Aquaphotomics: a novel diagnostic tool for biotic and abiotic stress of soybean reveals the importance of water

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Introduction

Soybean (*Glycine max* Merrill 1917) is often called the "miracle" crop, and is one of the world's leading providers of protein and oil. Soybean mosaic disease, caused by soybean mosaic virus (SMV), is considered one of the most important soybean diseases in many areas of the world.[1] SMV adversely affects both seed oil content and root nodulation, and reduces soybean yield by up to 50–100% in outbreaks.[2] Use of genetically-resistant cultivars is the most effective strategy for managing SMV but most of the commercially grown cultivars are susceptible.[3] A simple, rapid and non-destructive method for identifying SMV infected and stress-tolerant plants would be useful for soybean breeding and cultivation. Although several molecular biological methods, including enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and western blotting have been used for the diagnosis of viral infections, they demand specialised sample harvesting and preparations that involve extraction, maintenance in buffer solutions, centrifugation and purification for each assay.[4] Thus, none of them is ideal in terms of cost-effectiveness, speed, and accuracy.[5]

Use of the electromagnetic spectrum for detecting plant disease was possibly first reported by Bawden in 1933 by observing that a near infrared (NIR) film captures some virus symptoms before they appear to the human eye.[6] However, since then, most of the applications of this technology seems to have concentrated on long distance plant observation done by remote sensing.[7,8] In such long distance monitoring a variety of environmental interferences can negatively impact the detection performances. Thus, an emphasis on close-contact spectroscopic monitoring of plant leaves for finer scale information has emerged.

NIR spectroscopy has recently been used to distinguish transgenic and non-transgenic leaves and to discriminate tomato plant varieties.[9,10] The accuracy of detection is higher due to the direct contact of detectors with samples. Moreover, such methods are highly attractive to users because of the non-invasive and rapid nature of detection.

The objective of this investigation was to assess the use of a handheld NIR spectrometer for identifying SMV infected plants before the disease developed visual symptoms and for identifying stress tolerant plants.

Materials and Methods

Samples

Twenty soybean plants (well grown seedlings; Japanese Hyuga variety) were placed in different locations inside a growth chamber and maintained at 28°C. Ten plants were randomly selected and inoculated with SMV strain B eight days after seed germination as previously described by Walkey.[11] A standard double door entry procedure was followed to prevent cross-infections by insects,. Spectra from all healthy plants were taken prior to the infected plants to avoid virus transmission through probes. After completing the scan of all infected plants, the adjacent box was removed from the spectrometer and washed with 70% ethanol, then washed with running water and soap, and left to dry. Leaf samples from all plants were assayed for SMV infection two times by ELISA, as previously described by Edwards and Cooper (1985), once on the first day and again on the last day of the experiment: all inoculated plants were positive for SMV and all control plants were negative over the total period of spectral acquisition.[12]

A subsequent experiment considered five soybean cultivars with different cold stress tolerances: Kitamusume (A), Toyoharuka (B), Toyocomachi (C), Toyomusume (D), Hokkaihadaka (E). Twenty plants per group were grown in phytothron at 27°C for 2 weeks. Plants were then moved to a 22°C phytothron for in situ cold stress perturbation for one week.

An FQA-NIR Gun NIR spectrometer (Shizuoka Shibuya Seiki, Hamamatsu, Japan) was used to acquire daily transflectance spectra of SMV-inoculated and healthy plants, and to collect spectra from cold-stressed plants after one week at 22°C (588–1025 nm at 2 nm steps). Spectra were collected from five leaves of each studied plant. A soft independent modeling of class analogy (SIMCA) prediction model was constructed (Pirouette 3.11 Infometrix, Inc, WA, USA) and optimised by mean centered 2nd derivative spectra. The models were validated by an independent validation set.

Reference paper as:
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Instrument and spectra acquisition

An NIR spectrometer FQA-NIR Gun (Shizuoka Shibuya Seiki, Hamamatsu, Japan) was used to acquire the reflectance spectra from 730 nm up to 1025 nm at 2 nm steps. Studying the water-specific spectral response at the latent stage of the disease required the elimination of background noise. The rigid steel probe in the original instrument was not suitable for leaf spectra measurements and did not prevent interference from environmental light. Instead, support was added in the form of a small (0.06 m^3) rubber block with a central inner cylindrical path (0.025 m diameter) for light transmission. To keep a constant background below the lower surface of the leaves, a movable hinge-type bottom plate with a white polypropylene sheet was attached. The modified probe provided constant measuring conditions for all leaves (Figure 1). The acquired reflectance spectra (R) were converted to Log 1/R and the corresponding leaf absorbance spectra were obtained.

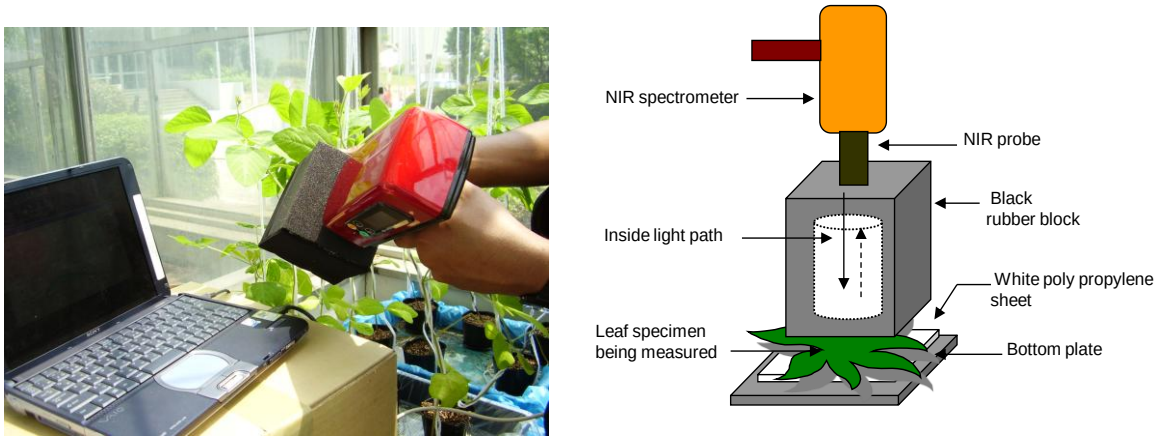

Figure 1. NIR spectrometer with modified probe for measuring leaf spectra.

Ten leaf spectra were taken per plant, from 10 fully grown leaves every other day within a 24 days period accumulating 2400 spectra from the 20 plants. The spectra taken from 14 plants (7 from each group: 10 spectra x 14 plants x 12 times = 1680 spectra) were used in the model set. The rest of the spectra from 6 plants (3 plants from each group: 10 spectra x 6 plants x 12 times = 720 spectra) were used in the prediction set for the model evaluation process.

Data analysis

Spectral data were studied by principal component analysis (PCA) to identify outliers according to the Mahalanobis distance.[13] 1652 spectra remained in the dataset, equally distributed between the two classes of infected and non-infected plants (826 each). Similarly, 708 spectra were in the prediction sets, with 354 spectra representing each class group. Predictions of plant health were made with SIMCA, in which healthy and infected plants were assigned as discriminant class 1 and 2 respectively. SIMCA models were optimised for different model specifications including number of factors, data pre-treatments and mathematical transformations. The prediction results of all combinations were used to calculate disease diagnosis sensitivity and specificity to define the accuracy of diagnosis using the following equations:

$$\text{Disease diagnosis specificity} = \frac{\text{Number of HEALTHY plants identified by NIRS}}{\text{Number of HEALTHY plants identified by ELIZA}}$$

$$\text{Disease diagnosis sensitivity} = \frac{\text{Number of INFECTED plants identified by NIRS}}{\text{Number of INFECTED plants identified by ELIZA}}$$

Results and Discussion

Raw spectra of infected and healthy plants

NIR spectra of infected plants showed higher absorbances when compared with spectra of healthy plants (Fig. 4). Plants in abnormal conditions, such as with nutrient deficiency, have been reported to absorb less light and maintain less photosynthetic activity.[14] However, similar to our findings, bacteria-infected cabbage and virus-infected *Nicotiyana spp*., were reported to increase their light absorbance, probably due to leaf moisture variations in damaged plant cells.[15,16] Hence, increased water content increased light absorption. In our study, both infected and non-infected plants showed their highest light absorbance around 680 nm (Fig. 2) which

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corresponds to chlorophyll. However, studies have shown that this spectral region provides unreliable information as it gives similar responses for different conditions, such as herbicide, heat stress, nitrogen and potassium deficiencies.[17,18] As such, we focused on the short wave NIR (SWNIR; 730 nm-1025 nm) region to seek information specific to SMV.

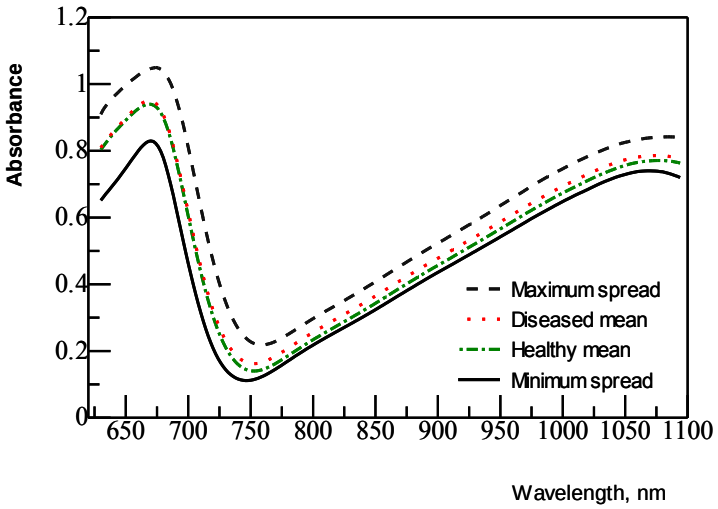

Figure 2. Spread of raw spectra and average category of healthy and infected plant leaves.

The prediction performance of SIMCA

The first models of SIMCA based on different pre-treatments indicated that mean centering produced the most accurate models. Thus, further data analyses were done with mean centered spectral transformation data. Table 1 shows the necessity of 1st and 2nd derivatives and the baseline correction when compared to the other options. Using a mean centering and 2nd derivative mathematical transformation, 303 healthy plant spectra of a total number of 354 were correctly identified (85.6% accuracy for disease specificity; Table 1). 299 infected plants were correctly identified (84.4% accuracy for disease sensitivity; Table 1). These results are consistent with several other studies in which canopy structural and background effects in plant leaf spectra were overcome using second derivative transformation.[18,19]

Table 1. Results of different mathematical treatments for primary spectra models.

Math treatment	Category	Total spectra	Correct classifications	Error classification	No match	Specificity	Sensitivity
1st derivative	Healthy	354	304	49	1	85.5	
	Diseased	354	298	47	9		84.1
2nd derivative	Healthy	354	303	51	0	85.6	
	Diseased	354	299	47	8		84.4
Baseline correct	Healthy	354	286	67	1	80.8	
	Diseased	354	282	62	10		79.7
Smoothing (point5)	Healthy	354	297	57	0	83.8	
	Diseased	354	261	80	13		73.7
Normalize	Healthy	354	305	48	0	86.1	
	Diseased	354	275	70	9		77.6
MSC	Healthy	354	297	57	0	83.8	
	Diseased	354	270	66	18		76.2
Non	Healthy	354	297	57	0	83.8	
	Diseased	354	277	61	16		78.2

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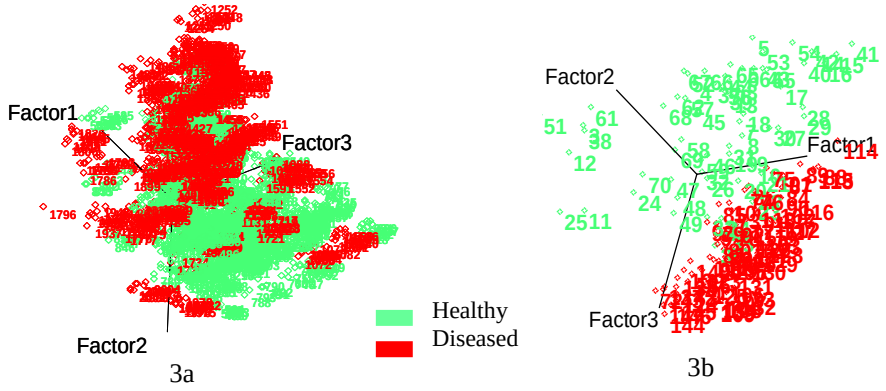

Figure 3. Unaveraged (3a) and averaged (3b) spectra in 3D principal component space.

Using average spectra in the models increased the prediction of disease sensitivity by 8.53% (from 84.4% to 91.6%) and disease specificity by 11.91% (85.6% to 95.8%; Table 2). Moreover, we did not have any classification error in the average spectra models, although there were several "no match" classifications. The suspected reason is in the limited number of samples available in the multi-dimensional principal component space after averaging, as shown in the score plot diagrams (Fig. 3). Such shortage of spectra could be easily overcome by increasing the number of data samples. These results show the benefit of taking many samples of spectra from leaves of a single plant and calculating an average to use in models.

The positive results from in vivo diagnosis of SMV indicated the possibility of studying cultivar genetic potential.[20] Here, leaf spectra were acquired before imposing cold stress (i.e. continuously grown at 27 °C) and after imposing cold stress (fixed low temperature stress at 22 °C) to identify cold tolerant cultivars.

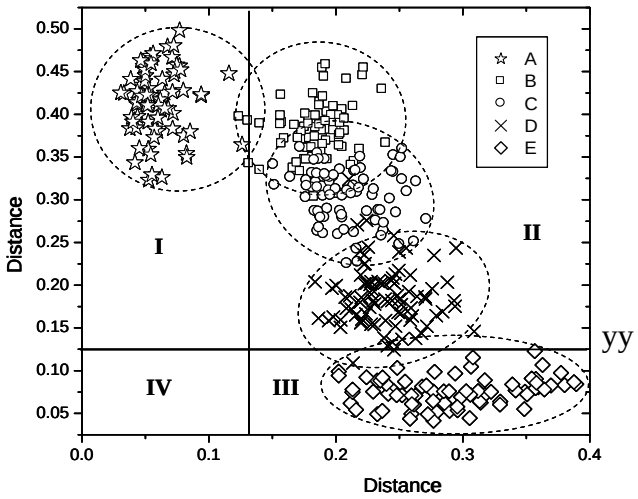

Figure 4. Potential of cold tolerance prediction based on Coomans plot configurations.

Cultivars were well-predicted (> 92%) while configuring their order of cold tolerance in the model. It was found that lowering temperature by only 5°C activated cold protection mechanisms in the plants and provided enough spectral information at NIR water specific bands for successful discrimination of cultivar cold tolerance. Moreover, an excellent arrangement of soybean cultivars in their order of cold tolerance was observed in the Coomans plot space after cold stress perturbation; this can be used as decision making diagram to identify the relative level of cold tolerance in any new variety of interest.

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Conclusions

Near infrared spectroscopy with a specific focus on water bands (i.e. aquaphotomics) was applied as a rapid, non-invasive and reagent-free method for understanding biotic and abiotic stress of soybean (*Glycine max* Merrill 1917). Infection and cold tolerance in soybean cultivars were successfully identified. These results proved that plant reactions towards biotic and abiotic stress regarded as polygenic and multi-factorial syndromes are strongly correlated with plant water activity dynamics.

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