

Fourier-Transformed Infrared Spectroscopy Applied for Studying Compatible Interaction in the Pathosystem *Phytophthora infestans*-*Solanum tuberosum*

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Abstract

In this study we used the FOURIER-TRANSFORMED INFRARED (FT-IR) technique to examine the compatible reaction of potato (*Solanum tuberosum*) to infection by the late blight agent *Phytophthora infestans*. Three virulent isolates have been used, different by their level of pathogenicity on R2 potato. The response was dependent on the pathogenicity of the isolate. The Infrared spectra in the middle infrared region (MIR) of infested versus healthy (control) leaves showed that controls absorb (intensity) mostly in the range 650-950 cm⁻¹. The absorbance was higher for the most virulent isolate in the range 950-1650 cm⁻¹. In the domain 3000-4000 cm⁻¹ for the control and less pathogenic interactions, have absorbed more than the highly infested samples. The shape of spectra has generally been the same shape. However, the number of peaks suppressed/induced after inoculation and in function of the isolate differs indicating a perturbation of plant metabolism. FTIR technique provides important information about the events occurring during the interaction.

Keywords: *Phytophthora infestans*, *Solanum tuberosum*, compatible interaction, resistance, FT-IR

Introduction

Potato (*Solanum tuberosum*) is the fourth world crop, after wheat, rice and corn. In Romania, potato occupies the second place. *Phytophthora infestans* (Mont.) de Bary (1876) the agent of late blight is the most important pathogen on potato crop. It attacks also other *Solanaceae* species, for instance, tomatoes or eggplants. During the last century (1845 and 1847) the well known great Irish famine was caused by this pathogen. Before that, in the early 1840s it was also problematic in the northern United States (Stevens, 1933; Fry and Smart, 1999). Currently, *P. infestans* is responsible for multibillion-euro losses annually in both potato and tomato (Razukas *et al.*, 2008).

Potato breeding for late blight resistance was based on introgression of resistance (*R*) genes from the wild species *Solanum demissum*. Eleven *R* genes have been introgressed (*R1*, *R2*... *R11*). This type of resistance has been easily overcome by the pathogen. Actually the attention is paid to other wild relatives such as: *S. bulbocastanum*, *S. phureja*. The resistance genes from *S. bulbocastanum* actually offer a broad spectrum resistance to late blight. Until now, 5 *R* genes have been mapped from *S. bulbocastanum* (Simko *et al.*, 2007).

Being a hemibiotroph, *P. infestans* adopts a two step infection procedure: in the first stage the pathogen needs living host cells; the second is represented by an extensive

necrosis resulting in colonization and sporulation (Kamoun and Smart, 2005). A successful infection involves the secretion of proteins and other molecules (Huitema *et al.*, 2004). Some of these molecules participate to the pathogen attachment to plant surface, others to breaking physical obstacles to infection (cell wall membranes), and several modify the plant physiology by suppressing the plant defence (Kamoun and Smart, 2005).

Generally the interaction study between late blight agent and potato is made by the molecular technique such as microarray, differential display in the gene expression level. The majority of the studies in this field had focused on the incompatible interaction. We considered that it is important also to study the interaction in the case of compatibility also. In this study, we tried to monitor the reaction of potato to late blight agent infection, in the case of compatible interaction by the FTIR technique.

FTIR is a simple and rapid technique. It could be used directly and in non-destructive manner. It measures the total composition of an organism cell, producing an IR spectrum with bands from all cellular components (membranes, proteins, nucleic acids) (Oust *et al.*, 2004). However, matrix heteropolymers, pectins and hemicelluloses, phenolic oxidation products, dyes, alcohols, terpenes, tannins, waxes, and other plant components make it difficult to use nondestructive spectroscopic methods for plant status evaluation (Ivanova and Singh, 2003).

Materials and methods

R2 Potato (potato plant with the R2 resistance gene to *P. infestans*. R1, R3, and R5 are potato plant with the resistance gene R1, R3, and R5 respectively; ' Bintje' and ' Désiree' are two potato cultivars R genes free) plant grown in green house has been used in this experiment. The infestation has been performed using three *P. infestans* isolates (A1.1 (NL08045), A1.3 (80029), and A2.2 (NL08009)) kindly send to us by W.G. Flier, G.B.M. van den Bosch and G.J.T Kessel from Plant Research International BV. All three isolates were able to colonize potato leaves. For this experiment we used the detached leaf test as described by the CIP manual (Cip, 1997). The control (uninfested leaves) was inoculated with sterile distilled water.

In the fifth day, the leaves have been ground into a fine powder in a mortar and pestle under liquid nitrogen, and then transferred to an eppendorf 2 ml tube. 1 ml of 70 % methanol was added, and mixed by sonication for 15 minutes, then centrifuged. 100 µl of supernatant was used for analysis. Infrared profile was realized using the Shimadzu Prestige 2, Apodization: Happ-Genzel spectrophotometer, the profile was recorded in the wavelength range of 4000-500 cm⁻¹. For notification, R2 A1.1, R2 A1.3, and R2 A2.2 are the potato leaves inoculated by the isolate A1.1, A1.3, and A2.2 respectively.

Results and discussions

All the three isolates have been able to infest potato leaves, as it has been expected. However, the intensity of symptoms (Fig. 1) differs in function of the pathogen isolate. The isolate A1.1 has been the less virulent and the

isolate A2.2 has been the most virulent. The A2.2 has been able to grow mycelium as seen in the Fig. 1 in the 5th day post inoculation. In the case of the other isolates, the mycelium could not be detected. The symptoms just start to appear in these treatments.

FTIR Analysis

The infection of R2 potato plant by different isolates has had different consequences on the potato leaves, either induction of some absorbance band (1), or suppression of others (2) as shown in the Tab. 1, Fig. 2 represents the FTIR spectra of R2 potato 5 days post inoculation of healthy and infested leaves by the 3 isolates A1.1, A1.3, and A2.2, and Fig. 3 the FTIR spectra in the region 650-1800 cm⁻¹. The spectra shape is almost the same. However the intensity of absorbance differs. In the interval 600-1800 cm⁻¹, the most intense absorbance has been registered when the leaves are inoculated with the isolate A1.1.

According to the fingerprint obtained, we registered the absorption values of healthy (R2) and infested potato leaves (R2 A1.1, R2 A1.3, R2 A2.2) in the IR range 662-3900 cm⁻¹, as presented in Tab. 1.

The implication of amino acids in plant reaction to pathogen infection generally was not intensely studied, especially in the pathosystem *P. infestans*-*S. tuberosum*. Recently, Abu-Nada *et al.* (2007) have found that nine amino acids (L-aspartic, L-threonine, L-alanine, L-proline, L-valine, L-isoleucine, L-tyrosine, glutamine and L-phenylalanine) are up-regulated when potato was infested by *P. infestans*, in early stage of infection (after two days) and after four days, the induction became not significant compared to the control. These data indicates a significant

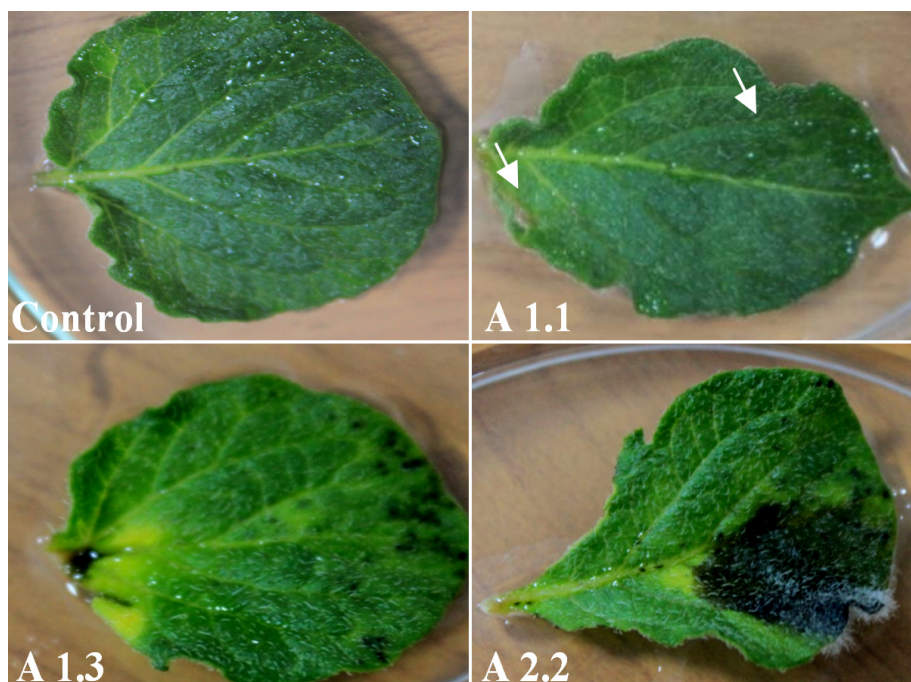


Fig. 1. Symptoms observed in the 5th day post inoculation of R2 potato leaves. The control was inoculated with distilled sterile water; A1.1, A1.3, and A2.2 are isolates of *P. infestans*

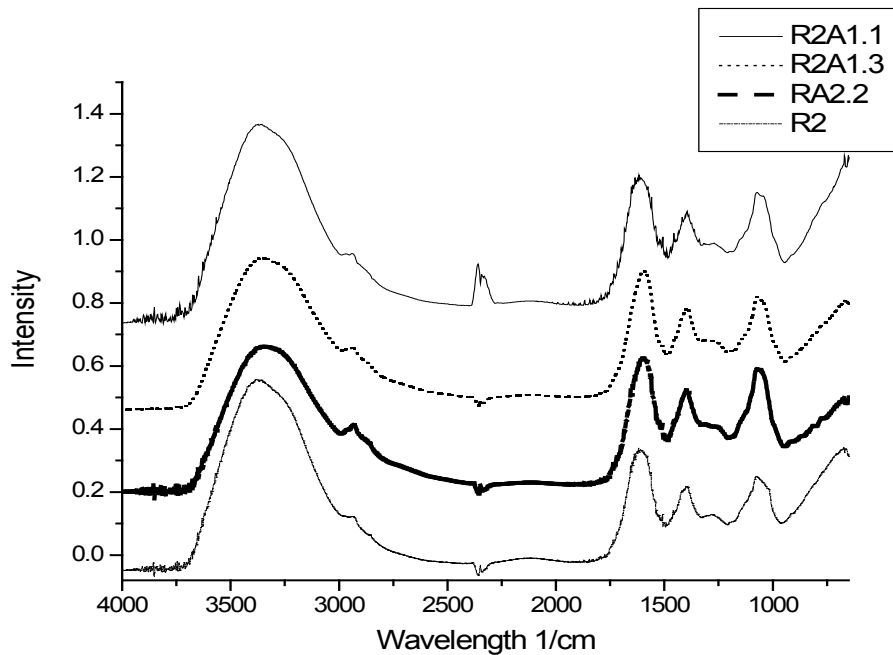


Fig. 2. FTIR spectra of R2 potato 5 days post inoculation of healthy and infested leaves by the 3 isolates A1.1, A1.3, and A2.2

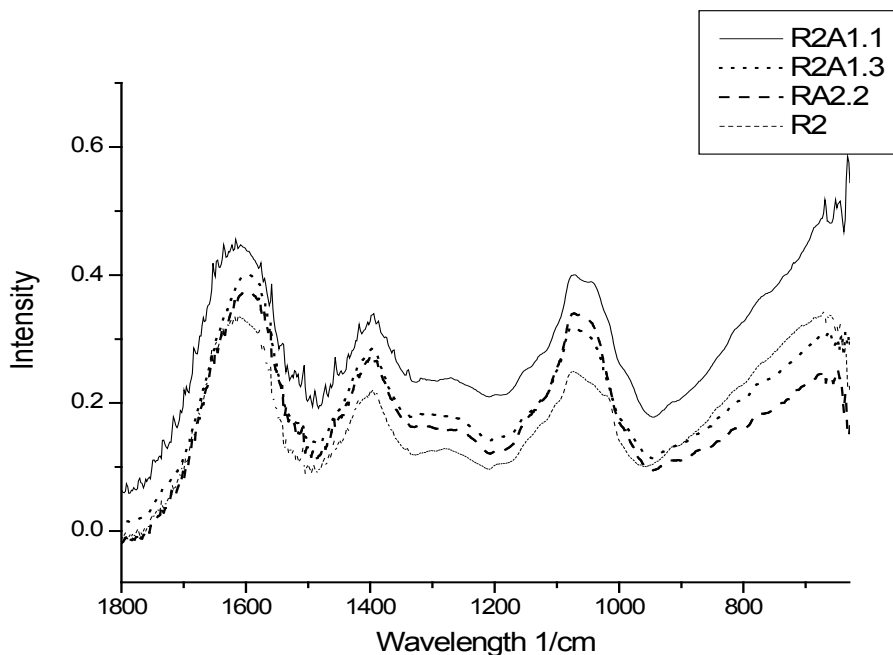


Fig. 3. FTIR spectra in the zone 650-1800 cm^{-1}

role of amino acids in primary defence response against *P. infestans* (Abu-Nada *et al.*, 2007).

In our case, we used three *P. infestans* isolates (all virulent on potato plant used R2). The FTIR analysis was made in the 5th day post inoculation. Many peaks which have been attributed to different amino acids have been detected. The absorbance peak at 662 cm^{-1} , has been detected in the case of healthy and A2.2 treatment. The absorbance has been more intense in the case of healthy leaves (0.2561 for non-infested and 0.1812 for infested R2). This peak was assigned by Wolpert and Helling (2006) to amidazole

group. Amidazole can be incorporated into many important biological molecules, such as amino acid histidine. The peak at 775 cm^{-1} , which has been induced by A2.2, has been assigned to the amino acid histidine (Wolpert and Helling, 2006). Many other peaks have been attributed to amino acids, e.g. 1319 cm^{-1} to leucine, found only after infection by A1.3 and A2.2. The same amino acid has been found in the healthy treatment but at 1341 cm^{-1} , the same peak has been observed also after infection by A2.2. Leucine with valine and isoleucine are important amino acids for the synthesis of plant defence secondary metabolites

Tab. 1. The FTIR absorbance peaks characteristic for healthy and infested potato leaves in the IR range 662-3900 cm⁻¹

Wavelength	R2	A1.1	A1.3	A2.2	Wavelength	R2	A1.1	A1.3	A2.2
662	0.2561	-	-	0.1812	2162	0.109	-	-	-
671	0.2625	-	0.1508	-	2170	-	0.1118	-	-
775	-	-	-	0.1379	2201	-	0.1103	-	-
814	-	-	-	0.1192	2270	-	0.1107	-	-
1063	-	-	-	0.3318	2280	0.0997	-	-	0.1026
1072	-	0.1611	-	0.3354	2284	-	-	0.104	-
1074	0.2296	-	-	-	2330	-	0.1967	-	-
1076	-	-	0.2386	-	2344	-	0.2028	-	-
1196	0.1029	-	0.0891	0.137	2359	-	0.2363	-	-
1281	0.1398	-	-	-	2384	0.099	-	-	0.1078
1302	0.1399	-	-	-	2401	-	-	0.1068	-
1319	-	-	0.1487	0.1955	2421	-	-	-	0.1087
1339	-	-	-	-	2473	0.1003	-	-	0.1153
1341	0.1494	-	-	0.2042	2502	-	0.1144	-	-
1362	-	0.1344	-	-	2509	0.1027	-	-	-
1387	-	0.1876	-	-	2521	-	0.1161	-	-
1391	0.2421	-	-	0.3057	2538	-	0.1178	-	-
1396	-	0.2017	0.2657	0.3138	2581	0.108	-	-	-
1418	-	0.1918	-	-	2859	0.1946	-	-	-
1508	-	-	0.1682	0.2195	2934	-	-	-	0.2987
1524	-	-	-	0.2282	2938	0.255	-	0.2738	-
1547	0.2277	-	-	-	2940	-	0.2878	-	-
1553	-	-	-	0.327	2967	-	-	0.2702	-
1558	0.3139	0.2993	-	-	2968	0.2556	0.2874	-	-
1572	0.345	0.3115	-	0.4111	3267	-	-	-	0.5345
1597	-	-	-	0.4432	3287	-	-	-	0.5421
1601	-	-	0.4206	-	3296	-	0.6825	-	-
1609	0.3943	0.376	-	-	3300	-	-	-	0.5457
1624	0.391	-	-	-	3302	0.6612	-	-	-
1717	-	0.1413	-	-	3304	-	-	0.5543	-
1923	-	-	0.0841	-	3321	-	-	0.5605	-
1969	0.0939	-	0.0931	-	3331	-	-	-	0.5514
1992	0.1001	0.1008	-	-	3335	-	0.7021	0.5645	-
2010	-	-	0.1008	-	3347	0.6868	-	0.5662	0.5527
2018	-	0.1049	-	-	3354	-	-	0.5675	0.5524
2041	0.1044	0.1065	-	-	3356	0.6931	0.7105	-	-
2066	0.1071	0.1089	0.1047	0.1046	3374	-	0.7111	0.5663	-
2091	-	0.1103	-	0.1054	3379	0.6958	-	-	-
2116	0.1095	0.1118	0.107	-	3836	-	0.1227	-	-
2139	-	-	0.1072	-					

such as cyanigenetic glycosides, and glucosinolates (Coruzzi and Last, 2000).

The peak at 1072 cm⁻¹, detected in the infested R2 by A1.1 and A2.2, was assigned to methionine (Wolpert and Helling, 2006). The intensity of absorbance has been doubled, from 0.1611 to 0.3354. The same amino acid has been found in the case of healthy R2, at 1281 cm⁻¹ with lower intensity (0.1398) indicating the degradation of cell

membranes and/or defense enzymes used by potato for defense. The amount of released methionine is proportional to the evolution of symptoms.

The band at 1341 cm⁻¹ was attributed to serine (Pawlukojc *et al.*, 2001), and the one at 1339 cm⁻¹ they was assigned to threonine. While serine has been detected in the case of healthy R2, threonine was identified at infested R2 by A2.2. The same authors have attributed the peaks at

1387 and 1319 cm^{-1} to threonine observed after infection by A1.1 and A1.3 respectively. So, the amino acid threonine has been detected in all the infested potato *R2* and not in the case of the healthy one. The peak at 1363 cm^{-1} , was attributed to glutamine (Ramirez *et al.*, 1998), which has been induced by the A1.1.

The absorption bands between 1200-950 cm^{-1} have been assigned to the deformation modes of C-O and O-H groups of secondary alcohols, and can be regarded as a spectroscopic marker of the cellulosic skeleton of the leaves (cellulosic domain); therefore, this region can be indicative of the presence of polysaccharides and of their structural modification (Bertoluzza *et al.*, 1999). The peak at 1062 cm^{-1} was attributed to the P-O-C terminal phosphate by Cagnasso *et al.* (2010) found in *R2* infested by A2.2 (1063 cm^{-1} in our case). Also, Bertoluzza *et al.* (1999), considered the band at 1300 cm^{-1} as a marker for healthy horse chestnut, absent in diseased or abiotic stressed leaves. In our studies, the peak at 1302 cm^{-1} was identified in the case of healthy *R2* and absent in all infested *R2*. However we are not sure that this band could be considered as a marker for healthy plant specially potato; we did not find this band in the case of the other healthy treatment (Bintje, Desiree, *R1*, and *R5*) (unpublished data).

The infection of potato by *P. infestans* has induced the peak at 1396 cm^{-1} . It could be assigned to COO^- in the amino acids and fatty acid chains. Quilles *et al.* (2010) assigned the peak 1400 cm^{-1} to COO^- . 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 *et al.*, 2004). Abu-Nada *et al.* (2007) identified many fatty acids which were up regulated when potato was infected by *P. infestans*: palmitic acid, linoleic acid, and oleic acid, one day after infection. In the second day after infection, three down regulated and three up-regulated fatty acids: 9-octadecanoic acid, hexadecanoic acid, eicosanoic acid, 9-hexadecenoic acid, 7, 10, 13-hexadecatrienoic acid, 7, 10-hexadecadienoic acid respectively. In the fifth day post infection, only eicosanoic acid has been up-regulated, when 9-hexadecenoic acid, 9, 12, 15-octadecatrienoic acid, 7, 10, 13-hexadecatrienoic acid, and 7, 10-hexadecadienoic acid have been down-regulated. Abu-Nada *et al.* (2007) noticed that following pathogen invasion, over time, their total abundance was reduced. In our case, for *R1*, the infection by A2.2 suppressed the peak. In the same way, in the infested *R3*, the peak did not appear. In case of Desiree and Bintje, the infection has induced a decrease of peak intensity by 30% (unpublished data). In these cases, either the production of fatty acids was reduced, as a result of the chloroplasts dysfunction, due to pathogen infection (Soulie *et al.*, 1989) or due to the use of unsaturated fatty acids was increased (Abu-Nada *et al.*, 2007). However, infection of *R2* has induced the peak. In the case of *R5*, the

absorbance has doubled after infection (from 0.2324 to 0.4218) (unpublished data). Wolpert and Helling (2006) assigned this peak to valine.

The peak at 1547 cm^{-1} which has been observed in the non-infested *R2*, this peak was identified to belong to the amid II band (Quilles *et al.*, 2010). Also this peak could be assigned to hydroxyproline. 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 *et al.*, 1984). The cell wall hydroxyproline increased beginning within 12-18 h after inoculation of a resistant cultivar but not until after 48 h in a susceptible cultivar. This initial increase in hydroxyproline content of the resistant cultivar corresponded with the time of initial penetration of the pathogen into all cultivars (Hammerschmidt *et al.*, 1984). In our case, the hydroxyproline has been detected only in the case of healthy potato leaves.

Another plant category of plant metabolites which is largely implicated in disease resistance are phenolic compounds. Many authors have discussed their role in resistance (Bennett and Wallsgrove, 1994, Dixon *et al.*, 2002). The set of bands between 1650 and 1500 cm^{-1} is due to vibration modes of aromatic rings probably in aromatic derivatives, unsaturated conjugated compounds, phenols, chlorophyll, etc (Ramirez *et al.*, 1992). Bertoluzza *et al.* (1999) have noticed a gradual weakening of the intensity of the aromatic domain band (1500-1650 cm^{-1}) passing from healthy to pathological and to physiologically stressed leaves. The *R2* has not respected the model of Bertoluzza *et al.* (1999), as we can see in the Tab. 1 and Fig. 2 and 3. The absorbance peak has been the most intense in the case of *R2* infested by A2.2 (the most virulent isolate on *R2*, Fig. 1). The weakest intensity has been occurred in the case of *R2* infested by A1.1, even the healthy *R2* leaves taken as control.

If we take the number of peaks as a criteria (between 1500-1650 cm^{-1}), the healthy *R2* has had 5, 3 for infested by A1.1, 2 for infested by A1.3 and 5 for infested by A2.2. In the case of A1.1, all peaks are common with *R2*. In the case of A1.3 no one and only the peak at 1572 cm^{-1} has been common between *R2* and infested *R2* by A2.2. In function of infection progression, *P. infestans* has suppressed two peaks (case of A1.1), and induced two others (case of A1.3), in the case of A2.2, four peaks have been suppressed and other four have been induced. The same reaction was seen in the interval 600-950 cm^{-1} .

The peak at 1508 cm^{-1} , was attributed to lignin (Stuart, 2004). This has been induced after infection by A1.3 and A2.2, the absorbance intensity has been more important in the case of A2.2. The accumulation of lignin after infection is a known phenomenon in plant reaction after infection (Agrios, 1997; Schmelzer, 2002). The lignification of the cell wall is a particularly important response by plants to pathogens (Stewart *et al.*, 1994). The peak at 1601 cm^{-1} was assigned by Stuart (2004) to pectin; however this

peak was detected only in the case of A1.3. In the case of A2.2 we have found it at 1597 cm^{-1} . In their study of wheat resistance to *Puccinia graminis* f.sp. *trici*, Moerschbacher *et al.* (1988) have demonstrated that the resistant and the susceptible iso-line both exhibit a first maximum in the coordinately regulated enzyme activities of phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL) at a time when the fungus is still growing on the surface of the leaves. This maximum is followed by a decrease to the levels of controls. In the resistant isolate, a second increase is observed at the time of the hypersensitive resistant reaction. In contrast, enzyme activities in susceptible plants continue to decline, even falling below control levels until the onset of sporulation when a second, late increase can be detected.

In tomato, under salinity stress, the region $2100\text{-}2300\text{ cm}^{-1}$ was attributed to saturated and unsaturated nitrile compounds (Schulz and Baranska, 2009). The increased occurrence of these substances is attributed to the detoxification of hydrogen cyanide, a by-product produced during the biosynthesis of ethylene, which is enhanced in response to stress conditions, such as salinity (Mizrahi, 1982). In infested *R2* by A2.2, two peaks have been suppressed. However, the infection by A1.1 has induced three peaks and no one has been in common with the infested *R2* by A2.2. The infection by A1.3 has induced two new peaks. A peak has been identified as being in common with the healthy infested *R2* by A1.1 and A1.3.

The double peaks at 2925 and 2850 cm^{-1} are attributable mainly to the asymmetric and symmetric stretching modes of the CH_2 methylene group, the most abundant structural unit in plant products (Bertoluzza *et al.*, 1999). However, Quilles *et al.* (2010) have assigned these bands to fatty acids in the membranes. In the case of the infestation by A1.1 one band has been induced at 2940 cm^{-1} while peaks at 2859 and 2938 cm^{-1} have been suppressed. When infested by A1.3, the peak at 2859 cm^{-1} has been suppressed. The infection by A2.2 suppressed all the peaks cited above, and induced a new peak at 2934 cm^{-1} . If we link the apparition and suppression of bands with the symptoms evolution, this phenomenon could be explained by the destruction of membranes, especially in the case of A2.2. This is in concordance with the apparition of the peak at 1396 cm^{-1} in all the infested *R2*, which was assigned to amino acids and fatty acids chains, resulting from membrane degradation.

FTIR is largely used for bacterial identification. Certain authors used it for enzyme activity study (Kumar and Barth, 2010; Chis, personal communication). We used the FTIR technique for monitoring the interaction in the pathosystem *P. infestans-S. tuberosum* in the case of compatible interaction. We consider the technique very useful. It is very informative, rapid, simple and cost effective. Stewart *et al.* (1994) used this method for studying the infection of potato tuber infection by *Erwinia carotovora* spp. *carotovora*. Ivanova and Singh (2003) used it

for monitoring leaf senescence and the changes induced by elicitor in plant leaves. They considered that FTIR can provide important information about the physiological events occurring in plant tissue. It allows an overview of the general changes in the leaves when inoculated, with very low costs, and maximum rapidity. Beside its simplicity and rapidity, FTIR technique offers an overview of the physiological and pathological changes that are happening in the plant. This overview could not be obtained by the molecular techniques such as microarray and differential display. The last two technologies show the induced and/or suppressed genes but not their final outcome. An enzyme could be produced, and its gene activated, but its activity could be guaranteed, because the pathogen can use effectors which stop its activity.

Conclusions

In this study we monitored the response of potato to infection by the late blight agent in the case of compatible interaction, using the FTIR fingerprint and specific absorptions (in the range $650\text{-}1800\text{ cm}^{-1}$) of key-molecules involved in defense.

The FTIR spectra indicates modifications in the amino acid biosynthesis (under 1000 cm^{-1} and 1400 cm^{-1}), 1547 cm^{-1} (hydroxyproline), fatty acid metabolism, cellulosic domain ($1200\text{-}950\text{ cm}^{-1}$) and aromatic rings probably in aromatic derivatives, unsaturated conjugated compounds, phenols, chlorophyll, etc (region $1650\text{-}1500\text{ cm}^{-1}$).

All the isolates used were virulent on *R2* potato plant. However, symptom apparition and evolution have been dependent on the isolate, so the potato reaction has been different for each isolate. Potato has been very susceptible in the case of the isolate A2.2, and the less susceptible in the case of A1.1. This reaction could be explained by two reasons: the resistance gene *R2* causes a delay of infection, in comparison with the case of A2.2, or a specialization of *P. infestans* isolates. The isolate A2.2 has been isolated from a potato plant with the gene *R2*.

Finally, we consider this method to be useful method in the study of the interaction in the pathosystem *P. infestans-S. tuberosum*.

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