

ATR-FTIR VIBRATIONAL SPECTROSCOPY AS A TOOL TO INVESTIGATE THE GRAPEVINE (*VITIS VINIFERA* L.) SANITARY STATUS

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Abstract

A nondestructive, efficient, and accurate fingerprinting method using Fourier Transform Infrared (FTIR) spectroscopy has been used to study some differences between fleck virus infected and sanitized grapevine by biotechnological methods. Various functional groups present in grapevine virus free leaves were identified. The collection of FTIR spectra was carried out at 100 scans with resolution of 4 cm⁻¹ using Cosine apodization in the frequency regions of 4000 - 400 cm⁻¹. The amide I, amide II and carbohydrate regions of the spectrum have been shown to be the most significant in terms of disease identifying. FTIR spectroscopic method is effective in distinguish virus infected from healthy plants.

Key words: spectrum, frequency, vine, virus, healthy

1. INTRODUCTION

Fourier Transform Infrared (FTIR) is a simple and rapid spectroscopic technique that permits to study structure and intermolecular interactions. It enables to study objects at the tissue level without disturbing the biological sample and it is possible to receive information about their chemical composition. The FTIR method measures the vibrations of bonds within chemical functional groups and generates a spectrum that can be regarded as a biochemical or metabolic “fingerprint” of the sample. By obtaining IR spectra from plant samples, it might be possible to detect the minor changes of primary and secondary metabolites (Surewicz, Mantsch & Chapman 1993).

The advantages of Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) technique include a small amount of material required to experiment, and can be repeated many times very quickly to verify results, short time to obtain spectra, high sensitivity, and in the case of tissue it is not necessary to do preparation of samples (Topală 2013; Topală, Oprescu & Tătaru 2013). ATR-FTIR measures the total composition of an organism cell, producing an IR spectrum with bands from all cellular components (membranes, proteins, nucleic acids) (Oust 2004). It has been demonstrated that each kind of biological material has unique infrared signatures in the spectrum. From these spectra it is possible to reveal changes in biochemical properties of samples, which are studied at the molecular level (Olsztyńska-Janus et al. 2011).

Vitis species include *Vitis vinifera*, the cultivated grapevine, used for wine and grape agricultural production and considered the world's most important fruit crop. Grapevine, horticultural species of high national interest, is strongly attacked by different pathogens, including viruses, leading to both quantitative and qualitative yield losses. The most dangerous viruses in vineyards are fanleaf virus (GFLV), leafroll - associated viruses (GLRaVs), fleck virus (GFkV) and virus A (GVA, in single infection or in combinations. In order to obtain virus-free propagating grapevine material, numerous biotechnological methods are available. Thermotherapy and/or *in vitro* culture are known as conventional methods of virus elimination (Buciumeanu & Vișoiu 2000), but they are energy and time consuming. In the last period, alternative and environmental friendly methods of grapevine sanitation have been developed: *in vitro* chemotherapy, electrotherapy (Guță & Buciumeanu 2012) and cryopreservation (Wang et al. 2008).

A GFkV infection produces fleck disease, which is latent in European grapevine varieties and in most American rootstock. Symptoms are expressed in *Vitis rupestris* and consist of clearing of the veins of third and fourth order, producing localized translucent spots. Leaves with intense flecking are wrinkled, twisted and may curl upward. Fleck is ubiquitous disease reported from most viticultural countries in the world (Martelli & Boudon-Padieu 2006). GFkV can be eliminated by heat therapy, meristem, fragmented shoot or apex culture (Buciumeanu & Vişoiu 2000), *in vitro* chemotherapy (Guță, Buciumeanu & Vişoiu 2014), electrotherapy (Guță et al. 2011), somatic embryogenesis (Popescu, Buciumeanu & Vişoiu 2003).

The objective of this study was to determine if correlation could be made between FTIR spectra and grapevine health status, with regard the GFkV presence or the virus absence in the foliar tissue. The experiment has been done on GFkV-infected and GFkV-free grapevine, sanitized by *in vitro* chemotherapy and thermotherapy.

2. MATERIALS AND METHODS

The biological material consisted of grapevine plants belonging to *Vitis vinifera* L., Tămâioasă românească 3-2-2, quantitatively selected clone, currently under approval, but which has been identified as GFkV infected by enzyme-linked immunosorbent assay (ELISA). The necessity to promote in the culture the virus free propagating material led to the implementation of biotechnological method of virus elimination, *in vitro* thermotherapy and chemotherapy. The use of chemotherapeutics in media was other method for GFkV-free grapevine plants obtaining. In order to block the virus multiplication, a mixture of ribavirin + oseltamivir had been used in concentration variants V1 – 40+40 mg/L and V2 – 20+40 mg/L. The grapevine plants regenerated after applying of both methods of virus elimination have been ELISA analyzed; in order to confirm the diagnostic, the plants selected as GFkV- free have been retested by polymerase chain reaction (PCR) (Guță, Buciumeanu & Vişoiu 2014). *In vitro* thermotherapy consisted in maintaining the axillary buds culture at 37°C for 7 days; after that, the viable inocula have been transferred on fresh media and incubated at 22°C in the growth chamber. In the present experiment leaves collected both from GFkV infected and sanitized grapevine have been used as sample in the spectral analyze (Fig 1).



Fig 1. Grapevine plants of Tămâioasă românească 3-2-2 variety, sanitized by *in vitro* chemotherapy

A JASCO 6300 FTIR instrument containing Diamond ATR accessory with a diamond crystal (Pike technologies) was used for ATR-FTIR measurements and no further sample preparation was necessary. The spectra were acquired with 4 cm^{-1} resolution and 100 scans. The sample spectra were background subtracted using a spectrum collected in the absence of any sample. A small amount of fresh plant leaf was placed directly on the diamond crystal of the infrared spectrometer with constant pressure applied and data of infrared absorbance collected over the wave number ranged from 4000 cm^{-1} to 400 cm^{-1} , detector TGS, apodization Cosine, software Spectra Manager II. All spectra were performed by ATR corrected. The results recorded as absorbance.

The specific vibrations of functional groups were assigned to the bands and spectral parameters were determined. Some changes vibrations of bonds in $1000\text{--}1050\text{ cm}^{-1}$ region were noticed.

3. RESULTS

Typical ATR spectra have been used to study GFkV infected comparatively with virus free grapevines, regenerated by *in vitro* chemotherapy (GFkV free – V1 and GFkV free - V2) and *in vitro* thermotherapy (GFkV free - T).

Results of FTIR spectroscopic studies have revealed the existence of various chemical constituents in leaves (Fig 2, 3). The band at $3836\text{--}3831\text{ cm}^{-1}$ was characteristic for water. That was not surprising since leaves have high water content. The hydroxy groups at these frequencies may be within the same molecule (intramolecular hydrogen bonding) or they most likely exist between neighboring molecules (intermolecular hydrogen bonding) (Dent & Chalmers 1997). The impact of the hydrogen bonding is to produce significant band broadening and to lower the mean absorption frequency. The lowering of the frequency tends to be a function of the degree and strength of the hydrogen bonding (Coates 1996). NH absorption was observed at $3350\text{--}3296\text{ cm}^{-1}$. Bands appearing in the region of $2918\text{--}2916$ and $2850\text{--}2849\text{ cm}^{-1}$ were assigned to the asymmetric (ν_{asym}) and symmetric (ν_{s}) CH_2 stretching vibrations.

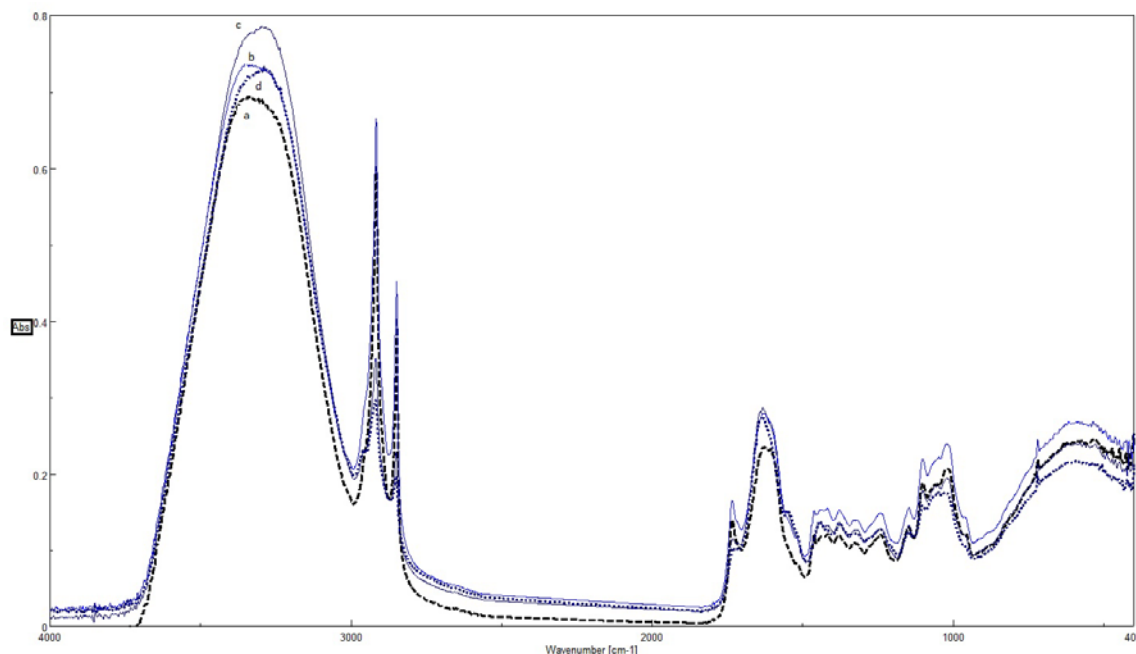


Fig 2. ATR-FTIR spectra of (a) dashed line GFkV infected leaf, (b) GFkV free - V1, (c) GFkV free - V2, (d) GFkV free -T

Table 1. FT-IR analysis of plant leaves, peak frequency (cm^{-1})

Sample	$\nu_{\text{asym}}\text{CH}_2$	$\nu_s\text{CH}_2$	$\delta_s\text{CH}_2$	νCO	$\nu\text{CH}_2\text{--O--CO}$	ρCH_2
GFkV-infected	2916	2849	1461	1733	1147	719
GFkV free - V1	2917	2850	1441	1731	1147	719
GFkV free - V2	2916	2849	1461	1733	1147	719
GFkV free - T	2918	2850	1433	1731	1147	719

The infrared spectra of protein are characterized by a set of absorption regions known as the amide region and the C–H region. The most widely used modes in protein structure studies in the amide region are amide I, amide II and amide III. The amide I band arises principally from the C=O stretching vibration of the peptide group. The amide II band is primarily N–H bending with a contribution from C–N stretching vibrations. The amide III absorption is normally weak and arises primarily from N–H bending and C–N stretching vibrations. The amide absorptions are considered sensitive to protein conformation; hence an increase or a decrease in the ratio of the intensities of the bands at $\sim 1549\text{ cm}^{-1}$ (amide II) and $\sim 1626\text{--}1634\text{ cm}^{-1}$ (amide I) could be attributed to a change in the composition of the whole protein pattern (Khani Baseri & Baker 2011; Komal & Kumar 2011). Amide I appears at 1633 cm^{-1} as a very strong band and amide II bands at 1549 cm^{-1} , as a medium broad shoulder (for GFkV free - T).

In addition, the peak at $1733\text{--}1731\text{ cm}^{-1}$ assigned to the C=O stretching vibration means that some carbonyl compounds: wax esters, ketones or of the carboxylic acid group possibly of galic acid and malic acid present in the grape leaves. Cuticular waxes constitute the main barrier limiting the transport across the plant–atmosphere interface. This property allows waxes to control cuticular transpiration, foliar uptake of xenobiotics resistance against fungi (Casado & Heredia 1999). The epicuticular waxes of grape berry fruit constitute about 25% of their cuticular membrane weight. They are mainly formed by oleanolic acid, a cyclic triterpenoid acid, which can constitute up to 60% of the total wax (Comménil, Brunet & Audran 1997). The $\delta_s\text{CH}_2$ absorption at 1461 cm^{-1} is a medium intensity band.

Band at 719 cm^{-1} which appeared in all samples is attributed to the methylene rocking vibration ρCH_2 . Some attention was also paid to the C–O stretching region between 1300 and 1100 cm^{-1} . As shown in Table 1, the spectra of each sample showed the presence of bands due to the C–O stretching vibration of esters. These bands are slightly broader and weaker than the corresponding carbonyl absorption bands.

GFkV infection induced in FTIR spectra of vine leaves some absorbance band or suppression of other. According to the fingerprint obtained, were registered the absorption values of infected grapevine virus free samples in the range $3836\text{--}719\text{ cm}^{-1}$, as presented in Table 2.

The comparison of FT-IR reflectance spectra of examined plant leaves, showed differences in their amide region (1626 in GFkV infected leaves to 1634 cm^{-1} for virus free grape leaves and 1549 cm^{-1} in GFkV free - T) and significant in the carbohydrate region as presented in Table 2. The peak at 1549 cm^{-1} , which has been observed only in the virus free grape leaves, was identified to belong to the amide II band. Also this peak could be assigned to hydroxyproline (Quilles, Humbert & Delille 2010; Taoutaou et al. 2010). In cucumber, increased hydroxyproline content of cucumber cell walls were associated with resistant, but not susceptible, responses of ten cucumber cultivars to *Cladosporium cucumerinum* (Hammerschmidt, Lamport & Muldoon 1984).

The carbohydrate region of the spectrum, between 1000 and 1147 cm^{-1} , is the region that has been shown to be the most significant in terms of identifying GFkV virus (disease). All manner of carbohydrates have peaks in these regions: starches, cellulose, sugars, and polysaccharides (Lammers, Arbuckle-Keil & Dighton 2009; Vasko, Blackwell & Koenig 1971). Polysaccharides are one of the obvious functions on enhancing body immunity, antitumor and resisting radiation damage.

In healthy plants, the spectra have a carbohydrate region that has a broad flat peak centered around 1049 cm^{-1} and weak shoulder at 1026 cm^{-1} . The GFkV virus infected plants have spectra with a large, sharp peak at 1017 cm^{-1} instead of 1049 and 1026 cm^{-1} . The spectra shape is almost the same. However the intensity of absorbance differs. In the interval $1800\text{--}600\text{ cm}^{-1}$, the most intense absorbance has been registered when the leaves are free of virus (GFkV free – V1 and GFkV free - V2) (Table 2).

Comparison of the FTIR spectrum of virus free leaves to that of grapevine infected leaves reveals a clear difference in the carbohydrates that are present in each plant (Figure 2).

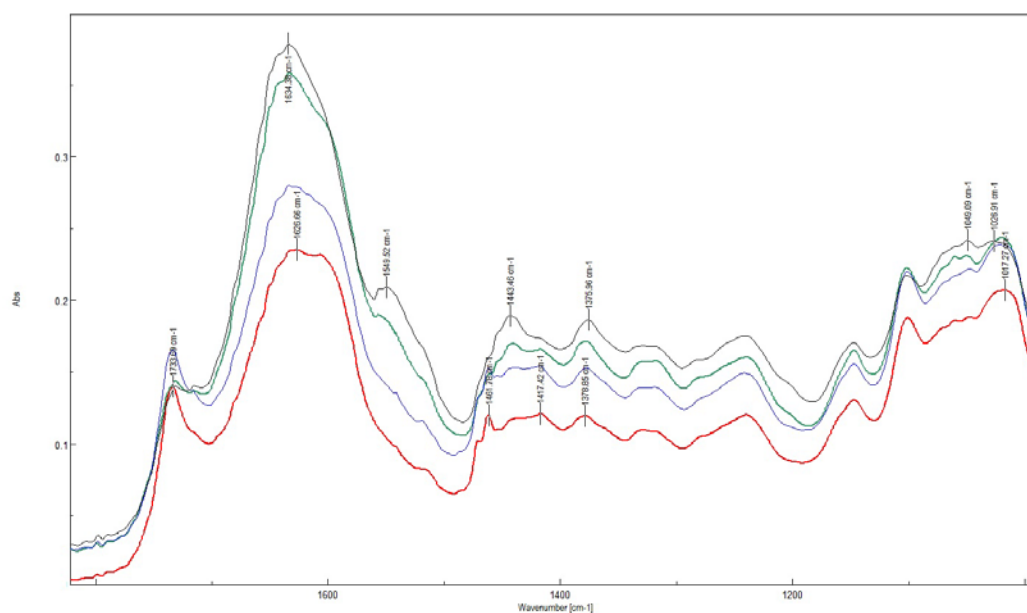


Fig 3. The FTIR-ATR spectra for virus free leaves (V1-green, V2-blue, T-black) and GFkV infected grapevine leaves (red)

The GFkV presence has induced the peak of 1417 cm^{-1} . It could be assigned to COO^- in the amino acids and fatty acid chains (Quilles, Humbert & Delille 2010). The role of fatty acids in disease resistance is that are major components of triglycerides, cutin, suberin, and waxes, plasma, plastid and mitochondrial membranes (Somerville et al. 2000), and about 70% of the membrane lipids in chloroplasts are represented by unsaturated fatty acids (Yaeno, Matsuda & Iba 2004). Abu-Nada et al. (2007) identified many fatty acids which the leaves were infected: palmitic acid, linoleic acid, and oleic acid, one day after infection.

Table 2. The ATR-FTIR absorbance peaks characteristic for GFkV infected and virus free grapevine leaf

Wave-length	GFkV-infected	GFkV free -V1	GFkV free - V2	GFkV free - T	Wave-length	GFkV-infected	GFkV free - V1	GFkV free -V2	GFkV free - T
3836	-	0.020	0.028	0.034	1378	0.119	-	-	-
3350	-	-	0.736	-	1377	-	0.170	0.154	-
3343	0.693	-	-	-	1375	-	-	-	0.186
3296	-	-	0.982	-	1328	0.109	-	-	0.167
3288	-	-	-	0.980	1319	-	-	0.139	-
2918	-	-	-	0.407	1317	-	0.157	-	-
2917	-	0.439	-	-	1241	-	-	0.148	-
2916	0.600	-	0.644	-	1240	0.119	0.159	-	0.174
2850	-	0.286	-	0.269	1147	0.130	0.164	0.155	0.170
2849	0.403	-	0.451	-	1102	-	0.222	-	0.216
1733	0.139	-	0.164	-	1101	0.187	-	0.219	-
1731	-	0.143	-	0.140	1050	-	0.203	-	-
1634	-	0.375	0.279	0.377	1049			0.221	0.241
1549	-	-	-	0.208	1026	-	-	-	0.240
1556	-	0.189	-	-	1022	-	-	0.237	-
1626	0.234	-	-	-	1020	-	0.242	-	-
1461	0.119	0.169	0.151	-	1017	0.206	-	-	-
1443	-	0.169	-	0.188	719	0.218	0.265	0.244	0.270
1417	0.120	-	0.154	-					

CONCLUSIONS

The virus infected grapevine plants revealed signs of change in the FTIR spectra, in the absence of visual symptoms as indicated in this study. The presence of disease induced by GFkV in grapevine plants had a distinctive spectrum in carbohydrates ($1147\text{-}1000\text{ cm}^{-1}$) and amide region (amide I: $1634\text{-}1626\text{ cm}^{-1}$ and amide II: 1549 cm^{-1}) that could be used to distinguish an infected plant from a healthy plant.

The ability of FTIR spectroscopy to show the changes in the chemistry of the plant, along with its ease of use, speed, and relative cost, could lead to its increased use as a predictive diagnostic method.

FTIR fingerprinting has the potential to be a fast and reliable analytical methodology for the discrimination between healthy and attacked crop plants by various viruses or other pathogens. The spectra of disease and healthy plants collected for the future work will be investigated with the aim to develop a chemometric model to be used for the prediction of the different diseases presence in the plants.

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