

Research Article

Potential of spectroscopy for differentiation between PVY infected and healthy potato plants

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Abstract: Spectroscopy in visible and part of near infrared region was assessed as a non-destructive technique for the detection of plants infected with Potato virus Y (PVY). The aim of our research was to recognize spectral signatures that indicate PVY infected plants. In this assay, we studied spectral reflectance of potato leaves showing different PVY symptoms in cultivars Agria and Milva. Virus titer of leaves that showed different disease symptoms, were estimated using enzyme-linked immune sorbent assay. The means of spectral data obtained from different leaves in each experimental plant were used for spectral analysis. Analyses showed that spectral region in 900-1100nm was markedly sensitive to the PVY infection and could be useful for developing a good spectral signature for detection of the infection. Based on the X loading weights obtained from principal component analysis (PCA), sensitive wavelengths were screened, some wavelengths in this region have most positive or negative loading and based on linear discriminant analysis, they could discriminate infection status with high accuracy. The reflectance variation in this region is related to changes in cell structure and water activity due to viral infection. Results indicate that spectroscopy has a suitable potential to detect virus-infected plants; which could be further developed for more accurate potato field inspection aimed at controlling the spread of viral infection.

Keywords: detection, non-destructive, PVY, spectra, virus

Introduction

Remote sensing as a rapid, non-destructive, and inexpensive method could be used for detection of biotic and abiotic stresses in the visible and near-infrared (Vis/NIR) regions (Sirisomboon *et al.*, 2009). The reflectance of light from plant depends on the biophysical and biochemical characteristics such as leaf pigment content in the visible region and leaf structure and water content in NIR region (Mahlein *et al.*, 2016)

that could be altered by biotic stresses such as viral contaminations.

Potato Virus Y (PVY) is an important virus in the family Potyviridae that has a wide range of hosts including Solanaceae family such as potato (Kerlan, 2008). Limiting the initial inoculum is one of the efficient management strategies for controlling PVY infection. The vectors (Aphids) spread the virus from plants emerging from the infected tubers to healthy plants and develop the infection of PVY in that season and the next generations. Seed certification programs through using virus-free seed tubers efficiently control the development of the disease (Karasev and Gray, 2013). Seed growers must produce seed with low PVY infection levels to meet state standards for

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commercial seed (Strand, 2006). Tuber-borne diseases such as PVY require special attention in field inspection steps. Visual identification of PVY in different potato cultivars is difficult. Mildly symptomatic recombinant PVY strains or asymptomatic infections and late season asymptomatic infections could misdirect the inspectors and contribute to their inability to accurately detect the PVY infection (Hamm *et al.*, 2010). Development of new methods for fast, real-time, and accurate detection of disease in the field facilitates management decisions. The spectroscopic techniques have efficient potential in detection of various infected plants (Jinendra *et al.*, 2010; Naidu *et al.*, 2009; Martinelli *et al.*, 2014). Morphological, physiological, and phytochemical changes can lead to an alteration of normal spectral behavior of plants (Krezhova *et al.*, 2014; Kogovšek and Ravnikar 2013). Symptoms due to the virus infection can be explained by destruction of leaf structure and alteration of chemical composition so that the plant spectral characteristics in infected leaves can develop the specific signature that can be used for diagnosis of the disease (Naidu *et al.*, 2009). In this study, we investigated the spectral reflectance data obtained from PVY infected and healthy potato plants (Agria and Milva cultivars) for the development of a suitable spectral detector for rapid and non-destructive PVY detection system.

Materials and Methods

Plant material

Two different potato *Solanum tuberosum* L. cultivars (Agria and Milva) obtained from seed and plant certification and registration institute were grown in pots containing a mixture of field soil, peat moss and perlite (50: 25: 25) under the greenhouse controlled condition at 20-25 °C and photoperiod of 16: 8 (L: D). The healthy plants with eight expanded leaves were inoculated with potato virus Y via mechanical inoculation. Before inoculation, leaves of experimental plants were dusted with carborundum and then sap of infected plant in extraction buffer was rubbed into the leaves (Piche *et al.*, 2004). Three plants in each cultivar without any viral infection

(tested by RT-PCR) were treated with buffer and used as negative control. The severity and kind of disease symptoms were recorded at 7 and 14 days after inoculation. Experiments were repeated three times.

Verification of PVY infection using RT-PCR

The infection of experimental plants was confirmed by RT-PCR techniques, 14 days after inoculation. Total RNA was extracted from inoculated and control plants using the RNX-Plus Kit (Sinajen, Iran). Reverse transcription was performed using HyperScriptTM Master Mix (GeneAll, Korea) according to the manufacturer's instructions. For RT-PCR reaction in 25µl volume, 12.5µl Taq 2x Master Mix Red (Ampliqon, Denmark), 1µM of each primer (forward and reverse), 2µl cDNA and 8.5µl distilled water were used in each reaction. The primers used in the RT-PCR assays were PVYF (5' ATACTCGRGCAACTCAATCACA 3') and PVYR (5' CCATCCATCATAACCCAAACTC 3') (Du *et al.*, 2006). The thermal cycling conditions were: 94 °C for 5 min, 35 cycles of 94 °C for 1min, 56 °C for 1 min, 72 °C for 1min and a final extension at 72 °C for 10min. An amount of 7µl of PCR products were analyzed by electrophoresis on 1% agarose gel.

Determination of virus concentration using TAS-ELISA

PVY concentration in apical leaves and leaves above and below inoculation site showing different severity of symptoms in each experimental plant were determined using TAS-ELISA (Clark and Adams, 1977). ELISA absorption values were measured at 405nm on an automated ELISA plate reader and then values were used for comparing virus concentration in leaves with different situation in each experimental plant.

Leaf reflectance measurements from PVY infected and non-infected plants

Reflectance spectra of infected and non-infected plants (36 leaf samples) were acquired at 200 to 1100nm by the AvaSpec-3648 Fiber Optic Spectrometers (Avantes, Netherlands)

with 0.75nm resolution and 161.79 ms integration time. Combined deuterium and halogen light source and leaf probe attachment device were used for measurements. The spectrometer was calibrated by AvaLight-DH-CAL. The average number of measurements was about 25 per sample. Three leaves from each plant were used for spectral analysis. The average reflectance of each plant in three replicates were considered for spectral analysis.

Analysis of spectral data

Primary spectral data and pretreated data by normalization, baseline correction, first and second derivatives of the Savitzky Golay method and full multiplicative scatter correction (MSC) were analyzed using the Unscrambler 10.5. For interpreting the data in a more meaningful form, principal component analysis was used to reduce the number of variables, and to obtain interpretable linear combinations of the data. Linear discriminant analysis also was used to classify the groups and discriminant infected from healthy plants (Sirisomboon *et al.*, 2009).

Our study was conducted to determine special waveband to discriminate infection status.

Results and Discussion

Symptom development

PVY symptoms appeared as light mosaic patterns, vein necrosis and crinkling on Agria leaves two weeks after inoculation. But on Milva, symptoms appeared as severe mosaic and necrotic line patterns on veins (Fig 1 and Table 1). Potato cultivars may react differently to PVY infection and each cultivar exhibits distinct symptoms (Shrestha *et al.*, 2014; Draper *et al.*, 2002) which further complicates detection of infection in field inspections. On the other hand, mosaic symptoms varied from severe in apical leaves to mild and absent, respectively in upper and lower sites of inoculated leaves, 14 days after inoculation. Necrotic line patterns appeared on upper leaves earlier than lower and apical leaves. Generally, virus symptoms developed faster on upper leaves than lower leaves. On non-inoculated plants, no symptoms of virus infection were observed.

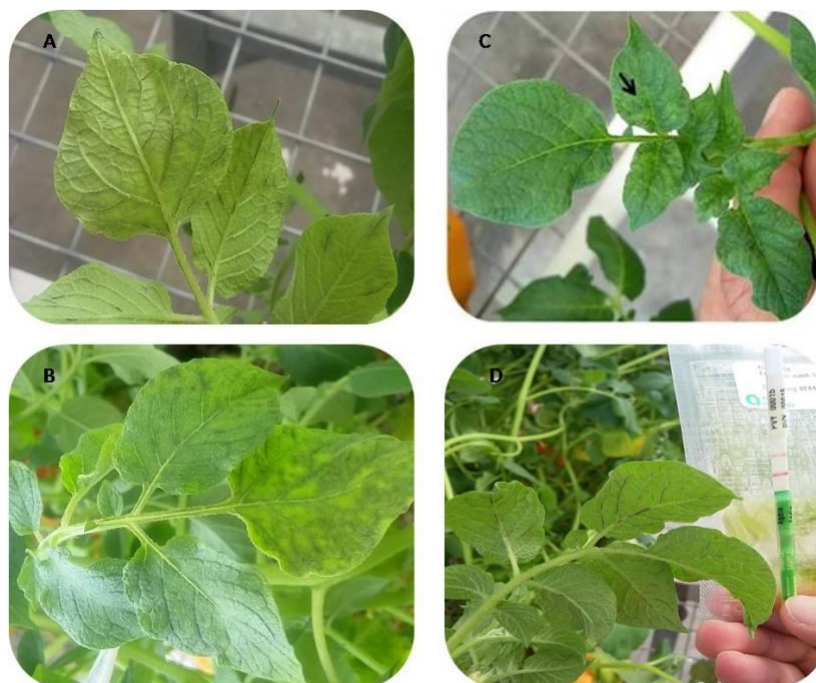


Figure 1 Different disease symptoms of PVY infection on potato cultivars. A, B: symptoms on Milva with severe mosaic and necrotic line patterns on veins and C, D: symptoms on Agria with light mosaic patterns, vein necrosis and crinkling.

Table 1 Disease symptoms of PVY infection on different leaves in each cultivar 14 days after inoculation.

Cultivar	Apical leaves		Upper leaves		Lower leaves	
	Vein necrosis	Mosaic	Vein necrosis	Mosaic	Vein necrosis	Mosaic
Agria	-	Mediocre	+	Mild	-	-
Milva	+	Severe	+	Mild	-	-

Verification of PVY infection

The RT-PCR assay was used to test different leaf samples exhibiting typical symptoms of PVY infection or leaves of infected plant without symptoms. RT-PCR successfully confirmed PVY infection in apical, upper and lower leaves of inoculated plants. Specific PCR product of PVY was observed at a position of approximately 166bp, in agreement with that reported by Du *et al.* (2006). In negative control plants, no band was detected (Fig. 2).



Figure 2 PCR products (166bp) of PVY from different leaves of infected Agria and Milva cultivars. NC: negative control; PC: Positive control, 100bp and 50bp DNA Ladder.

Virus titer

ELISA technique was used to estimate virus titers separately in leaves from different positions (apical, above and below the inoculation point) on each experimental plant. ELISA results are presented in Fig. 3. The apical leaves had a higher virus titer and showed more severe mosaic symptoms in both cultivars. The results presented here show that virus titers in different leaves can be varied, and development of the disease symptoms correlates with the cultivar sensitivity (Scholthof, 2007). Therefore, three different leaves with different virus titers in each experimental plant were used

for precise spectral analysis and the means of spectral data from different leaves in infected and control plants were compared in this research.

The averages of entire spectral reflectance dataset of PVY infected and non-infected potato plants (Agria and Milva cultivar) 14 days after inoculation are shown in Fig. 4. The differences between inoculated and healthy leaves (in Milva Cv.) seen in 550nm (green peak), indicated less chlorophyll absorption in disease case. Photosynthetic activity and chlorophyll content were decreased in Milva more than in Agria due to PVY infection. Reduction in photosynthetic pigments in symptomatic leaves (Kogovšek and Ravnikar, 2013) caused alterations in reflectance pattern in visible region (400-700) (Fig. 4). Biochemical constituents of plants as one of the dominant factors controlling leaf reflectance, are subjected to variation by different factors. Changes in the reflectance observed in Figure 4 are attributed to PVY infection as a biotic stressor that disrupts plant normal physiology, cell structure, and leaf pigmentation and causes alteration in the leaf reflectance pattern (Prabhakar *et al.*, 2012).

Wavelengths in NIR region (700-1200) showed maximum differences in reflectance pattern due to disease stress. The internal structure of non-infected leaves acts as excellent diffuse reflector in this region (Krezhova *et al.*, 2014). At the same time, the results obtained from the statistical analysis showed that among all the collected data, spectra in the wavelength range between 900-1100nm were most effective in predicting PVY infection in potato and the maximum change in spectra signature was observed in this region.

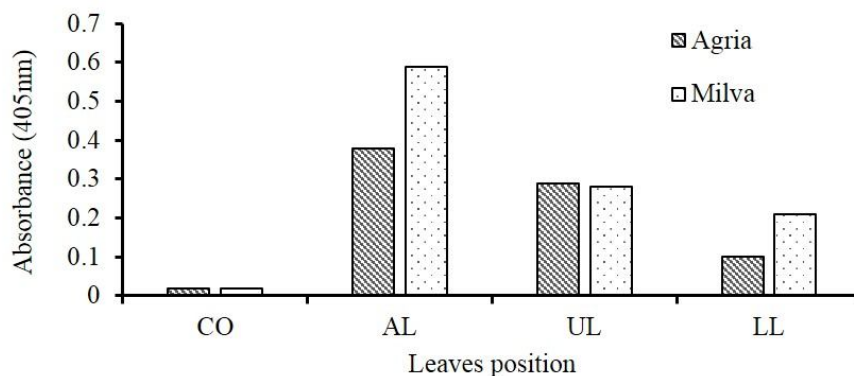


Figure 3 Comparison of PVY titer in different leaves of each experimental plant (Agria and Milva cultivars). Co: healthy plants; AL:apical leaves; UL:upper leaves and LL:lower leaves of infected plants.

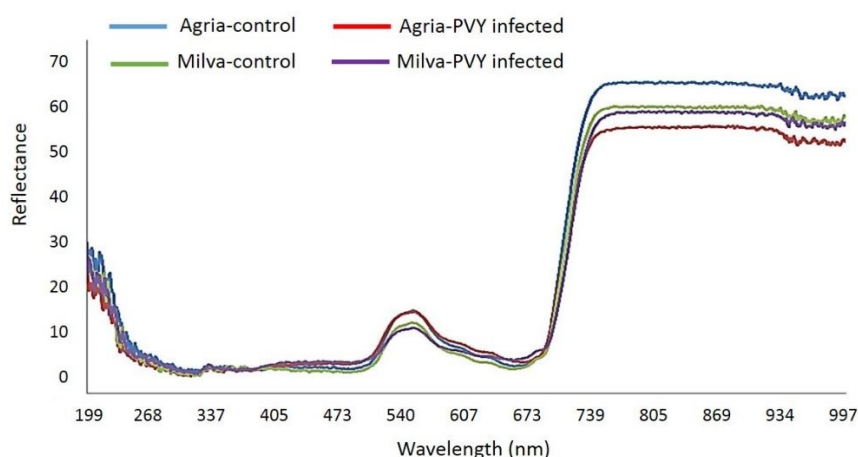


Figure 4 Spectral reflectance of *Solanum tuberosum* (Agria and Milva cultivars) two weeks after inoculation.

Principal component analysis (PCA) was used to identify which variables have the largest effect on each component. PCA score plots in Figure 5 give information about patterns in the samples. The closer the samples are the more similar they look with respect to the two components concerned. First and second derivative spectra provided a higher accuracy for classification than the other pretreatments namely normalization, baseline correction, full multiplicative scatter correction and raw spectra. Derivatives of spectra are more useful for spectral analysis because raw spectra that are similar in reflectance mode reveal significant differences in the derivative mode (Fig. 5.). PCA score

plots of first derivative spectra can clearly separate infected plants from healthy plants especially in Milva cultivar (Fig. 5). Virus titer and severity of symptoms are more noticeable in Milva, so high variation in the leaf reflectance pattern is due to its susceptibility to PVY. On the other hand, infected samples in both cultivars are closer to each other than healthy plants. It can be interpreted that potato infected plants have similar reflectance pattern in both cultivars.

Cell structure is a dominant factor controlling leaf reflectance in 900-1100nm regions

(Sankaran *et al.*, 2010). High rate replication of virus particles, disrupts normal metabolic

processes of plants (Agrios, 1988) that influence water molecules activities. Previous research has indicated that spectra variations in the 890-1025nm region belong to different vibration patterns of water molecule due to viral infection (Jinendra *et al.*, 2010) and is interpreted as new proteins created by virus, with an increase in free water activity in infected plants, so paying attention to these spectral regions could be useful for discrimination of virus-infected plants.

Based on line plot (plot of x-loading) results in PCA analysis, some wavelengths such as 1020, 1037, 1040, 1048, 1052, 1058, 1067, 1070, 1079, 1083, 1091nm have most positive or negative loading, these specific wavelengths are more important for the component concerned and they are responsible for the greatest differences between the samples and could be used as the fingerprints of the spectra.

Selected wavelength bands were used for classification based on linear discriminant analysis (LDA) to test the detection accuracies. LDA is a well-known method in pattern recognition. It intends to find a linear combination of features which separates two or more classes of samples. The classification results indicated same determination accuracy (100%) in wavelengths from 1020 to 1090nm but detection accuracy at other selected wavelengths such as 960, 985 and 996nm have 78.57, 92.86, 85.71% accuracy, respectively (Fig. 6). Based on the selective sensitive wavelengths analysis, the infection status of plants can be better identified than analysis of whole spectral region, because the whole spectra contain ineffective data and noise, while the sensitive wavelengths contain the most important information and less noise (Wu *et al.*, 2008).

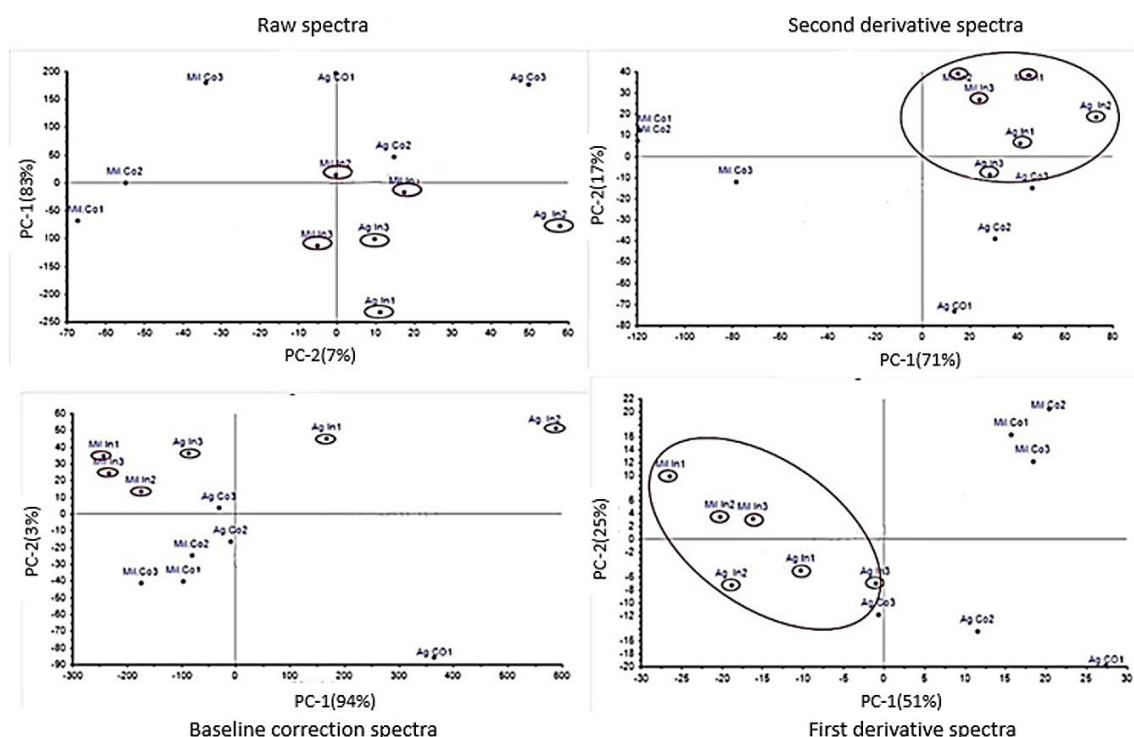


Figure 5 PCA score plot of PVY infected and healthy potato plant (Milva and Agria cultivars) for primary and pretreated spectra in the 900-1100nm. Indicated samples by circles belong to infected Milva and Agria. (Ag.Co): healthy plant of Agria; (Ag.In): infected plant of Agria; (Mil.Co): healthy plant of Milva; (Mil.In): infected plant of Milva.

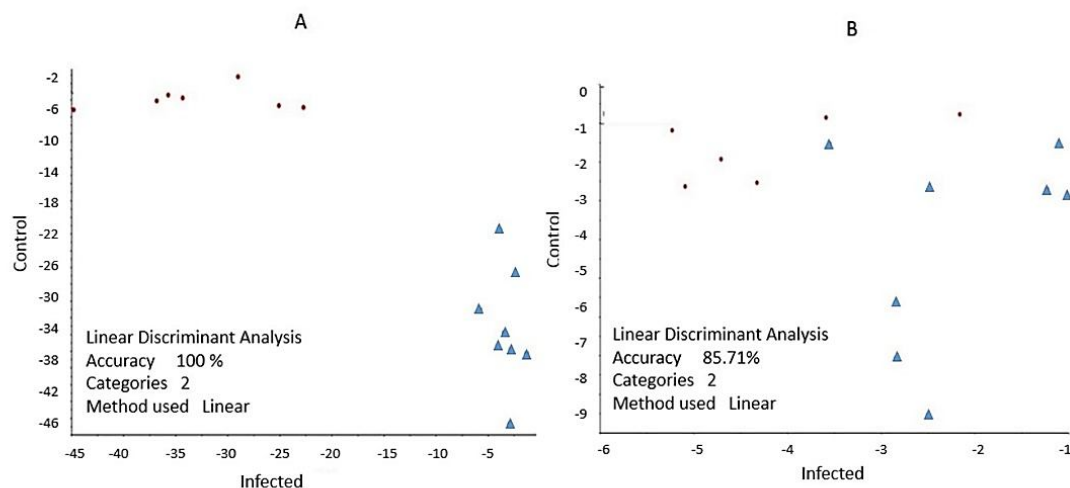


Figure 6 The scatter plot of linear discriminant analysis at wavelengths 1040nm (A) and 996nm (B) that separates two classes of infected (blue dots) and healthy plants (red dots), respectively with 100 and 85.71% determination accuracy.

Finally, it can be concluded that sensitive wavelengths in NIR region markedly discriminated infected plants and produced accurate precision for disease detection. The current research shows the possibility of developing a fast, non-destructive and economic method for determining viral infection in potato plants. Further studies should be considered with respect to environmental condition and other stresses in the fields.

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پتانسیل اسپکتروسکوپی در تفکیک گیاهان سیب‌زمینی آلوده به ویروس PVY از گیاهان سالم

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چکیده: اسپکتروسکوپی در ناحیه مرئی و قسمتی از ناحیه مادون قرمز نزدیک به‌عنوان روش تشخیصی غیرتخریبی برای تعیین آلودگی PVY در گیاه سیب‌زمینی مورد ارزیابی قرار گرفت. این پژوهش با هدف مطالعه تغییرات بازتابش طیفی برگ‌های گیاه سیب‌زمینی آلوده به PVY (با علائم متفاوت بیماری) در دو رقم آگریا و میلوا انجام شد. غلظت ویروس در برگ‌هایی که علائم متفاوت بیماری را نشان می‌دادند با استفاده از آزمون سرولوژیکی ELISA بررسی گردید. میانگین داده‌های طیفی حاصل از برگ‌های مختلف در یک گیاه در تکرارهای مختلف برای آنالیزهای طیفی مورد استفاده قرار گرفت. نتایج تجزیه تحلیل‌ها مشخص کردند که داده‌های طیفی در ناحیه ۹۰۰-۱۱۰۰ نانومتر به‌صورت معنی‌داری نسبت به آلودگی PVY تغییر نشان دادند. لذا از این دامنه طیفی می‌توان به‌عنوان ناحیه‌ای با ویژگی‌های بازتابشی منحصربه‌فرد برای توسعه سیستم تشخیصی ویروس PVY بهره برد. بر پایه نتایج آنالیز تحلیل مؤلفه‌های اصلی، طول موج‌های حساس که در اثر بیماری بیش‌ترین تغییرات (افزایشی یا کاهش) را نشان دادند، انتخاب شدند و به استناد نتایج آنالیز افتراقی خطی این طول موج‌ها به‌خوبی قادر به گروه‌بندی و تفکیک گیاهان سالم از آلوده بودند. تغییرات بازتابش در این محدوده طیفی به ساختار و فعالیت آب در گیاه بستگی دارد. اسپکتروفتومتری پتانسیل بسیار خوبی برای تشخیص گیاهان آلوده به ویروس دارد و می‌تواند به‌عنوان یک سیستم مناسب، کم‌هزینه و غیرتخریبی برای بازرسی مزارع سیب‌زمینی و کنترل آلودگی‌های ویروسی توسعه یابد.

واژگان کلیدی: طیف، PVY، تشخیص، غیرتخریبی، ویروس