Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(4): 27-31

Received: 07-05-2018
Accepted: 08-06-2018

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Volatile profiling and bio-efficacy of *Citrus hystrix* fruit peel as a seed protectant against *Callosobruchus maculatus*

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Abstract
Volatile chemical composition of the fruit peel of *Citrus hystrix*, its repellent activity and seed protectant ability against damage caused *Callosobruchus maculatus* were investigated. Repellent effect of the fruit peel was dose and time dependent, where maximum of 98% repellency was observed after only one hour of weevil exposure. *C. hystrix* fruit peel has the ability of significantly reducing both seed damage and weevil perforation index (31.01) and increasing percentage protectant ability (68.98) without affecting seed germination. Volatile fraction of *C. hystrix* fruit peel obtained from the HS-SPME medium polar fiber identified a total of 45 compounds. The major constituents were 3-Carene (18.310%), Citronellal (12.267%), D-limonene (11.538%), α-Pinene (9.244%), α-Cadinene (4.290%) and Copaene (4.290%), Linalool (4.020%), Caryophyllene (3.988%) and γ-Cadiene (3.544%), which accounted for about 71% of the total detected compounds. The results highly signify the strong seed protectant ability of *C. hystrix* fruit peel in controlling *C. maculatus* infestations.

Keywords: *Citrus hystrix, Callosobruchus maculatus*, repellency, damage, germination, volatile profile

Introduction

Cowpea (*Vigna unguiculata)* is an annual leguminous crop widely grown in the tropics and subtropics for the purpose of human food as well as for animal feeds [1]. It is more beneficial over other legumes by being a cheap protein source in addition being the most practical source of storage and transportable protein [2].

Callosobruchus maculatus, commonly referred to as cowpea beetle, is one of major pest of wide range of stored legume seeds including cowpea [3]. Larvae, the most destructive stage of *C. maculatus* is known to cause serious economic damage [4]. They are capable of causing damage with varying percentages at different levels of storage including the producer levels, the trader and the central store levels. This leads to quality deterioration of the crops, thereby affecting its market value and becoming unsuitable for human consumption and production of sprouts [1, 5].

Use of synthetic insecticides applied as a liquid or fumigant formulation is the most popular and successful method used in protecting stored grains from insect pest infestations. However, their indiscriminate and enormous use have created serious problems including development of genetic insect resistance to pesticides, disturbance of the environment, pest resurgence, lethal effects on non-target organisms in addition to direct toxicity to the user [6-8]. These drawbacks have necessitated the need for sustainable alternatives that are readily available, affordable and less detrimental to the environment [4].

Recently, considerable attention of scientists has been given towards screening plant secondary chemical compounds for developing insecticides for the control of stored pests [9]. Numerous investigations have confirmed that these compounds are capable of controlling pests extending sub lethal effects on oviposition deterrence, antifeedant activity and repellent actions as well as their effect on biological processes including growth rate, lifespan and reproduction [10]. The use of these botanical insecticides may offer sustainable, biodegradable environmentally friendly and safer alternative to synthetic insecticides with a specific mode of action [11, 5].

Among the numerous plant parts, a promising level of control over pulse pests have been attributed by fruit peels of Citrus species [12]. Citrus plants that are grown widely in Sri Lanka offer an opportunity for developing them as alternatives to chemical pesticides in protecting cowpea seeds from beetle attack.

The present study therefore, was aimed at investigating the volatile profiling and the bio-efficacy of powdered fruit peel of *Citrus hystrix* as a seed protectant against *C. maculatus*.

2. Material and Methods

2.1 Maintenance of laboratory cultures of *C. maculatus*

Cowpea seeds infested with *C. maculatus* were collected from the local market and insect cultures were maintained in the Laboratory under ambient laboratory conditions of 28±2°C and 84±2%RH.

2.2 Preparation of plant material

Healthy, ripened fresh fruits of *Citrus hystrix* (Kundalu dehi) were collected from Pallepola, Sri Lanka. Fruits washed thoroughly with running tap water and air dried were peeled into fine, uniform particles using a domestic grater immediately before the onset of each experiment.

2.3 Repellent Activity

Repellent activity of the powdered fruit peel was tested using a dual-choice olfactometer made up of two plastics cups (height 4cm, diameter 7cm) connected to a transparent plastic tube (length 12cm, diameter 1.5cm) to its two ends. A hole (1cm diameter) was made in the middle of the tube. Freshly grated fruit peels of *C. hystrix* (1, 4, 7g) mixed with 60 un-infested cowpea seeds were placed in the first cup. Seeds with no fruit peel in the second cup were served as the control. Twenty, one-day old, unsexed adult beetles were then introduced into the middle hole of the plastic tube using a small glass vial (Diameter 1cm). The number of insects that moved into treatment and control were counted 15, 30 and 60 minutes after the introduction of insects and percentage repellency was calculated for each treatment. All experiments were replicated five times.

2.4 Assessment of Damage

Damage assessment was made on both treated and untreated seeds by assessing seed weight loss and by number of seeds damaged.

Grated peels of *C. hystrix* (0.6g) were mixed with previously weighed cowpea seeds in a plastic cup (height 4cm, diameter 7cm) and five pairs of newly emerged males and females of *C. maculatus* were introduced. After emergence, all F1 adults were removed and the seeds were cleaned using a fine brush. Weight of the treated and untreated cowpea seeds was measured and five replicates each were made.

Percentage weight loss was determined using the following equation [13],

$$\%\text{Weight loss} = \frac{\text{Initial weight} - \text{Final weight} \times 100}{\text{Initial weight}}$$

Grated peels of *C. hystrix* (2.5g) were thoroughly mixed with 400g of un-infested cowpea seeds in a medium-sized plastic bottle (height 18cm, diameter 8cm). Thirty pairs of newly emerged males and females of *C. maculatus* were then introduced and allowed to oviposit. Observations were made for two generations while removing the dead insects. A similar setup without adding the peel served as the control. Three equal random samples (20ml) each were taken from treatment and control. Seeds with adult exit holes were counted to evaluate the extent of bruchid damage.

Percentage damage (PD), Weevil Perforation Index (WPI) and Percent Protectant Ability (PPA) were calculated according to the methods described by Rotimi & Ekperusi (2012) [12].

$$PD = \left(\frac{\text{Total number of treatment grains perforated}}{\text{Total number of grains}}\right) \times 100$$

$$WPI = \left(\frac{\text{\% of treatment grains perforated}}{\text{\% of control grains perforated} + \text{\% of treated grains perforated}}\right) \times 100$$

PPA = 100 – WPI

2.5 Seed Germination

Thirty un-infested cowpea seeds were admixed with 0.4g of grated fruit peels. After 72 hours, seeds were spread on a thick, moistened cotton wool layer in a glass petri dish. This set-up was kept under laboratory conditions (28±2°C and 84±2%RH) and subsequently, number of germinated seeds was recorded after 48 hours. Five replications each were made for the treatment and the control.

2.6 Volatile profiling of fruit peel

Volatile compounds of *C. hystrix* were analyzed using an Agilent Technologies 7890A gas chromatograph (Palo Alto, CA) equipped with an Agilent Technologies 5975C inert XL EI/CI mass selective detector. The HS-SPME medium polar fiber was conditioned for 15 minutes at 250°C, prior to the experiment to fully remove any contaminants. About 0.1g of fresh grated fruit peel was placed in the headspace vial (diameter 2cm, height 6.7cm, volume 12ml) and it was immersed in the water bath (60°C). SPME fiber was released manually through the needle towards the sample and allow to micro-extract volatile organic compounds for 30 minutes. Then fiber was inserted into the GC injector for 20 minutes in split less mode. Helium was used as the carrier gas at a flow rate of 1ml/min and detector gases were hydrogen and air. Initial oven temperature was 40°C for 3 minutes and then it was increased at 10°C/min up to 280°C, where it was held for 3 minutes and maintained constant for 30 minutes. Identification of volatile components in the sample was based on retention times and MS spectral library at Chemistry Department of University of Sri Jayewardenepura, Sri Lanka.

2.7 Analysis of Data

Data obtained for repellent effects of *C. hystrix* were subjected to two sample t-test and one-way analysis of variance (ANOVA). Damage assessment and seed viability data were subjected to two-way analysis of variance (ANOVA). Multiple comparison test (Tukey) was used to separate mean values of the experiments. Comparisons between plant species and doses were made using General Linear Model (GLM) procedure.

3. Results and Discussion

3.1 Repellent Activity

The current study revealed that the repellent activity of *C. maculatus* adults to different doses of *C. hystrix* peel showed that, the number of insects moving into the control was significantly higher at all doses (Table 1). The results clearly indicated that insect repellency increased with the increase of dose attaining the highest repellency of 19.60 ± 0.55 at the highest dose (7g). Also, even at the lowest dose (1g) more than three fourths of insects were repelled. Moreover, the cumulative effect of *C. hystrix* peel on *C. maculatus* was found to be not only dose but also time dependent (Figure 1).

In fact, adult repellency increased with the increase of the exposure time, reaching to a maximum of 98% after only one hour, signifying the extremely strong repellent effect of *C. hystrix* fruit peel on cowpea beetle. The results thus indicate good potential of using *C. hystrix* as an efficient repellent against *C. maculatus*.

Table 1: Repellent effects of fruit peel of *C. hystrix* on adult *C. maculatus* in a dual-choice olfactometer after 1 hour exposure period

Dose (g)	Number of insects moved to the treatment *Mean ±SD	Number of insects moved to the control *Mean ±SD	Probability (Two sample t test)
1	5.00 ± 0.71	15.00 ± 0.71^{a}	*P*<0.05
4	2.80 ± 0.84	17.20 ± 0.84^{b}	*P*<0.05
7	0.40 ± 0.55	19.60 ± 0.55^{c}	*P*<0.05
Probability (1 way ANOVA)		*P*<0.05	

*Means followed by different letters in the same column are not significantly different according to Tukey's test at *P*<0.05

*1 way ANOVA used to compare doses (g) in the same column; Two sample t test used to compare number of insects moved to the control and the corresponding treatment.

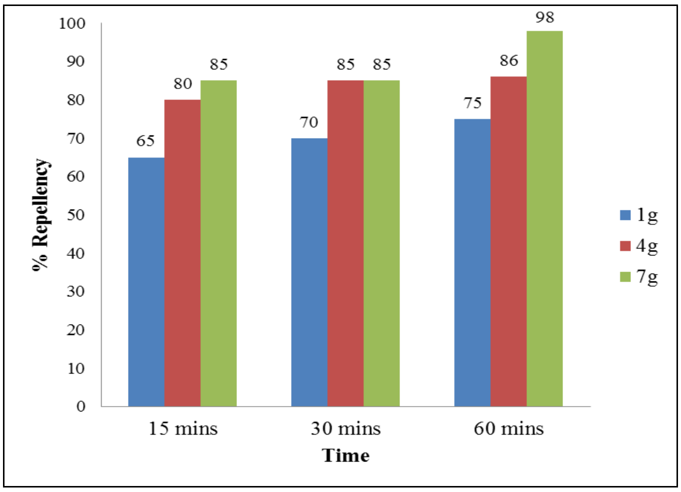

Fig 1: Percentage repellency of *Citrus hystrix* fruit peel on *Callosobruchus maculatus* after different times of exposure

Furthermore, repellent properties of peels of other Citrus species on post-harvest pests including *C. maculatus* have been documented by other researchers [14-16]. This ability may be due to the complex chemical composition of volatile materials present in the fruit peel playing an important role in repelling the beetles.

3.2 Assessment of Damage

In this experiment, weight loss which indicates the quantitative loss in stored grains due to larval feeding was taken as an index of damage assessment. When compared with the control, it was evident that fruit peel of *C. hystrix* significantly reduced the weight loss of cowpea seeds due to *C maculatus* damage (Table 2). In fact, percentage weight loss (9.88 ± 0.88) obtained for cowpea seeds treated with *C. hystrix* was approximately about four times lower than the weight loss obtained for the control.

Table 2: Effect of fruit peel of *C. hystrix* on seed weight loss caused by *C. maculatus*

Treatment	% weight loss *Mean ± SD
Control	36.13 ± 1.50^{a}
C. hystrix	9.88 ± 0.88^{b}
Probability	*P*<0.05

*Means followed by different letters are significantly different according to Tukey's test at *p*<0.05

In damage evaluation, weight loss which indicates the quantitative loss in stored grains due to insect larval feeding was taken first as an index of damage assessment. The reduction in seed weight loss could be a result of the reductive effect of the fruit peel observed in the low rate of F1 adult emergence. This is in accordance with the findings of Alemayehu & Getu (2015) [17], where plant parts of eight species including *Citrus lemon* have reported to significantly prevent emergence of F1 adults of *C. chinensis* and subsequent seed weight loss due to pest activity. Interference with the processes such as number of eggs present initially, number of eggs hatched [18] and larval mortality may lead to the reduction in the insect population thereby decreasing the rate of seed damage resulting in lower seed weight loss.

According to the results given in Table 3, percentage damage to treated cowpea seeds (40.19 ± 2.88) caused by the adult beetle was nearly 40% lower than the control (89.30 ± 1.65).

Table 3: Effect of fruit peel of *Citrus hystrix* on seed damage caused by *C. maculatus*

Treatment	*Percentage Damage (PD) ± SD	WPI	PPA
Control	89.30 ± 1.65^{a}		
C. hystrix	40.19 ± 2.88^{b}	31.01	68.98
Probability	*P*<0.05		

*Means followed by different letters are significantly different according to the Tukey's test at *p*<0.05

WPI =Weevil Perforation Index; PPA = Percent Protection Ability

The results also revealed that *C. hystrix* fruit peel has the ability of reducing damage to seeds, lowering the weevil perforation index (WPI) and increasing percentage protectant ability (PPA). As WPI value above 50 is usually considered as an indication of negative protectant ability [19], WPI value of the present study indicates a positive protectant capability of *C. hystrix* fruit peel (31.01). In this instance, the high effectiveness in protecting seeds could be arisen due to the coating of the seeds by the chemical constituents of the fruit peel [12]. The protective ability of citrus fruit peel could also be attributed to insect responses to peel constituents which possess repellent properties.

3.3 Seed Germination

Results indicated that when compared with the control, treatment with fruit peel of *C. hystrix* did not impair the germination of cowpea seeds (Table 4). Hence, it is evident from the observations that *C. hystrix* fruit peel does not afflict any adverse effect on the germination capacity of cowpea seeds. This bears out that mixing citrus fruit peel with cowpea maintains the seed quality while at the same time protecting seeds from cowpea beetle damage. Similarly Gselase and Getu (2009) [20] investigated botanical powders for the control of *Zabrotes subfasciatus* on haricot bean seeds and found that there was no adverse effect of botanicals on seed germination.

Table 4: Effect of fruit peel of *Citrus hystrix* on germination of cowpea seeds

Treatment (g)	Number of germinated seeds *Mean± SD
Control	29.60 ± 0.89
C. hystrix	29.60 ± 0.89
Probability	*NS

*Mean number of germinated seeds ± SD; 30 seeds each for five replicates
*NS – Not Significant (*P*>0.05)

3.4 Volatile Profiling of Organic Components of *C. hystrix* fruit peel by Headspace Solid- Phase Micro Extraction (HS-SPME)

According to the results of the volatile fraction obtained from the HS-SPME medium polar fiber of *C. hystrix* fruit peel, a total of organic volatile components were identified (Table 5).

Table 5: Solid Phase Micro Extraction (SPME) of *C. hystrix* fruit peel with Medium Polar SPME fiber

Volatile Compound [a]	RT [b]	Percentage %*
2-Hexenal	5.573	0.299
α-Pinene	7.093	9.244
3-Carene	8.140	18.310
Octanal	8.546	0.305
4-Carene	8.812	0.629
D-Limonene	9.216	11.538
β-Ocimene	9.328	0.457
γ-Terpinene	9.506	0.936
β-Terpineol	9.700	2.687
Linolool	10.162	4.020
Citronellal	11.308	12.267
Borneol	11.432	0.268
Terpinen-4-ol	11.526	0.318
Decanal	11.822	0.404
Citronellol	12.170	1.317
Geraniol / Lemonal	12.534	0.436
α-Citral / Geranial	12.764	0.138
(-)-Bornyl acetate	12.974	0.074
Tridecane	13.097	0.095
Tetradecanal	13.282	0.042
Elixene	13.561	0.062
Bicyclo[4.1.0]hept-2-ene,3,7,7-trimethyl	13.708	1.368
Cis-2,6-dimethyl-2,6-octadiene	13.895	2.217
Geranyl acetate	14.057	0.205
Cyclosativene	14.139	0.215
Copaene	14.338	4.290
γ-Cadiene	14.536	3.815
Dodecanal	14.663	0.085
α-Gurjunene	14.743	0.145
Caryophyllene	14.954	3.988
α-Guainene	15.081	0.619
1,4,7-Cycloundecatriene,1,5,9,9-tetramethyl-ZZZ	15.341	1.583
γ-Muurolene	15.701	3.069
α-Armophene	15.877	1.840
α-Bulnesene	15.951	0.613
α-Cadinene	16.240	4.290
1,4-Cadinadiene	16.316	0.379
Elemol	16.469	0.463
Nerolidol	16.566	0.137
Germacrene-D-4-ol	16.802	0.127
α-Eudesmol	16.959	0.122
Ledol	17.159	0.020
δ-Cadinene	17.399	0.052
Γ-Muurolol	17.563	0.046
Thujone	17.863	0.031
Total		93.565

[a]Compound listed in order of elution
[b]RT- Retention Time
*Data are expressed as percentage of the total peak area

The main volatile components identified were 3-Carene (18.310%), Citronellal (12.267%), D-limonene (11.538%), α-Pinene (9.244%), α-Cadinene (4.290%), and Copaene (4.290%), Linalool (4.020%), Caryophyllene (3.988%) and γ-Cadiene (3.544%) (Table 5) which accounted for about 71% of the total detected compounds. However, in another study, the major compounds of the essential oil of *C. hystrix* peel identified by GC- MS were reported as sabinene, α-pinene, D-limonene, myrcene, citronellal, and terpinen-4-ol [21]. Furthermore, some chemical constituents not recorded in their study such as 3-carene, 3-cyanopyrrole, borneol, geraniol and copaene were identified in the present study. The discrepancy in constituent characterization of *C. hystrix* may be mainly due to different techniques used for extraction, or/and due to different environmental and genetic factors, plant variety, and the geographical regions. It has also been revealed that *Citrus hystrix* leaf and peel essential oils can act as repellents against *Aedes aegypti* and *Anopheles minimus* [22]. Ikawati *et al*. in 2017 [15] reported the toxic and repellent activities of *Citrus hystrix* leaf extract against cigarette beetle, *Lasioderma serricorne*. Citronellal and geranial was reported to be a most effective repellent against *Sitophilus zeamais* [23]. *Cymbopogon giganteus* essential oil rich in D-limonene has exhibited insecticidal effects on *C. maculatus* [24]. D-limonene was also reported to be highly effective on *Sitophilus oryzae* and *T. castaneum* [25]. Moreover, α-pinene and D-limonene isolated from the essential oil from *Juniperus formosana* leaves strongly repelled *T. castaneum* adults [26].

It can be suggested from the observations, that the insecticidal and repellent properties of *C. hystrix* peel against *C. maculatus* might be attributed to the synergistic effects of its wide array of major and minor components. Finally, the overall results of the present study strongly indicate that *C. hystrix* peel has potential to be developed as a natural repellent and a seed protectant to manage *C. maculatus* populations.

4. Conclusion

According to the obtained results, fruit peel powder of *Citrus hystrix* has exhibited strong repellent effects against adult *Callosobruchus maculatus* and has demonstrated its high potential in the reduction of damage caused by *C. maculatus.* Moreover, no adverse effect was elicited by *C. hystrix* fruit peel on the viability of cowpea seeds. Several major components were identified from the volatile fraction of *C. hystrix* fruit peel which may be responsible for its bio-efficacy against the beetle. The overall research findings of this study reveal that *C. hystrix* fruit peel could be successfully incorporated as a grain protectant for the control of *C. maculatus* infestations.

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