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Potential for using pheromone trapping and molecular screening in phosphine resistance research

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Abstract

Phosphine resistance monitoring typically involves bioassays of beetles from population samples collected from grain storage facilities. Insects are classified into susceptible or resistant phenotypes based on mortality or survival at one or more discriminating doses. Although valuable, phenotype testing has several drawbacks. First, phenotype testing needs live insects, and considerable effort is required to collect and maintain them before testing. Second, population samples may contain multiple genotypes expressing different levels of resistance that may not be distinguishable using discriminating dose bioassays. Third, collections are likely to be focussed around grain storages to maximise sampling success. Recent research shows that several key pests are actively dispersing through flight. The availability of commercial pheromone lures and recent advances in molecular screening provide an opportunity to provide information on resistance gene frequencies more broadly across the landscape. This approach is proving to be a valuable adjunct to traditional resistance testing in Australia.

Keywords: pheromones, DNA markers, traps**,** phosphine resistance, allele frequency

1. Introduction

The development of resistance in stored grain beetles threatens the efficacy of phosphine fumigation (e.g. Daglish et al., 2002; Lorini et al., 2007; Kaur and Nayak, 2015). Generally, monitoring for resistance involves bioassays of beetles from population samples collected from grain storage facilities, and insects are classified into different susceptible or resistant phenotypes based on mortality or survival at one or more discriminating doses (e.g. Benhalima et al., 2004; Daglish et al., 2014; Cato et al., 2017; Konemann et al., 2017). This approach, also known as phenotype testing, is valuable but has several drawbacks. The first drawback is that live insects are needed for testing, and considerable effort is required to collect and maintain them before testing. Another drawback is that population samples may contain multiple genotypes (including heterozygotes), expressing different levels of resistance, that may not be distinguishable using discriminating dose bioassays. Finally, collections are likely to be focussed around grain storages to maximise sampling success. Recent research shows that several key pests are actively dispersing through flight. The availability of commercial pheromone lures and recent advances in molecular screening provide an opportunity to provide information on resistance gene frequencies more broadly across the landscape. We demonstrate this using results from eastern Australia on the lesser grain borer, *Rhyzopertha dominica* (F.).

Phosphine resistance in *R. dominica* is conferred by two major genes (*rph1* and *rph2*) and there could be up to nine genotypes to be present in sampled populations (Schlipalius et al., 2002). Beetles that are homozygous for resistance at *rph1* alone are widespread in eastern Australia and exhibit weak resistance. Beetles that are homozygous for both *rph1* and *rph2* are much less common and exhibit strong resistance. While the FAO discriminating dose is useful for discriminating between susceptible and resistant beetles, using a discriminating dose to distinguish between weak (*rph1*) and strong resistance (*rph1* + *rph2*) is more difficult because of the overlap of the dose-response curves of beetles with weak resistance and those with strong resistance (Lorini et al., 2007). The dose-response curves of many of the other seven genotypes is not known, and further prediction of their responses is complicated by the semi-recessive nature of phosphine resistance.

The development of the capacity to screen with a molecular resistance marker for *rph2* means that individual beetles (including heterozygotes) can be tested for the presence of absence of *rph2* alleles (Kaur et al., 2013). Because resistance at *rph1* is already widespread, the presence of the resistance marker at *rph2* provides a strong indication that strong resistance is already posing a pest control problem or that it will soon emerge in insects at the site where it was found. An advantage of molecular testing over phenotype testing is that it does not need live insects, provided the DNA of the dead insects has been preserved. Traps baited with commercial aggregation pheromone lures for *R. dominica* have been used in Australian and North America to show that flying adults can be caught not only near grain storages but also many kilometres away (Edde et al, 2006; Mahroof et al., 2010; Ridley et al., 2016; Daglish et al., 2017). Also, such trapping has provided *R. dominica* specimens with intact DNA suitable for gene flow analysis (Ridley et al., 2016). A trapping program using aggregation pheromone lures is under way in southern Queensland, Australia, providing information on infestation pressure from flying *R. dominica*, and specimens for molecular resistance screening. This approach is proving to be a valuable adjunct to traditional resistance testing.

2. Materials and Methods

Trapping

A trapping program is under way around two large storage depots in southeast Queensland, Australia, hereafter referred to as Depot 1 and Depot 2, respectively. The two depots are about 25 km apart. Ten Lindgren four-funnel traps (Contech Inc, Delta, BC, Canada) were set up at each site, 50-100 m from the silos and sheds. Each trap is baited with an aggregation pheromone lure for *R. dominica* (Trécé Inc, Adair, OK, USA) and lures for two other species not covered here. A small

amount of propylene glycol in the trap collection containers serves as a preservative. A sub-set of trapped *R. dominica* is used for molecular screening (see below).

Genomic DNA extraction

Genomic DNA (gDNA) was extracted from individual insects using a modified Hotshot DNA extraction method described by Montero-Pau et al. (2008). Individual insects were placed in 96 well PCR plates and single insect was lysed in 75 µL Alkaline lysis buffer (25 mM NaOH and 0.2 mM EDTA) (pH = 12) at 95°C for 30 min, cooled down at 4°C for 10 min; then solution was neutralized by 75 µL of 40 mM Tris–HCl (pH = 5). Samples were centrifuged and the supernatant gDNA from individual insects were stored at -20°C. Susceptible (QRD14) and resistant (QRD569) laboratory strains were also included in each 96 well PCR plate in gDNA extraction for resistance marker visualisation and valid interpretation.

Resistance marker visualisation

The *rph2* marker used a previously designed QRD569_*rph2* PCR marker, which visualises a K142E substitution in dihydrolipoamide dehydrogenase (DLD) first observed in the strongly resistant laboratory strain QRD569, originally collected from Millmerran, Queensland (Schlipalius et al., 2002; Kaur et al., 2013). The PCR amplification was performed using a Terra™ PCR polymerase kit (Clontech Laboratories, Inc.) in a reaction volume of 50 µL containing 25 µL of 2×Terra™ PCR buffer, 1.5 µL of 10 µM forward (5_-CGTGACTTCCGATCCAGT-3_) and reverse (5_-ACACAGTGGTGAATTAGCGG-3_) primers, 1.0 µL of Terra™ PCR polymerase and 4.0 µL of gDNA stock. PCR conditions were: denaturation for 2min at 98°C, followed by 35 cycles of 98°C for 10 s, 60°C for 15 s and 68°C for 1 min and a final extension at 68°C for 1 min. Amplified 327 bp product was digested with 1 U of *Hpy*188III at 37°C for 2 h in a reaction volume of 10 µL containing 5 µL of PCR product, 1.5 µL of 10× buffer and 0.15 µL of 100× BSA. Digested product was visualised in 2% agarose gel electrophoresis. The susceptible allele in the PCR product showed two fragments, 135 bp and 192 bp fragments after digestion with *Hpy*188III, while the resistant allele showed no cleavage.

Total number of susceptible and resistant alleles in *R. dominica* populations from Depots 1 and 2 were scored and allelic frequency was calculated.

3. Results and Discussion

As an example, the trapping and screening results for *R. dominica* trapped between January and February 2018 are shown in Tab. 1. Pheromone trapping is proving to be an effective means of obtaining *R. dominica* adults for molecular screening in southern Queensland. This is expected based on similar trapping studies in Queensland using the same types of trap and pheromone lure (Ridley et al., 2016; Daglish et al., 2017). The number of beetles caught varied greatly between traps (8-34 at Depot 1 and 2-241 at Depot 2), showing that multiple traps should be deployed at sites to ensure sufficient beetles are available for screening and analysis. The trapping reported here occurred during summer so it is possible that trapping during colder months would yield fewer beetles (Ridley et al., 2016; Daglish et al., 2017). Lindgren funnel traps baited with aggregation pheromone lures have also been effective at trapping *R. dominica* in the USA (Edde et al, 2006; Mahroof et al., 2010) showing the potential for using trapping in other countries to obtain beetles for molecular screening.

Screening of *R. dominica* for the QRD569_*rph2* marker confirmed the presence of this resistance allele in beetles caught between January and February 2018 (Tab. 1). Despite being only 25 k apart there were large differences between the two depots. Allele frequency at Depots 1 and 2 were 6.0 and 13.4%, respectively. In comparison, Kaur et al. (2013) reported QRD569_*rph2* allele frequencies of 3-26% in populations of *R. dominica* collected from grain on farms in southern Queensland in

2011. Most of the beetles that were carrying the QRD569_*rph2* allele were heterozygotes, and it is unlikely that these would have been detected in phenotype tests because of the semi-recessive nature of phosphine resistance. Our screening results provide no information on resistance *per se*, but they do provide information on the frequency of one of the two alleles required for expression of strong resistance to phosphine. Changes in the frequency of resistance alleles shown through regular or periodic trapping and screening are likely to be correlated with changes in phenotypic resistance frequencies.

Beetles from a reference susceptible and strongly phosphine resistant strains were screened for the QRD569_*rph2* allele as well. DNA was extracted from 12 susceptible beetles and 12 strongly resistant beetles. PCR amplification was successful in all reference beetles. All susceptible beetles were homozygous susceptible for this allele while all resistant beetles were homozygous resistant for this allele, providing confidence in the screening. Although PCR amplification was successful in the reference beetles, this was not the case with many of the trapped beetles (Tab. 1). This shows the need to optimise methods to ensure that every trapped beetle can be genotyped.

Our results show that trapping with aggregation pheromone lures, followed by molecular screening for resistance alleles is providing information on resistance gene frequencies, and is proving to be a valuable adjunct to traditional resistance testing in Australia. The beetles caught in the traps could have been beetles flying away from the depots, beetles flying towards the depots, or a combination of both types of beetles. Thus, trapped beetles cannot be directly attributed to a storage site, unlike beetles collected by sampling directly from infested grain. Nevertheless, screening of trapped beetles can provide valuable information on background frequencies of resistance alleles. There are multiple resistance allele variants (Schlipalius et al., 2012), and the current results are for only one of these. The capacity to screen beetles for more of these variants would increase the information value of this approach.

Tab. 1 Screening for *rph2* phosphine resistance allele in *Rhyzopertha dominica* caught in southeast Queensland using traps baited with aggregation pheromone lures.

Depot	Trapped	DNA extracted	PCR amplified	Genotypes			*r* (%)	Carriers of *r* (%)
				rr	*rs*	*ss*		
1	154	96	50	0	6	44	6.0	12.0
2	706	264	71	2	15	54	13.4	23.9

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Screening of Phosphine Resistance in *Sitophilus oryzae* (L.) (Rice Weevil) Populations in Turkey

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In this study, the status and prevalence of phosphine resistance in *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) populations collected from Mersin and Konya Province in Turkey were investigated by conducting the discrimination concentration tests and the concentration–mortality bioassays. Discriminating concentration tests showed that 89.9 and 83.3 % populations of tested total *S. oryzae* populations collected from Mersin and Konya province respectively were resistance to phosphine, which reveals high prevalence of phosphine resistance in the insect sampling locations of both provinces. Moreover, discrimination low concentration (0.04 mg/l) tests indicated that 62.5 and 33.3% of total *S. oryzae* populations collected from Mersin and Konya province respectively had 90% or above survival rate, which showed that the frequency of high phosphine resistance in *S. oryzae* populations collected from Mersin province was higher than that in *S. oryzae* populations collected from Konya province. The concentration–mortality bioassays indicated that there were significant differences in resistance levels of *S. oryzae* populations collected from different provinces. Based on the resistance factors (RF) calculated by LC_{50} values *S. oryzae* populations from Mersin and Konya province were 102- to 104-fold and 38- and 81-fold resistance to phosphine compared with susceptible *S. oryzae* population, respectively. The highest level of phosphine resistance was determined in *S. oryzae* populations from Mersin province, followed by those from Konya provinces, respectively. These results indicated that *S. oryzae* populations from Mersin province had higher phosphine resistance than those from Konya Province. In conclusion, this study showed that high levels of phosphine resistance in *S. oryzae* populations collected from different grain storages in Mersin and Konya province of Turkey were prevalent.

Key Words: Phosphine resistance, *Sitophilus oryzae*, populations, discrimination concentration, Turkey