



MOLECULAR MONITORING OF HORSE CHESTNUT LEAVES AFFECTED WITH BIOTIC AND ABIOTIC DISORDERS

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SUMMARY

Results are given of a preliminary research aimed at the molecular investigation of dryness of horse chestnut leaves incited by *Guignardia aesculi* and environmental pollution. Modifications due to pathology and physiological disorders are characterized. Leaves showing pathologies or a physiological disorder compared to sound leaves were investigated by FTIR Spectroscopy. The spectra showed a weakening of the aromatic domain bands (1650-1500 cm⁻¹) and intensity inversion of two bands of this domain (1650, 1610 cm⁻¹) passing from the sound leaves to those affected with a physiological disorder. Moreover in the spectrum of these leaves, the 1300 cm⁻¹ band disappears, the carbonylic ester band (1730 cm⁻¹) decreases while the band at 1400 cm⁻¹ is enhanced probably because of partial hydrolysis of the ester groups.

These bands could be a spectroscopic marker of the disease. The results show that FTIR spectroscopy is a promising technique helping to unravel the processes involving the biological macromolecules of plant tissues.

RIASSUNTO

MONITORAGGIO MOLECOLARE DI FOGLIE DI IPPOCASTANO SANE E AFFETTE DA PATOLOGIE E FISIOPATIE. Sono riportati i risultati di una indagine a livello molecolare su foglie di ippocastano affette da seccume indotto da avversità fungine ed ambientali e sulla caratterizzazione delle modificazioni patologiche rispetto a quelle fisiologiche. Sono state esaminate foglie affette da seccume dovuto a *Guignardia aesculi* e da fisiopatia in confronto con foglie sane mediante spettroscopia vibrazionale FTIR. L'analisi comparata degli spettri evidenzia: diminuzione progressiva delle bande del dominio aromatico (1650-1500 cm⁻¹) passando dalle foglie sane a

quelle con patologia fungina e a quelle con fisiopatia; inversione dell'intensità delle bande principali del dominio aromatico (1650-1610 cm⁻¹) passando dalle foglie sane a quelle con fisiopatia, indice di variazioni nella composizione dei gruppi costituenti di questo dominio; scomparsa, nello spettro delle foglie con fisiopatia, di una banda debole a 1300 cm⁻¹ presente invece nello spettro delle foglie sane ed in quelle con patologia; diminuzione dell'intensità della banda carbonilica dei gruppi esterei (1730 cm⁻¹) nelle foglie con fisiopatie e contemporaneo aumento di una banda a 1400 cm⁻¹, probabilmente per idrolisi parziale dei gruppi esterei. I risultati mostrano che la spettroscopia FTIR può fornire informazioni utili per la comprensione dei processi biochimici inerenti le macromolecole dei tessuti vegetali.

Key words: FTIR spectroscopy, FTIR-ATR, leaf dryness.

INTRODUCTION

The horse chestnut (*Aesculus hippocastanum* L.), still a very widespread species in urban areas, is often struck by a serious form of dryness induced not only by the fungus *Guignardia aesculi* PK., St., but by a physiological disorder connected to environmental pollution.

Previous research proved the possibility of chemical control of the disease (Zechini D'Aulerio *et al.*, 1984) or a general reduction in pollution injuries by injecting the trunk with biostimulant drugs (Marchetti *et al.*, 1994). Therefore, it is evident that of an insight into the biochemical alterations induced at the foliar level by the physiological injuries is necessary in order to formulate interventions aimed at the prevention or at least the control of dryness of the leaves.

The methodology used for monitoring the leaf samples was the vibrational Fourier transform infrared spectroscopy (FTIR). This is an advanced research technique at the molecular level, computerized for the accumulation and intensification of vibrational spectra obtained from very small sample amounts. The vibrational infrared spectrum is characterized by absorption

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bands of which the frequencies and intensity are peculiar to the functional groups present in the molecular structure of the sample. A structure modification induces a change of the frequency and/or of the intensity of the corresponding band. Recently the technique proved to be very useful for the study and molecular characterization of complex biological systems (Clark and Hester, 1986). Furthermore, the technique was applied to the study of the mechanism of chilling stress and of the monitoring of the best operative conditions for cold storage of fruit and vegetables (Bertoluzza *et al.*, 1994, 1995), and also for characterizing organic functional groups in vegetal cuticular membranes undergoing structural modifications as a consequence of interactions with chemicals (Villena *et al.*, 1998).

MATERIALS AND METHODS

The samples of horse chestnut leaves were from different areas, some from the city center of Bologna, others the country side. The samples were kept for a few days in a moist chamber and afterwards observed under the stereomicroscope for the identification of possible sporulating structure.

On the basis of these observations the leaves were divided into three groups, a hundred units per group, with the following features: sound leaves, leaves with

dryness symptoms due to *G. aesculi* and leaves showing alterations due to a physiological disorder.

The infrared spectra were recorded in the 4000-400 cm^{-1} spectral region by a Jasco FTIR 300E spectrometer, 2 cm^{-1} resolution; about 1000 scans were accumulated for each sample in order to enhance the signal to noise ratio. The spectra were obtained in transmission mode on leaf samples, previously lyophilized and finely powdered, using the potassium bromide pellet technique (1% in KBr). Spectra were also performed from single leaves on a KRS-5 crystal, 8 reflection path length, mounted into an attenuated total reflectance cell (ATR), especially designed for recording infrared spectra on films. With this apparatus it was possible to obtain spectra directly on small leaf pieces (0.5 x 2 cm).

RESULTS AND DISCUSSION

Fig. 1 shows the FTIR spectrum of a sample composed of lyophilized sound leaves in a KBr pellet. The spectrum is characterized by several absorption bands of which the broad one, centered at about 3400 cm^{-1} is due to the stretching vibrations of the OH functional groups of water, alcohols and phenols. The doublet at 2925 and 2850 cm^{-1} is attributable mainly to the asymmetric and symmetric stretching modes of the CH_2 methylene group, the most abundant structural unit in

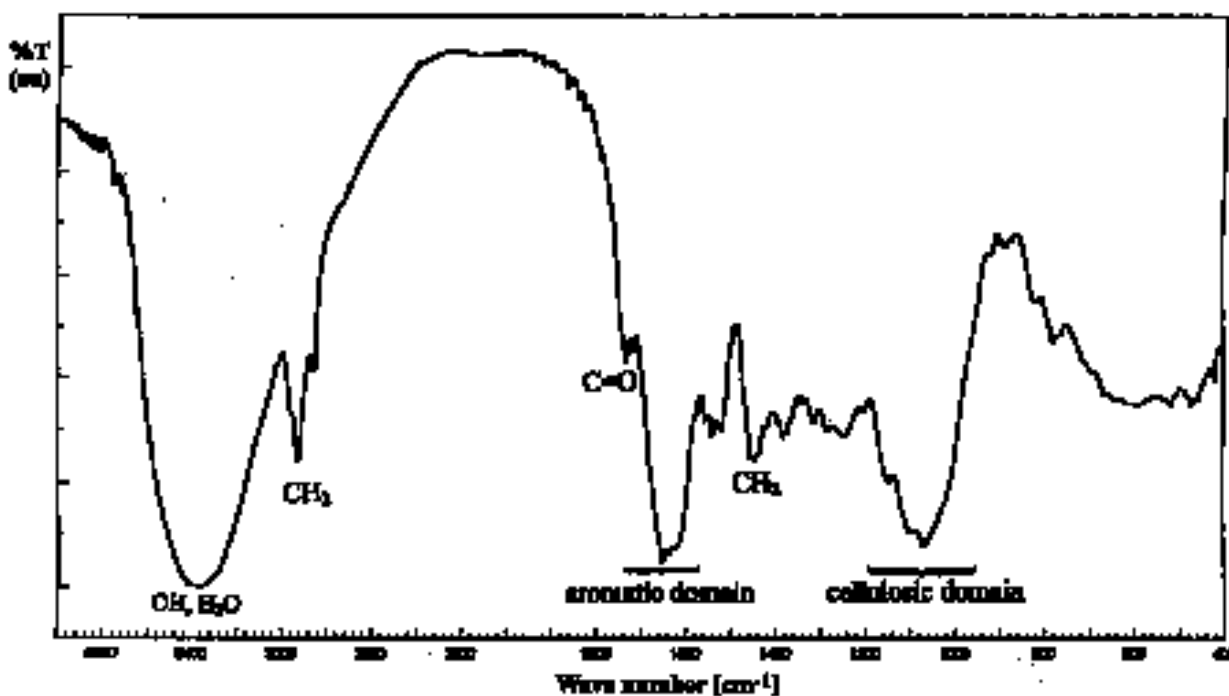


Fig. 1. FTIR absorption spectra of sound horse chestnut leaves.

plant products. The 1730 cm^{-1} band comes from the stretching vibration of the $\text{C}=\text{O}$ group indicative of the presence of carbonylic compounds mostly of esteric type (esters of fatty acids generally present in plant products). The set of bands between 1650 and 1500 cm^{-1} is due to vibrational modes of aromatic rings probably in aromatic derivatives, unsaturated conjugated compounds, phenols, chlorophyll, etc. (Ramirez, 1992). This set of bands is referred to as the aromatic domain. Hence, modifications of the structure of compounds accountable for the aromatic domain can be detected by analyzing this spectral region.

The medium intensity band at 1470 cm^{-1} is typical of the deformation mode of the CH_2 methylene groups and couples with the pair of bands of the same groups in the $2900\text{-}2800\text{ cm}^{-1}$ zone. The absorption bands between $1200\text{-}950\text{ cm}^{-1}$ have been assigned to the deformation modes of C-O and O-H groups of secondary alcohols (Fengel, 1993) and can be regarded as a spectroscopic marker of the cellulosic skeleton of the leaves (cellulosic domain). Therefore, this region can be in-

dicative of the presence of polysaccharides and of their structural modifications.

Fig. 2 reports the FTIR spectra of horsechestnut leaves with a physiological disorder (spectrum c) and fungus pathologies (spectrum b) compared to the spectrum of sound leaves (spectrum a).

The most evident modifications are: (i) gradual weakening of the intensity of the aromatic domain bands ($1650\text{-}1500\text{ cm}^{-1}$) passing from sound (Fig. 2a) to pathological (Fig. 2b) and to physiologically stressed leaves (Fig. 2c). In fact the decrease is highly noticeable when comparing the relative intensities of the aromatic domain bands with those of the cellulosic domain; (ii) the intensity of the two main bands of the aromatic domain ($1650, 1610\text{ cm}^{-1}$), marked by 1 and 2 in Fig. 2 and as yet not attributed, changes passing from spectrum a (sound leaves) to spectrum b (leaves with pathologies) and to spectrum c (leaves with a physiological disorders). In spectrum a the component 1 is more intense than the component 2, in spectrum b the two components are about the same intensity whilst in spec-

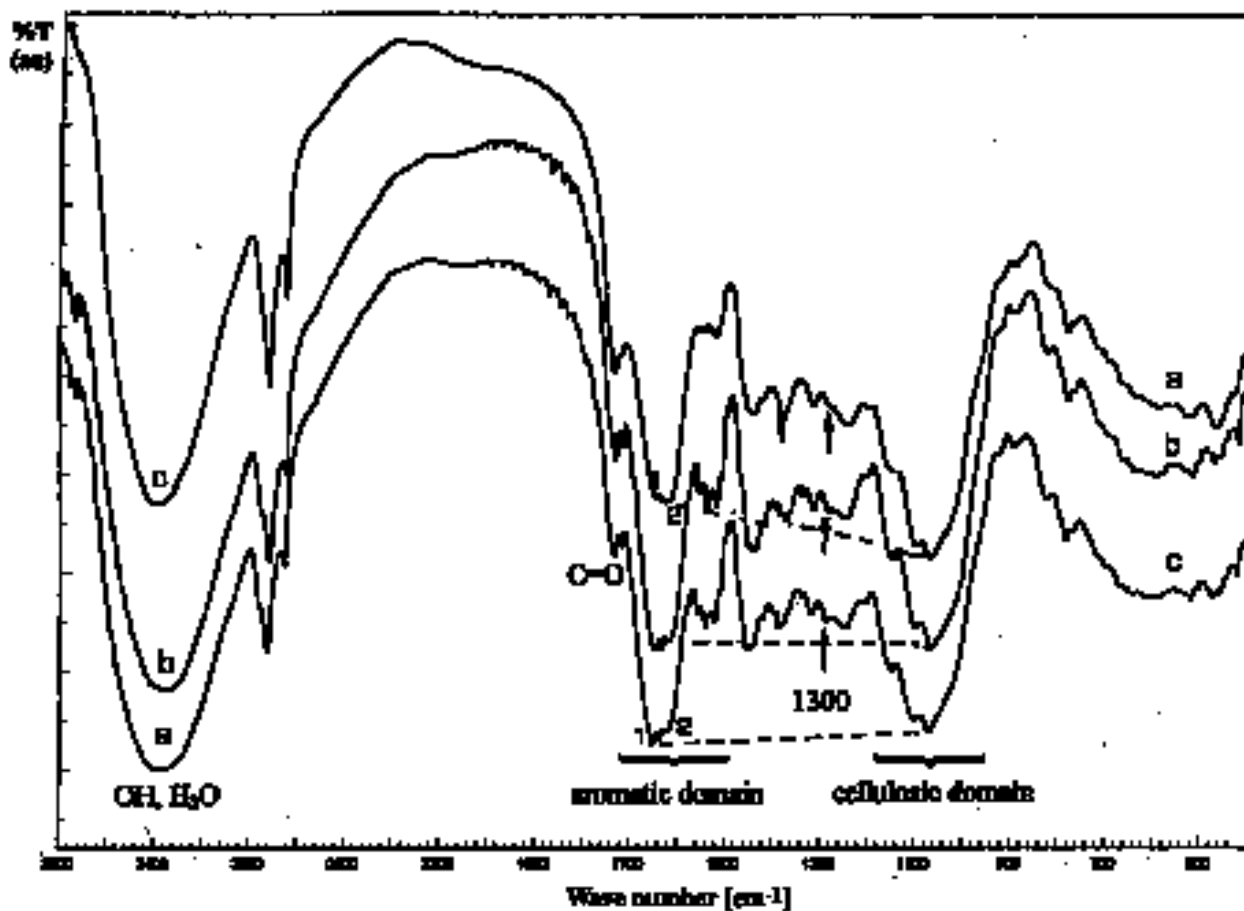


Fig. 2. FTIR absorption spectra of sound leaves (a), leaves affected with a fungus (b), leaves affected with a physiological disorder (c).

trum c the two components reverse their intensity with component 2 being more intense than component 1; (iii) disappearance in spectrum c (leaves with a physiological disorder) of a weak band which in contrast is present (marked by an arrow in Fig. 2) at about 1300 cm^{-1} in spectrum b (leaves with fungus pathologies) and in spectrum a (sound leaves).

The modifications observed in the spectra of the three different samples of horse chestnut leaves provide information on the main structural changes of the aromatic domain going from sound leaves to those affected by disease, whilst the bands of the cellulosic domain seem to remain almost unchanged. In the spectral region of the aromatic domain the different intensity of components 1 and 2 in the three groups of leaves means a different composition of the functional groups which contribute to the components of this domain. The weak band at 1300 cm^{-1} , not yet attributable with certainty, can however be considered the first and important spectroscopic marker of the presence of a physiological disorder.

In Fig. 3 the spectrum of the green (a) and of the dry brown fraction of the leaves (b) suffering from a physiological disorder are given. The comparison of the two spectra shows again a new band at about 1300 cm^{-1} [clearly present in the spectrum of the green part of the

leaf (a)] which undergoes, in contrast, a strong decrease in the spectrum intensity of the dry part of the leaf (b).

Fig. 4 shows the FTIR-ATR spectra recorded directly on a small piece of a single leaf, to be precise of the upper surface of a sound leaf (a) and of the upper green surface of a leaf with a physiological disorder (b). By comparing the two spectra a decrease in the relative intensity of the C=O carbonylic band of the esteric groups at 1730 cm^{-1} can be observed, passing from the sound to the diseased leaves together with an increase in the relative intensity of the broad band centered at about 1400 cm^{-1} . Such a trend leads to the hypothesis of a partial breakage process of the esteric bonds in the altered leaf (probably in consequence of a hydrolysis process) and the formation of carboxylate groups (COO^-), also confirmed by a band typical of these groups at about 1400 cm^{-1} .

CONCLUSIONS

The results of this preliminary research show that the vibrational FTIR spectroscopy can be a promising tool helping in monitoring, through spectroscopical molecular markers (aromatic, cellulosic domains) the structure of plant tissue constituents exposed to differ-

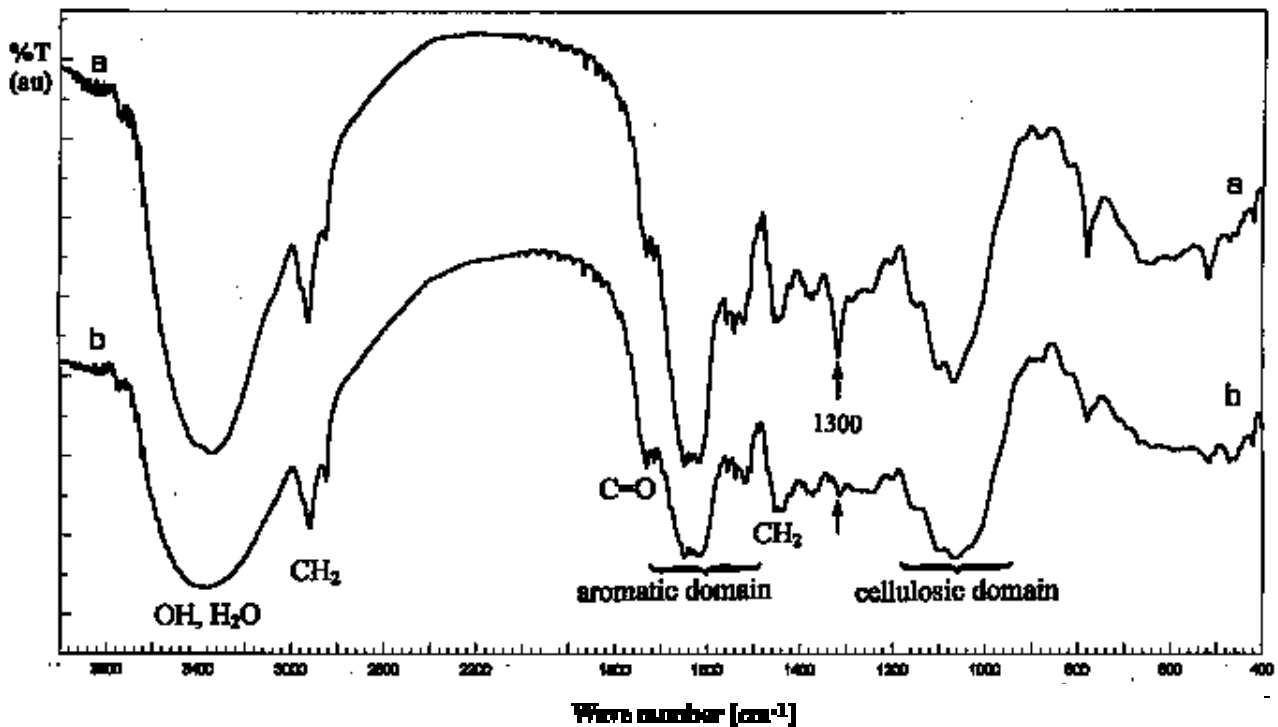


Fig. 3. FTIR absorption spectra of the green portion (a) and of the dry brown portion (b) of horse chestnut leaves affected with a physiological disorder.

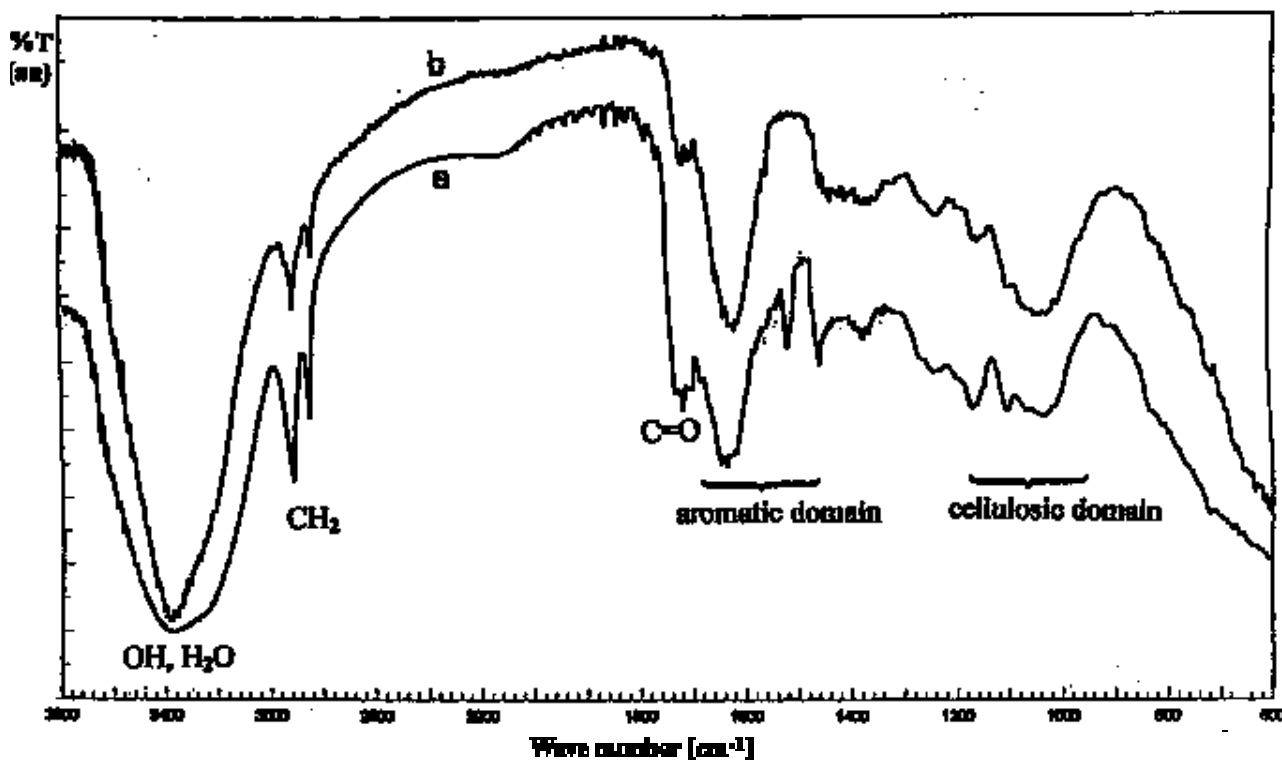


Fig. 4. FTIR-ATR spectra of the upper surface of a sound leaf (a) and of the upper surface of a leaf affected with a physiological disorder (b).

ent situations (sound plant tissues or affected with fungal or environmental adversities) and hence in understanding the processes involving the biomacromolecules of plants.

The study has also proved that the utilization of different techniques (KBr pellet, ATR cell) gives complementary results which provide a wider interpretation of the spectroscopic response.

As a whole, the research has defined several modifications in the functional groups of the foliar constituents which mainly are: (i) progressive weakening of the intensity of the bands characterizing the aromatic components as regards the cellulosic one, passing from the sound leaves to those affected with fungus pathology and a physiological disorder, together with an intensity inversion of the two main bands of the aromatic domain. This is an indication of structural changes following the occurrence of biodegradative processes due to the particular disease; (ii) progressive decrease either in the relative intensity of the aromatic domain bands with respect to the cellulosic ones or in the band at 1300 cm^{-1} regards also the leaves with a physiological disorder passing from the green to the dry fraction. The trend is similar to that observed in physiologically disordered leaves as compared to sound ones. The 1300

cm^{-1} bands, could act as a molecular marker of the presence of a physiological disorder; (iii) decrease in the intensity of the esteric carbonylic band passing from sound to leaves suffering from a physiological disorder and increase in the intensity of the band centered at 1400 cm^{-1} , probably owing to a partial hydrolysis process of the esteric groups followed by carboxylate group formation.

The research allows the analysis by vibrational FTIR spectra of a series of components (hydrophobic and hydrophilic fractions, lipids, sterols, unsaturation degree) of horse chestnut leaves, otherwise obtainable only by complex chemical separation techniques.

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