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Utility of biotechnology based decision making tools in postharvest grain pest management: An Australian case study

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Abstract

A major concern for the Australian grain industry in recent years is the constant threat of resistance to the key disinfestant phosphine in a range of stored grain pests. The need to maintain the usefulness of phosphine and to contain the development of resistance are critical to international market access for Australian grain. Strong levels of resistance have already been established in major pests including the lesser grain borer, *Rhyzopertha dominica* (F.), the red flour beetle, *Tribolium castaneum* (Herbst), and most recently in the rusty grain beetle *Cryptolestes ferrugineus* (Stephens). As a proactive integrated resistance management strategy, new fumigation protocols are being developed in the laboratory and verified in large-scale field trials in collaboration with industry partners. To aid this development, we have deployed advanced molecular diagnostic tools to accurately determine the strength and frequency of key phosphine resistant insect pests and their movement within a typical Australian grain value chain. For example, two major bulk storage facilities based at Brookstead and Millmerran in southeast Queensland, Australia, were selected as main nodes and several farms and feed mills located in and around these two sites at a scale of 25 to 100 km radius were selected and surveyed. We determined the type, pattern, frequency as well as the distribution of resistance alleles accurately for two major pests, *R. dominica* and *T. castaneum*. Overall, this information along with the phenotypic data, provide a basis for designing key intervention strategies in managing resistance problems in the study area.

Keywords: phosphine, molecular platform, grain value chain, resistance management

1. Introduction

Protecting harvested grain from insect infestations is essential for facilitating domestic and international trade. In Australia, for example, the industry strictly adheres to a 'nil tolerance' principle for live insects to gain competitive advantage in international trade. Over the last decade, there has been significant progress in pest and resistance management in Australia in response to the development of high level of resistance to phosphine in key pest species, the primary fumigant used to disinfest stored grain (Nayak et al., 2013; Kaur and Nayak, 2015). While the alternative fumigants sulfuryl fluoride is being evaluated as a 'resistance breaker' to alleviate phosphine resistance problems (Nayak et al., 2016), efforts are ongoing to extend the usefulness of phosphine through development of higher application rates to control strongly resistant populations (Nayak et al., 2013; Kaur and Nayak, 2015).

In any resistance management program, key components include proper determination of strength of resistance and its distribution along the value chain, and appropriate and timely control of resistant populations. Researchers in Australia and India are collaboratively engaged in the

deployment of advanced molecular diagnostic tools to accurately quantify resistance to phosphine, assess risks along the grain value chain and to implement appropriate intervention strategies to manage them. It is important to note that Australia and India share similar sub-tropical and tropical climates conducive to insect infestations and both countries have a long history of use of phosphine to disinfest stored grain. Over the last decade, both countries have faced a constant threat to stored grain through widespread development of resistance to phosphine in key pest species, leading to risk to food security and market access. While losses to stored food grain due to insect problems are conservatively estimated around US\$364 million (Boxall, 2001) in India, Australian losses are negligible. However, a 'nil tolerance' to live insects applies to all export and domestic grain trade, therefore, poor implementation of pest management practices can jeopardise the country's trade in grains worth AU\$9 billion annually (<https://www.graintrade.org.au>).

Here we present a brief account of progress made in gathering critical resistance data in Australia using advanced molecular diagnostics. We used a molecular screening assay on pest populations collected along a pre-determined grain value chain that has two major bulk storage sites and numerous farms in Southeast Queensland. Our overall aim is to utilise the molecular resistance detection method as a decision-making tool for accurate determination of problematic sites within each node of the grain value chain and to facilitate timely implementation of resistance management tactics. The current study focuses on two major grain insect pests, the lesser grain borer, *Rhyzopertha dominica* (F.), and the red flour beetle, *Tribolium castaneum* (Herbst). Resistance data generated through both phenotypic and molecular methods are presented and discussed in the context of managing these two species.

2. Materials and Methods

2.1 Study sites and sample collection

Our area of focus was on a typical grain supply chain containing several grain handling nodes in the township of Millmerran, located in south-east Queensland, Australia. The supply chain contains a cluster of several on-farm grain storage silos, feed and stored product processing mills in and around two bulk grain depots, Millmerran, and Brookstead, each with the storage capacity of 30,000 tonnes (Figure 1). These depots are located 25 km apart and the distance between the farms and feed mills are approximately within 100 km. Grain samples were collected in a consistent pattern across all the selected nodes, representing the entire grain supply chain during 2017-18. For example, 3-5 sites within each node were selected, depending upon the storage size and the structure of the site. Within each site, 5-10 grain samples were collected, each weighing approximately 2 kg. The grain samples were screened in the laboratory for live adults and progeny (in the form of eggs and other immature life stages generated from the collected parent populations). Although several pest species were collected through this study, here we present data only on *R. dominica* and *T. castaneum*.

2.2 Phenotypic testing

The collected live adults were subjected to a phosphine discriminatory dose of 0.25 mg L⁻¹ over 48 h for *R. dominica* and 20 h for *T. castaneum* to diagnose strongly resistant populations in each species as described previously for the resistance testing bioassays (Collins et al 2002 and Jagadeesan et al 2012). A cohort of approximately 150 insects were used for each site within each node for the resistance testing bioassays. In the case of eggs, all the eggs emerged as adults (progeny) after 6-8 weeks of incubation were fumigated. Both live and dead insects from the bioassays were subsequently preserved in 70% ethanol at -20°C before DNA extraction and molecular resistance screening.

2.3 High throughput molecular screening of resistance alleles

2.3.1 Genomic DNA extraction

Genomic DNA was extracted from individual insects using a modified Hotshot DNA extraction method described by Montero-Pau et al. (2008). Test insects in a 96 well PCR plate were lysed individually with 75 μ L 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 and then neutralized by addition of 75 μ L of 40 mM Tris-HCl (pH = 5). Samples were centrifuged and the supernatant containing gDNA from individual insects were stored at -20 °C for high-throughput sequencing. The susceptible and resistant reference strains in both *R. dominica* and *T. castaneum* were also included in each 96 well PCR plate in gDNA extraction for valid interpretation and used as positive controls.

2.3.2 Molecular resistance screening assay

The molecular assay is a genotyping-by-sequencing method comprising multiplex amplification and sequencing of the exons of the dihydrolipoamide dehydrogenase (DLD) gene of either *R. dominica* or *T. castaneum* (Schlipalius et al., 2012) using the Illumina MiSeq™ next-generation sequencing platform. The assay encompasses nearly the entire protein coding sequence of the gene by employing multiple primer pairs to amplify the gene in segments that are subsequently sequenced together. The forward and reverse primers for each exon region were tagged with individual 10-mer index sequences during synthesis to facilitate bioinformatic sorting of the sequences to the individual from which they had been amplified.

Each forward primer was tagged to be specific to a 96-well plate that was assayed 96 tagged reverse primers were specific to individual wells of a 96 well plate. As a result, each DNA amplification product could be traced back to the plate and well in which it had been amplified.

2.3.3 PCR conditions to amplify multiple alleles

The PCR reactions utilised Terra™ PCR mix (Clontech), which amplifies directly from tissue and samples with high protein content. Each sample reaction contained: 3 μ L template DNA (~5-10ng), 1X PCR Buffer, 10 μ M of each primer (5 forward, 5 reverse), 0.6U Taq polymerase and water to a final volume of 22 μ L. The PCR cycling conditions were: 98°C for 2 min; 4 cycles of 98°C for 15 s, 65°C for 30 s, 68 °C for 60 s; with a final 36 cycles of 98°C for 15 s, 55°C for 30 s and 68°C for 60 s.

Resulting amplicons were pooled and sent to the Australian Genomic Research Facility (AGRF) for sequencing on the Illumina MiSeq™ sequencing platform with the 250 bp paired-end read protocol.

2.3.4 Data processing and interpretation

Paired-end MiSeq data were demultiplexed using CLC Genome Workbench V9.5.4 (CLCBIO) using the forward and reverse tags. The data for each sample was then aligned against a genomic reference sequence (JX434608 or KF032715) using the parameters: mismatch cost=2, insertion cost =2, deletion cost=3, length fraction=0.5. Variants were then called using the CLC Genome Workbench basic variant detection algorithm (minimum coverage=10, min frequency 25%, minimum variant count=2).

3. Results and Discussion

Our results on insect sampling clearly indicated the existence of both target pests, *R. dominica* and *T. castaneum* within the selected grain value chain at different density levels. For example, the average number of *R. dominica* per sample (1430.5) was higher than that of *T. castaneum* (728.5) (Tables 1 and 2). Comparison of number of insects identified in each grain handling node across the grain value chain confirmed that infestation of *R. dominica* was prevalent across the entire grain value chain, except for Depot 1 and the processing feed mill, whereas infestation of *T. castaneum* was prevalent only in farm storages (Tables 1 and 2). The average number of *T. castaneum* recorded

in the only mill sampled was double of that of *R. dominica* and far much higher than the *T. castaneum* collected on both depots (Tables 1 and 2).

Our phenotypic resistance screening in collected insect populations across the grain value chain identified three strongly phosphine resistant populations of *R. dominica* (one each from Depot 1, Depot 2 and Farm 1) and a single resistant population of *T. castaneum* (from Farm 1). The molecular screening for single nucleotide variants (SNV) (that confers strong resistance to phosphine), in both *R. dominica* and *T. castaneum* supported the results of phenotypic screening. In addition, the assay has identified two other strongly resistant *R. dominica* populations from Farm 2 and Farm 3, and one strongly resistant *T. castaneum* population from Farm 3, which were initially scored as 'not strongly resistant' in the discriminating phenotypic testing (Table 1). In total, molecular screening has identified three different SNVs, P>49>S, G>135>S and K>142>E in *R. dominica*, and a single SNV in *T. castaneum*, P>45>S (a homologue of P>49>S in *R. dominica*). These resistant alleles were also previously detected in farms and bulk storages in south east Queensland (Schlipalius et al., 2012, Kaur et al 2013), and bulk grain storages in India (Kaur et al 2015), and USA (Chen et al., 2015). Unlike our current high-throughput methods, however, the earlier studies relied on low-throughput DNA marker assays, targeting a specific resistance allele.

The accurate discrimination of multiple genotypes (*rr*, *rs* and *ss*) in each SNV (resistance allele) in selected populations of *R. dominica* and *T. castaneum*, identified allele frequency (R %) as well as percentage of actual carriers of resistance (R%) in each node (i.e. the proportion of individuals having at least one copy of resistant allele in the population). Comparison of resistance allele frequency between the grain handling nodes, indicated that frequency was higher in *R. dominica* in bulk storage depots (10.6-11.3%) compared to farms (2.5-3.95%) (Table 1). In an earlier study Kaur et al. (2013) estimated frequency of one specific variant, K>142>E in populations of *R. dominica* from farms in southern Queensland in 2011 using traditional CAPS (Cleaved Amplified Polymorphic Sequence) marker analysis, which showed a much higher range of allele frequency (3-26%) compared to the range established in our current study. Daghli et al (*in press*, in this Proceedings), using the same method estimated resistance allele frequency in *R. dominica* between (6.0-13.4%), that were trapped at the bulk storage depot sites of the same study site as ours, which supports the findings of the current study. In the case of *T. castaneum*, resistance was confined to farms, and frequency of resistance alleles remained relatively low (1.27-6.25%) (Table 2).

The observed variation in resistance allele frequency among the *R. dominica* and *T. castaneum* populations within the study area indicates that insect populations at each grain handling node have been exposed to differential selection pressure to phosphine. For example, the higher resistance observed in *R. dominica* at depots suggests that populations at this node might have had undergone stronger phosphine selective pressure than populations that were collected from farms and the feed mill. However, this trend was not observed with *T. castaneum* and, in fact, resistance in this species appeared to be prevalent only on farms. This difference, perhaps related to their inherent strength of expressing the resistance phenotype (Jagadeesan and Nayak, 2017) or probably related to species biology and habitat (Daghli et al., 2017). For example, Jagadeesan and Nayak (2017) showed that adults and eggs of strongly resistant *R. dominica* populations exhibit nearly 2-3 fold higher resistance level than that of *T. castaneum*. Thus, the fumigation strategies (concentration x exposure period) that are currently adopted at bulk storage depots to control resistant *R. dominica* could have been extremely high for *T. castaneum*, leaving no survivors after the phosphine treatment.

The grain value chain studied here is ideal in a sense that there has been a high degree of grain movement over the years between farms and bulk storage depots in the region. Our results led us to conclude that there is a high degree of possibility that resistance alleles, can migrate from farms to bulk storages and get exposed to higher selective pressures to phosphine. There is also the possibility of two way insect movement within the study area, which may aggravate the resistance problems in this region. Australian studies have demonstrated that *R. dominica* and *T. castaneum* flight occurs across the broader farming landscape (Daghli et al., 2017), and there is also the

potential for human-aided movement of insects along the supply chain (Hernandez Nopsa et al. 2015).

In conclusion, the current study established base-line information on pest populations and the type and frequency of phosphine resistance alleles for two key grain insect species in a typical grain supply chain in Australia using both phenotypic and molecular resistance tools. Currently, we are in consultation with industry collaborators for implementation of suitable intervention strategies for each grain handling node in a systemic pattern across our study area. We strongly believe that such approach will facilitate achievement of a sustainable pest and resistance management program for stored grains in Australia.

Table 1 Frequency of phosphine resistance in *R. dominica* within a selected grain value chain.

Sites	Insects collected	Phenotype scoring*	DNA analysed	Resistant alleles			Genotypes			R (%)	Carriers of R (%)
				P49S	G135S	K142E	rr	rs	ss		
Depot 1	52	SR	52	-	-	9	2	7	43	10.6	17.3
Depot 2	5234	SR	637	-	1	96	47	50	540	11.3	15.2
Farm 1	220	SR	40	1	-	1	0	2	38	2.5	5.0
Farm 2	3000	Not SR	152	1	-	8	3	6	141	3.95	5.92
Farm 3	1100	Not SR	156	1	2	6	3	6	147	3.85	5.77
Farm 4	376	Not SR	80	-	-	-	-	-	80	0	0
Feed mill	32	Not SR	32	-	-	-	-	-	32	0	0

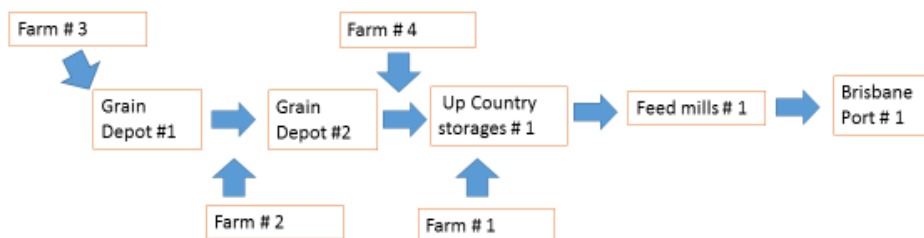
*SR – strongly resistant.

Table 2 Frequency of phosphine resistance in *T. castaneum* within a selected grain value chain

Sites	Insects collected	Phenotype scoring*	DNA analysed	Resistant alleles		Genotypes			R (%)	Carriers of R (%)
				P45S	G131S	rr	rs	ss		
Depot 1	25	Not SR	23	-	-	-	-	23	0	0
Depot 2	16	Not SR	15	-	-	-	-	15	0	0
Farm 1	199	SR	48	3	-	2	1	45	5.21	6.25
Farm 2	69	Not SR	24	-	-	-	-	-	0	0
Farm 3	4000	Not SR	315	4	-	0	4	311	0.63	1.27
Feed mill	62	Not SR	62	-	-	-	-	62	0	0

*SR – strongly resistant.

Figure 1. The model of bulk grain supply chain selected for this study.



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Australia's On-Farm Grain Storage Extension Project – a national initiative improving stored grain pest management and maintaining phosphine fumigation efficacy on-farm for the Australian grains industry.

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Abstract

Phosphine's continued use in Australia to control grain insect pests in on-farm and central storage systems is threatened through increased resistance in both frequency and strength in target insect pests. Effective fumigation combined with best practice integrated pest management is essential to the sustainability of grain biosecurity, food safety, quality assurance and market access for Australian post-harvest grain systems.

The National Stored Grain Extension Program (NSGEP) is an industry funded initiative developed to facilitate best practice in grain storage management within Australia's grains industry. The NSGEP uses a multi approach engagement strategy and a variety of adult learning principles and training techniques aimed at increasing awareness and knowledge to build capacity and support to enable farmers and industry to manage their grain storage systems and meet best practice and market requirements. These include: training workshops, field days, practical demonstrations, industry forums, multi-media and website development and building networks with grower groups, government agencies and agribusiness.