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ORIGINAL PAPER

Kernel volatiles of some pigmented wheats do not elicit a preferential orientation in *Sitophilus granarius* adults

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Abstract
The granary weevil, *Sitophilus granarius* (L.) (Coleoptera, Dryophthoridae), is a primary pest of stored cereals worldwide. To identify possible sources of wheat resistance toward this pest, two commercial durum and bread wheat varieties with yellow pericarp were compared with anthocyanin-pigmented durum and bread wheat genotypes that have never entered commercial production. The composition of the kernel head-space volatile organic compounds (VOCs) and the olfactory responses of granary weevil adults to these kernel VOCs were investigated. Head-space solid-phase micro-extraction and gas chromatography–mass spectrometry analysis highlighted 17 and 13 kernel VOCs from durum and bread wheats, respectively. These compounds mainly included aldehydes and alcohols, and to a lesser extent, terpenes and benzene derivatives. Quantitative and qualitative differences were seen between the odor profiles of yellow and pigmented wheat kernels. In two-choice behavioral bioassays, granary weevil adults were significantly attracted by the kernel odors from the yellow commercial wheat varieties and their hexane extracts, but not by those of the pigmented wheat genotypes and their hexane extracts. Electroantennography confirmed the presence of VOCs in all of the hexane extracts that stimulated the olfactory system of both sexes of the granary weevil in a dose-dependent manner. Thus, differences among the odor blends were responsible for the different olfactory responses of granary weevils to the yellow and pigmented wheat kernels. These differences in VOC emissions and olfactory responses induced in granary weevils by the yellow and pigmented wheat kernels can be exploited to characterize resistance mechanisms associated with different genotypes and to incorporate resistance into improved varieties.

Keywords Wheat · Granary weevil · Anthocyanins · Volatile organic compounds · VOCs · Electroantennography · EAG · Post-harvest losses

Key message

- Development of resistant wheat varieties is one of the most promising alternatives to insecticides in integrated management of stored grain pests.
- The identification of the sources and mechanisms of host-plant resistance to specific insect species represents the first step for the breeding of new varieties.
- In this study, marked qualitative and quantitative differences in VOCs emission by kernels of some pigmented durum and bread wheat genotypes and those of two yellow commercial durum and bread wheat varieties were found.
- These differences resulted in a lack of granary weevil orientation to kernel odors of pigmented wheat genotypes compared to the commercial varieties which elicited insect attraction.
- Pigmented wheat genotypes can be exploited to characterize resistance mechanisms associated with VOCs emission in wheat.

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Introduction

Wheat is the most widely grown crop in the world, with more than 700 million metric tons produced annually worldwide. This consists mainly of bread wheat (*Triticum aestivum* L.), but includes ~ 35–40 million metric tons of durum wheat (*Triticum durum* Desf.) (FAO 2016), which is adapted to the hot dry conditions of the Mediterranean basin (Ficco et al. 2014). Post-harvest wheat losses are estimated to be about 10% of the annual production worldwide, which are mainly due to insect and disease attacks during the wheat grain storage (Anon 1979; Ahmad et al. 1992; Khan and Kulachi 2002). Among these loss factors, insect pests during product storage have been recognized as an increasingly important problem worldwide (Phillips and Throne 2010), the attacks of which can result in extensive losses that can reach 50% of the total harvest in some developing countries (Fornal et al. 2007). Nowadays, control of stored-product insect pests has become more difficult than in the past, because of restrictions on the use of some fumigants and other broad-spectrum synthetic insecticides, and because of the increasing consumer demand for healthy and safe food (Bell 2000; Phillips and Throne 2010).

Development of resistant wheat varieties is one of the most promising alternatives to insecticides in the integrated management of stored grain pests (Throne et al. 2000). Here, the identification of the sources and mechanisms of host-plant resistance to specific insect species represents the first step toward the design of good assays and for the breeding of new varieties (Stoner and Shelton 1988; Jyoti et al. 2001).

However, compared to other cereals, in wheat these mechanisms remain poorly understood (Horber 1993). Evaluation of the resistance of wheat varieties to post-harvest insects has most commonly focused on various physiochemical factors, such as grain size, hardness, protein content, and oil content, whereas the roles of the kernel volatile organic compounds (VOCs) have received little attention (Kučerová and Stejskal 1994; Throne et al. 2000, and references therein; Nawrot et al. 2006; Trematerra and Colacci 2015). Indeed, because of the low genetic variability among commercial wheat varieties that has resulted in very similar VOC blends (Seitz 1995), the contribution of olfactory signals for host utilization by stored-wheat insect pests has become difficult to evaluate (Throne et al. 2000).

Pigmented wheat genotypes carry genes for purple pericarp or for blue aleurone, due to the accumulation of anthocyanins. This is an extremely rare trait that was reported for the first time in some forms of tetraploid wheat, and which has a high frequency in Ethiopian wheat germoplasm (Zeven 1991). These kinds of wheat genotype have never spread to commercial agricultural use for several reasons, although more recently they have received increasing attention due to their added nutritional value.

Phytophagous insects use plant VOCs to locate suitable hosts in their search for food, mate and oviposition sites, and also to avoid unsuitable habitats and hosts (Dickens 1984; Visser 1986; Agelopoulos et al. 1999). In some species of *Sitophilus*, both the physical and chemical properties of grain kernels have been shown to influence their host preference and acceptance by egg-laying females (Trematerra et al. 2013). The granary weevil, *Sitophilus granarius* (L.) (Coleoptera, Dryophthoridae), is one of the most damaging pests of stored cereals throughout the world whose larvae develop inside grains. Infestation by this species causes severe quantitative and qualitative losses due to insect feeding, alteration of nutritional and aesthetic value and contamination of commodities with insect bodies, excrements and mycotoxins that result from insect-promoted fungal growth during storage (Rajendran 2002; Sauer et al. 1984; Magan et al. 2003; Plarre 2010). Unlike other *Sitophilus* spp., the granary weevil is perfectly adapted to the man-made artificial grain storage systems and has to date not been observed in natural grain reservoirs. Therefore, non-anthropogenic populations of granary weevil might not exist (Plarre 2010).

Adult granary weevils are attracted to the odors emitted by commercial wheat with yellow kernels (Levinson and Kanaujia 1981; Germinara et al. 2007). Indeed, studies on behavioral responses of granary weevil adults to VOCs identified in the odors of different cereal species (Maga 1978) have shown that there are both attractant and repellent compounds, which suggests that insect attraction is mediated by the balance of positive and negative odor stimuli (Germinara et al. 2008). Therefore, the identification of wheat varieties that produce high levels of repellent compounds able to modify the insect behavior and increase the storability of wheat grains remains a very ambitious goal and represents the first step in the promotion of new breeding programs.

To date, to the best of our knowledge, no studies have been carried out on behavioral responses of granary weevil adults to odors emitted by the kernels of pigmented wheat varieties that carry genes for the purple pericarp or blue aleurone traits. We thus hypothesized that VOCs emission from the kernels of pigmented wheat genotypes might be different from those emitted by commercial yellow wheat kernel varieties and that this might affect the behavioral responses of granary weevils. To investigate this hypothesis, the kernels of two commercial yellow wheat varieties were compared with the kernels of a set of pigmented bread and durum wheat genotypes with different origins that had been characterized previously for their anthocyanin and polyphenol contents (Ficco et al. 2014). The aims were thus to: (1) determine the compositions and variability of the VOCs emitted by the kernels of these different wheat

genotypes; (2) investigate the olfactory responses of granary weevil adults to odors from kernels and their hexane extracts of these wheats; and (3) identify possible weevil-repellent and/or non-attracting wheat genotype(s) for use in breeding programs.

Materials and methods

Insects

Sitophilus granarius were collected from a wild population occurring in traditional storage facilities near Campobasso (central Italy) and reared on whole wheat kernels for several generations. The colonies were maintained in the dark in a climate chamber set at 23 ± 2 °C and 60 ± 5% relative humidity (r.h.). Adult weevils of mixed sex and age were used for the experiments.

Plant materials

The wheat kernel samples used in this study were from wheat grown simultaneously in a field trial carried out at the Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca per la Cerealicoltura e le Colture Industriali (CREA-CI), of Foggia, Italy (41°28′N, 15°32′E; 75 m a.s.l.), on a clay-loam soil (Typic Chromoxerert) during the 2015 and 2016 growing seasons, using standard agronomic practices. The harvested wheat samples were stored at low temperature until the time of the study. The samples comprised seven wheat genotypes representing two wheat species (*T. aestivum*, $2n = 6\times = 42$; *T. durum*, $2n = 4\times = 28$) and three kernel color classes including blue, purple/red, and yellow wheats. These were characterized previously for their anthocyanin composition and technological and nutritional properties (Ficco et al. 2014). More specifically, these comprised one commercial durum wheat variety with yellow kernels (Ofanto), four pigmented durum wheat genotypes with purple or red kernels (ELS6404-149-2, ELS6404-77-2, Mog, Worldseed-3), one commercial bread wheat genotype with yellow kernels (Mec), and one bread wheat genotype with blue kernels (Sebesta Blue-2). The yellow kernels of the Ofanto and Mec varieties were considered as the controls as these commercial varieties are grown widely in Italy. Three subsets of each genotype were used as replicates for subsequent analyses.

Volatile organic compounds (VOCs) analysis

To identify and quantify the VOCs emitted by wheat kernels, static head-space solid-phase micro-extraction (HS-SPME) technique was used according to Beleggia et al. (2009). Briefly, 3 g of each sample was subjected to solid-phase micro-extraction using a 50/30 µm DVB/Carboxen/PDMS Stable-Flex fiber that was inserted directly into the head-space for 24 h, using 40-mL amber vials with caps and PTFE/Silicon septa (Supelco, Co., Bellefonte, PA). These vials were maintained at 30 ± 0.1 °C in a water bath. After sampling, the SPME fiber was placed immediately into the injection port of the gas chromatography system (GC-QTOF 7200; Agilent Technologies). The VOCs were thermally desorbed at 230 °C for 15 min in splitless mode. Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min, and the VOCs were separated using an HP-5 ms column (30 m × 0.25 mm i.d.; 0.25 µm). The oven program, the transfer line, and the ion source temperature were as described in Beleggia et al. (2009). The spectrometer was operated in electron-impact mode, and the ionization voltage was 70 eV, with the mass tuning performed according to manufacturer recommendations, using tris-(perfluorobutyl) amine. The scan range was from 15 amu to 300 amu. The VOCs were identified by comparison of their spectra with those of pure compounds contained in a custom library and with the NIST11 databases. The relative concentrations of individual VOCs were determined by normalizing the peak areas of the compounds in the chromatograms with those of the internal standard (1 µL decane). Unless stated otherwise, all of the chemicals were purchased from Sigma-Aldrich Inc. (Milan, Italy).

Solvent extracts

To perform electrophysiological and behavioral bioassays, solvent extracts from the kernels of each wheat genotype and variety were prepared. A wheat kernel sample (10 g) was immersed in 6 mL hexane (purity > 98%; Sigma-Aldrich, Milan, Italy) in a glass vial (25 mL) that was stirred by hand for 30 s. After 30 min, the extract was transferred to a clean glass vial. For each wheat genotype and variety, three extractions were preformed separately and the resulting extracts were pooled. Extracts were stored at − 20 °C until needed.

Behavioral bioassays

The behavioral responses of granary weevil adults to odors emitted by kernels of the yellow and pigmented wheats and their hexane extracts were measured using a two-choice pitfall bioassay similar to that described by Germinara et al. (2008). The test arena was a plexiglas container (18 cm diameter, 2 cm high) with two diametrically opposed holes (2 cm diameter) located 1.5 cm from the side wall. Glass vials (50 mL) for the collection of the responding insects were positioned under each hole. The inside necks of the collection vials were coated with mineral oil to prevent insects from returning to the arena. The floor of the arena was covered in filter paper (Whatman No. 1) to provide a uniform

surface and to facilitate insect movement. Ten insects of mixed sexes that had been left for at least 4 h without food were placed under an inverted Petri dish (3 cm diameter; 1.2 cm high) at the center of the arena and allowed 30 min to acclimatize prior to release. The arena was covered with a plexiglas lid to prevent insects from escaping. Tests lasted 3 h and were carried out in the dark at 25 ± 2 °C and 60 ± 5% r.h. Each granary weevil was used only once.

In a first set of experiments, the attractiveness of the kernels of each wheat variety and genotype was investigated. Insects were presented with odors from a wheat kernel sample (4 g; moisture content, 11.5%) positioned in a collection vial and control air (empty vial). Eleven replicates were performed for each test.

In a second set of experiments, the attractiveness of the kernel hexane extract of each wheat variety and genotype was evaluated. Insects were given choices between test (100 µL of hexane extract) and control (100 µL of hexane) stimuli adsorbed onto a filter paper disk (2 cm^2) suspended at the center of each hole using a cotton thread taped to the lower surface of the arena. The 100 µL dose was chosen according to preliminary bioassays that showed maximum attraction for this dose among the different doses (25, 50, 100, 150 µL) of the hexane extracts obtained from the attractive wheat Mec variety. Ten replicates of each test were performed.

For each replicate, a response index (RI) was calculated according to Phillips et al. (1993). Positive values of RIs indicate attraction to the treatment, and negative RIs indicate repellence.

Electroantennography (EAG)

The antennal sensitivity of male and female granary weevils to solvent extracts of wheat varieties was assessed using the EAG technique described in previous studies (Germinara et al. 2007; Germinara et al. 2012a, b). Briefly, the head of the insect was excised from the prothorax and mounted between two properly pulled (PC-10 puller, Narishige, Tokyo, Japan) glass capillary electrodes (Microglass, Naples, Italy) filled with Kaissling saline (Kaissling and Thorson 1980). The recording electrode (diameter ~ 100 µm) was placed in contact with the dorsal surface of the terminal antennal segment, while the neutral electrode was inserted into the base of the head. AgCl-coated silver wires were used to maintain the electrical continuity between the antennal preparation and an AC/DC UN-6 amplifier in DC mode connected to a PC equipped with the EAG 2.0 program (Syntech Laboratories, Hilversum, The Netherlands).

Stimuli were increasing aliquots (25, 50, 100, and 150 µL) of each hexane extract. The test stimulus was applied to a filter paper (Whatman No. 1) strip (2 cm^2) inserted in a Pasteur pipette (15 cm long) after solvent evaporation. Using a disposable syringe, the vapor stimuli (3 cm^3) were blown for 1 s into a constant stream of charcoal-filtered humidified air (500 mL/min) flowing in a stainless steel delivery tube (1 cm i. d.) with the outlet positioned ~ 1 cm from the antenna. Control (100 µL of hexane) and standard (10 µL of a 100 µg/µL hexanal solution) stimuli were applied at the beginning of the experiment and after each group of 5 test stimuli. The intervals between stimuli were 1 min. The different volumes of each extract were tested on five antennae from different males and females.

The amplitude (mV) of the EAG responses to each of the test stimuli was adjusted to compensate for solvent and/or mechanosensory artefacts by subtracting the mean EAG response of the two nearest hexane controls (Raguso and Light 1998). To compensate for the decrease in the antennal responsiveness during the experiment, the resulting EAG amplitude was corrected according to the reduction of the EAG response to the standard stimulus (Den Otter et al. 1991). Dose–response curves were calculated based on the corrected EAG values. In the dose–response curves, the activation threshold was considered to be the lowest dose at which the lower limit of the standard error of the mean response was greater than the upper limit of the standard error for the lowest dilution tested (Sant'Ana and Dickens 1998). The saturation level was taken as the lowest dose at which the mean response was equal to or less than the previous dose (Germinara et al. 2009).

Data analysis

The relative concentrations of each VOC and chemical class detected in the durum wheat genotypes were submitted to the analysis of variance (ANOVA) followed by Tukey's HSD test for comparison of means, whereas the Student's *t* test for independent samples (2-tailed) was used to compare the two bread wheat genotypes. Levene's test was used to assess homogeneity of variances. To obtain a general and comprehensive characterization of the samples, the VOCs detected were subjected to principal component analysis (PCA), which is based on correlations, and which was followed by factor analysis. Significance of the mean RI to each treatment tested in behavioral bioassays was evaluated by Student's *t* test for paired comparisons (2-tailed). Male and female EAG responses to each stimulus tested were compared using Student's *t* test (2-tailed) for independent samples. Statistically significant differences were determined at the probability level of $P \leq 0.05$. Analyses were performed with JMP version 8.0 (SAS Institute Inc., Cary, NC, USA).

Results

Characterization of kernel volatile composition

The VOC head-space compositions of kernels of the Ofanto yellow commercial durum wheat control and the different pigmented durum wheat genotypes (6404-149-2, 6404-77-2, Mog, Worldseed-3) are given in Table 1. Similarly, those of the Mec yellow commercial bread wheat control and the blue bread wheat genotype (Sebesta Blue-2) are given in Table 2. Seventeen and 13 VOCs were identified, respectively, accounting in four classes of compounds that include aldehydes, alcohols, terpenes, and benzene derivatives.

For the durum wheat, the levels of the VOCs emitted by the pigmented genotypes were generally significantly higher than those of the yellow Ofanto variety used as control. The highest level of aldehydes was observed in Worldseed-3, which was twofold those of the other pigmented genotypes, and 3.8-fold those of the control variety. The highest levels of alcohols were found for the pigmented 6404-149-2, Mog and Worldseed-3 genotypes, without any significant differences among these. The level of alcohols of the 6404-77-2 genotype was significantly lower than those of the other pigmented genotypes and was comparable to the control. Instead, the 6404-77-2 genotype showed the highest level of terpenes. Finally, for the benzene derivatives identified, the highest levels were observed for Mog, while they were not detected in the 6404-149-2 and Worldseed-3 samples.

Similar to the durum wheat, the VOCs emitted by the pigmented bread wheat Sebesta Blue-2 contained higher aldehyde and alcohol levels in comparison with the Mec variety used as control. Limonene was the only terpene in the bread wheat, and it was present only in the control samples. For the benzene derivatives, there were no significant differences between Mec and Sebesta Blue-2.

These datasets were subjected to PCA and factor analysis. The number of factors that adequately described the data was determined on the basis of the eigenvalues and consequently on the percentages of the total variance explained by each factor. The two main factors that accounted for 51% of the total variance were selected, as PC1 and PC2, which explained 29.2% and 21.8%, respectively, of the total variance. Twelve and six of the VOCs showed positive and

Table 1 VOCs levels detected in the kernel head-space of a yellow commercial durum wheat (Ofanto) and different pigmented durum wheat genotypes (6404-149-2, 6404-77-2, Mog, Worldseed-3)

VOC group/ compound	VOC concentration (ng/g dry weight) (mean ± S.E.)					Sum of squares	F	*P* value
	Ofanto	6404-149-2	6404-77-2	Mog	Worldseed-3			
Total aldehydes	2.27 ± 0.09 d	5.94 ± 0.02 b	2.91 ± 0.33 d	4.68 ± 0.01 c	8.62 ± 0.39 a	77.53	120.07	< 0.0001
Hexanal	0.72 ± 0.02 c	0.91 ± 0.00 c	0.71 ± 0.02 c	1.87 ± 0.01 b	3.19 ± 0.42 a	13.75	32.00	< 0.0001
Benzaldehyde	0.00 ± 0.00 b	0.00 ± 0.00 b	0.49 ± 0.02 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.58	468.57	< 0.0001
Decanal	0.27 ± 0.03 c	2.21 ± 0.06 a	0.41 ± 0.05 c	1.51 ± 0.09 b	0.36 ± 0.02 c	9.06	217.74	< 0.0001
Nonanal	1.28 ± 0.15 c	2.81 ± 0.05 b	1.30 ± 0.27 c	1.29 ± 0.11 c	5.06 ± 0.05 a	32.74	119.94	< 0.0001
Total alcohols	3.38 ± 0.17 b	8.65 ± 0.34 a	3.83 ± 0.10 b	8.66 ± 2.11 a	8.62 ± 0.52 a	91.65	7.85	0.0040
1-Hexanol	0.22 ± 0.03 b	0.00 ± 0.00 c	0.09 ± 0.00 bc	0.49 ± 0.07 a	0.45 ± 0.01 a	0.56	43.46	< 0.0001
2,3-Butanediol	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.20 ± 0.03 a	0.00 ± 0.00 b	0.10	44.82	< 0.0001
Phenol	0.00 ± 0.00 b	0.56 ± 0.05 a	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.76	145.87	< 0.0001
Benzyl alcohol	1.45 ± 0.17 ab	4.41 ± 0.82 a	0.98 ± 0.01 ab	3.92 ± 1.57 a	0.00 ± 0.00 b	44.21	5.81	0.0111
2-Ethyl-1-hexanol	0.63 ± 0.02 b	1.43 ± 0.45 ab	1.17 ± 0.04 b	1.52 ± 0.25 ab	2.44 ± 0.04 a	5.18	8.16	0.0034
1-Octanol	0.42 ± 0.00 b	0.61 ± 0.03 b	0.51 ± 0.01 b	0.97 ± 0.09 b	4.56 ± 0.32 a	37.65	140.86	< 0.0001
1-Nonanol	0.65 ± 0.04 c	1.63 ± 0.02 a	1.08 ± 0.12 bc	1.57 ± 0.10 ab	1.16 ± 0.17 ab	1.92	14.32	0.0004
Total terpenes	0.79 ± 0.05 b	0.15 ± 0.01 d	0.94 ± 0.02 a	0.71 ± 0.01 b	0.41 ± 0.03 c	1.22	107.25	< 0.0001
Limonene	0.38 ± 0.03 a	0.00 ± 0.00 b	0.39 ± 0.02 a	0.42 ± 0.00 a	0.41 ± 0.03 a	0.39	57.87	< 0.0001
Aromadendrene	0.00 ± 0.00 c	0.15 ± 0.01 a	0.00 ± 0.00 c	0.07 ± 0.00 b	0.00 ± 0.00 c	0.05	150.14	< 0.0001
Isobornyl acetate	0.41 ± 0.02 b	0.00 ± 0.00 d	0.54 ± 0.01 a	0.22 ± 0.01 c	0.00 ± 0.00 d	0.71	395.48	< 0.0001
Total benzene derivatives	0.18 ± 0.01 c	0.00 ± 0.00 d	0.48 ± 0.02 b	0.66 ± 0.08 a	0.00 ± 0.00 d	1.06	65.78	< 0.0001
1,3-Dimethylbenzene	0.09 ± 0.00 bc	0.00 ± 0.00 c	0.24 ± 0.02 ab	0.38 ± 0.07 a	0.00 ± 0.00 c	0.32	26.51	< 0.0001
Ethylbenzene	0.09 ± 0.01 c	0.00 ± 0.00 d	0.24 ± 0.00 b	0.29 ± 0.01 a	0.00 ± 0.00 d	0.22	479.51	< 0.0001
2-Pentylfuran	0.10 ± 0.01 b	0.22 ± 0.01 b	0.00 ± 0.00 b	1.34 ± 0.16 a	0.00 ± 0.00b	3.90	64.04	< 0.0001

In a row, values were submitted to one-way ANOVA ($F = 5.81–468.57$; $df = 4, 10$; $P < 0.01$). Values with no letter in common are significantly different at $P \leq 0.05$ (Tukey's HSD test)

Table 2 VOCs levels detected in the kernel head-space of yellow commercial bread wheat (Mec) and pigmented bread wheat genotypes (Sebesta blue-2)

VOC group/compound	VOC concentration (ng/g dry weight) (mean ± S.E.)		Student's *t* test	
	Mec	Sebesta blue-2	*t* value	*P* value
Total aldehydes	0.91 ± 0.01	5.26 ± 0.26	16.56	< 0.001
Hexanal	0.18 ± 0.02	0.00 ± 0.00	9.22	0.012
Nonanal	0.45 ± 0.07	1.44 ± 0.07	10.00	0.001
Decanal	0.28 ± 0.03	3.83 ± 0.19	18.51	< 0.001
Total alcohols	1.89 ± 0.07	9.53 ± 0.25	28.94	< 0.001
1-Hexanol	0.09 ± 0.00	0.71 ± 0.03	18.20	< 0.001
2,3-Butanediol	0.00 ± 0.00	1.47 ± 0.09	15.50	< 0.001
1-Heptanol	0.00 ± 0.00	0.23 ± 0.00	156.69	< 0.001
Benzyl alcohol	0.84 ± 0.06	0.00 ± 0.00	14.55	< 0.001
2-Ethyl-1-hexanol	0.42 ± 0.03	1.40 ± 0.06	14.63	< 0.001
1-Octanol	0.53 ± 0.01	4.47 ± 0.00	410.35	< 0.001
1-Nonanol	0.00 ± 0.00	1.26 ± 0.06	21.03	< 0.001
Total terpenes	0.14 ± 0.01	0.00 ± 0.00	24.25	< 0.001
Limonene	0.14 ± 0.01	0.00 ± 0.00	24.25	< 0.001
Total benzene derivatives	0.15 ± 0.01	0.35 ± 0.01	1.70	0.164
1,3-Dimethylbenzene	0.07 ± 0.00	0.35 ± 0.01	21.73	< 0.001
Ethylbenzene	0.08 ± 0.0	0.00 ± 0.00	8.05	0.001

In a row, significant differences between bread wheat genotypes are indicated by Student's *t* test ($P \leq 0.05$)

negative loading on PC1, while nine showed positive and negative loading on PC2, as shown in Fig. 1, where the arrows indicate the loadings for each VOC along the first two factors. As shown in this PCA score plot, color/pigmentation had a large impact on the segregation of all of the samples in the multivariate space (Fig. 1) and hence on the global composition of the VOCs for both the durum and bread wheats.

Behavioral responses to wheat kernels

In the behavioral bioassays testing the responses of insects to kernel odors, the red (6404-149-2 and 6404-77-2) and purple (Mog and Worldseed-3) durum wheat and the blue (Sebesta blue-2) bread wheat genotypes did not elicit significant ($t = 0.27$–1.20, $df = 10$, $P > 0.05$) mean RIs, indicating no preferential orientation of insects toward the kernel odors of these genotypes (Table 3). In contrast, granary weevil adults showed positive and significant RIs, indicating actual attraction, in response to odors of the Ofanto ($t = 4.37$, $df = 10$, $P = 0.001$) and Mec ($t = 3.75$, $df = 10$, $P = 0.004$) varieties used as durum and bread wheat controls, respectively. The highest RI was observed in response to the grain odors of the Mec variety.

Behavioral responses to hexane extracts

The hexane extract from Mec kernels elicited positive and significant ($t = 2.41$–7.56, $df = 9$, $P < 0.05$) mean RIs in the aliquot range from 25 to 150 µL with the 100 µL

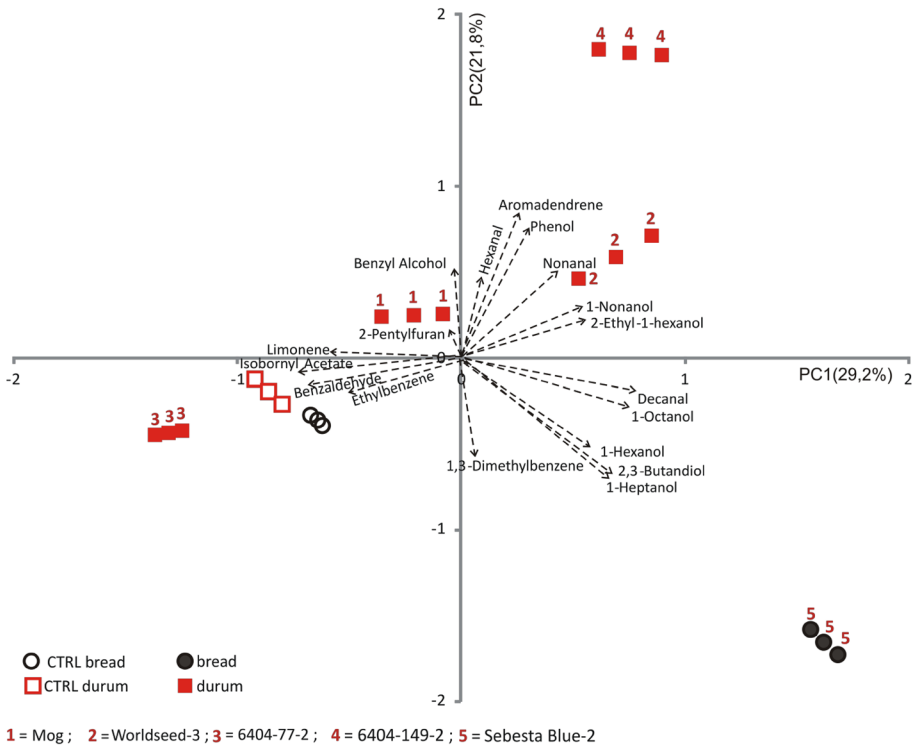

Fig. 1 Principal component analysis score plots for principal components 1 and 2 (PC1, PC2) and for the VOCs. Three analytical replicates are presented for each sample. Full symbols, pigmented wheat genotypes; empty symbols, control wheat cultivars

aliquot being the most attractive ($t = 7.56$, $df = 9$, $P < 0.001$) (Table 4). Based on these data, the other extracts were tested using 100 µL aliquots. At this dose, the hexane extract from kernels of the Ofanto variety used as control durum wheat was attractive ($t = 9.00$, $df = 9$; $P < 0.001$), whereas extracts from kernels of the red and purple durum wheat and the blue bread wheat genotypes did not induce a preferential orientation of granary weevil adults ($t = 0.92$–1.86, $df = 9$, $P > 0.05$) (Table 5).

EAG responses to hexane extracts

The mean EAG responses of granary weevil males and females to each aliquot of different kernel hexane extracts were not significantly different ($t = 0.208$–0.807, $df = 8$, $P > 0.05$); consequently, the data were pooled and analyzed together. The sensitivity of adult granary weevil antennae toward increasing aliquots of each hexane extract tested is reported in Fig. 2. For all of the extracts, the activation threshold was recorded at the 50 µL aliquot and the amplitudes of the EAG responses increased as the volume of the test aliquots increased. For each extract, the mean EAG amplitude for the 150 µL aliquot was higher than that for the previous aliquot tested, which indicated that the saturation of receptors was not reached in the range of extract aliquots tested.

Table 3 Behavioral responses of granary weevil adults to odors emitted by kernels (4 g) of pigmented durum (ELS6404-149-2, ELS6404-77-2, Mog, Worldseed-3) and bread (Sebesta blue-2) wheat genotypes and two yellow commercial durum (Ofanto) and bread (Mec) wheat control varieties, in two-choice pitfall bioassays

Wheat type	Genotype or variety	Kernel odors (mean ± S.E.)	Control air (mean ± S.E.)	Student's *t* test		Response index (mean ± S.E.)
				t value	*P* value	
Durum	ELS6404-149-2	3.8 ± 0.4	4.1 ± 0.6	0.27	0.793	−2.7 ± 10.1
	ELS6404-77-2	5.0 ± 0.6	3.5 ± 0.7	1.20	0.258	15.5 ± 12.9
	Mog	4.4 ± 0.7	3.9 ± 0.5	0.38	0.711	4.5 ± 11.9
	Worldseed-3	4.0 ± 0.5	3.4 ± 0.5	0.73	0.485	6.4 ± 8.8
	Ofanto (control)	5.2 ± 0.4	2.7 ± 0.3	4.37	0.001	24.5 ± 5.6
Bread	Sebesta blue-2	4.3 ± 0.6	3.4 ± 0.5	1.01	0.336	9.1 ± 9.0
	Mec (control)	5.9 ± 0.5	2.8 ± 2.8	3.74	0.004	30.9 ± 8.3

In a row, significant differences between treatment and control responses are indicated by Student's *t* test ($P \leq 0.05$)

Table 4 Behavioral responses of granary weevil adults to increasing aliquots (25, 50, 100, 150 µL) of kernel hexane extract of the attractive Mec variety, in two-choice pitfall bioassays

Test aliquots (µL)	Kernel hexane extract (mean ± S.E.)	Control hexane (mean ± S.E.)	Student's *t* test		Response index (mean ± S.E.)
			t value	*P* value	
25	4.4 ± 0.3	3.1 ± 0.3	2.41	0.039	10.0 ± 5.4
50	5.0 ± 0.3	3.1 ± 0.5	2.97	0.016	20.0 ± 6.4
100	5.2 ± 0.4	1.6 ± 0.2	7.56	< 0.001	36.0 ± 4.8
150	4.8 ± 0.2	2.8 ± 0.6	4.74	0.001	20.0 ± 4.2

In a row, significant differences between treatment and control responses are indicated by Student's *t* test ($P \leq 0.05$)

Table 5 Behavioral responses of granary weevil adults to odors of kernel hexane extracts (100 µL) of pigmented durum (ELS6404-149-2, ELS6404-77-2, Mog Worldseed-3) and bread (Sebesta blue-2) wheat genotypes and two yellow commercial durum (Ofanto) and bread (Mec) wheat control varieties, in two-choice pitfall bioassays

Wheat genotype or variety	Kernel hexane extract (mean ± S.E.)	Control hexane (mean ± S.E.)	Student's *t* test		Response index (mean ± S.E.)
			t value	*P* value	
ELS6404-149-2	2.9 ± 0.3	2.3 ± 0.5	0.92	0.382	6.0 ± 6.5
ELS6404-77-2	3.3 ± 0.4	2.5 ± 0.4	1.86	0.096	13.0 ± 7.0
Mog	3.1 ± 0.4	3.2 ± 0.4	0.15	0.888	−1.0 ± 6.9
Worldseed-3	2.9 ± 0.6	2.5 ± 0.5	0.51	0.625	4.0 ± 7.9
Ofanto (control)	4.3 ± 0.2	2.2 ± 0.3	9.00	< 0.001	21.0 ± 2.3
Sebesta blue-2	3.0 ± 0.4	2.9 ± 0.4	0.16	0.879	1.0 ± 6.4
Mec (control)	5.2 ± 0.4	1.6 ± 0.2	7.56	< 0.001	36.0 ± 4.8

In a row, significant differences between treatment and control responses are indicated by Student's *t* test ($P \leq 0.05$)

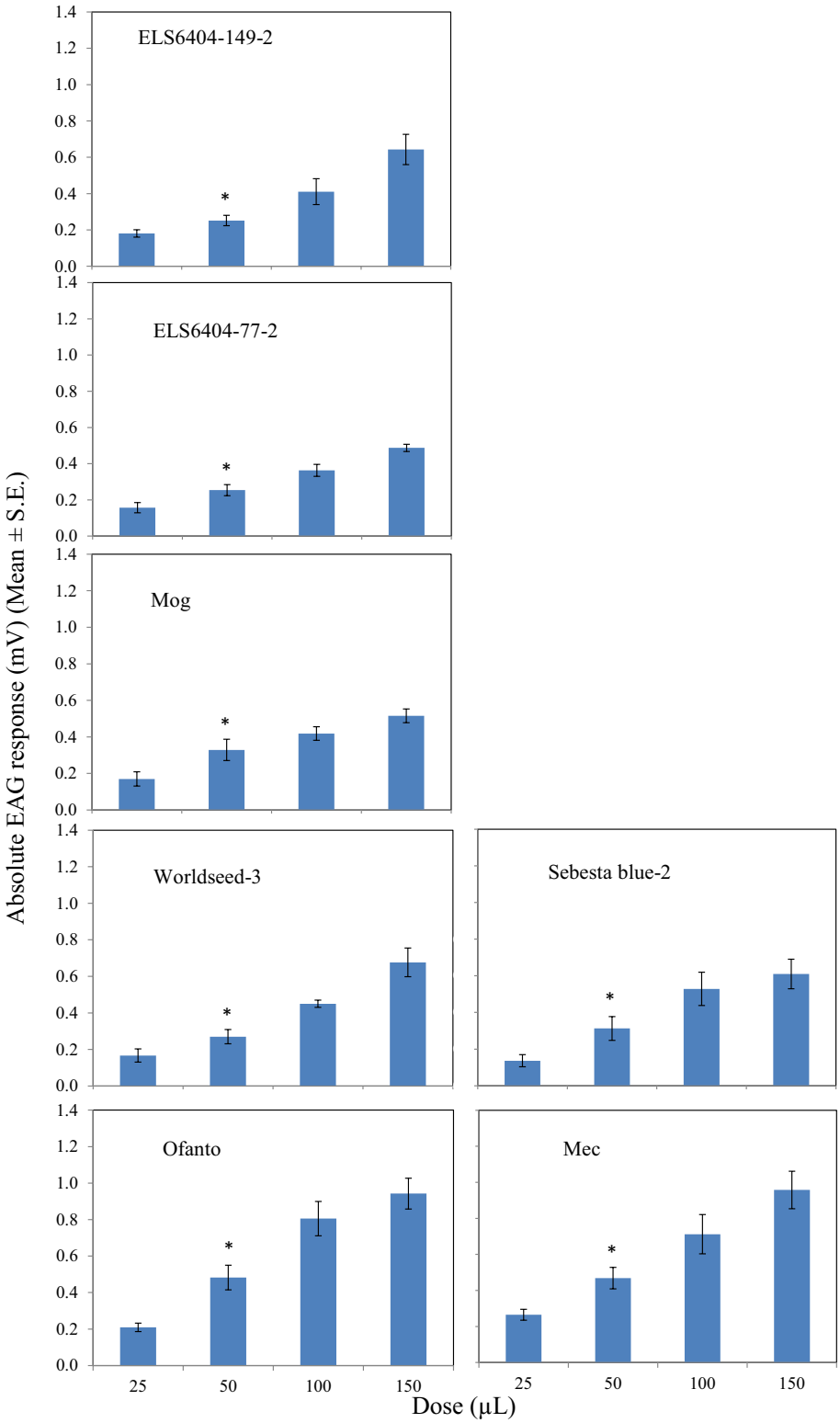

Fig. 2 Electroantennogram dose–response profiles of granary weevil adults ($n = 10$) to kernel hexane extracts of pigmented durum (ELS6404-149-2, ELS6404-77-2, Mog, Worldseed-3) and bread (Sebesta Blue-2) wheat genotypes and two yellow commercial durum (Ofanto) and bread (Mec) wheat varieties. Asterisks indicate the activation thresholds

Discussion

Elucidation of the mechanisms involved in wheat resistance to post-harvest insect pests is very important for the breeding of new varieties. To date, the roles of VOCs have received little attention, mainly due to the low genetic variability among commercial wheat varieties, which has resulted in great similarity among the blends of the VOCs they emit (Throne et al. 2000).

In the present study, the composition of the kernel headspace VOCs of some pigmented durum (ELS6404-149-2, ELS6404-77-2, Mog, Worldseed-3) and bread (Sebesta blue-2) wheat genotypes were characterized in comparison with commercial durum (Ofanto) and bread (Mec) wheat varieties with yellow kernels, respectively. Chemical analysis identified 17 and 13 VOCs from the durum and bread wheats, respectively, distributed in the four chemical classes of aldehydes, alcohols, terpenes, and benzene derivatives. For all of these genotypes and commercial varieties, the aldehydes and alcohols were the most abundant chemicals, as has also been reported previously for other commercial wheat varieties (Beleggia et al. 2009; Mattiolo et al. 2017). On the contrary, terpenes and benzene derivatives were present in small quantities, mainly in the bread wheats. Marked differences were found within each group of durum and bread wheat between the control and pigmented wheats, and among the pigmented durum wheats, in terms of the numbers and quantities of the individual compounds identified. These differences suggest high genetic diversity, which is potentially useful to study the role of chemical cues in host utilization by granary weevils and to understand resistance mechanisms that might be incorporated into improved wheat varieties (Bergvinson and Garcia-Lara 2004).

In the behavioral bioassays, *S. granarius* adults were significantly attracted by the kernel odors of both of the commercial durum and bread wheat varieties, but they did not exhibit a preferential orientation toward the odors of pigmented durum and bread wheat genotypes. The granary weevils also showed a similar behavioral pattern when presented with the odors of the kernel hexane extracts of the same wheat genotypes and varieties. EAG experiments confirmed that all of the hexane extracts contained VOCs that were perceived by the peripheral olfactory system of both. Therefore, this strongly suggests that differences in composition among the different blends of the VOCs emitted by the pigmented wheat genotypes and the related commercial wheat varieties account for the differences observed in the granary weevil behavioral responses.

As indicated above, the aldehydes and alcohols were the major VOCs identified, and these also showed the most significant differences between the yellow and pigmented wheats. Hexanal, nonanal, and decanal were the most represented aldehydes in all of the samples, and with the exception of durum wheat genotype 6404-149-2, they showed the highest levels for all of the pigmented durum and bread wheats, compared with the commercial yellow varieties. Short-chain aliphatic aldehydes are enzymatically produced by plant tissues through the hydroperoxide lyase pathway of oxylipin metabolism (Feussner and Wasternack 2002; Matsui 2006), and these can be converted into the corresponding alcohols by the action of alcohol dehydrogenase (Hubert et al. 2008). In a previous study, short-chain aliphatic aldehydes, including hexanal, showed strong repellent effects against adult *S. granarius* adults (Germinara et al. 2008), and some of them effectively disrupted granary weevil orientation to kernels of the attractive yellow durum wheat variety Simeto (Germinara et al. 2015). Short-chain aliphatic aldehydes have also been shown to have fumigant and contact toxicity against various stored-product insect pests (Ferguson and Pirie 1948; Hammond et al. 2000; Hubert et al. 2008; Germinara et al. 2012a; Anfora et al. 2014). Therefore, the literature data strongly suggest a role for the volatile aldehydes in the inhibition of granary weevil preferential orientation to pigmented wheat genotypes.

However, many studies have highlighted the importance of ratios and concentrations of host-plant VOCs for host location by herbivorous insects (Najar-Rodriguez et al. 2010; Webster et al. 2010; Cha et al. 2011). Therefore, the contributions of other, even minor, VOCs that are released by the kernels of pigmented wheat genotypes cannot be excluded. Indeed, these become even more important considering that granary weevil behavioral responses to host odors appear to depend on the balance of attractant and repellent stimuli (Germinara et al. 2008).

For three of the four pigmented durum wheats examined here, with the exception of genotype 6404-149-2, benzyl alcohol, 2-ethyl-1-hexanol, and 1-octanol were the most abundant alcohols identified; in the blue bread wheat, the most representative compound was 1-octanol. Benzyl alcohol has acaricidal properties against house dust and stored crops mites (Castagnoli et al. 1996), and Juneja et al. (1972) suggested benzyl alcohol as a possible resistance factor in a greenbug-resistant barley selection than in a greenbug-susceptible one. 2-Ethyl-hexanol was recently reported as a potential attractant for the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera, Curculionidae). 2-Ethyl-hexanol is generally ubiquitous, but it is more commonly released by leguminous plants (Ajayi et al. 2015) that are an uncommon host of the granary weevil. 1-Octanol has been reported as an egg-laying stimulant for female *S. granarius* (Niewiada et al. 2005); however, being a major fungal metabolite in spoilage of stored commodities (Jelen and Wasowicz 1998) it may also serve as a reliable cue for habitat quality assessment and to avoid suboptimal substrates by granary weevils, as proposed for some other fungal metabolites (Germinara et al. 2012b). Further behavioral bioassays testing different doses of benzyl alcohol, 2-ethyl-hexanol, and 1-octanol are required to clarify the bioactivities of these compounds toward granary weevil adults.

For the terpenes and the benzene derivatives, although these appeared to have little importance in the VOC profiles of the different wheats, some of these compounds are major components of many plant extracts and essential oils with strong repellent and insecticidal activities against stored-product insect pests (Isman 2006; Nerio et al. 2010),

including *S. granarius* (Lazink et al. 2012; Germinara et al. 2017). Therefore, more behavioral studies with these minor wheat VOCs are needed to better understand their contribution in the behavioral responses of granary weevils in this study.

From an ecological perspective, it can be speculated that the granary weevil behavioral responses to the pigmented wheat VOCs might reflect a lack of coevolution. Indeed, this pest is perfectly adapted to man-made artificial storage systems, and it has not been observed in natural reservoirs (Plarre 2010). At the same time, the pigmented wheats used in the present study have never spread to agriculture. As a consequence, *S. granarius* orientation to odors of the commercial varieties of durum and bread wheats might reflect their host preference, whereas the lack of granary weevil orientation to pigmented wheats suggests that they do not recognize these genetic materials as a food source. These differences confirm the key role played by chemical signals in the host selection process by the granary weevil (Germinara et al. 2008) and demonstrate its great capability to discriminate possible host substrate based on their VOC profiles. Moreover, being these differences in insect behavior related to intrinsic characteristics of pigmented wheat genotypes, they appear to represent a good starting point for deepening the genetic basis of VOCs and their role in mechanisms of wheat resistance to this pest.

In conclusion, the lack of granary weevil orientation to pigmented wheats contrasts sharply with their attraction to the commercial yellow varieties investigated here. However, further studies are needed to identify the key compounds of pigmented wheats that affect the granary weevil behavior. The genetic variability of these genotypes with regard to VOCs emission can potentially then be exploited to characterize resistance mechanisms to post-harvest pests, and to incorporate resistance into improved wheat varieties using conventional and genetic engineering approaches.

Author contributions

GSG and PDV conceived and designed research. RB, FMG, and PDV performed chemical analysis. GSG and MOP conducted EAG and behavioral bioassays. GSG, RB, and PDV wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent The authors accepted that the paper is submitted for publication in the *Journal of Pest Science* and report that this paper has not been published or accepted for publication in another journal, and it is not under consideration at another journal.

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