Asian Journal of Plant Pathology

ISSN 1819-1541

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Asian Journal of Plant Pathology

ISSN 1819-1541
DOI: 10.3923/ajppaj.2017.167.173

Research Article
Spectral and Molecular Studies on Gray Mold in Strawberry

[1]Hala Abdel Wahab, [2]Mohamed Aboelghar, [2]Abdelraouf Massoud Ali and [2]Mona Yones

[1]Laboratory of Molecular Diagnostic of Plant Diseases, Department of Plant Pathology, Faculty of Agriculture, Ain Shams University, 11241 Cairo, Egypt
[2]National Authority for Remote Sensing and Space Sciences, Cairo, Egypt

Abstract

Background and Objective: Gray mold is a serious problem in strawberry production worldwide. Early diagnostic of diseased strawberry fruits allows export decision. The aim of the study was to test the capability of spectral measurement for detecting the asymptomatic *Botrytis* infection. **Materials and Methods:** Asymptomatic and symptomatic samples of 25 strawberry fruits were measured for gray mold using two reliable systems, qPCR and spectroradiometer, to detect the causal pathogen, *Botrytis cinerea*. **Results:** Molecular results showed the presence of gray mold in many asymptomatic fruits. Moreover, spectral analysis demonstrated a higher reflectance in healthy fruit than that of infected ones throughout the visible near infra red (VNIR) spectral range, while the short wave infra red (SWIR) spectral zone showed different degrees of gray mold infection. **Conclusion:** The VNIR found to be the best spectral zone that could differentiate between healthy and infected strawberry fruits due to infection impact on the cellular pigments of the fruit, while SWIR was the best spectral zone to classify infection degrees because of the changes in cellular structure and water content due to infection.

Key words: *Botrytis cinerea*, molecular diagnostic, remote sensing, spectral diagnostic, spectral variation

Citation: Abdel Wahab H., M. Aboelghar, A.M. Ali and M. Yones, 2017. Spectral and molecular studies on gray mold in strawberry. Asian J. Plant Pathol., 11: 167-173.

Corresponding Author: Abdel Wahab H., Laboratory of Molecular Diagnostic of Plant Diseases, Department of Plant Pathology, Faculty of Agriculture, Ain Shams University, 11241 Cairo, Egypt Tel: +20-112551100

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gray mold threatens all strawberry production areas: The field, storage, transit, marketing and export of strawberry fruits, due to the severe rot when the fruits begin to ripen. *Botrytis cinerea* Pers., is a wide host range necrotrophic fungus causing rot symptoms[1] and can be difficult to be combated. Early detection of diseased strawberry fruits, before observation of symptoms, allow accurate diagnosis[2-4], especially before fruit export. Molecular techniques have previously been used for pathogenic characterization[4-7]. Quantitative real time PCR (qPCR) application has been developed and offered the ability of simultaneous detection and quantification of pathogenic DNA based on its sequence and concentration[8,9]. The qPCR system has many advantages: high sensitivity, quantitative properties and specificity, which make this method suitable for routine diagnostic and disease management decisions, but it is highly expensive and time consuming.

The measurements of spectral reflectance of vegetation contain information on plant pigment concentration, cellular structure, plant anomalies[10] and moisture content[11]. Spectral reflectance was previously reported to measure powdery mildew infection in wheat under greenhouse condition. In addition, VIS-NIR reflectance spectroscopy (325-1075 nm) had been used for early detection of many diseases like *B. cinerea* infection in eggplant leaves at symptomless stages[10], powdery mildew disease in wheat[12], *Sclerotinia* rot infection on celery[13] and Huanglongbing (HLB) bacterial infection on citrus[14,15]. Delalieux *et al.*[16] had also investigated the detection of apple scab using hyperspectral spectroscopy and found that the spectral domain between 1300-1700 nm is the most effective region to separate stressed from healthy plant leaves. This is the first study using both diagnostic techniques, spectroradiometer and qPCR, to distinguish *Botrytis* infected strawberries from healthy ones. The aim of this study was to investigate an alternative inexpensive methodology for early detection of gray mold using spectroradiometer.

MATERIALS AND METHODS

Sampling: Asymptomatic and symptomatic samples were collected, during 2013-2014 at Faculty of Agriculture, Ain Shams University, from two varieties of strawberry fruits (*Fragaria ananassa* Duchesne var. Sweet Charlie and Festival), which have been grown under natural condition. Fifteen samples from sweet charlie (samples: A, B, C, D, E, F, G, H, I, J, K, L, M, N, O) and ten samples from Festival (1, 2, 3, 4, 5, 15, 16, 17, 18, 19) were tested microscopically for their infection with gray mold and then kept at 4°C for further analysis.

Spectral reflectance measurements: An indoor black house laboratory was set up in the National Authority for Remote Sensing and Space Sciences (NARSS) Station in order to measure the spectral reflectance of healthy and infected samples using the ASD FieldSpec® 3 JR spectroradiometer. According to Zhou *et al.*[17] and Zhang *et al.*[18], measurements have been carried out in the whole spectral range: Visible, near-infrared and short wave infrared, starting from 350-2500 nm (Table 1) with a spectral resolution of 3 nm from 350-1000 and 30 nm from 1000-2500 nm. A halogen light (50 W) was used as the light source for such indoor measurement. A white reference (spectralon) was used every 2 min in order to calibrate the spectral measurements. For each measurement, the number recorded was based on an average of five individual spectral measurements.

DNA extraction and molecular analysis preparation: Samples from all fruits were lyophilized and stored at -20°C. The extraction of DNA was performed by Plant Genomic DNA extraction KIT® (GeneJET, Thermo Scientific), according to the manufacturer's manual. DNAs were quantified and adjusted to 10 ng μL^{-1} before molecular amplification.

Real-time quantitative PCR (qPCR): Two specific primers, Bc424f: 5'-GCT TCC CCC GTA TCG AAG A-3'; Bc491r: 5'-CGA ACG GCC AGG TCA TCT-3', were used according to Saito *et al.*[19]. The qPCR assay based on SYBR® green was performed on all DNA samples. Five microliter from each DNA sample (10 ng μL^{-1}) was mixed in a final volume of 25 µL with 2X Fermentas SYBR green master-mix. Amplification program

Table 1: Spectral regions of the electromagnetic radiation under study

Spectral regions	Wavelength nanometers (nm)
Ultra violet	
UVA	315-400
Visible (VIS)	
Blue light	400-525
Green light	525-605
Yellow light	605-655
Red light	655-725
Far red	725-750
Near-infrared (Near-IR)	
Short wave infrared (SWIR)	750-1100
SWIR1	1000-1800
SWIR2	1800-2500
Near infrared (NIR)	1000-2500

was performed on a PicoReal™ system as the following: 95°C for 10 min, followed by 45 cycles for 30 sec at 95°C, 55°C for 45 sec and 72°C for 45 sec. Standard curve samples for qPCR were obtained by isolating DNA from *B. cinerea* mycelia. Negative control (NTC, no *B. cinerea* DNA) was included in each run. This amplification test was performed twice.

Dissociation curve analysis: The specificity of the qPCR reaction was confirmed using melting curve analysis in order to establish that differences observed in C_t values between reactions are valid and not due to the presence of non-specific products or contamination.

DNA quantification: Absolute quantification was performed by plotting unknown concentration of fruit samples on a standard curve of a serial dilution of known concentration. The standard curve was typically a plot of the cycle quantification (Cq, C_t) against the logarithm of the amount of pathogen's DNA. A linear regression analysis of the standard plot was used to calculate the amount of pathogen's DNA in unknown fruit samples. The slope was related to the PCR efficiency of the samples which was determined by performing serial dilutions of such samples. For a graph where log (DNA copy) was represented on the y-axis and C_t on the x-axis: PCR efficiency = $((10^{-1/slope}) 1) \times 100\%$. (©2013 Thermo Fisher Scientific, Inc.) (http://www.thermoscientific.com/onebio).

RESULTS

Screening of gray mold degrees in strawberries: Nine fruits (samples: A, B, C, D, E, F, G, H and I) from sweet charlie and only one from festival (sample 1) appeared healthy. Whereas, six samples (J, K, L, M, N and O) from sweet charlie and nine (2, 3, 4, 5, 15, 16, 17, 18 and 19) from festival showed *Botrytis* infection. Some of asymptomatic fruits upon collection were revealed gray mold within 2 days in the laboratory. The symptomatic fruits were grouped under the two infection degrees of gray mold: Low infection without rot and high infection associated with rot (Fig. 1).

Spectral detection of gray mold in strawberry fruit: All strawberry fruits, taken from two varieties, demonstrated the same trend of spectral reflectance for healthy and infected ones despite the slightly difference of spectral pattern due to plant variety (Fig. 2a, b). Results showed a higher spectral reflectance for the healthy fruit at NIR spectral zone: 700-1200 and 700-900 nm in festival and sweet charlie, respectively. However, such healthy fruit has always a clear distinguished spectral pattern located between low and severely infected samples at SWIR spectral zone: 1200-2500 nm. At SWIR spectral zone, the lowest spectral reflectance was observed for the fruit with a low infection level, while severely infected fruit demonstrated the highest spectral reflectance at such spectral zone for both strawberry varieties (Fig. 2a, b).

Fig. 1(a-b): Infection degrees of gray mold in two strawberry varieties (natural infection), (a) Sweet charlie and (b) Festival. From left to right: healthy, low infection, high infection

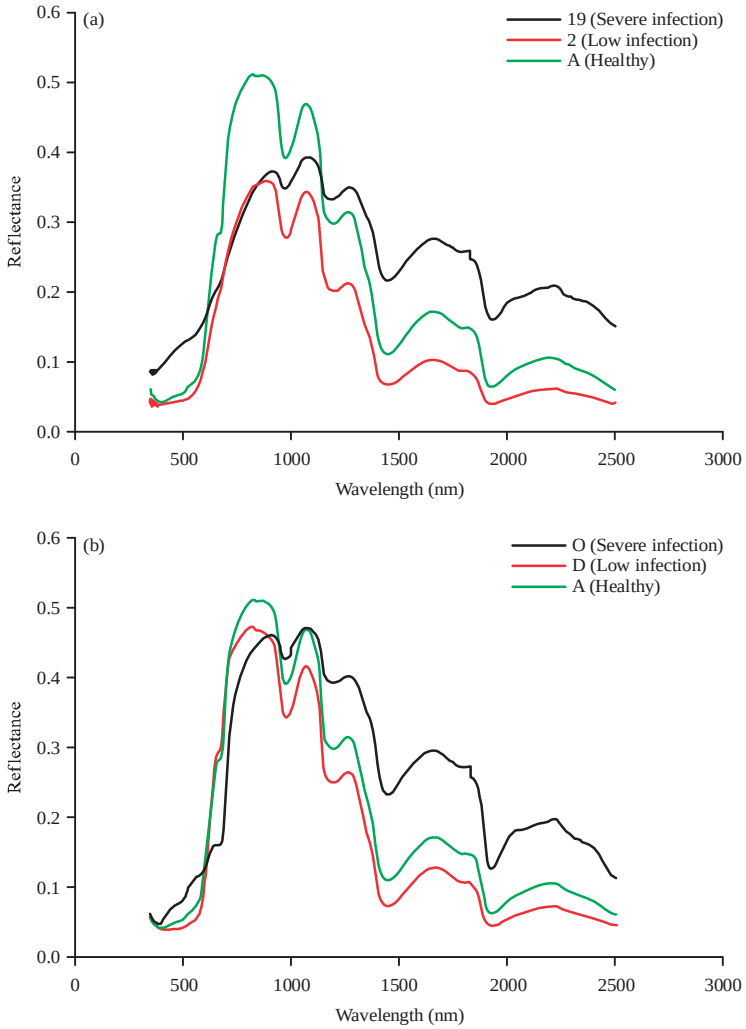

Fig. 2(a-b): Spectral reflectance pattern of healthy, low and severely infected strawberry fruits in two varieties (a) Festival and (b) Sweet charlie

Calibration of qPCR curves: Genomic DNA obtained from *B. cinerea* was used as a positive template (Cont+) for qPCR with primers Bc424f and Bc491r. The PCR product melting temperature was 77±1.00°C and no contaminating products nor primer dimers shown in this reaction. The standard curve for *B. cinerea* was generated by plotting the log of DNA quantity against the C_t value determined by qPCR (Fig. 3). The slope of the standard curve was -0.297, corresponds to DNA amplification efficiency of 98%. The detection limit was defined as the lowest population of *B. cinerea* that could be detected using SYBR Green qPCR method.

Molecular detection of gray mold in strawberry fruits: Amplification of genomic DNAs of samples using qPCR, taken from all symptomatic and asymptomatic strawberry fruits (Var. Festival and Sweet Charlie), was performed by SYBR green. All symptomatic samples (2-3-4-5-15-16-17-18-19-J-K-L-M-N-O) showed positive amplification curves at different C_t values depending on *Botrytis* quantity within each infected fruit sample (Table 2). The asymptomatic sample, A, did not show any amplification curve, while the other 9 asymptomatic samples (1, B, C, D, E, F, G, H, I) showed amplification curves at different C_t values, indicating that they have a quiescent infection (Table 2). The maximum C_t values: 31.2 and 33.6 were observed in samples: D and 18, respectively and defined as the lowest pathogen quantities (0.4 and 0.1pg, respectively) that were detected using SYBR green real time PCR method (Table 2).

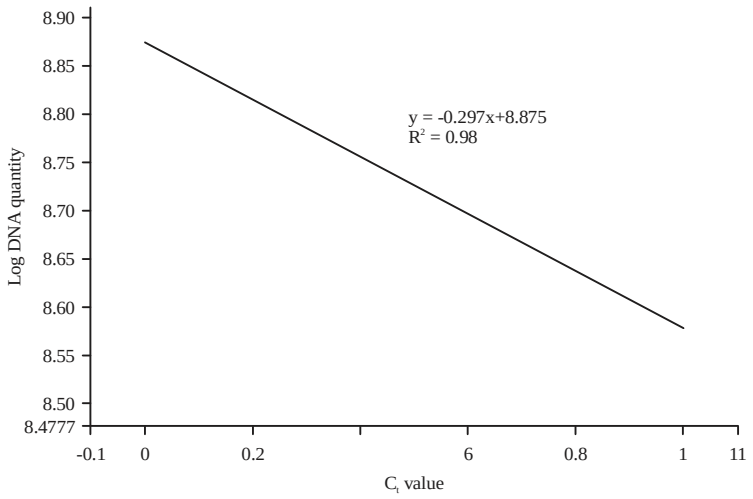

Fig. 3: Standard curve revealed a linear relationship ($R^2 = 0.98$) between the DNA quantity of serial dilutions of target genomic DNA of *B. cinerea* and the cycle threshold (C_t)

Table 2: Quantification of DNAs of *B. cinerea* in symptomatic and asymptomatic strawberry fruits using qPCR

Variety	Sweet charlie			Festival	
Samples	C_t	Q (pg)	Samples	C_t	Q (pg)
Cont+	18.8	1880.0	Cont+	21.1	500.0
A	0.0	0.0	1	23.4	84.5
B	30.3	0.8	2	28.3	2.8
C	30.8	0.5	3	22.3	178.1
D	31.2	0.4	4	25.3	22.9
E	29.4	1.4	5	24.2	48.8
F	29.3	1.4	15	27.4	5.4
G	29.6	1.1	16	33.2	0.1
H	28.5	2.4	17	26.0	13.7
I	28.6	2.3	18	33.6	0.1
J	28.4	2.7	19	25.6	18.5
K	29.0	1.7			
L	28.1	3.2			
M	28.0	3.4			
N	27.9	3.6			
O	25.4	20.9			

C_t: Cycle threshold, Q (pg): Quantity of Pathogen's DNA (picogram), Cont+: Positive control (Pathogen's DNA)

DISCUSSION

The present study tests the capability of using spectral reflectance of strawberry fruits to detect asymptomatic *Botrytis* infection. The presence of *B. cinerea* in asymptomatic plant varieties had been previously reported[4,5,9], concluded that alternative reliable and accurate techniques should be tested in parallel for early diagnostic of gray mold. This study used qPCR as a calibrated molecular system for early detection of *B. cinerea* to evaluate the accuracy of spectral reflectance for gray mold detection. Such alternative model could be potentially used to provide inexpensive synergic verification for early diagnostic. In the current study, post harvested fruits taken from naturally infected strawberry have been tested for gray mold infection. The measurements were in good agreements with existing data published in literature for asymptomatic and symptomatic plants, even if the hyperspectral reflectance levels differ. Generally, results showed that the healthy fruit having a higher spectral reflectance than that of infected ones throughout VNIR spectral range (700-900 nm) and showed a slightly difference of the spectral pattern between two strawberry varieties, may due to a different plant behavior according to the amount of water content[20]. Likewise, the typical spectral signature for healthy plant had been investigated throughout VNIR spectral range (VIS700-NIR1200 nm). This was in accordance with the previous studies on infected beans with *Botrytis fabae* which produced a decrease in NIR reflectance, at 800 nm due to infection[21]. In fact, VIS-NIR spectroscopy was previously applied to detect *B. cinerea* disease at spectral range 325-1075 nm in eggplant leaves at asymptomatic stages[10] and also investigated *Sclerotinia* rot disease on celery[13]. However, the current study investigated the potential for the use of spectral information for gray mold detection at red bands (700 nm) in the visible zone and NIR bands which revealed a good potential to discriminate *Botrytis* infection. In contrast, spectral reflectance pattern of infected fruits was inversed at SWIR spectral zone (1200-2500 nm) which refers to the effect on fruit composition due to *Botrytis* infection (negative relation of reflectance pattern was found between VNIR and SWIR spectral zones). Accordingly, hyperspectral spectroscopy was found to be useful in classifying infected trees by *Venturia inaequalis* at the spectral regions,

1350-1750 and 2200-2500 nm[16]. Indeed, it was confirmed that VNIR (700-900 nm) was the best spectral zone for differentiation between healthy and infected strawberry fruits due to infection effect on the cellular pigments of the fruit, while SWIR (1200-2500 nm) was the best spectral zone to classify infection degrees due to change in cellular structure and water content. Moreover, results showed that SWIR zone could distinguish between spectral patterns of *Botrytis* populations within the infected fruits at SWIR2 spectral zone (2055-2315 nm) according to Aboelghar and Abdel Wahab[22]. In fact, vegetation stress was a result of complex physiological processes, such changes affect the spectral behavior of plants through spectral reflectance, producing changes in the normal spectral reflectance patterns of plants[23,24]. Therefore, using remote sensing for detection of plant diseases assumes that stress induced by the infection interferes with physical plant structure and affects the light energy absorption, therefore, altering the characteristics of spectral reflectance of the plants. Previous studies had shown that changes in cell composition created a qualitative or quantitative variance in the spectral reflectance between healthy and infected plants[21]. Furthermore, it was shown that *Botrytis* removes water from the grape, leaving behind solids with a higher percent, such as fruit acids, sugars and minerals, thus the shortwave infrared (1400-2500 nm) was strongly influenced by the water in plant tissue, particularly at 1450 and 1940 nm.

Visible and near infrared (VIS-NIR) spectroscopy had been used extensively to detect plant anomalies[10,13,16], while other works had highlighted the importance of the red edge (maximum slope of plant reflectance at 680-720 nm) in order to predict plant stress. In addition, several studies had shown that spectral reflectance at 600-710 and 750-900 nm, the red and near infra-red wavelengths, respectively, were correlated with disease severity estimates. In the visible range, reflectance was considered to be influenced by plant pigments and in the near-infrared range reflectance is affected by changes in the anatomical structure of plant[25].

The differences in the reflectance measurements at specific wavelength intervals between *Botrytis*-infected and healthy strawberries and their correlation with qPCR results for the presence of *B. cinerea* suggested spectral reflectance method as a promising application for non-cost-effective and non-destructive diagnostic method for gray mold indoors as well as outdoors.

CONCLUSION

The two diagnostic applications, spectroradiometer and qPCR, were evaluated to compare their reliability to distinguish *Botrytis* infected fruits from healthy ones. Both different systems discriminated between the healthy and infected strawberry fruits and demonstrated their accordance in measurement results. Generally, the qPCR cycle and the spectral reflectance values of healthy fruit were higher than those of infected ones along with the whole sample collection. The results demonstrated that VNIR is the best spectral zone that would discriminate between infected and healthy fruits, while SWIR-2 is the best spectral zone to distinguish between population patterns of *Botrytis* within the infected fruits.

SIGNIFICANCE STATEMENTS

This study carried out the possible synergistic usage of an economic technique (remote sensing) and will help the researcher to early detect gray mold without sample preparation prior to measurements. Thus, a new methodology on gray mold diagnostic and possibly other combinations, would be used as a routine work.

ACKNOWLEDGMENT

This study was supported financially by the Science and Technology Development Fund (STDF), Egypt with Grant No. 2131.

REFERENCES

1. Elad, Y., B. Williamson, P. Tudzynski and N. Delen, 2007. *Botrytis* spp. and Diseases They Cause in Agricultural Systems-An Introduction. In: Botrytis: Biology, Pathology and Control, Elad, Y., B. Williamson, P. Tudzynski and N. Delen (Eds.)., Chapter 1. Springer, Netherlands, pp: 1-8.
2. Abdel Wahab, H., N.A.H. Aly and M.K. Ali, 2010. Improving detection means for strawberry gray mold caused by *Botrytis cinerea* in Egypt. Egypt. J. Phytopathol., 38: 107-119.
3. Abdel Wahab, H. and N.A.S. Helal, 2013. Evaluation of preharvest bioagent applications for both production and biological control of onion and strawberry under natural *Botrytis* infections. Afr. J. Plant Sci. Biotechnol., 7: 64-69.
4. Abdel Wahab, H., 2015. Characterization of Egyptian *Botrytis cinerea* isolates from different host plants. Adv. Microbiol., 5: 177-189.
5. Abdel Wahab, H. and N. Balabel, 2011. Detection and molecular characterization of *Ralstonia solanacearum* race 3 biovar2 isolates, using Insertion Sequence (IS) analysis in Egypt. Egypt. J. Phytopathol., 39: 154-168.
6. Dufresne, M., A. Hua-Van, H.A. El Wahab, S.B. M'Barek and C. Vasnier *et al*., 2007. Transposition of a fungal miniature inverted-repeat transposable element through the action of a Tc1-like transposase. Genetics, 175: 441-452.

7. Lopez-Berges, M.S., A. Di Pietro, M.J. Daboussi, H.A. Wahab and C. Vasnier *et al*., 2009. Identification of virulence genes in *Fusarium oxysporum* f. sp. *lycopersici* by large-scale transposon tagging. Mol. Plant Pathol., 10: 95-107.
8. Zeng, Q.Y., S.O. Westermark, A. Rasmuson-Lestander and X.R. Wang, 2007. Detection and quantification of *Cladosporium* in aerosols by real-time PCR. J. Environ. Monit., 8: 153-160.
9. Abdel Wahab, H. and R.A.A. Younis, 2012. Early detection of gray mold in grape using conventional and molecular methods. Afr. J. Biotechnol., 11: 15241-15245.
10. Wu, D., L. Feng, C. Zhang and Y. He, 2008. Early detection of *Botrytis cinerea* on eggplant leaves based on visible and near-infrared spectroscopy. Trans. ASABE., 51: 1133-1139.
11. Borengasser, M., T.R. Gottwald and T. Riley, 2001. Spectral reflectance of citrus canker. Proc. Fla. State Hortic. Soc., 114: 77-79.
12. Graeff, S., J. Link and W. Claupein, 2006. Identi cation of powdery mildew (*Erysiphe graminis* sp. *tritici*) and take-all disease (*Gaeumannomyces graminis* sp. *tritici*) in wheat (*Triticum aestivum* L.) by means of leaf reflectance measurements. Cent. Eur. J. Biol., 1: 275-288.
13. Huang, J.F. and A. Apan, 2006. Detection of *Sclerotinia* rot disease on celery using hyperspectral data and partial least squares regression. J. Spat. Sci., 51: 129-142.
14. Mishra, A.R., R. Ehsani, W.S. Lee and G. Albrigo, 2007. Spectral characteristics of citrus greening (Huanglongbing). Proceedings of the ASABE Annual Meeting, Paper No. 073056, June 17-20, 2007, American Society of Agricultural and Biological Engineers, St. Joseph, Michigan.
15. Mishra, A.R., R. Ehsani, D. Karimi and L.G. Albrigo, 2009. Potential applications of multiband spectroscopy and hyperspectral imaging for detecting HLB infected orange trees. Proc. Fla. State Hortic. Soc., 122: 147-151.
16. Delalieux, S., J. van Aardt, W. Keulemans, E. Schrevens and P. Coppin, 2007. Detection of biotic stress (*Venturia inaequalis*) in apple trees using hyperspectral data: Non-parametric statistical approaches and physiological implications. Eur. J. Agron., 27: 130-143.
17. Zhou, Q.F., Y.L. Zhan and J.F. Huang, 2010. Detection of nitrogen-overfertilized rice plants with leaf positional difference in hyperspectral vegetation index. J. Zhejiang Univ. Sci. B, 11: 465-470.
18. Zhang, C., J.M. Kovacs, M.P. Wachowiak and F. Flores-Verdugo, 2013. Relationship between hyperspectral measurements and mangrove leaf nitrogen concentrations. Remote Sens., 5: 891-908.
19. Saito, S., K.J. Dunne, K.J. Evans, K. Barry, L. Cadle-Davidson and W.F. Wilcox, 2013. Optimisation of techniques for quantification of *Botrytis cinerea* in grape berries and receptacles by quantitative polymerase chain reaction. Aust. J. Grape Wine Res., 19: 68-73.
20. Fabre, S., A. Lesaignoux, A. Olioso and X. Briottet, 2011. Influence of water content on spectral reflectance of leaves in the 3-15-µm domain. IEEE Geosci. Remote Sens. Lett., 8: 143-147.
21. Malthus, T.J. and A.C. Madeira, 1993. High resolution spectroradiometry: Spectral reflectance of field bean leaves infected by *Botrytis fabae*. Remote Sens. Environ., 45: 107-116.
22. Aboelghar, M. and H. Abdel Wahab, 2013. Spectral footprint of *Botrytis cinerea*, a novel way for fungal characterization. Adv. Biosci. Biotechnol., 4: 374-382.
23. Zarco-Tejada, P.J., J.R. Miller, G.H. Mohammed and T.L. Noland, 2000. Chlorophyll fluorescence effects on vegetation apparent reflectance: I. Leaf-level measurements and model simulation. Remote Sens. Environ., 74: 582-595.
24. Krezhova, D., 2011. Spectral Remote Sensing of the Responses of Soybean Plants to Environmental Stresses. In: Soybean-Genetics and Novel Techniques for Yield Enhancement, Krezhova, D. (Ed.). Chapter 11, InTech Publisher, Rijeka, Croatia, ISBN: 978-953-307-721-5, pp: 215-256.
25. Grant, L., 1987. Diffuse and specular characteristics of leaf reflectance. Remote Sens. Environ., 22: 309-322.