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Phytochemical Mediated Resistance in Sweetpotato to Sweetpotato Weevils

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DEDICATION

To my Dad, my Mum, dear wife Maureen and sons, Joshua, Jonathan and Moses

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ABSTRACT

This study evaluated sweetpotato resistance to sweet potato weevils (*Cylas* spp.) and investigated the phytochemicals that mediated this defence. New Kawogo, LIR302 and ARA228 were shown to be the most resistant based on stem and root damage. Significant differences were observed on the number of faecal droppings, feeding holes and eggs laid on the root in a choice and no-choice bioassays confirming that New Kawogo, LIR302 and ARA228 affected development and emergence of adult weevils. Six hydroxycinnamic acid esters, including hexadecylcaffeic, hexadecylcoumaric, octadecylcaffeic, octadecylcoumaric, heptadecylcaffeic and 5-O-caffeoylquinic acid esters were identified on the root surface and amounts of these differed significantly between resistant and susceptible varieties. The mean number of *C. puncticollis* and *C. brunneus* feeding holes, faecal droppings and egg laid on the root core were significantly different among the root cores treated with synthetic hydroxycinnamic acid esters. The study also showed that there were significant differences in the root volatiles of resistant and susceptible variety both before and after infestation. The larval survival of sweetpotato weevil was significantly affected by hydroxycinnamic acid esters treatment and Bt-toxin applied on the diet. There was also significant differences in percentage sweetpotato weevil root infestation among genotypes of the segregating population. The genotype by environment (GxE) interaction effect was also significant on the sweetpotato weevil damage on the stem portion of the sweetpotato vine indicating that weevil stem damage is dependent on the season. The mean number of sweetpotato weevil feeding holes differed significantly on the root of the genotypes of the segregating population in the feeding and oviposition

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bioassay. There was significant difference in total hydroxycinnamic acid (HCA) esters among the genotypes of the segregating population. The distribution of genotype mean total HCA ester concentration was skewed to the left and only one progeny, NKB257, had higher total HCA ester concentration than New Kawogo, the resistant mother used in the crossing. A weak but significant correlation between total HCA ester concentration and sweetpotato weevil root damage was observed signifying that resistance to sweetpotato weevils depended on other factors as well. The results are discussed in terms of how they might be incorporated into integrated pest management of sweetpotato weevils.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION TO THE SWEET POTATO CROP

1.1.1 Taxonomy and Morphology of Sweetpotato

Sweetpotato (*Ipomoea batatas* (L.) Lam.; (Convolvulaceae) is a perennial plant cultivated as an annual crop (Martin, 1970; Huaman, 1992). Sweetpotato is grown throughout the tropical world for its storage roots which provides an important source of carbohydrate, food security and income generation (Diaz *et al*., 1996; Mwanga *et al*., 2001; Tairo *et al*., 2004). This plant produces an herbaceous perennial vine, bearing alternate heart-shaped or palmate lobed leaves and medium-sized sympetalous flowers (Figure 1.1). The crop is propagated vegetatively and the vines are used as planting materials (Gibson *et al*., 2009; Namanda *et al*., 2011). The edible root is long and tapered, with a smooth skin whose colour ranges between red, purple, brown and white. It is not known what ecological significance the root colour has but this could influence sweetpotato weevil behaviour. The genus *Ipomoea* consists of about 600 to 700 species including the cultivated sweetpotato (Vaeasey *et al*., 2008; Cao *et al*., 2009). The series batatas which contains the sweetpotato consists of 13 wild species closely related to cultivated sweetpotato (Orjeda *et al*., 1990; Diaz *et al*., 1996; Huang and Sun, 2000) and could provide novel traits that enhance resilience to climate, pests or diseases. In the present study the research focussed on the cultivated species.

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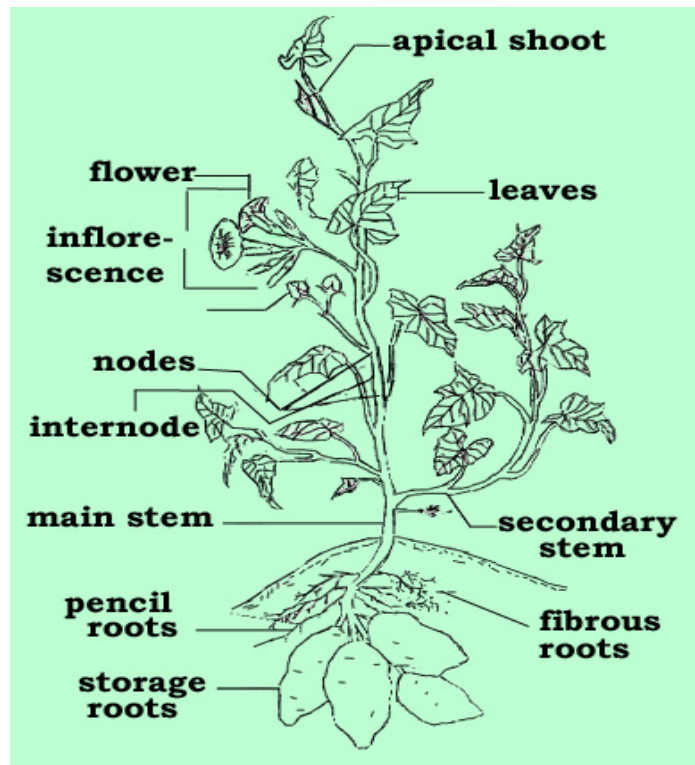
Plate 1.1: Morphology of sweetpotato plants adapted from Purseglove (1991)

1.1.2 Origin and Ecological Requirements of Sweetpotato

Central America has been documented as the origin and the primary centre of diversity of the cultivated species *I. batatas* (Zhang *et al*., 2000; Gichuki *et al*., 2003; Srisuwan *et al*., 2006; Low *et al*., 2009). However, historically, sweetpotato is thought to have originated in north-western South America. Accordingly, the domestication was associated with the development of tropical forest agricultural cultivation systems (Austin, 1988; Zhang *et al*., 2004). Sweetpotato is believed to have been introduced to Africa by Portuguese explorers during 16th and 17th century (Zhang *et al*., 2000; Gichuki *et al*., 2003).

Sweetpotato is grown from 48°N to 40°S of the equator with altitudes ranging from 0 to 3000 m above sea level (Woolfe, 1992; Vaeasey *et al*., 2008; Low *et al*., 2009; Troung *et al*., 2011). It requires ambient day and night temperatures from 15 to 33°C for optimum growth and root development. Temperatures above 25°C are considered optimal for maximum growth (Woolfe, 1992). However, temperatures below 12°C and above 35°C retard sweetpotato growth and resultant yield (Kuo, 1991). Dry matter production increases with increasing temperatures from 20 to 30°C, but declines at temperatures beyond 30°C (Kuo, 1991). The crop grows and yields best with a well distributed annual rainfall of 600 to 1600 mm (Low *et al*., 2009). Excess rainfall at early stages of establishment may aggravate weed problems reducing yield due to competition for nutrients (Harrison and Jackson, 2011). The crop is grown extensively under rainfed conditions and is relatively drought tolerant. It is therefore a highly suitable crop to be cultivated with current climate change and weather variability in developing countries including Uganda. However, prolonged and frequent dry spells or drought and erratic rainfall cause substantial yield reduction (Low *et al*., 2009; Schafleitner *et al*., 2010). Sweetpotato requires well-drained soil with a pH of 5.5 to 6.5 (Woolfe, 1992). It also requires full sunlight; although, it can tolerate a 30 to 50% reduction of full solar radiation (Troung *et al*., 2011).

1.2   ECONOMIC IMPORTANCE OF SWEETPOTATO

Sweetpotato is the seventh most important food crop globally. According to FAO (2012), the estimated production stands at 135 million metric tonnes. Indeed, more than 95% of the total 135 million metric tonnes of sweetpotato comes from

developing countries emphasizing the importance of the crop in meeting food requirements in poorer households (Scott *et al.* 1999*)*. In fact, in developing countries, sweetpotato ranks third in caloric contribution in human diet. It is grown in different agro-ecological conditions including semi-arid, high altitude and temperate climates (Woolfe, 1992). Sweetpotato is more widely grown than all the other root crops in the world (CIP, 1999). It plays a central role in the food security for millions of people across South America, Africa and Asia and has had historical importance in disaster relief in many countries. For instance; the Japanese used it when hurricanes demolished their rice fields, it provided a crucial food source in China during a famine in the early 1960s, and the crop saved rural Ugandans from hunger when *African cassava mosaic virus* (ACMV) epidemics hit the country in the late 80's and early 1990s (Abidin *et al*., 2005). These examples demonstrate the importance of sweetpotato and justification for the research to increase the productivity of the crop that includes studying traits for improved resilience such as the resistance of the crop to insect pests for food security and livelihood improvement. Sweetpotato is grown predominantly by smallholder farmers in East Africa, its cultivation is mainly done by women and the youth (Abidin *et al*., 2005). Production of sweetpotato meets the needs for both home consumption and sale to local markets and urban areas to supplement household income. Funding support and finding solutions towards improving the crop therefore indicates a huge potential for future economic growth especially for African women farmers.

1.3   PROPAGATION AND USES OF SWEETPOTATO

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Sweetpotato production in Uganda has a long tradition and there are many local landraces/cultivars that are grown. Improved varieties have been released to enhance production (Mwanga *et al.*, 2003). The crop is easy to propagate through cuttings and is highly adaptable in the farming system. The vines also constitute a major livestock feed component (Andrade *et al.*, 2009). Additionally, sweetpotato leaves constitute an important and abundant vegetable consumed by numerous communities in the world (Islam, 2002). Sweetpotato roots have high human nutritive value supplying carbohydrates, fibre, protein and vitamins. Orange fleshed varieties are particularly valued for their high content of bio-available vitamin A (retinol) precursors (carotenoids) and thus provides one of the most economic means of combating vitamin A deficiency which is a public health problem in the developing world (Andrade *et al.*, 2009). It also has considerable amounts of iron, potassium, zinc and essential trace elements such as manganese, chromium, selenium and molybdenum (Yamakawa and Yoshimoto, 2002). 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) contributing to alleviation of heart problems (Woolfe, 1992). The improvement of sweetpotato is of high importance due to its nutritive value and role alleviating diseases associated with malnutrition and its purported immune boosting properties for example for persons with HIV/Aids and oxidative stress related diseases. Sweetpotato is chiefly grown for its storage roots which are utilized in diverse ways: as a vegetable, snack food and animal feed. Sweetpotato also has non-culinary uses, for instance, it can be used as a commodity for industrial starch extraction and in fermented form as a brew (Lin *et al.*, 1985). Kurata *et al.* (2007) reported that sweetpotato leaves have high amounts of 5-O-caffeoylquinic acid

(CQA) that can suppress cancer cell proliferation in the colon. Some sweetpotato varieties also have high levels of antioxidant anthocyanins which can substitute for synthetic colouring agents (Andrade *et al.*, 2009; Woolfe, 1992).

According to Bashaasha *et al.* (1995), the sweetpotato cropping systems in Uganda are diverse. For instance, in north-eastern Uganda, sweetpotato is often followed by finger millet or groundnut, while maize is grown after sweetpotato in the northern moist farmland. This indicates that sweetpotato fits in agro-ecological intensification context and it is being advocated for climate change mitigation and adaptation. Sorghum, millet, maize, beans, sesame and groundnut are also often cropped before sweetpotato (Ebregt *et al.*, 2004a). Indeed, the rotation system is known to helps ensure the cycle of sweetpotato weevils is broken when sweetpotato is follow by a non-host crop.

1.4   SWEETPOTATO PRODUCTION CONSTRAINTS

The production of sweetpotato is affected by several biotic constraints such as viral diseases, insect pests and weeds (Ndunguru *et al.*, 2009; Lou *et al.*, 2010; Schafleitner *et al.*, 2010; Harrison and Jackson, 2011). Diseases and insects of significant importance are sweetpotato virus diseases (SPVD) and sweetpotato weevils, respectively. SPVD caused by the dual infection and synergistic interaction of sweetpotato chlorotic stunt virus and sweetpotato feathery mottle virus is distributed worldwide (Gibson *et al.*, 1998; Mukasa *et al.*, 2006). It is the most devastating disease causing reduction in plant growth and storage root yields (Gibson *et al.*, 1997; Karyeija *et al.*, 2000; Gibson *et al.*, 2004; Gibson, 2005; Kapinga *et al.*, 2009a).

Sweetpotato weevils, *Cylas* spp., are another major sweetpotato production constraint which can cause between 60-100% yield losses in sweetpotato in a susceptible variety during dry spells in sub-Saharan Africa (SSA) (Smit 1997). The sweetpotato weevil species, *C. puncticollis* and *C. brunneus* predominantly occurring in Africa, reduce the production and market value of sweetpotato in SSA (Stathers *et al*., 2003a). The major damage is inflicted on the storage roots by larval feeding (Cockerham *et al*., 1954; Jansson *et al*., 1987). Secondary pathogen infection and ensued induction of defence sesquiterpenes in the plant in response to sweetpotato weevil feeding makes the storage roots bitter and unacceptable for both human and animal consumption (Uritani *et al*., 1975; Sato and Uritani 1981) (Plate 1.2).

Plate 1.2: Sweetpotato roots damaged by weevil larvae feeding and pupation (Photo Otema .A .M)

1.5 ECONOMIC IMPORTANCE, BIOLOGY, ECOLOGY AND MANAGEMENT OF SWEETPOTATO WEEVILS

1.5.1 Economic importance of Sweetpotato Weevils (*Cylas* spp.)

Sweetpotato weevils are the single most important pest of sweetpotato in both tropical and sub-tropical regions. According to Ray and Ravi (2005) and Stathers *et al.* (2003b), there are no control measures or resistant varieties.

Weevil damage is reported to increase sharply between 24 and 30 weeks after planting. However, this coincides with the period of root expansion, the effect being aggravated by dry and hot conditions, with both weevil species co-existing on the same root. According to International Potato Center (CIP) (1999), there are three species of sweetpotato weevils, namely, *Cylas puncticollis* Boheman, *C. brunneus* Fabricius and *C. formicarius* Fabricius. They all complete the entirety of their life cycle on the host sweetpotato plant (Smit, 1997). Females feeding produce small pits in the vines near the plant's crown above the soil or in exposed sweetpotato roots, in which to lay their eggs, one egg is laid per pit. Once the egg hatches, the larva tunnels within the host plant's tissue causing damage that can severely affect market value. Pupation then occurs within these tunnels. Adults emerge and begin feeding on the host plant leaves, leaf petioles and stems. Infested plants may wilt or even die because of extensive stem damage due to larval tunnels that can reduce the size and number of storage roots due to the imbalance it creates on transportation of translocation materials from the shoot as well as water and mineral nutrients absorbed by the roots from the soil. Weevil feeding on the inside of the

vines is characterized by 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 (Mullen, 1984; Sutherland, 1985; Stathers *et al.*, 1999). Storage root shows little swelling or lateral growth. This growth of storage roots is inhibited by weevil feeding (Plate 1.3).

Plate 1.3. Growth of storage roots are inhibited due to weevil damaged vascular system of sweetpotato (Photo by Otema .A.M.)

Although this is not considered as important as damage to the storage root, it has been found that yields decrease with increased attack to the crown or stems (Palaniswami and Chattopadhyay, 2006). This damage interferes with the plant's ability to transfer water and nutrients within the crown and from foliage to developing

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storage roots (Plate 1.3). According to Mwanga *et al.* (2007), weevil damage increases with the length of time the crop remains un-harvested in the field.

1.5.2 Biology of sweetpotato weevils

Weevil eggs are usually laid on root surfaces and stem bases, singly in an egg hole made by the adult females during feeding. After the egg is laid, the hole is covered with a greyish mass of faecal materials which hardens to form a protective cap over the developing egg. The egg incubation period ranges from 4-8 days depending on temperature (Ekobu *et al.*, 2010). This knowledge is important because resistance of sweetpotato to weevils will be evaluated based on the egg laying on different varieties. The hatched larvae feed internally within the root or vine for 12-15 days during which they complete three larval instars. The developing larvae are legless, curved and white, tunnelling extensively as they feed in the vines and storage roots while depositing faecal droppings within the tunnels (Smit, 1997). In response to the damage, the storage roots secretes terpene- like chemicals which tarnish the root colour and induce bitter taste even at low levels of insect damage (Jannson and Raman, 1991). However, the chemicals do not affect the insects so that even when the whole root is brown and horrible, the weevils are still happy to feed on it (Plate 1.2).

Pupation takes place within the roots or stems; the pupal stage lasting 4-8 days. Immediately after emergence, the adults mate but oviposition does not occur until after 5-8 days. Mean life cycle duration from egg to egg may be up to 45 days. However, the mean development of the weevil from egg to adult takes an average of 32-33 days (Sutherland, 1986).

Adult sweetpotato weevil survival varies greatly from 63-120 days under field conditions. Similarly, fecundity also varies greatly but a single female can lay up to 340 eggs depending on the number of matings and crowding (Smit, 1997) illustrating the potential scale of infestations.

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 sweetpotato weevils have characteristic differences in the shape of their antennae and these are helpful when selecting weevils of specific gender for bioassay experiments (Korada *et al*., 2010). The antennae of the males are straight while those of the female are round and club-shaped. Infestation by weevils is determined by digging or uprooting the storage roots of sweetpotato and splitting them open to expose the tunnels or galleries containing different stages of the weevil (Stathers, 2003a & Stathers, 2003b) (Plate 1.3).

1.5.3   Ecology of sweetpotato weevils

Sweetpotato is the preferred host for sweetpotato weevil and under ideal conditions it has the ability to go through the entire life cycle from eggs to adult in as little as 32-33 days (Sutherland, 1986; Smit, 1997; CIP, 2009). This can result in very large populations developing in a single sweetpotato cropping cycle. Although, the three sweetpotato weevil species are pests of economic importance in many sweetpotato growing areas of the world (Downham *et al*., 2000; Kuriwada *et al*., 2013), *C. puncticollis* and *C. brunneus* are confined to Africa (Smit and Van Huis, 1998) whereas *C. formicarius* is widespread in Asia, USA and the Caribbean (Korada *et al*., 2010)

According to Muyinza *et al*. (2012), both *C. puncticollis* and *C. brunneus* have some differences in their biology, but their ecological interactions in the field are similar and they may co-exist in the same roots. Adult female weevils usually feed on the epidermis of the vines, scraping oval patches off young vines and petioles. They also feed on external surfaces of storage roots resulting in round feeding punctures. According to Smit and Van Huis (1998), adult weevils are not usually seen on the crop, the soil surface under the vines or in the soil around the base of the 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. The two species; *C. puncticollis* and *C. brunneus* are found throughout Africa where sweetpotato is grown (Figure 1.1).
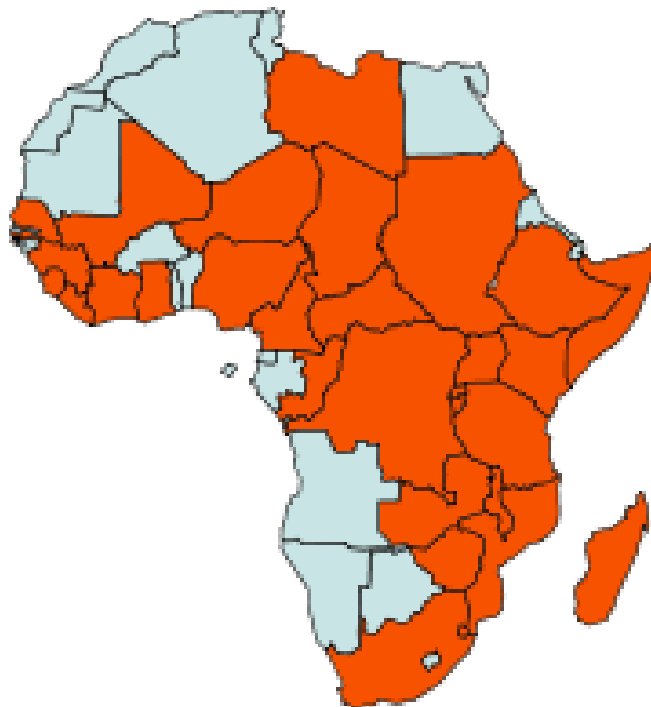
Figure 1.1. Geographical distribution of sweetpotato weevils in Africa marked in red (source: http://www.infonet-biovision.org/default/ct/97/pests)

Influence of temperature on sweetpotato weevil development

Jansson *et al*., (1990) reported that the duration of the life cycle of sweetpotato weevils depends on weather conditions and takes between 28 and 49 days. A warm to hot temperature increase the rate of life cycle and shortens the duration to completion for sweetpotato weevil (*C. formicarius*). This species has similar characteristics with *C. puncticollis* and *C. brunneus* being described in this thesis. *C. formicarius*, like *C. puncticollis* and *C. brunneus* is a tropical/sub-tropical insect; therefore cooler temperatures decrease the rate of development from one stage to the next. Kimura *et al*., (2006) surveyed the cold tolerance of *C. formicarius* in Japan and found females still actively laying eggs between 16 and 18 °C. In Papua New Guinea the life cycle was shorter in the warmer lowlands than the cooler highlands (Sutherland, 1985). Under favourable conditions sweetpotato weevils can produce 13 generations a year and live for 3-4 months and can produce up to 340 eggs per female during their lifetime (Smit, 1997a).

Influence of wet and dry season on sweetpotato weevil infestation and damage

Studies in Papua New Guinea noted a significant variation in the intensity of sweetpotato weevil infestation between the wet and dry seasons. According to Bourke (1985), the weevil caused economic damage in areas with marked dry season or in unseasonably dry years. Arguably sweetpotato weevil is a problem wherever the crop is grown and often worse during dry months. Heavy rain reduces air circulation to the subterranean environment whereas high levels of weevil incidence generally correspond with lower rainfall levels. This explains why the weevil was reported to be most serious in areas with a marked dry season or in drier

parts of the highlands such as the Benabena and Henganofi areas of the Eastern Highlands province of Papua New Guinea. According to Stathers *et al.*, (2003b), weevils generally fail to penetrate wet soils but can penetrate dry soils. In fact, weevils are really poor at excavating the soil and that explains why the damage is so much less in the wet season in the absence of soil cracking. Consequently, weevils are a particularly serious problem under dry conditions, because the insects, can access roots more easily through cracks that appear because of expansion and contraction due to heat in the soil as it dries out (Stathers *et al* 2003a). It is for this reason that during the dry season, unlike cassava, sweetpotato roots cannot be stored in-ground for any significant period of time without significant damage realized under farmers' socio-economic and biophysical environments (Abidin *et al.*, 2005).

1.5.4 Host range and geographical distribution of sweetpotato weevil.

According to Pinese (2001) *Cylas formicarius,* the sub-tropical weevil species has a wide host range including relatively common wild hosts that are botanically related to the commercial sweetpotato. Alternate host plants in Australia for example include: *Ipomoea polpha*, *I. aquatic*, *I. pes-caprae*, *I saintronaensis*, *I. cairira*, *I. nil*, *I. angulate*, *Meremia quinata* and *Parchyrhizus* species. However, the host range of *C. puncticollis* and *C. brunneus* is not as well documented compared to *C. formicarius*. Therefore, *I. batatas* is the only known primary host of these two weevil species (Smit, 1997).

Kumar (1992) reported that other host plants of *C. formicarius* include, coffee, maize, cowpea, sesame, *Cassia acutifolia* and wild species of Convolvulaceae. Pinese (2001) suggested that while wild hosts for *Cylas formicarius* increase the risk of migration of weevils into commercial crops, migration from within cultivated crops, particularly of sweetpotato left on top of the ground after harvest, is a more significant threat. This is significant because sweetpotato plants and crop residues are accessible to the sweetpotato weevils throughout the year.

1.5.5 Flight behaviour

Adults of *C. formicarius* are observed to be active fliers and are usually noticed in the field especially when storage root formation begins (Jansson *et al*., 1990). Moriya and Hiroyoshi (1998) found that males of *C. formicarius* had greater flight ability than females of the same age. Locomotion was also higher in males than in females (Moriya and Hiroyoshi, 1998). Flight behaviours are not well studied with *C. puncticollis* and *C. brunneus*.

1.5.6 Sweetpotato   pest   management   strategies

One of the most practiced traditional weevil management strategies involves the use of cultural management techniques. This includes the implementation of cultivation practices targeted at preventing infestation such as field sanitation involving the removal and destruction of infested vines and residues (Jannson and Raman, 1991).

Also, rotating sweetpotato with non-host crops to sweetpotato weevils for 2-3 seasons has been recommended to break the pest cycle. However, for small holder farmers this is not so practical as the sweetpotato is a staple so must be grown and land is too scarce to make effective rotation possible (Abidin *et al*., 2005). Other cultural management measures included use of clean planting materials, timely planting and prompt harvesting to avoid the dry period is recommended (Mansaray *et al*., 2013).

Earthing 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 during weeding to break the soil clod and loosen the soil to improve aeration and 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 (Smit and Matengo, 1995). It is recommended that care should be taken to ensure that the mulching material used is from a non-host plant that weevils cannot feed and develop on. But all these measures are time costs to farmers so are not always practical or implemented.

Chemical Control

Chemical control of the sweetpotato weevil is an option that has been adopted in some countries such as India and Japan. This involves dipping planting materials into a synthetic pesticide such as chloropyrifos (Dursban) granules at 2.25 kg a.i./ha; monochrotophos 36% SL, phoimidaclopid 17.8% SL and profenos 50% EC (Tarafdar and Sarkar, 2006). Where it has been praticed, the treatment has been

found to result in delayed pest infestation for several months. However, the high cost of the pesticides and the resulting environmental contamination such as non-target effects and health hazards to man and animals makes its use unsustainable for control of *C. puncticollis* and *C. brunneus* in SSA (Jackson *et al*., 2002). The cryptic nature of the adult and feeding by the root tunnelling weevil larvae within a subterranean root makes chemical control otherwise less applicable and therefore reduces reliance on insecticides (Tarafdar and Sarkar, 2006). Because the larval period is spent within vines or tubers, and the adults are nocturnal, chemical control is not recommended to resource poor farmers (Reddy *et al*., 2012). The use of chemicals for weevil management is therefore only limited to large scale commercial root production or vine multiplication nurseries (CIP, 2009).

Use of pheromone traps

Pheromone traps have been developed for the management of and monitoring population dynamics of sweetpotato weevil. Pheromone-baited light-red unitraps, 13 x 17.5 cm, installed 50cm above the crop canopy, were effective at catching *C. formicarius* adults and demonstrated the greatest potential for use in trap-and-kill strategies and eradication programs (Reddy *et al*., 2012). However, pheromone-based trappings do not appear to reduce the damage level. The pheromones of *C. puncticollis* and *C. brunneus* were identified as decyl- and dodecyl (*E*)-2-butenoate respectively (Downham *et al*., 1999). Lures and traps for these species were developed (Downham *et al*., 2001). Male adults attracted by the sex pheromone fall into the pail of water and are easily collected and removed from the field (CIP 2009). The formulation used contained the synthetic pheromone of *C. brunneus* as

this attracted both species (Downham *et al*., 1999). Mass trappings of male weevils has therefore 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 prevented yield loss (Braun and Van Den Fliert, 1997). Mass trapping using sex pheromone traps in effect did not lead to reduction in weevil damage to roots and had minimal adoption for *Cylas* weevil management.

Biological Control

Sweetpotato weevils have several natural enemies including predators, parasites and pathogens. However, use of biological control methods have not been exploited much for sweetpotato weevil management. However, recent advances have resulted in the identification of a fungus (*Beauveria bassiana*) as a potential fungal pathogen and have been isolated and used for management of *C. puncticollis* and *C. brunneus* in East Africa (Allard 1990). However, this approach is very limited in the absence of favourable conditions for fungal proliferation and distribution like high humidity at the peak of weevil infestation that typically occurs during drought periods (Ignoffo 1985). 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).

Entomopathogenic nematodes of the family *Steinernematide* and *Heterorhabditidae* were isolated in *C. formicarius* (Jannson and Raman, 1991). Other nematodes including *Rhabditis* sp., *Aphelenchus avenae*, *Acrobeloises* sp. and *Steinernema* sp. were reportedly isolated from *C. formicarius* in the U.S.A., with *Steinernema*

reportedly being particularly pathogenic to *C. formicarius* in the laboratory (Jansson, 1991). However, some environmental factors such as soil moisture, solar radiation and temperature may limit the effectiveness of entomopathogenic nematodes as biological control agents of insects (Kaya, 1985). None of these have been found effective on *C. puncticollis* and *C. brunneus* and in the field against *C. formicarius*. Thus, their potential as components of integrated management of *Cylas* spp. has not yet been attained (Muyinza, 2010).

Transgenic *Bacillus thuringiensis* var. *Tenebrionis* Berliner (Bt) has been expressed in sweetpotato with limited success in the weevil management (Moran *et al*., 1998). Bt toxin acts by interfering with insect digestion through creation of holes in the larval insect gut membrane, leading to leakage of gut contents and eventually the death of susceptible weevils (Gill *et al*., 1992). Recent efforts in implementing transgenic weevil resistance in Uganda have not been successful. Seven Bt cry proteins from Bayer Crop Science and Monsanto were tested for toxicity against *C. puncticollis* and *C. brunneus* on an artificial diet at NaCRRI and have been found to be toxic to the *Cylas* spp. at an LC_{50} of less than 1 ppm (Ekubo *et al*. 2010). However, transgenic loci introduced into higher plant species have been reported to display unsuitable patterns of inheritance and expression (Yin *et al*., 2004). As a result, transgenes may deviate from Mendelian segregation depending on several genetic factors. Some of these deviations can occur when a transgene encounters a biochemical product that significantly interferes with the endogenous biochemical pathways for gamete development and transmission (Hood *et al*., 1997). In addition, transgene expression can also be affected by a variety of factors influenced by plant genetic background (Schmidt *et al*., 2004). Sweetpotato with many chromosomes may explain the lack of success of Bt toxins in transformed roots.

Host Plant Resistance

Host plant resistance is a viable strategy, since smallholder farmers using resistant varieties do not require additional skill or investment. Work on developing host-plant resistance has resulted in cultivars with moderate levels of resistance to sweetpotato weevils (Nottingham and Kays, 2002). However, host plant resistance has not been successful in sweetpotato weevil management over the last four decades mainly because of a lack of an understanding of the genetic basis of resistance (Talekar, 1987; Stevenson *et al*., 2009). Most of the resistance in sweetpotato has been associated with escape (Stathers *et al*., 2003b) and high dry matter content (Hahn and Leuschner, 1981). Though some weevil resistant lines were identified in the US (Jackson and Bohac, 2006), classical breeding has not delivered weevil resistant varieties for the farming community in the USA or SSA to date (Stevenson *et al*., 2009).

The identification of the chemical basis of resistance against *C. puncticollis* presents a new opportunity for sweetpotato weevil management in SSA. The high level of hydroxycinnamic acid esters from the root latex was recently proposed as a possible chemical mechanism of resistance to sweetpotato weevils in the Ugandan land race, New Kawogo (Stevenson *et al*., 2009). Hydroxycinnamic acid esters are phenolic compounds constitutively synthesized in plants that may play a role in plant defence against pathogens and herbivory. Earlier research showed that sweetpotato latex contained hexadecyl, octadecyl, and eicosylcoumaric acid esters that were associated with sweetpotato resistance to *C. formicarius* (Snook *et al*., 1994; Data *et al*., 1996) although no evidence was provided that these compounds actually affected weevils. Nevertheless, selection for increased levels of these

compounds in breeding populations has been proposed as a method to enhance the levels of resistance to sweetpotato weevil for improvement and variety development programs in SSA. In Uganda, there exists a wide variation in levels of susceptibility, with some varieties such as New Kawogo reported by farmers as resistant (Stevenson *et al*., 2009), while others are highly susceptible. Earlier studies found that where there were observed cultivar differences in susceptibility to *Cylas* spp. , 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 weevil (Stathers *et al*., 2003a). Early maturing cultivars enabled farmers to harvest roots before the onset of the dry season and subsequent increase in *Cylas* population (Mao *et al*., 2004). Stathers *et al*., (2003b) also suggested, however, that some form of active resistance to sweetpotato weevils likely explained their field observations although they were unable to identify what this was. 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). Phenolic compounds in roots were also associated with less preference for feeding by *C. formicarius* (Nottingham *et al*., 1989). More recently, Wang and Kays (2002) reported variation 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 not yet elucidated. The present research therefore aimed to gain a deeper understanding of the mechanisms of resistance to the weevil, to provide robust evidence of the biological activity of potential target compounds and to provide evidence that could guide the breeding effort to develop resistant varieties.

1.6 POTENTIAL FOR SWEETPOTATO ROOT RESISTANCE TO WEEVILS

1.6.1 Phytochemistry of sweetpotato

The process of co-evolution between plants and their natural enemies is believed to have generated much of the earth's biological diversity including chemical diversity. Consequently, plants produce a wide range of secondary metabolites which are a mixture of volatile and non-volatile compounds (Karban and Baldwin, 1997). Plant surface chemistry is known to play an active role in plant-insect interactions. Earlier, Wilson *et al*., (1988) indicated that surface chemical factors in sweetpotato may play a role in susceptibility or resistance to the weevil. As a result, several studies into factors mediating resistance to the weevil have been conducted through varietal screening of germplasm against the weevil to identify resistance mechanisms. Work on developing host-plant resistance resulted only in the identification of cultivars with moderate levels of resistance (Wang and Kays, 2002). Some of the resistance investigated has been shown to be caused by antibiosis and antixenotic effects or non-preference shown by low infestation and less oviposition on local varieties (Magira, 2003). However, progress in breeding varieties with resistance to sweetpotato weevils has not been successful partly due to inconsistent expression of the resistance (Collins and Mendoza, 1991).

Sweetpotato is a lactiferous crop and therefore produces latex in the roots, vines and leaves which exudes in large quantities on wounding, an indication that there is a possible role for latex in plant defence (Data *et al*., 1996). It was further reported that the area of greatest latex production by the vines is the young apical tissue. Weevil feeding and damage tend to be lower at the vine apex than in the older part

of the plant base. These facts collectively suggest that latex plays a protective role against insect feeding in sweetpotato and varieties that produce more latex could be targeted for host plant resistance against sweetpotato weevil. Insect resistance induced by latex may occur via chemical and/or physical means. The physical means may be due to the sticky nature of the latex. On the other hand, there is increasing evidence of the presence of heritable resistance to sweetpotato weevils that involves mediating processes of oviposition and feeding (Stathers *et al.*, 2003b).

1.6.2 Occurrence of phytochemicals conferring insect resistance in sweetpotato

Phytochemicals are reported to play a role in insect pest host-finding behaviour, oviposition and feeding responses where they can act as deterrents or attractants (Wang and Kays, 2002; Korada *et al.*, 2013). Additionally, boehmeryl acetate and boehmerol are oviposition stimulants in sweetpotato and where these compounds differed in sweetpotato cultivars with varied resistance to the *C. formicarius* it was proposed they might influence resistance or susceptibility (Snook *et al.*, 1994)*.*

Sweetpotato is known to produce several phenolics as discussed above, some of which are believed to contribute to resistance against herbivores insects in other plant pest interactions but also in sweetpotato with sweetpotato cultivars showing high or low levels of caffeic acid and 5-O-caffeoylquinic acid (Son *et al.*, 1991a). The compounds occur widely among many plant species in the plant kingdom (Takenaka *et al.*, 2003) and the levels vary widely in food crops (Clifford, 2000). 5-

O-caffeoylquinic acids are among numerous phenolic compounds found in plants that are thought to function in defence against herbivory (Summers and Felton, 1994; Stevenson *et al.*, 1993). These compounds are also implicated in the resistance of the tomato plant to tomato blight and are known to form orthoquinones in damaged plant tissues. Orthoquinones have been reported to alkylate-NH_2 and –SH groups of proteins and amino acids altering solubility and digestibility as well as infectivity of some pathogenic insect viruses (Isman and Duffy, 1982).

Extending this work on phenolic compounds research on sweetpotato in the USA into resistance mechanisms in sweetpotato to *C. formicarius* reported high levels of hexadecyl, octadecyl, and eicosyl esters of *p*-coumaric acid in the vine and root latex of resistant USA sweetpotato varieties to *C. formicarius* (Snook *et al.*, 1994). However, none of the above compounds and associated resistance mechanisms was proven by their evaluation in artificial bioassays since neither the artificial diets nor sufficient quantities of the compounds were available to test (Mao *et al.*, 2001).

1.7   STATEMENT OF THE PROBLEM

The basis of resistance to sweetpotato weevils in Ugandan germplasm is not known. However, there are varieties with varying levels of resistance to the weevils while the latex of sweetpotato has been reported to contain phenolic compounds that may influence levels of resistance as discussed above in studies on *C. formicarius.* It was indicated that phenolic esters of hydroxycinnamate found in resistant varieties were toxic to larvae of *C. puncticollis* but this didn't fully explain the resistance and their role against adults was unknown and unproven (Stevenson *et al.*, 2009). The occurrence of these compounds in the latex also did not explain the reported reduced oviposition on root surfaces. Consequently, the collection of wider

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germplasm source materials was needed to identify representative genotypes with stable resistance to sweetpotato weevil infestation.

1.8 CONCEPTUAL FRAMEWORK

Variation in levels of resistance to *C. puncticollis* and *C. brunneus* can be explained by the 'escape' where some varieties could avoid being damaged by the weevil. For example, where varieties produce a thick canopy cover which enhances soil moisture conservation, this results in reduced cracking of the soil by storage roots hence hindering sweetpotato weevils from accessing sweetpotato roots. 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 constituting resistance. Also with deep rooted varieties it implies that the soil temperature around storage roots will be lower resulting in reduced weevil development and damage and thus lower susceptibility to *Cylas* spp.

The mechanisms of resistance on the other hand could be active. 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* weevil spp.

Of the different resistant mechanisms thus far proposed, this study targeted characterizing the inherent biochemical sweetpotato compounds for resistance to *Cylas* spp. The research questions addressed were: what are these compounds? Where do they occur? How do they affect adult weevils? This was due to growing evidence that resistance in sweetpotato could be inherent and capable of use for breeding against *Cylas* spp. This approach could also provide a basis on which

quantitative trait loci for resistance could be identified and biochemistry assisted

selection tool developed to enhance breeding for weevil resistance.

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┌────────────────────────────────────────────────────────────────┐
│   Variation in weevil infestation and damage levels in sweetpotato │
│                           varieties                            │
└────────────────────────────────────────────────────────────────┘

      ┌──────────────────┐        ┌──────────────────────────┐
      │      Escape      │        │   Non-preference of some  │
      │                  │        │  sweetpotato varieties for feeding │
      │                  │        │   and oviposition by Cylas │
      └──────────────────┘        └──────────────────────────┘

  ┌──────────────────┐  ┌──────────────────┐
  │   Deep rooting   │  │       High       │
  │ sweetpotato varieties │  │   sweetpotato    │
  │                  │  │  vegetative cover │
  └──────────────────┘  └──────────────────┘

                                          ┌──────────────────────────┐
┌──────────────────────┐ ┌──────────────┐ │  Variation in root chemical │
│  High soil moisture around │ │ Low soil temperature │ │  content with effects on  │
│   sweetpotato roots   │ │  around roots │ │  preference for sweetpotato │
│                      │ │              │ │    by Cylas spp. and on   │
└──────────────────────┘ └──────────────┘ │       weevil biology      │
                                          └──────────────────────────┘

┌──────────────────────┐
│  Low soil cracking around │
│   sweetpotato roots   │
└──────────────────────┘

┌──────────────────┐ ┌──────────────────┐ ┌──────────────────────────┐
│  Less exposure of │ │ Reduced sweetpotato │ │   Bioassays to determine  │
│ sweetpotato roots to │ │ weevil multiplication │ │   antibiosis or antixenosis │
│     Cylas spp.    │ │   and damage     │ │  effects of phytochemicals │
│                  │ │                  │ │      against Cylas spp.    │
└──────────────────┘ └──────────────────┘ └──────────────────────────┘

┌────────────────────────────────────────────────────────────────┐
│  Understanding phytochemical mediated resistance of Cylas spp. in sweetpotato │
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Figure 1.2. Conceptual framework

1.9 JUSTIFICATION

The concealed nature of *Cylas* spp. means that applications of most management options do not effectively reduce weevil damage. Therefore, the identification and deployment of resistant sweetpotato varieties is the most viable option for sweetpotato weevil management especially for resource poor farmers in Uganda and other sub-Saharan African regions. Although variation in damage by sweetpotato weevils exists among sweetpotato varieties, the mechanisms of these varietal differences are not known. Therefore, developing technologies that manage sweetpotato weevils such as the one that this study is reporting is essential as this would increase resilience in the crop and sustain production. Consequently, this is envisaged to have a positive impact on the livelihoods of millions of resource poor farmers across sub-Saharan Africa. Accordingly, this calls for the urgent development of an environmentally benign control approach for this weevil.

Although the importance of sweetpotato chemistry for the development of cultivars resistant to the weevil and other soil insects has been advocated for decades, this has still not been fully exploited. In fact, understanding possible phytochemical mediated resistance mechanisms and their genetic inheritance would facilitate targeted breeding to develop resistant varieties. Suspected among the factors that may confer this variation is the type, quantity and location of root chemicals that may influence preference of African sweetpotato weevils for feeding, oviposition and larval development. This altogether makes investment in sweetpotato research to control weevil damage and increase the crop productivity a worthwhile venture.

1.10 OBJECTIVES

The major objective of this study was to establish and evaluate the chemical mechanisms of resistance to sweetpotato weevils in Ugandan sweetpotato landraces and varieties. Specifically the study was conducted to:

1. Evaluate the response of improved sweetpotato varieties and landraces to *C. puncticollis* and *C. brunneus.*

2. Identify, locate and quantify the chemical compounds in varieties and landraces that vary in their resistance to *C. puncticollis* and *C. brunneus.*

3. Identify sweetpotato volatile compounds that might influence host location, oviposition and feeding of *C. puncticollis* and *C. brunneus.*

4. Determine the biological effects of sweetpotato phytochemicals against *C. puncticollis* and *C. brunneus.*

5. Determine interactions between sweetpotato phytochemicals and Bt-proteins on *C. puncticollis* and *C. brunneus.*

6. Assess the correlation of the field resistance, biological activity and chemistry data in the segregating population of a cross between a fully mapped US variety and a resistant Ugandan land race.

1.11 HYPOTHESES

The following hypotheses were tested in this study:

1. Improved sweetpotato varieties and landraces respond differently to *C. puncticollis* and *C. brunneus* infestation

2. Chemistry of sweetpotato varieties and landraces that vary in their resistance to sweetpotato weevils differ.

3. Sweetpotato volatile compounds influence host location, oviposition and feeding of *C. puncticollis* and *C. brunneus.*

4. Toxicity and deterrence of sweetpotato phytochemicals affect *C. puncticollis* and *C. brunneus* in a dose dependent manner.

5. Sweetpotato phytochemicals complement the effects of Bt-protein on *C. puncticollis* and *C. brunneus.*

6. The segregating population of New Kawogo and Beauregard sweetpotato clones differ in their chemical composition associated with resistance.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. WEEVIL CULTURING AND SEXING

Sweetpotato weevils used during the study were reared on a susceptible sweetpotato variety NASPOT 1 storage roots in cages made of metal and wire meshes with cloth meshes at the entrance to allow free air circulation at Sweetpotato Entomology laboratory at National Crops Resources Research Institute (NaCRRI), Namulonge, Wakiso district in central Uganda (Plate 2.1). The colonies were maintained at a temperature of 25 to 28ºC and 55 to 75% relative humidity. Weevils were allowed to feed and oviposit on sweetpotato storage roots for 24 hours and transferred to fresh storage roots every day. The old roots were then incubated until a new generation of weevils emerged after approximately 4 to 5 weeks. Weevils were collected as they emerged and kept for 2-3 weeks. The weevils were separated into male and female based on the distinct antennal characteristics of either species. Two-week-old female adults were used in the bioassay as described by Stevenson *et al*. (2009).

Plate 2.1. Sweetpotato weevil rearing at NaCRRI sweetpotato Entomology laboratory (Photo by Otema, A.M.)

2.2. CHROMATOGRAPHY

Chromatography describes the techniques used to separate and/or to analyze complex chemical mixtures (Harbone, 1998). The compounds that need to be separated are distributed between a stationary and mobile phase. A mixture of different compounds is placed on the solid phase and enters the chromatographic process once the solid phase is exposed to the mobile phase which is a solvent in liquid chromatography (LC) and gas in gas chromatography (GC). In LC different components pass through the system at different rates depending on the differential

and relative affinity for the stationary phase and willingness to solubilise in the organic solvent or aqueous mobile phase. The lower the affinity a molecule has for the stationary phase, the shorter the time it will spend on the stationary phase. If the right adsorbent, mobile fluid and operating conditions are used, almost all soluble or volatile components can be separated using chromatography. Among the number of chromatographic methods used in this study was high performance liquid chromatography (HPLC) linked to Mass Spectrometry and gas chromatography (GC). The operation of each technique is described in the relevant experimental section below.

2.2.1. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is an analytical technique that evolved from preparative flash chromatography. It is an adaptable natural product separation technique where the mobile phase elutes through the solid phase in a column at high pressure (up to 4000 p.s.i.) and where the stationary phase is very fine, high-precision particles of silica d (2-10 µm diam.) that is either substituted with hydrocarbon chains of varying length (typically C18) (reverse phase) or unsubstituted (normal phase). In the present study only reverse phase chromatography was used. The column is stainless steel to withstand the high pressure. The eluting compounds can be detected using their different physical and structural properties by connecting a detector (ultra violet/visible UV/VIS, refractive index or mass spectrometer). High Performance Liquid Chromatography is mainly used for compounds that are non-volatile such as flavonoids, large terpenoids, phenolic acids, alkaloids, lipids and sugars as well as for detecting using a

Photodiode array (PDA). PDA is best for compounds that have a strong and characteristic chromophore and can thus be detected in the ultraviolet and visible regions of the spectrum (Harborne, 1998).

In this study HPLC was performed on a Waters system with 600E quaternary pump, 717 autosampler and 996 diode array detector. Chromatographic separation was performed on 150 mm x 4.6 mm internal diameter (5µm particle size) Zorbax Eclipse C18 columns using a linear mobile phase gradient of 0.400 ml/min flow rate with (A): H_2O (B): MeOH (C): Acetonitrile with 1% formic acid using A=90, B=0 and C=10 at T=0 and A=0, B=90 and C=10 at t= 20 mins. Injection was 10µl and data analysis was performed using Millennium software. LC-MS was carried out using an Agilent 1200 LC system with single quadrupole mass spectrometer (Agilent technologies 2000 series) interfaced with a single quad mass spectrometer using electrospray ionization (ESI) source operating in positive mode under standard conditions and source voltages tuned for optimal transmission of rutin and the same column and chromatographic conditions described above.

2.2.2. Gas Chromatography

Gas Chromatography (GC) technique to analyze volatile compounds where the compounds are carried by an inert gas (N_2, He) through a heated (50-350ºC) stationary phase fused silica tubing with bonded polar or non-polar phase). Either a flame ionization detector (FID) or electron capture detector (ECD) detects the compounds eluting from the column producing a signal which can transform into a peak on a chromatogram. The technique is sensitive thereby facilitating low concentration samples (less than nanograms) to be analysed. The individual volatile

compounds were identified by comparison of their mass spectra with National Institute of Science and Technology (NIST) spectral libraries.

CHAPTER 3

RESPONSES OF SELECTED UGANDAN SWEETPOTATO VARIETIES TO SWEETPOTATO WEEVIL INFESTATION AND DAMAGE

3.1. INTRODUCTION

Sweetpotato weevils (*Cylas* spp.) are the most harmful insect pests of sweetpotato world-wide (Rees *et al*., 2003). In fact, sweetpotato weevils can cause up to 100% loss depending on the season and the variety (Smit 1997, Nottingham and Kays, 2002). In Uganda, a survey on the socio-economic impact of weevils done in Uganda, indicated an average yield loss of over 28% depending on the seasons (Kiiza *et al*., 2009).

The major damage is caused by sweetpotato weevil larvae which through tunnelling inside the root, cause unacceptable microbial contamination and production of a sesquiterpene toxicant by the sweetpotato root, making it unfit for sale or human consumption (Uritani *et al*.1975, Sato and Uritani 1981, Woolfe 1992). In addition, fungal rotting occurring as a consequence of weevil tunnelling in the storage roots produces several compounds including ipomeamarone which is particularly toxic to animals and possibly humans (Pandey, 2008).

Therefore, the potential of sweetpotato to achieve its yield potential can only be realized if weevils can be controlled and their damage minimized. Accordingly, the

development of weevil resistant cultivars has been suggested as an essential component of Integrated Pest Management of sweetpotato weevil (Muyinza *et al*., 2012). However, attempts to develop host plant resistance have only been moderately successful due to limited source of resistance (Wang and Kays, 2002). Currently, sweetpotato breeders select for resistance using insect damage as an index of susceptibility. However, the most reliable approach to developing sweetpotato varieties with sustainable levels of resistance to the sweetpotato weevil involves understanding the resistance mechanisms deployed by the plants. Therefore, if the phytochemicals responsible for resistance are identified and quantified mapping out the genes controlling the production of the chemicals that confer resistance would help in developing biochemistry assisted selection (BAS). The present study aimed to identify phenotypic characters that could help identify quantitative trait loci (QTL) for resistance and facilitate breeding for resistant varieties to sweetpotato weevils. Previously, Stevenson *et al*. (2009) identified a possible mechanism of resistance in one Ugandan variety New Kawogo. Compounds were identified in the latex that were associated with resistance and reduced development in larvae. Therefore, this study was conducted to evaluate levels of resistance to help understand the mechanism of resistance to sweetpotato weevils in Ugandan sweetpotato germplasm. If this mechanism of resistance was associated with field resistance in several varieties then it might provide greater evidence for breeding traits.

3.2.   MATERIALS AND METHODS

3.2.1. Field Trials

Seven improved sweetpotato varieties indicated as having lower levels of field infestation by Muyinza (2010) were selected for this study. The varieties included: HMA 519, ARA 230, LIR 302, APA 356, ARA 228, RAK 865, and New Kawogo. These cultivars were evaluated for resistance to weevils in a field trial and compared with three susceptible varieties: NASPOT 1, Kakamega, and Tanzania planted in field plots at the National Semi-Arid Research Institute (NaSARRI), Serere, (1°32′N, 3°27′E), 1,085 m.a.s.l. This area was selected because it has a dry and hot environment that favours weevil population build up and damage to sweetpotato storage roots.

The trial was planted in a randomized complete block design (RCBD) with 10 replicates to minimise the effects of variation, on 1.0 m ridges with 3 vines placed 30 cm apart and 1.0 m space between ridges (Plate 3.1). First planting was done in September and second planting done in June to allow root maturity period to coincide with the peak for weevil activity in the field during the dry season. Earthing up of the soil was done twice with hand hoes during weeding at 28 and 56 days after planting (DAP) to control for the possibility that root damage could be a consequence of soil cracking. Further weed removal was carefully done using hand pulling at 10 WAP to avoid damage to the vines and young roots which would occur when hand hoes are used.

Plate 3.1 Field experiment planted in NaSARRI, Serere (Photo: Otema A.M.)

Artificial infestation was done at 90 and 120 days after planting (DAP) with adult *C. puncticollis* and *C. brunneus* to augment the natural field infestation. The infestation was done by releasing two week old *C. puncticollis* and *C. brunneus* adults (7 females and 3 males of each) on each ridged plot following the procedure of Muyinza *et al*. (2012). The weevils were obtained from a culture that was established at NaCRRI sweetpotato entomology rearing facilities with initial cultures obtained from infested roots collected from farmer's fields in Soroti.

The trial was harvested 5 months after planting, the normal time of crop maturity. Fresh weight of the vines of each plant was recorded for each variety per replication. The roots from each plant were carefully dug up for each ridged plot, counted, weighed and sorted into damaged and undamaged roots. A root was considered damaged if it had characteristic scarred spots, typical of weevil feeding and

oviposition, whereas those without damage were considered clean. The number of infested and non-infested roots were counted and recorded.

Basal damage was assessed by cutting the first 10-15 cm woody portion above ground level for two of the three vines per ridged plot. The stems were then dissected longitudinally and damage rated on a 1-5 score where 1= 0-20%; 2= 21-40%; 3= 41-60%; 4= 61-80%; and 5= 81-100% of stem damaged (Muyinza *et al.*, 2012). The means of the internal and external damage scores for each variety were then used to evaluate the varieties for levels of damage using the stem base indicator.

3.2.2. Development of sweetpotato weevils on roots of the different clones.

Bioassay to Evaluate the Feeding and Oviposition by C. puncticollis on the Selected Sweetpotato Varieties.

Freshly harvested roots from each of the seven improved varieties and the three local checks were brought into the laboratory, washed under running water to remove soil particles and air-dried under at temperature. Root cores from each of the 7 varieties and 3 local checks were cut to approximately 10 mm depth using a 24 mm diameter cork borer No. 15 as described by Stevenson *et al.*, (2009). The weevils used in the bioassay were starved for 3 hrs prior to the experiments. The root cores were precisely fitted into wells of a 12- well plate presenting only the periderm to the weevils. Two–week-old gravid *C. puncticollis* female adult weevils were introduced into the feeding trays at the rate of two weevils per root core. The lid was cemented in place with clay to prevent the weevils from escaping from the

test arena and perforated to allow air circulation. *Cylas puncticollis* was preferentially used in this experiment because of its high feeding rate compared to *C. brunneus*. The experiment was set in a completely randomized design in 12 replicates repeated four times. The weevils were allowed to feed and oviposit for 48 h (Plate 3.2).

Plate 3.2. Feeding and oviposition bioassay in sweetpotato Entomology laboratory at NaCRRI (Photo: Otema A.M.).

Data Collection and Analysis of Bioassay Data.

The number of feeding punctures and faecal droppings on the root plug were counted for each treatment after 48 hours to evaluate the resistance of different cultivars as described by Stevenson *et al*., (2009). The root periderm was gently removed with a scalpel and the number of eggs deposited was observed and counted under a magnifying glass.

Multiple choice tests were also conducted in which insects were provided with 3 root cores from each of four different varieties: NASPOT 1, Tanzania, New Kawogo and LIR302. The 3 root cores of the four varieties were placed in the 12-well feeding trays in a complete randomized design. The experiments were replicated four times in a completely randomized design (CRD) and repeated five times. This bioassay technique was an adaptation of one previously used by Stevenson *et al*., (2009) that has been used in several SPW feeding and oviposition studies. The data obtained were analysed using one-way ANOVA. Where there was significant difference at P < 0.05, means were separated using Tukey's honesty significant test. This was used because the data was normally distribution. The relationship between stem and storage root damage, vine weight and root damage was described using correlation coefficient.

3.3. RESULTS

3.3.1. Variation in resistance and susceptibility of selected sweetpotato varieties to sweetpotato weevil infestation and damage in the field.

The mean fresh vine weight, number of total root and number of infested roots of different sweetpotato varieties were significantly different (ANOVA, $P \leq 0.001$, $F=7.22$ and df= 9,90; ANOVA, $P \leq 0.01$, $F=2.49$ and df= 9,90; ANOVA, $P \leq 0.01$, $F=2.45$ and df= 9,90) under field infestations. New Kawogo, RAK 865 and Tanzania had significantly higher fresh vine weight compared to NASPOT1 and Kakamega. In contrast, ARA 230 had the least fresh vine weight compared to the weight of vines in control varieties (Table 3.1).

Tanzania recorded the highest number of roots followed by RAK865. However, the number of roots yielded by New Kawogo was low (Table 3.1).

The mean number of roots infested by sweetpotato weevils was higher in ARA230 compared to the local checks. Interestingly, New Kawogo, ARA228 and LIR302 showed no damage (Table 3.1).

Table 3.1 Fresh vine weight, total number of roots and number of damaged roots (Mean ± SEM) per plant of selected sweetpotato varieties harvested 5 months after planting in NaSARRI

Variety	N	Vine weight (kg)	Number of roots	Damaged roots
APA356	10	1.9 ± 0.3ab	5.7 ± 0.9b	0.5 ± 0.3b
ARA228	10	1.1 ± 0.3b	5.1 ± 1.1b	0.0 ± 0.0
ARA230	10	0.5 ± 0.1b	4.4 ± 0.6b	2.3 ± 0.5a
HMA519	10	2.8 ± 0.3a	7.3 ± 1.0ab	1.4 ± 0.6ab
LIR302	10	2.2 ± 0.3ab	5.3 ± 1.0b	0.0 ± 0.0
NASPOT1(L)	10	1.8 ± 0.3ab	8.3 ± 1.0a	1.0 ± 0.4ab
New Kawogo	10	3.3 ± 0.3a	7.9 ± 0.8ab	0.0 ± 0.0
RAK865	10	3.3 ± 0.4a	7.3 ± 1.4ab	0.5 ± 0.4b
Kakamega(L)	10	1.7 ± 0.3ab	9.3 ± 2.0 a	1.6 ± 0.4ab
Tanzania(L)	10	3.2 ± 0.5a	12.3 ± 1.3a	1.9 ± 0.8ab
LSD		0.6	3.3	0.4
P		0.001	0.001	0.001

[a] Means accompanied by same letters are not significantly different from each other (ANOVA, $P \leq 0.05$, Tukey's test). N is the number of samples and L are the susceptible, local control varieties.

There were significant differences in mean sweetpotato weevil damage (ANOVA, P ≤ 0.001, F=8.22 and df= 9,90 and ANOVA, P ≤ 0.001, F=7.22 and df= 9,90) on both internal and external stem bases of sweetpotato varieties. Similarly, the mean number of sweetpotato larvae found inside the stem at harvest among the selected varieties differed significantly (ANOVA, P ≤ 0.015, F=9.8 and df=9,90). However, sweetpotato weevil damage on both the external and internal stem base was lower in New Kawogo, RAK865, LIR302, APA356 and ARA228 compared to the local checks. Among the experimental materials selected on the basis of resistance, ARA230 damage was not significantly different (P>0.05). As with the number of infested roots, New Kawogo, LIR302 and ARA228 had no larvae developing inside the stem base (Table 3.2). Significantly more weevil larvae were found inside the stem of local varieties NASPOT 1 and Tanzania (Table 3.2).

Table 3.2 External and internal stem damage score, and number of larvae (mean ± SEM) in the stems of selected sweetpotato germplasm after artificial infestation with *Cylas puncticollis* and *C. brunneus*.

Variety	N	External	Internal	Number of larvae
APA356	10	1.5 ± 0.1b	1.3 ± 0.1b	0.1 ± 0.0a
ARA228	10	1.7 ± 0.1b	1.5 ± 0.1b	0.0 ± 0.0b
ARA230	10	2.2 ± 0.3a	2.2 ± 0.3ab	0.9 ± 0.6b
HMA519	10	1.7 ± 0.2ab	1.6 ± 0.2b	0.5 ± 0.2b
LIR302	10	1.6 ± 0.1ab	1.6 ± 0.1b	0.0 ± 0.0b
NASPOT 1(L)	10	2.8 ± 0.3a	2.8 ± 0.2b	3.1 ± 1.5a
New Kawogo	10	1.3 ± 0.2b	1.2 ± 0.2b	0.0 ± 0.0b
RAK865	10	1.4 ± 0.1b	1.3 ± 0.1b	0.1 ± 0.0b
Kakamega (L)	10	1.9 ± 0.1ab	1.9 ± 0.1ab	0.5 ± 0.3b
Tanzania (L)	10	2.5 ± 0.2a	2.5 ± 0.2a	2.7 ± 1.4a
LSD		0.5	0.5	1.0
P<F		0.01	0.001	0.01

[a]Means followed by the same letters are not significantly different from each other (ANOVA, P ≤ 0.05 Tukey's test). N is sample size and L are susceptible local control varieties.

3.3.2. Relationships between sweetpotato weevil damage parameters and sweetpotato varieties.

There was significant (R = 0.94, P<0.004) positive correlation between external stem damage and root damage in the field indicating that stem damage is linked to storage root damage. Significant relationship between sweetpotato vine weight and sweetpotato weevil root damage was recorded (ANOVA, P ≤ 0.05, R = -0.86) suggesting vine weight and weevil root damage are contrasting.

3.3.3. Feeding and oviposition of sweetpotato weevil on different sweetpotato varieties.

Significant differences (ANOVA, P ≤ 0.001, F= 10.04 and df= 8,195; ANOVA, P ≤ 0.001, F=5.11 and df= 8,195 and ANOVA, P ≤ 0.001, F=5.62 and df= 8,195 were observed on the number of faecal droppings, feeding holes, and eggs laid on the roots respectively. The mean number of faecal droppings was lower on the root cores of New Kawogo compared to the other varieties such as NASPOT 1, Tanzania, Kakamega, RAK 865, and ARA230 (Table 3.3). Similarly, the number of feeding holes was lowest on New Kawogo and LIR 302 (Table 3.3). The mean number of eggs laid was lower on the root cores of New Kawogo than all other varieties and was highest on NASPOT 1, Kakamega, Tanzania, and RAK 865 indicating that they were more preferred (Table 3.3).

Table 3.3 Number of faecal droppings, feeding holes, and eggs (mean ± SEM) on the root core of different varieties of sweetpotato in a No-Choice Bioassay.

Variety	N	Faecal dropping	Feeding hole	Egg laid
APA356	48	13.9 ± 1.8ab	18.5 ± 1.4b	10.2 ± 0.7b
ARA230	48	20.5 ± 1.1b	18.6 ± 1.0b	10.7 ± 0.6b
HMA519	48	14.7 ± 1.0ab	15.0 ± 1.5ab	9.1 ± 0.5b
LIR302	48	13.0 ± 1.8ab	14.5 ± 2.8ab	7.3 ± 1.2b
NASPOT 1(L)	48	23.6 ± 1.9b	26.8 ± 2.0b	13.1 ± 1.0b
New Kawogo	48	11.6 ± 1.2a	12.8 ± 1.6a	5.6 ± 0.9a
RAK865	48	20.4 ± 1.1b	19.9 ± 5.6b	12.2 ± 1.2b
Kakamega (L)	48	20.0 ± 1.2b	20.6 ± 1.7b	12.3 ± 1.1b
Tanzania (L)	48	23.6 ± 2.0b	20.7 ± 1.9b	12.2 ± 1.2b
P≤		0.001	0.001	0.001

[a]Means followed by the same letters are not significantly different from each other (P ≤ 0.05 Tukey's test). N is the sample size and L are susceptible, local control varieties.

There were significant differences in the mean number of faecal droppings (ANOVA, P ≤ 0.05, F= 6.36 and df= 3,57), feeding holes (ANOVA, P ≤ 0.001, F= 3.48 and df= 3,57 and eggs (ANOVA, P ≤ 0.01, F= 4.52 and df= 3,57) in a multiple choice

bioassay. NASPOT1 had the highest mean number of faecal droppings while New Kawogo had the lowest mean number of faecal droppings on root cores compared to other varieties though did not differ significantly from the faecal materials recorded on LIR302 (Table 3.4). The number of feeding holes was significantly greater on NASPOT 1 compared to New Kawogo and LIR302 (Table 3.4). The number of eggs laid was highest on NASPOT 1 and Tanzania and lowest on New Kawogo and LIR302 which did not differ significantly from each other (Table 3.4).

Table 3.4 Number of faecal droppings, feeding hole and eggs laid (mean ± SEM) on the root cores in a multiple-choice bioassay.

Variety	N	Faecal droppings	Feeding hole	Eggs
LIR302	60	14.0 ± 3.2a	12.0 ± 2.9a	7.7 ± 1.0a
NASPOT1(L)	60	26.7 ± 7.0c	18.0 ± 4.5c	11.7 ± 2.0b
New Kawogo	60	10.0 ± 5.0a	6.0 ± 4.6a	6.3 ± 3.0a
Tanzania (L)	60	18.0 ± 1.2b	16.3 ± 1.2b	11.0 ± 0.3b
LSD		4.65	2.192	3.1
P<		0.0179	0.015	0.033

[a]Means followed by the same letters are not significantly different from each other (P ≤ 0.05 Tukey's test). N is the sample size and L are control varieties.

3.4. DISCUSSION

The study was conducted to show whether sweetpotato varieties and landraces vary in resistance to sweetpotato weevil. Damage on internal and external stem bases of sweetpotato varieties differed between clones. These data relates closely to findings reported by Muyinza *et al*. (2007) that significant correlation exists between external stem and root damage in the field indicating that stem damage is an important and potentially easier measurable parameter for determining weevil resistance in sweetpotato and avoids the need to uproot whole plants. The field data showed that resistance exists in land races but from these data it was not yet clear whether this effect was active or escape.   Field evidence of resistance is important since this demonstrates that any mechanisms identified under laboratory conditions are manifested under field conditions. However, these results did not prove that the mechanisms was active since as has been discuss earlier escape can mediate reduced damage in field trials (Stathers *et al*., 2003a).

In no-choice bioassays, the number of faecal droppings and other evidence of feeding and egg laying were significantly lower on the root cores of New Kawogo compared to the number on NASPOT 1, Tanzania, Kakamega, RAK 865, and ARA230 indicating that weevil feeding was reduced in this variety. Thus in the absence of field parameters, such as soil cracks, this result shows that resistance in New Kawogo was active. Similarly, the mean number of eggs laid on the root cores of New Kawogo was significantly lower than on all other varieties but was highest on NASPOT 1, Kakamega, Tanzania, and RAK865. A range of feeding responses by *Diabrotica* species feeding on sweetpotato from different cultivars have been reported by Jackson and Bohac (2007) suggesting a quantitative

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resistance to this insect that could be related to the effect reported in this study from *Cylas* spp. The fact that both studies presented adults with root surfaces and showed a similar effect supports this. Perhaps the result of greatest importance in terms of pest control was that even when presented in a no-choice experiment. *C. puncticollis* lays significantly fewer eggs on the root cores of New Kawogo than on all other cultivars indicating that the resistance would be effective even where this variety was the only material in the field. The lower the infestation the lower the internal damage and reduced loss. Two other varieties, LIR302 and HMA519, shown to be resistant in the field, also recorded significantly fewer eggs laid than on the most susceptible variety NASPOT 1 so these varieties would also be worth following up.

A similar observation was made on feeding and oviposition recorded in choice bioassays. Weevils feeding on NASPOT 1 produced significantly more faecal droppings than weevils on any other variety followed by Tanzania but produced least on New Kawogo indicating that weevils fed less on New Kawogo. The number of feeding holes was also different among varieties with most occurring on NASPOT 1 and New Kawogo having the least feeding holes of all the varieties. The number of eggs laid on the root cores was also different among the sweetpotato varieties being highest on NASPOT 1 and lowest on New Kawogo. The variability in the mean number of faecal droppings, feeding holes, and eggs laid observed on the root cores of different varieties indicate that sweetpotato weevils have a preference for particular varieties when presented with a choice. Significantly fewer faecal droppings and feeding holes and lower oviposition on New Kawogo indicated that this variety was less preferred than susceptible varieties and demonstrates an active resistance. In both the choice and no choice tests NASPOT 1, Tanzania, ARA230,

and Kakamega were most susceptible to damage and egg laying by weevils. This accepts the hypothesis that sweetpotato varieties vary in their susceptibility to sweetpotato weevils. Earlier reports that New Kawogo was resistant (Mwanga *et al*., 2001) are supported by these laboratory bioassays but also indicate that the mechanism is not based on escape but is an active mechanism, as proposed by earlier work of Muyinza *et al*., (2007) with New Kawogo, LIR302 and ARA228 expressing lower weevil root damage than other varieties evaluated. An absence of field damage on roots with low stem damage together with low feeding and oviposition in the laboratory bioassay indicated that the resistance expressed by these varieties was active and not simply escape as suggested for resistance earlier (Stathers *et al*., 2003a and b). Thus a mechanism of resistance was apparent and might be explained by chemical differences among the varieties since earlier work had suggested plant chemistry might play a role in mediating resistance (Data *et al*., 1996; Wang and Kays, 2002; Stevenson *et al*., 2009).

CHAPTER 4

ISOLATION AND IDENTIFICATION OF PHENOLIC COMPOUNDS FROM SWEETPOTATO VARIETIES WITH VARYING LEVELS OF RESISTANCE TO SWEETPOTATO WEEVILS

4.1. INTRODUCTION

Phenolic compounds are a group of secondary metabolites found in many plants species (Harborne, 1998). Phenolic compounds are reported to play a number of roles in plants including protection against ultraviolet radiation or defence against antagonists such as pathogens and herbivorous insects (Manach *et al*., 2004). Phenolic acids are molecules with a phenol ring containing one or more hydroxyl groups and are frequently found in fruits, vegetables and other products derivatives (Liu, 2004; Farah and Donangelo, 2006). One of the major groups of phenolic acids is the hydroxycinnamic acids most commonly represented by caffeic, *p*-coumaric and ferulic acids and are one of the largest classes of phenolic compounds in plants of economic importance (Crozier *et al*., 2009). Previous work on sweetpotato has reported phenolics to be associated with resistance (Snook *et al*., 2004; Son *et al* 1994). Hydroxycinnamic acid esters in the latex were found previously to be biologically active against the larvae (Stevenson *et al*., 2009). However, the latex vessels sit below the root surface, which is the first point of contact for adult weevils, so latex chemistry may not directly influence adult weevils' behaviour or are toxic to

them. In the present study, the occurrence of hydroxycinnamic acid esters on the root surface, in the periderm and epidermal tissues were investigated as well as root and leaf latex of sweetpotato storage root and leaves and peeled whole roots were isolated, identified and correlated to the resistance that was observed in Chapter 3. Identification of compounds that confer resistance would provide important phenotypic characters and facilitate breeding for resistant against sweetpotato weevils (Wang and Kays, 2002).

4.2. MATERIALS AND METHODS

4.2.1. Extraction of root surface compounds

Freshly harvested roots of the above 10 varieties reported in Chapter 3 were extracted in analytical grade hexane (Fisher, Loughborough, UK) for analysis of the variation in surface chemistry between varieties. Prior to the extraction, roots were weighed, and the surface area was estimated by wrapping them in graph paper and cutting away extra paper folds, leaving only paper that was in contact with the surface. The graph paper was then removed and laid flat, and squares remaining on the graph were counted to give the surface area of the root sample used. The surface area to weight relationship was plotted to allow root surface areas to be estimated according to their weight based on the measurement of three roots from each of the 10 varieties following procedure described earlier (Muyinza *et al*., 2012).

Three roots from each variety were extracted individually by complete immersion of the roots in hexane (500 mL) for 1 min. This reduced the extraction of compounds from deeper layers of the root. More polar compounds were extracted by the same

procedure but for 24 h in methanol. The crude extracts were evaporated to dryness *in vacuo* on a rotary evaporator and re-dissolved in a few mL of methanol. The supernatant was transferred into LC-MS vials, labelled, and stored in the refrigerator prior to LC-MS analysis.

4.2.2. Extraction of chemicals from freeze-dried samples

Sweetpotato storage roots harvested from the field were washed under tap water. Two transverse root disks were cut from the middle of each root and freeze-dried (True-Ten Industrial Co.) for 72 h. The periderm and epidermal sections of the freeze-dried root disks were separated using the edge of a kitchen knife. The separated portions were powdered using laboratory blender. The powdered material of each plant part (50 mg) was weighed (Mettler AT 201) and transferred into Eppendorf tubes. Methanol (1 mL) was added and allowed to extract for 24 h. The crude mixture was spun using a Mini-centrifuge (Costar) at 1300 rpm for 5 min and the supernatant collected for analysis.

4.2.3. Root latex extraction

Root latex from New Kawogo, ARA228, APA356, HMA519, Tanzania and ARA230 was extracted in methanol, and extracts were compared by high-performance liquid chromatography (HPLC) analysis. The chemical components were identified by liquid chromatography-mass spectrometry (LC-MS) and synthesized for bioassays. Freshly harvested roots of the two test varieties were broken in two by hand, which optimized the flow of latex as opposed to cutting using a knife, which resulted in low

latex production. The latex was collected from the broken exposed surface immediately with a melting point/capillary tube. An aliquot of the latex was transferred to a vial, weighed, dissolved in methanol, and stored at 3 ± 3 °C.

The mixture was spun using a mini-centrifuge (Costar 10m-08446, USA) at 1,300rpm for 5 min for uniform mixing of the latex in methanol. The supernatant was drawn using pipette and placed into LC-MS vials labelled and stored prior to LC-MS analysis using the procedure described in section 4.2.1.2.

4.2.4. Leaf latex extraction

Leaf latex was obtained by breaking off the shoot tips and the latex was collected using capillary tubes as described above. The resultant latex was dissolved in methanol and the solid particle was centrifuged and the resultant aliquot drawn in HPLC vial for analysis.

4.2.5. Whole root extraction

The roots of the ten sweetpotato varieties were harvested as in 4.2.2, cleaned under running water and air dried under room temperature. The roots were peeled using a kitchen knife and the flesh was sliced and packed in polythene bags, frozen and freeze-dried using a vacuum freeze dryer (True-ten Biotech and Food dryer/cooling equipment) for 72 hours as in 4.2.2. The samples were taken to Natural Resources Institute (NRI) for chemical analysis.

4.2.6. Sample Analysis using Liquid Chromatography-Mass Spectrometry (LC-MS)

All extracts from the above samples applied to HPLC columns were first passed through 0.45 μm nylon Acrodisc filters as described in section 2.2.1. Chemical analysis of the filtered samples was carried out using a LC−MS detector (Agilent Technologies 1200 series) interfaced with a single quadrupole mass spectrometer using an electrospray ionization (ESI) source operating in positive mode under standard conditions and source voltages tuned for optimal transmission of rutin (Anyanga *et al*., 2013). Extracts were separated with a 150 mm ×4.0 mm i.d. Zorbax Eclipse C18 column with 5 μm particle size (Agilent Technologies, UK) operating under gradient conditions, with A = MeOH, B = H_2O, C = 1% HCO_2H in MeCN; A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min. Column temperature was 30°C and flow rate 0.5 ml/min. Injection volume was 10 μL and data analysis was performed using Chemstation software (Agilent Technologies 1200 series).

Compounds were detected by their photodiode array ultraviolet (PDA-UV) and MS spectra, where fragmentation in ESI-MS (+) was characterized by the molecular ion of each compound accompanied by two fragments representing the two ion forms of the cinnamate moiety (e.g., coumarate and coumaroyl). Compound **1**: UV (LC−PDA) λmax 325 nm; MS m/z 163.5, 181.3, 403.2 [M + H]$^+$. Compound **2**: UV (LC−PDA) λmax 315 nm; MS m/z 165.2, 147.3, 389.4 [M + H]$^+$. Compound **3**: UV (LC−PDA) λmax 325 nm; MS m/z 181.3, 163.5, 417.2 [M + H]+. Compound **4**: UV (LC−PDA) λmax 325 nm. MS m/z 181.3, 163.2, 433.1 [M + H]$^+$. Compound **5**: UV

(LC−PDA) λmax 315nm; MS m/z 165.2, 147.3, 417.2 [M + H]$^+$. Compound **6:** UV (LC− PDA) λmax 325 nm; MS m/z 355.4 [M + H]$^+$ (Stevenson *et al*., 2009).

The integrated peak areas extracted from single ion chromatograms based on [M + H]$^+$ were quantified against calibration curves of synthetic standards of **1−5** prepared as described below and against 5-*O*-caffeoylquinic acid (**6**) (Sigma Aldrich, Dorset, UK).

4.2.7. Synthesis of hydroxycinnamic acid esters

Synthetic standards for compounds (**1−5**) were prepared in high yield from the corresponding acids by acetylation, esterification, and deacetylation as described earlier by Stevenson *et al*. (2009).

4.2.8. Data analysis.

The data obtained was analysed using one-way Analysis of variance (ANOVA) in R-statistical software generalized linear model. Where differences were significant, means were separated using Tukey's honest test.

4.3. RESULTS

4.3.1. Presence of hydroxycinnamic acid esters on the root surface of sweetpotato cultivars obtained from combined hexane and methanol extracts.

The study identified six hydroxycinnamic acid esters from the sweetpotato root surface. The compounds identified were hexadecylcaffeic acid (**1**), hexadecylcoumaric acid (**2**), heptadecylcaffeic acid (**3**), octadecylcaffeic acid (**4**), octadecylcoumaric acid (**5**) (Figure 4.1), and 5-*O*-caffeoylquinic acid (chlorogenic acid) (**6**).
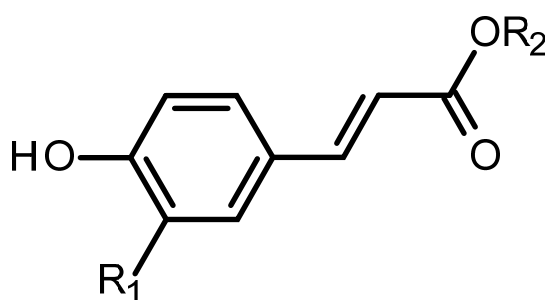
	R_1	R_2
Hexadecylcaffeic acid (**1**)	OH	$-(CH_2)_{15}CH_3$
Hexadecylcoumaric acid (**2**)	H	$-(CH_2)_{15}CH_3$
Heptadecylcaffeic acid (**3**)	OH	$-(CH_2)_{16}CH_3$
Octadecylcaffeic acid (**4**)	OH	$-(CH_2)_{17}CH_3$
Octadecylcoumaric acid (**5**)	H	$-(CH_2)_{17}CH_3$

Figure 4.1. Compounds identified in sweetpotato roots

The concentration of the compounds isolated overall varied significantly (P≤ 0.001) among the sweetpotato varieties. There were significant differences in concentration of hexadecyl caffeic acid (ANOVA, P≤ 0.001; F=9.82 and df= 9,18), hexadecylcoumaric acid (ANOVA, P≤ 0.005; F=3.96 and df= 9,18), heptadecylcaffeic acid (ANOVA, P≤ 0.003; F=4.39 and df= 9,18), octadecylcaffeic acid (ANOVA, P≤ 0.011; F=3.41 and df= 9,18), octadecylcaffeic acid (ANOVA, P≤ 0.003; F=9.77 and df=9,18) and 5-O-caffeoylquinic acid (ANOVA, P≤ 0.001; F=6.31 and df= 9,18) respectively on root surface of the selected sweetpotato varieties.

Figure 4.2. Mean concentration of hydroxycinnamic acid esters on the root surface of selected sweetpotato varieties assayed at NRI.

Abbreviations: C16 Caffeic = hexadecylcaffeic acid (**1**); C16 Coumaric = hexadecylcoumaric acid (**2**); C17 Caffeic = heptadecylcaffeic acid (**3**); C18 Caffeic = octadecylcaffeic acid (**4**); C18 Coumaric =octadecylcoumaric acid (**5**); CQA= 5-O-caffeoylquinic acid (**6**).

The concentration of hexadecylcoumaric acid was highest on the root surface of New Kawogo and ARA228 (Figure 4.2). Similarly, the concentration of heptadecylcaffeic acid differed significantly (ANOVA, P≤ 0.003; F=4.39 and df= 9,18) among the varieties.

The concentration of hexadecylcoumaric acid (**2**) was highest on the root surface of New Kawogo and ARA228 (Figure 4.2). The highest concentration of heptadecylcaffeic acid (**3**) was recorded by New Kawogo compared to the other varieties. Additionally, the highest and lowest concentrations of octadecylcaffeic acid ester (**4**) were recorded from New Kawogo and Kakamega, respectively. Furthermore, New Kawogo and Kakamega had the highest and lowest octadecylcoumaric acid esters (**5**) on the root surface compared to all the varieties (Figure 4.2). In contrast, ARA228 and Kakamega had the highest and lowest concentration of 5-O-caffeoylquinic acid ester (**6**) on its root surface, respectively (Figure 4.2). Overall the two resistant varieties from Chapter 3, ARA228 and New Kawogo, had the highest total concentration of hydroxycinnamic acid esters on the root surface while the susceptible varieties NASPOT 1, Tanzania and Kagamega had the lowest concentrations.

4.3.2. Hydroxycinnamic acid esters in the root periderm extract of different sweetpotato varieties.

There were significant differences in concentrations of hexadecylcaffeic acid (**1**), hexadecylcoumaric acid (**2**), heptadecylcaffeic acid (**3**), octadecylcaffeic acid (**4**), octadecylcoumaric acid (**5**) and 5-O-caffeoylquinic acid (**6**) in the root periderm extracts of the selected sweetpotato varieties (ANOVA, P≤ 0.001, F=3.59 and

df=9,18; ANOVA, P≤ 0.01, F=3.61 and df= 9,18; ANOVA, P≤ 0.001, F=2.62 and df=9,18; ANOVA, P≤ 0.001, F=5.39 and df=9,18; ANOVA, P≤ 0.001, F= 4.28 and df=9,18; ANOVA, P≤ 0.001, F=2.68 and df=9,18, respectively).

The concentration of hexadecylcaffeic acid (**1**) was highest in the periderm of New Kawogo compared to other varieties. However, the lowest concentration was recorded in ARA230 and NASPOT 1, respectively (Figure 4.3). The concentration of hexadecylcoumaric acid ester (**2**) was lowest in the root periderm of NASPOT1 (Figure 4.3). Similarly, the resistant variety New Kawogo had the highest concentration of 5-O-caffeoylquinic acid (**6**) compared to the other varieties. 5-O-caffeoylquinic acid (**6**) was lowest in NASPOT 1 and ARA230 (Figure 4.3). In contrast, octadecylcaffeic acid (**4**) concentration in the root periderm was highest on New Kawogo followed by ARA228 and lowest on NASPOT1, respectively (Figure 4.3). The concentration of octadecylcoumaric (**5**) acid was highest in RAK865 followed by New Kawogo and was lowest in the susceptible variety NASPOT 1 (Figure 4.3).

Figure 4.3 Mean concentration of hydroxycinnamic acid esters on the root peridermal tissues of selected sweetpotato varieties.

Abbreviations: C16 Caffeic = hexadecylcaffeic acid (**1**); C16 Coumaric = hexadecylcoumaric acid (**2**); C17 Caffeic = heptadecylcaffeic acid (**3**); C18 Caffeic = octadecylcaffeic acid (**4**); C18 Coumaric =octadecylcoumaric acid (**5**); CQA= 5-O-caffeoylquinic acid (**6**).

4.3.3. Hydroxycinnamic acid esters in the epidermal extracts of different sweetpotato varieties

There was a significant difference in the concentrations of hexadecylcaffeic acid (**1**), heptadecylcaffeic acid (**3**), octadecylcaffeic acid (**4**) and octadecylcoumaric acid (**5**) respectively in the epidermal extracts of the selected sweetpotato varieties (ANOVA, P≤ 0.01, F= 18.46and df = 9,18; ANOVA, P≤ 0.001, F= 18.46 and df=9,18;

ANOVA, P≤ 0.001, F= 89.41 and df=9,18; and ANOVA, P≤ 0.001, F= 23.28 and df=9,18, respectively). Hexadecylcoumaric acid (**1**) and 5-O-caffeoylquinic acid (**6**), however, did not differ significantly in the epidermal extracts of the selected varieties respectively (ANOVA, P≥0.05, F= 1.99 and df=9,18 and ANOVA, P≥0.05, F= 2.33 and df= 9,18, respectively).

New Kawogo had the highest concentration of hexadecylcaffeic acid ester (**1**) compared to other varieties (Figure 4.4). The concentration of heptadecylcaffeic (**3**) acid ester was highest in New Kawogo root epidermal extracts followed by ARA228. Hexadecylcoumaric acid ester (**2**) was lower in the root epidermis similar to that which was found in the periderm (Figure 4.4). In contrast, the concentration of octadecylcaffeic acid (**4**) was highest in ARA228 followed by LIR302 and lowest in Tanzania, respectively (Figure 4.4). The highest and lowest concentration of octadecylcoumaric acid (**5**) from the epidermis and roots was recorded from LIR302 and ARA230 and RAK865, respectively (Figure 4.4). The concentration of 5-O-caffeoylquinic acid ester (**6**) was highest in the epidermal extracts of Tanzania compared to the rest of the varieties. On the other hand, ARA230, Kakamega and NASPOT 1 had least 5-O-caffeoylquinic acid ester (**6**) on its root epidermal tissues (Figure 4.4).
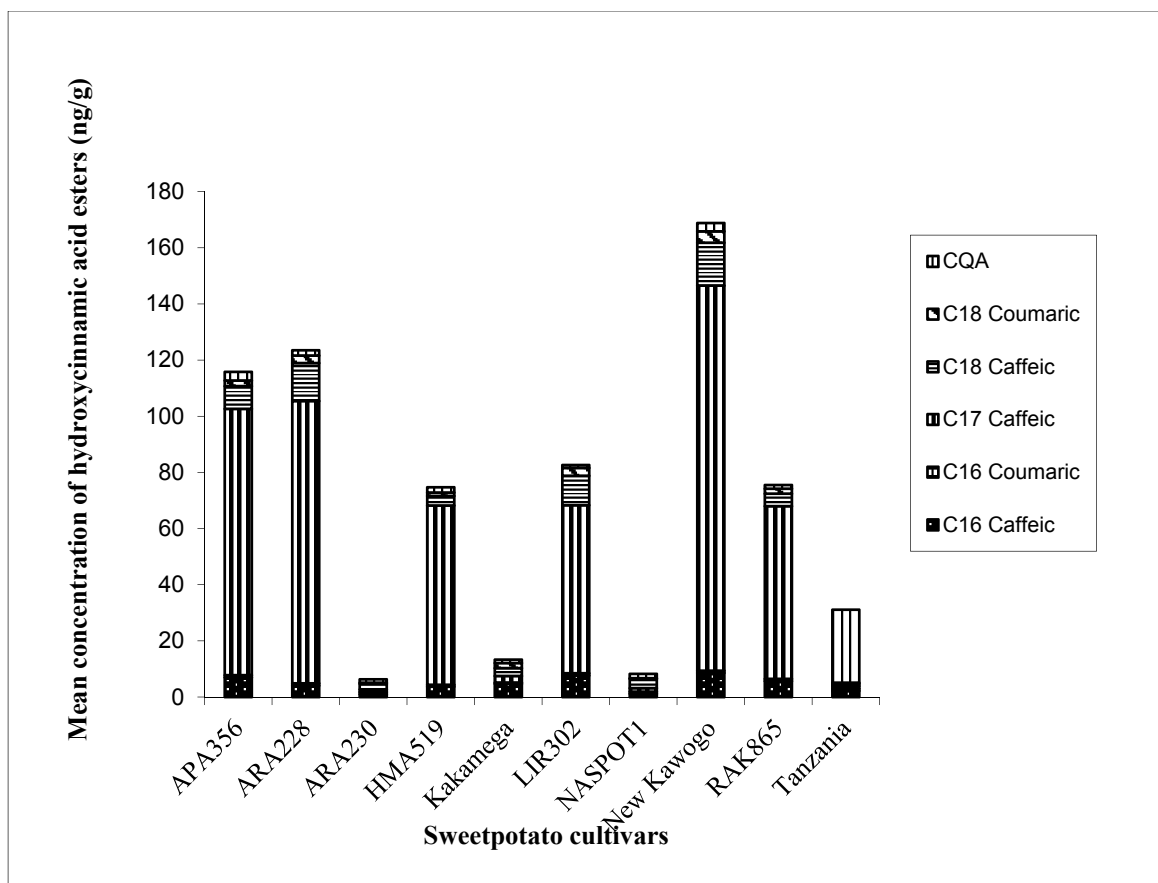
Figure 4.4 Mean concentration of hydroxycinnamic acid esters in the root epidermal tissues of selected sweetpotato varieties.

Abbreviations: C16 Caffeic = hexadecylcaffeic acid (**1**); C16 Coumaric = hexadecylcoumaric acid (**2**); C17 Caffeic = heptadecylcaffeic acid (**3**); C18 Caffeic = octadecylcaffeic acid (**4**); C18 Coumaric =octadecylcoumaric acid (**5**); CQA= 5-O-caffeoylquinic acid (**6**).

4.3.4. Hydroxycinnamic acid esters in the root latex of sweetpotato varieties

Hydroxycinnamic acid esters were also identified in the root latex of the selected sweetpotato varieties. There was a significant difference (ANOVA, P≤ 0.05, F= 5.36; df= 5,12 and ANOVA, P≤0.05, F= 4.48; df= 5,12 respectively) in the concentration of hexadecylcaffeic acid (**1**) and octadecylcaffeic acid (4) in the root latex of the selected sweetpotato varieties. However, there was no significant difference (ANOVA, P≥0.05, F= 3.38; df= 5,12; ANOVA, P≥0.05, F= 1.88; df= 5,12 and

ANOVA, P≥0.05, F= 2.14; df= 5,12) in the concentration of hexadecylcoumaric acid (**2**), octadecylcoumaric acid (**5**) and 5-O-caffeoylquinic acid (**6**), respectively. New Kawogo and APA356 had the highest and lowest concentration of hexadecylcaffeic acid (**1**) and octadecylcaffeic acid (**4**), respectively (Figure 4.5).
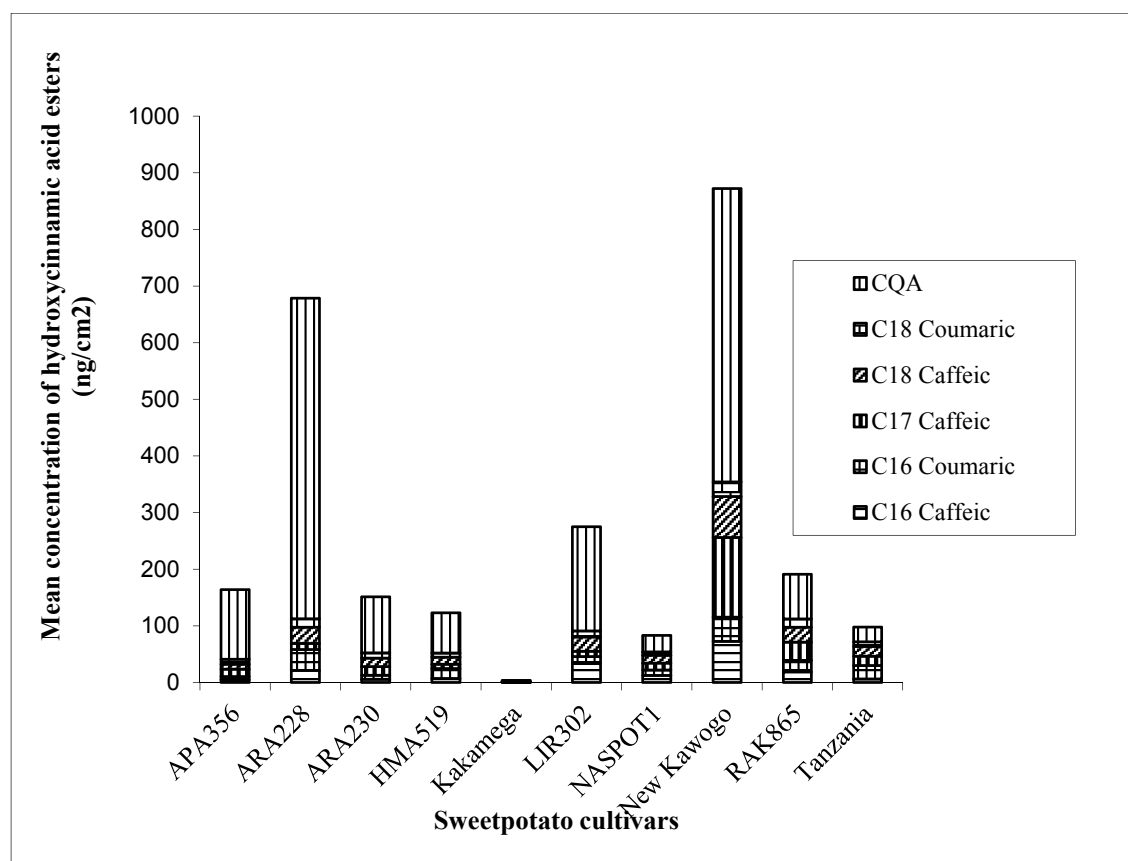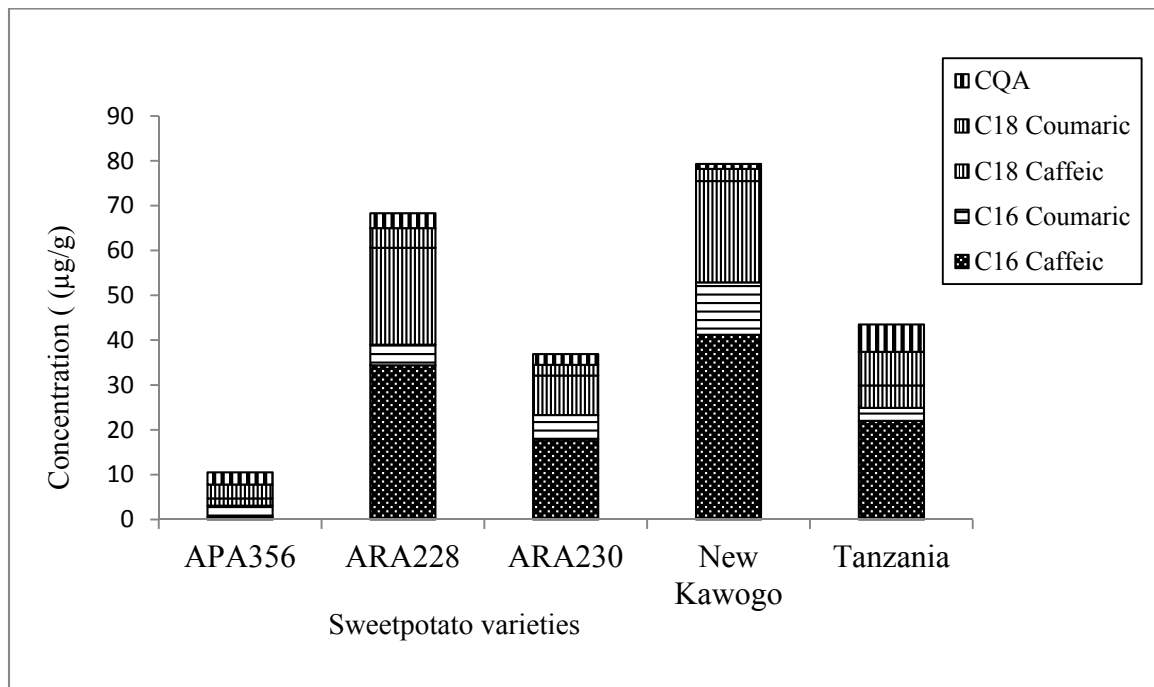

Figure 4.5 Mean concentration of hydroxycinnamic acid esters in the root latex of sweetpotato varieties.

Abbreviations: C16 Caffeic = hexadecylcaffeic acid (**1**); C16 Coumaric = hexadecylcoumaric acid (**2**); C17 Caffeic = heptadecylcaffeic acid (**3**); C18 Caffeic = octadecylcaffeic acid (**4**); C18 Coumaric =octadecylcoumaric acid (**5**); CQA= 5-O-caffeoylquinic acid (**6**).

4.3.5. Hydroxycinnamic acid esters in the leaf latex of different sweetpotato varieties.

There was a significant difference (ANOVA, P ≤ 0.001, F=5.36; df=5,12 and ANOVA, P ≤ 0.05, F=2.99; df=5,12) in the concentration of hexadecylcaffeic acid

(**1**) and octadecylcaffeic (**4**) acid in the leaf latex of some sweetpotato varieties. However, there was no significant difference (P>0.05) in the concentration of hexadecylcoumaric acid (**2**), octadecylcoumaric acid (**5**) and 5-*O*-caffeoylquinic acid (**6**) among these varieties suggesting that the concentration of these compounds in latex may have been less significant than previously suggested (Stevenson *et al*, 2009).

New Kawogo and RAK865 had the highest and lowest concentrations of hexadecylcaffeic acid (**1**) and octadecylcaffeic acid (**4**), respectively (Figure 4.6). The concentration of 5-O-caffeoylquinic acid (**6**) was highest in the susceptible variety suggesting this compound does not play a significant role in resistance when it occurs in the latex.
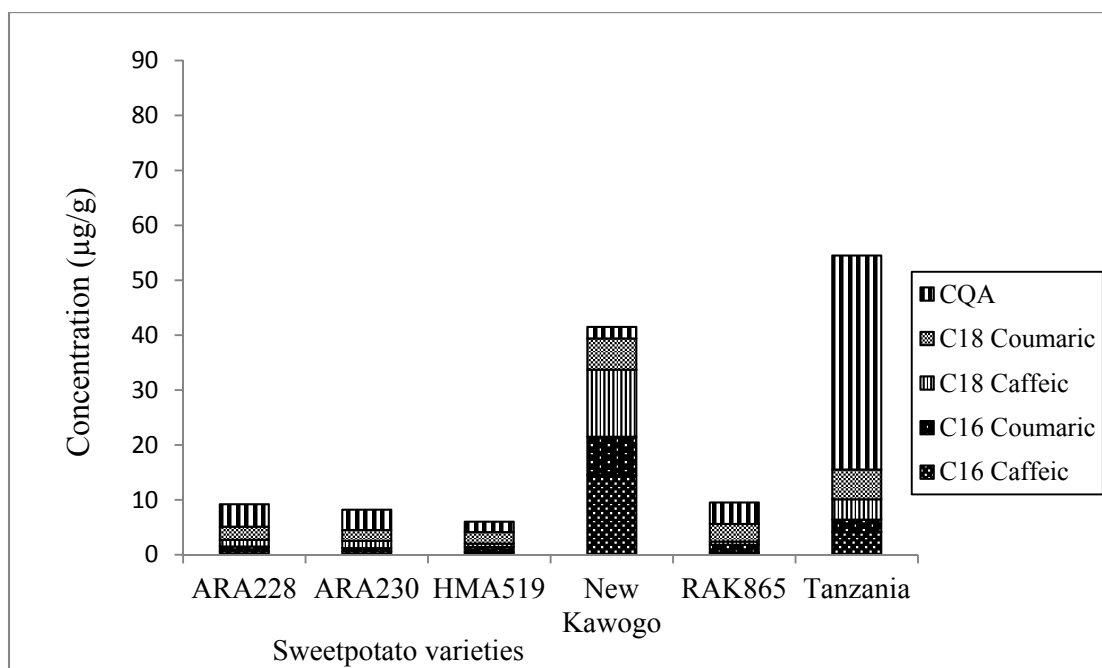
Figure 4.6 Mean concentration of hydroxycinnamic acid esters in the leaf latex of sweetpotato varieties.

Abbreviations: C16 Caffeic = hexadecylcaffeic acid (**1**); C16 Coumaric = hexadecylcoumaric acid (**2**); C17 Caffeic = heptadecylcaffeic acid (**3**); C18 Caffeic = octadecylcaffeic acid (**4**); C18 Coumaric =octadecylcoumaric acid (**5**); CQA= 5-O-caffeoylquinic acid (**6**).

4.3.6. Hydroxycinnamic acid esters in peeled whole root of different sweetpotato varieties

Hydroxycinnamic acid esters were characterized and identified in the peeled whole root flesh of different sweetpotato varieties that includes the cortex. There was a significant difference in the concentration of octadecylcaffeic acid (**4**) (ANOVA, $P \leq 0.001$, F= 4.8; df= 5,12) in the peeled whole root of selected sweetpotato varieties but the lower concentrations were recorded in the resistant varieties. However, there was no significant difference ($P > 0.05$) in the concentration of hexadecylcaffeic acid (**1**), hexadecylcoumaric acid (**2**), octadecylcoumaric acid (**5**) and 5-O-caffeoylquinic acid (**6**) in the peeled whole root of different varieties. The highest and lowest concentrations of octadecylcaffeic acid (**4**) in the peeled whole root were recorded from Tanzania and LIR302, respectively (Figure 4.7).
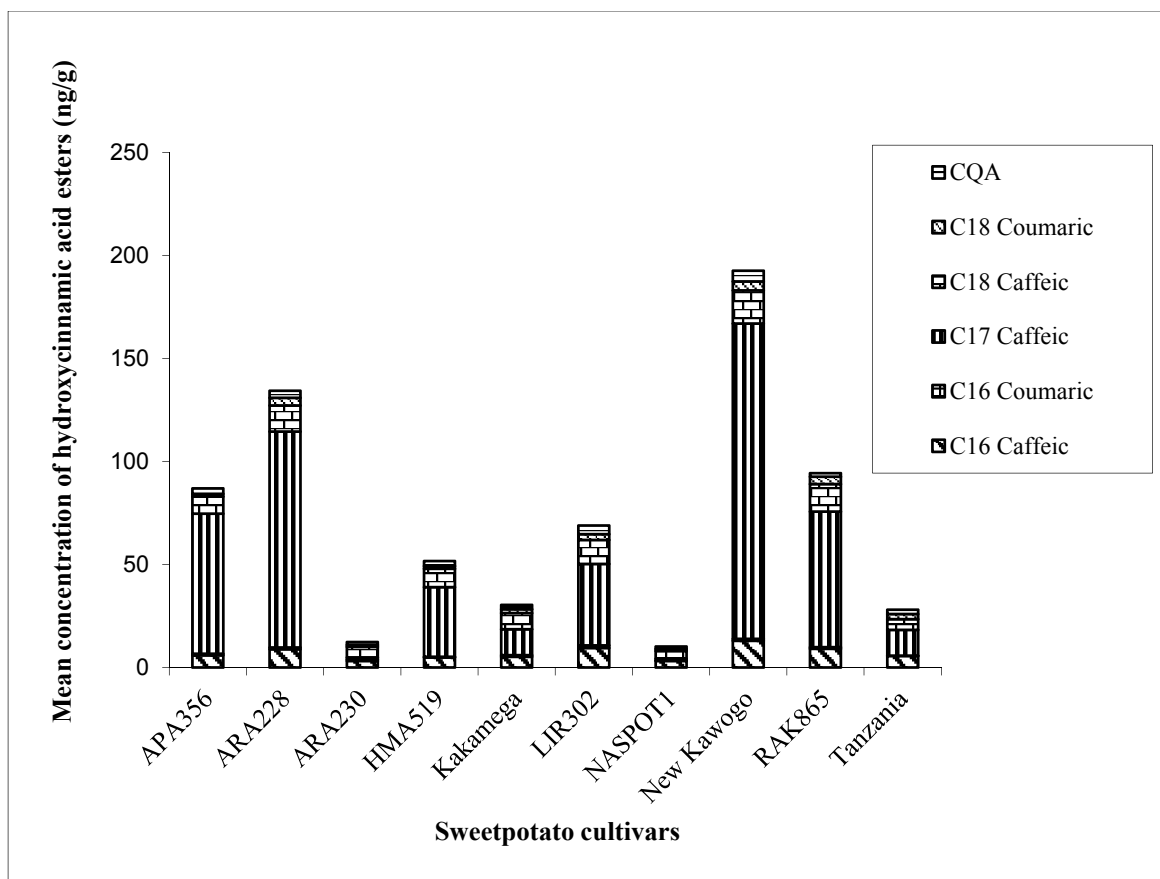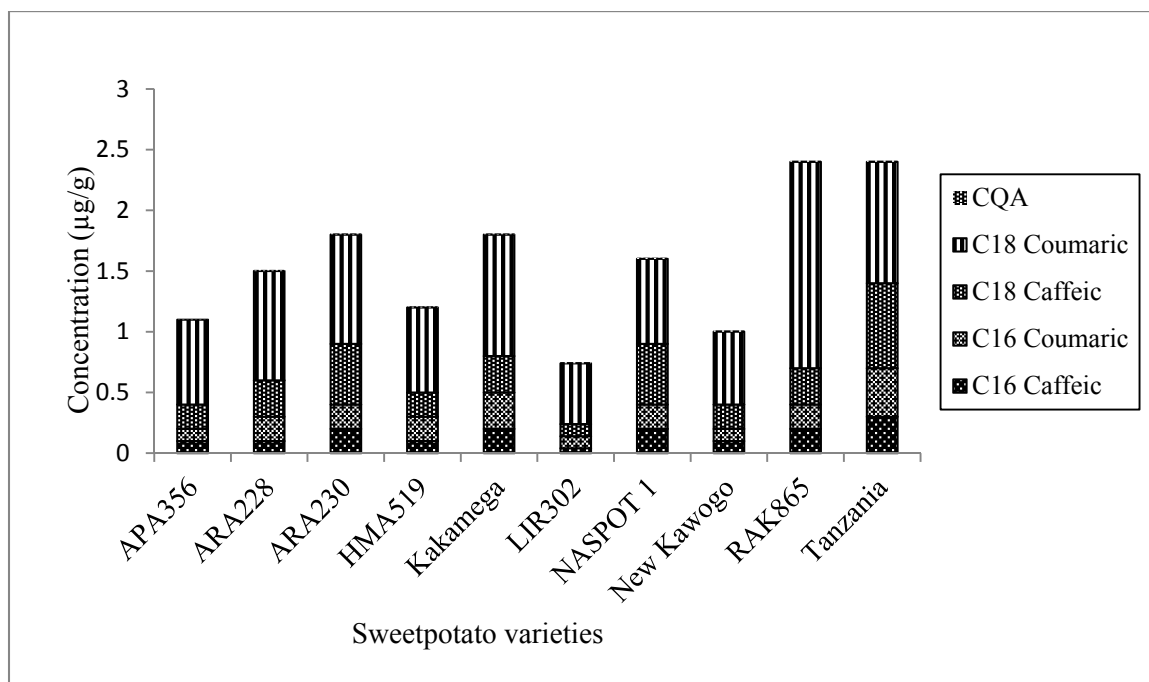
Figure 4.7 Mean concentration of hydroxycinnamic acid esters in the root flesh of sweetpotato varieties.

Abbreviations: C16 Caffeic = hexadecylcaffeic acid (**1**); C16 Coumaric = hexadecylcoumaric acid (**2**); C17 Caffeic = heptadecylcaffeic acid (**3**); C18 Caffeic = octadecylcaffeic acid (**4**); C18 Coumaric =octadecylcoumaric acid (**5**); CQA= 5-O-caffeoylquinic acid (**6**).

4.4. DISCUSSION

The analyses described in this chapter were conducted to determine whether the chemistry of sweetpotato varieties and landraces with varying susceptibility to sweetpotato weevils differed. Six hydroxycinnamic acid esters including hexadecylcaffeic acid (**1**), hexadecylcoumaric acid (**2**), heptadecylcaffeic acid (**3**), octadecylcaffeic acid (**4**), octadecylcoumaric acid (**5**) and 5-O-caffeoylquinic acid (**6**) were identified. The concentrations of these hydroxycinnamic acid esters differed significantly on the root surface, periderm and epidermal tissues but not in the whole peeled root flesh and only for some compounds in the latex. In both the root and

leaf latex only the concentrations of hexadecylcaffeic (**1**) and octadecylcaffeic acid (**4**) differed significantly whereas the concentrations of hexadecylcoumaric acid (**1**), octadecylcoumaric acid (**5**) and 5-O-caffeoylquinic acid (**6**) did not differ significantly. Heptadecylcaffeic acid (**3**) was not identified in the latex extracts from the root and leaves and peeled whole root. Only octadecylcaffeic acid (**4**) concentration differed significantly in the peeled whole root flesh of the selected varieties with the highest concentration occurring in RAK 865 and Tanzania a susceptible variety. The concentration of caffeoyl and coumaroyl acid esters was highest in the cortex of susceptible variety suggesting the occurrence of these compounds in the cortex do not play a significant role in resistance despite previous suggestions about the importance of caffeic acid in the cortex of sweetpotato (Harrison *et al*., 2003; Stevenson *et al*. 2009)

The difference in the concentration of these compounds near the root surface, however, was strongly associated with the varietal resistance and susceptibility to sweetpotato weevils occurring in the field. The susceptible checks including Tanzania, NASPOT1, Kakamega and ARA230 had low concentrations of these hydroxycinnamic acid esters on the root surface, peridermal, epidermal tissues and root and leaf latex. Earlier work by Stevenson *et al*. (2009) showed that the concentration and diversity of compounds in sweetpotato latex in New Kawogo differed from those in Tanzania. These results therefore supported earlier findings that chemical differences in latex could mediate resistance to sweetpotato weevils (Data *et al*., 1996) or at least there occurrence in latex could mediate their occurrence in the root surface tissue. The present study has also shown that the differences in latex were not as high as in the surface tissues indicating that resistance may be most emphatically expressed at the surface and low

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concentrations of these compounds on the root surface, periderm and epidermis may explain the susceptibility of these varieties to sweetpotato weevils. This is particularly poignant since the primary point of interaction between fecund females and sweetpotato is the root surface, periderm and epidermis because the insects make their feeding holes in these tissues where the eggs are laid. The results indicated that the concentration of hexadecylcaffeic acid (**1**), hexadecylcoumaric acid (**2**) and octadecylcaffeic (**4**) and heptadecylcaffeic acid (**3**) and 5-O-caffeoylquinic acid (**6**) occurred in higher amount on the root surface, peridermal and epidermal tissues of the two resistant varieties New Kawogo and ARA228 while the lowest concentrations were recorded in the most susceptible varieties. This finding therefore could support the link between resistance and concentration of hydroxycinnamic acid esters. New Kawogo, LIR302 and ARA228 were found to be resistant to sweetpotato weevil infestation and damage compared to Tanzania, NASPOT1, Kakamega and ARA230 which were shown to be susceptible under field conditions (Chapter 3).

According to Muyinza *et al*. (2012) and Stevenson *et al*. (2009), hydroxycinnamic acid esters especially hexadecyl and octadecyl-ρ-coumaric and caffeic acids are biologically active against sweetpotato weevil larvae. However, we show here that the overall concentrations of compounds between cortex tissue the principal food of the larvae did not differ greatly so perhaps the significance of earlier findings are less significant in terms of explaining how sweetpotato mediate resistance. The occurrence of these compounds in this study therefore at higher concentration in the root surface tissues of resistant varieties suggests that they may be active against adults too and that this activity is more likely to differ between the varieties based on qualitative differences in chemistry and so may account for the resistance

reported in earlier studies and in Chapter 3. This results correlate with the study reported by Son *et al*. (1991a) of the significant differences in the levels of caffeic acid and 5-O-caffeoylquinic acid (**6**) reported among sweetpotato cultivars expressing varying levels of resistance to sweetpotato weevils. In contrast, this study 5-O-caffeoylquinic acid (**6**) was not present at higher concentrations in the resistant variety. The potency of these compounds may need to be tested in the feeding and oviposition bioassay to ascertain its effect in the next chapter.

The concentration of hydroxycinnamic acids esters in the latex was similar across the cultivars despite earlier reports of differences between resistant and susceptible varieties (Stevenson et al., 2009) but in the present study the concentration was higher in the periderm of the resistant varieties. The difference in the levels of hydroxycinnamic acids was earlier reported to affect the levels of acceptability of sweetpotato varieties by *C. formicarius* (Wilson *et al*., 1990; Snook *et al*., 1994). The low concentration of these compounds in the whole root flesh suggests that once the weevil lays eggs successfully, the larvae can develop in the deeper tissues of the roots without any problem.

The presence of compounds that have toxicity against an invertebrate in the part of the plant that is consumed may set alarm bells ringing among some about the possibility that these compounds might also have some toxic effects against consumers. According to Clifford (2003), phenylpropanoids and their derivatives like those reported in this study are ubiquitous in plants and are unlikely to have adverse impacts on consumers anyway, but the fact that the concentrations of these compounds were similar in the root flesh of both the susceptible variety Tanzania and the resistant varieties suggests there is little difference in the main consumed component among the varieties. Accordingly, resistant varieties will present no

greater potential issues for consumers than those already widely grown and consumed.

Recent evidence suggests that these compounds may also act by other mechanisms in addition to the antioxidant capacity as modulating the activity of some specific enzymes and inhibiting cancer cell growth (Manach, 2004). In general, hydroxycinnamic acids have been consistently associated with reduced risk of cardiovascular disease, cancer and other chronic diseases in humans (Spencer *et al*., 2008). This knowledge together with the information about the overall concentrations in the roots of different varieties might help alleviate any concerns that hydroxycinnamic acids in sweetpotato as a mechanisms of resistance are harmful to human consumers.

CHAPTER 5

EFFECTS OF SYNTHETIC HYDROXYCINNAMIC ACID ESTERS ON BEHAVIOUR OF SWEETPOTATO WEEVILS

5.1. INTRODUCTION

Plants produce a variety of chemical defenses that inhibit feeding by herbivorous organisms or are toxic to them (Schafer *et al*., 2009). Plant chemicals can have insecticidal and ovicidal activity or developmental- and behavior-modifying effects such as repellence, antifeedancy, growth regulation, oviposition deterrents and delayed hatching among others. These properties can be used for Integrated Pest Management (IPM) (Bernays and Chapman, 1994) if enhanced in crops that are otherwise susceptible to specific insect pests. Therefore, understanding the mechanisms of host plant insect interactions would be important for the development of environmentally benign crop protection approach (Akhtar *et al*., 2008). In Chapter 4 of this thesis, it was found that hydroxycinnamic acid esters including hexadecyl and octadecyl-*p*-coumaric and caffeic acid esters and 5-O-caffeoylquinic acid occurred in greater quantities on the root surface and in the peridermal and epidermal tissues of New Kawogo and other resistant sweetpotato varieties resistant to weevils than on the susceptible varieties Tanzania, NASPOT 1, Kakamega and ARA230. According to Stevenson *et al*. (2009), these compounds were found to increase the mortality of weevil larvae but their effect against the most important life stage in terms of pest control, the gravid female, was not tested.

Therefore, the objective of the study reported in this Chapter was to assess the effects of hydroxycinnamic acids on feeding and oviposition by weevils.

5.2.   MATERIALS AND METHODS

5.2.1. Bioassay procedures

Roots of the susceptible sweetpotato variety NASPOT1 were used for the experiment as the testing medium for plant compounds. Root cores were obtained by cutting sweetpotato roots using a 24 mm diameter cork borer No. 15 as described in Chapter 3. The root cores were cut to approximately 10 mm deep to fit precisely into the wells of a- 12 well assay plate presenting only the root surface to the weevils as described by Stevenson *et al*. (2009). The root core was used as a medium for testing compounds. Four of the six hydroxycinnamic acid esters: octadecylcaffeic (**4**) and coumaric (**5**) acids, heptadecylcaffeic acid (**3**) and 5-O-caffeoylquinic acid (**6**) that were initially identified on sweetpotato root surface were tested. Heptadecylcaffeic acid (**3**) had the highest concentration on the periderm and epidermis but its effect was not previously tested. The effect on feeding and oviposition was tested to relate with its occurrence on the root surface.

Twelve root cores of the susceptible variety NASPOT1 placed in a 12-well tray were treated with three different concentrations of octadecylcaffeic (**4**) and coumaric acid (**5**), heptadecylcaffeic acid (**3**) and 5-O-caffeoylquinic acid (**6**) synthesised by Professor David Hall. Approximately one milligram (mg) of the pure compounds was weighed and dissolved in 1mL of acetone as a carrier solvent to obtain a concentration of 1 mg/ml. This concentration was serially diluted to 0.10, 0.01, and

0.001 ng/ µL to correlate with natural concentrations found on the sweetpotato root surface and control (0.0 ng/ µL). To relate this concentration to the natural concentrations on the root surface, the diameter of the root core was measured and the surface area was determined by using the formula for deriving the area of a circle. The compounds were applied using pipette as a 100, 10, 1 µL aliquot, and these concentrations represented 10, 1 , 0.1, and 0 ng/cm^2 on the surface of the root cores of a susceptible variety NASPOT 1 from calculation of the 24mm diameter root core. Compounds were applied in acetone on to the root surface 3 hours before weevils were introduced to allow the solvent to evaporate and leave the compound on the root core surface.

Both *Cylas puncticollis* and *C. brunneus* weevils used in this experiment were reared in the laboratory on NASPOT1 sweetpotato storage roots in cages held at approximately 28°C and 70% Relative Humidity as described in Chapter 2 of this thesis. On each root core cut with a cork borer (no. 15) and placed in a 12 well feeding trays, 2 adult (2-weeks-old) gravid females of both weevil species were introduced 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 the periderms, using clay into which tiny holes were made to allow air circulation in a similar way as shown in Plate 3.3. The experiment was set up in a completely randomized design. Each experiment setup was replicated 5 times and the whole experiment repeated 3 times. The weevils were allowed to feed and oviposit for 24 h.

5.2.2. Data collection and analysis

The number of feeding holes and faecal droppings produced in 24 h were recorded. The root periderm was gently removed with a scalpel and the number of eggs observed using magnifying glass and counted. Data obtained was analysed using one way analysis of variance (ANOVA) linear model in R-statistics. Where there were differences, means were separated using Least Significant Difference at 5%.

5.3. RESULTS

5.3.1. Effects of hydroxycinnamic acids treatment on the number of feeding holes produced by adults of *Cylas puncticollis* and *C. brunneus*

Effects of octadecylcaffeic acid treatment on C. puncticollis and C. brunneus feeding holes.

The mean number of *C. puncticollis* and *C. brunneus* feeding holes was significantly different among the root cores treated with octadecyl caffeic acid (ANOVA, $P \leq 0.001$, F= 9.29, df= 3,8 and ANOVA, $P \leq 0.001$, F=8.42, df= 3,8 respectively) (Figure 5.1). Overall, the mean number of *C. puncticollis* and *C. brunneus* feeding holes decreased gradually with an increase in the concentration with fewer feeding holes on root cores treated with 0.1 ng/µL octadecyl caffeic acid (Figure 5.1). The mean number of feeding holes was significantly different between control and treated root cores. The difference in the number of feeding holes between root cores treated with 0.01 ng/µL and 0.1 ng/µL did not differ implying they were all not suitable for feeding by *C. puncticollis* (Figure 5.1). The mean number of *C. brunneus* feeding

holes, however, differed significantly between control and treated root cores and within the treatment levels applied on the root cores (Figure 5.1).

Figure 5.1. Mean number of feeding holes produced by *C. puncticollis* and *C. brunneus* on sweetpotato root plugs treated with octadecylcaffeic acid

Cp: *C. puncticollis* and Cb: *C. brunneus,* N=15, ±SEM.

Effects of octadecylcoumaric acid treatment on C. puncticollis and C. brunneus feeding holes.

The mean number of *C. puncticollis* feeding holes differed significantly (ANOVA, $P \leq 0.001$, F= 30.47 and df= 3,8) among the treatments. Mean number of *C. puncticollis* feeding holes was significantly higher ($P \leq 0.05$) on the control compare to those treated with octadecyl coumaric acid. The mean number of *C. puncticollis* feeding holes decreased as the concentration of octadecyl caffeic acid applied on

the root surface increased and it differed significantly between control and within the treatment (Figure 5.2).

The mean number of *C. brunneus* feeding holes was significantly different among treatments (ANOVA, P≤0.001, F= 16.22 and df= 3,8) on root surface treated with octadecyl coumaric acid. Similarly to *C. puncticollis*, the mean number of *C. brunneus* was significantly higher (P≤0.05) on the control root than the mean number on the roots treated with 0.01 and 0.1 ng/μL octadecylcoumaric acid (Figure 5.2).
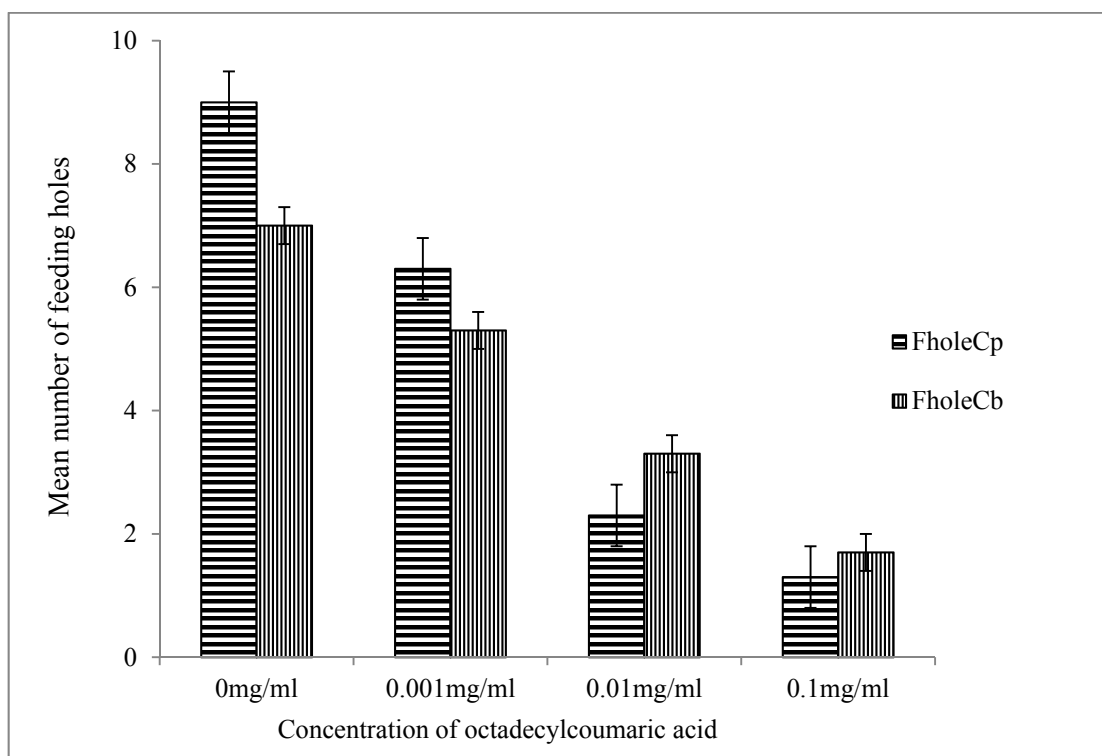
Figure 5.2 Mean number of feeding holes produced by *C. puncticollis* and *C. brunneus* on sweetpotato root plugs treated with octadecyl coumaric acid, N=15, SEM.

Effects of heptadecylcaffeic acid treatment on C. puncticollis and C. brunneus feeding holes.

There was significant difference (ANOVA, P≤0.001, F= 16.16, df= 3,8) in the mean number of feeding holes caused by *C. puncticollis* and *C. brunneus* on the sweetpotato root core treated with heptadecylcaffeic acid, respectively. The mean number of feeding hole of both weevil species decreased significantly with increasing levels of hexadecylcaffeic acid concentrations with highest mean number recorded on the control root plug (Figure 5.3). The mean number of *C. puncticollis* and *C. brunneus* were similar at 0.1 ng/μL concentration of heptadecylcaffeic acid applied on the root core (Figure 5.3).

Figure 5.3. Mean number of feeding holes produced by *C. puncticollis* and *C. brunneus* on sweetpotato root plugs treated with heptadecylcaffeic acid.

Cp: *C. puncticollis*, Cb: *C. brunneus,* Fhole: feeding hole, N=15, ±SEM.

Effects of 5-O-caffeoylquinic acids treatment on C. puncticollis and C. brunneus feeding holes

The mean number of *C. puncticollis* feeding holes differed significantly (ANOVA, P≤0.001,F= 14.27, df= 3,8) between treated root cores and control. The mean number of feeding holes was significantly (P≤0.05) higher on untreated than on the 5-O-caffeoylquinic acid treated sweetpotato root cores (Figure 5.4). The number of feeding holes was lower for *C. brunneus* than on the roots where *C. puncticollis* were placed to feed suggesting that the two species were differentially sensitive to any effect of this compound (Figure 5.4).

Figure 5.4: Mean number of feeding holes produced by *C. puncticollis* and *C. brunneus* on sweetpotato root plugs treated with octadecylcaffeic acid.

 N=15, SEM. Feeding holes for both *C. puncticollis* and *C. brunneus* was significantly different between control and treated root cores and not among treatments.

5.3.2. Effects of hydroxycinnamic acids treatment on *Cylas puncticollis* and *C. brunneus* faecal droppings

Biological activity of octadecylcaffeic acids from sweetpotato on production C. puncticollis and C. brunneus faecal droppings

The mean number of *C. puncticollis* faecal droppings recorded on sweetpotato root cores treated with octadecylcaffeic acid was significantly different (ANOVA, $P \leq 0.001$, F= 3.09 and df= 3,8). Similarly, the mean number of faecal droppings of *C. brunneus on* the root cores treated with octadecylcaffeic acid was significantly different (ANOVA, $P \leq 0.001$, F=9.29 and df= 3,8). The mean number of *C. puncticollis* faecal droppings was higher on the control root compared to those treated with 0.1ng/μL of octadecylcaffeic acid but not significantly different (Figure 5.5). The mean number of *C. brunneus* faecal droppings was higher and significantly different from control and within treatments (Figure 5.5).

Figure 5.5. Mean number of faecal droppings produced by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with octadecylcaffeic acid

Effects of octadecylcoumaric acid treatment on production of C. puncticollis and C.

brunneus faecal droppings.

The mean number of *C. puncticollis* faecal droppings differed significantly (ANOVA,

P≤0.001, F=30.47 and df= 3,8) between treatments and control. Similarly, *C.*

brunneus faecal droppings on the root surface treated with octadecylcoumaric acid

differed significantly (ANOVA, P≤0.001, F= 10.5 and df=14) among the treatments.

The mean number of *C. puncticollis* and *C. brunneus* faecal droppings on the control

root was significantly higher (P≤0.05) than on any treated roots (Figure 5.6). The

mean number of faecal droppings of both weevil species was higher on control root

core and lower on root core treated with 0.1 ng/μL octadecylcoumaric acid (Figure 5.6) and the effect was dose dependent.
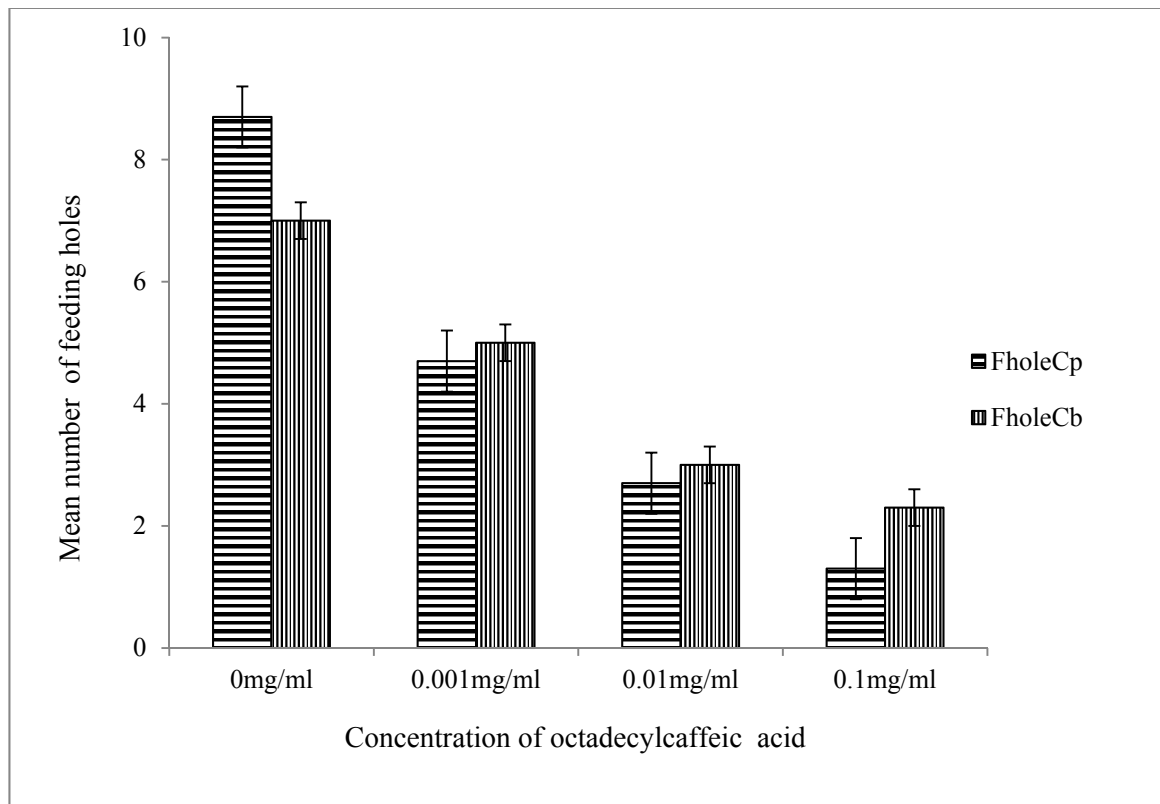

Figure 5.6. Mean number of faecal droppings produced by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with octadecyl coumaric acid.

Effects of heptadecylcaffeic acid treatment on C. puncticollis and C. brunneus faecal droppings.

The number of *C. puncticollis* and *C. brunneus* faecal droppings on the surface of sweetpotato root core treated with heptadecylcaffeic acid was only significantly different (ANOVA, $P \leq 0.05$, F=0.61, df= 3,8) for *C. brunneus* at high concentration (0.1 ng/μL) from the control treatment. The number of *C. puncticollis* faecal droppings was lowest on the root treated with 0.1 ng/μL heptadecylcaffeic acid (Figure 5.7).

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The number of faecal droppings produced by either *C. puncticollis* or *C. brunneus* decreased with increasing concentration of heptadecylcaffeic acid treatment (Figure 5.7).
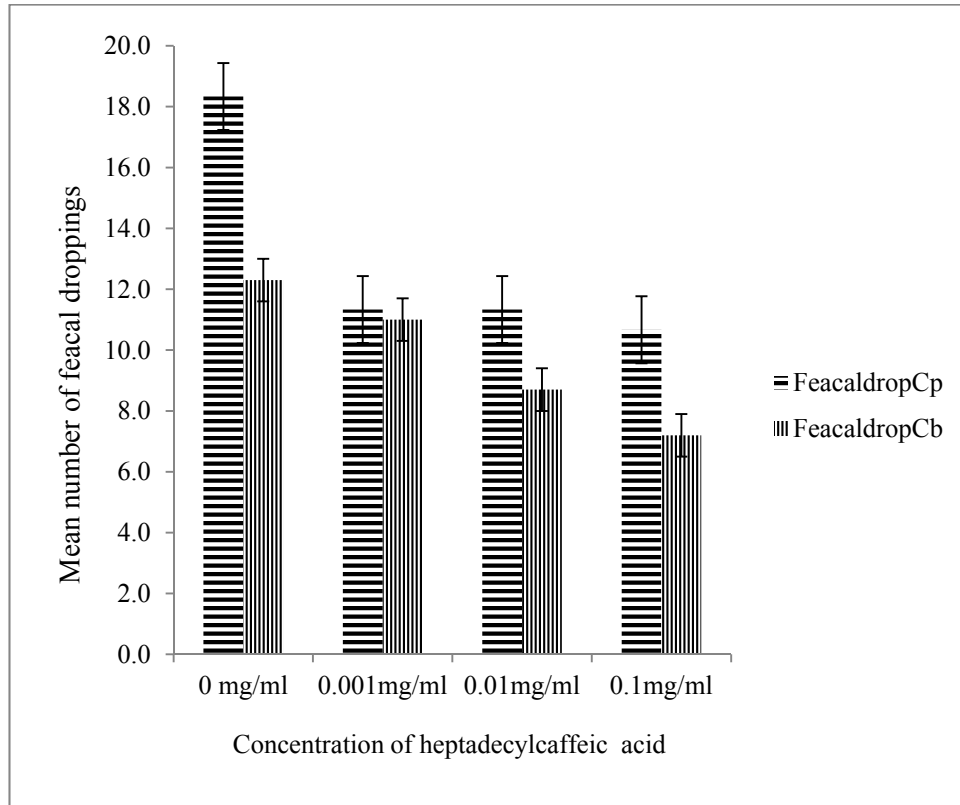
Figure 5.7. Mean number of faecal droppings produced by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with heptadecylcaffeic acid.

Cp: *C. puncticollis*, Cb: *C. brunneus,* N=15, ±SEM.

Biological activity of 5-O-caffeoylquinic acid treatment on production of C. puncticollis and C. brunneus faecal droppings

There was a significant difference (ANOVA, P≤0.019, F= 6.06 and df= 3,8) in the mean number of *C. puncticollis* faecal droppings among treatments. Conversely, the number of *C. brunneus* faecal droppings was not significantly different (ANOVA, P≤0.626, F=0.93, df= 14) among the treatments. Mean number of faecal droppings

was significantly (P≤0.05) higher on the control than on the root surface treated with 5-O-caffeoylquinic acid (Figure 5.8). There were no faecal droppings produced by *C. brunneus* on 0.1 ng/μL 5-O-caffeoylquinic acid.

Figure 5.8. Mean number of faecal droppings produced by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with 5-O-caffeoylquinic acid.

N=15,± SEM.

5.3.3. Biological activity of hydroxycinnamic acids at different concentrations on oviposition by *C. puncticollis* and *C. brunneus*

Effects of octadecylcaffeic acid on C. puncticollis and C. brunneus oviposition

C. puncticollis egg laying was significantly (ANOVA, P≤0.023, F=8.54, df= 3,8) affected by octadecylcaffeic acid treatment. Similarly, there was a significant difference (ANOVA, P≤0.05, F=9.29; df= 3,8) in the number of *C. brunneus* eggs laid on the sweetpotato root core treated with octadecylcaffeic acid. The mean number of *C. puncticollis* eggs laid on the control root was significantly higher than on the root core treated with different concentrations of octadecylcaffeic acid *C. puncticollis* did not lay eggs at 0.01 and 0.1 ng/μL respectively (Figure 5.9). The mean number of eggs laid by *C. brunneus* was significantly lower with increasing levels of octadecylcaffeic acid dose applied on the root core (Figure 5.9). The root core treated with 0.1ng/μL of octadecylcaffeic acid prevented *C. brunneus* females from laying eggs completely (Figure 5.9).
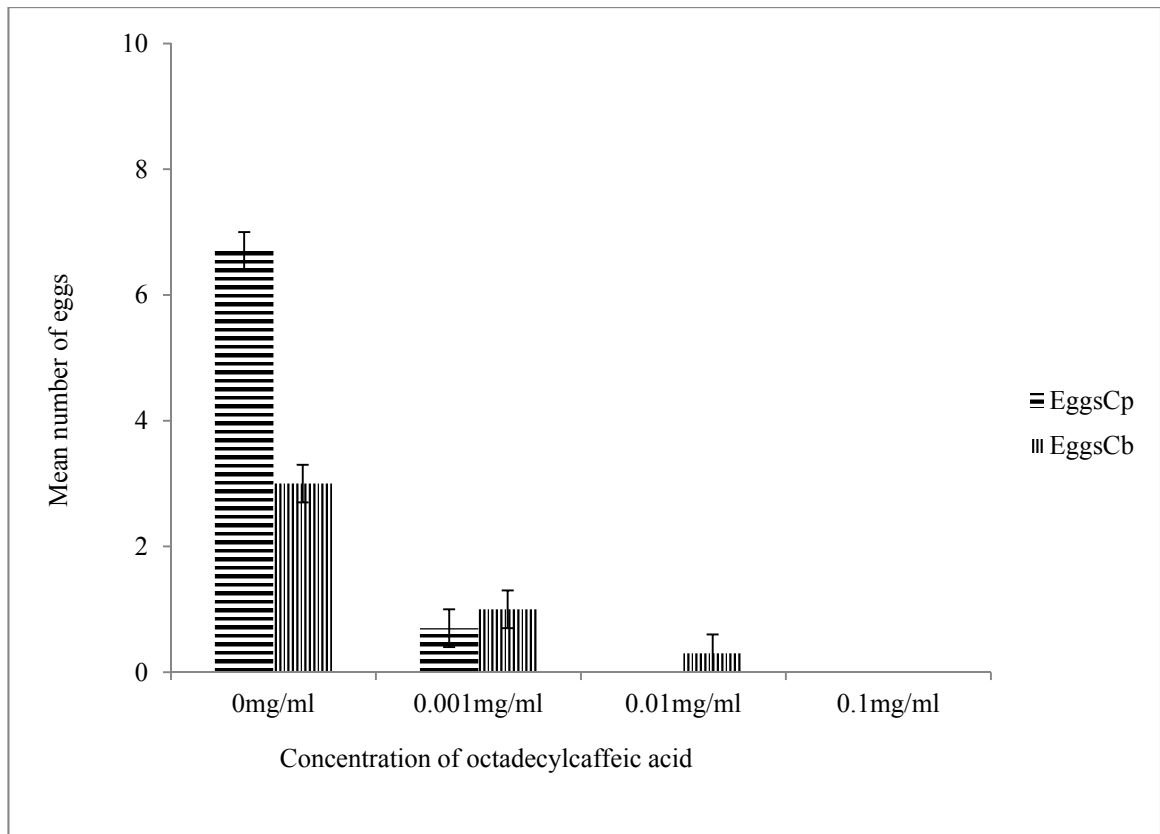
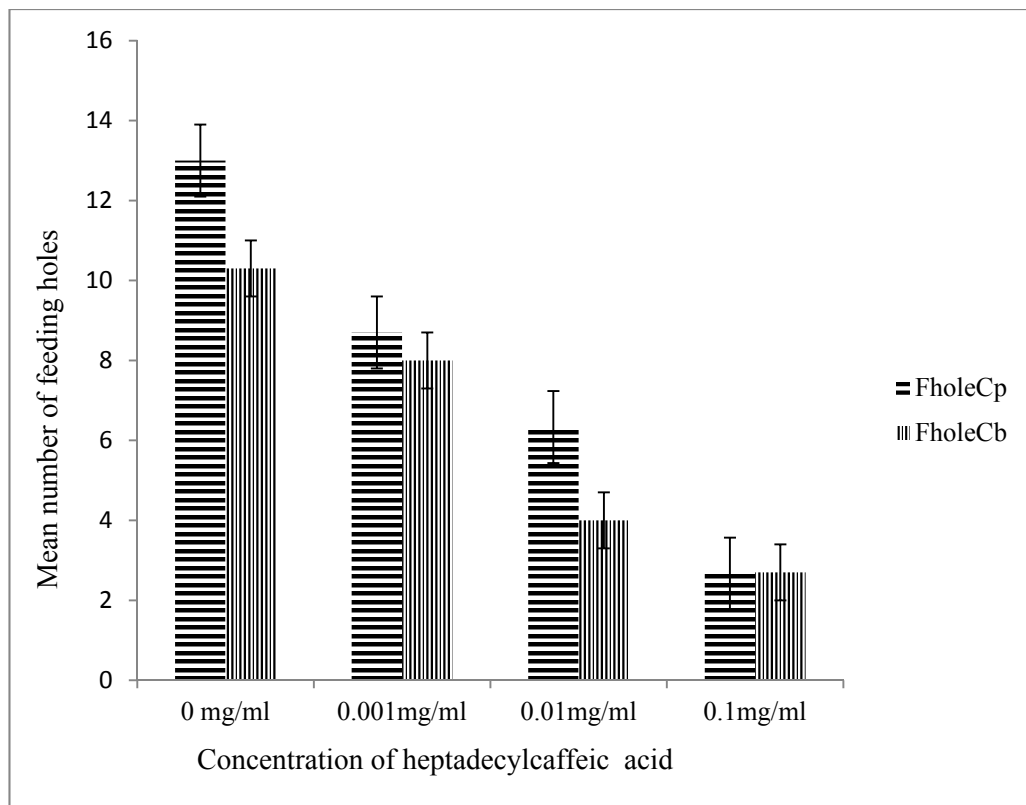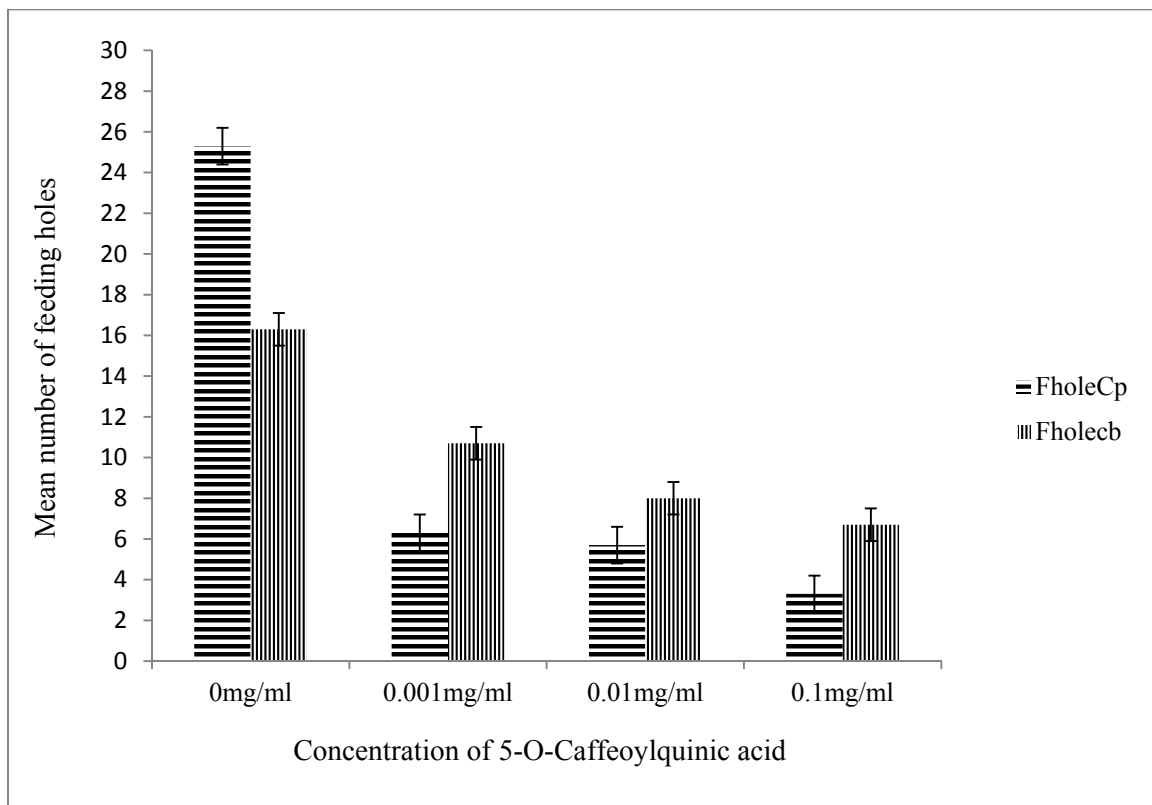**Figure 5.9** Mean number of eggs laid by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with octadecylcaffeic acid. Cp: *C. puncticollis* and Cb: *C. brunneus.*

N=15, SEM.

Biological activity of octadecylcoumaric acid on C. puncticollis and C. brunneus oviposition

Octadecylcoumaric acid treatment significantly (ANOVA, P≤0.001, F=8.54 and df= 3,8) affected *C. puncticollis* egg laying on the sweetpotato root core among the treatments. Similarly, the mean number of *C. brunneus* eggs laid on the root core treated with octadecylcoumaric acid was significantly different (ANOVA, P≤0.001, F=8.54 and df= 3,8). The mean number of *C. puncticollis* eggs laid on the control root was significantly (P≤0.05) higher than on roots treated with octadecylcoumaric acid (Figure 5.10). However, the mean number of eggs laid on the root plug

decreases with increased dosage on the periderm treated with octadecylcoumaric acid (Figure 5.10). The lowest mean number of eggs was laid on the root core treated with 0.1ng/μL octadecylcoumaric acid (Figure 5.10).

The mean number of *C. brunneus* eggs laid on the control root was significantly ($p < 0.05$) higher than on root core treated with octadecylcoumaric acid (Figure 5.10). The mean number of *C. brunneus* egg laid was lowest on root core treated with 0.1ng/μL octadecylcoumaric acid (Figure 5.10).
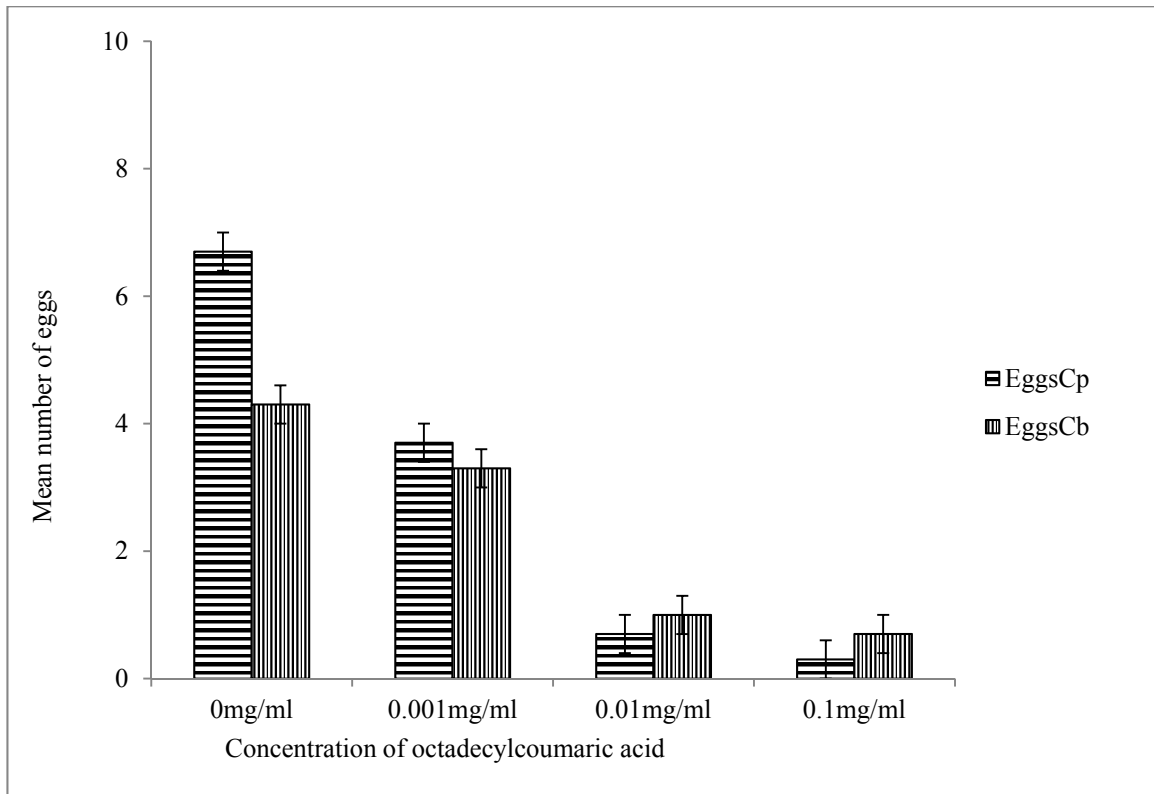
Figure 5.10. Mean number of eggs laid by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with octadecylcoumaric acid.

Cp: *C. puncticollis* and Cb: *C. brunneus,* N=15, SEM.

Biological activity of heptadecylcaffeic acid on C. puncticollis and C. brunneus oviposition

There was significant difference (ANOVA, P≤ 0.019, F=6.0 and df= 3,8) in the mean number of eggs laid by *C. puncticollis* on the roots treated with heptadecylcaffeic acid among the treatments. Similarly, the mean number of eggs laid by *C. brunneus* was significantly different (P≤0.001) on the root cores treated with heptadecylcaffeic acid. The mean number of eggs on control root was significantly (P≤0.05) more compared to the number that was recorded on root cores treated with heptadecylcaffeic acid (Figure 5.11). *C. puncticollis* did not lay eggs on the root core treated with 0.1ng/μL of heptadecylcaffeic acid (Figure 5.11).

The mean number of eggs laid by *C. brunneus* was significantly (P≤0.05) more on the control root cores than on root core treated with 0.001 mg/ml of heptadecylcaffeic acid (Figure 5.11). In contrast, *C. brunneus* did not lay eggs completely on the root cores treated with 0.001 and 0.01 and 0.1 ng/μL respectively (Figure 5.11).
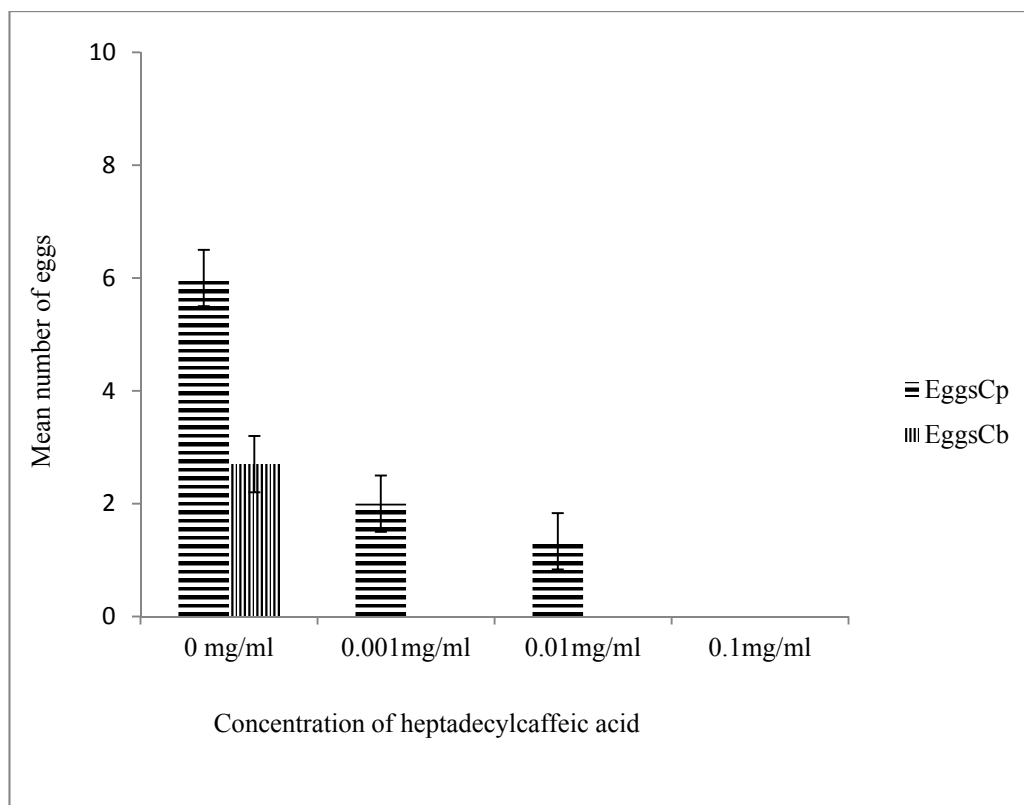
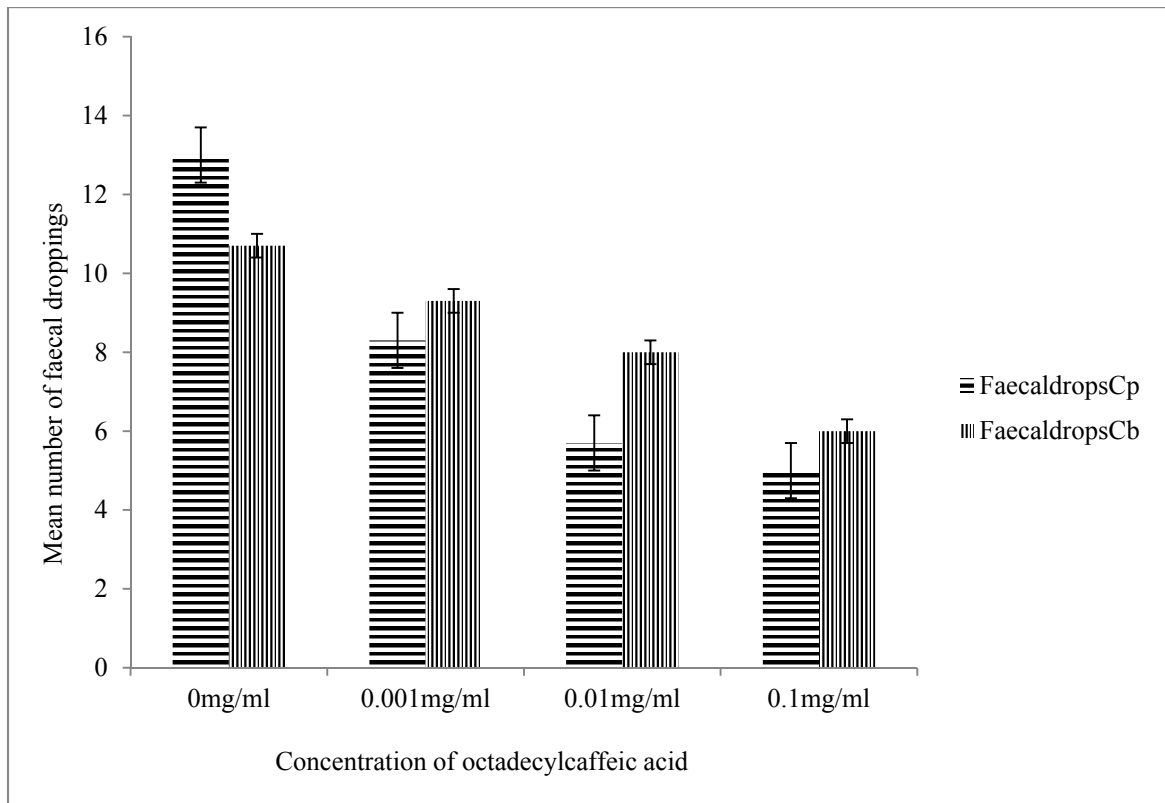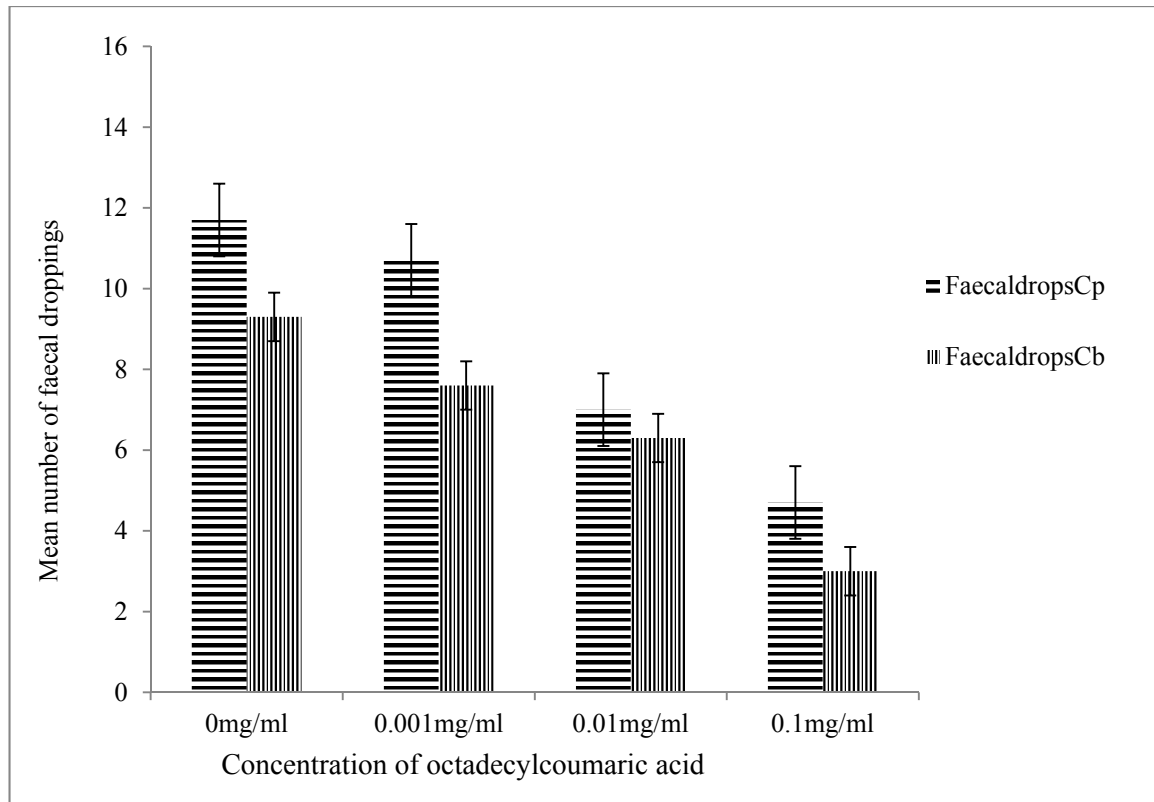**Figure 5.11**. Mean number of eggs laid by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with heptadecylcaffeic acid.

Cp: *C. puncticollis*, Cb: *C. brunneus,* N=15, SEM.

Biological activity of 5-O-caffeoylquinic acid on C. puncticollis and C. brunneus oviposition

There was significant difference (ANOVA, P≤0.001, F=27.42, df= 3,8) in the mean number of *C. puncticollis* eggs laid among treatments. Similarly, the mean number of eggs laid by *C. brunneus* was also significantly different (ANOVA, P≤0.005, F= 9.50, df = 3,8) among treatments. The mean number of eggs laid was significantly higher (P≤0.05) on the control root cores than on the root core treated with 5-O-caffeoylquinic acid (Figure 5.12) and the effect was dose dependent. Additionally, the mean number of eggs laid by *C. brunneus* was significantly (P≤0.05) more on control root plugs than it was on the treated root plugs treated with 5-O-

caffeoylquinic acid (Figure 5.12). Egg laying in both *C. puncticollis* and *C. brunneus* was completely repressed at 0.01 and 0.1 ng/μL 5-O-caffeoylquinic acid respectively (Figure 5.12).
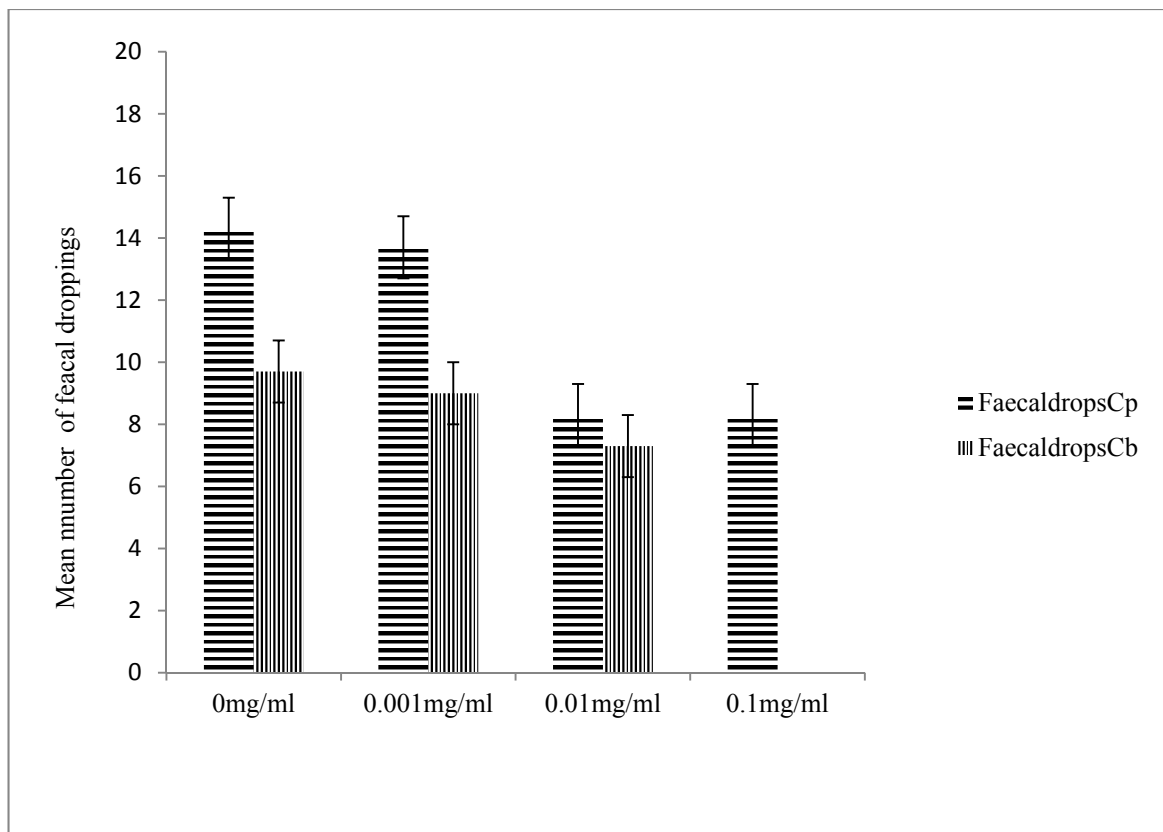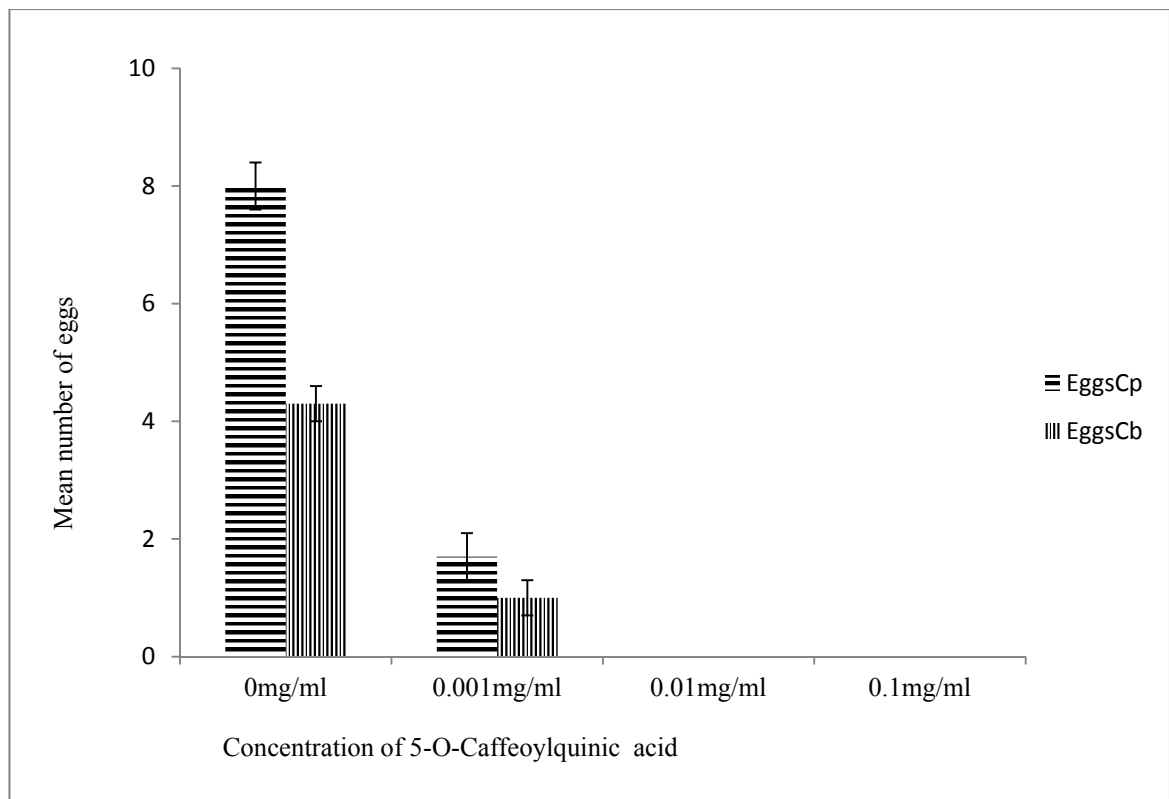

Figure 5.12. Mean number of eggs laid by *C. puncticollis* and *C. brunneus* on sweetpotato root cores treated with 5-O-caffeoylquinic acid.

5.4. DISCUSSION

The surface treatment of sweetpotato root plugs with octadecylcaffeic acids, octadecylcoumaric acids, heptadecylcaffeic acid and 5-O-caffeoylquinic acid affected the feeding and oviposition of *C. puncticollis* and *C. brunneus.* This suggests that these compounds from sweetpotato are biologically active on sweetpotato weevils as feeding punctures and oviposited eggs may be good indicators of resistance to *Cylas* weevils. The effect of these hydroxycinnamic acid esters were profound but were potent at higher concentrations. This indicates that the differences in the concentration of these compounds at the root surface are likely to be responsible for the reduced oviposition recorded on the resistant varieties New Kawogo, LIR302, and ARA228 and thus confer resistance to sweetpotato weevils. It is also interesting to note that the compounds have similar activities but occur as several different structures, potentially reducing the possibility of developing tolerance to their effects in the pest insect (Akhtar *et al*., 2008). The biological significance of this study is that the combined effect of the hydroxycinnamic acid esters can reduce feeding and oviposition by C*. puncticollis* and *C. brunneus* adults in a sweetpotato variety expressing higher concentrations of these compounds on the root surface.   This was demonstrated by the treated periderm significantly affecting weevil feeding compared to the control. In fact, the feeding of the weevils decreased with increasing concentration of these compounds; an indication that increases in the concentrations of these compounds is correlated with resistance as suggested in chapter 4 of this thesis.

The mean number of faecal droppings was lower on the chemical treated periderm compared to untreated ones. The number of faecal droppings is an indicator of the

amount of time spent feeding. Accordingly, the biological significance of this finding is that if sweetpotato varieties with higher levels of these compounds are developed it could greatly reduce the time spent producing feeding holes in which to lay eggs. In general, sweetpotato weevil preferred varieties support feeding hence making it susceptible.

There was a major effect on oviposition on the root plug of a susceptible variety NASPOT1 when treated with the compounds identified at higher concentrations on resistant varieties with no eggs laid at some concentrations of hydroxycinnamic acid esters applied on the root plugs indicating that the compounds deterred oviposition effectively. No oviposition by either *C. puncticollis* or *C. brunneus* was recorded on periderms treated with some hydroxycinnamic acid esters at the highest concentration of 0.1ng/μL, indicating that the effect of this compound is dose dependent. Therefore, higher concentrations of these compounds on resistant roots may accounts for the lower weevil damage in New Kawogo and ultimately explain resistance reported in field where roots were observed with no feeding damage.

This observation is expected if the constitutive occurrence of these compounds on the storage root surface was naturally high and may explain further the mechanisms of resistance and reduction in oviposition by *C. puncticollis* and *C. brunneus*, respectively. Female weevils are known to oviposit eggs just below the surface of the storage roots and at the base of the stem (Muyinza, 2010). Thus, the occurrence of these compounds in the surface, periderm and epidermal tissue may be well placed to demonstrate the resistance expressed by some varieties such as New Kawogo. Dramatic reductions in the oviposition on the root plug of NASPOT1 occurred due to the application of higher doses 0.01 and 0.1 ng/μL octadecylcaffeic acid, octadecylcoumaric acid, heptadecylcaffeic acid and 5-O-caffeoylquinic acid to

the root surface. Since, this variety typically is oviposited on by *Cylas* this indicated that weevil susceptibility for feeding as well as oviposition can be arrested by the presence of these compounds (Anyanga *et al*., 2013, Ekobu *et al*. (2010).

This study therefore showed that additive effect of the hydroxycinnamic acid esters can reduce feeding and oviposition by *C. puncticollis* and *C. brunneus* adults in a sweetpotato variety expressing higher concentrations of these compounds on the root surface.

Earlier work reported that hexadecyl, octadecyl, and eicosyl esters of coumaric acid were associated with *Cylas* resistance although the compounds identified were not directly tested against weevils (Snook *et al*. 1994) so this present study provides the proof missing from earlier predictions of activity. Elsewhere, caffeoyl and coumaroyl esters were shown to reduce development of larvae and so possibly contribute to resistance (Stevenson *et al*., 2009). The present work adds significantly to this earlier work by demonstrating that these hydroxycinnamic acid esters occur on the root surface and, when applied to roots of otherwise susceptible varieties NASPOT 1, reduce feeding and egg laying by adults. This presented an indication of the deterrent effect of compounds, where insects were allowed to choose between root cores treated with different concentrations of the pure compounds as described by Anyanga *et al*. (2013). The additive effects of these compounds on adults and larvae explain the resistance in New Kawogo, LIR302 and ARA228 reported in chapter 3 of this thesis. Additionally, 5-O-caffeoylquinic acid was also associated with lower feeding and egg laying, so this compound may also contribute to the observed effects in field and laboratory bioassays and supports earlier reports that caffeoylquinic acids are associated with defence in sweetpotato (Jackson and Bohac, 2006).

The significant differences in response of *Cylas* species to sweetpotato were due to the presence of higher concentrations of hydroxycinnamic acid esters on the root surface and in peridermal and epidermal tissues. This is the demonstration, since they occur at higher concentrations on resistant roots and the application of these compounds to root plugs of susceptible varieties led to reduced oviposition and feeding as recorded on resistant varieties. Thus, sweetpotato varieties with higher levels of hydroxycinnamic acids, particularly in the surface root tissue and on the surface, could be selected for to optimize the development of resistance to sweetpotato weevils. This will involve screening of potentially hundreds of breeding progeny using freeze-dried samples of roots and analysis by LC-MS as described in chapter 4 of this thesis.

CHAPTER 6

BEHAVIOURAL AND ELECTROPHYSIOLOGICAL RESPONSES OF SWEETPOTATO WEEVILS TO VOLATILE COMPOUNDS FROM SWEETPOTATO PLANTS

6.1. INTRODUCTION

Many volatile organic compounds are emitted from plants and because they disperse easily through air (Pichersky and Gang, 2000) can be perceived by foraging herbivores to indicate a source of food (Metcalf, 1987). Sweetpotato produces a variety of volatile organic compounds (Korada *et al.,* 2010) that could provide this function to weevils. Volatile organic compounds comprise a chain of 5-20 carbon atoms with a variety of functional groups some of which mediate insect-insect and insect-plant interactions. Accordingly, the differences in volatile chemistry among sweetpotato clones may be related to the variability in susceptibility or resistance to sweetpotato weevils. For instance, Wang and Kays (2002) demonstrated that the sweetpotato host plant chemistry can potentially modify behaviour of the weevil from host finding (volatiles) through feeding (surface chemicals) to larval development in the roots (internal root chemistry). In fact, one or a mixture of these volatile organic compounds produced from either the leaves, root surface or in the root of sweetpotato could change the behaviour of sweetpotato weevils. Volatile phytochemicals could play a crucial role with respect to host finding and oviposition. For example, initial work in the United States of America on volatile

chemicals released from sweetpotato showed that adult male and female *C. formicarius* were not equally attracted to root and leaf volatiles (Mullen, 1984). Therefore, the knowledge of the qualitative and/or quantitative differences in the volatile compounds between the aerial plant parts and the storage roots might be useful in the selection of lines for breeding for resistance to sweetpotato weevils. This chapter aims to establish the differences in the volatile chemistry of resistant and susceptible varieties and if there are any effects on sweetpotato weevils.

6.2.   MATERIALS AND METHODS

6.2.1. Collection of volatiles from shoots and roots

Clean roots of the resistant variety New Kawogo and a susceptible variety Tanzania used in the field and laboratory experiments in chapter 3 were harvested from a field planted at the National Crops Resources Research Institute (NaCRRI), Namulonge, Uganda (00° 31 30N, 32° 36 54 E). Volatiles from shoots infested with twenty weevils and non-infested shoots were collected. Storage roots were also infested with similar number of weevils. Volatiles from both infested and non-infested roots were trapped separately by air entrainment for 24 hours (Plate 6.1). Volatile components emitted into polythene bags (oven bags, Sainsbury's) were trapped at the outlet on to Porapak-Q (Waters Corp, U.S.A.), by drawing purified air (filtered through charcoal dust filled column) 1.0 L min^{-1} across the enclosed sample of either the shoot or roots. Compounds were desorbed by eluting the adsorbent with 1.5 ml of methylene chloride into a drum vial sealed with parafilm, labelled and refrigerated. Both shoot and root volatile collection was done eight times.
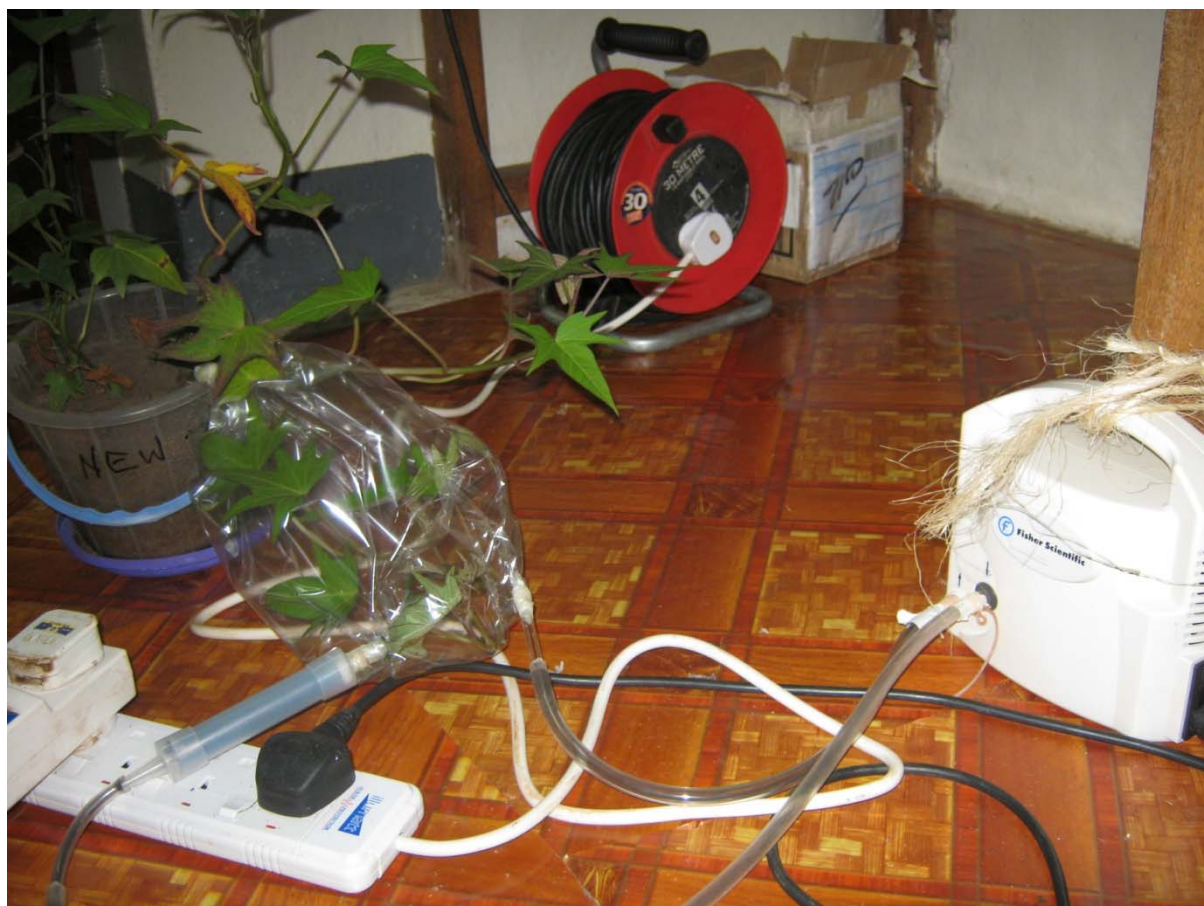
Plate 6.1. Collection of sweetpotato leaf and root compounds for volatile chemical analysis.

A set up for headspace volatile extraction equipment is shown in Plate 6.1. Samples (aerial part and roots) were placed in the oven bags on either side that was closed tied to the Teflon tube fitted in with Porapak-Q.

6.2.2. Volatile analysis

Samples were analysed at the Natural Resources Institute, University of Greenwich with the technical assistance of Dudley Farman. Gas Chromatography-Mass Spectrometry was carried out on a Perkin-Elmer GC Autosystem 2000 fitted to a Perkin-Elmer Mass Spectrometer QMass-910. The GC was equipped with a 25 m., 0.18 mm ID, DB-5 fused silica capillary column. The GC oven temperature was programmed from 40°C (4 min) to 150°C at 6°C/min, and final temperature was maintained for 15 min. The individual volatile compounds collected were identified by comparison of their mass spectra with National Institute of Science and Technology (NIST) spectral libraries.

6.2.3. Behavioural responses of sweetpotato weevils to α-caryophyllene and Germacrene D

Behavioural response studies were conducted in a linear flow olfactometer. A synthetic form of two compounds prominent in the volatile metabolome of sweetpotato plants: α-caryophyllene and germacrene D were purchased from Boots, Nottingham, UK and tested with male and female weevils separately in a dual-choice olfactometer. Caryophyllene was made up in a solution of 1 mg/ml solution of purified caryophyllene in hexane prepared by weighing 5 mg of caryophyllene and making a solution in 5 ml of hexane. A series of dilution was made to test a lowest concentration of 10 ng/µl solutions while germacrene-D obtained from Ylang ylang oil extract was used in bioassays. Twenty 14-day-old adult weevils were introduced singly in the bioassay after being deprived of food for one day before the start of the bioassay. A vacuum pump was used to generate an

air volume of 1.0 L min^{-1} adjusted using airflow meter on a silicon tube that was connected to the pump to pass air through an air inlet chamber fitted with a charcoal filter column. A 30 µl aliquot of each compound was placed using a pipette and released on to filter paper and the carrier solvent was allowed to evaporate. Carbon filtered air was drawn from the chamber using a vacuum pump at a flow rate of 1.0 L min^{-1}. This process delivers the odour towards the entry point for the insect and enabled the test insect to choose to move towards the odour source or blank control. Single insect introduction to the arena was done to avoid for insects being attracted by aggregation pheromones secreted by weevils when put together or groups. The weevil movement was monitored for their response to the stimulus. The movement of the weevils was observed from the time they were introduced into olfactometer chamber. The position of weevils was observed and recorded after 30 minutes. Fresh insect were brought in after flushing out the arena by blowing in fresh air. Data was collected 5 times and the experiment replicated five times with a fourteen day old weevils of either sex in each experiment. The numbers of weevils that responded to odour and those that did not respond were pooled together and the response of the weevils was computed using attractive index from the relationship; attractive index (a.i) = n/N (Starr *et al*., (1991) where; n= number of weevils in the arm or outside the buffer zone orienting towards the direction of the odour and N= total number of weevils introduced in to the olfactometer arena. This index was converted into percentage by multiplying by 100.

6.2.4. Bioassay on response of sweetpotato weevils to sweetpotato volatiles

The bioassay was conducted using a linear flow olfactometer which comprised a long glass tube connected by Teflon tubing, and plastic lunch boxes where the plant samples were placed in one box and pure water in the other box as control. The set up was connected to the olfactometer chamber and lunch boxes on either end with Teflon tubes. The either ends of the plastic boxes were connected to a charcoal filter to clean incoming air. A 15 cm buffer zone was marked off on either side from the weevil's point of entry to the olfactometer so that the weevils had to move more than 30cm towards the odour source for this to be considered attracted. This was designed to ensure any record of attraction to an odour represented a commitment to seek that source of food and was not a measure of potential random exploratory behaviour. Roots and leaves were used as odour sources in experiments investigating the possible attraction or repellency of both weevil sexes for one of the two host plants leaves and storage roots. The youngest shoot or the roots for both Tanzania and New Kawogo were selected and used for the bioassay. The response of males and females to sweetpotato volatiles was tested in two (2) experiments to elucidate whether there was *Cylas* preference for: i) shoot volatiles from Tanzania or New Kawogo compared to a blank control; ii) and sweetpotato root volatiles versus sweetpotato shoot volatiles. Each experiment was replicated five times with either male or female adult weevils at a time to see if they respond to the odour in the same way. The data obtained was pooled as in section 6.2.2.

6.2.5. Electroantennography (EAG) analysis of the electrophysiological responses to specific odours.

The sweetpotato weevils (herein referred to as SPW), used for Electroantennography (EAG) recording were *C. puncticollis*, and maintained in a controlled environment room at 22-25^{0}C with a 14L:8D cycle on sweetpotato roots purchased within the UK (Asda, UK).

GC-EAG recordings were carried out by M. Fernandez-Grandon at NRI on behalf of the author using an integrated unit (INR-02; Syntech, Germany) consisting of electrode holders, micromanipulators and amplifier. This was connected to one detector of an Agilent 6890 gas chromatograph to digitise the output and data were collected and processed using EZChrom Elite v3.0 software (Agilent). Electrodes were glass capillary tubes (o.d. 1.50 mm, i.d. 1.17 mm) pulled to a fine point and filled with electrolyte (0.1 M potassium chloride with 1% polyvinylpyrrolidine to reduce evaporation). These were placed over silver wires in the electrode holders. The unit had built-in amplification x10.

Gas Chromatography Equipment (GC) used for EAG

A 6890N Agilent GC was used for the separation of compounds for EAG. Oven temperature was set to 80°C starting temperature ramping by 20°C every 2 minutes until reaching the 250°C. In total the run lasted 17 minutes. These oven temperatures were selected over longer runs which may provide greater separation to ensure that the response of SPW did not deteriorate dramatically during the run and allow more replicates to be completed. At the start of each day of testing

standards of acetates and hydrocarbons were run through the equipment to ensure that separation was consistent and that no contamination was present on the column. A clean, continuous airflow was humidified through distilled water and delivered the odours over the antenna at a rate of ~150 ml/min.

EAG recordings from male and female Sweetpotato weevil

EAG recordings were made from male and female SPW between 0-14 days old. Individuals were chilled in ice before having the head and one antenna removed using fine dissection scissors. Anaesthetisation with CO_2 was experimented with in early trials, though was not found to confer any additional advantage in the preparation. The most distal segment of the antenna was removed and the proximal end was placed in the indifferent electrode. The distal end was then gently fitted into the recording electrode ensuring a complete circuit with the olfactory receptors exposed.

A consistent response could be seen of the SPW to decyl-*E*-2-butenoate from male antenna. Once the response was ascertained this pheromone was used as an internal positive control to test the volatiles from sweetpotato resistant and susceptible varieties. Entrainment samples from three varieties were tested and were: # 01 Tanzania and #03 New Kawogo with # 01 Tanzania representing susceptible variety and # 03 New Kawogo an example of the SPW- resistant sweetpotato variety. The numbers represented the sample codes tested during entrainment.

6.3. RESULTS

6.3.1. Volatile components of the storage roots of Tanzania and New Kawogo

Uninfested storage roots of Tanzania and New Kawogo emitted volatile chemicals in the absence of weevils. The roots of Tanzania emitted quantitatively fewer compounds than the roots of New Kawogo before weevil infestation. The chemical profile of the volatiles differed between the storage roots of the two sweetpotato varieties and indicated that β-caryophyllene, α-farnesene and germacrene B were produced exclusively by the roots of New Kawogo and not by the roots of Tanzania prior to infestation (Figure 6.1).
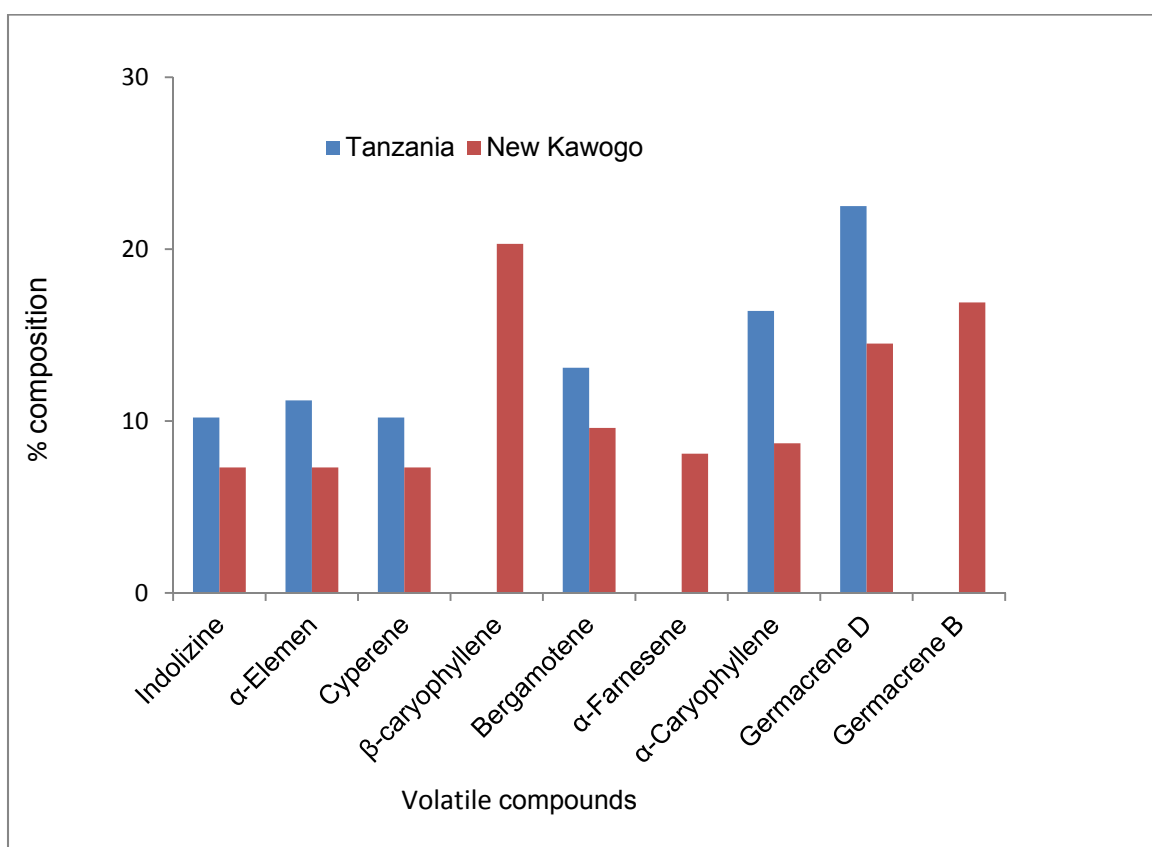
Figure 6.1. Mean percentage volatile chemical compound composition produced by the storage root of Tanzania and New Kawogo before weevil infestation

The emission of volatiles from the storage roots of Tanzania increased six fold after the weevils were introduced to feed on the roots for 24 hours. Four volatile compounds: α-elemene, β-caryophyllene, germacrene D and germacrene B were emitted in greater amounts by the storage roots of Tanzania (susceptible) compared to New Kawogo (Figure 6.2). Emission of α-caryophyllene was also lower in New Kawogo than Tanzania. Another interesting observation was that volatile emissions by New Kawogo roots were quantitatively lower in infested roots compared to uninfested root and the compound (*Z*)-3-hexen-1-ol acetate was only emitted by Tanzania and not by New Kawogo after the roots were infested with sweetpotato weevils and allowed to feed for 24 hours (Figure 6.2).
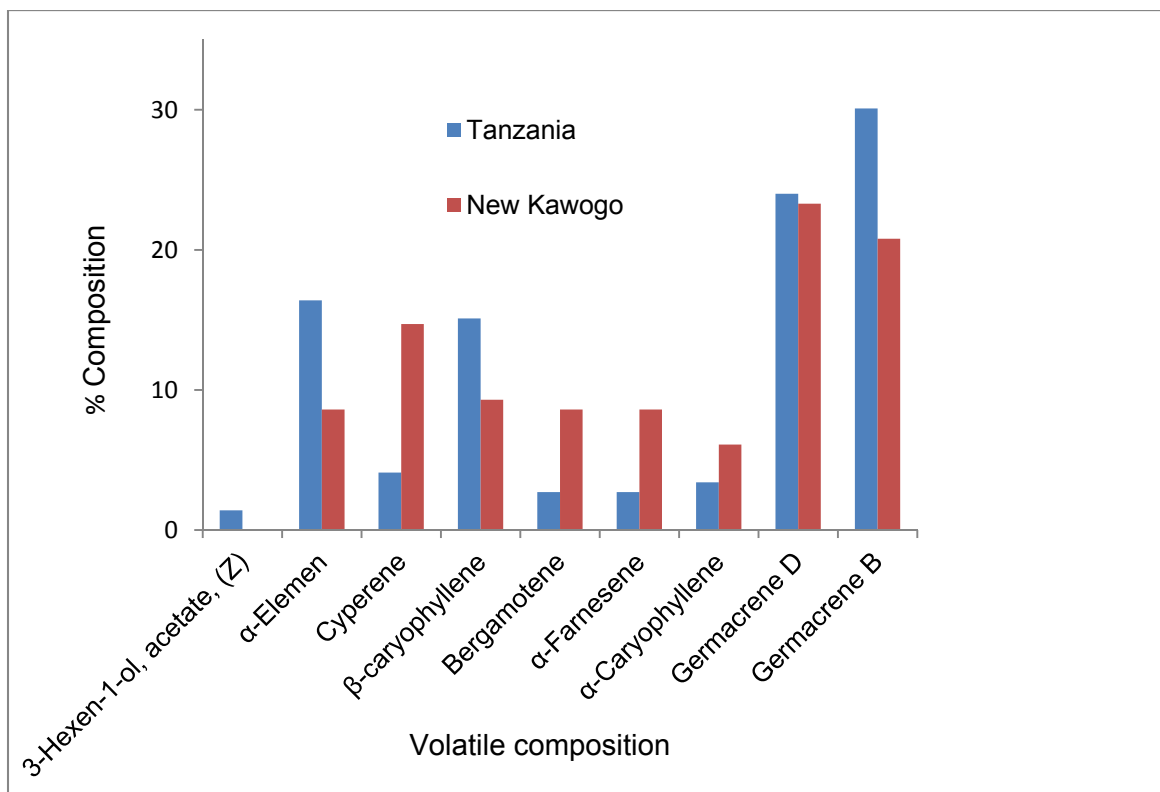
Figure 6.2. Mean percentage volatile chemical compound composition produced by the storage root of Tanzania and New Kawogo after weevil infestation

6.3.2. Volatile components of the aerial parts of New Kawogo and Tanzania

The volatile emissions from undamaged shoots of New Kawogo and Tanzania showed that overall similar compounds were emitted by both varieties. However, the results showed that the aerial parts of Tanzania emitted α-caryophyllene which was not produced by the aerial part of New Kawogo before infestation (Figure 6.3).
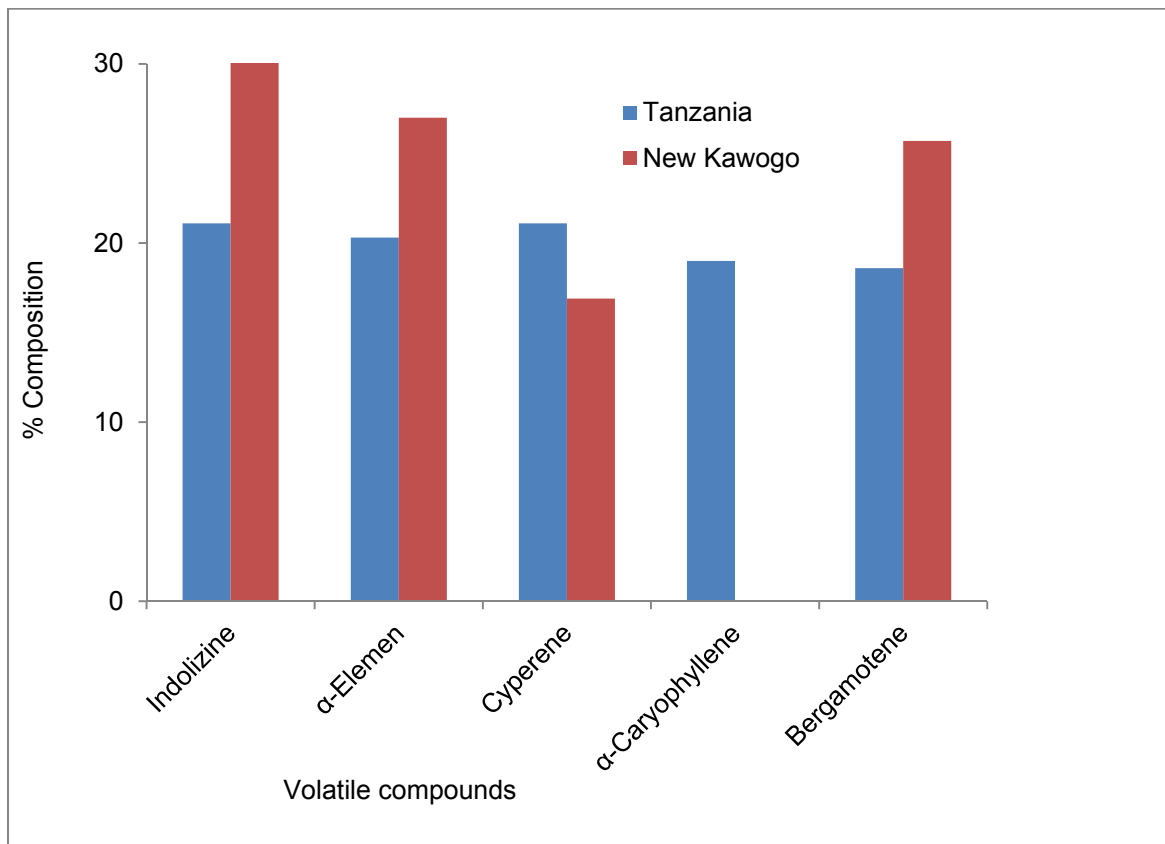
Figure 6.3. Mean percentage volatile chemical compound composition produced by the aerial parts of Tanzania and New Kawogo before weevil infestation

After sweetpotato weevil infestations, the emission of α-caryophyllene and germacrene D increased in the above ground shoots of Tanzania whereas in New Kawogo these two volatile compounds were not emitted. The emission of the rest of the compounds did not change before and after sweetpotato weevil infestation

(Figure 6.4). Emission of α-caryophyllene and germacrene D before infestation as recorded from the roots and shoot of Tanzania seems to be associated with location cues for the weevil whereas the production of the same compounds in New Kawogo are triggered after the weevils feeding. The emission could be a defence response in an attempt to deter further damage by the weevils.
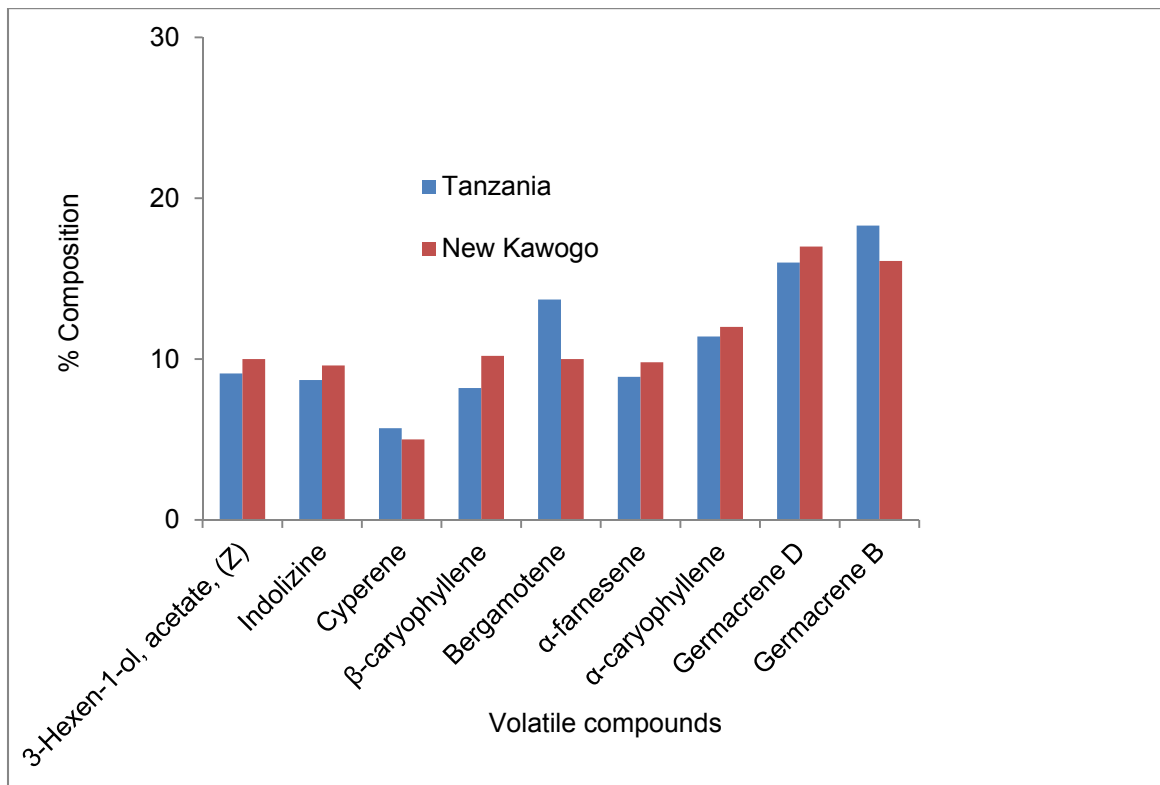
Figure 6.4. Mean percentage volatile chemical compound composition produced by the aerial parts of Tanzania and New Kawogo after weevil infestation

6.3.3. Response of the weevils to α-caryophyllene and ylang ylang oil

There was no significant difference in the response of sweetpotato weevils to sweetpotato volatiles in the linear flow olfactometer experiment. Fifty two percent of the weevils were lured by α-caryophyllene while germacrene D trapped 60.2% of the weevils during the study (Figure 6.5).
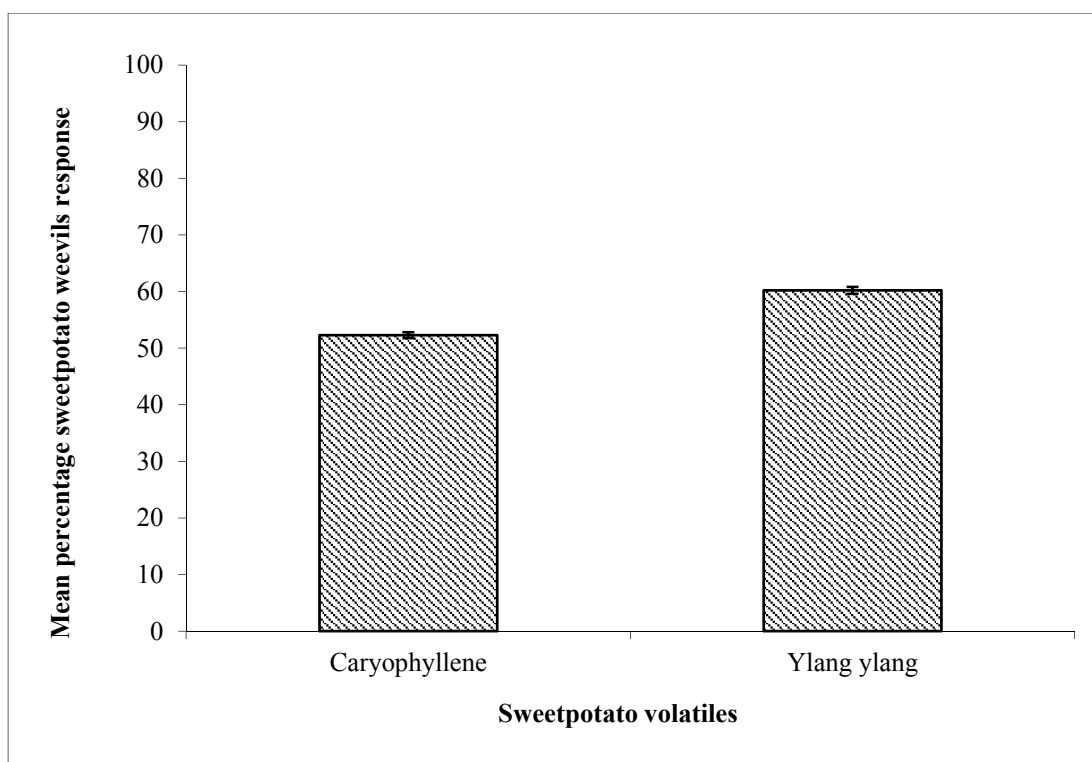
Figure 6.5. Mean percentage attraction of sweetpotato weevils to caryophyllene and ylang ylang (n=20, replicated 5 times).

6.3.4. Response of the weevils to sweetpotato leaf odours

The attraction of male sweetpotato weevils to New Kawogo leaf odour was significantly ($P \leq 0.04$) greater than the attraction of female weevils to the same odour. Similarly significantly more ($P \leq 0.05$) males (41%) were also lured to leaves

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of Tanzania compared to females (23%) (Figure 6.6). However, there was no significant differences (P>0.05) in the number of each sex attracted to the above ground plant parts indicating the insects were responding equally to the stimulus in the leaves of both New Kawogo and Tanzania .

Forty seven percent of the male weevils were lured to the odour compared to only 16% female weevils that responded to the volatile (Figure 6.6).
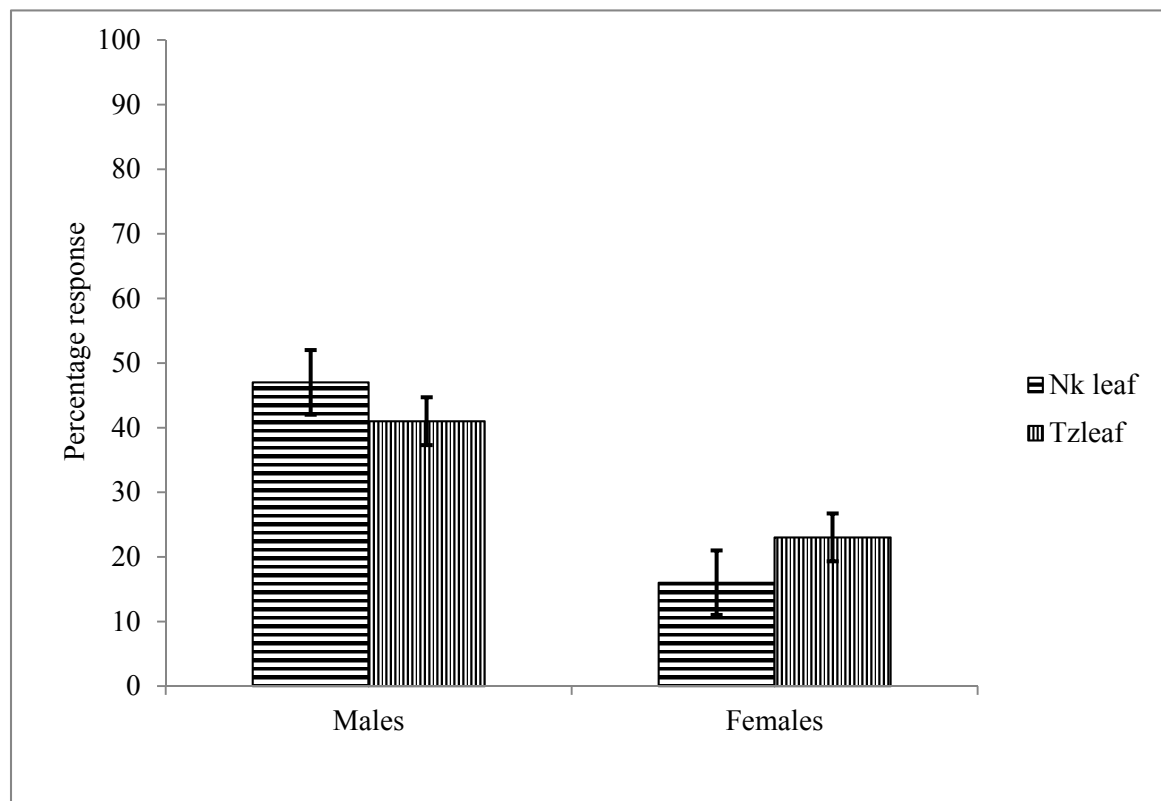
Figure 6.6. Mean attraction of male and female sweetpotato weevil to sweetpotato leaf odours (n=20, replicated 5 times) of New Kawogo (NK) and Tanzania (Tz).

6.3.5. Response of the weevils to sweetpotato root odours.

The responses of sweetpotato weevils to root volatiles were not as profound as their responses to above ground plant parts. The data suggested that males were less able to recognize root volatiles compared to females although the difference was

not significant (P ≥ 0.05).   For instance, New Kawogo root volatiles attracted 2% males while 8% females responded to the volatiles and were attracted to the root of New Kawogo in the olfactometer study (Figure 6.7). The response changed when the root of Tanzania was used.   The root volatiles from Tanzania attracted 6% males and 15% females respectively (Figure 6.7). The response of female weevils to the root volatiles of both New Kawogo and Tanzania was surprisingly low and not significantly different.
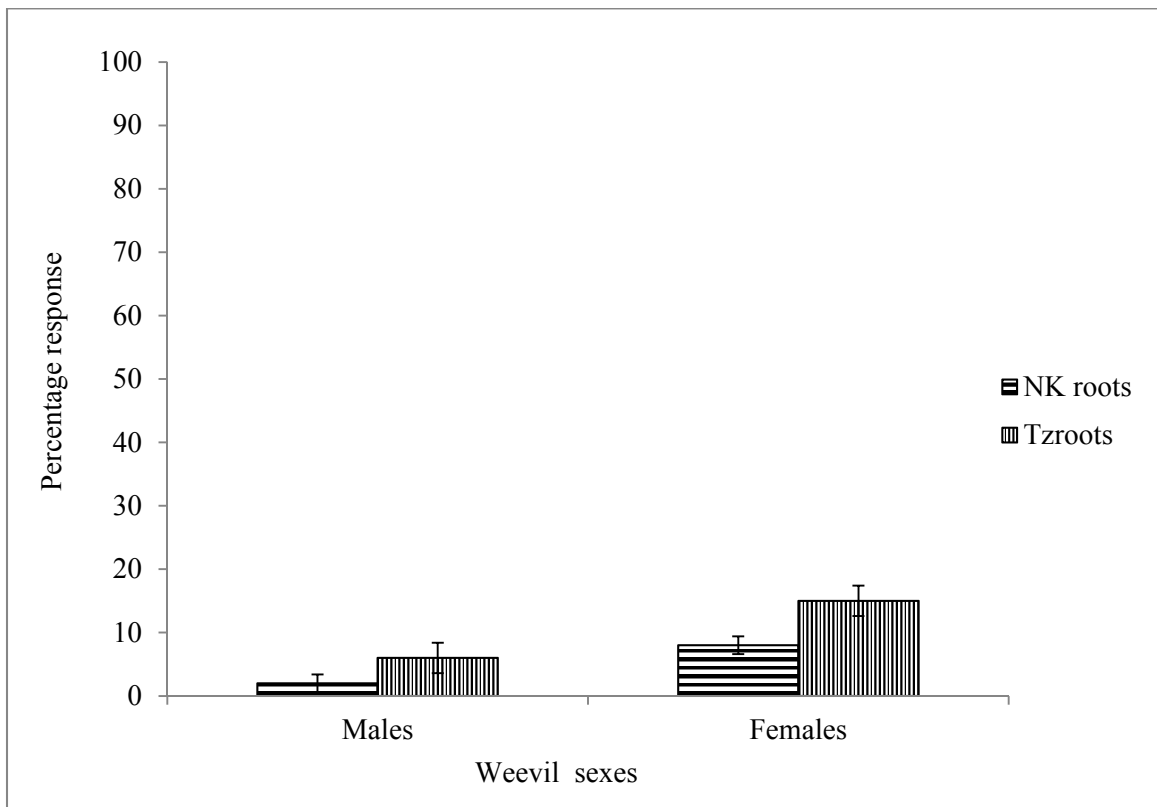
Figure 6.7. Mean attraction of male and female sweetpotato weevil to sweetpotato root odours (n=20, replicated 5 times) of New Kawogo (NK) and Tanzania (Tz).

6.3.6. Electrophysiological responses

The only antennal response observed with any consistency was that of the pheromone sample (decyl-*E*-2-butenoate) with a peak produced in all replicates at ~9.8 minutes (Figures 6.8, 6.9, 6.10, 6.11, 6.12 and 6.13). Due to the diverse chemical content of the entrainment samples it would be difficult to ascertain the response of individuals to specific compounds without repetition. Although responses in the antenna were occasionally observed to plant volatile compounds, repetitions have shown these not to be consistent with the release of compounds from the column and are more likely to be false positives, which are not uncommon in EAG.

Around 54 replicates were completed with the SPW using concentrated samples, males and females, antenna-only preparations and SPWs of different ages. The most useful of these are probably the series of trials included in which male SPW are tested against the volatile blends and the pheromone as an internal positive control in which the only responses were recorded to the pheromone, which can be seen around 9.8 minutes, are included. Three replicates are completed for each of the volatile blends:
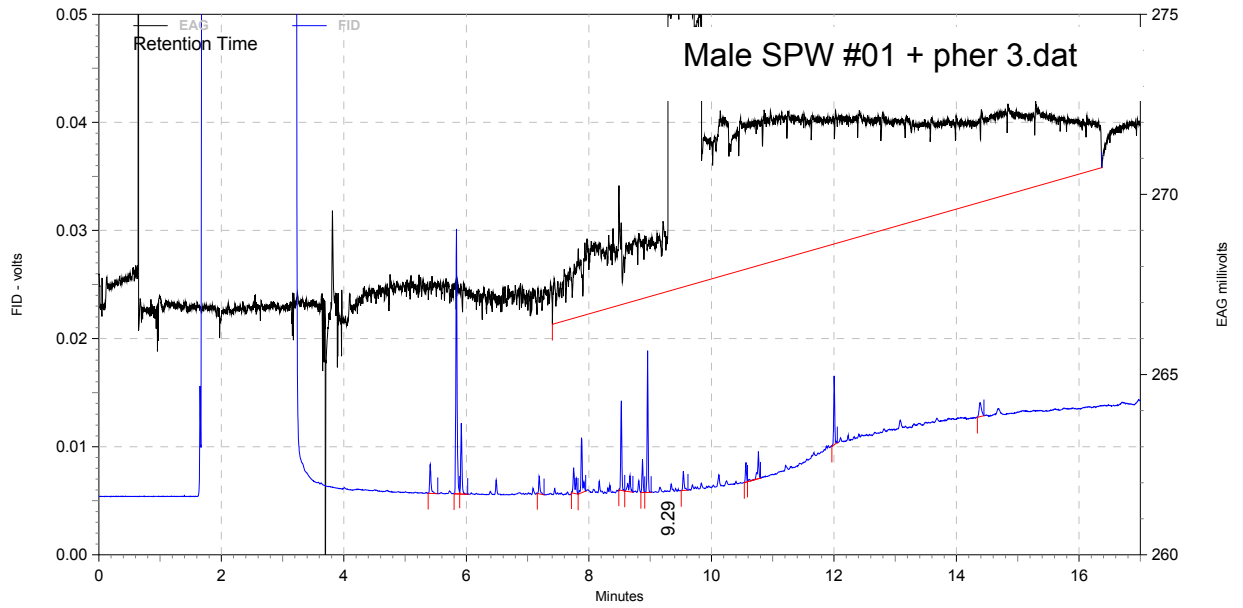
Figure 6.8. Chromatogram of Tanzania volatiles plus pheromone and EAG response using a male *C. puncticollis* antenna with the tip cut and the second antenna removed.
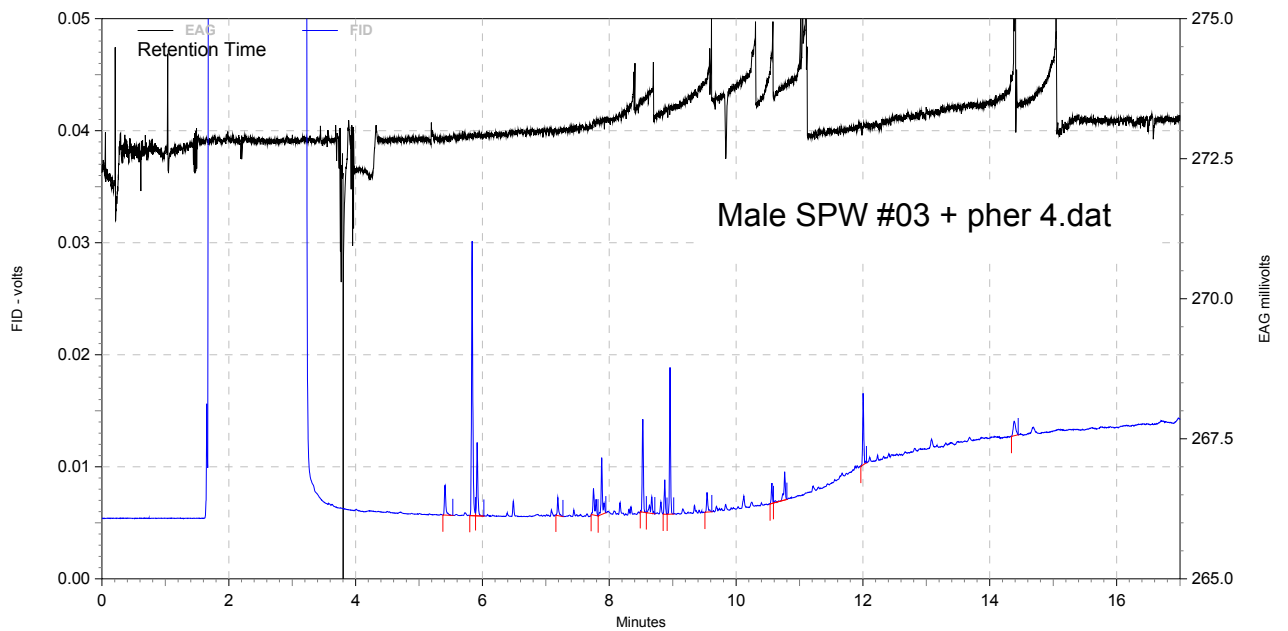
Figure 6.9. Chromatograms of Tanzania volatiles plus pheromone and EAG response using a male *C. puncticollis* antenna with the tip cut and the second antenna removed
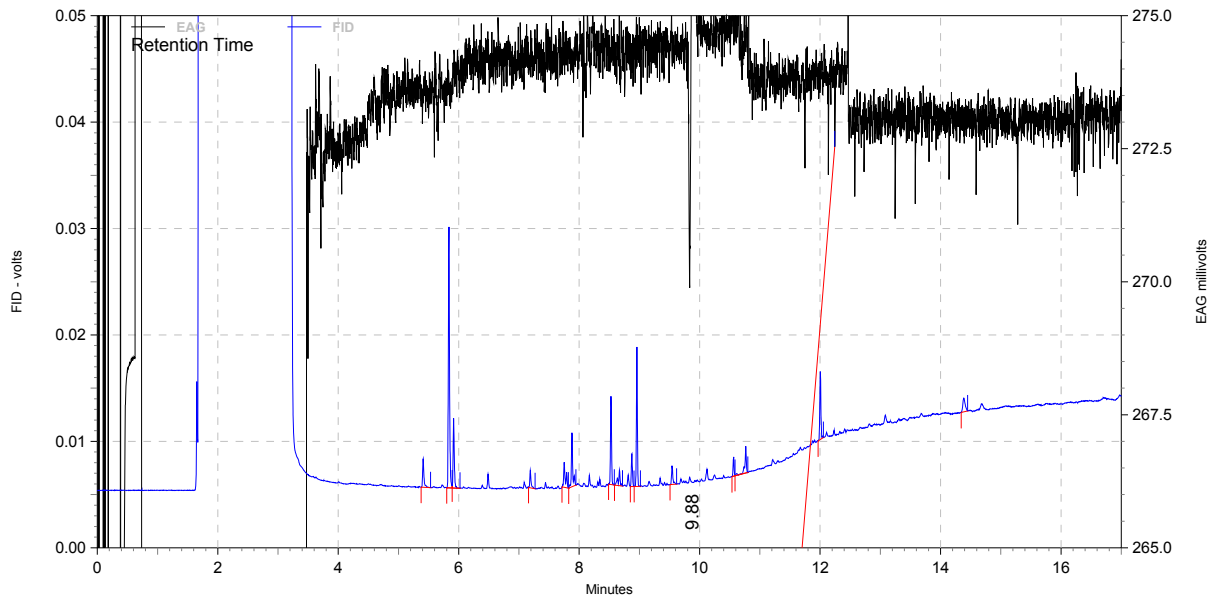
Figure 6.10. Chromatograms of Tanzania volatiles plus pheromone and EAG response using a male C. puncticollis antenna with the tip cut and the second antenna removed
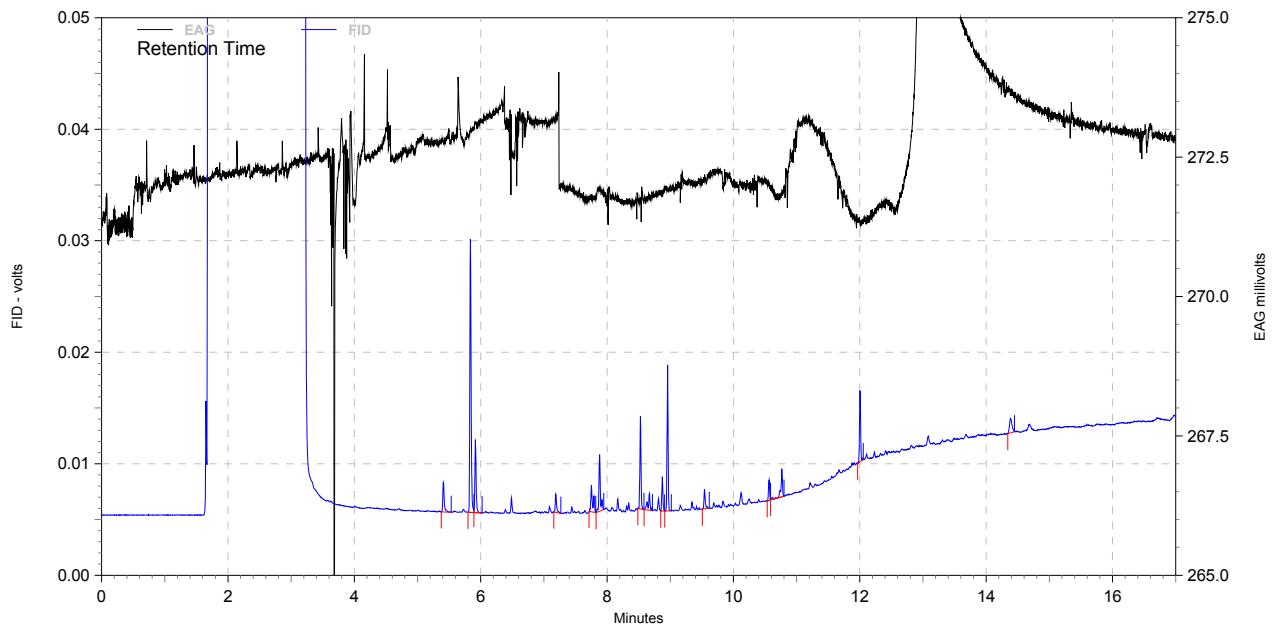
Figure 6.11. Chromatograms of New Kawogo volatiles plus pheromone and EAG response using a male *C. puncticollis* antenna with the tip cut and the second antenna removed
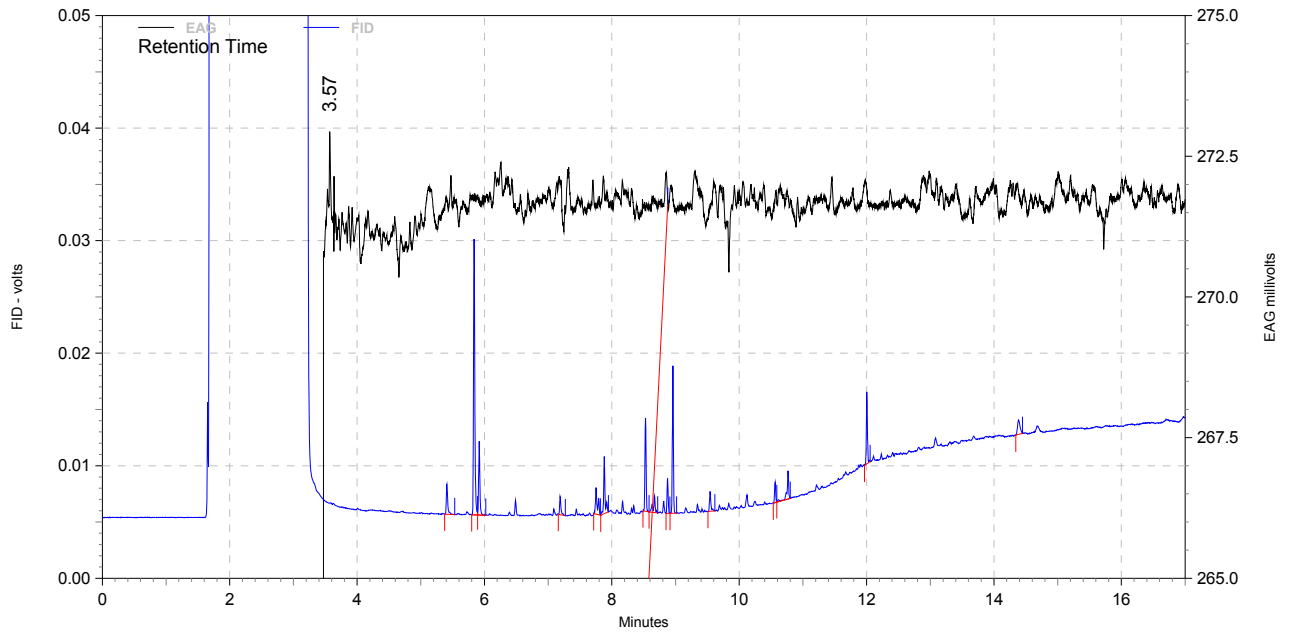
Figure 6.12. Chromatograms of New Kawogo volatiles plus pheromone and EAG response using a male *C. puncticollis* antenna with the tip cut and the second antenna removed
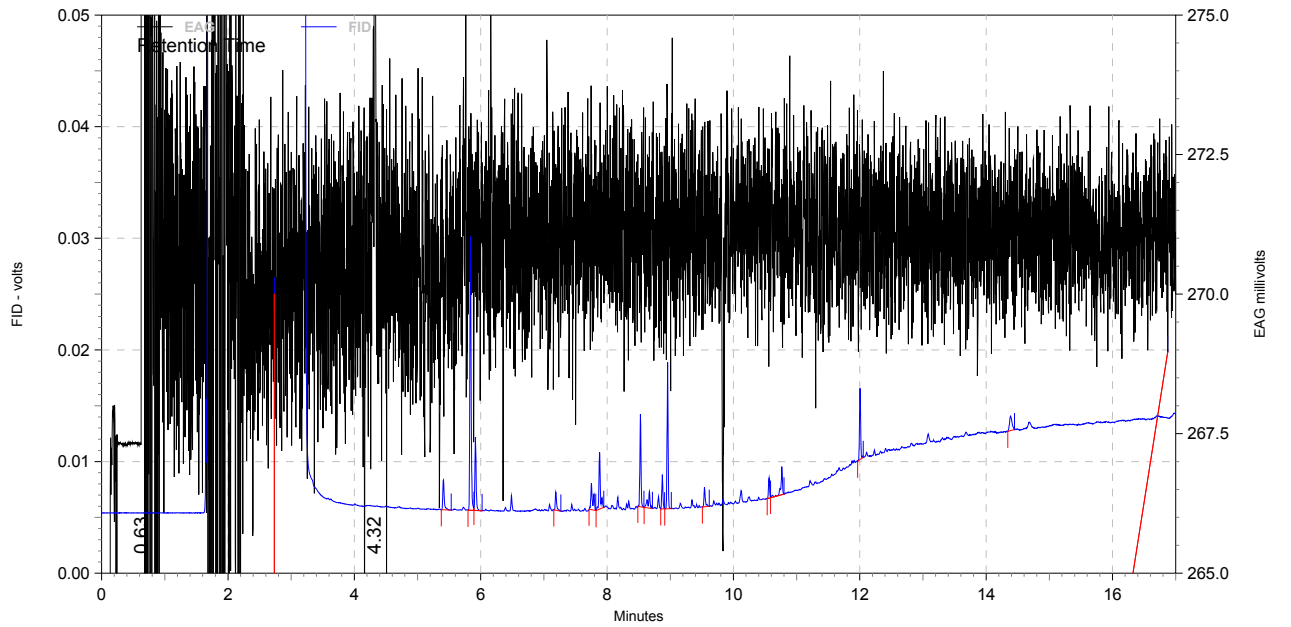
Figure 6.13. Chromatograms of New Kawogo volatiles plus pheromone and EAG response using a *C. puncticollis* antenna with the tip cut and the second antenna removed.

6.4. DISCUSSION

Host location and electrophysiological behaviour studies of *C. puncticollis* and *C. brunneus* for oviposition sites and feeding on resistant and susceptible sweetpotato varieties were conducted. The study showed that there were differences in the sweetpotato root volatile both before and after weevil infestation. This suggests that weevil feeding may induce biochemical processes that trigger the production of sesquiterpenes. For instance, α-elemen, β-caryophyllene, germacrene B and germacrene D were produced in greater quantity by Tanzania root after infestation compared to the New Kawogo. If these compounds were active then such chemical activity could influence host location and therefore susceptibility to herbivory.

Olfactometer study showed that although more *C. puncticollis* females oriented towards the roots of Tanzania than New Kawogo, this orientation was not significantly different. In other words the weevils did not differentiate between the volatiles from the roots of Tanzania and that New Kawogo in the olfactometer chamber. According to Lewis *et al*., (1991) when female insect receive a stimuli, they learn to recognise the volatile odours associated with the plants. This finding was supported with the report of Korada *et al*. (2010) that volatile compounds in susceptible sweetpotato varieties play a significant role in plant recognition by *C. formicarius.*

The roots of New Kawogo (the resistant variety) produced carophyllene, α-farnesene, germacrene B and 4-methyl-1,5-heptadiene which were not produced by Tanzania (the susceptible variety) but only before it was infested by sweetpotato weevils implying these compounds are produced for a physiological need that is not responding to herbivory although may still be repellent. According to Pare and

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Tomlinson (1999) leaves normally release small quantities of volatile chemicals for different physiological roles but upon wounding by external agent, many more volatiles are released. Lewis and Tomlinson (1988) suggested that these induced volatiles may either attract natural enemies of the insect or induce alert responses in neighbouring plants.

Emission of caryophyllene and germacrene D before infestation as recorded from the roots and shoot of Tanzania seems to be associated with location cues for the weevil whereas the production of the same compounds in New Kawogo are triggered after the weevils feeding. It is possible that this response could alert a natural enemy of sweetpotato weevil. A large number of chemical compounds have been implicated in signalling to herbivores, predators and parasitoids. According to Rasman *et al*., (2005) root worm larvae (*Diabrotica virgifera virgifera*) feeding on maize roots induced (*E*)‑β‑caryophyllene production in the roots and attracted entomoparasitic soil nematodes to orientate toward damaged plant roots in tests with a sand‑filled olfactometer. This appears to relate with the release of caryophyllene by New Kawogo after weevil infestation in this study.

Although there were differences in volatile production in the root and shoot, weevils were not significantly attracted to these two volatile odours implying they do not influence the host locating behaviour of the herbivores. The response observed between caryophyllene and ylang ylang oil could have been a result of other chemical odours not sweetpotato volatiles themselves. Electrophysiological responses were observed to the pheromone, decyl-*E*-2-butenoate, in male SPWs. However no other response was clear for any of the plant volatiles of either resistant or susceptible varieties. Plant volatiles appear be a response to alert a natural

enemy of sweetpotato weevil for the presence of a prey. Terpenes such as germacrene D, (*Z,E*)-α-farnesene, (*E,E*)-α-farnesene which are similar to the ones identified in sweetpotato roots after infestation have been reported as constituents of the alarm pheromone released when pea aphids are attacked by a predator, triggering escape responses in the aphid colony (Francis *et al*., 2005); Pickett and Griffiths, 1980). This could play a similar role in alerting sweetpotato weevils of the presence of their natural enemies since the response of weevils to volatile components was not detected by EAG suggesting they are not involved in attracting sweetpotato weevils, *C. puncticollis* in the plants but reported luring *C. formicarius* to the plants by Korada *et al*., (2013).

Elsewhere, sex pheromone were found effective in reducing *C. formicarius* populations in mass trapping trials in Taiwan (Hwang and Hung, 1991), Vietnam (Braun and Van de Flieart, 1997) and Cuba (Alcazar *et al*., 1997). Unfortunately in Uganda, mass trapping using these sex pheromone traps did not lead to a reduction in weevil damage to roots (Smit *et al*., 2001).

However the absence of a positive control made testing of females unreliable. It is also worth noting that the amplitude of the responses may indicate a problem with the equipment used. The EAG response to pheromone while present is of low amplitude which may suggest that a failure to identify any volatile components from the plant is an issue of sensitivity rather than indicating that a volatile component does not play role in sweetpotato weevil behavioural response. Overall, New Kawogo and Tanzania traces looked appeared very similar in the EAG indicating that the differences in the volatile chemistry of New Kawogo and Tanzania do not to influence the behaviour of sweetpotato weevils and does not account for the resistance in New Kawogo. This therefore contrasts with the original hypothesis that

sweetpotato volatile odours influence host location, oviposition and feeding of *C. puncticollis* and *C. brunneus.*

CHAPTER 7

INTERACTIVE EFFECTS OF HYDROXYCINNAMIC ACID ESTERS AND Bt PROTEINS ON MORTALITY OF SWEETPOTATO WEEVIL LARVAE

7.1. INTRODUCTION

Sweetpotato weevils (*C. puncticollis* and *C. brunneus)* are the most important biological threat to sweetpotato productivity in sub-Saharan Africa (Kiiza *et al*., 2009). Sweetpotato weevil control using conventional pesticides is challenged by the fact that they are subterranean and spend the majority of their lifecycle inside the roots. As described in Chapters 4 and 5 natural resistance provides one option for the development of effective pest control but this could be complemented by the use of biologically active natural toxins expressed in the tissue and produced through transformation. Numerous crops have already been transformed to express various *Bacillus thuringiensis* (Bt) Cry proteins, including crops that express coleopteran-active Cry proteins in roots against *Diabrotica* spp. in maize, *Zea mays* L. (Cry3Bb1 and Cry34Ab1/35Ab1), and sweetpotato weevil, *C. formicarius,* in sweetpotato (Cry3A) (Moran *et al*. 1998; Vaughn *et al*., 2005; Storer *et al*., 2006). Sweetpotato expressing Cry3A was not developed further, partly because the Cry3A protein expressed within the sweetpotato root results in relatively low *C. formicarius* control (Moran *et al*., 1998). The development of Bt sweetpotato resistant to *Cylas* spp. could, however, provide the most cost effective control

against these pests, assuming a highly *Cylas*-active Bt Cry protein could be expressed at levels required to control *Cylas* spp.

The generation of insect-resistant, transgenic crop plants which express insecticidal Bt toxins is a standard crop improvement approach (de Maagd *et al*., 2001). To date, many plants have been transformed with different *cry* genes and showing differing levels of resistance to insect feeding (De Villiers and Hoisington, 2011). Chinese japonica rice (*Oryza sativa L*.) expressing a synthetic *cry1Ab* transgene showed significant resistance to feeding by the striped stem borer (*Chilo suppressalis*) (Wang *et al*., 2002). Similar results have been observed with resistance to insect feeding in *cry* expressing transgenic eggplant (Arpaia *et al*., 1997), broccoli (Cao *et al*., 2002) and potato (Nault, 2001). In addition, Williams *et al.* (2006) also reported significant resistance of maize to southwestern corn borer (*Diatraea grandiosella*) fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*). Successes such as these with regard to insect feeding resistance have led to the development and release of a number of commercial transgenic plants. The most prevalent types of commercial Bt crops are currently maize and cotton with many commercial lines available (James, 2012).

Expression of *Cylas*-active *Bacillus thuringiensis* (Bt) Cry proteins in sweetpotato could also provide an effective control strategy and seven coleopteran active Bt Cry proteins were incorporated into diet and toxicity data were generated against neonate *C. puncticollis* and second-instar *C. brunneus* (Ekobu *et al.,* 2010). All Bt Cry proteins tested had toxicity greater than the untreated control. Cry7Aa1, ET33/34, and Cry3Ca1 had LC_{50} values below 1µg/g diet against both species.

Chapter 4 of this thesis showed that high levels of field resistance showed low weevil damage and oviposition in laboratory bioassay experiments and these varieties had high concentrations of hydroxycinnamic acids esters (hexadecyl, heptadecyl, octadecyl, and 5-*O*-caffeoylquinic acid) on the root surface, while the susceptible varieties had low hydroxycinnamic acid esters levels and high weevil damage. The same hydroxycinnamic acid esters were reported to increase the mortality of weevil larvae (Stevenson *et al*., 2009). The potentially useful Cry7a proteins produced by Bt transformed varieties could have interactive effects with the natural defence components hydroxycinnamic acid esters that could complement their activities when expressed in cortex and may produce complementary effects if used together in roots. Alternatively their complementary use could reduce the likelihood of pests developing Bt resistance which otherwise can happen rapidly in insects (Tabashnik *et al*., 1997; Griffiths and Aroian, 2005; (Bravo and Soberon, 2007). Similarly they could also have negatively interactive effects so may counteract their benefits; however, this is an area of study that is surprisingly poorly studied. Therefore the objective of this study was to evaluate the interactive effects of hydroxycinnamic acids found occurring naturally in sweetpotato and Bt proteins that might be used in the transformation of sweetpotato for weevil resistance.

7.2. MATERIALS AND METHODS

7.2.1. Chemicals

NASPOT1 sweetpotato flour, agar and other ingredients and Bt protein Cry7Aa1 was kindly provided by Professor W.J. Moar, Auburn University, USA (now at Monsanto). The hydroxycinnamic acid esters used were hexadecylcaffeic acid and hexadecylcoumaric acid, octadecylcoumaric acid and were kindly synthesised as described previously (Stevenson *et al.*, 2009) and provided by Professor David Hall, (NRI, University of Greenwich) while 5-O-caffeoylquinic acid was obtained from Sigma Aldrich (Gillingham, Dorset, UK).

7.2.2. Preparation of diet

Diet was prepared for the bioassays following procedures developed by Ekobu *et al.* (2010). Agar (40g) was measured using Mettler PM2000 and was dissolved in 900mls of distilled water in a 1000 ml conical flask and boiled. A magnetic stirrer was placed in the bottom of the conical flask to keep on stirring the mixture during boiling. A heat temperature of 200-300ºC was used to fasten the boiling process as agar boils at 100ºC. The boiled mixture was cooled to 55ºC before mixing other ingredients. Other ingredients measured were sweetpotato powder (180g), casein (24g), cellulose (16g), sucrose (40g), yeast (10g), salt mixture (3.0 g) and ascorbic acid (2.0g). These ingredients were added to the boiled mixtures and blended to obtain uniform mixtures using a magnetic stirrer place on a non-heat shaker.

Additional ingredients: B-vitamin mixture (40mg), choline chloride (400mg), inositol (320mg), cholesterol/stigmasterol (640mg), potassium sorbate (600mg),

tetracycline (200mg) and methylhydroxybenzoate (675mg) was mixed in 20 ml of 100% ethanol by stirring on non-heat shaking equipment at NaCRRI tissue culture laboratory. The ethanolic mixture was added to agar mixture and blended for 10 minutes. Ninety grams (90g) of the diet was poured in the petri dish and allowed to solidify.

A stock solution of pure compounds was prepared by weighing 10mg of hydroxycinnamic acids and dissolving in 10 ml of acetone to make 1mg/ml of solution. The solution was serially diluted to make 0.1 and 0.0l ng/μL solution for the experiment. Refrigerated Bt-cry protein was prepared by diluting with water to obtain 1 µg/g that was used in the study, the effective concentration reported by Ekobu *et al.*, (2010). This was added into the diet to solidify inside the diet.

Seven treatment combinations were used in the experiment as follows:

  a) Diet only
  b) Diet + 1 ml of acetone
  c) Diet + 1 ml Bt-protein (1ppm)
  d) Diet + 1 ml of 0.1 ng/µL hydroxycinnamic acid ester
  e) Diet + 1 ml of 0.01 ng/µL hydroxycinnamic acid ester
  f) Diet + 1 ml of 0.1 ng/µL hydroxycinnamic acid ester + 1 µg/g Bt-protein
  g) Diet + 1 ml of 0.01 ng/µL hydroxycinnamic acid ester +1 µg/g Bt-protein

7.2.3. Bioassay procedure

Ten first instar larvae of *C. puncticollis* (8 days) and second instar larvae of *C. brunneus* (11 days) used in the experiment were initially obtained by allowing gravid female weevils to oviposit on the roots for 24 hours. The age of the weevils were recorded from the first day of incubation. After the diet had solidified, ten small burrows were excavated using spatula edge. One larva was placed into each diet

burrow with ten burrows on each petri dish. When the larvae were placed in the burrow the displaced diet was replaced on top to minimise desiccation. The diets were then covered with a disc of filter paper to absorb excess moisture from the diets and the lids were covered on top. The bioassay was left to stand for 15 days. The experiment was conducted at 25± 2 °C and 70 ± 10% Relative Humidity in a completely randomized design and repeated 4 times in four replications.

7.2.4. Data collection and analysis

The number of dead and surviving larvae was evaluated by observing no larval movement following the procedure of Ekobu *et al.* (2010) and Rukarwa *et al.* (2013a) and recorded. The percentage larval mortality was obtained by calculating the proportion of the dead larvae from the total number of larvae applied in the diet. The data was squared root transformed and analysed using generalized linear model one way analysis of variance using R-statistics. Mean percentage mortalities were generated and separation of means was done using Tukey's test. The mean percentage mortality of *Cylas puncticollis* and *C. brunneus* was combined and presented in figures.

7.3. RESULTS

7.3.1. Effects of hexadecyl caffeic acid and Bt toxins on sweetpotato weevil larvae

The larval mortality of sweetpotato weevils, *C. puncticollis* and *C. brunneus* were significantly different among the hexadecylcaffeic acid treatments applied on the diet compared to control (ANOVA, $P<0.001$, $F=6.14$ and $df=6,14$). *C. puncticollis* and *C. brunneus* larvae feeding on Bt toxin in diet suffered 56.7% and 57% mortality respectively (Figure 7.1). When a 0.1 ng/µL hexadecylcaffeic acid was incorporated with Bt toxins there was 80% mortality in both *C. puncticollis* and *C. brunneus* larvae (Figure 7.1). This level of mortality was significantly ($P<0.05$) higher than when both Bt toxins and the highest dose of hexadecylcaffeic acid treatments were individually applied (Figure 7.1) indicating there was additive effect of the two treatments on weevil mortality.

The low dose application of hexadecylcaffeic acid caused 56.9% mortality in *C. puncticollis* and *C. brunneus* larvae, respectively (Figure 7.1). The mortality of *C. puncticollis* and *C. brunneus larvae* caused by 0.01 ng/µL of hexadecylcaffeic acid was similar to the mortality of larvae of both weevil species caused by Bt-toxins alone in the diet (Figure 7.1). However, when Bt-toxin was incorporated with the low concentration of hexadecylcaffeic acid the resultant mortality was only 66.9% in the larvae of both *C. puncticollis* and *C. brunneus,* respectively (Figure 7.1). The 10% increase in the mortality in the larvae of both weevil species was not significantly different ($P<0.05$) from the mortality in either Bt toxins or low dose hexadecylcaffeic acid individually applied in the diet indicating that there was neither additive nor synergistic but no antagonistic effect of the two compounds.

Figure 7.1 Mean mortality (±SEM) of *Cylas puncticollis* and *C. brunneus* larvae fed on diets treated with hexadecylcaffeic acid and Bt toxins.

C16Cafflow; 0.01 ng/µL of hexadecylcaffeic acid treated diet: C16CafflowBt; 0.01 ng/µL hexadecylcaffeic acid and Bt treated diet: C16Caffhigh; 0.1 ng/µL hexadecylcaffeic acid treated diet: C16CaffhighBt; 0.1ng/µL and Bt treated diet: Bt; Bt treated diet.

7.3.2. Effects of hexadecylcoumaric acid and Bt-toxins on sweetpotato weevil larvae

Application of treatments in the diet led to significant differences in weevil larval mortality among the treatments (ANOVA, $P<0.001$, $F= 20.28$ and $df= 6,14$). The presence of Bt-toxins alone in the diet caused 57% death of the weevil larvae of both weevil species (Figure 7.2). Incorporating an additional high dose (0.1 ng/µL of hexadecylcoumaric acid resulted in an increase in mortality of *C. puncticollis* and *C. brunneus* larvae by 15.5% (Figure 7.2) above that recorded when feeding on Bt

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alone. However, when a similar dose of hexadecylcoumaric acid was incorporated in the diet with Bt toxin, it caused an increase of 4% mortality in the larvae of both *C. puncticollis* and *C. brunneus* compared to the mortality caused by high dose hexadecylcoumaric acid esters incorporated in the diet alone (Figure 7.2). This indicated that effectiveness of Bt toxins on larval mortality is compromised by increasing the concentration of hexadecylcoumaric acid.

The presence of a low dose (0.01 ng/µL) of hexadecylcoumaric acid alone caused 59% mortality in *C. puncticollis* and *C. brunneus* larvae (Figure 7.2). Incorporation of this low dose of hexadecylcoumaric acid and Bt-toxin, on the other hand, caused an increase of 12.5% mortality in the larvae of *C. puncticollis* and *C. brunneus* indicating that the combination of Bt and hexadecylcoumaric acid is more potent when ingested by the larvae than when 0.01 ng/µL was applied alone in the diet (Figure 7.2). The combined effect of low dose of hexadecylcoumaric acid and Bt was additive but incorporation of higher dose of hexadecylcoumaric acid esters and Bt treatment was weakly additive.
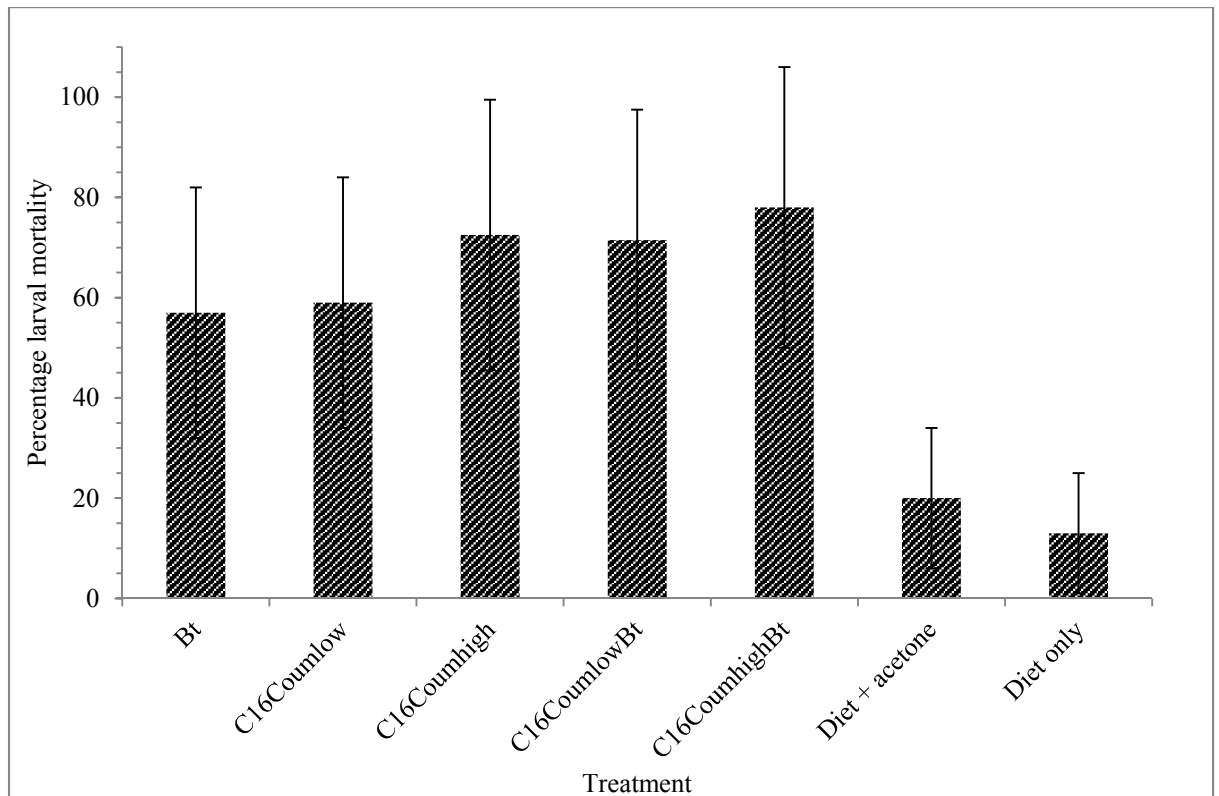
Figure 7.2 Mean mortality (±SEM) of *C. puncticollis* and *C. brunneus* larvae fed on diets treated with hexadecylcoumaric acid and Bt toxin.

C16Coumlow; 0.01 ng/µL of hexadecylcoumaric acid treated diet: C16CoumlowBt; 0.01 ng/µL hexadecylcoumaric acid and Bt treated diet: C16Coumhigh; 0.1 ng/µL hexadecylcoumaric acid treated diet: C16CoumhighBt; 0.1ng/µL hexadecylcoumaric acid and Bt treated diet: Bt; Bt treated diet.

7.3.3. Effects of octadecyl coumaric acid and Bt-toxins on sweetpotato weevil larvae

When octadecylcoumaric acid was incorporated in the diet this significantly affected the larval survival (ANOVA, P<0.001, F=12.1 and df=6,14). Bt-toxins alone in the diet caused 57% mortality of larvae of both *C. puncticollis* and *C. brunneus* (Figure 7.3). A high dose (0.1 ng/µL) of octadecylcoumaric acid in the diet increased the mortality with 66.5% both *C. puncticollis* and *C. brunneus* larvae recorded dead (Figure 7.3). When a high dose of octadecylcoumaric acid was incorporated in the

diet with Bt-toxins there was a resultant 75% mortality in both C. *puncticollis* and *C. brunneus* larvae, respectively (Figure 7.3). The difference in larval mortality when Bt-toxin was combined in the diet was, however, not significantly different (P>0.05) from that mortality caused by high dose (0.1 ng/µL) octadecyl coumaric acid (Figure 7.3) indicating that there was no additive or synergistic effect of the two treatment together at higher level of octadecylcoumaric acid.

The lower dose (0.01 ng/µL) application of octadecylcoumaric acid in the diet caused 60% mortality in both *C. puncticollis* and *C. brunneus* larvae (Figure 7.3). Incorporation of both low dose octadecylcoumaric acid and Bt-toxins in the diet caused 73.5% larval mortality in both weevil species (Figure 7.3). The difference in larval mortality of both *C. puncticollis* and *C. brunneus* in the combined treatment of Bt-toxins and low dose octadecylcoumaric acid was more than the resultant increase in mortality when the higher dose octadecylcoumaric acid was mixed with Bt-toxins in the diet implying activity of Bt is compromised in varieties with higher concentrations of octadecylcoumaric acid esters (Figure 7.3).
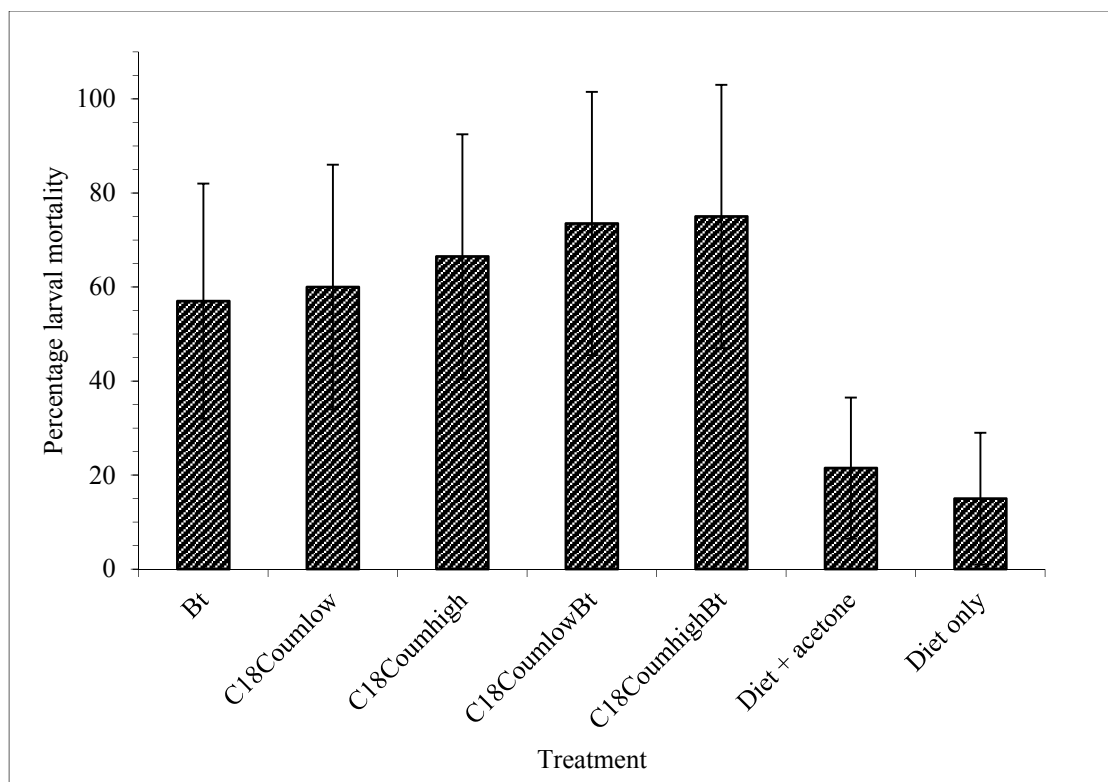
Figure 7.3 Mean mortality (±SEM) of *C. puncticollis* and *C. brunneus* larvae feeding on diets treated with octadecylcoumaric acid and Bt-toxins.

C18Coumlow; 0.01 ng/µL of octadecylcoumaric acid treated diet: C18CoumlowBt; 0.01 ng/µL octadecylcoumaric acid and Bt treated diet: C18Coumhigh; 0.1 ng/µL octadecylcoumaric acid treated diet: C18CoumhighBt; 0.1ng/µL octadecylcoumaric acid and Bt treated diet: Bt; Bt treated diet.

7.3.4. Effects of 5-*O*-caffeoylquinic acid and Bt-toxins on sweetpotato weevil larvae

The larval survival of sweetpotato weevil was significantly affected by the 5-*O*-caffeoylquinic acid treatment applied on the diet (ANOVA, $P<0.001$, $F=5.14$ and df $=6,14$). Bt-treated diet resulted in 56.8% mortality of the larvae of both *C. puncticollis* and *C. brunneus* (Figure 7.4). However, the higher dose of 5-O-caffeoylquinic acid (0.1 ng/µL) in the diet caused 66.9% mortality of the larvae of both weevil species. When a high dose (0.1 ng/µL) of 5-O-caffeoylquinic acid was

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incorporated with Bt- cryproteins into the diet, 70% mortality recorded for both *C. puncticollis* and *C. brunneus* larvae.

Application of the lower dose (0.01 ng/µL) of 5-O-caffeoylquinic acid in the diet resulted in 53.2% death in *C. puncticollis* and *C. brunneus (*Figure 7.4)*. When Bt-toxins were incorporated with low dose (0.01 ng/µL) 5-O-caffeoylquinic acid into the diet, 63.2% mortality was observed in both *C. puncticollis* and *C. brunneus* larvae (Figure 7.4). These increased mortality recorded as a result of incorporating the two treatments was high but not significantly ($P \geq 0.05$) different from when Bt, high and lower doses of 5-*O*-caffeoylquinic acids were applied individually.
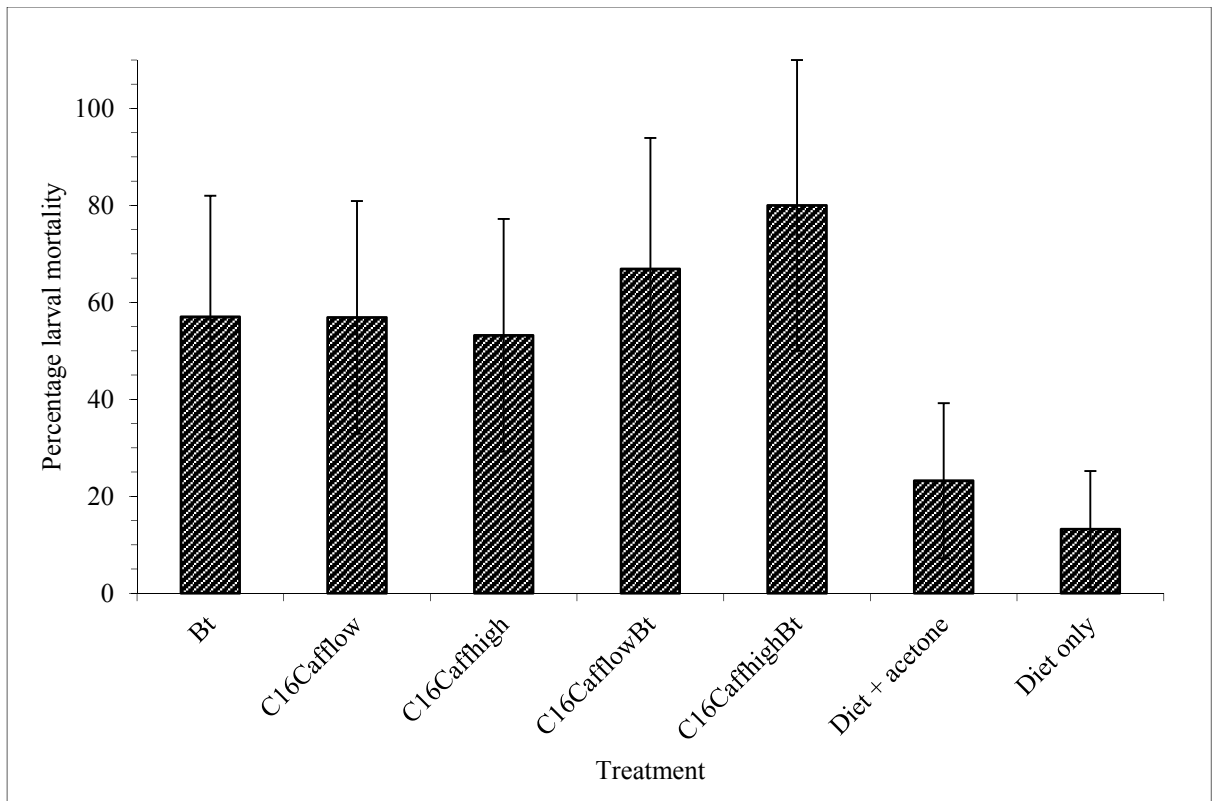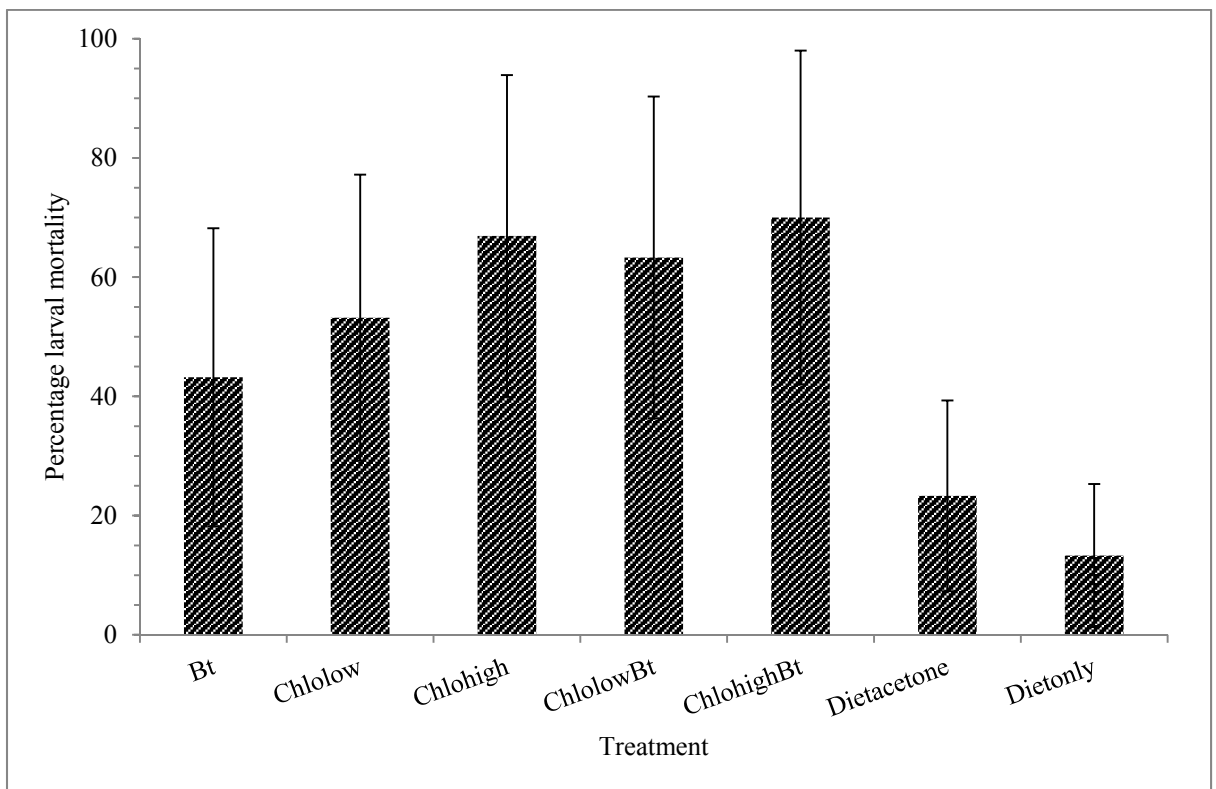

Figure 7.4 Mean mortality (±SEM) of C. *puncticollis* and *C. brunneus* larvae feeding on diets treated with 5-O-caffeoylquinic acid and Bt toxins.

Chlolow; 0.01 ng/µL of 5-O-caffeoylquinic acid treated diet: ChlolowBt; 0.01 ng/µL 5-O-caffeoylquinic acid and Bt treated diet: Chlohigh; 0.1 ng/µL 5-O-caffeoylquinic acid treated diet: ChlohighBt; 0.1ng/µL 5-O-caffeoylquinic acid and Bt treated diet: Bt; Bt treated diet.

7.4. DISCUSSION

The larval survival of sweetpotato weevil was significantly (P<0.001) affected by hydroxycinnamic acid esters treatment and Bt-toxin applied on the diet. This study examined whether there was an interactive effect of hydroxycinnamic acid esters and Bt proteins on sweetpotato weevil larval survival. The inclusion of hydroxycinnamic acids in the diet caused significant mortality in the larvae of both weevil species in all experiments compared to control while Bt-toxins also caused larval mortality of both *C. puncticollis* and *C. brunneus*. Earlier work reported resistance against sweetpotato weevils and was linked to latex chemicals (Snook *et al.*, 1994) but with little evidence for their action on weevil biology. This has been largely attributed to absence of a suitable artificial diet capable of supporting the development of weevil larvae to adulthood (Stevenson *et al.*, 2009), facilitating incorporation of synthesized root chemical in bioassays. The observed successful development and increased survivorship of the larvae in controls compared to treated diets of both hydroxycinnamic acid esters and Bt toxins suggests an active host plant resistance mechanism with potential uses in sweetpotato breeding can be developed and reiterates the biological activity of these compounds as reported earlier (Stevenson *et al.*, 2009).

In Chapter 4 of this thesis, resistant sweetpotato varieties were found to have higher quantities of hydroxycinnamic acid esters on the root surfaces compared to minimal quantities of these compounds on the surfaces of susceptible varieties indicating that when weevils encounter these compounds at the root surfaces, there is resultant larval mortality, reduced development and thus resistance. This observation relates with previous studies of Snook *et al.*, (1994), which reported

differences in the presence of coumarates with variation in susceptibility of different sweetpotato cultivars to *C. formicarius*. According to Stevenson *et al*., (1993), the biological activity of caffeic acid esters may be due to their dihydroxy phenolic groups which bind covalently to proteins so can interfere with digestive processes or reduce availability of proteins in food and thus reduces insect development. It has also been suggested earlier by Jones (1986) that phenolic compounds limit the digestion of carbohydrates although the metabolic role of phenolics in the plant remained unclear. Studies by Gill *et al*., (1992) indicate that Bt also acts by interfering with insect digestion through creation of holes in the insect larval gut membrane leading to leakage of gut content and eventually the death of susceptible insects.

Work on interaction of Bt-toxins and phytochemicals have been studied in other crops and pests with significant adverse effects on the larvae. Ludlum *et al* (1991) found that *Bacillus thuringiensis* subsp.*kurstaki* (BTk) incubated with chlorogenic acid and polyphenol oxidase and fed to larval *Heliothis zea* was more toxic than untreated BTk. Similar but less dramatic results arose when BTk was incubated with polyphenol oxidase alone.

Navon *et al* (1993) reported that leaf feeding by the larvae of *Heliothis virescens* and larval survival and weights decreased with an increase in Bt concentration. Antifeedant effects of acid exudates reduced food consumption and hence might reduce the efficacy of Bt sprays on insect-resistant chickpea genotypes or Bt-transgenic chickpeas, although the combined effect of plant resistance based on organic acids, and Bt had a greater effect on survival and development of *H. armigera* than Bt alone. This study agrees with the findings of Navon *et al*., (1993) only for the interaction of Bt and low dose (0.01 ng/µL) of the hydroxycinnamic acid

when applied together in the diet. Higher concentrations of the hydroxycinnamic acid esters applied in the diet did not result to significant mortality. This signify that transforming sweetpotato varieties with this high concentration of hydroxycinnamic acids will compromise the activity of Bt toxins on weevil larvae mortality.

Similar work by Surekha *et al*., (2011) indicated that biological activity of *Bt* was lower on artificial diets with leaf or pod powder of resistant chickpea genotypes, which might be because of a low intake of *Bt* toxins due to antifeedant effects of acid exudates in the chickpea or reduction in biological activity of *Bt* due to the interaction of biochemical constituents in chickpea with the *Bt* toxins. Larval survival, larval and pupal weights, pupation and adult emergence were significantly lower on diets with leaf or pod powder of the *H. armigera* resistant genotypes than on the susceptible check. Chickpea genotypes with resistance to *H. armigera* acted in concert with *Bt* to cause adverse effects on the survival and development of this insect. This result concurs with our findings on interaction of Cry7Aa1 and hydroxycinnamic acid esters although it indicated neither additive nor synergistic interaction but no antagonism on sweetpotato larvae mortality.

Work by Rukarwa *et al* (2013a) developed ten transgenic events expressing Cry7Aa1, Cry3Ca1, and ET33-34 proteins for weevil resistance but none were effective against *C. puncticollis* Since the concentration of hydroxycinnamic acid esters decreased with deeper tissue layer, incorporation of Bt-toxins through genetic transformation being done by CIP would complement the resistance to sweetpotato weevil by knocking down weevil larvae as the toxins are ingested if the root expression of the toxins can only be quantified.

CHAPTER 8

CHEMICAL ANALYSIS AND BIOLOGICAL EVALUATION OF A CROSS BETWEEN WEEVIL-RESISTANT NEW KAWOGO AND WEEVIL-SUSCEPTIBLE BEAUREGARD

8.1. INTRODUCTION

The concealed feeding behaviour of sweetpotato weevils makes their management difficult (Nottingham and Kays 2002; Odongo *et al.*, 2003). Host plant resistance is a major component of any integrated pest management (IPM) programme but the development of weevil resistant varieties through classical breeding has not been successful in SSA (Talekar, 1987). Recently Stevenson *et al.*, (2009) and Anyanga *et al.*, (2013) evaluated weevil-resistant sweetpotato genotypes along with susceptible cultivars to identify the biochemical basis of sweetpotato weevil resistance. There were differences in the concentrations of hydroxycinnamic acid esters among the screened genotypes with the highest concentration observed in the resistant variety New Kawogo, a Ugandan landrace variety. The five hydroxycinnamic acid esters associated with sweetpotato weevil resistance are shown in Figure 4.1 in Chapter 4 where the constitutive production of these hydroxycinnamic acid esters was proposed as the chemical basis of sweetpotato weevil resistance. Hexadecyl, octadecyl, and eicosylcoumaric acid esters were associated with resistance to *C. formicarius* in the US sweetpotato genotypes (Data *et al.*, 1996; Snook *et al.*, 1994) but never proved through bioassay as has been

achieved in the present study. To fully understand the potential of this mechanism in breeding, however, it is necessary to evaluate inheritance in a segregating population of a cross between a resistant and a susceptible variety, so that the resistance can be combined with the best other agronomic traits.

The objective of this study was therefore to determine the heritability of resistance reported in New Kawogo through a study of the segregating population from a cross with Beauregard, a US weevil-susceptible variety that has been mapped (Cervantes *et al.*, 2008).

8.2.   MATERIALS AND METHODS

The study evaluated an F1 progeny population of 287 offspring from a bi-parental cross between New Kawogo (NK) and Beauregard (B). The crossing was done at the National Crops Resources Research Institute (NaCRRI), Namulonge, Wakiso district, 0º 32'N, 33º35'E, 1,160 m.a.s.l. New Kawogo (female) is a sweetpotato weevil resistant (SPW), Sweetpotato Virus Disease (SPVD) tolerant, high dry matter content and white fleshed released Ugandan landrace (Mwanga *et al.*, 2001; Mwanga *et al.*, 2003; Mwanga *et al.*, 2007; Stevenson *et al.*, 2009). Beauregard (male) is a weevil and SPVD susceptible, low dry matter content and orange-fleshed popular US cultivar (Rolston *et al.*, 1987).

8.2.1. Evaluation of weevil damage in field experiments at three locations in Uganda.

The 287 progeny population and the two parents were evaluated for SPW resistance at three field sites and across two seasons in Uganda during 2012. All the experiments were planted in a randomized complete block design (RCBD) with 3 replications per genotype per site on a 1.5 m ridged plots following the procedure used in Chapter 3 of this thesis. Five plants were planted in each experimental plot at a spacing of 30 cm between plants and the ridges were separated by 1.0 m walk way. The trials were conducted at: National Crops Resources Research Institute (NaCRRI), Namulonge: 0º 32'N, 33º35'E, 1,160 m.a.s.l. National Semi-Arid Resources Research Institute (NaSARRI), Serere: 1º 32'N, 3º 27'E, 1,085 m.a.s.l. and Ngetta Zonal Agricultural Research and Development Institute (NgeZARDI): 2º 202' N, 33º 62' E, 1,080 m.a.s.l.

The trials were artificially infested with 10 weevils per plot (7 female and 3 male sweetpotato weevils each of *C. puncticollis* and *C. brunneus*) at 90 days after planting to increase the weevil population and ensure an effective exposure using procedures of Muyinza *et al*., (2012). The weevils used in the trials were reared as described in Chapter 2. During the first season (2012A), the trials were planted in June and harvested in November, 2012. The second season trails (2012B) were planted in November and harvested in May, 2013. The harvesting of both trials was done at 5-6 months after planting to allow ample time for sweetpotato weevil infestation to occur.

8.2.2. Evaluation of weevil stem damage in field experiments at 3 locations in Uganda

Plant vine weight, number of infested roots was recorded at harvest. SPW root damage was assessed by estimating the percentage damage inflicted on roots and basal damage by cutting the first 10−15 cm of two of the three vines per plot following the methods used in Chapter 3 of this thesis for evaluating root and stem base damage.

8.2.3. Phytochemical analysis of the root samples of the segregating population of New Kawogo and Beauregard.

Fresh roots of 289 different progenies obtained from the field in NgeZARDI, NaSARRI and NaCRRI were washed by dipping in water to loosen and remove soil particles and left to dry by air circulation at room temperature and cut transversely into 2-3 root discs for chemical analyses. Phytochemical analysis of root samples was done following the methodology described in Chapter 4 section 4.2.1.2.

8.2.4. Evaluation of feeding and oviposition bioassay

Weevil feeding and oviposition study was conducted on the genotypes of the segregating population of New Kawogo and Beauregard by infesting the roots with *C. puncticollis*. Clean roots were obtained from the field, washed by dipping in water to remove soil and dried at room temperature. The roots were placed in plastic tins and infested with ten two weeks old (gravid) females for 24 hours. The weevils used for this study were reared on sweetpotato variety NASPOT 1. The weevils were

removed after 24 hours and the number of feeding holes were counted and recorded. The eggs laid in the roots during the 24-hour feeding period were left to incubate until emergence. The number of adult emerging were counted from 25th day after infestation and continued after every five days until 50th day after infestation.

8.2.5. Data collection and analysis.

Data was collected from the field evaluation, laboratory bioassay and phytochemical analysis. All data analysis was performed on just 284 different offspring and the parents. Three progeny were dropped because their field establishment was poor with 2-3 plots dead making them not suitable for robust analysis. The data for the two seasons were pooled together for analysis. Field data was pooled across seasons and locations. Analysis of variance (ANOVA) of sweetpotato field resistance data was conducted using R statistics by the Research Method team of the McKnight Foundation. The genotypes were treated as fixed effects and block, site and season as random effects. The overall sweetpotato weevil damaged root and stem least significant (LS) means of parents and that of their progeny was compared. The genotype means were used to assess the level of transgressive segregation for sweetpotato weevil resistance.

For total hydroxycinnamic acid ester concentration, analysis of variance using linear and Pearson correlation analysis of field sweetpotato weevil infestation and hydroxycinnamic acid ester production was done using R-statistics.

8.3. RESULTS

8.3.1. Evaluation of weevil damage in field experiments at 3 locations in Uganda.

There was significant difference (P≤ 0.001) in the percentage sweetpotato weevil root infestation among genotypes of the segregating population. Twenty five of the genotypes had low storage root damage of below 10% weevil infested roots observed. The low storage root weevil infested genotype in this range included New Kawogo used as a resistant mother in the crossing indicating there was transgressive segregation (Figure 8.2). Beauregard, the susceptible father on the other hand, had mean storage root infestation of 100%. The rest of the genotypes had varying root infestation between that of New Kawogo and Beauregard (Figure 8.1). There was a significant spatial effect (P≤ 0.001) on sweetpotato weevil infestation of the genotypes as the mean sweetpotato weevil infestation was higher in NaSARRI than the mean root infestation at NaCRRI and Ngetta ZARDI. In fact, weevil root infestation in NaCRRI and Ngetta ZARDI were similar including their standard error of the means (Figure 8.2).
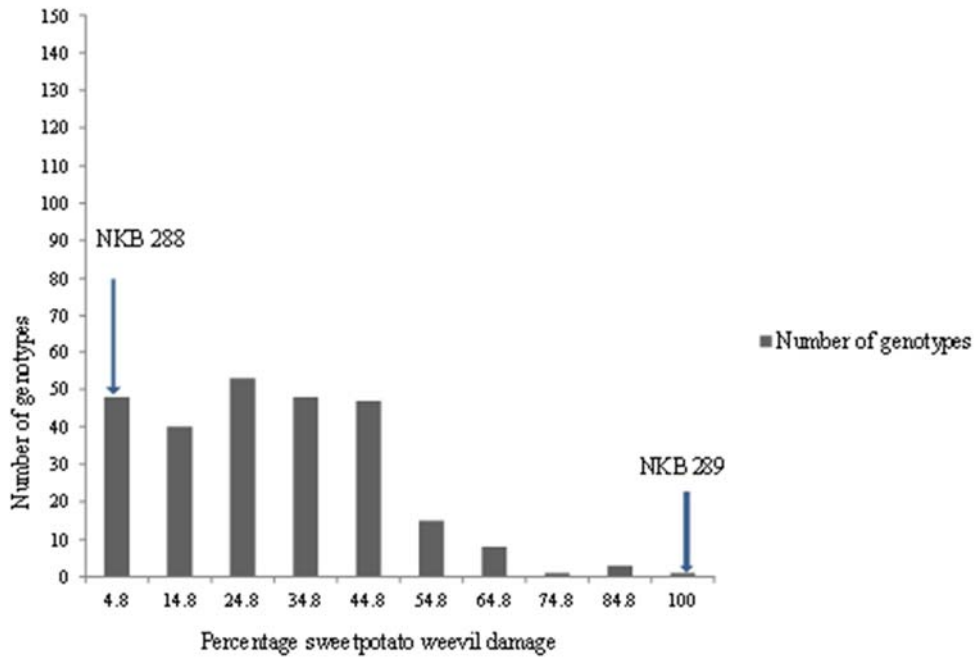
Figure 8.1. Percentage sweetpotato weevil root infestation in the segregating population of New Kawogo (NKB288) and Beauregard (NKB289).
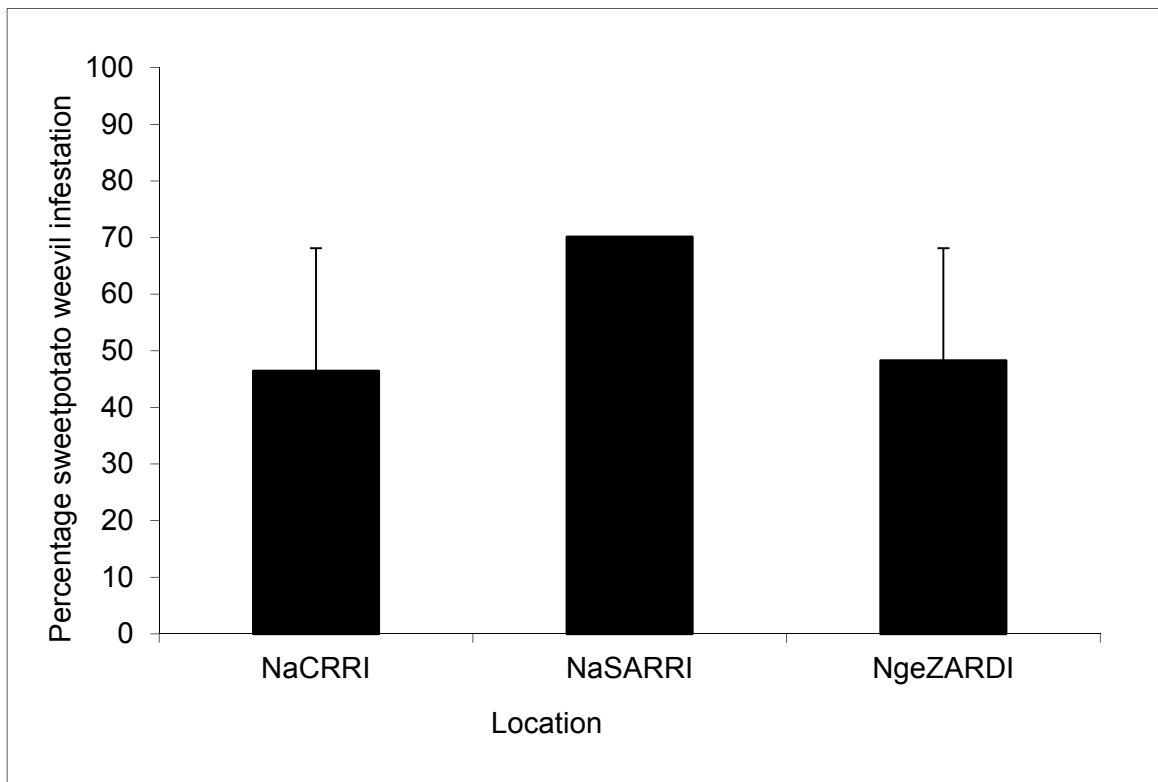
Figure 8.2 Mean percentage (± SEM) sweetpotato weevil root infestation in 3 locations in Uganda.

The effect of genotype by season interaction was significant (P≤ 0.001) on sweetpotato weevil root infestation. Mean sweetpotato weevil root infestation was significantly higher (P≤0.05) in the experiment planted in season 2 than the infestation that was observed in the experiment planted in the first season of the study (Figure 8.3).
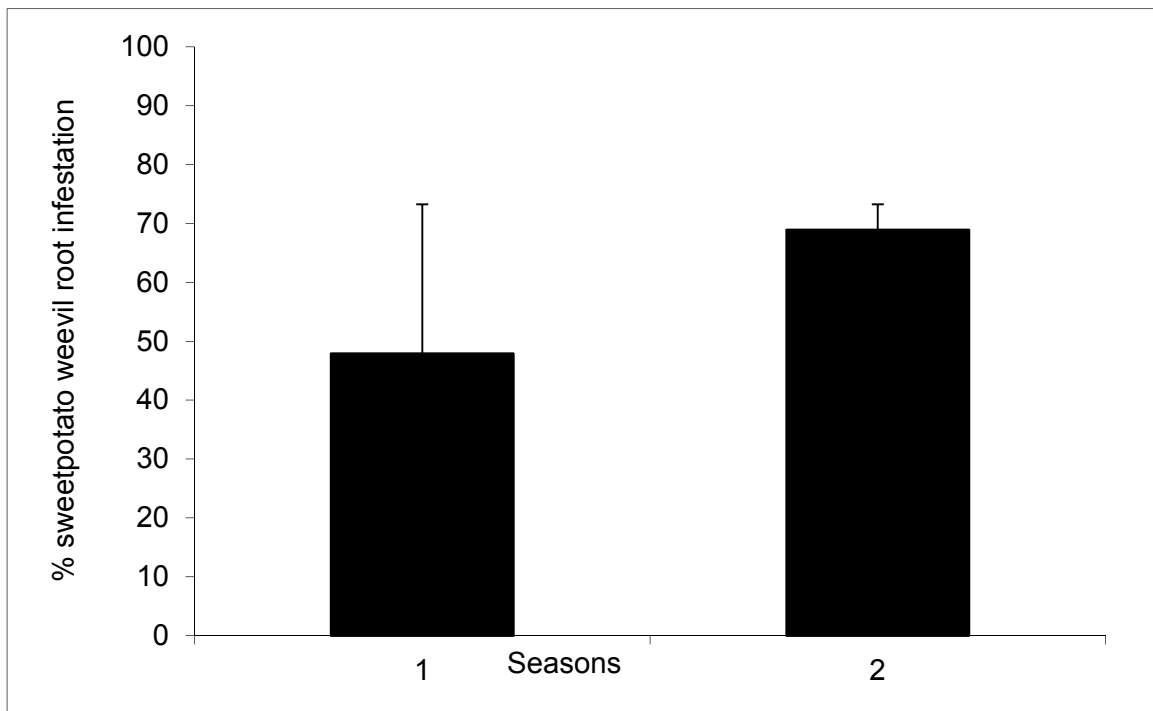
Figure 8.3 Mean percentage (± SEM) sweetpotato weevil root infestation across seasons

The genotype x environment (GXE) interaction effect was also significant (P≤ 0.001) on the weevil damage on the stem portion of the sweetpotato vine indicating that weevil stem damage is dependent on the season. The mean sweetpotato weevil stem damage score was higher in the experiment planted in November and harvested May (season 2) than it was on the experiment planted in June and harvested in November (season1) (Figure 8.4). This might be explained by the fact that the second season experiment planted at the close of the dry season exposed

the plants to heat stress earlier in the crop's growth cycle causing poor establishment and reduced plant's resilience to weevil attack with similar effects observed on the storage root infestation shown in Figure 8.3 above.
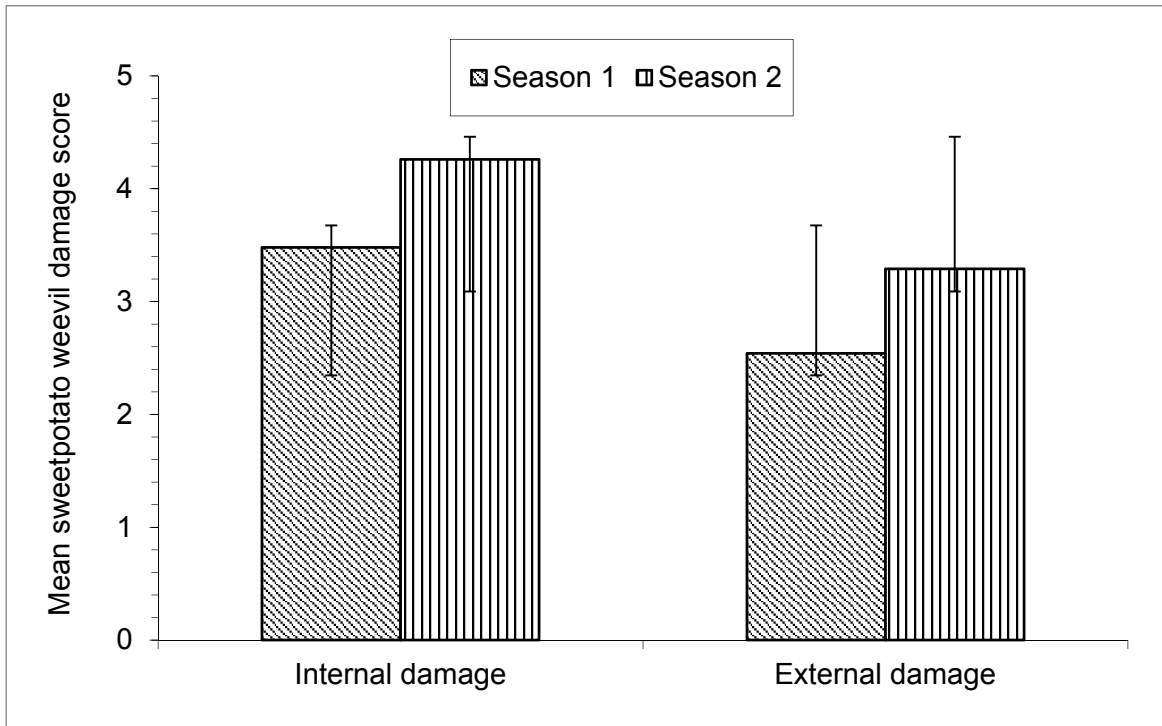
Figure 8.4 Mean sweetpotato damage score (±SEM) on the internal and external stem base of sweetpotato vine in two seasons.

There was a significant difference ($P \leq 0.001$) in sweetpotato weevil stem damage on the sweetpotato vine in the 3 locations. The mean sweetpotato stem damage score on the sweetpotato vine was higher in NaSARRI than it was in NaCRRI and Ngetta ZARDI (Figure 8.5).
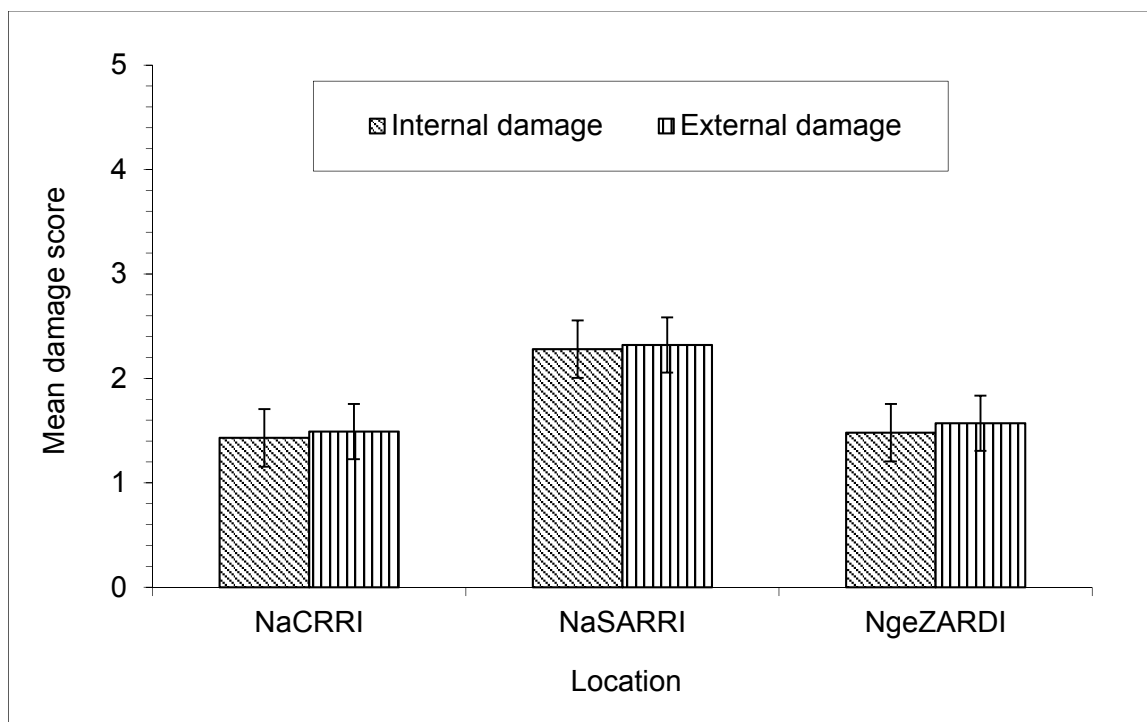
Figure 8.5 Mean sweetpotato weevil damage (±SEM) on the stem base portion of sweetpotato vine recorded at 3 locations.

8.3.2. Effect of sweetpotato weevil in feeding and oviposition bioassay

The mean number of sweetpotato weevil feeding holes differed significantly (P≤0.001) on the root of the genotypes of the segregating population in the feeding and oviposition bioassay. Fifty seven genotypes had lower mean number of less than 20 feeding holes on the root (Figure 8.6). The mean number of *C. puncticollis* feeding holes on the roots of New Kawogo (NKB288) and Beauregard (NKB289) were 29 and 42 respectively and were higher than in some genotypes indicating that some genotypes were more resistant than New Kawogo (Figure 8.6). Progeny such as NKB152, NKB257, NKB72, NKB59, NKB260, NKB225, NKB108, NKB52, NKB158 and NKB279 are the ten genotypes that had the lowest overall *C. puncticollis* mean number of feeding holes indicating that there was transgressive

segregation in these genotypes for weevil resistance. The mean number of adult emergence was not significantly different (P≥0.05) at 25, 35, 40 and 45 days after infestation with *C. puncticollis* indicating that gravid females were able to feed and lay eggs on the root. This situation under study could be described as a "suicide" oviposition situation because the weevils selected for the study were already mated and gravid female. The eggs laid inside the root were capable of developing into larvae and finally emerge as adults. This observation of adults emerging from all the genotypes is not a surprise because in Chapter 4 of this thesis the concentration of hydroxycinnamic acid esters in the peeled root did not differ between New Kawogo and the control varieties. This indicates that resistant chemical compounds occurs on the sweetpotato root surface affected feeding and oviposition but once the eggs are laid inside the likelihood of their development to adults to occur is high.
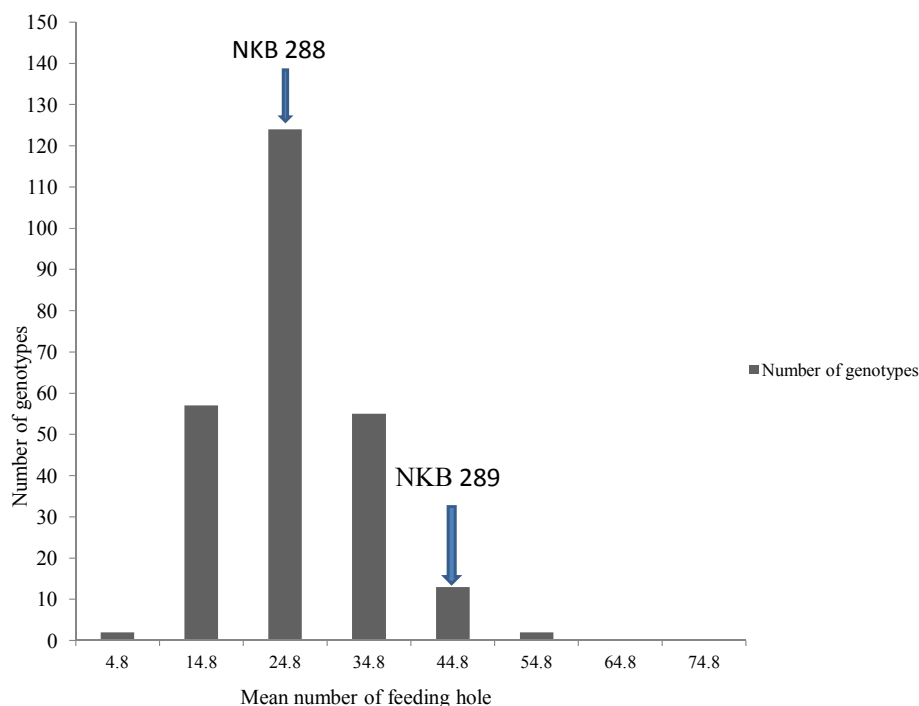
Figure 8.6 Mean number of feeding holes in a segregating population of New Kawogo (NKB288) and Beauregard (NKB289).

8.3.3. Phytochemical analysis of the segregating population.

There was significant difference (P≤0.001) in total hydroxycinnamic acids (HCA) among the genotypes (progeny and parents) of the segregating population. The mean total HCA hydroxycinnamic acid esters in New Kawogo and Beauregard were 282 ng/g and 70.4 ng/g, respectively. Genotype mean total HCA ester concentrations ranged from 4.9- 366.5 ng/g in progeny NKB175 and New Kawogo, respectively. The distribution of genotype mean total HCA ester concentration was skewed to the left and only one progeny NKB257 had higher total HCA ester concentration than New Kawogo, the resistant parent (Figure 8.7). Over 70% of the progeny had less than 100 ng/g mean total HCA ester concentrations; an indication of low levels of sweetpotato weevil resistance (Figure 8.7). There was significant but correlation between genotype total HCA ester concentrations and sweetpotato field infestation (r= 0.103, P=0.015) implying that other factors also contribute to resistance recorded in the materials tested. The 10 genotypes with highest mean total hydroxycinnamic acid ester concentration were: NKB257 (366.5 ng/g), NKB152 (357.5 ng/g), NKB108 (268.9 ng/g), NKB256 (254.0 ng/g), NKB265 (237.1 ng/g), NKB100 (228.8 ng/g), NKB258 (205.1 ng/g), NKB254 (185.5 ng/g), NKB59 (183.5 ng/g) and NKB60 (181.5 ng/g). On the other hand, the progeny that showed the lowest concentration of mean total hydroxycinnamic acid esters were: NKB175 (4.9 ng/g), NKB285 (5.0 ng/g), NKB182 (6.3 ng/g), NKB29 (8.1 ng/g), NKB223 (8.7 ng/g), NKB138 (8.8 ng/g), NKB195 (9.2 ng/g), NKB28 (10.3 ng/g), NKB8 (11.9 ng/g) and NKB68 (12.1 ng/g).

Out of the top ten best performing progeny for mean total hydroxycinnamic acid ester concentrations and sweetpotato root infestation, only five genotypes (NKB152, NKB257, NKB108, NKB59, and NKB60) showed high consistent performance on the basis of sweetpotato weevil root infestation and total hydroxycinnamic acid ester concentrations. Out of the 29 progeny that performed better than New Kawogo from the field weevil root infestation, only 9 had total hydroxycinnamic acid ester concentrations greater than 140 ng/g suggesting that hydroxycinnamic acids did not account for resistance in all genotypes. Another interesting observation made was that some progeny which were highly susceptible to sweetpotato weevil infestation in the field such as NKB80, NKB151, and NKB283 had high total hydroxycinnamic acid ester concentrations of 163.9 mg/g, 160.5 ng/g and 140.6 ng/g respectively.
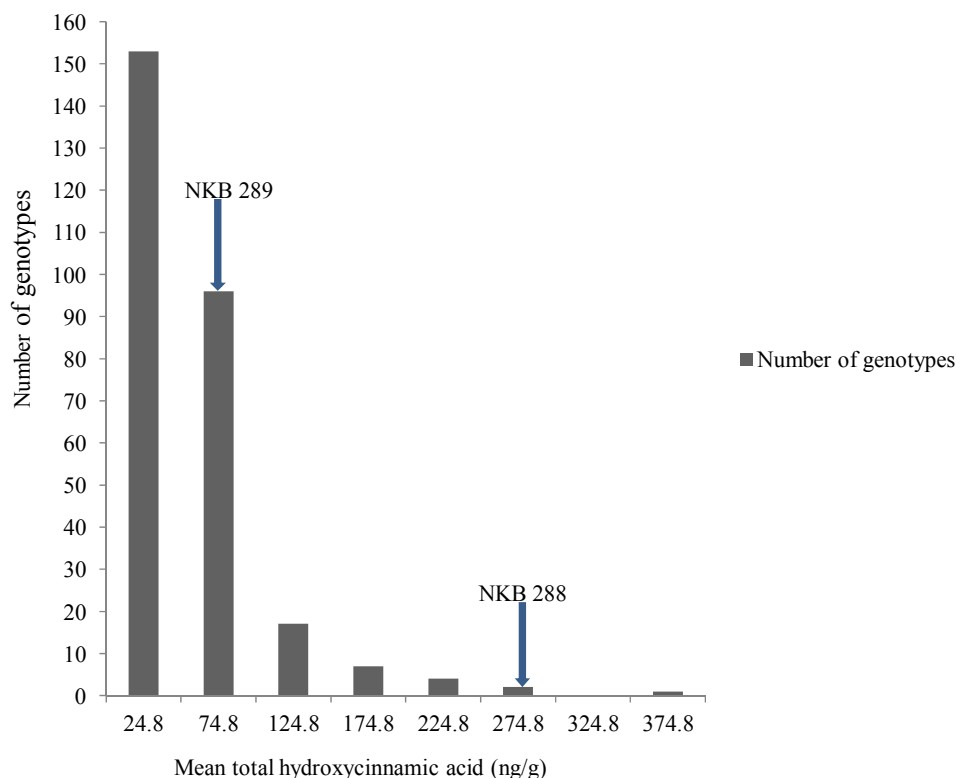
Figure 8.7. Mean total hydroxycinnamic acid (ng/g) in the segregating population of New Kawogo (NKB288) and Beauregard (NKB289).

The effect of location was significant ((P≤0.001) on total hydroxycinnamic acid esters produced on the sweetpotato roots. The mean total hydroxycinnamic acid was higher on sweetpotato roots from the experiments planted in NaSARRI an area with higher weevil infestation than on sweetpotato roots planted in NaCRRI and NgeZARDI respectively (Figure 8.8) indicating that while hydroxycinnamic acid esters are biosynthesized constitutively by sweetpotato its production may be moderated by environmental factors that also favours weevil development.
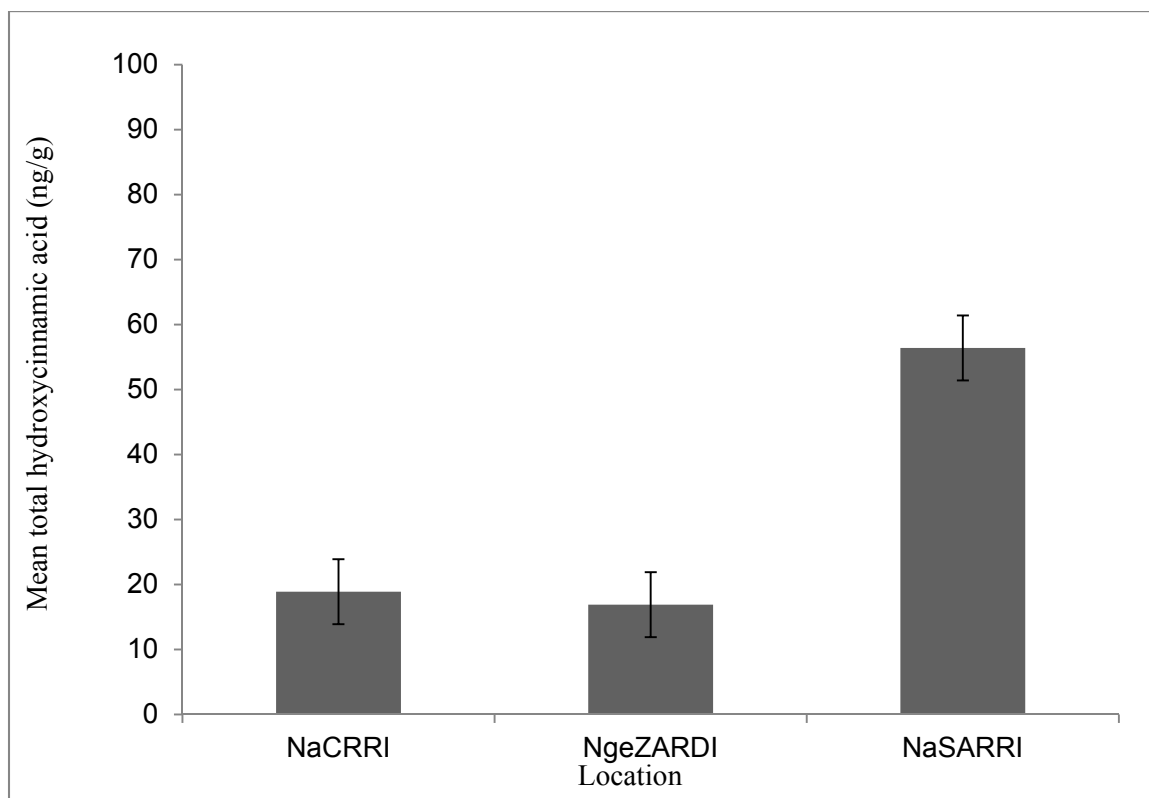
Figure 8.8 Mean total hydroxycinnamic acid (ng/g) (±SEM) in the roots a segregating population of New Kawogo and Beauregard across locations

8.4. DISCUSSION

Sweetpotato weevil damage escalates in sweetpotato fields at the onset of dry spells (Stathers *et al.*, 2003a). Our results relate with this study as there were significant Genotype by Environment (GxE) interactions. In the 3 locations where we conducted our field experiments, NaSARRI, located in a semi-arid part of Uganda had the highest mean weevil root infestation. Typically at NaSARRI, dry spells begin at the end of the each growing season and are characterized by high levels of soil cracks on the sweetpotato mounds or ridges as the storage roots matures. The soil cracks around the roots create entry routes for gravid weevils to lay eggs in exposed storage roots, which then hatch into larvae, the most destructive

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stage of the sweetpotato weevils (Jansson *et al*., 1987). Severe damage by larvae occurs through concealed feeding within the storage roots. This could have affected the results obtained for incubation bioassay for adult emergence as the weevils were presented with the roots in the tins with no significance difference recorded at 25, 35, 40, 45 and 50 days after infestation with *C. puncticollis.*

During drought the amount of water in the soil is low so less water will be absorbed and transported throughout the plant and the plants consequently dry up (Mao *et al*., 2004). But these conditions exacerbate sweetpotato weevil infestation and the transportation of plant defence phytochemicals throughout the plant may therefore, be hindered due to the low amount of water flowing through the vascular tissues (Ni *et al.,* 2009). This may explain why even the resistant variety New Kawogo can be infested by sweetpotato weevils if it remains in the field during such environmental conditions.

The parental genotypes in this study differed significantly in their mean weevil infestation across location and seasons. There was significant correlation between field stem infestation and field root infestation among the genotypes implying stem damage can be an indicator for screening sweetpotato varieties for resistance to sweetpotato weevil (Muyinza *et al*., 2007) and (Table 8.1).

Table 8.1 Correlation coefficients between sweetpotato weevil damage in the field, in laboratory studies and concentration of hydroxycinnamic acid esters for a segregating population of New Kawogo and Beauregard

Damage parameters	Total hydroxycinnamic acid esters concentration	Field root infestation	Field stem infestation
Field root infestation	0.103 (P≤ 0.0105)		
Field stem infestation	-0.318 (P≤ 0.05)	0.497 (P≤0.001)	
Mean number of feeding holes in lab study	-0.64 (P≤0.05)	0.501 (P≤ 0.05)	0.205 (P≤0.05)

New Kawogo was reported to have high levels of field resistance (Stevenson *et al*., 2009, Muyinza *et al*., 2012)), whereas Beauregard was reported to be highly susceptible to a host of insect pests including sweetpotato weevils (Roston *et al*., 1987).

Variation in the sweetpotato weevil infestation was observed in this population. Progenies that were more resistant than New Kawogo are potential candidates for selection and used as sources of genes for future population improvement. According to Grüneberg *et al*., (2009) variation in progeny from a bi-parental cross is attributed to the diverse nature of the parents selected from heterotic gene pool suggesting occurrence of transgressive segregation. Significant negative correlation between mean number of feeding holes in a laboratory study and total hydroxycinnamic acid ester concentrations was observed suggesting that varieties with higher concentrations of these compounds deter feeding by weevils (Table 8.1). A moderately high level of genetic diversity was observation in the New Kawogo x Beauregard population reported in this chapter, therefore supports the observation

of the large number of progeny exhibiting transgressive segregation for resistance to sweetpotato weevils.

The significant differences in the total hydroxycinnamic acid esters among the genotypes of the segregating population could be attributed to random re-assortment of alleles at multiple loci in sweetpotato genome for this trait in this cross. New Kawogo (female) was reported to have a high concentration of hydroxycinnamic acid esters in Chapter 4 and high level of field resistance to sweetpotato weevil in Chapter 3. Hydroxycinnamic acid esters was reported also to reduce feeding and oviposition in *C. puncticollis* and *C. brunneus* in feeding and oviposition bioassay in Chapter 5. Hydroxycinnamic acid esters were reported to have an effect on the mortality of sweetpotato weevils on artificial diet in a dose dependent response, which is the reason they were hypothesized to be the chemical basis of resistance in New Kawogo to sweetpotato weevils (Stevenson *et al*., 2009 and Anyanga *et al*., 2013).

The highly skewed distribution of mean concentration of total hydroxycinnamic acid esters could be an indication of a recessive inheritance but which qualitatively influence the biochemistry of sweetpotato and a trait that can be exploited in sweetpotato breeding. Further evaluations are required to confirm this possibility. Hydroxycinnamic acid esters have been shown to play key roles in the biosynthesis pathway of lignin, a key mode of plant defence against pathogenic attack and herbivory (Boerjan *et al*., 2003). Ramos *et al*., (2001) isolated single genes and were shown to encode the enzymes for lignin biosynthesis including hydroxycinnamate CoA ligase (4CL) in sugarcane. The mode of inheritance of hydroxycinnamic acid esters in sweetpotato needs to be understood for enhancing their application to sweetpotato weevil resistance through breeding.

Although for the whole population there was not a negative correlation between sweetpotato weevil root infestation and total hydroxycinnamic acid ester concentration, five genotypes (NKB152, NKB257, NKB108, NKB59, and NKB60) showed high and consistent performance on the basis of sweetpotato weevil root infestation and total hydroxycinnamic acid ester concentrations. These clones could be selected for evaluation for use as breeding lines in population improvement for development of sweetpotato weevil resistant varieties. The low sweetpotato weevil infestation in NKB72, NKB225, NKB52, NKB158 and NKB279 that had contrastingly very low hydroxycinnamic acid esters concentration could be attributed to other resistance mechanism different from hydroxycinnamic acid esters synthesis such as deep rooting and high sweetpotato vegetative cover. Sweetpotato varieties with deep rooting and high vegetative cover have been reported to sustain low sweetpotato weevil damage in SSA (Stathers *et al*., 2013a).

Significant positive correlation between mean number of sweetpotato weevil feeding holes and field root infestation and field stem infestation was observed (Table 8.1) indicating that varieties with high stem damage and root damage support weevil feeding and oviposition and therefore to weevil attack in the field. The low mean number of *C. puncticollis* feeding holes on clones NKB152, NKB257, NKB72, NKB59, NKB260, NKB225, NKB108, NKB52, NKB158 and NKB279 was consistent with low sweetpotato weevil damage in the field indicating that there was transgressive segregation in these genotypes for weevil resistance.

In conclusion, our study showed that sweetpotato weevil resistance is an active and quantifiable trait that can be inherited. Field resistance to sweetpotato weevil is significantly influenced by environment; therefore further evaluation of this population should be undertaken in multi-location sites for weevil resistance. The

transgressive segregating clones need to be selected and screened further to confirm the level of sweetpotato weevil resistance for use in population improvement. Even when a resistant variety is developed it will be important to support this by integrating alongside additional crop management practices to enhance the stability of resistance in farming.

CHAPTER 9

SUMMARY DISCUSSIONS, CONCLUSIONS AND

IMPLICATION FOR FUTURE SWEETPOTATO WEEVIL

RESEARCH FOR SSA

9.1. SUMMARY

The main goal of sweetpotato research is the development of high-yielding, nutritious varieties resistant to the numerous biotic constraints but that continue to meet consumer demand. This is important in enhancing food security, nutrition and income among the resource poor farmers whose livelihoods are dependent on sweetpotato. However, prevalence of sweetpotato weevils has hampered sweetpotato improvement and a lack of understanding of the genetic mechanism of resistance has also impedes effort in the development of weevil resistant varieties through breeding. This study was conducted to understand and enhance the application of phytochemical mediated resistance for selection and use in future sweetpotato improvement program through 5 experimental chapters.

Chapter 3, reported the evaluation of the response of sweetpotato landraces and improved varieties to controlled field infestation and damage by sweetpotato weevils. The trial was conducted for two seasons at the National Semi-Arid Resources Research Institute (NaSARRI) and bioassay at National Crops Resources Research Institute (NaCRRI) respectively.

Earlier reports by farmers that one of these varieties, New Kawogo (Mwanga *et al*., 2001) was resistant were supported by the results of the field study but also indicated that the mechanism was not based on escape which had earlier been proposed as the likely principal mechanisms (Stathers *et al*., 2003a). This was because the resistance was measurable under laboratory conditions indicating the mechanism was independent of field conditions that might otherwise facilitate access through soil cracking so was an active mechanism. Similar feeding responses by *Diabrotica* species on different cultivars of sweetpotato have been reported (Jackson and Bohac, 2007) suggesting a quantitative resistance to this pest that could be related to the effect reported here for *Cylas* spp. This was corroborated by the fact that when presented in a no-choice situation *C. puncticollis* still laid significantly fewer eggs on the root cores of the resistant variety New Kawogo than all other cultivars. This demonstrated that sweetpotato weevils were able to selectively feed on sweetpotato substrates, with the levels of acceptability of these materials determined by their biochemical composition. This contrasted with previous studies (Stathers *et al*., 2003b) which attributed resistance in sweetpotato to 'escape' as a result of deep rooting thus supporting the hypothesis that improved sweetpotato varieties and landraces respond differently to *C. puncticollis* and *C. brunneus* infestation.

It was further shown that even when New Kawogo, the resistant variety was exposed to weevils in the laboratory there was less preference for oviposition and feeding in choice and no choice bioassay. This provided more evidence for an active resistance against *C. puncticollis* and *C. brunneus.*

In Chapter 4 plant chemicals were shown to vary among improved and landraces and also varied in their susceptibility to *C. puncticollis* and *C. brunneus* suggesting that phytochemicals might mediate resistance.

Six hydroxycinnamic acid esters: hexadecylcaffeic acid, hexadecylcoumaric acid, heptadecylcaffeic acid, octadecylcaffeic acid and octadecylcoumaric acid and 5-O-caffeoylquinic acid were identified from the sweetpotato root surface that was reported earlier. The concentration of the individual compounds varied among the varieties, but overall, total hydroxycinnamic acid esters were highest in the resistant varieties particularly New Kawogo.

Earlier work identified hexadecyl, octadecyl, and eicosyl esters of coumaric acid that were reportedly associated with resistance to SPW, but in these earlier studies the compounds were not tested directly against weevils (Snook *et al*. 1994) while caffeoyl and coumaroyl esters are reported to reduce development of larvae and so possibly contribute to resistance Stevenson *et al*. (2009). The present work, however, adds to this earlier work by showing that the hydroxycinnamic acid esters previously identified do not differ among varieties in the cortex but do so on the root surface and are present at higher concentrations on the surface of resistant varieties. Furthermore, when these compounds are applied to roots of varieties that were shown in chapter three to be susceptible (notably NASPOT 1) the compound led to reduced feeding and oviposition. It is likely that the combined effects of these compounds on different life stages of SPW explain the observed resistance in the Ugandan land races. The presence of 5-O-caffeoylquinic acid was also shown to have similar effects including reduced feeding and oviposition so this compound also contributes to the field and laboratory effects and supports earlier reports that caffeoylquinic acids were associated with defence in sweetpotato (Harrison *et al*.,

2003). Our study characterised the root surface compounds and clearly provided evidence of qualitative and quantitative variation in the chemistry of sweetpotato varieties. It also provided evidence for the existence of a direct link between chemical mediation and levels of acceptability by *C. puncticollis* and *C. brunneus* for feeding, oviposition and development. This indicates the feasibility of selecting sweetpotato parents based on chemistry for breeding or mapping for resistance against *Cylas* spp. It also supports the hypothesis that chemistry of sweetpotato varieties and landraces that vary in their resistance to sweetpotato weevils differs and the levels of field resistance in these varieties are active and quantifiable and capable of being targeted for sweetpotato breeding.

In Chapter 5, *C. puncticollis* and *C. brunneus* were tested in a choice bioassay. Root periderm treated with hydroxycinnamic acid recorded significantly lower feeding and oviposition than the controls for both *Cylas* spp. indicating that higher concentrations on resistant roots account for lower insect damage. The effect of hydroxycinnamic acid on oviposition also implied that differences in the concentrations of these compounds at the root surface are likely to account for the reduced oviposition recorded on the resistant varieties New Kawogo, LIR302 and ARA228 and thus mediate resistance to the weevils reported in Chapter 3 of this thesis. It was also shown that the hydroxycinnamic acid esters have similar activities but occur as several different structures, potentially reducing the chance of the pest insect developing tolerance to their effects. The biological significance of this study is that the combined effect of the hydroxycinnamic acid esters as it is occurring in New Kawogo can confer resistance *C. puncticollis* and *C. brunneus* adults by reducing feeding and oviposition. Similar study reported that hexadecyl, octadecyl, and eicosyl esters of coumaric acid were associated with *Cylas* resistance, but the

compounds identified were not tested directly against the weevils (Snook *et al*., 1994). In addition, the effect of these compounds on the biology of *Cylas* spp was not known. In this study it has been found that the hydroxycinnamic acid esters present in the roots of the different lines, landraces and varieties, varied in quality and/ or quantity and reduce feeding and egg laying by adults. Our finding is novel and adds significantly to the understanding of resistance to weevil suggested from other earlier work by demonstrating that these hydroxycinnamic acid esters occur on the root surface and when applied to roots of otherwise susceptible varieties, reduce feeding and egg laying by adults.

The varieties investigated in this study varied in their response to sweetpotato weevil under field and laboratory conditions supported by earlier reports that herbivorous pests respond to different varieties in ways that strongly indicate varying levels of susceptibility or resistance (Mwanga *et al*., 2001; Jackson and Bohac, 2006). Laboratory bioassays indicated that no sweetpotato was completely immune to attack by sweetpotato weevils, however, this study demonstrated that the levels of susceptibility vary widely across varieties sufficiently to measure resistance under field and laboratory conditions. In the field trials New Kawogo, ARA228 and LIR302 recorded no damage, yet when presented in the laboratory under no choice conditions this variety was damaged suggesting that the resistance is adequate to minimise field effects despite being below a level of expression that could be considered immunity. The optimal defence theory predicts that the role of plant traits in providing resistance against herbivores came with a recognition that secondary metabolites and plant physical traits influence the feeding patterns of arthropod herbivores (Akhtar *et al*., 2008) so when weevils are imposed in a no-choice situation they will be forced to encounter resistant varieties.

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The significant differences in response of *Cylas* species to sweetpotato shown in this study is determined by the presence of higher concentrations of hydroxycinnamic acid esters on the root surface and in epidermal and peridermal tissues. Sweetpotato varieties with higher levels of hydroxycinnamic acid esters, particularly in the peridermal, epidermal tissues and root surface could be selected for to optimize the development of resistance to sweetpotato weevils. Based on the findings in this study, resistance to sweetpotato weevil could be studied in a mapping population of a cross between the resistant variety New Kawogo and a widely grown susceptible variety, such as Beauregard, an important variety in the United States for which detailed quantitative trait loci (QTLs) have been identified already for several traits. This approach would facilitate the identification of QTLs for weevil resistance and assist breeding for good agronomic and other culinary traits already established as well as weevil resistance discussed in chapter 8.

The results Chapter 6 of the study report the identification of sweetpotato volatile compounds and tried to illustrate whether volatile odours from sweetpotato could influence host location and thus, oviposition by sweetpotato weevils. The preference for a determined plant through its volatile cues (Singer, 2000) is an important step in the selection process and in the search for a source of food and a place for oviposition and refuge of herbivorous insects (Nieminen *et al*., 2003). Volatile phytochemicals that modify insect oviposition behaviour could be useful as part of an integrated control strategy or have other practical applications (Grant and Langevin, 2002).

Volatile components of the root and aerial parts of New Kawogo and Tanzania were collected before and after weevil infestation for 24 h in Porapak Q for chemical analysis at NRI. The study showed that there were differences in the sweetpotato

root volatile both before and after weevil infestation. Bioassay on the responses of sweetpotato to the volatiles from the root and the aerial parts was conducted in the olfactometer study and there was no significant response. Electrophysiological behavioural studies of male and female *C. puncticollis* were conducted using electroantennography. The response of male sweetpotato weevil antenna to the sex pheromone decyl-*E*-2-butenoate was consistent with previous work (Downham *et al*., 2001) in that the antenna produced an electrophysiological response and indicated the method was working.

Chapter 7, examined whether hydroxycinnamic acid esters interacted with Bt proteins to affect sweetpotato weevil larvae mortality. The application of hydroxycinnamic acids in the diet caused significant mortality in the larvae of both weevil spp. and supported earlier work that these compounds reduce survival when presented in diet (Stevenson *et al*., 2009). Thus the hydroxycinnamic acids not only have antixenotic properties (Chapter 6) but antibiotic properties too indicating that the mechanisms of resistance to *Cylas* spp are a result of a combination of antixenosis and antibiosis responses due to sweetpotato root chemical content on weevil stages. Dual effects of plant chemicals on insects are not unknown. Chemical analysis in chapter 5, however, indicated that the concentration of hydroxycinnamic acids in the cortex did not vary among varieties suggesting that the presence of a toxin in the cortex of SP would not differ between the resistant and susceptible varieties. However, their presence does not enhance or counteract the effects of a variety genetically modified with Bt. Bt has recently been considered to be a strong candidate option for addressing sweetpotato weevil (Lou *et al*., 2010)) and Bt-toxins caused larval mortality in both *C. puncticollis* and *C. brunneus* but until the present study it was unknown what the interactive effects of this would be with

hydroxycinnamic acid esters also known to occur in the cortex. Interactive effects of plant chemicals and biological pest control are not unknown and can have negative effects. Therefore, efforts to introduce weevil Bt resistant genes into sweetpotato with high levels of inherent hydroxycinnamic acid esters based resistance may provide opportunities for the development of varieties with higher chances of sustained resistance. This study found that hydroxycinnamic acid esters did not interact negatively with Bt- Cry 7a proteins on weevil larvae mortality. This suggests that if sweetpotato could be transformed successfully that the mechanisms would not be compromised by the presence of phenolic compounds in the latex, cortex or on the surface. Although there was no measurable synergistic effect the Cry 7a protein and hydroxycinnamic acids together led to a greater effect on weevils than the two components alone. Further work using lower concentrations might give more information on possible beneficial interactive effects of the two components. The regeneration and transformation protocol developed for Uganda landraces by Sefasi *et al.,* (2012) would be useful in transforming other more susceptible varieties using weevil resistance genes; to obtain weevil-resistant Bt transformed African sweetpotato varieties in the knowledge that there are no interactive effects with the principal plants products. Antifeedant effects of phytochemicals reduced food consumption and efficacy of Bt sprays on insect-resistant chickpea genotypes or Bt-transgenic chickpeas, but the combined effect of plant resistance and Bt had a greater effect on survival and development of *H. Armigera* than Bt alone Suredha *et al*., (2011)

Chapter 8, a bi-parental mapping population of 287 progeny developed from the New Kawogo x Beauregard was evaluated at 3 locations. 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 also showed that resistance in sweetpotato is mediated by root chemicals which are measurable and thus could be used for selection of material from breeding programmes of identifying quantitative trait loci. Yada (2014) identified five SSR markers which were associated with field-based sweetpotato weevil resistance and seven SSR markers which were associated with hydroxycinnamic acid ester-based sweetpotato weevil resistance in this population. This finding indicates that the segregating population of New Kawogo and Beauregard sweetpotato clones differ in their chemical composition associated with sweetpotato weevil resistance and that these traits can be identified in the mapping population and ultimately to breed improved varieties. Previous work by Cervantes *et al* (2011) constructed two parental maps Tanzania and Beauregard using a population of 240 clones identified quantitative trait loci (QTL) for dry-matter, starch, and β-carotene content in a hexaploid sweetpotato mapping population improved traits from a white-fleshed, high dry-matter African landrace, and an orange-fleshed, low dry-matter sweetpotato cultivar popular in the USA.

Thus the identification of resistance markers for resistance breeding will facilitate the development of sweetpotato varieties for weevil resistance. The identification of resistance genes to *C. puncticollis* and *C. brunneus* weevils could be of value to identify a full technology package for development of sweetpotato weevil resistance across the world.

Twenty-nine of the F1 progeny showed transgressive segregation for sweetpotato weevil resistance. Clones NKB225 and NKB219 had stable performance for sweetpotato weevil resistance across sites. The top performing clones from these crosses could be screened to identify the next set of parental genotypes. Multi-

location trials especially from NaSARRI data provided evidence that the hydroxycinnamic acid ester is constitutive and there is ecological interaction of the chemicals in sweetpotato weevil resistance.

9.2. CONCLUSIONS

Overall, the findings from this study have shown that the level of susceptibility to sweetpotato weevils varies among the landraces and improved sweetpotato germplasm grown in different agro-ecological zones of Uganda. Hydroxycinnamate mediated resistance in sweetpotato re-creates defence mechanisms in plants by possessing traits that are generally separated into those that lower plant attractiveness to insects (nonpreference or antixenosis) and impair development (antibiosis). This was demonstrated in field evaluation and feeding and oviposition bioassay. The use of resistance will have to be applied in conjunction with other direct control tactics to sustain the resistance. By re-creating plant defences, genetic resistance to insect pests plays a vital role in the attempt to enhance ecological stability in agricultural crops in an environmentally compatible manner. In particular, this work demonstrated that resistance in sweetpotato to sweetpotato weevils *C. puncticollis* and *C. brunneus* is not simply escape but is active and the root surface compounds including hexadecylcaffeic, hexadecylcoumaric, heptadecylcaffeic, octadecylcaffeic and octadecylcoumaric acids esters and 5-O-caffeoylquinic acid are heritable was through a bi-parental cross between a resistant and a susceptible variety, New Kawogo and 'Beauregard'. Plant resistance to insects is one of several cultural control methods. It involves the use of agronomic practices to reduce insect

pest abundance and damage below that which would have occurred if the practice had not been used.

Recommended direct control tactics that are advised in the use with resistance include selection of healthy and clean planting materials, crop rotation, good land preparation, early planting and mound re-hilling during weeding and piece meal harvesting. Soil amendment is required although sweetpotato is largely categorised as low input crop and farmers' tendency is to apply no input at all. This reduces plant vigour and resilience in case of insect pest attack. Integration of pest resistant crop varieties and Integrated Pest Management (IPM) has long lasting impact of technologies in enhancing crop yields. Compared to sweetpotato weevils, sorghum midge *Stenodiplosis sorghicola* (Coquillett), a ubiquitous and serious sorghum pest challenge in the USA with no solution for a long time (Sharma *et al*., 2003).

A major mistake has been with the effort to develop Bt resistant sweetpotato weevil lines to use as a sole control tactic rather than a component of integrated pest management (IPM) making it a repeat of the mistakes of the insecticide era. Understanding the genetics of resistance in plants will provide the knowledge to improve resistance deployment strategies. Regardless of the technology used to develop insect resistant plants, the technology will have to be used as a component of IPM in the same way traditionally developed tactics that, in combination with others, provides desired protection of the crop from insect pest.

IPM and plant resistance was also successfully and sustainably used in the control of Greenbug, *Schizaphis graminum* (Rodani) in sorghum. Greenbug-resistant sorghum with tolerance resistance mechanism was developed (Royer *et al*., 2015). This intervention was ecologically stable because natural enemies are maintained

that complement the resistance and suppressed Greenbug abundance during the groups growing cycle with reduction in secondary pest outbreaks (Burd, 2002; Burton *et al*., 1990).

The associated molecular study at North Carolina State University later showed there was transgressive segregation of the compounds in the mapping population. If the specific Quantitative Trait Loci corresponding to these compounds are identified, more targeted breeding can be done to attain weevil resistant varieties and provide a genetic base for use in improvement of the breeding process, then enhancing the generation of resistant varieties for Uganda and elsewhere in the world.

9.3.   RECOMMENDATIONS AND IMPLICATIONS FOR FUTURE RESEARCH

Sweetpotato resistance compounds are present in sweetpotato varieties grown in other areas of the world.

- More research aimed at screening larger sweetpotato germplasm accessions from different agro-ecological areas and regions where sweetpotato weevils are a problem will broaden sources of weevil resistance.
- There is need to test whether *C. formicarius* is susceptible to these hydroxycinnamic acid esters. Resistant sweetpotato germplasm collected can then be evaluated further for field resistance and chemical analysis to identify if there are similarities in sweetpotato root chemical profiles to what has been reported in this study.

- Where these compounds will not be found, then efforts will be required to improve existing germplasm and also include other attributes of resistance in the selection of the parents for use in the breeding scheme. Resistant sweetpotato varieties identified could be evaluated further at multi-location sites to suit the ecological conditions and meet consumer needs for different regions.

- Selection of resistance should be based on field evaluation, comparing the resistant materials identified in choice and no choice bioassays and profiling of hydroxycinnamic acid esters concentrations in the clones.

- Other traits such as yield, dry matter, sweetpotato virus and Alternaria blight disease resistance and micronutrients needs to be combined with SPW resistant traits to produce multiple resistant and nutritious varieties that continue to be demanded by the consumers.

- The study found that hydroxycinnamic acid esters did not interact negatively with Bt Cry 7a proteins on weevil larvae mortality. Further work using lower concentrations might give more information on possible beneficial interactive effects of the two components.

- The transgenic weevil management approach alone used by International Potato Center (CIP) under the Sweetpotato Action for Security and Health in Africa (SASHA) project was not effective. This was due to low expression of Cry proteins from *Bacillus thuringiensis* (Bt) in sweetpotato storage roots (Rukarwa *et al*., 2013a). Integrating transgenic sweetpotato for weevil resistance and other existing IPM practices will help create a stable ecological conditions for sustainable weevil resistant management.

- There is also a need for more comprehensive studies to gain more understanding on the biochemical pathway of hydroxycinnamic acid esters, factors triggering its production and effect of the relationship between phytochemical compositions and how their production could be affected by the environment in which sweetpotato is grown. This present study showed higher concentrations of hydroxycinnamic acids esters in NaSARRI, a hot and dry environment compared to NaCRRI, a moist environment with tall grassland in the central region.

- It is further recommended that heritability of the biochemical mediated resistance mechanisms indicated by transgressive segregation of the New Kawogo x Beauregard mapping population be further evaluated for selection of resistant genotypes. The effect of genotype x environment interaction should be further studied through phenotypic studies in additional agro-ecologies. This can be implemented through increased replications and locations to produce more data and hasten the development of resistant weevil resistant varieties.

- Studies to identify the best methods of integrating biochemical based resistance into new and existing management options against sweetpotato weevils should conducted. This will provide a more reliable and stable management strategy for the control of *Cylas* spp. in Africa and elsewhere in the world and ensure there is sweetpotato available for multiple uses.

- Breeder friendly and advanced equipment for acquisition of pest and disease damage data are needed since advances will need to be made in the use of plant biochemistry for identifying the basis of pest and disease resistance in sweetpotato. Plant biochemistry is now being used by breeders for screening

sweetpotato weevil resistance. However, low cost analysis of plant chemistry through calibrating equipment such as NIRS for analysis of hydroxycinnamic acid esters are needed since chemical profiling of resistance by wet chemistry through equipment such as liquid-chromatography mass spectrometry (LC-MS) is still expensive for the breeders and researchers in SSA who screen large number of clones.

- All the advances in sweetpotato genomics, phenomics and biochemistry will pave way for the use of novel and next generation approaches for future sweetpotato improvement including generation sequencing in wild relatives of sweetpotato species for new resistance traits for sweetpotato weevil management.

- Finally, a functional collaboration between the sweetpotato breeding programs in SSA with breeding programs and researchers in the developed countries and other advanced laboratory will be critical to enhance the quality of research to unveil the full potential of sweetpotato in the region.

CHAPTER 10

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