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Detection of phosphine resistance in major stored-product insects in Greece and evaluation of a field resistance test kit

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ABSTRACT

The use of phosphine has been effective against a wide range of stored-product pests in different types of commodities and facilities. However, its continuous and improper use has led to resistance development in -several major insect species. Although phosphine resistance has been reported from many countries across the globe, reports from Europe have been very limited. In the present study, we determined phosphine resistance in insect populations that had been collected from a range of storages across Greece, using two different diagnostic protocols. Apart from the traditional Food and Agriculture Organization (FAO) protocol, a field test kit (known as the Detia Degesch Tolerance Test Kit, DDTTPK) was utilized, for "same day" determination of the resistance status of field collected insects. In total, 53 populations belonging to *Rhyzopertha dominica*, *Sitophilus oryzae*, *Sitophilus granarius*, *Cryptolestes ferrugineus*, *Tribolium confusum*, *Tribolium castaneum* and *Oryzaephilus surinamensis* were tested. For the majority of the species and populations tested, both FAO and DDPTTK provided similar results, for the susceptibility to phosphine and thus, the quick test could be used with success for an initial same day screening of phosphine resistance. Among the tested species, the populations recorded with the most frequent survival at the FAO testing dose of phosphine was that of *R. dominica*. The dissimilar evaluation and characterization of resistance to phosphine between diagnostic protocols is particularly important, as it poses risks in the over or underestimation of the resistance status of a given population. Our data indicate that the DDPTTK could be used to determine resistance to phosphine in the field, before the initiation of fumigations to disinfest stored commodities.

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1. Introduction

Phosphine gas, or hydrogen phosphide (PH_3), is the most commonly used fumigant insecticide for the disinfestation of a wide range of durable commodities including grains, dried fruits and tobacco in warehouses and processing facilities globally (Benhalima et al., 2004; Daglish, 2004; Collins et al., 2005; Wang et al., 2006). The phase-out of methyl bromide (UNEP, 1995) has increased the reliance on phosphine significantly, as a fumigant for stored-product protection (Bell, 2000; Chandhry, 2000; Nayak et al., 2010). Phosphine has several advantages that are compatible with wide industrial use, including its relatively ease in application, low price, suitability for a wide range of storage types and commodities and global acceptance as a residue-free treatment (Emery et al., 2003; Nayak and Collins, 2008; Kaur and Nayak, 2015). Moreover, phosphine has been proved effective against most major stored product insect and mite pests (UNEP, 1995; Emery et al., 2003; Opit et al., 2012; Cato et al., 2017).

The dominance of phosphine as a disinfestant over several decades has led to the development of resistance in several pest species, that is posing a continuous threat to the sustainability of this key treatment (Rajendran and Gunasekaran, 2002; Nayak et al., 2003, 2013; Collins et al., 2005; Lorini et al., 2007). The first extensive global surveillance on phosphine resistance was undertaken by Champ and Dyte (1976). Later, Zettler and Cuperus (1990) investigated failure of phosphine fumigations in the US and correlated these to either development of phosphine resistance or inadequate fumigation practices. Many key pest species in Australia have now demonstrated resistance to phosphine, including the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera:

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Bostrychidae) (Collins et al., 2005), the psocid *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae) (Nayak and Collins, 2008), the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Holloway et al., 2016), the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Jagadeesan et al., 2012) and the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) (Nayak et al., 2013). Furthermore, detection of resistant populations of key pest species has also been reported from Brazil (Pimentel et al., 2009, Pimentel and Guedes, 2010; Lorini et al., 2007), China (Song et al., 2011), Morocco (Benhalima et al., 2004), India (Rajendran and Narasimhan, 1994; Kaur et al., 2015), Pakistan (Alam et al., 1999; Ahmad et al., 2013) and USA (Opit et al., 2012; Chen et al., 2015; Saglam et al., 2015; Cato et al., 2017; Afful et al., 2018). Compared to these reports from several continents across the globe, there is very limited information available on the existence of phosphine resistance in Europe. In a recent study, Aulicky et al. (2015) characterized a population of the confused flour beetle, *Tribolium confusum* Jacquelin Du Val (Coleoptera: Tenebrionidae) from Czech Republic to be far less susceptible than a laboratory population. Prior to this, the only report on any European resistance data was included in the global resistance survey by Champ and Dyte (1976) that showed resistance to phosphine in some major stored product insect species.

The protocols to determine the resistance in key storage pests have evolved significantly from the first recommendations put forward by the FAO Method (Food and Agricultrure Organization, 1975). Basically, the FAO method involves a bioassay that simply discriminates between susceptible and resistant individuals, when adults of a field population are exposed for 20 h exposure to roughly 30–50 ppm (depending on the species) of phosphine, followed by mortality assessments at the end of the exposure period and 14 days post-fumigation. This method has been used recently with slight modification to diagnose resistance in field populations in the USA (Opit et al., 2012; Cato et al., 2017; Afful et al., 2018). With the characterization of two levels of resistance (weak and strong) in the major pest species, researchers in Australia have significantly modified the FAO method and established two discriminating dosages for their regular monitoring (Daglish and Collins, 1999; Nayak et al., 2013; Holloway et al., 2016). More recently, a 'quick knock down test' was specifically established to detect strong resistance in *C. ferrugineus* that involves a 5 h exposure to the very high concentration of 1436 ppm of phosphine (Nayak et al., 2013). In similar line, the company Detia Degesch GmbH (Laudenbach, Germany) has developed the Detia Degesch Phosphine Tolerance Test Kit (DDPTTK), which is based on a rapid knock down bioassay that can diagnose resistance in a pest population in less than 30 min. This protocol has been modified from knock down tests developed by previous researchers (Mills, 1986; Chandhry, 2000; Reichmuth, 1992). In an effort to obtain data for Europe, the aim of the current study was to survey stored product insect populations from several storage and processing facilities in Greece; in order to evaluate and quantify the potential existence of phosphine resistance. While determining the resistance frequency in key stored product pests for the first time in some Greek storages, we have attempted to evaluate the utility of the commercial test kit (DDPTTK) compared with the traditional FAO method.

2. Materials and methods

2.1. Insects

A total of 53 stored product insect populations were sampled from a range of storage facilities across different geographic regions in Greece (Fig. 1). These include flour mills, feed mills, pasta factories, silos and farm warehouses. The populations were collected

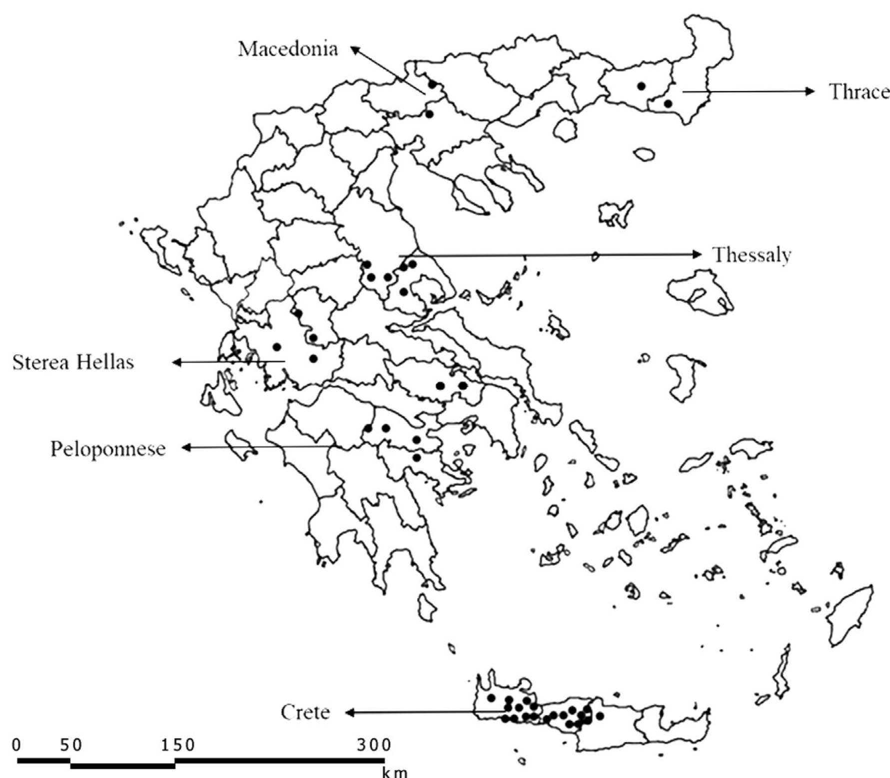

Fig. 1. Map of Greece showing approximate geographic locations of stored product insects analyzed for phosphine resistance; study regions are labeled with the arrows.

over the period November 2014–June 2017. The distance of these storage sites from our laboratory (Laboratory of Entomology and Agricultural Zoology, Department of Agriculture Crop Production and Rural Environment, University of Thessaly) ranged from 350 km in the east, 330 km in west (Sterea Hellas), 722 km in south (Crete) and 350 km in north (Thrace). The insects were collected in samples of infested commodities and transferred to the laboratory. Once received in the laboratory, all samples were screened individually for insects, which were then sorted to species and individual populations of each species was put into culturing jars with its preferred rearing media. The place and commodity from where these populations were collected are presented in Table 1. In total, populations belonging to 7 major stored product pest species were established, each of which were given a unique code. The substrate for insect rearing was hard wheat for *R. dominica*, *S. oryzae* and the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), cracked hard wheat with wheat flour for *C. ferrugineus*, wheat flour for *T. confusum and T. castaneum*, and oat flakes for the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae). In all experiments, the standard laboratory reference cultures of individual species with known susceptibility to phosphine were included as “controls”. These susceptible populations have been maintained in the laboratory for more than 20 years with no exposure to phosphine. All insect cultures were kept in incubator chambers set at 25 °C, 55% relative humidity (RH) and continuous darkness.

2.2. Phosphine resistance test protocols

Both FAO and DDPTTK protocols were followed for resistance testing of all populations. Bioassays were conducted under laboratory conditions set at 25 °C and 55% RH.

2.2.1. DDPTTK

The use of DDPTTK is described by Steuerwald et al. (2006), but here the test kit was used with some modifications. In brief, 20 adults of the tested populations (separate sets of adults each time) were placed in a plastic kit syringe with 100 ml capacity. Phosphine was produced by adding 2 kit tablets to 50 ml of water, within a flexible gas-tight plastic canister of 5 L capacity. Concentration of the gas produced inside the canister was determined as suggested by Steuerwald et al. (2006). Then, a specific quantity was removed from the canister with the syringe in order to achieve a concentration of 3000 ppm. Individual insects inside the syringe were monitored and classified as ‘active’, ‘knocked down’ and “dead”. In this context, we used the term ‘active’ for insects that were able to

Table 1
Insect species sampled from various commodities stored across different geographic regions in Greece.

Strain code	Area sampled	Commodity sampled	Species found
ASC3	Sterea Hellas	Wheat flour	*T. confusum*
ASC7	Thrace	Wheat flour	*S. oryzae*
ASC8	Thrace	Wheat flour	*S. granarius, C. ferrugineus*
ASC9	Thessaly	Wheat flour	*T. confusum*
ASC10	Thessaly	Wheat flour	*T. castaneum, O. surinamensis*
ASC11	Peloponnese	Wheat	*T. castaneum, R. dominica, O. surianemensis, S. oryzae*
ASC14	Sterea Hellas	Cereals	*T. confusum, R. dominica*
ASC15	Peloponnese	Semolina	*T. castaneum*
ASC16	Peloponnese	Wheat flour	*T. confusum*
ASC19	Thessaly	Wheat flour	*T. castaneum*
GA1	Peloponnese	Barley flour	*S. oryzae, O. surinamensis*
GA2	Macedonia	Wheat	*R. dominica,C. ferrugineus, S. oryzae*
GA3	Thessaly	Wheat	*R. dominica*
GA6	Thessaly	Wheat	*R. dominica*
GA12	Thessaly	Wheat	*T. confusum, R. dominica*
U1	Macedonia	Wheat	*S. oryzae*
V1	Crete	Wheat flour	*T. confusum*
V2	Crete	Wheat flour	*T. confusum*
P1	Crete	Bran Flour	*T. confusum*
PP1	Crete	Residues	*T. confusum*
P2(1)	Crete	Residues	*T. confusum*
P2(2)	Crete	Residues	*T. confusum*
P3	Crete	Residues	*T. confusum*
P4	Crete	Residues	*T. confusum*
F1	Crete	Residues	*T. confusum*
F2	Crete	Residues	*T. confusum*
F3	Crete	Residues	*T. confusum*
F4	Crete	Residues	*T. confusum*
F5	Crete	Residues	*T. confusum*
S1	Crete	Residues	*T. confusum*
S2	Crete	Residues	*T. confusum*
S3	Crete	Residues	*T. confusum*
SK1	Crete	Residues	*T. confusum*
SK2	Crete	Residues	*T. confusum*
SKG1	Crete	Residues	*T. confusum*
SKG2	Crete	Residues	*T. confusum*
AGR-01	Sterea Hellas	Grain byproducts	*S. oryzae, R. dominica, T. confusum*
AGR-03	Sterea Hellas	Rice	*R. dominica*
AGR-04	Sterea Hellas	Barley flour	*T. castaneum*
AGR-05	Sterea Hellas	Wheat flour	*T. confusum*
EXT1	Macedonia	Rice	*O. surinamensis*
LAB	University of Thessaly	Preferred rearing media	*S. oryzae, S. granarius, O. surinamensis, T. confusum, T. castaneum, R. dominica, C. ferrugineus*

walk normally, 'knocked down' for insects that had abnormal or uncoordinated movement and "dead" for insects that had no any movement. By using the term "dead" in this test we indicate complete absence of movement and not necessarily "death" of the exposed individuals. The exposed insects were observed at the exposure intervals of 5, 10, 15, 20, 25, 30, 45, 60 and 90 min. For each population, there were three replicates (canisters) with three sub-replicates (syringes), with new phosphine production each time. Based on the current kit instructions, the presence of active insects after 8, 11, 12 and 13 min of exposure, indicate presence of tolerance to phosphine in *T. castaneum*, *O. surinamensis*, *S. granarius*, and *C. ferrugineus*, respectively, as recommended by Steuerwald et al. (2006).

2.2.2. FAO

In this case, we used the standard FAO protocol, as described by FAO Plant Protection Bulletin (Food and Agricultrure Organization, 1975) with a slight modification to the assessment period. Twenty adults (two weeks after eclosion) of a population were placed in an air-tight 1 L glass jar. Using a glass syringe, 30 ppm of phosphine was taken from the freshly generated gas source (as described for the DDPTTK protocol) and injected through a gas tight rubber septum of the 1 L glass jar with the test insects. The insects were exposed to phosphine for 20 h. After the termination of this interval the adults were classified as above and were transferred to an untreated petri dish, with food for 7 days and all dishes were placed in incubators set at 25 °C and 55% RH. After this period end point mortality was estimated. The 7 days end point mortality was used here instead of the original FAO recommended recovery period of 14 days, due to recent recommendations by some researchers (Holloway et al., 2016; Nayak et al., 2017). For each population, we considered three replicates with three sub-replicates, with new phosphine source generated each time. Resistance to phosphine was determined on the basis of presence of active insects after the termination of the 20 h exposure followed by the 7 d recovery period.

2.3. Statistical analysis

For the DDPTTK protocol, the data were analyzed, separately for each species and population, by using probit analysis to estimate KDT_{99} values, which was based on the sum of knockdown and dead insects. For this purpose, we applied Regression Analysis by using SPSS Statistical Analysis (IBM SPSS v.24). For FAO protocol, we calculated the mean number of active adults and standard error (SE) values for each population. Moreover, for each species, we calculated the correlation coefficient values between the percentage of adults that were knocked down after exposure to DDPTTK, i.e. for 90 min at 3000 ppm and the percentage of adults that were knocked down after exposure to FAO, i.e. for 20 h at 30 ppm. Similarly, the percentage of adults that have been knocked down after exposure to DDPTTK was correlated with the percentage of adults that were dead 7 d after the termination of their exposure to FAO. The coefficient values were tested for departure from zero by using the two-tailed *t*-test, at n-2 df and at 0.05, where n was the number of populations per species.

3. Results

3.1. DDPTTK

Based on this protocol, all laboratory populations tested were classified as susceptible (Table 2). Among the 53 field populations tested, two populations of *O. surinamensis*, two of *S. oryzae*, five of *T. confusum* and one of *T. castaneum* were found to be susceptible to phosphine (Table 2). Based on the kit diagnosis, one field population of *S. granarius* was diagnosed as resistant to phosphine, whereas two populations of *O. surinamensis*, four of *S. oryzae*, 22 of *T. confusum*, four of *T. castaneum*, both populations of *C. ferrugineus*, and all field populations of *R. dominica* were diagnosed as resistant (Table 2). In terms of KDT_{99}, the highest values were recorded for *O. surinamensis* GA1, *S. oryzae* ASC11 and *R. dominica* AGR-01.

3.2. FAO

Based on this protocol, all laboratory populations tested were classified as susceptible (Table 3). Among the 53 field populations tested, in total 43 populations spread across the species spectrum were diagnosed as resistant to phosphine, as there were surviving individuals (Table 3). In 17 out of these 43 populations, more than 80% of the exposed individuals were classified as active after 20 h of exposure. Furthermore, 45 populations were found to have surviving adults after the 7-d post exposure interval, while in 12 of these populations more than 80% of the exposed individuals were characterized as active (Table 3).

Forty-one of the populations tested had adults that were knocked down after 20 h of exposure, whereas for 32 of these populations we detected dead adults after the 7-post exposure interval (Table 3). Regardless of the performance of specific populations to phosphine, *R. dominica* populations showed high percentages of adults that were still alive, in contrast with most of the other species/populations examined here.

By comparing DDPTTK and FAO protocols, we see that among the 53 populations tested, both protocols gave 96% matching in their diagnosis for resistance, with some few exceptions [*T. castaneum* ASC15 and *T. confusum* P2(2)] (Table 3). Moreover, the correlation coefficients' values indicated that there was a positive and significant correlation between the two protocols for all species tested, with the exception of *S. granarius* (Table 4). This was recorded for both cases, i.e. correlation between knocked down adults after exposure to DDPTTK for 90 min with either knocked down adults after exposure to FAO for 20 h or dead adults at the 7 d post-exposure period.

4. Discussion

To our knowledge, this is the first study in which extensive sampling has been conducted in a European country, in order to estimate resistance to phosphine of several stored product insects. In this study, our field samples covered the whole species spectrum that generally infest the stored products in an attempt to estimate the spread of resistance in these species along the stored commodity value chain across a wide geographic landscape in Greece. Simultaneously, we used two established diagnostic protocols, the FAO and DDPTTK to validate our resistance diagnosis data. Although determining the strength of resistance was not planned for and investigated in this study, based on the results that are reported here, 43 field populations belonging to seven major stored product pest species were diagnosed as resistant to phosphine. This constituted 81% of the 53 populations collected in total. With the exception of two results [P2(2) *T. confusum* and ASC15 *T castaneum*] the diagnosis of susceptible and resistant populations determined by the FAO and DDPTTK protocols matched perfectly. This high incidences of phosphine resistance across a wide geographic landscape should be taken seriously by the storage operators, specifically in terms of using phosphine as a fumigant to control pests.

The current results are notably different than the results that had been obtained in surveillances from other areas, where strong resistance was more commonly found (Nayak et al., 2013; Holloway

Table 2
Probit analysis for KDT_{99} (confidence intervals) of adults that exposed to 3000 ppm of phosphine and monitored for 5–90 min, using the DDPTTK (df = 7).

Species	Populations	KDT_{99}	χ^2	P	Diagnosis[a]	Time based on DDPTTK (min)
S. granarius	LAB	[b]	[b]	[b]	Susceptible	12
	ASC8	34.2 (24.4–64.7)	0.2	0.99	Resistant	
O. surinamensis	LAB	[b]	[b]	[b]	Susceptible	11
	ASC10	5.1[c]	0.1	0.99	Susceptible	
	ASC11	536.9[c]	8.4	0.30	Resistant	
	GA1	3917.1[c]	0.5	0.99	Resistant	
	EXTR1	[b]	[b]	[b]	Susceptible	
S. oryzae	LAB	[b]	[b]	[b]	(Susceptible)	
	GA2	298.3[c]	30.9	<0.01	(Resistant)	
	GA1	28.9[c]	0.6	0.99	(Resistant)	
	ASC7	[b]	[b]	[b]	(Susceptible)	
	ASC11	2351.9[c]	1.3	0.98	(Resistant)	
	U1	103.6 (83.9–151.1)	4.9	0.66	(Resistant)	
	AGR-01	[b]	[b]	[b]	(Susceptible)	
T. confusum	LAB	[b]	[b]	[b]	(Susceptible)	
	ASC3	49.7 (36.9–81.6)	8.5	0.28	(Resistant)	
	ASC9	12.0 (8.7–39.5)	0.7	0.99	(Susceptible)	
	ASC14	151.4 (104.5–280)	1.8	0.96	(Resistant)	
	V1	133.3 (97.5–224.8)	4.0	0.77	(Resistant)	
	V2	112.1 (81.4–189)	4.2	0.74	(Resistant)	
	P1	86.1 (64–140)	4.6	0.70	(Resistant)	
	AGR-01	12.9 (9.1–57.9)	0.9	0.99	(Susceptible)	
	AGR-05	44.5 (36.7–62.9)	4.7	0.68	(Resistant)	
	ASC16	46.1 (33.8–78.4)	2.2	0.94	(Resistant)	
	GA12	624.9 (288–2902)	2.7	0.90	(Resistant)	
	F1	64.0 (41.5–315)	24.0	0.01	(Resistant)	
	F2	120.0 (84.7–213.8)	1.5	0.98	(Resistant)	
	F3	102.5 (69.9–194.6)	1.7	0.97	(Resistant)	
	F4	57.9 (45.2–88.7)	1.9	0.96	(Resistant)	
	F5	80.7 (60.2–131.7)	1.8	0.96	(Resistant)	
	PP1	74.8 (52.5–135.4)	8.6	0.28	(Resistant)	
	P2(1)	7.8[c]	0.03	0.99	(Susceptible)	
	P2(2)	7.5[c]	0.08	0.99	(Susceptible)	
	P3	8.1[c]	0.1	0.99	(Susceptible)	
	P4	7.1[c]	0.08	0.99	(Susceptible)	
	S1	163.5 (75.6–1168)	3.0	0.87	(Resistant)	
	S2	39.5 (26.8–84.8)	2.0	0.95	(Resistant)	
	S3	78.1 (53.3–167.2)	1.3	0.96	(Resistant)	
	SKG1	71.0 (50.5–124.8)	8.0	0.32	(Resistant)	
	SKG2	70.5 (51.3–118.7)	8.0	0.84	(Resistant)	
	SK1	88.3 (67–139.7)	3.3	0.84	(Resistant)	
	SK2	50.3 (36.7–85.3)	2.5	0.92	(Resistant)	
C. ferrugineus	LAB	[b]	[b]	[b]	Susceptible	13
	ASC8	33.4 (29.3–42.8)	2.7	0.90	Resistant	
	GA2	452.0[c]	2.6	0.92	Resistant	
T. castaneum	LAB	[b]	[b]	[b]	Susceptible	8
	ASC10	1835.0[c]	1.3	0.98	Resistant	
	ASC11	2057.4[c]	2.3	0.93	Resistant	
	ASC15	18.5 (15.3–27.4)	0.8	0.99	Resistant	
	ASC19	92.2 (55.1–253.9)	7.9	0.36	Resistant	
	AGR-04	76.8 (57.9–122.3)	10.2	0.17	Resistant	
R. dominica	LAB	[b]	[b]	[b]	(Susceptible)	
	ASC11	381.7 (195.8–1326.3)	2.4	0.92	(Resistant)	
	GA6	115.6 (76.9–289.9)	11.5	0.11	(Resistant)	
	GA3	23.9[c]	322.8	<0.01	(Resistant)	
	GA2	66.3 (50.2–106.2)	5.8	0.55	(Resistant)	
	AGR-03	108.2 (77.9–187)	4.2	0.74	(Resistant)	
	GA12	58.4 (45.9–88.5)	2.5	0.90	(Resistant)	
	ASC14	152.7 (108.8–267.7)	4.8	0.67	(Resistant)	
	AGR-01	3338.4[c]	0.4	0.99	(Resistant)	

[a] Based on time periods to determine the susceptibility of insects. Parenthesis indicates our estimation based on the maximum time in the DDPTTK instructions (= 15min), given that there are no instructions for these species in the DDPTTK guidelines.
[b] Could not estimate KDT values.
[c] Confidence intervals could not be estimated accurately.

et al., 2016; Gautam et al., 2016; Cato et al., 2017; Afful et al., 2018). For example, in a survey that was carried out between 1982 and 2002 in Australia, it was reported that the weak resistance frequency increased from 5% to 34% of the total number of populations tested (Emery et al., 2003). Moreover, in USA, Cato et al. (2017) and Afful et al. (2018) found a considerable proportion of the sampled populations of *T. castaneum* and *R. dominica*, respectively, to be resistant to phosphine. Furthermore, Lorini et al. (2007) detected

Table 3
Mean number (% ± SE) of adults that were found active after 20h of exposure to 30 ppm of phosphine and after the termination of a 7 d post-exposure period.

Species	Populations	20h	7d	Diagnosis [a]
S. granarius	LAB	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC8	41.6 ± 16.1	35.5 ± 13.9	Resistant
O. surinamensis	LAB	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC10	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC11	100.0 ± 0.0	99.4 ± 0.5	Resistant
	GA1	100.0 ± 0.0	99.4 ± 0.5	Resistant
	EXTR1	0.0 ± 0.0	0.0 ± 0.0	Susceptible
S. oryzae	LAB	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	GA2	96.1 ± 2.6	80.5 ± 3.0	Resistant
	GA1	2.2 ± 1.4	3.8 ± 2.6	Resistant
	ASC7	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC11	90.0 ± 5.7	53.8 ± 15	Resistant
	U1	96.1 ± 1.8	78.8 ± 4.8	Resistant
	AGR-01	0.0 ± 0.0	0.0 ± 0.0	Susceptible
T. confusum	LAB	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC3	23.3 ± 14.5	70.5 ± 10.1	Resistant
	ASC9	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC14	45 ± 12.7	43.3 ± 10.4	Resistant
	V1	50.0 ± 8.0	57.2 ± 8.1	Resistant
	V2	28.9 ± 7.0	31.6 ± 6.6	Resistant
	P1	29.4 ± 6.6	36.1 ± 8.3	Resistant
	AGR-01	0.0 ± 0.0	6.1 ± 2.7	Susceptible
	AGR-05	26.1 ± 14.4	41.1 ± 13.5	Resistant
	ASC16	6.1 ± 2.4	15.5 ± 3.2	Resistant
	GA12	100.0 ± 0.0	100.0 ± 0.0	Resistant
	F1	16.6 ± 8.2	46.6 ± 12.4	Resistant
	F2	17.2 ± 6.7	42.7 ± 11.9	Resistant
	F3	22.2 ± 8.8	43.8 ± 9.9	Resistant
	F4	17.2 ± 3.8	43.8 ± 8.4	Resistant
	F5	5.0 ± 2.6	37.7 ± 5.9	Resistant
	PP1	12.7 ± 4.5	33.3 ± 7.3	Resistant
	P2(1)	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	P2(2)	7.2 ± 7.2	7.2 ± 6.6	Resistant [a]
	P3	0.0 ± 0.0	0.5 ± 0.5	Susceptible
	P4	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	S1	71.1 ± 5.2	74.4 ± 3.1	Resistant
	S2	10.5 ± 4.4	23.8 ± 5.7	Resistant
	S3	3.3 ± 1.4	32.2 ± 6.2	Resistant
	SKG1	8.8 ± 2.9	40.0 ± 5.2	Resistant
	SKG2	6.6 ± 2.2	60.0 ± 4.7	Resistant
	SK1	50.0 ± 8.9	61.1 ± 8.8	Resistant
	SK2	1.1 ± 1.1	45.5 ± 3.4	Resistant
C. ferrugineus	LAB	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC8	12.2 ± 5.4	9.4 ± 3.6	Resistant
	GA2	88.8 ± 4.8	84.4 ± 14.6	Resistant
T. castaneum	LAB	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC10	87.2 ± 10.9	90.5 ± 1.3	Resistant
	ASC11	100.0 ± 0.0	96.6 ± 0.8	Resistant
	ASC15	0.0 ± 0.0	0.0 ± 0.0	Susceptible [a]
	ASC19	0.5 ± 0.5	10.5 ± 4.2	Resistant
	AGR-04	30.0 ± 2.7	56.1 ± 6.5	Resistant
R. dominica	LAB	0.0 ± 0.0	0.0 ± 0.0	Susceptible
	ASC11	96.6 ± 2.2	88.3 ± 4.4	Resistant
	GA6	99.4 ± 0.5	85.0 ± 5.1	Resistant
	GA3	100.0 ± 0.0	75.5 ± 10.8	Resistant
	GA2	88.3 ± 11.0	77.7 ± 11.8	Resistant
	AGR-03	98.8 ± 1.1	97.2 ± 1.2	Resistant
	GA12	100.0 ± 0.0	71.1 ± 4.9	Resistant
	ASC14	97.7 ± 1.2	91.6 ± 2.0	Resistant
	AGR-01	84.4 ± 7.1	84.4 ± 4.2	Resistant

[a] Diagnosis reported here for the FAO assay was the same as the diagnosis determined by DDPTTK, except for the two populations marked with "*".

strong resistance to phosphine in a large number of *R. dominica* populations collected in Brazil. In view of the above reports from several continents, the importance of characterization of phosphine resistance in pest populations in Greece as well as other European countries is paramount. During our field collections, as reported from other countries such as Australia and USA, we experienced the irregularities in fumigation practices across the facilities visited. Currently, there are no standardized protocols available for farmers in Greece, in terms of undertaking phosphine fumigation. Given that there are data which indicate the existence of many resistant populations in farm-stored grains (Opit et al., 2012; Cato et al., 2017), additional background work is needed in Greece to determine the effectiveness of the fumigations at the farm level, as compared to larger fumigations in high-capacity facilities.

From the current results, the most commonly-found resistant populations were those of *R. dominica*, a species which is very common in Greek storage facilities (Buchelos and Athanassiou, 1999; Athanassiou and Buchelos, 2001). Highly resistant *R. dominica* populations have been reported from many regions of USA (Opit et al., 2012; Chen et al., 2015), Australia (Collins et al., 2005) and Brazil (Lorini et al., 2007). Given that the frequency of resistance of *R. dominica* populations from Greece was high as all eight populations collected were diagnosed as resistant, future samplings, specifically for this species may enable us to characterize the strength of resistance.

In this work, we attempted to use DDPTTK and FAO protocols for the evaluation of resistance, in order to draw the inferences necessary for the potential development of a harmonized protocol in the future. Despite the fact that the protocols tested here were qualitatively different, there were some noticeable similarities regarding the diagnosis of resistance. The DDPTTK is considered as a rapid diagnostic/discrimination tool that uses the movement behavior of adult insects as an indication of resistance. In this context, 'knocked down' is considered as an indication of presence of resistance. In an earlier study, Nayak et al. (2013) used the term "knockdown" as an indicator of resistance, and defined different times-to-knockdown under short exposures to phosphine to separate susceptible, weakly resistant and strongly resistant populations of *C. ferrugineus*. In this context, based on the data of the present work, we can consider short exposures through the DDPTTK protocol as a valid tool for the evaluation of resistance, by using immobilized individuals as indicators. Currently, the test kit is able to diagnose the presence of phosphine resistance in a field collected insect sample. To make the test kit more efficient, we need to establish two different 'time to knock down' protocols to diagnose both the 'weak' and 'strong' level of resistance to phosphine, that are now accepted globally. Information on both levels of

Table 4
Correlation coefficient values for each species between a) the percentage of adults that were knocked down after exposure to DDPTTK, i.e. after 90 min to 3000 ppm, and the number of adults that were knocked down after exposure to FAO, i.e. after 20 h to 30 ppm and b) the percentage of adults that were knocked down after exposure to DDPTTK, i.e. after 90 min to 3000 ppm, and the number of adults that were dead 7 d after the termination of their exposure to FAO (n represents the number of populations per species).

Species	n	r_1 [a]	t_1 [b,g]	P_1 [c]	r_2 [d]	t_2 [e,g]	P_2 [f]
S. granarius	9	0.131	−1.7	0.36	0.116	−1.4	0.38
S. oryzae	54	0.828	−2.9	<0.01	0.706	−0.7	<0.01
R. dominica	72	0.225	−8.8	0.02	0.306	−6.4	<0.01
O. surinamensis	36	0.991	−1.0	<0.01	0.995	−1.7	<0.01
C. ferrugineus	18	0.561	1.2	<0.01	0.598	2.2	<0.01
T. confusum	243	0.442	4.5	<0.01	0.488	−2.6	<0.01
T. castaneum	45	0.528	0.8	<0.01	0.393	−0.2	<0.01

[a] Correlation coefficient value between knocked down adults after exposure to DDPTTK and knocked down adults after exposure to FAO (20 h).
[b] t value for r_1.
[c] *P* value for r_1.
[d] Correlation coefficient value between knocked down adults after exposure to DDPTTK and dead adults 7 d after the termination of their exposure to FAO (20 h).
[e] t value for r_2.
[f] *P* value for r_2.
[g] One-tailed *t*-test at 0.05.

resistance would then be used to develop fumigation protocols to manage the resistant pest populations appropriately. For example, although the registered rates of phosphine can control the weak resistant pest populations, sometimes these rates fail to manage strong resistant populations as previously shown in the case of *C. ferrugineus* populations in Australia (Nayak et al., 2013), that required the development of new protocols (concentration x time profiles) to manage them (Kaur and Nayak, 2015). We definitely look forward to subject the resistant populations from Europe to further research in this respect so as to manage them appropriately.

The FAO protocol that is based on the mortality response of adult insects has been modified by several researchers over recent years, especially in terms of concentrations that are used and the interval at which mortality is recorded (Pimentel et al., 2009; Gautam et al., 2016; Holloway et al., 2016). For example, Gautam et al. (2016) recorded mortality after 7 or 14 days depending on the species, while Cato et al. (2017) and Afful et al. (2018), recorded only delayed mortality at a 14-d post-exposure period. Holloway et al. (2016) and Nayak et al. (2017) recorded the mortality at 7 days from the initiation of the fumigation for their resistance testing. In the present study, we found that the mortality level at the 7 d post-exposure period correlated well with the immobilization that was observed right after the exposure. Thus, delayed mortality gave similar results to those obtained at a quick 'knock down' assay using the DDPTTK kit. By taking into account the correlation of the results between the FAO protocol and the DDPTTK, it became evident that both protocols gave the same results, for most of the species tested, which has strengthened our approach for the development of quick diagnostic bioassays. Apparently, DDPTTK can provide results on site, without the need to transfer the insects to a specialized laboratory to carry out bioassays that can last for days or weeks. In general, populations that were knocked down earlier than others after exposure to DDPTTK indicated higher mortality at the post-exposure of the FAO protocol.

Our study provides the first extensive data set for the evaluation of resistance to phosphine in Greece and probably in Europe. From the species tested, *R. dominica* strains were more often diagnosed as resistant in comparison with other species. Furthermore, there are interesting similarities among the two protocols used here. For the majority of the species and populations tested, the kit and the FAO protocol gave similar results, suggesting that the utilization of a rapid test that lasts for a short period of time (few hours or even less than 1 h) can provide realistic results for the detection of resistance to phosphine on site. Additional samplings will help to map the potential existence of resistance in Greece and to enhance knowledge on the similarities of the two diagnostic protocols. We suggest that future research should be focused on expanding the utility of the test kit to diagnose both the 'weak' and 'strong' level of phosphine resistance. Surveys of phosphine resistance should also be undertaken in other countries in Europe where recent information on phosphine resistance is lacking.

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Appendix A. Supplementary data

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