Cotton Catchment Communities CRC

FINAL REPORT

Part 1 - Summary Details

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Part 3 – Final Report Guide (due at 31st May 2012)

Background

The key insect pests of cotton in Australia are *Helicoverpa armigera, H. punctigera* and secondary pests such as spider mites (*Tetranychus* spp.) and whiteflies (*Bemisia tabaci*). Although *Helicoverpa* spp. can be controlled by the application of insecticides such as synthetic pyrethroids, resistance against them is a problem. Furthermore, dependency on chemical pesticides has created enormous economic, environmental and ecological problems in Australia. The Australian cotton industry has benefited greatly from the introduction of the transgenic Bollgard II® cotton. Bollgard II® expresses Bt toxins which kills larvae after feeding and has subsequently reduced the use of chemical insecticides to control insect pests. As resistance to the Bt toxins in *Helicoverpa* spp. remains a threat, extensive science-based Resistance Management Plans (RMPs) have been developed.

These RMPs involve, among other measures, growing refuge crops to breed genetically susceptible moths, pupae busting using cultivation to disrupt overwintering pupae in the soil, trap crops to retain the offspring of moths selected for Bt resistance, and planting windows. A major gap in the science on which the tactics in these RMPs are based is that there has been no reliable way of determining whether moths infesting Bt crops, refuges or trap crops are derived from larvae which were reared on cotton (and therefore exposed to Bt toxin), or on other crops such as pigeonpeas (widely used in refuge and trap crops) or other components of the farming system, including non-crop vegetation.

In order to assess the effectiveness of RMPs we have attempted to develop an assay based on the detection of biomarkers in the adult moth which are specific to the host plants in which they fed as larvae. Previous research has identified some markers of host origin, such as stable isotopes of carbon, fatty acid ratios and the compound gossypol in cotton, but they cover a limited host range and/or are sometimes unreliable. An effective assay would provide a tool for more effective RMPs for Bt cotton and would also contribute to better pest management in other crops affected by *Helicoverpa* spp.

Objectives

To identify stable leaf wax compounds of Bt cotton not found in refuge crops. (complete)

The first objective for this project was to identify leaf wax compounds found in cotton but not in other refuge crops. This required that the components found in cotton and the refuge crops were first identified. Leaf wax components of cotton, pigeon pea and sorghum were examined by extracting the leaves and a variety of compounds identified. As the cuticular leaf wax of plants is known to vary depending on environmental factors such as soil water content, both field raised plants and greenhouse raised plants were compared. Compounds were identified by MS on the basis of molecular weight and fragmentation pattern by comparison with database spectra and then authentic

samples of the material. Both the presence of a compound and its relative abundance were considered potential points of differentiation between plants.

Compounds identified as unique to cotton.

- The relative abundance of the linear alkanes from $C_{23}H_{48}$ to $C_{35}H_{72}$ present in the leaves was quantified. Of note was the relative abundance of $C_{29}H_{60}$ and $C_{31}H_{64}$ linear alkanes.
- α-Tocopherol was identified in the leaf extract of cotton and was found to be relatively abundant. Trace quantities of γ-tocopherol were also identified in the leaf extract of cotton. Only trace γ-tocopherol was present in cotton seeds.
- β-Amyrin was identified in the leaf extract of cotton.
- A large number of other components were seen in the GC-MS trace of cotton leaves, however, as we were unable to unambiguously identify the component, and the compounds were not present in the GCMS traces of moth extract, the GCMS peaks are not listed.
- The relative quantities of triacyl glycerides in cotton leaves was characterised by RP-HPLC-MS.

Compounds identified as unique to pigeon pea.

- Linear alkanes present in the leaves were quantified and the relative abundance of the C_{29} and C_{31} *n*-alkanes were found to be significantly different to cotton.
- Lupeol and lupenone were identified in the leaves of pigeon pea.
- α-Tocopherol was identified in the leaf extract of pigeon pea and significant quantities of γ-tocopherol were also identified. Trace amounts of β-tocopherol were detected.
- The relative quantities of triacylglycerides in pigeon pea leaves was characterised by RP-HPLC-MS.

Compounds identified as unique to sorghum, tomato and soybean.

Linear alkanes present in the leaves were quantified and the relative abundance quantified.

The relative quantities of triacylglycerides in soybean was characterised by RP-HPLC-MS.

To detect leaf wax compounds and other potential markers from cotton in the adult moth. (complete)

A range of linear alkanes present in the adult moth were detected following homogenisation and solvent extraction. The relative abundance of the leaf wax components was determined but the quantity did not reflect the relative abundance of linear alkane present in the host plant, or differences were not significant enough to detect.

We were unable to detect the unique triterpenes lupeol, lupenone or β-amyrin in the adult moth.

We were able to detect both α- and γ-tocopherols in trace quantities in the adult moth. The relative abundance of the type of tocopherol in the host plant was reflected in the adult moth. Insignificant

amounts of γ-tocopherol was found in cotton raised moths while pigeon pea moths had much greater relative amounts of γ-tocopherol.

Triacylglycerides present in the adult moth have been extracted, separated and both the substitution pattern and fatty acid identity determined. An insufficient number of samples have been analysed to make conclusions, however, preliminary results are favourable and it may be possible to differentiate pigeon pea, cotton and soybean using principal component analysis.

To validate the detection assay (incomplete)

While expanding the data set of moths for the detection of γ- and α-tocopherol, a significant number of moths raised on pigeon pea were found to have only a small amount of γ-tocopherol. Thus, the use of the ratio of γ- and α-tocopherol to detect pigeon pea as a host plant would lead to a significant number of false negatives. Futhermore, it would not be possible to use the lack of γ-tocopherol to assign a moth as reared on cotton.

A large number of moths (n=15) raised on each host plant are currently being analysed to determine their triacylglyceride profile. A larger dataset may provide conclusive results.

Methods

3.1 Materials

Plant materials including cotton, pigeon pea and tomato of various ages were collected from greenhouse specimens and sorghum, soybean and mungbean were collected from the field. Field samples of cotton and pigeonpea were also collected for comparison.

Insects (*Helicoverpa* Spp. were grown on whole plants in the Department of Agronomy, University of New England.

Methods for the analysis of alkane content

3.2.1 Extraction of plant materials

As we were initially interested in the cuticular wax components of the different host plants, an extraction protocol tailored for the surface of the leaf was used. This had the advantage that a less complex extract was obtained from the leaves containing mainly semivolatile hydrocarbons. Plant materials (cotton, pigeonpea, tomato, sorghum and soybean leaves) were extracted by successive dipping in chloroform (2 x 30 mL) for 30 seconds. Removal of the chloroform under reduced pressure left a residue which was purified by a solid phase extraction protocol using a silica SPE cartridge (100 mg/1 mL) eluting with hexanes. The samples were made up to 2 mg/mL in chloroform and analysed by GCMS.

We also wished to compare whether surface components were similarly distributed throughout the rest of the leaf and so a whole leaf extract was also prepared using several different protocols.

Protocol 1. The dried and homogenized plant materials (500 mg) were extracted using chloroform (40 ml) for three hours. The extracts were filtered and the solvents removed under reduced pressure on a rotary evaporator. The wax components were separated using a silica gel column (6 cm x 1.5 cm) with hexanes (40 mL) then 20% hexanes in chloroform as eluant (40 mL). The hexanes fraction and chloroform fraction in chloroform (2 mg/ml, 1 µL) were separately analysed by GCMS.

Protocol 2. The dried and homogenized plant materials were extracted using chloroform and methanol solvent mixture (2:1). The wax components were separated using a silica gel column (6 cm x 1.5 cm) with hexanes then 20% hexanes in chloroform as eluant (40 mL).

Protocol 3. Acid catalysed transeserification of the crude extract from protocol 2 above (5 mg) was conducted. Samples were dissolved in toluene (1 ml) in a round bottom flask fitted with a stopper and methanolic-hydrogen chloride (2 mL) was added and the mixture was kept overnight at 50^{o}C. Water was added (5 mL) and samples were extracted with hexane (5 mL). The hexane layer was removed and dried over anhydrous sodium sulphate then filtered. The solvent was removed under reduced pressure and the residue taken up in hexane (2 mg/ml) and analysed using GCMS.

A comparison of the different extraction protocols showed that the leaf wax composition detected was dependant on the extraction protocol used. A true representation of the alkane content of leaves is best determined using whole leaf extracts as some hydrocarbons, in particular the $C_{31}H_{64}$ linear alkane, showed different abundances depending on the extraction protocol used. The results for the extraction is further discussed later in the results section.

3.2.2 Extraction of Insects

Freeze dried and individually homogenized insects (200 mg) were extracted using 20% hexanes in chloroform (40 ml) for three hours. The extracts were filtered and the solvent was evaporated under reduced pressure. Samples were prepared at a concentration of 2 mg/mL in chloroform prior to GC column injection.

3.2.3 Gas Chromatography-Mass Spectrometry

GC-MS analysis was performed on an Agilent 6890 Gas Chromatograph with splitless injection, coupled to an Agilent mass spectrometer.

A Zebron ZB-1HTr Capillary Column (30m x 0.25 mm i.d.) was used. The oven temperature was programmed from 80°C to 330°C, initially at the rate of 15°C per min from 80°C to 300°C,

and from 300°C to 330°C at the rate of 25°C/min and with the final 330°C temperature held for at least 7 min. Helium was used as the carrier gas. The MS data were collected under full-scan mode in the range of m/z 50-550.

3.2.4 Identification

A mixture of 13 standard alkanes (C24-C36) was analysed by GCMS and GC with flame ionisation detection (GC-FID) and a clear separation was observed among the different alkanes after optimizing the temperature gradient. *n*-Alkane retention times, molecular ions and fragment ions were used to identify n-alkanes present in the plant and insect extracts. Alkanes are easy to identify using GCMS as fragmentation occurs through the sigma-bonding framework of the molecule leading to sequential loss of methylene (CH_2, *m/z* 14) units. The most abundant peak for all *n*-alkanes is *m/z* 57 and thus the signal at *m/z* 57 could be used to quantify the alkanes. As this fragment ion is also present in other linear hydrocarbons such as alkanols and esters, a silica gel purification step ensured that only alkanes were seen if observing at this molecular weight.

Identification of other components was achieved using NIST (the National Institute of Standards and Technology) MS Search 2.0d reference database (NIST 2005, Ringoes, USA). Retention time and mass spectra of authentic samples were used to confirm the assignments made using the NIST reference database.

3.3 Methods for the analysis of lipid content in plants and insects

3.3.1 Lipid extraction

Lipids were extracted using a modified Folch method. Briefly, 150 mg of sample was extracted with 60 ml of solvent mixture (chloroform/methanol, 2:1, v/v) and the solvent mixture was converted to chloroform/methanol/water (1:1:0.9, v/v/v) for phase separation. The lower chloroform layer was collected and evaporated to dryness under reduced pressure. Extracts were saturated with nitrogen and stored at -20 ^{o}C.

3.3.2 High performance liquid chromatography (HPLC)

A Varian ProStar chromatographic system (Varian, Palo Alto, USA) was used with Alltima^{TM} HP C18 Column (150 mm × 2.1 mm, i. d. × 3 μm, particle size (Alltech Associates, Deerfield, USA). The solvent gradient was initially dichloromethane/acetonitrile (30:70; v/v) and changed to 50:50 v/v over 25 min, to 70:30 v/v over 36 min, converted to the initial gradient (30:70 v/v) for a further 37 min, and finally re-equilibrated for another 10 min. The flow rate was 0.32 ml/min and the analyses were performed with the column kept at room temperature (20 ^{o}C). Samples were prepared at a concentration of 10 mg/ml in

dichloromethane and filtered prior to HPLC column injection. Samples (5 µl) were injected manually at room temperature (20 ^{o}C).

3.3.3 Mass spectrometry

Mass Spectrometric analysis was conducted on a Varian 1200L Quadrupole MS with an atmospheric pressure chemical ionization module set in positive ion mode. The MS data were collected under full-scan mode in the range of 500-1000 *m/z*. Nitrogen was used as drying gas at 150ºC, 12 psi pressure. The corona current was 10 µA, 1400 V and shield voltage was 600 V. The capillary induced dissociation was set at 50 V. The scan method was centroid with sim width 0.7 amu and scan time 1 sec.

3.3.4 Quantification of results

Mass spectra were interpreted and quantified using Varian MS Data Review 6.4.2. Triacylglycerols (TAG) were identified by their relative retention times and diagnostic TAG molecular and diacylglycerols (DAG) ions. Standard TAGs, 1,2-distearoyl-3-oleoyl-glycerol (TAG-SSO) and 1,3-dipalmitoyl-2-oleoyl-glycerol (TAG-POP) were purchased from Sigma-Aldrich (Sydney, Australia). HPLC results were quantified by calculating the sum of the integrated areas of the protonated TAG molecular ion $[M + H]^{+}$, the protonated molecular ion plus acetonitrile ion $[M + H^{+} + 41]^{+}$ and the respective DAG fragment ions.

Results

4.1 Optimization of the extraction protocol for n-alkanes

Experimental design first focused on the identification and quantification of n-alkanes in both plants and insects as alkanes were considered possible biomarkers. The cuticular wax of leaves was extracted by repeatedly dipping the leaf material in chloroform and then directly analysing the extract by GCMS, Figure 1.

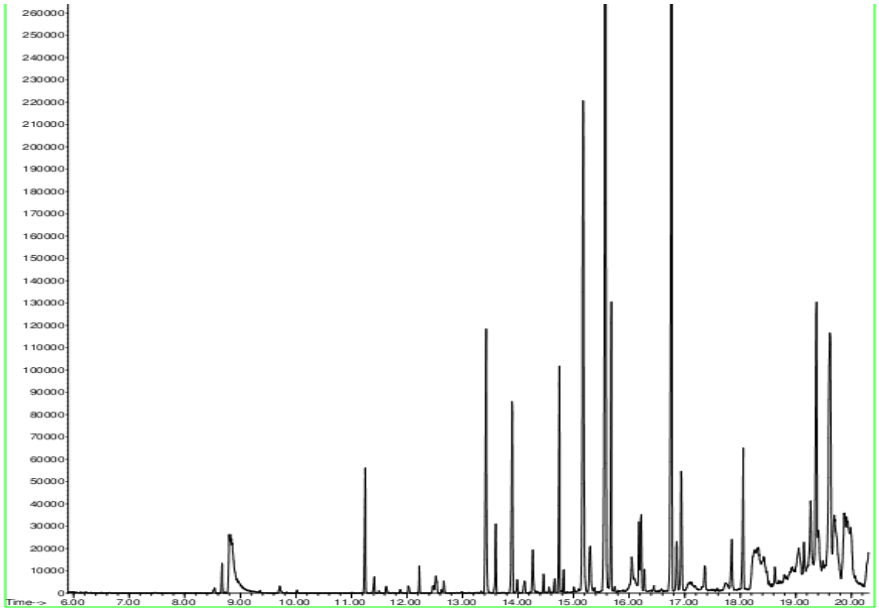


Figure 1. A typical GCMS trace showing the complexity of the leaf cuticular wax extract obtained from cotton.

Due to the large number of components present in the leaf cuticular wax, resolution of peaks was poor and alkanols overlapped some alkanes. Thus it was necessary to develop a protocol for the separation of the non-polar n-alkanes from other components. A simple silica gel SPE separation eluting with hexanes removed moderately polar components giving a concentrated extract of n-alkanes, Figure 2.

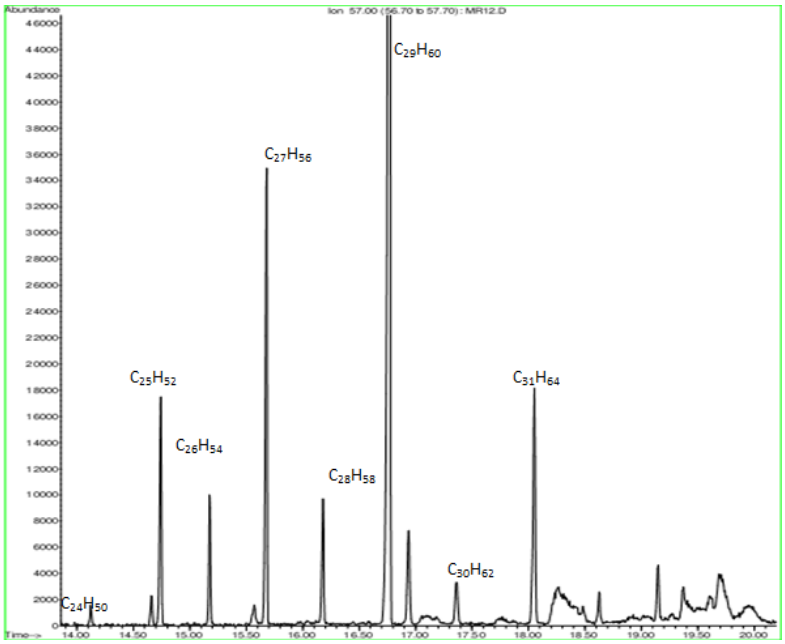


Figure 2. *n*-Alkanes present in the cuticular wax of cotton following SPE separation.

An alternative procedure for the analysis of n-alkanes compared the whole leaf extract obtained by chloroform extraction of leaf homogenate followed by purification by silica gel chromatography eluting with hexanes. The two extraction protocols yielded different relative

amounts of n-alkanes but the whole leaf extract was thought to more accurately represent the diet of the larvae when feeding.

4.2 Alkane compositions

4.2.1 *Alkane composition of plant materials*

The relative compositions of n-alkanes using whole leaf extraction protocols and cuticular wax extraction protocols in pigeon pea and cotton are shown in Figures 3-6.

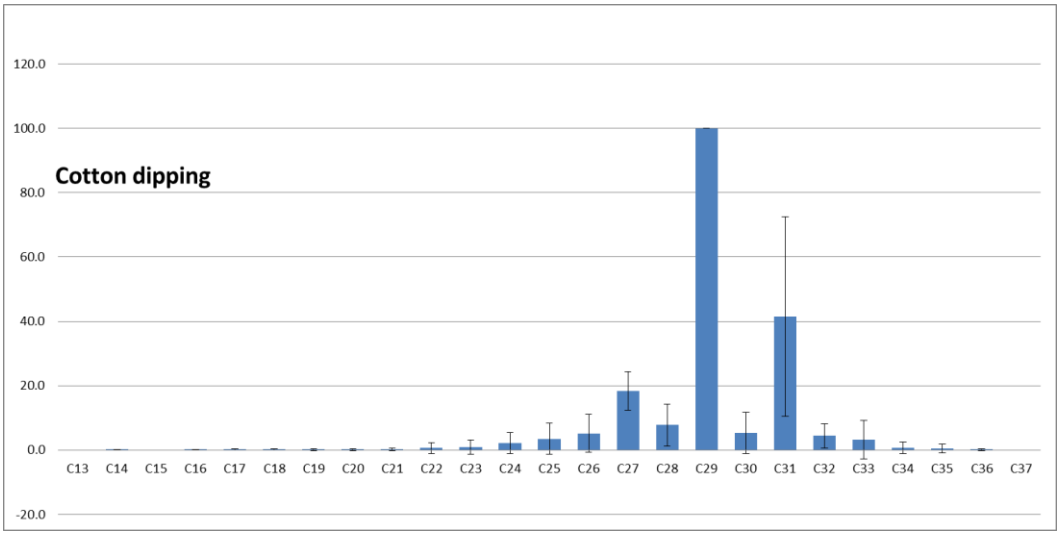


Figure 3. Relative abundance of n-alkanes present in cotton leaf cuticular wax (n=6).

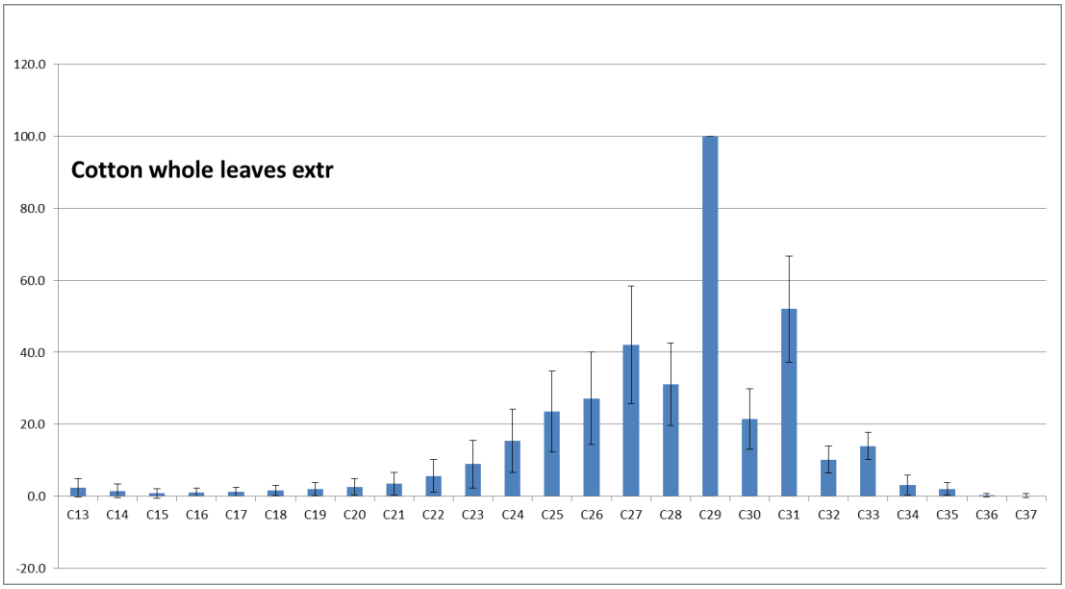


Figure 4. Relative abundance of n-alkanes present in cotton whole leaf homogenate (n=8).

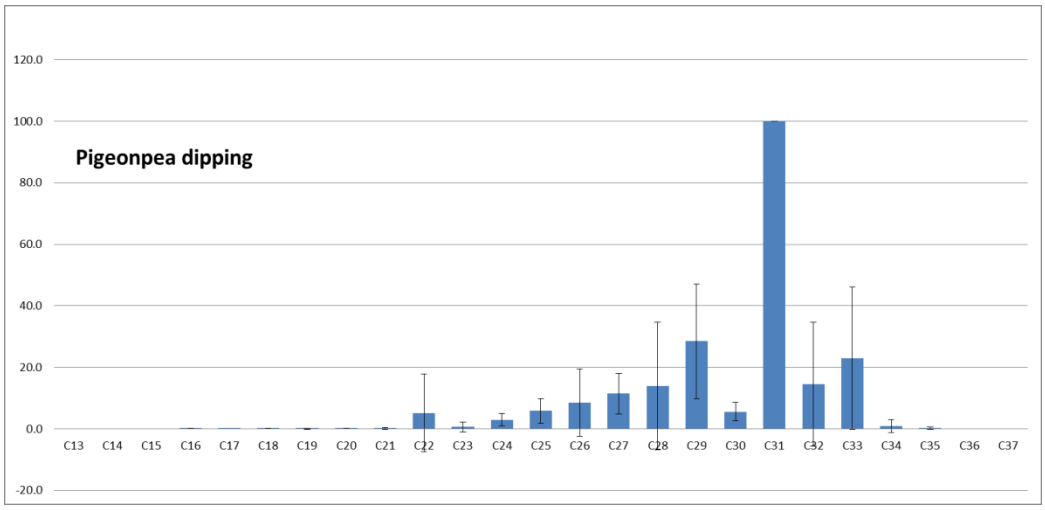


Figure 5. The relative abundance of n-alkanes in pigeon pea leaf cuticular wax (n=6).

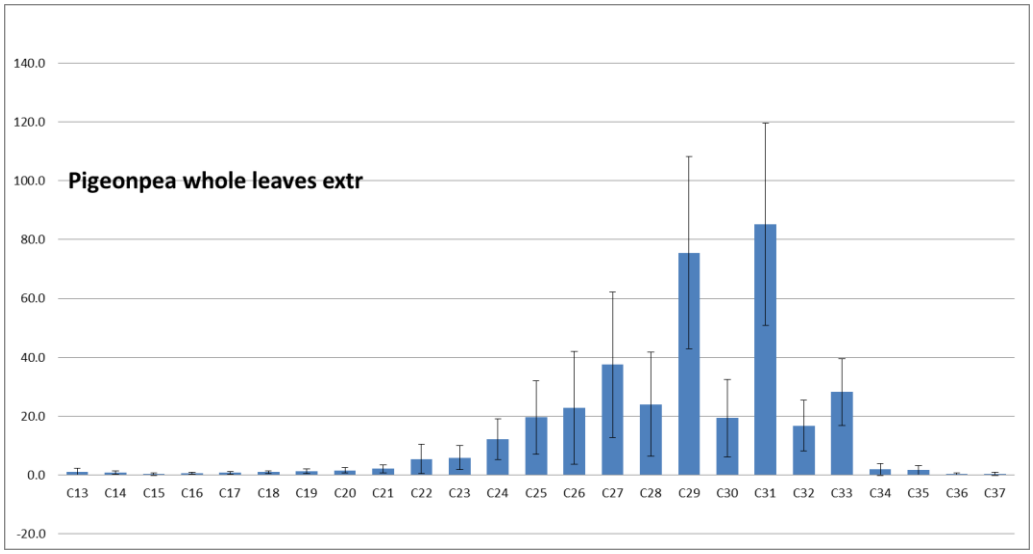


Figure 6. The relative abundance of n-alkanes in pigeon pea whole leaf homogenate (n=8).

There was some variability in the relative abundance of the n-alkanes, however, some clear trends were observed. Importantly, the relative abundance of the $C_{29}H_{60}$ and $C_{31}H_{64}$ *n*-alkanes were significantly different in cotton and pigeon pea. This provided a potential biomarker to differentiate cotton and pigeon pea raised moths if the difference was also detected in the hydrocarbon content of the moths. Interestingly, the relative abundance of the *n*-alkanes was not significantly altered by the extraction protocol in cotton, however, was significantly different for pigeon pea.

Not surprisingly, the most abundant alkanes were those with odd numbered chains which is a consequence of the biosynthesis which involves decarboxylation of even numbered linear fatty acids. The most abundant alkane in the extract of whole leaves of cotton was nonacosane (C29, relative abundance 100%) and the relative amounts of the other alkanes were tetracosane (C24; 16.6%), pentacosane (C25; 25.6%), hexacosane (C26; 29.4%),

heptacosane (C27; 45.0%), octacosane (C28; 33.1%), triacontane (C30; 22.6%), hentriacontane (C31; 51.0%), dotriacontane (C32; 10.6%) and tritriacontane (C33; 14.0%). Significant quantities of low molecular weight alkanes were seen using the whole leaf extraction protocol but not when using the cuticular wax extraction protocol. For comparison, the cuticular wax extraction protocol gave C24 (2.9%), C25 (4.4%), C26 (6.1%), C27 (18.9%), C28 (9.1%), C29 (100.0), C30 (6.4%), C31 44.%), C32 (4.8%) and C33 (4.8%).

In comparison, the most abundant alkane in pigeon pea leaves was the $C_{31}H_{64}$ *n*-alkane for both extraction protocols. The relative abundance of the other alkanes were C24 (13.1%), C25 (21.3%), C26 (25.4%), C27 (41.9%), C28 (27.1%), C29 (87.8), C30 (22.4%), C32 (20.5%) and C33 (35.4%).

Sorghum leaves

The alkane composition of sorghum; leaves were also determined and the most abundant alkane was C29 and the others were C24 (12.2%), C25 (20.0%), C26 (20.9%), C27 (41.8%), C28 (18.3%), C30 (11.8%), C31 (60.4%), C32 (8.6%) and C33 (27.1%). C27, C29 and C31 have been previously identified as major n-alkanes in sorghum leaves (Bianchi et al 1977).

Mungbean leaves

The alkane composition of mungbean; leaves were also determined and the most abundant alkane was C31 (100%) and the others were C24 (11.4%), C25 (17.5%), C26 (19.7%), C27 (21.5%), C28 (16.9%), C29 (28.8%), C30 (13.5%), C32 (13.7%) and C33 (11.9%).

Soybean leaves

The alkane composition of sorghum; leaves were also determined and the most abundant alkane was C31 and the others were C24 (42.1%), C25 (54.9%), C26 (64.0%), C27 (75.0%), C28 (54.0%), C29 (70.4%), C30 (33.8%), and C33 (32.2%).

4.2.2 *Hydrocarbon composition of insects:*

Cotton and pigeonpea reared moths

Although alkanes are chemically unreactive, they are substrates for oxidising enzymes such as CYP450 and may exhibit low bioavailability due to their size and insoluble nature. Freeze dried homogenised moths raised on conventional cotton or pigeon peas gave virtually identical GCMS traces, Figure 7. It was only by searching traces for the molecular ions of interest that differences in the moth extract could be detected which required the prior identification of analytes of interest in the larval host plant.

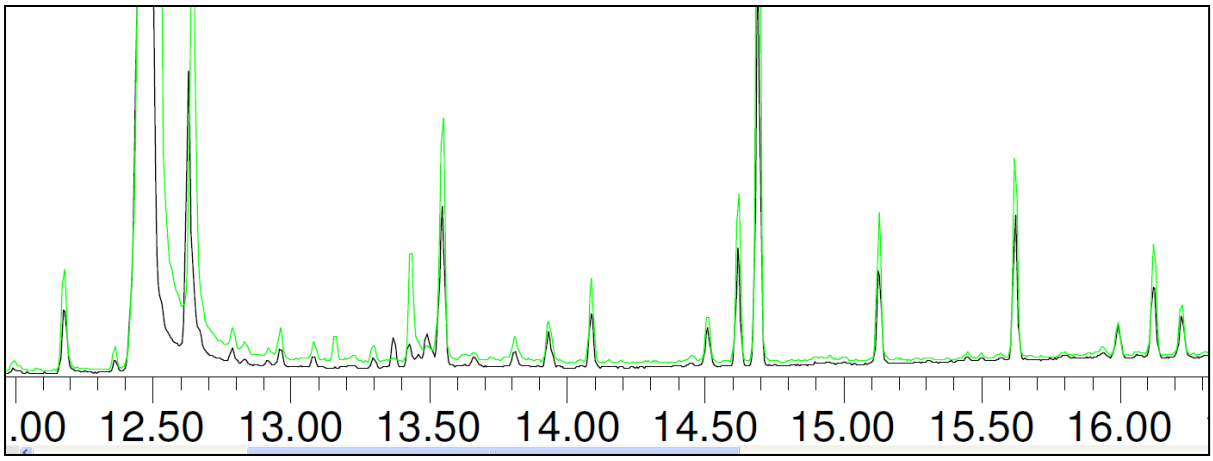


Figure 7. An overlay of GCMS traces from extracts of moths raised on cotton and pigeon pea (green = cotton, black = pigeon pea).

The *n*-alkanes in pigeon pea and cotton raised moths were quantified and the results from a series of individual specimens are shown in Figure 8. Little difference was seen between moths raised on cotton or pigeon pea (n=4 of each type, 2 x male and 2 x female).

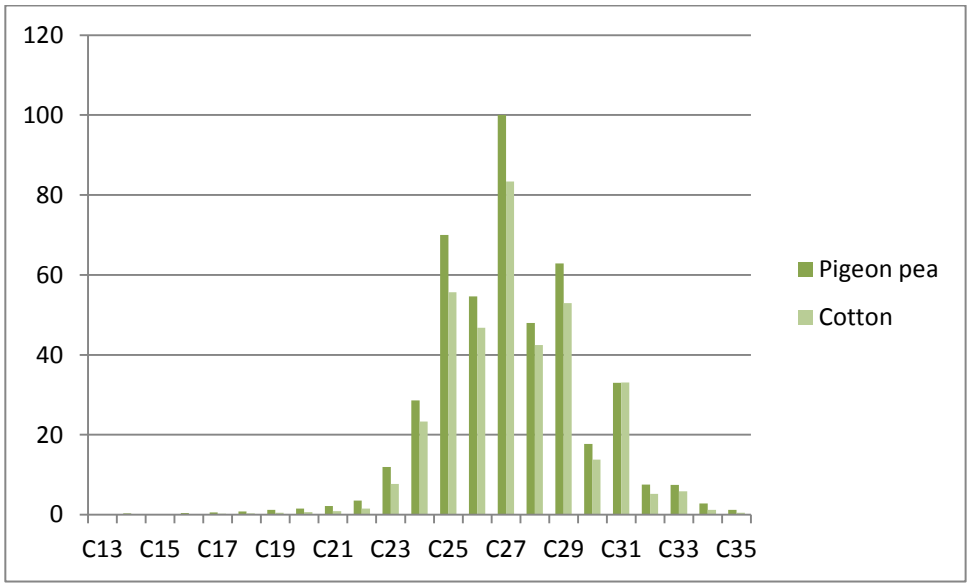


Figure 8. Alkane content in pigeon pea and cotton raised moths

Mungbean reared moths

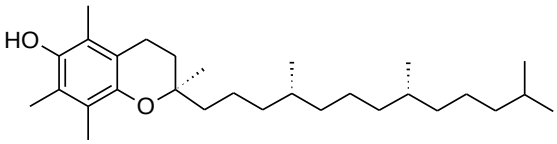
The composition of alkanes in the mungbean reared moths was slightly different from the cotton and pigeonpea reared moths. Although the most abundant alkane is C27, lower molecular weight alkanes were also abundant. The relative compositions of these alkanes were C19 (11.9%), C20 (29.9%), C21 (62.6%), C22 (74.2%), C23 (74.2%), C24 (59.3%), C25 (78.8%), C26 (63.8%), C28 (51.5%), C29 (69.1%), C30 (29.4%), C31 (48.3%), C32 (14.9%) and C33 (12.3%).

Soybean reared moths

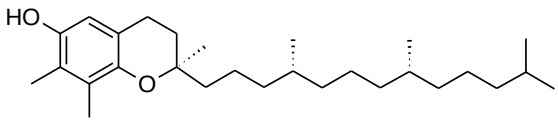
The soybean reared moths showed slight differences as relatively higher amounts of C25 and C27 alkanes (~100%) were observed. The relative abundances of the other alkanes were C19 (11.4%), C20 (15.4%), C21 (27.9%), C22 (54.8%), C23 (69.3%), C24 (62.9%), C26 (68.7%), C28 (52.3%), C29 (59.0%), C30 (28.6%), C31 (30.1%), C32 (14.4%) and C33 (10.1%).

4.2.3 *Tocopherol composition in leaves and insects*

Authentic samples of α-, β- and γ-tocopherols were used to identify and quantify tocopherols in leaf and insect material. The relative abundance of α- and γ-tocopherols was calculated in cotton and pigeonpea leaves as well as in the cotton and pigeonpea reared moths. It was observed that a relatively lower abundance of γ-tocopherol was present in cotton leaves as well as cotton reared moths relative to moths raised on pigeon pea. In many cases, it was almost undetectable in cotton reared moths (9 of 12 samples had > 10:1 α /γ) while a clear signal for γ-tocopherol was observed in most pigeon pea reared moths (5 of 12 samples had < 5:1 α /γ).



α-Tocopherol



γ-Tocopherol

Extraction protocols for the analysis of alkanes were not appropriate for the analysis of tocopherol content due to the increased polarity in the molecules and thus a more polar solvent system was required to elute these compounds on silica. Although variation in the ratios of γ- and α-tocopherols was observed, there was significant variability in the results for pigeon pea raised moths and a significant percentage did not contain > 10% γ-tocopherol relative to α-tocopherol.

Tocopherols were also identified in soybean and mungbean leaves as well as the moths reared on their leaves. Mung bean leaves had a low γ-tocopherol concentration relative to α-tocopherol while Soybean leaves were enriched in γ-tocopherol. The content of γ-tocopherol in Soybean reared moths was also high reflecting the diet. Due to the variability in the sample content, the tocopherol ratios alone were insufficient to identify larval host of a moth.

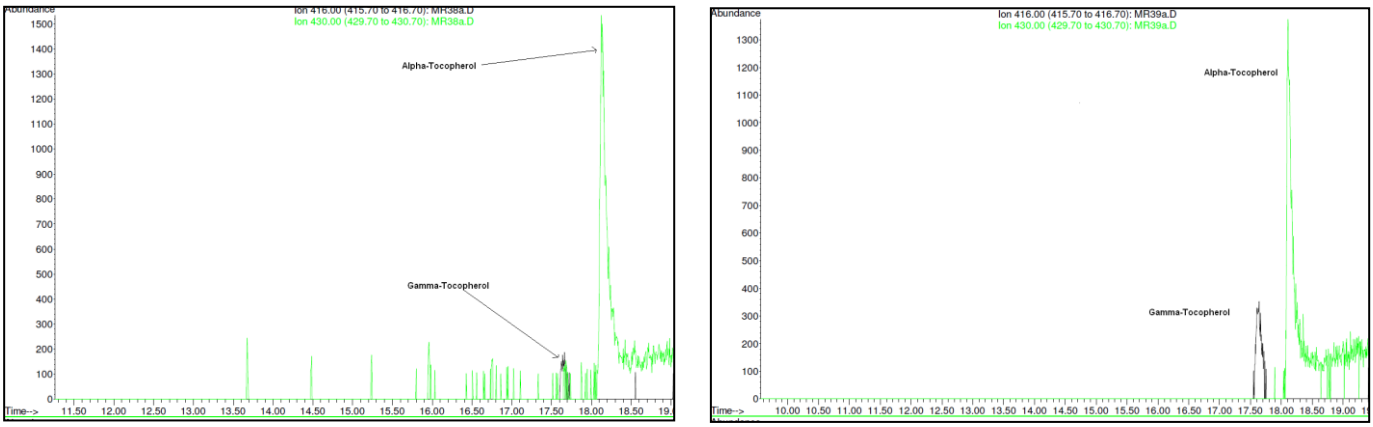


Figure 9. Tocopherol content in moths raised on cotton (left) and pigeon pea (right).

4.3 Composition of diacylglycerols and triacylglycerols in cotton, pigeon pea and the abundance of triacylglycerides in moths raised on each host.

The composition of triacylglycerides and diacylglycerides was identified as a potential difference in insects raised on cotton and other crops. Whereas the content and identity of alkanes can be determined by GCMS, triacylglycerides are best analysed using HPLC with MS detection. Previous studies have examined fatty acid content in *helicoverpa* moths and have seen differences in the length and unsaturation of fatty acids correlated with larval host plant. When examining fatty acid composition, the entire complement of fatty acids in the insect is examined following transesterification and includes triacylglycerides, diacylglycerides, phospholipids and any other fatty acid present in the insect. A large number of triacylglycerides can be formed in the insect from the combinations of different chain length fatty acids leading to complex data sets.

4.3.1 ***Principal Component Analysis (PCA)***

PCA is a procedure used to reduce the dimensionality of complex data sets, making the data easier to visualize and interpret. It relies on the assumption that, in data containing many variables, some of these variables may be correlated and hence new variables (called principal components) can be calculated as a linear combination of the correlated variables. This is achieved by firstly centering the data (by subtracting the mean value for each variable from each value of the variable), which places the origin at the centre of the data and then transforming the data set to a new set of axis. In theory, number of principal components can be the number of original variables but in practice the variance captured by latter PCs is generally only noise and a smaller number of principal components are generally considered. Often the first three PCs can account for over 80% of the variance in a data set, allowing the data to be well described with a much smaller number of variables, if there is a high degree of correlation between some variables. The contribution of each of the original variables to a

principal component is called its loading. The score for a case is calculated by summing the values of the original variables for that case, after correction by multiplying the variable by the loading for that variable in that principal component. A plot of the scores for each case (usually presented as a two dimensional scatter plot of the scores with respect to a pair of principal components) allows an easy visualization of structure in the data. For example, cases that are close together in the plot of scores for two principal components share some similarities. A plot of the loading for the same components then allows the variables that are responsible for these similarities to be identified, as they occupy a corresponding position in the loadings plot to that of the cases in the scores plot i.e. cases which group together in the top right quadrant (say) of the score plot have relatively higher values for the variables that are in the top right quadrant of the loading plot.

4.3.2 *Triacylglyceride and diacylglyceride content*

Three cotton, three pigeonpea and two soybean moths were extracted for acylglycerols (DAGs and TAGs) and the lipids were analysed using HPLC-APCIMS to identify and quantify DAG and TAG molecular species. Cotton and pigeonpea leaves were also extracted for lipids and analysed using HPLC-APCIMS. The HPLC-MS method used in this experiment was one which was previously developed at UNE. The average of the composition of the individual molecular species of the respective moths is presented in Figure 9 and the average composition in leaves is presented in Figure 10**.**

Abbreviations used in the assignment of TAGs and DAGs: My: Myristic 14:0, P: Palmitic 16:0, Po: Palmitoleic 16:1, Ma: Margaric 17:0, Mo: Heptadecenoic 17:1, S: Stearic 18:0, O: Oleic 18:1, L: Linoleic 18:2, Ln: Linolenic 18:3, A: Arachidic 20:0.

A total of 35 acylglycerol (DAG and TAG) molecular species were identified in cotton moths, including 10 DAG molecular species. In moths raised on pigeonpea, 36 acylglycerol molecular species were identified and 7 of these were DAGs. The number of acylglycerol molecular species in soybean moths was 32 with 8 DAG molecular species. Comparatively higher content of DAG was observed in cotton moths than pigeonpea and soybean moths and the content of DAG was 10.2% in cotton moth, 5.2% in pigeonpea moth, 4.3% in soybean moths, respectively. The most abundant DAGs in cotton and pigeonpea moths were OP and LPo whereas in soybean moths LnL, LL and OP were the most abundant DAG molecular species.

A total of 25 TAG molecular species were identified in cotton moths and predominant molecular species were POP (17.7%), OOP (14.1%), OLnP (10.1%), LOP (8.4%), POPo (8.0%) and LnLnP (6.5%).

Pigeonpea moths produced a total of 29 TAG molecular species and predominant molecular species were POP (21.5%). OOP (18.4%), POPo (15.0%) OLnP (8.3%), LOP (6.8%), and PLnP (5.3%).

A total of 24 TAG molecular species were isolated from soybean reared moths and the major molecular species were LOP (17.0%), LLP (14.7%), OOP (8.7%), OLL (8.3%), POP (6.8%), PLP (6.2%) and OOL (6.1%)

It was observed that the relative composition of acylglycerol molecular species of cotton and pigeonpea moths were similar, whereas there was significant variation in composition of acylglycerols in soybean moths compared to the cotton and pigeonpea moths.

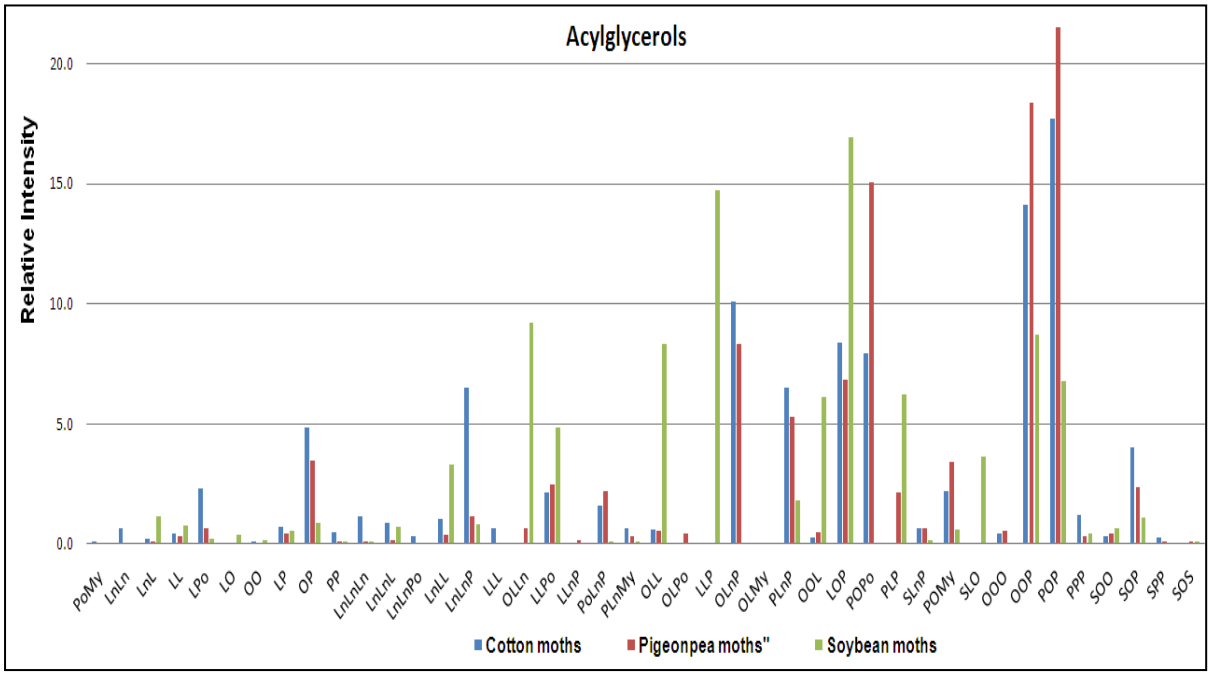


Figure 10. Acylglycerol molecular species composition of cotton, pigeonpea and soybean moths

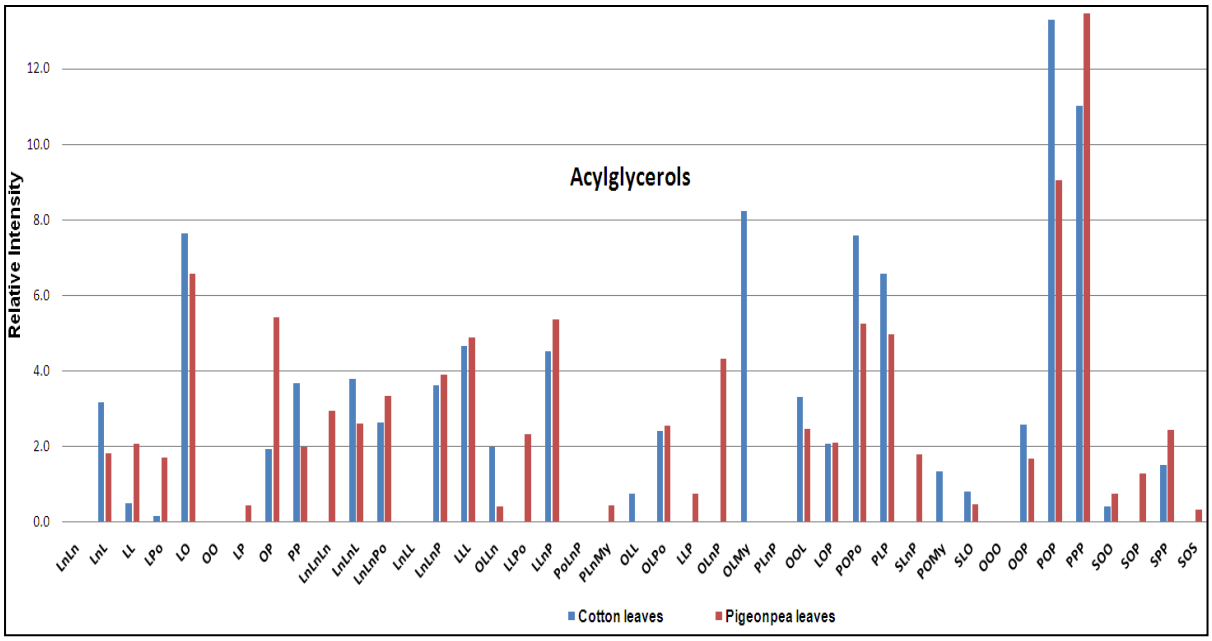


Figure 11. Acylglycerol molecular species composition of cotton and pigeonpea leaves

The principal component analysis of the composition of DAGs and TAGs was performed using SPSS software version 20.0. In this analysis, all DAGs and TAGs were considered and showed that PC1, PC2 and PC3 explained 83% variance of the data set. The score plot showed a clear separation of soybean moth from cotton and pigeonpea moths by PC1, Figure 11 The loading plot of PC1 against PC2 showed that the soybean moths clustered separately due to the presence of higher amounts of LLP, SLO, OOL, OLLn, OLL and PLP molecular species, Figure 12. The separation of cotton from pigeonpea moths was not obvious, however pigeonpea and cotton moths clustered separately. The loading plot also indicated that the cotton and pigeonpea moths clustered separately because of higher content of SOP, OLnP and OP in cotton moths in comparison to pigeonpea moths. There was no separation between cotton and pigeonpea leaves, however, cotton and pigeonpea leaves from green house and field clustered separately. Additional samples may therefore improve detection of the larval host and therefore a larger number of moths are being examined to improve the data set and provide bounds against which additional moths can be blind screened

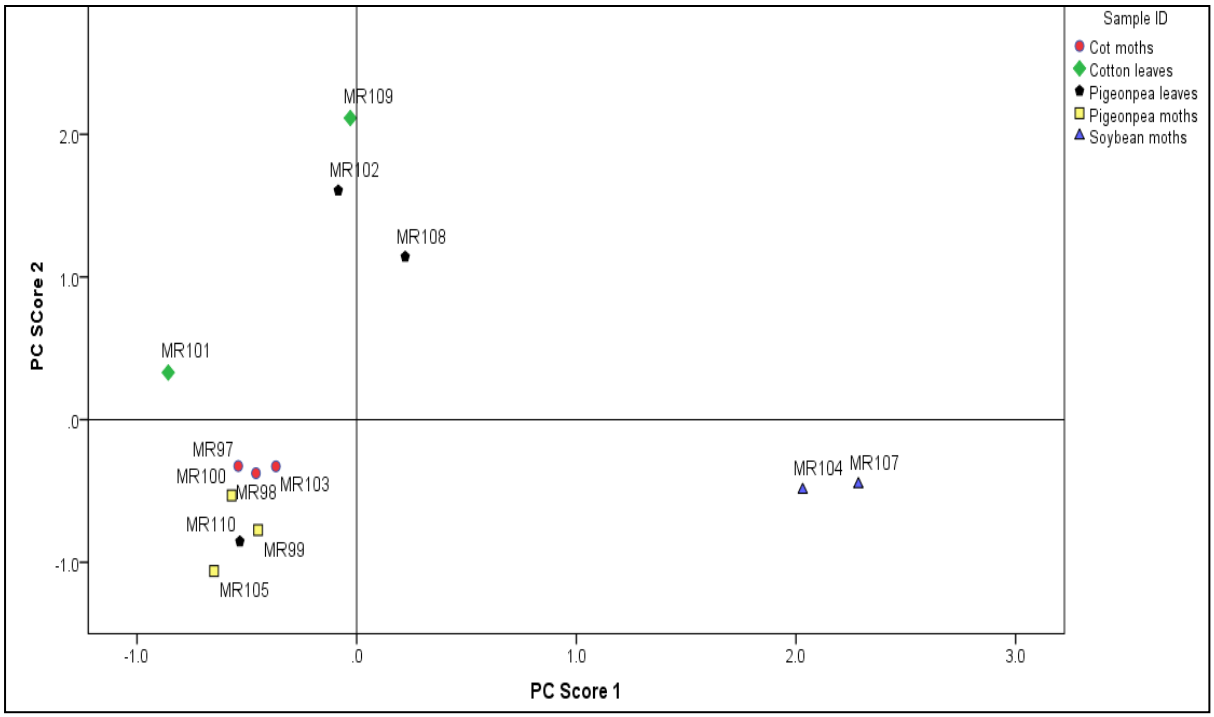


Figure 12: Score plot of Principal Component 1 (PC1) against Principal Component 2 (PC2) for the most abundant DAG and TAG molecular species

Cotton leaves: MR101 (Field), MR109 (Green house); Pigeonpea leaves; MR102, MR108 (Field), MR110 (Green house).

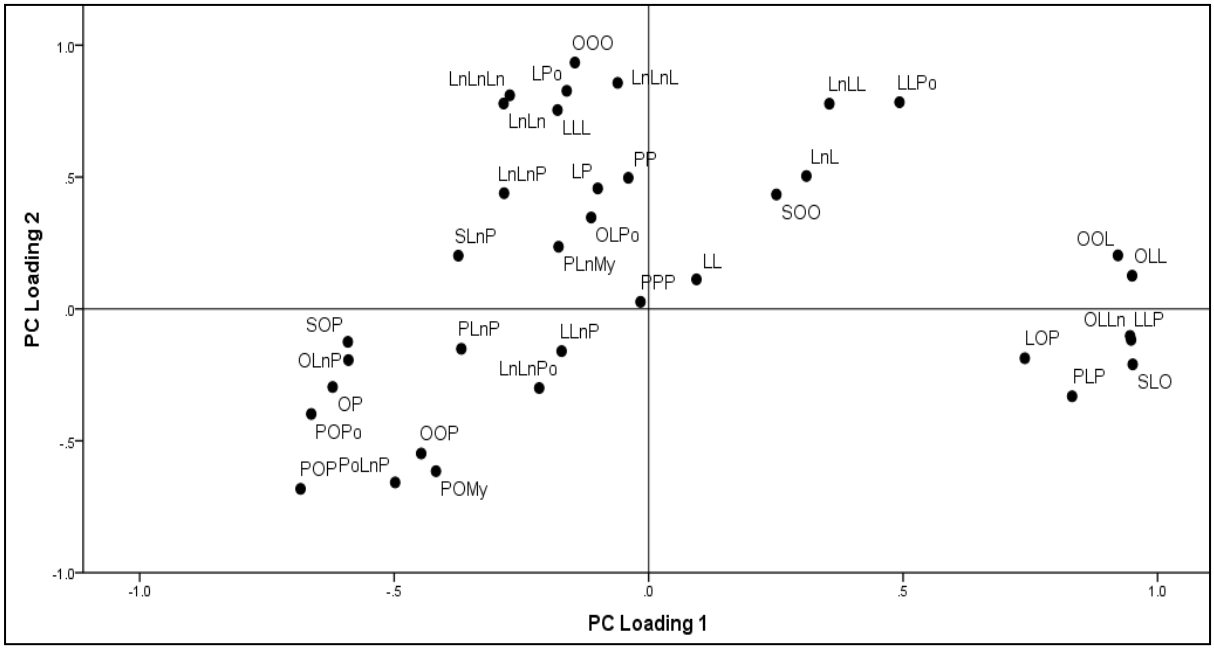


Figure 13. Loading plot of PC1 against PC2 for the most abundant DAG and TAG molecular species

5 Outcomes

5.1 Knowledge on surface wax composition of several host plants, incorporated in reports and publications

Linear alkanes as potential biomarkers of host plant origin have been examined and have been eliminated as they do not reflect the hydrocarbon content of the larval host plant. We have quantified the leaf wax and whole leaf composition of cotton and pigeon pea with respect to *n*-alkanes and found that the method of analysis can significantly alter the quantities of *n*-alkanes detected. The cuticular wax and whole leaf composition of *n*-alkanes present in the plants will be published in due course.

5.2 Knowledge of surface wax compounds and secondary metabolites such as phenolics and flavonoids in adult moths, incorporated in reports and publications.

Tocopherols have been identified in adult moths and result from larval feeding and we have examined the relative isomeric ratio as a potential biomarkes for larval feeding. The ratio of isomers present in the plant is reflected in the adult moth and therefore, the ratio of γ-tocopherol relative to α-tocopherol is an indicator that the moth was not raised on cotton. Analysis of multiple samples demonstrated that this alone is insufficient to differentiate the larval host and will not capture all moths which fed on non-cotton crops. This information will be presented in publication format in due course.

The use of triacylglycerides and diacylglycerides is a promising avenue of research and has the potential to differentiate moths raised on a variety of host plants including soybean, pigeon pea and cotton. However, the number of samples analysed to date is insufficient to make conclusions regarding the accuracy of the method. From the limited number of samples analysed, the TAG and DAG content in the plant alters the DAG and TAG content of moths which fed on the plant as larvae.

5.3 ***Assay methods for determining suitable marker compounds***

Assay methods for the analysis of detection of plant specific metabolites have been developed. These include the solid phase extraction protocol used for the concentration of *n*-alkanes and removal of alkanols. The application of triacylglyceride analysis by MS fragmentation pattern for the determination of TAGs and DAGs in insects.

6 Technical advances achieved

We have identified several chemicals present in adult moths from larval feeding. As tocopherol biosynthesis has only been observed in photosynthetic organisms, its presence must be a product of larval feeding. The relative abundance of the different tocopherols reflected the isomeric ratios found in the plants, however, this alone is insufficient to assign larval host due to the variability in isomeric ratios found in individual plants.

We have found that the methods used in extraction of *n*-alkanes from plants will affect the *n*-alkanes detected and the abundance of alkanes in cuticular wax does not accurately represent the abundance of cuticular wax in the whole leaf.

We have applied for the first time to the best of our knowledge, TAG and DAG analysis methods to *Helicoverpa* moths and have preliminary evidence that the abundance of TAGs and DAGs in the larval host plant affects the quantities and identity of TAGs and DAGs in the moth.

7 Conclusion

The identification of the larval host of helicoverpa moths is not possible using linear *n*-alkanes. The isomeric ratio of tocopherols present in the larval host is reflected in the adult moth, however, there is significant variability in the quantities of tocopherols present in individual host plants.

The quantities of diacyl glycerides and triacylglycerides in the adult moth are potential biomarkers for larval feeding. If further experiments confirm these results,

this will constitute a robust and transferrable assay for the determination of larval host origin.

8 Extension Opportunities

The work in this project as aimed at developing a tool for other researchers, so they are the primary target of communication and extension efforts, rather than growers or consultants. The work will be presented at the next meeting of REFCOM, and published in the scientific literature. Non-technical summaries will be presented to grower groups at field days and workshops, for example, two field days planned for the upper Namoi area in June 2012.

Further research will be needed to develop the triacyglyceride findings into a usable test to identify moths from the field. This work can be continued at a low level through Peter Gregg's new CRDC project on moth busting (for which markers of origin are crucial), but we also plan on submitting a further application to CRDC in the 2012 round.

9 Publications

No publications have arisen from this project as yet. It is envisaged that tocopherol and alkane content will be published separately to the TAG and DAG analysis of cotton and pigeon pea raised moths.

No online resources have been developed from this project.

Part 4 – Final Report Executive Summary

The Australian cotton industry has benefited greatly from the introduction of the transgenic Bolgard II cotton. Bolgard II expresses the Bt toxins which kill *Helicoverpa* larvae after feeding and has subsequently reduced the use of chemical insecticides to control insect pests. As resistance to the Bt toxins in *Helicoverpa* remains a threat, resistance managment plans for Bollgard II have been developed. These measures include, growing refuge crops to breed genetically suscpetible moths, pupae busting using cultivation to disrupt overwintering pupae in the soil and trap crops to retain the offspring of moths selected for Bt resistance.

A limitation in evaluating these measures is that there has been no reliable way of determining whether trapped *Helicoverpa* adults are derived from larvae which were reared on cotton (and therefore exposed to Bt toxin), or on other crops such as pigeonpeas (widely used in refuge and trap crops). To address this shortcoming, we have reared moths on cotton and other host crops and then examined the adult moths for the presence of plant secondary metabolites. The secondary metabolites identified in moths were then correlated with the levels found in the different host plants.

Extraction and analysis of *n*-alkanes in cotton and pigeon peas identified 15 linear alkanes and it was found that the relative abundance of hentriacontane and nonacosane in the plants was significantly different. Furthermore, a series of plant compounds, namely triterpenes and tocopherols were detected that were unique to cotton and pigeon peas. Moths reared on either conventional cotton, pigeon pea and other host crops were then analysed for the presence of these secondary metabolites. Alkane levels in the moths did not reflect the levels found in the plants indicating that the moths may biosynthesise their own alkanes. Tocopherols were identified and quantified in the moths and the levels found were an indicator of the plant on which the larvae fed.

A series of other plant products were analysed in the moths and it is expected that using these biomarkers, along with the amount and type of tocopherol present, will allow for the determination of the larval host plant. The successful development of this assay will allow for the quantitative evaluation of resistance management plans by determining the host plant of adult moths.

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