Bulletin of Insectology ***64*** *(2): 223-227, 2011*
ISSN 1721-8861

Identification of predation by spiders on the diamondback moth *Plutella xylostella*

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Abstract

The diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera Plutellidae), is one of the most destructive cosmopolitan pests of brassicaceous crops, and spiders (Araneae) are important predators to control DBM in the fields. A specific DNA marker was developed for detecting predation on DBM by four species of spiders, *Ebrechtella tricuspidata* (F.), *Pardosa astrigena* Koch, *Pardosa laura* Karsch, and *Pardosa pseudoannulata* (Boesenberg et Strand). A distinct 275-bp product was amplified by polymerase chain reaction (PCR) from the internal transcribed spacer (ITS-1) of the ribosomal gene of *P. xylostella*. This 275-bp sequence was not found in 16 other species of arthropods collected from crucifer fields. When the spiders were analyzed after they were fed a single fourth-instar *P. xylostella*, 50% of *E. tricuspidata* individuals were positive for the 275-bp PCR product up to 36 h after feeding. Likewise, the 275-bp PCR product was present in 60% of the individuals of *P. astrigena*, 70% of *P. laura*, and 70% of *P. pseudoannulata* up to 72 h after feeding on a single fourth-instar *P. xylostella*. A study in a cabbage and a rape field found that *P. astrigena* showed the highest incidence of positivity for the 275-bp PCR product, assayed by using primers of DBMITSF3/DBMITSR3; and the species of spiders and the two different fields were correlated with the positivity incidences of these spiders.

Key words: Araneae, *Plutella xylostella*, predation, PCR, internal transcribed spacer.

Introduction

The diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera Plutellidae), is one of the most destructive cosmopolitan insect pests of brassicaceous crops (Sarfraz *et al.*, 2006). It is also one of the most difficult pests to manage because of its propensity to evolve resistance to chemical and biological insecticides (Shelton and Wyman, 1992; Talekar and Shelton, 1993). However, the role of natural enemies in controlling DBM in agricultural ecosystems is largely unknown. In order to conduct integrated pest management (IPM) programs for crops, investigation of the influence of natural enemies on DBM is necessary.

Spiders may play an important role as predators, but this role is presently little understood (Öberg *et al.*, 2011). Spiders are the most numerous invertebrate predators in terrestrial habitats, and feed mostly on insects, so they play important roles in ecological systems of pest control. Ecologists assign important roles to spiders as biological indicators and in pest control (Prieto-Benítez and Méndez, 2011). In China, many species of spiders occur in fields of cruciferous plants (Wang and Li, 2006), and the spiders are natural enemies of DBM in the field (Zhao, 1995). The significance of spiders for biological control of DBM needs to be investigated.

The exact determination of the prey range of a predator species, let alone the quantification of ratios of consumed prey, can be difficult under field conditions (Schmidt *et al.*, 2009). The identification of predator-prey interactions relies heavily on field observation (Pimm *et al.*, 1991). Among the many methods of studying predation are direct field observations, dissecting the digestive tracts of the natural enemies (Hengeveld, 1980), radiolabeling the prey (McDaniel *et al.*, 1978), protein electrophoresis (Traugott, 2003), and immunological testing using polyclonal and monoclonal antibodies (Nemoto *et al.*, 1985; Nemoto, 1986; Liddell and Cryer, 1991; Hagler *et al.*, 1993). Kiritani and Dempster (1973) compared several of the major evaluation methods for natural enemies. However, these methods are inadequate. In recent years, many ecologists have applied molecular detection to study predator-prey interactions (Symondson, 2002). The use of a molecular method to detect the presence of small amounts of prey DNA in the digestive tracts of predators can elucidate the linkages between generalist predators and their prey in the field (Weber and Lundgren, 2009).

The present study used a specific DNA marker from the internal transcribed spacer (ITS-1) of the ribosomal gene region of DBM to evaluate the predation on DBM by four species of spiders, *Ebrechtella tricuspidata* (F.), *Pardosa astrigena* Koch, *Pardosa laura* Karsch, and *Pardosa pseudoannulata* (Boesenberg et Strand). The aim of this study was to evaluate the roles of the spiders in predating on DBM in the laboratory and in crop fields.

Materials and methods

Sample collecting and rearing

Seventeen species of arthropods were collected to test the specificity of DBM-primers from fields of cruciferous plants on the campus of Huazhong Agricultural University, Wuhan (114°31′N - 30°52′E), China, from October 2009 to May 2010 (table 1). Four species of spiders, *E. tricuspidata*, *P. astrigena*, *P. laura* and *P. pseudoannulata*, were selected for assaying of DBM DNA in gut contents, as these species are all abundant and are most

Table 1. Arthropod species screened by the DBM-specific primers. Positive: having the 275-bp DBMITSF3/DBMITSR3 PCR product; Negative: lacking the 275-bp DBMITSF3/DBMITSR3 PCR product.

Species	Order Family	Test result
Plutella xylostella (L.)	Lepidoptera Plutellidae	Positive
Spodoptera exigua (Hubner)	Lepidoptera Noctuidae	Negative
Pieris rapae (L.)	Lepidoptera Pieridae	Negative
Lipaphis erysimi (Kaltenbach)	Homoptera Aphididae	Negative
Eurydema gebleri Kolenati	Hemiptera Pentatomidae	Negative
Apis florea F.	Hymenoptera Apidae	Negative
Tetramorium caespitum (L.)	Hymenoptera Formicidae	Negative
Phyllotreta striolata (F.)	Coleoptera Chrysomelidae	Negative
Colaphellus bowringi Baly	Coleoptera Chrysomelidae	Negative
Acrida cinerea (Thunberg)	Orthoptera Acrididae	Negative
Gryllus chinensis Weber	Orthoptera Gryllidae	Negative
Eristalis tenax (L.)	Diptera Syrphidae	Negative
Drosophila melanogaster Meigen	Diptera Drosophilidae	Negative
Ebrechtella tricuspidata (F.)	Araneae Thomisidae	Negative
Pardosa astrigena Koch	Araneae Lycosidae	Negative
Pardosa laura Karsch	Araneae Lycosidae	Negative
Pardosa pseudoannulata (Boesenberg et Strand)	Araneae Lycosidae	Negative

likely to affect the dynamics of DBM in the field (Song *et al*., 1999). The DBM were fed with cabbages. The spiders were kept individually in clear Plexiglas® enclosures (5.0 × 5.0 × 7.5 cm, length × width × height) (SAIFU Inc., Ningbo, Zhejing, China) and fed with adults of *Drosophila melanogaster* Meigen. All arthropods were reared at 25 ± 0.5 °C, 60-70% RH and under laboratory conditions of 13L:11D photoperiod.

DNA extraction and the selection of the primers

For species-specific tests, DNA was extracted from the whole body of each arthropod. All arthropods were fasted and provided with only water for 72-96 hours before the extraction of DNA. The DNA was extracted from the whole body by standard phenol-chloroform extraction (Kocher *et al.*, 1989). After the DNA concentrations were measured spectrophotometrically, the final concentrations were diluted to 50 ng/µl.

The efficacy of diagnostic DNA markers for gut-content analysis of predators depends on a number of properties, including species specificity and a level of sensitivity sufficient to detect the product for reasonable periods of time after feeding on prey (Ma *et al.*, 2005). Eukaryotic ribosomal RNA genes (rDNA) are arranged in clusters repeated in tandem, each cluster containing the conserved genes for 18S, 5.8S, and 28S ribosomal RNAs (Mukha *et al*., 2000). This means that unique primers for amplification from a wide range of species can be developed. In contrast to rDNA regions, the non-coding rDNA spacer sequences are highly variable between species (Hackett *et al*., 2000). In our study, 17 species of arthropods were chosen to test the specificity of DBM-specific primers. The 275-bp DBMITSF3/DBMITSR3 PCR product was not detected in any of the species except DBM. Therefore, species-specific markers can be successfully developed for the detection of predation on DBM.

The primers that were described by Ma *et al.* (2005) to amplify a part of specific ITS-1 sequences from DBM DNA, i.e., a distinct 275-bp fragment, were modified and improved in the present study. The primer pairs are as follows:

DBMITSF3 5'-CTGCGGAAGGATCATTAACG-3'
DBMITSR3 5'-ATGCGGTGGATGAGTGACG-3'.

Specificity test of DBM-specific primer and PCR amplification of ITS-1 gene

Two primers, DBMITSF3 and DBMITSR3, for screening the ITS-1 region of DBM and DNA from 17 species of arthropods were used for the specificity test. Polymerase chain reaction amplifications were performed in 25 µl reaction volumes, containing 2.5 µl of 10x reaction buffer, 1.5 µl of 25 mM $MgCl_2$ (Promega), 0.5 µl each of forward primer (50 ng/µl) and reverse primer (50 ng/µl), 0.5 µl dNTPs (15 mM), and 0.16 µl of 5.5 U/µl Taq DNA polymerase (Promega) and 10 µl (500 ng) resuspended DNA. After 5 min at 95 °C, 35 amplification cycles were run, including denaturing at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. The final extension was carried out for 5 min at 72 °C. PCR amplification always included DBM DNA and water (no DNA) as positive and negative controls, and the samples that indicated poor quality were excluded from the study. Eight microliters of PCR products were electrophoresed on 1.2% agarose gels at 110 mA, stained with ethidium bromide, and observed under UV light.

Detection of the period of DBM DNA in feeding experiments

Feeding experiments were designed to determine whether DBM DNA could be detected after the spiders (*E. tricuspidata*, *P. astrigena*, *P. laura* and *P. pseudoannulata*) consumed DBM with specific primers. Field-collected spiders were fed with *D. melanogaster* for at least 2 weeks before the test. Mature spiders were selected for the assay. Each spider was fasted for 7 days and then allowed to consume one or two fourth-instar

DBM. Control spiders were fed with *D. melanogaster* only. Once the spiders had completely consumed one DBM larva, they were either immediately frozen (t = 0) or held without prey for 2, 4, 6, 8, 16, 24, 36, 48, 72 or 96 h and then frozen at −20 °C for subsequent molecular assay.

Collection of the spiders and estimation of the densities of DBM and the spiders in the fields

To further explore whether DBM was preyed upon by the spiders in the fields, four species of spiders (*E. tricuspidata*, *P. astrigena*, *P. laura*, and *P. pseudoannulata*) were collected from cabbage and rape fields on the campus of Huazhong Agricultural University, in October 2010. Ten spiders of each species were randomly selected and stored in Eppendorf tubes at −20 °C before DNA extraction. Meanwhile, we investigated the densities of DBM and the spiders in the cabbage and rape fields. The densities of DBM were estimated by counting the number of DBM larvae on 30 randomly selected plants. The densities of the spiders were defined as the number of individuals found along 30 m of two adjacent rows (about 30 m^2 of crop), using methods similar to Ma *et al.* (2005). The DNA of the spiders was extracted as described above. The predation incidences were determined by PCR, using the 275-bp DBMITSF3/DBMITSR3 PCR product.

Statistical analysis

Variables influencing the incidences of positivity of the spiders, including DBM density, crop type, and predator species, were evaluated in a multinomial logistic regression. Statistical analyses were performed with SPSS for Windows (version 14.0; SPSS Inc., Chicago, IL, USA).

Results

Specificity of primers and detection periods

The 275-bp DBMITSF3/DBMITSR3 PCR product was successfully amplified in *P. xylostella*. This fragment was not found in the DNA samples from the other 16 species of arthropods evaluated (table 1).

A time-course analysis was performed, where the spiders were assayed, using primers of DBMITSF3/DBMITSR3, at various time periods after consuming prey (figure 1). DBM DNA was detected in all individuals of all species of spiders up to 24 h, with a 100% incidence of positivity of the spiders (figure 2). The 275-bp PCR product could be detected in the wolf spiders, *P. astrigena*, *P. laura* and *P. pseudoannulata*, for up to 48 h. The detection periods for wolf spiders were longer than those of the crab spider (*E. tricuspidata*) (figure 2). The half-life (the time after feeding at which only half of the individuals tested positive for the target species; Greenstone and Hunt, 1993) for detection of DBM DNA using the primers DBMITSF3/DBMITSR3 was 36 h for *E. tricuspidata*. The half-life for the three species of wolf spiders (*P. astrigena*, *P. laura* and *P. pseudoannulata*) was 72 h.

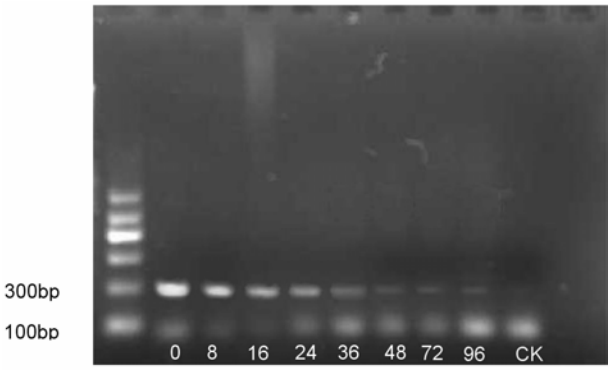

Figure 1. Agarose gel showing the 275-bp DBMITSF3/DBMITSR3 PCR products amplified from *P. pseudoannulata* fed on a single fourth-instar DBM larva for 0, 8, 16, 24, 36, 48, 72 and 96 h at 24 °C. Negative control (CK) was water.

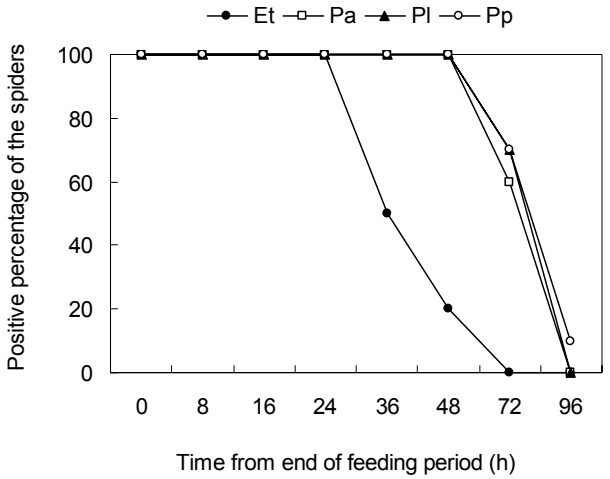

Figure 2. The positive percentage of the spiders after feeding on *P. xylostella* for different time periods, assayed by using the primers of DBMITSF3/DBMITSR3 (n = 10). Et: *E. tricuspidata*; Pa: *P. astrigena*; Pl: *P. laura*; Pp: *P. pseudoannulata.*

Field results

In the two fields, DBM was found to be the most abundant lepidopteran insect, followed by *P. laura* and *P. astrigena*. The density of DBM in the cabbage field was nearly five times higher than in the rape field. In the cabbage field, *P. astrigena* and *P. laura* were abundant, and their densities were much higher than those of the other two species of spiders. In the rape field, *P. laura* was the most abundant species, and its density was higher than those of the other three species of spiders (table 2).

Of the spiders collected from the cabbage field, *P. astrigena,* assayed using the DBMITSF3/DBMITSR3 primers, showed the highest incidence of positivity for DBM DNA, followed by *P. laura.* Of the spiders from the rape field, *P. laura* showed the highest positivity incidence for DBM DNA (table 2). Therefore, the most effective natural enemies of DBM in the cabbage and rape fields were *P. astrigena* and *P. laura* respectively. The multinomial logistic regression showed that the

Table 2. Densities (mean ± SD) of *P. xylostella* and spiders in fields of two crops, and percentage of spiders that were positive for the 275-bp PCR product, assayed by using DBMITSF3/DBMITSR3 primers.

Species of arthropods	Cabbage field		Rape field	
	Density	% positive	Density	% positive
P. xylostella	14.3 ± 2.2		2.4 ± 0.5	
E. tricuspidata	0.7 ± 0.3	20.0 ± 11.1	2.2 ± 0.7	11.5 ± 5.0
P. astrigena	4.3 ± 0.5	44.2 ± 6.6	2.5 ± 0.7	16.5 ± 6.0
P. laura	3.0 ± 0.6	40.0 ± 11.2	5.2 ± 0.8	28.7 ± 4.5
P. pseudoannulata	0.4 ± 0.2	5.0 ± 5.0	1.0 ± 0.3	9.2 ± 3.2

species of spiders and the two different fields were significantly correlated with the positivity incidences of these spiders (four species of spiders: $\chi^2 = 43.586$, $P < 0.001$; two fields: $\chi^2 = 5.116$, $P = 0.024$).

Discussion

Evaluation of the frequency of predation on prey by DNA techniques requires an understanding of the persistence of detectable prey DNA in the gut of each predator species (Ma *et al.*, 2005). Parameters such as temperature, starvation period before feeding, and subsequent feeding on alternative prey, or the lack of it, have profound effects on the detection periods (Agustí *et al.*, 1999). In our study, the feeding test demonstrated that a single DBM could be detected in *E. tricuspidata* for at least 24 h, and in *P. astrigena*, *P. laura* and *P. pseudoannulata* for at least 48 h in 100% of the predators tested. One advantage of the use of primers to detect DNA is that the rates at which the target sequences break down during digestion are likely to be far less variable than is the case for different protein epitopes (Agustí *et al.*, 2003). The latter may be either labile or refractory to digestion, resulting in detection periods varying from almost instantaneous denaturing to survival for many days (Symondson, 2002). In this study, the detection periods for the three species of wolf spiders were longer than that for the crab spider, which indicates that the rates of digestion and absorption may vary among spiders from different families.

The field results demonstrated that the spiders predate intensively on DBM. Harwood *et al*. (2001; 2003) found that linyphiids locate their webs in areas of high collembolan density in the field, and these invertebrates represent a potentially major food resource. Increased density of Collembola in response to the addition of detritus can lead to increased numbers of spiders and other predators (Halaj and Wise, 2002); contrariwise, removal of spiders can cause the numbers of Collembola to increase (Lawrence and Wise, 2000).

Our results clearly showed a relatively high incidence of positivity for DBM DNA of all the spiders except *P. pseudoannulata*, as assayed by the DBMITSF3/DBMITSR3 primers. The positivity incidence of *P. astrigena* for the occurrence of the 275-bp PCR product was the highest among the spiders in the cabbage field. Similarly, the positivity incidence of *P. laura* for the occurrence of the 275-bp PCR product was the highest among those found for spiders in the rape field (table 2). This indicated that *P. astrigena* and *P. laura* are most likely to choose DBM as an alternative prey, and that these species are effective natural enemies for pest control in the fields. Meanwhile, the species of spiders and the two different fields were correlated with the incidences of positivity of these spiders. One possible explanation is that the densities of DBM and of the species of spiders in different crop fields can vary widely. Nemoto (1986) showed that the application of methomyl might cause a resurgence of the moth population, through stimulation of the reproductive potential and differential mortality between predators and prey. We suggest that *P. astrigena* and *P. laura* may be the key DBM predators, important natural enemies of DBM, and potentially useful for biological control.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No: 31071895, 30970405) and the Natural Science Fund of Hubei Province (2008CDA082). We are grateful to two anonymous reviewers for their comments and suggestions to the manuscript. We also thank Dr. Janet W. Reid (Biological consulting and editing services, JWR Associates, New York, USA) to help us edit the English for the manuscript.

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Received March 17, 2011. Accepted September 5, 2011.