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Repellency of anethole- and estragole-type fennel essential oils against stored grain pests: the different twins

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Abstract

Aromatic plants essential oils (EOs) are promising alternatives to chemical insecticides and insect repellent for the post-harvest protection of crops. Fennel (*Foeniculum vulgare* Mill.) is a highly aromatic plant, cultivated worldwide of which several chemotypes can be distinguished on the basis of the relative content of its main compounds (*E*)-anethole and estragole. Fennel is well known for its pharmacological, antioxidant antimicrobial and acaricidal activities, and several studies showed its effectiveness as insecticidal and repellent against insects. In this study the repellency of the EOs extracted from two chemotypes, anethole- and estragole-type of *F. vulgare* fruits, against *Rhyzopertha dominica* (F.), *Sitophilus zeamais* Motschulsky and *Tribolium confusum* Jaqcquelin du Val, three of the major worldwide post-harvest grains insects was assessed by *in vitro* bioassays. Along with the EOs, we also tested the repellency of their major chemical components (*E*)-anethole, estragole, limonene and, fenchone and we evaluated the co-repellency effect of (*E*)-anethole and estragole. Finally, the repellence of the fennel EOs in the presence of maize was tested by a two-choice pitfall bioassay. (*E*)-anethole and estragole content in the anethole-type was 78.4 and 8.0%, respectively while in the estragole-type fennel EO anethole and estragole content was 0.9 and 85.5%, respectively. RD_{50} values showed that the estragole-type EO was the most effective repellent against the three insect species with values of 0.007, 0.051 and 1.124 mg cm^{-2} for *R. dominica*, *T. confusum* and *S. zeamais*, respectively. Consistently, relative median potency analysis showed that estragole was significantly more repellent to the three pest insect species than (*E*)-anethole. Interestingly, in the EOs, (*E*)-anethole and estragole showed a synergistic co-repellent effect. The strongest synergy was observed against *R. dominica* (CRC = 634.51). The repellency of fennel EOs also in the presence of maize was confirmed by a two-choice bioassay. Also in this case, the most overall effective EO resulted to be the estragole-type. The results highlight the importance of a chemical standardization based on the bioactivity of the fennel EOs and indicate the estragole-type fennel EO as suitable for the development of eco-friendly repellents for the post-harvest protection of grain crops.

Key words: post-harvest pest, anethole; estragole, insect repellency, synergy, fennel.

Introduction

Insects are major pests of stored food causing losses estimated around 20% of the annual world crop production (Sallam, 1999). Essential oils (EOs) of aromatic plants are effective natural products as contact and fumigant insecticides and as repellents against stored food pests (Isman, 2000; Nerio *et al*., 2009; Conti *et al*., 2011; Athanassiou *et al*., 2013; Bougherra *et al*., 2015). Moreover, due to their high biodegradability and low mammalian toxicity, EOs are regarded as very promising tools for the formulation of rational low-toxic, eco-friendly food preservative (Isman, 2006). However, a major difficulty for the implementation of aromatic plant EOs is that their bioactivity varies considerably depending to their chemical composition. EOs composition is reported to vary depending on a number of factors such plant genetic structure (Telci *et al*., 2009), phenological stage and site of origin of the plants (Shaaya and Kostyukovysky, 2006). Since the biological activities of EOs depend on their chemical composition, the chemical characterization coupled with the assessment of their biological properties is essential to standardize the chemical parameters that ensure the EOs biological effects. Such standardization that has already been successfully introduced for the Australian Tea tree oil (Callander and James, 2012), could strongly reduce the potential variability of the EOs effectiveness.

Fennel, *Foeniculum vulgare* Mill. (Apiaceae), is an aromatic plant indigenous of the Mediterranean region well known for its pharmacological properties as carminative, digestive, lactogogue and diuretetic (Manzoor *et al*., 2012). The fennel, is largely distributed, especially in dry soils, near the sea coast and on river banks, both as wild and cultivated plant (Napoli *et al*., 2010). The species shows a large morphological and chemical diversity. Some of the fennel genotypes that have been entered in the European Pharmacopeia, are impossible to be discriminated in relation to only morphological data. However, several different chemotypes have been identified on the basis of the EOs relative presence of the main compounds, the two enantiomers (*E*)-anethole and estragole (Krüger and Hammer, 1999).

Fennel EO has been already shown to have acaricidal (Lee, 2004), antifungal (Singh *et al*., 2006), as well as antibacterial activity (Ruberto *et al*., 2000) and, recently, its effectiveness as insecticidal and repellent against insects has been evaluated as well (Bertoli *et al*., 2012). However, to our knowledge, no information is available about the fennel EO effectiveness in relation to the chemical composition of its chemotypes.

The aim of the work was to assess the repellent activity of different fennel EO chemotypes and of their main compounds against three of the major grain pests: the

lesser grain borer *Rhyzopertha dominica* (F.) (Bostrichidae), the maize weevil *Sitophilus zeamais* Motschulsky (Dryophthoridae), and the confused flour beetle *Tribolium confusum* Jaqcquelin du Val (Tenebrionidae) in order to detect the relevant chemical parameters useful for a bioactivity based standardization of the fennel EOs.

Materials and methods

Plant material

Estragole-type *F. vulgare* fruits were manually harvested, at full ripeness, in Kabylia (Algeria) in the summer 2012, dried in the shade, at room temperature (20-25 °C) until constant weight and stored in polyethylene bags in the dark at 4 °C. Seeds were then transported in insulated polystyrene boxes on ice to the laboratory of the University of Pisa for the EO extraction.

Anethole-type *F. vulgare* fruits, were purchased from Biochimica srl (Arezzo, Italy).

Essential oil extraction and GC-MS analyses

Fennel fruits were coarsely grounded in a mortar with a pestle and then subjected to hydro-distillation in a modified Clevenger-type apparatus for 3 h (Guenter, 1949). The resulting essential oil was dried over anhydrous sodium sulphate and stored in a glass vial at 4 °C until use.

Gas chromatography (GC) analyses were carried out with an Hewlett Packard -5890 Series II instrument equipped with HP-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 µm film thickness), working with the following temperature program: 60 °C for 10 min, ramp of 3 °C min^{-1} up to 220 °C; injector and detector temperatures 250 °C; carrier gas helium (2 ml min^{-1}); detector dual FID; split ratio 1:30; injection of 0.5 µl (10% hexane solution). Components identification was carried out, for both columns, by comparing their retention times with those of pure authentic samples and by means of their linear retention index (LRI), relative to the series of *n*-hydrocarbons. Gas chromatography-electron impact mass spectroscopy (GC-EIMS) analyses were performed with a Varian CP-3800 gas chromatograph, equipped with a HP-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector with the following analytical conditions: injector and transfer line temperatures 220 °C and 240 °C respectively; oven temperature programmed from 60 °C to 240 °C at 3 °C min^{-1}; carrier gas helium at 1 ml min^{-1}; injection of 0.2 µl (10% hexane solution); split ratio 1:30. Constituents identification was based on the comparison of retention times with those of authentic samples, comparing their LRIs with the series of *n*-hydrocarbons and using computer matching against commercial (Adams, 1995) and home-made library mass spectra (built up from pure substances and components of known oils and MS literature data (Adams, 1995). Moreover, molecular weights of all identified substances were confirmed by gas chromatography-chemical ionization mass spectrometry (GC-CIMS), using methanol as the chemical ionizing gas.

Chemicals

(*E*)-anethole, estragole, limonene and, fenchone were purchased from Sigma-Aldrich (Milano, Italy). Chemicals were of analytical standard grade.

Insect cultures and rearing conditions

R. dominica, *S. zeamais* and *T. confusum* were reared at the Department of Agriculture, Food and Environment of the University of Pisa, since 2000. Insects were reared at room temperature (20-24 °C), 45-65% R.H., in the dark, in (20 × 27 × 11 cm) plastic boxes containing broken corn and wheat and covered by a nylon net allowing air exchange. Insects homogeneous in age for the bioassays were obtained by removing the adults present in the rearing boxes by sieving the grain and collecting the newly emerged insects (*S. zeamais*, 0-3 days old; *R. dominica* and *T. confusum*, 0-1 day) the following day.

Repellence bioassays

Area preference bioassays

The repellence of the anethole- and estragole-type fennel EOs and of their main chemical compounds (*E)*-anethole, estragole, limonene and, fenchone was assessed by the area preference method described by Tapondjou *et al*. (2005). In detail, half filter paper disks (8 cm ⌀) were treated with 500 µL of *F. vulgare* EO or pure chemical compounds as ethanolic solutions. Control half filter paper disks were treated with 500 µL of ethanol. Ethanol of the treated and control filter paper disks were evaporated under a fume hood. Each Petri dish's bottom (8 cm ⌀) was covered with half filter paper treated with the EO or the chemical solution, while the other half, was covered with a control half filter paper disk. Repellency bioassays were performed with both *F. vulgare* EOs at doses ranging from 0.005 to 0.385 mg cm^{-2}. Chemicals were tested at doses ranging from 0.01 to 0.74 mg cm^{-2}. Twenty unsexed adults were introduced in each Petri dish, and the lid was sealed with Parafilm®. The Petri dishes were maintained in climatic chamber at 25 ± 1 °C, 65 ± 5% R.H., in the dark, covered by black plastic pots. Five replicates were performed for each assay, and insects were used only once. The number of insects on the two half of the Petri dish was recorded after 24 h from the beginning of the test. The percent repellence (PR) of the EO and of each volatile compound was calculated by the formula: PR (%) = [(Nc − Nt) / (Nc + Nt)] × 100 where Nc is the number of insects present in the control half paper and Nt the number of insects present in the treated one.

Two-choice pitfall bioassay

The potential protection of grains due to repellency of the main volatile compounds of the fennel EOs, (*E)*-anethole and estragole was evaluated against *R. dominica*, *S. zeamais*, and *T. confusum* adults, using the two-choice bioassay described by Germinara *et al*. (2007). The two-choice pitfall bioassay for the presence of grains and because insects are never in direct contact with the repellent substance, allows to test the repellency of substances in conditions that are much more close to a real situation respect to the area preference

method. The bioassay was conducted in a steel arena (32 cm ⌀ × 12 cm high) with two diametrically opposed holes (3 cm ⌀) located 3 cm from the sidewall, in the bottom. 10 µl of EO or ethanol (control) were adsorbed onto a filter paper disk (1 cm ⌀) suspended at the centre of each hole by a cotton thread taped to the lower surface of the arena. Glass flasks (500 ml) filled with 100 g of maize grains (hybrid Eleonora, Pioneer Hi-Bred, Italy) were positioned under each hole, and the inside surface of their necks were coated with paraffin oil to prevent insects, that have previously chosen, from returning to the arena. Preliminary trials allowed us to exclude any repellent or attractant effect of paraffin oil. The floor of the arena was covered with filter paper to provide an uniform surface and to facilitate insect movements. Fifty insects, deprived of food for at least 4 hours, were placed under an inverted Petri dish (3 cm ⌀ × 1.3 cm high) at the centre of the arena and allowed to acclimate for 30 min. The arenas were covered with steel lids and sealed with Parafilm® to prevent insects from escaping and were left for 24 h in the dark at 25 ± 1 °C and 65% R.H. Three replicates were performed for each assay, and insects were used only once. The number of insects in the flasks was recorded 24 h from the beginning of the test. The percent repellence (PR) of each volatile was then calculated after 24 h using the formula: PR (%) =[(Nc − Nt) / (Nc + Nt)] × 100 where Nc was the number of insects present in the control flask and Nt the number of insects present in the treated flask.

Table 1. Chemical composition (%) of the anethole- (AEO) and estragole-type (EEO) *F. vulgare* essential oils used in the repellency assays.

Constituents[a]	*LRI*	AEO	EEO
α-pinene	941	0.6	0.2
sabinene	978	0.3	0.2
myrcene	993	0.1	0.3
p-cymene	1028	0.4	0.4
limonene	1032	6.2	7.5
1,8-cineole	1034	0.3	0.2
(*Z*)-*β*-ocimene	1042	0.2	0.5
γ-terpinene	1063	0.1	0.1
fenchone	1089	3.1	3.8
linalool	1101	0.1	-
trans-*p*-mentha-2,8-dien-1-ol	1123	0.2	-
cis-limonene oxide	1136	0.1	-
cis-*p*-mentha-2,8-dien-1-ol	1139	0.1	-
trans-limonene oxide	1142	0.1	-
camphor	1145	0.2	0.1
4-terpineol	1179	-	0.1
estragol*	1197	8.0	85.5
carvone	1244	0.5	-
p-anisaldehyde	1255	0.5	-
(*E*)-anethole*	1285	78.4	0.9
dill apiole	1623	-	0.1
Total identified		99.5	99.9

[a] Chemical constituents ≥ 0.1%; *LRI*, linear retention index on DB-5 column; * chemicals tested for insect pests repellency.

Statistics and data analyses

Median repellent dose (RD_{50}) was calculated by Log-probit regression. Significant differences between RD_{50} values were determined by estimation of confidence intervals of the relative median potency (rmp). Differences among RD_{50} values were judged to be statistically significant when 1.0 was not found in the 95% confidence interval of rmp. All the analyses were performed by the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Co-repellency of (*E*)-anethole and estragole was evaluated using co-repellency coefficient (CRC) according to the co-toxicity coefficient calculation by Islam et al. (2010) on the basis of the EOs components above 1% [(*E*)- anethole, estragole, limonene and, fenchone] using RD_{50} instead of LD_{50} values as follows:

- Repellency index (RI) of (*E*)-anethole = 100;
- Repellency index (RI) of estragole = (RD_{50} of (*E*)-anethole / RD_{50} of estragole) × 100;
- Actual RI of EO = (RD_{50} of (*E*)-anethole / RD_{50} of EO) × 100;
- Theoretical RI of EO = RI of (*E*)-anethole × % of (*E*)-anethole in EO + RI of estragole × % of estragole in EO + RI of limonene × % of limonene in EO + RI of fenchone × % of fenchone in EO;
- CRC = (Actual RI of EO / Theoretical RI of EO) × 100.

The effect was considered synergistic when CRC > 120; indifferent when the CRC is > 80 to < 120 and antagonistic when the CRC is < 80 (Islam *et al*., 2010).

Results

Essential oil extraction and GC-MS analyses

EOs yield (w/w) from the anethole-type fennel was 2.32%, whereas the yield from the estragole-type one was 2.25% dry weight. The EOs colour was pale yellow and the smell long-lasting and very aromatic.

In the anethole-type fennel essential oil (AEO), 24 constituents were identified, accounting for 99.5% of the whole oil. In the estragole-type fennel essential oil (EEO), 19 constituents were identified, accounting for 99.9% of the whole oil (table 1). The principal chemical constituent of the AEO was (*E*)-anethole (78.4%) whereas estragole (85.5%) was the main chemical in the EEO (table 1). Other important volatiles were for both EOs limonene and fenchone (table 1). All other chemical constituents were below 1%.

The main chemical class was represented by phenylpropanoids (86.5 and 86.4% for AEO and EEO, respectively). The other classes were non-terpene derivatives, monoterpene hydrocarbons and oxygenated sesquiterpenes (table 2).

Repellence bioassays

Area preference bioassays

Both fennel EOs showed a good repellent activity against the three insects *R. dominica*, *S. zeamais* and *T. confusum*. AEO RD_{50} ranged from 0.056 to 0.1 mg cm^{-2} against *R. dominica* and *T. confusum*, respectively while, EEO RD_{50} values ranged from 0.007 to 0.051 mg cm^{-2} against *R. dominica* and *T. confusum*, respectively (table 3).

Table 2. Principal chemical classes (%) in the anethole- (AEO) and estragole-type (EEO) *F. vulgare* essential oils used in the repellency assays.

Chemical classes	AEO	EEO
Monoterpene hydrocarbons	9.2	7.9
Non-terpene derivatives	-	0.5
Oxygenated monoterpenes	4.2	4.7
Phenylpropanoids	86.5	86.4
Not identified	0.1	0.5

Consistently with fennel EOs, their main components (*E*)-anethole and estragole, exerted a clear repellent activity against insect pests. The RD_{50} of (*E*)-anethole ranged from 0.612 to 0.094 mg cm^{-2} for *R. dominica* and *T. confusum*, respectively, while the RD_{50} of the estragole ranged from 0.038 to 0.060 mg cm^{-2} for *R. dominica* and *S. zeamais*, respectively (table 4). A strong repellency was also observed for fenchone and limonene against *T. confusum* and *R. dominica* (table 4).

Interestingly, rmp analyses showed a significant different sensitiveness among species to estragole. In detail, rmp analyses indicate that the most sensitive species to estragole was *R. dominica* while the less sensitive was *S. zeamais* (table 5). On the contrary, no significant differences of sensitiveness to (*E*)-anethole were found among species (table 5). The comparison of the EOs components bioactivity by rmp analyses showed that estragole was significantly more active than (*E*)-anethole against *S. zeamais* and *R. dominica* with Log rmp values (estragole vs (*E*)-anethole) ranging from −1.206 to −0.146 for *R. dominica* and *T. confusum*, respectively (figure 1). CRC values showed an overall synergistic effect (CRC values > 120) of (*E*)-anethole and estragole against the three insects species with the exception of estragole-type EO against *S. zeamais* and (*E*)-anethole-type EO against *T. confusum* whose effects are to be considered as addictive with CRC values of 118.27 and 108.13, respectively. The strongest synergistic effect was observed against *R. dominica* with CRC values of 537.71 and 634.51 for the anethole- and estragole-type, respectively (table 3).

Two-choice pitfall bioassay

The effect of the fennel EOs main components in the presence of maize was tested by a two-choice pitfall bioassay. In line with the area preference bioassay, the most overall effective EO resulted to be the estragole-type with repellency values of 71.9 ± 24.48, 28.5 ± 9.95 and 58.1 ± 7.66% for *R. dominica*, *S. zeamais* and *T. con-*

Table 3. Repellency of anethole- (AEO) and estragole-type (EEO) *F. vulgare* essential oils (EOs) against adults of *R. dominica*, *S. zeamais* and *T. confusum.*

	EOs	CRC[a]	RD_{50}[b]	95% CI[c]	Slope ± SE	Intercept ± SE	χ^2 (df)[d]
R. dominica	AEO	537.71	0.056	0.019-0.080	1.353 ± 0.460	1.353 ± 0.439	**0.23** (1)
	EEO	634.51	0.007	0.003-0.014	0.651 ± 0.125	1.386 ± 0.227	**1.49** (2)
S. zeamais	AEO	168.09	0.176	0.156-0.198	4.672 ± 0.662	3.447 ± 0.493	**0.38** (1)
	EEO	118.27	0.124	0.078-0.265	0.896 ± 0.217	0.798 ± 0.258	**0.13** (2)
T. confusum	AEO	108.13	0.100	0.082-0.122	2.681 ± 0.470	2.640 ± 0.481	**0.01** (1)
	EEO	136.68	0.051	0.018-0.075	1.245 ± 0.343	1.589 ± 0.338	**2.20** (2)

[a] Co-repellency coefficient (CRC). CRC< 80 is considered as antagonistic, 80 < CRC < 120 as additive, CRC > 120 as synergistic; [b] Concentration of the extract that repels 50% of the exposed insect. Data are expressed as mg cm^{-2}; [c] Confidence Interval; [d] Chi-square (df) degrees of freedom; Values in bold indicate $P > 0.05$.

Table 4. Repellency of (*E*)-anethole and estragole, limonene and fenchone against adults of *S. zeamais*, *T. confusum* and *R. dominica*.

Repellent	Pest target	RD_{50}[a]	95% CI[b]	Slope ± SE	Intercept ± SE	χ^2 (df)[c]
(*E*)-anethole	*R. dominica*	0.612	0.232-34.890	0.542 ± 0.187	0.116 ± 0.210	**0.83** (2)
	S. zeamais	0.271	0.163-0.682	0.838 ± 0.201	0.47 ± 0.200	**2.71** (2)
	T. confusum	0.094	0.072-0.118	2.247 ± 0.329	2.311 ± 0.337	**1.16** (1)
Estragole	*R. dominica*	0.038	0.000-0.849	0.580 ± 0.252	0.826 ± 0.299	**0.01** (1)
	S. zeamais	0.126	0.035-0.234	0.869 ± 0.342	0.782 ± 0.294	**1.51** (1)
	T. confusum	0.060	0.040-0.084	1.315 ± 0.194	1.607 ± 0.230	**0.09** (2)
Limonene	*R. dominica*	0.099	0.058-0.176	0.904 ± 0.270	0.906 ± 0.289	**0.33** (2)
	S. zeamais	0.213	0.148-0.654	1.414 ± 0.458	0.950 ± 0.433	**1.71** (1)
	T. confusum	0.409	0.165-967.012	1.007 ± 0.415	0.391 ± 0.504	**0.54** (2)
Fenchone	*R. dominica*	0.310	0.128-9.698	0.825 ± 0.269	0.419 ± 0.317	**1.78** (1)
	S. zeamais	0.417	0.096->1000	0.428 ± 0.200	0.163 ± 0.311	**0.02** (1)
	T. confusum	0.062	0.043-0.095	1.347 ± 0.272	1.615 ± 0.358	**1.36** (1)

[a] Concentration of repellent that repel 50% of the exposed insects. Data are expressed as mg cm^{-2}; [b] Confidence Interval; [c] Chi-square (df) degrees of freedom; Values in bold indicate $P > 0.05$.

Table 5. Relative susceptibilities of adults of *R. dominica*, *S. zeamais* and *T. confusum* to (*E*)-anethole and estragole.

Component		*T. confusum*	*R. dominica*
(*E*)-anethole	*S. zeamais*	0.325	1.139
	R. dominica	0.285	
Estragole	*S. zeamais*	**0.420**[a]	**0.393**
	R. dominica	1.069	

[a] relative median potency (rmp) analyses values of the comparisons: *T. confusum* vs *S. zeamais* and *R. dominica*; *R. dominica* vs *S. zeamais*. Values < 1 indicates more susceptibility; Values > 1 indicates less susceptibility. In bold the significant values (95% CI ≠ 1).

fusum, respectively, while the repellency of (*E*)-anethole was 48.9 ± 9.9, −20.5 ± 3.1 and 37.7 ± 10.6% for *R. dominica*, *S. zeamais* and *T. confusum*, respectively (figure 2). The negative value of (*E*)-anethole indicates that, at the doses tested, it had an attractive activity for *S. zeamais* (figure 2). Consistently with the area preference method, the two choice tests confirmed *R. dominica* as the most susceptible species to the repellency of fennel EOs and *S. zeamais* the less one. Two-ways ANOVA showed significant differences between the two EOs chemotypes, as a function of species ($F_{2, 18}$ = 4402.542, $P < 0.001$) the EO ($F_{1, 18}$ = 34.834, $P < 0.001$) and that there were no interaction between species and EO ($F_{2, 18}$ = 8.419, P = 0.005). Besides, we observed a different number of insects that did not choice either of the two treated and non-treated grain chambers. The non-choosing individuals in the tests with the estragole were 38.0 ± 3.1, 11.0 ± 1.2 and 5.0 ± 2.6 for *R. dominica*, *S. zeamais* and *T. confusum*, respectively, while non-choosing individuals of (*E*)-anethole were 39.0 ± 3.5, 12.3 ± 2.4, and 10.7 ± 1.2 for *R. dominica*, *S. zeamais* and *T. confusum*, respectively. Significant differences of the number of non-choosing individuals were found, as a function of species ($F_{1, 18}$ = 64.926, $P <$ 0.001) but not of the EO ($F_{2, 18}$ = 44.290, P = 0.093) and there were no interaction between species and EO ($F_{2, 18}$ = 1.805, P = 0.206).

Discussion

Chemical analyses showed considerable quantitative rather than qualitative differences in the chemical composition of the two EOs. In particular, EEO and AEO exhibited a very different proportion of the two isomers estragole and (*E*)-anethole. While (*E*)-anethole was the main component of the AEO, the main component of the EEO was estragole. Overall, yields and chemical composition of *F. vulgare* EOs match with previous studies on fennel (Napoli *et al*., 2010; Diao *et al*., 2014;

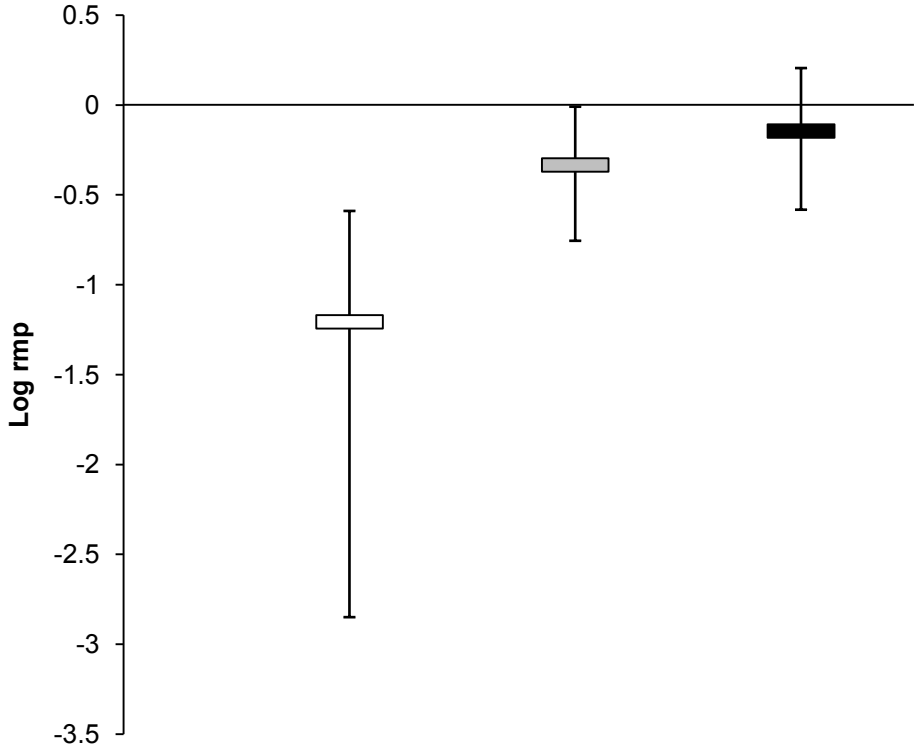

Figure 1. Relative median potency (rmp) comparison between the repellency of (*E*)-anethole and estragole against the insect pests assessed by area preference bioassay. Values < 0 indicates a stronger repellency of estragole respect to anethole. Bars crossing the zero line indicate that the difference of effectiveness is not statistically significant. *R. dominica*, white; *S. zeamais*, grey; *T. confusum*, black.

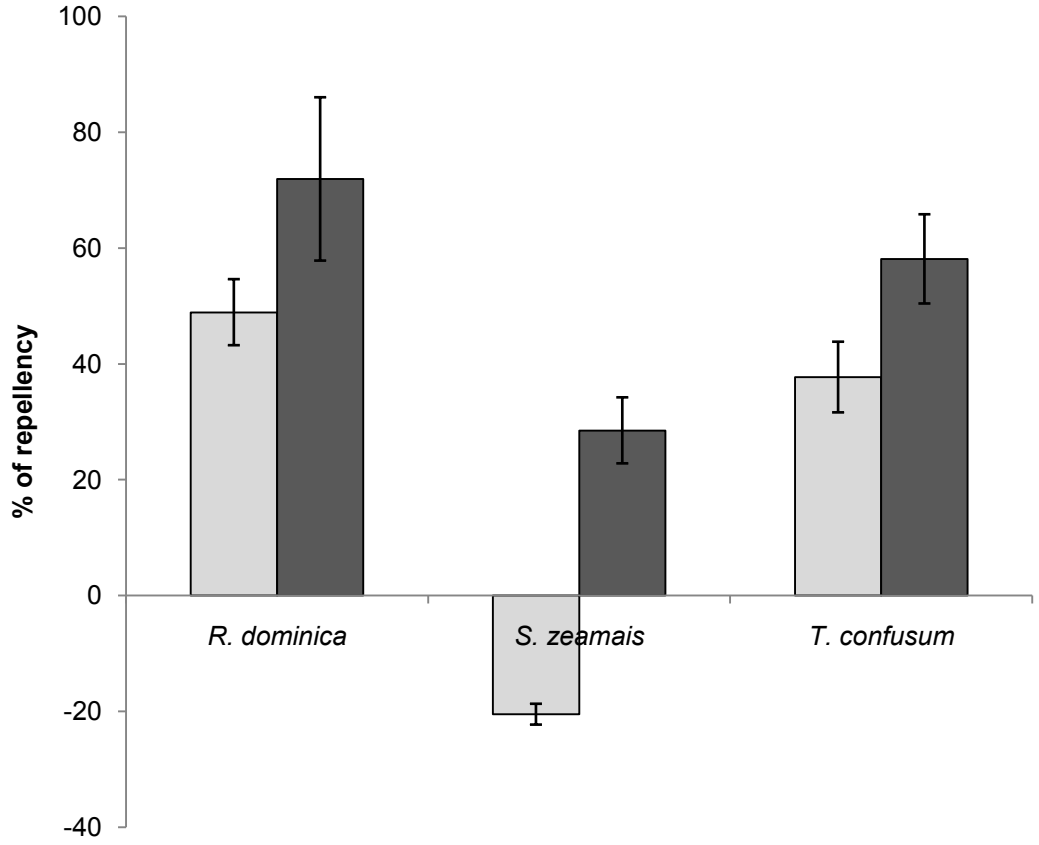

Figure 2. Repellency of anethole- and estragole-type fennel essential oils against the insect pests assessed by two-choice pitfall bioassay. Anethole-type, light gray; estragole-type, dark gray. Bars indicate standard errors.

Özcan *et al*., 2006) confirming a strong difference in the chemical composition of fennel EOs chemotypes (Badoc *et al*., 1998).

Although at different intensity, both the fennel chemotypes AEO and EEO, were able to exert a repellent activity against the three pest insect species. To that regard, our data are overall in accordance with previous studies showing a repellent effect of fennel EO against food-stuff pests. Cosimi *et al*. (2009) found that an anethole-rich fennel EO exerted a moderate level of repellency against *S. zeamais*, lower than the repellency of bergamot and lavandin. Similarly, insecticidal activity of an anethole-rich fennel EO was also observed against the granary weevil *S. granarius* L. by Zoubiri and Baaliouamer (2011) and by Bertoli *et al*. (2012) who, comparing six aromatic plant EOs, observed that an estragole rich fennel essential oil showed the highest contact toxicity rates, at any concentrations.

In comparison with the repellent activity of other aromatic plants essential oils, both the fennel EOs resulted in line with the effectiveness of Algerian *Pistacia lentiscus* L. and *Laurus nobilis* L. EOs (RD_{50} = 0.037 and 0.033 µL cm^{-2}, for *P. lentiscus* and *L. nobilis*, respectively) against *R. dominica* (Mediouni Ben Jemâa *et al*. 2012; Bougherra *et al*., 2015) but lower than the one reported for Algerian *P. lentiscus* EOs against *S. zeamais* and *T. confusum* (RD_{50} = 0.010 and 0.025 µL cm^{-2}, respectively) (Bougherra *et al*., 2015).

Even if both repellent against the insects, significant differences in the effectiveness between the two EOs chemotypes were observed. Such differences can be explained on the basis of the different effectiveness observed between the two enantiomers (*E*)-anethole and estragole. In fact, rmp analysis showed that estragole is more effective as insect repellent than (*E*)-anethole particularly against *R. dominica* (rmp value = 0.062). Accordingly to our results, Kim and Lee (2014) found that estragole was the most active compound also of basil and orange EOs, as adulticidal, against *S. zeamais* and the red flour beetle *Tribolium castaneum* (Herbst). Similarly, estragole was found to be more effective as adulticidal agent than (*E*)-anethole against *Sitophilus oryzae* (L.), *Callosobruchus chinensis* (L.), and *Lasioderma serricorne* (F.) (Kim and Ahn, 2001). Similar differences between enantiomers were previously evidenced also for two enantiomeric forms of limonene, which although similar in the LC_{50}, showed significant differences in their repellent activity against the Asian tiger mosquito *Aedes albopictus* (Skuse) (Diptera Culicidae) (Giatropoulos *et al*., 2012). Besides (*E*)-anethole and estragole, in our experiment, we observed a strong repellent activity also by limonene and fenchone against *R. dominica* and *T. confusum*, respectively. Consistently, a similar repellent activity of limonene against

R. dominica was previously observed by Bedini *et al*. (2015) who also observed that limonene was the most active component of hops EO against *S. granarius*.

Our results also showed a different susceptibility of the three insect species to fennel EOs and their major chemical components. Among the three species tested, *R. dominica* was the overall most susceptible specie while *S. zeamais* the less sensitive one. These results are in contrast with the findings of Bougherra *et al*. (2015) who observed a higher susceptibility of *S. zeamais* respect to *R. dominica* to the *P. lentiscus* EO, but they are consistent with the bioactivity of spent hops (*Humulus lupulus* L.) EO (Bedini *et al*., 2015) that was about 24 time higher against *R. dominica* than against *S. granarius*. These findings indicate that the efficacy of EOs as repellent depends not only on their chemical composition but also on the target species that may be differently susceptible to the different chemical compounds. Accordingly, in this experiment, the rmp analyses showed that estragole was not only the overall most effective compound of fennel EOs but also the compound whose activity is more clearly dependent on the species.

In addition we observed that the effectiveness of the fennel EOs against *R. dominica* was much higher than the one of (*E*)-anethole and estragole alone. Interestingly, CRC calculation showed a strong synergistic effect of (*E*)-anethole and estragole against *R. dominica*. Such synergistic effect could explain the stronger effectiveness of the fennel EOs against *R. dominica* respect to the other two species. To our knowledge, this is the first experiment evaluating the contribution of the synergistic effect of essential oil components in the insect repellency. However, several previous experiments on insect toxicity are consistent with our results showing that the combined effect of bioactive substances on insects is synergistic, addictive or antagonistic depending on the substances and on the insect species (Sun and Johnson, 1960; Jiang *et al*., 2009; Pavela, 2014). Similarly, Savelev *et al*. (2003) found a complex interaction among the constituents of *Salvia lavandulaefolia* Vahl EO in the inhibition of acetylcholinesterase with both synergistic and antagonistic effects between the component terpenes, while Hummelbrunner and Isman (2001) observed a synergistic effect of (*E*)-anethole with thymol, citronellal, and α-terpineol, in acute toxicity and feeding deterrence on the tobacco cutworm, *Spodoptera litura* (F.) (Lepidoptera Noctuidae).

Hence, our study confirms that the repellency of EOs is due to the combined action of their single chemical constituents (Bakkali *et al*., 2008) whose effects, on the basis of our results, appears to depend not only to their relative quantities but also to the target species. In the case of fennel EOs, the strongest repellent activity of the estragole-type EO against *R. dominica* can be explained both by the higher effectiveness of estragole respect to (*E*)-anethole and the strong synergistic effect of the two chemicals.

Similarly, also the two choice tests showed that also in the presence of maize the fennel EO is able of maintain a significantly repellent effect whose intensity vary depending on the EO chemotype and on the insect species with the best result obtained with estragole-type fennel EO against *R. dominica*. Besides, we observed a different percentage of individuals among species that did not choice any of the two flasks containing maize. A high percentage of non-choosing individuals for *R. dominica* was already observed and could be due to characteristic behavior of this species (Bougherra *et al*., 2015).

Conclusions

Overall, this experiment contributes to the knowledge about the chemical composition and bioactivity of the fennel EOs chemotypes. Even if, in a real world scenario, repellence alone would probably be insufficient to provide complete protection to stored grains, by lowering the insect infestation level, it could highly facilitate the action of other parallel control methods. In this regard, estragole-type fennel EO also because of the synergistic effect between its main components, resulted significantly more effective that anethole-type fennel EO. Thus, the standardization of fennel EOs by estragole content could be a useful step toward the development of reliable low-toxic eco-friendly repellents able to reduce the post-harvest grain losses caused by insect pests.

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