Inheritance of resistance to phosphine in the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens)

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Abstract

Strains of the rusty grain beetle, *Cryptolestes ferrugineus*, a cosmopolitan pest of stored grain, have developed very high levels of resistance to phosphine in Australia, potentially threatening market access and food security. We undertook a genetic analysis of this resistance as part of a research program aimed at management of this pest. Resistance was investigated in three field-collected strains, Susceptible (S-strain), Weakly Resistant (Weak-R) and Strongly Resistant (Strong-R) to phosphine. Three mass inter-crosses were established, S-strain x Weak-R, S-strain x Strong-R and Weak-R x Strong-R, and the dose mortality responses of the F_1, F_2 and F_1-back cross were tested against the hypothesis of monogenic inheritance. The fumigations were undertaken at 25°C and 65% r.h. for 72 hours. Weak-R and Strong-R were 6.3× and 505× resistant compared with the S strain at their LC_{50} levels. Both Weak and Strong resistance were expressed as incompletely recessive with degrees of dominance of -0.404 and -0.411, respectively. However, the analysis indicated that a significant proportion (~15 to 40%) of F_1 individuals survived at higher than predicted concentrations, suggesting the presence of a semi-dominant factor. Results of F_2 and F_1-back cross responses rejected the hypothesis of single gene inheritance in both the Weak-R and Strong-R strains, and indicated the existence of one major gene in Weak-R, and at least two major genes in Strong-R, one of which was allelic with the major factor in Weak-R. The second gene unique to Strong-R accounts for ~80× resistance, confirming that it interacts synergistically with the first gene in conferring a high level of resistance. Neither gene was sex linked. Our results will help in understanding the process of selection for phosphine resistance in the field which will, in turn, inform resistance management strategies. In addition, this information will provide a basis for the identification of the resistance genes.

Keywords: genetics, gene interactions, selection pressure, management

1. Introduction

The rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) is a cosmopolitan pest that infests a wide range of stored cereals and processed commodities (Throne, 1987; Throne and Culik, 1989). Until recently, phosphine fumigation has been effective in controlling this species, however, populations have now been detected that can survive current registered rates of this fumigant, threatening market access of infested commodities (Nayak et al., 2012). Resistant populations in Australia are capable of developing very high levels of resistance up to 1200×, much higher than the levels reported in other grain insect pests (Nayak et al., 2012).

Resistance is micro evolutionary process, thus a heritable change occurs within the insect populations, as an intrinsic response to selection imposed by humans (Roush and McKenzie, 1987). So understanding the genetics of phosphine resistance in *C. ferrugineus* may help us to identify the factors driving the development of strong resistance in this species, which is crucial for developing an effective management strategy. Strong resistance to phosphine in

the lesser grain borer, *Rhyzopertha dominca* (Fabricius), and the red flour beetle, *Tribolium castaneum* (Herbst) is mediated by two major autosomal recessive genes, *rph1* and *rph2* (Collins et al., 2002; Jagadeesan et al., 2012). In homozygous isolation, each of these genes confers only weak (4-15x) resistance, however when they co-occur in one individual, they interact synergistically and provide very high level of resistance up to 600x (Collins et al., 2002; Jagadeesan et al., 2012). In the case of the rice weevil, *S. oryzae* (Linneaus), the genetic dissection of weakly resistant strain indicated the existence of one major gene, in agreement with weakly resistant *R. dominca* and *T. castaneum* (Daglish et al., 2014; Li and Li, 1994). There is no such information available regarding the inheritance of weak or strong resistance phenotypes in *C. ferrugineus*. Therefore, to support the development of effective resistance management strategy, this study was conducted to determine the mode of inheritance, dominance and sex linkage of phosphine resistant alleles and their interaction, if any, in two field-collected, phosphine-resistant strains of *C. ferrugineus*.

2. Materials and Methods

2.1. Insect strains

Three field-collected strains of *C. ferrugineus* were used, phosphine-susceptible, QCF31, weakly resistant, QCF 37 and strongly resistant, QCF73 (Nayak et al., 2012). Throughout this report we refer to these strains as S-strain, Weak-R and Strong-R, respectively. Both the Weak-R and Strong-R strains were selected with phosphine for at least three generations to promote homozygosity at resistant loci. All insects were cultured on a standard diet of rolled oats + cracked sorghum + yeast (75:20:5%) at 30ºC and 65% r.h. (Jagadeesan et al., 2013b).

2.2. Inheritance of phosphine resistance

2.2.1. Genetic crosses

To determine the mode of inheritance, three reciprocal mass inter-crosses were established by crossing the parental strains; S x Weak-R, S x Strong-R and Weak-R x Strong-R. The F_1 hybrids, F_2 and backcross progeny (F_1-BC), crossed to recessive resistant parent (test cross) were obtained from each cross as previously described (Jagadeesan et al., 2012). Sexing was done on adults (Lefkovitch, 1959).

2.3. Phosphine susceptibility tests

Phosphine gas was generated and the source gas concentration measured using a gas chromatograph (Perin-Elmer, Clarus 580) according to the standard method described earlier (Daglish, 2004). Phosphine susceptibility in adults was assessed by exposing them to series of concentrations of phosphine in gas-tight desiccators for 72 hours at 25ºC and 55% r.h., following the methods previously described (White and Lambkin, 1990). Thereafter, insects were provided with fresh diet and allowed to recover for 7 days at which time mortality was assessed.

2.4. Data analysis

2.4.1. Resistance factor, dominance and sex linkage

The response of parental strains and reciprocal F_1 progeny were analysed and fitted to log-dose-probit mortality response curves (Finney, 1979) using the software Genstat version 16 (VSN, 2013). Resistance factor was calculated as the ratio between LC_{50} value of resistant parental strains or F_1 hybrids with LC_{50} value of S-strain or Weak-R (Robertson et al., 2007), depending on the crossing scheme. The 95% fiducial limits of LC_{50} values of reciprocal F_1 crosses were tested for overlap and non-overlap of fiducial limits to indicate the presence of sex-linkage (maternal factors) (Finney, 1979). The degree of dominance was estimated from

the response of parental and reciprocal F_1 hybrids in each cross, according to the method of stone (Stone, 1968).

2.4.2. Number of genes conferring resistance

Two methods were used to determine the number of genes conferring resistance. First, the shape of the observed response of the F_2 and F_1-BC progeny to a full range of concentrations of phosphine was compared visually to the response expected assuming monogenic inheritance. According to Tsukamoto, (1963), if resistance is conferred by a single recessive gene, then a plateau or point of inflection should occur in the log dose-probit response line of F_2 at around 75% and F_1-BC at around 50%. Second, the null hypothesis of monogenic inheritance of resistance was tested on the basis of goodness-of-fit (Sokal and Rohlf, 1995) between observed mortality and the theoretical expectations of F_2 and F_1-BC curves according to the method of Georghiou, (1969) using modified chi-square (Preisler et al., 1990).

3. Results

3.1. Inheritance of weak resistance to phosphine (S-strain X Weak-R)

3.1.1. Resistance levels, maternal effects and degree of dominance

The Weak-R strain was 6.3× more resistant than the S-strain at LC_{50}, whereas the reciprocal F_1 hybrids showed resistance factors of 1.8× and 1.4×. There is no significant difference between the responses of reciprocal F_1 hybrids (Fig. 1), suggesting that weak resistance was autosomal and maternal factors were absent. The data of reciprocal F_1 hybrids was pooled for further interpretation. The response of the pooled F_1 lay close to the S-strain with the degree of dominance of -0.404, indicating the resistance is expressed as an incompletely recessive trait. However, a significant proportion (~15%) of individuals survived at higher doses (0.01 to 0.1 mg/L^{-1}) and their response was very close to the resistant parent suggesting the possible presence of some dominant factors in the F_1 hybrids that are expressed only at higher doses (Fig. 1).

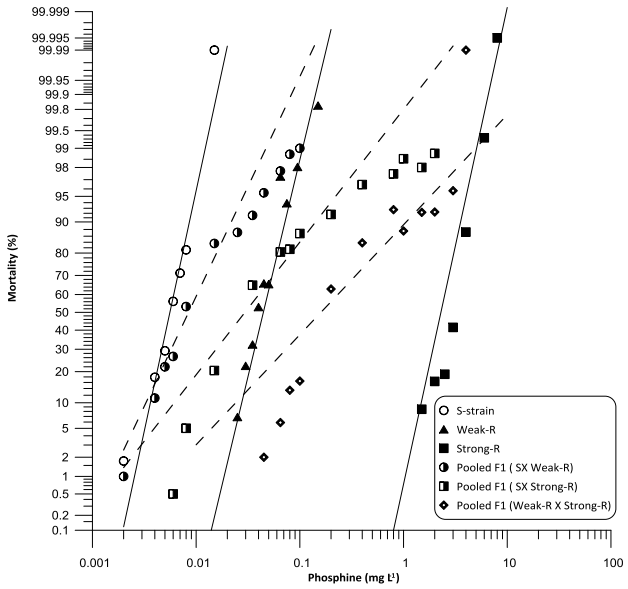

Figure 1 Probit mortality response lines of phosphine susceptible, resistant strains and their pooled F_1 hybrids at 72 h at 25 °C and 65 % r.h.

3.1.2. Number of genes conferring weak resistance

Visual examination of the observed F_2 and F_1-BC progeny response curves showed plateaus at ~ 75% (0.008 to 0.025 mg L^{-1}) and ~50% (0.018 to 0.035 mg L^{-1}) mortality levels, respectively, in conformity with predicated single gene inheritance. However, modified chi-square analysis of individual concentrations of the observed F_2 and F_1-BC response curves identified significant differences at lower concentrations from 0.004 to 0.008 mg L^{-1} (P<0.001, df = 1) for F_2 and 0.01 to 0.014 mg L^{-1} (P = 0.01, df = 1) for F_1-BC. These differences were reflected in the overall chi-square analysis of the F_2 (χ^2 = 32.3, df = 14, P = 0.001) and F_1-BC (χ^2 = 29.3, df = 14, P= 0.04) curves, rejecting null hypothesis of single gene inheritance.

3.2. Inheritance of strong resistance to phosphine (S-strain x Strong-R)

3.2.1. Resistance levels, maternal effects and degree of dominance

At LC_{50}, the Strong-R parent and the reciprocal F_1 hybrids were 505× and 5.6× more resistant than the S-strain, respectively. The responses of the reciprocal F_1 hybrids were overlapping at all concentrations tested and remained statistically indistinguishable, suggesting the absence of sex-linked inheritance, and therefore the responses of F_1 reciprocals were pooled for further analysis. The degree of dominance of the pooled F_1 was -0.411, showing that strong resistance was expressed as an incompletely recessive trait. However, as observed with the F_1 hybrids of the S-strain x Weak-R cross, a significant proportion (~ 30%) of the individuals within F_1 cohort survived at higher concentrations of phosphine (0.06 to 1.0 mg L^{-1}), suggesting the expression of additional dominant factor(s) in the F_1 hybrids at higher concentrations of phosphine.

3.2.2. Number of genes conferring strong resistance

Visual examination of both the observed F_2 and F_1-BC progeny response curves revealed significant deviations from the single gene inheritance model. A significant plateau occurred at ~90% mortality level in the F_2 response curve at concentrations 0.3 to 2 .0 mg L^{-1}. Lack of a plateau at the 75% mortality level in the observed F_2 response curve supported rejection of the null hypothesis of monogenic inheritance. Similarly, the observed F_1-BC curve exhibited two plateaus at ~25 and ~75% mortality level, at concentrations of 0.08 to 0.1 mg L^{-1} and 0.8 to 5.0 mg L^{-1}, respectively, instead of a major plateau at 50%. These results show strong conformity to multifactorial control of strong resistance, and match closely with the phenotypic categories anticipated for two major incompletely recessive genes. The chi-square analysis of the observed responses of F_2 and F_1-BC at individual concentrations showed significant deviations at high concentrations, 0.6 to 4.0 mg L^{-1}, for F_2 (χ^2 = 4.58, df = 1, P = 0.032) and at both lower, 0.06 and 0.08mg L^{-1}, and higher concentrations, 0.6 to 3.0 mg L^{-1}, for F_1-BC (χ^2 = 16.33, df = 1, P = 0.000) curves. The overall chi-square values of F_2 (χ^2 = 25.2, df = 14, P = 0.014) and F1-BC (χ^2 = 76.4, df = 14, P = 0.000) also supported rejection of the null hypothesis of single gene inheritance.

3.3. Interactions between weak and strong resistance genes (Weak-R X Strong-R)

3.3.1. Resistance levels, maternal effects and degree of dominance

Mortality testing revealed that the Strong-R was 80× more resistant to phosphine than Weak-R. There was a significant overlap between the dose response curves of the reciprocal F_1 hybrids at almost all concentrations, except at very high doses, indicating the absence of maternal factors in the inheritance of the gene, which is exclusively present Strong-R. Thus, the response data of F_1 reciprocal crosses were pooled for further analyses and interpretation.

The pooled F_1 mortality curve showed variation in degree of dominance in response to the increase in phosphine concentrations, as observed with F_1 hybrids of other crosses, S-strain x Weak-R and S-strain x Strong-R, confirming the expression of some dominant factors, in addition to the incomplete recessivity estimated at LC_{50} (-0.092). The comparison of mortality responses of all the pooled F_1 hybrids of the crosses, S-strain x Weak-R, S-strain x Strong-R and Weak-R x Strong-R, indicated a steady increase in the proportion of survivors, ~15, 30 and 40%, respectively, confirming the observed trend of change in degree of dominance in F_1 hybrids at high concentrations of phosphine was a real phenotypic effect and the quantum of this effect was influenced by genetic background of parental strains.

3.3.2. Number of genes shared between weak and strong resistance phenotypes

The observed F_1 and F_1-BC (Strong-R x Weak-R) response curves showed strong conformity to the single gene inheritance model with major plateaus at ~75% and ~50%, respectively, at phosphine concentrations from 0.3 to 2.0 mg L^{-1}. Similarly, the modified chi-square analysis for individual concentrations as well as the overall deviation in the response of the observed F_2 curves (χ^2 = 9.6, df = 14, P = 0.65) and F_1-BC (χ^2 = 18.3, df = 14, P = 0.1), aligned with the null hypothesis of monogenic inheritance. These results confirm the existence of a unique factor in Strong-R, which accounts for 80× resistance, in addition to gene (s) that are present in Weak-R, conferring weak resistance. Therefore, it is evident that the single gene present in Strong-R (80×) interacts synergistically with the weak resistance gene (6.3×) that was shared between both Weak-R and Strong-R and conferred a very high level of resistance, up to 505×.

4. Discussion

Resistance factors of 6.3× and 505× for Weak-resistance and Strong-resistance in *C. ferrugineus* resemble those reported for Weak (~3.2 to ~23.4×) and Strong resistance levels (~431 to ~600×), reported previously for *R. dominica* (Collins et al., 2002) and *T. castaneum* (Jagadeesan et al., 2012). Analysis of reciprocal F_1 hybrids from the crosses, S-strain x Weak-R, S-strain x Strong-R and Weak-R x Strong-R, revealed the absence of maternal factors, thus the genes in both the weak and strong resistance phenotypes are inherited autosomally, as also observed with weakly and strongly resistant strains of *R. dominica* and *T. castaneum* (Jagadeesan et al., 2012). These results indicate that phosphine resistance is not conferred from the genetic changes within mitochondrial genome, although key pathways in mitochondria have been proposed as a primary target site for phosphine toxicity (Nakakita, 1976; Schlipalius et al., 2012).

Our analysis of degree of dominance indicated that both weak and strong resistance genes were expressed as incompletely recessive traits. However, examination of the mortality response curves of pooled F_1 hybrids in each cross, S-strain x Weak-R, S-strain x Strong-R and Weak-R x Strong-R, indicated the survival of a significant proportion of F_1 hybrids in each cross, specifically at very high concentrations, reflecting the variation in the expression of recessive inheritance (dominance) at high concentrations of phosphine in *C. ferrugineus*. This is an important result showing that a significant percentage of both weakly and strongly resistant heterozygotes survive at high concentrations of phosphine. In the field fumigations such survival would favour rapid development and spread of high level resistance. Thus, more research on this aspect is essential to develop effective management strategies that can effectively suppress the expression of dominance in heterozygotes in the field. We believe a holistic research approach including the interaction of key selection factors, genetic, operational, and biological on the development and expression of strong resistance in *C. ferrugineus* would be essential for developing a sustainable management strategy for this pest species.

The results of the F_2 and F_1-BC progeny responses from the crosses, S x Weak-R and S x Strong-R, showed strong concurrence to single gene and two genes inheritance for weak and strong resistance, respectively, for the majority of the resistance factor. In addition, the response of F_2 and F_1-BC progeny of the cross between the two resistant parents, Weak-R x Strong-R, indicated that both the weak and strong resistance phenotypes share the weak resistance factor, while the Strong-R has an additional major gene that accounts for 80× resistance, which is absent in Weak-R. Thus, the very high level of resistance exhibited by Strong–R phenotype (505×) appears to be a result of strong synergistic interactions of the two major genes. These results are consistent with inheritance of weak and strong resistance to phosphine in *R. dominica,* and *T. castaneum,* where the weak resistance phenotype was predominantly controlled by a single major gene, *rph1* and strong resistance was governed by the synergistic interaction of two major genes, *rph1* and *rph2*, with one of them being co-allelic with weak-R (Jagadeesan et al., 2012; Jagadeesan et al., 2013a; Schlipalius et al., 2008). Our results and those of previous work (Nayak et al., 2012) indicate clearly that strongly resistant *C. ferrugineus* has unique ability to withstand a very high dose of phosphine, 1 mg L^{-1}, even over a lengthy exposure period of 144 h, which would kill almost all known strongly resistant phenotypes of other species. We suspect that this specific tolerance effect could be due to a natural basal tolerance level of *C. ferrugineus*, or the possible involvement specific background genes, in addition to the two major genes, in contributing to high level resistance in *C. ferrugineus*. This aspect needs further investigation as it has direct relevance to resistance management.

The observed similarity in number of genes conferring weak and strong resistance to phosphine among the weakly and strongly resistant phenotypes across the three key grain insect pests, *R. dominca, T. castaneum, S. oryzae* and *C. ferrugineus,* indicates the possible existence of a highly conserved genetic response to phosphine selection in these pest species, and in such case, one would expect that the underlying biochemical resistance mechanisms could be similar. To answer this question, currently, we are working on molecular characterization of phosphine resistance genes, *rph1* and *rph2* in *C. ferrugineus* using the advent of next generation sequencing. We believe the results will provide special insight into specific genetic changes (mutations) in the resistance genes and its associated mechanisms of resistance in resistant phenotypes.

5. Conclusions

- Strong resistance to phosphine in *C. ferrugineus* is conferred by the synergistic interaction of two major autosomal genes, one of which is also found in weakly resistant strain.
- Both weak and strong resistances were inherited and expressed as incompletely recessive traits in heterozygotes, however, there is a strong indication of the presence of some dominant factors that were expressed only at higher concentrations, giving survival advantage at fumigations and favouring rapid selection within the resistant populations.
- Complementary crosses between weakly and strongly resistant strains confirmed that inheritance of weak and strong resistance to phosphine in *C. ferrugineus* resembled the inheritance pattern seen in *R. dominica* and *T. castaneum,* thus the differences in resistance level across these species is due to their differences in basal tolerance to phosphine and not because of an additional major gene effect.

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