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Proximal remote sensing to differentiate nonviruliferous and viruliferous insect vectors – proof of concept and importance of input data robustness

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Proximal remote sensing is being widely studied as a noninvasive method to partially automate diagnostics of plants and insects. The hypothesis that proximal remote sensing can be used to differentiate specimens of adult beet leafhoppers (*Circulifer tenellus*) that were nonviruliferous or viruliferous for beet curly top virus (BCTV) was tested. A key aspect of applications of proximal remote sensing is the 'robustness' or repeatability of input reflectance data. Many factors may contribute to low input reflectance data robustness; these include: (i) issues related to the consistency of proximal remote sensing conditions (light intensity and spectral composition, ambient temperature), (ii) insect specimen preparation (projection angle, storage and handling), and (iii) insect specimen characteristics (age, growing conditions, variety/biotype, host plant). This study demonstrates that nonviruliferous and viruliferous specimens of adult beet leafhoppers possess unique body reflectance features and, therefore, can be differentiated. However, insect specimen preparation (removal of wings and placement) markedly affected the classification accuracy. Addition of experimental noise to input reflectance data was conducted to simulate varying degrees of input reflectance data robustness. The potential of developing reflectance-based diagnostic tools for detection of plant pathogenic viruses in insects is discussed, with an emphasis on input data robustness.

Keywords: curtovirus, detection methods, hyperspectral imaging, integrated pest management, reflectance profiling, virus detection

Introduction

Proximal remote sensing consists of acquiring and classifying reflectance or transmittance data at one or multiple wavelengths from target objects placed within a short distance (<1 m and typically much less) from an imaging sensor (Nansen, 2016). In addition to several important reviews on use of reflectance-based technologies in plant science (Prabhakar *et al.*, 2012), a rapidly growing number of studies describe the use of proximal remote sensing technologies to detect and diagnose infection of plants by plant pathogens, including: cercospora leaf spot (*Cercospora beticola*), sugar beet rust (*Uromyces betae*) and powdery mildew (*Erysiphe betae*) in sugar beet (*Beta vulgaris*; Rumpf *et al.*, 2010); powdery mildew (*Blumeria graminis* f. sp. hordei, isolate K1) in barley (*Hordeum vulgare*; Kuska *et al.*, 2015; Thomas *et al.*, 2017); and fusarium head blight in wheat (*Triticum* spp.; Bauriegel & Herppich, 2014). There are also recent reviews describing the rapidly growing body of research into the use of proximal remote sensing in entomological studies (Nansen, 2016; Nansen & Elliott, 2016). For instance, insect body reflectance data have been used to: age-grade mosquito species (*Anopheles* spp.; Sikulu *et al.*, 2014), biting midges (*Culicoides sonorensis*; Reeves *et al.*, 2010) and two species of fruit flies (*Drosophila melanogaster* and *Drosophila simulans*; Aw *et al.*, 2012); determine whether two species of fruit flies were infected with *Wolbachia* (Aw *et al.*, 2012); differentiate mated and unmated honey bee queens (Webster *et al.*, 2009); determine developmental stages of blowfly puparia (Voss *et al.*, 2016); and characterize levels of 'terminal stress' imposed by killing agents on maize weevils (*Sitophilus zeamais*) exposed to an insecticidal plant extract, and larger black flour beetles (*Cynaus angustus*) exposed to entomopathogenic nematodes (Nansen *et al.*, 2015a). These studies highlight that, although groups of plants or insect specimens may be indistinguishable by the human eye, internal physiological changes and/or marked alterations of metabolic processes can potentially be detected via extraction and classification of body reflectance features. It is important to emphasize that, although reflectance features are acquired from the surface of objects

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(such as insects, seeds and green leaves), a recent study has clearly demonstrated how 'penetration' (particularly in the near-infrared portion of the radiometric spectrum) leads to reflectance data being at least partially influenced by the structure and composition of tissues below the surface (Nansen, 2018).

Proximal remote sensing proposed as a diagnostic tool for detection of plant pathogens in insect vectors is based on two fundamental assumptions. First, acquisition of plant pathogens by insect vectors causes physiological changes in insect vectors; that is, the pathogen may only be present in very specific tissues or organs (such as salivary glands), but it may elicit systemic physiological changes/responses in the insect vector in response to the pathogen (Kaur *et al.*, 2017; Hasegawa *et al.*, 2018). Secondly, pathogen-induced changes to insect physiology can be detected based on body surface reflectance features, even after insect specimens have been killed and stored in 70% ethanol. The latter assumption has recently been investigated, and it was demonstrated that specimen storage in 70% ethanol (compared to 50% or 90%) should be recommended (Li *et al.*, 2017). This study showed that storage time up to several weeks in 70% ethanol had negligible effects on reflectance features acquired from insect specimens. These technical details are very important because they highlight that insect specimens can potentially be collected at field sites, stored in 70% ethanol, and shipped before arriving at diagnostic imaging facilities, although such effects should be evaluated for each insect species prior to beginning extensive experimentation.

An increasing body of research into deployment of proximal remote sensing technologies to detect and diagnose plant pathogens in both plants and insect vectors suggests that such reflectance-based technologies may be used to greatly improve quarantine and inspection efforts and regional crop disease monitoring programmes. That is, similar to PCR- and ELISA-based services currently provided by commercial diagnostic laboratories, it seems reasonable to expect that reflectance-based technologies can be developed, so that agricultural stakeholders can ship insect samples to these laboratories and obtain rapid, reliable and cost-effective data on infection rates. With such potential, it is understandable and certainly justifiable that many research groups are studying advanced classifications of proximal remote sensing data as part of resistance breeding of crop plants and plant disease diagnostics (Mahlein, 2015; Wahabzada *et al.*, 2016).

As part of developing reflectance-based diagnostic tools, the 'robustness' or repeatability of input reflectance data must also be considered (Nansen, 2011). Low robustness implies a high level of variability in data acquired from the same object at multiple time points, different portions of the same object, or several objects in the same category or class. Many factors contribute to low input reflectance data robustness (Nansen, 2018) and include issues related to the consistency of proximal remote sensing conditions (light intensity and spectral composition, ambient temperature, etc.), insect specimen preparation (projection angle, storage and handling, etc.), and insect specimen characteristics (age, sex, growing conditions, variety/biotype, host plant etc.). Moreover, low data robustness implies that both the sensitivity (ability to differentially detect low levels of disease-induced stress) and the repeatability (ability to accurately classify a wide range of datasets) of reflectance-based classification algorithms are jeopardized. In short, if the robustness of input data is low, then there is an increased risk of classification errors (false positive (Type I) and/or false negative (Type II)). As part of the development and testing of reflectance-based classification algorithms to be used in studies of insect vectors of plant pathogens, it is therefore important to include quality control of classification algorithms to obtain better insight into their performance when applied to input data with varying degrees of robustness.

Beet curly top virus (BCTV) is a plant-infecting virus with a circular single-stranded DNA genome, which is encapsidated in small twinned quasi-isometric virions that measure 18 × 30 nm. It is a member of the family *Geminiviridae* and the type species of the genus *Curtovirus*. BCTV induces curly top disease in a large number of crops, including sugar beet, tomato, melon and pepper (Soto & Gilbertson, 2003; Munyaneza & Upton, 2005). BCTV is transmitted by beet leafhoppers (*Circulifer tenellus*), which are sap-sucking insect pests (Nault & Ammar, 1989). The mode of BCTV transmission by the beet leafhopper is circulative and nonpropagative (Chen & Gilbertson, 2016). The virus can be acquired and transmitted in minutes, but longer periods of feeding result in higher rates of transmission (Bennett, 1971; Thomas & Boll, 1977). The current standard for detection of BCTV in beet leafhoppers is a PCR-based method (Soto & Gilbertson, 2003; Chen & Gilbertson, 2008, 2016). Although PCR-based methods for detection of BCTV are highly accurate, sensitive and specific, cost and processing time are major constraints. A noninvasive diagnostic tool to rapidly, accurately and cost-effectively screen large numbers of beet leafhoppers for the presence of BCTV would be beneficial as a way to forecast epidemics and to target infected insect vector populations, both spatially and temporally.

The current study is based on the hypothesis that proximal remote sensing can be used to acquire and analyse insect body reflectance features to accurately differentiate nonviruliferous and viruliferous specimens of adult beet leafhoppers. A negative association between input reflectance data robustness and accuracy of reflectance-based classification of adult beet leafhopper specimens was predicted. Separate analyses of reflectance data acquired from processed (wings removed and each beet leafhopper carefully placed on the side) and unprocessed (wings intact and placed haphazardly) insect specimens were conducted. As a framework of quality control to quantify the importance of input reflectance data robustness, average reflectance profiles acquired from beet leafhopper specimens were experimentally manipulated by adding stochastic ranges of noise to simulate varying degrees of input reflectance data robustness. The potential of developing reflectance-based diagnostic tools for detection of important plant pathogenic viruses in insects

as a tool to determine the prevalence of viruliferous vector insects in a population is discussed, with an emphasis on input data robustness.

Materials and methods

Beet leafhoppers

Specimens of beet leafhopper were obtained from colonies reared on uninfected sugar beet plants (nonviruliferous) or on BCTV-infected sugar beet plants (viruliferous), which had been maintained at UC Davis for more than 24 months. Beet leafhopper colonies were maintained on sugar beet plants inside cages in separate greenhouses. It was assumed that the level of specimen processing could potentially affect the ability to differentiate nonviruliferous and viruliferous specimens based on body reflectance. Consequently, separate analyses of what is referred to as processed and unprocessed insect specimens were conducted. In the first dataset, beet leafhoppers were processed by removing wings and carefully placing each beet leafhopper on its side prior to proximal remote sensing (Fig. 1a). Removal of wings was done by gently pressing the wings of each specimen onto a piece of sticky tape and pulling off the wings. In addition to removal of wings, all specimens were carefully laid on their side, so that all specimens were imaged in the same position. The second and third datasets are referred to as unprocessed because wings were not removed, and, during acquisition of proximal remote sensing data, the specimens were placed haphazardly (not in any specific position; Fig. 1b). The first and second datasets consisted of specimens sampled on two different dates to include variability over time into the analysis. The first dataset consisted of 70 adult beet leafhopper specimens, with 35 specimens each from BCTV-free and BCTV-infected sugar beet plants (this was done by collecting 15 beet leafhoppers from each colony on 10 April 2017 followed by 20 from each colony on 17 April 2017). The second dataset consisted of 100 adult beet leafhopper specimens, with 50 specimens each collected from uninfected and BCTV-infected sugar beet plants (this was done by collecting 25 beet leafhoppers from each colony on 7 and 15 December 2016). Regarding the third dataset, acquired proximal remote sensing and PCR detection was performed on beet leafhoppers collected at five time points: 0 (baseline, control not exposed to BCTV-infected plants) and 1, 2, 3 and 4 days after exposure to BCTV-infected sugar beet plants. Here, *c.* 200 adult beet leafhoppers from the colony reared on noninfected sugar beet plants were transferred to BCTV-infected sugar beet plants, and subsamples of 10 adult specimens were collected daily after 0–4 days. As a positive control, 12 adult beet leafhopper specimens taken directly from the colony reared on BCTV-infected sugar beet plants (viruliferous beet leafhoppers) were sampled. With data acquired in two time series for the five time points (0–4 days), and 12 individuals as positive controls, a total of 112 adult beet leafhopper specimens were included in this third dataset. Thus in total, this study consisted of analysis of 282 adult beet leafhopper specimens from three datasets and with each dataset including samples collected at two separate dates (dataset 1 = 70 specimens, dataset 2 = 100 specimens, and dataset 3 = 112 specimens).

For all three datasets, adult nonviruliferous and viruliferous specimens were transferred directly to vials containing 70% ethanol, which was chosen because it was previously identified as being superior to other killing methods and ethanol concentrations when proximal remote sensing is deployed for studies of insects (Li *et al.*, 2017). Adult beet leafhopper specimens were stored in 70% ethanol for 2–5 days prior to acquisition of proximal remote sensing data. Thus, the processing of adult beet leafhopper specimens was designed to simulate field sampling and subsequent shipment of specimens for analysis to a diagnostic laboratory.

Extraction of DNA and PCR detection of BCTV in adult beet leafhoppers

DNA extraction methods described in previously published studies were used (Soto & Gilbertson, 2003; Chen & Gilbertson, 2008, 2016). Briefly, individual adult beet leafhoppers were ground in 150 μL STE buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) containing RNase A (10 μg mL^{-1}) in a 1.5 mL Eppendorf tube with a minipestle. After the addition of 350 μL STE buffer, the tubes were incubated at 37 °C for 10 min. Then, 2.5 μL of proteinase K (100 μg mL^{-1}) and 25 μL of 10% sodium dodecyl sulphate (SDS) solution were added to these suspensions, and tubes were incubated at 37 °C for 1 h. The suspensions were clarified by centrifugation (5 min at

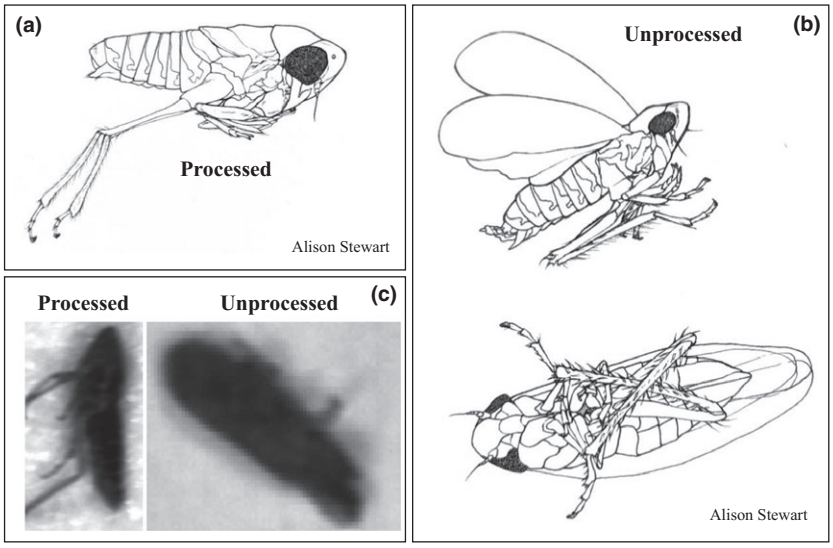

Figure 1 Drawings of adult beet leafhoppers (*Circulifer tenellus*) when either processed (a; leafhopper wings were removed and each leafhopper was placed carefully on its side prior to proximal remote sensing) or unprocessed (b; wings were not removed, and, during acquisition of proximal remote sensing data, the specimens were placed haphazardly and not in any specific position). Representative images of processed and unprocessed adult beet leafhoppers are also shown (c).

12 000 *g*), and the supernatant was extracted with an equal volume of phenol:chloroform. Nucleic acids were recovered by ethanol precipitation, suspended in 20 μL sterile distilled water, and 2 μL was used in the PCR.

To detect BCTV, the primer pair BGc396 (5′-CAACTGGTC GATACTGCTAG-3′) and BSCTVv2688 (5′-GAGCTGGTACT TCGATGTTG-3′), designed to detect severe strains of BCTV (including the BCTV-Svr-CFH isolate used in the present study) was used to direct the amplification of *c.* 0.7 kb BCTV DNA fragments, which includes parts of the overlapping V2/V3 and C1/C4 genes flanking the entire intergenic region (Chen *et al.*, 2010). The PCR parameters were as follows: an initial denaturing step at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s (denaturing), 61 °C for 40 s (annealing), and 72 °C for 1 min (extension); with a final extension step of 72 °C for 7 min. PCR-amplified DNA fragments were analysed by electrophoresis in 1% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained with ethidium bromide and visualized with UV light.

Proximal remote sensing data acquisition

Proximal remote sensing data were acquired from individual adult beet leafhopper specimens under environmental conditions similar to those described in previous studies (Nansen *et al.*, 2014; Voss *et al.*, 2016; Nansen, 2018). Adult specimens were of unknown age, sex and mating status, as intended to simulate the variation encountered during field sampling of adult beet leafhoppers. A push-broom hyperspectral camera (PIKA XC; Resonon Inc.) mounted 20 cm above the specimens was used, and hyperspectral images were acquired with the spatial resolution of about 50 pixels mm^{-2} under artificial lighting (four 15 W 12 V light bulbs with two on either side of the lens). The main specifications of the hyperspectral camera were FireWire IEEE 1394b interface, 14 bit digital output, and 7° angular field of view. The objective lens had a 17 mm focal length (maximum aperture of f1.4), optimized for the near-infrared and visible near-infrared spectra. Reflectance data were acquired in 240 spectral bands from 383 to 1036 nm (spectral resolution = 2.1 nm), but only 210 spectral bands from 435 to 1008 nm were included. Spectral data in both ends of the acquired spectrum were omitted due to concerns about low signal-to-noise ratio. With the number of spectral bands exceeding the number of samples (dataset 1 = 70 specimens, dataset 2 = 100 specimens, and dataset 3 = 112 specimens), a major concern is model over-fitting caused by the Hughes phenomenon or violation of the principle of parsimony (Defernez & Kemsley, 1997; Hawkins, 2004). Consequently, spectral binning was conducted, so that the original 210 spectral bands were averaged and converted into 70 spectral bands (spectral resolution = 6.3 nm).

Data analysis

During acquisition of proximal remote sensing data, the relative humidity was 30–40% and the ambient temperature 19–22 °C. A piece of white teflon (K-Mac Plastics) was used for white calibration, and the light saturation level was adjusted to the white teflon. Background colour has been shown to affect reflectance data acquired from insect specimens, such as adult mosquitoes (Nansen, 2018). In this study, adult beet leafhoppers were placed on top of white paper to maximize the difference in reflectance between insects and background in all spectral bands across the examined spectral range. Data processing and analyses were conducted in PC-SAS v. 9.4 (SAS Institute). Similar to previously published studies (Nansen *et al.*, 2014; Voss *et al.*, 2016; Nansen, 2018), a dichotomous radiometric filter was developed to automate exclusion of white background. After exclusion of white background, the average number of pixels per specimen was 510 ± 16 SE. A single average reflectance profile was generated for each beet leafhopper specimen.

A key objective of this study was to assess the effect of input reflectance data robustness, and this was addressed through experimental addition of stochastic noise, i.e. the average reflectance value in each spectral band was experimentally manipulated. The following briefly describes how stochastic noise was added. It was assumed that the reflectance value in a single spectral band acquired from one adult beet leafhopper is 0.150000. If the stochastic noise range is 0–0.5%, then a random value ranging from −0.5% to 0.5% is added/subtracted. Thus, experimental manipulation of the actual reflectance value, 0.150000, by adding/subtracting 0–0.5% noise would generate a new reflectance value of 0.14925–0.150075. The experimental addition of stochastic noise to reflectance values was performed so that the manipulation in one spectral band was independent of the noise added to the reflectance value in other spectral bands. In addition, the manipulation of reflectance values was independent across average reflectance profiles. Multiple ranges of stochastic noise were examined for each dataset.

Classification of nonviruliferous and viruliferous specimens of adult beet leafhoppers was based on linear discriminant analysis (Fisher, 1936), which has been used in similar classification studies of average reflectance profiles acquired from insects and seeds (Nansen *et al.*, 2015a,b; Li *et al.*, 2017). Initially, stepwise linear discriminant analysis was used to select only the spectral bands (out of the 70 spectral bands) with significant contribution to the linear discriminant classification model. The selected subset of explanatory variables was used to generate linear discriminant classification models.

There are numerous approaches for validation of reflectance-based classifications of objects, such as insects, and the three most commonly used are: (i) jack-knife cross-validation (leave one out and using it for validation and repeating this for all observations), in which a single observation is removed from the training dataset and is used for validation. This method is then repeated with all observations to calculate an average classification accuracy. (ii) The entire dataset is divided into several groups, for instance four, then data in three of these groups are used as training data to generate the classification model, and the remaining fourth is used for validation. In both of these methods, all proximal remote sensing data are essentially collected under the same conditions, so possible noise from inconsistency in proximal imaging conditions is completely ignored. (iii) A third validation method consists of collecting training data on some days and then an independent validation dataset is collected on other days. The latter validation method does allow possible noise caused by inconsistency in proximal imaging conditions that may affect the classification accuracy, but factors associated with specimen conditions are kept constant, because the validation data are typically collected from a replication of the experiment conducted to generate the training dataset.

In this study, jack-knife cross-validation was used to quantify the accuracy of the linear discriminant classification model of processed specimens. To quantify the accuracy of the linear discriminant classification model derived from unprocessed insect specimens, the linear discriminant classification model was developed using the second dataset, and the third dataset was used for independent validation.

Results

Processed insect specimens

To investigate possible effects of insect specimen preparation, a high level of specimen processing was included, which consisted of removing wings to expose the insect abdomen and also placing each specimen carefully on its side prior to proximal remote sensing (Fig. 1a,c). Such a level of processing would probably be unfeasible to perform if this technology were to be implemented as a commercial and large-scale operation with high throughput. However, the dataset was included as an important comparison. Average reflectance profiles showed that viruliferous adult beet leafhoppers had consistently higher reflectance compared with nonviruliferous conspecifics in spectral bands from about 600–1004 nm (Fig. 2a). Figure 2b shows the variance as a percentage of average reflectance values, and it is seen that variance in all spectral bands was relatively low (<0.14% of average reflectance values). Figure 2b also shows that proximal remote sensing data acquired from viruliferous adult beet leafhoppers had slightly higher variance compared to nonviruliferous conspecifics. Due to considerable differences of average reflectance values and low within-class variance, a combination of only four spectral bands (indicated by circles in Fig. 2a) was selected in the stepwise discriminant analysis, and the classification accuracy of the linear discriminant model was very high, 97%. To examine the robustness of the linear discriminant model, 0–8% noise was added to the data. This analysis revealed that when more than 1% stochastic noise was

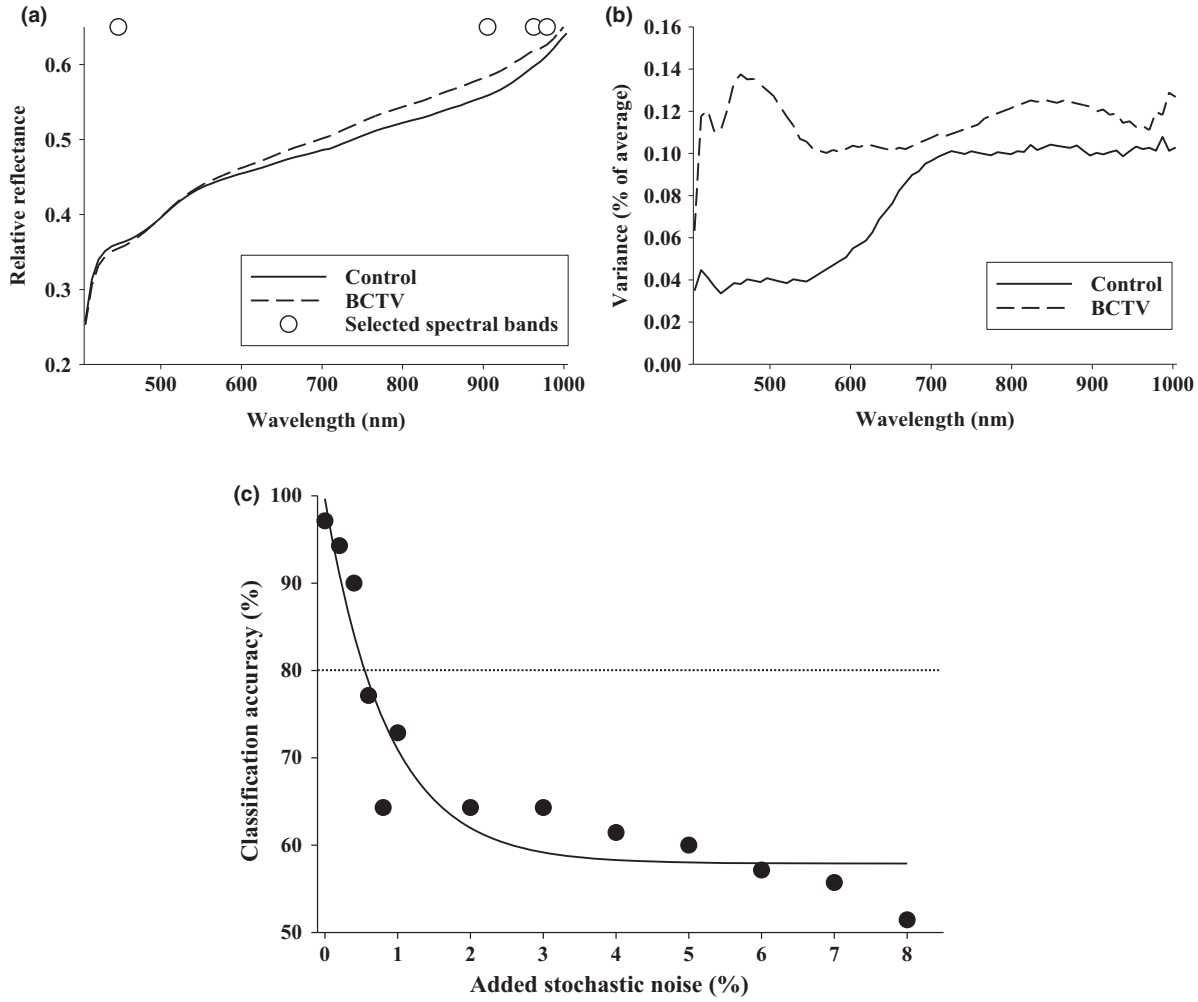

Figure 2 Average reflectance profiles of processed adult beet leafhoppers raised on noninfected sugar beet (control) and on sugar beet infected with beet curly top virus (BCTV) in 70 spectral bands from 435 to 1008 nm (a), and variance as a percentage of average reflectance values (b). Stochastic noise was added to average reflectance profiles as a way to experimentally reduce input reflectance data robustness. Reflectance data were analysed based on linear discriminant analysis, and the relationship between range of stochastic noise and classification accuracy was examined (c). The dotted horizontal line represents classification accuracy of 80%. Open circles in (a) represent spectral bands selected for the linear discriminant function based on stepwise forward selection.

added, the classification accuracy decreased below 80% (Fig. 2c).

Unprocessed insect specimens

Individual adult beet leafhoppers were subjected to proximal remote sensing without taking into consideration their position when placed under the hyperspectral camera and without any processing (i.e. removal of wings and without careful placement of insect individuals) (Fig. 1b,c). For commercial application of proximal remote sensing-based diagnosis of adult beet leafhoppers, this would be the most feasible procedure. As seen in Figure 3a, there was little visual separation between average reflectance profiles of nonviruliferous and viruliferous adult beet leafhoppers. However, the trend was similar to that observed with processed adult beet leafhoppers: that viruliferous specimens had higher reflectance than nonviruliferous conspecifics. Figure 3b shows that the variance as percentage of average reflectance in individual spectral bands was again highest in viruliferous adult beet leafhoppers. Moreover, the overall variance was similar to that of processed adult beet leafhoppers, although the location of the highest level of variance was different (*c.* 600–900 nm). Due to higher similarity between average reflectance profiles of nonviruliferous and viruliferous adult beet leafhoppers and slightly higher variance, the ability to accurately classify unprocessed adult beet leafhoppers was slightly lower (91%) compared to processed beet leafhoppers (97%). The 24 spectral bands included in the linear discriminant model of unprocessed specimens are represented as circles in Figure 3a, and it is seen that these spectral bands were not restricted to a specific spectral region. In other

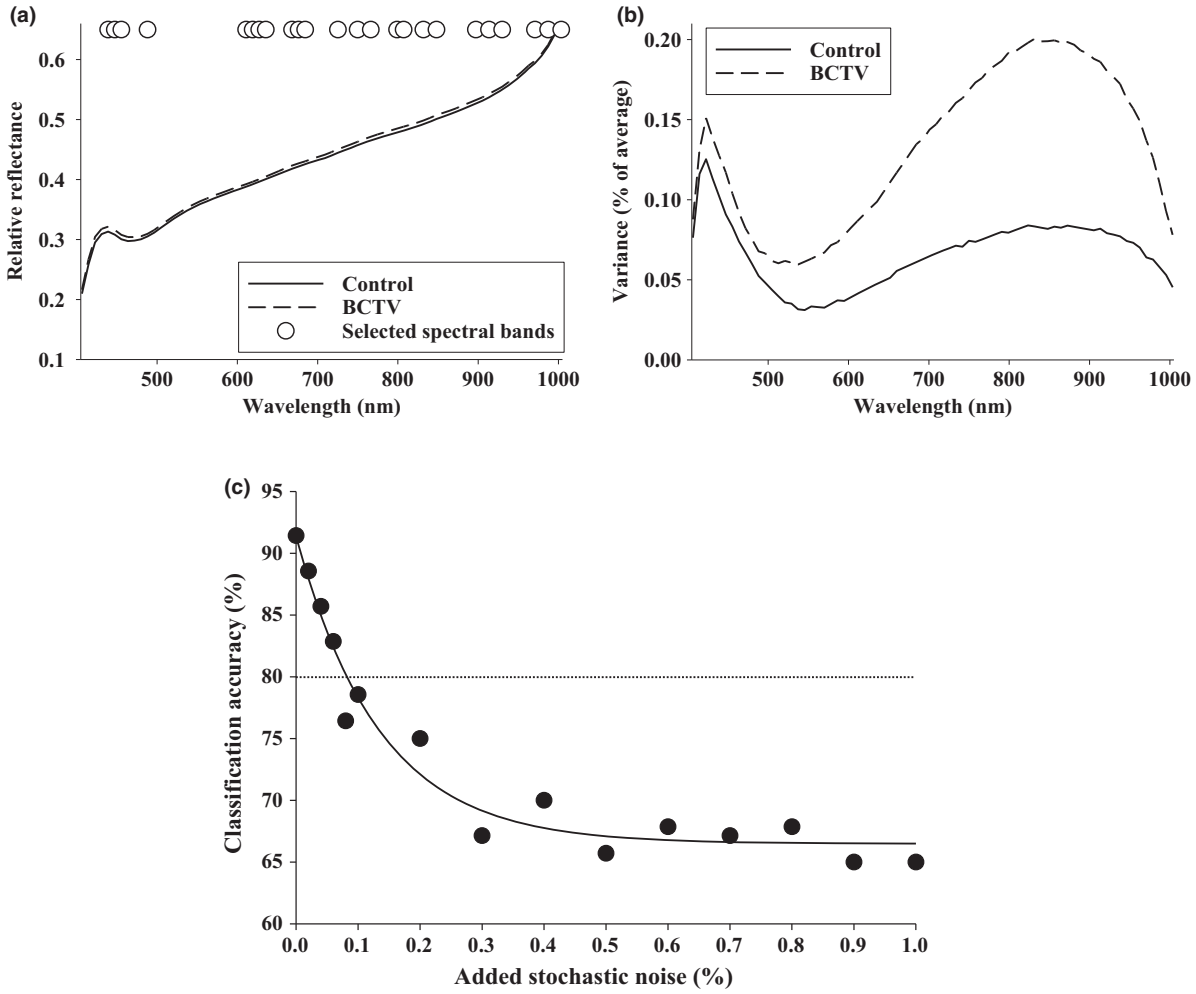

Figure 3 Average reflectance profiles of unprocessed adult beet leafhoppers raised on noninfected sugar beet (control) and on sugar beet infected with beet curly top virus (BCTV) in 70 spectral bands from 435 to 1008 nm (a), and variance as a percentage of average reflectance values (b). Stochastic noise was added to average reflectance profiles as a way to experimentally reduce input reflectance data robustness. Reflectance data were analysed based on linear discriminant analysis, and the relationship between range of stochastic noise and classification accuracy was examined (c). The dotted horizontal line represents classification accuracy of 80%, and it was considered the threshold for acceptable classification accuracy. Open circles in (a) represent spectral bands selected for the linear discriminant function based on stepwise forward selection.

words, accurate classification of nonviruliferous and viruliferous adult beet leafhoppers was possible based on spectral data from individual bands within the entire spectral range. To test the robustness of the linear discriminant model, 0–1% noise was added to the data; when more than 0.1% stochastic noise was added, the classification accuracy decreased below 80% (Fig. 3c).

BCTV expression over time in unprocessed insect specimens

An important aspect of diagnostic tools is to determine how long insect vectors need to feed on an infected plant before acquiring a detectable amount of the pathogen. Thus, a third dataset was evaluated in which nonviruliferous adult beet leafhoppers were transferred to a BCTV-infected sugar beet plant and beet leafhoppers sampled daily after 0–4 days of feeding. As a positive control, 12 adult beet leafhoppers were also sampled from the colony reared on BCTV-infected sugar beet plants. PCR data confirmed that all 12 of these adult beet leafhoppers were positive for BCTV, i.e. viruliferous. Beet leafhoppers from the 0 day time point, i.e. collected from the colony reared on uninfected sugar beet plants, were all negative for BCTV based on the PCR test (Fig. 4). The PCR data also showed a gradual increase in the percentage of beet leafhoppers positive for BCTV, exceeding 98% after 3 days (Fig. 4). The classification of the exact same adult beet leafhoppers for the presence of BCTV based on proximal remote sensing data showed: (i) about 25% false positive error for the day 0 beet leafhoppers, (ii) a gradual increase in percentage of beet leafhoppers positive for BCTV following 1–3 days of feeding on BCTV-infected plants, and (iii) a substantial reduction (*c.* 40%) in predicted BCTV-positive beet leafhoppers following 4 days of feeding on BCTV-infected sugar beet plants. To the authors' knowledge, the results presented in Figure 4 represent the first report of truly independent validation of a reflectance-based classification of insect vectors to determine their status as either nonviruliferous or viruliferous for a plant virus. That is, one dataset was used as training data to develop the classification model and another dataset was used for validation. Importantly, the training dataset was derived from data acquired from two colonies of adult beet leafhoppers reared on noninfected or BCTV-infected plants, but the age and sex of the collected beet leafhoppers were unknown. Despite important discrepancies between PCR-based and reflectance-based diagnoses, the results presented in Figure 4 are very encouraging as a proof-of-concept. To obtain a higher level of classification accuracy of the reflectance-based diagnosis, a larger dataset is needed. In addition, effects of host plant should be investigated with reflectance-based methods, as host plants may affect beet leafhopper health and longevity (Thomas & Boll, 1977). There are also indications of geographical origin (affecting life traits of both insects and host plants) and host plants may affect the consistency of proximal remote sensing data (Li *et al.*, 2017).

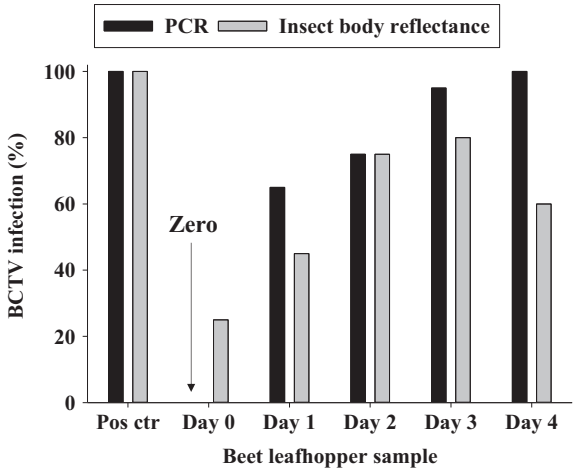

Figure 4 Analysis of adult beet leafhoppers for the presence of beet curly top virus (BCTV) based on PCR and body reflectance (proximal remote sensing). Adult beet leafhoppers from a colony reared on uninfected sugar beet plants were transferred to BCTV-infected sugar beet plants and subsamples of adult beet leafhoppers were collected daily for 0–4 days. As positive control (pos ctr), viruliferous adult specimens were collected from a colony reared on BCTV-infected sugar beet plants.

Discussion

The results presented in this study hinge upon the assumption that acquisition of plant pathogens by insect vectors cause physiological changes in insect vectors. Physiological and/or molecular data were not collected to confirm a BCTV-induced response, but this was recently demonstrated for other virus–vector systems. For example, significant and temporally regulated differences in gene expression have been shown between whiteflies (*Bemisia tabaci*) that fed on tomato plants infected with either tomato chlorosis virus (genus *Crinivirus*, family *Closteroviridae*) (Kaur *et al.*, 2017) or tomato yellow leaf curl virus (genus *Begomovirus*, family *Geminiviridae*) (Hasegawa *et al.*, 2018) and those that fed on virus-free host plants. The latter study is particularly relevant as BCTV is also a member of the *Geminiviridae* and has a similar mode of virus translocation through the insect vector. In a study on tomato spotted wilt virus (TSWV) (genus *Orthotospovirus*, family *Tospoviridae*), which replicates in its thrips vector, Zhang *et al.* (2013) compared the differences in gene expression between western flower thrips (*Frankliniella occidentalis*) reared on healthy tomato plants and viruliferous thrips reared on tomato plants infected with TSWV. Interestingly, the authors found that TSWV infection caused decreased protein synthesis and amino acid and carbohydrate metabolism in the infected insects, and that genes often associated with inhibition of virus replication were up-regulated in *F. occidentalis* upon infection (Zhang *et al.*, 2013). It has also been demonstrated, based on time series data, that insect body reflectance increased significantly in response to killing agents (entomopathogenic nematodes and an insecticidal plant

extract) at the time the killing agent is believed to induce terminal stress (Nansen *et al.*, 2015a). However, in all studies involving insect body reflectance data, direct causal relationships between physiological and/or molecular responses and insect body reflectance may be difficult to establish, due to probable complex and cascading effects.

The results presented in this study suggest, as a proof-of-concept, that proximal remote sensing technologies can be developed to diagnose whether insect vectors carry important plant pathogens. However, successful large-scale commercial applications of this technology will depend on the ability to maximize input reflectance data robustness. Low and inconsistent reflectance data robustness (Nansen, 2011) has been highlighted in several articles as a major challenge adversely affecting accuracies of reflectance-based classifications of different objects, including plants and insect specimens (Nansen *et al.*, 2008; Nansen & Elliott, 2016). However, to the authors' knowledge, this is one of the first studies in which reflectance data robustness has been examined experimentally. Due to potentially lower sample costs and shorter handling time than current diagnostic methods, it may also be possible for region-wide pest management organizations to develop comprehensive databases with high spatial and temporal resolutions, so that the spatiotemporal epidemiology of emerging disease outbreaks can be characterized and ultimately prevented.

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