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Sensing tomato's pathogen using Visible/Near infrared (VIS/NIR) spectroscopy and multivariate data analysis (MVDA)

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Abstract: Quality of agricultural products is a very important issue for consumers as well as for farmers in relation to price, health and flavour. One of the factors that determine the quality is the absence of pathogens that can cause diseases for products and also for consumers. An advanced method to sense pathogens and their antagonists is the use of Visible/Near Infrared (VIS/NIR) spectroscopy. In this paper, the VIS/NIR spectroscopy, with the help of two techniques of multivariate data analysis (MVDA); namely principal component analysis (PCA) and support vector machine (SVM)-classification; showed very reliable results for sensing two artificially inoculated fungi (*Fusarium oxysporum* f. sp*. Lycopersici* and *Rhizoctonia solani*), and two antagonistic bacteria (*Bacillus atrophaeus* and *Pseudomonas aeruginosa*). The two fungi cause loss of quality and quantity for tomatoes. The results showed that the lowest classification rates using VIS/NIR spectroscopy for pathogens, antagonistic and their combinations were 90%, 85% and 74%, respectively. These results open a wide range for using VIS/NIR spectroscopy sensor technology for agricultural commodities quality at quality control checkpoints.

Keywords: principal component analysis (PCA), support vector machine (SVM)-classification, fungi, antagonistic, quality control.

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is considered one of the most popular vegetables worldwide. It is ranked eighth worldwide according to the production value. The top five countries producing tomatoes are China, India, United States of America, Turkey and Egypt (FAO, 2014). Moreover, it plays an important health role, due to its contribution in prevention of heart and cancer disease (Temple, 2000; Hamid *et al.*, 2010).
In Palestine, tomato is considered as the most important and popular vegetable crop, and it is a part of many traditional meals and folkloric medicine (Sawalha, 2014). It is mostly grown in green houses (Angioni et al., 2012). Its production reached more than 205 thousands metric tons, with more than 75 million dollars value in 2012 (FAO, 2014).

Quality of agricultural products which is defined as the "degree to which a set of inherent characteristics fulfills requirements of the customer", plays an essential factor for consumers and farmers in price, health and flavour issues. One of the important factors that determine the quality of tomato is pathogens absence, and it is important to detect whether the crop has some of these pathogens, that can affect their quality and shelf life in pre- and post-harvest stages. Detection of pathogens can be done in many destructive ways, such as plating method. One of the trends and new methods is to detect quality using non-destructive methods, such as Visible/Near infrared red (VIS/NIR) spectroscopy, which is an optical sensor (Nicolai *et al.*, 2014).
VIS/NIR spectroscopy method has been used for quality evaluation of agricultural products. It is based on overtone and combination bands of spe-

cific functional groups, *e.g.* C-H, N-H, and O-H bands, which are the primary structural components of organic molecules. This opens the possibility of using spectra signatures to determine complex attributes of foods. The VIS/NIR method has many advantages, *i.e.* simplicity, rapidity, requires minimal sample processing prior to analysis, ease or total absence of sample preparation, can be easily automated, and ability to obtain the information about different product properties in a single measurement (Abu-Khalaf and Bennedsen, 2002a; Abu-Khalaf and Bennedsen, 2002b; Abu-Khalaf and Bennedsen, 2004; Xie and Ying, 2009; Guidetti *et al.*, 2010; Giovenzana, 2013; Sanchez, 2013; Chaparro and Pena-Rodriguez, 2014; Opara and Pathare, 2014; Sanchez *et al.*, 2014; Nicolai *et al.*, 2014). Moreover, it is stated that optical methods; like VIS/NIR; offer advantages of high reproducibility and good long-term stability (Winquist *et al.*, 2006).

For detecting pathogen, many authors reviewed the methods for plant diseases detection, and VIS/NIR spectroscopy was one of the indirect remote sensing methods (Sankaran *et al.*, 2010; Nezhad, 2014; Martinelli *et al.*, 2014). Spectroscopy method has been used for detecting pathogens in different crops, *e.g.* olives (Abu-Khalaf and Salman, 2014; Moscetti *et al.*, 2015), wheat (Li *et al.*, 2014; Yuan *et al.*, 2014), cereals (Levasseur-Garcia, 2012) and herbaceous flowering plant (Nilsson *et al.*, 1994).

VIS/NIR spectroscopy was used for detecting some of tomato quality parameters, *e.g.* soluble sugar content (SSC), firmness and titratable acidity (He et al., 2005; Saad et al., 2014). Also, it was used to detect stress in tomatoes induced by late blight disease in California, USA (Zhang *et al.*, 2003) and also to detect *Rhizopus stolonifer,* that causes significant postharvest losses (Hahn *et al.*, 2004).

Tomato is affected by many pathogens that affect quality and quantity of the crop. *Fusarium oxysporum* f. sp. *Lycopersici* which causes fusarium wilt (Shanmugam *et al.*, 2011), and *Rhizoctonia solani* causing root and crown rot disease on tomato (Thomashow and Bakker, 2015).

The bacteria on the other hand are acting as control agents against the fungi and supposed not to cause any symptoms on the fruits. The antagonistic Bacillus spp. inhibits the activity of some fungus in tomatoes. *Bacillus atropheus* was used to control gray mold disease caused by *Botrytis cinerea* on tomato (Abu-Khalaf and Salman, 2012). It was also found that it inhibits the activity of *Fusarium oxysporum* f.sp. *lycopersici* and *Alternaria solani* infecting tomato (Shanmugam *et al.*, 2011).

Pseudomonas aeruginosa also has been used as a biological control, it has *e.g.* alone or with mineral fertilizers showed a significant ability to inhibit root diseases in tomato (Parveen *et al.*, 2008).

The aim of this study is to investigate the feasibility of using VIS/NIR spectroscopy and multivariate data analysis (MVDA) (*i.e.* chemometrics) for detecting two pathogenic fungi and two antagonistic bacteria that were artificially inoculated into tomato. Both fungi cause rots and molds on tomato fruits in the green houses and causing severe market losses.

Material and methods

Tomato fruits

A 45 red, homogenous in shape and colour and pathogen free tomato fruits were chosen from a batch purchased from a local market. The fruits were kept at room temperature. The fruits were inoculated with 9 different treatments, including control samples. Each treatment had 5 tomato fruits. Each fruit (except the control samples) was inoculated with microorganisms, either a fungus and/or bacterium.

The treatments (**Table1**) were: control (C), *F. oxysporum f. sp. lycopersici* (F1), *R. solani* (F2), *B. atrophaeus* (B1), *P. aeruginosa* (B2), *F. oxysporum + B. atrophaeus* (F1B1), *F. oxysporum + P. aeruginosa* (F1B2), *R. solani + B. atrophaeus* (F2B1), and *R. solani + B. atrophaeus* (F2B2).

Experiment was carried in two trials, and the data of first trial is shown in this paper. However, both trials gave the same results.

Fungal strains growth conditions

F. oxysporum f. sp. *Lycopersici and R. solani* were grown and maintained on potato dextrose agar (PDA). For inoculum preparation, 5 mm diameter of PDA disks grown with 3 days old fungi were inoculated in 250 ml Erlenmeyer flasks containing 50 ml potato dextrose broth (PDB). The flasks were incubated at 25℃ on a rotary shaker at 150 rpm for three days. Spores were then harvested after filtration using cheese clothes. Spore suspension was adjusted to $2x10^6$ sporangiospores/ml using autoclaved water. A 80 µl of spores suspension were inoculated into tomato fruits (in which 8 pores were done and in each pore 10 µl of the suspension were added), in total 80 µl of spore suspension were injected (inoculated) into tomato fruits.

Bacterial growth conditions

B. atrophaeus and *P. aeruginosa* were grown in Nutrient agar media, stored at 4℃ and subcultured routinely every two weeks. For inoculum, the bacteria were grown in nutrient broth (NB) media for

overnight on a rotary shaker at 150 rpm at 28℃. (the bacteria were centrifuged and pellets were re-suspended in100 ml of autoclaved water then 10 µl (~ 10^9 cfu/ml) of bacterial suspension were added to each pore in tomato fruit).

Spectroscopy

A VIS/NIR spectroscopy, a USB2000+ miniature fiber optic spectrometer (OceanOptics, USA) with a 50 µm fiber optics probe and Vivo light source, was used for reflectance spectra acquisition. The spectroscopy has a 550-1100 nm wave length and a resolution of 0.35 nm full width at half maximum (FWHM). It has 2-MHz analog-to-digital (A/D) converter, a 2048-element CCD-array detector, and a high-speed USB 2.0 port. The USB2000+ can be controlled by SpectraSuite software. Vivo system contains 4 tungsten halogen bulbs that can be turned on or off individually. The risk of overheating the sample is mitigated through active fan cooling. This protects the sample and ensures accuracy every time. The 4 halogen tungsten light sources make the Vivo a high-powered VIS/NIR source, which allows a shorter integration time than conventional methods (OceanOptics, USA). The integration time used in this investigation was 1.37 ms.
Tomato was placed on the top of Vivo light source, and three reflection spectra (550-1100 nm) were taken at three equidistant positions around the equator (approximately 120º) of each tomato.
A diffuse reflectance standard WS-1 (OceanOptics, USA) was used as the optical reference standard for the system every 10 minutes during the experiment. The goal of using was to ensure the stability of the measurements.
The dark reference was done once in the beginning of each experiment, through closing the entrance of incoming light from probe to the USB2000+ miniature fiber optic spectrometer by a plastic cap.
After completing all spectral measurements, the acquired data were properly stored for later use.

Multivariate Data Analysis (MVDA)

Two MVDA techniques; namely principal component analysis (PCA) and support vector machine (SVM)-classification; were used to analyse, unravel and interpret the optical properties of VIS/NIR signatures and allow classification of samples. Before analysing normalized and centred spectra signatures with PCA and SVM-classification, the spectra were gone through a first order Savitzky-Golay derivative, employing a one-point smoothing window of a second order polynomial. The goal was to remove the baseline offset (Lomborg *et al*., 2009), and it was found that this pre-processing technique gave a good classification models.
Unscrambler software (version 10.3, CAMO Software AS, Oslo, Norway) was used for MVDA.
PCA is a non-supervised linear multivariate technique that uses a mathematical procedure to transform a set of correlated response variables into principal components (PCs), generating a new set of non-correlated variables. PCs represent in pattern of observations in plots. The score plot explains the relation between samples, and the loading plot explains the relation between variables. PCA plots provide information about structure of data. In PCA, VIS/NIR spectra represented matrix (Abu-Khalaf and Bennedsen, 2004). The goal from carrying PCA was to show the linear relationship between different samples and variables, and the possibility of classifying different treatments during the experiments, through investigating scores plot of different treatments during experiment period (*i.e.* five days). Random cross validation with 10 segments was used.
SVM is a powerful multivariate methodology for supervised non-linear classification and regression problems. It was developed by Vapnik's group in 1995 (Vapnik, 2000). There are many advantages of SVMs, *i.e.* don't need a large number of samples to be trained, not affected by the presence of outliers, and there are very few parameters to tune or select a priori when compared with other methods. Moreover, the main advantages of SVMs are mainly refer to their generalization ability, which is achieved by using the maximum margin hyperplane for separation and the application of non-linear discriminant functions. SVMs can handel convexit discrimination problems. SVM classification used cross validation method (Acevedo *et al*., 2007; Xie and Ying, 2009; Rumpf *et al*., 2010; Suphamitmongkol *et al*., 2013). SVMs were used in several agricultrual and biological applications (Abu-Khalaf and Salman, 2013; DomInguez *et al*., 2014; Gromski *et al*., 2014; Mokhtar *et al*., 2015).
In SVM-classification, VIS/NIR spectra represented X-data matrix, and treatments names as categories were used as the response factor (Y-data matrix, label classes). Unscrambler uses one column for a classifier factor. For SVM-classification, nu-SVC, with radial basis function (RBF) as the kernel type was used. The different parameters were: *gamma* $6*10^{-4}$, *nu* value: 0.5 and weights: all 1. The cross validation with 10 segments was used.

Table1: Different nine treatments of tomato fruits with fungus and bacteria that were used in the experiment.

Treatments	Name	Abbreviation (category name)	Nr. of inoculation days	Number of samples/day	Number of spectra/day (spectra were taken in triplicates)	Number of spectra during experiment (samples X spectra/day X days of experiment)
1	Control	C	-	5	5 (15)	60
2	*F. oxysporum f. sp. lycopersici*	F1	4	5	5 (15)	60
3	*R. solani*	F2	2*	5	5 (15)	30
4	*B. atrophaeus*	B1	4	5	5 (15)	60
5	*P. aeruginosa*	B2	4	5	5 (15)	60
6	*F. oxysporum + B. atrophaeus*	F1B1	4	5	5 (15)	60
7	*F. oxysporum + P. aeruginosa*	F1B2	4	5	5 (15)	60
8	*R. solani + B. atrophaeus*	F2B1	2*	5	5 (15)	30
9	*R. solani + B. atrophaeus*	F2B2	2*	5	5 (15)	30

*At the end of second day after inoculation, samples with *R. solani* (F2) and its combinations, *i.e. R. solani* + *B. atrophaeus* (F2B1) and *R. solani* + *B. atrophaeus* (F2B2) were decayed, and spectra for them were not taken in the next days.

Results and discussion

In this study, ranges of Visible/Short-Wave Near-Infrared (VIS-SWNIR) was used, *i.e.* 550-1100 nm. The SW-NIR region, 700-1100 nm, is more suitable for non-destructive or non-invasive analysis for intact foods and biological materials, compared with long-wave NIR region (1100-2526 nm). SW-NIR has several advantages. It can penetrate more deeply into a sample with less heating effect and the interference arising from the intense water bands can be diminished. There are strong evidences, that the range from 700-900 nm constitutes a "diagnostic window" in which chemical compositions of samples can be investigated. Moreover, the low cost of SW-NIR spectroscopy is a big advantage (Osborne *et al.*, 1993; Archibald *et al.*, 1999; Abu-Khalaf *et al.*, 2004; Fu *et al.*, 2012; Melendez-Pastor *et al.*, 2013; Lapchareonsuk and Sirisomboon, 2014).

PCA and SVM-classification were used for spectra data analysis. It was clear that there was non-linearity in the PCA models, and that's was one of the reasons to use SVM to carry out the classification. The results for each day of experiments after inoculation are explained below.

In the first day of inoculation

The PCA is shown in **Figure 1**. The first and second PCs explained 54% and 11% of the variation, respectively. Six PCs were the optimum number of PCs for the model, and they explained 76.8% of the variation.

The loading plot is shown in **Figure 2**. The most important wavelengths ranges in the range of 550-1100 nm that contribute to the model; *i.e.* have the highest loading that is greater than |0.02| in the loading plot; are around 580-670, 675-690, 700-950, and 1000-1050 nm. **Table 2** shows the SVM-classification rates for the first day after inoculation. It shows good classification rates for all treatments. The classification rates were 100% for all treatments, except for *R. solani + B. atrophaeus* (F2B2) treatment, which was 93%. However, the later classification rate is still high. The accuracies were 99.3% and 80.7% for training and validation sets, respectively.

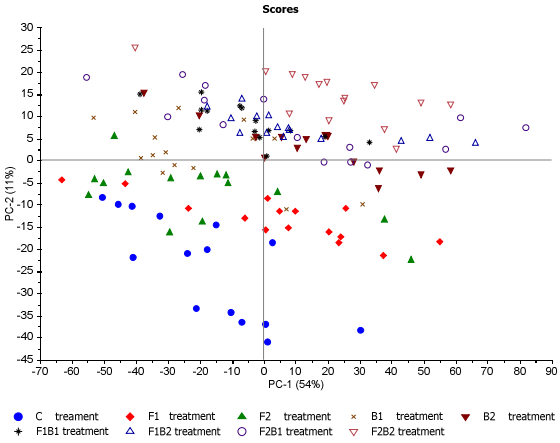

Figure 1. Scores plot for first and second principal components (PC1 and PC2) for samples in the first day after inoculation.

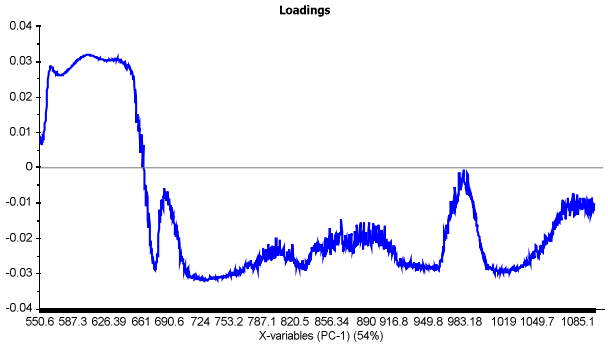

Figure 2. Loading plot for the first principal component (PC) for the first day after inoculation.

In the second day of inoculation

The PCA is shown in **Figure 3**. The first and second PCs explained 41% and 21% of the variation, respectively. Six PCs were the optimum number of PCs for the model, and they explained 81.8% of the variation. The loading plot is shown in **Figure 4**. The most important wavelengths ranges that contribute to the model are 550-570, 590-650, and 700-1100 nm. **Table 2** shows that the classification rates were 100% for all treatments, except for *R. solani + B. atrophaeus* (F2B2) treatment, it was 93%. However, 93% classification rate is also still high rate. The accuracies were 99.3% and 81.3% for training and validation sets, respectively. Moreover, **Figure 3** shows a very clear linear classification between.

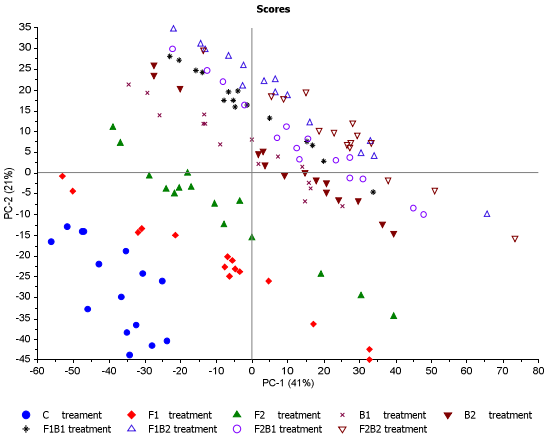

Figure 3. Scores plot for first and second principal components (PC1 and PC2) for samples in the second day after inoculation.

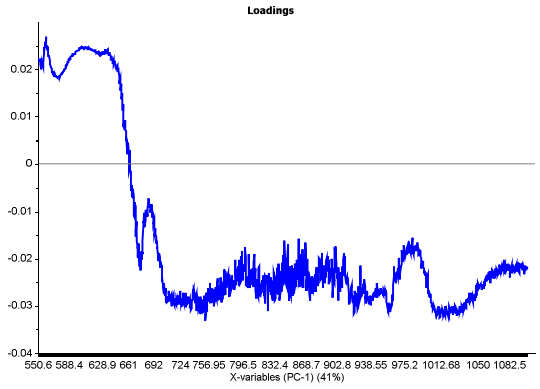

Figure 4. Loading plot for the first principal component (PC) for the second day after inoculation.

control, *F. oxysporum f. sp.* (F1) and *R. solani* (F2) samples At the end of second day after inoculation, samples with *R. solani* (F2) and its combinations, *i.e. R. solani + B. atrophaeus* (F2B1) and *R. solani + B. atrophaeus* (F2B2) were decayed, and their spectra for them were not taken in the next days. The total number of tomato samples in the third and fourth day after inoculation were 30 samples (*i.e.* spectra were: 6 treatments including control X 5 tomatoes X spectra for each tomato in triplicate = 90 with triplications).

In the third day of inoculation

The PCA is shown in **Figure 5**. The first and second PCs explained 46% and 20% of the variation, respectively. Five PCs were the optimum number of PCs for the model, and they explained 77.6% of the variation. The loading plot is shown in **Figure 6**. The most important wavelengths ranges that contribute to the model are 550-560, 580-660 and 700-1100 nm.

Table 2 shows that the classification rates were 100% for all treatments. The accuracies were 100% and 86.7% for training and validation sets, respectively. It should be noticed that scores plot in **Figure 5** shows a very clear linear classification between control and *F. oxysporum f. sp.* (F1) samples.

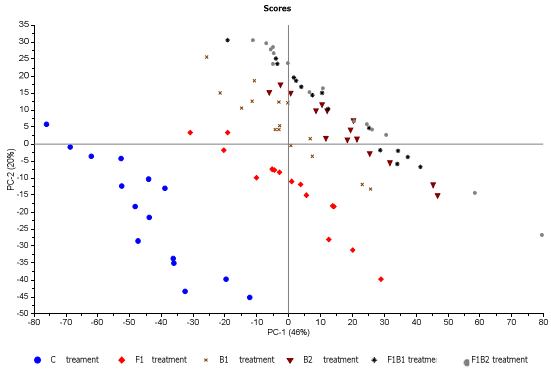

Figure 5. Scores plot for first and second principal components (PC1 and PC2) for samples in the third day after inoculation.

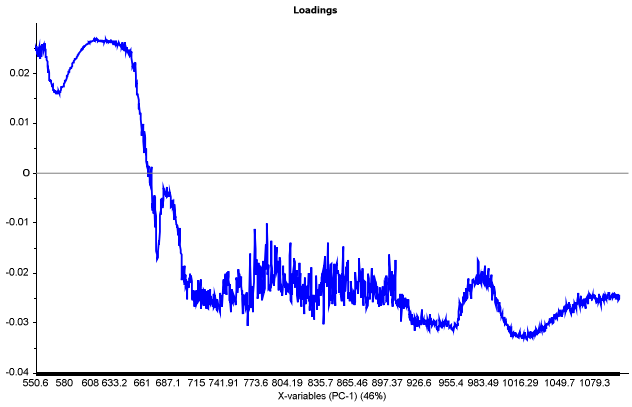

Figure 6. Loading plot for the first principal component (PC) for the third day after inoculation.

In the fourth day of inoculation

The PCA is shown in **Figure 7**. The first and second PCs explained 45% and 23% of the variation, respectively. Six PCs were the optimum number of PCs for the model, and they explained 80.1% of the variation.

The loading plot is shown in **Figure 8**. The most important wavelengths ranges that contribute to the

model are 550-660, 670-680, 690-960 and 1000-1100 nm. **Table 2** shows that the classification rates were 100% for all treatments except for *P. aeruginosa* (B2) and *F. oxysporum + B. atrophaeus* (F1B1), which were 93% for both of them. The accuracies were 97.8% and 90% for training and validation sets, respectively.

Figure 7 shows a very clear linear classification between control and *F. oxysporum f. sp.* (F1) samples.

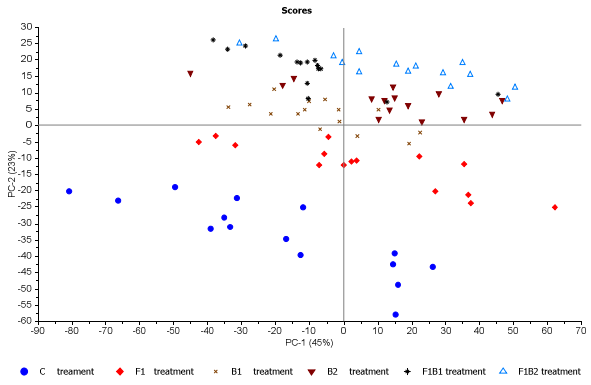

Figure 7. Scores plot for first and second principal components (PC1 and PC2) for samples in the fourth day after inoculation.

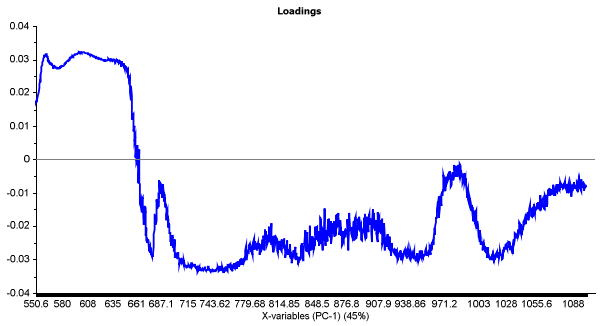

Figure 8. Loading plot for the first principal component (PC) for the fourth day after inoculation.

It can be seen from the above results that PCA and SMV-classification are able to reveal the spectra signatures and carry out classification of the two fungi and/or two antagonists in different treatments during 4 days of inoculation. The SVM-classification were powered enough to detect the treatment categories, despite the non-linearity of the models. Moreover, high classification rates were obtained.

In the results above, SVM-classification models for each day of inoculation were shown. Taking into consideration all samples during the four days of inoculation, and making one SVM-classification model, the result is shown in **Table 3**. It can be seen that the classification rates for all treatments were higher than 73%. The classification rates of control, *F. oxysporum f. sp. lycopersici* (F1), *R. solani* (F2), *B. atrophaeus* (B1) and *P. aeruginosa* (B2) were 97%, 92%, 90% and 85%, respectively. The accuracies were 88% and 67.5% for training and validation sets, respectively.

The classification rates of the two fungi and two antagonistic bacteria were higher when carrying out SVM-classification model for each day of inoculations (*i.e.* reached 100% in most cases), rather than making one SVM-classification for all samples in all days of experiment. This can be explained by that when considering one model for all samples in all days, then the SVM-classification are influenced by higher variation of all samples, than samples going through classification in each day of inoculations.

It can be concluded from the results above, that both VIS and NIR wavelengths had influence in the PCA loading plots, and not just VIS neither NIR ranges alone.

VIS/NIR showed a high potential for classification samples according to two fungi and two antagonistic inoculations, and this gives a potential for using VIS/NIR spectroscopy as a tool for detecting pathogens in tomatoes. This opens a wide range of possibility of using VIS/NIR spectroscopy in monitoring quality of agricultural and food products. Also possible using of portable spectroscopies, and this agrees with other researchers' viewpoint, who supported the use of portable spectroscopies for agricultural and food applications (dos Santos *et al.*, 2013).

It is important to underline that SVM-classification is a supervised classification method, and this made it easier to classify the spectra according to the treatments that they had. However, it is not easy to classify when we made a non-supervised classification (Casale and Simonetti, 2014), and that was clear when carrying PCA models. However, the PCS scores plot showed to some extend the distinguished treatments. However, non-linearity was clear.

It is envisaged that this research is a step in a long research aiming to use spectroscopy signatures to detect the pathogens on fruits and vegetables, to save time, monitor the quality and prevent any harm for consumers.

Table 2. Support vector machine (SVM)-classification rate (%) and accuracy (%) for training and validation sets of control and different tomato fruit samples that were treated by fungi and /or bacteria using visible/near infrared (VIS/NIR) spectroscopy.

			Classification rate (%)								
			Treatments								
Days after inoculation		Accuracy (%)	C	F1	F2	B1	B2	F1B1	F1B2	F2B1	F2B2
1	Training	99.3	100	100	100	100	100	100	100	100	93
	Validation	80.7									
2	Training	99.3	100	100	100	100	100	100	100	100	93
	Validation	81.5									
3	Training	100	100	100	-	100	100	100	100	-	-
	Validation	86.7									
4	Training	97.8	100	100	-	100	93	93	100	-	-
	Validation	90									

Table 3. SVM-classification rate (%) for different samples during four days of inoculation.

Classification rate (%)								
Treatments								
C	F1	F2	B1	B2	F1B1	F1B2	F2B1	F2B2
97	92	90	90	85	95	93	74	77

Conclusion

It can be concluded that:

- VIS/NIR was able to classify (distinguish) tomatoes samples that were artificially infected by two fungi (*F. oxysporum* f. sp. *Lycopersici* and *R. solani*), and two antagonistic bacteria (*B. acillus atrophaeus* and *P. aeruginosa*).
- The lowest classification rate for the two fungi was 90%.
- The lowest classification rate for the two antagonistic was 85%.
- The lowest classification rate for fungi and antagonistic combinations was 74%.
- PCA and SVM-classification showed powerful ability to reveal the VIS/NIR spectra signatures.
- Both VIS and NIR wavelengths played a rule in the success of pathogens classification.

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