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Orginal araştırma (Original article)

Determining phosphine resistance in rust red flour beetle, *Tribolium castaneum* (Herbst.) (Coleoptera : Tenebrionidae) populations from Turkey[1]

Türkiye Un Biti *Tribolium castaneum* (Herbst.) popülasyonlarında fosfin direncinin belirlenmesi

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Summary

Fumigation with phosphine gas is the primary method of controlling stored grain pests. In Turkey, phosphine has been used extensively since the 1950's. Even though high levels of phosphine resistance have been detected in several key stored products pests across the world, it has never been studied in Turkey despite this long history of phosphine use. High-level phosphine resistance has been detected and genetically characterised previously in the rust red flour beetle, *Tribolium castaneum* in other countries. Since this pest is also a common problem in stored grain environment in Turkey, the current study was undertaken for the first time, to investigate the distribution and strength of phosphine resistance in *T.castaneum*. Four strains of *T. castaneum* were tested through bioassays for determining the weak and strong phosphine resistance phenotypes on the basis of the response of adults to discriminating phosphine concentrations of 0.03 mg/L and 0.25 mg/L, for 20 hour exposures respectively. Phenotype testing showed all strains exhibited some level of phosphine resistance with a maximum level of 196 fold. Sequencing and genetic testing of seven field-collected strains showed that all of them carried a strong resistance allele in at the *rph2* locus similar to the one previously reported. Our results show that strong resistance to phosphine is common in Turkish strains of *T. castaneum.*

Keywords: Wheat, phosphine resistance, *Tribolium castaneum,* molecular diagnostic, dihydrolipoamide dehydrogenase, *dld*

Özet

Depolanmış hububattaki zararlılarla mücadeledeki ana yöntem fosfin gazı ile fumigasyondur. Fosfin gazı Türkiye'de 1950'li yıllardan beri yoğun olarak kullanılmaktadır. Fosfinin uzun yıllardan beri depolanmış hububatta kullanımına ve dünyada da pek çok ülkede yüksek seviyelerde fosfin direncinin belirlenmiş olmasına rağmen Türkiye'de bugüne kadar bu konuda herhangi bir çalışma yapılmamıştır. Yüksek seviyelerde fosfin direnci, un biti *Tribolium castaneum*'da belirlenerek direnç yapısı genetik olarak tanımlanmıştır. Bu zararlı Türkiye'de depolanmış buğdayda yaygın bir zararlı konumunda olduğundan, bu ilk çalışma fosfin direncinin *T. castaneum'*daki durumunu ve ülkedeki dağılımını ortaya koymak üzere yürütülmüştür. Dört popülasyon, zayıf ve kuvvetli direnç için sırasıyla ayırıcı fosfin konsantrasyonları olan 0.03mg/L ve 0.25mg/L dozları ile 20 saat süreyle uygulama temeline dayanan bioassaylerle test edilmişlerdir. Bu testlerde popülasyonların tümünde direnç olduğu ve direnç oranının 196 kata kadar ulaştığı belirlenmiştir. Yapılan genetik çalışmalar ve sekans, tüm popülasyonların kuvvetli direnç allellerine sahip olduklarını ve bu popülasyonların daha önce literatürde rapor edilenlere benzer olarak *rph2* lokusundaki direnç allelini taşıdıklarını göstermiştir. Çalışma sonuçlarımız, *T. castaneum*'un Türkiye popülasyonlarında fosfine karşı güçlü bir direncin yaygın olduğunu göstermektedir.

Anahtar sözcükler: Buğday, fosfin direnci, *Tribolium castaneum,* moleküler tanımlama, dihydrolipoamide dehydrogenase, *dld*

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Introduction

The Rust Red Flour Beetle, *Tribolium castaneum* (Herbst.) is a serious pest of stored grains and grain products across the world (Bell, 2000). Currently, fumigation with phosphine is the primary method of control of this species across the world. Phosphine remains the fumigant of choice throughout the world including Turkey over several decades for disinfestation of stored products because of its low cost, residue free treatment, lack of viable alternative and ease of application (Heseltine & Thompson, 1957; Zettler & Arthur, 2000; Anonymous, 2014).

Extensive use of phosphine has led to the development of resistance in key pest species (Collins et al., 2002; Daglish et al., 2002). Phosphine resistance in *T. castaneum* was first reported through the global survey by FAO in 1972-73 (Champ & Dyte, 1976). Ten years later, resistance levels in *T. castaneum* were increased to 48.1% in developing countries (Taylor & Halliday, 1986). Resistance to phosphine is an increasing problem and there have been widespread reports of resistance in *T. castaneum* in Australia (Attia & Greening, 1981; Daglish & Collins, 1999; Jagadeesan et al., 2012), Brasil (Sartori et al., 1990; Pimentel et al., 2007), Morocco (Benhalima et al., 2004), India (Rajendran, 1999), China (Champ & Dyte, 1976; Cao et al., 1999; Zeng, 1999), Bangladesh (Mills, 1983), Malaysia (Rahim & Sulaiman, 1999; Rahim et al., 2004), Pakistan (Taylor, 1986; Ansell, 1992; Ahmad et al., 2013), USA (Zettler et al., 1989; Opit et al., 2012) and Thailand (Jittanun & Chongrattanameteekul, 2014).

Only recently, Schlipalius et al. (2012) discovered that mutations in the dihydrolipoamide dehydrogenase (DLD) gene in *Rhyzopertha dominica* (F.) and *T. castaneum* are the cause of phosphine resistance at the *rph2* locus. This breakthrough research has enabled us to directly detect resistance frequencies in field collected populations at the *rph2* locus.

In the current research, we aimed to investigate the distribution and strength of phosphine resistance on *T. castaneum* for the first time in Turkey.

Materials and Methods

T. castaneum samples were collected from grain storages in Ankara (Sincan and Ayaş distric), Konya (Karatay distric), Şanlıurfa, Elazığ, Karaman and Mersin provinces (Table 3). At least 30 individuals from each province were collected, out of which 10 adults were preserved in 96% ethanol for DNA extraction. Remaining insects were cultured on wheat flour + yeast (20: 1 w/w) at constant regimes of 25°C±1 and 60±5% RH. Both live and ethanol preserved materials were imported to a QC-3 Quarantine Laboratory of Postharvest Grain Protection Unit - Ecosciences Precinct (Agri-Science Queensland, Department of Agriculture, Fisheries and Forestry, Brisbane, Qld, Australia) through appropriate quarantine permit. On arrival, the live samples were put into cultures in whole wheat flour and yeast 20:1 and maintained at constant regimes of 30±1°C and 55±5% relative humidity (RH).

Phenotype characterisation by bioassay

All bioassay studies were carried out at the Postharvest Grain Protection Unit of the Department of Agriculture Fisheries and Forestry in Brisbane, Queensland, Australia. Phosphine gas was generated by dissolving commercially available aluminium phosphide tablets (Fumitoxin®, Nufarm Australia Limited) in 5% sulphuric acid solution and its concentration was determined by Perkin Elmer Clarus 580 gas chromatography (USA) using a thermal conductivity detector (TCD) with Nitrogen (N_2) as the standard. Bioassays were undertaken by placing plastic cups containing 50 adults (1-2 weeks old) inside gastight desiccators (4.0-6.0 L) and injecting phosphine through a rubber septum in the lid using a gastight syringe (Manual Gas Chromatography Syringe, Hamilton® Company, USA).

Phenotypic resistance levels were determined for progeny of field collected adults from Ankara, Konya and Şanlıurfa provinces using a modified FAO method (FAO, 1975). Response of field strains to phosphine were examined by phosphine fumigation at low dose (0.03 mg/L) and high dose (0.25 mg/L) at 25±1°C and 70±5 % RH for 20 h. Each assay was replicated twice. After fumigation, mortality was assessed following a recovery period of seven days in whole wheat flour at 25±1°C and 55±5% RH. The results were evaluated and presented in Table 1.

Table 1. Interpretation of Phosphine bioassay results

Low dose (0.03 mg/L)	High dose (0.25 mg/L)	Classification
No survivors	No survivors	Susceptible
Survivors	No survivors	Weak resistance
Survivors	Survivors	Strong resistance

Probit analysis

After the initial resistance screening strains from Şanlıurfa and Ankara (Ayaş district), which showed a strong resistance phenotype were reared at 25±1°C and 55±5% RH. Thereafter, these populations were subjected to a series of doses of phosphine to determine their relative strengths of resistance by using probit analysis. The doses for probit analysis were: control (air), 0.5, 1.0, 3.0, 5.0, 7.0, 9.0, 10.0, 12.0 and 14.0 mg/L of phosphine for 20 h. For each strain, a total of 1000 individuals were divided into 50 individuals per dose with two replications per dose. After fumigation, mortality was assessed following a recovery period of seven days in whole wheat flour at 25±1°C and 55±5% RH. The percentage responding to all test levels were corrected using Abbott's formula (Abbott, 1925). If control mortality was greater than 10%, the results were discarded and that test was repeated.

Probit analysis was performed and the LC_{50} and $LC_{99.99}$ values were calculated using the Genstat 9 (PC/Windows XP) program (Payne, 2004). Resistance ratios of field strains were calculated using Australian susceptible *T. castaneum* strain (QTC4) as a reference.

Determination of nucleotide variants in the *dld* gene

This process followed the same as described previously by Schlipalius et al. (2012). Total RNA was extracted from 15 individuals of the Ankara (Ayaş) and Şanlıurfa strong resistant strains using an Isolate mini RNA kit (Bioline). Complementary DNA (cDNA) synthesis was generated using a Superscript III cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. The coding region of the *T. castaneum* dld gene was amplified from the cDNA (5x Buffer 4 µl, 50 mM $MgCl_2$ 0.6 µl, 2.5 mM dNTPs 0.6 µl, H_2O (Gibco®) 10.55 µl,Taq 0.25 µl, 1 µl forward (AGAGGTCACTCGATAATG) and reverse (CGGAAAAAAATGGGCAGC) RPH2 primers using the following PCR conditions: denaturation for 3 min at 95°C, followed by 40 cycles of 95 ºC for 20 s, 50 ºC for 30 s and 72 ºC for 2 min, and a final extension at 72 °C for 5 min. The PCR product was visualised using 1% agarose gel with TAE buffer. This was run in the gel at 150 volts for 40 min.

Following amplification the DNA fragment was purified and prepared for sequencing using a QiagenQIAquick PCR Purification Micro-centrifuge column according to the manufacturer's protocol. Sequencing was performed in AGRF (Australian Genome Research Facility).

Determination of *rph2* allele frequencies in Turkish populations

Molecular characterisation was carried out in the School of Biological Sciences, University of Queensland, Australia. The *rph2* genotype that is responsible for resistance was tested from strains collected from Ankara (Ayaş and Sincan district), Konya, Karaman, Mersin, Elazığ and Şanlıurfa. The marker amplification allowed us to calculate genotype frequency (homozygous and heterozygous resistant, homozygous susceptible) in the samples.

Genomic DNA was extracted from individual insect samples using a Chelex-100 DNA extraction protocol (Schlipalius et al., 2001). A 368 bp fragment of the *dld* gene containing the nucleotide variant corresponding to P45S variant previously reported (Schlipalius et al., 2012, Kaur et al., 2015) was amplified by PCR in a reaction containing reaction concentrations of Terra PCR buffer 6 µl, restriction enzyme (Mbo*I*) 1 µl, ddH_2O 3 µl and primers Mdu *rph2* Fwd and Rev. The cycling conditions were: denaturation for 3 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 30 s, and a final extension at 7 min. The MboI restriction enzyme was subsequently used to determine resistance genotypes in a cleaved amplified polymorphic sequence (CAPS) marker assay which recognizes the specific nucleotide variation that corresponds to the P45S variant that has been reported to confer resistance at the *rph2* locus and gives two fragments 296 bp and 72 bp in length (Kaur et al., 2015).

Results and Discussion

Phenotype characterisation of resistance in Turkish strains

The discriminative dose assays showed that all strains exhibited some level of phosphine resistance. While the Ankara (Sincan) strain showed weak resistance phenotype, the Ankara (Ayaş district), Konya and Şanlıurfa strains exhibited strong resistance. The resistance ratios of Şanlıurfa and Ankara (Ayaş district) strains, which showed strong resistance, were at least 196.43 and 136.23-fold, respectively (Table 2). However, since these strains were not selected to homozygosity for resistance, indicated by the broad 95% fiducial limits, these resistance ratios are indicative only and would be expected to increase under further selection. The first FAO worldwide survey found that the most resistant strain was only 12 times more resistant than the susceptible reference (Champ & Dyte, 1976). During the following decades, it has been revealed that high levels of phosphine resistance were found to be 80-fold in Pakistan (Ahmad et al., 2013), 186-fold in Brasil (Pimentel et al., 2007), 119-fold in USA (Opit et al., 2012), and 400-fold in Australia (Jagadeesan, 2012). However, the calculation of resistance ratios is a function of both the response of the susceptible reference strains used as well as the frequency of the resistance alleles in the strains being assayed and so it is difficult to interpret whether these resistances are in fact similar or not. However, given that the *rph1* gene appears to confer only a weak (~4-fold) resistance phenotype, would be reasonable to assume that all these strains would have strong resistance alleles present at the *rph2* locus.

Table 2. Dose –response results of *Tribolium castaneum* strains with phospine

Location	No. tested[a]	Slope ± SE	LC_{50} (95% FL) (mg/L)	$LC_{99.9}$ (95% FL) (mg/L)	df[b]	χ^2	P	RR[c]
Şanlıurfa	900	3.030 ± 0.282	1.375 (1.081-1.699)	14.390 (9.463-27.010)	7	*17.32*	0.015	196.43
Ankara (Ayaş)	360	2.940 ± 0.331	0.954 (0.767-1.155)	10.730 (6.989-20.550)	7	*1.110*	0.993	136.23

[a]Number of insects subjected to phosphine bioassay, excluding control.
[b]Degrees of freedom.
[c]Resistance Ratio (RR) = Resistance Ratio (LC_{50} of resistant /LC_{50} of susceptible strain).
RR was calculated wrt QTC4 (LC_{50} = 0.007 mg/L)

Determination of nucleotide variants in the *dld* gene

Sequencing of the expressed *dld* gene showed that there was an amino acid variant (P45S) present in the Şanlıurfa (TC 38) and Ankara - Ayaş (TC 48) strains that have previously been reported (Figure 1). We used a CAPS marker assay that discriminated the genotypes at this particular *rph2* resistance variant to determine the presence of this allele in *T. castaneum* collected from seven different locations within Turkey: Ankara (Ayaş - Sincan distric), Elazığ, Karaman, Konya, Mersin and Şanlıurfa (Table 3).

The action mechanism of phosphine in insects is to reduce the impact of acethylcholinesteras enzyme in the nervous system, block the dehydrogenes glycerophosphate enzyme in mitochondria and deactivate "Complex IV". Also, there is a direct redox relationship between phosphine and cysteine in reactive disulfide, breaking down the sulfide redox bonds in cysteines by blocking the glutathione reductase enzyme (Nisa et al., 2011). One nucleotide changing (Cysteine to Threonine) between susceptible and resistant individuals resulted to transformation of proline to serine (Figure 1). Because of this mutation, phosphine resistance has been occurred.

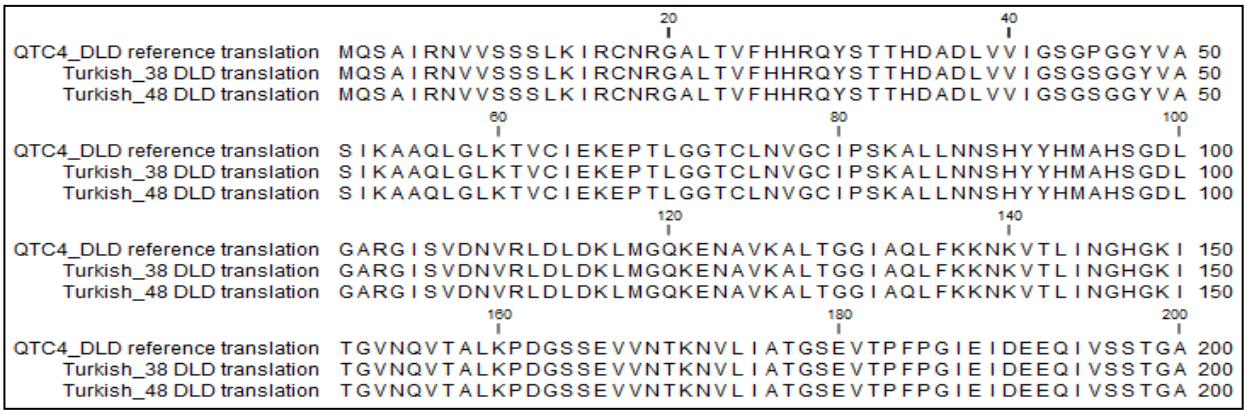

Figure 1. Sequencing of the expressed *dld* gene showing an amino acid variant (P45S) in the Şanliurfa (TC 38) and Ankara - Ayaş (TC 48) strains.

Determination of *rph2* allele frequencies in Turkish populations

The P45S resistance variant was detected from all the strains assayed. It was observed that 77.4% of all the individuals assayed carried at least one *rph2* resistance allele (32.3% homozygous resistant + 45.1% heterozygous resistant). This result shows that the specific P45S allele of *rph2* responsible for strong phosphine resistance previously reported to be in other regions of the world (Schlipalius et al., 2012; Jagadeesan et al., 2012; Kaur et al., 2015; Chen et al., 2015) is in relatively high frequency in Turkish strains of *T. castaneum* collected from grain storages of Konya, Şanlıurfa, Mersin, Elazığ, Karaman and Ankara provinces (Figure 2).

Table 3. The resistance allele in *Tribolium castaneum* collected from seven different locations within Turkey

Province	Result	Genotype
Ankara – Ayas	Heterozygous	Resistant
	Heterozygous	Resistant
	Heterozygous	Resistant
	Homozygous	Susceptible
	Homozygous	Susceptible
Ankara – Sincan	Heterozygous	Resistant
	Heterozygous	Resistant
	Homozygous	Susceptible
	Homozygous	Susceptible
	Homozygous	Susceptible
Elazığ	Heterozygous	Resistant
	Heterozygous	Resistant
	Homozygous	Resistant
	Homozygous	Susceptible
Karaman	Heterozygous	Resistant
	Heterozygous	Resistant
	Heterozygous	Resistant
	Heterozygous	Resistant
	Homozygous	Susceptible
Konya - Karatay	Homozygous	Resistant
	Homozygous	Resistant
	Homozygous	Resistant
	Homozygous	Resistant
	Heterozygous	Resistant
Mersin	Homozygous	Resistant
	Heterozygous	Resistant
Şanlıurfa	Homozygous	Resistant
	Homozygous	Resistant
	Homozygous	Resistant
	Homozygous	Resistant
	Heterozygous	Resistant

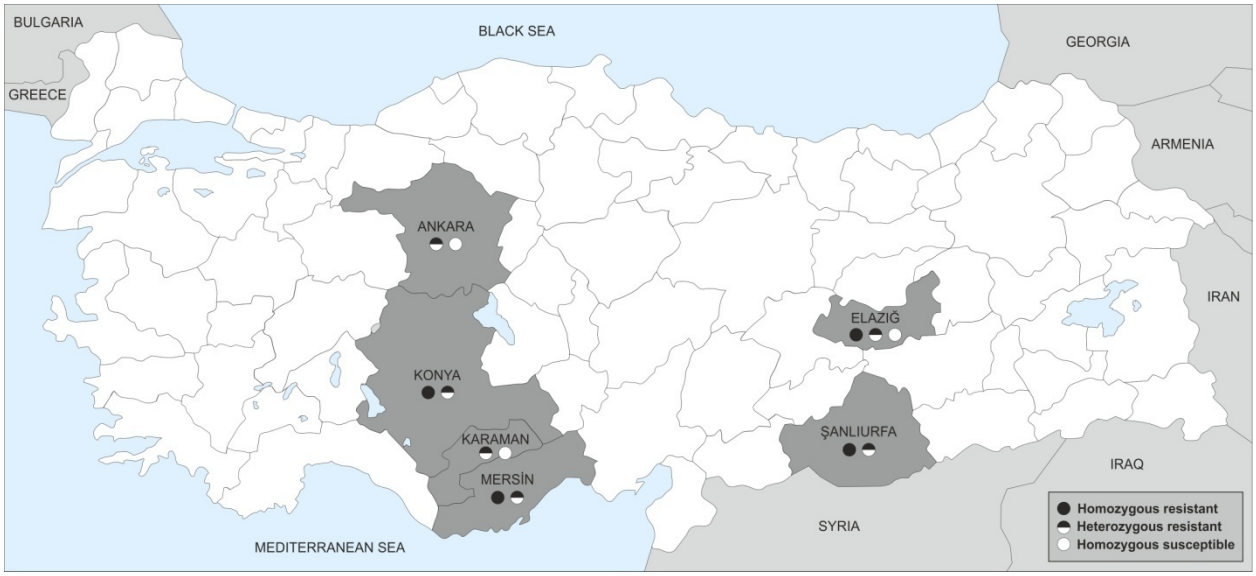

Figure 2. The presence of resistance allele in *Tribolium castaneum* collected from different provinces within Turkey.

The high frequency of the P45S resistance allele in the Eurasian region suggests that this allele may be the most common allele worldwide, even though it is likely to independently arising in all the multiple regions it has been found. The reasons for this phenomenon may include the suggestion that the P45S allele confers the strongest phosphine resistance phenotype, and also possibly confers the least fitness cost. This would allow survival under strong selection pressures and maintenance in insect populations that breed outside of storages and are not exposed to phosphine. It has been noted that some *rph2* alleles reported in *T. castaneum* do carry significant fitness costs (Jagadeesan et al., 2013), however this has yet to be studied in detail for the P45S allele.

In present research, it was highlighted for the first time that strong level of resistance has been developed in *T. castaneum* in Turkey over these years. Due to the limited number of populations involved in the current study, we suggest that a more comprehensive study on characterisation of phosphine resistance is urgently needed across Turkey; that should involve other key pest species such as *R. dominica*, *Cryptolestes ferrugineus* (Stephens), *Sitophilus oryzae* (L.) and *S. granarius* (L.). It is important to note that without this basic information, it is hard to undertake any resistance management strategies.

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