*The biochemical correlation between the epicuticular wax of upland cotton (*Gossypium hirsutum *L.) and the wax of different mealybug species*

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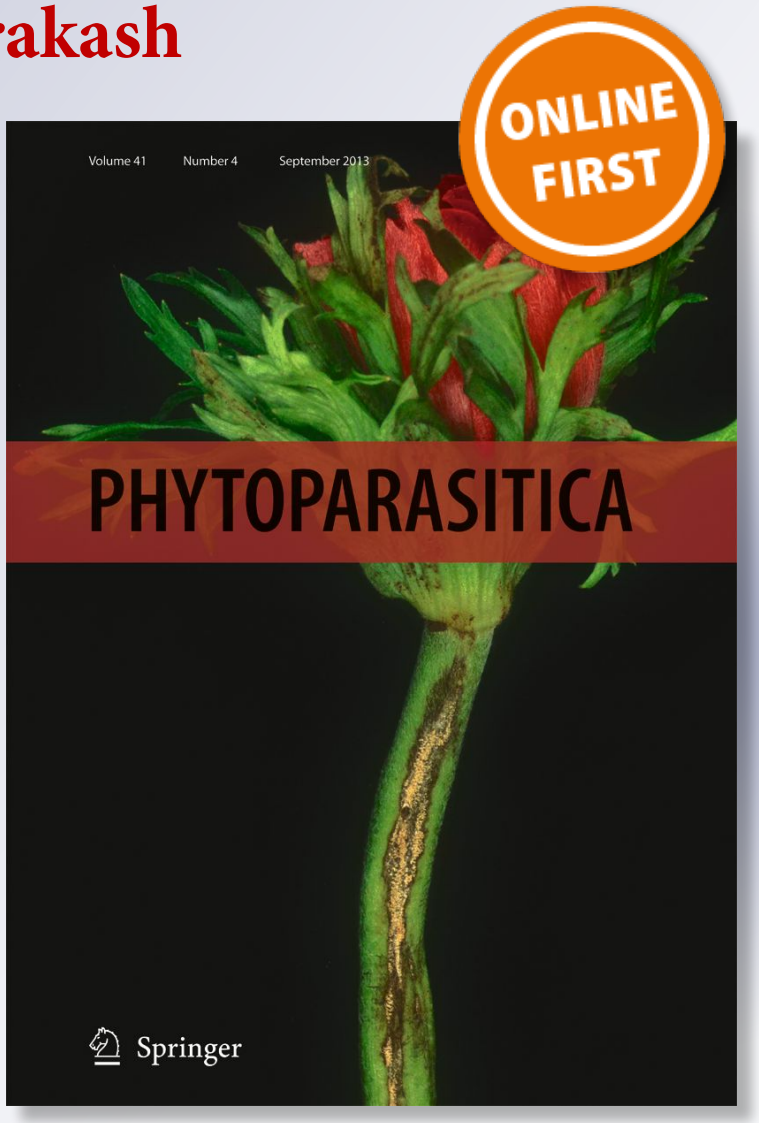

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The biochemical correlation between the epicuticular wax of upland cotton (*Gossypium hirsutum* L.) and the wax of different mealybug species

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Abstract The study aimed to find the possible differences, existing between the cuticular waxes of cotton and mealybug insects, using advanced analytical studies. The biochemical composition of the leaf wax of upland cotton (*Gossypium hirsutum* L.) and the cuticular wax of the different mealybug species, including *Phenacoccus solenopsis* Tinsley, *Ferrisia virgata* Cockerell, *Paracoccus marginatus* Williams and Granara de Willink, and *Drosicha mangiferae* Green were analyzed in detail by Gas Chromatography-Mass Spectrometry (GC-MS). The results clearly confirmed that the cotton wax is dominated by the six-carbon alkanes, while the mealybug wax is a mixture of both the five-carbon alkanes and the six-carbon alkanes. Apart from these differences, the common hydrocarbons such as hexadecane, icosane, and heneicosane, the uncommon hydrocarbons such as ethane, cyclobutanone, decane, and cyclododecane, the species-specific compounds of mealybugs such as myristyl alcohol, quinoline, hexacosane, and pentacosane were also identified and their retention times (RT) were listed out in detail. The outcome of this study will be useful to develop pest management techniques targeting the waxy cuticle of mealybugs without obstructing the normal physiology and growth of the cotton crop.

Keywords Cotton · Mealybug wax · Composition · Correlation · Identification · GC-MS

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Introduction

Wax, a type of lipid contains a wide variety of the long-chain alkanes, esters, polyesters, and hydroxyesters of the long-chain primary alcohols and fatty acids. The surface waxes of plants are the complex mixtures of the relatively nonpolar, aliphatic, and cyclic compounds (Jetter et al. 2006). The surface waxes of plants consist of the various groups of the long-chain lipids such as hydrocarbons, wax esters (the esters of the long-chain alcohols and fatty acids), alkyl esters, fatty acids, long-chain alcohols, aldehydes, ketones, beta-diketones, and hydroxy-beta-diketones (Hansen et al. 1997). As the plants cover much of the earth's surface, it seems likely that the plant waxes are the most abundant of all the natural lipids. The chemistry of the waxes secreted by insects has been studied over the last 100 years. The cuticular waxes of the insect species contain the following chemical classes: hydrocarbons, fatty acids, alcohols, triacylglycerols, and wax esters. The waxes of

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some of the insect species also contain aldehydes, ketones, esters, and sterols (Gołębiowski et al. 2011).

The plant waxes limit the diffusion of water, resist drought, control the release of volatiles, deter from pest attack, render protection against diseases and insects or attract the pollinating insects. In the leaves of cotton, both the upper and lower surfaces are protected with an amorphous layer of cuticle with the abundant ridges of the epicuticular wax (Wullschleger and Oosterhuis 1989). Typically, the long-chain alkanes such as n-octacosane, n-nonacosane, n-triacontane, dotriacontane, and n-tetracontane are the chief constituents of the epicuticular wax of cotton (Bondada et al. 1996). Likewise, the major polyphagous pest, mealybug possesses a waxy coating on the dorsal side that protects it from the insecticides and the natural factors of mortality. The different mealybug species, belonging to the Pseudococcidae and Monophlebidae families of the order Hemiptera are recorded as the pests of cotton in India among which, the striped mealybug, *Ferrisia virgata* Cockerell (Hemiptera: Pseudococcidae), the solenopsis mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae), and the papaya mealybug, *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae) are prevalent in Tamil Nadu, India (Nagrare et al. 2014). The mango mealybug, *Drosicha mangiferae* Green (Hemiptera: Monophlebidae) is a serious pest of mango but it also causes considerable damage to the plants such as cotton, mulberry, guava, papaya, jamun, citrus, tamarind, okra, eggplant, and hibiscus (Sathe et al. 2014).

However, information regarding the biochemical constitution of the mealybug wax is very limited. The modern analytical techniques such as the High-Performance Liquid Chromatography (HPLC), the Gas Chromatography-Mass Spectrometry (GC-MS), the Supercritical Fluid Chromatography (SFC), and the Liquid Chromatography-Mass Spectrometry (LC-MS or alternatively, HPLC-MS) help in the general detection, separation, and potential identification of the chemicals of particular masses in the presence of other chemicals (Siddiqui et al. 2013). The Gas Chromatography-Mass Spectrometry (GC-MS) is an efficient tool for the identification of the organic, volatile, and semivolatile compounds. Gas Chromatography (GC) separates mixtures into their individual components according to their boiling points, whereas Mass Spectrometry (MS) is used to identify the various components from their mass spectra, using the NIST/EPA/NIH Mass Spectral Library. Therefore, the aim of the present study is to determine the biochemical composition of the epicuticular wax of upland cotton (*Gossypium hirsutum* L.) and its correlation with the cuticular wax of the various mealybug species by the GC-MS analysis so that the pest control techniques, targeting the mealybugs of cotton by the degradation of the wax cannot hinder the normal physiology and growth of the crop in any way.

Materials and Methods

Collection of plant samples

The samples of the fully expanded leaves of *G. hirsutum* L. (variety Surabhi) were collected from the Indian Council for Agricultural Research-Central Institute for Cotton Research (ICAR-CICR), Regional Station, Coimbatore, Tamil Nadu, India (11.014327° N latitude, 76.929456° E longitude). The collected samples were packed instantly in polyethylene bags, transported to the laboratory, and kept at room temperature for processing. Under laboratory conditions, the fully expanded leaf samples of *G. hirsutum* L. were washed in sterilized water and dried at room temperature.

Extraction of wax from cotton leaves

The leaf samples of *G. hirsutum* L. were weighed (typical sample weight 5 g), cut, and immersed in hexane for 30 s (15 s more than the standard procedure of Bakker et al. 1998) at room temperature for maximum extraction of wax. The resulting solution of the cuticular wax was concentrated by keeping in a hot air oven at 40 °C. The solution, containing hexane, wax, and the impurities, was filtered through a Whatman™ 1001–090, Grade 1 Qualitative Filter Paper, having a diameter of 9 cm and a pore size of 11 μm, in order to remove the impurities. The filtrate, containing the wax was transferred into test tubes and allowed to dry off. A white film of the wax was seen at the bottom of each test tube. After the solvent had dried off completely, a solution containing 10 μL each of pyridine and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + Trimethylchlorosilane (TMCS) was added to each sample, using a glass syringe to convert the free hydroxyl groups into their trimethylsilyl ethers and the free carboxyl groups into their esters, respectively. After a few minutes, the solution, containing the wax was resuspended in 100–200 μL of fresh hexane, filtered through a cellulose acetate membrane syringe filter, having a

diameter of 28 mm and a pore size of 0.2 μm, and transferred into a GC vial.

Collection of insect samples

The adult females of *P. solenopsis*, *F. virgata*, and *P. marginatus* were collected from the ICAR-CICR, Regional Station, Coimbatore, Tamil Nadu, India, while *D. mangiferae* was collected from the mango orchard of the Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The mealybug samples were transported to the laboratory in sealed polyethylene bags. The live insects were anesthetized and killed subsequently by freezing on dry ice for 1 min.

Extraction of the mealybug wax

The cuticular lipids of mealybugs were extracted by soaking the thawed insects in hexane for 60 s. The volume of hexane, used for the extraction and elution varied among the mealybug species, depending on the amount of hexane required to completely submerge the insects. A total of 15 mealybugs from each mealybug species were used in each of the extraction replicates, in order to obtain enough wax for the subsequent GC-MS analysis. The dried extracts were eluted with 10 μL of pyridine and a mixture, containing 10 μL of BSTFA+ TMCS and hexane for 40 min. The remaining solution, containing the wax was filtered through a cellulose acetate membrane syringe filter, having a diameter of 28 mm and a pore size of 0.2 μm and transferred to a GC vial.

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis

For the GC-MS analysis, the electron impact mass spectra (70 eV) were acquired with an Agilent 5975 C Mass Selective Detector, interfaced to an Agilent 7890A Gas Chromatograph, fitted with a DB–5 column (30 m × 0.32 mm i.d., 0.25 μm). Helium gas of 99.999% purity was used as a carrier gas at a constant flow rate of ±1 mL/min. The samples were injected into the column at 50 °C, held isothermal at 50 °C for 2 min, and then desorbed by increasing the temperature according to the following profile: 40 °C/min to 200 °C, held at 200 °C for 2 min, 3 °C/min to 310 °C, and held isothermal at 310 °C for 30 min. The individual hydrocarbon peaks were identified by comparing the retention times and mass spectra with those of the synthetic standards, matching with the spectra published previously, and studying the fragmentation patterns, using the NIST Mass Spectral Library 2005 (Wang et al. 2006).

Results and Discussion

The plant waxes reduce water loss, limit the release of solutes and volatiles, deter from pest attack, and possibly attract the pollinating insects. Likewise, the wax layers of insects limit water loss, offer protection from desiccation, and prevent the penetration of the pathogens and toxic chemicals through the insect integument (Arunkumar et al. 2017). The plants and the insects exhibit a lot of differences, ranging from their mode of evolution to the process of decomposition (Grimaldi and Engel 2005). Despite their differences, both the plants and insects have evolved a functionally similar cuticle layer, in order to cover their external organs (Kenrick and Crane 1997). Though the functional roles of the waxes are the same in both the kingdoms, there exists a difference in their composition. The plant waxes are a combination of the aliphatic hydrocarbons and they also contain the secondary metabolites such as triterpenoids, phenylpropanoids, and flavonoids, whereas the insect hydrocarbons are formed of the saturated, unsaturated, and methyl-branched hydrocarbons (Nguyen et al. 2014). Detailed literature exists about the composition of the plant waxes, including that of cotton but a correlation study between the cotton wax and the mealybug wax is still missing. The modern-day advanced techniques such as the GC-MS analysis can fill this gap and provide detailed information for the development of target-specific pesticides or biocontrol agents for degrading the mealybug wax.

The biochemical composition of the wax of cotton leaf

Using the GC-MS analysis in the current study, the biochemical constituents of the cuticular wax, within the retention times of 0.904–16.002 min were detected and quantified. The sum of the areas of the identified peaks ranged between 95 and 97% of the total area. The GC-MS analysis revealed that the dominant hydrocarbon class in the leaf epicuticular wax of upland cotton (*G. hirsutum* L.) was cyclohexane (63.13%), a six carbon cycloalkane, followed by the saturated fatty acids such as palmitic acid, myristic acid, and stearic acid and

some higher alkanes, having nine or more carbon atoms such as cyclohexane, hexadecane, triacontane, and tetracontane (Table 1; Fig. 1). The long-chain alkanes such as n-octacosane, n-nonacosane, n-triacontane, dotriacontane, and n-tetracontane were the chief constituents of the epicuticular wax of cotton leaf (Bondada and Oosterhuis 2000).

The biochemical composition of the mealybug wax

After hexane extraction, the effectiveness of the procedure was confirmed. Neither the internal layers nor the structural integrity of the mealybugs was affected by the extraction process (Fig. 2). The cuticular wax obtained from the different mealybug species in this study revealed

Table 1 Biochemical composition of wax from upland cotton (*Gossypium hirsutum* L.) and mealybugs by GC/MS analyses (compounds were identified on the basis of the retention time through NIST library)

Compound identified	Comparison of chemical compounds present in the Samples									
	G. hirsutum		*P. solenopsis*		*F. virgata*		*P. marginatus*		*D. mangiferae*	
	RT	PA(%)	RT	PA(%)	RT	PA(%)	RT	PA(%)	RT	PA(%)
Hexane C_6H_{14}	1.762	25.62	0.904	24.40	1.068	25.12	1.490	21.49	1.444	19.58
Pentane C_5H_{12}	2.245	0.16	0.958	9.62	1.119	7.74	1.917	7.52	1.464	9.12
Cyclobutanone C_4H_6O	–	–	–	–	1.175	3.83	–	–	–	–
Cyclopentane C_5H_{10}	–	–	1.857	21.70	1.680	20.31	1.827	23.59	1.822	21.27
Decane $C_{10}H_{22}$	–	–	–	–	1.850	3.66	–	–	–	–
Cyclohexane C_6H_{12}	2.534	63.13	2.505	25.82	2.628	28.03	2.505	38.94	2.496	31.38
Heptane C_7H_{16}	–	–	2.943	8.88	–	–	–	–	–	–
Pentanol $C_5H_{11}OH$	–	–	3.175	4.00	–	–	–	–	–	–
Ethane C_2H_6	–	–	4.517	0.25	–	–	–	–	–	–
Hexanoic acid $C_5H_{11}COOH$	7.044	0.10	–	–	–	–	–	–	–	–
Quinoline C_9H_7N	–	–	–	–	–	–	–	–	7.109	2.16
Cyclooctane C_8H_{16}	–	–	–	–	–	–	7.197	1.19	–	–
Cyclododecane $C_{12}H_{24}$	–	–	–	–	7.204	3.76	–	–	–	–
Hexadecane $C_{16}H_{34}$	8.164	0.14	–	–	–	–			9.729	0.68
2-methyl-Heptadecane $C_{17}H_{36}$	–	–	–	–	–	–	–	–	8.927	4.27
Pentadecane $C_{15}H_{32}$	8.928	0.29	–	–	–	–	–	–	8.973	0.17
Myristyl alcohol $C_{14}H_{30}O$	–	–	–	–	8.948	1.09	–	–	–	–
Dodecyl acrylate $C_{15}H_{28}$	–	–	–	–	–	–	8.992	0.39	–	–
1-Pentadecene $C_{15}H_{30}$	–	–	–	–	–	–	9.256	0.30	–	–
Myristic acid $CH_3(CH_2)_{12}COOH$	9.397	0.8	–	–	–	–	–	–	–	–
2-methyl-Octadecane $CH_3(CH_2)_{16}CH_3$	–	–	9.652	0.35	9.094	1.01	9.650	0.60	9.037	0.26
Heneicosane $C_{21}H_{44}$	9.726	0.23	–	–	–	–	9.975	0.40	9.796	0.14
Icosane $C_{20}H_{42}$	10.345	0.60	–	–	10.016	0.33	10.344	0.60	9.654	1.06
Tetracontane $C_{40}H_{82}$	9.911	0.50	–	–	–	–	–	–	–	–
Triacontane $C_{30}H_{62}$	10.346	0.63	–	–	–	–	–	–	–	–
Nanodecane $C_{19}H_{40}$	10.491	0.74	–	–	–	–	–	–	–	–
Palmitic Acid $CH_3(CH_2)_{14}COOH$	10.786	2.15	10.787	0.70	10.790	0.83	10.783	1.22	10.788	5.51
Stearic acid $C_{17}H_{35}COOH$	11.944	0.60	12.060	0.11	11.682	0.13	11.945	0.77	–	–
Heptacosane $C_{27}H_{56}$	12.239	0.14	–	–	–	–	–	–	–	–
Hexacosane $C_{26}H_{54}$	–	–	13.859	0.17	–	–	–	–	–	–
Pentacosane $C_{25}H_{52}$	–	–	14.009	0.27	–	–	–	–	–	–

RT- Retention time, PA- Peak area

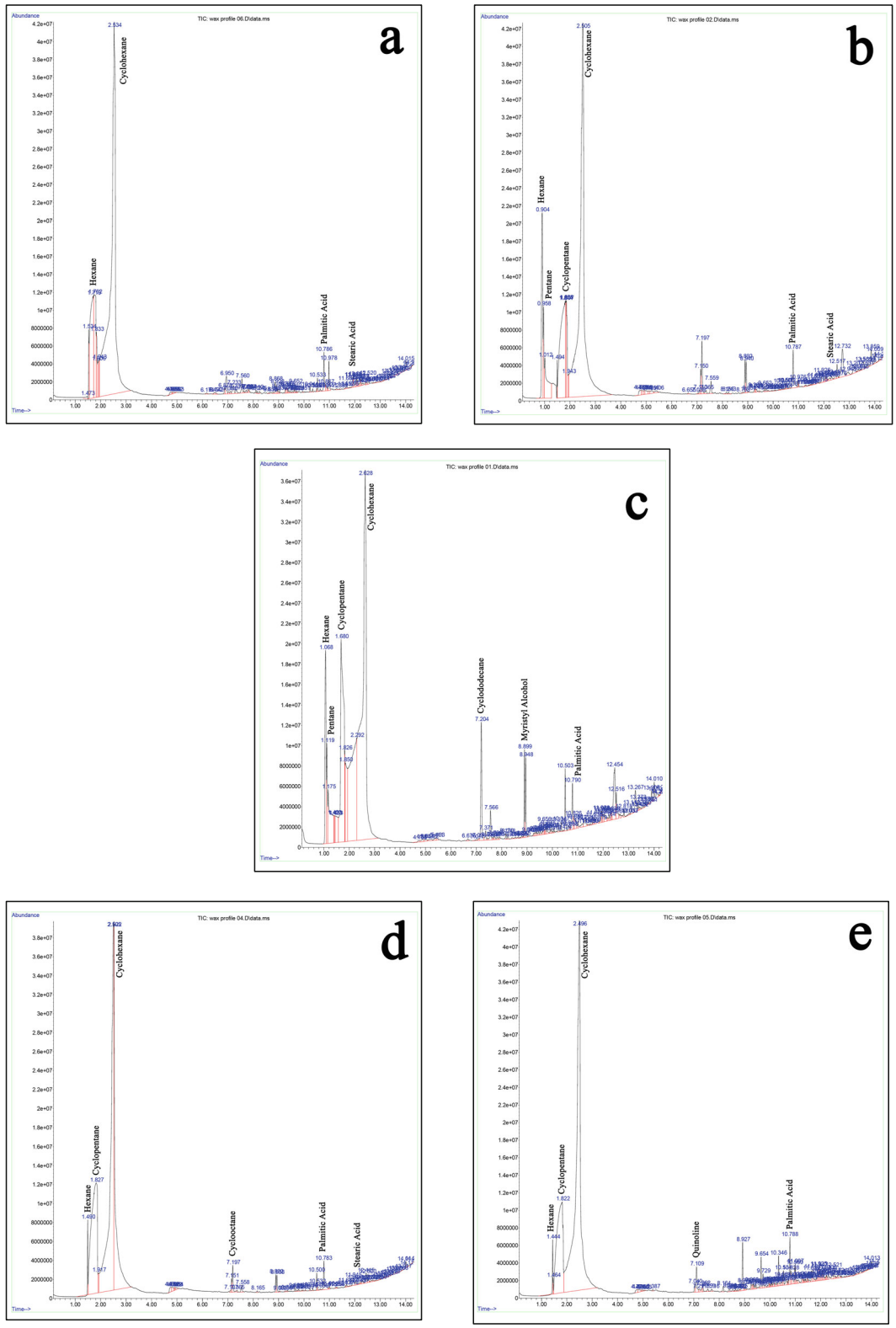

Fig. 1 GC/MS analysis of the compounds present in the isolated wax of **a** *Gossypium hirsutum* L., **b** *Phenacoccus solenopsis* Tinsley, **c** *Ferrisia virgata* Cockerell, **d** *Paracoccus marginatus* Williams and Granara de Willink and **e** *Drosicha mangiferae* Green

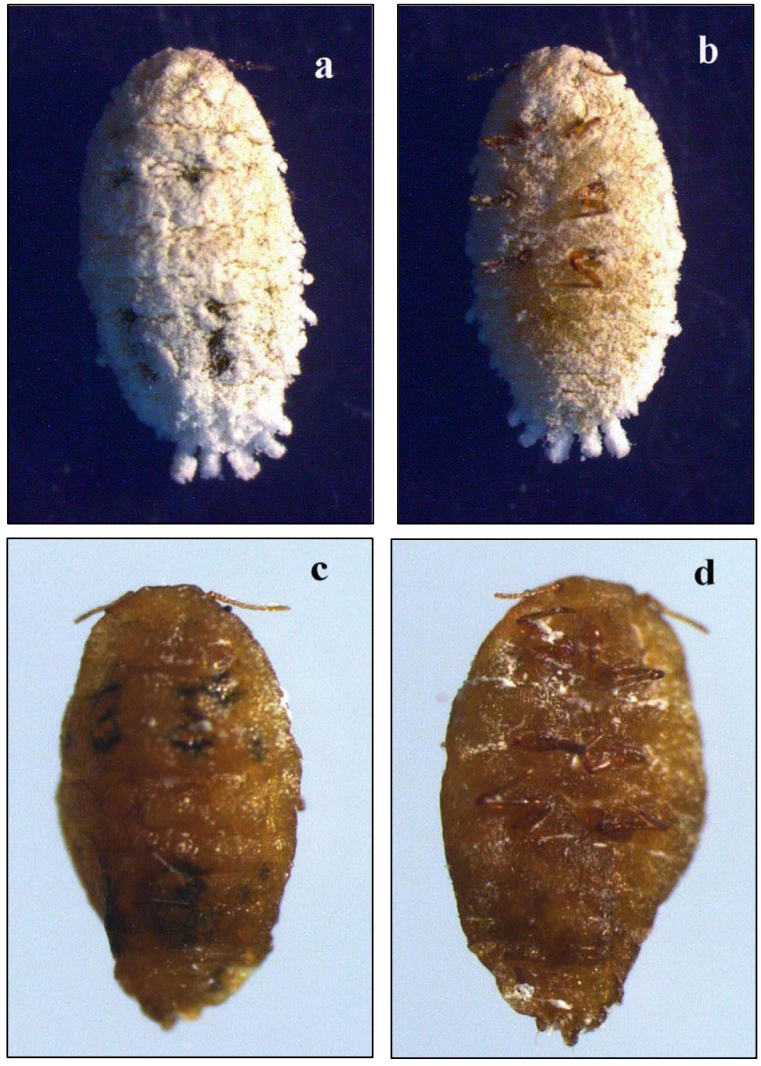

Fig. 2 Mealybug (*Phenacoccus solenopsis* Tinsley) after hexane extraction **a** dorsal view before extraction, **b** ventral view before extraction, **c** dorsal view after extraction, **d** ventral view after extraction

the presence of the three major alkanes such as pentane, cyclopentane, and cyclohexane at higher concentrations. The alkane, cyclohexane was also the dominant hydrocarbon in the mealybug wax but the composition ranged between 25 and 38% of the total peak area. The composition of the mealybug revealed the presence of the five-carbon compounds such as pentane and cyclopentane together up to 31.32% in *P. solenopsis*, up to 28.05% in *F. virgata*, up to 31.11% in *P. marginatus* and up to 30.39% in *D. mangiferae,* respectively, whereas a meager 0.06% of pentane was identified in the epicuticular wax of the leaves of *G. hirsutum* L. The methyl alkane, 2-methyl-Octadecane [$CH_3(CH_2)_{16}CH_3$] was found in all the four mealybug species. Similar studies, conducted on the cuticles of various insects confirmed that the saturated alkanes and the methyl-branched alkanes are present only in the insect wax and not in the plant wax (Nguyen et al. 2014). In our study, the concentration of the lipid extraction solvent, hexane in the GC-MS analysis of the mealybug wax ranged between 19.58–25.12% of the total peak area.

The biochemical correlation between the cotton wax and the mealybug wax

The results of the present study clearly confirmed that the wax of cotton leaf mostly consists of the six-carbon alkanes and the mealybug wax is dominated by both the five-carbon alkanes and the six-carbon alkanes. The saturated fatty acids such as palmitic acid, myristic

acid, and stearic acid were found in the epicuticular wax of *G. hirsutum* L. However, only palmitic acid and stearic acid were identified in the wax of all the tested mealybug species. On the other hand, *D. mangiferae* revealed the presence of only palmitic acid. Weete et al. (1978) reported that palmitic acid is the principal substrate for the synthesis of wax in cotton under stress conditions. In the present study, nonadecane, an alkane hydrocarbon with the chemical formula $[CH_3(CH_2)_{17}CH_3]$ was found only in the wax of *G. hirsutum* L. and this has also been documented by Khan et al. (2015). In our study, the higher alkanes or the n-alkane family, similar to both the plant and insect waxes were hexadecane, icosane, and heneicosane. Apart from these, certain higher hydrocarbons such as ethane, cyclobutanone, decane, cyclododecane, cyclooctane, 1-pentadecene, heneicosane, quinoline, heptadecane, pentadecane, and hexadecane were found only in the mealybug wax. The other notable chemical compounds, specific to certain mealybug species such as myristyl alcohol, quinoline, hexacosane, and pentacosane were found at lower concentrations, thereby making less considerable changes between the wax profiles of the mealybugs. Previously, Kun et al. (2014) reported the presence of stearic acid, pentacosane, and myristic acid in the wax of the Chinese insects by the GC-MS analysis.

Conclusion

The present study has shown considerable differences existing in the profiles of the epicuticular wax of cotton leaf (*G. hirsutum* L.) and the mealybug wax. The GC-MS analysis confirmed that the six-carbon alkanes were dominant in the wax of cotton leaf, whereas a mixed composition of the five-carbon alkanes and the six-carbon alkanes were found in the mealybug wax. Therefore, these results will pave the way for developing biological and chemical pesticides, targeting the insect wax without hindering the composition of the plant wax. However, in order to achieve utmost success, experiments in live plants and field studies should also be conducted for the absolute confirmation of any target-specific pest control formulation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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