Impact of transgenic legumes expressing an α-amylase inhibitor on storage pests and their natural enemies

Inauguraldissertation
der Philosophisch-naturwissenschaftlichen Fakultät
der Universität Bern

vorgelegt von

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Leiter der Arbeit:

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Forschungsanstalt Agroscope Reckenholz-Tänikon ART

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Der Dekan:
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ABSTRACT

Grain legumes have been transformed to express αAI-1, an α-amylase inhibitor of the common bean, to protect their seeds against bruchid beetles. Those genetically modified (GM) seeds are highly resistant against several important bruchid species. The combination of GM legumes and hymenopteran parasitoids might be a sustainable and effective strategy for a sustainable bruchid management. A prerequisite for this combination is that the introduced insecticidal trait does not interfere with the control services provided by the parasitoids. In the present study, we investigated the impact of αAI-1 expressed in GM chickpea (*Cicer arietinum*) and cowpea (*Vigna unguiculata*) on bruchids and their parasitoids.

The presence of αAI-1 made chickpea and cowpea seeds essentially immune to the bruchids *Callosobruchus chinensis* and *C. maculatus*. Larvae of those species suffered from a significantly increased mortality. In contrast, the αAI-1 tolerant bruchids *Acanthoscelides obtectus* and *Zabrotes subfasciatus* developed normally in GM seeds.

Parasitoids attacking αAI-1 tolerant bruchids developing in GM seeds might be exposed to the inhibitor. An *in vitro* characterization of the α-amylase activity showed that bruchid parasitoids do possess α-amylases and that those α-amylases are inhibited by αAI-1. Direct feeding assays with purified αAI-1 revealed that harmful effects on parasitoids cannot be discounted.

The impact of αAI-1 on parasitoids was subsequently assessed *in vivo* in experiments including several αAI-1 GM chickpea and cowpea lines. Exposure of parasitoids to αAI-1 seems to be negligible. Furthermore, no impact on the bruchid larvae and thus no reduction in their quality as hosts for the parasitoids could be detected. This implies that both direct (via host-mediated exposure) and indirect (via reduced host quality) impacts on parasitoids are unlikely. Consistently, fitness of parasitoids offered hosts developing in GM or control seeds was not significantly different. We conclude that the αAI-1 GM legumes should not interfere with biological control services provided by bruchid parasitoids.

αAI-1 could also be expressed in other crops to control true bugs (Hemiptera). The quantification of the *in vitro* activity of α-amylases of 12 Hemiptera species and the

inhibition of those α-amylases by αAI-1 indicate that αAI-1 has the potential to protect GM crops against several hemipteran families. The susceptible families contain many important pest species but also potential biological control agents. We suggest that researchers assess the potential of αAI-1-expressing crops to control true bugs *in vivo* and also assess impacts on non-target Hemiptera.

CHAPTER 1

General Introduction and Thesis Outline

Legume seeds are an important source of nutrition for both humans and livestock. Their seeds are rich in proteins, carbohydrates and lipids, and they can be stored over extended periods. Additionally, the nitrogen-fixing abilities of the plants are important for the management of soil fertility. All these properties match perfectly with the requirements of small-scale, low-income farmers in developing countries.

Bruchid beetles (Coleoptera: Chrysomelidae) are responsible for the largest post-harvest losses to stored seeds, directly through consumption of the resource and, secondarily, through the qualitative deterioration of the commodity or the reduced stock viability. Females lay their eggs on the seed surface and the larvae burrow into the seed, where they feed and complete their development (Southgate, 1979). The beetles usually continue to multiply during seed storage, which can lead to extensive or even total losses, especially if the seeds are stored for long periods. Surface and fumigant chemical applications are thought to be the most effective methods for managing bruchid infestations. However, prohibitive costs, which limit their application to large scale or extended storage, and the risks of adverse secondary effects from such treatments, have driven the exploration of alternative strategies to manage bruchid infestations. These include biological control (Sanon et al., 1998; Gauthier et al., 1999; Schmale et al., 2006) and host plant resistance factors (Ignacimuthu et al., 2000; Schmale et al., 2003; Appleby and Credland, 2004).

A crop protection tool with high potential for small-scale farmers is genetic engineering. Although the largest areas of genetically modified (GM) crop production have been in industrial countries, it's the small-scale farmers in developing countries that might benefit the most from this technology (Wambugu, 1999; Thomson, 2008). In 2011, 90% of the farmers planting GM crops were in developing countries (James, 2011). To date, all commercially cultivated insect-resistant GM crops are expressing Cry proteins derived from the soil bacterium *Bacillus thuringiensis* (Berliner) (so called *Bt* crops). The potential of insects to evolve resistance against the deployed *Bt* Cry toxins, their narrow spectrum of activity and the risk of infringing existing patents

have driven the development of alternative insecticidal traits for genetic engineering, including inhibitors of digestive enzymes (Malone et al., 2008).

Using a *Phaseolus vulgaris* resistance factor for genetic engineering

The common bean (*Phaseolus vulgaris* L.) and other *Phaseolus* species possess a family of evolutionary related defence proteins including phytohemagglutinin (PHA), arcelin (Arc) and α-amylase inhibitors (αAI) (Chrispeels and Raikhel, 1991). The genes encoding these three proteins are located in a single locus in the *P. vulgaris* genome (Nodari et al., 1993) and it is likely that these homologous genes have arisen by duplication of an ancestral gene. The proteins feature different modes of action and insecticidal properties against bruchids (Leavitt et al., 1977; Liener, 1986; Osborni et al., 1988; Velten et al., 2007; Janarthanan et al., 2008). αAI can strongly inhibit the activity of α-amylases. These enzymes hydrolyse starch or glycogen and play a key role in the carbohydrate metabolism of microorganisms, plants and animals. Several insects, especially those feeding on starchy seeds during any period of their life cycle like bruchid beetles, depend on α-amylases for survival (Grossi de Sa et al., 1997; Franco et al., 2002). This feature has attracted notice to αAIs, making them a promising candidate for genetic engineering. The αAI of *P. vulgaris* exists in at least two different allelic variants. The isoform found in cultivated beans is called αAI-1 (Moreno and Chrispeels, 1989); a second isoform, αAI-2, is found in some wild accessions of the common bean that contain Arc as the major storage protein instead of phaseolin (Suzuki et al., 1993). Of particular interest is that the two isoforms differ in their specificity towards α-amylases. With the exception of one major storage pest, *Acanthoscelides obtectus* (Say), one or both αAI from the common bean have been shown to inhibit midgut amylases of major bruchid pests found worldwide (Ishimoto and Kitamura, 1992; Franco et al., 2002) (Tab. 1). The successful transfer of common bean αAI into tobacco plants (Altabella and Chrispeels, 1990) indicated that they could also be engineered to other plant species. Subsequently, genes encoding αAI-1 and/or αAI-2 from *P. vulgaris* were introduced by methods of genetic engineering into peas (*Pisum sativum* L.), cowpeas (*Vigna unguiculata* L.), azuki beans (*Vigna angularis* [Wildenow]) and chickpeas (*Cicer arietinum* L.) (Shade et al., 1994; Schroeder et al., 1995; Ishimoto et al., 1996; Morton et al., 2000; Solleti et al., 2008). The *αai* gene construct introduced in these plants is regulated by flanking sequences from the seed-specific bean

phytohemagglutinin PHA (*dlec*) gene. This promoter regulates the expression of the αAI restrictively to the cotyledon and embryonic axis of the developing seed (Schroeder et al., 1995), targeting exclusively seed feeding herbivores like bruchids.
The potential of GM legumes expressing αAI to control bruchids has been confirmed in several studies. In peas, levels of expression of αAI ranged between 1.5 and 3.5% of total soluble protein, providing 100% control of *Bruchus pisorum* (L.) under glasshouse (Schroeder et al., 1995) and field conditions (Morton et al., 2000). Azuki beans expressing 0.9% αAI-1 (per dry weight) provided 100% control of the two bruchids *Callosobruchus chinensis* L. and *Callosobruchus maculatus* F., both important pest species in East Asia (Ishimoto et al., 1996). Similarly, αAI-1-GM cowpeas and chickpeas have been reported to strongly inhibit the development of *C. chinensis* and *C. maculatus* (Sarmah et al., 2004; Solleti et al., 2008).

Compatibility of αAI with biocontrol agents

The potential of combining plant resistance factors together with biological control agents, especially parasitoids, has been shown to be a powerful method to control storage pests like bruchids (Schmale et al., 2003; Velten et al., 2008). One major concern regarding the use of insect-resistant GM plants is the effect that the expressed insecticidal protein might have on non-target organisms. This is especially relevant for traits with a broad spectrum of activity such as αAIs where any organism relying on α-amylases for carbohydrate digestion is potentially affected by the inhibitor. The potential impact of αAIs on bruchid parasitoids has never been evaluated. Kluh et al. (2005) extensively screened the inhibitory activity of αAI-1 against 24 insect species from eight different orders. The most sensitive species belonged to the orders of Coleoptera, Diptera and Hymenoptera. The Hymenoptera tested included an endoparasitic wasp, *Venturia canescens* (Gravenhorst) (Ichneumonidae). However, no bruchid parasitoids were included and whether they rely on α-amylases is still unknown.

Assessing the impact of legumes expressing αAI-1 on bruchid parasitoids

Environmental risk assessment for non-target organisms is a required step in the evaluation process of new GM plants (Sanvido et al., 2012). This is particularly true for GM crops expressing insecticidal proteins. The assessment typically follows a

tiered framework which is conceptually similar to that of pesticides (Hill and Sendashonga, 2003; Romeis et al., 2008).

In an initial step one has to define what we do not want to see harmed by the GM trait. In the case of αAI-1 expressing legume seeds this is the biological control function provided by parasitic wasps that contribute to the control of bruchid species which are not or insufficiently affected by the introduced insecticidal trait. We can then develop a conceptual model describing a pathway how the presence of αAI-1 in the GM seeds can cause harm to biological control (Fig. 1). Following the different steps of the conceptual model allows a comprehensive evaluation of the risk for the parasitoids.

Outline of the thesis

The aim of the thesis was to assess the compatibility of biological control and GM legume expressing αAI-1 in their seeds for a sustainable control of bruchids. We used GM chickpea and cowpea as study plants, which are the predominant grain legumes in the Indian subcontinent and the savannah of tropical Africa, respectively.

Chapter 2 describes the resistance of the GM cowpea and chickpea to three important bruchid species and compares it with non-transformed genotypes.

Chapter 3 presents the results of the characterization of digestive enzymes in bruchid parasitoids and of the assessment of the *in vitro* susceptibility of those species to αAI-1. Additionally, the impact on adult females was assessed in direct feeding assays.

Chapter 4 reports the impact of αAI-1 on the αAI-1 tolerant bruchid *Z. subfasciatus* developing in GM cowpea, and how the parasitoid *D. basalis* is affected when attacking hosts that have ingested αAI-1.

Chapter 5 presents the results of tritrophic experiments with GM chickpea expressing αAI-1, the αAI-1 tolerant bruchid *A. obtectus*, and three different parasitoid species.

Chapter 6 looks beyond legumes, bruchids, and parasitoids and provides results on the assessment of the potential of αAI-1 to control true bugs in GM crops.

In **Chapter 7**, the results of the thesis are discussed and general conclusions drawn on the impact of αAI-1 expressed in GM crops on target and non-target insects.

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Table 1. Present known distribution of major bruchid pests and their sensitivity to αAI-1 and αAI-2 from *P. vulgaris.*

Species	Distribution Worldwide						Inhibition of midgut α-amylase	
	North America	South America	Africa	Asia	Australia	Europe	αAI-1	αAI-2
Acanthoscelides obtectus	(+)	(+)	+	+	+	+	n[3]	n[3]
Bruchus pisorum	+	+	+	(+)	+	+	y[2]	p[2]
Callosobruchus analis	+		+	(+)			y[2]	
Callosobruchus chinensis	+	+	(+)	(+)	+	+	y[1,2,3]	n[1,3]
Callosobruchus maculatus	+	+	(+)	(+)	+	+	y[1,2]	y[3]
Zabrotes subfasciatus	(+)	(+)	+	+		+	n[1,2,3]	y[1,2,3]

(+) indigenous; + established

y = yes; n = no; p = partial

[1] artificial diet; [2] transgenic seeds; [3] amylase inhibitory activity

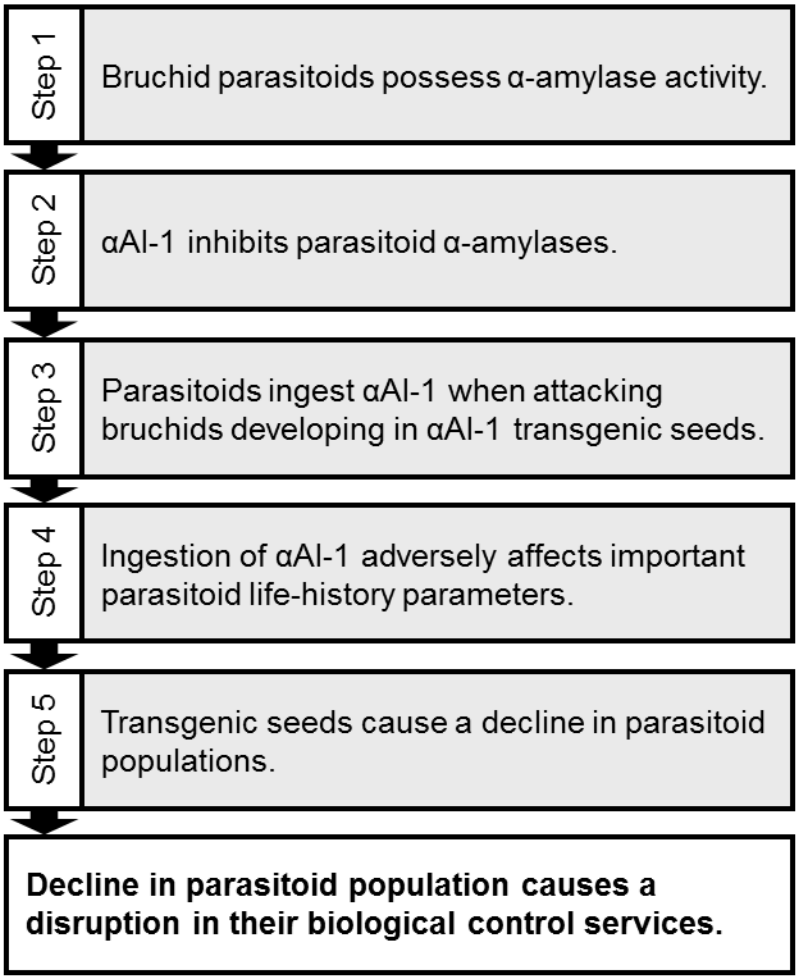

Figure 1. Pathway on how αAI-1 from *P. vulgaris* expressed in transgenic legume seeds could harm the biological control function provided by bruchid parasitoids.

CHAPTER 2

Resistance of αAI-1 Transgenic Chickpea (*Cicer arietinum*) and Cowpea (*Vigna unguiculata*) Dry Grains to Bruchid Beetles (Coleoptera: Chrysomelidae)

ABSTRACT

Dry grain legume seeds possessing αAI-1, an α-amylase inhibitor from common bean (*Phaseolus vulgaris*), under the control of a cotyledon-specific promoter have been shown to be highly resistant to several important bruchid pest species. One transgenic chickpea and four cowpea lines expressing αAI-1, as well as nine conventional chickpea cultivars were assessed for their resistance to the bruchids *Acanthoscelides obtectus* (Say), *Callosobruchus chinensis* L. and *Callosobruchus maculatus* F.

All transgenic lines were highly resistant to both *Callosobruchus* species. *Acanthoscelides obtectus*, known to be tolerant to αAI-1, was able to develop in all transgenic lines. Whereas the cotyledons of all non-transgenic lines were highly susceptible to all bruchids, *C. chinensis* and *C. maculatus* larvae suffered from significantly increased mortality rates inside transgenic seeds. The main factor responsible for the partial resistance in the non-transgenic cultivars was deduced to reside in the seed coat.

The αAI-1 present in seeds of transgenic chickpea and cowpea lines significantly increases their resistance to two important bruchid pest species (*C. chinensis* and *C. maculatus*) essentially to immunity. To control αAI-1 tolerant bruchid species such as *A. obtectus* and avoid the development of resistance to αAI-1, varieties carrying this transgene should be protected with additional control measures.

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INTRODUCTION

Grain legumes play a crucial role in agricultural areas with semi-arid climate. They are a major source of protein for humans, but also a source of fodder and help maintain soil fertility of cereal-based cropping systems due to their ability to fix nitrogen from the atmosphere and tolerance to heat and drought (Graham & Vance, 2003). Subsistence farmers in developing countries profit from the fact that dry grain legume seeds are storable over extended periods and are thus available for consumption or sale throughout the year. Storability is important because dramatic seasonal price variation of many grain legumes means farmer returns can be substantially higher when sales are commenced off-season, when the prices are considerably higher than at harvest (Moussa et al., 2011).

The most important pests of stored grain legume seeds are bruchid beetles (Coleoptera: Chrysomelidae: Bruchinae). Even low initial infestation rates can cause tremendous damage because of the high fertility and short generation times of bruchid beetles (Southgate, 1979). Most subsistence farmers in developing countries rely on traditional storage structures, which are especially vulnerable to bruchid attacks (van Huis, 1991; Nukenine, 2010). Application of residual insecticides or fumigants to protect the seeds from bruchids is not reasonable under such circumstances for economic and health reasons (Keneni et al., 2011). Alternative controls that can be applied include cultural, physical, biological, biorational, and genetic measures (van Huis, 1991; Murdock et al., 2003; Phillips and Throne, 2010). However, their effective implementation is often hindered by the lack of equipment and expertise of the farmers, and non-acceptance of newly proposed techniques (van Huis, 1991). The protection against bruchids could be improved by growing varieties featuring an inherent seed resistance to bruchid beetles. Despite intensive conventional breeding efforts, bruchid-resistant varieties of chickpea (*Cicer arietinum* L.) and cowpea (*Vigna unguiculata* L.), the predominant grain legumes in the Indian subcontinent and the savanna of tropical Africa, respectively, have not been achieved (Keneni et al., 2011). Screening of more than 5000 chickpea lines for resistance to *Callosobruchus chinensis* L. did not reveal any useful resistance (Singh, 1997). A similar screening of more than 8000 cowpea lines for resistance to the bruchid *Callosobruchus maculatus* F. revealed only three lines with moderate resistance, including the promising landrace TVu 2027 (Singh, 1977; Singh et al., 1985). However, the moderate resistance of this particular line, for example, lasted

only for about 90 days post-infestation (Murdock et al., 2008). This does not meet the requirements of subsistence farmers that need to store the seeds at least until the next sowing season, i.e., for about 9 months. Transgenic approaches offer a capable path to obtain varieties with substantially higher resistance than that available in the crop germplasm resources.

Genetic engineering has been used to transfer the gene coding for the α-amylase inhibitor αAI-1, a bruchid resistance factor from the common bean (*Phaseolus vulgaris* L.), into other grain legumes including pea (*Pisum sativum* L.), azuki bean (*Vigna angularis* (Wildenow)), chickpea, and cowpea (Ishimoto et al., 1996; Sarmah et al., 2004; Ignacimuthu & Prakash, 2006; Solleti et al., 2008). α-Amylases, the target of αAI-1, are key enzymes for starch digestion and have been shown to be vital for bruchid development. The gene construct transferred to the transgenic legumes is regulated by the seed-specific promoter phytohemagglutinin-L gene (*dlec2*) of *P. vulgaris*, resulting in expression restricted to the cotyledon and embryonic axis of the developing seeds (Altabella & Chrispeels, 1990). Following egg-hatch, bruchid larvae chew into the seed on which they are laid until completion of development. In seeds expressing αAI-1, bruchid infestation of transgenic seeds proceeds normally until the larvae are exposed to αAI-1 in the cotyledons. The development of susceptible bruchid species ceases rapidly and the larvae starve in the first or second instar. At this early stage of development, physical damage to and weight loss of the seed is minimal (Schroeder et al., 1995). In all the grain legumes expressing αAI-1 in their cotyledons, there is high resistance to the bruchid species *C. chinensis*, *C. maculatus*, *C. analis,* and *Bruchus pisorum* (L.) (Shade et al., 1994; Schroeder et al., 1995; Ishimoto et al., 1996; Morton et al., 2000; Sarmah et al., 2004; Ignacimuthu & Prakash, 2006; De Sousa-Majer et al., 2007; Solleti et al., 2008). The potential of this approach has been demonstrated even under field conditions, where transgenic pea seeds were completely resistant to *B. pisorum* (Morton et al., 2000).

In the present study, we assessed the resistance of αAI-1 transgenic cowpea and chickpea lines and several conventional chickpea cultivars to the bruchid species *C. chinensis*, *C. maculatus*, and *Acanthoscelides obtectus* (Say). The latter is known to be tolerant to αAI-1 (Ishimoto & Kitamura, 1992). The two *Callosobruchus* species are considered to be major pests of chickpea and cowpea. *Acanthoscelides obtectus* has spread throughout the world and, although primarily attacking the common bean,

it has also become a pest of both cowpea and chickpea (CABI crop protection compendium, available on www.cabi.org/cpc). For the first time, we did a simultaneous evaluation of the resistance of different αAI-1-expressing legume species, lines and/or cultivars to three major bruchid pests providing a thorough assessment of the potential of this resistance trait.

MATERIALS AND METHODS

Insects

The experiments were carried out with three bruchid species, all provided by C. Adler (Julius Kühn-Institut, Germany): *A. obtectus*, *C. chinensis,* and *C. maculatus.* The strains are colonized in the laboratory since 1967 (*A. obtectus*, *C. chinensis*) or 1998 (*C. maculatus*), their geographical origin is unknown. Colonies were maintained on both *C. arietinum* and *V. unguiculata* seeds for the respective experiments for at least five generations in a climate chamber at 25°C, 50% RH, and total darkness.

Seeds

Twelve chickpea and ten cowpea genotypes were included in this study (Table 1). Transgenic chickpea seeds of the cultivar Semsen expressing αAI-1 have been described (Sarmah et al., 2004). The corresponding Semsen non-transgenic parental line was included as a control. Additionally, the following conventional chickpea cultivars were included: the Desi type cultivars ‘Vijay’ (a high yielding cultivar released in central India; resistant to *Fusarium oxysporum* and *Helicoverpa armigera*), ICCC 37 (a high yielding cultivar released in Andhra Pradesh, India; resistant to *F. oxysporum*, moderately resistant to *H. armigera*, and moderately tolerant to root rot), ICCV 10 (a high yielding cultivar released in southern and central India; resistant to *F. oxysporum* and drought tolerant), ICC 506 (a cultivar resistant to *H. armigera*) and the Kabuli type ICCV 2 (a cultivar resistant to *F. oxysporum* and tolerant to drought, salinity, and heat stress), with hitherto unknown resistance to bruchids, as well as four Desi cultivars with reported resistance to *C. maculatus* (ICC 12422, ICC 4969, ICC 14336, and ICC 4957) (Erler et al., 2009). These conventional cultivars and the respective information were provided by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India.

Transgenic cowpea lines expressing αAI-1 were developed in two diverse cowpea genotypes (Popelka et al., 2006; Higgins et al., in press). The cowpea parental genotypes from which the transgenic lines were developed were breeding line IT86D-

1010, developed at the International Institute of Tropical Agriculture (IITA), Nigeria, and the Japanese cultivar ‘Sasaque’. The transgenic αAI-1 expressing and non-expressing lines developed from IT86D-1010 were TCP 14A and NTCP 14A, respectively, while from cultivar Sasaque, three independently transformed lines expressing αAI-1 (T 170, T 239, and T 310) and their corresponding non-transformed null-pair lines (NT 170, NT 239, and NT 310) were assayed.

As an additional control, the commercially available chickpea and cowpea seeds used to rear the bruchids, purchased from a local supermarket, were also included in the experiments.

Physical characteristics of the seeds are presented in Table 1. Seed weight is the average of 100 seeds. To determine the average seed coat thickness, ten dried seeds per genotype were peeled, and the thickness of the coat at the side of the seed was measured using an Absolute Digimatic 500-181U micrometer (Mitutoyo, Urdorf, Switzerland).

Experimental set-up

Experimental conditions were identical to the rearing conditions, i.e., 25°C, 50% RH, and total darkness. The experiments were carried out with each combination of bruchid species and seeds from chickpea and cowpea separately. For the experiments with *C. chinensis* and *C. maculatus*, 30 seeds of each genotype were placed individually in an open Petri dish (2.2x2.2x1 cm) and arranged randomly in a large box (100x50x20 cm). Approximately 2000 newly emerged adult beetles were released into the box and allowed to oviposit for 24 h. Embryonic development, which is visible through the egg chorion, was inspected on a daily basis and as soon as the first larva started chewing into the seed, all other larvae on the same seed were removed with a scalpel to avoid interference among multiple larvae developing in a single seed.

Because *A. obtectus* does not attach the eggs to the seeds, eggs were collected by carefully sieving the seeds on which adult beetles had been depositing eggs for 24 hours. Into each seed, a hole of 1 mm depth was pierced with a needle. After hatching, one larva per seed was carefully introduced into the hole with a fine brush, checked for physical integrity, and observed until it started chewing into the seed. Emerging adults were collected daily from individual seeds, transferred into a 0.2 ml cup and immediately frozen and stored at -20°C until they were dried and weighed.

Chickpea and cowpea resistance to bruchids and stage-specific mortality

Resistance of each chickpea and cowpea genotype to bruchids was calculated as the percentage of seeds in which no adult bruchid emerged. Seeds where no adult bruchid emerged were dissected and the stage-specific mortality determined. We distinguished whether the bruchid (i) failed to perforate the seed coat and enter the cotyledons; (ii) died inside the seed in the larval (further referred to as within-seed larval mortality) or (iii) pupal stage; or (iv) failed to emerge from the seed after successfully completing development. Because only bruchid larvae feeding on the cotyledons and embryonic axis of a seed are exposed to αAI-1, within-seed larval mortality in the different transgenic and corresponding non-transgenic chickpea and cowpea genotypes was analyzed.

Impact of host seeds on bruchid life-history parameters

For all bruchid adults emerging from the different non-transgenic chickpea cultivars, within-seed developmental time (WSD) and adult dry weight (ADW) were determined to assess sublethal effects on the bruchids. The WSD was calculated by measuring the time from a larva starting to chew into the seed until the emergence of the adult. Emerged adults were sexed and their ADW determined after drying them at 60°C for 72 h using a MX5 microbalance (Mettler Toledo, Greifensee, Switzerland). For both sexes of each bruchid species, the impact when feeding on the different chickpea cultivars was evaluated by correlating WSD and ADW with mean seed weight, seed coat thickness, resistance, and the within-seed larval mortality rate.

We did not assess the impact of the seed characteristics for cowpea, because the parental lines came from only two genetic backgrounds.

Data analyses

All data were analyzed using the software R (version 2.13.2). Resistance and within-seed larval mortality rates of the transgenic and corresponding null-pair chickpea and cowpea lines, respectively, were analyzed pairwise using Fisher's exact test. In case of the cowpea breeding line IT86D-1010, three pairwise comparisons between the parental, transgenic, and null-pair line were conducted and the α level adjusted according to the Bonferroni method, resulting in $\alpha = 0.017$.

RESULTS

Chickpea genotypes

Results for the chickpea genotypes with respect to resistance and within-seed larval mortality of the three bruchid species are presented in Fig. 1. The parental Semsen line had, compared to the other non-transgenic cultivars, a relatively high resistance to all three bruchid species. The transgenic Semsen line was completely resistant to *C. maculatus* and nearly so to *C. chinensis*. However, the difference to the parental line was only significantly different for the latter bruchid species ($p < 0.01$). As expected, resistance to *A. obtectus* was not increased in the transgenic line. Resistance of the other non-transgenic cultivars was highly variable within each of the three bruchid species, but also varied among species. Resistance was highest against *C. maculatus* (10-87%), followed by *A. obtectus* (3-67%), and *C. chinensis* (0-33%).

Within-seed larval mortality of all bruchid species was low in the non-transgenic chickpea cultivars. Highest mortality rates for *A. obtectus*, *C. chinensis,* and *C. maculatus* were 20%, 13%, and 26%, respectively. In contrast, within-seed larval mortality in the transgenic line was 100% and 97% for *C. maculatus* and *C. chinensis*, respectively, and, in both cases, significantly higher than the parental Semsen line ($p < 0.001$). The resistance of the transgenic Semsen line to *C. chinensis* was exclusively due to within-seed larval mortality. In the case of *C. maculatus*, some mortality was caused by the fact that larvae failed to perforate the seed coat. However, all larvae reaching the cotyledon subsequently died in the larval stage. There was no difference in within-seed larval mortality of *A. obtectus* between the transgenic line and its control.

In all species, there were differences between the overall resistance, (i.e., total mortality rate) and the within-seed larval mortality rate in most genotypes (results are illustrated in Supplementary Fig. 1). For *A. obtectus* and *C. chinensis*, this difference was exclusively due to adults failing to emerge from the seed after successfully completing their development. In contrast, *C. maculatus* larvae frequently failed to perforate the seed coat. Additionally, there was a single case of mortality in the pupal stage in the latter species.

In the case of the chickpea seeds, the WSD of the emerging beetles was positively correlated with seed coat thickness (except for *A. obtectus* males) and the overall resistance (Table 2). The ADW was negatively correlated with seed coat thickness

and resistance (except for *C. maculatus* males in both cases) (Table 2). With one exception, neither the WSD nor the ADW data correlated with the within-seed larval mortality rate. The WSD and ADW data are provided in detail in Supplementary Table 1.

Cowpea genotypes

Results for the cowpea genotypes with respect to resistance and within-seed larval mortality of the three bruchid species are presented in Fig. 2. The transgenic IT86D-1010 line (TCP 14A) was completely resistant to the two susceptible *Callosobruchus* species, significantly more than both the corresponding null-pair line (NTCP 14A) and parental line (IT86D-1010) (for both, $p < 0.001$). In contrast, the transgenic line was significantly more susceptible to *A. obtectus* than the parental line ($p = 0.007$), but did not differ from the null-pair line. Furthermore, the null-pair line was significantly more susceptible to all bruchid species than the parental line (for all, $p < 0.001$). All transgenic Sasaque lines were completely resistant to both *Callosobruchus* species, significantly more than their corresponding null-pair lines (for all, $p < 0.001$). As expected, none of the transgenic lines was completely resistant to *A. obtectus*. However, the transgenic line T 170 was more resistant ($p = 0.030$) and the transgenic line T 310 more susceptible ($p < 0.001$) than their corresponding null-pair lines.

Within-seed larval mortality of the *Callosobruchus* species was 100% in all transgenic lines, significantly higher than in their corresponding null-pair lines and the parental IT86D-1010 line, respectively (for both, $p < 0.001$). No significant differences could be detected between the IT86D-1010 parental and its null-pair for these two bruchids. Mortality of *A. obtectus* was mostly due to within-seed larval mortality (Supplementary Fig. 1). Mortality was significantly higher in the parental IT86D-1010 line compared to the corresponding transgenic ($p = 0.015$) and the null-pair ($p < 0.001$) line. The two latter lines did not differ significantly from each other. In the Sasaque lines, a significantly higher within-seed larval mortality was observed in the transgenic line T 170 compared to the corresponding null-pair line ($p = 0.030$) and in the null-pair line NT 310 compared to the corresponding transgenic line ($p = 0.005$).

In cowpea, few adults from *A. obtectus* and *C. maculatus* failed to emerge from the seeds while this was not observed at all for *C. chinensis* (Supplementary Fig. 1). *Acanthoscelides obtectus* mainly died in the larval stage inside the seeds. In contrast,

larvae of the two *Callosobruchus* species frequently failed to enter the seeds. Mortality in the pupal stage was observed only once in *C. chinensis*.

DISCUSSION

All transgenic cowpea lines expressing αAI-1 were completely protected from the bruchid species *C. chinensis* and *C. maculatus*. The single chickpea line expressing the inhibitor was also completely resistant to *C. maculatus* and highly resistant to *C. chinensis*. This is not surprising, as the two *Callosobruchus* species are known to be susceptible to αAI-1(Ishimoto & Kitamura, 1989) and it confirms earlier reports of increased resistance of αAI-1 transgenic legumes to these bruchids (Ishimoto et al., 1996; Sarmah et al., 2004; Ignacimuthu & Prakash, 2006; Solleti et al., 2008). The significant increase in within-seed larval mortality clearly demonstrates that αAI-1 was the cause of this effect. The fact that the transgenic chickpea line was not completely resistant to *C. chinensis* is likely to be due to a lower expression level of αAI-1 in the transgenic chickpea line compared to the cowpea lines tested (Higgins TJV, unpublished). It is a common observation that independent transgenic legume lines display varying levels of transgene expression (Shade et al., 1994; Sarmah et al., 2004; Solleti et al., 2008). αAI-1 expression level-dependent resistance of susceptible bruchids has been reported for pea (Shade et al., 1994; Morton et al., 2000), and other transgenic chickpea and cowpea lines had only detrimental, but not lethal impacts on *C. chinensis* and *C. maculatus* (Sarmah et al., 2004; Ignacimuthu & Prakash, 2006; Solleti et al., 2008). Although the experiment was conducted only for a single bruchid generation, we can assume that the initial level of resistance would not decrease during seed storage. αAI-1 is a seed storage protein, which are known to be highly stable, not likely to be changed in dry mature seeds, and only broken down during germination and seedling growth (Ladizinsky & Hymowitz, 1979; Chrispeels & Raikhel, 1991). Nevertheless, a high expression level is required to achieve a complete protection of the stored seeds and to prevent development of resistance in susceptible species, but also that αAI-1 has a significant detrimental effect on the survival of susceptible bruchid larvae. The recent finding that bruchids rely heavily on water produced during carbohydrate metabolism (Murdock et al., 2012), in combination with the fact that grain legume seeds are also rich in proteins, implies that αAI-1 not only limits energy production in susceptible larvae, but also deprives them of water.

As expected, the transgenic chickpea and cowpea lines were not resistant to *A. obtectus*, whose α-amylase is not inhibited by αAI-1 (Ishimoto & Kitamura, 1992). However, in two out of four cowpea lines there were significant differences in both resistance and within-seed larval mortality between the transgenic lines and their respective control lines. Whereas the cause of the observed differences remains subject to speculation, it suggests that the transformation procedure has caused some changes to the cowpea seed that affect the bruchids. For example, it is known that the process of tissue culture, used in the generation of transgenic plants, can lead to phenotypic changes often called somaclonal variation (Larkin & Scowcroft, 1981; Pellegrineschi, 1997).

Comparing the performance of the different bruchid species on the non-transgenic chickpea cultivars, it became evident that certain cultivars were more resistant than others to all three bruchid species. This included the four cultivars reported to be partially resistant to *C. maculatus* by Erler et al. (2009). But whereas in the previous study three out of these four cultivars were completely resistant, none of them was completely resistant to *C. maculatus* in our experiment and the resistance against *C. chinensis* and *A. obtectus* was even lower. In our study, both chickpea and cowpea seeds were more susceptible to *C. chinensis* than to *A. obtectus* and *C. maculatus*. This illustrates the difficulty of extrapolating the results obtained with a single bruchid strain; resistance not only varies among bruchid species, but also between strains of a species. For example, the cowpea landrace TVu 2027, denoted as bruchid resistant, was in fact only tested with *C. maculatus* (Singh, 1977; Singh et al., 1985). Whether this landrace is also reasonably resistant to other bruchid species is not known. Furthermore, this genotype was not only shown to be resistant for a limited period only, but was also highly susceptible to another strain of *C. maculatus* found in Nigeria (Shade et al., 1999; Murdock et al., 2008).

Aside from the expression of αAI-1, the major source of resistance to all three bruchid species was the seed coat. In chickpea, the difference between within-seed larval mortality and resistance for *C. chinensis* and *A. obtectus* was exclusively due to adults failing to emerge from the seeds. Larvae failing to enter the seeds occurred regularly in *C. maculatus* only. The role of the seed coat in defense against bruchids is also supported by the fact that WSD and ADW was significantly correlated with seed coat thickness in chickpea. Unfortunately, a thicker seed coat also makes the chickpea less desirable for human consumption (Moreno & Cubero, 1978; Gil &

Cubero, 1993). Whereas there is consensus that the seed coat has an impact on bruchid resistance in chickpea, its value in cowpea is controversial. While Edde & Amatobi (2003) claim that the seed coat has no value in protecting cowpea against *C. maculatus*, Lattanzio et al. (2005) state that resistance factors in the seed coat must also be considered in the biochemical defense of cowpea against *C. maculatus*. Finally, Souza et al. (2011) demonstrated that defense compounds in the seed coat of non-host legumes can significantly contribute to protection against bruchids.

The significant correlation of WSD and ADW with resistance in chickpea indicates that the resistance factor(s) also cause sublethal effects on the surviving beetles, leading to a reduced fitness which will contribute to a delay in bruchid population growth in the stored seeds. But even though resistance in a range of 80-90%, as observed in those non-transgenic cultivars with the highest resistance, may increase the period until a certain damage threshold is exceeded, multivoltinism, short generation time, and high fertility of the bruchid species means significant loses will occur under common storage scenarios, where farmers would store their crops for six months or more (Southgate, 1979). According to Erler et al. (2009), only genotypes with a resistance higher than 90% can be considered as practically resistant. Hence, none of the non-transgenic chickpea cultivars tested in our study would be considered resistant to any of the three tested bruchid species. Furthermore, the beetles in our experiment developed in the respective seeds only for a single generation. Bruchids are known to be able to quickly adapt to new hosts. This has for example been reported for *A. obtectus* infesting chickpea (Tucić et al., 1997) and for *C. maculatus* infesting cowpea (Fricke & Arnqvist, 2007; Zhu-Salzman & Zeng, 2008). Especially for the two *Callosobruchus* species, which have been attacking both chickpea and cowpea for thousands of generations, the efforts to find new resistance traits in wild relatives and transfer them to domesticated legumes, which was already found to be difficult *per se* (Sarmah et al., 2004; Murdock et al., 2008), may not provide long-term control. Shade et al. (1999) argue that it is likely that *C. maculatus* has encountered most resistance genes present in both wild and domesticated *Vigna* species and resistance achieved by conventional breeding will therefore be of low durability. The situation should be different for introduced bruchid species, such as *A. obtectus*. Pelegrini et al. (2008) identified an α-amylase inhibitor in cowpea called VuD1, which efficiently inhibits α-amylases from the αAI-1 tolerant bruchids *A. obtectus* and *Zabrotes subfasciatus*, both new world species, but not *C.*

maculatus. The authors suggested that the gene coding for VuD1 could be transferred into other plants to control these bruchids, but it should also be possible to develop cowpea cultivars with a VuD1-based resistance to *A. obtectus* and *Z. subfasciatus* by conventional breeding.

Independent of whether the resistance is achieved by conventional breeding or genetic engineering, bruchid management should not be based on a single resistance factor alone, but a combination of different approaches to maximize efficiency and sustainability of bruchid management (Lüthi et al., 2010). This would not only reduce damage, but also prevent or delay development of resistance to αAI-1. Hermetic storage of transgenic seeds in drums or bagging utilizing triple plastic bags (Murdock et al. 2003), or releasing natural enemies (Sanon et al., 1998, Schmale et al., 2003; Velten et al., 2008) are powerful approaches that could be combined with the transgenic seeds. For the combination with natural enemies, this means, however, that the insecticidal trait in the αAI-1 transgenic seeds should not interfere with the biological control services provided by natural enemies, in particular hymenopteran parasitoids (Romeis et al., 2004). Hosts developing in transgenic seeds have ingested αAI-1, therefore parasitoids of the larval and pupal stages of bruchids might be exposed to the inhibitor when attacking such hosts. The potential interference with these biological control organisms should thus be considered in the non-target risk assessment of αAI-1 transgenic legumes prior to commercial release (Romeis et al., 2008). A conceptual model describing how transgenic legume seeds expressing αAI-1 could interfere with bruchid control by parasitoids has been developed (Lüthi et al., 2010). An initial non-target risk assessment of αAI-1 transgenic legumes revealed that harmful effects on the inhibitor on parasitoids cannot be discounted (Álvarez-Alfageme et al., 2012). Further research will be required to determine whether αAI-1 expressing chickpea and cowpea have a negative impact on this important group of non-target organisms. But if the impact on bruchid parasitoids can be shown to be minimal, we believe that αAI-1 transgenic legumes are a leap in the development of bruchid-resistant legume seeds and could significantly contribute to food security in developing countries.

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Table 1. Chickpea and cowpea genotypes included in the experiment, their plant background, average seed weight, and seed coat thickness. Transgenic lines are indicated with an asterisk.

	Genotype	Plant background	Weight per seed (mg)	Seed coat thickness (mm)
CHICKPEA	*Semsen TG	Desi	228	0.15
	Semsen PL	Desi	253	0.16
	ICCV 2	Kabuli	232	0.06
	Vijay	Desi	180	0.14
	ICCG 37	Desi	184	0.13
	ICCV 10	Desi	165	0.11
	ICC 506	Desi	155	0.17
	ICC 12422	Desi	163	0.15
	ICC 4969	Desi	119	0.13
	ICC 14336	Desi	164	0.12
	ICC 4957	Desi	127	0.14
	Rearing var.	Kabuli	447	0.03
COWPEA	IT86D-1010		164	0.06
	*TCP 14A	IT86D	151	0.06
	NTCP 14A	IT86D	122	0.06
	*T 170	Sasaque	149	0.07
	NT 170	Sasaque	157	0.07
	*T 239	Sasaque	138	0.07
	NT 239	Sasaque	128	0.07
	*T 310	Sasaque	151	0.07
	NT 310	Sasaque	145	0.07
	Rearing var.		217	0.03

Table 2. Pearson correlation coefficients for mean within-seed developmental time (WSD) and mean adult dry weight (ADW) of females (f) and males (m) of the bruchid species *A. obtectus*, *C. chinensis,* and *C. maculatus* emerged from different non-transgenic chickpea cultivars correlated to seed weight, seed coat thickness (see table 1), bruchid-resistance (see Fig. 1), and the within-seed larval mortality rate (see Fig. 2). *p < 0.05, ***p < 0.01, “n.s.” indicates that the correlation was not significant.

	Species	Sex	Seed weight	Seed coat thickness	bruchid resistance	Larval mortality rate
WSD	*A. obtectus*	f	n.s.	0.685*	0.601*	0.733*
		m	n.s.	n.s.	0.748***	n.s.
	C. chinensis	f	n.s.	0.824***	0.758***	n.s.
		m	n.s.	0.782***	0.795***	n.s.
	C. maculatus	f	n.s.	0.736*	0.744***	n.s.
		m	n.s.	0.684*	0.676*	n.s.
ADW	*A. obtectus*	f	n.s.	-0.734***	-0.800***	n.s.
		m	n.s.	-0.835***	-0.770***	n.s.
	C. chinensis	f	n.s.	-0.841***	-0.710*	n.s.
		m	n.s.	-0.772***	-0.842***	n.s.
	C. maculatus	f	n.s.	-0.754***	-0.815***	n.s.
		m	n.s.	n.s.	n.s.	n.s.

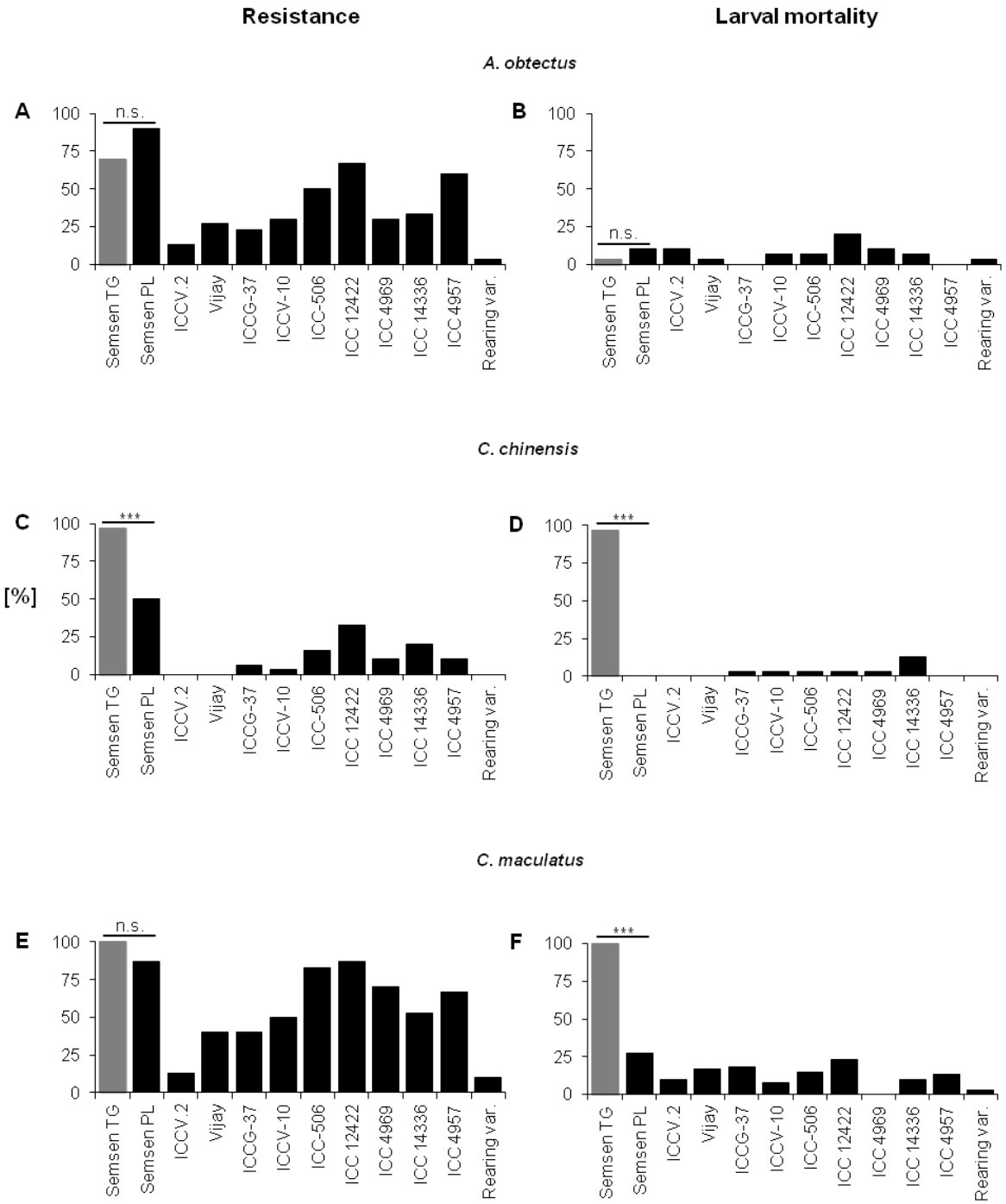

Figure 1. Resistance (percentage of seeds from which no adult beetle emerged) and within-seed larval mortality in different chickpea genotypes for *Acanthoscelides obtectus*, *Callosobruchus chinensis*, and *C. maculatus*. A pairwise comparison was made among the transgenic (TG, bar in grey) and parental (PL) Semsen line using Fisher's exact test (*p < 0.05, ***p < 0.01, n.s. = not significant).

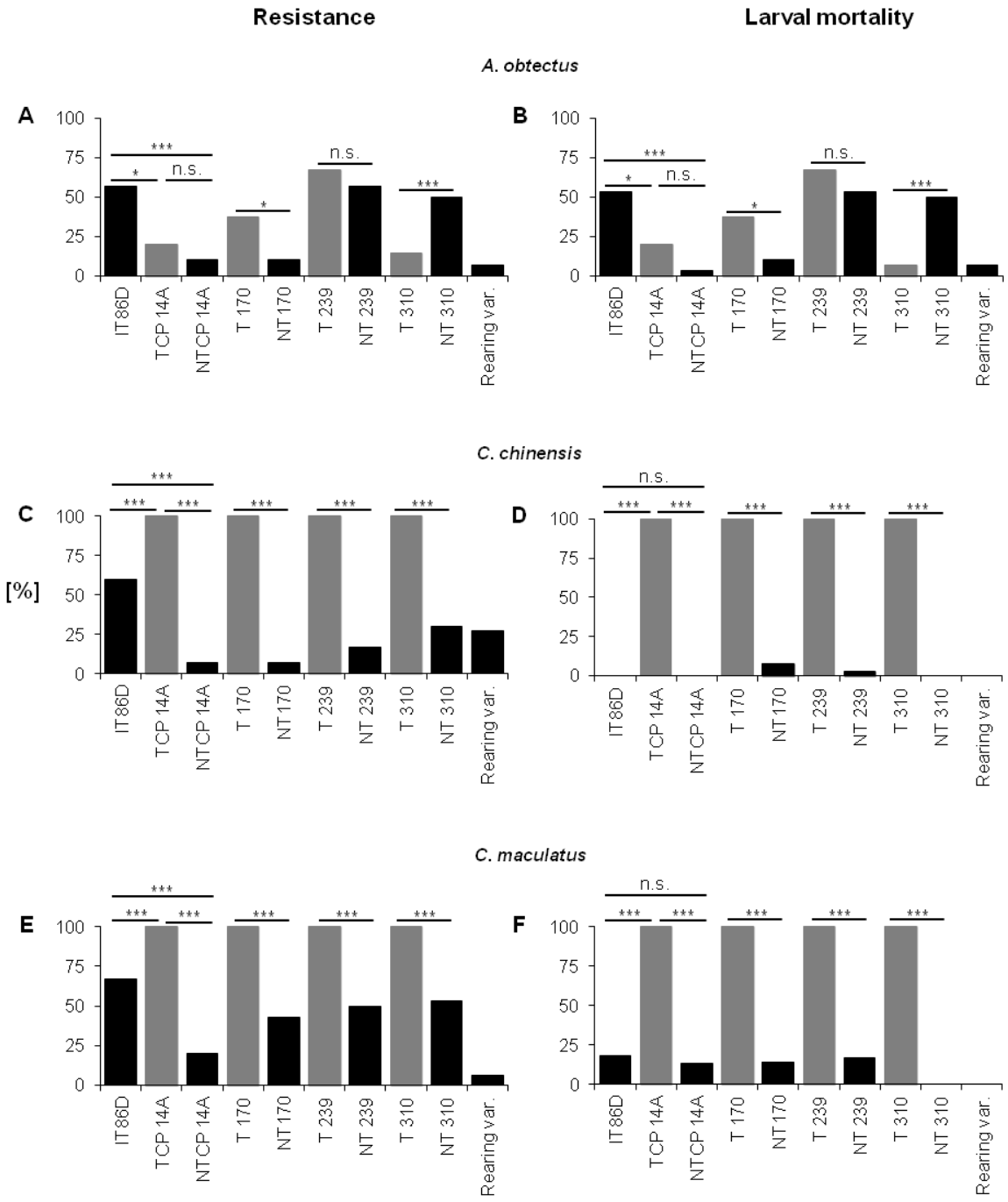

Figure 2. Resistance (percentage of seeds from which no adult beetle emerged) and within-seed larval mortality in different cowpea genotypes for *Acanthoscelides obtectus*, *Callosobruchus chinensis*, and *C. maculatus*. Comparison was made among the three IT86D lines (IT86D: parental line; TCP14A: transgenic line; NTCP14A: null-pair line) and pairwise among the transformed (T, bar in grey) and respective non-transformed (NT) Sasaque lines 170, 239 and 310 using Fisher's exact test (*$p < 0.05$, ***$p < 0.01$, n.s. = not significant; for the IT86D lines, the α level was adjusted for three pairwise comparisons using the Bonferroni method, resulting in $\alpha = 0.017$).

Table 1S. Number of emerged adult beetles (N), means and standard deviation (SD) of adult dry weight (ADW) and within-seed developmental time (WSD) for females and males of *Acanthoscelides obtectus*, *Callosobruchus chinensis,* and *C. maculatus* emerging from different chickpea and cowpea genotypes. Transgenic lines are indicated with an asterisk.

			females			males		
			N	DW ± SD	WSD ± SD	N	DW ± SD	WSD ± SD
A. obtectus	Chickpea	Semsen TG*	4	2.928 ± 0.417	49.5 ± 4.4	5	2.700 ± 0.176	47.8 ± 4.8
		Semsen PL	2	3.297 ± 0.127	46.5 ± 2.1	1	2.702 ± 0	45 ± 0
		ICCV 2	15	4.132 ± 0.374	41.3 ± 1.7	11	3.369 ± 0.250	41.2 ± 2.8
		Vijay	11	3.847 ± 0.422	42.5 ± 2.6	11	2.963 ± 0.379	42.5 ± 3.1
		ICCG 37	15	3.663 ± 0.310	43.7 ± 3.1	8	2.951 ± 0.305	43.1 ± 5.9
		ICCV 10	12	3.679 ± 0.475	44.4 ± 3.2	9	2.967 ± 0.527	42.9 ± 5.1
		ICC 506	8	3.533 ± 0.443	42.7 ± 1.8	7	2.891 ± 0.338	44.5 ± 4.6
		ICC 12422	6	2.791 ± 0.572	52.7 ± 5.6	3	2.352 ± 0.533	49.3 ± 3.2
		ICC 4969	10	3.393 ± 0.304	42.6 ± 2.4	10	2.964 ± 0.296	39.5 ± 1.8
		ICC 14336	10	3.628 ± 0.426	44.2 ± 3.6	10	3.051 ± 0.267	42.5 ± 2.8
		ICC 4957	6	3.357 ± 0.318	42.8 ± 2.6	6	2.573 ± 0.212	43.3 ± 4.9
		Rearing var.	16	4.026 ± 0.474	42.4 ± 5.5	13	3.429 ± 0.171	39.2 ± 1.6
	Cowpea	IT86D-1010	7	3.709 ± 0.401	40.7 ± 5.6	6	3.289 ± 0.308	37.5 ± 3.4
		TCP 14A*	16	3.635 ± 0.256	38.7 ± 1.8	8	3.017 ± 0.301	37 ± 2.3
		NTCP 14A	10	3.771 ± 0.387	38.9 ± 3.8	17	3.024 ± 0.402	38.9 ± 5.7
		T 170*	6	3.549 ± 0.640	41.2 ± 8.9	13	2.773 ± 0.482	39.5 ± 8.8
		NT170	13	3.623 ± 0.404	38.9 ± 3.1	14	2.990 ± 0.484	37.7 ± 3.5
		T 239*	5	3.602 ± 0.175	39.4 ± 2.3	5	2.859 ± 0.269	36.8 ± 1.3
		NT 239	6	3.535 ± 0.160	40.5 ± 3.2	7	3.014 ± 0.216	41.3 ± 10.6
		T 310*	11	3.550 ± 0.543	39.6 ± 4.5	15	3.055 ± 0.294	37.3 ± 2.3
		NT 310	5	3.699 ± 0.148	40.2 ± 4.0	11	2.912 ± 0.574	41.3 ± 6.8
		Rearing var.	14	3.275 ± 0.508	39.1 ± 2.1	13	3.072 ± 0.416	36.8 ± 2.3
C. chinensis	Chickpea	Semsen TG*	1	2.218 ± 0	42.0 ± 0	0		
		Semsen PL	7	2.240 ± 0.239	36.7 ± 2.4	8	1.504 ± 0.181	34.5 ± 5.1
		ICCV 2	13	2.871 ± 0.133	29.4 ± 0.9	17	1.790 ± 0.073	29.1 ± 1
		Vijay	19	2.707 ± 0.153	31.1 ± 1.3	11	1.715 ± 0.095	30.2 ± 0.9
		ICCG 37	16	2.653 ± 0.185	31.9 ± 1.4	12	1.687 ± 0.148	31.3 ± 1.3
		ICCV 10	18	2.741 ± 0.221	30.7 ± 1.1	11	1.779 ± 0.117	29.9 ± 0.8
		ICC 506	10	2.521 ± 0.295	32.6 ± 2.3	12	1.546 ± 0.159	32.0 ± 2.3
		ICC 12422	11	2.436 ± 0.244	34.0 ± 3.1	9	1.495 ± 0.247	33.8 ± 4.5
		ICC 4969	19	2.538 ± 0.187	31.5 ± 1.8	8	1.550 ± 0.158	30.6 ± 0.9
		ICC 14336	12	2.496 ± 0.213	32.0 ± 2.2	12	1.621 ± 0.092	30.4 ± 1.7
		ICC 4957	19	2.425 ± 0.256	32.9 ± 2.1	8	1.519 ± 0.076	32.3 ± 1.8
		Rearing var.	17	3.023 ± 0.190	29.1 ± 0.7	12	1.766 ± 0.135	28.2 ± 0.9
	Cowpea	IT86D-1010	5	2.642 ± 0.066	28.2 ± 1.3	7	1.784 ± 0.092	28.7 ± 3.2
		TCP 14A*	0			0		
		NTCP 14A	15	0.686 ± 0.123	29.1 ± 2.0	13	1.725 ± 0.233	31.1 ± 4.7
		T 170*	0			0		
		NT170	15	2.592 ± 0.126	29.7 ± 2.8	13	1.685 ± 0.114	29.0 ± 3.4
		T 239*	0			0		
		NT 239	9	2.698 ± 0.119	29.0 ± 2.2	16	1.743 ± 0.136	29.1 ± 3.4
		T 310*	0			0		
		NT 310	15	2.665 ± 0.104	31.1 ± 3.9	6	1.738 ± 0.098	27.8 ± 1.2
		Rearing var.	18	2.629 ± 0.154	27.8 ± 0.6	4	1.804 ± 0.162	27.0 ± 0
C. maculatus	Chickpea	Semsen TG*	0			0		
		Semsen PL	4	2.099 ± 0.398	51.5 ± 7.6	0		
		ICCV 2	12	3.256 ± 0.332	36.8 ± 1.5	13	1.979 ± 0.291	38.7 ± 8.9
		Vijay	8	2.400 ± 0.388	43.3 ± 3.3	7	1.626 ± 0.314	45.1 ± 4.4
		ICCG 37	9	2.590 ± 0.522	42.4 ± 4.8	8	1.614 ± 0.321	46.4 ± 12.0
		ICCV 10	8	2.596 ± 0.473	46.0 ± 8.7	7	1.711 ± 0.192	42.1 ± 3.5
		ICC 506	4	2.188 ± 0.579	50.0 ± 5.5	1	1.897 ± 0	60.0 ± 0
		ICC 12422	2	2.813 ± 0.520	46.5 ± 0.7	1	1.825 ± 0	43.0 ± 0
		ICC 4969	5	2.161 ± 0.232	42.4 ± 3.6	4	1.648 ± 0.296	45.3 ± 4.2
		ICC 14336	9	2.416 ± 0.459	42.2 ± 3.5	5	1.604 ± 0.417	44.8 ± 11.9
		ICC 4957	3	2.640 ± 0.349	44.7 ± 4.7	4	1.369 ± 0.308	54.5 ± 12.2
		Rearing var.	13	3.009 ± 0.603	42.8 ± 11	14	1.842 ± 0.210	38.8 ± 3.8
	Cowpea	IT86D-1010	6	3.109 ± 0.372	40.0 ± 5.2	4	1.978 ± 0.162	37.0 ± 1.8
		TCP 14A*	0			0		
		NTCP 14A	13	3.137 ± 0.301	36.6 ± 2.0	11	2.152 ± 0.278	39.3 ± 4.3
		T 170*	0			0		
		NT170	13	3.224 ± 0.314	37.4 ± 4.4	4	2.063 ± 0.207	35.3 ± 0.5
		T 239*	0			0		
		NT 239	11	3.166 ± 0.605	36.5 ± 0.9	4	2.159 ± 0.157	34.8 ± 1.0
		T 310*	0			0		
		NT 310	7	3.145 ± 0.336	37.5 ± 2.4	7	2.069 ± 0.430	36.8 ± 2.2
		Rearing var.	13	3.255 ± 0.398	38.2 ± 7.3	15	2.111 ± 0.381	35.7 ± 2.7

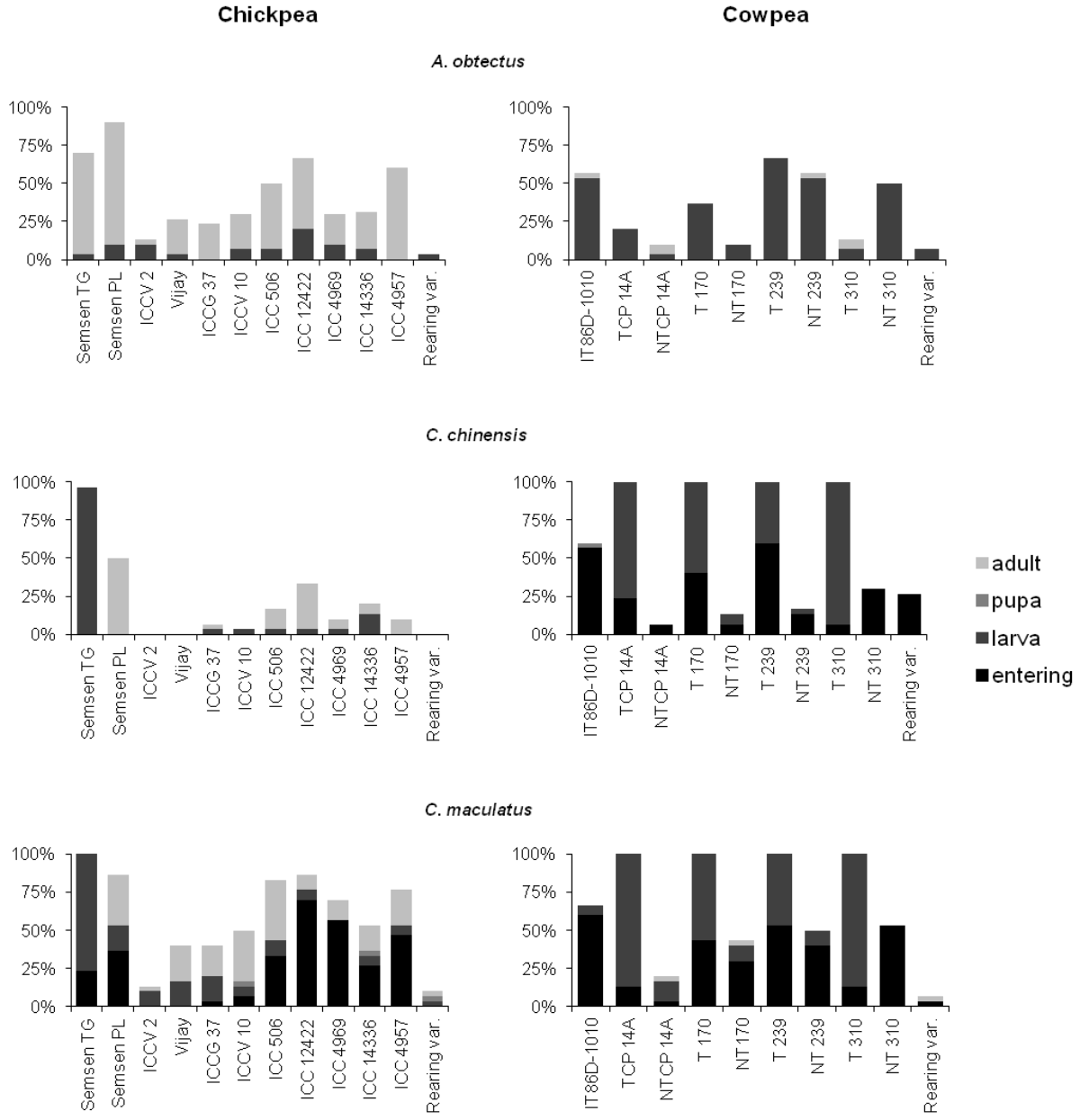

Figure 1S. Bruchid mortality rates in chickpea and cowpea genotypes. Stacked bars represent total mortality, single stacks indicate mortality rates for each respective stage (“Entering”: larva failed to enter seed; “Larva”, ”Pupa”: beetle died in the respective stage inside the seed; “Adult”: adult beetle failed to emerge from the seed).

CHAPTER 3

Characterization of Digestive Enzymes of Bruchid Parasitoids – Initial Steps for Environmental Risk Assessment of Genetically Modified Legumes

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ABSTRACT

Genetically modified (GM) legumes expressing the α-amylase inhibitor 1 (αAI-1) from *Phaseolus vulgaris* L. or cysteine protease inhibitors are resistant to several bruchid pests (Coleoptera: Chrysomelidae). In addition, the combination of plant resistance factors together with hymenopteran parasitoids can substantially increase the bruchid control provided by the resistance alone. If the strategy of combining a bruchid-resistant GM legume and biological control is to be effective, the insecticidal trait must not adversely affect bruchid antagonists. The environmental risk assessment of such GM legumes includes the characterization of the targeted enzymes in the beneficial species and the assessment of the *in vitro* susceptibility to the resistance factor. The digestive physiology of bruchid parasitoids remain relatively unknown, and their susceptibility to αAI-1 has never been investigated.

We have detected α-amylase and serine protease activities in all five bruchid parasitoid species tested. Thus, the deployment of GM legumes expressing cysteine protease inhibitors to control bruchids should be compatible with the use of parasitoids. *In vitro* inhibition studies showed that sensitivity of α-amylase activity to αAI-1 in the parasitoids was comparable to that in the target species. Direct feeding assays revealed that harmful effects of α-amylase inhibitors on bruchid parasitoids cannot be discounted and need further evaluation.

Based on: Álvarez-Alfageme, F., Lüthi, C., Romeis, J., 2012. Characterization of digestive enzymes of bruchid parasitoids – initial steps for environmental risk assessment of genetically modified legumes. PLOS ONE e36862.

INTRODUCTION

Grain legumes, also known as pulses or food legumes, are mainly cultivated in developing countries, where they are essential for nutrition. Pulses represent a source of income and livestock feed and meet the requirements of small-scale, low-income farmers in developing countries (Popelka et al., 2006). Grain legumes are commonly stored over extended periods to ensure supplies of household food and seed for sowing (Nukenine, 2010). Several coleopteran and lepidopteran pests are responsible for extensive losses to stored grain legumes because these pests develop and reproduce rapidly, completing multiple generations in the storage. In addition, insect pests increase the temperature and humidity of the stored pulses, which increases grain respiration and thereby reduces grain quantity and quality (Odogola, 1994). The average grain-weight loss for pulses due to insect pests is 20% (Phillips and Throne, 2010), although it can be up to 100% and is generally much higher than the loss caused by rodents, microorganisms, and other pests (Nukenine, 2010). Larvae of several *Acanthoscelides*, *Zabrotes*, and *Callosobruchus* spp. (Coleoptera: Chrysomelidae) are among the most important insect pests of pulses worldwide.

Many insects, especially those like bruchids that feed on starchy seeds, depend on α-amylases for survival (Franco et al., 2002). Because these enzymes are active in the digestive tract and play a key role in carbohydrate metabolism, they are ideal targets for seed-based pest management approaches. Genetically modified (GM) legumes (i.e., cowpeas, peas, chickpeas, and azuki beans) expressing the α-amylase inhibitor 1 (αAI-1) from the common bean, *Phaseolus vulgaris* L., are resistant to several bruchid species under laboratory (Schroeder et al., 1995; Ishimoto et al., 1996; Sarmah et al., 2004; Solleti et al., 2008) and field conditions (Morton et al., 2000). The deployment of GM legumes expressing other types of digestive enzyme inhibitors to control bruchids, such as plant protease inhibitors, has also been suggested (Koiwa et al., 1998; Amirhusin et al., 2004, 2007). Robust, reproducible, and efficient transformation procedures are available for many legumes species (Popelka et al., 2004). In addition, the combination of plant resistance factors together with biological control agents, especially hymenopteran parasitoids, can substantially increase the bruchid control provided by host-plant resistance alone (Schmale et al., 2001; Schmale et al., 2003; Velten et al., 2008). If the strategy of combining a bruchid-resistant GM legume and biological control is to be effective and

sustainable, the insecticidal trait expressed by the resistant crop must not adversely affect bruchid antagonists. A conceptual model describing how GM legume seeds expressing αAI-1 could harm the biological control service provided by parasitoids of bruchids has been developed by Lüthi et al. (Lüthi et al., 2010). The model consists of five sequential steps and could be applied for protease inhibitor-expressing plants as well. In the first two steps, the model (i) characterizes the targeted digestive enzymes in the beneficial species and (ii) assesses the *in vitro* susceptibility to the plant resistance factor. The information required to satisfy these two steps of the model are not available for bruchid parasitoids. In the case of bruchid parasitoids, the physiological and biochemical aspects of their nutrition remain relatively unknown, and their susceptibility to αAI-1 has never been investigated.

In this study, we have characterized the α-amylase and protease activities in extracts of larvae and adult females of five common hymenopteran exoparasitoids of last instar larvae or pupae of bruchid pests. We then conducted *in vitro* experiments to assess the susceptibility of the exoparasitoid α-amylases to αAI-1 from *P. vulgaris*; for comparison, these biochemical assays also included extracts of three bruchid species. Finally, we used direct feeding assays to evaluate the effects of a commercial wheat α-amylase inhibitor and a serine protease inhibitor on females of two parasitoid species.

MATERIALS AND METHODS

Insects

Bruchids

The following bruchids were obtained from C. Adler (Julius Kühn-Institut, Germany) and were maintained for several years on chickpea (*Cicer arietinum* L.) seeds (Kabuli type) at 24 ± 2°C, 60 ± 5% r.h., and complete darkness: *Acanthoscelides obtectus* (Say), *Callosobruchus chinensis* (L.), and *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae).

Parasitoids

Seeds infested with bruchids and parasitoids were sent to us by several investigators. *Heterospilus prosopidis* Viereck (Hymenoptera: Braconidae) parasitizing *C. chinensis* reared on Azuki bean [*Vigna angularis* (Willd.)] seeds were provided by M. Shimada (University of Tokyo, Japan). *Anisopteromalus calandrae*

(Howard) (Hymenotpera: Pteromalidae) and *Lariophagus distinguendus* (Först.) (Hymenoptera: Pteromalidae) reared on wheat (*Triticum aestivum* L.) seeds infested with *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) were obtained from J. Steidle (Hohenheim University, Germany). *Dinarmus basalis* (Rond.) (Hymenoptera: Pteromalidae) and *Eupelmus vuilleti* (Crw.) (Hymenoptera: Eupelmidae) on cowpea [Vigna unguiculata (L.) Walp.] seeds infested with *C. maculatus* were provided by J.P. Monge (Tours University, France). Upon arrival, seeds were kept in a climate chamber at 24 ± 2 °C, 60 ± 5% r.h., and complete darkness. Emerging adults were then transferred to plastic containers (10.5 cm diameter, 15 cm high) containing last-instar larvae and/or pupae of *C. chinensis* on chickpea seeds and were reared in the laboratory for at least two generations before the start of the experiments.
Females of all species but *H. prosopodis* are synovigenic, i.e., they are born with immature eggs and must feed on their host to sustain egg production.

Characterization of digestive enzymes in parasitoids and bruchids and *in vitro* inhibitory activity of purified *P. vulgaris* αAI-1

Preparation of insect extracts

Fourth-instar larvae of all bruchid species and third-instar larvae and 1-week-old females of all parasitoid species from the rearing colonies were used for the *in vitro* characterization of digestive enzymes. Insects were homogenized in ice-cold 0.15 M NaCl (100 insects ml^{-1}). The homogenates were centrifuged at 10,000 *g* for 5 min, and the supernatants were pooled and stored frozen at -20°C to obtain soluble protein extracts for determination of enzymatic activity. Total protein content was determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

Characterization of α-amylase activity

A series of overlapping buffers was used to generate a pH range from 3 to 11. Reaction buffers were: 0.1 M citrate (pH 3–6), 0.1 M phosphate (pH 6–7), 0.1 M Tris-HCl (pH 7–9), and 0.1 M glycine-NaOH (pH 9–11). All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$. Assays were performed using a modified version of the Bernfeld assay (Bernfeld, 1955). The standard assay volume was 100 µl, which contained 5 µg of insect protein extract and potato starch as substrate added to a final

concentration of 0.5% (w/v). After the samples had been incubated at 30 °C for 45 min, the reaction was stopped by the addition of 100 µl of dinitrosalicylic acid (DNSA) reagent, followed by boiling for 5 min in a thermoblock (1102, SKS Bio-medical Instruments Ltd., Luton, England). Then, 1 ml of distilled water was added to the solution, which was mixed and left at room temperature for 15 min. Finally, the absorbance was read at 540 nm in a spectrophotometer, and α-amylase activity was expressed as mg of maltose liberated min^{-1} mg $protein^{-1}$. A standard curve with maltose was constructed to calculate α-amylase activity.

The α-amylase activity of each bruchid and parasitoid extract was further characterized at its optimum pH in the presence of the specific inhibitors acarbose and wheat α-AI. Both inhibitors were incubated with the extracts at room temperature for 15 min before the substrate was added. The inhibitors were added in 10 µl of distilled water to a final concentration of 0.001% (w/v).

In vitro inhibitory activity of purified P. vulgaris αAI-1

The susceptibility of extracts of bruchid larvae and of parasitoid larvae and females to purified αAI-1 was determined by *in vitro* assay. αAI-1 purified from the seeds of *P. vulgaris* as described by Marshall and Lauda (1975) was provided by T.J.V. Higgins (CSIRO, Australia). Previous studies have indicated that the two *Callosobruchus* species used in the current investigation are highly susceptible to αAI-1 (Ishimoto and Kitamura, 1989) whereas the α-amylase activity of *A. obtectus* is not (Ishimoto and Kitamura, 1992). The α-amylase activity in the extract of each parasitoid and bruchid species was assayed at its optimum pH as described above. αAI-1 at concentrations ranging from 0 to 1 µg ml^{-1} was preincubated with the insect extracts for 15 min at room temperature before the substrate was added. Results were expressed as the percentage of protease activity relative to that in the absence of the inhibitor.

Characterization of general proteolytic activity

For determination of general proteolytic activity in the bruchid *C. chinensis* and in the parasitoids *H. prosopidis*, *E. vuilleti*, and *A. calandrae*, a series of overlapping buffers (see section 2.2.2) was used to generate a pH range from 3 to 11. The standard assay volume was 100 µl, which contained 10 µg of insect protein extract and sulfanilamide-azocasein as substrate added to a final concentration of 0.5% (w/v).

Samples were subsequently incubated overnight at 30°C. The reaction was stopped by the addition of 50 µl of ice-cold 10% TCA. Samples were then centrifuged at 10,000 *g* for 5 min, and 120 µl of the supernatant was added to 18 µl of 5 M NaOH. Finally, absorbance was measured at 450 nm using a SpectrafluorPlus plate reader (Tecan, Männedorf, Switzerland), and general proteolytic activity was expressed as Abs_{450} min^{-1} mg $protein^{-1}$.

The proteolytic activity of the *C. chinensis* extract and of each parasitoid extract was further characterized at its optimum pH in the presence of the following class-specific protease inhibitors: the serine protease inhibitors SKTI (soybean Kunitz trypsin inhibitor) and PMSF (phenylmethylsulphonyl fluoride), the cysteine protease inhibitors E-64 (L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane) and IAA (iodoacetamide), the aspartic protease inhibitor pepstatin-A, and the metalloprotease inhibitor EDTA (ethylenediaminetetraacetic acid); final assay concentrations were 1 mM, 10 µM, 10 µM, 1 mM, 10 µM, and 10 mM, respectively. Protease inhibitors were incubated at room temperature for 15 min before the substrate was added. All compounds were added in 10 µl of 0.15 M NaCl, except PMSF and pepstatin-A, which were added in 10 µl of DMSO (dimethyl sulfoxide). The doses tested were selected according to the concentrations recommended by Beynon and Salvesen (Beynon and Salvesen, 1989).

All enzymatic assays were carried out in triplicate, and appropriate blanks were used to account for spontaneous breakdown of substrates. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Effect of wheat α-AI and SKTI on female parasitoid survival

Bioassays were conducted to test the effect of the commercial inhibitors wheat α-AI and SKTI on the survival of females of the parasitoids *A. calandrae* and *E. vuilleti*. α-Amylase and serine protease inhibitors are known to have detrimental effects on pests of stored products. The two parasitoid species were selected because they are synovigenic and belong to different hymenopteran families. Females of *A. calandrae* and *E. vuilleti* from the rearing colonies were collected after emergence (< 24 h), individually kept in plastic boxes (2 x 2 x 1.5 cm), and provided with two 0.5-µl droplets of a 2 M sucrose solution containing different concentrations of either wheat α-AI or SKTI (0, 0.1, and 1%, w/v). In the bioassay with wheat α-AI, potato starch (20 µg ml^{-1}) was added to the sucrose solution to stimulate α-amylase, whereas in the

bioassay with SKTI, BSA (20 µg ml^{-1}) was added to stimulate protease activity. Thirty females were tested per treatment. The food solutions were changed three times per week and were never completely consumed during this time period. Survival of the females was recorded daily. Bioassays were conducted in a climate chamber at 25 ± 2 °C, 70 ± 5% RH, and 16:8 h L:D. Previous assays had shown that both parasitoids ingest the sucrose solution containing either of the two inhibitors (unpublished observations).

Data analysis

The survival response of female parasitoids to wheat α-AI or SKTI was analyzed using the Kaplan-Meier procedure and Logrank test. The α-level was set at 5%. Statistical analyses were conducted with the software package Statistica (Version 9, StatSoft Inc., Tulsa, OK, USA).

RESULTS

Characterization of α-amylase activity

We analyzed the optimum pH, the specific activity, and the effect of two specific α-amylase inhibitors on the hydrolysis of potato starch to characterize α-amylase activity in several bruchid and hymenopteran parasitoids (Table 1). α-Amylase activity was detected over a broad range of pH in both bruchids and parasitoids. In general, α-amylase activity was much higher in bruchids than in the parasitic wasps, and in females than in larvae of parasitoids. Hydrolysis of potato starch was greatest at pH 5.5 in all bruchid larvae and at pH 5 to 7 in parasitoid larvae and females. Hydrolysis of potato starch was greatly inhibited by acarbose and wheat αAI in extracts from all insects.

In vitro inhibitory activity of recombinant *Phaseolus vulgaris* αAI-1

In vitro inhibition studies were conducted to assess the susceptibility of bruchids and parasitoids to purified αAI-1. α-Amylase activity in extracts from all parasitoid larvae and females was reduced by the inhibitor (Fig. 1B–F). Except for *E. vuilleti* (Fig. 1C), inhibition was greater in females than in larvae of parasitoids. α-Amylase inhibition caused by αAI-1 in the susceptible bruchid species *C. chinensis* and *C. maculatus* was similar to that in the hymenopteran parasitoids (Fig. 1A). As expected, the α-amylase activity of *A. obtectus* was not inhibited by αAI-1 (Fig. 1A).

Characterization of general proteolytic activity

We characterized proteolytic activity in protein extracts of the bruchid *C. chinensis* and the parasitoids *H. prosopidis*, *E. vuilleti*, and *A. calandrae*. Hydrolysis of the general substrate azocasein occurred over a broad range of pH in all species, although the profile differed for *C. chinensis* vs. the three parasitoids (Table 2). While azocasein was hydrolyzed mainly at acidic pH values by the bruchid, and bruchid proteolytic activity was highest at pH 5.0, proteolysis in parasitoid larvae and females was highest at alkaline pH values. Proteolytic activities were further characterized with specific diagnostic protease inhibitors (Table 2). Proteolytic activity of *C. chinensis* was inhibited by the cysteine-like protease inhibitors E-64 and IAA, and by the aspartic protease inhibitor pepstatin-A. In contrast, general proteolysis was inhibited by the serine proteases inhibitors SKTI and PMSF in larvae of the parasitoids *H. prosopidis* and *A. calandrae,* and only by SKTI in *E. vuilleti* larvae. Hydrolysis of azocasein was reduced by SKTI and the metalloproteases inhibitor EDTA in females of *A. calandrae* and *E. vuilleti*. Together, these results indicate that the proteolytic mechanisms differ for the bruchid *C. chinensis* and the three parasitoids: whereas *C. chinensis* relies on cysteine and aspartic proteases, parasitoid larvae rely on serine proteases, and parasitoid females rely on serine proteases and metalloproteases.

Effect of two digestive inhibitors, wheat αAI and SKTI, on female parasitoid survival

The effect of wheat α-AI and the serine protease inhibitor SKTI on the survival of *E. vuilleti* and *A. calandrae* females was investigated *in vivo*. For *E. vuilleti*, female longevity in the control treatments was 51.0 ± 2.64 days (mean ± SE) in the wheat α-AI assay and 31.9 ± 1.67 days in the SKTI assay. For *A. calandrae*, female longevity in the control treatments was 75.8 ± 2.32 days in the wheat α-AI assay and 66.2 ± 3.65 days in the SKTI assay. The survival of *E. vuillleti* and *A. calandrae* fed with 1% wheat α-AI was significantly lower than in the control (P = 0.007 and P = 0.035, respectively) (Fig. 2A,C). SKTI significantly decreased the survival of *A. calandrae* at 0.1% and 1% concentration (P < 0.001 and P = 0.044, respectively) (Fig. 2D) whereas no effect was observed in *E. vuilleti* females (Fig. 2B).

DISCUSSION

Characterization of α-amylase activity

α-Amylase activity has been mainly studied in lepidopteran and coleopteran (including bruchids) storage pests. Only a few studies have characterized the α-amylase activity in insect predators (Ferreira and Terra, 1989; Zeng and Cohen, 2000; Boyd et al., 2002; Oliveira et al., 2006) and parasitoids (Kluh et al., 2005), and to our knowledge α-amylase activity has never been characterized in any parasitoid of bruchids. In our study, significant α-amylase activity was measured in extracts of all parasitoid species tested. Hydrolysis of potato starch was highest at pH 5–6 for larval extracts and at pH 6–7 for female extracts. Similar pH values for optimal α-amylase activity were recorded by Kluh et al. (2005) for three hymenopteran species that included adults of the ichneumonid *Venturia canescens* Grav., which is an endoparasitoid of lepidopteran larvae. As previously reported by Podoler and Applebaum (1971), Campos et al. (1989), and Franco et al. (2005), α-amylase activity in the bruchid larvae in the current study was optimal under acidic conditions. α-Amylase activity in parasitoid extracts was usually greater in females than in larvae. This difference might be due to the ability of female parasitoids to feed on carbohydrate-rich sources, like floral and extra-floral nectar (Jervis and Kidd, 1986). Except for extracts from *A. calandrae* and *L. distinguendus* females, α-amylase activity was lower in parasitic wasp extracts than in bruchid larval extracts. This is not surprising because bruchid larvae exclusively feed on starchy legume seeds and therefore rely mainly on α-amylases for food digestion.

In vitro inhibitory activity of recombinant *Phaseolus vulgaris* αAI-1

In our study, the α-amylase activity of all hymenopteran parasitoids was reduced when exposed to purified αAI-1. With the exception of *E. vuilleti*, *in vitro* inhibition was higher in females than in larvae. For comparison, larval extracts of the bruchids *A. obtectus*, *C. chinensis*, and *C. maculatus* were also exposed to purified αAI-1. The α-amylase activity of *C. chinensis* and *C. maculatus* larvae was reduced by αAI-1, and the reduction was greater for *C. maculatus*. This is in agreement with previous reports that neither species was able to grow on seeds of the common bean (Ishii, 1952; Janzen et al., 1976). In contrast, the α-amylase activity in larval extracts of *A. obtectus* was insensitive to αAI-1. The reasons for which *A. obtectus* can survive on common beans remain unknown (Ishimoto et al., 1996). Our *in vitro* results with

larvae of the three bruchid species are in agreement with those published by Ishimoto and Kitamura (1989; 1992). Our results also revealed that the inhibition of α-amylase activity by αAI-1 in the parasitoids was similar to that in their bruchid hosts.
The inhibitor αAI-1 has been shown to have a broad spectrum of *in vitro* activity against arthropods. Kluh et al. (2005) screened the *in vitro* inhibitory activity of purified αAI-1 against α-amylases from 30 arthropod species representing nine orders. The α-amylases from coleopteran, dipteran, and hymenopteran species, including that from the parasitoid *V. canescens*, were susceptible to the inhibitor. Together with our results, it appears that coleopteran and hymenopteran species are highly sensitive to αAI-1 in general. In contrast, α-amylase activities of lepidopteran larvae as well as of those species belonging to Blattodea, Psocoptera, Orthoptera, Hemiptera, and Acari were not inhibited by αAI-1 at concentrations up to 1 μM (Kluh et al., 2005).

Characterization of general proteolytic activity

We partially characterized the proteolytic enzymes in larval and female extracts of the parasitic wasps *H. prosopidis*, *E. vuilleti*, and *A. calandrae*, and in larval extracts of the bruchid *C. chinensis*. General proteolytic activity in the parasitic wasps was highest under alkaline conditions and was higher in larvae than in females. This might be due to different nutritional requirements of females vs. larvae: while females consume the small amounts of haemolymph that exude from punctures in the host cuticle (Jervis and Kidd, 1986), the larvae consume most of their host. The ability of extracts to hydrolyze the general substrate azocasein, the optimal pH for hydrolysis, and the sensitivity to a range of protease inhibitors demonstrated that larvae predominantly rely on serine proteases for protein digestion while *E. vuilleti* and *A. calandrae* females contain both serine proteases and metalloproteases. Because females are host-feeders, they feed from the same resource as the larvae. Such serine-protease based digestive metabolism has been previously reported from other hymenopteran species, including ants (Whitworth et al., 1998), bees (Moritz and Crailsheim, 1987), bumblebees (Malone et al., 2000), and parasitic wasps. Trypsin- and chymotrypsin-like activities were detected in larvae of the ectoparasitoid *Eulophus pennicornis* (Nees) (Hymenoptera: Eulophidae) (Down et al., 1999). Similarly, the predominant proteases detected in larvae of the ectoparasitoid *Habrobracon hebetor* Say (Hymenoptera: Braconidae) and in larvae and adults of the

aphid parasitoids *Aphelinus abdominalis* Dalman (Hymenoptera: Aphelinidae) and *Aphidius ervi* Haliday (Hymenoptera: Braconidae) belonged to the serine protease class (Baker and Fabrick, 2000; Azzouz et al., 2005a; Azzouz et al., 2005b). In contrast to parasitic wasps, maximal hydrolysis of azocaseine in larval extracts of the bruchid *C. chinensis* occurred at acidic pH. Indeed, the proteolytic enzymes detected differed between *C. chinensis* larvae and their hymenopteran parasitoids in that both cysteine and aspartic proteases were detected in *C. chinensis* larvae but not in the parasitoids. Our results agree with those of similar studies conducted with several bruchid species, including *C. chinensis* (Kitch and Murdock, 1986; Silva and Xavier-Filho, 1991; Kuroda et al., 1996; Melo et al., 2003).

Effect of digestive inhibitors (wheat αAI and SKTI) on female parasitoid survival

The two parasitoids tested in our study differed in susceptibility to digestive enzyme inhibitors. The survival of the *E. vuilleti* females was reduced by wheat αAI, while the survival of *A. calandrae* was reduced by wheat αAI and the serine protease inhibitor SKTI. Although serine protease activity in extracts of *E. vuilleti* was inhibited *in vitro* by SKTI, no effect was observed when the protease inhibitor was administered *in vivo*. This could be explained by the ability of some natural enemies to adapt their digestive metabolism to the presence of plant antidigestive proteins (Alvarez-Alfageme et al., 2007).

The negative effects of the test compounds on both parasitoids are unlikely to be biologically relevant because females that fed on αAI and SKTI survived much longer than required for oviposition. Survival of susceptible females that consumed αAI and SKTI was not affected until after day 50, but in previous studies, female *A. calandrae* and *E. vuilleti* kept on seeds infested with bruchid larvae, and thus able to oviposit, lived for only 10 and 13 days, respectively (Schmale et al., 2001; Giron et al., 2004). Only a few studies have assessed the *in vivo* effects of serine protease inhibitors on hymenopteran parasitoids, and the impact of digestive enzyme inhibitors on bruchid parasitoids has never been previously evaluated. Negative, host-mediated effects of the cowpea trypsin inhibitor (CpTI) and the soybean Bowman-Birk inhibitor (SbBBI) on adult *E. pennicornis* and *A. abdominalis* were reported by Bell et al. (2004) and Azzouz et al. (Azzouz et al., 2005a), respectively. When those parasitoid species were directly fed with sugar solutions containing the protease inhibitor, however, no detrimental effects were observed, suggesting that the negative effects were due to

reduced host quality (because the hosts are known to be susceptible to the test compound) rather than to a direct effect of the inhibitor.

Conclusions

This is the first study to characterize the α-amylase and proteolytic enzymes in parasitoids of bruchids and to assess the *in vitro* susceptibility of the parasitoids to αAI-1. The results of our study also provide information required by the model of Lüthi et al. (2010), which assesses the potential effects of GM legumes on non-target species.

The results of this study indicate that serine proteases are the main digestive enzymes in all the parasitoids of bruchids that were tested, whereas cysteine proteases are the main digestive enzymes in bruchid larvae. It follows that the use of GM legumes expressing cysteine protease inhibitors to control bruchids should be compatible with the control provided by hymenopteran parasitoids. We have also detected significant levels of α-amylase activity in larval and female extracts of several hymenopteran parasitoid species used for bruchid control. Subsequent *in vitro* inhibition studies showed that sensitivity of α-amylase activity to αAI-1 in the bruchid antagonists was comparable to that in the target species, suggesting that parasitoids might be negatively affected by αAI-1-expressing legumes if they consume this inhibitor when feeding on their hosts. Direct feeding assays performed in our study under worst-case exposure conditions revealed that harmful effects of αAI on bruchid parasitoids cannot be discounted. Future bioassays should therefore be conducted under more realistic exposure conditions to evaluate the compatibility of αAI-1-expressing GM legumes with biological control agents for bruchid management.

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Table 1 α-Amylase activity (mg of maltose liberated min^{-1} mg^{-1} protein) and inhibition of potato starch hydrolysis by the specific inhibitors acarbose and wheat αAI in extracts of bruchids and hymenopteran parasitoids

Source of extract			α-Amylase activity		Inhibition (%)[c]	
Family	**Species**	**Stage [a]**	**Optimum pH**	**Specific activity[b]**	**Acarbose (10^{-3} %)**	**Wheat αAI (10^{-3} %)**
Chryomelidae	*A. obtectus*	L	5.5	0.68 ± 0.010	98.9 ± 0.55	83.9 ± 1.43
	C. chinensis	L	5.5	0.84 ± 0.023	98.0 ± 1.09	51.2± 3.31
	C. maculatus	L	5.5	0.51 ± 0.032	97.2 ± 0.33	91.4 ± 0.17
Braconidae	*H. prosopidis*	L	6.0	0.07 ± 0.006	35.0 ± 1.77	71.7 ± 0.43
		F	6.0	0.14 ± 0.001	97.9 ± 0.17	24.8 ± 1.78
Eupelmidae	*E. vuilleti*	L	6.0	0.17 ± 0.000	77.8 ± 0.26	73.0 ± 1.31
		F	6.0	0.25 ± 0.003	95.6 ± 0.61	40.4 ± 4.13
Pteromalidae	*A. calandrae*	L	5.0	0.02 ± 0.000	99.2 ± 0.20	86.1 ± 1.96
		F	6.0	0.81 ± 0.023	68.2 ± 0.80	87.4 ± 2.77
	D. basalis	L	5.0	0.21 ± 0.001	60.4 ± 3.37	63.7 ± 4.21
		F	6.0	0.38 ± 0.017	94.4 ± 0.30	49.6 ± 1.08
	L. distinguendus	L	5.0	0.04 ± 0.003	96.2 ± 0.80	82.2 ± 0.00
		F	7.0	0.84 ± 0.032	61.6 ± 1.91	75.9 ± 4.57

[a] L = larvae; F = females

[b] Values are means ± SE of triplicate measurements for a unique pool of extracts

[c] The percentage of inhibition was calculated as [1-(activity with an inhibitor /activity in control) × 100]. Inhibition was measured at the optimum pH

Table 2 Proteolytic activity (as indicated by Abs_{450} min^{-1} mg $protein^{-1}$) and inhibition of azocasein hydrolysis by specific protease inhibitors in extracts of *Callosobruchus chinensis* and three parasitoid species

Source of extract			Proteolytic activity		Inhibition (%)[c]					
Family	**Species**	**Stage**[a]	**Optimum pH**	**Specific activity**[b]	**PMSF (10 mM)**	**SKTI (10 µM)**	**E-64 (10 µM)**	**IAA (1 mM)**	**EDTA (10 mM)**	**Pepstatin-A (10 µM)**
Chrysomelidae	*C. chinensis*	L	5.0	22.2 ± 0.12	ni	ni	68.2 ± 1.03	52.6 ± 0.39	ni	34.9 ± 2.56
Braconidae	*H. prosopidis*	L	9.0	216.4 ± 17.56	24.4 ± 4.18	75.4 ± 2.78	ni	ni	ni	ni
		F	7.0	3.1 ± 0.55	nd	nd	nd	nd	nd	nd
Eupelmidae	*E. vuilleti*	L	10.0	196.7 ± 1.15	ni	83.1 ± 1.20	ni	ni	ni	ni
		F	8.0	31.0 ± 1.56	ni	78.6 ± 0.89	ni	ni	40.6 ± 3.95	ni
Pteromalidae	*A. calandrae*	L	9.0	147.2 ± 9.88	36.3 ± 4.18	75.4 ± 2.78	ni	ni	ni	ni
		F	8.0	144.2 ± 4.10	ni	69.3 ± 1.24	ni	ni	86.5 ± 0.98	ni

[a] L = larvae; F = females

[b] Values are means ± SE of triplicate measurements for a unique pool of extracts

[c] The percentage of inhibition was calculated as [1-(activity with an inhibitor /activity in control) × 100]. Inhibition was measured at the optimum pH

Protease inhibitors abbreviations: PMSF (phenylmethylsulphonyl fluoride), SKTI (soybean Kunitz trypsin inhibitor), E-64 (L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane), IAA (iodoacetamide), EDTA (ethylenediaminetetraacetic acid)

"ni": inhibition < 10%

"nd": not determined

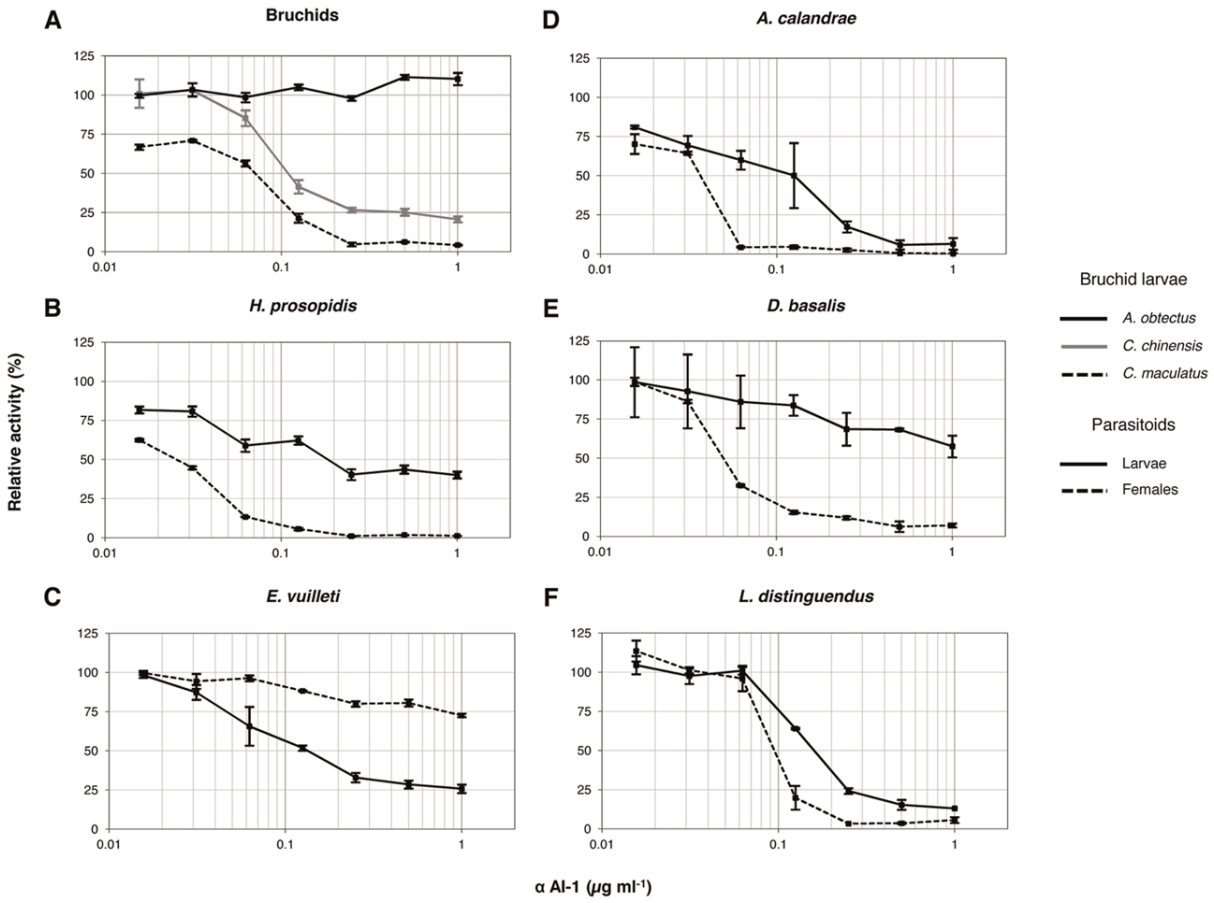

Figure 1 *In vitro* activity of αAI-1 from *Phaseolus vulgaris*. Inhibitory activity of αAI-1 against α-amylase activity in extracts of (A) three bruchids (*Acanthoscelides obtectus*, *Callosobruchus chinensis*, and *Callosobruchus maculatus*) and in extracts of larvae and adult females of the hymenopteran parasitoids (B) *Heterospilus prosopidis*, (C) *Eupelmus vuilleti*, (D) *Anisopteromalus calandrae*, (E) *Dinarmus basalis*, and (F) *Lariophagus distinguendus*. Relative activity was calculated as [(activity with αAI-1 /activity without αAI-1) × 100]. Bars represent means ± SE of three measurements from a unique pool of extracts.

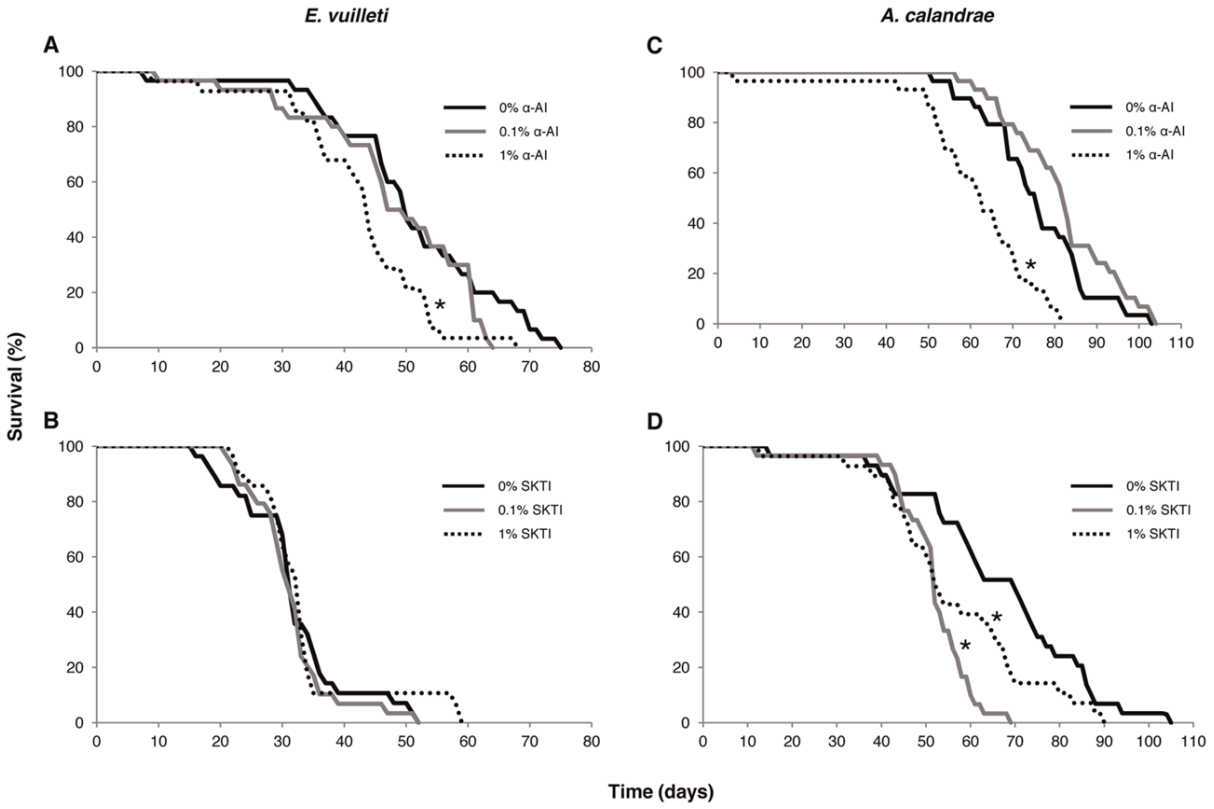

Figure 2 Effect of wheat αAI and SKTI on bruchid parasitoids. Survival of females of the parasitoids *Eupelmus vuilleti* (2A, 2B) and (2C, 2D) *Anisopteromalus calandrae* fed with a 2 M sucrose solution containing different amounts of either wheat αAI or the serine protease inhibitor SKTI. (*N* = 28–30). Potato starch or BSA (20 µg ml^{-1}) was added to the sucrose solution to stimulate α-amylase and protease activity, respectively. An asterisk indicates that survival was significantly lower with wheat αAI or SKTI than with the control (Logrank test, $P < 0.05$)

CHAPTER 4

Impact of αAI-1 Expressed In Genetically Modified Cowpea on *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae) and its Parasitoid, *Dinarmus basalis* (Hymenoptera: Pteromalidae)

ABSTRACT

Genetically modified (GM) cowpea seeds expressing αAI-1, an α-amylase inhibitor from the common bean, have been shown to be immune against several bruchid species, the major pest of the stored seeds. An effective control of such pests by growing GM cowpea could promote the spread of αAI-1 tolerant bruchid species. It has therefore been suggested that the GM seeds could be combined with hymenopteran parasitoids for a more sustainable control of bruchid pests. However, there are concerns that αAI-1 could interfere with the biological control provided by parasitoids. Here, we assess the impact of GM cowpea seeds expressing αAI-1 on the αAI-1 tolerant bruchid *Zabrotes subfasciatus* and its parasitoid *Dinarmus basalis*. We show that αAI-1 in cowpea seeds does not increase resistance to *Z. subfasciatus* or affect the larval mortality rate. Parasitism by *D. basalis* and offspring fitness are not affected by the presence of αAI-1. Thus, αAI-1-expressing cowpeas and parasitoids should be compatible to control bruchid pests.

Based on: Lüthi, C., Álvarez-Alfageme, F., Romeis, J. Impact of αAI-1 expressed in genetically modified cowpea on *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae) and its parasitoid, *Dinarmus basalis* (Hymenoptera: Pteromalidae) (submitted to PLOS ONE).

INTRODUCTION

Cowpea (*Vigna unguiculata*) is the predominant legume crop in West Africa, a region that is responsible for more than 80% of the global cowpea production (FAOSTAT: http://faostat.fao.org). The adaptation of the plant to the local climate, its high nutritional quality, and the storability of the dry seeds make cowpea a staple food for small-scale subsistence farmers. The major pests of stored cowpea seeds and other stored legume seeds are bruchid beetles (Coleoptera: Chrysomelidae: Bruchinae). In the case of cowpea, the two predominant bruchid pests are *Callosobruchus maculatus* and *C. chinensis* (Solleti et al., 2008). The observation that seeds of the common bean (*Phaseolus vulgaris*) are resistant to several bruchid species, including the two *Callosobruchus* spp., led to the identification of the storage protein αAI-1 as a main resistance factor (Huesing et al., 1991). αAI-1 is an α-amylase inhibitor and is toxic to bruchids because it inhibits their α-amylases, which are key enzymes in their digestion of long-chain carbohydrates. The gene coding for αAI-1 has been transferred into other legumes, including cowpea, which were subsequently protected against several bruchid species (Shade et al., 1994; Schroeder et al., 1995; Ishimoto et al., 1996; Morton et al., 2000; Sarmah et al., 2004; Ignacimuthu and Prakash, 2006; De Sousa-Majer et al., 2007; Solleti et al., 2008; Higgins et al., *in press*). Genetically modified (GM) cowpea expressing αAI-1 under the seed-specific promoter of the common bean phytohemagglutinin gene (*dlec2*) is resistant to the two *Callosobruchus* spp. (Solleti et al., 2008; Lüthi et al., *2012;* Higgins et al., *in press*). However, at least two cosmopolitan bruchid species considered as secondary cowpea pests, *Zabrotes subfasciatus* and *Acanthoscelides obtectus* (CABI, 2007) are tolerant to αAI-1 (Ishimoto and Kitamura, 1992; Ishimoto and Chrispeels, 1996) and αAI-1-expressing GM chickpea and cowpea seeds are as susceptible to *A. obtectus* as non-transformed seeds (Lüthi et al., *2012*). Progress in management of the predominant *Callosobruchus* spp. by growing αAI-1 GM cowpea might therefore be erased by the spread of secondary bruchid pests or by the development of resistance in the hitherto susceptible species. Researchers have therefore suggested that bruchid management might be more sustainable if control by αAI-1 GM cowpea were combined with control by hymenopteran parasitoids, which are important natural enemies of bruchids (Lüthi et al., 2010). However, Álvarez-Alfageme et al. (2012) demonstrated that under *in vitro* conditions, the α-amylases of several important bruchid parasitoids are as susceptible as those of the *Callosobruchus* spp. to αAI-1.

Accordingly, parasitoids attacking bruchid larvae tolerant to αAI-1 and developing in GM seeds might be directly harmed (by host-mediated exposure) or indirectly harmed (by reduced host quality) by the presence of αAI-1 in the seeds. This might lead to a decline in the control provided by the parasitoids and might ultimately promote the spread of secondary bruchid pests.

In this study, we investigated the compatibility of αAI-1 GM cowpea and bruchid parasitoids. The study included three independently transformed cowpea lines expressing αAI-1 and their respective controls, the αAI-1 tolerant bruchid *Z. subfasciatus*, and the αAI-1 susceptible parasitoid *Dinarmus basalis* (Hymenoptera: Pteromalidae). *Dinarmus basalis* is a solitary ectoparasitoid of bruchid larvae and pupae.

MATERIALS AND METHODS

Insects

Individuals of *Z. subfasciatus* were provided by I. Zaugg (University of Fribourg, Switzerland), who collected them from a wild population on wild common bean plants in Mexico. Before the experiment, the beetles were maintained for at least three generations on cowpea seeds.

Dinarmus basalis was provided by J.P. Monge (Tours University, France). The parasitoids were reared on *C. chinensis* larvae in chickpea seeds. Before the start of the experiment, the parasitoids were reared on *Z. subfasciatus*-infested cowpea seeds for at least two generations. The insects were maintained and the experiments were conducted in a climate chamber at 24 ± 2 °C, 40 ± 5% r.h, and complete darkness.

Cowpea seeds

GM cowpea seeds expressing αAI-1 were developed at CSIRO Plant Industry (Australia) and provided together with the corresponding control lines by T.J.V. Higgins (CSIRO Plant Industry, Australia) (Popelka et al., 2006; Higgins et al., *in press*). Seeds with two different plant backgrounds (cultivars IT86D-1010 and Sasaque) were used. IT86D-1010 was the parent for the GM line TCP 14A and the corresponding null-pair line NTCP 14A. Sasaque was the parent for three independently transformed lines (T170, T239, and T310) and their corresponding null-pair lines (NT170, NT239, and NT310). Experiment 1 (the “bitrophic” experiment)

included all nine lines described earlier in this paragraph (plus the cowpea seed that was used to breed *Z. subfasciatus*, which was purchased from a local supermarket). Experiment 2 (the "tritrophic" experiment) included the following pairs of GM and corresponding null-pair lines: TCP 14A and NTCP14A, T170 and NT170, and T239 and NT 239.

Resistance of cowpea seeds to *Z. subfasciatus* (experiment 1)

A bitrophic experiment was conducted with *Z. subfasciatus* and all cowpea lines. Thirty seeds of each cowpea line were placed individually in open plastic containers (2.2 x 2.2 x 1.0 cm), and the 300 plastic containers were arranged randomly in a large box (100 x 50 x 20 cm). Approximately 2000 newly emerged adult beetles were released into the box. Seeds were inspected daily for 3 days. Infested seeds (i.e., seeds with eggs attached) were removed from the box and kept individually in plastic containers. Seeds without eggs after 3 days were discarded. Infested seeds were inspected daily, and as soon as the first larva began chewing into the seed, all other larvae on the same seed were removed with a scalpel to avoid interference among multiple larvae developing in a single seed. Seeds were inspected daily for adult emergence until the experiment was terminated after 70 days. For each line, resistance was calculated as percentage of infested seeds without adult emergence. Seeds without adult emergence were dissected, and the stage of the dead bruchid was determined. We determined whether the bruchid failed to penetrate the seed coat; whether the bruchid penetrated the seed coat but died inside the seed in the larval or pupal stage; or whether the bruchid penetrated the seed coat and successfully completed development but failed to emerge from the seed. Because only bruchid larvae feeding on the cotyledons and embryonic axis of a seed are exposed to plant-expressed αAI-1, larval mortality within the different cowpea lines was analyzed.

Host-mediated impact of αAI-1 GM cowpea seeds on *D. basalis*

A tritrophic experiment was conducted with *D. basalis*, *Z. subfasciatus*, and the following three pairs of GM and null-pair cowpea lines: TCP 14A / NTCP 14A, T170 / NT170, and T239 / NT239. In the initial bitrophic experiment, we observed a strong effect of the seed coat on the development of *Z. subfasciatus* (Supplementary Figure 1). Preliminary experiments also revealed that the seed coat affects the parasitism

rate of *D. basalis*. The seed coats were therefore replaced by an artificial coat according to Shade et al. (1986) to exclude this factor in the tritrophic experiment. After 50 seeds per line were soaked in distilled water for 60 to 90 min, their coats were removed. The seeds were dried again for 24 h at 35°C and then dipped into an 8% gelatin solution maintained at 55–60°C**.** The seeds were then put on a glass Petri dish on ice for 5-10 min to quickly solidify the gelatin. The seeds were finally kept in the climate chamber under experimental conditions for 3 days before the start of the experiment.

To infest the seeds with *Z. subfasciatus* larvae, all seeds from one line were placed in a plastic container (10.5 cm diameter, 15 cm high), and approximately 300 adult bruchids were released into the container. After 2, 4, and 6 days, the seeds were inspected, and those with eggs attached were placed individually in a plastic container (2.2 x 2.2 x 1.0 cm). Seeds without eggs after 6 days were considered unsuitable for oviposition and were excluded from the experiment. Infested seeds were inspected regularly, and as soon as a single larva entered a seed, additional eggs and larvae were removed with a scalpel. Once the bruchid larvae reached the fourth larval instar, one young (<24 h) and mated *D. basalis* female was introduced into each container (which contained a single seed and host) and was allowed to oviposit for 24 h. Subsequently, seeds were inspected daily, and if and when the adult parasitoid emerged, its developmental time and sex were recorded. Parasitism rate was calculated as the percentage of seeds from which an adult parasitoid emerged. Filial generation 1 (F_1) males were immediately frozen, and their individual dry weights were measured with a MX5 microbalance (Mettler Toledo, Greifensee, Switzerland) after they had been dried at 60°C for 72 h. Each newly emerged F_1 female and a single male from the rearing colony were introduced into a plastic container (6 cm diameter, 10 cm high) containing 100 cowpea seeds from the variety that was used to maintain *Z. subfasciatus*. All seeds came from a single pool of seeds that had been exposed to *Z. subfasciatus* over a period of 14 days; this was done to provide suitable hosts for the entire oviposition period of *D. basalis*. For each F_1 female, realized fecundity (i.e., the total number of offspring) and the offspring sex ratio were recorded.

Data analyses

All data were analyzed using the software R (version 2.14.1). In the bitrophic experiment, differences in resistance and larval mortality between the GM and the corresponding null-pair lines were analyzed pairwise using Fisher's exact test. For the lines based on IT86D-1010, three pairwise comparisons between the parental, GM, and null-pair line were conducted, and the α-level adjusted according to the Bonferroni method, resulting in $\alpha = 0.017$. In the tritrophic experiment, differences in parasitism rate and offspring sex ratio of *D. basalis* females were analyzed pairwise using Fisher's exact test. Developmental time, dry weight, and realized fecundity of F_1 individuals were compared pairwise using the parametric Student's *t* test because the data were normally distributed and the variances were homogenous. Data for the offspring sex ratio of F_1 females were arcsine transformed and compared pairwise using the non-parametric Mann-Whitney U test.

RESULTS

Resistance of cowpea seeds to *Z. subfasciatus* (experiment 1)

The resistance of the cowpea lines to *Z. subfasciatus* is presented in Fig. 1A. The parental IT86D-1010 line was significantly more resistant than the GM line TCP14A ($p = 0.004$) and was borderline more resistant than the null-pair line NTCP14A ($p = 0.019$); the difference between the null-pair line NTCP14A and the GM line TCP14A was not significant. Resistance did not differ between the GM and null-pair lines of Sasaque 170 or 239, but resistance was greater for GM line 310 than for its control line ($p < 0.001$). In all lines except the breeding variety (the line used to maintain *Z. subfasciatus*), most mortality was caused by the failure of larvae to chew through the seed coat (Supplementary Figure 1).

Results for the within-seed larval mortality of *Z. subfasciatus* in the different cowpea lines are presented in Fig. 1B. Within-seed larval mortality was generally low in all lines and was absent in the parental IT86D-1010 line. None of the pairwise comparisons revealed significant differences in mortality rate.

Host-mediated impact of αAI-1 GM cowpea seeds on *D. basalis*

Results of the tritrophic experiment are presented in Table 1. The number of seeds per line containing a suitable host, i.e., the sample size per line, ranged from 28 to 39. Parasitism rate ranged from 89.3 to 94.4% and did not significantly differ between

GM lines and their respective controls. The sex ratio in F_1 was female-biased in all lines, ranging from 0.56 to 0.77 but did not significantly differ between GM lines and their respective controls.

In all cases, developmental time of *D. basalis* larvae was significantly longer for females than for males (Student's *t* test, $t = 11.7$, $p < 0.01$) and was therefore separately analysed for the two sexes. The comparison for females and for males was not significant in any of the three pairwise comparisons.

The impact of the GM vs. non-GM lines on the F_1 adults was analyzed by comparing the dry weight of males and the realized fecundity of females. Male adult dry weight did not significantly differ between the GM lines and their respective controls. Realized fecundity or the offspring sex ratio of the females also did not significantly different between the GM lines and their respective controls.

DISCUSSION

Zabrotes subfasciatus is known to be tolerant to αAI-1 (Ishimoto and Kitamura, 1992; Ishimoto and Chrispeels, 1996) and should therefore not be affected by the presence of this inhibitor in the GM cowpea seeds. Indeed, none of the GM lines expressing αAI-1 was immune to this bruchid, and mortality of larvae, the stage that is exposed to αAI-1, was low in all lines. Nevertheless, resistance did differ in two of the comparisons of GM and null-pair lines. A similar result has been found for the other αAI-1 tolerant bruchid, *A. obtectus* (Lüthi et al., *2012*). The low mortality during the larval stage inside the seeds indicates that factors other than αAI-1 are responsible for the observed differences in seed resistance. Researchers have argued that the seed coat of non-host legumes can contribute to the resistance against bruchids (Souza et al., 2011), and in the current study, the failure to chew through the seed coat accounted for the highest larval mortality in experiment 1. Cowpea is not a primary host of *Z. subfasciatus*, and the cowpea seed coat contains resistance factors such as tannins and α-amylase inhibitors (Lattanzio et al., 2005). The concentrations of resistance factors in the seed coat might have been affected by tissue culture, which was used in the generation of GM plants, because tissue culture can lead to phenotypic changes, so called somaclonal variation (Larkin and Scowcroft, 1981; Pellegrineschi, 1997). Seed coat thickness can also contribute to bruchid resistance but was shown to be similar in all IT86D and Sasaque lines in our

study (Lüthi et al., *2012*). We therefore infer that seed coat thickness was not responsible for the observed differences in resistance to *Z. subfasciatus*.

The similar larval mortality observed in all lines investigated in the bitrophic experiment and the use of an identical artificial seed coat in the tritrophic experiment indicate that the quality of *Z. subfasciatus* larvae as hosts for *D. basalis* in the tritrophic experiment did not differ between the GM and their corresponding null-pair lines except for the presence or absence, respectively, of αAI-1. Thus host-quality–mediated (indirect) effects on bruchid parasitoids can be excluded.

Females of hymenopteran parasitoids are able to evaluate host quality and adjust their egg-laying behavior accordingly (Quicke, 1997). Female *D. basalis* are synovigenic, i.e., they rely on nutrient intake to develop mature eggs sequentially, and they therefore feed on host hemolymph. Because host-feeding kills the host or at least reduces its quality, it is assumed that hosts are usually used either as a food source or for oviposition only (Jervis and Kidd, 1986; Heimpel and Collier, 1996). Upon finding hosts, *D. basalis* females therefore must decide whether to feed or oviposit and, when ovipositing, whether to deposit an egg that produces a male or a female. An influence of host quality on sex ratio has documented for *D. basalis*, with lower host quality resulting in a male-biased offspring ratio (Gauthier et al., 1997; Lacoume et al., 2006). The ingestion of αAI-1 by the host might influence host quality and hence the behavior of *D. basalis* females encountering it. In the current study, however, the percentage of parasitized hosts or the offspring sex ratio did not differ between GM lines and their controls. It seems that hosts developing in αAI-1 GM and null-pair seeds were of similar quality and did not affect the parasitic behavior of *D. basalis*. Additionally, a negative impact on host-feeding females is unlikely because direct feeding studies with αAI-1 have shown that *Anisopteromalus calandrae*, a parasitoid that is closely related with *D. basalis*, is relatively tolerant of αAI-1 (Álvarez-Alfageme et al., 2012). Taken together, the effect of αAI-1 GM seeds on oviposition and on host-feeding females appears to be negligible.

Parasitoid larvae depend greatly on the host quality because the host is their only source of nutrition. This is particularly true for idiobiont species like *D. basalis*, where the host does not grow after parasitism and the quality and quantity of nutrients available for the developing parasitoid larva are therefore fixed. Although the *D. basalis* larvae were likely exposed to αAI-1 when consuming a host in a GM seed, male and female larval developmental time, which is one of the most important

fitness parameters of parasitoids (Roitberg et al., 2001), did not differ between GM lines and their control lines. This indicates that the consumption of αAI-1 by *Z. subfasciatus* did not influence parasitoid development. Similarly, parasitoid larvae that developed into adults on hosts in GM seeds did not seem to suffer from reduced fitness in terms of adult male dry weight, male and female developmental times, realized fecundity, and offspring sex ratio. The dry weight was similar for adult males emerging from GM or null-pair seeds. Lacoume et al. (2006) showed that host size, which is an important component of host quality, affects the body size of *D. basalis* males and that smaller males suffered from mating disadvantages compared to larger males. The absence of a significant difference in dry weight and developmental time is strong evidence that males are not affected by αAI-1 in the GM seeds. In the case of the F_1 females, developmental time, realized fecundity, and offspring sex ratio were not significantly different when the females had developed on hosts in GM or control seeds. Because F_1 females were provided hosts in similar quantity and of similar quality for oviposition, we conclude that the fitness of the females was not affected by the presence of αAI-1 in the GM seeds.

Although host-mediated impacts of several plant metabolites on hymenopteran parasitoids have been described (e.g., reviewed in Ode, 2006), we did not observe significant effects of αAI-1 expressed in the GM seeds on *D. basalis*. Yet, the level of exposure of *D. basalis* to host-mediated αAI-1 remains unclear. Because α-amylases of both larvae and females of *D. basalis* are strongly inhibited by αAI-1 (Álvarez-Alfageme et al., 2012), the exposure was probably too low to cause a detectable effect. Additionally, *in vitro* assays showed that midgut extracts of *Z. subfasciatus* larvae rapidly digest αAI-1 and thus inactivate the inhibitor (Ishimoto and Kitamura, 1992; Ishimoto and Chrispeels, 1996). If such inactivation of αAI-1 also occurs *in vivo*, it could at least partially explain why GM cowpea, expressing αAI-1 and infested with *Z. subfasciatus*, did not negatively affect the parasitoid. Finally, *D. basalis* is a common parasitoid of *Z. subfasciatus* and *A. obtectus*, which are both pests of the common bean (CABI, 2007). Thus, *D. basalis* might have encountered αAI-1, which is naturally present in common bean seeds, in its evolutionary history and found ways to cope with it. In this context, a second plant resistance factor from the common bean, arcelin, did not to interfere with the control of *A. obtectus* by *D. basalis* (Velten et al., 2007). Furthermore, the combination of plant resistance factors and *D. basalis* has been found to improve the control of *A. obtectus* and *Z. subfasciatus* (Schmale et

al., 2003; Velten et al., 2008). Therefore, the evidence indicates that αAI-1 GM cowpeas are not only compatible with biological control services provided by *D. basalis*, but that their combination could provide an improved and sustainable management of bruchid pests.

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Table 1. Performance of *Dinarmus basalis* on GM or non-GM cowpea lines (experiment 2). Performance was assessed as parasitism rate and offspring sex-ratio of *D. basalis* parental generation females (P) as well as by the developmental time and dry weight of males and by the developmental time, realized fecundity, and offspring sex ratio of females of the first filial generation (F_1). P females were provided a single *Zabrotes subfasciatus* larval host developing in a αAI-1 GM or corresponding null-pair control line seed. F_1 females were provided *Z. subfasciatus* larvae developing in cowpeas of the breeding variety *ad libitum*. Means are presented with standard error.

	Females P		Males F_1		Females F_1		
Cowpea line[a]	Hosts provided (n) / parasitism rate (%)[b]	Sex ratio (f:m)[b]	Developmental time (d)[c]	Dry weight (µg)[c]	Developmental time (d)[c]	Realized fecundity (n)[c]	Mean offspring sex ratio (f:m)[d]
NTCP14A	39 / 89.7	27:8	18.5 ± 0.5	281.6 ± 16.1	21.7 ± 0.2	20.6 ± 1.2	0.68 ± 0.052
TCP14A*	36 / 94.4	23:11	18.7 ± 0.4	267.2 ± 9.7	21.8 ± 0.3	19.6 ± 1.2	0.66 ± 0.042
	p = 0.68	p = 0.43	p = 0.72 (*t* = -0.37)	p = 0.43 (*t* = 0.81)	p = 0.84 (*t* = -0.20)	p = 0.58 (*t* = 0.56)	p = 0.57 (U = 340)
NT170	36 / 94.1	23:9	19.6 ± 0.4	261.6 ± 11.7	21.6 ± 0.2	19.7 ± 1.3	0.69 ± 0.067
T170*	37 / 91.7	19:15	18.8 ± 0.4	251.5 ± 15.3	21.4 ± 0.2	20.7 ± 1.4	0.53 ± 0.069
	p = 0.71	p = 0.21	p = 0.20 (*t* = 1.32)	p = 0.65 (*t* =0.46)	p = 0.67 (*t* = 0.42)	p = 0.57 (*t* = -0.57)	p = 0.24 (U = 265)
NT239	28 / 89.3	17:8	20.0 ± 0.6	262.9 ± 16.1	21.9 ± 0.4	16.9 ± 1.4	0.64 ± 0.043
T239*	32 /90.6	18:11	19.0 ± 0.4	262.5 ± 7.1	21.9 ± 0.3	18.8 ± 1.5	0.61 ± 0.081
	p = 1	p = 0.78	p = 0.15 (*t* = 1.52)	p =0.98 (*t* =0.02)	p = 0.99 (*t* = -0.01)	p = 0.36 (*t* = -0.92)	p = 0.95 (U = 156)

[a] GM lines are indicated with an asterisk.

[b] Values are compared pairwise using Fisher's exact test.

[c] Values are compared pairwise using Student's *t* test.

[d] Values are compared pairwise using Mann-Whitney U test.

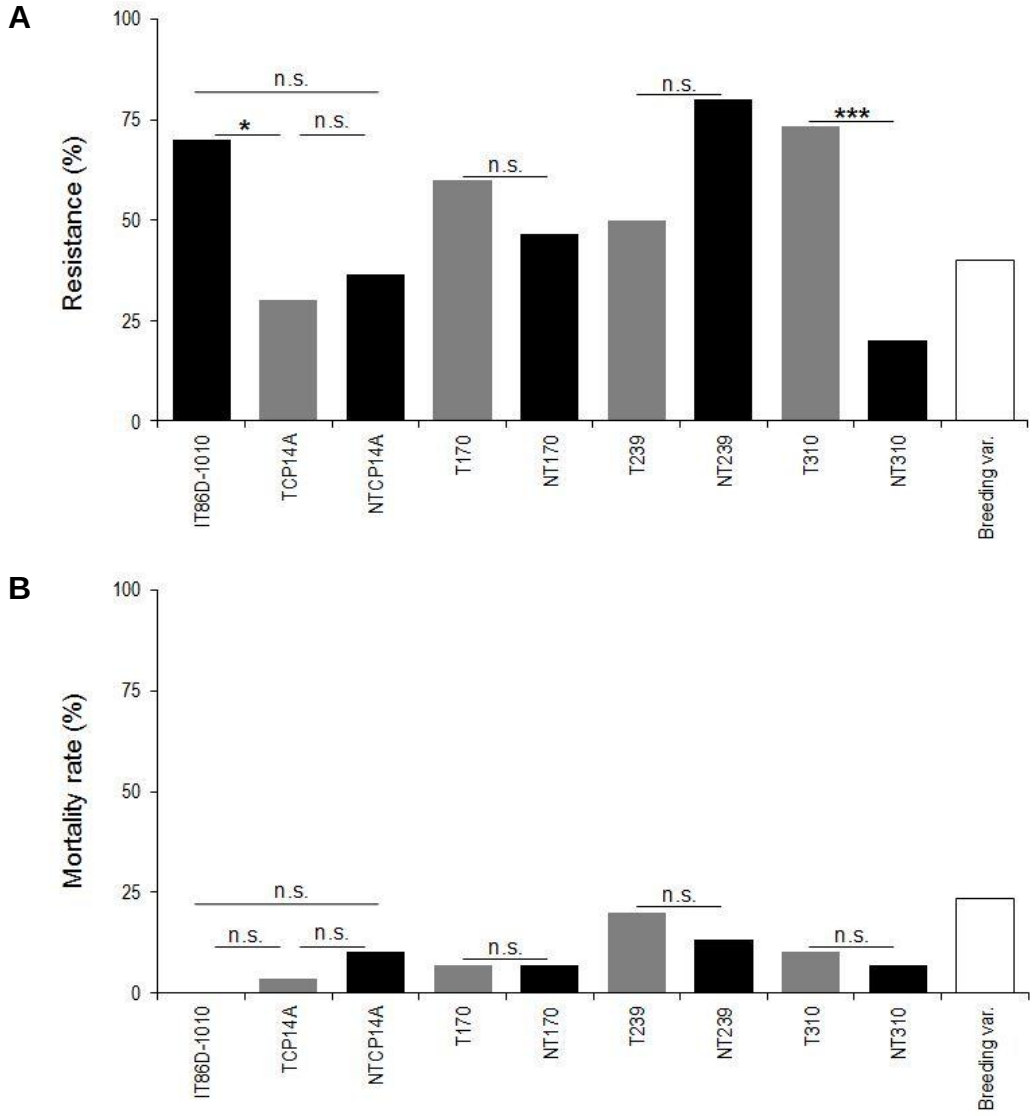
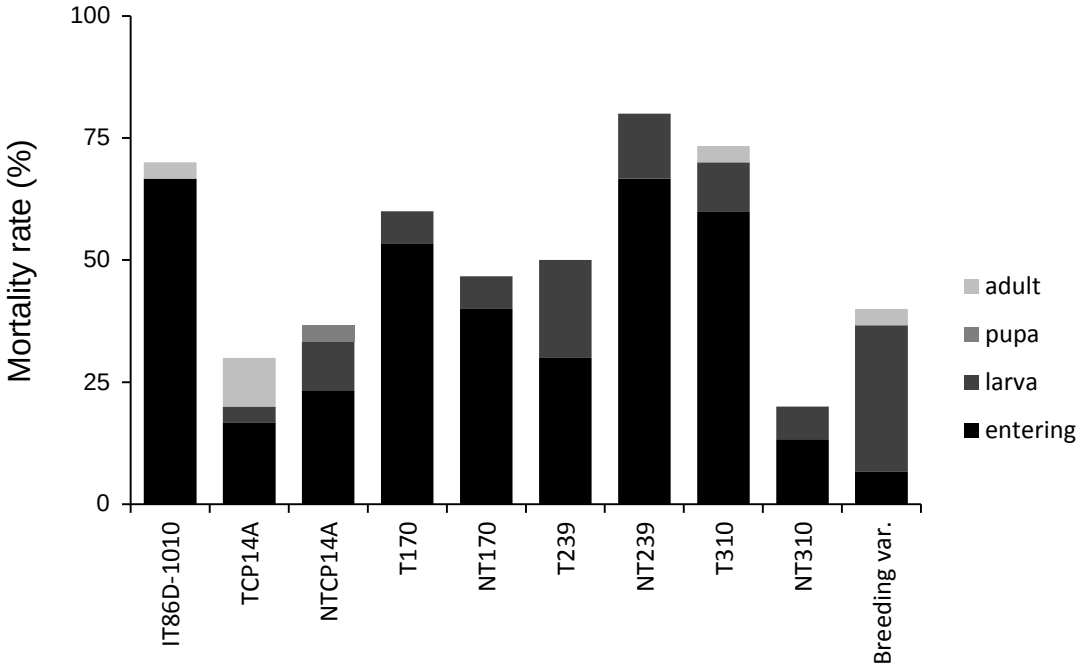


Figure 1. Effects of GM and non-GM cowpea lines on the bruchid pest *Zabrotes subfasciatus* (experiment 1). (A) Resistance (percentage of infested seeds from which no adult beetle emerged) and (B) within-seed larval mortality of *Zabrotes subfasciatus* in different cowpea lines (experiment 1).

Comparisons were made among the three IT86D lines (IT86D-1010: parental line; TCP14A: GM line; NTCP14A: null-pair line) and between the transformed (T) and the respective non-transformed (NT) Sasaque lines 170, 239, and 310 using Fisher's exact test (*$p < 0.05$, ***$p < 0.01$, n.s. = not significant; for the IT86D lines, the α level was adjusted for three pairwise comparisons using the Bonferroni method, resulting in $\alpha = 0.017$). Grey bars indicate transformed lines and black bars indicate nontransformed lines. "Breeding var." refers to the variety that was used to breed *Z. subfasciatus*.



Supplementary Figure 1. Mortality rates of *Zabrotes subfasciatus* developing in the three IT86D lines (IT86D: parental line; TCP14A: GM line; NTCP14A: null-pair line); in the transformed (T) and respective non-transformed (NT) Sasaque lines 170, 239, and 310; and in the breeding variety (experiment 1). Stacked bars represent total mortality, and single stacks indicate mortality rates for each respective stage ("entering": larva failed to drill into seed; "larva", "pupa": beetle died in the respective stage inside the seed; "adult": adult beetle failed to emerge from the seed). "Breeding var." refers to the variety that was used to breed *Z. subfasciatus*.

CHAPTER 5

The Bean α-Amylase Inhibitor αAI-1 in Genetically Modified Chickpea Seeds Does Not Interfere with Biological Bruchid Control Provided by Hymenopteran Parasitoids

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ABSTRACT

Different legumes have been genetically engineered to express αAI-1, an α-amylase inhibitor of the common bean, in their seeds. Whereas the genetically modified (GM) seeds are immune to certain bruchid pest species, the cosmopolitan bruchid *Acanthoscelides obtectus* is tolerant to αAI-1 and thus develops normally. Hymenopteran parasitoids, the most important natural enemies of bruchids, might thus be exposed to αAI-1 when attacking *A. obtectus* developing inside GM seeds. Exposure might reduce parasitoid fitness, resulting in a decline of the natural control of *A. obtectus* and promote its spread.

We investigated the exposure to αAI-1 and the impact of the presence of αAI-1 in chickpea seeds on parasitoid fitness in tritrophic experiments with αAI-1 GM and non-transformed chickpea seeds, *A. obtectus*, and three bruchid parasitoid species. We show that exposure of parasitoids to αAI-1 is negligible and that their fitness is not affected when using *A. obtectus* in GM chickpea seeds as hosts. We conclude that the release of αAI-1 GM chickpea should not harm this important group of non-target insects.

Based on: Lüthi, C., Álvarez-Alfageme, F., Romeis, J. The bean α-amylase inhibitor αAI-1 in genetically modified chickpea seeds does not interfere with biological control provided by hymenopteran parasitoids (in preparation).

INTRODUCTION

Grain legume crops are essential for the food security in many developing countries because of the high protein content and the storability of their dry seeds. The storage of legume seeds in traditional grain stores, which are mostly used by small-scale subsistence farmers, is especially vulnerable to post-harvest pests. Bruchid beetles (Coleoptera: Chrysomelidae) are the most important pest of stored legume seeds. Bruchid pests owe their status to the multivoltinism, high fecundity, short generation time, and hidden development inside the seeds. Typically, bruchid females lay their eggs on the maturing pods in the field and the infested seeds arrive in the store unnoticed. Murdock et al. (2003) estimated that a kilogram of cowpea (*Vigna unguiculata*) seeds infested with a single gravid female of the cowpea bruchid, *Callosobruchus maculatus*, can be destroyed in less than three months. Conventional breeding failed to develop bruchid resistant varieties in major grain legumes like cowpea or chickpea (*Cicer arietinum*) (Keneni et al., 2011). In contrast, seeds of the common bean (*Phaseolus vulgaris*) have been long known to be resistant to several major bruchid pest species, such as *Bruchus pisorum*, *Callosobruchus analis*, *C. chinensis*, and *C. maculatus* (Ishimoto and Kitamura, 1989; Schroeder et al., 1995; Ishimoto et al., 1996). Huesing et al. (1991) identified αAI-1, an inhibitor of mammalian and insect α-amylases, as the responsible resistance factor. α-Amylases are digestive enzymes degrading long-chain carbohydrates. They are particularly vital for bruchids, because they require α-amylases not only for production of energy, but also metabolic water (Murdock et al., 2012). The gene coding for αAI-1 has been successfully transferred into other legumes, which were subsequently protected against bruchids (Shade et al., 1994; Schroeder et al., 1995; Ishimoto et al., 1996; Morton et al., 2000; Sarmah et al., 2004; Ignacimuthu and Prakash, 2006; De Sousa-Majer et al., 2007; Solleti et al., 2008; Higgins et al., *in press*). Shade et al. (1994) found that concentrations of 0.1% and 1.0% αAI-1 in pea (*Pisum sativum*) seeds were sufficient to provide complete resistance to *C. chinensis* and *C. maculatus*.

Two cosmopolitan bruchid species, *Acanthoscelides obtectus* and *Zabrotes subfasciatus*, are known to be tolerant to αAI-1 (Ishimoto and Kitamura, 1992). In order to control also such tolerant species and to delay development of resistance in susceptible species, it has been suggested that αAI-1 GM seeds could be combined with other strategies, such as natural enemies (Lüthi et al., 2010). Hymenopteran solitary ectoparasites of the larval and pupal stages of bruchids are the most

important group of natural enemies (Southgate, 1979). Parasitoids attacking αAI-1 tolerant bruchids developing in GM seeds might be exposed to the inhibitor via host-mediation. α-Amylases of several bruchid species have been shown to be strongly inhibited by αAI-1 at *in vitro* conditions (Álvarez-Alfageme et al., 2012). Thus, a potential exposure could have detrimental effects on the parasitoids, leading to a decline in control of such bruchid species and ultimately promote their spread.

In our study, we evaluated the impact of αAI-1 GM chickpeas on three bruchid parasitoids. In a first tritrophic experiment, we investigated the exposure of the hymenopteran bruchid parasitoid *Dinarmus basalis* (Pteromalidae) to host-mediated αAI-1. We used chickpea seeds of an αAI-1 GM and a corresponding parental line at the first and *A. obtectus* at the second trophic level. In a second tritrophic experiment, we assessed the impact of αAI-1 GM seeds on the bruchid parasitoids *D. basalis*, *Eupelmus vuilleti* (Eupelmidae) and *Heterospilus prosopidis* (Braconidae). In this experiment, we used chickpea seeds of an αAI-1 GM chickpea line, the corresponding parental line, and two conventional varieties as additional controls at the first, and *A. obtectus* at the second trophic level.

MATERIALS AND METHODS

Insects

Acanthoscelides obtectus was provided by C. Adler (Julius Kühn-Institut, Germany) and maintained on chickpea seeds for at least 10 generations. *Heterospilus prosopidis* parasitizing *C. chinensis* reared on Azuki bean seeds were provided by M. Shimada (University of Tokyo, Japan). *Dinarmus basalis* and *E. vuilleti* on cowpea seeds infested with *C. maculatus* were provided by J.P. Monge (Tours University, France). Upon arrival, all parasitoid species were reared on *C. chinensis* larvae as hosts in chickpea seeds. Before the start of the experiment, the parasitoids were reared on *A. obtectus* for at least three generations. All insects were maintained and the experiments conducted in a climate chamber at 24 ± 2 °C, 40 ± 5% r.h., and complete darkness.

Chickpea seeds

Four different ‘desi’ type chickpea genotypes were used in the experiments. Chickpea seeds expressing αAI-1 GM and the corresponding non-transformed parental line of the variety Semsen (Sarmah et al., 2004) were provided by T.J.V.

Higgins (CSIRO Plant Industry, Australia). Seeds of the varieties ICCV 10 and ICCC 37 were provided by H.C. Sharma (ICRISAT, India). Preliminary experiments revealed that the seed coat of the chickpea seeds strongly affects the parasitism rate and emergence of the parasitoid adults. Therefore, the seed coat of all seeds was removed manually prior to all experiments.

Chemicals

The Megazyme Ceralpha assay kit and additional blocked *p*-nitrophenyl maltoheptasoide were purchased from Megazyme (Wicklow, Ireland). All other chemicals were purchased from Sigma Aldrich (St. Louis, USA).

Exposure experiment

In vitro α-amylase activity of *D. basalis* larval and female extracts was quantified to indirectly evaluate the host-mediated exposure to αAI-1. *Dinarmus basalis* was provided *A. obtectus* larvae developing in GM or parental line seeds (i.e., two different treatments). Larvae and females of *D. basalis* and unparasitized *A. obtectus* larvae were collected and analyzed in three replicates.

Chickpea seeds infested with *A. obtectus* adults from the laboratory colony were sieved to obtain eggs. Fifty seeds of either the GM or the parental line were put in a plastic jar (12x9.5 cm) covered with fine mesh, and infested with 50 *A. obtectus* eggs. Development of *A. obtectus* larvae was observed visually. When the larvae reached the 4th larval instar, 15 seeds were removed and frozen at -20°C to later collect larvae. Five pairs of *D. basalis* adults were introduced in each char containing the remaining 35 seeds. Adults were removed from the jar after 72h. Females were frozen at -20°C whereas males were discarded. Development of *D. basalis* larvae was observed by dissecting seeds regularly. As soon as the first larvae were observed to void the meconium, the seeds were frozen at -20°C. Frozen seeds were dissected on ice to collect *A. obtectus* and *D. basalis* larvae. *Dinarmus basalis* larvae that had already voided the meconium and thus potentially excreted their αAI-1 were discarded. All specimens were carefully cleaned in ice-cold 0.15M NaCl to avoid contamination with seed-borne αAI-1. Specimens collected from the same replication were pooled and homogenized in 10 µl ice-cold 0.15 M NaCl per specimen. The homogenates were centrifuged at 10000 *g* for 5 min, and the supernatants were pooled and stored at -20°C. Total protein content was determined according to the

method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard. All extracts were diluted to 1 µg protein μl^{-1}.

In vitro α-amylase activity in the extracts was measured using a modified version of the Megazyme Ceralpha assay (McCleary et al., 2002). Analyses were done in 96-well microplates with a standard assay volume of 160 µl, which contained 5 µl of insect protein extract, 105 µl of assay buffer, and 50 µl of blocked *p*-nitrophenyl maltoheptasoide (BPNPG7) (27.25 µg μl^{-1}) as a specific substrate. Samples were incubated for 16 h at 37°C. The reaction was stopped by adding 50 µl of 5% Tris (unbuffered). Absorbance was read at 405 nm at room temperature with a Spectrafluor Plus plate reader (Tecan, Männedorf, Switzerland), and α-amylase activity was quantified as mg of *p*-nitrophenol released from BPNPG7 min^{-1} mg^{-1} protein. All enzymatic assays were carried out in duplicate from the same pool of extracts. Blanks not containing buffer instead of insect extract were used to account for spontaneous breakdown of substrates.

Parasitoid fitness experiment

The impact of αAI-1 GM chickpea on parasitoid fitness was evaluated in a tritrophic experiment. The parasitoids *D. basalis*, *E. vuilleti*, and *H. prosopidis* were provided *A. obtectus* larvae developing in one out of four chickpea genotypes, GM Semsen line, parental Semsen line, ICCV 10, or ICCC 37 (i.e., four treatments per species). For each parasitoid species, each treatment was replicated 15 times.

Chickpea seeds infested with *A. obtectus* adults were sieved daily over a period of 14 days to collect eggs. Eggs of each day were kept separately in glass containers until emergence of the 1st instar larvae. On a daily basis and over a period of 14 days, 172 seeds of each chickpea genotype were put individually in a 1.5 ml cup with a perforated lid. Three *A. obtectus* larvae from the pool were carefully placed on each seed with a brush. Infested seeds were kept in the cup for 48 h to allow the larvae to chew into the seed.

Freshly emerged parasitoids were collected from the rearing and pooled for 24 h for mating. 60 females of each species were individually transferred into a plastic container (2.2x2.2x1 cm) and randomly allocated to one of the four treatments. Each female was provided three infested seeds per day for 14 days or until its death. Seeds were replaced every 24 h. The three removed seeds, were transferred into a 1.5 ml cup with perforated lid. Cups were inspected daily for adult offspring

emergence. Sex, developmental time, and dry weight of each emerging offspring was recorded. Emerged adults were collected in 0.2 ml cups and frozen at -20°C. Dry weight was measured after drying adults at 60°C for 72 h with a MX5 microbalance (Mettler Toledo, Greifensee, Switzerland). For each female, realized fecundity (i.e., the total number of offspring produced), offspring sex-ratio, mean male and female offspring developmental time, and mean male and female offspring dry weight was calculated.

Data analyses

Data from the parasitoid fitness experiment were analyzed with Statistica version 9.1. Sex-specific differences in developmental time and dry weight were analyzed using Student's t test. Because all comparisons were highly significant in all three species, developmental time and dry weight were analyzed for each sex separately. Fitness parameters of the parasitoid females were compared among treatments using one-way analysis of variance (ANOVA). Means were subsequently separated using the Tukey Honestly-Significant-Difference (HSD) test. Offspring sex-ratio was arc sin transformed before ANOVA. Data with inhomogeneous variances were analyzed using Welch's ANOVA. Data not fulfilling the normal distribution requirement were transformed to the second (sex-ratio *E. vuilleti*) or third power (realized fecundity and male dry weight *E. vuilleti*), or square root transformed (realized fecundity *D. basalis*). Where transformation failed to generate normally distributed data, data were analyzed using Kruskal-Wallis ANOVA.

RESULTS

Exposure experiment

In vitro α-amylase activity was assessed indirectly in larvae of *A. obtectus* developing in GM or parental line seeds and in *D. basalis* females and larvae feeding on those hosts (Fig.1). Mean α-activity was highest in extracts of *A. obtectus* larvae and higher in extracts of *D. basalis* females than in extracts of *D. basalis* larvae. Extracts of the same species / stage collected from GM or parental seeds had similar α-amylase activity. Mean α-amylase activity of larval extracts of *A. obtectus* larvae and *D. basalis* developing in GM seeds was 1.7% and 0.5%, respectively, higher than of those developing in parental line seeds. Mean α-amylase activity in *D. basalis*

females attacking hosts in parental line seeds was 0.8% higher than those from hosts in GM seeds.

Parasitoid fitness experiment

Dinarmus basalis

Fitness parameters of *D. basalis* are shown in Table 1. Mean realized fecundity of *D. basalis* was ranging from 12.6 to 16.6 and was female-biased in all treatments and did not differ significantly among chickpea seeds. Females had a significantly longer developmental time ($t = -11.53$, $p < 0.001$) and dry weight ($t = -20.12$, $p < 0.001$) than males, but both parameters were almost identical for each sex among treatments.

Eupelmus vuilleti

Fitness parameters of *E. vuilleti* are shown in Tab. 2. Realized fecundity was ranging from 39.6 to 47.5. Females produced significantly more offspring when offered hosts in the parental line Semsen seeds than when offered hosts in ICCV 10 seeds (ANOVA, $F_{3,56} = 4.01$, $p = 0.01$). Differences in realized fecundity between all other treatments (including GM and its corresponding parental line) were not significant. Sex-ratio was female-biased in all treatments, but not significantly different among treatments. Females had a significantly longer developmental time than males ($t = -10.91$, $p < 0.001$), but differences between treatments were not significant for males and females, respectively. Females had a significantly higher dry weight than males ($t = -62.63$, $p < 0.001$). While dry weight of males was similar among treatments, females emerging from GM Semsen seeds had a significantly lower dry weight than those emerging from ICCC 37 seeds ($F_{3,54} = 3.22$, $p = 0.03$). Differences in female dry weight were not significant between the other treatments.

Heterospilus prosopidis

Fitness parameters of *H. prosopidis* are shown in Tab. 3. Realized fecundity of *H. prosopidis* was lower than in the other two parasitoid species. Mean fecundity ranged from 6.5 to 8.3 in the different treatments and was always female-biased. Differences in fecundity and sex-ratio were not significant among treatments. Females had a significantly longer developmental time ($t = -8.12$, $p < 0.001$) and were significantly

heavier ($t = -5.13$, $p < 0.001$) than males but differences among treatments were not significant for both males and females.

DISCUSSION

Hymenopteran parasitoids and resistant seeds are important components of an integrated management of bruchid pests in storage systems (Schmale et al., 2003; Velten et al., 2008). A prerequisite for the use of αAI-1 GM legumes in the field should thus be that the introduced insecticidal trait does not interfere with the biological control services provided by the parasitoids (Lüthi et al., 2010). α-Amylases of *A. obtectus* have been shown to be tolerant to αAI-1 (Ishimoto and Kitamura, 1992). Indeed, this bruchid does not only develop normally in seeds of the common bean, but also in those of αAI-1 expressing chickpea and cowpea (Lüthi et al., 2012). It is thus not surprising that the *in vitro* α-amylase activity of extracts of *A. obtectus* larvae collected from GM seeds was similar to that of extracts of larvae collected from parental line seeds.

The fate of αAI-1 after ingestion by *A. obtectus*, however, remains unclear. The transfer of legume seed resistance proteins into internal organs of bruchids after ingestion has been documented in at least two cases. Arcelin has been found in the haemolymph of *Z. subfasciatus* feeding on cowpea seeds (Paes et al., 2000) and Vicilin in haemolymph, fat body, and malpighian tubes of *C. maculatus* also feeding on cowpea seeds (Uchôa et al., 2006). Whether αAI-1 arrives in the internal organs of *A. obtectus* larvae and whether it accumulates there is still unknown. In any case, parasitoids attacking those larvae could be exposed to the inhibitor. Because their α-amylases seem to be highly susceptible to αAI-1 (Álvarez-Alfageme et al., 2012), an exposure could have an impact on both larvae and females feeding on such hosts. Such a host-mediated impact of plant resistance factors on natural enemies has been described in several tritrophic systems (Ode, 2006). Studies with GM oilseed rape (*Brassica napus*) expressing protease inhibitors reported a prey-mediated impact on the gut proteolysis of two coleopteran generalist predators (Ferry et al., 2003; Ferry et al., 2005). In our system, we found that the *in vitro* α-amylase activity of *D. basalis* females provided hosts in seeds of the GM line was similar to that observed in the control. Thus, an exposure to the inhibitor is at most very low. To assess an impact on parasitoid females *in vivo*, we recorded the realized fecundity and the offspring sex-ratio. Besides exposure to αAI-1 via host-feeding, the presence of αAI-1 in the

seeds could also influence the oviposition behavior of the females. Synovigenic parasitoids are assumed to adjust their offspring sex-ratio actively. Host quality has been shown to be an important factor affecting both the decision whether using a host for feeding or oviposition, which are usually non-concurrent (Jervis and Kidd, 1986; Heimpel and Collier, 1996), and whether depositing an egg producing a male or females offspring (Quicke, 1997). An influence of host quality on offspring sex-ratio has not only been described for the two synovicenic species *D. basalis* (Gauthier et al., 1997; Lacoume et al., 2006) and *E. vuilleti* (Terrasse et al., 1996), but atypically also for the proovigenic *H. prosopidis* (King, 1989; Kobayashi and Shimada, 2000). If αAI-1 reduces host quality, this could result in more host-feeding instead of oviposition and in deposition of more eggs producing male than female offspring. We found that *E. vuilleti females* had a significantly higher fecundity (by 20%) when provided hosts in seed of the parental Semsen line compared to hosts in the variety ICCC 37. This demonstrates that seed characteristics influence host quality. However, realized fecundity and offspring sex-ratio of parasitoids offered hosts in GM seeds were not significantly different from those offered hosts in seeds of the conventional varieties and particularly in seeds of the parental Semsen line. This confirms the results of the exposure experiment; a direct impact of host-mediated αAI-1 seems to be negligible. Additionally, it shows that *A. obtectus* larvae that have ingested αAI-1 are not of inferior quality for the parasitoids.

Larvae of solitary ectoparasitoids typically consume the entire host during their development (Quicke, 1997). In contrast to adult females, larvae might thus be exposed to αAI-1 even in the case that the inhibitor is only present in the gut, but not in the internal organs of *A. obtectus*. Despite the fact that the α-amylases of their larvae have been shown to be strongly inhibited by αAI-1 under *in vitro* conditions (Álvarez-Alfageme et al., 2012), *in vitro* α-amylase activity of *D. basalis* larval extracts collected from hosts in GM seeds was similar to those collected from hosts in parental line seeds. Analogue to adult females, an eventual exposure seems to be negligible. This finding is again supported by the results of the fitness experiments. Developmental time and adult dry weight of both males and females of all three parasitoid species was not significantly different in the GM seeds compared to the non-GM seeds. There was only a single significant difference in larval fitness parameters between the treatments; the dry weight of *E. vuilleti* females emerging from seeds of the parental Semsen line was significantly higher (by 5%) than of those

emerging from seeds of the variety ICCC 37. As in the case of the females, this indicates that differences in the seed characteristics can influence parastoid fitness in the larval stage.

Taken together, our results suggest that exposure of parasitoid females and larvae to αAI-1 is negligible. We did not find an impact on *in vitro* α-amylase activity or on fitness of parasitoid females or larvae. In this context, Velten et al. (2007) reported that Arcelin, another resistance factor in bean seeds, did not interfere with biological control by *D. basalis*. Because of the quantitative character of our exposure assay, we can, however, not unerringly exclude an exposure to αAI-1 for parasitoid larvae and females. Moreover, the presence of αAI-1 does not seem to reduce quality of *A. obtectus* larvae as hosts for the parasitoids and thus does not influence parasitoid fitness indirectly. *Acanthoscelides obtectus* is originally a pest of the common bean, whose seeds contain αAI-1 naturally. The tolerance to αAI-1 is rather exceptional for coleopterans and has only been descried in a second bruchid attacking the common bean as primary host, *Z. subfasciatus* (Ishimoto and Kitamura, 1992; Kluh et al., 2005). This suggests that *A. obtectus* has specifically adapted to the presence of αAI-1 in the seeds and thus explain why no reduction in host quality has been observed.

Overall, αAI-1 GM chickpea seeds do not have a detrimental impact on parasitoids and could thus be combined for an improved and more sustainable bruchid management.

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Table 1. Fitness parameters of *Dinarmus basalis* females and larvae using *A. obtectus* developing in different chickpea seeds as hosts. Values are means ± SE.

Fitness parameter	test statistic[a]	p	Semsen GM	Semsen PL	ICCV 10	ICCC 37
Realized fecundity (n)	$F_{3,56} = 0.57$	0.63	16.1±2.9	14.9±2.4	16.6±2.1	12.6±1.8
Offspring sex-ratio (proportion females)	$H_{3,60} = 3.90$	0.27	0.64±0.07	0.72±0.07	0.69±0.03	0.70±0.08
Developmental time males (d)	$H_{3,56} = 0.31$	0.96	18.6±0.2	18.5±0.2	18.5±0.2	18.6±0.2
Developmental time females (d)	$H_{3,56} = 5.16$	0.16	20.0±0.2	19.8±0.2	20.3±0.2	20.3±0.2
Adult dry weight males (µg)	$F_{3,53} = 0.31$	0.82	247±9	258±9	251±10	248±9
Adult dry weight females (µg)	$H_{3,57} = 0.58$	0.90	359±10	371±6	364±4	368±7

[a]F: one-way ANOVA, H: Kruskal-Wallis ANOVA

Table 2. Fitness parameters of *Eupelmus vuilleti* females and larvae using *Acanthoscelides obtectus* developing in different chickpea seeds as hosts. Values are means ± SE. Significant p values and significantly different means (identified with Tukey HSD test) are in bold.

Fitness parameter	test statistic[a]	p	Semsen GM	Semsen PL	ICCV 10	ICCC 37
Realized fecundity (n)	$F_{3,56} = 4.01$	**0.01**	45.7±3.6	**47.5±3.9**	**39.6±2.3**	38.7±3.8
Offspring sex-ratio (proportion females)	$F_{3,56} = 1.68$	0.18	0.52±0.07	0.64±0.04	0.65±0.04	0.69±0.03
Developmental time males (d)	$WF_{3,52} = 0.61$	0.61	26.2±0.1	26.1±0.2	26.3±0.1	26.5±0.3
Developmental time females (d)	$F_{3,52} = 0.66$	0.58	28.0±0.2	27.7±0.2	28.0±0.2	27.6±0.3
Adult dry weight males (µg)	$F_{3,53} = 2.43$	0.08	523±7	500±11	530±7	513±7
Adult dry weight females (µg)	$F_{3,53} = 3.22$	**0.03**	962±8	**930±12**	961±8	**976±13**

[a]F: one-way ANOVA, WF: Welch's ANOVA

Table 3. Fitness parameters of *Heterospilus prosopidis* females and larvae using *Acanthoscelides obtectus* developing in different chickpea seeds as hosts. Values are means ± SE.

Fitness parameter	test statistic[a]	p	Semsen GM	Semsen PL	ICCV 10	ICCC 37
Realized fecundity (n)	$F_{3,56} = 0.52$	0.67	7.9±0.9	6.5±1.1	7.7±1.1	8.3±1.0
Offspring sex-ratio (proportion females)	$H_{3,58} = 4.82$	0.19	0.64±0.08	0.53±0.10	0.57±0.07	0.74±0.05
Developmental time males (d)	$F_{3,52} = 1.81$	0.16	18.4±0.3	19.3±0.4	18.5±0.3	18.1±0.3
Developmental time females (d)	$F_{3,50} = 0.68$	0.57	20.5±0.3	20.1±0.3	20.3±0.2	20.1±0.2
Adult dry weight males (µg)	$F_{3,52} = 0.82$	0.82	1339±134	1274±121	1194±101	1.04±97
Adult dry weight females (µg)	$F_{3,50} = 0.55$	0.65	1648±111	1668±64	1608±53	1541±84

[a]F: one-way ANOVA, H: Kruskal-Wallis ANOVA

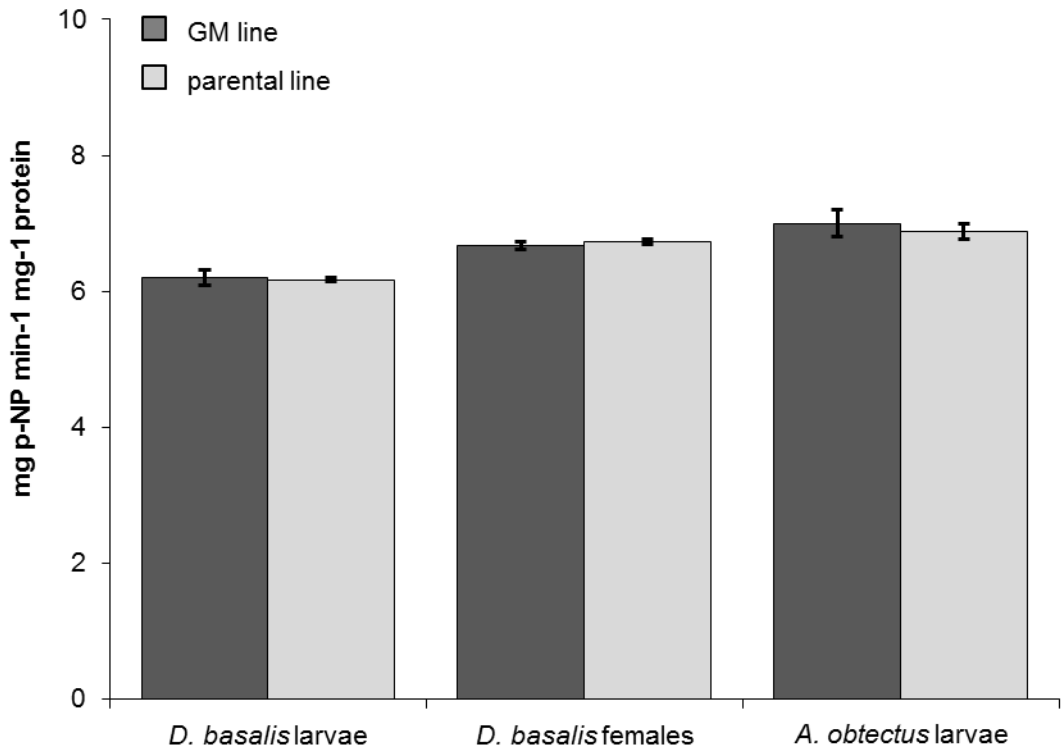

Figure 1. *In vitro* α-amylase activity of extracts of *Dinarmus basalis* larvae and females and *Acanthoscelides obtectus* larvae, expressed as mg of *p*-nitrophenol released from BPNPG7 min^{-1} mg^{-1} protein. Larvae of *A. obtectus* were developing either in chickpea seeds of the αAI-1 GM or the parental Semsen line and *D. basalis* was provided hosts developing in seeds of one of the two lines. Values are mean ± SE of 3 independent replicates. Each replicate was measured in duplicate.

CHAPTER 6

The Potential of the Bean α-Amylase Inhibitor αAI-1 to Control True Bugs (Hemiptera)

ABSTRACT

True bugs (Hemiptera) are an important pest complex not controlled by Bt crops. An alternative source of resistance includes inhibitors of digestive enzymes. αAI-1, an α-amylase inhibitor from the common bean, has been shown to inhibit α-amylases of bruchid pests of grain legumes. Here we quantify the *in vitro* activity of α-amylases of 12 Hemiptera species and the *in vitro* inhibition of those α-amylases by αAI-1.

α-Amylase activity was present in all species. Susceptibility to αAI-1 varied among the different groups. α-Amylases of species in the Lygaeidae, Miridae, and Nabidae were highly susceptible, whereas those in the Auchenorrhyncha (Cicadellidae, Membracidae) had a low susceptibility, and those in the Pentatomidae seemed to be tolerant to αAI-1. The susceptible families contain many important pest species but also potential biological control agents. We suggest that researchers assess the potential of αAI-1-expressing crops to control true bugs *in vivo* and also assess impacts on non-target Hemiptera.

Based on: Lüthi, C., Álvarez-Alfageme, F., Li, Y., Naranjo, S.E., Higgins, T.J.V., Romeis, J. The potential of the bean α-amylase inhibitor αAI-1 to control true bugs (Hemiptera) (submitted to Scientific Reports).

INTRODUCTION

Since their first commercial release in 1995, insect-resistant genetically modified (GM) crops have been increasingly adopted. In 2011, GM crops with insect resistance and with insect resistance/herbicide tolerance were grown on 66.1 million hectares worldwide (James, 2011). All these crops express lepidopteran- or coleopteran-active Cry toxins from *Bacillus thuringiensis* (Bt). An important pest complex not controlled by Bt crops are true bugs (Hemiptera). Moreover, the changes of pest management in Bt crops may contribute to the rise of minor or secondary hemipteran pests that were once controlled by the use of broad-spectrum synthetic insecticides applied to suppress pests now targeted by Bt crops (Naranjo, 2011). For example, the wide-scale adoption of Bt cotton in China increased the likelihood of mirid (Hemiptera: Miridae) outbreaks in cotton and surrounding crops (Lu et al., 2010).

True bugs are characterized by their piercing-sucking mouthparts and extra-oral digestion, which allows them to exploit a wide spectrum of plant hosts. When feeding on crops, Hemiptera cause not only direct damage but also indirect damage by acting as vectors of numerous plant pathogens, making them an important challenge for insect pest management (Chougule and Bonning, 2012). Despite the promise of hemipteran-active Bt toxins under development (Baum et al., 2007), alternative sources of genes should be considered in transgenic approaches for crop protection against hemipteran pests. A recent review of transgenic resistance to hemipteran pests (Chougule and Bonning, 2012) discusses the use of lectins and protease inhibitors as the most likely candidates. An additional group of plant-derived insecticidal compounds not considered by Chougule and Bonning (2012) are α-amylase inhibitors. α-Amylases are key digestive enzymes in the degradation of long-chain carbohydrates. α-Amylase activity has been demonstrated in herbivorous Hemiptera (e.g., Zeng and Cohen, 2000a, b), indicating that α-amylase inhibitors might be an option for the control of hemipteran pests. The same reports, however, also document α-amylase activity in predatory Hemiptera.

The α-amylase inhibitor αAI-1 from the common bean, *Phaseolus vulgaris*, has been studied extensively, and the gene coding for the inhibitor has been transferred into other legumes to confer resistance against bruchid beetles (Coleoptera: Chrysomelidae) (Shade et al., 1994; Ishimoto et al., 1996; Sarmah et al., 2004; Ignacimuthu and Prakash, 2006; Solleti et al., 2008) and into Arabica coffee, *Coffea*

arabica, to confer resistance against the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) (Barbosa et al., 2010). An *in vitro* screening of the inhibitory specificity of αAI-1 to various insects from eight different orders included two true bugs, *Eurydema oleracea* and *Graphosoma lineatum* (Hemiptera: Pentatomidae) (Kluh et al., 2005). However, α-amylases of both species had a low susceptibility to αAI-1.

In our study, we screened the *in vitro* activity of α-amylases from 12 Hemiptera species, and we measured the *in vitro* inhibition of the 12 α-amylases by αAI-1. The 12 Hemiptera species represent six families, including both herbivorous and predatory species. As a reference species, we included the bruchid *Callosobruchus chinensis,* which is highly susceptible to αAI-1 (Ishimoto and Kitamura, 1989). The data constitute the initial step in evaluating the potential of αAI-1 to control hemipteran pests in crops.

MATERIALS AND METHODS

Insect samples

In China, specimens of *Adelphocoris fasciaticollis, A. lineolatus, A. suturalis,* and *Apolygus lucorum* were collected from cotton fields at the experimental station of the Institute of Plant Protection, CAAS, near Langfang City, Hebei Province (39.5°N; 116.7°E), and the specimens of *Cyrtorhinus lividipennis* were collected from rice fields near Xiaogan City, Hubei Province (30.56°N; 113.54°E). In the USA, specimens of *Empoasca solana, Geocoris pallens, G. punctipes, Nabis alternatus,* and *Spissistilus festinus* were collected from alfalfa and cotton fields at the University of Arizona, Maricopa Agricultural Center farm (33.07°N; 111.97°W) in Maricopa, Arizona. Specimens of *Bagrada hilaris* were obtained from a greenhouse colony reared on broccoli at the USDA-ARS facility in Maricopa, Arizona. Upon transport from the field to the laboratory, living insects were frozen at -20°C, shipped to Switzerland on dry ice, and stored at -20°C. For comparison, the αAI-1 susceptible bruchid beetle *C. chinensis* (Coleoptera: Chrysomelidae), taken from a colony reared in our laboratory, was included in the study.

Chemicals

The Megazyme Ceralpha assay kit and additional blocked *p*-nitrophenyl maltoheptasoide were purchased from Megazyme (Wicklow, Ireland).

Diethylaminoethyl (DEAE) ion exchange and size fractionation column matrices were purchased from GE Healthcare (Upsala, Sweden). All other chemicals were purchased from Sigma Aldrich (St. Louis, USA).

Characterization of α-amylase activity

Frozen samples of the same species and stage were pooled (at least 15 specimens) and homogenized in ice-cold 0.15 M NaCl. The homogenates were centrifuged at 10000 *g* for 5 min, and the supernatants were pooled and stored at -20°C. Total protein content was determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The protein concentration in all samples was subsequently adjusted to 7 ± 2 µg $µl^{-1}$.

A pH range from 4.0 to 8.0 was generated by using a series of overlapping buffers. The buffers were 0.1 M malate (pH 4.0–5.5), 0.1 M maleate (5.5–6.5), and 0.1 M Bis-Tris propane (6.5–8.0). All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$. Assays were performed using a modified version of the Megazyme Ceralpha assay (McCleary et al., 2002) in 96-well microplates with a standard assay volume of 160 µl, which contained 2 µl of insect protein extract, 108 µl of buffer, and 10 µl of blocked *p*-nitrophenyl maltoheptasoide (BPNPG7) (27.25 µg $µl^{-1}$) as a specific substrate. Samples were incubated between 45 min to 24 h at 37°C. The reaction was stopped by adding 50 µl of 5% Tris (unbuffered). Absorbance was read at 405 nm at room temperature with a Spectrafluor Plus plate reader (Tecan, Männedorf, Switzerland), and α-amylase activity was quantified as mg of *p*-nitrophenol released from BPNPG7 min^{-1} mg^{-1} protein. All enzymatic assays were carried out in triplicate from the same pool of extracts, and appropriate blanks were used to account for spontaneous breakdown of substrates.

***In vitro* inhibitory activity of purified αAI-1**

The susceptibility of the α-amylases to αAI-1 was determined by *in vitro* assays. αAI-1 was purified from GM cowpea seeds of the variety Sasaque. Transformation of cowpea expressing αAI-1 has been described elsewhere (Popelka et al., 2006; Higgins et al., *in press*).). αAI-1 was purified from 50 g of cowpea meal as described by Marshall & Lauda (1975) with some modifications. Protein was extracted in a 1% NaCl solution. The solution was centrifuged, and the supernatant was collected and incubated in a water bath at 70°C for 30 min. The solution was centrifuged, and the

supernatant was collected. Unwanted protein in this solution was precipitated by adding 20% (w/v) ammonium sulfate. The solution was stirred and centrifuged, and the supernatant was collected and treated with 55% (w/v) ammonium sulfate to precipitate out αAI-1 from other proteins. The solution was stirred and centrifuged, and the pellet was collected and re-suspended in deionized water. All subsequent purification steps were carried out at 4°C. For all dialyses against water, deionized water containing 0.2% sodium azide was used. The solution was dialyzed against water and centrifuged, and the supernatant was collected. The supernatant was dialyzed against 10 mM citrate/phosphate buffer before it was loaded onto a DEAE-Sepharose CL-6B ion exchange column. Unbound protein was washed out with 10 mM citrate/phosphate buffer. Bound protein was washed out using a salt gradient of 100–500 mM NaCl in 10 mM citrate/phosphate buffer. Fractions of the outflow containing αAI-1 were identified using the Megazyme Ceralpha assay as described above; the fractions were pooled, dialyzed against water, freeze-dried, resuspended in 50 mM acetate buffer (pH = 5) with 5 mM $CaCl_2$, and run through a Sephadex S-200 size fractionation column. Collected outflow fractions containing αAI-1 were identified as above, pooled, dialyzed against water, freeze-dried, re-suspended in the same acetate buffer used in the previous step, and fractionated on a Sephadex S-25 column. Collected outflow fractions containing αAI-1 were identified as above and run separately on a 15–25% PAGE gel. Purest fractions were pooled, dialyzed against water, freeze-dried, and stored at -20°C.

For calculation of IC_{50} and IC_{90} values (the concentrations of αAI-1 required to inhibit the maximal α-amylase activity by 50 and 90%, respectively), dose-response curves using eight αAI-1 concentrations ranging from 0 to 2 µg ml^{-1} were constructed. For *G. punctipes*, *S. festinus*, *B. hilaris, Euschistus heros*, and *C. chinensis*, two additional concentrations up to 8 µg ml^{-1} were tested. The α-amylase activity of the different extracts were assayed at their respective optimal pH values, and the extracts were incubated with αAI-1 for 15 min at 37°C before adding the substrate. Dose-response curves were constructed, and IC_{50} and IC_{90} values were calculated using the software ReaderFit (Advanced Desktop version, Hitachi Solutions America Ltd., San Francisco, USA).

RESULTS

Characterization of α-amylase activity

We characterized the *in vitro* α-amylase activity in 12 Hemiptera species from six families; eight species are pests and four are considered generalist predators. Details on species, their stage, source, and host plant are provided in Table 1. α-Amylase activity was detected in extracts of all species assayed (Table 2). The pattern of the α-amylase activity over the range of pH was similar in all extracts. The activities of the α-amylases were nearly zero at pH 4.0 and were low at pH 4.5 but then quickly increased and reached their maximum at pH 5.0 to 6.5 (Supporting Figure 1). Beyond the optimal pH, the activity continuously decreased and was nearly zero at pH 8.0. The pH supporting maximal α-amylase activity never differed by more than 0.5 units for nymphs and adults of the same species and never by more than 1.0 unit within the same family.

α-Amylase activity ranged from 0.5 to 40.0 mg of *p*-nitrophenol released from BPNPG7 min^{-1} mg^{-1} protein (Table 2). α-Amylase activity was lower in the hemipteran extracts than in the extract of the reference species, *C. chinensis*. The only exception in this regard was the extract of *E. heros* adults, which had a similar activity as the latter. α-Amylase activity of the extracts was similar within the families Lygaeidae and Miridae and within the groups of Auchenorrhyncha and the predatory species. α-Amylase activity differed greatly between species and also between nymphs and adults of the two pentatomids. However, difference in the α-amylase activity between life stages of a species seemed to be species-specific, i.e., no general pattern was evident.

In vitro inhibitory activity of purified αAI-1

α-Amylase activity was inhibited by αAI-1 in all extracts with the exception of those of the two pentatomids, *B. hilaris* and *E. heros.* IC_{50} and IC_{90} values are given in Table 2. Concentrations of αAI-1 as high as 8 µg ml^{-1} did not inhibit α-amylase activity in extracts of adults or nymphs of the pentatomids (Figure 1F). Low susceptibility was also observed in extracts of *S. festinus* (Figure 1I) and *G. punctipes* (Figure 1H). Extracts of *A. fasciaticollis* nymphs and adults and *E. solana* had relatively high IC_{50} values but low IC_{90} values. The most susceptible extracts were those of *G. pallens* and *N. alternatus* (Figure 1H). Susceptibility to αAI-1 was greater for all other extracts than for the *C. chinensis* extract, which served as the highly susceptible control.

DISCUSSION

α-Amylase activity of Hemiptera has been mainly studied to understand hemipteran diversity and flexibility in feeding habits (Cohen, 1996, 1998; Zeng and Cohen, 2000b, a; Zeng and Cohen, 2000c; Boyd et al., 2002), but this important group of digestive enzymes might also be a target for pest management. Our results demonstrate that α-amylase activity is present in all species in our study regardless of their taxonomic or functional group. The pH profile of activity was similar in all extracts, and the optimal pH ranged from 5.0 to 6.0, which is consistent with reports of other Hemiptera species (Zeng and Cohen, 2000c; Kluh et al., 2005; Ramzi and Hosseininaveh, 2010). Given that the optimal pH for the formation of the enzyme-inhibitor complex (α-amylase-αAI-1) is 5.0 to 6.0 (Powers and Whitaker, 1978), αAI-1 seems to be very well suited for the potential use against hemipteran pests in GM crops. However, the similar profile of predatory species indicates that impacts on this group of non-target insects must also be considered.

Whereas the pH profile was similar in all extracts, the levels of α-amylase activity varied considerably. Nymphs and adults of all mirid species had similar levels of activity. The adults of the three predator species investigated had activity levels in the same range as the mirids. In contrast, the α-amylases of the two Auchenorrhyncha, *E. solana* and *S. festinus,* had the lowest activity, indicating that α-amylases play a minor role in their digestive system. These are only two species, however, in a large taxonomic group that contains many pests; determining whether Auchenorrhyncha in general possess a low-level of α-amylase activity will require the assay of additional species. The variation among species was greatest between the two pentatomids. Adults of the pentatomid *Euschistus heros* had the highest α-amylase activity of all extracts tested, even higher than for the bruchid *C. chinensis.* Bruchid beetles are well known to require these enzymes to obtain energy and metabolic water from carbohydrates (Southgate, 1979; Murdock et al., 2012). Furthermore, the α-amylase activity was more than five times higher in adult *E. heros* than in the nymphs. Although the nymphs and adults were reared on different hosts (soybeans and peanuts, respectively), it is unlikely that this difference in activity is due to an adaptation to the different food source because both sources have similar carbohydrate levels and are rich in protein and lipids. Remarkably, α-amylase activity also differed greatly in adults vs. nymphs of the second pentatomid, *B. hilaris*, but in the other direction: activity was higher in nymphs than adults. Here, both life stages

were taken from the same greenhouse host. In the case of the mirids, activity was similar for nymphs and adults of the herbivorous species but differed substantially for nymphs vs. adults of the predatory species *C. lividipennis*. These findings suggest that differences in the digestive systems of nymphs and adults might be species-specific.

Agusti and Cohen (2000) suggested that the high activity in the saliva of two herbivorous mirids, *Lygus lineolaris* and *L. hesperus*, indicates an adaptation to exploit insoluble starch from plant material via extra-oral digestion. Zeng and Cohen (2000a) reported that the α-amylase activity was dramatically lower in the salivary gland of the predator *G. punctipes* than in those of the herbivorous *L. lineolaris* and *L. hesperus*; they concluded that these are adaptations to their respective feeding behaviors. Surprisingly, we found that the α-amylase activity in extracts of whole insects of *G. punctipes*, i.e., extracts of both salivary glands and midgut, was higher than in any mirid species in our study. This indicates that it is important to distinguish between salivary and midgut α-amylase activity if α-amylase activity is to be used as an indicator of feeding habit.

Although α-amylase activity was detected in all species analyzed, not all α-amylase extracts were inhibited by αAI-1. Interestingly, the only extracts not inhibited were from nymphs and adults of the two pentatomid species. This is in line with the results from a previous study reporting a lack of inhibitory activity of αAI-1 on the pentatomids *Eurydema oleracea* and *Graphosoma lineatum* (Kluh et al., 2005). However, the authors of this previous study concluded that Hemiptera in general might be tolerant to αAI-1. Our results show that while this may be true for Pentatomidae, it is not true for Hemiptera in general because α-amylases of all other species were inhibited by αAI-1. Whereas susceptibility was similar in most of these species, there were two remarkable exceptions. First, *S. festinus* was much less susceptible than all other species. This species was also the one with lowest α-amylase activity. Second, α-amylase activity of the predator *G. punctipes* did not fall below 40% of original activity even at the highest concentrations of αAI-1 tested. Either the α-amylases of this species are only partially inhibited or it has multiple α-amylases and not all are susceptible to αAI-1. This pattern was not observed in the second predator from this genus, *G. pallens*.

Unexpectedly, all other species were at least as susceptible to αAI-1 as the bruchid *C. chinensis*. This is especially interesting because these species include both

herbivorous as well as predatory species from four different families. It is encouraging that all species in the families Lygaeidae and Miridae were highly susceptible to αAI-1. These two families contain several important cotton pest species not controlled by Bt-transgenic varieties (Naranjo et al., 2008).

To further evaluate whether GM crops that express α-amylase inhibitors can be used to manage hemipteran pests, researchers should answer the following questions with *in vivo* experiments: (i) To what extent are Hemiptera dependent on α-amylases? and (ii) What α-AI-1 concentrations in plant tissues are required to achieve a sufficient level of protection? Both questions could be answered by feeding insects artificial diets (Cohen, 1985; Cohen, 2000) containing purified αAI-1. Because the susceptibility of non-target Hemiptera to αAI-1 was similar to that of target Hemiptera, the impact on non-target species, especially the important group of predatory bugs, should be assessed using the same approach.

The development of non-legume GM crops expressing αAI-1 also requires research concerning αAI-1 levels in plant tissues. Because αAI-1 is a storage protein in beans, it is accumulated during seed maturation, resulting in levels of 1.00–1.25% (w/w) (Huesing et al., 1991). Although this amount of αAI-1 is sufficient to protect mature seeds against bruchid beetles (Ishimoto and Kitamura, 1989; Huesing et al., 1991), whether levels in other plant tissues can be increased sufficiently to suppress sap-feeding hemipteran pests is not known. Furthermore, expression of αAI-1 in transgenic legumes is restricted to the cotyledons and the embryonic axis of the developing seeds because the bean phytohemagglutinin-L (dlec2) promoter is used (Shade et al., 1994) and because the pro-protein requires posttranslational modifications to become active (Pueyo et al., 1993). For αAI-1 to be expressed in other tissues, an alternative promoter must be used, and a gene coding for the enzyme cleaving the pro-protein into the two subunits must be transferred together with the gene coding for αAI-1. However, these are technical problems that can be solved. Our encouraging initial results on the potential of αAI-1 to protect crops from true bugs indicate that this approach is worth investigating.

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Table 1. Overview of the hemipteran samples included in the study.

Suborder	Family	Species	Stage[a]	Functional group	Host[b]	Origin
Auchenorrhyncha	Cicadellidae	*Empoasca solana*	A	Herbivore	Alfalfa/cotton	USA
	Membracidae	*Spissistilus festinus*	A	Herbivore	Alfalfa	USA
Heteroptera	Lygaeidae	*Geocoris pallens*	A	Generalist predator	Alfalfa/cotton	USA
		Geocoris punctipes	A	Generalist predator	Alfalfa/cotton	USA
	Miridae	*Adelphocoris fasciaticollis*	N A	Herbivore	Cotton	China
		Adelphocoris lineolatus	N A	Herbivore	Cotton	China
		Adelphocoris suturalis	N A	Herbivore	Cotton	China
		Apolygus lucorum	N A	Herbivore	Cotton	China
		Cyrtorhinus lividipennis	N A	Generalist predator	Rice	China
	Nabidae	*Nabis alternatus*	A	Generalist predator	Alfalfa/cotton	USA
	Pentatomidae	*Bagrada hilaris*	N A	Herbivore	Broccoli	Greenhouse colony
		Euschistus heros	N A	Herbivore	Soy Peanut	Lab colony

[a] A= adult, N= nymph.

[b] Plant on which the insects were collected in the field or greenhouse or reared in the laboratory.

Table 2. Optimal pH and specific α-amylase activity (mg of *p*-nitrophenol released from BPNPG7 min^{-1} mg^{-1} protein) of the various Hemiptera species and stages, and *in vitro* inhibition of the α-amylases by αAI-1 expressed as IC_{50} and IC_{90}. IC_{50} and IC_{90} values lower than in the reference species *Callosobruchus chinensis* are in bold.

Source of extract			α-Amylase activity		Inhibitory concentration[c]	
Family	**Species**	**Stage[a]**	**Optimal pH**	**Specific activity[b]**	**IC_{50}**	**IC_{90}[d]**
Cicadellidae	*Empoasca solana*	A	6.5	1.99±0.04	**0.67**	**(2.35)**
Lygaeidae	*Geocoris pallens*	A	5.5	8.54±0.09	**0.12**	**0.42**
	Geocoris punctipes	A	5.5	13.01±0.01	0.99	n.d.
Membracidae	*Spissistilus festinus*	A	6.5	0.50±0.01	2.78	(76.06)
Miridae	*Adelphocoris fasciaticollis*	N	5.5	5.46±0.25	**0.60**	**(2.18)**
		A	5.5	5.46±0.46	0.93	**(2.99)**
	Adelphocoris lineolatus	N	5.5	8.34±0.27	**0.23**	**1.87**
		A	5.0	4.25±0.03	**0.20**	**(2.08)**
	Adelphocoris suturalis	N	5.0	8.25±0.05	**0.17**	**(2.50)**
		A	5.0	10.58±0.22	**0.22**	**(3.96)**
	Apolygus lucorum	N	5.0	9.21±0.02	**0.34**	**(3.31)**
		A	5.5	7.78±0.05	**0.16**	**(4.03)**
	Cyrtorhinus lividipennis	N	6.0	12.43±0.11	**0.32**	**(3.13)**
		A	6.0	5.70±0.10	**0.35**	**(6.63)**
Nabidae	*Nabis alternatus*	A	6.0	9.95±0.58	**0.26**	**0.83**
Pentatomidae	*Bagrada hilaris*	N	5.5	9.71±0.18	n.i.	n.i.
		A	5.5	3.29±0.04	n.i.	n.i.
	Euschistus heros	N	6.5	7.44±0.10	n.i.	n.i.
		A	6.0	40.02±1.47	n.i.	n.i.
Chrysomelidae	*Callosobruchus chinensis*	L	6.5	30.29±0.89	0.82	7.2

[a] A = adult; N = nymph; L = larva.

[b] Values are means ± SE of triplicate measurements from a unique pool of extracts.

[c] µg αAI-1 ml^{-1}, n.d. = not determinable; n.i. = not inhibited.

[d] IC_{90} values in parentheses are beyond the highest αAI-1 concentration tested and were calculated using the equation of the best fit model determined by ReaderFit software.

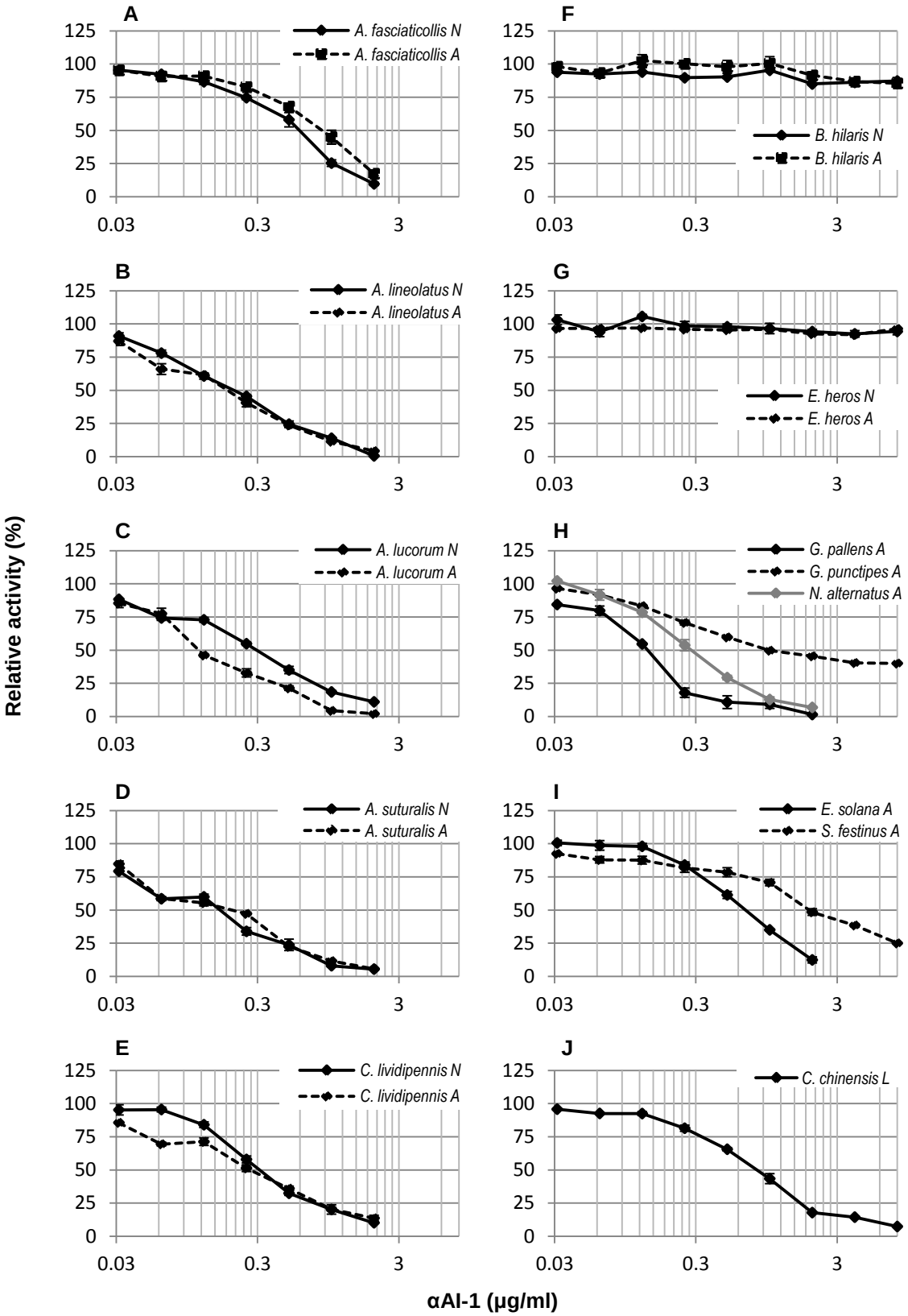


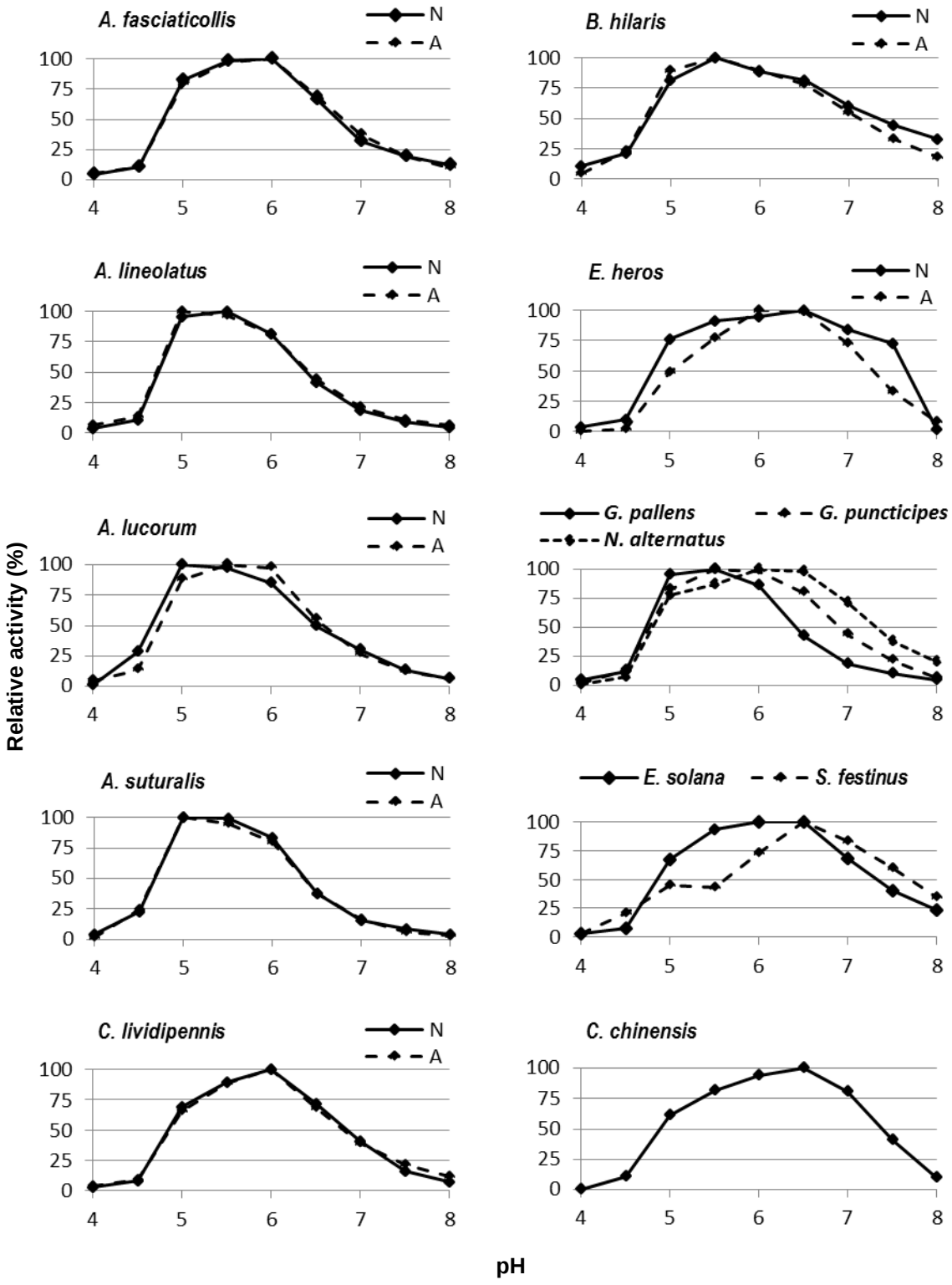
Figure 1. Effect of αAI-1 on the relative *in vitro* activity of α-amylases in extracts from herbivorous Miridae (A-D), a predatory Miridae (E), Pentatomidae (F,G), predatory

Lygaeidae and Nabidae (H), Auchenorrhyncha (I), and the bruchid *C. chinensis* (J). Activity is expressed relative to that in the absence of the inhibitor. Bars represent means ± SE of three measurements from a unique pool of extracts. N=nymph, A=adult, L=larva.

Supplementary Table 1. Total amount of protein used and incubation time at 37°C in the *in vitro* α-amylase activity and inhibition assays for all samples included in the study.

Family	Species	Stage[a]	Protein (µg)	Incubation time (min)
Cicadellidae	*Empoasca solana*	A	12.42	480
Lygaeidae	*Geocoris pallens*	A	9.20	120
	Geocoris punctipes	A	9.74	120
Membracidae	*Spissistilus festinus*	A	13.44	1440
Miridae	*Adelphocoris fasciaticollis*	N	12.08	120
		A	17.60	180
	Adelphocoris lineolatus	N	11.66	120
		A	12.64	180
	Adelphocoris suturalis	N	14.18	180
		A	12.82	180
	Apolygus lucorum	N	14.42	180
		A	12.42	120
	Cyrtorhinus lividipennis	N	12.16	120
		A	10.56	180
Nabidae	*Nabis alternatus*	A	11.54	120
Pentatomidae	*Bagrada hilaris*	N	10.08	120
		A	9.92	360
	Euschistus heros	N	11.54	180
		A	15.20	45
Chrysomelidae	*Callosobruchus chinensis*	L	13.18	45

[a]A = adult; N = nymph; L = larva.



Supplementary Figure 1. Relative α-amylase activity of insect extracts as affected by pH. pH was adjusted using a series of overlapping buffers (malate: pH = 4.0–5.5, maleate: pH = 5.5–6.5, Bis-Tris propane: pH = 6.5–8.0). Maximum activity was set to 100%. N=nymph, A=adult. Where no stage is indicated, adults were assayed (except that larvae were assayed for *C. chinensis*).

CHAPTER 7

General Discussion

Grain legumes expressing αAI-1 in their seeds are highly resistant to several important bruchid pest species. In Chapter 2, we demonstrate the power of this approach in GM chickpea and cowpea expressing this inhibitor. Within-seed mortality rates of the three bruchids *Acanthoscelides obtectus*, *Callosobruchus chinensis* and *C. maculatus* was low or absent in all non-GM genotypes tested. In contrast, the *Callosobruchus* spp. suffered from significantly increased mortality rates inside the GM seeds. There are several important advantages of seed-borne resistance factors. Firstly, there is no special knowledge or equipment required for their application, farmers only need access to the seeds. Secondly, genetic engineering allows to introduce the trait of resistance in basically any existing variety. Robust, reproducible, and efficient transformation procedures are available for many legume species (Popelka et al., 2004). Varieties with desirable nutritional characteristics and / or adaptation to local environmental conditions can thus be transformed to express αAI-1 to make them immune to αAI-1-susceptible bruchid species. Thirdly, the seeds can be combined with basically any other bruchid management approach available. Simple, yet effective methods available to small-scale subsistence farmers in developing countries are the triple bagging system, the drum storage technology, or the release of natural enemies (Sanon et al., 1998; Schmale et al., 2001; Murdock et al., 2003). The combination with additional management strategies is particularly relevant to protect the seeds against the αAI-1 tolerant bruchids *A. obtectus* and *Zabrotes subfasciatus* (Ishimoto and Kitamura, 1992) which are not affected by the presence of αAI-1 in the legume seeds and to prevent the development of resistance in susceptible species (Chapter 2 and 4).

The combination of resistance factors and parasitoid of the larval and pupal stages of bruchids, the most important group of natural enemies, has been shown to be a powerful approach to control bruchids (Schmale et al., 2003; Velten et al., 2008). There are, however, concerns that the parasitoids could be harmed by the introduced

insecticidal trait in GM seeds. We assessed the risk of αAI-1 to bruchid parasitoids according to the conceptual model presented in Chapter 1.

The first two steps of this model, whether bruchid parasitoid possess α-amylases and whether those α-amylases are inhibited by αAI-1, were both confirmed by the experiments presented in Chapter 3. Hence, in case of an exposure to αAI-1, the parasitoids might actually be harmed by the inhibitor.

The next step was assessing the exposure of the parasitoids to αAI-1. Both parasitoid larvae and adult females feed on bruchid larvae and are thus potentially exposed to αAI-1. In Chapter 5, we show that the *in vitro* α-amylase activity in extracts of *Dinarmus basalis* larvae and females collected from hosts developing in GM or control seeds was very similar. This allows to conclude that the exposure to αAI-1 is negligible.

The results concerning the next step in the model, i.e., whether the ingestion of αAI-1 affects the parasitoids, confirmed our assumption that the exposure is negligible. The impact of αAI-1 on parasitoids was investigated in two tritrophic systems. In the first system, we used seeds of several corresponding pairs of GM and control cowpea lines and *Z. subfasciatus* as host for the parasitoid *D. basalis* (Chapter 4). In the second system, we used a GM chickpea line and three non-transformed control lines and *A. obtectus* as hosts for the parasitoid species *D. basalis*, *Eupelmus vuilleti* and *Heterospilus prosopidis* (Chapter 5). The results in both systems were concordant: the presence of αAI-1 in the seeds does not affect the fitness of the parasitoids. There were no direct (i.e., via host-mediated exposure to αAI-1) nor indirect (i.e., via reduced host quality) effects detectable. Moreover, direct-feeding assays with adult female parasitoids reveled toxic effects only at αAI-1 concentrations and at adult age that are unlikely to occur in the field (Chapter 2).

Because we could show that the pathway in the conceptual model is interrupted, we have good evidence to conclude that GM seeds containing αAI-1 will not cause a decline in the parasitoid population (step 5 in the model) and thus that the biological control services provided by the parasitoids will not be disrupted. In a nutshell: the αAI-1 GM legumes do not seem to pose a risk for this important group of non-target insects.

The number of other bruchid enemies that could be harmed by αAI-1 is very limited. Because only bruchid larvae ingest the inhibitor, the risk is restricted to natural enemies attacking this particular life-stage. Thus, another important group of

parasitoids in the genus *Uscana* (Hymenoptera: Trichogrammatidae) (van Huis et al., 1990) are not at risk because they are parasitizing the bruchid eggs. Additionally, some generalist predators seem to prey on bruchids occasionally, but they only attack the free-living stages, i.e., eggs and adults (Van Alebeek, 1996; Sing and Arbogast, 2008). Taken together, the impact of αAI-1 GM legumes on bruchid natural enemies will generally be negligible.

Because of its broad range of activity (Franco et al., 2002; Kluh et al., 2005), αAI-1 could be an alternative to the widely deployed *Bt* crops. An important pest complex not controlled by *Bt* crops are true bugs (Hemiptera). Our *in vitro* screening revealed that α-amylases of several Hemiptera families are strongly inhibited by αAI-1. Those families contain many important pest species, but also potential biological control agents (Chapter 6). The potential of αAI-1-expressing crops to control true bugs and impacts on non-target hemipterans now needs to be assessed *in vivo*. Additionally, there are some technical problems for αAI-1 to be expressed in other tissues than cotyledons and embryonic axis of legume seeds. However, the urgent need for alternative to *Bt* crops and our encouraging initial results on the potential of αAI-1 to protect crops from true bugs indicate that this approach is worth investigating.

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gemäss Art. 28 Abs. 2 RSL 05

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Bachelor ☐ Master ☐ Dissertation ☒

Titel der Arbeit: Impact of transgenic legumes expressing an α-amylase inhibitor on storage pests and their natural enemies

Leiter der Arbeit: PD Dr. Jörg Romeis

Ich erkläre hiermit, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen benutzt habe. Alle Stellen, die wörtlich oder sinngemäss aus Quellen entnommen wurden, habe ich als solche gekennzeichnet. Mir ist bekannt, dass andernfalls der Senat gemäss Artikel 36 Absatz 1 Buchstabe o des Gesetztes vom 5. September 1996 über die Universität zum Entzug des auf Grund dieser Arbeit verliehenen Titels berechtigt ist.

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Ort/Datum

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Unterschrift

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