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Research Paper

Diagnosis of freezing stress in wheat seedlings using hyperspectral imaging

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Low-temperature freezing stress of wheat seedlings adversely influences growth and the final yield of wheat. Imaging spectrometry and image integration were used to study the extent of freezing injury on wheat seedlings on three dates in 2010. Thirty wheat cultivars with limited freezing resistance that are usually grown in Southern China were grown in pots located outdoors in Beijing. Imaging spectra of potted wheat samples were acquired in the spectral range of 450–900 nm, and a polyphenol tester was used to determine the nitrogen balance index of wheat samples. Increased freezing injury was related to increased spectral reflectance in the 450–650 nm wave band and decreased spectral reflectance in the 700–900 nm wave band. Average spectral reflectance of wheat seedling canopies was negatively correlated (−0.7) with nitrogen balance index in the red edge area between 650 nm and 700 nm. Absolute values of correlation coefficients under freezing stress at three measuring dates reached a maximum at 680 nm, and this wavelength was used as the characteristic wavelength for freezing-injury diagnosis of the wheat seedlings. From spectral images at this characteristic wavelength, it was feasible to intuitively observe the area and extent of freezing-injured wheat seedlings. Our results show that it is feasible to monitor freezing stress of wheat seedlings by use of hyperspectral imaging which could accurately reflect freezing-injured parts of wheat seedlings.

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1. Introduction

High and dependable wheat yields result from a combination of cultivar, environment, and cultivation technology. Low-temperature freezing injuries are one of the major natural problems influencing wheat growth (Sofalian, Mohammadi, Aharizad, Moghaddam, & Shakiba, 2006). Freezing injury occurs particularly in the seedling stage and wheat crops have suffered from serious freezing injury in the USA (Skinner & Mackey, 2009), Canada (Nemetha, Paulleyb, & Preston, 1996), and Russia (Kolesnichenko et al., 2003), causing great loss of wheat production to these countries. Low-temperature freezing injury is also a common problem for wheat in Northern China. Recently, wheat freezing injury has occurred, to different extents, almost yearly with increased areas of high-quality wheat affected each year. This is likely because existing high-quality wheat cultivars are spring or late-spring cultivars and/or they are southern wheat cultivars. Freezing

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Nomenclature

SOD	superoxide dismutase
qP	fluorescent photochemical quenching coefficient
qN	non-photochemical quenching coefficient
NOAA	National Oceanic and Atmospheric Administration
PIS	pushbroom imaging spectrometer
DN	digital number
CCD	charge-coupled device
BMP	bitmap
BIL	band interleaved by line
ENVI	the environment for visualizing images
ROIs	the regions of interest
ASD	analytical spectral devices

injury can also be increased by selection of an unfavourable seeding time, pour seeding quantity control, and inadequate soil preparation.

Slight freezing injury results in different degrees of yield reduction, but serious freezing injury causes low yield and significant loss in agricultural production (Bjarko & Line, 1988). Gao, Wang, Liu, Ji, and Du (2006) investigated physiological changes of maize seedlings under low-temperature stress. Their results showed that variations of physiological indicators in maize seedlings, such as malonaldehyde, proline, and soluble sugar content, could accurately reflect the extent of low-temperature injury to maize seedlings. Ye, Jin, Qin, and Song (2009) used radish, maize, and wheat seedlings as experimental materials to study the influence of low-temperature stress on superoxide dismutase (SOD) activity. They controlled temperature stress and stress time variations by use of a freezing injury simulation method, and determined SOD activity by means of nitroblue tetrazolium autoxidation method. Their results showed that appropriate low-temperatures could increase SOD activity. At temperatures below the range of plant tolerance, SOD activity was reduced due to inhibition. These methods identified the influence of low-temperature stress on crops by judging variations of chemical elements in seedlings. However, these methods were time-consuming and strenuous and this hindered their promotion and application. Therefore, it is important to develop a quick and non-destructive method to diagnose low-temperature stress in wheat.

Optical methods have advantages in fast and non-destructive detection. Therefore, many researchers have tried various optical sensors to evaluate crop growing status. Thenkabail and Smith (2000) determined spectral bands that were best suited for characterising biophysical variables of agricultural crops. Their data came from ground-level hyperspectral reflectance measurements of cotton, potato, soybeans, maize, and sunflower. Reflectance was measured in 490 discrete narrow bands between 350 nm and 1050 nm. Malthus and Maderia (1993) used high resolution reflectance spectra from field bean leaves to diagnose the *Botrytis fabae* infection. As for the freezing injury detection, Li, Chen, Yang, and Zhang (2006) carried out preliminary investigation on chlorophyll fluorescence characteristics of cotton seedlings under low-temperature stress. Their results showed that low-temperature stress reduced the fluorescent photochemical quenching coefficient (qP) and increased the non-photochemical quenching coefficient (qN) of cotton seedlings. For the large scale measurement of freezing injury, remote sensing has shown to have potential for measurement of freezing injury. Tang and Sun (1989) synthesised a greenness image by use of US National Oceanic and Atmospheric Administration (NOAA) spectral data with lower spatial resolution. They also evaluated freezing injuries in winter wheat in Jiangsu province, China in 1978 by comparing greenness differences at different times. Yang, Wang, and Pei (2002) conducted remote sensing monitoring and research for late-spring freezing injury of winter wheat in Shandong province, China in 1995 by use of vegetation index of NOAA data plus meteorological data. Remote sensing monitoring has also been used for assessing freezing injury in spring wheat and summer maize (Zhang, Chen, Su, & Zhou, 2001) and winter wheat (Zhang et al., 2006) in Ningxia, China. However, these studies used low spectral and spatial resolution remote sensing data, thus the monitoring effectiveness was not very satisfactory.

Hyperspectral imaging has often been used for monitoring the quality of food products (Kim, Chen, & Mehl, 2001), such as tomato (Polder, van der Heijden, van der Voet, & Young, 2004), apple (Mehl, Chen, Kim, & Chan, 2004), and pork (Qiao, Ngadi, Wang, Gariépy, & Prasher, 2007). Some of the major challenges in hyperspectral imaging-based plant disease detection are the selection of a disease-specific spectral band and selection of a statistical classification algorithm for a particular application, which depends on the data acquisition setup under field conditions. Bravo, Moshou, West, McCartney, and Ramon (2003) investigated the application of visible-NIR hyperspectral imaging for the early detection of yellow rust disease (*Puccinia striiformis*) in winter wheat.

Freezing-injury diagnosis is important for crop management. Therefore, we investigated monitoring the freezing injury of wheat seedlings under low-temperature stress using hyperspectral imaging technology and high resolution image integration.

2. Materials and methods

2.1. Experimental design

Our field study was conducted within Beijing Academy of Agriculture and Forestry (North latitude 40.17°, east longitude 116.433°) from November, 2010 to March, 2011. Thirty cultivars of Yangzhou southern winter wheat with low cold resistance, such as Yang 11, Yang13, Yang 15, and 18 cultivars of northern winter wheat with medium or high cold resistance such as Jingdong 12, Jing 411, Yannong 19, were provided by Xiahe Agriculture Science Institute in Jiangsu province. Seeds were planted in outdoor pots on October 5, 2010. Imaging spectra of the wheat seedling canopy were acquired on one to two week intervals from seedling emergence to the occurrence of freezing injury.

2.2. *Instrument introduction*

A second generation Pushbroom Imaging Spectrometer (PIS) was used for measurement of spectral images. The PIS was jointly developed by Beijing Agricultural Information Technology Research Center and the University of Science and Technology of China. Its purpose was to closely detect spectral information of individual crops and crop sections to facilitate ground-based quantitative agricultural remote sensing research. The complete hyperspectral imaging system includes a spectrometer, motor, guide rail, carriage, and an external notebook PC (Fig. 1). Before use, the spectrometer was calibrated indoors by Anhui Institute of Optics and Fine Mechanics, Chinese Academy of Sciences. An integrating sphere source was used as the radiation source; the radiation intensity was changed by opening different numbers of lamps of the integrating sphere (1, 2, 4, and 8 lamps). The spectral response was calculated as the ratio between the Digital Number (DN) values measured by the image spectrometer and the brightness value of the integrating sphere source at each wavelength. When using the imaging spectrometer in the field, the exposure time and aperture were changed to make the ambient light be close to the radiation intensity of the integrating sphere with 8 lamps open. In order to further decrease the influence of radiation calibration accuracy, the imaging spectrometer only recorded DN values in the field experiment, spectral relative reflectivity was used for calculation and the absolute radiation intensity was not used. The CCD camera used in this study had a wide operation range of 0–50 °C; therefore, the effects of temperature on the sensitivity of the camera could be ignored. The key performance parameters of the spectrometer are shown in Table 1.

2.3. *Acquiring and processing of imaging spectra of wheat seedlings under freezing injury*

Imaging spectra from wheat seedling canopies were acquired from 9:30 A.M. to 15:00 P.M. on sunny calm days. In order to eliminate the influence of the different colour temperatures of sunlight during the study, the spectra values (DN values) of a standard $BaSO_4$ board were recorded simultaneously, and relative reflectivity was used to construct the prediction model. Measurements were primarily conducted on the 30 southern wheat cultivars. The vertical height from samples to the spectrometer lens was 1130 mm. Motor speed was 20 mm s^{-1}. After setting various parameters of the acquiring software, the integration time of the imaging spectrometer was set to 113 ms, and the frame frequency was 8 frame s^{-1}. The PIS was used to acquire spectral images until all the southern wheat seedlings died due to freezing injury. Seedling death was confirmed in the spring season of the following year, noting that the wheat was blasted. Four observational periods, were selected for this experiment according to the physiological stages of freezing injury, namely, 3 occasions before the traditional Chinese Spring Festival (February 2nd, 2011) and once after Spring Festival. Temperatures in China around the Spring Festival in 2011 were very low. Since all the wheat died after the Spring Festival, and only soil and dead seedling tissue remained in the pots, only three of the planned four spectral images of wheat seedlings were acquired. Thus, the quantity of acquired samples in each test was 30.

Table 1 – Key performance parameters of spectrometer.

Parameters	Value
Spectrum range	400 ~ 1000 nm
Spectrum resolution	3 nm
Spatial resolution	≧0.5 mm
Sampling interval	0.7 nm
Pixel dimension	7.4 μm × 7.4 μm
Field of view	24°
Image resolution	1400 (Spatial) × 1024 (Spectrum)

Fig. 1 – Pushbroom imaging spectrometer.

Since the raw spectral data were in Bitmap (BMP) format, it was spliced into Band Interleaved by Line (BIL) format before use. The process used was: (1) the BMP format images were spliced into a number of BIL format images using self-developed programs in MATLAB 2009a software (The Math Works Inc., MA, USA); (2) spectra were extracted from the original spectral images using remote sensing image processing software EVNI 4.4 (Exelis Visual Information Solutions, Inc. Pearl East Circle Boulder, CO, USA). The spectra of the leaf base, middle leaf, and tip of leaves were extracted, and averaged, as the original spectral values of the target leaves. The regions of the leaves were selected by visual inspection.

The hyperspectral reflectance values for the canopy, single plant, and single leaf were obtained from:

$$Ref_{object} = Rad_{object}/Rad_{whiteboard} \times Ref_{whiteboard} \times 100\% \quad (1)$$

where, Ref_{object} represents the spectral intensity data of the object obtained by whiteboard reflectance; Rad_{object} represents the radiance of object measured by spectrometer; $Rad_{whiteboard}$ represents the radiance of the whiteboard measured by spectrometer; $Ref_{whiteboard}$ represents the known reflectance ratio of whiteboard.

2.4. Determination of nitrogen balance index

A plant polyphenols tester Multiplex 3 (Force-A Company, Orsay Cedex, France) was used to synchronously determine nitrogen balance index of wheat canopies at three measurement dates. The Multiplex 3 is a hand-held, non-contact and multi-parameter instrument for measuring the fluorescence of leaves, and it can provide quick and easy measurements in the field. Since Multiplex 3 had a self-contained light source as the UV excitation source, it was not influenced by ambient light. Consequently, the instrument did not need to be calibrated in the field. It has an option to restore factory settings. The LCD touch screen provided an interface for users and displayed real-time data (Fig. 2).

The Multiplex 3 provided accurate and comprehensive data on crop physiological status. The equation for determining the nitrogen balance index is:

$$NBI\_G = FRF\_UV/RF\_G \quad (2)$$

where, NBI_G represents the nitrogen balance index; FRF_UV represents Far-Red fluorescence excited by ultra violet; RF_G represents red fluorescence excited by green light. NBI_G ratios depend both on epidermal phenolic compounds and chlorophyll. Equation (2) is based on the work on wheat (Cartelat, Cerovic, Goulas, Meyer, Lelarge, & Prioul, 2005) and several ligneous species (Demotes-Mainard, Boumaza, Meyer, & Cerovic, 2008; Meyer, Cerovic, Goulas, Montpied, Mainard, & Bidel, 2006). NBI has been shown to respond to nitrogen nutrition of plants and can be used directly in comparison studies, provided illumination and the developmental stage are kept constant among samples.

Fig. 2 – Plant polyphenols tester Multiplex 3.

3. Results and analysis

3.1. Images and spectral reflectance curve of wheat seedling canopy before and after freezing injury

Fig. 3 shows the images of southern Yangfu wheat 7091 in the different physiological periods from the occurrence of freezing injury to death. It was found that the four physiological periods for wheat seedlings were between December 8, 2010, December 28, 2010, January 17, 2011 and March 29, 2011 respectively (shown in Fig. 3). They were, respectively, the initial stage of wheat seedling freezing stress, serious stage of wheat seedlings freezing injury, and death stage of wheat seedling freezing injury. Imaging spectrum data were acquired during these periods. All 30 Yangzhou wheat cultivars (Yangfu wheat 7091 is representative) died, but the freezing injury of 18 northern wheat seedlings used as control was mild with 90% of cultivars growing well in next-year reviving period and all surviving.

The spectra from the wheat seedling canopies under freezing stress were acquired within the wavelength range of 400–1000 nm, and then spectral characteristics were analysed. The spectral acquisition process is shown in Fig. 4. The ENVI software could automatically calculate the average spectra by selecting the wheat area in the flowerpot (by red signs), as shown in the right part of Fig. 4. Specifically, the regions of interest (ROIs) in the specific area of the image were defined according to human opinion or created automatically with a threshold value. The ENVI software then extracted the spectra of each pixel in the ROIs, and calculated their mean spectra.

After acquiring the average spectral intensity of wheat, it was compared with the spectral intensity of whiteboard for calculating the relative reflection spectrum. On the three observation dates comparative analysis was conducted to acquire average spectral values for the 30 southern wheat cultivars under freezing stress (Fig. 5). In the spectral range of 450–900 nm, a reflection absorption peak occurred at 550 nm, and reflection absorption valley occurred at 680 nm, this may be due to the absorption of chlorophyll. At wavelengths greater than 750 nm, there was a high reflection platform. We found that the spectral reflectance curves for distinct characteristics of plants were not high in the spectral range of 490–600 nm. A strong reflection peak for chlorophyll occurred close to 550 nm. Chlorophyll had strong absorption in the spectral range of 600–700 nm. Therefore, most plants had minimum reflectance around 680 nm or 670 nm. In the spectral range of 700–750 nm, the curves were steep and almost linear, which is typical of vegetation (Xue, Luo, Cao, & Tian, 2003). The average spectra of wheat in the visible range of 450–600 nm on the three measuring dates (December 8, 2010; December 28, 2010; January 17, 2011) were analysed. At a wavelength of 550 nm, reflectance of acquired averages spectral curves on the three measuring dates was reduced with the exacerbation of freezing injury. The average spectral

Fig. 3 – Images of Yangzhou southern wheat seedlings suffering from freezing injury in different physiological periods.

reflectance acquired on January 17, 2011 was the highest and that acquired on December 8, 2010 was the lowest. In the near infrared region of 700–900 nm, reflectance in a red edge region of 680–750 nm rose quickly and reached about 0.60, and then reflectance remained stable. The average spectral reflectance curves for the three measuring dates were different to those in the visible region. The spectral reflectance of seeding wheat samples acquired on December 8, 2010 was higher than that of the other two measuring dates, and spectral reflectance of seeding wheat samples acquired on January 17, 2011 was lowest, which is in line with the results of Li, Zhou, Lu, Lin, and Li (2008) who investigated the hyperspectral characteristics of winter wheat after freezing injury at the jointing stage. Variations in the spectral curves on the three measuring dates possibly lay in the decrease of chlorophyll activity due to freezing stress which caused blue green leaves and shrivelled leaves that presented high reflectance. The freezing injury of wheat seedlings acquired on January 17, 2011 was very serious and most leaves were withered. Therefore, spectral reflectance in the visible region was lower than previously reported for wheat seedlings. Our results demonstrate that the PIS imaging spectrometer was suitable for these types of measurements in that it could acquire spectral characteristics and integrate images and spectra to reflect the growth status of wheat seedlings under freezing stress.

3.2. *Variations of nitrogen balance index of wheat seedlings under freezing injury*

It was found from comparative analysis that with exacerbation of freezing stress of wheat seedlings, average nitrogen balance index values of seedlings acquired on the three measuring dates (December 8, 2010, December 28, 2010 and January 17, 2011) were respectively 3.02, 3.51 and 3.58, and curve variations were in a rising trend. When the wheat seedlings suffered from freezing injury from December 8, 2010 to December 28, 2010, the nitrogen balance index curve rose greatly. With increasing freezing injury the trend decreased. Meyer et al. (2006) showed that polyphenol content is significantly correlated with the concentration of leaf nitrogen. When plant leaves are under freezing stress, particularly when a reduction in nutrient elements limits plant growth, polyphenol content rises significantly (Poutaraud et al., 2007). Consequently, it was possible to evaluate variations of wheat seedlings under freezing stress by use of polyphenol content. Generally, polyphenol content was negatively correlated with nitrogen content (Kandil, Grace, Seigler, & Cheeseman, 2004; Peckol, Yates, & DeMeo-Anderson, 1992). The Multiplex 3 polyphenols tester calculated the nitrogen balance index only according to polyphenol content.

3.3. *Correlation between spectral reflectance of wheat seedling canopy before and after freezing injury and nitrogen balance index*

In this study, average spectral reflectance of seedling canopies of 30 of wheat samples were acquired respectively on three measuring dates (December 8, 2010, December 28, 2010 and January 17, 2011). Correlation analyses conducted between average spectral reflectance and synchronously-determined nitrogen balance index values in the spectral range of 450–900 nm, and their correlation coefficients are shown in

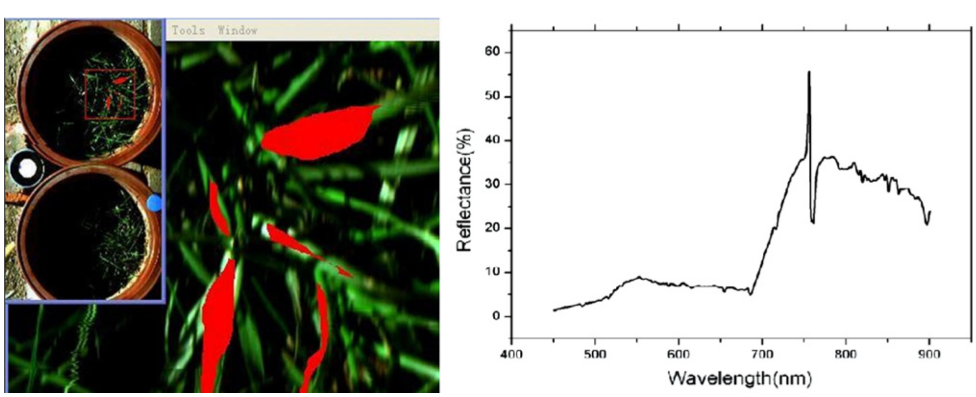

Fig. 4 – Schematic diagram of acquiring spectral reflectance of wheat canopy.

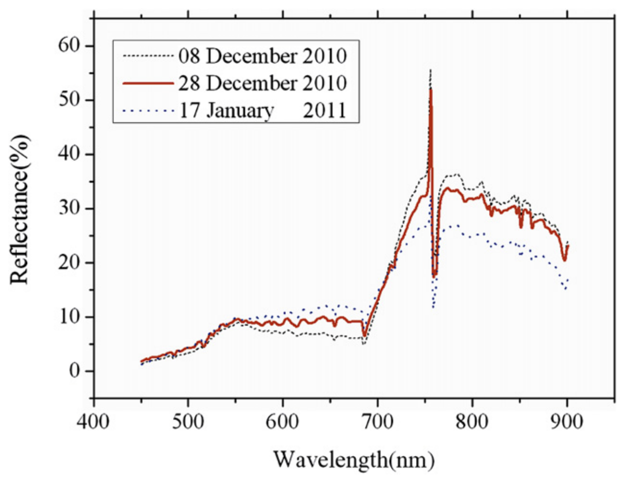

Fig. 5 – Average spectra of 30 wheat samples under freezing stress in different periods.

Fig. 6. It was found that under freezing stress, the average spectral reflectance was significantly positively associated with nitrogen balance index in spectral range of 700–900 nm, except for our data acquired on December 8, 2010 and that average spectral reflectance data of wheat seedling canopy acquired on December 28, 2010 and January 17, 2011 were significantly negatively associated with nitrogen balance index values in red edge regions, obviously presenting a blue shift after environmental stress. This was in line with research results of Huang et al. (2004) and Pu and Gong (2000), pp. 89–90. The largest negative correlation coefficient was about 0.7. The wavelength of maximum correlation coefficient was selected as the characteristic wavelength. Results showed that spectral characteristic wavelengths of wheat canopy acquired in the three measuring dates were in the red edge region between 650 nm and 700 nm, and the maximum was about 680 nm.

3.4. *Image characteristics of wheat seedling canopy before and after freezing injury*

In ENVI software, images at three original colour wavelengths of R, G and B (680 nm, 550 nm and 450 nm) were extracted to

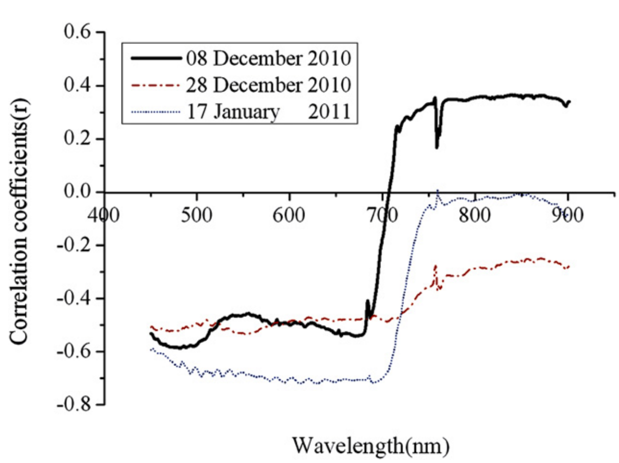

Fig. 6 – Correlation between spectral reflectance of wheat canopy and nitrogen balance index.

obtain RGB images. From the acquired RGB images, it was possible to conduct colour analysis of canopy seedlings. The leaves of wheat seedlings changed from normal green into dark green, and finally faded and died due to freezing injury, respectively, on three measuring dates December 8, 2010, December 28, 2010 and January 17, 2011. It was found from acquired images at characteristic wavelength of 680 nm that as freezing injury increased the leaves changed from wide, thick, dense shapes into slim, long and sparse shapes, and finally curled, faded and died. Thus, the freezing-injured parts of leaves and relative injured parts could be identified. Therefore, it was feasible to intuitively analyse the freezing injury of wheat by means of hyperspectral imaging technology.

4. Discussions

Previous researchers have conducted a number of investigations on freezing stress of crops by use of an Analytical Spectral Devices (ASD) Spectrometer. Li et al. (2008a) used a frost box to simulate freezing injury and used a hyperspectral spectrometer to determine the chlorophyll content of leaves of winter wheat grown in pots and the hyperspectral reflectance curves of a wheat canopy. Differences between different regions or different time phases were compared to evaluate differences in freezing injury of crops. Information on differences between canopy spectra of winter wheat suffering from late-spring freezing injury at jointing stage and normal spectra was acquired to provide appropriate evaluation indicators for remote sensing identification of freezing injury and judging freezing injury extent. Li et al. (2008b) used hyperspectral reflectance to study spectral characteristics of cotton seedlings. The results showed that the chlorophyll content, photosynthetic rate, transpiration rate of freezing-injured cotton leaves were significantly lower than that of control samples which did not suffer from freezing injury. It was established that Log(1/R) values of single wave band at 638 nm, 682 nm, 720 nm and 768 nm could be used as the sensitive wavelength indicators for freezing injury. Li et al. expected that the hyperspectral vegetation index could be used to differentiate the extent of freezing injury. However, the chlorophyll content and the various spectral characteristic value differences between different freezing injury treatments had no significant difference, suggesting that it is difficult to quantitatively estimate freezing injury extent in cotton leaves. At present, seedling monitoring studies primarily use a non-imaging Pushbroom spectrometer. Generally, the dis-advantages of traditional non-imaging spectroscopy are: (1) its acquired spectral information of wheat canopy was mixed, and the acquired data were affected by soil background; (2) traditional spectrometer combined with fibre provide 'point' data, therefore we have to measure several points if we would like to analyse information from seedlings in different local area.

The effects of outdoor freezing stress on 30 potted southern wheat seedlings were analysed by use of imaging spectral data and integration of the image and spectrum of hyperspectral imaging to identify the location of injury and the extent of freezing stress on wheat. Preliminary results showed that it is feasible to monitor freezing stress of wheat seedlings by use of hyperspectral imaging. Future work could be implemented in the following aspects: (1) improvement of the experimental protocol. This experiment was a test of freezing stress on plants grown in pots, and measurement results could likely be different from a large-field situation. We believe that a real-time site measurement study in a large field is warranted; (2) in this study we only conducted a qualitative analysis on extent of freezing injury of wheat on different measuring dates. Future studies should include a quantitative analysis on freezing injury extent and/or a comparative analysis on cold resistance of different cultivars of wheat.

5. Conclusions

Imaging spectrum and nitrogen balance index data of wheat seedlings under freezing stress in three measuring dates (December 8, 2010, December 28, 2010 and January 17, 2011) were acquired to analyse variations of average spectral reflectance curve of wheat seedlings under freezing stress. After correlation analysis between the average spectral reflectance and the nitrogen balance index was completed, it was concluded that wheat seedlings suffering from the most freezing injury, expressed the highest spectral reflectance in visible region and to the contrary in the near infrared region. By intuitively identifying the extent and location of freezing injury, and by extracting images under the characteristic spectrum, it is suggested that it was feasible to monitor the freezing stress status of wheat seedlings by means of hyperspectral imaging technology which could better reflect the growth of wheat seedlings under freezing stress.

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