

The Role of Protease Inhibitors and Parasitoids on the Population Dynamics of *Sitotroga cerealella* (Lepidoptera: Gelechiidae)

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ABSTRACT The Angoumois grain moth, *Sitotroga cerealella* (Oliver), is one of the major storage pests of cereals, and no antibiotic resistance in wheat against this insect has been identified to date. Midgut proteases are vital to insects that digest food in the midgut and have been considered as targets for the control of insect pests. Protease inhibitors are attractive for their potential use in developing insect-resistant plant varieties via genetic engineering. Characterization of the midgut proteases of *S. cerealella* larvae revealed the major digestive proteases were trypsin-like and α -chymotrypsin-like serine proteases. The partial inhibition of proteolytic activity by pepstatin A, however, suggested the presence of another protease in the midgut sensitive to this inhibitor. The potential value of naturally occurring plant protease inhibitors as resistance factors for *S. cerealella* was assessed in bioassays using artificial seeds prepared by freeze-drying a flour paste in Teflon molds and then coating the seeds with gelatin. Soybean trypsin inhibitor (Kunitz inhibitor) had an adverse effect on the development of the insect and suggested a protease inhibitor might serve as a transgenic resistance factor. To evaluate the potential value of seed resistance in conjunction with an egg parasitoid on *S. cerealella* population dynamics a predictive model was developed. The model was directed toward grain storage in developing countries. While the model was hypothetical, outputs supported the use of resistant seed in conjunction with parasitoids to control the population growth of *S. cerealella* in a small seed storage room.

KEY WORDS *Sitotroga cerealella*, Angoumois grain moth, protease inhibitors, parasitoids, population dynamics

CEREAL GRAINS REMAIN one of mankind's principal sources of food today, especially in the developing countries. In the developing world, 68-98% of the cereals produced are used for human consumption. However, losses because of improper postharvest handling and processing are large, ranging from 2 to 40% for rice, 1-100% for maize, 2-52% for wheat, and 1-68% for legumes in various developing countries (Multon 1988). The use of resistant seed and parasitoids may be important for protection of stored grains in developing countries where harvesting, drying, and storing of grains are still done in traditional ways. Additionally, with the World's population increasing in the 21st century, problems associated with food supply and the environment, and the banning of methylbromide for food preservation (Mitsuda 1999) such research can be of value in developed countries.

The Angoumois grain moth, *Sitotroga cerealella* (Oliver), is a major insect pest of stored grains and can

infest grains in the field or in storage (Cogburn and Vick 1981, Barney and Weston 1994, Weston and Rattlingourd 1999). Females deposit eggs on the surface of seeds and first-instar larvae burrow into the seed where they complete their development. Not only are wheat, corn, barley, sorghum, and rice hosts for *S. cerealella*, but seeds of oat, rye, cowpea, chickpea, buckwheat, and beans also are subject to infestation. Currently, no effective resistance in common wheat, *Triticum aestivum* L., to *S. cerealella* has been reported.

A possible approach to developing wheat cultivars with resistance to *S. cerealella* is to incorporate, via genetic engineering, the gene for a specific protease inhibitor that can act as a resistance factor. Altpeter et al. (1999) demonstrated that barley trypsin inhibitor CMe (BTI-CMe) reduced the survival rate of early-instar larvae of Angoumois grain moth reared on transgenic wheat seeds expressing the inhibitor compared with the untransformed control. However, BTI-CMe did not provide a protective effect against leaf-feeding insects. To select effective inhibitors of digestive proteases as transgenes for resistance, knowledge of the character of midgut proteases in larvae of *S. cerealella* and the effects of the inhibitors on their activities is needed.

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Development and implementation of efficacious crop production and protection practices has resulted in higher yields for many crops. Of equal importance, particularly for grain, is the development of effective storage practices to reduce losses in stores. In industrialized countries losses in stored grain caused by insect pests are estimated at 1%, which contrasts with 10–30% in developing countries (Scholler et al. 1997). The use of biological control with parasitoids as a potential component of pest management in stored product protection has been proposed (Brower 1990, Brower and Press 1990, Flinn et al. 1994, Prozell et al. 1995, Scholler et al. 1997). The use of transgenes for resistance consolidated with parasitoids could provide a useful strategy for pest management in stored grain particularly in developing countries.

To assess the potential effect of seed resistance and parasitoids, such as *Trichogramma* spp., on control of *S. cerealella* in a storage room, a model describing *S. cerealella* population dynamics is a useful tool. Such a mathematical hypothesis will allow development of additional hypotheses concerning the effects of various parameters and conditions on the population growth of *S. cerealella* in storage facilities. This will bring together biochemical and bioassay work directed toward developing seed resistance and practical control of the pest. The current work was directed to 1) characterize the digestive proteases of *S. cerealella* larvae, 2) identify inhibitors of the proteases, 3) evaluate by bioassay the inhibitors as resistance factors, and 4) develop a predictive model to describe the population dynamics of *S. cerealella* in a small storage room of the type potentially used in agricultural systems of developing countries.

Materials and Methods

Experimental Insect. *S. cerealella* larvae were reared on wheat seeds (cultivar 'Monon') in an environmental chamber at 27°C with a 12-h photoperiod and 70% RH in plastic jars (16 cm in diameter) containing 300 g of wheat seeds mixed with 4 g of brewers yeast. Newly emerged adults were collected from 42- to 48-d old cultures and 50 moths were transferred to one of the containers with seeds. Under environmental chamber conditions, ≈45 d were required for the insect to complete a generation. *S. cerealella* voucher specimens deposited in the Purdue Entomological Research Collection.

Midgut Extracts. Midguts were dissected from early last-instar larvae by making a mid-dorsal incision and excising the posterior and anterior ends of the larva. The midgut was then removed from the body and suspended in 0.15 M NaCl. Fifty midguts were collected in 50 μ l of 0.15 M NaCl contained in a 1.5-ml polypropylene centrifuge tube kept on ice. The midguts were stored at -70°C until assayed for proteolytic activity. To prepare midgut extracts, frozen midguts were homogenized in the centrifuge tube with a microcentrifuge sample pestle (Sigma, St. Louis, MO). The homogenate was diluted to 500 μ l with buffer at the pH for the enzyme assay to be performed. The

diluted homogenate was centrifuged at 14,000 rpm for 10 min at 4°C in a microcentrifuge (Eppendorf model 5415, Eppendorf, Westbury, NY) and the supernatant removed for use in enzyme assays.

pH of Midgut Content. The pH of midgut content was determined as described by Wolfson and Murdock (1990). Midguts were dissected as described above and the associated hemolymph absorbed on a piece of filter paper. The contents were expelled onto pH indicator sticks (Sigma, St. Louis, MO: range pH 4.0–7.0 and pH 6.5–10.0). Colors developed were compared with the standards provided by the manufacturer.

Enzyme Assays. Continuous spectrophotometric assays for the hydrolysis of tosyl-L-arginine methyl ester (TAME) and benzoyl-L-tyrosine ethyl ester (BTEE) were carried out at room temperature with a split-beam UV-visible spectrophotometer as described by Hummel (1959). The reaction volume was 1 ml, and reaction rates were determined from the linear portion of the change in absorbance per min recorded. For spectrophotometric assays, one enzyme unit was defined as the amount of material required to hydrolyze 1 μ mol of substrate per min under the conditions of the reaction. Micromoles of substrate hydrolyzed were determined from the change in absorbance per min using the following molar extinction coefficients: TAME ($E_{247} = 540$; Decker 1977), BTEE ($E_{256} = 964$; Decker 1977). Radiometric assays for protein hydrolysis were carried out as described by Kitch and Murdock (1986). The substrate consisted of 0.4 μ Ci/ml [3 H]methemoglobin (Kumarasamy and Symons 1979) and 2 mg/ml of unlabeled methemoglobin. Reactions were initiated by the addition of 50 μ l of enzyme, diluted in buffer, to 50 μ l of substrate solution in a 1.5-ml centrifuge tube. A blank was included to determine the amount of trichloroacetic acid (TCA)-soluble material in control reactions without enzyme action. In the radiometric assay, the amount of material required to release 2,000 dpm (corrected for the blank) from [3 H]methemoglobin at 37°C during a 10-min incubation was defined as one unit.

Distribution of Midgut Proteases. The distribution of proteolytic activity between midgut lumen content and midgut tissue was determined after separating lumen content from midgut tissue. The contents from 50 midguts were collected in 50 μ l of 0.15 M NaCl kept on ice. Midgut tissue was washed in ice cold 0.15 M NaCl and homogenized in 50 μ l of 0.15 M NaCl in a 1.5-ml centrifuge tube. Lumen content and homogenate of midgut tissue were clarified by centrifugation in a microcentrifuge (*vide supra*), and the supernatants were removed for enzyme activity assays.

Characterization of Proteases. The effects of pH on the hydrolysis of TAME, BTEE, and [3 H]methemoglobin were determined by spectrophotometric and radiometric assays. Buffers used were: 0.05 M 1,4-piperazinediethanesulphonic acid, pH 5.0–7.4; 0.1 M Tris-HCl, pH 7.4–8.9; 0.2 M glycine-NaOH, pH 8.9–11.2; 0.05 M $\text{Na}_2\text{HPO}_4/\text{Na}_3\text{PO}_4$, pH 11.2–12.4. Ionic

strength of all buffers was adjusted to that of 0.2 M NaCl with NaCl.

The effects of diagnostic inhibitors on the activity of midgut proteases were determined by the spectrophotometric and radiometric assays for enzyme activity described above. Diagnostic inhibitors of serine proteases included diisopropyl fluorophosphate (DFP), N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK), an inhibitor of trypsin-like enzymes, and L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), an inhibitor of chymotrypsin-like enzymes. Iodoacetamide (IAAm) was used as a cysteine protease inhibitor (Barrett 1977a) and pepstatin A was used as a carboxyl protease inhibitor (Barrett 1977b).

The effects of two naturally occurring plant protease inhibitors on the proteolytic activity of midgut proteases were also assessed. Inhibitors used were the Bowman-Birk inhibitor (BBI) and the Kunitz soybean trypsin inhibitor (STI) (both from Sigma, St. Louis, MO). Midgut extracts were incubated on ice for 10 min with inhibitors before they were assayed for enzyme activity.

Bioassay of Inhibitors. Artificial seeds used to assess the effects of protease inhibitors on development of *S. cerealella* were prepared by the method of Shade et al. (1986). The basic diet used was flour milled from seeds of wheat cultivar 'Monon'. To reduce the possible effects of salts, inhibitors were dialyzed against distilled water for 18 h, lyophilized, and then dissolved in 25 mM potassium phosphate buffer (pH 7.0). Inhibitors were dissolved at various concentrations and incorporated at 1.0 ml of solution per 1.0 g of flour. Final concentrations of inhibitor in the diet were 1% and 5% (wt:wt). These concentrations were chosen because proteins considered having potential function as defensive factors in plants (protease inhibitors, α -amylase inhibitors, lectins, and lipoxygenases) occur within this concentration range in seeds (Shukle and Murdock 1983). To control for salt in the diet, artificial seeds with the same amount of potassium phosphate present with the inhibitor were used. To control for added protein in the diet, casein was added as a general protein at concentrations of 1% and 5% (wt:wt).

Eggs at a similar developmental stage (one day before hatching) deposited on paper strips by adults from the laboratory culture reared on wheat seeds were used to infest artificial seeds. Two eggs were transferred to an artificial seed in a glass culture tube (13 by 100 mm) plugged with a cotton ball. Twenty artificial seeds for each treatment or artificial seed type were used and the bioassay was replicated twice. Development of *S. cerealella* in wheat seeds (cultivar 'Monon') under the same environmental conditions (*vide supra*) was used as controls for development in artificial seeds. Data from development of *S. cerealella* in artificial seeds were analyzed by Student's *t*-test, and means were considered significantly different at $P \leq 0.05$.

Model Structure. The model was constructed to 1) describe *S. cerealella* egg population dynamics on wheat seeds in a small storage room and 2) determine

the potential effects of seed resistance, parasitoids, and removal of seeds on *S. cerealella* egg population growth. The model was to represent conditions in developing countries where farmers would remove seed from storage for personal use.

These assumptions were made in construction of the model. It was assumed wheat was planted in November and harvested in May. The normal seed storage period was six months from 1 June (integer date 152) to 30 November (integer date 334). The seed storage room was cylindrical, 2.4 m in diameter and 2.5 m high. These dimensions were selected to represent a small seed storage room that might be used in developing countries. Infestation by *S. cerealella* started in the field before harvest, and eggs were brought in with the seeds.

At the beginning of the storage period, there were fifty host eggs on seeds in the upper surface area of the grain in the storage room. No *S. cerealella* adults were present at the beginning of the storage period. Adults emerged from seeds in the upper surface area of the stored grain. Oviposition by females was limited to seeds in the upper surface area of the grain. Over time host eggs were evenly distributed on the upper surface of the stored grain. Therefore, egg density was expressed in terms of area.

Twenty percent of the stored seeds had resistance. The proportion of resistant seeds can be varied, but for initial conditions in construction of the model twenty percent was selected as representing a mix of resistant and susceptible seeds placed into storage in agricultural systems of developing countries. Ten adult parasitoids were introduced at the beginning of the storage period. This was selected as representing a minimum number of adults for successful inoculation of parasitoids into the storage room. Parasitoid search range was limited to the upper surface area of the stored grain, and resistant seeds had no direct effect on the parasitoid. Twenty percent of the stored seeds were removed at 30-d intervals from the top of the grain in the storage room. This value was selected to accommodate expected removal of seeds from the storage room for production of flour by farmers in the agricultural systems of developing countries.

The system included four mini-models 1) a parasitoid search model, 2) a host egg model, 3) a host adult model, 4) a parasitoid adult model. Model output was the number of host eggs per m^2 versus time in days; the programming language was FORTRAN. Model parameters and variables are listed and defined in the text and in Table 1.

Parasitoid Search model. The parasitoid (*Trichogramma* spp.) search model was expressed as $S_t = 0.01e^{(-0.00005 \times Et)} + 0.001$. The search model considered search area of parasitoids as related to host density and followed a negative exponential function (O'Neil 1988, Wiedenmann and O'Neil 1992) as $S_t = C_1 e^{(-C_2 \times N/A)} + C_3$, where N/A was prey density expressed as the number of host eggs per m^2 at time t (E_t), C_1 was the maximum search area, C_2 was the density constant (rate at which C_1 goes to C_3), and C_3 was the minimum search area.

Table 1. List and definition of model parameters and variables

Symbol	Definition
S_t	Search area of parasitoid adult females at time t
C_1	Maximum search area for the parasitoid
C_2	Density constant, rate at which C_1 goes to C_3
C_3	Minimum search area for the parasitoid at high prey density
E_t	Number of host eggs per m^2 at time t
A_t	Number of host adults at time t
P_t	Number of parasitoid adults at time t
R	Host daily reproductive rate
H	Daily host egg hatching rate
M_A	Daily host adult mortality rate
M_E	Host egg mortality
M_L	Mortality of host larvae from seed resistance
M_P	Daily parasitoid mortality rate
I	Daily increase rate of host adults
G	Daily growth rate of the parasitoid
K	Proportion of resistant seeds to total amount of seeds
W	Removal of host eggs, adults, and parasitoids with seeds
t	Time in days starting with integer date 152, June 1
Dt	Time step (one day)

In host-parasitoid systems the actual amount of search time allocated may vary from patch to patch even in patches of equal host density and field measurements of searching time allocation may be required to validate significant searching time aggregation by parasitoids. (Morrison 1986). In *Trichogramma* spp., host population density has been shown to be an important factor in host acceptance and in the percentage of females parasitizing at least one host (Reznik and Umarova 1991). The spatial distribution of parasitism in relation to the host distribution has been shown to be inversely density dependent for *Trichogramma papilionis* Nagarkatti (Hirose et al. 1976). Variation in the travel speed of females of different *Trichogramma maidis* Pintureau and Voegele strains also has been related to the potential for parasitism in the field (Bigler et al. 1988). Measurements of parasitoid search time allocation, parasitoid locomotion, and host density influence on parasitoid host acceptance were beyond the scope of the current study. Maximum search area (C_1) for the parasitoid within the storage room at zero prey density was assumed to be $0.01 m^2$ and minimum search area (C_3) at high prey density was assumed to be 10% of C_1 or $0.001 m^2$.

The density constant (C_2) was estimated by the time constant (t) method: $\tau = 1/k$ (confer Odum 1983 for time constant, turnover time, in estimating the rate at which one parameter approaches another in systems and simulations). C_2 was a constant inversely proportional to prey density that described how quickly search area approached a minimum, asymptotic amount at greater prey densities. Prey density at one host egg per seed area was calculated as follows. Mean per seed area was calculated as $10 \times 10^{-6} m^2$. Upper surface area of the stored grain was $4.5 m^2$. When host egg density reached one egg per seed area, the number of host eggs on the upper surface area

would be $4.5 m^2/10 \times 10^{-6} m^2 = 4.5 \times 10^5$ eggs. The number of host eggs per m^2 of stored grain surface area = $4.5 \times 10^5/4.5 m^2 = 10^5$ eggs per m^2 . If $\tau = 1/k$ and 5τ is required for host egg density to reach one egg per seed area or $5\tau = 10^5$, then $k = 1/\tau = 1:1.5 \times 10^5 = 5 \times 10^{-5}$. Substituting $k = C_2 = 5 \times 10^{-5}$.

Host Egg Model. The governing equation for the host egg model was $E_{t+1} = E_t + (\text{one} - M_L K) R A_t - H E_t - M_E E_t - E_t S_t P_t - W E_t$. Host egg number per m^2 at a specific time (day) was expressed as follows. Host egg number per m^2 of the previous day (E_t) plus the daily egg increase [(one - $M_L K$) $R A_t$] minus the daily egg decrease because of the hatching ($H E_t$) and mortality ($M_E E_t$), minus the number of eggs attacked by parasitoids ($E_t S_t P_t$) and the number of eggs removed with seeds ($W E_t$). Daily increase of host eggs was related to host daily reproductive rate (R), which may be affected by seed resistance. Number of eggs attacked by the parasitoid was related to host egg density (E_t), parasitoid search area (S_t) and parasitoid density (P_t). Abbreviations are as follows: M_L = mortality of host larvae from seed resistance, K = proportion of resistant seeds to total amount of seeds, A_t = number of host adults at time t , H = daily host egg hatching rate, M_E = host egg mortality, S_t = search area of parasitoid adult females at time t , P_t = number of parasitoid adults at time t , W = removal of host eggs, adults, and parasitoids with seeds.

Host Adult Model. The equation was $A_{t+1} = A_t + I E_t - I M_L K E_t - M_A A_t - W A_t$. Host adult number at a specific time was expressed as adult number of the previous day (A_t) plus the daily increase of adults from eggs ($I E_t$), minus mortality because of seed resistance during development ($I M_L K E_t$) adult background mortality ($M_A A_t$) and number of adults removed with seeds ($W A_t$). Abbreviations are as follows: I = daily increase rate of host adults, M_A = daily host adult mortality rate.

Parasitoid Model. The equation was $P_{t+1} = P_t + G E_t S_t P_t - M_P P_t - W P_t$. Parasitoid adult number at a specific time was expressed as parasitoid number of the previous day (P_t) plus the daily conversion of hosts to parasitoids ($G E_t S_t P_t$), which was related to daily growth rate of the parasitoid (G), host density (E_t), parasitoid search area (S_t) and parasitoid density (P_t), minus background mortality of the parasitoid ($M_P P_t$) and parasitoids removed with seeds ($W P_t$). Abbreviation: M_P = daily parasitoid mortality rate.

Initial Conditions of the Model. Number of host eggs on seeds in the upper surface area of grain in the storage room (E) was set to 50.0. Number of host adults in the storage room (A) was 0.0. Host daily reproductive rate (R) was 2.5. Each host female can produce ≈ 50 eggs over an average lifespan of 10 d that become larvae (fecundity is 95 and fertility is 53%) (Santos 1977). Host female:male ratio was 1:1. Therefore, total female larvae produced per host adult female in 10 d would be 25 and reproductive rate per day would be 2.5 (no age effect is included).

Daily host egg hatch rate (H) was 0.14. Average host egg development period is 7 d (Santos 1977). Therefore, daily host egg hatch rate would be one-

sevenths = 0.14 (assumes a distribution of egg age at any time). Host egg mortality (M_E) was 0.05 (Santos 1977). Daily host adult mortality rate (M_A) was 0.10. Adult life span is ≈ 10 d, therefore, estimated daily host adult mortality rate was $1/10 = 0.10$. Daily increase rate of host adults under normal conditions (I) was 0.029. Time from egg to adult is ≈ 35 d. Therefore, increase rate of host adults was estimated at $1/35 = 0.029$.

Initial number of parasitoids introduced into storage room (P) was 10.0. Life table data for parasitoids has only occasionally been collected (Hassell and Waage 1984). However, comparative biology and life tables of two *Trichogramma* spp. with the lepidopteron *Eplestia elutta* (Hubner) as host have been reported (Scholler and Hassan 2001). For construction of the present model, daily conversion or growth rate (G) and daily mortality rate (M_p) of the parasitoid were assumed to be 5% (0.05).

Initially, removal of host eggs, adults, and parasitoids with seeds (W) was set at 0.00. The model assumed a proportion of host eggs, adults, and parasitoids would be removed with seeds ($W = 0.20$) at 30-d intervals from integer date 153 (days 183, 213, 243, 273, 303). Proportion of resistant seeds to total amount of seeds (K) was 0.20. Mortality of host larvae due to seed resistance (M_L) was 0.80. On some resistant Brazilian maize germplasm lines and on some millet seed lines mortality of 80% can occur (Santos 1977, Seifelnasr and Mills 1985). Starting time (t) in days for the model was integer date 152, 1 June. Time step (Dt) was one day.

Model Testing. The model developed by Wiedenmann and O'Neil to describe predator search area as a function of prey density has been tested (confer Wiedenmann and O'Neil 1992). Collection of data for testing of the four mini-models comprising the *S. cerealella* egg population model was omitted for these reasons. Measurements of parasitoid behaviors influencing search area were outside the scope of the current study. There is no resistance within wheat to *S. cerealella* and little resistance in other cereals (confer Santos 1977). Seed of transgenic wheat expressing the Barley trypsin inhibitor CMe has shown resistance to *S. cerealella* (confer Altpeter et al. 1999). Seed of this transgenic line is present at IPK Gatersleben, Gene Transfer Group, Corrensstrasse 3, 06466 Gatersleben, Germany, but is no longer available within the United States.

The artificial seed developed by Shade et al. (1986) for bioassay is significantly larger than the seed of wheat and slightly larger than that of cowpea or maize. When the size of the artificial seed is reduced to that approaching wheat, the seeds fracture easily upon removal from the mold. While artificial seed allowed us to assess the effects of STI on development of *S. cerealella* larvae, the difference in seed size, a difference in development of *S. cerealella* larvae in control artificial seeds relative to whole-wheat seeds, and difficulty in producing a sufficient number of artificial seeds suggested problems in testing the model with artificial seeds. Resistance to *S. cerealella* has been

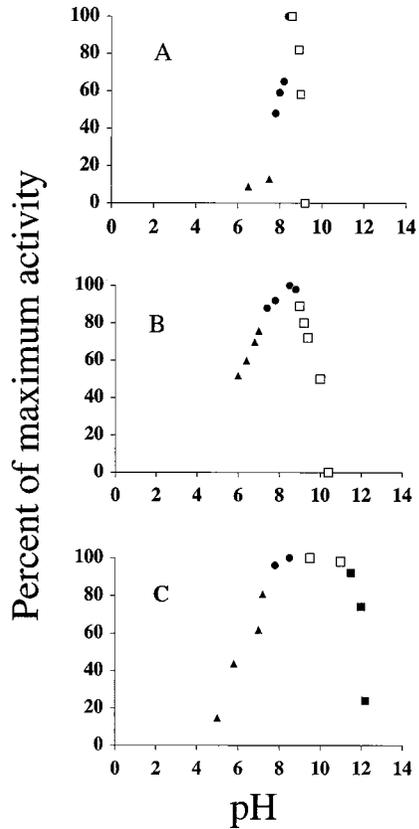


Fig. 1. Effects of pH on the hydrolysis of ester and protein substrates by midgut proteases of *Sitotroga cerealella*. (A) Effect of pH on the hydrolysis of the ester substrate for trypsin-like enzymes tosyl-L-arginine methyl ester (TAME). (B) Effect of pH on the hydrolysis of the ester substrate for α -chymotrypsin-like enzymes benzoyl-L-tyrosine ethyl ester (BTEE). (C) Effect of pH on the hydrolysis of the protein substrate [3 H]methemoglobin. Buffers used were: 0.05 M 1,4-piperazinediethanesulphonic acid, pH 5.0–7.4 (filled triangles); 0.1 M Tris-HCl, pH 7.4–8.9 (filled circles); 0.2 M glycine-NaOH, pH 8.9–11.2 (open squares); 0.05 M $\text{Na}_2\text{HPO}_4/\text{Na}_3\text{PO}_4$, pH 11.2–12.4 (filled squares).

found in some Brazilian maize germplasm lines and in some millet lines (Santos 1977, Seifelnasr and Mills 1985). Obtaining these lines and increasing the seed for testing the model was not considered.

Results

Characterization of Midgut Digestive Proteases. The effects of pH on the hydrolysis of the ester substrates TAME and BTEE as well as the protein substrate [3 H]methemoglobin by midgut extract are shown in Fig. 1. The optimum pH for hydrolysis of both protein and ester substrates was pH 8.5. Additionally, the pH of midgut content was determined to be between pH 8.5 and 9.0. The hydrolytic activities toward [3 H]methemoglobin, TAME and BTEE were detected mainly in the midgut lumen. The activities toward these substrates in the gut wall were only 5%

Table 2. Effects of various diagnostic inhibitors on the proteolytic and esterolytic activities of *Sitotroga cerealella* midgut proteases

Inhibitor	Percent activity remaining toward substrate ^a		
	[³ H]-Hb	TAME	BTEE
2 mM DFP	8 ^b	0 ^c	0 ^c
1 mM TLCK	38 ^b	2 ^b	100
0.2 mM TPCK	68	87	7 ^b
10 mM IAAM	90	83	100
1 mM pepstatin A	30 ^b	79	100

[³H]-Hb = [³H]-methemoglobin; TAME = tosyl-L-arginine methyl ester; BTEE = benzoyl-L-tyrosine ethyl ester; DFP = diisopropyl fluorophosphate; TLCK = N-alpha-p-tosyl-L-lysine chloromethyl ketone; TPCK = L-1-tosylamide-2-phenylethylchloromethyl ketone; IAAM = iodoacetamide.

^a Percent activity remaining was relative to 100% in buffer controls. 100% activities as enzyme units were 1.5 ± 0.1 for proteolytic activity and 0.15 ± 0.01 for esterolytic activity. Inhibitors were incubated with proteases for 10 min on ice prior to enzyme assays. Data represent the means for quadruplicate assays.

^b Differences between activity remaining and 100% activity in buffer controls was significant at $p < 0.05$ (Student's *t*-test).

^c No detectable activity remaining.

that of lumen content. The pepstatin A-sensitive proteolytic activity also was mainly detected in the lumen content (94% of the activity present in lumen content).

The effects of various diagnostic inhibitors on the esterolytic and proteolytic activities of midgut proteases are shown in Table 2. DFP, an inhibitor of serine proteases, inhibited the hydrolysis of [³H]methemoglobin, TAME and BTEE by midgut proteases. TLCK, an inhibitor of trypsin-like serine proteases, and TPCK, an inhibitor of α -chymotrypsin-like serine proteases, inhibited the hydrolysis of TAME and BTEE, respectively, and reduced the hydrolysis of [³H]methemoglobin. The cysteine protease inhibitor, IAAM, had little effect on the hydrolysis of TAME and [³H]methemoglobin and had no effect on the hydrolysis of BTEE. The carboxyl protease inhibitor, pepstatin A, had no effect on hydrolysis of BTEE by midgut extract and little effect on hydrolysis of TAME. However, the hydrolysis of [³H]methemoglobin by midgut extract was inhibited by pepstatin A.

The effects of the naturally occurring plant protease inhibitors on the proteolytic activity of midgut proteases are shown in Table 3. Kunitz soybean trypsin inhibitor (STI) inhibited 60% of the proteolytic activity of midgut proteases; however, Bowman-Birk inhibitor (BBI) did not effectively inhibit proteolytic activity. In contrast to these results, STI inhibited bovine trypsin 90% under the same conditions and BBI inhibited bovine trypsin and α -chymotrypsin $\geq 90\%$.

Bioassay of Protease Inhibitors. Artificial seeds supported the development of *S. cerealella*; however, there was a 10-d delay in development in artificial seeds compared with that in whole-wheat seeds. To test whether STI, which inhibited the proteolytic activity of midgut proteases, would affect the development of *S. cerealella*, the inhibitor was incorporated at concentrations of 1% and 5% (wt:wt) in artificial seeds.

Table 3. Effects of two naturally occurring plant protease inhibitors on the proteolytic activity of *Sitotroga cerealella* midgut proteases

Inhibitor ^a	Percent activity remaining toward [³ H]-Hb ^b
	STI
BBI	95

[³H]-Hb = [³H]-methemoglobin; STI = Kunitz soybean trypsin inhibitor; BBI = Bowman-Birk inhibitor.

^a Inhibitors tested at a concentration of $3\mu\text{g}/100\mu\text{l}$ reaction volume, which was determined to inhibit bovine trypsin and α -chymotrypsin $\geq 90\%$. Inhibitors were incubated with proteases for 10 min on ice prior to enzyme assays.

^b Percent activity remaining was relative to buffer controls. 100% activities as enzyme units were 1.5 ± 0.1 . Data represent the means for quadruplicate assays.

^c Difference between activity remaining and 100% activity in buffer controls was significant at $p < 0.05$ (Student's *t*-test).

At both concentrations, STI caused a significant ($P \leq 0.05$) delay in the development of *S. cerealella* (Table 4). At a concentration of 1%, the inhibitor caused approximately a 6-d delay, and at 5% a 12-d delay in development. There was no significant difference ($P > 0.2$) between the control salt-free artificial seeds and control seeds containing the same amount of potassium phosphate present with STI in supporting the development of *S. cerealella*. The addition of casein as a general protein to artificial seeds at concentrations of 1% and 5% had no significant effect on the development of *S. cerealella*. Addition of BBI, which had little effect on the proteolytic activity of midgut proteases, to artificial seeds at a concentration of 1% did not significantly delay development of *S. cerealella*.

Model Behavior Analysis. Output of the model as the number of host eggs per m^2 versus time in days over a six-month storage period (time in days starting with integer date 152, 1 June) with initial parameters and condition of the variables is shown in Fig. 2A. If the proportion of host eggs, adults, and parasitoids removed with seeds (*W*) at 30-d intervals from the storage room was increased from 0.20 to 0.40, the decrease in host egg number was greater at the removal points, but the magnitude of the subsequent host egg peak increased (Fig. 2B). An increase in the proportion of resistant seeds (*K*) from 0.20 to 0.40 caused a delay in the first host egg peak occurrence from day 207 (Fig. 2A) to day 267 (Fig. 2C), but the magnitude of the peak was not suppressed. When the initial parasitoid number was increased from $P = 10.0$

Table 4. Effects of Kunitz soybean trypsin inhibitor (STI) on the development time of *Sitotroga cerealella*

Artificial seed type	n ^a	Development time (larva + pupa stage) in days ^b
Control	56	46.6 \pm 5.6
1% STI	54	52.4 \pm 5.1*
5% STI	52	58.8 \pm 6.3*

^a n = number of adults emerged

^b values represent mean \pm S.D. The development times followed by an asterisk (*) are significantly different ($p \leq 0.05$) from the control.

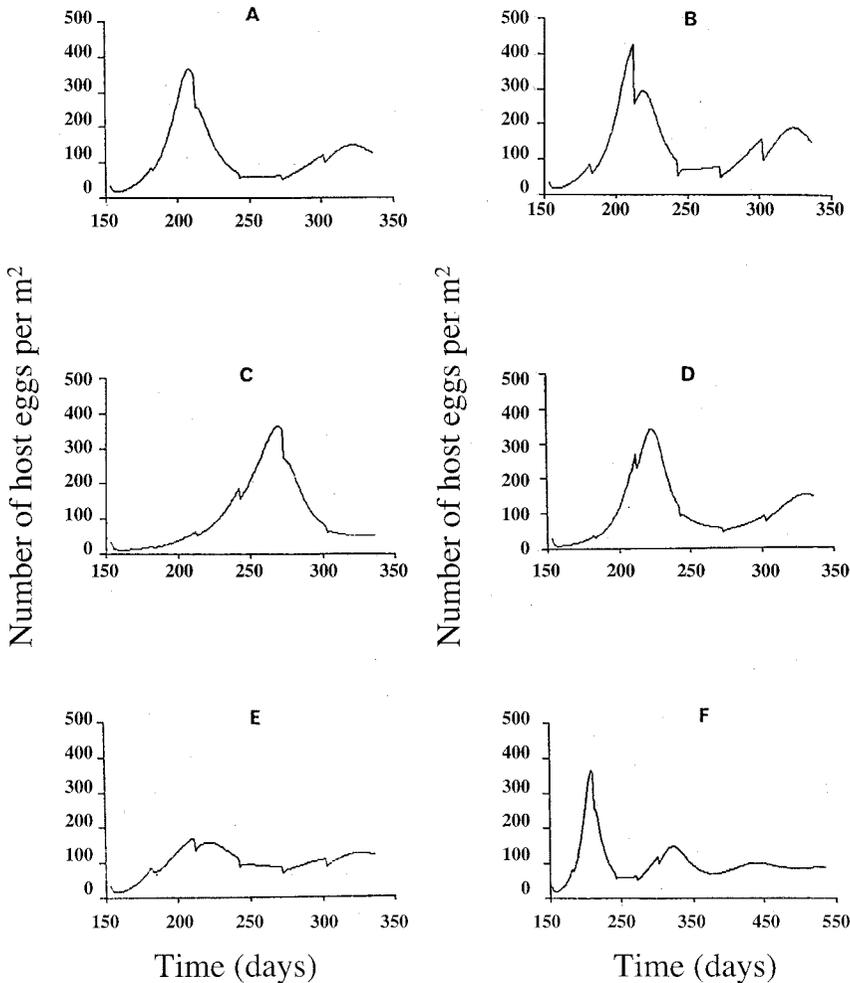


Fig. 2. Results of behavior analysis of the *Sitotroga cerealella* egg population model. Output is number of host eggs per m^2 versus time in days starting with integer date 152, 1 June. (A) Egg population dynamics over a six-month storage period with initial parameters and condition of the variables. (B) Egg population dynamics with an increase in removal of host eggs, adults, and parasitoids with seeds (W) from 0.20 to 0.40. (C) Egg population dynamics with an increase in proportion of resistant seeds to total amount of seeds (K) from 0.20 to 0.40. (D) Egg population dynamics with an increase of initial parasitoid number from $P = 10.0$ to $P = 20.0$. (E) Egg population dynamics with an initial parasitoid number of $P = 10.0$ and an additional introduction of parasitoids ($P = 10.0$) at day 183. (F) Egg population dynamics over an extended storage period (twelve months).

to $P = 20.0$, it caused a delayed occurrence of the first host egg peak to day 222 (Fig. 2D), but the peak was only slightly suppressed. However, the peak was greatly decreased (i.e., by 54%) if the initial parasitoid number was kept at 10.0, and another 10.0 parasitoids were released at day 183 just after the first removal of seeds and before the first host egg peak (Fig. 2E). If no parasitoids were released at any time, host egg population grew exponentially (graphic not shown). The model output with an extended storage period of twelve months showed the host egg population was kept at a low level during the extended period (Fig. 2F).

Model Sensitivity Analysis. Plus and minus 10% changes were made for each parameter and initial condition of variables in the sensitivity analysis. Based

on change in output, the model was most sensitive to changes in host daily reproductive rate (R) (Fig. 3A and B), daily increase rate of host adults (I) (Fig. 3C and D), and daily growth rate of the parasitoid (G) (Fig. 3E and F). With an increase in host daily reproductive rate (R) or daily increase rate of host adults (I) the first host egg peak occurred six days earlier (day 201, Fig. 3A and C) compared with the initial condition (day 207, Fig. 2A) and with a decrease in R or I the peak was delayed six days (day 213, Fig. 3B and D). When daily growth rate of the parasitoid (G) was increased the first host egg peak was suppressed (320 eggs per m^2 , Fig. 3E) compared with the initial condition (370 eggs per m^2 , Fig. 2A) and when G was decreased the peak increased (420 eggs per m^2 , Fig. 3F). The model was less sensitive to changes in maxi-

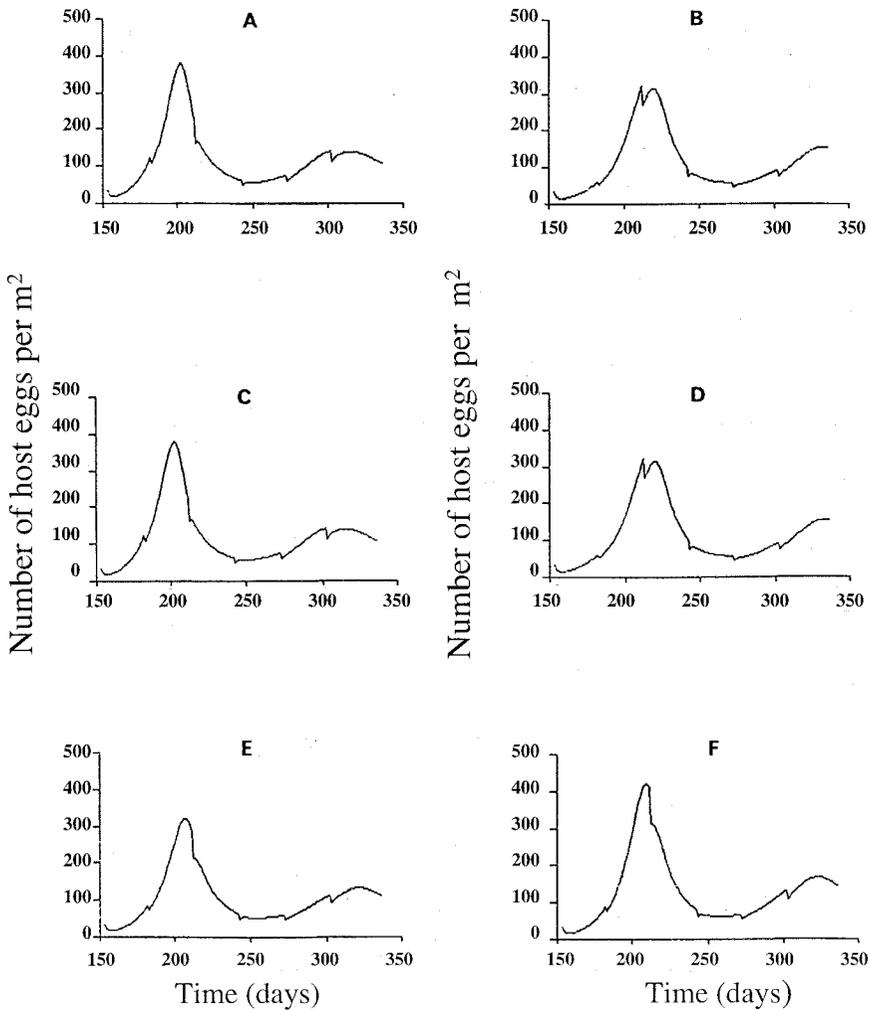


Fig. 3. Results of sensitivity analysis of the *Sitotroga cerealella* egg population model with plus and minus 10% changes for each parameter or initial condition of the variables for which the model was most sensitive. Output is number of host eggs per m^2 versus time in days starting with integer date 152, 1 June. (A) Egg population dynamics with a 10% increase in host daily reproductive rate (R). (B) Egg population dynamics with a 10% decrease in R . (C) Egg population dynamics with a 10% increase in daily increase rate of host adults (I). (D) Egg population dynamics with a 10% decrease in I . (E) Egg population dynamics with a 10% increase in daily growth rate of the parasitoid (G). (F) Egg population dynamics with a 10% decrease in G .

num search area for the parasitoid (C_1), density constant (C_2), minimum search area for the parasitoid (C_3), daily host adult mortality rate (M_A), host egg mortality (M_E), mortality of host larvae from seed resistance (M_L), daily host egg hatching rate (H), daily parasitoid mortality rate (M_P), initial condition of parasitoid number (P), initial condition of host eggs per m^2 on the upper surface of the stored grain (E), proportion of resistant seeds to total amount of seeds (K), and removal of host eggs, adults, and parasitoids with seeds (W).

Discussion

The major digestive proteases in the midgut of *S. cerealella* larva are serine proteases with trypsin-like

and α -chymotrypsin-like specificity. This was suggested by hydrolysis of the synthetic substrates TAME (an ester substrate for trypsin-like enzymes) and BTEE (an ester substrate for α -chymotrypsin-like enzymes). The pH optimum for proteolytic and esterolytic activities of midgut proteases was pH 8.5, which is consistent with what has been reported in many lepidopterous species that possess serine digestive proteases (Applebaum 1985). Additionally, the overall pH of midgut content was determined to be between pH 8.5 and 9.0, which is in the range for optimum activity by the major midgut proteases. The distribution of proteolytic activity between the midgut lumen and midgut tissue indicates that proteases in midgut extract are involved in the digestion of ingested proteins in the lumen.

Results with DFP, an inhibitor of serine proteases, TLCK, an inhibitor of trypsin-like enzymes, and TPCK, an inhibitor of α -chymotrypsin-like enzymes, further suggest the trypsin-like and α -chymotrypsin-like character of the major midgut digestive proteases. The minor inhibition of midgut proteolytic activity toward [^3H]methemoglobin by the cysteine protease inhibitor, IAAM, plus the alkaline pH optimum for proteolytic activity of midgut proteases suggest the major digestive proteases in the midgut are not cysteine proteases. However, inhibition of the proteolytic activity of midgut extract but not the esterolytic activities by pepstatin A, an inhibitor of carboxyl proteases, suggests that a pepstatin A-sensitive protease is present in the midgut. While the overall pH of midgut content was in the range of pH 8.5–9.0, some regions within the midgut might have pH values outside this range. Thus, it is probable that a carboxyl digestive protease is also present in the midgut of *S. cerealella* larva.

Although the major digestive proteases of *S. cerealella* larvae were trypsin-like and α -chymotrypsin-like serine proteases, the proteases showed differences from bovine trypsin and α -chymotrypsin with respect to their interaction with the plant protease inhibitors. Only STI inhibited the proteolytic activity of the midgut proteases. While BBI has two inhibitory domains, one for bovine trypsin and one for α -chymotrypsin, it did not effectively inhibit the proteolytic activity of the midgut proteases. These results suggest that one or more of the midgut serine proteases of *S. cerealella* do not interact with the inhibitory domains of BBI in the same manner as bovine trypsin and α -chymotrypsin.

The artificial seed system developed by Shade et al. (1986) was found to function with *S. cerealella*. While there was a 10-d delay in development for larvae in the artificial seeds compared with larvae in whole-wheat seeds, the system enabled the detection of significant delays in the development of *S. cerealella* on inhibitor-incorporated seeds compared with controls (Table 4). The delay in development of *S. cerealella* larvae feeding on artificial seeds containing STI was probably because of inhibition of midgut digestive proteases by the inhibitor. The addition of casein, a general protein, and the addition of BBI, which did not effectively inhibit *S. cerealella* digestive proteases, did not significantly delay development time. These results suggest that certain protease inhibitors can be used as resistance factors in wheat to control *S. cerealella*. If the inhibitor is a direct gene product, it should be possible to incorporate the gene into the wheat genome and, if appropriately expressed, produce a delay in development or mortality of *S. cerealella* larvae. Such a result has been demonstrated by Altpeter et al. (1999) with the barley trypsin inhibitor CMe (BTI-CMe) expressed in transgenic wheat seed. However, while BTI-CMe was effective against early-instar *S. cerealella* larvae, it did not provide protective effect against leaf-feeding insects. A possible explanation for this was that the leaf-feeding insects possess a different suite of digestive proteases that were not inhibited

by BTI-CMe. This underscores the importance of having basic biochemical and bioassay data concerning the nature of the system being targeted in an insect pest by transgenes for resistance.

A combination of seed resistance and biological control may provide enhanced control of stored grain pests under conditions in developing countries. To evaluate the potential of seed resistance and parasitoids on *S. cerealella* egg population dynamics in a small seed storage room, a predictive model was constructed. The model was directed toward seed storage in developing agricultural systems where farmers remove seed from storage for personal use. It described host egg population dynamics under given conditions in the storage room. The model represented a mathematical hypothesis, however, it should allow development of additional hypotheses concerning the effects of various parameters and conditions on *S. cerealella* population dynamics.

Model behavior analysis showed that the proportion of resistant seeds, the time and number of parasitoids released, and the way of removing seeds were important for control of *S. cerealella* egg population growth. Model sensitivity analysis revealed the model was most sensitive to plus and minus 10% change in host daily reproductive rate, daily increase rate of host adults, and daily growth rate of the parasitoid.

Increasing the proportion of resistant seeds in the storage room from 0.20 to 0.40 delayed the occurrence of the first *S. cerealella* egg peak by 60 d (day 207 to day 267), but did not suppress the magnitude of the first egg peak (confer Fig. 2A and C). While not modeled here, the reproductive ability of *S. cerealella* adults that survive from resistant seeds may also be affected, and resistance can also cause delay in development of larvae that survive from resistant seeds. Two of the parameters the model was most sensitive to plus and minus 10% change in were host daily reproductive rate and daily increase rate of host adults. The effect of resistant seeds only in storage on *S. cerealella* egg population growth was not modeled. The focus of the present work was directed toward an agricultural system where a mixture of resistant and susceptible seed was considered most likely to be placed into storage. Additionally, placing only resistant seeds expressing antibiosis toward pest larvae into storage should place a stringent selection pressure on the pest population, and genotypes that were able to overcome the resistance would be selected particularly under conditions in a seed storage room.

The way of removing grain from the storage room was of importance in control of *S. cerealella* egg population growth. Increasing the proportion of host eggs, adults, and parasitoids removed by increasing the proportion of seeds removed decreased host egg number at the removal points, but the magnitude of the subsequent peak increased (confer Fig. 2A and B). Removing too much grain from the upper surface can reduce the parasitoid number and decrease the suppressive effect on the pest population. Additionally, because *S. cerealella* females lay eggs on seeds in the

upper surface of the stored grain, there would be more fresh seeds exposed to the pest for oviposition.

While releasing more parasitoids at the beginning of the storage period resulted in a 15-d delay (day 207 to day 222) in the occurrence of the first *S. cerealella* egg peak, and a slight reduction in magnitude of the peak, it was not the best strategy for parasitoid release. The most efficacious control of *S. cerealella* egg population growth occurred with a second release of parasitoids at day 183 just after the first removal of seeds and before the occurrence of the first host egg peak (confer Fig. 2A and D, and E). If no parasitoids were released at any time, model output showed the *S. cerealella* egg population would grow exponentially under the storage conditions. Model analysis also indicated daily growth rate of the parasitoid was a significant parameter in the biology of the *Trichogramma* spp. for efficacious control of host population growth.

In summary, we have characterized the major digestive proteases of *S. cerealella* larvae. These proteases were serine proteases with trypsin-like and α -chymotrypsin-like specificity. Additionally, it was likely that a carboxyl protease was also present in the midgut of *S. cerealella* larva. Two plant protease inhibitors were evaluated in enzyme assays to determine their effect on the proteolytic activity of midgut proteases. STI demonstrated effective inhibition of proteolytic activity and bioassays with this inhibitor revealed that it caused a significant delay in development of *S. cerealella* larvae when incorporated into the diet. These results indicated that a plant protease inhibitor could provide antibiotic resistance toward *S. cerealella* larvae. This has been demonstrated by Altpeter et al. (1999) with the barley trypsin inhibitor CMe (BTI-CMe). If used properly such antibiotic resistance in conjunction with other methods, such as biological control, could keep pest populations below economic threshold.

While the *S. cerealella* egg population model was hypothetical, we feel it was based on reasonable biological principles and with appropriate testing it could predict pest population dynamics under various conditions and benefit decisions for pest control. The model output suggested that a combination of seed resistance and parasitoids could provide control of *S. cerealella* population growth on grain in a small storage room with an extended storage period. Validation of the model and further refinement can be the goals of additional research particularly if transgenic wheat seeds containing BTI-CMe, or another transgene for resistance, can be obtained.

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