Imaging Chlorophyll *a* Fluorescence to early monitor Plant Pathology

Following a pathogen attack, in order to stop or limit the spread of the pathogen plants start several metabolic modifications. To understand the mechanisms of plant-pathogen interaction it has an important role for both plant physiology research and for early diagnosis of diseases. Among these metabolic changes the affect on photosynthetic performance is included and that can modify leaves optical properties and consequently the fluorescence emission. Indeed a lot of studies showed that under stress conditions the reduction of the photosynthetic quantum yield was observed. The induced chlorophyll fluorescence emission is a non-destructive technique widely applied in plant research to monitor the health of plants. In the last years this technique has been improved by processing Imaging. This characteristic allows to show the plant-pathogen interaction on whole surface just immediately after infestation. In this way it is possible to highlight the spatial and temporal variation on leaves, due to no uniform alteration in plant metabolism. In the present work, by means of Imaging-PAM fluorometer, the effects on photosynthetic quantum yield and the photochemical processes of photosynthesis on Brassica oleracea var. Italica Plenk, inoculated with *Phoma lingam* was investigated. The inoculation was carried out on leaf and after 4 days after inoculation the photochemical parameters as well as Fv/Fm, Y(PSII), photochemical (q_p) and non-photochemical quenching (NPQ) were collected on different days both in fungi-infected and uninfected plants. Imaging analysis have allowed to visualize the heterogeneity in plant response. The results, in fact showed different responses depending if the area was directly affected by the pathogen or not. More than to heterogeneity the development of the disease was also observed. Already on the 4th day following inoculation the Fv/Fm was significantly reduced compared with the plants before inoculation. All photochemical parameters considered in this study have changed even if the symptoms were not evident. The areas of infection, far from inoculation point, corresponding to disease development, were evident in imaging analysis. These results demonstrate that Imaging PAM Fluorescence is an essential tool for mapping the development of plant disease useful for physiological study of host-pathogen interaction, as well as for early and non-destructive detection of disease

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Fluorescenza della clorofilla *a* e analisi di immagine per la diagnostica precoce di patologie in pianta

In una pianta, a seguito dell'attacco di un patogeno, l'ospite va incontro ad una serie di cambiamenti metabolici che cercano di arrestare o limitare la propagazione del patogeno. Lo studio dei meccanismi che sono alla base di tale interazione riveste un ruolo importante sia nella ricerca di base, che in campo agronomico ed in particolare per la diagnostica precoce delle malattie. L'emissione di fluorescenza della clorofilla a indotta è un metodo non distruttivo ampiamente utilizzato sia per il monitoraggio dello stato di salute delle piante, che per gli studi di base della fotosintesi. Nel presente lavoro si è valutato, attraverso il fluorimetro Imaging-PAM, l'effetto sui processi fotochimici della fotosintesi in piante di Brassica oleracea var. Italica Plenk, inoculate, in condizioni controllate di laboratorio, con *Phoma lingam*, uno dei più importanti ed aggressivi patogeni fungini del suolo delle Brassicacee. L'inoculazione effettuata su foglia stata seguita sulle stesse piante nel tempo. I risultati ottenuti hanno mostrato diverse risposte fra le aree direttamente colpite dal patogeno e quelle lontane, ma influenzate dalla sua presenza. È stata osservata oltre alla eterogeneità nella risposta sulla superficie fogliare, anche la possibilità di monitorare, fin dagli stadi iniziali, l'evoluzione della malattia. La massima efficienza quantica del PSII subisce una riduzione già a 4 giorni dall'inoculazione, con valori significativamente inferiori rispetto alle foglie delle piante analizzate prima dell'inoculazione. Si è inoltre osservata una maggiore attività fotosintetica nell'area adiacente a quella direttamente colpita dal patogeno. L'analisi di immagine dei parametri di fluorescenza e gli stessi parametri correlati ai parametri fotochimici della fotosintesi si confermano come metodologie utili sia per lo studio di base della fisiologia della fotosintesi che per la diagnostica precoce di malattia, in grado di rilevare la patogenesi prima che sia evidente. Inoltre, la possibilità di studiare ogni singola pianta in modalità non distruttiva e quindi seguita nel tempo, permette l'approfondimento negli studi di base per la comprensione di meccanismi fisiologici e molecolari dell'interazione ospite-parassita.

Introduction

Plant–pathogen interaction induces drastic physiological changes in the host that may lead to metabolic damage to cells, tissues and organs, and finally to the expression of apparent symptoms. The metabolic modifications interest several zones: the point of infection hosting the pathogen, and the area far from infection but affected by the pathogen's metabolism of. This is the reason why the leaf could display a heterogeneous response. Many studies emphasize the possibility to evaluate the damages of infected plant during infection[1,2] and the usefulness to follow the disease development particularly at its early stages.[3,4] Understanding the disease development mechanisms correlated to plant response is very important to study host-pathogen interaction. Among the physiological processes affected by pathogen infection as fungi, viruses or bacteria, the photosynthesis activity is heavily influenced, as well as the Photosystem II (PSII) and the Electron Transport Rate (ETR). Photosynthesis decreases as the infection progresses. This is mainly evident in diseases that evolve through chlorotic and necrotic symptoms on leaf.[3-7] The induced chlorophyll a fluorescence emission is a useful and widely employed tool to investigate the metabolic changes during the first stages of infection. This methodology is particularly functional in plant and agronomic research because it is possible to perform a rapid and non-destructive screening of healthy plants.[8-10] Following the first observation of the changes of the chlorophyll fluorescence emission correlated with the primary photochemical reaction of photosynthesis by Kaustsky and Hirsch (1931)[11], numerous studies demonstrate that the induced stress significantly modifies the kinetic of fluorescence emission. As a result, the activity of PSII, i.e. photosynthetic metabolism is mainly influenced.[12,13] Additional information on the efficiency of PSII and the photosynthetic activity can be obtained by applying a saturation pulse on dark-adapted leaf (*Pulse Amplitude Modulated method*).[14,15] This technique allows to assess the quantum yield of energy conversion at the PSII reaction centre by other fluorescence parameters such as: maximum PSII efficiency in the dark–adapted leaf (Fv/Fm), operating quantum

efficiency (Y(II)) in the light-adapted leaf, Electron Transport Rate (ETR), Photochemical quenching (qP) and Non-Photochemical Quenching (qN, NPQ). Although the increase in current fluorescence emission (F_L) is related to the decrease in photosynthesis efficiency[8], it is known that there is a relationship among the efficiency of light harvested by PSII, the ETR, the photoinhibition or the increase in qN, NPQ and various stress factors.[16,17] The assessment of photosynthesis by means of chlorophyll fluorescence emission can be used as an early diagnostic tool for disease detection. The PSII of tomato plants *(Lycopersicon esculentum L.)* cv. Kunera, inoculated with Fusarium oxysporum, is early affected and its activity is heavily reduced.[18] Once more in the interaction between *Fusarium oxysporum* and *Lycopersicon esculentum Mill.* cv Roma, 31 days after inoculation, a significant decrease in Y(II), ETR, and qP (by 27%, 50% 28% respectively) was observed whereas Fv/Fm decreased by approximately 25% after 35 days.[19] During the infection of *Colletotrichum lindemuthianum on Phaeseolus vulgaris* cv. Carioca, a fall of 70-80% in fluorescence and 38% in ETR, corresponding to the 50% decrease in photosynthesis, was observed in the necrosis zone.[20]

In the last years remarkable technological upgrading led to the improvement of that technique by the development of an Imaging Chlorophyll a fluorescence system *(Imaging)*.[1,2,21] Moreover *Imaging* was able to give information about photochemical parameters in real time and in a non-destructive manner. The most essential new information is represented by the simultaneous detection of leaf heterogeneity of these fluorescence parameters which reflects a physiological heterogeneity. Indeed, it is demonstrated that even in healthy plants there is patchiness in correspondence to the stomata opening. Bassanezi et al. (2002)[5] showed how the photosynthesis activity variation depends on the kind of infection also. *Imaging* has been used to study spatial and temporal heterogeneity of the photosynthetic efficiency in response to different levels of biotic and abiotic stress.[2,22-24] When the leaf is infected, several metabolic damages, including photosynthesis, are not uniformly distributed over the whole leaf area. Similarly, uniform visible symptoms are not expected to develop. Therefore by *Imaging* it is possible to analyse the photochemical process in a whole area of a large number of leaves, i.e., plants. It may be a helpful tool for early detection of stress-induced damage. Since the *Imaging* acquisition is non-destructive and rapid, it is immediately possible to compare the metabolic changes due to stress before symptoms are evident.[3,23,25-27] This is particularly useful for screening in plant stress physio-pathology.[2,21,23] On grapevine leaves inoculated with Plasmopara viticola, the *Imaging* system shows a heterogeneity response corresponding to the spread of the pathogen. In particular, the significant changes in Fv/Fm and Y(II) were observed 3 days before the symptoms were evident.[4]

Also in *Pseudomonas syringae*, *both* pv *phaselicola* and pv *tomato-Phaseolus vulgaris* interaction, *Imaging* shows significant changes in *Y(II)* and *qN* before the appearance of the symptoms[25]. In particular, the *Images* of *Y(II)* did not show any differences among the two pathogens, whereas significant *differences* exist for qN[25]. The *Imaging* system did perform well on viruses infections including *Tobacco Mosaic Virus*, where a decrease in Fv/Fm was observed in the inoculated areas immediately a few hours following the inoculation.[27]

Several studies of plant-pathogen interaction and its effect on photosynthesis are reported, but there is little information on *Brassica oleracea- Phoma lingam*, Tode ex Fr. interaction. P. lingam is one of the most aggressive soil pathogens that mainly infects brassicaceae (*stem cancer*; *Phoma leaf spot*; Figure 1) and can be particularly destructive for its virulence and propagation.

The pathogen hits all host parts but the symptoms are

FIGURE 1
Phoma leaf spot symptoms
Source: ENEA

not immediately visible. Commonly, soil pathogens cause extensive damages because of their long life in the temperate climate zone. They can live up to 4 years in the resistance form.[28] Thanks to the variety of adaptive strategies of their life cycle and the development of different ways of infection, soil pathogens are the most interesting to study plant-pathogen interaction. The greatest damage is caused by the necrotrophic and biotrophic spread which usually does not destroy the plant, though it deeply influences its physiology. In necrotrophic broadcast the pathogen destructs the tissues by causing wide lesions and radical changes in their physiology.[28] The biotrophic and necrotrophic stages could alternate into the life cycle depending on climate conditions.[28-30] The infection leads to a mesophyll a biotrophic intercellular colonization on the leaf, following the biotrophic invasion of the xylem tissue. This process ends with a necrotophic step on the stem. In *P. lingam* the necrotrophic stage is known as *crown canker* and leads to the enhancement of the damages due to the biotrophic stage. In order to stop or reduce the pathogen propagation during infection, the plant accumulates a large amount of lignin both in the xylem vessels and in the parenchyma cells[30]. This causes the unbalanced water transport in the plant[5], with a reduction of the transpiration due to stomatal closure.

In this work the *Imaging* system was used to investigate the fungus *P. lingam* effect on the photosynthetic activity of Cauliflower (*Brassica oleracea*, convar. *Botrytis L.*), var. *Italica* Plenk, a widespread plant also used as test plant. The aim is to obtain the characterization of plant-pathogen interaction for early diagnosis by providing the disease mapping. The plant's response was also evaluated as peroxide (H_2O_2) production (oxidative stress).

Materials and methods

Plants and pathogen

Plantlets of *Brassica oleracea* var. *Italica* cv. Calabrese tardivo obtained from organic seed and *Phoma lingam*, race UWA P30, kindly given from Faculty of Natural and Agricultural Sciences, Australia, were used. In order to avoid any foreign contamination, the pots and the soil were sterilized before transferring plantlets. The plants were grown in a Grown Chamber at T=24±1C°, RH=60%, 16000 lux, 16:8 h photoperiod, and regularly watered.

The pathogen was cultured *in vitro* on PDA (Potato Destrosio Agar)–Oxoid at T=23± 1C°, and transferred onto fresh medium every 20 days. Since *P. lingam* is a semi-obligate pathogen, to preserve its virulence it was recurrently necessary to isolate it from the plant infected with pycnidiospores. To help the pycnidiospores develop, *P. lingam* was kept in the dark. The pycnidiospores originated after the 7th day and reached maturity when the production of pink exudates is evident (Figure 2).

Inoculum preparation and pathogen test

According to Gugel et al., (1990) within a modified protocol the inoculum with pycnidiospores suspension was prepared. The 20-day-old mycelium was separated by filtration and the concentration of pycnidiospores was determined by counting in a Burker chamber (Fortuna, Germany). In order to assure the virulence of fungi, inoculations on cotyledons of 25 plantlets were

FIGURE 2
Mature picnidiospores of *Phoma lingam* with exudates (arrow)
Source: ENEA

FIGURE 3
Inoculated plants protected with the plastic sheet
Source: ENEA

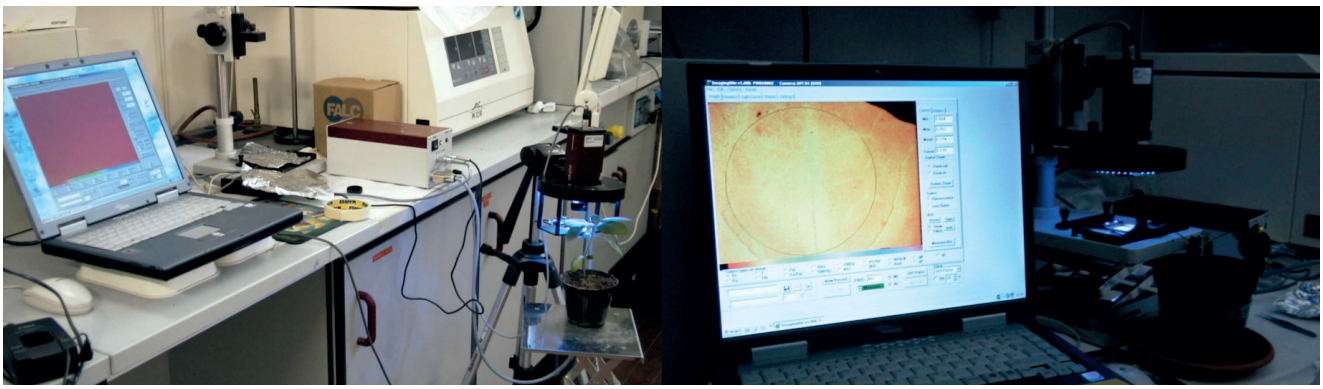

FIGURE 4 Imaging PAM fluorometer
Source: ENEA

practised. After 10 days from inoculation the severity of symptoms is determined according to scale of Bansal et al., (1994).[31] The severity index (SI) was also calculated.[31]

Leaf inoculation

The inoculation was practised on 48-day plants. Each leaf was inoculated with 2 drops of 10 µl of suspension (1 x 10^7 pycnidiospores/ml). The wound was obtained by piercing the leaf with a needle of 200 µm of diameter. Each infected plant was compared with plants inoculated with sterile distillated water (control). Just to ensure a positive outcome, the infection plants were closed into a plastic sheet with the RH 100% (Figure 3). Then the plants were put into a growth chamber at T=28±1 C°, photoperiod 16:8. After 5 days the plastic was removed.

Imaging Chlorophyll a fluorescence analysis

Chlorophyll a fluorescence was measured by IMAGING-PAM Chlorophyll fluorometer (Walz GmbH, Effeltrich, Germany) (Figure 4).
The IMAGING-PAM applies a Pulse Amplitude Modulated (PAM) measuring light according to Schreiber (1986)[14]. The variation of the induced fluorescence shows the changes of photochemical efficiency and the energy dissipation. In order to determine Fv/Fm and calculate the photochemical parameters, the instrument is provided of the measuring light and saturation pulse. The IMAGING-PAM real-time processing both induces chlorophyll a fluorescence and generates two-dimensional images.
The system is made up of the following components (Figure 5):

- Control Unit containing a rechargeable Li-ion battery (it connects the CCD-camera to the PC and harvest data to it)
- LEDs-Array Illumination (96 blue LEDs (λ=470 nm), 8 red LEDs (λ=650 nm), and 8 Near-InfraRed LEDs, (λ=780 nm))
- CCD-camera (640x480 pixel, equipped with two lenses passing the red fluorescence as well as 650 and 780 nm)
- PC with Win-software *Imaging*.

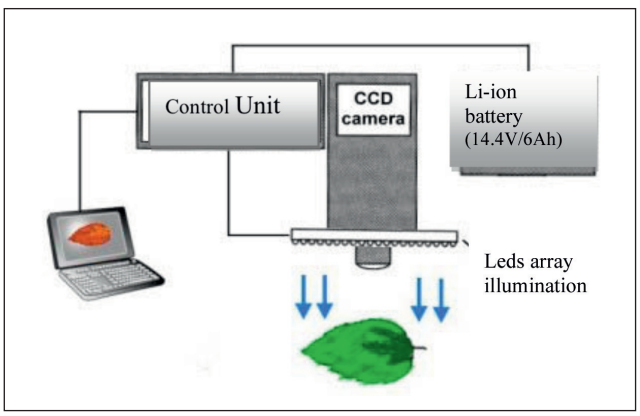

FIGURE 5 Set-up of Imaging-PAM
Source: ENEA

The intensity of the blue-light excitation is 0,5 µmol quanta m^{-2} s^{-1}; the actinic light is 1500 µmol quanta m^{-2} s^{-1}. The intensity of the saturation pulse is 2400 µmol quanta m^{-2} s1. The calibration of the CCD-camera was carried out before the measurements.

Processed parameters:

- **F0** (dark fluorescence yield);
- **Fm** (dark maximal fluorescence yield); **Fm'** (maximal fluorescence yield on light);
- **F** (current fluorescence yield in switched-on Measuring light, **FL** in the text);
- **Fv/Fm** (maximal PSII quantum yield after dark adaptation);
- **Y(II)**, (effective PSII quantum yield);
- **qN** (non-photochemical quenching coefficient);
- **qP** (photochemical quenching coefficient);
- **ETR** (Electron Transport Rate).

In order to properly determine the dark-light induction curve, which is fundamental to give information on the various steps of the complex photosynthetic process, the plants were placed in the dark for 20 min, so that all PSII reaction centers are open. Then the saturation pulse was applied to them. After that, the fluorescence yield of the same plants at measuring light was observed. Concerning the light curve, to obtain right ETR values the plant was first adjusted to actinic light for at least 10 min, and then submitted to increasing light intensity. Because of its non-destructive characteristic, the same inoculated and non-inoculated leaves of the same plant were analyzed during all the experiment. The 4th and 5th leaves of 15 plants were inoculated and compared with plants inoculated with sterile water only. The plants were analyzed at 4, 7, 8, 11, 14, and 18 days after inoculation (DAI). In order to be sure to analyze healthy plants, before inoculation (T0) the first screening was carried out by the Fv/Fm value.

In order to obtain information of possible fluorescence patchiness, reflecting stomata opening and physiological heterogeneities due to pathogen were identified (Figure 6):

- Point of inoculation area (*area 1*)
- Far from the inoculated area (*area 2*)
- New reaction area far from the inoculation point (*area 3*).

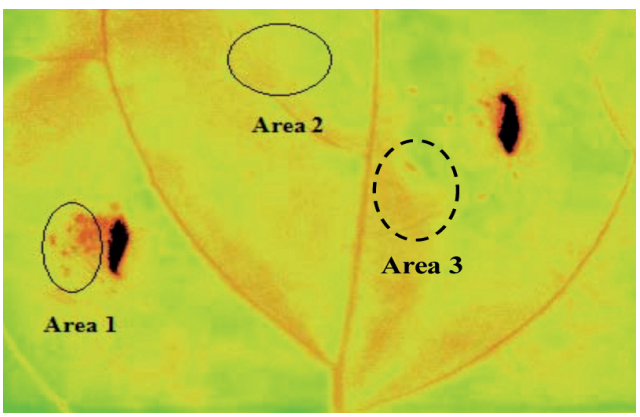

FIGURE 6 Selected areas on the inoculated leaf for Imaging analysis
Source: ENEA

Data Processing

Imaging data were processed by Imaging Win-software (V 0.55, Walz). The statistical analysis was carried out by SPSS for Windows, Release 11.0.

Results

Imaging plant response

Table 1 shows the results obtained for Fv/Fm, Y(II), ETR, and qP (PAR=460 µmol quanta m^{-2} s^{-1}).

Already after 4 DAI, the Fv/Fm values are significantly different in both areas 1 and 2 compared to T0 (F=8,229; P≤0,001). However, between the two areas the Fv/Fm values are not significantly different until 18 DAI, where area 1 is significantly lower (about 4,5%) compared to area 2. The Fv/Fm values of area 1 decrease significantly after 11 DAI compared to 4 DAI (Fv/Fm=0,739ce; Table 1). Conversely, the Fv/Fm values of area 2 are never significantly different during all experiments (Table 1). The ETR of area 1 after 4 DAI is higher (F=2,933; P≤0,001) compared to area 2 (ETR_2=54,9*a* and ETR_1=48,0*bc*), even if it is not related with qP at the same time (qP_2=0,779*ce* and qP_1=0,748*e*; Table 1). As expected at 8 DAI, qP values are significantly higher in area 1 (0,822*ac*) than in area 2 (0,770*de*) (F=6,497; P≤0,001) (Table 1). The highest values observed for ETR and qP are possibly due to the

Photochemical Parameter	DAI	Area 2 (± S.E.)	Area 1 (± S.E.)
F_v/F_m	0	0,788 ± 0,004 a	
	4	0,752 ± 0,005 bc	0,765 ± 0,004 b
	7	0,740 ± 0,004 ce	0,740 ± 0,008 ce
	8	0,748 ± 0,004 bd	0,748 ± 0,005 bd
	11	0,729 ± 0,013 ce	0,739 ± 0,007 ce
	14	0,723 ± 0,004 e	0,725 ± 0,016 de
	18	0,733 ± 0,007 ce	0,700 ± 0,011 f
F		4,351 ***	
Ψ (II)	4	0,533 ± 0,014	0,567 ± 0,007
	7	0,560 ± 0,011	0,591 ± 0,014
	8	0,534 ± 0,012	0,579 ± 0,012
	11	0,596 ± 0,009	0,611 ± 0,009
	14	0,574 ± 0,014	0,565 ± 0,017
	18	0,573 ± 0,015	0,538 ± 0,023
F		2,840 n. s.	
ETR	4	48,0 ± 3,4 bc	54,9 ± 3,7 a
	7	47,4 ± 2,8 bc	43,0 ± 3,6 bd
	8	41,0 ± 2,2 cde	43,9 ± 2,6 cde
	11	48,7 ± 3,9 bc	48,3 ± 4,4 bc
	14	44,8 ± 3,2 bcd	42,0 ± 3,9 cde
	18	35,5 ± 3,0 de	33,5 ± 2,6 e
F		2,933 **	
qP	4	0,748 ± 0,017 e	0,779 ± 0,011 ce
	7	0,809 ± 0,014 bc	0,849 ± 0,019 ab
	8	0,770 ± 0,016 de	0,822 ± 0,015 ac
	11	0,855 ± 0,009 ab	0,860 ± 0,015 a
	14	0,829 ± 0,011 ab	0,832 ± 0,016 ab
	18	0,844 ± 0,012 ab	0,819 ± 0,018 ac
F		F=6,497 ***	

TABLE 1 Maximal PSII quantum yield after dark adaptation (Fv/Fm) and effective PSII quantum yield (Y(II)), Electron Transport Rate (ETR), photochemical quenching (qP) on Day After Inoculation (DAI). Area 1= Inoculated area; Area 2= far from the inoculation point. The values with the same letter are not significantly different according to Duncan test (P≤0,05). Significantly: **= for P≤0,01; ***= P≤0,001
Values followed by the same letters are not significantly different at P<0.05according to Duncan's Test
Source: ENEA

raise photosynthetic activity. The *B. oleracea-P. lingam* is a biotrophic interaction. For this reason in the area 1 there can be a movement and accumulation of photosynthetic products as observed also in other plant-pathogen interactions[32]. The Y(II) parameter doesn't show significant differences (F=2,840; P=0,257).

Imaging

The images were carried out using PAR=460 µmol quanta m^{-2} s^{-1} previously obtained from the induction curve. The *Imaging* of FL, Y(II), qP, at 4, 7, 8 and 11 DAI are shown from figure 7 to 11. The leaf heterogeneity response is immediately evident in the fluorescence

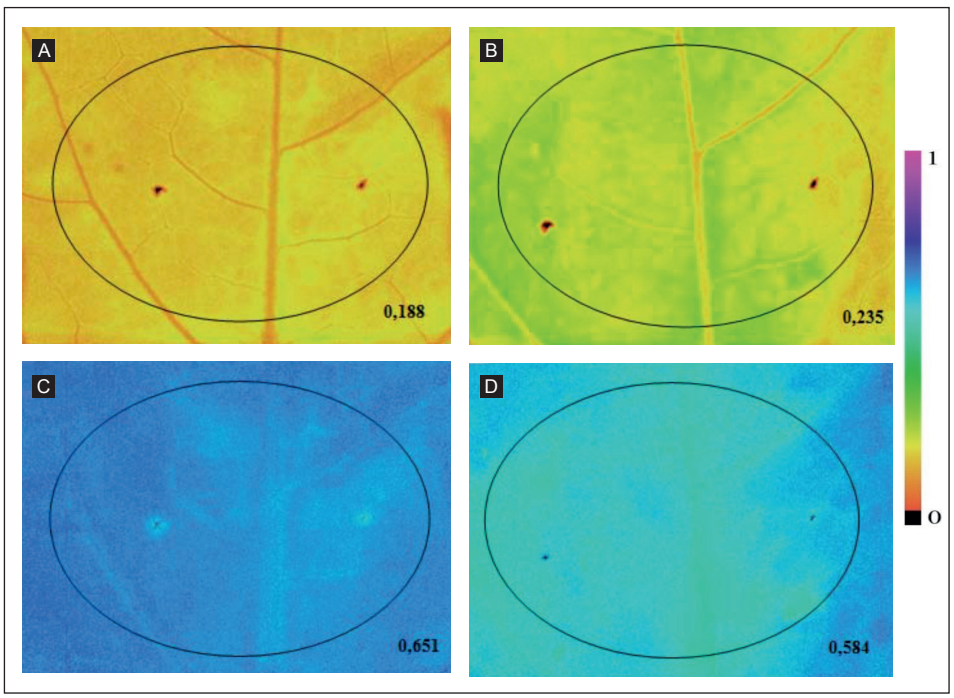

FIGURE 7

F_L and Y(II) *Imaging* (PAR 460 µmol quanta m^{-2} s^{-1}) of leaves inoculated with sterile water (control):
F_L at 4(a) and 18 days (c);
Y(II) at 4 (b) and 18 days (d)

Colorimetric bar:
0 = low fluorescence;
1 = high fluorescence

Source: ENEA

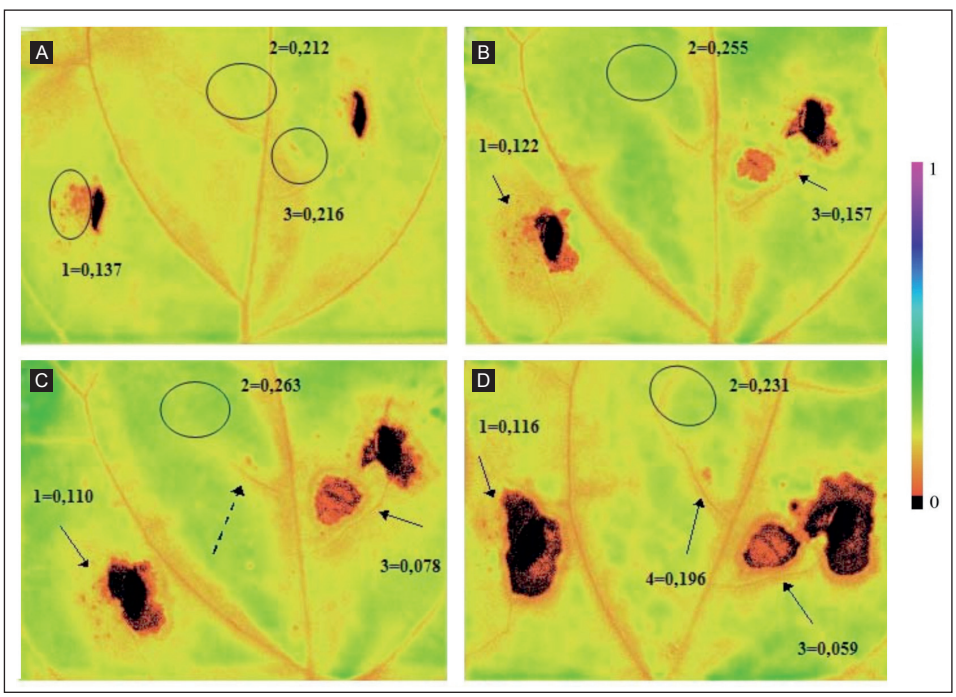

FIGURE 8

Imaging of F_L (PAR di 460 µmol quanta m^{-2} s^{-1}) of inoculated leaves:
A. 4 days
B. 7 days
C. 8 days
D. 11 days

Colorimetric bar:
0 = low fluorescence
1 = high fluorescence

Source: ENEA

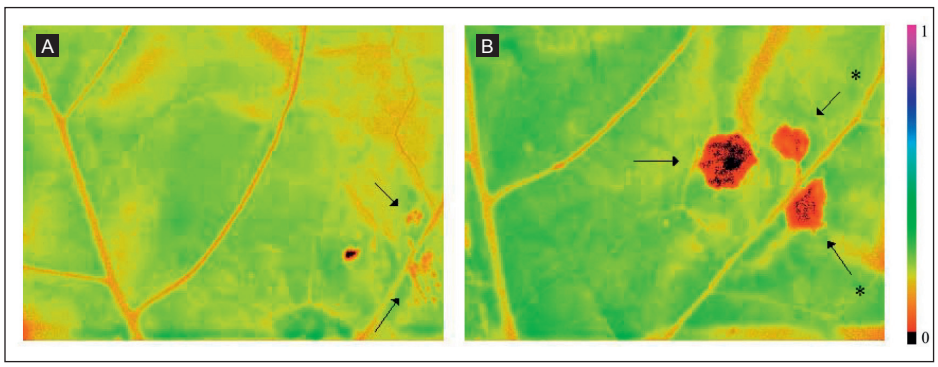

FIGURE 9
Imaging of FL (PAR di 460 µmol quanta m^{-2} s^{-1}) of inoculated leaves:
A. 4 days
B. 7 days
Colorimetric bar:
0 = low fluorescence
1 = high fluorescence
Source: ENEA

emission, in the inoculated plant with *P. lingam*, even if the symptoms are not visible.
The imaging of the following days (14, 18, and 21 DAI) is not reported because the symptoms as well as necrosis are visible. In Figure 7 FL and Y(II) leaf imaging of the inoculated plant with sterile water after 4 and 18 days is shown.
As expected, in all days of analysis the differences were not observed neither in the inoculation point nor in the closed areas, though the little necrosis due to the needle is evident (figure 7). The increasing FL observed (about 20%) and the decreasing Y(II) (nearly 10%) (Figure 7a,c; and Figure 7b,d) are due to natural physiological ageing.
Figures 8÷11 show the images of leaves inoculated with *P. lingam* after different DAI. During the experiment a new reaction are, far from the inoculation (area 3) was identified (Figure 6). As showed in figure 8, already 4 DAI the different emission of fluorescence in area 1 compared to 2 and 3 was observed (Figure 8a)

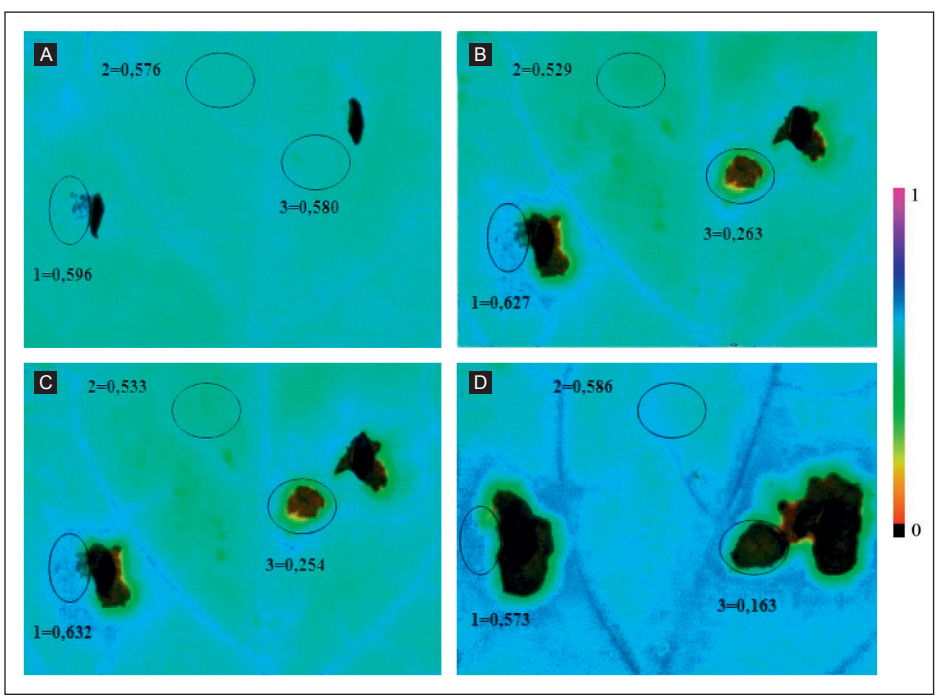

FIGURE 10
Imaging of Y(II) (PAR=460 µmol quanta m^{-2} s^{-1}) on inoculated leaf
A. 4 days
B: 7 days
C. 8 days
D. 11 days
Colorimetric bar:
0 = small fluorescence
1 = maximal fluorescence
Source: ENEA

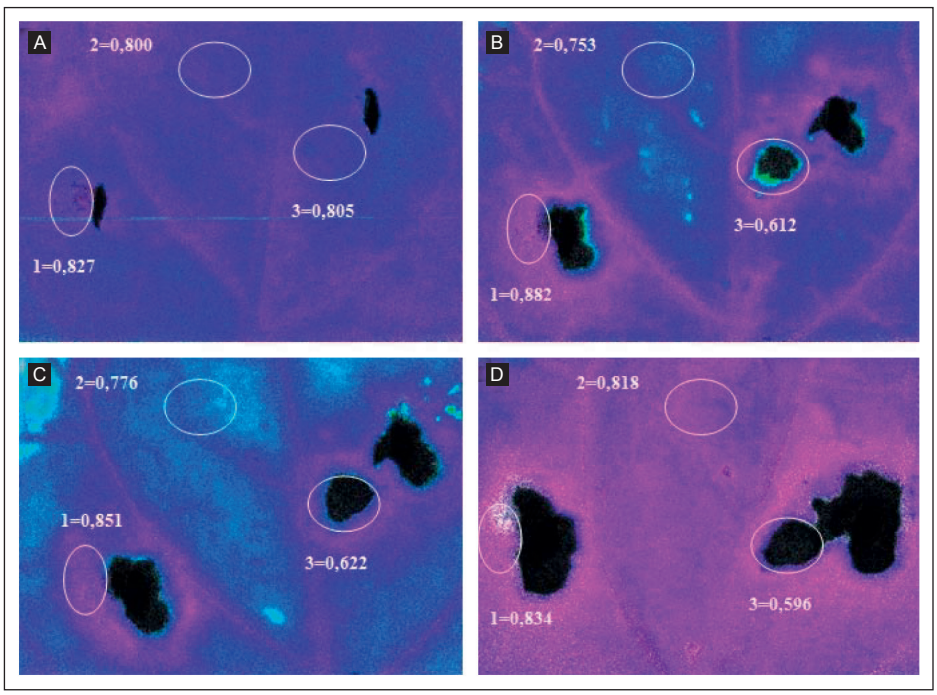

FIGURE 11
Imaging of qP (PAR=460 µmol quanta m^{-2} s^{-1}) on inoculated leaf
A. 4 days
B. 7 days
C. 8 days
D. 11 days
Colorimetric bar:
0 = small fluorescence
1 = maximal fluorescence
Source: ENEA

(F_L=0,137, 0.212 and 0,216 respectively). The response of plant on area 1 causes immediately lowering fluorescence, much more evident at 7 DAI (F_L=0,122 Figure 8b). The same day, in area 3 the formation of the reaction zone with reduction of fluorescence caused by the pathogen, though not directly there, was also observed (Figure 8b). This result confirms the pathogen effect on the whole metabolic leaf surface even without visible symptoms. In Figure 8c the FL highlights the additional spread of the disease. In fact a new reaction zone is also originating far from the inoculation point (dashed arrow), which becomes more evident after 11 DAI (Figure 8d, number 4). This result shows the possibility to apply the non-destructive method following the propagation of the disease on the leaf tissues day by day. The same results were almost expressed in most of the inoculated leaves (Figure 9a). Here the imaging shows the several reaction zones far away from the inoculation point (arrows) that spread after 7 DAI from inoculation (Figure 9b, arrows with star). The same points showed visible necrosis after 11 DAI.

In Figure 10 images of Y(II) are shown. The differences are mainly visible after 7 DAI. The 50% lower value in area 3 and after 11 DAI is decreased by 72% highlighting the decrease of the photosynthetic activity also in an area far from inoculation. Instead, close to area 1 it was observed the Y(II) increasing (Y(II)=0,627) which remains the same until 8 DAI. This result already observed for ETR (Table 2) confirms the rise of photosynthetic activity due to the biotrophic plant-pathogen interaction.

The images of qP are given in Figure 11. The little but progressive increase of the qP in area 1 was observed from 4 to 7 DAI. On area 3, not directly affected by the presence of the pathogen, a decrease of the qP was instead observed from 7th till 11th day according to the results obtained for Y(II). This result confirmed the decrease of the photosynthetic activity.

Discussion and conclusions

The leaves of the *Brassica oleracea* inoculated with *Phoma lingam*, Tode ex Fr. showed a different response on the whole surface depending on whether the area

is directly affected by the pathogen or far from it but with its metabolism being pathogen-influenced. Already on the 4th and 11th DAI the *Imaging* highlighted several areas, distinct in area 3 and area 4 (both distant from the inoculation site) with a different fluorescence emission (Figure 8). The *Imaging* is able to monitor the development of the disease from the very early stage and allows to follow the spread of the pathogen not only in the area next to the inoculation point but also in the distant areas (Figure 8 e Figure 9).

The heterogeneity of the plant response is confirmed by the appearance of a different fluorescence emission, a marker of physiological response linked to the pathogen action in distant areas from the inoculation point. The pathogen presence is confirmed by the visible symptoms in the following 11 DAI. As showed in Table 2 the Fv/Fm value (*maximal PSII quantum yield*) of infected plant is significantly lower already on the 4th DAI compared to the plant analyzed before inoculation (T0). This result showed that the PSII is promptly damaged, although the Fv/Fm is not significantly different in both areas: area 1 where the pathogen is inside, and area 2 where the pathogen is temporary absent (Table 1).

The results obtained for ETR and qP are very interesting. Both values are significantly higher in area 1 than in area 2, after 4 and 8 DAI (Table 2). This result is confirmed by *Imaging* the Y(II), where in area 1 the increase of activity is also observed (Figure 10b and 10c). The *Imaging* of qP shows the same result until 7 DAI (Figure 11b).

These results suggest an increased photosynthetic activity due to pathogen presence according to Pomar et al., (2004)[32] the observation being made during the early growth of *Verticillium dahaliae* on *Capsicum annuum*. Actually this plant-pathogen interaction is biotrophyc-type so that in area 1, the pathogen induces metabolic changes including the new synthesis of the photosynthetic products. This interaction can lead to a temporary bigger photosynthetic activity[32]. The oxidative stress was confirmed by the H_2O_2 production (data not shown). Already 2hs after inoculation the microscopy observation confirms the H_2O_2 production which increases up significantly after 8hs.

After 24hs the H_2O_2 production is detected also in other areas far from the inoculation point demonstrating the formation of new infection focus. In conclusion Imaging is confirmed to be an excellent tool for early and non-destructive detection of different plant stresses being able to detect pathogenesis before the symptoms are visible. Besides its characteristics, the *Imaging* system allows to make observation on the same single plant following the disease development. Moreover, thanks to the several and complex information acquired, this technique provides a precious background for further physiological and molecular analysis to study the mechanisms of the host-pathogen interaction.

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