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SHORT COMMUNICATION

Efficacy of *Ocimum tenuiflorum* essential oil as grain protectant against coleopteran beetle, infesting stored pulses

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Abstract In the present investigation, essential oil (EO) of *Ocimum tenuiflorum* and its principal constituent (eugenol) was evaluated for its toxicity and mode of action against *Callosobruchus maculatus*. Furthermore, fumigant toxicity and germination studies on the application of *O. tenuiflorum* EO and eugenol against *C. maculatus* on different pulses was also studied. Fumigant activity studies revealed that EO toxicity was significantly ($p < 0.05$) influenced by concentration and exposure time. In fumigant toxicity assay without food, *O. tenuiflorum* EO and eugenol showed LC_{50} value of 278.6 and 256.5 µL/L air, respectively, at one hour exposure. Further, *O. tenuiflorum* EO displayed fumigant toxicity via inhibiting acetylcholinesterase activity. Pulses treated with *O. tenuiflorum* EO showed 70% of *C. maculatus* mortality at 250 µL/L air concentration after 24 h. Furthermore, these treatments didn't affect the seed viability of the pulses tested. Hence, the application of *O. tenuiflorum* EO has potential scope as a botanical insecticide.

Keywords Acetylcholinesterase activity · Biopesticide · Bruchid · *Callosobruchus maculatus* · Leguminous seeds · Germination

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Introduction

Pulses have excellent nutritional value, but the occurrence of insect pests during storage, leads to severe qualitative and quantitative loss such as weight loss, decreased nutritional and aesthetic value, increased mould growth and failure of seed/grain germination (Iturralde-García et al. 2016). Major threatening insects infesting the pulses, known as pulse beetles are the genus *Callosobruchus* of which, *C. maculatus* is the most pestilential (Gbaye et al. 2012). Synthetic chemical insecticides like organophosphates, pyrethroids, and fumigants such as phosphine, methyl bromide are apparently available for effective control of stored product insects (Bomzan et al. 2018). However, the best approach for pest management is the application of biopesticides that are eco-friendly and relatively safe alternatives (Isman 2000; Bhavya et al. 2020). Most plant extracts are characterized by low mammalian toxicity, selectivity, rapid degradation, and minimal influence on the environment, and seed germination (Nenaah 2014). Plant essential oils (EOs) are potential alternative to synthetic pesticides, used as contact/fumigant pesticides against various stored product insects (Isman 2000), because of its rich source of bioactive compounds. Numerous plant species used in the form of powder or EO have exhibited protection against pulse beetle with toxic, antifeeding, ovicidal, repellence, larvicidal, and growth inhibitory activities (Kedia et al. 2015). The bioactives of some plants with insecticidal property lead to different mode of action through GABA, octopamine synapses, and by inhibiting acetylcholinesterase (AChE), which are generally due to the synergistic effects of various active compounds (Koul et al. 2008).

Promising control of *Callosobruchus* species has been reported by the application of EO and their components

(monoterpenoids, cyanohydrins, sulphur compounds, thiocyanates) as fumigants (Kedia et al. 2015; Sanon et al. 2018). Though there are a few reports on insecticidal activities of *Ocimum* species against *Callosobruchus* species, comprehensive assessment on the efficacy of *Ocimum tenuiflorum* EO as a biopesticide in stored pulses has not been studied. In this regard, the present investigation focuses on the evaluation of insecticidal activity of *O. tenuiflorum* EO and its major bioactive component (eugenol) against the major pulse beetle, *C. maculatus* in four stored pulses using fumigation procedure. Further, studies on the mode/mechanism of action of *O. tenuiflorum* EO and its constituent, against *C. maculatus* were carried out. Additionally, effect of the application of these EO on the seed germination was also investigated.

Materials and methods

Materials and chemicals

O. tenuiflorum EO was purchased from Falcon Essential oils, Bengaluru, India. Pulses: green gram (*Vigna radia*), Bengal gram (*Cicer aritinum L*), cowpea (*Vigna unguicalata*) and lablab bean (*Lablab purpureus*) were procured from local market in Mysuru, India. Eugenol standard was purchased from Himedia, Mumbai, India. Acetylthiocholine iodide (ATCI), bovine serum albumin (BSA) and 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) were procured from Sigma–Aldrich Chemical Co., USA.

Rearing of stored product insect

C. maculatus (cowpea seed beetle) was cultured at Department of Food Protection and Infestation Control, CSIR-CFTRI, Mysuru, India using conditioned green gram (12–14% moisture content). The cultures were maintained under the laboratory conditions set at 28 ± 2 °C, 70 ± 5% RH and 16:8 light: dark photoperiod without any exposure to insecticides.

Vapour toxicity assay (without food) against *C. maculatus*

The vapour toxicity/fumigant activity of *O. tenuiflorum* EO and its major component (eugenol) without food against *C. maculatus* was determined according to Negahban et al. (2007) with slight modifications. Briefly, in 30 mL screw cap glass vial (lined with Insect-A-slip, 1 cm below the stopper rim), nine different concentrations of the EO and eugenol (20–500 µL/L of air) were impregnated on Whatman filter paper strips (1 cm × 5 cm) and pasted to the lower side of stopper. Then, fifteen beetle adults (0–1 day old) were released into each vial including untreated control and triplicates were kept for each experiment according to Tripathi et al. (2009). After 1 and 3 h of treatment, adult mortality was recorded. Percentage of beetle mortality was calculated using Abbott's correction method (Abbott 1925) and $LC_{50,}$ LC_{90} values for the tested concentration were calculated using Probit analysis (Finney 1971).

Evaluation of in vivo acetylcholinesterase (AChE) activity

The in vivo AChE inhibitory action of *O. tenuiflorum* EO and its major compound, eugenol against *C. maculatus* was estimated as described by Bhavya et al. (2018). In brief, beetles were exposed to different concentrations (LC_{25}, LC_{50} and LC_{75}) of *O. tenuiflorum* EO and eugenol for 1 h. Further, live beetles were homogenized in sodium phosphate buffer (pH 8, 100 mM) in a glass-Teflon homogenizer. Then, homogenate was centrifuged at 10,000 rpm at 4 °C for 10 min and the supernatant was further used. For the assay, sodium phosphate buffer (5.6 mL), enzyme supernatant (40 µL) were mixed with 100 µL of DTNB reagent (10 mM) and further, by the addition of substrate (100 µL ATCI), the reaction was initiated. The coloured product formed, 5-thio-2-nitrobenzoate anion, was measured at 412 nm using UV spectrophotometer (UV-1700, Pharma Spec, SHIMADZU). Protein content in the supernatant was determined by Lowry's method (Lowry et al., 1951) using BSA as the standard.

Vapour toxicity bioassay (with food) against *C. maculatus*

The vapour toxicity of *O. tenuiflorum* EO and eugenol was also studied in pulses (green gram, Bengal gram, cowpea and lablab bean) according to Vendan et al. (2017) with few modifications against *C. maculatus,* at five different concentrations (50–250 µL/L of air). In 50 mL glass screw cap vials, 15 g of conditioned pulses were taken separately and fifteen *C. maculatus* adults were introduced including untreated control. Later, the known concentration of the EO and eugenol were added onto the filter paper strip and immediately introduced into the glass vials as mentioned above. At 24 and 48 h post exposure, mortality of the beetles were noted down and percentage mortality was calculated.

Seed germination studies

After 60 days of treatment, the viability of control and treated pulses were tested according to Dalvi et al. (1972) with slight modifications. For germination assay, 25 seeds

from each replicate of treated and control group were placed separately on glass Petriplates containing moist filter paper. The germination of the seeds was assessed for each treatment by keeping the Petriplates in a dark incubator maintained at 27 ± 2 °C. The plates were observed for the germination of seeds for one week and later the percentage germination, root and shoot length were estimated.

Statistical analysis

All the experiments were conducted in triplicates and values were presented as mean ± SD. Further, statistical analysis was performed using SPSS statistical software (SPSS version 20.0). One-way ANOVA with Tukey's HSD posthoc test was used to find significance between the groups and value of $p < 0.05$ was considered as significant. Probit analysis was also performed using SPSS statistical package to calculate the lethal concentrations.

Results and discussion

Vapour toxicity against *C. maculatus* without food

In the fumigation study, *O. tenuiflorum* EO and eugenol showed toxicity against *C. maculatus* in dose dependent manner (Fig. 1). The response of *C. maculatus* to both the EO (Fig. 1a) and eugenol (Fig. 1b) in fumigant toxicity assay showed significant ($p < 0.05$) lethality at 1 h, leading to 93.33 and 96.67% mortality at 500 µL/L air respectively. Ke´ita et al. (2001) observed ~ 70 and 80% fumigant toxicity with *O. gratissimum* and *O. basilicum* EO against *C. maculatus,* respectively, at 25 µL concentration in 8 mL vial after 12 h treatment. While, *O. gratissimum* EO and eugenol (1 µL/L air) showed 100% mortality of *C. chinensis* after 24 h (Ogendo et al. 2008). Additionally, EO and its components showed strong species specific toxicity, dependent on the concentration (dosage) and exposure period (Ogendo et al. 2008). Abd El-Salam (2010) observed 100% mortality of *C. chinensis* after 3 days of exposure to *O. basilicum* EO at 1.0 mL/38.5 mL air. Mann (2012) also mentioned that hexane fraction of *O. gratissimum* EO containing eugenol has shown grain protectant action against *C. maculatus*. However, the differences in the bruchid mortality among different studies can be attributed to the variation in *Callosobruchus* species as well as basil species tested, which are of different compositions.

The GC-MS analysis of *O. tenuiflorum* EO used comprised of phenylpropenes (61.99%) like eugenol (50.39%), methyl eugenol (8.36%), estragole (2.99%); terpenes (5.87%) and sesquiterpenes (22.53%) like β-caryophyllene

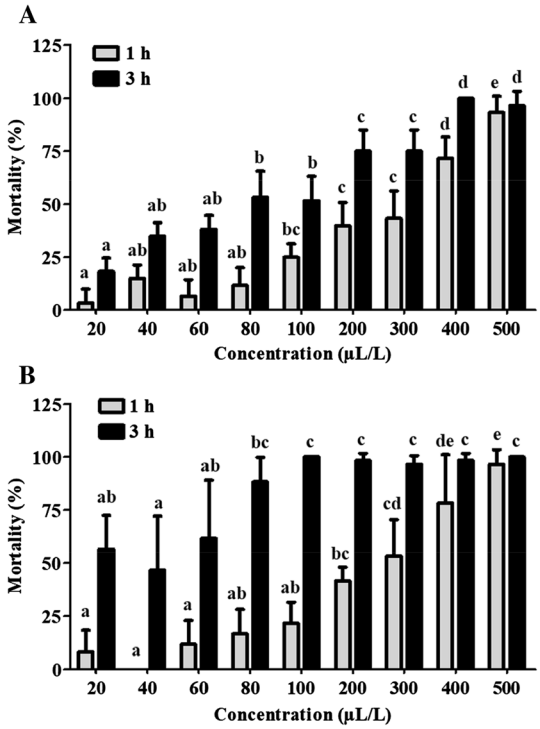

Fig. 1 Fumigant action of *O. tenuiflorum* essential oil (**a**) and eugenol (**b**) against *C. maculatus*. Different letters on the bar chart indicate statistically significant values (ANOVA, Tukey HSD posthoc test, $p < 0.05$)

(20.07%), humulene (1.92%) as per our previous work (Bhavya et al., 2018). In the present study, the lethal concentrations, LC_{50} and LC_{90} values estimated for *O. tenuiflorum* EO and eugenol against *C. maculatus* are presented in supplementary Table 1. It was found that LC_{50} of *O. tenuiflorum* EO (consisting of 50.4% eugenol) and eugenol were 279 and 256 µL/L air, respectively at 1 h exposure. The strong fumigant toxicity of *O. tenuiflorum* EO against *C. maculatus* could be attributed to the distinct and synergistic effect of eugenol (50.39%) and β-caryophyllene (20.07%). Ke´ita et al. (2001) found the LC_{50} values (12 h) of *O. basilicum* and *O. gratissimum* EOs were 660 and 1060 µL/L, respectively, against *C. maculatus*. Contrastingly, Ogendo et al. (2008) reported LC_{50} values (24 h) of 0.20 and 0.01 µL/L air of *O. gratissimum* EO and eugenol, respectively against *C. chinensis*. Abd El-Salam (2010) calculated the LC_{50} values to be 1.88 µL/38.5 mL air for *O. basilicum* EO against *C. chinensis* after 24 h treatment.

In vivo acetylcholinesterase (AChE) activity against *C. maculatus*

Acetylcholinesterase inhibition is the potential, well explored mode of action of EOs and their bioactive compounds against the stored product insects (Isman 2000).

Hence, in the current study, effect of *O. tenuiflorum* EO on in vivo AChE inhibition of *C. maculatus* was studied. Pulse beetle exposed to EO and eugenol at LC_{25}, LC_{50} and LC_{75} for 1 h caused ~ 10, 19 and 48% AChE inhibition, respectively compared to control. Further, a direct correlation was also noticed, highlighting the dose-dependent inhibition, between the anti-AChE activity and the exposure dose (Fig. 2). It suggests that the tested EO and its bioactive compound targets the key site, AChE.

Eugenol (50.4% in *O. tenuiflorum* EO) was found to possess potential anti-AChE activity even, when tested individually. However, the EO showed the same inhibitory activity to that of eugenol, even though it constituted only for ~ 50% of the total composition. Hence, the AChE inhibitory activity of the *O. tenuiflorum* EO may be attributed to the synergistic effect of the major bioactive compounds such as eugenol (50.39%), and β-caryophyllene (20.07%). Similarly, Dohi et al. (2009) also reported that eugenol being the major compound accounted for 25% of the observed AChE inhibitory activity (in vitro) of the *O. sanctum* EO. Even, our previous studies showed that the *O. tenuiflorum* EO exhibited anti-AChE activity against *S. oryzae* adults (Bhavya et al. 2018). Therefore, the *O. tenuiflorum* EO was responsible for the mortality of *C. maculatus,* possessing neurotoxic effect on pulse beetle, *C. maculatus*.

Vapour toxicity against *C. maculatus* with food (pulses)

The observed mortality caused by *O. tenuiflorum* EO was positively dependent on the concentration of oil/bioactive compound and the exposure time (Supplementary Fig. 1). The green gram treated with the EO and eugenol showed a range of 25–73.3% and 31.7–71.7% mortality, respectively at 50–250 µL/L air after 24 h of exposure (Supplementary Fig. 2). Similar trend was also observed for Bengal gram and lablab bean treated with the EO and eugenol. While, cowpea treated with *O. tenuiflorum* EO and eugenol showed 25–70% and 11.7–55% mortality, respectively after 24 h. In a previous study by Pascual-Villalobos and Ballesta-Acosta (2003), *O. basilicum* EO (50.5% linalool and 18.5% eugenol) showed 48% mortality of *C. maculatus* after 24 h treatment on kidney beans at 5 µL concentration.

The LC_{50} and LC_{90} values estimated for *O. tenuiflorum* EO and eugenol in presence of pulses against *C. maculatus* are listed in supplementary Table 2. It clearly shows that the fumigant toxicity and the lethal dose of the EO and eugenol decreased when treated with-pulses (with-food) compared to that of without-food. This difference can be due to the low vapour pressure and high sorption of the botanical components onto the food grains thereby reducing the efficacy of the test compounds (Vendan et al. 2017; Bhavya et al. 2018).

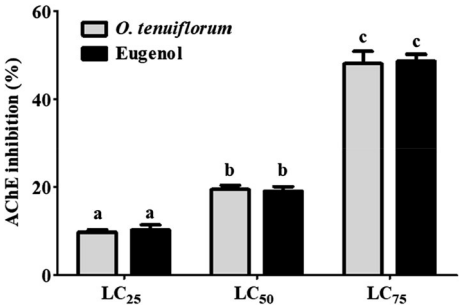

Fig. 2 In vivo acetylcholinesterase activity of *O. tenuiflorum* essential oil and eugenol. Different letters on the bar chart indicate statistically significant values (ANOVA, Tukey HSD posthoc test, $p < 0.05$)

Germination studies of treated pulses

The pulses, green gram, Bengal gram, cowpea and lablab beans, treated with *O. tenuiflorum* EO and eugenol were subjected to germination studies to check its viability/toxicity on the propagation capacity. Tested EO and the major bioactive compound did not show any adverse effect on germination of these pulses compared to control (Fig. 3). Similarly, Ke´ita et al. (2001) also reported that cowpea seeds treated with *O. basilicum* and *O. gratissimum* aromatized powder exhibited 79% of germination, while control showed 97% of germination. Even, the effect of *O. tenuiflorum* EO and eugenol did not show any significant change on the length of the root and the shoot (Supplementary Fig. 3). These results implicate that *O. tenuiflorum* EO exhibiting significant fumigant toxicity without affecting the seed germination, can be applied for pulses stored for sowing purpose also.

Conclusion

To summarize, *O. tenuiflorum* EO and its major constituent, eugenol showed significant vapour toxicity (93–97% mortality at 500 µL/L air for 1 h) against *C. maculatus* via AChE inhibition (~ 48% at LC_{75}). The application of *O. tenuiflorum* EO has shown insecticidal activity against *C. maculatus* in pulses, without affecting its germination ability. Hence, *O. tenuiflorum* EO, rich in eugenol can be exploited as a rational, safer fumigant substitute to conventional pesticide for the management of *C. maculatus* (pulse beetle) and other stored product insect-pests with an appropriate formulation and dose. However, scale-up studies are required further to test the efficacy of *O. tenuiflorum* EO at a larger extent to be used as stored product pest control agent.

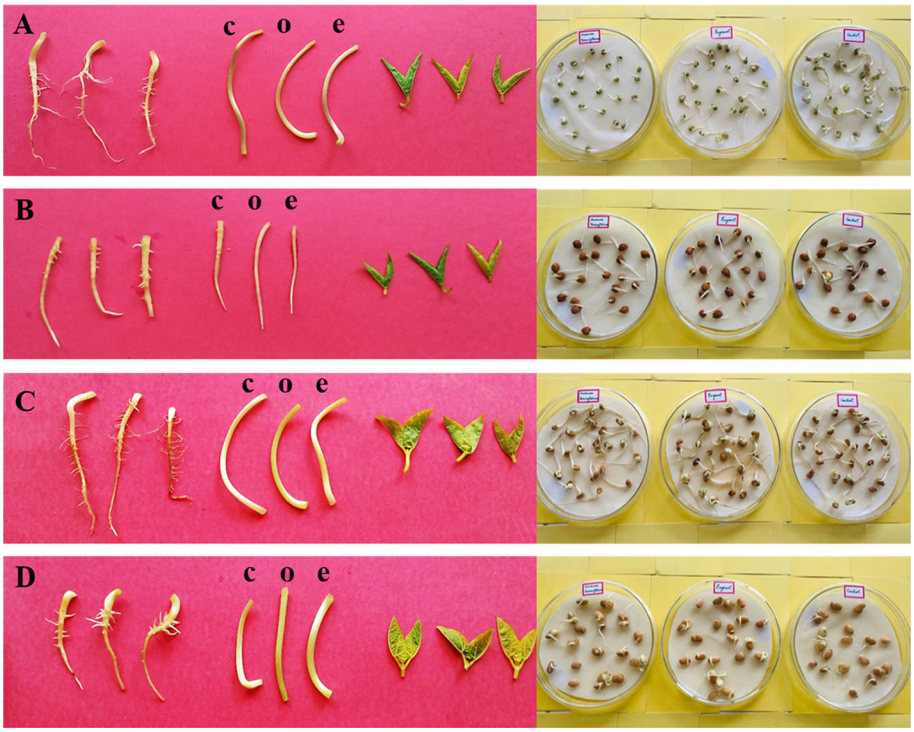

Fig. 3 Germination of pulses treated with *O. tenuiflorum* essential oil and eugenol. Green gram (**a**), Bengal gram (**b**), Cow pea (**c**), Lablab beans (**d**), (c—control untreated, o—*O. tenuiflorum* essential oil and e—eugenol)

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Compliance with ethical standard

Conflict of interest The authors declare that there is no conflict of interest.

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