Research Article

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Efficacy of bioactive compounds and their association with different cowpea cultivars against their major stored pest

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Abstract

Background: Stored grain insects are controlled with fumigant insecticides which can select resistant insect populations and cause environmental and applicator contamination. Thus, resistant cultivars and chemical constituents of essential oils are an alternative to the almost exclusive use of these insecticides. The effects of the combination of cowpea cultivars *Vigna unguiculata* (L.) Walp. with chemical constituents of essential oils against *Callosobruchus maculatus* were determined. Four cowpea cultivars: BRS Tracuateua, BR 17 Gurgueia, Epace 10 and Sempre Verde (insect rearing) untreated were used in the experiments and combined with chemical constituents of essential oil: eugenol, geraniol and trans-anethole. The biological parameters observed were: total egg number and eggs per grain, egg viability (%), insects emerged and insects per grain, immature stage viability (%), instantaneous rate of growth (ri), insect dry weight (mg), grain weight loss (%) and egg-adult period.

Results: When comparing all biological parameters, the cultivars BRS Tracuateua and BR 17 Gurgueia were harmful to *C. maculatus*. In the toxicity tests, the results showed that LC_{30} and LC_{50} of the chemical constituents ranged from 54.77 to 103.48 ppm and 60.99 to 125.18 ppm, respectively. In most of the biological parameters, LC_{50} had adverse effects significantly higher than LC_{30} and BR 17 Gurgueia treated were harmful to *C. maculatus*.

Conclusions: Overall, the findings showed that BR 17 Gurgueia combined with eugenol and geraniol more significantly affected the biological parameters of *C. maculatus* than when associated with trans-anethole, reducing egg number, insects emerged and egg viability.

Keywords: cowpea weevil; *Vigna unguiculata*; plant resistance; botanical insecticides

1 INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is a legume cultivated in semi-arid of Africa, Brazil and the United States.[1] It is a valuable source of dietary protein, vitamins and minerals. Cowpea has some losses in the postharvest, the quantitative and qualitative loss of food value in food crops until they reach the consumer, is a leading cause of food insecurity in some countries.[2]

The cowpea weevil, *Callossobruchus maculatus* Fabr., (Coleoptera: Chrysomelidae: Bruchinae) is the major pest in stored cowpea, especially in tropical countries.[3] It causes substantial quantitative and qualitative losses resulting from the perforation of seeds and consequent weight and germination reductions.

Effective control of these insect pests can be accomplished with the use of insecticides (pyrethroids and organophosphates) and fumigants.[4,5] However, these pesticides can cause adverse effects on applicators and consumers. Thus, it is important to use alternative methods of control, such as the use of resistant cultivars and botanical insecticides,[6] microwave and ionizing irradiation, pheromone baited traps, IGRs and use of entomopathogens which have been highly effective against stored grain insects.[7]

The development and release of cowpea resistant cultivars to *C. maculatus* present several advantages, such as ease of use, low cost, and compatibility with other control tactics.[8]

The botanical insecticides are also important in the control of stored grain pests, some of the main botanical families with insecticidal potential and that essential oils can be extracted are Anacardiaceae, Apiaceae (Umbelliferae), Araceae, Asteraceae (Compositae), Brassicaceae (Cruciferae), Chemopodiaceae, Cupressaceae, Lamiaceae (Labiatae), Lauraceae, Pinaceae, Liliaceae and Zingiberaceae, from which essential oils may be

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extracted.[9] A large number of substances derived from plants cause behavioral and physiological effects on stored product insects, also becoming an alternative to the use of synthetic insecticides. Some of these substances such as terpenoids (mainly mono and sesquiterpenes), which are volatile essential oils and low molecular weight have been effective on *C. maculatus* management.[10] The enormous structural diversity of the terpenoids is almost matched by their functional variability.[11] Terpenoids have important roles in almost all basic plant processes, including growth, development, reproduction and defense. Some of these compounds such as eugenol, geraniol and trans-anethole can be found in plants as *Illicium verum*, *Citrus latifolia* and *Pimpinella anisum*, respectively, which have insecticidal activity.[3,12,13]

Essential oils and their constituent concentrations, necessary to control insect pests, and their mechanisms of action are potentially safe for humans and vertebrates.[14] The compounds of the essential oils exert their activities on insects through neurotoxic effects involving several mechanisms, notably through GABA, octopamine synapses, and the inhibition of acetylcholinesterase.[15] Eugenol acts on octopaminic receptors,[16] thymol acts on GABA-modulating and GABA-mimetic,[17] carvacrol binds to a membrane containing nicotinic acetylcholine receptors (nAChRs),[18] Eugenol, thymol and carvacrol decrease the insect nervous system activity,[19] camphene, camphor, carvone, 1-8-cineole, cuminaldehyde, (l)-fenchone, geraniol, limonene, linalool, menthol and myrcene as acetylcholinesterase (AChE) inhibitors.[20] Major compounds on essential oils can cause effects at a cellular level, such as apoptosis, and can affect nutrition and reproductive parameters of the insects yet *et al.* 2017.[21,22]

There is no record of the combination of resistant cultivars and constituents of essential oils in the control of *C. macultaus*, therefore, this research is unprecedented, being possible to provide important information about the interaction of these two control methods. In this context, this study aimed to evaluate the lethal and sublethal effects of the combination of cowpea cultivars with chemical constituents of essential oils on *C. maculatus*.

2 MATERIAL AND METHODS

2.1 Rearing of insects

The insects were reared for several generations in cowpea (*V. unguiculata* cv. Sempre Verde) in 400 mL glass containers, sealed with perforated plastic lids internally lined with transparent voile-type fabric to allow ventilation.[3] The insects were confined for 4 days for oviposition, being afterward removed. The containers were kept at 26.0 ± 2.0 °C, 63.08 ± 2.6% RH and 12-h photoperiod until adult emergence, these conditions were observed daily with the aid of a thermohygrometer.

2.2 Compounds

The standard synthetic constituents of essential oils were purchased from Sigma-Aldrich® Brazil company (Torre Eiffel, São Paulo, Brazil). We used the constituents: eugenol, geraniol and trans-anethole with a purity of 98%.

2.3 Cowpea cultivars

The cultivars used were: BRS Tracuateua, BR 17 Gurgueia, Epace 10 and Sempre Verde (insect rearing). These cultivars were obtained from the Brazilian Agricultural Research Corporation (Embrapa Meio-Norte) and used because they are commonly adopted by local farmers (Brazil) and presented resistance to *C. maculatus*.[23-26]

2.4 Toxicity test

The experiments were conducted at 26.0 ± 2.0 °C, 63.08 ± 2.6% RH and 12-h photoperiod. The tests were performed using cowpea (cv. Sempre Verde) and the compound of essential oils individually, using a completely randomized design with four replications. For dilution of the compounds was used 1 mL of acetone. Preliminary tests were performed with the acetone solvent to determine the volume and time required for evaporation that did not affect the insects, either on oviposition or mortality.

For each test recipients were used with 20 g of grains infested with 10 females (0–48 h old) of *C. maculatus* in 250 mL glass containers, sealed with perforated plastic lids lined with transparent cloth. The acetone was added to the grains with the aid of manual glass pipettor after the constituents of essential oils were added to the grains with an automatic pipettor, and manually stirred for 2 min. Insects were added to the grains after a total time of 4 min of stirring and drying to evaporate the solvent. Adult mortality indices were determined after 48 h of confinement.

The concentrations of the constituents eugenol (82.5; 110; 165; 220 and 275 ppm), geraniol (43; 79.98; 109.65; 149.64; 204.68 and 279.5 ppm), and trans-anethole (47.5; 57; 66.5; 76 and 85.5 ppm) were established after preliminary tests. The control for each test consisted of 20 g of cowpea (without constituent/ solvent only) and 10 females of *C. maculatus*. Mortality was evaluated after 48 h, and females were eliminated. Eggs were counted at 12 days and the insects emerged at 32 days after the beginning of experiments.

Lethal concentrations (LC_{30} and LC_{50}) values were determined by PROC PROBIT of the program SAS version 8.02.[27] Toxicity ratios (TR) were obtained by the quotient between the LC_{30} and/or LC_{50} of less toxic with most toxic.

2.5 Test with untreated cultivars

The same recipients, quantity of grains and females of the toxicity test were used in this test. The parameters tested were total egg number and eggs per grain (after 12 days), egg viability (%), insects emerged and insects per grain, immature stage viability (%), instantaneous rate of growth (r_i), grain weight loss (%) and egg-adult period. For each treatment (four cowpea cultivars) were used a completely randomized design with five replications.

Immature stage viability was obtained by the quotient between insects emerged and viable egg number. Instantaneous rate of growth (r_i) was estimated through the formula[28] $ri = [\ln(N_f/N_0)]/\Delta t$, where Nf = final number of insects; N0 = initial number of insects; and Δt = number of days in which the insects emerged (32 days).

For the calculation of the egg-adult period was used: [Σ(daily number of insects emerged ×number of days after infestation)/ total of insects emerged].[29] The insects emerged counts were performed daily, ceasing after 4 days without emergence.

The data were submitted to Shapiro–Wilk normality test and ANOVA, after the means were compared by Tukey test at 5% probability, through software SAS version 8.02.[27]

In addition, the similarity between cowpea cultivars was determined by hierarchical cluster analysis, using the method of single linkage, comparing the similarity through Euclidean distances. A dendrogram of similarity between cowpea cultivars was done according to biological parameters, through the program IBM SPSS STATISTICS 19.

2.6 Combination of cowpea cultivars with constituents of essential oils

Were used LC_{30} and LC_{50} estimated from toxicity test, and associated with cowpea cultivars BR 17 Gurgueia, BRS Tracuateua, Epace 10 and Sempre Verde (used in the rearing). Recipients with 20 g of cowpea (all cultivars) infested with 10 females (0–48 h old) of *C. maculatus* in 250 mL glass containers, sealed with perforated plastic lids lined with transparent cloth were used for each test. Acetone was added to the grains with the aid of manual glass pipettor after the constituents of essential oils were added to the grains with an automatic pipettor, and manually stirred for 2 min. Insects were added to the grains after a total time of 4 min of stirring and drying to evaporate the solvent. Adult mortality indices were determined after 48 h of confinement. For each combined treatment was used a completely randomized design with four replications.

In the evaluation of the effects of the combination of cowpea cultivars with constituents the parameters used were: total of eggs and eggs per grain, egg viability (%), insects emerged and insects per grain, immature stage viability (%), instantaneous rate of growth (r_i), insect dry weight (mg), grain weight loss (%) and egg-adult period. Except for insect dry weight, all other parameters were tested as in the experiment with untreated cultivars.

To determine insect weight, after emergence the insects were placed in glass bottles of 120 mL capacity and placed in a freezer (−5 °C) to die. The containers were opened and placed in an oven (40 °C) for 48 h and weighed on a precision balance.

The data were submitted to Shapiro–Wilk normality test and ANOVA in a factorial scheme with four (cultivars) × three (constituents) × two (lethal concentrations), and the means compared by Tukey test at 5% probability, through SAS version 8.02.[27]

3 RESULTS

3.1 Toxicity test

The values of LC_{30} and LC_{50} of the constituents were estimated in 103.48 and 125.18; 54.77 and 77.42; 55.98 and 60.99 ppm, respectively, for eugenol, geraniol and trans-anethole. The last two presented the lowest values for LC_{30} and LC_{50}, distinguishing from eugenol by the confidence interval. However, geraniol showed the highest toxicity ratio for LC_{30} (1.89 times) and trans-anethole presented the highest toxicity ratio for LC_{50} (2.05 times) (Table 1). The chi-square values ranged from 0.64 to 8.42, which are relatively low, showing the adjustment to the Probit model. The compound trans-anethole had a higher slope (14.11 ± 1.62), showing that this compound has toxicity to the insect in a period lower than the other compounds.

3.2 Test with untreated cultivars

There was no difference in total amount of eggs and insects emerged between cowpea cultivars. The cultivars Epace 10 and BR 17 Gurgueia presented fewer eggs per grain and egg viability (Table 2).

In the parameter insects per grain and immature stage viability (%) the cultivar BR 17 Gurgueia showed lower values, while in the instantaneous rate of growth the cultivars Epace 10, BRS Tracuateua and BR 17 Gurgueia presented less increase of population than Sempre verde (Table 3).

Grain weight loss was lower in the cultivar Epace 10, BRS Tracuateua and BR 17 Gurgueia. The egg-adult period was bigger in Epace 10 (Table 4).

Cluster analysis allowed the cultivars to be separated into two groups, where group 1 includes the cultivars BRS Tracuateua and BR 17 Gurgueia, whose performance of biological parameters was lower while another group only contemplates to cultivar control. This way, these cultivars were harmful to *C. maculatus*, presenting better results when compared to the cultivar Sempre Verde used as a control (Fig. 1).

3.3 Combination of cowpea cultivars with constituents of essential oils

In this combination, there was a significant interaction ($P < 0.05$) among the three factors (cultivars, constituents, and concentrations) for the parameters: total egg number and egg per grain, total insects emerged and insects emerged per grain, egg-adult period and weight loss. In most parameters, LC_{50} showed a significantly greater adverse effect ($P < 0.05$) than LC_{30}.

The totals of eggs and eggs per grain at LC_{30} and LC_{50} were lower in eugenol and geraniol in each of the four cultivars. BRS Tracuateua presented a lower egg average than Sempre verde, except for geraniol where BRS Tracuateua provided 68.0 eggs, however, it did not differ statistically from Sempre Verde ($P > 0.05$) (Table 4).

Egg viability (%) was lower in BR 17 Gurgueia, compared to other cultivars in the LC_{30} and LC_{50} in each of the three constituents and provided viability of 49.86% when combined with LC_{50} of eugenol and 49.83% when combined with LC_{30} of trans-anethole (Table 4).

When observing each of the four cultivars separately (lines), the total insects emerged and insects per grain were lower when combined with eugenol and geraniol (Table 5). In general, BR 17 Gurgueia provided a lower percentage of immature stage viability in both concentrations (Table 6).

In relation to the insect dry weight, there was no statistically significant difference among cultivars, with the exception of in trans-anethole ($P < 0.05$), when combined with each constituent, with the insects emerged from BR 17 Gurgueia having an average weight of 1.35 mg at LC_{30} (Table 6).

Table 1 Toxicity of chemical constituents of essential oils on *Callosobruchus maculatus* in cowpea grains

Treatment	n	DF	Slope (±SE)	LC_{30} (CI95%)	TR_{30}	LC_{50} (CI95%)	TR_{50}	χ^2
eugenol	200	3	6.34 ± 0.72	103.48 (92.17–113.20)	-	125.18 (114.55–135.98)	-	3.32
geraniol	240	4	3.48 ± 0.61	54.77 (26.81–74.59)	1.89	77.42 (49.43–100.84)	1.62	8.42
trans - anethole	200	3	14.11 ± 1.62	55.98 (53.09–58.31)	1.84	60.99 (58.58–63.29)	2.05	0.64

n = number of insects used in the test; DF = degrees of freedom; SE = standard error; CI = confidence interval; TR = toxicity ratio, χ^2 = chi-square. TR = LC_{30} and/or LC_{50} of eugenol/other compounds.

Table 2 Values (±SE) of total of eggs, egg per grain, egg viability (%) and total of insects emerged of *Callosobruchus maculatus* in different cowpea cultivars

Cultivar	Total of eggs†	Egg per grain†	Egg viability (%)†	Total of insects emerged‡
Sempre Verde‡	353.20 ± 35.52a	4.45 ± 0.45a	64.53 ± 2.69a	227.60 ± 24.6a
Epace 10	279.20 ± 48.79a	2.75 ± 0.47b	60.30 ± 1.80b	166.20 ± 26.93a
BRS Tracuateua	231.60 ± 24.03a	3.52 ± 0.36a	63.54 ± 3.03a	148.20 ± 18.70a
BR 17 Gurgueia	292.60 ± 95.96a	1.72 ± 0.55b	43.69 ± 4.47c	131.60 ± 42.01a

†Means followed by same letter do not differ in columns by Tukey test at 5% probability.
‡Control.

Table 3 Values (±SE) of insects per grain, instantaneous rate of growth (r_i), immature stage viability (%), grain weight loss (%) and egg-adult period of *Callosobruchus maculatus* in different cowpea cultivars

Cultivar	Insects per grain†	Instantaneous rate of growth (r_i)†	Immature stage viability (%)†
Sempre Verde‡	2.85 ± 0.30a	0.07753 ± 0.002a	79.24 ± 2.65a
Epace 10	1.64 ± 0.26c	0.06889 ± 0.004b	76.26 ± 1.81a
BRS Tracuateua	2.25 ± 0.28b	0.06651 ± 0.003b	78.02 ± 2.26a
BR 17 Gurgueia	0.68 ± 0.03d	0.05836 ± 0.009b	68.49 ± 3.99b
Cultivar	**Grain weight loss (%)†**	**Egg-adult period†**	
Sempre Verde‡	35.79 ± 1.12a	27.41 ± 0.12b	
Epace 10	14.78 ± 3.85b	30.83 ± 0.16a	
BRS Tracuateua	11.06 ± 0.58b	27.60 ± 0.18b	
BR 17 Gurgueia	13.83 ± 0.85b	29.94 ± 0.43a	

†Means followed by same letter do not differ in columns by Tukey test at 5% probability.
‡Control.

When comparing insect dry weight of the constituents within each cultivar, it was observed that Sempre Verde combined with LC_{30} presented less insect weight (1.47 mg) (Table 6).

Sempre Verde, Epace 10 and BR 17 Gurgueia provided less weight loss when combined with geraniol and eugenol in the two concentrations tested, compared the constituents in each cultivar (lines) (Table 6).

The egg-adult period in BR 17 Gurgueia and Epace 10 was longer than other cultivars when compared with cultivars in each constituent. However, when the comparison was made with the constituents in each cultivar there was a statistical difference only in Epace 10 at LC_{30}, with trans-anethole providing 28.99 days of the egg-adult period (Table 6).

4 DISCUSSION

Several studies have been developed to evaluate the bioactivity of essential oils and their constituents in the control of *C. maculatus*. For the essential oil of *Cinnamomum aromaticum* (Nees) (5.36% of eugenol) was determined LC_{50} of 23.16 μg cm^{-2} after 48 h.[30] Monoterpenes and phenylpropanoids that naturally occurring in essential oils were tested and eugenol was one of the most effective fumigants against *C. maculatus* and *S. zeamais*.[31] In the present study, eugenol was less toxic than geraniol and trans-anethole (Table 1). There is no evidence in the literature that terpene compounds, such as limonene and eugenol are toxic and carcinogenic to humans. The US EPA (United States Environmental Protection Agency) does not list these constituents as toxic, and the FDA (US Food and Drug Administration) considers limonene as GRAS (Generally Recognized as Safe), allowing its use in human food.[32]

Essential oils may contain hundreds of different constituents, but certain components are present in larger quantities. For example, 1.8-cineole is prevalent in the essential oil of *Eucalyptus spp*., limonene in *Citrus spp*., Myrcene in *Curcuma longa*, carvone in *Carumcarvi* and asarone in *Acorus calamus*. Among essential oil components, terpenoids have attracted most of the attention for fumigant activity against stored grain insects.[9] It is possible that cowpea also absorbs essential oils and their compounds, so this aspect needs to be investigated in relation to the potential use of these products in cowpea,[12] so the toxicity of geraniol an acyclic monoterpenoid alcohol[33] in the present study may have been influenced by this hypothesis.

The constituents of essential oil can act synergistically or not with other components, depend on which insect pest is being studied in relation to toxicity, for example, two constituents d-limonene and α-terpineol presented significant correlation and toxicity to the cabbage looper, but no significant correlations between constituents and toxicity to the armyworm.[34] In the present study, geraniol and trans-anethole (a phenylpropanoid)[35] presented higher toxicity to *C. maculatus*, however, more studies need to be made to determine the synergistic effect between these compounds.

The compounds eugenol and trans-anethole are phenylpropanoids, differentiating in their structure because the former has a hydroxyl portion, these being of the same chemical class can more easily present a synergistic effect. There is evidence for the mechanism underlying the synergistic interaction between 1,8-

Table 4 Values (±SE) of total egg number, egg per grain and egg viability (%) of *Callosobruchus maculatus* on different cowpea combined with constituents of essential oils

Cultivars	Total of eggs†		
	LC_{30}		
	eugenol	geraniol	trans - anethole
Sempre Verde	204.75 ± 4.55aB	94.25 ± 4.42aC	333.75 ± 8.13aA
Epace 10	125.25 ± 2.89bB	81.50 ± 1.10aB	299.75 ± 23.41abA
BRS Tracuateua	104.00 ± 3.80bB	68.00 ± 1.65aB	241.25 ± 12.20bA
BR 17 Gurgueia	130.75 ± 6.47bB	79.75 ± 2.49aB	280.00 ± 32.08abA
Cultivars		Total egg number†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	142.50 ± 13.37aB	91.25 ± 5.15aB	256.25 ± 30.53aA
Epace 10	113.00 ± 1.91aB	78.25 ± 2.25aB	230.50 ± 55.52aA
BRS Tracuateua	99.25 ± 2.59aAB	65.50 ± 5.11aB	152.75 ± 6.08bA
BR 17 Gurgueia	111.50 ± 4.44aB	75.75 ± 3.75aB	257.75 ± 26.50aA
Cultivars		Egg per grain†	
		LC_{30}	
	eugenol	geraniol	trans - anethole
Sempre Verde	1.940 ± 0.13aB	0.85 ± 0.04aC	3.16 ± 0.07abA
Epace 10	1.16 ± 0.02bcB	0.73 ± 0.01aB	2.76 ± 0.24bA
BRS Tracuateua	1.52 ± 0.05abB	0.97 ± 0.07aC	3.57 ± 0.17aA
BR 17 Gurgueia	0.75 ± 0.03cB	0.47 ± 0.02aB	1.64 ± 0.18cA
Cultivars		Egg per grain†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	1.35 ± 0.03aB	0.89 ± 0.03aB	2.44 ± 0.25Aa
Epace 10	1.03 ± 0.02abB	0.72 ± 0.01aB	2.12 ± 0.50aA
BRS Tracuateua	1.45 ± 0.03aB	0.94 ± 0.02aC	2.23 ± 0.11aA
BR 17 Gurgueia	0.65 ± 0.02cB	0.44 ± 0.01aB	1.520 ± 0.16bA
Cultivars		Egg viability (%)†	
		LC_{30}	
	eugenol	geraniol	trans - anethole
Sempre Verde	72.53 ± 1.46aA	72.98 ± 3.18aA	73.61 ± 1.34aA
Epace 10	63.64 ± 0.83bA	63.50 ± 0.54bA	62.77 ± 0.60bA
BRS Tracuateua	72.95 ± 3.25aA	73.72 ± 2.59aA	73.03 ± 2.87aA
BR 17 Gurgueia	52.46 ± 2.37cA	53.34 ± 3.19cA	49.83 ± 0.73cA
Cultivars		Egg viability (%)†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	67.43 ± 0.68aB	73.63 ± 1.00aA	67.35 ± 0.88aB
Epace 10	62.64 ± 0.61aA	63.25 ± 1.05bA	61.84 ± 0.65aA
BRS Tracuateua	66.29 ± 0.79aA	66.87 ± 1.08bA	65.31 ± 0.43aA
BR 17 Gurgueia	49.86 ± 0.997cA	50.81 ± 0.53cA	50.55 ± 2.88bA

†Means followed by the same lower letter in the column and capital letter in the lines, do not differ by Tukey test at 5% probability.

cineole and camphor two terpenoids major constituents of the rosemary oil against cabbage looper 1,8-cineole facilitates the entry of camphor through the insect's integument into the bloodstream, where the latter compound is more toxic than the former.[36] There is no record related to the combination of the compounds used in the present study, but the combined effect of trans-anethole and limonene on *Spodoptera frugiperda* is known, where the mixture is more toxic than limonene individually.[22]

The relation between compound and toxicity was observed in geraniol, where this constituent was more insecticidal to *Musca domestica* than the monocyclic monoterpenoid alcohols menthol, terpineol and carveol.[33] In the present study geraniol also showed high toxicity to the tested insect, but with an effect similar to phenylpropanoid trans-anethole.

The protection against bruchids could be improved by growing varieties featuring an inherent seed resistance to bruchid beetles.[37] The use of improved cultivars may represent an important tool to improve seed production, reducing the use of pesticides and promoting increased productivity, efficiency, profitability, and sustainability of crop production.[38] The cowpea bruchid spends its larval life feeding within the seed, so it is difficult to

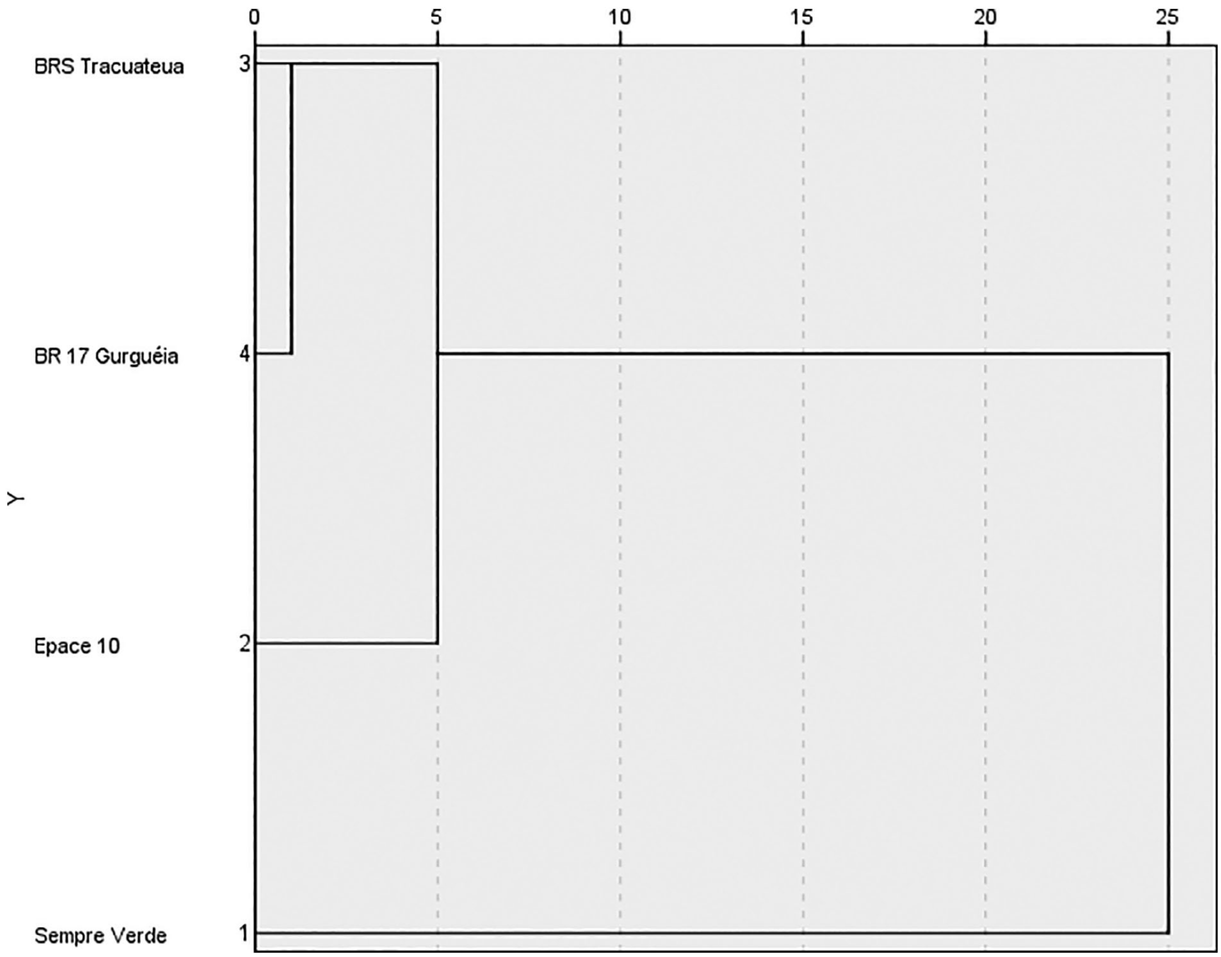

Figure 1 Dendrogram comparing the similarity (Euclidean distances) of cowpea cultivars in relation to biological parameters of *C. maculatus*.

describe its behavior or to ascertain how its behavior may differ in resistant *vs.* susceptible seeds.[39] An elevated level of cowpea trypsin inhibitor (CPTI) is responsible for resistance, and the CPTI is concentrated just below the seed testa.[40] So, the resistance presented in the cultivars used in this research can be due to trypsin inhibitor (CPTI) or other protein.

There are several possible explanations for the different feeding patterns in susceptible and resistant seeds. There may be: (i) a physical barrier in the interior of seeds that the insects cannot penetrate; (ii) a zone in the interior of the seed that is poor in nutritional value, and thus does not support normal larval growth and development; (iii) a toxin that is more concentrated toward the interior of the cotyledon; (iv) a repellant factor in the interior of seeds; or (v) a combination of the foregoing.[39] A loss of mass in stored beans is an important parameter to measure both from an economical point of view and as an indicator of cultivar resistance to pests.[41] In the present research, all cultivars (untreated) tested, presented a lower loss of mass than the cultivar uses as control (Table 4).

In general, in the present study the cultivars Epace 10, BRS Tracuateua and BR 17 Gurgueia untreated presented better results against *C. maculatus* than the cultivar Sempre verde used as a control (Fig. 1 and Tables 2 and 3).

The compound trans-anethole was toxic to *S. frugiperda* reducing the number of eggs, oviposition period and adult longevity.[22] Five compounds when tested against *S. zeamais* and *C. maculatus* showed that eugenol (a phenylpropanoid)[35] was one of the most effective insecticides and the functional and positional isomerisms of the pairs appears to exert little or no influence on their effects.[31] Several monoterpenes were tested against *Sitophilus orizae* and *T. castaneum* with the concluding result being that geraniol and cuminaldehyde showed the highest toxicity against *S. oryzae*.[20] In the present study, BRS Tracuateua and BR 17 Gurgueia, when associated with LC_{50} geraniol, showed a number of eggs of 65.50 and 75.75, respectively, however, they did not differ from other cultivars (Table 5).

In the present study, BR 17 Gurgueia provided just 0.75 eggs per grain when combined with LC_{30} and 0.65 eggs when combined with LC_{50} of eugenol, and 1.64 and 1.53 eggs when combined with LC_{30} and LC_{50} of trans-anethole, respectively, when the cultivars were compared in each constituent (Table 5). The mortality threshold recommended for the use of essential oils in integrated pest management was estimated at 30%, this way, in the present study the high mortality contributes to egg reduction.[42]

The potential of the combination of neem (*Azadirachta indica*) with resistant cowpea cultivars showed that the Kanannado cultivar provided an average of three emerged insects when combined with neem oil at 100 mg/5 g of seeds.[43] In the present study, BR 17 Gurgueia combined with LC_{50} of geraniol presented an average 38.5 insects emerged (Table 6). The compound 1,8-cineole (the major constituent of *Alpinia calcarata* Rosc.) at 0.060 g L^{-1} provided 2.16 insects emerged when used against *C. maculatus*.[44]

Table 5 Values (±SE) of total insects emerged, insects per grain and instantaneous rate of growth (r_i) of *Callosobruchus maculatus* on different cowpea combined with constituents of essential oils

	Total of insects emerged†		
	LC_{30}		
Cultivars	eugenol	geraniol	trans - anethole
Sempre Verde	148.50 ± 10.34aB	68.50 ± 2.98aC	245.50 ± 5.20aA
Epace 10	79.75 ± 2.49bB	51.75 ± 1.49aB	188.00 ± 14.07bA
BRS Tracuateua	75.50 ± 0.64bB	49.75 ± 2.13aB	176.75 ± 13.64bcA
BR 17 Gurgueia	68.25 ± 2.28bB	42.25 ± 1.37aB	140.00 ± 17.35cA
Cultivars		Total of insects emerged†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	96.00 ± 2.27aB	67.25 ± 3.72aB	172.75 ± 20.91aA
Epace 10	70.75 ± 0.62abB	49.50 ± 1.19aB	143.50 ± 35.29abA
BRS Tracuateua	65.75 ± 01.25abAB	43.75 ± 0.47aB	99.75 ± 3.90cA
BR 17 Gurgueia	55.50 ± 1.65bB	38.50 ± 1.44aB	129.00 ± 10.10bcA
Cultivars		Insects per grain†	
		LC_{30}	
	eugenol	geraniol	trans - anethole
Sempre Verde	1.40 ± 0.1Ba	0.62 ± 0.02abC	2.32 ± 0.05aA
Epace 10	0.74 ± 0.02bcB	0.46 ± 0.01abB	1.72 ± 0.14bA
BRS Tracuateua	1.10 ± 0.01abB	0.71 ± 0.02aC	2.62 ± 0.21aA
BR 17 Gurgueia	0.39 ± 0.01cB	0.25 ± 0.09bB	0.82 ± 0.10cA
Cultivars		Insects per grain†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	0.91 ± 0.01aB	0.65 ± 0.03aB	1.65 ± 0.17aA
Epace 10	0.65 ± 0.01abB	0.45 ± 0.01abB	1.32 ± 0.31aA
BRS Tracuateua	0.95 ± 0.01aB	0.63 ± 0.01aB	1.45 ± 0.07aA
BR 17 Gurgueia	0.32 ± 0.01bB	0.22 ± 0.06bB	0.76 ± 0.06bA
Cultivars		Instantaneous rate of growth (r_i)†	
		LC_{30}	
	eugenol	geraniol	trans - anethole
Sempre Verde	0.076883 ± 0.001aB	0.054898 ± 0.001aC	0.091433 ± 0.0006aA
Epace 10	0.059280 ± 0.0009bB	0.046933 ± 0.0008abC	0.083583 ± 0.002abA
BRS Tracuateua	0.057758 ± 0.0002bB	0.045763 ± 0.001bC	0.081793 ± 0.002bA
BR 17 Gurgueia	0.054830 ± 0.0009bB	0.041125 ± 0.0009bC	0.074740 ± 0.003bA
Cultivars		Instantaneous rate of growth (r_i)†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	0.064598 ± 0.0006aB	0.054320 ± 0.001aC	0.080783 ± 0.003aA
Epace 10	0.055898 ± 0.0002abB	0.045675 ± 0.0006abC	0.072590 ± 0.008abA
BRS Tracuateua	0.053795 ± 0.0005bB	0.042160 ± 0.0003bC	0.065648 ± 0.001bA
BR 17 Gurgueia	0.048925 ± 0.0008bB	0.038455 ± 0.001bC	0.072783 ± 0.002abA

†Means followed by the same lower case letter in the column and capital letter in the lines, do not differ by Tukey test at 5% probability.

Geraniol in both lethal concentrations provided the lowest instantaneous rate of growth, comparing the constituents within each cultivar, however, all rates were positive (Table 6). Instantaneous rate of growth has been widely used in toxicity studies, since it allows assessing lethal and sublethal effects of insecticides and acaricides for a population after a predetermined time, integrating survival values and fecundity. The instantaneous rate of growth is an important parameter of the population improvement. This way if the compound affects this parameter the population reduces. Instantaneous rate of growth estimated in BR 17 Gurgueia when tested in relation to resistance against *C. maculatus* was 0.058.[23] In the present research, this cultivar combined with LC_{50} of Geraniol presented instantaneous rate of growth of 0.038455. BR 17 Gurgueia also affected the immature stage viability of *C. maculatus*.

The immature stage viability can be affected by insecticidal proteins present in the grain. The chemical components of plant defense include antibiotics, alkaloids, terpenes, cyanogenic glycosides, and proteins.[45] Proteins usually associated with defense mechanisms are lectins, alpha-amylase inhibitor, proteinase inhibitors, protein inactivating ribosomes, reserve proteins (vicilins) modified, lipid transport proteins, glucanases and chitinases. Among the relevant anti-nutritional factors found in legume seeds and cowpea are lectins and protease inhibitors. Other

Table 6 Values (±SE) of immature stage viability (%), insect dry weight (mg), grain weight loss (%) and egg-adult period of *Callosobruchus maculatus* on different cowpea combined with constituents of essential oils

	Immature stage viability (%)†		
	LC_{30}		
Cultivars	eugenol	geraniol	trans - anethole
Sempre Verde	87.00 ± 0.91aA	85.00 ± 0.71aA	87.25 ± 0.75aA
Epace 10	77.75 ± 1.03bA	78.50 ± 0.96bA	78.50 ± 1.19bA
BRS Tracuateua	80.75 ± 0.85bA	80.75 ± 1.11abA	82.75 ± 0.63abA
BR 17 Gurgueia	69.50 ± 1.71cA	70.50 ± 1.55cA	68.25 ± 0.85cA
Cultivars		Immature stage viability (%)†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	86.25 ± 1.11aA	85.00 ± 1.15aA	87.00 ± 1.15aA
Epace 10	76.50 ± 1.55bA	76.75 ± 0.85bA	76.750 ± 1.11bA
BRS Tracuateua	77.00 ± 2.74bA	79.50 ± 1.55bA	79.50 ± 1.94bA
BR 17 Gurgueia	68.75 ± 0.75cA	68.50 ± 0.96cA	69.00 ± 1.08cA
Cultivars		Insect dry weight (mg)†	
		LC_{30}	
	eugenol	geraniol	trans - anethole
Sempre Verde	1.95 ± 0.17aAB	1.47 ± 0.06aC	2.45 ± 0.40aA
Epace 10	1.77 ± 0.30aB	2.00 ± 0.25aAB	2.52 ± 0.11aA
BRS Tracuateua	1.67 ± 0.15aA	2.00 ± 0.16aA	2.20 ± 0.18aA
BR 17 Gurgueia	1.45 ± 0.13aA	1.57 ± 0.10aA	1.35 ± 0.05bA
Cultivars		Insect dry weight (mg)†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	1.45 ± 0.05aA	1.25 ± 0.10aA	1.30 ± 0.06aA
Epace 10	1.60 ± 0.17aA	1.52 ± 0.14aA	1.60 ± 0.18aA
BRS Tracuateua	1.55 ± 0.15aA	1.85 ± 0.21aA	1.60 ± 0.20aA
BR 17 Gurgueia	1.40 ± 0.08aA	1.42 ± 0.09aA	1.60 ± 0.14aA
Cultivars		Grain weight loss (%)†	
		LC_{30}	
	eugenol	geraniol	trans - anethole
Sempre Verde	14.65 ± 0.28aB	11.35 ± 0.42aC	35.59 ± 0.01aA
Epace 10	7.94 ± 0.18bB	9.74 ± 0.58abB	14.97 ± 0.80bA
BRS Tracuateua	8.55 ± 0.48bB	9.25 ± 0.27abB	14.40 ± 1.69bcA
BR 17 Gurgueia	6.85 ± 0.23bB	8.23 ± 0.52bB	11.63 ± 0.68cA
Cultivars		Grain weight loss (%)†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	12.18 ± 0.53aB	10.60 ± 0.28aB	26.835 ± 2.80aA
Epace 10	7.21 ± 0.29bB	9.50 ± a0.29bB	12.52 ± 0.51bcA
BRS Tracuateua	7.81 ± 0.39bA	7.57 ± 0.49abA	9.75 ± 0.85cA
BR 17 Gurgueia	6.40 ± 0.46bB	7.32 ± 0.30bB	13.91 ± 1.05bA
Cultivars		Egg-adult period†	
		LC_{30}	
	eugenol	geraniol	trans - anethole
Sempre Verde	28.29 ± 0.23abA	27.91 ± 0.06bA	27.85 ± 0.09cA
Epace 10	28.36 ± 0.05abB	28.00 ± 0.03abB	28.99 ± 0.09aA
BRS Tracuateua	27.91 ± 0.01bA	27.99 ± 0.10abA	27.59 ± 0.07cA
BR 17 Gurgueia	28.76 ± 0.03aA	28.41 ± 0.08aA	28.37 ± 0.30bA
Cultivars		Egg-adult period†	
		LC_{50}	
	eugenol	geraniol	trans - anethole
Sempre Verde	27.78 ± 0.07bA	27.98 ± 0.04abA	27.53 ± 0.22bA
Epace 10	28.27 ± 0.01abA	28.06 ± 0.05abA	28.42 ± 0.22aA
BRS Tracuateua	27.91 ± 0.02bA	27.74 ± 0.04bA	27.80 ± 0.08bA
BR 17 Gurgueia	28.75 ± 0.03aA	28.44 ± 0.13aA	28.74 ± 0.34aA

†Means followed by the same lower letter in the column and capital letter in the lines, do not differ by Tukey test at 5% probability.

non-protein factors such as tannins and phytic acid have also been detected in seeds of different cowpea cultivars, acting directly in the gastrointestinal system, others still acting on the nervous system, hormonal balance and metabolism of its consumers.[46]

In some resistant cultivars tested on *C. maculatus* was verified that AM-61-1-Costela de Vaca had the lowest dry weight (1.751 mg).[47] The association of cowpea genotypes with essential oils was tested and presented lower weight loss when combined *Vitex agnus castus* and *Piper callosum* with BRS - Urubuquara, which provided a consumption of 0.010 g.[48] In the present research, BR 17 Gurgueia combined with LC_{50} of geraniol and eugenol provided weight loss of 7.32 and 6.40%, respectively.

In the cultivar Epace 10, *C. maculatus* presented an egg-adult period of 28 days.[49] In the present study, the same cultivar with LC_{30} of trans-anethole provides a similar egg-adult period (Table 7).

Studies related to the effect of insecticides on insect pests and nontarget organisms, such as natural enemies, are traditionally accessed by the estimative of lethal effects, through mortality data. Due to the limitations of the traditional methods, recent studies in the past three decades are assessing the sublethal effects of insecticides upon several important biological traits of insect pests and natural enemies. Besides mortality, the sublethal dose/concentrations of an insecticide can affect insect biology, physiology, behavior and demographic parameters.[50] In the present study, the compounds of essential oils associated with some cowpea cultivars have affected many biological parameters of *C. maculatus*, such as oviposition, insects emerged, immature stage viability (%), instantaneous rate of growth (r_i), insect dry weight (mg) and egg-adult period.

The validation of the insecticidal efficacy of isolated monoterpenes and the phenylpropanoid eugenol may permit a more advantageous, rapid, economic and optimized approach to the identification of promising oils or its compounds for commercial formulations when combined with ethnobotanical strategies.[31] The insecticidal activity of essential oils is based on the high concentrations of major compounds that belong to the classes of terpenes, phenolics and alkaloids. Thus, the combination of these essential oils compounds with resistant cultivars can be an important alternative of control to the stored grain insects, between them, and *C. maculatus*.

In the present study, the cowpea cultivars used can also cause mortality due to the effect of insecticidal proteins. Some studies have shown that resistance is associated with vicillin polypeptides, which are expressed mainly in cotyledons of resistant seeds, and that there is an association of vicillins with chitin present in the midgut of insects. The interaction capacity of cowpea vicillins with chitin, a property that is directly related to the defense of plants against insects.[49]

Essential oils and their compounds must undergo a series of studies, before they can be recommended for the treatment of grains for human consumption, such as toxicology of volatile components, the cost of treatment, the effect on the odor and taste of the processed grains and the formulations and registration.[51] In addition, effects of these products on humans and non-target organisms also need to be made and large-scale application also suffers from problems with persistence in the storage environment.

Regarding the findings of the present research, the combination of resistant cultivars with constituents of essential oils can be a promising control method, following the principles of integrated pest management and with the potential to be an important alternative to the exclusive use of chemical insecticides. In general, BR 17 Gurgueia combined with eugenol and geraniol affected more significantly the biological parameters of *C. maculatus* than in combination with trans-anethole, reducing egg number, insects emerged and egg viability.

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AUTHOR CONTRIBUTIONS

Barbosa, DRS participated in all stages of manuscript production; Oliveira JV and Silva PHS guided the research and contributed to the revision of the manuscript; Dutra KA and Lopes FSC participated in the practical implementation of the bioassays; Araujo AMN and Breda MO helped with statistical analysis and wrote part of the manuscript.

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