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Enhanced proteolytic and cellulolytic activity in insecticide-resistant strains of the maize weevil, *Sitophilus zeamais*

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ABSTRACT

Insecticide resistance is frequently associated with fitness costs in the absence of insecticides, but extended and intense past selection with these compounds may favor the evolution of fitness modifier genes that mitigate such costs. Insecticide resistance without fitness cost was associated with greater accumulation of total proteins and carbohydrates in a strain of the maize weevil (*Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae)). Increased energy reserves may be due to an accumulation of carbohydrates and proteins because of increased digestive efficiency. To test this hypothesis, proteases and enzymes (cellulase and the pectinases polygalacturonase and pectin lyase), which enable insects to access the nutrients, were used to compare digestive efficiency in insecticide-susceptible and -resistant strains of the maize weevil. A canonical variate analysis indicated significant differences among the strains in enzyme activities, and kinetic parameters were calculated. Serine- and cysteine-proteinases as well as cellulase activities were smaller in susceptible than resistant strains. In addition, the esterolytic activity of serine-proteinases was most increased in the insecticide-resistant strain exhibiting a fitness disadvantage associated with insecticide resistance. Overall, enzymes in the insecticide-resistant strains had increased serine- and cysteine-proteolytic and cellulolytic activity, and kinetic parameters suggested that cysteine-proteinase and cellulase activities were more important in mitigating the cost of insecticide resistance in maize weevil strains.

1. Introduction

The pleiotropic modifications associated with insecticide resistance usually lead to fitness costs in the absence of insecticides, which is a frequent assumption in models of insecticide resistance evolution (Roush and McKenzie, 1987; McKenzie, 1996; Coustau et al., 2000). As a result, an increase in the metabolic rate is expected in the insecticide-resistant individuals to maintain their resistance mechanism. If such increased metabolism does not take place, the energy reallocation necessary for the individual protection against insecticides may impair fundamental physiological processes in the individual, such as development and reproduction (Hostetler et al., 1994; Chown and Gaston, 1999; Harak et al., 1999). Nonetheless, the occurrence of fitness costs associated with insecticide resistance in the absence of insecticides is not universal, although frequent (Beeman and Nanis, 1986; Haubruge and Arnaud, 2001; Raymond et al., 2001).

Demographic and competition studies carried out with insecticide-susceptible and insecticide-resistant strains of *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), the maize weevil, indicated the existence of fitness costs associated with insecticide resistance in some strains, but not in others (Fragoso et al., 2005; Oliveira et al., 2007). Furthermore, the insecticide-resistant strain of *S. zeamais* lacking fitness disadvantage led to higher grain loss than the resistant strain exhibiting fitness disadvantage. In addition, the energy reserve cells (i.e., trophocytes) in the former strain were larger suggesting a higher potential for accumulation of energy reserves, which was confirmed by the greater body mass of insects from this strain (Guedes et al., 2006). The activity of carbohydrate-digestion enzymes, particularly amylase, was also significantly higher in the resistant strain without fitness disadvantage suggesting that a more efficient energy accumulation may mitigate the physiological costs associated with the expression of insecticide resistance mechanisms (Araújo et al., 2008). If so, the activities of

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proteinases and of enzymes enabling insects to utilize nutrients (e.g., cellulase and the pectinases polygalacturonase and pectin lyase) are also likely to be higher in insects from insecticide-resistant strains not showing a fitness disadvantage associated with this trait. The present study aimed to test this hypothesis and therefore provide further evidence about the underlying mechanisms mitigating the cost of insecticide resistance.

Polygalacturonase and pectin lyase are insect pectinases and are therefore involved in the breakdown of pectin, the major component of the plant cell wall (Shen et al., 1996). These enzymes are usually present in the insect salivary glands and provide access for the insect to its food source. Cellulose is a major energy source for several insects, particularly xylophagous and phytophagous insects, but the presence of its digestive enzyme cellulase also increases the energy and nutrient availability (Wei et al., 2005). Proteinases cleave peptide bonds and are involved in several physiological processes including protein activation, immune responses, production of bioactive peptides, besides digestion, etc. (Kanost and Clarke, 2005; Terra and Ferreira, 2005). Among the proteinases, serine-proteinases are the best-studied digestive proteases and are present in the majority of insect species, including *S. zeamais* (Baker, 1982; Houseman and Thie, 1993; Reeck et al., 1999; Terra and Ferreira, 2005). However, cysteine-proteinases are also recognized as important digestive proteinases in beetles, particularly grain beetles such as *S. zeamais* (Houseman and Thie, 1993; Reeck et al., 1999; Zhu-Salzman et al., 2003).

The objective of the present study was to further test the hypothesis that differences in digestion do exist between insecticide-resistant strains of *S. zeamais*, some of which favor energy accumulation and the consequent mitigation of the costs associated with insecticide resistance in some strains of this species. Previous studies on grain loss, insect weight, carbohydrate and lipid digestion and population growth in strains of *S. zeamais* provide support for this contention (Fragoso et al., 2005; Guedes et al., 2006; Oliveira et al., 2007; Araújo et al., 2008). However, such a trend should also extend to enzymes providing food access and protein digestion, which has not been tested. Therefore, the activity of proteinases, cellulases and pectinases was determined in one insecticide-susceptible and two representative insecticide-resistant strains (one with and the other without fitness disadvantage) of *S. zeamais*. Higher activity levels of these enzymes were expected in the insecticide-resistant strain not showing an associated fitness disadvantage, in contrast with the insecticide-susceptible strain and the insecticide-resistant strain with fitness disadvantage.

2. Material and methods

2.1. Insects and chemicals

Three strains of *S. zeamais* were used in the present study. These strains are termed here as 'susceptible', 'resistant cost' and 'resistant no-cost'. The susceptible strain was collected in Sete Lagoas county and provided by the National Center of Maize and Sorghum from the Brazilian Agricultural Research Corporation (EMBRAPA Milho e Sorgo). This strain has been maintained for nearly 20 years without insecticide exposure and its susceptibility to pyrethroids and organophosphates is well known (Guedes et al., 1994, 1995; Fragoso et al., 2003; Ribeiro et al., 2003; Araújo et al., 2008).

Both insecticide-resistant strains are pyrethroid-resistant (>100-fold resistant and subjected to periodical checks before and during the present study) (Guedes et al., 1994, 1995; Ribeiro et al., 2003; Oliveira et al., 2007; Araújo et al., 2008). The resistant no-cost strain was collected in Jacarezinho county (state of Paraná, Brazil) in the late 1980s and the resistant cost strain was collected in Juiz de Fora county (state of Minas Gerais, Brazil) in 1999 (Guedes et al., 1995; Fragoso et al., 2003). The resistant cost strain shows a fitness disadvantage in the absence of pyrethroids, unlike the resistant no-cost strain (Fragoso et al., 2005; Oliveira et al., 2007). In addition, the insects from the resistant no-cost strain are heavier, show smaller departures from perfect symmetry (i.e., are more symmetric) and have higher energy reserves than the susceptible and resistant cost strains (Guedes et al., 2006; Oliveira et al., 2007; Ribeiro et al., 2007). Both pyrethroid-resistant strains share the same major pyrethroid resistance mechanism, altered target-site sensitivity, with secondary involvement of enhanced detoxification by glutathione-*S*-transferases (Guedes et al., 1995; Fragoso et al., 2003, 2007).

All three insect strains were maintained on whole maize grains free of insecticides under controlled temperature (25±2 °C), relative humidity (70±5%) and photoperiod (LD 12:12). All reagents were purchased from Sigma-Aldrich Química Brasil (São Paulo).

2.2. Preparation of enzyme extracts

Three random lots of either 40 or 100 adult insects (not sexed) of each strain were collected from the laboratory colonies for the determination of general proteolytic and serine-proteinase activity (amidolytic and esterolytic) (40 insects per lot) and cysteine-proteinase activity (100 insects per lot). The insects in each lot were surface sterilized with 1.5% KCl and subsequently homogenized in 5.0 ml of 0.1 M Tris–HCl buffer (pH 8.0). Whole bodies, rather than guts and salivary glands, were used as enzyme sources due to the inherent difficulties in the dissection of small insects with heavily sclerotized cuticle, as usually carried out for stored-grain beetles (e.g., Shen et al., 1996; Fragoso et al., 2003, 2007; Araújo et al., 2008). The crude (whole body) homogenate was filtered through glass–wool and centrifuged at 100,000 × g_{max} for 15 min. The pellet was discarded and aliquots of the supernatant were used for determination of protein content and enzyme activity. Three lots of 500 non-sexed adult insects of each population were used for the determination of cellulase, polygalacturonase and pectin lyase activity using the same buffer as for proteinase activity determination, but in higher volumes, 6.0 ml for cellulase and 7.0 ml for both pectinases.

2.3. Protein determination and enzyme assays

Protein concentration was determined following Warburg and Christian (1941). Total protease activity was determined using azocasein as substrate (2%, w/v) at 37 °C following Tomarelli et al. (1949). The reaction mixture encompassed 250 μl substrate and 300 μl enzyme extract, which was incubated at 37 °C for 30 min. The reaction was stopped by adding 1.2 ml of 10% trichloroacetic acid (TCA) and then rested on ice for 15 min, after which 1.4 ml of 1.0 M NaOH was added before the absorbance was read at 440 nm.

The activity of serine-proteinases was assessed using two substrates: *N*-α-benzoyl-L-Arg-*p*-nitroanilide (L-BApNA) as a substrate for determination of amidolytic activity, and *N*-α-*p*-tosyl-L-Arg methyl ester (L-TAME) as a substrate for determination of esterolytic activity. The amidolytic activity was determined using the methods of Erlanger et al. (1961) using 60 mM L-BApNA in 0.1 M Tris–HCl buffer (pH 8.2) containing 20 mM $CaCl_2$. The reaction mixture encompassed 5 ml substrate and 0.6 ml enzyme extract, which was incubated at 25 °C for 2.5 min. The absorbance reading was carried out at 410 nm and the extinction coefficient 8800 M^{-1} cm^{-1} was used to calculate the enzyme activity. The