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Original article (Orijinal araştırma)

Comparing bioassay and diagnostic molecular marker for phosphine resistance in Turkish populations of *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae)[1]

Rhyzopertha dominica (F., 1792) (Coleoptera: Bostrichidae)'nın Türkiye popülasyonlarındaki fosfin direncinde bioassay ile moleküler markörün karşılaştırılması

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Abstract

Phosphine gas is the major pesticide applied to stored cereal grains against insects across the world and has been used in Türkiye since the 1950s. Increasing resistance to this fumigant is a problem in stored grain pests worldwide. This study determined the phosphine resistance ratios of the lesser grain borer, *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) in 18 populations from 12 provinces of Türkiye between 2013 and 2017. Discriminating dose studies showed 3 of 15 populations comprise phosphine-resistant specimens. Dose-response bioassays established that resistance ratios were between 96 and 533-fold. The current molecular resistance marker, which detects the amino acid mutation P49S in the DLD (dihydrolipoamide dehydrogenase) gene, were assayed in phosphine-resistant populations. The R allele occurred at a high frequency (83.7%) in 15 highly resistant populations and was absent in three susceptible populations. For 324 individuals from the resistant populations the average proportion of homozygous resistant, heterozygous resistant and homozygous susceptible alleles were 62.0, 18.9 and 19.1%, respectively. The genetic marker detection results were comparable to bioassay results in relation to the resistance status of Turkish populations of *R. dominca*. So, genetic testing for phosphine resistance will simplify resistance management in Türkiye.

Keywords: Bioassay, DLD, lesser grain borer, P49S, phosphine

Öz

Fosfin gazı depolanmış hububattaki böceklere karşı dünya genelinde kullanılan ana pestisittir. Türkiye'de de 1950'lerden itibaren kullanılmaktadır. Bu fumiganta karşı dünya genelinde böceklerde direnç artışı önemli bir problemdir. Bu çalışmada ekin kambur böceği, *Rhyzoperta dominica* (F., 1792) (Coleoptera: Bostrichidae)'nın ülkemizde 12 ilden 18 popülasyonundaki fosfin dirençleri 2013-2017 yılları arasında belirlenmiştir. Ayırıcı doz çalışmaları 15 popülasyonda fosfin direnci geliştiğini göstermiştir. Bu popülasyonlarda doz-yanıt bioassayleri, direnç oranlarının 96-533 kat arasında değiştiğini göstermiştir. Ayrıca, fosfin direncine sahip bu popülasyonlarda DLD (dihydrolipoamide dehydrogenase) geninde amino asit mutasyonunu gösteren mevcut moleküler direnç markörü P49S test edilmiştir. R direnç alleli bu 15 popülasyonda yüksek frekansta (%83.7) belirlenmişken hassas olan üç popülasyonda ise belirlenmemiştir. Dirençli popülasyonlardaki 324 bireyden elde edilen genetic sonuçlara göre homozigot direnç, heterozigot direnç ve homozigot hassas allel oranları sırasıyla %62.0, 18.9 ve 19.1 olarak belirlenmiştir. Türkiye *R. dominica* popülasyonlarında genetik markör ile fosfin direncini belirleme sonuçlarının bioassay sonuçlarıyla kıyaslanabilir olduğu görülmüştür. Sonuçta, fosfin direncinin genetik olarak testlenmesi Türkiye'de direnç yönetimini kolaylaştıracaktır.

Anahtar sözcükler: Bioassay, DLD, ekin kambur biti, P49S, fosfin

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Introduction

Phosphine is a widely used fumigant insecticide for effective protection of stored products (Cato et al., 2017). After the phasing-out of methyl bromide (UNEP, 1995), the reliance on phosphine increased substantially (Nayak et al., 2010). The use of phosphine solely over several decades has led to the development of resistance in many insect species (Champ & Dyte, 1977; Collins et al., 2005; Lorini et al., 2007). The lesser grain borer, *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) is one of the most damaging stored-product insects in Türkiye and worldwide, causing substantial economic loss to stored cereal grains. High-level phosphine resistance in *R. dominica* has been recorded in Australia (Collins et al., 2002, 2016), Bangladesh (Tyler et al., 1983; Hasan et al., 2018), Brasil (Lorini et al., 2007; Pimentel et al., 2010), Burkina Faso (Hasan et. al., 2018), China (Cao et al., 2004; Song et al., 2011), Greece (Agrafioti et al., 2019), India (Kaur et al., 2015; Muralitharan et al., 2016), Malaysia (Hasan et al., 2018), Morocco (Benhalima et al., 2004), Pakistan (Ahmad et al., 2013; Wakil et al., 2021), Philipinnes (Acda et al., 2000) and the USA (Opit et al., 2012; Cato et al., 2017; Afful et al., 2018). Nayak et al. (2015) indicated that the resistant phenotype had 100-fold or greater LD_{50}.

Phosphine has been used in Türkiye since the 1950s. About 6.3 kt of wheat were imported annually between 2012 and 2017 years in Türkiye. So, many phosphine resisted different pest species that could be entered the country. Also, poorly isolated storage and false dose applications of phosphine cause the development of resistant species. Recently, some bioassays for phosphine resistance of coleopteran insects were performed on Turkish populations of the rust-red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) (Koçak et al., 2015), the lesser grain borer, *R. dominica* (Yilmaz & Koçak, 2017), the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens, 1831) (Coleoptera: Laemophloeidae) (Koçak et al., 2018a), the rice weevil, *Sitophilus oryzae* (L., 1763) (Coleptera Curculionidae) (Işıkber et al., 2017), the grain weevil, *Sitophilus granarius* (L., 1758) and the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L., 1758) (Coleoptera: Silvanidae) (Koçak et al., 2018b). These studies have shown that phosphine resistance is more common and at high levels in Türkiye than detected previously. Phosphine resistance is a serious problem and current resistance bioassays are labor-intensive and time-consuming. So, it is important to determine the genetic resistance factors so that selection for even higher resistance levels can be avoided (Schlipalius et al., 2008). The genetic of phosphine resistance in *R. dominica* was first characterized by Schlipalius et al. (2002). Subsequently, Schlipalius et al. (2012) discovered that mutations in the gene coding for DLD in *R. dominica* is a cause of phosphine resistance at the *rph2* locus. Mau et al. (2012) showed that the same *rph2* (DLD) locus was responsible for the development of high phosphine resistance in multiple strains of *R. dominica*. Kaur et al. (2013) developed a DNA marker to determine the distribution of phosphine-resistance of *R. dominica.* Subsequently, the amino acid substitutions on the DLD gene were determined as P49S (the most frequent), P85S, G135S, K142E and N506H (Schlipalius et al., 2018). The most frequent variants have been found to be, P49S, K142E, P85S and G135S (Nayak et al., 2018, 2021) in Australia. The widespread presence of P49S in several populations of *R. dominica* across Australia (Kaur et al., 2013; Nayak et al., 2018, 2021; Schlipalius et al., 2019), the USA (Chen et al., 2015) and India (Kaur et al., 2015) have been reported recently. Molecular resistance markers have been developed for P49S (Kaur et al., 2013; Chen et al., 2015). A resistant allele in *T. castaneum*, P45S (a homolog of P49S in *R. dominica*) has also been detected in Türkiye (Koçak et al., 2015). So, we determined whether the marker for P49S was appropriate for Turkish phosphine-resistant *R. dominica* populations by determining resistance levels among field populations using discriminating dose and detailed bioassays for resistance levels. The bioassay results were compared with the resistance allele marker for determining the allele frequencies in the same populations.

Materials and Methods

Sample collection and rearing

Beetles were collected in 2013-2014 from 18 grain storage facilities in 12 provinces of Türkiye (Figure 1). They were identified according to Mason & McDonough (2012). Sampling was conducted at five locations and depths in each facility, over the conveyor belt in large silos, and from the grain stored in bulk, approximately 4 kg wheat sample was taken using a 2-m grain probe. Each sample was brought to the laboratory in nylon bags, after being labeled with the date of taking, crop type, production year and sampling place. One kg subsamples were taken from each 4-kg samples and transferred to 1-L glass jars, then mouths of the jars covered with gauze. After keeping the glass jars in climate cabinets at 27°C, 65 ± 5% RH and 16:8 h L:D photoperiod, samples were passed through metal sieves (Retsch, Haan, Germany), beetles collected, identified, counted and subsequently cultured on a mixture of 95% whole soft wheat grains and 5% cracked grain admixture (w/w) (Chen et al., 2015). The nutrient mixture (~100 g) was added to 1-L glass jars with perforated lids. To prevent contamination, the jars were placed on plastic bases in tubs filled with liquid vaseline (Pimentel et al., 2008; Opit et al., 2012). Individuals emerging from the eggs transferred to these jars completed their development in approximately 30-35 days to become adults. The 0-24-hour eggs were collected and placed in nutrient a medium containing wheat flour and yeast. Adult emergence was observed daily in jars for about 30-40 days after the addition of eggs, and the first adult emergence date was recorded. From the first adult emergence to the week 7, all adults were taken from the jars and 1-3-week-old males and females of the first generation were used as mixed in the experiments (Esin, 1971; Şayeste, 1971; FAO, 1975; Işıkber, 2005; Opit et al., 2012).

Figure 1. The provinces where collected *Rhyzopertha dominica* populations across Türkiye.

Fumigation

Phosphine gas was produced from phosphine tablets (57% $AlPH_3$) in the gas generator. The aluminum phosphide tablets were added to the water in a 1-L glass cylinder containing 5% H_2SO_4 (FAO, 1975). Glass desiccators with a volume of 3 L with closed circuit gas circulation were used for the experiments. KOH solution was placed in the desiccator in order to provide the desired humidity at a level of 60-65% before the experiment (Solomon, 1951). The phosphine gas collected in the upper part of the desiccator was sampled through a septum with a 100 ml syringe. The gas pipes in the desiccator were connected to the gas outlet and gas suction parts of the phosphine gas measuring device (Ati PortaSense, Analytical Technology Inc., Collegeville, PA, USA). The required amount of phosphine gas was given with a syringe from the desiccator gas inlet. After reaching the required gas concentration in the setup, the phosphine measuring device was turned off. In addition, after the pipes connected to the desiccator were removed from the device, the desiccators were placed in the incubator at 26°C, 60 ± 5% RH and 16:8 h L:D photoperiod (FAO, 1975; Kahraman, 2009; Opit et al., 2012). Dräger Pac 7000 gas measuring device (Draeger Arabia Co. Ltd., Riyadh, Saudi Arabia) was placed in the incubator, leak proofness was measured in the desiccators and leaky desiccators were removed. PVC containers (3 x 3 x 3 cm) with 25 adults as mixed and 1-2 g of cracked wheat were placed in each desiccator. Experiments were set up with four replicates of up to five phosphine doses. After the insects were exposed to phosphine for 20 h, they were transferred to jars containing food and kept in the incubator for 14 days and viability counts were made.

Phenotypic resistance levels were determined on the progeny of field-collected adults according to the standard method (FAO, 1975) at discriminating doses of phosphine of 20 ppm for 20 h to detect weak resistance using Ati PortaSense gas measuring device. Mortality responses to PH_3 of the resistant strains were modified from Kaur et al. (2015) and measured against a range of PH_3 concentrations, 0.025-5.0 mg/l. Fumigation was undertaken by placing 25 unsexed adults (1-3 weeks post eclosion) in a 30-ml plastic cup containing 5 g whole grain with four replicates per dose. Samples were placed inside gas-tight desiccators and PH_3 was injected through a rubber septum in the lid using a gas-tight syringe. Insects were exposed to PH_3 for 48 h, then removed from the desiccators and kept until endpoint mortality was assessed following a recovery period of 7 days at 25°C and 65% RH. Both live and dead insects from the bioassays were subsequently preserved in 70% ethanol at -20°C before DNA extraction and molecular resistance screening.

Data analysis

The mortality data were corrected using Abbott's correction for control mortality (⩽10%; Abbott, 1925) before the probit analysis (Finney, 1971). The analysis was performed using LeOra Software, PoloPlus 2002-2009 statistical package. The resistance ratio for the resistant strains was calculated by dividing the LC_{50} of the resistant strain by the LC_{50} value of a reference Australian strain, a susceptible *R. dominica*, QRD14 (Collins et al., 2002).

Genomic DNA extraction and PCR conditions

The live and dead insects from the bioassays were subsequently preserved in 70% ethanol at -20°C until the genetic study. At least 15 individuals representing each population were used in molecular studies. Insects were arbitrarily sampled proportionally from both live and dead samples to avoid bias, and insects were tested from the progeny of the field sample (Schlipalius et al., 2018). Genomic DNA was extracted from the beetle samples from the field using Qiagen (Hilden, Germany) DNeasy Blood &Tissue Kit according to the manufacturer's instructions with some revisions. One adult was crushed with tissue lysis buffer (ATL) in an Eppendorf tube, proteinase K was added and kept at 56°C for 24 h. It was shaken by adding AL buffer, and ethanol (96%) was mixed on it. This mixture was transferred to a special filtered Eppendorf, centrifuged at 8,000 rpm for 1 min. The filtered Eppendorf was placed in a new tube and AW1 was added on the filter and centrifuged at 8,000 rpm for 1 min. The plastic tube was changed and AW2 buffer was added and centrifuged at 14,000 rpm for 3 min. An Eppendorf tube (1.5 ml) was placed under filter Eppendorf tube, AE buffer was added and waited for 1 min, then centrifuged at 8,000 rpm for 1 min. The gDNAs obtained were stored at -20°C during the study. The coding region of the *R. dominica dld* gene was amplified from the DNA (12.5 µL PCR direct buffer (Mg + dNTP), 0.5 µL Taq, 6.5 µl H_2O (Gibco, Thermo Fisher Scientific, Glasgow, UK), 0.75 µL forward Rd-MM (5'-AGGTCCAAGCGTAGGGTTTT-3') and 0.75 µL reverse (5'-AACTGGGAGAATTCGGCTTT-3') RPH2 primers (Chen vet al., 2015) using the following PCR conditions: initial denaturation for 3 min at 95°C, followed by 27 cycles of 95°C for 20 s, 55°C for 20 s and 68°C for 30 s, and a final extension at 68°C for 7 min. The PCR product was visualized using 1.5% agarose gel with TAE buffer (Schlipalius et al., 2012).

Determination of *rph2* allele frequencies

Detection of the P49S allele was determined by a restriction digestion assay. A 20 µL mixture containing 10 µL PCR product, 2 µl reaction buffer, 0.2 µl restriction enzyme (MboI), and 7.8 µL ddH2O was prepared and incubated at 37°C for 12 h. The PCR product consisted of a 375 bp fragment of the *dld* gene containing the nucleotide variant corresponding to the P49S variant that has been reported to confer resistance at the *rph2* locus and gives two fragments of 236 and 139 bp long (Chen et al., 2015) when digested with MboI. The resulting digestion product was run on 1.5% agarose gel with TAE buffer at 100 V for 60 min (Schlipalius et al., 2012).

Results and discussion

Phenotype characterization of resistance

The discriminative dose assays showed that while three strains (Diyarbakır RD55, Karaman RD19, and Batman RD56) exhibited no phosphine resistance, because of dying of all individuals. The other 15 strains from the nine provinces had high resistance (Table 1). The average resistance ratio was about 325-fold for the 15 resistant populations. The LC_{50} resistance ratios of the highly resistance strains were between 96- and 537-fold. The strain RD32 showed the highest resistance among the other highly resistant strains tested. This population was collected from grain storage facility with a high frequency of phosphine use in Şanlıurfa Province, which has a dry and hot climate. In contrast, the Samsun population (RD54) had the lowest resistance ratio of 96-fold. It can be easily said that high resistance to phosphine has developed and is now common in *R. dominica* in Türkiye. High resistance ratios have been previously revealed globally as 600-fold in Australia (Collins, 1998), 595-fold (Afful et al., 2017), and 1,520-fold in the USA (Opit et al., 2012), 86-fold (Ahmad et al., 2013) and 126-fold (Wakil et al., 2021) in Pakistan, >200-fold in Brasil (Lorini & Collins, 2006) and >80-fold in Bangladesh and Burkina Faso (Hasan et al., 2018).

Table 1. Resistance ratios in *Rhyzopertha dominica* populations

Population		n	h	Slope ± SE	LC_{50} ppm (95% confidence limits)	Resistance ratio
Province	Strain					
Australian*	QRD14				1.25	
Ankara	RD46	600	2.91	3.07 ± 0.25	305 (241-362)	244
	RD47	600	2.37	5.14 ± 0.55	446 (362-509)	357
Hatay	RD13	600	3.29	9.60 ± 1.62	471 (291-541)	377
İzmir	RD37	600	2.43	4.38 ± 0.30	357 (314-400)	286
	RD36	600	2.09	5.19 ± 0.44	396 (342-442)	316
Konya	RD6	600	3.58	3.85 ± 0.36	419 (316-502)	335
	RD17	600	5.86	2.40 ± 0.25	322 (176-436)	257
Kütahya	RD45	600	3.71	1.83 ± 0.29	124 (105-143)	99
Mersin	RD7	600	2.06	8.42 ± 0.58	666 (629-704)	533
Samsun	RD54	600	4.48	5.08 ± 0.33	120 (120-213)	96
Şanlıurfa	RD32	600	2.91	13.3 ± 1.80	671 (618-721)	537
	RD33	600	5.16	3.21 ± 0.27	394 (289-491)	315
	RD38	600	3.85	6.00 ± 0.52	481 (410-540)	384
Tekirdağ	RD21	600	4.16	6.54 ± 0.73	602 (490-681)	482
	RD44	600	7.36	2.12 ± 0.22	324 (152-455)	259

Genotype characterization of resistance

We estimated the frequency of one specific variant, the P49S resistance allele, in *R. dominica* populations across Türkiye. This allele has been previously detected at high frequencies in Australia (Schlipalius et al., 2012), India (Kaur et al., 2013), the USA (Chen et al., 2015) and Türkiye (as homolog allele P45S in *T. castaneum*) (Koçak et al., 2015). Nayak et al. (2018) identified three single nucleotide variants (SNVs), P49S, G135S, and K142E in *R. dominica*. These authors found that the frequency of

resistance allele K142E was extremely dominant over the other two SNVs. In an earlier study, Kaur et al. (2013) estimated the frequency of the K142E allele was 3-26%. Schlipalius et al. (2019) found that P49S was very common and the most resistance phenotype recorded for *R. dominica* in Australia and indicated that the variant is likely to be advantaged over alternative alleles in response to selection. When we analyzed 324 individuals from the 15 resistant populations, the average ratios of homozygous resistance, heterozygous resistance and homozygous susceptible alleles were 62.0, 18.9 and 19.1%, respectively. A total of 18 populations from 12 provinces showed an average of 69.8% R allele frequency. The R allele occurred at a high frequency (average 83.7%) in the 15 highly resistant populations and it ranged between 25 and 100% (Table 2). The R allele was absent in the three susceptible populations from Diyarbakır (RD55), Karaman (RD19) and Batman (RD56) Provinces because phosphine has not been used by the farmers for an extended period. Government silos have generally high phosphine-resistant populations because of routine and frequent phosphine use. It was determined that the populations with surviving individuals after phosphine exposure have resistance alleles. The SS alleles were not found in 11 populations of the 18 populations. It was shown that the marker for P49S works for Turkish phosphine-resistant or -susceptible *R. dominica* populations (Figure 2). It should be noted that the bioassay responses are a product of both strength and frequency of the resistance alleles, it is likely that the variance in resistance ratios is due to different frequencies of the *rph2* resistance allele. All the phosphine-resistant strains exhibited high frequencies of resistance compared to the susceptible strain and the research also has shown that populations with high frequencies of resistant individuals display higher resistant phenotype responses. For example, Mersin (RD7) and Şanlıurfa (RD32) populations had only homozygous resistant alleles (RR) and their resistance ratios were both about 535-fold. It is also remarkable that Samsun (RD54) population had no RR alleles and it had the lowest resistance of the highly resistant populations. When the resistance ratio exceeded 100X, R allele frequency ratio generally become high. So, phosphine application managements like dose and exposure time increase should be applied.

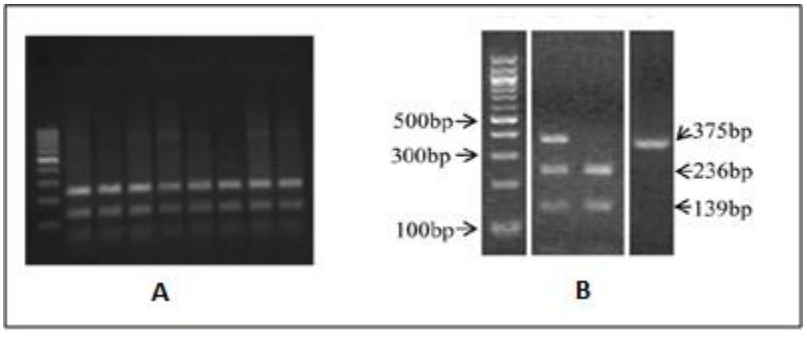

Figure 2. A) Representative gel on demonstration of utility of CAPS marker in *Rhyzopertha dominica* individuals from Türkiye; B) PCR amplicons of genomic DNA coding the DLD gene were digested with restriction enzyme, MboI. Homozygous resistant RR, 236 and 139 bp; susceptible SS, 375 bp; and heterozygous resistant, 375 and 236 bp.

No resistance alleles were found in Karaman (RD19), Diyarbakır (RD55) and Batman (RD56) populations, which were already been determined as susceptible according to discriminative dose studies (Tables 1 & 2). We found that the resistance ratios correlated with *rph2* allele frequencies in the highly resistant populations (Figure 3) and *rph2* alleles were absent in susceptible populations according to discriminative dose (Table 2). Therefore, we have demonstrated that the CAPS marker for P49S will readily detect phosphine-resistant individuals in Turkish *R. dominica* populations. This assay will inform and facilitate the implementation of phosphine resistance management strategies in Türkiye.

Table 2. Resistance related genotypes and allel frequencies in *Rhyzopertha dominica* populations

Population		n	Resistance statement			Allel frequency	
Province	Strain		RR (%)	RS (%)	SS (%)	R (%)	S (%)
Australian*	QRD14						
Ankara	RD46	19	16 (84.2)	3 (15.8)	0	92.1	7.89
	RD47	18	16 (90.0)	2 (11.1)	0	94.4	5.55
Hatay	RD13	19	10 (53.0)	8 (42.1)	1 (5.3)	73.7	23.7
İzmir	RD37	18	18 (100)	0	0	100	0
	RD36	18	15 (83.3)	3 (16.7)	0	91.7	8.33
Konya	RD6	19	15 (78.9)	4 (21.1)	0	89.5	10.5
	RD17	15	7 (46.7)	7 (46.7)	1 (6.7)	70.0	30.0
Kütahya	RD45	16	5 (31.2)	10 (62.5)	1 (6.3)	62.6	37.5
Mersin	RD7	18	18 (100)	0	0	100	0
Samsun	RD54	16	0	8 (50.0)	8 (50.0)	25.0	75.0
Şanlıurfa	RD32	18	18 (100)	0	0	100	0
	RD33	17	13 (76.5)	4 (23.5)	0	88.2	11.8
	RD38	14	12 (85.7)	2 (14.3)	0	92.8	7.14
Tekirdağ	RD21	18	15 (83.3)	3 (16.7)	0	91.7	8.33
	RD44	19	13 (68.4)	6 (31.6)	0	84.2	15.8
Batman	RD56	18	0	0	18 (100)	0	100
Diyarbakır	RD55	18	0	0	18 (100)	0	100
Karaman	RD19	18	0	0	18 (100)	0	100

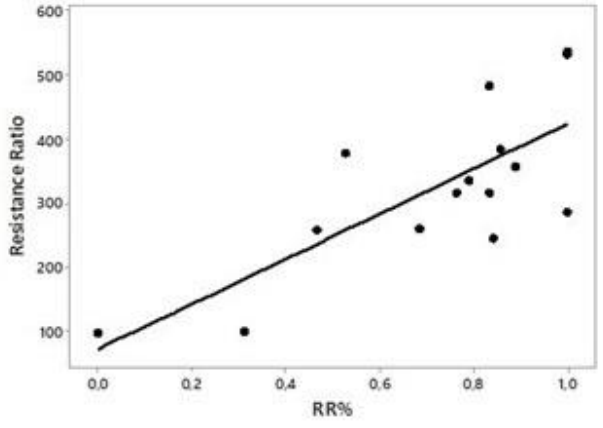

Figure 3. The regression equation is Resistance ratio = 71.4 + 352 RR% regression equation, Multiple R = 0.76, R-Sq = 0.57, R-Sq(adj) = 0.54.

This study showed the necessity of transition to phosphine use management, determination and implementation of a national phosphine resistance management strategies in order to ensure sustainable use of phosphine. In this framework, it is important to determine the factors contributing to resistance development, establish a resistance monitoring system, ensure the use of alternative control methods, evaluate the use of alternative fumigants, limit the use of phosphine according to regions, to update the phosphine usage instructions, to regulate the number of applications and to regulate the phosphine application doses according to the status of individual grain storage facilities.

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