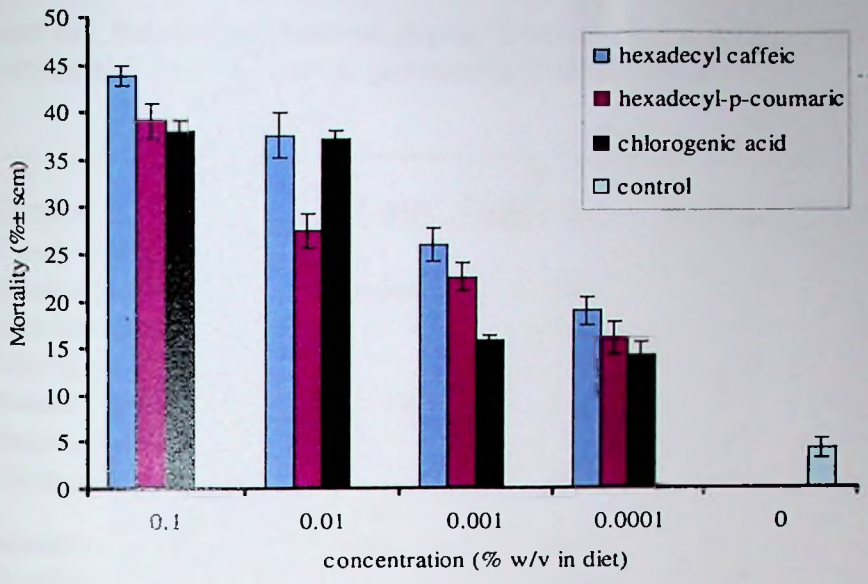
*P values are means significantly different at $P \leq 0.05$

6.4.2 Effect of cinnamic acid esters and chlorogenic acid on larvae development in diet-incorporated bio-assays

All the acids evaluated affected the larval development and survival of both *Cylas* species. Significantly ($P < 0.05$) higher mortality was observed in weevils fed with diets incorporated with phenolic compounds and this was dose dependent; high mortality was observed with high concentration of phenolic compounds applied to periderms (Fig 6.1). The highest larval mortality was obtained at 0.1 mg/ml treatment and least mortality in the controls for both weevil species. Larvae exposed to hexadecylcaffeic acid had higher mortality than to hexadecyl-*p*-coumaric and chlorogenic acids for both *C. brunneus* and *C. puncticollis* weevils.

There was a strong and positive relationship between dosage of compounds and % larval mortality for hexadecylcaffeic acid, hexadecyl-*p*-coumaric acid and chlorogenic acid for *C. brunneus and C. puncticollis* (Table 6.4.). The relationship between dosage and pupation was also significant ($P< 0.001$) and negative. Thus higher concentrations of the compounds result in reduced larval pupation. Mortality increased significantly ($P< 0.05$) with dosage in diets treated with all the compounds for *C. puncticollis;* however, this was not significant for chlorogenic acid on *C. brunneus* (Table 6.4).

Further, pupation of *C. puncticollis* reduced significantly ($P< 0.01$) in diets treated with hexadecylcaffeic and hexadecyl-*p*-coumaric acid. However, there was no significant dose response due to treatment with chlorogenic and hexadecylcaffeic acids on pupation of *C.brunneus* (Table 6.4).



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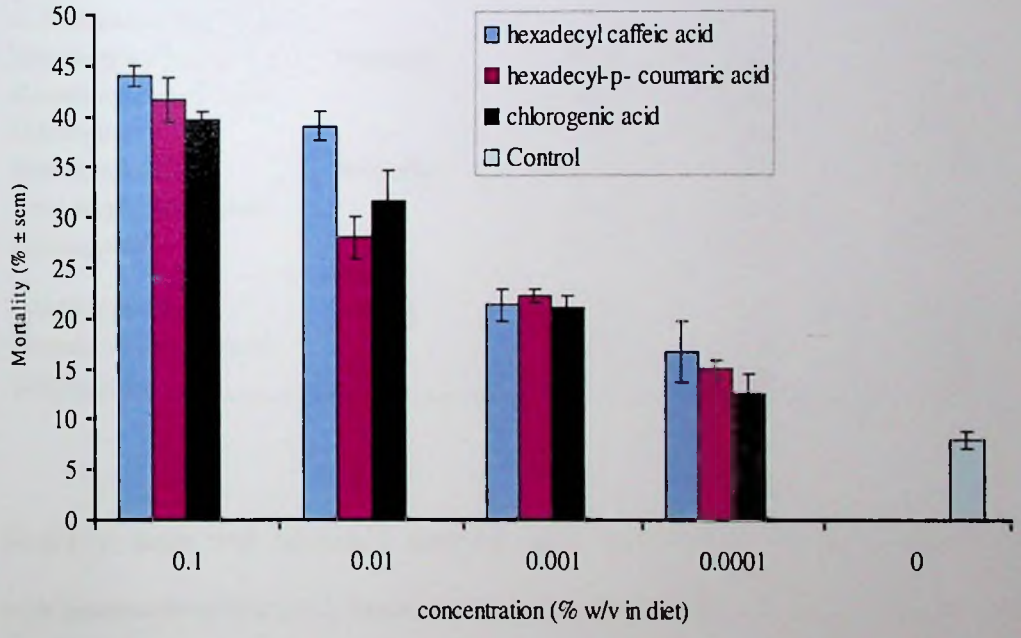


B

Fig. 6.1. Effect of hexadecylcaffeic acid and hexadecyl-*p*-coumaric in artificial diets on the mortality of larvae of *C. puncticollis (A)* and *C. brunneus (B)*

Table 6.4. Relationship between dosage levels and larval weights, mortality and levels of pupation of *C. brunneus* and *C. puncticollis* in treated diets

Chemical	Parameter	β-Coefficient	Intercept	R-Squared	P
C. puncticollis					
Hexadecylcaffeic	Pupation	-15.0	2.61	0.20	*0.02*
Hexadecyl-*p*-coumaric		-11.9	2.97	0.25	*0.01*
Chlorogenic		-13.3	2.95	0.27	*0.01*
Hexadecylcaffeic	Mortality	11.3	1.11	0.45	*0.00*
Hexadecyl-*p*-coumaric		9.7	0.92	0.55	*0.00*
Chlorogenic		10.6	0.92	0.45	*0.01*
Hexadecylcaffeic	Larval weight	-10.7	12.0	0.16	*0.00*
Hexadecyl-*p*-coumaric		-27.8	12.4	0.37	*0.00*
Chlorogenic		-6.8	11.9	0.02	0.47
C. brunneus					
Hexadecylcaffeic	Pupation	-14.2	2.43	0.17	0.07
Hexadecyl-*p*-coumaric		-34.8	4.75	0.23	*0.03*
Chlorogenic		-9.6	2.62	0.11	0.16
Hexadecylcaffeic	Mortality	12.6	1.03	0.42	*0.00*
Hexadecyl-*p*-coumaric		11.4	0.84	0.51	*0.00*
Chlorogenic		11.7	0.85	0.44	0.00
Hexadecylcaffeic	Larval weight	-8.1	11.8	0.05	0.28
Hexadecyl-*p*-coumaric		-15.7	12.4	0.05	*0.04*
Chlorogenic		-5.9	11.8	0.02	0.52

Similarly, there was increased mortality on *C. puncticollis* larvae in diets incorporated with hexadecylcaffeic acid, hexadecyl-*p*-coumaric acid and chlorogenic acids respectively. Thus further confirming the finding that higher amounts of these compounds resulted in increased weevil mortality.

The compounds caused significant reduction ($P < 0.05$) in the weights of developing larvae in diet bio-assays (Table 6.5). The mean larval weights of both species were lower

than in the controls for *C. puncticollis* on hexadecylcaffeic and hexadecyl-*p*-coumaric acid treatments. This effect however, was not seen on chlorogenic acid for both species.

Table 6.5. Variation in mean larval weights with acid concentration per treatment

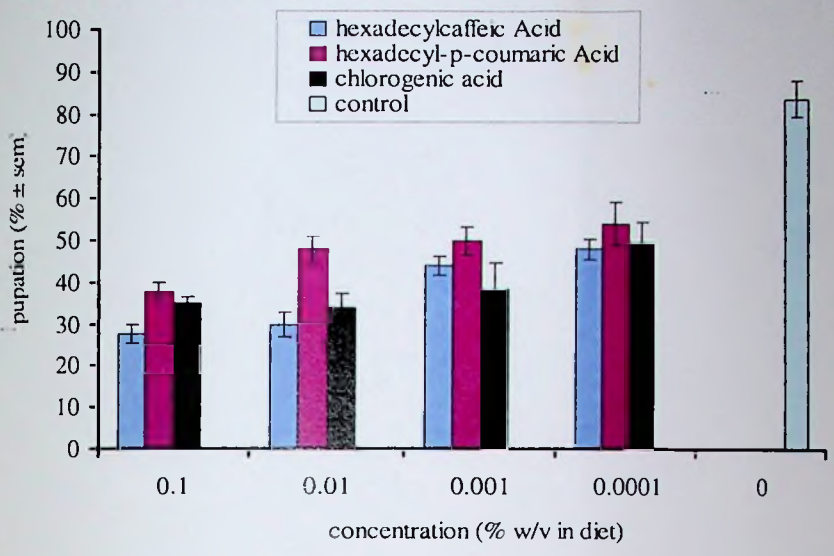
		*Larval weights (mean ± se) (mg)	
Dosage	Phenolic acids	*C. brunneus*	*C. puncticollis*
0.1	Hexadecylcaffeic acid	11.1 ± 1.3	11.0 ± 0.8
	Hexadecyl-*p*-coumaric acid	10.9 ± 0.4	9.70 ± 1.3
	Chlorogenic acid	11.3 ± 0.7	9.9 ± 0.2
	Control	14.3 ± 0.3	14.1 ± 0.2
	sed	0.70 (df=9)	0.97 (df= 11)
0.01	Hexadecylcaffeic acid	10.6 ± 0.5	10.6 ± 0.4
	Hexadecyl-*p*-coumaric acid	11.3 ± 0.7	11.0 ± 0.6
	Chlorogenic acid	11.7 ± 0.4	11.3 ± 0.1
	Control	14.3± 0.3	14.1 ± 0.2
	sed	0.75 (df=17)	0.65 (df=18)
0.001	Hexadecylcaffeic acid	11.1 ± 0.4	10.9 ± 0.4
	Hexadecyl-*p*-coumaric acid	12.2 ± 0.5	12.7 ± 0.5
	Chlorogenic acid	11.9 ± 0.6	11.4 ± 0.1
	Control	14.3 ± 0.3	14.1 ± 0.2
	sed	0.54 (df=19)	0.48 (df=15)
0.0001	Hexadecylcaffeic acid	11.9 ± 0.4	11.7 ± 0.4
	Hexadecyl-*p*-coumaric acid	11.6 ± 0.9	11.9 ± 0.7
	Chlorogenic acid	12.8 ± 0.2	12.1 ± 1.2
	Control	14.3± 0.3	14.1 ± 0.2
	sed	0.70 (df=19)	0.72 (df=19)

*Means in the same column for each *Cylas* spp. are significantly different when the difference between them is greater than twice *sed* values ($P \leq 0.05$)

Similarly, hexadecylcaffeic acid resulted in reduced larval weights of *C. brunneus* compared to the control, but this was not significantly different (Table 6.4). Where the compounds caused differences in rates of development, it is possible that they affect feeding and or impair normal weevil physiological functions affecting overall larval

development and cold thus reduce their survival. This effect however, seems to vary with treatment and weevil species.

Additionally, cinnamic acid incorporation affected the levels of weevil pupation with fewer numbers of insects pupating in treated diets compared to the controls for both *C. brunneus* and *C. puncticollis* weevils (Fig 6.2). This effect was highly profound ($P < 0.05$) for *C. puncticollis* and *C. brunneus* for hexadecylcaffeic acid, hexadecyl-*p*-coumaric acid and chlorogenic acids (Table 6.4). Thus increase in dosage of all the compounds resulted in reduced pupation across all compounds for both spp. although hexadecylcaffeic acid had a slightly higher effect on levels of pupation when compared to both hexadecyl-*p*-coumaric and chlorogenic acids (Fig 6.2).



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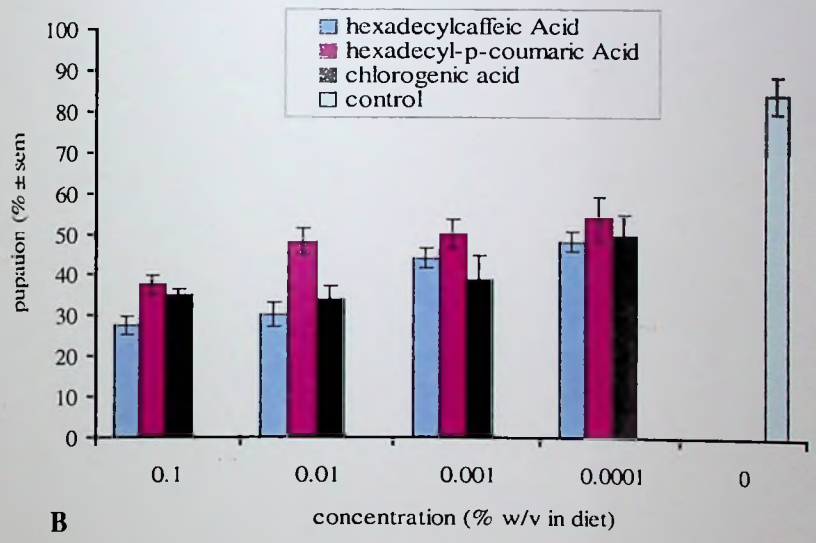

B

Fig 6.2. Effect of hexadecylcaffeic and hexadecyl-*p*-coumaric acid in artificial diets on pupation of *C. puncticollis* (A) and *C. brunneus* (B) weevils

6.5 Discussion

The significantly reduced weevil survival, larval weights and percent pupation levels by both *C. puncticollis* and *C. brunneus* when exposed to the individual cinnamic esters and chlorogenic acids in this study, provide evidence of an active biological effect by the sweetpotato root compounds on *Cylas* weevil biology. Resistance against the weevil has been linked to latex chemicals (Snook *et al.,* 1994), root physical factors and location (Stathers *et al.*, 2003a), and surface oviposition attractants (Nottingham *et al*, 2002), but with little evidence for their action on weevil biology. This has largely been due to absence of a suitable artificial diet capable of supporting the development of weevil stages to adulthood (Stevenson *et al.*, 2009), and facilitate incorporation of synthesised root chemical in bio-assays. The observed successful development and increased survivorship of the larvae in controls compared to the treated diets of both compounds suggests an active plant-host resistance mechanism with potential uses in breeding.

Reduction in larval weights and survival as a result of hexadecylcaffeic acid and hexadecyl-*p*-coumaric acid ingestion, are characteristics of antibiosis mechanisms (Gold *et al.*, 2001). On the other hand, these compounds also significantly deterred weevil feeding and oviposition suggesting presence of a multiple effect on weevil biology. This confirms earlier reports of reduced feeding punctures by *C. formicarius elengatulus* on root surfaces of semi-artificial diets when latex compounds were incorporated; and reduced feeding on root surfaces coated with sweetpotato latex compounds (Data *et al.*, 1996), and reduced oviposition when surface treated to root surfaces (Snook *et al.,* 1994). Chemical mediation of feeding and oviposition therefore may occur across the two *Cylas* spp. and is accounted for by specific chemicals that can be quantified.

It is possible that the compounds work additively or synergistically to produce the observed resistance behaviour in sweetpotato. Resistant sweetpotato varieties were found to have higher quantities of hexadecylcaffeic acid and hexadecyl-p-coumaric acids compared to minimal quantities of these compounds on the surfaces of these varieties. These compounds were also less per unit gram of root in susceptible varieties compared to more resistant ones. This could mean that weevils encounter these compounds at the root surface with resulting reduction in feeding and oviposition. Larvae then ingest higher amounts of these compounds as they feed on root tissues, resulting in larval mortality, reduced development and thus resistance. The reverse is true for the susceptible varieties with lower amounts of these compounds. This is also in agreement with previous studies, which associated differences in the presence of coumarates with differences in susceptibility of different varieties to *C. formicarius* (Snook *et al.*, 1994).

The biological activity of caffeic acid esters, recorded here may be due to their dihydroxy phenolic groups which bind covalently to proteins which can interfere with digestive processes or reduce availability of proteins in food and thus reduces insect development (Stevenson *et al.*, 1993). Furthermore, they may be converted to a more potent moiety in the presence of polyphenol oxidases and peroxidases that are released when plants are damaged by insect feeding. The importance of the caffeic acid group in resistance of sweetpotato to the *Cylas* species was also suggested by Harrison *et al.,* (2003b) who noted that the caffeic acid content of storage root periderm and cortex tissues (although they did not identify the compounds reported here in the latex) of genetically diverse sweetpotato cultivars and breeding clones ranged widely from almost absent to up to 8 mg/g root. Thus the reduction in larval development, increased larval mortality and reduced pupation

for both weevil species in bio-assays on artificial diets treated with hexadecyl caffeic acids and hexadecyl–*p*-coumaric acids could mean that they have similar effects on developing stages of the weevil in sweetpotato. This is further supported by the relative resistance of varieties such as New Kawogo, where octadecyl caffeic acid occurs and susceptibility where it is absent.

It has been suggested that phenolic compounds limit the digestion of carbohydrates (Jones, 1976). It was reported that the metabolic role of phenolics in the plant was still not clear. However, it has also been suggested that phenolics probably serve as chemical defense agents through their astringent taste and by interfering with the digestive enzymes of the pests. Similarly, Bernays and Chapman (1994) stressed the variability of effects of polyphenolics on insect herbivores although generalization concerning their ecological significance, need to be made with caution. These reports suggest an active role of plant compounds in mediating physiological processes of herbivores. The demonstrated action of the root chemicals and their effect on host acceptance for oviposition, development and feeding, provides further insight into the mechanisms of resistance in sweetpotato to *Cylas* spp., and suggest their potential in targeted breeding for resistance to the pests.

6.5.1 Conclusions

This study demonstrates a probable link between resistance and susceptibility of the roots of different sweetpotato varieties to *Cylas* species and their chemical composition. It provides strong evidence that root compounds affect the development of both *C. brunneus* and *C. puncticollis*, thus demonstrating occurrence of antibiosis factors occur in sweetpotato germplasm and contribute to differences in levels of resistance to *Cylas* spp.

The observed variation in chemical potency of the acids with dosage clearly shows that this effect will depend on the amounts of these compounds in the different tissues of the roots of sweetpotato and could thus explain the higher levels of susceptibility to *Cylas* spp. shown on varieties such as Tanzania and NASPOT 1 which had lower amounts of these compounds, and higher levels of resistance of New Kawago variety with high concentrations of these esters. Findings of this study thus directly corroborate earlier reports by other investigators of the presence of chemically mediated host plant resistance to *Cylas* spp. and could provide the long needed breakthrough to finding resistant varieties to *C. puncticollis* and *C. brunneus* weevils in sweetpotato germplasm.

This implies that identification of quantitative trait loci responsible for the identified chemicals in resistant sweetpotato, needs to be done, so as to facilitate development of varieties with bio-chemical resistance to *Cylas* spp. Additionally, since this study was conducted on *C. puncticollis* and *C. brunneus*, there is need to investigate their effect on *C. formicarius* and develop a genetic base for improvement of varieties to all the *Cylas* species through breeding. It is also possible that the resistance compounds identified may vary in sweetpotatoes with location and environment. Thus the developed resistant materials may need to be screened at various geographical locations to investigate their stability across site and with changing environmental conditions. Additionally, since chemical content of plants may vary with plant age, it would be of interest to breeders to identify the peak compositions of resistant compounds in the varieties with plant age so as to develop complementary integrated pest management packages for the pests in line with these changes.

These phenolic compounds are important not only as sources of resistance but as anti-oxidants capable of providing health and therapeutic effects in medicine (Padmaja, 2009). The promotion of sweetpotato varieties with high phenolic content and demonstrated resistance such as New Kawogo with resistance to *Cylas* species can provide a rich and easily affordable source of food, vitamin A and natural anti-oxidants, which can enhance the health of resource-poor communities in Africa and thus positively impact on their livelihoods.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion

The results of this study provided proof that there are valid and observable differences in the level of susceptibility to *Cylas* spp. weevils among the local landraces and improved sweetpotato germplasm grown in the various agro-ecological zones of Uganda. It also showed that observed differences in the level of susceptibility to *Cylas* spp. weevil among the local landraces and improved sweetpotato germplasm in Uganda is due to the presence of different phytochemicals in the roots of the different lines, landraces and varieties.

Prior to this study, sweetpotato root latex was assumed to be a contributing factor in sweetpotato resistance and could contain esters with biological activity against *C. formicarius* (Data *et al.*, 1996). There was however, no knowledge about the nature and type of phytochemical compounds in varieties of sweetpotatoes with varying levels of resistance to *Cylas* spp. Moreover, the effect of these compounds on the biology of *Cylas* spp. was not known. In this study it has been found that the phytochemicals present in the roots of the different lines, landraces and varieties, varied in quality and/or quantity and affect sweetpotato weevil biology: attraction to roots, feeding, oviposition and development.

Findings from this study provide evidence of qualitative and quantitative variation in the chemistry of sweetpotato varieties. It also provides evidence for the existence of a direct link between chemical mediation of levels of acceptability by *C. brunneus* and *C.*

puncticollis for feeding and oviposition and development. This suggests the feasibility of deploying sweetpotato chemicals for breeding or mapping resistance against *Cylas* spp.

It was shown that sweetpotato weevils are able to actively identify and selectively feed on sweetpotato substrates, with the levels of acceptability of these materials varying levels based on their biochemical composition. This therefore meant that materials which are most appropriate to weevil feeding and oviposition and development are preferred by *Cylas* spp. This to some extent, negated previous studies (Stathers *et al.*, 2003b), which attributed resistance in sweetpotato to be due to 'escape' as a result of deep rooting. Thus providing evidence to the hypothesis that weevil resistance in sweetpotato shown in the differing levels of field resistance in varieties is active and quantifiable and capable of being targeted for sweetpotato breeding.

It was further shown that field damage by the weevil was also consistently sustained even when resistant varieties such as New Kawogo and Dimbuka 2 were exposed to weevils in the laboratory, being less preferred for oviposition and feeding in choice and no choice biossays. This further gave more evidence for the existence of an active resistance against *C. puncticollis* and *C. brunneus*.

The results in this study, showed distinctive and clear linkage between levels of field and laboratory resistance to *Cylas spp.* and sweetpotato root chemistry. The study has shown that resistance in sweetpotato is likely mediated by root chemicals which can be quantified and thus targeted for further breeding using quantitative trait loci. Numerous crops have been improved using techniques targeting the enhancement of certain desired traits for plant protection against different biotic stresses. However, this approach has for

long been constrained by the absence of proper characters linking sweetpotato root characteristics to resistance to *Cylas* spp. The observed efficacy of compounds such as hexadecyl–*p*-coumaric and hexadecylcaffeic acid on the biology of larval and adult stages of both *Cylas* spp. points to the existence on an active biochemical based resistance against these pests, in sweetpotato. This could thus enable identification of traits for targeted breeding for sweetpotato resistance to *Cylas* spp.

There were distinct differences in chemical content of resistant and susceptible varieties. Compounds including hexa-, hepta-, octa and nona decyl caffeic acids found in resistant New Kawogo variety and absent in susceptible NASPOT 1 and Tanzania varieties, showed that weevils especially female when faced with the two varieties will have to encounter these compounds as they select oviposition and feeding sites. The observed reduction in feeding, oviposition and pupation increased larval mortality when weevils were exposed to the cinnamic esters validated for the first time that these differences in root chemical affect *Cylas* weevil biology. This means that resistance displayed by varieties with these compounds, must to some considerable extent, be due their presence and could result in antibiosis effects and cause antixenotic responses by the weevil at oviposition, thus explaining variation in levels of resistance *Cylas* spp. Thus, the results of this study could mean that mechanisms of resistance to *Cylas* spp. are a combination of antixenosis and antibiosis responses due to sweetpotato root chemical content on weevil stages.

Further, it is also possible that the compounds identified in sweetpotato during this study, could have additive or synergistic effects on *Cylas* weevil development. This could then explain the resistance of varieties with higher diversity of the compounds such as New

Kawogo compared to those which were less endowed with these compounds; this should be investigated in future studies.

Other compounds identified in the resistant compounds including hepta-, octa- and nonadecyl caffeic acids absent on the surfaces of the very susceptible varieties, could also have similar deterrent effects on *Cylas* spp. It is also possible that these being particularly present on root surfaces could also influence weevil preferences for varieties and thus resistance. Studies could thus be done to identify their specific effects on weevil biology. Additionally, previously research on *C. formicarius* had offered some evidence of variation in resistance to *Cylas* spp. among US varieties (Nottingham and Kays 2002; Jones *et al.*, 1983) but no conclusive evidence of active resistance mechanisms against *C. brunneus* and *C. puncticollis*, although reports of field performance of some East African varieties showed variation in field varietal damage (Magira, 2003; Stathers *et al.*, 2003b). In the present study, some varieties such as New Kawogo, reported by farmers to be resistant, were found to express resistance to both *C. puncticollis* and *C. brunneus* weevils and had clear differences in resistance compared to the more susceptible Tanzania and NASPOT 1 varieties. Resistance to *Cylas* spp. was shown to occur in field and was demonstrated in the laboratory bio-assays. It was demonstrated that even when varieties are grown in different locations and across different agro-ecologies, differences in levels of varietal damage by the pest occurred. This finding provides evidence of the existence of a stable form of resistance across sites, and provides an opportunity for breeders target this resistance for breeding for resistance to *Cylas* spp. Thus resistant lines can then be evaluated based on quantification of amounts of root compounds and varietal performance compared to those of the susceptible varieties.

Additionally, according to Collins and Mendoza (1991), sweetpotato is a hexaploid with 90 chromosomes and acts as a functional diploid with most traits of commercial importance quantitatively inherited, with additive gene effects usually being more important than dominance effects (Jones *et al.*, 1983; Collins and Mendoza, 1991). Thus some studies showed that resistance to certain insects exists in sweetpotato germplasm (Collins and Mendoza, 1991), including resistance to southern potato wireworm, *Conoderus falli* Lane, banded cucumber beetle *Diabrotica balteata* LeConte, spotted cucumber beetle *D. undecimpunctata hoardi* Barber among others (Jones *et al.*, 1976). The potential for increasing sweetpotato weevil resistance through conventional plant breeding by mass selection can be exploited (Collins and Mendoza 1991). This requires the identification identifying inherent plant characteristics associated with cultivars with higher levels of resistance to the weevils. Thus by identifying phenolic acids and cinnamic esters with demonstrated effects on weevil biology and development, this study has provided basis on which further research can be undertaken to develop resistant germplasm against *C. puncticollis* and *C. brunneus*. Hexadecyl-*p*-coumaric acids, hexa- and octa- decyl caffeic acids identified for the first time in sweetpotato in this study, with significant relationship between their amounts and damage levels by *C. brunneus* and *C. puncticollis,* could be targeted for the identification of molecular markers for resistance to these pests, thus enhancing the development of weevil resistant germplasm.

Further, the demonstrated effect of identified cinnamic esters in this study on weevil biology provided insight into the mechanisms of resistance in sweetpotato to the two African weevil species; and is in agreement with findings which reported possible linkage between levels of caffeic acid, caffeoylquinic acid and rutin among cultivars and resistance to *C. formicarius* (Son *et al.*, 1991a).

However, hydroxycinnamic esters found in sweetpotato varieties in this study may be present for some time in tissues and change with plant maturity. It is also possible that the biosynthesis of these chemicals in plants may depend on chemical changes in bio-chemical synthesis of phenolic compounds with time (Isman *et al.*, 2003a). For instance, the moiety of chlorogenic acid (3-0-caffeoyl-D-quinic) has been reported to be derived mainly from cinnamic compounds produced from L-phenlyalanine. Thus, research on the effect of maturity time and plant stage of development could be of use in identifying changes if any in compounds of sweetpotato varieties.

The importance of sweetpotato as a nutritive food, and source of micro-nutrients such as vitamin A and of anti-oxidative compounds used in the health industry, highlight its significance in the food and nutrition security of millions of people throughout the world (Andrade *et al.*, 2009). Since identified biochemicals from this study occurred in very small quantities and with a possible mode of action requiring chemical protein bonding by the compounds in sweetpotato weevils, with resultant larval mortality, these compounds cannot have any deterrent effects on consumers of sweetpotato. Thus their enhancement in sweetpotato varieties, to levels similar or higher than that identified in resistant varieties will not affect culinary characteristics of sweetpotato, or cause any deterrent effect on sweetpotato nutritive qualities. However, future varieties improved with biochemical compounds, may be analysed to further validate this.

Thus identification of resistance markers capable of being used for resistance breeding will enable researchers across Africa and elsewhere in the world, to develop varieties suitable for their unique locations and thus enable enhancement of the crop. The identification of resistance genes against *C. puncticollis* and *C. brunneus* weevils could

also be of value against *C. formicarius* spp., thus finally identifying a full technology package for sweetpotato resistance across the world. It is, however, important that bio-assays on the biology of *C. formicarius* be conducted to demonstrate and provide more evidence on this possibility. Also, multi-location trials to screen varieties of different regions to isolate identified root chemicals could provide more insight into the ecological interactions of the chemicals in sweetpotato weevil resistance and possibly identify even more resistant varieties for use in different regions for breeding.

Further, *C. puncticollis* and *C. brunneus* weevils from different parts of Uganda consistently responded differently to resistance compounds and showed preference for susceptible varieties in choice and no choice bio-assays. It is expected that weevils from other parts of the world will respond similarly when exposed at different stages to resistant compounds. However, to further validate this, studies to confirm consistency of the behaviour of weevils of different biotypes of *Cylas* spp. to cinnamic acids, could be done. Research to establish persistence of the chemical compounds with plant maturity and across sites, in addition to evaluating the effect of the compounds on the levels of weevil damage, when other Integrated Pest Management tactics are used could be done.

Breeding for weevil resistance may require non-conventional breeding techniques such as gene transformation and through quantitative trait loci identification and restriction fragment length polymorphism (RFLP) analysis (Collins and Mendoza 1991). Sweetpotato has been successfully transformed through Agrobacterium-mediated DNA transfer with incorporation of a high protein gene. This opened the possibility for the development of a transformation system which can be used to transfer genes directly to *I. batatas* if single genes for resistance to the weevil can be located in related sweetpotato

germplasm (Low *et al.*, 2009). Recent efforts in genetic engineering have shown its potential to confer insect resistance to several crops. In particular crops that express toxins from *Bacillus thuringiensis* (*Bt*) currently are being developed to confer weevil resistance in some plants (CIP, 2009). In sweetpotato, recent advances in research in this field have identified 3 distinct *Bt* proteins exhibiting weevil toxicity at levels similar to proteins expressed in commercial *Bt* crops, and already successfully managed to transfer these genes into sweetpotato (Ekobu *et al.*, 2010). Therefore, attempts to introduce weevil resistant genes into sweetpotato with high levels of inherent bio-chemical-based resistance may provide opportunities for the development of varieties with higher chances of sustained resistance to the weevil. There is, however, need for research to investigate how bio-chemical based resistance compliments the *Bt* sweetpotato transformation for resistance effort, and other components of the integrated pest management package for weevil control.

Success in the accomplishment of this research will ensure a double pronged approach which will merge biochemical-based resistance and sweetpotato transformation to generate weevil resistant varieties that will be of significant importance in the management of the weevil, especially, among the locally-grown varieties in sub-Saharan African countries. In line with this, future work should target the identification of the regeneration and transformation efficiencies of local varieties such as those identified in this study with resistance to the weevil, with a view of transforming other more susceptible varieties using weevil resistance genes, until weevil-resistant transformed African varieties are obtained.

Further, resistance to sweetpotato weevils was reported to be a quantitatively inherited trait and may have low heritability (Collins and Mendoza, 1991). Specific RFLP patterns have been linked to genes which control quantitative traits in certain crops including sweetpotato. These function just as normal conventional markers but have a heritability of 1.0 since they are a product of DNA digestion and are not affected by temperature, plant age or other environmental factors which may affect genes with which the RFLP is linked. Since large numbers of RFLP can be generated using specific restriction endonucleases (REN's) they can serve as indirect selection tools for the quantitative traits controlling weevil resistance to *Cylas* spp. Direct selection for RFLP marker loci associated with resistance QTLs may result in a correlated response for increase of the frequency of favourable alleles for weevil resistance and offer an opportunity for promoting resistance.

7.2 Conclusions

Thus the findings of this study confirmed that there are valid and observable differences in the level of susceptibility to *Cylas* spp. weevils among the local landraces and improved sweetpotato germplasm grown in the various agro-ecological zones of Uganda. This study also proved that the observed differences in the level of susceptibility to *Cylas* spp. weevil among the local landraces and improved sweetpotato germplasm in Uganda is due to presence of different phytochemicals which vary in quality and/or quantity among the varieties. It showed that the phytochemicals present in the roots of the different varieties affect sweetpotato weevil biology: attraction to roots, feeding, oviposition and development.

This study has provided the much needed information on which breeding for resistance to *Cylas* spp. can be enhanced. It has showed that resistance in sweetpotato to *C. puncticollis* and *C. brunneus* is not simply escape but is active and mediated by root biochemicals such as hexadecylcaffeic and hexadecyl-p-coumaric acids, whose heritability can be studied, focused and incorporated in the on-going efforts to breed for resistance to *Cylas* spp. in sweetpotato germplasm. If the specific Quantitative trait Loci corresponding to the availability of these compounds are identified, then more targeted breeding can be done to attain weevil resistant varieties. The resistant varieties identified in this study can then provide the needed genetic base for use in improvement of the breeding process, thus enhancing the generation of resistant varieties for the country and elsewhere in the world.

7.3 Recommendations

Sweetpotato resistance compounds could be present in sweetpotato varieties from other areas of the world. As such, more research aimed at screening larger germplasm accessions from different agro-ecological areas and regions, to identify if there are similarities in sweetpotato root chemical profiles, in these areas as identified in this study, should be undertaken. In cases where these compounds will not be found, then efforts are needed to improve existing genepools by introducing varieties with hexadecyl caffeic acids and hexadecyl-*p*-coumaric acids through breeding. Developed varieties could then be further developed to suit the ecological conditions and meet sweetpotato consumer requirements for different regions. However, other attributes such as yield, disease resistance and other varietal attributes preferred by consumers may also need to be taken into consideration.

There is however, need for more follow up studies to investigate presence if any additive and synergistic effects of the identified compounds on *Cylas* weevil biology and thus sweetpotato resistance. Also more comprehensive studies could be done to gain even further understanding of the effect of the relationship between pytochemical composition levels of resistance on one hand, and how this could be affected by the environment in which sweetpotato is grown.

More studies should also be done involving the identified compounds on their effect on *C. formicarius* and for weevil biotypes of *C. brunneus* and *C. puncticollis* found in different parts of the world.

Quantitative trait Loci for the identified resistance compounds should be studied and identified. These could then be mapped out in new sweetpotato populations and thus enhance resistance to *Cylas* weevil.

Thus it is recommended that heritabilities of the biochemical mediated resistance mechanisms identified in this study, as well as the environmental and genotype x environment interaction effects are further studied. This can be implemented through increased replications, locations and years to hasten the development of resistant varieties. This is, therefore, recommended and is already being initiated in new initiatives such as 'the Sweetpotato Action for Security and Health in Africa (SASHA)' project; which target the development of safe weevil-resistant sweetpotato varieties for SSA, through the use of local capacities and biotechnology. This will provide the long awaited breakthrough for the identification of sweetpotato weevil resistant varieties suitable for the region and capable for use elsewhere in the world.

Further, studies to identify the best methods of integrating biochemical based resistance into new and existing integrated pest management options against *Cylas* spp., should be done. This will then provide a reliable and stable management strategy for the control of *Cylas* spp. in Africa and elsewhere in the world.

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