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Detection of parasitism in diamondback moth, *Plutella xylostella* (L.), using differential melanization and coagulation reactions

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Abstract

Diamondback moth (DBM), *Plutella xylostella*, is known for causing damage to *Brassica* crops and developing resistance to chemical and biological pesticides; it has become one of the most difficult pests to manage in many regions around the world. The only way to reduce reliance on pesticides is to maximize the role of natural control agents for integrated pest management programs and be able to incorporate the mortality from control agents into pest control decision-making. More than 90 hymenopterous parasitoids are associated with DBM worldwide; among them, *Diadegma semiclausum*, is a major endoparasitoid of *P. xylostella*. To optimize parasitism of pests in pest control decision-making, it is necessary to develop rapid and simple methods for distinguishing parasitized from non-parasitized larvae in the field. Here we report on a number of diagnostic tools to identify parasitized larvae. One is based on differential melanization reactions in hemolymph due to immune suppression in parasitized larvae. The lack of coagulation reactions in hemolymph provides a simple initial test, where squashing a non-parasitized larva onto nitrocellulose membrane traps chlorophyll-containing gut content on the membrane leaving a green dot of clotted gut material. However, in immune-suppressed parasitized larvae, the gut content was washed away in absence of coagulation reactions and the membrane lacks a green dot. This tool alone or combined with others, allows quick detection of parasitized caterpillars in the field. We further showed that the antibody MAb 9A5 can be used to detect *D. semiclausum* parasitized larvae of DBM in Western blots.

Keywords: parasitization, melanization, coagulation, diamondback moth, *Diadegma semiclausum*, monoclonal antibody
Introduction

The diamondback moth (DBM), *Plutella xylostella* (L.), is considered a major pest of Brassicaceae in the world (Talekar and Shelton, 1993). A number of DBM populations resistant to chemical and biological pesticides have been recorded (Tabashnik et al., 1987; Talekar and Shelton, 1993; Baker and Kovaliski, 1999; Zhao et al., 2002). Sustainable strategies for the management of this destructive *Brassica* pest rely on reduced application of chemical pesticides and the use of biological control agents in integrated pest management (IPM) programs (Talekar and Shelton, 1993). Usage of parasitoids to biologically regulate the density of pest insects (IPM) programs (Talekar and Shelton, 1993). A number of DBM populations resistant to chemical and biological pesticides have been recorded (Tabashnik et al., 1987; Talekar and Shelton, 1993; Baker and Kovaliski, 1999; Zhao et al., 2002). Sustainable strategies for the management of this destructive *Brassica* pest rely on reduced application of chemical pesticides and the use of biological control agents in integrated pest management (IPM) programs (Talekar and Shelton, 1993). Usage of parasitoids to biologically regulate the density of pest insects (IPM) programs (Talekar and Shelton, 1993). A number of DBM populations resistant to chemical and biological pesticides have been recorded (Tabashnik et al., 1987; Talekar and Shelton, 1993; Baker and Kovaliski, 1999; Zhao et al., 2002).

**Phenoloxidase activity**

Hemolymph samples from *P. xylostella* larvae was collected in PBS (1.47 mM KH2PO4, 7.3 mM NaH2PO4, 138 mM NaCl, 2.7 mM KCl, pH 7.5) by piercing an abdominal proleg with a pair of forceps. Hemolymph samples (10 μl) from an individual larva was mixed with 10 μl of 18 mM DOPA, and then mixed with 20 μl Micrococcus lysodeikticus (Sigma) solution (in 0.1 M KPO4, pH 7.0). Colour change was observed at 1 h intervals for 3 h.

To test whether the larval body weight plays a role in the melanization reactions, the weight of an individual larva was measured before bleeding, and then the hemolymph was mixed with DOPA and *M. lysodeikticus* solutions. The hemolymph mixture, which turned to black within 3 h, was regarded as positive; the percentages of the larval hemolymph blacking were calculated against the total number tested. The data were further analysed statistically using the web-based $\chi^2$-test (http://schnoodles.com/cgi-bin/web_chi_form.cgi). In the experiment, about 60 non-parasitized and parasitized larvae each were tested.

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About 100 each of non-parasitized and 7-days parasitized larvae were individually squashed onto Nitrocellulose membrane. Then the membrane was washed with TBS (10 mM Tris-HCl pH 8, 150 mM NaCl) for 30 min and the green dots (cross-linking) were recorded. The percentages of the cross-linking were calculated and their difference was tested by web-based χ²-test.

**Hemolymph protein collection**

Hemolymph from a single 4th instar larva was collected from an abdominal proleg into PBS buffer. Care was taken to avoid rupturing the gut. Hemolymph was centrifuged at 5000 g for 5 min to pellet hemocytes. Cell-free hemolymph was collected and diluted 1:2 with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 50 mM Tris-HCl pH 6.8, 5% (v/v) β-Mercaptoethanol, 2% SDS and 0.2% bromophenol blue, heated at 65°C for 5 min and stored at −20°C until use.

Hemolymph proteins were collected from single parasitized and non-parasitized individuals, and the protein extracts were tested individually or pooled together. For an individual larva, 10 μl buffer containing hemolymph was centrifuged and cell-free hemolymph was then mixed with 10 μl 2× loading buffer. For the pooled samples, ten individual larvae were bled in 100 μl buffer with equal volume of 2× loading buffer added after removing hemocytes.

**Electrophoresis of hemolymph proteins**

Hemolymph proteins were analyzed by SDS-PAGE using the buffer system of Laemmli (1970). Frozen samples were thawed and reheated to 65°C for 5 min and 10 μl loaded onto a 15 cm thick 10% and 15% discontinuous polyacrylamide gels (Laemmli, 1970). Electrophoresis was performed at constant voltage (100 V) at room temperature using the Bio-Rad Mini gel system. The protein molecular weight marker was used according to the supplier’s instructions (Sigma).

**Coomassie staining and destaining of polyacrylamide gel**

Gels were stained by immersion in Coomassie staining solution (0.25 g Coomassie Brilliant Blue R-250 (Sigma) in 50% methanol and 10% glacial acetic acid) and incubated at room temperature for approximately 30 min with gentle agitation. Gels were destained by overnight incubation in destaining solution (10% glacial acetic acid, 30% methanol), with tissue paper to absorb free dye, before being placed on wet Whatman filter paper and wrapped in plastic wrap for image capture.

**Western blots**

After being separated by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (Amersham) in a Mini Trans-Blot cell (Bio-Rad) using 220 mA/100 V for about 45 min in a transfer buffer containing 192 mM glycine, 25 mM Tris-base and 20% (v/v) methanol (Towbin et al., 1979). Membranes were washed in PBS for 10 min then preincubated in the first blocking solution (8% w/v non-fat milk powder and 0.02% sodium azide in PBS) for 1 h at RT with gentle agitation. The primary antiserum MAb 9A5 was added to a final concentration (10 μg ml⁻¹), and the incubation was continued for an additional 2 h or overnight. Then the filters were washed four times, each for 15 min with moderate shaking in TBS-T. The secondary anti-mouse IgM antibodies (Sigma), which were either alkaline phosphatase-conjugated (diluted at 1:10,000) or peroxidase-conjugated (diluted at 1:1000), were added to membranes in the second blocking solution (5% w/v non-fat milk powder, 150 mM NaCl, 50 mM Tris-HCl; pH 7.5) and membranes were incubated for 1–2 h at RT. The membranes were washed four times, each for 15 min in TBS-T with moderate shaking. Finally, for the visualisation of the protein bands, the filters were stained in developing solution with Nitro-Blue Tetrazolium (NBT) and 5-Bromo-4-Chloro-3-Indolyphosphate (BCIP) (sigma) for alkaline phosphatase-conjugated or 4-chloronaphthol (4-CN), diaminobenzidine and hydrogen peroxide (sigma) for peroxidase-conjugated antibodies, as described by (Sambrook et al., 1989). For dot blot, 5 μl of hemolymph was loaded directly onto the nitrocellulose membrane, and the membranes were then treated as for the gel blots above.

**Results and discussion**

**Inhibition of the melanization reaction**

Reduced levels of hemolymph-derived melaninization in parasitized insects has been described as a precondition for successful parasitism (Shelby and Webb, 1999; Beck et al., 2000; Shelby et al., 2000). The inhibition of hemolymph melanization in DBM after parasitization by D. semiclausum was observed in non-parasitized larvae after 3 h but was not observed in the parasitized larvae. The hemolymph of non-parasitized larvae was completely black when left overnight, while hemolymph from parasitized larvae was virtually unchanged. These results indicate that melanization of hemolymph in DBM larvae was inhibited after parasitized by D. semiclausum (data not shown).

Reduced melanization in the parasitized larvae might be due to the inhibition of PO activity and of PO-activating enzymes in the melanization cascade. When H. virescens was parasitized by C. sonorensis, the key enzymes and precursors in the melanization pathway were dramatically reduced (Shelby et al., 2000). For example, PO and dopachrome isomerase (DI) were absent or inactivated in the plasma of parasitized larvae. While addition of M. lysodeikticus cells quickly induced melanization in plasma of non-parasitized larvae, it had no effect on plasma from parasitized larvae (Shelby et al., 2000). Our results also showed that a quicker colour change, due to melanisation, was indeed observed when DOPA and a M. lysodeikticus cell suspension were mixed together with hemolymph from non-parasitized larvae compared to hemolymph from parasitized larvae. This indicates that the bacteria induce the melanization reaction of non-parasitized larvae in the presence of DOPA, while induction is precluded in the parasitized larvae.

The inhibition of the hemolymph melanisation of the parasitized DBM larvae from non-parasitized ones was further tested. The difference between the non-parasitized and parasitized was compared according to their body weight (fig. 1). The results showed that differences in melanization between parasitized and non-parasitized larvae were significant in 3rd instar with an average body
weight of 2–5 mg (In3, $\chi^2 = 4.55, p \leq 0.005$; fig. 1) and early 4th instar with an average body weight of 5–7 mg (In4E, $\chi^2 = 9.81, p \leq 0.001$; fig. 1). In contrast, the differences in colour change in the later 4th instar larvae with a body weight of 7–10 mg were not significant (In4L, $\chi^2 = 0.03, p \geq 1$; fig. 1). There may be several reasons for this; melanization in later instars may not be suppressed by some parasitoids or may be suppressed but not to the low levels that preclude initiation of melanization in the presence of oxygen. Our results indicate that hemolymph melanization in the presence of DOPA and *M. lysodeikticus* could be used to distinguish parasitized 3rd and early 4th instar DBM larvae from non-parasitized ones.

A blackening was observed in both parasitized and non-parasitized larvae within 30–60 min of when the epidermis was placed into DOPA solutions (data not shown). This suggests that melanization pathways in the hemolymph were inhibited, whereas activity in the cuticle was not affected, which is consistent with the observation of Shelby et al. (2000). These authors showed that the activities of dopamine N-acetyltransferase (DAT) and plasma quinone methide isomerase, involved in cuticular sclerotin formation (Saul and Sugumaran, 1990), were not affected by parasitism (Shelby et al., 2000).

**Inhibition of coagulation reaction**

PO is also involved in coagulation reactions together with other abundant storage proteins, such as the lipid carriers lipophorin (Ma et al., 2006; Rahman et al., 2006) and hexamerin (Li et al., 2002; Theopold et al., 2002; Scherfer et al., 2004; Theopold et al., 2004). We observed a green signal on the membrane from the remains of non-parasitized DBM larvae (fig. 2a; NP), but not from larvae 7-days post-parasitization when the membranes were washed with TBST overnight (fig. 2a; P7d). It appears that the coagulation reactions include debris from the gut contents that are trapped in the coagulum and, thus, become attached to the membrane. Further tests showed that a difference between the parasitized and non-parasitized larvae could be observed after 30 min washing with TBST (b) and TBS (c) buffer for 30 min produced a clear difference between the parasitized and non-parasitized larvae. NP, non-parasitized larvae; P7d, 7 days post parasitization.

**Fig. 1.** Inhibition of melanization after parasitization. Hemo-
lymph melanization of parasitized and non-parasitized DBM were tested in presence of DOPA and *M. lysodeikticus* solutions. Third to 4th instar DBM larvae (about 20 in each group) were used, the body weight of each larva was measured and melanization was tested. The percentage of melanization and its relationship with body weight was compared. Results showed that 3rd and early 4th instar larvae showed significant differences between parasitized and non-parasitized larvae. NP, non-parasitized larvae; P, parasitized larvae; In3/2–5 mg, 3rd instar larvae with a body weight of 2–5 mg; In4E/5–7 mg, 4th instar larvae with a body weight of 5–7 mg; In4L/7–10 mg, later 4th instar larvae with a body weight of 7–10 mg. $\chi^2$-test showed that the difference is significant ($\chi^2 = 86.35, p \leq 0.001$).

**Fig. 2.** Inhibition of coagulation after parasitization. Whole larvae, both parasitized and non-parasitized, were squashed onto nitrocellulose membranes, and the membranes were incubated with the MAb 9A5 antibody. No specific reaction was detected in proteins from parasitized or non-parasitized larvae (data not shown). However, strong green signals (due to chlorophyll-containing coagulum being bound to the membrane) were mainly observed in the non-parasitized larvae (a). Therefore, to confirm the difference between the non-parasitized and parasitized larvae, individual larva was squashed onto membrane, and washed with different buffers at different times (data not shown). It turned out that membranes washed with TBST (b) and TBS (c) buffer for 30 min produced a clear difference between the parasitized and non-parasitized larvae. NP, non-parasitized larvae; P7d, 7 days post parasitization.
Parasitoids that develop in the hemocoel of their hosts are able to suppress the host’s immune system, inducing a decline in the responsiveness to microbial elicitors of a variety of cellular and humoral components (Carton and Nappi, 1997; Shelby and Webb, 1999; Bae and Kim, 2004). The situation in the D. semicaudata/P. xylostella system was explored. Hemolymph proteins from the non-parasitized and parasitized (day 1, 3, 4, and 7 of post-parasitisation) larvae were subjected to SDS-PAGE and the electrophoresed proteins were visualized by Coomassie Brilliant Blue. This analysis showed that proteins about the 70–80 kDa range are abundant and a series of high molecular weight proteins ranging from 100–250 kDa were present in the non-parasitized and parasitized (1–4 days) larvae (fig. 3). In hemolymph samples of larvae 7 days after parasitization (P7d), proteins ranging from 70–80 kDa were dramatically reduced, and a series of high molecular weight proteins disappeared leaving only weak bands (fig. 3; arrow). Several groups demonstrated that parasitism induced hemolymph protein production in various parasitoid/host systems, including the Cortesia congregata/Pieris brassicaceae systems (Beckage et al., 1987; Rolle & Lawrence, 1994; Ockroy et al., 2002). Bae and Kim (2004) reported the reduction of total hemolymph protein, especially an 80 kDa protein in P. xylostella larvae after parasitized by Cotesia plutellae. Our results also showed that the 70–80 kDa proteins, which are involved in coagulation (Li et al., 2002; Theopold et al., 2004; Scherfer et al., 2004; Theopold et al., 2004) are reduced in parasitized larvae. The disappearance of these proteins in P7d larvae might suggest that their coagulation capacity was inhibited, which provides further support to our previous results (fig. 2; P7d) and the paper of Bae and Kim (2004).

Based on the differences in food-containing coagulum bound to membranes and the protein pattern in the PAGE gel, we assume that the green signal produced by non-parasitized larvae is due to the bound coagulum proteins from the hemolymph of non-parasitized larvae, which have trapped chlorophyll-containing gut debris. The coagulation reaction was inhibited after larvae were parasitized for 7 days with less protein complexes formed in these larvae, and less chlorophyll-containing coagulum bound on membranes. Therefore, we can use the signal produced by bound coagulum to identify the parasitism status of larvae in the field.
the antibody reacts exclusively with proteins from parasitoids and not with proteins from noctuids examined (Stuart and Greenstone, 1997; Stuart, 1998), it may target a hymenopteran-specific antigen. The specificity of the antibody in the Diadegma/Plutella system was tested by homogenizing a single parasitized and non-parasitized DBM larva, separated in SDS-PAGE and probed with MAb 9A5. Multiple bands were detected in the DBM larvae 6 days after parasitization (fig. 4b; P6d) but not with the DBM larvae 3 days after parasitization (fig. 4b; P3d) and non-parasitized larvae (fig. 4b; NP).

Trowell et al. (2000) reported that intractable endogenous phosphatases may confound the detection when using phosphatase-conjugated secondary antibody. To avoid the cross-interaction of the endogenous enzyme, phosphatase- and peroxidase-conjugated secondary anti-mouse IgM were used in dot blots. A substrate, for developing the membrane in peroxidase-conjugated secondary antibody, was used at pH7.2 in which endogenous peroxidase, particularly in larvae, displayed little or no activity at neutral pH. In the dot blot, using the phosphatase-conjugated secondary antibody (fig. 5a), a very strong signal was observed in the Diadegma adult and slightly stronger signals were observed in the P6d than in the NP. In contrast, the dot blot with the peroxidase-conjugated IgM secondary antibody (fig. 5b) showed nearly nothing in the NP and moderate signals in the P6d and Diadegma. These results indicated that the peroxidase-conjugated IgM should be used in the dot blot, since it gave more distinguishable signals between the NP and P6d larvae in the dot blot tests.

Attempts were made to make the tests easier; dot blots containing proteins from single squashed parasitized and non-parasitized larvae were conducted. Unfortunately, under these conditions, signals were detected in both the parasitized and non-parasitized larvae (data not shown), which may be due to the high protein concentration on the blot that non-specific binding from MAb 9A5 produced in the non-parasitized larvae. Therefore, the MAB 9A5 antibody can only be used to detect parasitism of DBM in dot blots of protein extracts, but not when the whole larva was squashed onto the membrane.

Here we demonstrate that the inhibition of the melanization and coagulation pathways in parasitized DBM larvae by D. semiclausum provides a basis for simple and relatively reliable determination of parasitism rates. There are several advantages in the methods: (i) quick and easy to perform; (ii) no specific skills needed; and (iii) no expensive equipments required. In contrast, the method of rearing DBM larvae until the emergence of parasitoids is a slow process and needs at least a number of days to obtain the results; and the microscopic dissection needs microscope and specific skills to perform. Thus, the melanization of hemolymph and protein cross-linking in DBM can be used to distinguish parasitized from non-parasitized larvae in the field.

In conclusion, several methods have been developed to detect the parasitism of DBM. The inhibition of the hemolymph melanization and coagulation provides easy and rapid diagnosis which can be used in the field. The monoclonal antibody is more specific, but takes longer and has to be performed in the laboratory.

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