

# Hyperspectral and molecular analysis of *Stagonospora nodorum* blotch disease in durum wheat

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**Abstract** *Stagonospora nodorum* blotch (SNB) is an important wheat disease caused by *Parastagonospora nodorum* (Berk.) *Studies in Mycology*, 75: 307–390 (2013). This necrotrophic pathogen can attack common and durum wheat, causing grain yield, quality and safety worsening. This work aims to studying a novel diagnostic technique for early detecting *P. nodorum* attack and infection progression in three Italian commercial varieties of durum wheat. Plants of these wheat varieties were inoculated in field with a spore suspension of *P. nodorum*. Different experimental conditions were used: *P. nodorum* was inoculated in plants at different

growth stage (GS); GS32 (Inf A) and GS 73 (milk development - Inf B). To consider natural contaminating mycoflora interference for the subsequent analysis, non-inoculated controls, fungicide-treated (T) or non-treated (NT) were used. Inf A produced symptoms both on leaves and ears which appeared to be more severe compared to those induced by Inf B, even if the latter affected more thoroughly wheat yield in at least one variety of wheat. An early detection of SNB could be helpful to manage a targeted control strategy. Hyperspectral imaging analyses showed that this rapid and non-invasive technique allows differentiation between spectra of healthy and diseased tissues already 48 h after inoculation. qPCR confirmed hyperspectral results. The results showed that marked differences emerge in the ability of the varieties to tolerate the SNB disease especially regarding grain yield and quality. Compared with classical approaches, the hyperspectral imaging analysis may represent a useful tool for early detection of *P. nodorum* on wheat.

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## Introduction

*Parastagonospora nodorum* (Berk.) a necrotrophic fungal phytopathogen, causes *Stagonospora nodorum* (SNB) blotch, a widespread disease in common (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L.) affecting major wheat-growing areas (El-Bana and

Galal 2007; Eyal 1999). The pathogenic fungus originally identified and classified as belonging to the *Septoria* genus was recently moved to the genus *Parastagonospora* and its teleomorphic stage from *Leptosphaeria* to *Phaeosphaeria* (Cunfer and Ueng 1999). *P. nodorum*, a major pathogen of wheat, is currently indicated as a model for a specific class of necrotrophic pathogens, the *Pleosporales* (Oliver et al. 2012). In Italy, SNB disease is observed every year on wheat (Iori et al. 2012; Pasquini et al. 2012). Preliminary studies have been performed on durum wheat to evaluate the effects of the disease on some quantitative and qualitative traits of the wheat yield (Iori et al. 2011a, b). This pathogen causes devastating foliar diseases and typical symptoms are lens-shaped necrotic and chlorotic lesions (Faris and Friesen 2009). The genetic and physiological characterization of this severe pathology allowed the breeders to individuate varieties of wheat more resistant to *P. nodorum* (Oliver et al. 2012). In temperate areas, the fungus overwinters in seeds, plant residues, in *Poaceae* weeds and in wheat. *P. nodorum* spreads from infected wheat and triticale seeds, airborne plant debris, through ascospores and pycnidiospores (Arseniuk et al. 1998). SNB affects the quality of grain and can cause yield losses in wheat production that may reach up to 50 % of the potential harvest (Bhathal et al. 2003; Fried and Meister 1987). Resistance to SNB in wheat is under polygenic control, however, in some hybrids, authors found a monogenic inheritance (Ali et al. 2008; Liu et al. 2004a). QTLs for SNB resistance have identified on almost every wheat chromosome (Xu et al. 2004). The use of foliar fungicides, crop rotation, and debris management or, more recently, the selection of SNB-resistant genotypes represent the current strategies to control the disease (Oliver et al. 2012). Some varieties are partly resistant to foliar infection but seed-transmitted inoculum may initiate an epidemic (El-Bana and Galal 2007; Shah et al. 2000). The reduction of seed-born inoculum represents a possible and sustainable way for managing SNB (Arseniuk et al. 1998; Shah et al. 2000).

In general, the possibility to early detect the pathogen, before its visual evidence, represents a critical point to target a suitable control strategy. Although, visual and microscopic assessments are generally well accepted, the pathogen biomass, in asymptomatic plants, can be also quantified by qPCR (Oliver et al. 2008). In previous studies, authors used qPCR to detect pre-symptomatic *P. nodorum* in order to best direct fungicide applications

(Oliver et al. 2008). Recent studies have tried non-destructive, spectral techniques to achieve simple, rapid and inexpensive methods for the detection of fungal contamination on cereals. Hyperspectral imaging techniques represent an attractive solution for characterization, classification and quality control of cereals and other food, including early detection of mycotoxigenic fungi (Del Fiore et al. 2010, Zhang et al. 2012, 2012a, 2012b; Yuan et al. 2014). HSI is based on the use of integrated hardware and software architecture able to digitally capture and handle spectra, as an image sequence. In each image of the sequence, each column represents the discrete spectrum values of the corresponding element of the sensitive linear array. Such an architecture allows, with a “simple” arrangement of the detection device (“scan line” perpendicular to the moving direction of the objects) to realize a full and continuous control (Geladi et al. 2007; Hyvarinen et al. 1998). The spatial and spectral information, obtained simultaneously from the investigated object, are contained in a “hypercube”, a 3D dataset characterized by two spatial dimensions and one spectral dimension. Considering that in a hyperspectral image the spectrum of each pixel can be analyzed, HSI is the non-destructive technology providing the most accurate and detailed information extraction. According to the different wavelengths of the source and the spectral sensitivity of the device, several physical-chemical characteristics of a sample can be investigated and analysed. In this paper, HSI working in the NIR range (1000–1700 nm), coupled with chemometric analysis, was applied and evaluated to detect the fungal presence in durum wheat leaves.

The aim of this study is to propose and validate hyperspectral imaging by means of highly specific qPCR, for early detecting *P. nodorum* in durum wheat in field experiments, and to correlate fungal presence with wheat disease severity and yield.

## Materials and methods

### Fungal strain and plant materials

*Parastagonospora nodorum* (Berk.) was isolated from naturally infected durum wheat leaves (cv. Ciccio) cultivated in Italy. Leaf segments with pycnidia were placed in Petri dishes with water agar (Agar-Agar, Merck KGaA, Germany) (15 g/l) and incubated at

20 °C under 12 h photoperiod. A cirrhus of *P. nodorum* was transferred to potato dextrose agar plate (PDA, HiMedia, India), 39 g/L and incubated at 20 °C for 7 days. After incubation, the fungal conidia collected as described by (Eyal et al. 1987). The strain submitted to the Culture Collection Agro-Food ImportantToxigenic Fungi-Item, Institute of Science of Food Production (ISPA), National Research Council (CNR), Via Amendola, 122/O, 70126 Bari, Italy (<http://server.ispa.cnr.it/ITEM/Collection/>).

The commercial wheat varieties used for the *in-field* experiments were coded C1, C2 and C3, respectively, and are some of the main varieties grown in Italy (Quaranta et al. 2009, 2012) and produced by PRO.SE.ME and Produttori Sementi Bologna (Italy). The commercial wheat cultivars used for the experiments were: Ciccio, Simeto and Svevo named with the code C1, C2 and C3, respectively. No clear indications on the resistance phenotype of these varieties to Septoriosi and SNB have been provided by the 2 companies.

#### In field experiments

In-field inoculations were performed with an inoculum of *P. nodorum* 10<sup>6</sup> conidia/ml as previously indicated (Zadoks et al. 1974). The three wheat varieties were grown in experimental field (Montelibretti, Rome, Italy) with a completely randomized design (plots of 2×1.5 m) during crop season 2010–2011. Viable seeds (#450) were sown in 1 m<sup>2</sup>. One square meter area separated the rows of plots and wheat plots were alternated with barley to reduce the contamination between plots. In the field trials, the experimental conditions were:

- 1) Plants non-inoculated and treated (T) with commercial fungicide (Folicur – tebuconazole - Bayer Crop Science, DE) at ear emergence growth stage (GS 55, according to the scale of Zadoks (Zadoks et al. 1974));
- 2) Plants non-inoculated and non-treated with Folicur (NT);
- 3) Plants non-treated with Folicur and inoculated by spraying (80 ml/plot) with *P. nodorum* spore suspension at stem elongation-second node detectable growth stage GS 32 (Inf A);
- 4) Plants non-treated with Folicur and inoculated by spraying (80 ml/plot) with *P. nodorum* spore

suspension at the early milk development growth stage GS 73 (Inf B).

A transparent sheet covered the plants for 24 h to provide high humidity and to favour infection (McKendry et al. 1995). To protect the same plants from direct sunlight during moist incubation, plastic shade covered the plots. All the experiments were carried out in three field replicates, each analytical measure repeated three times ( $n=9$ ). For molecular and hyperspectral imaging analyses, the leaves were detached at 24, 48, 72, 168, 240 and 360 h post-inoculation (hpi). Visual identification of the disease and microscopic identification of *P. nodorum* pathogen were also done. In field, the SNB infection was visually assessed every 7–10 days post inoculation (dpi) as disease severity on flag leaf and as severity and incidence of the disease on ear. The final visual assessment of the disease at seeds maturity, in the field was performed 63 dpi for the plants of Inf A and 25 dpi for the plants of Inf B. SNB index was evaluated similarly to FHB (*Fusarium* Head Blight) index (Chen et al. 2012). On kernels, additional molecular and quality analyses were performed at maturity. Field plots were harvested at grain maturity and data concerning the production [Total production (kg/plot), Weight 1000 seeds (g) and test weight (Kg/hl)] of durum wheat varieties were analyzed in all the samples.

#### DNA extraction from wheat

DNA extracted from wheat leaves and seeds according to Färber method with minor modifications (Farber et al. 1997). Twenty gram-samples were grounded in liquid nitrogen and an amount of 100 mg was incubated with 500 µl of extraction buffer (Tris-HCl 200 mM pH 8.0, NaCl 250 mM, EDTA 25 mM, SDS 0.5 % w/v) for 60 min at 65 °C in an orbital shaker. After incubation, samples were put on ice for 10 min and centrifuged at 18,000×g for 15 min at 4 °C. The supernatant was collected in a 2-ml tube and 0.3 volume of sodium acetate 4 M was added. This solution was placed on ice for 30 min and centrifuged at 18,000×g for 10 min at 4 °C and the supernatant was transferred into new tube and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) was added shaken and centrifuged at 18,000×g for 10 min at 4 °C. The supernatant was transferred again and added with chloroform (1:1 v/v), shaken for 5 min, centrifuged for 15 min at 4 °C, and precipitated by adding 0.5 volume of cold 2-propanol.

One more precipitation step was carried out by adding 200  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , 20  $\mu\text{l}$  of  $\text{CH}_3\text{COONa}$  3 M pH 5.2, and 440  $\mu\text{l}$  of ethanol. After 20 min of incubation at  $-20^\circ\text{C}$  and centrifugation at  $18,000\times g$  for 30 min at  $4^\circ\text{C}$ , the pellet was washed with 70 % cold ethanol, centrifuged at  $18,000\times g$  for 10 min, dried at  $64^\circ\text{C}$ , and suspended in 30  $\mu\text{l}$  of sterile water containing 20 mg/ml RNase.

#### Real-time PCR assay

In order to monitor fungal growth into the wheat leaves and seeds, a specific SYBR green qPCR method was set by designing primers (for\_ TGGGTACGCTTTTGATCTCC; rev\_ AACGAGGTGGTTCAGGTCAC) in the  $\beta$ -tubulin of *P. nodorum* (NCBI Gene Bank Ac. No. AY 786332). Real-time PCR was prepared in 20  $\mu\text{l}$  reaction mixture which contains SYBR green JumpStart Taq Ready Mix 1X (Sigma-Aldrich, USA),  $\text{MgCl}_2$  3 and 0.5 mM each of primers. Real time PCR was performed in a LineGene K PCR detection systems (Bioer, PRC). The cycling conditions were:  $95^\circ\text{C}$  for 10 min, followed by 40 cycles of  $95^\circ\text{C}$  for 15 s,  $55^\circ\text{C}$  for 30 s and  $72^\circ\text{C}$  for 1 min. Standard calibration was performed plotting the real-time PCR signals obtained for *P. nodorum* genomic DNA, extracted from the same fungal strain used for the experimental studies, in the concentration range 50–5 pg.

Concerning the specificity, particular attention was given to the use of two field controls, one treated and one not treated with the pathogen *P. nodorum*, isolated and characterized in the laboratory and submitted to the Culture Collection Agro-Food Important Toxigenic Fungi-Item, Institute of Science of Food Production (ISPA), National Research Council (CNR).

This choice addressed to check eventual not specific amplification of the qPCR methodology towards fungal infections or fungal development on leaves and seeds. The untreated choice aimed at checking the presence of unwanted amplification of closely related species and fungi also commonly isolated on wheat leaves, and seeds.

The specificity of PCR amplification, was tested also by amplifying DNA of wheat plants not inoculated with *P. nodorum*, DNA of fungal pathogens such as *Alternaria alternata*, *Fusarium graminearum*, *Septoria tritici*, naturally occurring in durum wheat samples under our field condition as reported in figure S1. qPCR amplification reactions were carried out in triplicate

from three independent experiments ( $n=9$ ) and the results were expressed as ng of DNA/g of samples.

#### Phenological, molecular and yield data analysis

All the experiments were carried out in three field replicates, each analytical measure repeated three times ( $n=9$ ). Data obtained on seeds (*P. nodorum* DNA, seeds production in infected and control plots, etc.) were used for a series of comparisons and correlations aimed at the evaluation of the relationships among the variables and the significance of the observed differences among treatments and cultivars. One-way analysis of variance (ANOVA) was applied in the comparison of the treatments and significance of differences were tested at 95 % confidence. The ANOVA was followed by a test for comparing the means of categories (Tukey's HSD, Honestly Significant Difference, test) (Legendre and Legendre 1998; Sneath and Sokal 1973). The probability of making type I errors among all the hypotheses was tested before running the post hoc test, applying the Bonferroni correction. Pearson correlation between the variable DNA and all of the other variables considered in the study was calculated respect to each wheat variety. ANOVA and Pearson Correlation Analysis were performed using XLSTAT, 2009.4.06 software (Addinsoft, Paris, France).

#### Hyperspectral imaging

A hyperspectral imaging (HSI) based platform (DV srl, Italy) was utilized to perform all the analyses. The HSI based detection architecture is designed to carry out tests on material flow streams transported on a conveyor belt in order to perform, at laboratory scale, on-line detection in a sorting and/or quality control perspective. The platform, in terms of hardware components, is based on a controlled conveyor belt (width=26 cm and length=160 cm) with adjustable speed (variable between 0 and 50 mm/s). The utilized acquisition system is a NIR Spectral Camera<sup>TM</sup> (Specim, Finland), embedding an ImSpector N17E<sup>TM</sup> imaging spectrograph working in spectral range from 1000 to 1700 nm, with a spectral resolution of 7 nm, coupled with a Te-cooled InGaAs photodiode array sensor. The spectrometer was coupled to a 50 mm lens. The illumination system is constituted by a diffused light cylinder, aluminum internal coated, embedding five halogen lamps producing a continuous spectrum signal optimized for spectra

acquisition in the NIR wavelength range. The device works as a push-broom typeline scan camera allowing the acquisition of spectral information for each pixel in the line. The transmission diffraction grating and optics provided high light throughput and high quality and distortion-less image for the device. The result of acquisition was constituted by a digital image where each column represented the discrete spectrum values of the corresponding element of the sensitive linear array. A PC unit equipped with the Spectral Scanner™ v.2.3 acquisition/pre T processing software controls the device. Hyperspectral imaging was applied to selected leaves of the 3 durum wheat varieties (C1, C2 and C3), collected at different time intervals from the inoculation. The images were acquired scanning the investigated sample line by line. The acquired hypercube size was 320×280 (pixels)×101 (wavelengths). Each pixel corresponds to a NIR spectrum. Calibration was performed recording two images for black and white references. The black image (B) was acquired to remove the effect of dark current of the camera sensor, turning off the light source and covering the camera lens with its cap. The white reference image (W) was acquired for a standard white ceramic tile under the same condition of the raw image. Image correction was thus performed adopting the following equation (I):

$$I = \frac{I_0 - B}{W - B} \times 100 \quad (1)$$

Where I is the corrected hyperspectral image in a unit of relative reflectance (%),  $I_0$  is the original hyperspectral image, B is the black reference image (~0 % reflectance) and W is the white reference image (~99.9 % reflectance). All the corrected images were then used to perform the HSI based analysis, that is to extract spectral information, to select the effective wavelengths and for the final classification purposes.

### Spectral data analysis

Spectral data analysis was carried out adopting standard chemometric methods, utilizing the PLS\_Toolbox (Version 6.5.1, Eigenvector Research, Inc.) running inside Matlab® (Version 7.11.1, The Mathworks, Inc.).

First, the raw spectra were cut at the beginning and at the end of the wavelength range in order to eliminate unwanted effects due to background noise. The number of wavelengths was thus

reduced from 101 to 92 and the new investigated interval was 1013–1650 nm.

Data were first pre-processed applying the “generalized least squares weighting” (GLSW) algorithm, that calculates a filter matrix based on the differences between pairs or groups of samples which should otherwise be similar. The goal of GLSW is to remove the within-class variance as much as possible without reducing at the same time the between-class one. More in detail, the algorithm proceeds by centering the data coming from the different categories to their own class mean and use this class-centered data matrix to compute the filter (Serranti et al. 2013a, b). An exploratory analysis of the spectral data was carried out through the help of principal component analysis (PCA) (Wold et al. 1987)). From the hyperspectral images of leaves, several regions of interest (ROIs), representative of the different inoculation times, were selected and the corresponding spectra were arranged in a two-dimensional matrix which constituted the training set for this investigation. Starting from the training matrix, made up of the spectra from the selected ROIs, exploratory data analysis was performed. The training data, pretreated as previously described applying the GLSW algorithm, were then used to build a PCA model for exploratory purposes. Therefore PLS-DA was used to classify samples into predefined groups by forming discriminant functions from input variables (wavelengths) to yield a new set of transformed values that provides a more accurate discrimination than any variable (wavelength) alone (Barker and Rayens 2003). A discriminant function is then built using samples with known groups to be used later to classify samples with unknown group membership. Therefore, once the model is obtained, it can be applied to an entire hypercube and for classification of new hypercubes. The result of PLS-DA applied to hyperspectral images is a “prediction map”, where the class of each pixel can be identified using color mapping. The purpose of PLS-DA applied to leaves collected at different times was to validate their correct classification for the recognition of the fungal growth with time using a fast technique. The PLS-DA model was built using all the 92 acquired wavelengths collected in the NIR spectral range. A cross-validation method



based on a “Random Subset” (RS) algorithm was utilized, that is a selection method rather versatile, used effectively in a wide range of situations, allowing running multiple iterations of subset selections.

## Results

### Flag leaf and ear infection

The disease was recorded on the flag leaf and ear of all three varieties for all the treatments. The visual detection of the disease after an artificial inoculation with this *P.nodorum* isolate was 6 days post inoculation. The disease symptoms on flag leaves can be observed both on Inf A and Inf B plants even though with a different severity (Table 1). Notably, Inf A determined an higher impact of infection, concerning the symptoms manifested in the flag leaf and up to the ear, compared to Inf B and, more obviously to T and NT samples. Nevertheless, Inf B resulted in an overall lower yield compared to Inf A thesis with statistically significant differences in cultivar C2 (Table 1). The SNB index values indicated that C3 had an apparent higher susceptibility to infection, both at the early (Inf A) and the late stage of infection (Inf B), in comparison with C1 and C2 and the disease resulted more severe on the flag leaf respect to the ear, as also reported by other authors (Arseniuk et al. 1998) (Table 1). A qPCR assay performed to unravel whether the fungal mycelia were present in the kernel (Table 1). It was possible to detect *P. nodorum* in all the kernels of Inf A. C2 kernels of Inf A thesis presented more abundant amount of fungal DNA (~4.2 ng/g wheat seed) compared to C1 (~0.02 ng/g wheat seed) and C3 (~0.3 ng/g wheat seed) (Table 1).

### Comparison and correlation between grain yield, varieties and infection time (Inf A and Inf B)

A positive correlation should be expected between fungal DNA and variables linked to infection estimates - Severity on flag leaf (%), Severity on ear (%), Incidence (%) and the SNB index - while a negative correlation should be expected between fungal DNA and variables of wheat productivity -Total production, Weight 1000 seeds (g) and test Weight (Kg/hl). The Pearson correlation results (Table 2) followed what was formerly hypothesized only when considering C1 and C3 varieties.

A different performance of the DNA variable was highlighted for the C2 variety. ANOVA analysis (Table 1) showed that the three varieties, and in particular C2, behave in a significantly different way towards the infection with *P. nodorum*, but also respect to the productivity in the absence of fungal infection.

### Analysis and modeling of the fungal infection progression

In previous results, we have shown that early-stage infection (namely, Inf A) induced the most dramatic effects on flag leaf and on ear. In relation to this and, since SNB is one of the devastating foliar diseases of *T. aestivum* and *T. turgidum* (Faris and Friesen 2009), we decided to try to describe fungal contamination progression by matching hyperspectral images with molecular data related to fungal DNA amount into plant leaves.

The average raw and preprocessed spectra of the second leaves collected at 6 different time intervals (from 24 up to 360 h post inoculation) from inoculated C1, C2 and C3 Inf A thesis and not treated with fungicide (T) and not inoculated (NT) with *P. nodorum*, were reported in Fig. 1. In this figure, the reflectance level of the spectra increased with time, especially for C1 and C2 leaves, whereas for C3 it was not so evident. This result was confirmed by the application of PCA, as shown in Fig. 2. In fact, concerning the varieties C1 and C2, the spectral data of the six time-classes were clustered into distinct groups according to their spectral signatures, allowing a clear discrimination between the classes of wheat leaves. On the contrary, for C3 variety, only the 24 h inoculated leaves were clustered in a group separated from the others. Following the PCA, it was possible to obtain graphs of loadings and through a processing of the reflectance data, to select the wavelengths in which the most information regarding progression of fungal infection were accumulated. The loadings resulting from PCA were considered as an indication of the effective wavelengths that could contribute to classification of progression of fungal infection. The selected wavelengths were those contributing most to the loadings of the PCs. Thereafter, the significant wavelengths, i.e. those which are able to represent all the information useful to delineate the trend of growth and progression of the disease in Inf A samples, may be extrapolated and used for quantifying it (Table 3a–c). Notably,  $\lambda=1650$  seemed the most

**Table 1** One-way ANOVA/ Tukey’s HSD (honestly significant difference) test comparisons

DURUM WHEAT VAR.	Total production	Weight 1000 seed	Test weight	DNA	Severity on flag leaf %	Severity on ear%	Incidence %	SNB index
C1	INF A	1.91 A	51.24 A	76.87 A	0.02 A	46.67 B	23.33 A	9.67 B
	INF B	1.68 A	47.41 A	75.77 A	ND	36.67 B	16.67 A	1.83 AB
	NT	2.12 A	51.61 A	79.10 A	ND	8.33 A	4.67 A	0.57 A
	T	2.17 A	54.86 A	79.37 A	ND	3.33 A	3.00 A	0.20 A
C2	INF A	2.23 B	52.61 A	77.07 A	4.18 B	40.00 BC	26.67 A	9.00 A
	INF B	1.59 A	53.46 A	73.13 A	ND	46.67 C	23.33 A	4.67 A
	NT	2.11 AB	53.87 A	76.10 A	ND	10.00 AB	5.00 A	0.40 A
	T	2.21 AB	60.15 A	73.73 A	0.22 A	5.00 A	3.67 A	0.20 A
C3	INF A	2.05 AB	43.46 A	72.40 A	0.33 B	46.67 B	50.00 B	23.67 B
	INF B	1.35 A	39.63 A	69.20 A	0.02 A	40.00 B	73.33 B	36.67 B
	NT	2.55 B	52.61 A	78.50 A	ND	11.67 A	7.00 A	1.23 A
	T	2.47 B	48.65 A	80.10 A	ND	5.00 A	4.33 A	0.45 A

Total production (Kg/plot), Weight 1000 seeds (g), Test Weight Kg/hL, DNA (ng/g wheat seeds), Severity on flag leaf (%), Severity on ear (%), Incidence (%), SNB index were reported in Table. Statistically significant differences ( $p < 0.05$ ) between the treatments are marked with different letters (A, B, C). The ANOVA and relevant comparisons were separated for each variety (C1, C2 and C3). ND values are below the limit of confidence within the standard curve generated. (plants non-inoculated and treated with commercial fungicide (T), plants non-inoculated and non-treated with Folicur (NT), plants non-treated and inoculated with *P. nodorum* spore at growth stage GS 32 (Inf A), plants non-treated and inoculated with *P. nodorum* spore at growth stage GS 73 (Inf B)

performing in illustrating the alteration of reflectance caused by fungal disease progression. In fact, this  $\lambda$  was able to significantly measure alterations in C2, starting from 48 hpi and from 72 hpi in the other 2 varieties. An attempt to correlate the variation in reflectance with the real quantity of the fungus present into the leaf tissues was performed by qPCR on the same leaf analysed by hyperspectral imaging. The three varieties

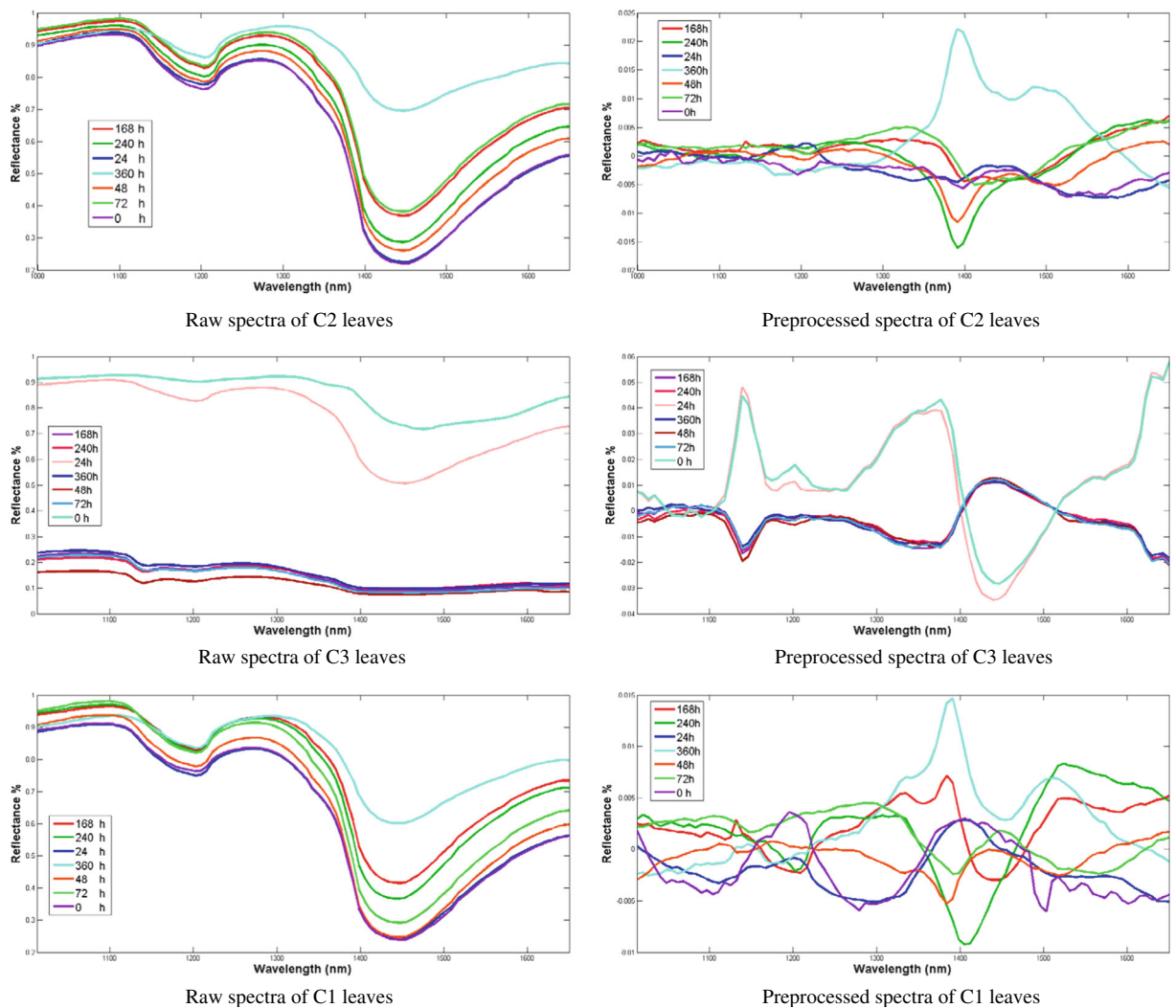
of wheat presented different quantities of fungal DNA (Table 4) during the whole time course of the experiment. Notably, C2 presented the highest amount of *P. nodorum* DNA with C1 the lowest.

The two matrix, i.e. the panel of DNA amount and  $\lambda = 1650$  values in all samples and in all the time intervals, were positively and significantly correlated ( $R^2 = 0.67$ ;  $p < 0.05$ ), suggesting that the variation in

**Table 2** Correlation (Pearson) between the variable “DNA” and all the other variables considered

	DNA all varieties	DNA only C2	DNA without C2
Total production	0.130	0.329	-0.039
Weight 1000 seeds (g)	0.087	-0.271	-0.333
Test Weight kg/hL	0.055	0.512	-0.333
Severity on flag leaf (%)	0.247	0.385	<b>0.492</b>
Severity on ear (%)	0.141	0.491	<b>0.530</b>
Incidence (%)	0.104	0.533	<b>0.455</b>
SNB index	0.059	0.475	<b>0.457</b>

Values in bold are different from 0 with a significance level  $\alpha = 0.05$ . The correlation was calculated considering data obtained from all the cultivars (C1-C2-C3), only from the cultivar C2, and from all the cultivars excluding C2. Significant correlation values between the variable DNA and some infection variables were obtained only when the cultivar C2 was treated separately, thus indicating a very different behavior between cultivars towards the infection



**Fig. 1** Acquired (raw) and pre-processed reflectance spectra of the different analysed leaf samples, collected at six different time intervals for the three different wheat varieties C1, C2 and C3 in

reflectance at this wavelength may be indicative of fungal presence in leaf tissue, thus providing a hyperspectral marker of SNB.

Moreover, an attempt of a PLS-DA (Partial Least Squares Discriminant Analysis) based classification was applied, in order to verify the possibility to identify the leaves with different time from inoculation. The same pre-treatment technique adopted to perform PCA was used (GLSW - see Methods section). The results shown in Fig. 3 indicated that the PLS-DA model allowed to achieve a good recognition/classification, according to the six different inoculation time for the leaf samples of C1 and C2 varieties; whereas for C3

the NIR field. Spectra pre-processing was carried out adopting a GLSW (generalized least squares weighting) procedure ( $\alpha=0.02$ )

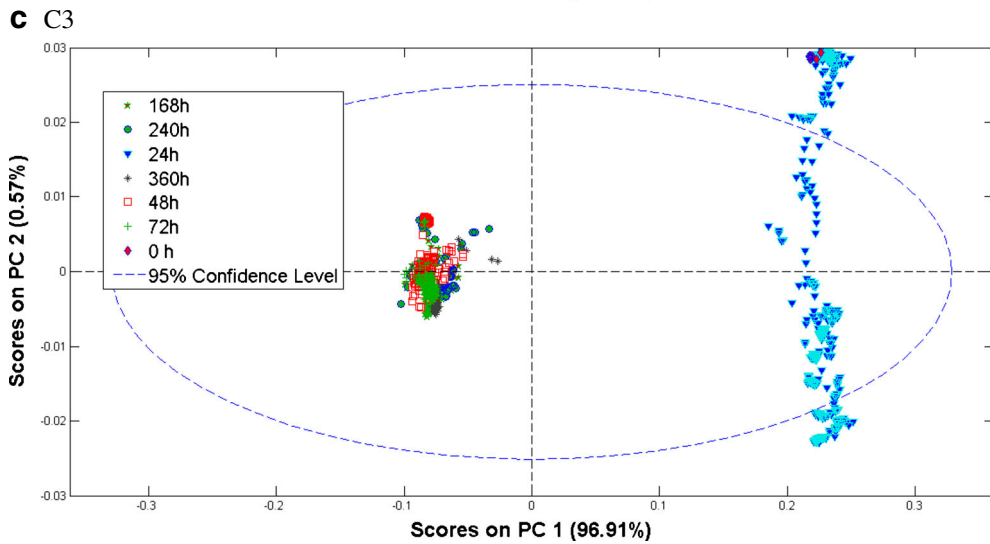
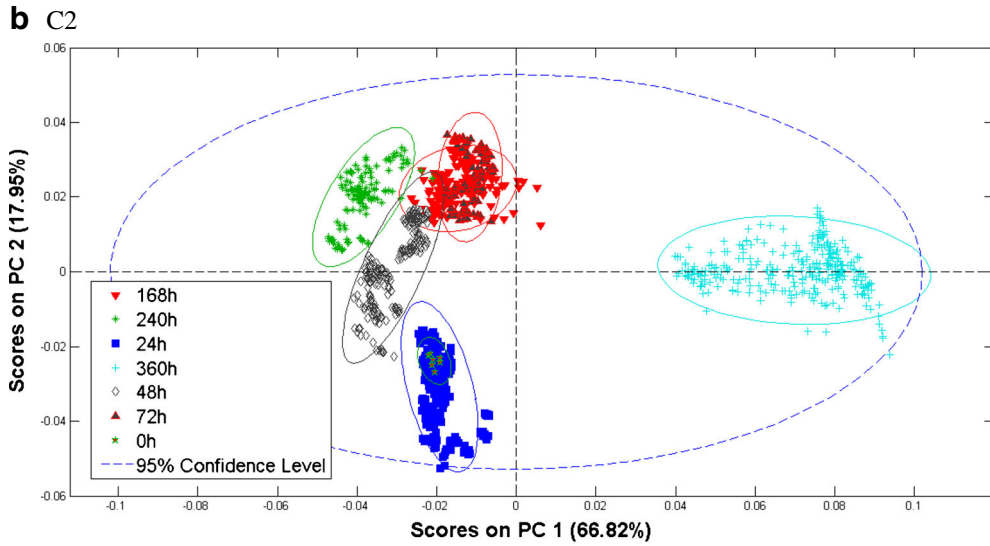
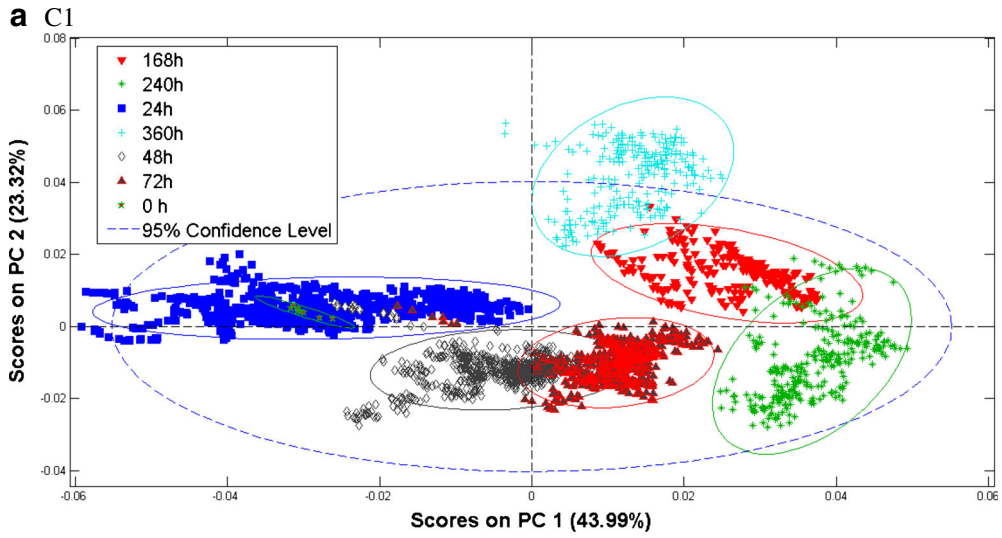
leaves it was possible to separate two groups: before and after 72 h from inoculation.

## Discussion

Durum wheat cultivation represents a major entry in the Mediterranean agro-industry (Quaranta et al. 2012).

**Fig. 2** Score plot (PC1 versus PC2), based on average HSI spectral signatures in the NIR wavelength region for the leaves of the three different wheat varieties collected at different times from inoculation. Each *score point* corresponds to a pixel of the selected ROI extracted from the image hypercube





**Table 3** Reflectance value at 1384 nm (A), 1510 nm (B) and 1650 nm (C) selected after the preprocessing of the reflectance raw data on Inf A samples

Reflectance values at $\lambda$ 1384								
a)	NT	0 hpi	24 hpi	48 hpi	72 hpi	168 hpi	240 hpi	360 hpi
C1	0.56±0.05	0.57±0.04	0.51±0.03	0.52±0.02	0.57±0.02	0.68±0.02	0.63±0.05	0.79±0.06
C2	0.50±0.02	0.51±0.02	0.49±0.01	0.55±0.03	0.67±0.03	0.67±0.03	0.83±0.05	0.79±0.07
C3	0.54±0.06	0.54±0.06	0.52±0.07	0.53±0.03	0.48±0.03	0.65±0.01	0.83±0.04	0.79±0.04
Reflectance values at $\lambda$ 1510								
b)	NT	0 hpi	24 hpi	48 hpi	72 hpi	168 hpi	240 hpi	360 hpi
C1	0.41±0.03	0.42±0.05	0.36±0.04	0.37±0.08	0.41±0.01	0.55±0.04	0.50±0.05	0.71±0.03
C2	0.34±0.04	0.34±0.03	0.34±0.06	0.39±0.04	0.53±0.03	0.54±0.03	0.76±0.08	0.71±0.08
C3	0.40±0.02	0.39±0.04	0.39±0.05	0.33±0.05	0.51±0.02	0.51±0.01	0.75±0.02	0.72±0.02
Reflectance values at $\lambda$ 1650								
c)	NT	0 hpi	24 hpi	48 hpi	72 hpi	168 hpi	240 hpi	360 hpi
C1	0.64±0.02	0.65±0.05	0.58±0.04	0.61±0.01	0.64±0.04	0.75±0.02	0.71±0.05	0.81±0.03
C2	0.56±0.02	0.57±0.03	0.56±0.02	0.62±0.04	0.73±0.01	0.73±0.03	0.85±0.05	0.83±0.07
C3	0.60±0.06	0.62±0.03	0.59±0.05	0.57±0.03	0.72±0.02	0.71±0.01	0.85±0.06	0.82±0.02

Results are presented as the mean ( $\pm$  SE) of  $n=9$  replications

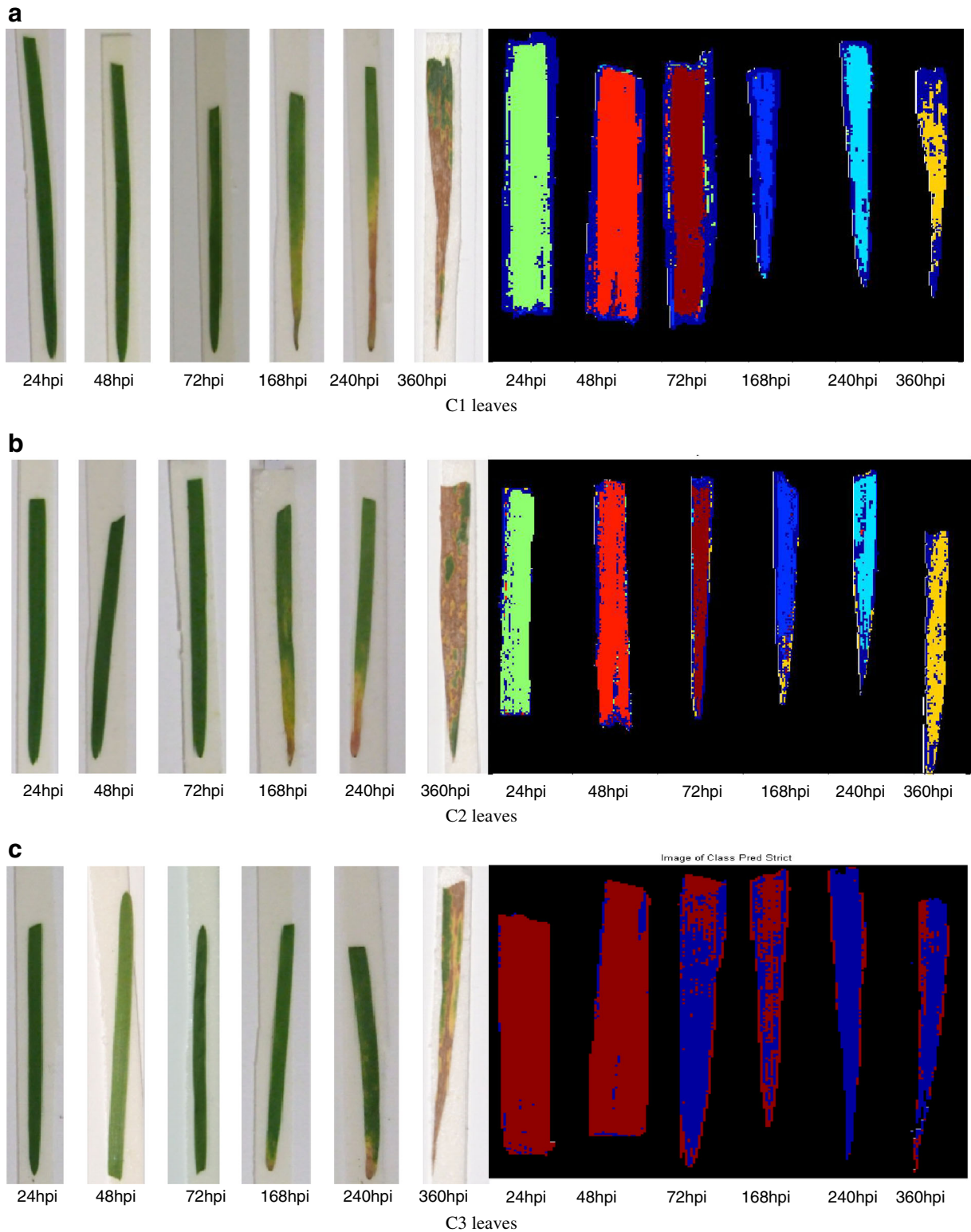
Nevertheless, the main use of durum wheat is for pastry, a small amount is indeed destined to bakery products. Durum wheat in the Mediterranean diet is very important as it provides carbohydrates, proteins, fibers and certain minerals and vitamins. Considering the valuable aspects of nutritional features and economic value of durum wheat, several studies are devoted to monitor the incidence and diffusion of fungal diseases in this crop (Pasquini et al. 2003). Durum wheat may be severely affected by fungal contaminations that cause seeds spoilage and accumulation of mycotoxins. Over recent years, SNB disease is more and more incident

worldwide, reducing harvest yield of durum wheat (Bhathal et al. 2003) and some authors reported that *P. nodorum* can produce a secondary metabolite identified as alternariol, a well-known mycotoxin (Tan et al. 2009). In this paper, we have used three Italian varieties of durum wheat commonly infected by *P. nodorum* (Pasquini et al. 2003) to mimic *P. nodorum* infection at different phenological stages, an artificial contamination was done at two different times of development of the plant in the field. The artificial contamination in field indicated that the early stage infection fully expresses disease symptoms. The macro- and microscopic

**Table 4** Fungal growth expressed as  $\beta$ -tubulin ng/g wheat leaves (Inf A) at different time intervals (0–360 hpi) and in 3 commercial varieties (C1, C2, C3)

$\beta$ -tubulin (ng DNA/g wheat leaves)								
	NT	0 hpi	24 hpi	48 hpi	72 hpi	168 hpi	240 hpi	360 hpi
C1	ND	ND	ND	ND	0.012±0.001	0.087±0.012	1.756±0.252	3.093±0.42
C2	ND	ND	ND	0.093±0.004	0.573±0.085	0.671±0.002	5.116±0.015	19.51±3.51
C3	ND	ND	ND	ND	0.345±0.062	0.566±0.001	4.598±0.652	10.00±2.01

Results are presented as the mean ( $\pm$  SE) of  $n=9$  replications. ND values are below the limit of confidence within the standard curve generated



**Fig. 3** Prediction images based on PLS-DA classification model built for C1, C2 and C3 leaves collected at different times from inoculation. In the *colour map* images, the different classes were attributed at different colours: *Green*-24 h; *Orange*-48 h; *Red*-72 h; *Blue*-168 h; *Cyan*-240 h; *Yellow*-360 h; *Dark blue*-not assigned pixel class

inspection confirmed the presence of *P. nodorum* only in infected samples (Inf A and Inf B).

Indeed, the progression of the diseases in the wheat leaves and ears was different in the three wheat varieties studied. *P. nodorum* develops rapidly in C2 compared to C1 and C3. Our results indicate that, particularly in the early stage infection (Inf A samples), the pathogen is able to reach the kernel. Nevertheless, even if *P. nodorum* is present in the kernel, it does not thoroughly affect wheat yield (Table 1). In fact, ANOVA analysis confirms that the values of yield in the Inf A samples are not significantly different from untreated (NT) and fungicide-treated samples (T). Intriguingly, Inf B (late stage infection) samples present less macroscopic disease symptoms and a lower fungal development in the kernels, but are hugely and significantly affected in yield compared to early stage inoculated samples (Inf A). The results indicate that wheat yield seems to be affected mostly by late stage than early stage infection processes. Nevertheless, the lower fungal growth on adult plant observed in our experiments may be related to what observed by other authors which report how the resistance towards pathogens in adult plant is major than in the juvenile one (Robert et al. 2004; Develey-Riviere and Galiana 2007; Whalen 2005) even if the production was affected. Notably, focusing on the different responses to SNB disease, C2 presents the higher amount of fungal DNA both in the leaves and in the kernels, but the effect of infection to its yield is similar to that of C1 and C3. This consideration may lead to hypothesize that C2 may tolerate *P. nodorum* presence better than the other varieties. The results of this study depict a scenario in which a prompt detection of the disease, performed straight in situ, may contribute to drive tailored intervention to reduce fungal contamination, considering the importance of an early pathogen detection in plant pathology.

In this study, we tried to anticipate the detection of SNB in durum wheat at an early stage of infection at field level, by matching hyperspectral imaging with molecular analysis of infected leaves. In this approach, the most performing DNA quantification by qPCR was used for validating the more erratic data originated by hyperspectral imaging. This approach has been used previously in maize and wheat for trying to correlate

the DNA amount of mycotoxigenic fungi in seeds with specific wavelength originated by hyperspectral imaging (Del Fiore et al. 2010).

According to the results, the severity of the damage caused by *P. nodorum* is not directly correlated to fungal growth and could be addressed to variety-specific necrotrophic effectors of the fungus described by several authors (Oliver et al. 2012; Faris and Friesen 2009; Ali et al. 2008; Liu et al. 2004a, b; Ciuffetti et al. 1997). From the results of hyperspectral data analysis it is evident that reflectance values increased over the time of infection, especially from 24 to 72 hpi, which corresponds with the early stages of *P. nodorum* development into plant tissues. The application of PLS-DA classification to all the samples confirmed these results indicating also that hyperspectral data are positively correlated with the *P. nodorum* DNA amount. Our data suggest that it is possible to detect SNB wheat disease in the early stage of infection with *non-invasive* methods.

Even if further investigations are needed to improve these findings, a better knowledge of this phenomenon could lead to safer and economically sound strategies in the control of SNB in wheat. Moreover, this study defines at least one diagnostic wavelength for early detecting SNB in durum wheat. The application of a fast, non-destructive and low-cost technique, that is NIR-HSI, using a sensing device, mounted on a vehicle, could be useful for the early detection of fungal contamination directly in situ, significantly reducing time and costs of the routine analyses for checking the healthiness of cereals, improving the quality and reducing economic loss.

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