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Chemical basis for resistance in sweetpotato *Ipomoea batatas* to the sweetpotato weevil *Cylas puncticollis**

Philip C. Stevenson[1,4,‡], Harriet Muyinza[2,3], David R. Hall[1], Elaine A. Porter[4], Dudley I. Farman[1], Herbert Talwana[2], and Robert O. M. Mwanga[3]

[1]Natural Resources Institute, University of Greenwich, Chatham Maritime, Kent, ME4 4TB, UK; [2]Department of Crop Science, Makerere University, P.O. Box 7062, Kampala, Uganda; [3]National Crops Resources Research Institute, Namulonge, Box 7084 Kampala, Uganda; [4]Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, UK

Abstract: The aim of this work was to determine the basis of resistance in a sub-Saharan sweetpotato variety, New Kawogo, to the African sweetpotato weevil *Cylas puncticollis.* This insect feeds on the roots, reducing quality and yield, and is the most important production constraint of sweetpotato in Africa. Laboratory bioassays were designed to determine how the performance of weevils differed on susceptible and resistant roots. Subsequently, liquid chromatography-mass spectrometry (LC-MS) analysis of the root surface and root latex identified quantitative and qualitative differences in the chemical profiles with higher levels of octadecyl and hexadecyl esters of hydroxycinnamic acids reported in the resistant variety. The compounds were synthesized to confirm their identity and incorporated into artificial diets for bioassays on *C. puncticollis.* High levels of mortality and developmental inhibition were recorded for larvae feeding on treated diets, and the effect was dose-dependent. Thus, in contrast to previous work on resistant African sweetpotato cultivars, resistance in New Kawogo is not only active, but is quantifiable and manageable for breeding. Work is underway to determine what effect these compounds have on the weevils at a molecular level. The inheritance of the root latex esters will be studied in new crosses and mapped in new populations using quantitative trait loci (QTLs) that are currently being developed.

Keywords: hexadecyl esters; octadecyl esters; hydroxycinnamic acid; caffeic acid ester; coumaric; root latex; *Ipomoea batatas*; *Cylas brunneus*; resistance.

INTRODUCTION

Sweetpotato [*Ipomoea batatas* (L.) Lam.] is an important crop in East Africa where it is grown as a staple food. For some farmers, the crop also supplements family income, and, thus, strategies to reduce losses to pests and diseases provide opportunities to enhance food security and improve livelihoods. In Africa, the sweetpotato weevils *Cylas puncticollis* Boheman and *C. brunneus* Fabricius are the major

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‡Corresponding author

production constraints, whereas in North America, *C. formicarius elegantulus* (Summers) is the major pest [1]. Technologies to manage weevils would boost production dramatically and have a positive impact on the livelihoods of millions of poor farmers across sub-Saharan Africa.

One approach that has been targeted is host plant resistance. Progress in breeding varieties with resistance to *C. puncticollis* and *C. brunneus* has, however, been slow [2] largely due to the scarcity of varieties with significant levels of resistance. Where cultivar differences in susceptibility to *Cylas* spp. were observed [3], the mechanism was reportedly escape. Specifically, the depth of roots and the tendency of shallow swollen roots to crack dry soil and so provide access for the insects were reportedly correlated with a root's tendency to be infested [4].

Progress toward resistance-based management of sweetpotato weevil in the United States has, however, been more successful and owes much to the apparent presence of higher levels of multiple insect species resistance in some varieties, including against *C. formicarius elegantulus* [5]. The potential for breeding improved African varieties with resistance to *C. puncticollis* and *C. brunneus* will be greatly enhanced if parental material can be identified with similarly active and robust resistance and if the mechanisms of resistance can be determined. Research in the United States has provided considerable insight into mechanisms of resistance in sweetpotato to *C. formicarius elegantulus*. For example, significant differences among sweetpotato cultivars in levels of caffeic acid, caffeoylquinic acid, and rutin have been reported with the suggestion that they may be associated with resistance [6]. These compounds are characterized by an orthodihydroxy group associated with insect toxicity in other plant–pest interactions [7,8]. Plants were also analyzed for the organic acids, malic, citric, and quinic acids, but no differences were found among resistant and susceptible varieties [6]. The levels of two triterpenoid components, boehmeryl acetate and boehmerol, which are known ovipositional stimulants [9], also differed significantly in sweetpotato cultivars that differed in susceptibility to the *C. formicarius elegantulus*, suggesting that variation in susceptibility of cultivars displaying moderate levels of resistance may be due, in part, to seasonal variation in the level of these two triterpenoids [10,11]. Thus, selecting varieties with low levels of these components may be a route to the selection of less preferred and therefore less susceptible cultivars, although there was also reportedly considerable chemical variation within a cultivar, suggesting that simple selection based on the presence of these compounds was not so straightforward [12].

Wang and Kays [13] identified three oxygenated monoterpenes—nerol, *Z*-citral, and methyl geranate—in storage roots (the primary site of oviposition) but not aerial plant parts, and these compounds were shown to be attractants to adult weevils. Several sesquiterpenes including α-gurjunene, α-humulene, and ylangene were also identified but were shown to be repellents. Differences in attraction among four sweetpotato cultivars to female sweetpotato weevil were inversely correlated with the concentration of these sesquiterpenes, and, thus, volatile behavior-modifying chemicals offer more options in chemical profiling for resistance.

Data et al. [14] reported that sweetpotato root latex could be a contributing factor in sweetpotato resistance to *C. formicarius*. The number of feeding punctures in semi-artificial diets incorporating the root latex were fewer than untreated diets and root cores recorded lower oviposition when coated with latex. The latex of sweetpotato has been reported to contain hexadecyl, octadecyl, and eicosylcoumaric acid esters [15], and it is possible but was never shown that these components were responsible for the biological activity of the latex.

The projected outcome of all these studies is that these components might help guide the development of resistant clones for managing weevils. Although none of these characters is currently reported to be used in the selection of clones for farmers, many useful factors have been identified and associated with differing levels of infestation and thus moved forward the understanding of host plant–weevil interactions considerably. By comparison, no progress in this respect has been made in the determination of factors responsible for resistance in African varieties against the African weevil species, *C. puncticollis* and *C. brunneus*. This paper describes preliminary studies that show that chem-

ical components in the root latex of African sweetpotato varieties are strongly associated with resistance.

MATERIALS AND METHODS

Development of sweetpotato weevils on roots of Tanzania and New Kawogo

Bioassay to evaluate feeding and oviposition by C. puncticollis *on Tanzania and New Kawogo*

Farmers in Uganda consistently report that a sweetpotato variety, New Kawogo, suffers lower sweetpotato weevil damage by harvest time compared to a popular and commercially important variety, Tanzania, widely grown in sub-Saharan Africa [16]. Consequently, we used these two varieties to evaluate differences in feeding behavior and development of adults on sweetpotato roots and for subsequent chemical analysis to determine if any components could be associated with this apparent varietal resistance. Root plugs from the two varieties were obtained using a 24-mm-diameter cork borer. The plugs were cut to be approximately 10 mm deep and to fit precisely into wells of a 12-well plate. The root surface faced upper-most being the surface on which insects would be placed. Mated adult females (2 weeks old) were placed individually on the plugs, and the plate lid was carefully replaced. The weevils were allowed to feed and oviposit for 24 h and then removed. The numbers of feeding holes, eggs laid, and droppings were recorded. The number of droppings was recorded to give an indication of the amount of feeding by each adult.

Bioassay to evaluate the development of C. puncticollis *on New Kawogo and Tanzania*

Ten undamaged roots of varieties of New Kawogo and Tanzania, respectively, were placed in 1 l aerated polystyrene jars that were covered to reduce the illumination of the roots. Each root was inoculated with 20 two-week-old mated *C. puncticollis* female weevils. They were allowed to oviposit for 48 h after which the adults were removed. The roots were then incubated at room temperature (22–25 °C) until weevils emerged. The number of adults that emerged from each root was recorded.

Chemistry of New Kawogo and Tanzania root latex

Root latex collection and root surface extraction

Root latex from New Kawogo and Tanzania 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. Whole undamaged roots of New Kawogo were submerged in 1 l hexane for 1 min. The extract was filtered through Whatman Grade 1 paper and evaporated to dryness under reduced pressure on a rotary evaporator and redissolved in methanol.

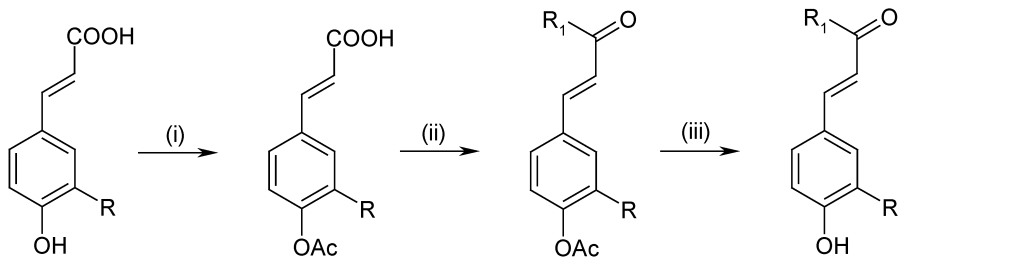
LC-MS and HPLC

HPLC was performed on a Waters system with 600E quaternary pump, 717 autosampler, and 996 diod-array detector. Chromatographic separation was performed on 150 × 4.6 mm i.d. (5 mm particle size) Zorbax Eclipse C18 column using a linear mobile-phase gradient of 0.94 ml/min flow rate with 5 % acetic acid (A); MeOH (B); 18:1:1 MeOH:water:acetic acid (C). Initial conditions were 80 % A, 0 % B, and 20 % C, changing to 80 % B and 20 % C at t = 20 min and finally 100 % B at 21 min and maintained until 41 min. Injection volume was 5 µl, and data analysis was performed using Millennium software.

LC-MS was carried out on a Thermo-Finnigan LC/MS/MS system consisting of a "Surveyor" autosampling LC system interfaced to a LCQ Classic quadrupole ion trap mass spectrometer. The ion trap MS was fitted with an atmospheric pressure chemical ionization (APCI) source operated under standard conditions; i.e., vaporizer temperature 450 °C, needle current 5 μA, heated capillary temperature 150 °C, sheath and auxiliary nitrogen gas pressure 80 and 20 psi, and the source voltages tuned for the optimal transmission of protonated rutin. The ion trap was set to monitor ions from *m/z* 125–1200 with collision energy of 45 %. Chromatographic separation was performed on a 150 × 4.6 mm i.d. (5 μm) Phenomenex Luna C18 column using a linear mobile-phase gradient of 1 ml/min flow rate, water (A); MeOH (B); 5 % HOAc in MeOH (C) with 80 % A and 20 % C at $T = 0$ changing to 80 % B and 20 % C at $T = 20$ and to $T = 25$). Injection volumes were 10 μl, and data analysis was performed using Xcalibur 1.2 software.

Synthesis of hexadecyl esters of hydroxycinnamic acids

Compounds **1** and **2** were prepared in quantitative yields from corresponding acids (**1a** and **2a**) as shown in Scheme 1. The hydroxyl group(s) were acetylated to give the acetoxy acids, and these were then coupled to the appropriate alcohol, R_1OH, using *N*,*N*'-dicyclohexylcarbodiimide catalyzed by *N*,*N*-dimethylaminopyridine in dichloromethane [17]. The acetoxy groups were then removed with potassium carbonate in methanol to give the esters (**1d** and **2d**) which precipitated out on acidification. The products were recrystallized from ethyl acetate/petroleum spirit and characterized by IR, LC-MS, and NMR spectroscopy. Up to 10 g of hexadecylcaffeic acid and hexadecyl-*p*-coumaric acid were synthesized for bioassays and analysis.



1a R=OH (caffeic acid) **1b** R=OAc **1c** R=OAc; R1=$OC_{16}H_{33}$ **1d** R=OH; R1=$OC_{16}H_{33}$
2a R=H (coumaric acid) **2b** R=H **2c** R=H; R1=$OC_{16}H_{33}$ **2d** R=H; R1=$OC_{16}H_{33}$

Scheme 1 Synthesis of hexadecyl esters of hydroxycinnamic acids. Reagents: (i) acetic anhydride/pyridine; (ii) *N*,*N*'-dicyclohexylcarbodiimide/*N*,*N*-dimethylaminopyridine/R_1OH/CH_2Cl_2; (iii) potassium carbonate/methanol.

Biological activity of phenolic esters on weevils

Effect of synthesized hexadecylcoumaric and hexadecylcaffeic acid on larval development

A diet developed by Ekobu [18] to rear larval stages of *C. puncticollis* and C. *brunneus* to adulthood was used to investigate the biological activity of **1** and **2**. Compounds were incorporated into diets as solutions in a minimum of ethanol. Control diets were treated with the same volume of ethanol only, to demonstrate no adverse effect of this diet component on the development of the larvae. To 90 g of diet, **1** and **2** were added respectively at concentrations of 0.1, 0.01, 0.001, and 0.0001 % w/w and mixed using a magnetic stirrer. The diet was then poured when still hot into sterile plastic Petri dishes with 7 replicates per treatment. After the diet had cooled and set, 5 small burrows were excavated in the diet surface using a plastic spatula by scooping out a small piece of diet. When larvae were placed in the burrow, the displaced diet was replaced on top to provide protection from desiccation.

Second instar *C. puncticollis* larvae (11 days old) were obtained by allowing adult mated weevils to oviposit on the roots for 24 h. The age of the weevils was recorded from the first day of incubation. One larva was placed into each diet burrow with 5 burrows on each Petri dish. The Petri dishes were then covered with a disc of filter paper to absorb excess moisture from the diets, and the lids were replaced and the bioassay left to stand for 10 days. The percentage larval mortality, larval weight, and mean number of surviving insects were recorded.

RESULTS AND DISCUSSION

Development of sweetpotato weevils on roots of Tanzania and New Kawogo

There were significantly more feeding punctures, droppings, and eggs laid by insects feeding on root plugs of Tanzania compared with insects feeding on New Kawogo root plugs (Fig. 1), indicating that an active and quantifiable mechanism of resistance was present in the roots of New Kawogo. This result reciprocates deterrent effects reported by Data et al. [14], who showed that (a) latex-coated root cores were less preferred for oviposition and (b) fewer feeding punctures were recorded in semi-artificial diets containing the latex. This suggests that the resistance of New Kawogo may be associated with components in the latex. The difference in the number of droppings reflects differences in consumption between adults feeding on the two varieties and suggests that fewer feeding punctures indicate reduced consumption and thus damage rather than the fewer feeding punctures actually being more substantial individual feeds.

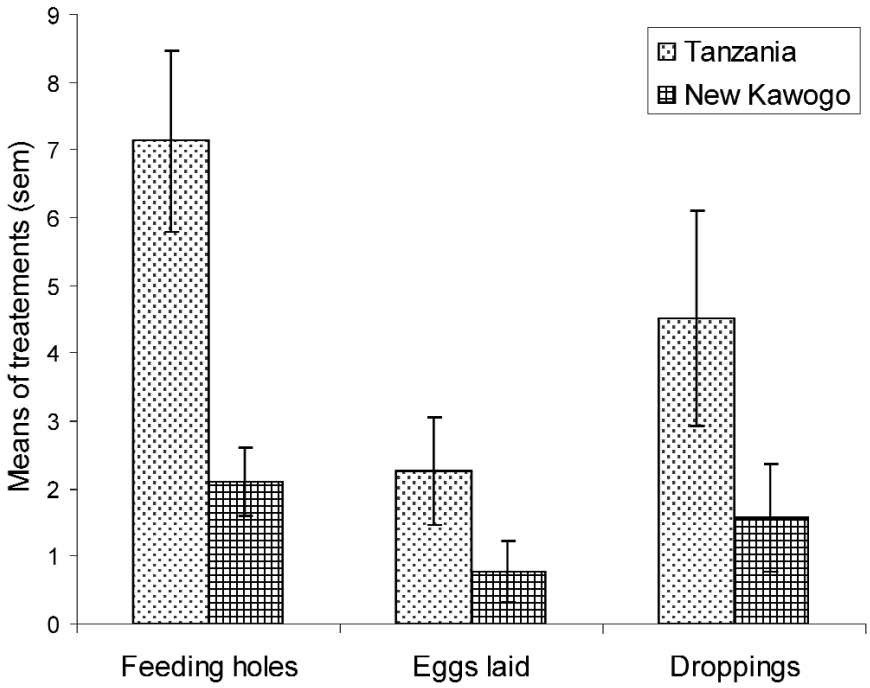


Fig. 1 Feeding holes, eggs laid, and droppings of adult *Cylas puncticollis* on root plugs of two varieties of sweetpotato (n = 16) after 24 h.

There were also significant differences in the numbers of weevils that emerged on these varieties with significantly more emerging from Tanzania. Those insects emerging from New Kawogo also took longer to develop (Fig. 2) than those on Tanzania. This is most clearly illustrated with data for adults emerging from 30 to 35 days (Fig. 2). These results indicate both deterrent and toxic effects on weevil adults and larvae, respectively, thus, resistance may be complex and consist of several components.

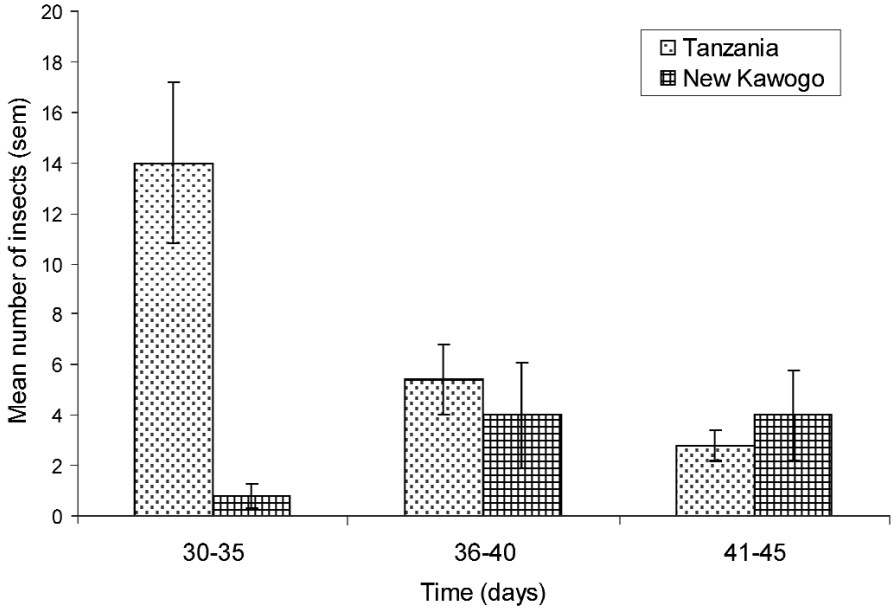
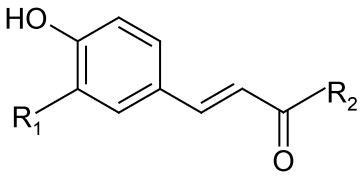


Fig. 2 Variation in numbers of *C. puncticollis* adults and time taken to emerge from Tanzania and New Kawogo roots.

Chemistry of New Kawogo and Tanzania root latex

The four most abundant compounds in the root latex of the sweetpotato roots were identified by comparison with standards synthesized in our laboratory as octadecyl and hexadecyl esters of hydroxycinnamic acids (**1–4**) as illustrated in Fig. 3. Fragmentation in APCI-MS$^{(+)}$ was characterized by the molecular ion of each compound with two fragments representing the cinnamate moiety (e.g., coumarate and coumaroyl). For example, the molecular ion for hexadecyl-*p*-coumaric acid (**2**) was *m/z* 389 $[M + H]^+$ which is in agreement with a molecular weight of 388, with fragments at *m/z* 165 (coumaric acid) and *m/z* 147 (coumaroyl) consistent with losses of $C_{16}H_{33}$ and $[O–C_{16}H_{33}]^+$, respectively. The most abundant compounds in the latex of Tanzania were **2** and **4** with a small amount of **1** compared to New Kawogo (Fig. 4). The chemical profile of New Kawogo root latex, which is also presented in Fig. 4, contained peaks for **1**, **2**, and **4**, but also contained **3** with a putative identification as octadecylcaffeic acid. This compound appears to be absent from Tanzania. Compounds **1** and **3** have not previously been found in sweetpotato and so are reported here for the first time. When the synthetic derivative of **2** (100 % *E*-form) was left in solution for 3 days at room temperature a second compound appeared eluting approximately 30 s earlier which had a similar mass spectrum and a UV spectrum consistent with the *Z*-form of coumaric acid. Thus, it is presently proposed that the peak eluting in front of **4** in Fig. 4 is the *Z*-form of **4** since this peak has an identical MS to **4** and a UV spectrum consistent with the *Z*-form of coumaric acid.



		R_1	R_2
1	Hexadecylcaffeic acid	OH	$OC_{16}H_{33}$
2	Hexadecyl-*p*-coumaric acid	H	$OC_{16}H_{33}$
3	Octadecylcaffeic acid	OH	$OC_{18}H_{37}$
4	Octadecyl-*p*-coumaric acid	H	$OC_{18}H_{37}$

Fig. 3 Compounds extracted from root latex of sweetpotato cultivars New Kawogo and Tanzania.

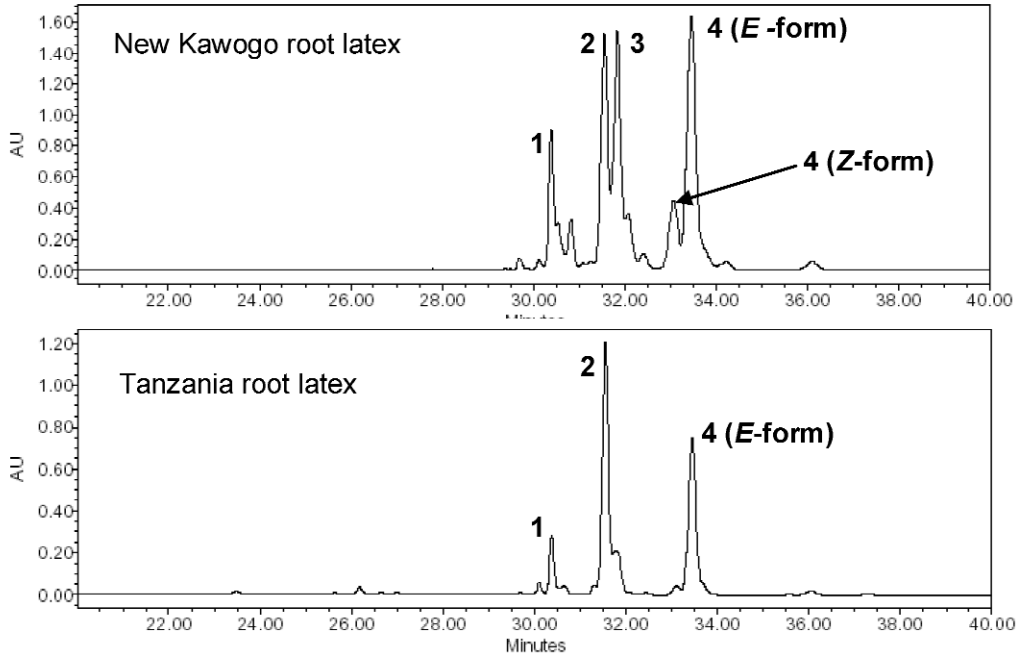


Fig. 4 HPLC chromatograms at 325 nm of New Kawogo and Tanzania root latex (40 mg/ml) illustrating differences in chemical profile for each variety.

The compounds were quantified by comparison with UV absorbance of the synthetic standards of known concentration. The combined concentrations of caffeic acid esters (**1** and **3**) and coumaric acid esters (**2** and **4**) in Tanzania latex were 7 mg/ml and 23 mg/ml, respectively, but were 43 mg/ml and 42 mg/ml in New Kowogo, indicating a much greater concentration of these components in the latter, most notably of the caffeic acid esters. Clearly, quantification of the latex in the whole roots needs to be determined and is presently being evaluated. However, the concentration in the latex of these target compounds is greater in the resistant variety and no obvious differences in latex exudation were noted between the two varieties.

The root surface extract of New Kawogo had a similar chemical profile to the latex, suggesting that the latex is exuded on to the surface where it may confer protective properties. Data et al. [14] reported that treatment of roots with the latex reduced oviposition by adults and that inclusion of latex into diets reduced feeding. If New Kawogo has higher concentrations of root latex components on the root surface as it has in the latex, this may explain why in the present study oviposition on the root plugs was lower on New Kawogo than on Tanzania and why fewer feeding punctures were recorded on New Kawogo.

Biological effects of cinnamic acid esters on weevils

Survival to pupae on the artificial diet controls was approximately 95 %, indicating that the diet is a suitable medium for rearing at least one generation of weevil and for testing plant compounds. Significantly higher mortality was recorded for weevil larvae feeding on diets treated with **1** and **2** compared to the control diets at all concentrations, and the effect was dose-dependent (Fig. 5). Overall, the effect on larval size was also dose-dependent. Furthermore, the compounds significantly reduced larval development. The biological effect of **1** was more pronounced than **2** although these differences were not significant (Fig. 6). While compounds **3** and **4** were not tested in these preliminary bioassays, their effect on the insects is likely to be similar since they differ from **1** and **2** only in the length of the alcohol moiety. Since the overall occurrence of these compounds was greater in the resistant variety, New Kawogo, the activity of the compounds on larval development could explain, at least in part, why New Kawogo suffers lower infestation and less damage by *Cylas* spp. in the field.

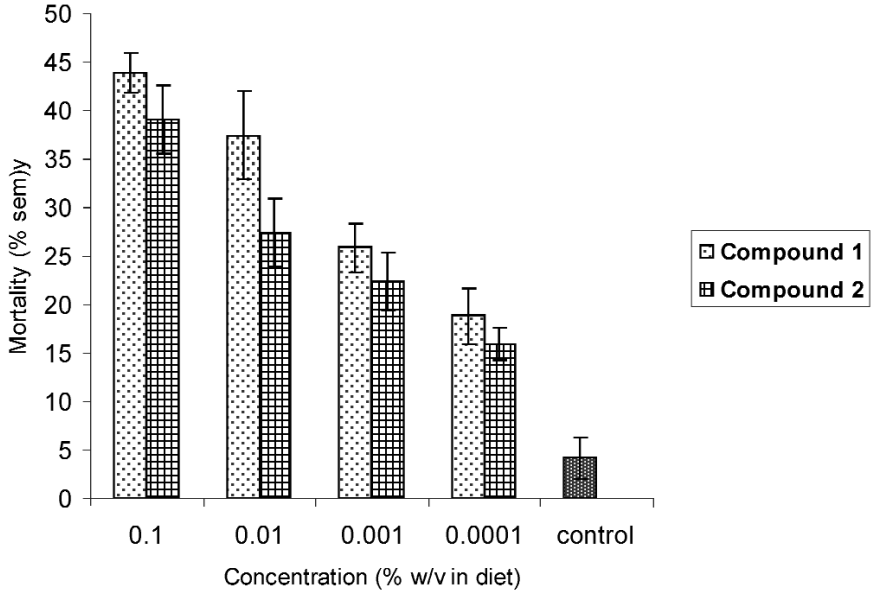


Fig. 5 Effect of diets incorporating compounds **1** and **2** at different concentrations on the mortality (% ± sem) of *C. puncticollis* larvae.

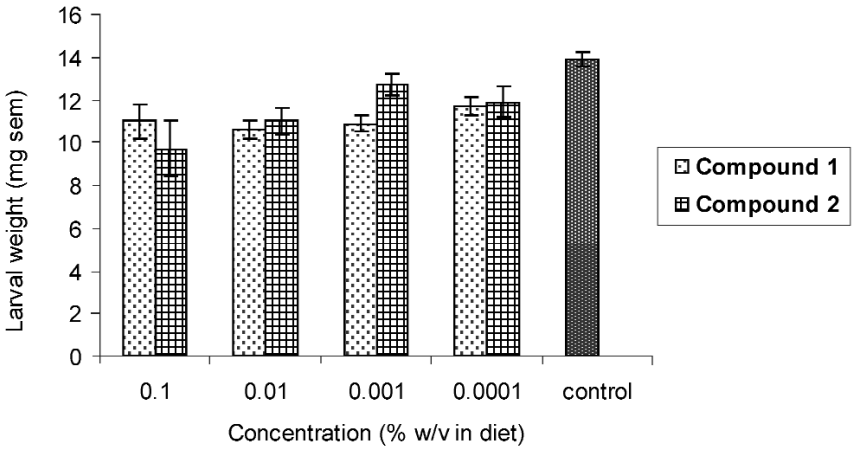


Fig. 6 Effect of diets incorporating compounds **1** and **2** at different concentrations on the larval weight (± sem) of *C. puncticollis*.

As touched on in the introduction and above, previous work has investigated the potential role sweetpotato latex might play on feeding and oviposition by sweetpotato weevils but with particular emphasis on *C. formicarius elegantulus*. All parts of the sweetpotato plant that are fed upon by the weevil including the roots, leaves, and vines can exude copious amounts of latex upon wounding [14]. Furthermore, young vegetative parts of sweetpotato produced more latex and tend to show reduced weevil feeding damage than older more mature portions of the vine. Latex incorporated into semi-artificial diets reduced the number of feeding punctures and when coated onto root surfaces resulted in lower oviposition. Earlier work had already shown that components of the latex included hexadecyl, octadecyl, and eicosyl esters of coumaric acid [15], suggesting that it may be the presence of these compounds that are responsible for the deterrent effects on adults feeding and oviposition. In the present study, we have shown that the number of feeding holes differed significantly between the resistant and susceptible variety. Since the two varieties differed quantitatively and qualitatively in the presence of octadecyl and hexadecyl esters of coumaric and caffeic acid, these chemical differences may well explain, at least in part, the observed responses of the insects in this study and also previous work [14]. We are presently undertaking work to determine categorically if this is the case by allowing adults to feed and oviposit on whole roots that have been surface-treated with the synthesized compounds.

While larvae do reach adulthood on the artificial diet used in the bioassays reported here, the insects are still likely to be under greater stress than when feeding on whole roots. Thus, the effects of the compounds may not reciprocate exactly their effect when encountered in the living root. Furthermore, the latex is compartmentalized in certain vessels and may be avoided by discerning insects to some extent. Nevertheless, the specific identification of **1** and **2** in New Kawogo and their observed effects on larval development are evidence that resistance in sweetpotato is active and quantifiable and thus useful in breeding for resistant varieties to *C. puncticollis* spp. In addition, the higher levels of these compounds obtained from the roots of New Kawogo compared to Tanzania strongly suggest that varieties with higher concentration of these compounds in the roots will be less damaged by *Cylas* spp. The identification on the root surface of all these esters is also significant in our objective of identifying resistance mechanisms in African sweetpotato to *C. puncticollis*; particularly since they affect early development of larvae and may also affect egg-laying behavior. Thus, breeding for varieties with higher quantities of hexadecylcaffeic and hexadecyl-*p*-coumaric acids offers a viable option in the effort to develop varieties with resistance against *C. puncticollis* weevil. We now also intend to determine what effect these compounds have on other species of *Cylas*.

From the present work, it is not possible to say categorically how the compounds affect the insects at a molecular level. However, it is reasonable to speculate that the phenylpropanoid moiety is critical since previous work on other insect species has demonstrated that this group of compounds plays a role in plant defense [7,8]. The effect on these compounds has been investigated for other insect groups. For example, caffeic acid and caffeoyl quinic acid have been shown to induce oxidative stress in *Helicoverpa zea* by increasing lipid peroxidation products, oxidized protein, and free iron while reducing levels of ascorbic acid in larvae ingesting these compounds [19]. Furthermore, there is evidence that caffeoyl groups can bind covalently to proteins containing the nucleophillic centers $–NH_2$ and SH such as lysine, histidine, cystein, and methionine and that the level of protein binding increases in the presence of the ubiquitous plant enzyme polyphenol oxidase owing to the enzymatic conversion of orthodihydroxy to dihydroquinone [20]. Thus, the compounds found here in sweetpotato latex and on the root surface may cause oxidative stress, affect protein uptake by the weevils and so their development, or may even bind insect proteins such as gut enzymes. The long-chain alcoholic moiety of the phenolic esters may facilitate the movement of the active moiety across cell membranes.

This paper reports that, in contrast to earlier reports on other resistant African sweetpotato cultivars [3,4], resistance in New Kawogo is more than simply escape but is active, quantifiable, and potentially manageable for breeding purposes. Furthermore, the effect on adults feeding on the resistant roots is demonstrated to be deterrent or toxic. Analysis of the roots determined that quantitatively and qualitatively there were significantly more hydroxycinnamic acid esters in the resistant variety New Kawogo than the susceptible Tanzania and that these compounds have a potently toxic effect on weevil larvae when incorporated in artificial diets, a result that may explain the field resistance. The inheritance of the root latex esters is being studied in new crosses, and mapped in new populations using quantitative trait loci (QTLs) that are currently being developed. It is hoped that a full understanding of their inheritance will lead to the development of new varieties in which resistance can be optimized.

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