

Differentiating Tobacco Budworm and Corn Earworm Using Near-Infrared Spectroscopy

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ABSTRACT Near-infrared spectroscopy (NIRS) was used to develop a simple and quick technique to differentiate two economically important species, the tobacco budworm, *Heliothis virescens* (F.), and corn earworm, *Helicoverpa zea* (Boddie), which are major pests of cotton, *Gossypium hirsutum* L., in the southern United States. In practice, it is difficult to distinguish the two species during their immature stages using morphological characteristics unless expensive microscopy equipment or trained technicians are available. The current studies demonstrated that the two species could be quickly and readily differentiated during early developmental stages, including egg and young larval (younger than third instar) stages, by using NIRS technology with up to 95% accuracy. NIRS technology could significantly improve pest diagnosis in cotton pest management.

KEY WORDS *Heliothis virescens*, *Helicoverpa zea*, species identification

Tobacco budworm, *Heliothis virescens* (F.), and corn earworm, *Helicoverpa zea* (Boddie), are major pests of several crops, including cotton, *Gossypium hirsutum* L. Their larvae often are found feeding inside plant structures such as blossoms, buds, and fruits. Significant destruction to hosts occurs beginning with the third instar. Pyrethroids and *Bacillus thuringiensis* Berliner have traditionally been applied against the two species. However, tobacco budworm and corn earworm vary significantly in their susceptibility to the two insecticides (Luttrell et al. 1991, 1999).

The composition of tobacco budworm and corn earworm in field populations may change over the season and years. The species composition also is affected as a result of development of resistance to insecticides or with the increasing adoption of genetically modified crops such as cotton and corn with constitutive expression of insecticidal proteins derived from *B. thuringiensis* (Romeis et al. 2006). Therefore, developing an adequate control strategy with traditional insecticides or genetically modified crops depends on species composition identification, pest resistance status, and timely application of control

measures against young larvae before their development into destructive stages.

Conventional approaches to distinguish between tobacco budworm and corn earworm at egg and young larval stages may require expensive microscopy equipment or trained technicians. It is difficult to differentiate tobacco budworm and corn earworm eggs by using morphological characteristics under a light microscope, even though it is relatively easy to tell the morphological differences between them under an electron microscope (Bernhardt and Phillips 1985, Zeng et al. 1998). Additionally, morphological differences between young larvae of tobacco budworm and corn earworm are not always obvious (Neunzig 1964). When fully developed, tobacco budworm larvae have a tooth-like projection on the inside surface of the mandibles and fine short hairs on the first, second, and eighth abdominal projection (tubercle) that bear a single, prominent spine. Corn earworm larvae do not have such a projection or hairs. To observe these characteristics may require a microscope or experienced technicians. Thus, correct determination of the composition of these two species during the early development stages (third instar or younger) based on morphological features only may not be feasible in practice. Moreover, it is also time-consuming and may not always be accurate. Several alternative approaches, including molecular sequences (Roehrdanz 1997), chemical assay (Bailey et al. 2001), and surface hydrocarbons (Nelson and Buckner 1995), also may be able to distinguish both tobacco budworm and corn earworm at their early developmental stages. However, they are either extremely complex in sample

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preparation or time-consuming in screening process for extension professionals.

Zeng et al. (1998) developed monoclonal antibodies that are specific to tobacco budworm and corn earworm eggs. Diagnostic kits based on this technology have been used for monitoring population composition and trends (Zeng et al. 1999). Unfortunately, these same monoclonal antibodies are not able to differentiate tobacco budworm and corn earworm larvae, which are the destructive stages. Thus, an inexpensive and quick technique to distinguish the two species and to estimate their composition in field populations is needed for insecticide selection decisions for cotton pest management.

In recent years, near-infrared spectroscopy (NIRS) has been applied to entomological problems (Dowell et al. 1998, 1999, 2005). Using an automated NIRS system, uninfested wheat, *Triticum aestivum* L., kernels and kernels infested with larvae of different stored-product insect pests can be identified successfully (Dowell et al. 1998). This NIRS technique also has been used to manually scan insects to differentiate between closely related species at a high accuracy rate (Dowell et al. 1999, Cole et al. 2003).

The objective of the current study was to determine whether an NIR method could be developed to differentiate between corn earworm and tobacco budworm by using NIR spectra from eggs, young larvae, and pupae. Additionally, we also attempted to differentiate *Heliothis subflexa* (Guenée) from tobacco budworm and corn earworm by using NIRS. *H. subflexa* and tobacco budworm are two closely related lepidopteran species. Tobacco budworm has a broad host preference and *H. subflexa* has a relatively narrow host preference, primarily feeding on plants in the genus *Physalis* (Sheck and Gould 1993, 1996).

Materials and Methods

Insect Samples. *Differentiation of Second and Third Instars of Tobacco Budworm and Corn Earworm.* Eggs of tobacco budworm and corn earworm were provided by Rosie Ford (USDA-ARS, Stoneville, MS). The tobacco budworm and corn earworm eggs were shipped to Manhattan, KS, separately on diet in rearing trays. The rearing trays were held at 26°C and 50% RH. Larval development was monitored regularly by examination of molting. The sampled larvae were removed from the trays and stored in a growth chamber for 2–3 h at ≈6°C before scanning. Spectra of 150 larvae were collected for each species at the second and third instar stages, and each larva was scanned only once.

Differentiation of Eggs and Young Larvae of Tobacco Budworm and Corn Earworm. NIR spectra from ≈200 individual eggs for each species were collected within 4 h after receiving the samples from Stoneville. Once the eggs hatched, a spectrum of each tobacco budworm and corn earworm larvae was collected every 24 h for the first 4 d. The tobacco budworm and corn earworm larvae had developed into the late second instar by day 4.

Differentiation of Tobacco Budworm and Corn Earworm in a Mixed Population. Upon receipt from Stoneville, the tobacco budworm and corn earworm eggs were mixed and reared individually in a rearing tray. Thus, the species identity of each individual larva remained unknown until the adult stage. One hundred larvae at the second instar were chosen randomly from the rearing tray, and spectra were collected individually. The scanned larvae were reared at ≈26°C and 50% RH until adulthood, and they were identified as corn earworm or tobacco budworm based on the maculations of the wings (Mitter et al. 1993). The speciation for each individual was based upon a calibration model developed from data in *Differentiation of Second and Third Instars of Tobacco Budworm and Corn Earworm* above. We regarded an individual as misclassified if the NIR classification did not match the morphological characteristics of its wings.

Differentiation of Tobacco Budworm and Corn Earworm Pupae. The tobacco budworm and corn earworm pupae were supplied by the Department of Entomology Insectary, North Carolina State University, Raleigh, NC. Pupae were ≈5–7 d old when received and scanned. Because the pupae were larger than the fiber optic probe spot size used, spectral data of tail and head of each pupae were collected. In total, 85 pupae were sampled from each species. Each pupa was scanned only once.

Differentiation of H. subflexa, Tobacco Budworm, and Corn Earworm. Specimens of the three species were provided by Fred Gould (Department of Entomology, North Carolina State University). Spectral data of second instars of *H. subflexa*, tobacco budworm, and corn earworm were collected individually from a total of 88, 148, and 149 larvae, respectively.

Insect Rearing. In addition to the pupal specimens used in *Differentiation of Tobacco Budworm and Corn Earworm Pupae* above, all other insect specimens were received as eggs and reared individually in rearing trays in a growth chamber at ≈26°C. The rearing tray (40 by 80 cm) contains 400 1- by 1- by 2-cm cells, and each cell was filled with enough artificial diet for one tobacco budworm, corn earworm, or *H. subflexa* larva to be able to develop from the first to fourth instar. The artificial diet also was provided by Rosie Ford. Under these conditions, it takes ≈3 d to complete the development of the first instar, 2 d for the second instar, and 1.5 d for the third instar at 26°C.

Scanning Procedures. All specimens used in this study were live insect eggs, young larvae, or pupae that were randomly selected from the rearing trays. Before spectral data collection, all larval specimens were exposed to the compressed 100% CO₂ from a tank. Thus, the larvae remained under anesthesia while being scanned. The sample size included in the analysis for each treatment was at least 50 individuals.

Larval specimens were positioned dorsal side-up on a 7.5-cm-round spectralon diffuse reflectance plate (Labsphere, North Sutton, NH). Visible and NIR (400–2,500 nm) reflectance energy was transmitted to a QualitySpec Pro spectrometer (350–2,500 nm) (Analytical Spectral Devices, Inc., Boulder, CO) via a

Table 1. Classifying tobacco budworm and corn earworm larvae by using PLS regression models developed from near-infrared spectra

Calibration model	No. PLS factors	R^2	SECV	n	No. misclassified/ no. samples		% correct classification
					<i>H. virescens</i>	<i>H. zea</i>	
Second instar ^a	6	0.80	0.224	296	5/148	1/148	98.0
Third instar ^a	6	0.81	0.221	294	8/148	1/146	97.0
Pooled ^b	8	0.78	0.249	590	12/296	7/294	96.8

^a At 450–2,490 nm.

^b Second and third instars at 450–1,700 nm.

bifurcated reflectance probe. A fiber optic illuminator (Analytical Spectral Devices, Inc.) was used to illuminate the sample through the fiber optic cable. A 3-mm-diameter bifurcated reflectance probe oriented vertically 2 mm above the Spectralon was used to scan eggs. This small probe had 33 fibers used for illumination, and seven fibers for reflectance. The reflectance fibers are bundled in the center of the probe, and the illumination fibers are oriented in two rings around the reflectance bundle. Larvae and adults were scanned using a 6.3-mm-diameter bifurcated probe oriented vertically 6.8 mm above the Spectralon. This large probe had 78 fibers used for illumination, and 78 fibers for reflectance. The illumination and reflectance fibers were randomized in the probe tip. A baseline was measured using a 2.5-cm-diameter Spectralon plate.

For each insect specimen, the instrument automatically collected 20 spectra, and then stored an averaged spectrum. The whole procedure including preparing, positioning, and collecting the spectral data took <1 min per specimen. ASD software RS³ (Version 3.1) was used to collect spectra. Spectra were then converted using ASD ViewSpecPro to a format that could be imported into GRAMS (Thermo Galactic, Salem, NH).

Data Analysis. Spectral data were analyzed using partial least-squares regression (PLS) (Martens and Naes 1989) with GRAMS PLS/IQ software. Some spectra were removed due to larval specimen movement during scanning, which resulted in noisy spectra. Comparisons were made using PLS by assigning a value of one or two for each pairwise combination of tobacco budworm versus corn earworm, *H. subflexa* versus tobacco budworm, or *H. subflexa* versus corn earworm.

To differentiate tobacco budworm and corn earworm larvae in a mixed population, a test set of 100 individuals was selected randomly from the mixed population, scanned, and classified using the calibration model created from the second instar. If the predicted value was <1.5, then the larva was classed as tobacco budworm, otherwise it was classed as corn earworm. The wavelengths important in classifying the species of interest were determined based on PLS regression coefficients and differences in spectra. Accuracy of identification was determined using weighted correct classification; coefficient of determination (R^2), indicating the closeness of fit between the NIRS and reference data; and standard error of

cross validation (SECV) by a one-sample-out procedure (Williams 2001).

Results

Differentiation of Second and Third Instar Tobacco Budworm and Corn Earworm Larvae. Distinguishing tobacco budworm from corn earworm larvae at the second and third instar was possible and classification was correct $\approx 97\%$ of the time (Table 1). The model had R^2 ($P < 0.01$) of 0.80 and 0.81 for the second and third instars, respectively. For the second instars, six of 296 were misclassified, giving a correct classification rate of 98% when using six PLS factors and a wavelength range of 450–2,490 nm. For the third instars, a 97% correct classification rate was achieved; nine of 294 were misclassified at the same number of factors and wavelength range used for second instars.

The PLS regression coefficients for second and third larval instars (six factors) showed similar important wavelengths (figure not shown) with wavelengths above $\approx 1,700$ nm mainly resulting in noise and were of little use. Thus, spectral data of second and third larval instars were pooled and PLS analysis was done for 450–1,700-nm wavelength range. The regression coefficients indicating important wavelengths in the calibration model are shown in Fig. 1. The 500–650-nm regions indicate there is a slight color difference in the samples. The 900–1,200- and 1,250–1,350-nm

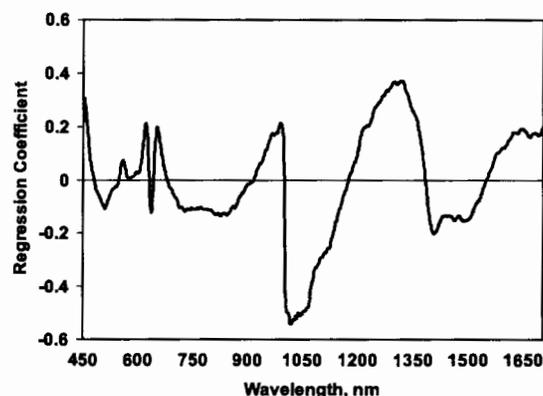


Fig. 1. Partial least-square regression coefficient plots used for indicating the important NIR wavelengths for detecting the differences between species *H. virescens* and *H. zea* at the second and third instar (PLS factors = 6).

Table 2. Classifying tobacco budworm and corn earworm eggs and larvae by using PLS regression models developed from near-infrared spectra (450–2,490 nm)

Calibration model	No. PLS factors	R ²	SECV	n	No. misclassified/ no. samples		% correct classification
					<i>H. virescens</i>	<i>H. zea</i>	
Day 1 (egg)	6	0.84	0.2	199	1/99	2/100	98.5
Day 2 (first instar)	6	0.77	0.24	187	3/89	1/98	98.0
Day 3 (first instar)	6	0.73	0.26	198	8/99	5/99	93.4
Day 4 (second instar)	6	0.78	0.24	199	4/100	3/99	96.5
Day 5 (second instar)	6	0.79	0.23	199	3/100	1/99	98.0

regions may be caused by differences in cuticular lipids between the two species, because insect cuticular lipids absorb strongest in this region (Dowell et al. 1999).

Differentiation of Egg and Young Larvae of Tobacco Budworm and Corn Earworm. Tobacco budworm and corn earworm can be distinguished in their early developmental stage (from egg to the second instar) by using this technique (Table 2). Correct classifications ranged from 93.4 to 98.5% over the 5-d period. A high rate of correct classification was obtained on day 1 while tobacco budworm and corn earworm were still in the egg stage. Overall, only three eggs of 199 were misclassified. The model had R² ($P < 0.01$) values ranging from 0.77 to 0.84. These findings suggest that the composition of tobacco budworm and corn earworm in field populations could be predicted with high accuracy at their early developmental stage by using NIRS.

Identification of Unknown Tobacco Budworm and Corn Earworm from a Mixed Population. A calibration model was developed using six PLS factors and used to predict the species of 102 unknown insects. After spectral data collection, the second instars were reared in the growth chamber to adulthood and identified based on maculations of the wings. Compared with the morphological identifications, we achieved a 96.1% correct classification rate by using NIRS (Table 3). Only one corn earworm larva of 52 was misclassified as tobacco budworm, and three tobacco budworm larvae of 50 were misclassified as corn earworm.

Differentiation of Tobacco Budworm and Corn Earworm Pupae. Generally, corn earworm pupae are bigger and heavier than those of tobacco budworm. However, the pupal size or weight is not a reliable indicator for species identification. Thus, we investigated whether NIRS could be used to differentiate species of pupae. Based on the spectral data, tobacco budworm, and corn earworm pupae were distinguishable with a correct classification rate of 96.5% by using

tail spectral data (Table 4). In contrast, we only obtained 68.1% of correct classification by using head spectral data. Our findings suggest both species may be distinguishable in the pupal stage and that use of the tail spectra provide better identification than spectra obtained from the head. We have no conclusive explanation for this difference to differentiate between the tail and head ends.

Differentiation of *H. subflexa* from Tobacco Budworm and Corn Earworm. Using NIRS, we were able to distinguish *H. subflexa* from tobacco budworm, and *H. subflexa* from corn earworm larvae at the second instar with 100% precision. There were no misclassifications between *H. subflexa* and corn earworm or *H. subflexa* and tobacco budworm (Table 5).

Discussion

As two primary cotton pests, tobacco budworm and corn earworm may occur simultaneously in the field. However, they vary considerably in their potential resistances to different insecticides. Thus, the first critical step in managing the two species is to estimate their compositions from the field population, helping to identify appropriate insecticide products for pest control. We have shown that NIRS can differentiate tobacco budworm from corn earworm at the egg, larval, and pupal stages.

NIRS technology, which detects the light energy diffusely reflected from the target object and therefore obtains information concerning its composition and other characteristics, has had a relatively short history in pest identification (Dowell et al. 1999). Recently, commercial NIRS instruments coupled with computers have allowed operators to collect and analyze digital data of the optimal wavelength of specimens. As a potential tool in helping differentiate two

Table 4. Classifying tobacco budworm and corn earworm pupae by using PLS regression models developed from near-infrared spectra (450–2,490 nm)

Calibration model	No. PLS factors	R ²	SECV	n	No. misclassified/ no. samples		% correct classification
					<i>H. virescens</i>	<i>H. zea</i>	
Pupa tail ^a	8	0.73	N/A	85	1/45	2/40	96.5
Pupa head ^a	8	0.12	N/A	85	13/45	14/40	68.1

^a *H. virescens* vs. *H. zea*.

Table 3. Classification of unknown corn earworm and tobacco budworm larvae by using a PLS regression model developed from near-infrared spectra (450–2,490 nm)

Calibration model	No. misclassified/ no. samples		% correct classification
	<i>H. virescens</i>	<i>H. zea</i>	
Second instar and 6 PLS factors	1/52	3/50	96.1

Table 5. Classifying *H. subflexa*, corn earworm, and tobacco budworm larvae by using PLS regression models developed from near-infrared spectra (450–2,490 nm)

Calibration model	No. PLS factors	R ²	SECV	n	No. misclassified/ no. samples		% correct classification
					<i>H. virescens</i>	<i>H. zea</i>	
<i>H. subflexa</i> vs. <i>H. virescens</i>	6	0.94	0.118	237	0/88	0/149	100
<i>H. subflexa</i> vs. <i>H. zea</i>	6	0.91	0.149	236	0/88	0/148	100

types of cotton pest, NIRS instrumentation (including hardware and software) need only to be purchased once, and the cost ranges from \$1,000 to \$100,000, depending on the wavelength regions covered and types of sensors used. A system to determine insect species may cost less than \$10,000, whereas the cost per sample is subject to justification of sampling frequency and number. On the condition that a cotton specialist needs to screen 450 samples annually (Scott Stewart, personal communication), the cost per sample is approximately \$2.00 over a 10-yr period. Moreover, the cost would be significantly reduced if several cotton professionals shared the same equipment.

Using NIRS techniques, tobacco budworm and corn earworm can be distinguishable in egg, larval, and pupal stages with up to 98% accuracy. Specimens of tobacco budworm and corn earworm from the different locations were differentiated using NIRS, demonstrating the potential utility of NIRS in cotton pest identification. It is also relatively quick, simple, and accurate. NIRS may detect differences that are the result of environmental and dietary causes, such as moisture or fat content. Thus, caution should be used when developing calibrations.

Additional research is needed to develop calibration models by using field populations of insects to predict the identification of individuals from different locations. Thus far, we have collected spectral data from only live specimens. In the future, we expect to use frozen, ethanol-preserved, or dried specimens to develop calibration and cross-validation models. In addition, we will explore the influence of host diets on principal absorptions of interest.

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