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Selection for tolerance in *Callosobruchus maculatus* to sub-lethal doses of essential oils of *Eugenia aromatica* and *Piper guineense*

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Abstract
Selection for tolerance to sub-lethal doses of essential oils of *Piper guineense* and *Eugenia aromatica* in population of *Callosobruchus maculatus* adult and effect of the selection bioassay on egg and larval stages was achieved through exposure to oil vapour (0.05ml, 0.1ml and 0.2ml) under laboratory conditions of 28 ± 3 ºC and 65% ± 5% relative humidity. The populations exposed to the oil vapour were termed "selected population" while the unexposed populations were termed "unselected population". All stages of the insect were monitored for four generations. The results show that adult individuals of the beetle exposed to essential oils for four generations showed no significant increase in percentage survival between the different generations. However, there was significant increase in the numbers of adult emergence from one generation to the next in *C. maculatus* selected as eggs and larvae, irrespective of the sub-lethal dose of the essential oils of *P. guineense* and *E. aromatica* respectively.

Keywords: *Callosobruchus maculatus*, selected and unselected population, sub-lethal dose, Essential oils, *Piper guineense*, *Eugenia aromatica*

1. Introduction
Synthetic insecticides are the quickest and pragmatic means of combating insect infestation; but their use has some major drawbacks in many parts of tropical Africa such as high cost and ability to contaminate the environment, harmful residues and induction of resistance in pest species. Several species of stored product beetle have developed resistance to different organophosphates. For instance, *Oryzaephilus surinamensis* has developed resistance to chlorp yrifos-methyl [11], and *Tribolium castaneum* and *Tribolium confusum* have developed resistance to malathion [13]. As a result of these major drawbacks, botanicals that may replace the insecticides have been identified and their efficacy confirmed for pest control properties [12, 1, 19, 20]. Many studies have demonstrated the insecticidal activity of various essential oils and their constituents on adult, egg and larval stages of stored product insects. However, relatively little attention has been given to the possibility of the insect developing tolerance to this essential oils. Few reports describe the development of tolerance by insects to essential oils or their major component [11, 22]. Therefore, this paper investigates the potential of development of tolerance by the cowpea seed beetle *Callosobruchus maculatus* to sub-lethal doses of essential oils derived from *Eugenia aromatica* and *Piper guineense* that have demonstrated insecticidal properties against storage insect pests.

2. Materials and Methods
2.1 Culturing of *C. maculatus*
The cowpea storage beetle, *C. maculatus* was cultured in No. 1 Kilner jars with meshed lids in an open laboratory throughout the period of the study. Standard laboratory procedure for culturing cowpea seed beetle was adopted [9]. Ife Brown cowpea, a well-known susceptible variety was used for culturing *C. maculatus*. Prior to the use of the cowpea seeds, any batch of the seeds to be used was first disinfested by deep-freezing for two weeks. After removal from the freezer, the seeds were left to equilibrate at room temperature for 12-24hrs to prevent moldiness before introduction of insects.

2.2 Extraction of essential oils of *E. aromatica* and *P. guineense*
E. aromatica and *P. guineense* essential oils were extracted separately from powders following the method of extraction described by [23].

The essential oil was extracted using an Essential Oil Steam Distillation apparatus, which is made of 2000ml capacity distilling flask with a thick round neck, condenser and graduated measuring tube with a collecting tap at the end. A known amount (150g) of each of the powders of *E. aromatica* and *P. guineense* was separately weighed into a distilling flask and mixed with 300ml of water. The apparatus was placed on a heating mantle for a period of 4hr. Thereafter, the volatile oil deposited on water was then collected through the attached graduated measuring tube by opening the tap. The essential oils were collected separately inside a bottle with tight fitted cover and stored in a cool refrigerator at a temperature of 4 ºC prior to the period of experimental bioassay.

2.3 Design of the fumigation chamber

Plastic containers (25ml) with a tight fitted screw cap that form the lid were used as fumigant chamber in this experiment. Under the lid was placed a dental woven pad neatly wrapped in a mesh through which the oil vapour diffuses into the chamber.

2.4 Selection for tolerance in Adult *C. maculatus* treated with sub-lethal dose of essential oil vapour of *E. aromatica* and *P. guineense*

The selection for tolerance in adult *C. maculatus* treated with different concentrations of *E. aromatica* essential oil was achieved through exposure of the insects to the oil vapour. Twenty adults (10 males and 10 females) of *C. maculatus* (0-2 day-old) were placed inside a fumigant chamber. The different concentration of essential oil, 0.05ml, 0.1ml and 0.2ml were injected separately onto a piece of dental woven pad underneath the surface of the cap of the fumigant chamber. This served as a vapour diffuser inside the chamber. A control treatment was also set-up, but the insects were not exposed to the oil vapour. All treatments and control were replicated four times. Adult survival was monitored in 24hr when exposed to *P. guineense* and 48hr when exposed to *E. aromatica*. Thereafter, insects that survived were counted and number recorded. The adult *C. maculatus* that survived were later transferred into a Petri dish containing clean Ife-Brown cowpea seeds in order to allow insect oviposition. The adults that emerged from the seeds were used to repeat the experiment. This procedure was repeated up to the fourth generation. The adult insects that were exposed to repective oils vapour were termed "selected population" while the insects that were not exposed to oil vapour (control treatment) were termed "unselected population".

2.5 Effect of selection bioassay of adult *C. maculatus* on eggs exposed to sub-lethal dose of essential oil vapour of *E. aromatica* and *P. guineense*

Fifty Ife-Brown cowpea seeds containing 100 eggs (0-2 day old), provided from the selected or unselected populations, were placed in fumigant chamber and exposed to sublethal concentration of *P. guineense* and *E. aromatica* essential oil vapour for 24hr and 48hr respectively. Thereafter, the cowpea seeds containing the eggs were transferred to a clean Petri-dish. Twenty one [21] days after oviposition, the seeds were examined daily for adult emergence for another 15days. The number of adults that emerged were counted and recorded. There were four replicates of 100 eggs for each concentration (0.05ml, 0.1ml, 0.2ml). A control treatment was also set-up and this was replicated four times.

2.6 Effect of selection bioassay of adult *C. maculatus* on larvae exposed to sub-lethal dose of essential oil vapour of *P. guineense* and *E. aromatica*.

One hundred larvae of 7 day old from selected or unselected population of adult *C. maculatus* was used in this experiment, following the procedure described for selection bioassay of eggs exposed to essential oil vapour of *E. aromatica.*

2.7 Data analysis

Data obtained in selection for tolerance by *C. maculatus* to essential oils of *P. guineense* and *E. aromatica* were subjected to Pearson Chi Square of "4x4" Contingency table using Stat Xact 4.0.

3. Results

Mean Percentage survival of adults *C. maculatus* subjected to selection by exposure to essential oil vapour *P. guineense* and the unselected over four successive generations is shown (Table 1). There was no significant relationship (χ^2 = 1.60; P>0.05) between adult survival from first to the fourth generation and the application of the essential oil. Adult survival decreased for the unselected population and increased for the selected adult population, however, the number of adult survival in the selected population, irrespective of the concentration of the essential oil from 0.05ml – 0.2ml was not significantly different from the unselected population.

Table 1: Mean percentage survival of adults from population of *C. maculatus* selected by exposure to essential oil vapour *of Piper guineense* and the unselected over four successive generations.

Mean percentage adult survival exposed to:					
Beetle generations	**0.0ml**	**0.05ml**	**0.1ml**	**0.2ml**	**Total**
1st	95.0	98.0	95.0	92.0	380
2nd	95.0	100.0	97.0	93.0	385
3rd	88.0	100.0	97.0	97.0	382
4th	82.0	100.0	98.0	98.0	378
Total	360	398	387	380	1525

χ^2=1.60; P (0.9962) >0.05

Mean number of adults emerging from eggs of *C. maculatus* selected by exposure to vapour of *P. guineense* essential oil and the unselected over four successive generations is shown (Table 2). There was a significant relationship (χ^2 = 24.80; P <0.05) between emergence from the first generation to the fourth, and the application of the essential oil. Adults emerged from eggs of the unselected populations decreased but increased significantly for the selected populations irrespective of the concentration of essential oil from 0.05ml – 0.2ml. Table 3 shows the mean number of adults emerging from larvae of *C. maculatus* subjected to selection by exposure to vapour of *P. guineense* essential oil and the unselected over 4 successive generations arranged in a 4 x 4 contingency table. There was a significant relationship (χ^2 = 30.01; P<0.05) between the adult emergence from first generation to the fourth, and the application of essential oil. Adult emergence from larvae decreased for the unselected population, but significantly increases for the selected larvae of *C. maculatus* population, irrespective of the concentration of the essential oil.

Table 2: Mean number of adults emerged from eggs of *C. maculatus* selected by exposure to vapour of *P. guineense* essential oil and the unselected over four successive generations.

Mean number of adults emerged for eggs subjected to:					
Beetle generations	**0.0ml**	**0.05ml**	**0.1ml**	**0.2ml**	**Total**
1st	63.0	19.0	11.0	6.0	99
2nd	50.0	20.0	13.0	11.0	94
3rd	52.0	26.0	15.0	14.0	107
4th	27.0	28.0	20.0	17.0	92
Total	192	93	59	48	392

$\chi^2 = 24.80$; P (0.0042) < 0.05

Table 3: Mean number adults emerged from larvae of *C. maculatus* selected by exposure to vapour Of *P. guineense* essential oil and the unselected over four successive generations.

Mean number of adults emerged from larvae subjected to:					
Beetle generations	**0.0ml**	**0.05ml**	**0.1ml**	**0.2ml**	**Total**
1st	54.0	40.0	37.0	35.0	166
2nd	43.0	62.0	60.0	58.0	223
3rd	34.0	67.0	62.0	62.0	225
4th	27.0	72.0	67.0	62.0	228
Total	158	241	226	217	842

$\chi^2 = 30.01$; P (0.0000) < 0.05

Percentage survival of adult with regards to adults of *C. maculatus* subjected to selection by exposure to essential oil vapour of *E. aromatica,* and the unselected is summarized in Table 4. There was no significant relationship ($\chi^2 = 2.40$; P > 0.05) between adult survival from first generation to the fourth and the application of essential oil, both for the unselected and selected populations.

Table 4: Percentage survival of adults from populations of *C. maculatus* selected by exposing adults to essential oil vapour of *E. aromatica* and the unselected over four successive generations.

Mean percentage adult survival treated with:					
Beetle generations	**0.0ml**	**0.05ml**	**0.1ml**	**0.2ml**	**Total**
1st	95.0	92.0	90.0	87.0	364
2nd	95.0	97.0	97.0	87.0	376
3rd	88.0	98.0	97.0	95.0	378
4th	82.0	98.0	98.0	95.0	373
Total	360	385	382	364	1491

$\chi^2 = 2.404$; P (0.9838) > 0.05

The mean numbers of adult emerging from eggs of *C. maculatus* selected by exposure to oil vapour of *E. aromatica* essential oil, and the unselected over four successive generations (Table 5). There was a significant relationship ($\chi^2 = 44.54$; $P<0.05$) between the adult emergence from the eggs and the exposure to *E. aromatica* essential oil from first to fourth generation. Adult emergence decreased from the unselected *C. maculatus* population but increased significantly for the selected irrespective of concentration of essential oil from 0.05ml-0.2ml. Mean number of adults emerging from larvae of *C. maculatus* subjected to selection by exposure to essential oil vapour of *E. aromatica* and the unselected, over four successive generations (Table 6). There was a significant relationship ($\chi^2 = 22.74$; $P<0.05$) between the adult emergence from larvae of *C. maculatus* from first generation to the fourth, and the application of the essential oil. Adult emergence decreased for the unselected larvae of *C. maculatus* populations but increased significantly for the selected larvae irrespective of the concentration of the essential oil from 0.05ml – 0.2ml.

Table 5: Mean number of adults emerged from eggs of selected populations of *C. maculatus* exposed to essential oil vapour of *E. aromatica* and the unselected over four successive generations.

Mean number of adults emerged for eggs subjected to:					
Beetle generations	**0.0ml**	**0.05ml**	**0.1ml**	**0.2ml**	**Total**
1st	71.0	8.0	5.0	4.0	75
2nd	52.0	10.0	9.0	4.0	43
3rd	38.0	14.0	12.0	10.0	121
4th	26.0	21.0	19.0	12.0	76
Total	187	53	45	30	315

$\chi^2 = 44.58$; P (0.0000) < 0.0

Table 6: Mean number of adults emerged from larvae of selected populations of *C. maculatus* exposed to essential oil vapour of *E. aromatic* and the unselected over four successive generations.

Mean number of adults emerged for larvae subjected to:					
Beetle generations	**0.0ml**	**0.05ml**	**0.1ml**	**0.2ml**	**Total**
1st	71.0	57.0	53.0	53.0	234
2nd	69.0	70.0	61.0	55.0	255
3rd	49.0	72.0	68.0	55.0	244
4th	38.0	79.0	73.0	72.0	262
Total	227	278	255	235	995

$\chi^2 = 22.74$; P (0.0068) < 0.05

4. Discussion

Data obtained in the selection experiments demonstrate that individuals of selected adult population *C. maculatus* exposed to sub-lethal doses of essential oils of *P. guineense* and *E. aromatica* for four generations showed no significant increase in percentage survival between the different generations. However, for the populations of *C. maculatus* selected as eggs and larvae respectively, irrespective of level of sublethal dose, there was a significant increase in numbers of adult emergence from one generation to the next. *Sitophilus ozyzae* (L.) and *Cryptolestes pusillus* (Schonherr) were reported to develop tolerance to volatile monoterpenoids such as linalool and estragole in plants after selection over seven generations [15]. Strain of the bruchids *Acanthoscelides obtectus* (Say) selected over eight generations for resistance to lavender essential oil vapour resulted in 8.6 and 4.7 times more tolerant females and males, respectively [22]. Similar phenomena for resistance to *Eucalyptus globules* Labill essential oil and cineole vapours among the adults of *Oryzaephilus surinamensis* was reported [11]. The hypothesis of development of resistance in eggs and larvae after adult selection in *A. obtectus* was also tested but no supporting results were found [22].This is contrary to the observations recorded in this study. The methodology employed in this study in which eggs and larvae of *C. maculatus* were exposed to the botanical powders and essential oils over four successive generations, may have elicited more selective pressure. Eggs and larvae obtained from selected adults of *A. obtectus* were exposed to the botanical oil once [22]. Besides, *C. maculatus* has been reported to be more sensitive to toxicity of insecticidal botanicals than many other stored products insects [4, 17]. Furthermore, consistent with the results of this study some earlier workers have shown that in various insect species, selection for resistance in one stage can also raise resistance in other stages [3, 5, 21]. Inbreeding depression has been observed in laboratory populations of *C. maculatus* [7, 8]. This may probably explain why unselected population of *C. maculatus* in this study completely showed a significant decreased in percentage adult survival in the experiment with adults and decreasing adult emergence with the eggs and larvae.

Counter responses of pest populations in the environment that diminish the effectiveness of management tactics have been termed ecological backlash [14]. Use of sublethal doses of essential oils of *P. guineense* and *E. aromatica* had earlier been observed to induce hormoligosis in *C. maculatus* [10]. Individuals of two different populations of *C. maculatus* cultured on seeds of resistant cowpea variety TVu 2027, for three generations was found to show a decrease in development time and a significant increase in percentage survival from one generation to the next [6]. Similarly it was observed that percentage adult emergence increased significantly with successive generations of *C. maculatus* raised on seeds of two resistant cowpea varieties, IT85F-2205 and IT90K-391 [2]. The resistance of seeds of TVu 2027 and its derivatives to *C. maculatus* has been attributed partly to chemical factors present in them [24, 16]. It was opined that the greater number of beetles surviving in the seeds of TVu 2027 after three generations may be due to their ability to overcome the adverse effects of the supposedly trypsin inhibitor responsible for expression of resistance [6]. The capacity of *C. maculatus* to overcome seed resistance may be associated with individuals possessing higher levels of midgut aspartic and cysteine proteinases [16]. Using synergism tests, obtained results suggested that the cytochrome P450, microsomal monooxygenases and glutathione S-transferases are involved in the observed resistance of *A. obtectus* to lavender essential oil vapour [22]. Study on transcriptional regulation in guts of individuals of *C. maculatus* during adaptation to plant defense protease inhibitor and observed over expression of insensitive protease as partly responsible for the adaptation [18]. The biochemical basis for tolerance of *C. maculatus* to toxicity of essential oils of *P. guineense* and *E. aromatica* was not investigated in this study.

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