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Feeding disruption tests for monitoring the frequency of larval lepidopteran resistance to Cry1Ac, Cry1F and Cry1Ab

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ABSTRACT

An alternative to traditional larval lepidopteran resistance-monitoring bioassays was developed. Feeding disruption tests were developed for detecting insects resistant to three *Bacillus thuringiensis* (Bt) proteins: Cry1Ac, Cry1F and Cry1Ab. The assays rely on a diagnostic dose of Bt toxin in 100-μl hydratable meal pads of artificial diet containing blue indicator dye. The assay was formatted as a portable (palm-sized) plastic plate containing an array of 16 test wells, each containing a single hydratable meal pad with one insect added per well. The diagnostic dose was the concentration of Bt in meal pad rehydration solution that reduced 24 h dyed fecal production of Bt-susceptible neonates to ≤2 fecal pellets per larva. Bt-resistant neonates were able to consume the diagnostic dose of the insecticidal protein and produce >2 blue fecal pellets. The feces were distinctly visible on the white background of the feeding disruption test plate. Diagnostic doses were determined with lab-strain Bt-susceptible *Heliothis virescens* and *Helicoverpa zea*. For *H. virescens*, the diagnostic doses were 10, 20 and 15 μg/ml for Cry1Ac, Cry1F and Cry1Ab, respectively. For *H. zea*, the diagnostic doses were 40, 200 and 500 μg/ml, respectively. The assays were validated against a lab-strain of Bt-resistant *H. virescens* and with susceptible larval *H. virescens* collected as eggs from field-grown tobacco in North Carolina.

1. Introduction

Since commercialization in 1996, the adoption of genetically modified (GM) crops expressing genes from *Bacillus thuringiensis* (Bt) that confer insect resistance has increased dramatically. In Bt cotton, *Gossypium hirsutum* L., the Bt transgene codes for proteins that are toxic *per os* to larval Lepidoptera, are nontoxic to mammals and other non-target organisms and reduce the use of chemical insecticides (Roe et al., 1985). The U.S. Bt cotton hectareage in 2008 was 2,540,852, an increase of more than 3-fold from the 749,127 ha planted in 1996 (Williams, 2009).

First-generation Bt cotton consists of Bollgard® cotton (Monsanto Company) which expresses the single protein toxin, Cry1Ac. The improved efficacy of Bt cotton strains registered since 2002 was obtained by the simultaneous expression of two genes. Bollgard II® cotton (Monsanto Company) expresses Cry1Ac and Cry2Ab. WideStrike® cotton (Dow AgroSciences) expresses Cry1Ac and Cry1F. VipCot™ cotton (Syngenta Seeds, Inc.) expresses Vip3Aa19 and modified Cry1Ab. Apart from its role in Bt cotton, Cry1Ab is also paired with supplemental Bt proteins in YieldGard® corn (Monsanto Company) and AgriSure™ corn (Syngenta Seeds, Inc.).

Insecticidal Bt proteins are expressed at high levels in the Bt cotton plant throughout most of its life. As a consequence, selection pressure favoring the evolution of resistance by Lepidoptera to Bt toxins is also high. Laboratory and field data demonstrated that target pests are capable of evolving high levels of resistance to the Bt toxins (Tabashnik et al., 1990, 2008, 2009; Tabashnik, 1994; Gould et al., 1992, 1995, 1997; Bauer, 1995). In a field study conducted before the commercial release of Bt crops in 1996, it was shown that diamondback moth, *Plutella xylostella* (L.) had developed resistance to Bt sprays (Tabashnik et al., 1990). Recently, fall armyworm, *Spodoptera frugiperda* (J. E. Smith) was found to be resistant to Cry1F corn grown in Puerto Rico (Moar et al., 2008). Tabashnik et al. (2009) reported the evolution of resistance in the field by populations of *Busseola fusca* (Fuller) to Cry1Ab corn grown in South Africa and by populations of *H. zea* (Boddie) to Cry1Ac and Cry2Ab cotton in the U.S.

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A common method of resistance monitoring for chemical insecticides with contact activity has been the adult vial test, in which the inside of vials is coated with insecticide (Plapp et al., 1987). This assay method is not applicable to Bt toxins that are only active *per os*. For lepidopteran pests, Bt resistance is often monitored using a diagnostic dose of the Bt toxin applied to the surface of artificial diet or incorporated into molten diet; the toxin is then introduced when the insect feeds (Gould et al., 1992; Ali et al., 2006; Mahon et al., 2007; Blanco et al., 2008; Ali and Luttrell, 2009; Anilkumar et al., 2009). The endpoint of these bioassays is mortality or growth inhibition observed 7 or-more days after test initiation. Test insects for these assays often come from laboratory colonies established from field-collected insects. In addition to the long assay time and the practice of establishing colonies of test insects in the laboratory from field-collected insects, the diet-incorporated bioassays are complicated by the difficulty of uniformly mixing insecticide into artificial diet, the need for relatively large amounts of insecticide for diet incorporation and the need for fresh artificial diet to conduct these assays.

A rapid alternative to traditional larval lepidopteran assays based on mortality or delayed development is an assay based on feeding disruption (Bailey et al., 1998, 2001; Roe et al., 2000, 2003). In feeding disruption tests (FDTs), insecticide activity is measured in terms of disrupted insect feeding resulting in reduced fecal production. In this paper, we describe the architecture for a novel, 16-well plastic plate specifically designed for FDT assays and the use of hydratable meal pads in this system for the diagnosis of resistance to Cry1Ac, Cry1F and Cry1Ab in neonates of *Heliothis virescens* and *Helicoverpa zea*.

2. Materials and methods

2.1. Architecture of the 16-well FDT plate

Fig. 1A shows a schematic of the FDT plate with 16 test wells in 4 rows and 4 columns. Fig. 1B is a photo of this plate containing a rehydrated (100-μl) meal pad per well and 1 larval insect per well. The infested plate is covered with a vented clear plastic lid with adhesive only in the areas that bind to the plate (not shown in this photo; part code BIO-CV-16, C-D International, Pitman, N.J., USA). The overall dimensions of the plate are 98 mm × 102 × 11 mm. The plates are made of rigid, 1.5 mm thick, opaque white styrene plastic (Raleigh Precision Products; Kenly, NC).Fig. 1C shows the schematic of a single test well consisting of two parts: an insect test arena on top and a 100-μl meal pad chamber below. The test arena tapers from a diameter of 17 mm at the top to 15 mm at the bottom. The depth of the test arena is 5 mm. The top of the meal pad chamber is in the bottom of the insect test arena. The diameter of the top of the meal pad chamber (in the base of the test arena) is 5 mm. The meal pad surface area in the bottom of the insect test arena is 19.6 mm^2. The total depth of the meal pad chamber is 6 mm. The shape of the meal pad chamber is cylindrical from the base of the insect test arena to a depth of 4 mm. At a depth of 4 mm, the diameter of the meal pad chamber is reduced to a circular opening with a 3 mm diameter. From the 4 mm depth of the meal pad chamber to the 6 mm total depth of the meal pad chamber, the diameter expands at an angle of 26.6° from perpendicular to 5 mm in diameter. This constriction in the cylindrical shape of the meal pad chamber fixes the hydratable (artificial diet) meal pad in each well for transport and handling before and after rehydration.

2.2. Hydratable meal pads

Agdia, Inc. (Elkhart, IN) had automated addition of artificial diet to the plates to form the meal pads but had ceased mass production

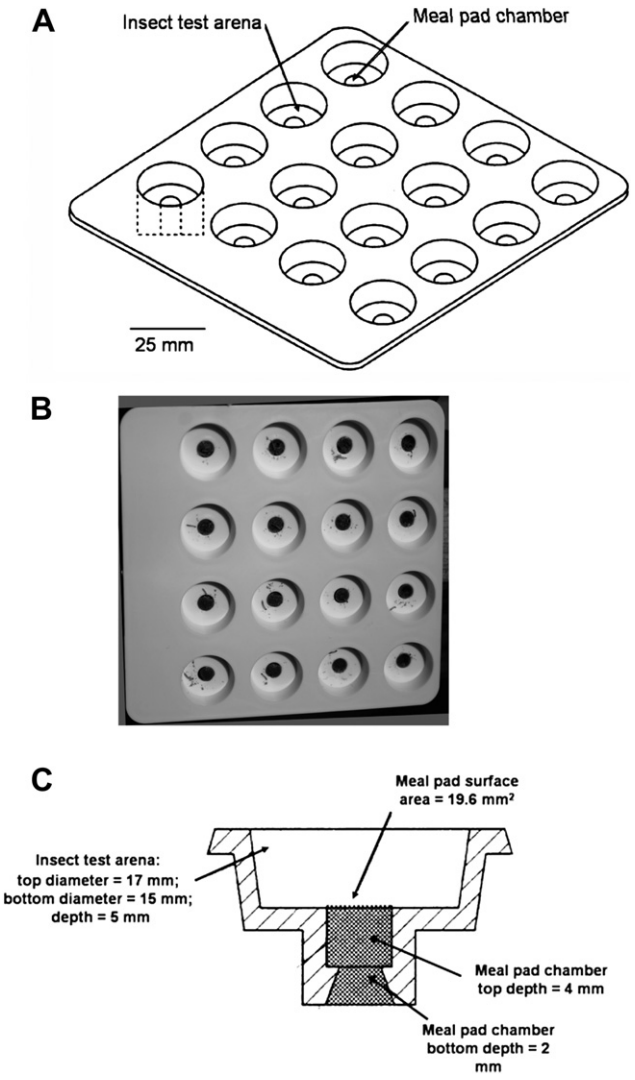

Fig. 1. Architecture of a 16-well feeding disruption test (FDT) plate with 100-μl rehydratable (artificial diet) meal pads. (A) Plate schematic; (B) Photo of plate with rehydrated meal pads and one first instar of *H. virescens* per test well; (C) Single test well schematic showing insect test arena and meal pad chamber.

of assay-ready plates. Thus, the FDT plates used for the work reported here were prepared manually in the lab of R.M. Roe at Dearstyne Entomology Building, Department of Entomology, North Carolina State University (Raleigh, NC). The 100-μl meal pad was a corn-soy based heliothine diet (Burton, 1970). Before mixing the dry ingredients with water and agar, they were ground for 24 h in a porcelain cylinder mill (Item # PC1451B2, Process Equipment Division, U.S. Stoneware, Akron, OH, USA). The additional milling results in molten diet that is flowable when dispensed into each well. Trypan Blue dye (40%; Direct blue 14; Sigma T-6146, Sigma Chemical Co., St. Louis, MO, USA) was added to the molten diet during preparation (0.08 mg dye/ml diet). The prepared diet was degassed and dispensed at a temperature hot enough to flow (≤100 °C). Degassing was conducted by placing the diet in a side-arm vacuum flask held in a water-bath sonicator (Branson Ultrasonic Cleaner, B-22-4; Branson Cleaning Equipment Company, Shelton, CT, USA) containing water at the same level as that of the diet. The side-arm flask was connected to a water faucet aspirator (Fisher Aire Ejector metal filterpump; ThermoFisher Scientific, Waltham, MA) and the diet degassed for 15 min. After degassing, the diet was transferred to a beaker of hot water (100 °C) on