LOCALIZED SEPTORIA LEAF BLOTCH LESIONS IN WINTER WHEAT FLAG LEAF DO NOT ACCELERATE APICAL SENESCENCE DURING THE NECROTROPHIC STAGE

R. Ben Slimane[1], P. Bancal[1], F. Suffert[2] and M.-O. Bancal[1]

[1] *INRA, UMR1091 EGC AgroParisTech, 78850 Thiverval Grignon, France*
[2] *INRA, UR1290 BIOGER-CPP, 78850 Thiverval Grignon, France*

SUMMARY

Mycosphaerella graminicola, the most damaging disease of wheat in Northern Europe, induces yield or grain quality losses usually related to green area losses. This work aims at clarifying whether induced senescence is due to an acceleration of apical senescence and thus to a modification of the leaf nitrogen remobilization rate. The effect on apical leaf senescence of a restricted diseased leaf area was investigated. The experiment involved four winter wheat cultivars varying in their susceptibility to four *M. graminicola* isolates. Flag leaves were inoculated at a dose representative of field epidemics. As soon as symptoms appeared, dynamics of apical senescence (*Sapi*) and around the inoculation zone (*Ssep*) were measured by repeatedly taking digital photos until senescent areas merged. *Ssep* trends were fit to a logistic function whose parameters depended on cultivar×isolate interactions. Disease severities ranged from 0 to 24% total leaf area. Apical senescence followed an exponential pattern in control and inoculated leaves. *Sapi* was twice as great in inoculated shoots as in controls, even when no disease occurred. The relative rate of *Sapi* depended on the cultivar, but no isolate effect was detected despite wide variations in *Ssep* extent. Paired comparisons between inoculated and control leaves showed that the relative rate of *Sapi* was not increased by inoculation. It is concluded, that over a wide range of Septoria disease, apical senescence was not accelerated in inoculated leaves as compared to control. Results suggest that the disease did not modify the regulation of leaf N remobilization rate.

Key words: *Septoria tritici*, *Mycosphaerella graminicola,* apical senescence, induced senescence, winter wheat, nitrogen remobilization.

Corresponding author: P. Bancal
Fax: +33.1.30815563
E-mail: bancal@grignon.inra.fr

INTRODUCTION

Septoria leaf blotch (SLB), caused by the hemibiotrophic fungus *Mycosphaerella graminicola* (Fückel) Schroeter (anamorph *Septoria tritici* Rob. ex Desm.), is among the most frequent and damaging foliar diseases of wheat (*Triticum aestivum*) in Northern Europe. The main damage to plant functioning caused by SLB is a decrease in green leaf area [GLA (Robert *et al.*, 2004)]. Disease usually expands after maximum GLA is reached, shortening the green leaf area duration (LAD) more specifically during grain filling. Gaunt (1995) stated that LAD shortening by pathogens is closely related to the lower photosynthesis balance after anthesis, and therefore to grain yield losses, which can reach 60% in susceptible cultivars (Cornish *et al.*, 1990). By contrast, the correlation between LAD and grain protein concentration (GPC) has been shown to vary with environment, genotype and disease severity (Dimmock and Gooding, 2002). In diseased crops, GPC (the main quality index in wheat trading) becomes unpredictable as it sometimes increases but more often decreases following SLB attacks. In this context, Bancal *et al.* (2008) pointed out that the decrease in GLA affected differently the two nitrogen (N) sources for grain filling, i.e. post-anthesis N uptake and N remobilization. The relative contribution of each of these to N grain filling can vary widely with environment and genotype. To make a robust prediction of GPC, GLA decrease should be separately compared to N uptake, N remobilization and carbon assimilation.

It may be worth modeling GLA dynamically in the absence and presence of SLB. After anthesis, leaves do senesce in healthy plants; N is remobilized to fill the grain, while GLA progressively declines. The mechanisms involved in the control of natural senescence have not been thoroughly described and are still not well understood. Most reports suggest that control is located in the vegetative parts, which are the main N sources for grain filling through remobilization (Gan and Amasino, 1997; Gregersen *et al.*, 2008; Masclaux-Daubresse *et al.*, 2008). Then when no disease occurs, N fluxes within the whole plant are related to natural leaf senescence, which in cereals progresses from the leaf apex to the ligulae (apical senescence). Most papers dealing with

leaf diseases are based on an overall assessment of senescence from which healthy control senescence is subtracted. In diseased leaves, however, apical senescence may itself be affected by diseases, and thus should be differentiated from local senescence around the infection point, as they involve different physiological perturbations.

SLB symptoms *sensu stricto* (necrotic area dotted with pycnidia) develop around the infection sites and can be directly attributed to damage by the fungus to the plant tissues. Tavernier *et al.* (2007) observed a considerable decrease in chlorophyll and protein content coinciding with the appearance of anthracnose symptoms in pea leaves when the fungus *Colletotrichum lindemuthianum* shifted from its biotrophic to its necrotrophic stage. The spread of local senescence beyond the pycnidia area has also been reported in SLB (Gooding *et al.*, 2005; Leitch and Jenkins, 1995). A possible scenario in this adjacent zone is that pathogens induce senescence through the release of toxic products causing pathogenesis near the infection points rather than through a direct interaction with plant tissues. Hence, Talbot *et al.* (1997) found that rice leaves showed a pronounced senescence 48 h after applying culture filtrate extracts of *Magnaporthe grisea*. The involvement of toxins in *M. graminicola*×wheat interaction is suspected, but to our knowledge it has not yet been firmly established. Local senescence is thus clearly induced by the disease, whereas the pathogen's influence on remote apical senescence is less clear. Even if apical senescence remains more or less controlled in diseased just as in healthy leaves, senescence control could itself be affected by external factors such as pathogen attack (Quirino *et al.*, 2000). One hypothesis that could support increased apical senescence in diseased leaves is that the pathogen diverts nutrients from the leaves for its own growth. The pathogen thus acts as an additional N sink and the plant might maintain the rate of grain N filling by accelerating N leaf depletion (Pageau *et al.*, 2006). While biotrophic fungi are the plant pathogens most cited as new sinks (Pageau *et al.*, 2006), hemibiotrophic fungi with a long biotrophic latent period like *M. graminicola* may also act as new sinks interfering with plant functioning.

At the leaf scale, the components of senescence should be separated into apical senescence and local senescence, because they result from different physiological processes: reaction to pathogen infection for local senescence versus whole plant N balances for apical senescence. However, independent assessments of apical senescence and SLB lesions are difficult to obtain in field experiments after the early stages of disease development. Infection points are scattered over the whole leaf area so that infected areas rapidly merge with apical senescence.

The aim of this article is to investigate the effect of a *M. graminicola* infection on the dynamics of apical senescence, using localized inoculation on a restricted leaf area and in the long term to model the effects of the disease on GLA loss. In the literature, inoculation frequently leads to explosive diseases with much higher infection rates than those encountered in the field. The inoculum concentrations used in artificial contamination are usually very high (10^7 conidia/ml) and are applied to the entire leaf area, which is hardly comparable with natural contamination events. In such conditions, Robert *et al.* (2004) observed an increase in apical senescence, but separation between senescences was obtained only at the very beginning of the rapidly advancing disease. In the study presented here, restricted areas on flag leaves of different winter wheat cultivars were inoculated using different *M. graminicola* isolates at a low inoculum concentration, probably close to natural conditions. A range of compatible interactions between plant and pathogen was thus obtained. Non destructive assessments were repeated to characterize the senescence kinetics of both inoculated zone and leaf apex on the same leaves. We tested whether or not apical senescence showed a pattern in diseased leaves different from that in healthy ones. If SLB affects apical senescence, then all N remobilization fluxes in the plant should be modified in future models. Otherwise the fungus controls senescence only in the pycnidia area and the adjacent zone.

MATERIALS AND METHODS

Greenhouse experiment design. The greenhouse experiment involved four winter wheat cultivars varying in their susceptibility to SLB: cvs Soissons and Apache are recorded as moderately susceptible (resistance rating 5), while cvs Koreli and Caphorn are classified as resistant and moderately resistant respectively (resistance rating 7 and 6).

Four isolates of *M. graminicola* were used. Three of them were recovered from typical SLB lesions on wheat leaves of cv. Soissons collected in 2008 in both Grignon (Yvelines, France) (isolate INRA08-FS0001 and INRA08-FS0002) and Le Rheu (Ille-et-Vilaine, France) (isolate INRA08-FS0003). The fourth one was the reference isolate IPO323 (Kema and Van Silfhout, 1997).

Plant preparation. Seeds of cvs Soissons, Apache and Caphorn were sown in Jiffy peat pots at a rate of two seeds per pot, on December 5th 2008; seeds of cv. Koreli were sown one week later. Seedlings were kept for eight weeks at 8°C with a 10 h light period and a 14 h dark period for vernalization. They were then transferred to square pots (1.1 l) filled with commercial compost (Klasmann substrate 4; Klasmann, France), fertilized with 4 g Osmocote Exact (16N 11P 11K 3MgO

Te), and placed in the greenhouse. The plants were fertilized twice with a commercial nutrient solution (1 l Hydrokani C2) diluted 1/100 and applied in the pot saucers five and seven weeks after the seedlings had been transferred to the greenhouse.

Daylight was complemented by 400-W sodium lamps to obtain 9 h of dark and 15 h of light. Temperature was regulated by a cooling system at 12°C at night and 20°C by day. Greenhouse air temperature was registered automatically every 15 min. Thermal time, expressed in degree-days (°Cd), was calculated by totaling daily mean air temperature using a 0°C base temperature.

Treatments against aphids (λ–Cyhalothrin, 1 mg/l; Pyrimicarb 20 mg/l) were applied regularly. A preventive treatment against powdery mildew (Ethyrimol, 2 ml/l; Quinoxyfen, 1 g/l) was applied immediately after the vernalization phase and repeated three weeks later. Secondary tillers were cut and only two shoots were kept per plant, from which the first (the main shoot) was inoculated and the second used as control.

Inoculum preparation and inoculation. Inoculation was performed at heading stage for cv. Caphorn, and at anthesis for Apache, Koreli and Soissons. The inocula were obtained from stock conidial suspensions of the four *M. graminicola* isolates. Subcultures were grown in Petri dishes containing potato dextrose agar (39 g/l) at 20°C in total darkness. Conidia suspensions were prepared on the day of inoculation by flooding the surfaces of 5-day-old cultures with sterile distilled water, then scraping the agar surface using a sterilized glass rod to release conidia. The resulting suspensions were adjusted to the desired concentration by dilution, using a haemocytometer (Malassez cell). Two drops of surfactant polyoxyethylene sorbitan monolaurate (Tween 20, Sigma, USA) were added per 100 ml of inoculum. Conidial suspensions were applied on April 14th 2009 to the adaxial face of tagged flag leaf blades of the main shoot. A paintbrush was used to apply the suspension, using a piece of plastic with a square opening (25×25 mm) to delimit the surface of inoculation. The leaf was thus inoculated along a 2.5 cm long section (3.5 to 4.5 cm^2 depending on the average width in the middle part of the leaf). After spraying, the inoculated side of the leaf was marked with a small white sticker, turned over to avoid inoculum suspension trickling, and enclosed for 72 h in a transparent polyethylene bag containing a small amount of distilled water, sealed to maintain high humidity to promote infection. Additional lamps were removed during the three days of bagging to avoid high temperature and increase air humidity in the bags. Each cultivar × isolate combination was replicated four times. This gave a set of 64 inoculated plants.

Assessment of diseased and senescent areas. From the appearance of the first symptoms (chlorosis, necrosis and pycnidia), the development of apical senescence on inoculated and controls leaves and SLB lesions on inoculated leaves were photographed and measured by numerical image analysis at a resolution at 1000 pixels/inch. The device consisted of a digital camera fitted in a closed body, the inside of which was equipped with light emitting diodes (LED) providing a polychromatic white light. The benefit of this device was to keep photos essentially free from the external monochromatic light from the sodium lamps of the greenhouse. Photos were taken on a blue ground for easily separating leaves by image analysis. The pictures were about 10 cm long, so several pictures were added to obtain each single leaf assessment. Assessments were carried out until all plants were completely senescent: inoculated shoots were assessed up to 10 times. In most cases, inoculated and control shoots were assessed on the same day. The assessments dates were expressed in °Cd after inoculation.

Image analysis. In order to eliminate color dominance, the white color was adjusted prior to any image analysis, using a control area within each picture. The resolution was then decreased to 700 pixels/inch, precise enough to allow analysis with Assess software. Results were obtained in pixels and ratios to whole leaf blade area (%LA) of green and senescent areas were calculated. Two kinds of symptoms were measured: senescent area at leaf apex (*Sapi*) and SLB lesions around the inoculation zone, with or without pycnidia (*Ssep*). Image analysis was performed according to each picture, and then results were added up for each leaf. At later stages senescent areas merged; results therefore are based on an average of only six fair assessments of *Sapi* and *Ssep* on the inoculated leaves.

Statistical analyses and data transformation. The data variances were compared between cultivars and between isolates using Cochran's and Bartlett's test, leading us in some cases to log-transform the raw data. The normality of the data sets was then checked using a skewness and kurtosis test. When data sets were not normal, the equality of medians was compared using the Kruskal-Wallis test to investigate the effects of cultivars and isolates. Normal data sets were compared by analysis of variance (ANOVA) and of covariance. Interaction between *Sapi* and *Ssep* kinetics was investigated by analysis of covariance using the logarithm of *Ssep* AUDPC as a covariate.

The *Ssep* kinetics versus time expressed after inoculation was adjusted for every shoot to a logistic function of time t with r^2 at 0.98 on average:

$$Ssep = Msep/[1 + e^{Rsep.(Tsep-t)}]$$

where *Msep* is the asymptotic plateau reached by *Ssep*, *Rsep* the initial relative growth rate, and *Tsep* the time at the inflection point when *Ssep* reached *Msep*/2. Statisti-

cal analyses were performed on these three parameters as well as on raw *Ssep* data.

The *Sapi* kinetics was adjusted for every shoot to an exponential function of time: data were log-transformed so that the parameters could be estimated from linear regression with r^2 at 0.97 on average. The slope represents the relative rate of apical senescence *Rapi* while the intercept is the logarithm of apical senescence at the inoculation date. Statistical analyses were performed on these two parameters as well as on raw *Sapi* data.

RESULTS

The first SLB symptoms occurred 24 days after inoculation (405°Cd). Senescent areas were assessed for the next 50 days. Some leaves still showed distinguishable senescent areas until 1244°Cd, but in most cases senescent areas merged long before that, impairing discrimination between *Ssep* and *Sapi* symptoms by image analysis. Averaged results per treatment are therefore presented in the present paper at 1003°Cd, using either raw data still available for 36 shoots, or extrapolations at this date for the 28 shoots exhibiting early area merging. Extrapolations were obtained from fitting of symptoms as described below. Hence, disease symptoms and apical senescence were analyzed at the two key dates: 405°Cd and 1003°Cd, before being fitted in order to analyze kinetics parameters.

Necrosis at the restricted inoculated area. ***Analysis of the beginning and the end of septoria development.*** (a) *At 405°Cd post-inoculation.* At 405°Cd, the first assessment date, one half of the inoculated shoots exhibited disease necrosis, but with wide variations between and within treatments (Table 1). At this stage, the resistant cvs Caphorn and Koreli (*Ssep* less than 0.1 cm^2) were already differentiated from the susceptible Apache and Soissons (*Ssep* 0.3 cm^2 on average, *i.e.* 0.8 %LA). Isolate IPO323 led to smaller *Ssep* (0.01 cm^2) than did INRA08-FS0001, INRA08-FS0002 and INRA08-FS0003, which did not differ from each other (0.21 cm^2).

(b) *At 1003°Cd post-inoculation.* At 1003°Cd, 51 of the 64 inoculated shoots exhibited SLB around the restricted inoculated area. With one exception, all shoots that did not show symptoms had been inoculated with IPO323 isolate. No necrosis was ever observed on resistant cvs Caphorn and Koreli inoculated with IPO323. Including inoculated shoots where necrosis did not oc-

Table 1. Necrosis at inoculation zone (*Ssep*): measurements at 405°Cd and 1003°Cd after inoculation and results of logistic fitting.

Cultivar/Isolate	Measurements (cm^2)		Parameters from logistic fitting			
	Ssep at 405°Cd (cm^2)	*Ssep* at 1003°Cd (cm^2)	*Msep* (cm^2)	*Rsep* ($°Cd^{-1}$)	*Tsep* (°Cd)	AUDPC to 1003°Cd (dm^2°Cd)
Apache	0.34 c	3.4 ab	5.0 ab	0.008 ab	825 a	10.8 b
Caphorn	0.06 b	2.0 a	3.5 a	0.006 a	953 ab	4.1 a
Koreli	0.00 a	2.0 a	4.7 ab	0.013 b	1024 b	2.1 a
Soissons	0.24 c	4.7 b	7.9 b	0.008 ab	849 ab	12.1 b
INRA08-FS0001	0.20 β	3.4 β	6.1 β	0.009 α	920 α	9.3 β
INRA08-FS0002	0.23 β	4.0 β	7.1 β	0.010 α	940 α	8.9 β
INRA08-FS0003	0.21 β	4.2 β	7.1 β	0.011 α	909 α	10.1 β
IPO323	0.01 α	0.6 α	0.9 α	0.006 α	719 α	1.4 α

Data averaged by cultivar or by isolate are indicated with results of variance analysis. The letter after the mean indicates no significant difference with the Bonferroni test at 5%. Greek letters refer to isolate effect, while Latin letters refer to cultivar effect.

cur, *Ssep* reached an average of 3.1 cm² (7.5 %LA). When *Ssep* and *Sapi* merged, a maximum *Ssep* level of 24 %LA was recorded. Data for *Ssep* were homoscedastic, permitting the use of the Kruskal-Wallis test without log-transformation. Statistical analyses on both *Ssep* level and AUDPC at 1003°Cd essentially confirmed what was observed at 405°Cd. Caphorn and Koreli were more resistant than Apache and Soissons ($p<10^{-4}$), while the isolate effect ($p<10^{-4}$) could be explained by the lesser virulence of IPO323.

Analysis of parameters of the fitted logistic function. The wide variation in earliness and severity within each treatment made statistical comparison between them difficult. However, the overall evolution of *Ssep* over time followed the ascending part of a sigmoid curve, which justifies the fitting of a logistic function to individual leaf data sets. When the different senescent zones merged, necrosis had extended beyond the 4 cm² initially inoculated. At that time however, *Ssep* accounted only for around two thirds of the asymptotic plateau *Msep*, which was actually never reached. Nonetheless, the *Msep* magnitude remained realistic (from 8 to 20 %LA). Moreover *Msep*, unlike *Rsep* and *Tsep*, was significantly correlated to *Ssep* AUDPC ($r^2 = 0.47$; $p<10^{-4}$, data not shown), suggesting it was adequately fitted. The parameters obtained from the fitting were then statistically compared (Table 1). Two-way ANOVA on *Msep* highlighted the weaker resistance of cv. Soissons compared to Caphorn ($p<0.05$) and the lesser virulence of IPO323 compared to INRA08-FS0001, INRA08-FS0002 and INRA08-FS0003 ($p<10^{-4}$), while no interaction was identified ($p>0.05$). The *Rsep* parameter, initial relative rate of *Ssep*, was very variable owing to the behavior of cv. Koreli (Table 1): in this cultivar, either no SLB symptom occurred and *Rsep* was then estimated as nil, or *Ssep* extended much more rapidly (median $18{\cdot}10^{-3}$°Cd^{-1}) than in other cultivars, which did not differ from each other (median $7{\cdot}10^{-3}$°Cd^{-1}; $p>0.05$). No significant isolate effect was observed on *Rsep* ($p>0.05$). Lastly, inflection time *Tsep* (data not shown) was not defined for the 11 inoculated shoots which did not exhibit disease. In the other cases *Ssep* was generally assessed until *Tsep* was bypassed; *Tsep* was then accurately fitted to values around 80°Cd before senescent areas merged. Two-way ANOVA on *Tsep* suggested that disease extended 200°Cd later in cv. Koreli (median at

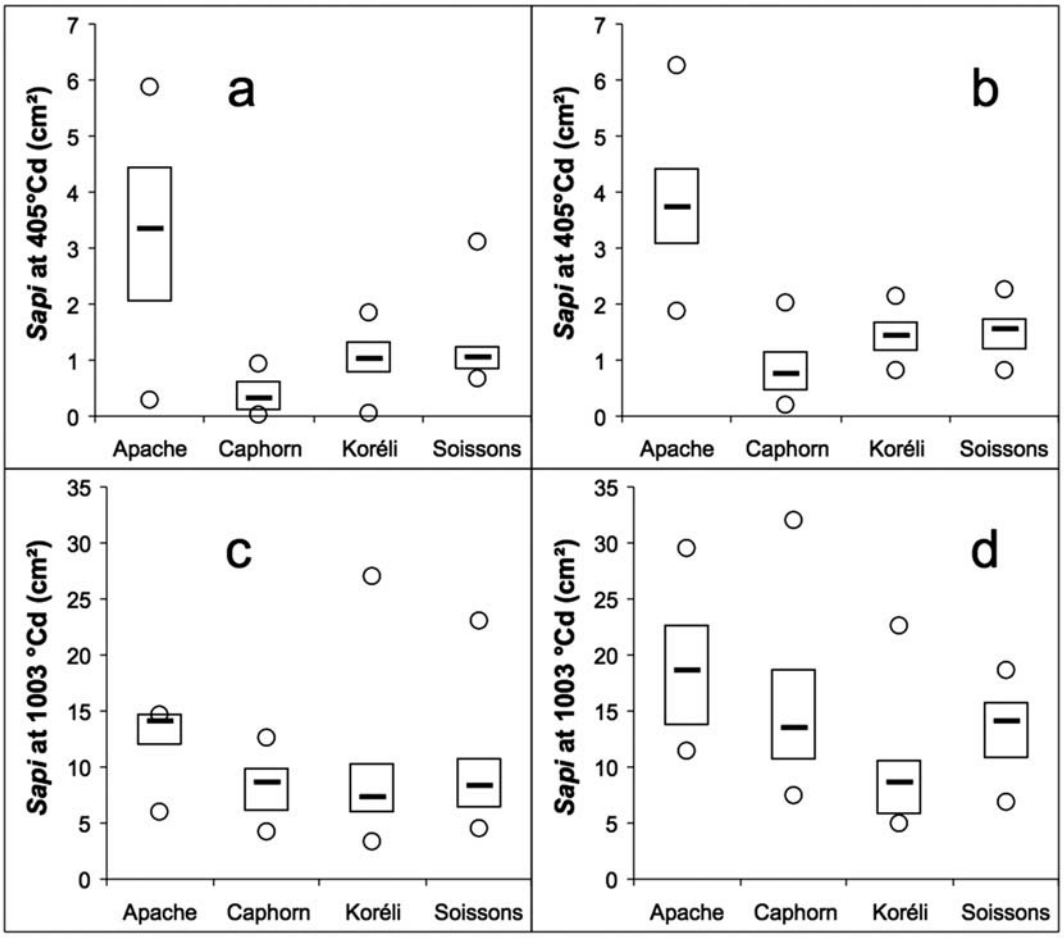

Fig. 1. Box plots for area of apical senescence (*Sapi*) in cm² at 405°Cd (a, b) and 1003°Cd (c, d) by control (a, c) and inoculated shoots (b, d) for each cultivar. The various isolates are pooled up. Boxes show the range between first and third quartile of the distribution. Medians are reported as thick lines and extreme recorded values as circles.

1018°Cd) than in cv. Apache (median at 820°Cd); however the difference was hardly significant ($p < 0.05$). No significant difference was assigned to isolates ($p>0.05$), while defective data sets did not allow us to test whether there was an interaction between cultivar and isolate on this parameter.

Apical senescence. Results of apical senescence kinetics follow the same outline than those of septoria kinetics shown above: at the beginning of the epidemics (at 405°Cd), when senescence started to merge with disease symptoms (at 1003°Cd) and analysis of the overall senescence kinetics.

Analysis of apical senescence at the beginning and the end of septoria development. (a) *At 405°Cd post-inoculation.* At 405°Cd, when septoria leaf blotch necrosis first appeared, apical senescence had already started (Fig. 1a, b); it was more developed in cv. Apache, with *Sapi* covering on average 3.5 cm^2 (8.6 %LA), and less in cv. Caphorn (0.7 cm^2; 1.5 %LA). This cultivar effect was highly significant ($p <10^{-4}$). Inoculated leaves were significantly more senescent than the control ones ($p < 0.01$), but with no isolate effect ($p > 0.05$). The higher *Sapi* of inoculated shoots at 405°Cd was also observed by paired comparison of the inoculated and control shoots in each plant ($p < 0.001$).

(b) *At 1003°Cd post-inoculation.* At 1003°Cd, inoculated shoots exhibited more *Sapi* than controls, according to ANOVA and paired comparison ($p<0.001$), with *Sapi* reaching an average of 9.5 and 14.5 cm^2 in control and inoculated shoot respectively (23 and 35 %LA) (Fig. 1c, d). Analysis of covariance of *Sapi* using *Ssep* AUDPC as covariate, led to the same conclusion as ANOVA though with no difference between isolates ($p>0.05$). Plotting *Sapi* against *Ssep* also implied the same result: the *Sapi* ratio of inoculated to control shoots was higher than 1 in most cases, but it did not correlate with septoria severity (Fig. 2a). The correlation was no more significant when *Sapi* was expressed directly as %LA (Fig. 2b), even though a very low correlation was detected when excluding shoots without disease: *Sapi* = 27%+0.8×*Ssep* ($r^2=0.12$; $p<0.05$). Paradoxically, paired comparison conducted only on cvs Caphorn and Koreli inoculated with isolate IPO323 still indicated a higher *Sapi* in inoculated than in control shoots ($p<0.05$) even though no SLB symptoms were detected in this case.

Both *Sapi* level and AUDPC (data not shown) showed that apical senescence was more advanced in cv. Apache than in the other cultivars, as confirmed by ANOVA ($p<10^{-4}$). Analysis of covariance of *Sapi* using *Ssep* AUDPC as covariate confirmed this finding ($p<10^{-4}$). Isolates were not found to differ in their effect on *Sapi* level or AUDPC ($p>0.05$) and no interaction was detected between the cultivar and isolate effects ($p>0.05$).

In conclusion, apical senescence was clearly more advanced in inoculated shoots at least after 405°Cd when the first SLB necrosis appeared in susceptible cultivars. Nevertheless, the wide *Ssep* variations observed in this experiment did not result in clear differences in apical senescence. This could be analyzed either as an absence of relationship between *Ssep* and *Sapi*, or as a too wide variability between and within cultivar×isolate combinations, preventing any significant correlation from showing up.

Apical senescence kinetics. Before it merged with *Ssep*, the *Sapi* kinetics followed an exponential curve; data were thus log-transformed and linearly related to

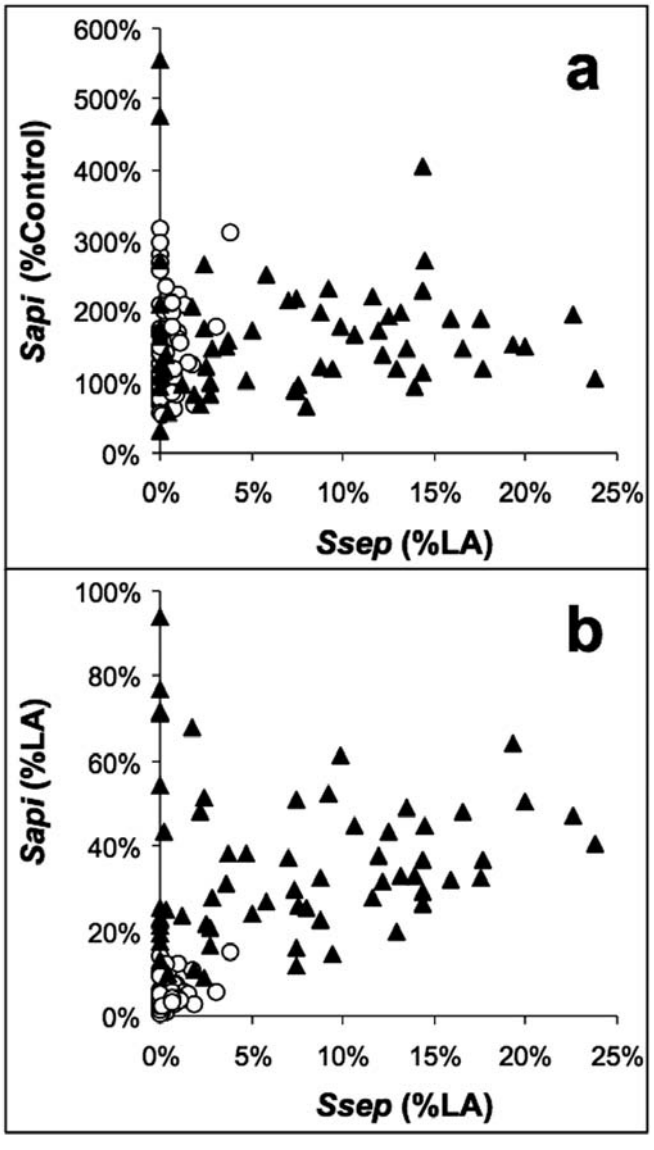

Fig. 2. Relationship between disease severity (*Ssep* in %LA) and apical senescence (*Sapi*) during necrotrophic stage. Apical senescence is compared either to *Sapi* in corresponding control shoot (a) or to total area of infected leaf (b). Data obtained 405°Cd after inoculation are reported as white circles, and data obtained at 1003°Cd as black triangles.

thermal time. The effects of cultivar and isolate on the intercept and slope of this relationship were then statistically analyzed. The slope of regression (Fig. 3a, b) was a measurement of *Rapi*, the relative rate of apical senescence, while intercept can be regarded as the logarithm of *Sapi* on the day of inoculation (Fig. 3c, d). A significant cultivar effect was found on the slope ($p<10^{-4}$), with apical senescence proceeding more slowly in cv. Apache and more quickly in cv. Caphorn (average $2.7 \cdot 10^{-3}$ and $5.0 \cdot 10^{-3}$ °Cd^{-1} respectively). However, no effect of inoculation ($p>0.05$) or interaction between the two factors ($p>0.05$) was found. Nor did paired comparison between inoculated and control shoots show any senescence acceleration in inoculated shoots ($p>0.05$). Neither *Ssep* level at 1003°Cd nor its AUDPC were accepted as covariate for *Rapi* ($p>0.05$), and no correlation appeared when plotting *Rapi* directly against either *Ssep* level at 1003°Cd or its AUDPC (data not shown); both results indicated that *Rapi* was not affected by *Ssep*. The intercept was linked to *Sapi* at inoculation day; the significant effect of cultivar on intercept ($p<10^{-4}$) thus suggests that *Sapi* was already more advanced in cv. Apache, scoring a median value of 1.2 cm^2, and less in cv. Caphorn (0.1 cm^2), than in cvs Soissons and Koreli (0.3 cm^2). Intercept also suggests that *Sapi* was already higher in inoculated shoots than in controls, amounting up to 0.4 and 0.2 cm^2 respectively. The difference was small enough to explain why it was not observed experimentally when the leaves were selected, but it was nevertheless significant ($p<0.001$). By contrast, the isolate effect was not significant at 5% level, and no interaction was found between cultivar and isolate ($p>0.05$). Paired comparison between inoculated and control shoots also suggested that *Sapi* was already less in the control shoots at the time of inoculation ($p<10^{-4}$). Hence, if the *Sapi* kinetic established during the necrotrophic phase already applied before, then apical senescence already differed between inoculated and control shoots when inoculation occurred.

Altogether, these results strongly show that apical senescence was not accelerated by disease once SLB necrosis was recorded. The higher *Sapi* level in inoculated shoots compared to control shoots throughout the experiment was probably due to a difference already observed at 405°Cd. The effect of inoculation on *Sapi* during the necrotrophic stage should therefore be re-

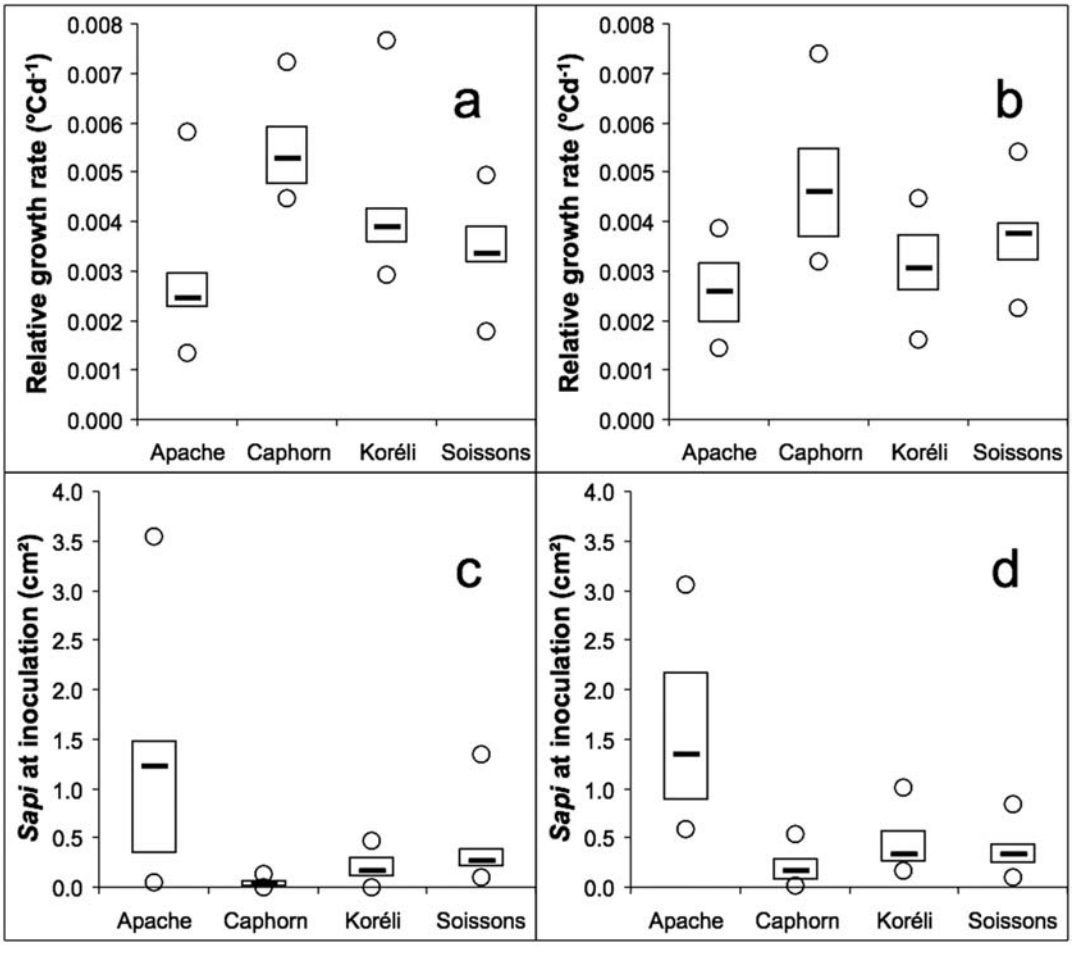

Fig. 3. Box plots of *Sapi* exponential parameters [relative growth rate (a, b) and *Sapi* extrapolation at inoculation time (c, d)] for each cultivar (in control (a, c) and inoculated (b, d) shoots. The various isolates are pooled. Boxes show the range between first and third quartile of the distribution. Medians are reported as thick lines and extreme recorded values as circles.

garded as hastening the start of apical senescence rather than accelerating it. The shift in apical senescence due to inoculation, *Tapi*, was then calculated for each plant by comparing the control and inoculated shoots. A significant effect of cultivar in *Sapi* anticipation was observed ($p<0.01$), with *Tapi* median at 2°Cd in cv. Koreli *vs*. 178°Cd in cv. Caphorn. The isolate effect was not significant at 5% level, and no interaction was found between cultivar and inoculation ($p>0.05$). Lastly, no correlation appeared between *Tapi* and either *Ssep* level at 1003°Cd or its AUDPC (data not shown). Senescence anticipation linked to inoculation was thus more or less affected by plant cultivar, but it could not be quantitatively related to *M. graminicola* symptoms.

DISCUSSION

The present study assessed the effect on apical leaf senescence of a localized *M. graminicola* inoculation resulting in a restricted area of diseased leaf (4 cm^2 on average). It involved several wheat cultivars varying in their susceptibility to SLB.

Symptom kinetics were in the same range of magnitude as those observed in the field. Relative rates *Rsep* ranged from $2{\cdot}10^{-3}$°Cd^{-1} to $31{\cdot}10^{-3}$°Cd^{-1}, as in Bancal *et al.* (2007), and *Msep* ranged from 1 to 39 %LA, as in Lovell *et al.* (2004). It contrasted with some previous studies where *M. graminicola* was inoculated to induce a massive, rapid disease development. In the present study, inoculum concentration was adjusted to a concentration 10^5 spores/ml, only one thousandth the dose used by Robert (2003) but close to that usually observed in natural conditions (Suffert and Sache, 2011). While disease severity is usually positively correlated with inoculum concentration at a given assessment point (Chungu *et al.*, 2001), increased inoculum concentration makes symptoms develop either faster or earlier. The median of fitted *Msep* in our work was 11 %LA, whereas severities of up to 100 %LA are reported in the literature. Such levels are attainable only when apical senescence is negligible, i.e. when disease develops soon after inoculation. For instance, Magboul *et al.* (1992) reported that symptoms appeared around 10 days after inoculation at 20°C (200°Cd) and the inflection point occurred 10 days later, twice as early as reported here. Logistic fitting reported by Magboul *et al.* (1992) also suggested *Rsep* was around $20{\cdot}10^{-3}$°Cd^{-1}, twice as high as the median of our data. In the present study, as the plants were not overwhelmed by sudden disease, varying resistance levels differentiated between the cultivars. *Rsep* was low in the case of cv. Caphorn, in other words disease developed more slowly in this cultivar. *Rsep* was much higher in cv. Koreli, but it was compensated by a later inflection point; disease developed rapidly, but late. In both cvs Caphorn and Koreli some of the plants failed to develop any *Ssep* symptoms; resistance involved not only disease development but also probably either the triggering of necrotrophic phase or the success of the initial infection. These differences in the ways plants resist, as well as in the virulence of the *M. graminicola* isolates, increased the variability in the ratio between SLB and natural senescence. For example *Ssep* AUDPC was one third of *Sapi* AUDPC for susceptible cv. Soissons compared to only 7% for resistant cv. Koreli.

During the necrotrophic stage the level of apical senescence in inoculated shoots was approximately twice that of controls at each assessment. However, this increase in *Sapi* was little or not at all related to the varying *Ssep* levels or AUDPC obtained by resistance or virulence tested. Moreover, exponential fitting of *Sapi* indicated no difference in its relative rate between controls and inoculated shoots. The difference in the development of *Sapi* comes only from its fitted intercept, suggesting that *Sapi* was anticipated rather than accelerated once necrotic lesions of disease had appeared. Higher intercepts of *Sapi* in inoculated shoots also suggested that corresponding *Sapi* was already greater on the day of inoculation. Two alternative hypotheses could explain this last result: (i) if extrapolation is valid from 0 to 405°Cd, an experimental bias should be suspected. For instance, the inoculation treatment was, unwittingly, always performed on the main shoot rather than on randomly selected shoots. As the main shoot is older than tillers, the difference in apical senescence between inoculated and control shoots, *Tapi*, would then reflect the age difference between main shoot and tillers; (ii) otherwise it could be that firstly *Sapi* had been accelerated before 405°Cd, i.e. during the asymptomatic phase, and then this acceleration had ended when the assessments began. This hypothesis may be corroborated by the fact that *M. graminicola* is associated with a long latent period, which can last several weeks after inoculation. This has been referred to as hemibiotrophic, assuming that the fungus is successfully acquiring sufficient nutrients from living tissue during the symptomless growth phase. *Sapi* could be affected during this early stage. However, it is difficult to attribute a precise physiological stage to 405°Cd. For instance, necrosis began at this time in some, but not all cultivar×isolate combinations. Remarkably, cvs Caphorn and Koreli inoculated with isolate IPO323 exhibited greater *Sapi* after inoculation despite they did not develop disease symptoms yet.

The consequences of the above two hypotheses differ in terms of leaf senescence and N mobilization, so further studies focusing on the biotrophic stage are needed to choose between them. In our study no clear relationship was observed between *Sapi* and *Ssep* during the necrotrophic stage. The first hypothesis would suggest that the rate of apical senescence was not affected by disease. However, apical senescence is the way leaves remobilize N to fill the grains. Thus the absence of varia-

tion in *Sapi* kinetics suggests that SLB does not directly influence the rate of N remobilization. Grain N filling in diseased plants would thus proceed unchanged until *Sapi* and *Ssep* merge. Only at this later stage would grain filling suddenly decline because N in *Ssep* area is probably unavailable for grain filling. These assumptions should be checked by further experiments, but they are supported by the high N content frequently found in diseased leaves at harvest, suggesting that N blockage occurs late in these tissues (Garry *et al.*, 1996; Bancal *et al.*, 2008).

By contrast, the second hypothesis implies that *Sapi* was increased at an early stage by inoculation. The literature reports that senescence is accelerated by biotrophic fungi, but to our knowledge this senescence occurs around the inoculation point, not at the leaf apex (Robert *et al.*, 2005). Some time before 405°Cd, *Sapi* was no longer accelerated, as shown by an unchanged *Rapi*, but it remained more advanced in inoculated leaves than in controls throughout the necrotrophic stage, owing to its exponential kinetic. However, the absence of a clear relationship between *Sapi* and *Ssep* is quite puzzling if we take this second hypothesis. If disease had increased apical senescence during the biotrophic stage, a relationship between the resulting *Sapi* and the level of *Ssep* developing later could be expected. It is possible that such a relationship was masked by plant-to-plant variations, as each cultivar×isolate combination was replicated only four times.

Even taking the first hypothesis in which apical senescence proceeds unaffected by disease, this should not suggest that leaf senescence was only located at its tip. In healthy leaves, N concentration remains quite homogeneous throughout blade length (Bertheloot *et al.*, 2008), although progressively declining as the grains fill. Leaf senescence is a highly integrated process, eliminating the green surface only at the apex but involving all leaf cells (Bernard *et al.*, 2008). Disease obviously removes some tissue from the natural senescence process; therefore if the latter proceeds unaffected, the missing tissue must somehow be compensated for, which could involve N metabolism in the local area of induced senescence. In an attempt to separate the components of induced senescence in the case of SLB, our work only considered the effects of the disease on the rate of apical senescence *Sapi*. However, our results suggest that local senescence induced around the inoculation zone *Ssep* was directly responsible for the loss of GLA by disease. Further research should concentrate on the determinants of local senescence.

Local senescence appears heterogeneous, as it probably includes both the necrotic area round the pycnidia and senescence developing in an adjacent zone all around. This would explain why *Ssep* extended beyond the inoculated area. Previous studies mentioning accelerated or anticipated senescence due to disease usually consider plants where apical senescence remains negligible. For those authors, induced senescence probably meant local senescence (pycnidia area+adjacent zone) with no apical senescence. Kema *et al.* (1996) suggest that necrosis in the pycnidia area and in adjacent zone might not respond to the same determinants. For instance, fungal toxins were previously supposed to play a role in the case of *Septoria tritici* (Shetty *et al.*, 2007) and have been characterized in several pathosystems that involve necrotrophs (van Baarlen *et al.*, 2004; Wang *et al.*, 1996; Navarre and Wolpert, 1999). On the other hand, necrosis in the adjacent zone resembles the hypersensitive response (HR) that has been characterized as a plant reaction with various pathogens (Pontier *et al.*, 1999). Other studies have mentioned HR-like cell death as a feature of disease susceptibility that may facilitate plant infection by various necrotrophic pathogens (Govrin and Levine, 2000; van Baarlen *et al.*, 2004), including SLB (Keon *et al.*, 2007). Study of local senescence will require efficient characterization of both the pycnidia area and the adjacent zone. An image analysis program would make it possible to separate the pycnidia area from the adjacent zone with some precision. The diseased area could be considered on the basis of pycnidia coverage (density, number). A complementary method would be to directly quantify the fungus biomass by qPCR, as this was recently shown to correlate well with area dotted by pycnidia (Guo *et al.*, 2006; Rohel *et al.*, 2002).

Under our experimental conditions SLB did not induce an acceleration of apical senescence during necrotrophic stage. The discrepancy of our results with previous works may be due to the inoculation method, leading to moderate severity and disease kinetics close to those observed in the field, and overall to the definition of induced senescence which in earlier studies has combined apical and local senescence. Apical senescence was nevertheless anticipated in inoculated shoots regardless of whether disease symptoms appeared. Observations suggest that apical senescence was not accelerated after the early biotrophic stage. Further investigations are needed to help model the perturbation of whole-plant N metabolism induced by foliar diseases, which determines yield or protein quality losses. This study did not discriminate between the pycnidia area and the adjacent zone, but this will be a key point in the study of senescence induced by disease, which is clearly seen from this study to be the main source of GLA decrease.

REFERENCES

Bancal M.-O., Roche R., Bancal P., 2008. Late foliar diseases in wheat crops decrease nitrogen yield through N uptake rather than through variations in N remobilization. *Annals of Botany* **102**: 579-590.

Bancal M.-O., Robert C., Ney B., 2007. Accounting for wheat

crop growth and yield losses by accelerated green leaf layer losses due to late leaf rust and blotch epidemics. *Annals of Botany* **100**: 777-789.

Bernard S.M., Moller A.L.B., Dionisio, G., Kichey T., Jahn T.P., Dubois F., Baudo M., Lopes M.S., Tercé-Laforgue T., Foyer C.H., Parry M.A.J., Forde B.G., Araus J.L., Hirel B., Schjoerring J.K., Habash D.Z., 2008. Gene expression, cellular localization and function of glutamine synthetase isozymes in wheat (*Triticum aestivum* L.). *Plant Molecular Biology* **67**: 89-105.

Bertheloot J., Martre P., Andrieu B., 2008. Dynamics of light and nitrogen distribution during grain filling within wheat canopy. *Plant Physiology* **148**: 1707-1720.

Chungu C., Gilbert J., Townley-Smith F., 2001. *Septoria tritici* blotch development as affected by temperature, duration of leaf wetness, inoculum concentration, and host. *Plant Disease* **85**: 430-435.

Cornish P., Baker G., Murray G., 1990. Physiological responses of wheat (*Triticum aestivum*) to infection with *Mycosphaerella graminicola* causing *Septoria tritici* blotch. *Australian Journal of Agricultural Research* **41**: 317-327.

Dimmock J.P.R.E., Gooding M.J., 2002. The influence of foliar diseases, and their control by fungicides, on the protein concentration in wheat grain: a review. *The Journal of Agricultural Science* **138**: 349-366.

Gan S., Amasino R.M., 1997. Making sense of senescence. Molecular genetic regulation and manipulation of leaf senescence. *Plant Physiology* **113**: 313-319.

Garry G., Tivoli B., Jeuffroy M.H., Citharel J., 1996. Effects of Ascochyta blight by *Mycosphaerella pinodes* on the translocation of carbohydrates and nitrogenous compounds from the leaf and hull to the seed of dried-pea. *Plant Pathology* **45**: 769-777.

Gaunt R.E., 1995. The relationship between plant disease severity and yield. *Annual Review of Phytopathology* **33**: 119-144.

Gooding M.J., Gregory P.J., Ford K.E., Pepler S., 2005. Fungicide and cultivar affect post-anthesis patterns of nitrogen uptake, remobilization and utilization efficiency in wheat. *Journal of Agricultural Science* **143**: 503-518.

Govrin E.M., Levine A., 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea. Current Biology* **10**: 751-757.

Gregersen P.L., Holm P.B., Krupinska K., 2008. Leaf senescence and nutrient remobilisation in barley and wheat. *Plant Biology* **10**: 37-49.

Guo J.-R., Schnieder F., Verreet J.-A., 2006. Presymptomatic and quantitative detection of *Mycosphaerella graminicola* development in wheat using a real-time PCR assay. *FEMS Microbiology Letters* **262**: 223-229.

Kema G.H.J., Annone J.G., Sayoud R., van Silfhout C.H., van Ginkel M., de Bree J., 1996. Genetic variation for virulence and resistance in the wheat *Mycosphaerella graminicola* pathosystem I. Interactions between pathogen isolates and host cultivars. *Phytopathology* **86**: 200-212.

Kema G.H.J., van Silfhout C.H., 1997. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. *Phytopathology* **87**: 266-272.

Keon J., Antoniw J., Carzaniga R., Deller S.N., Ward J.L., Baker J.M., Beale M.H., Hammond-Kosack K., Rudd J.J., 2007. Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. *Molecular Plant-Microbe Interactions* **20**: 178-193.

Leitch M.H., Jenkins P.D., 1995. Influence of nitrogen on the development of Septoria epidemics in winter wheat. *The Journal of Agricultural Science* **124**: 361-368.

Lovell D.J., Parker S.R., Hunter T., Welham S.J., Nichols A.R., 2004. Position of inoculum in the canopy affects the risk of *Septoria tritici* blotch epidemics in winter wheat. *Plant Pathology* **53**: 11-21.

Magboul A.M., Geng S., Gilchrist D.G., Jackson L.F., 1992. Environmental influence on the infection of wheat by *Mycosphaerella graminicola*. *Phytopathology* **82**: 1407-1413.

Masclaux-Daubresse C., Reisdorf-Cren M., Orsel M., 2008. Leaf nitrogen remobilisation for plant development and grain filling. *Plant Biology* **10**: 23-36.

Navarre D.A., Wolpert T.J., 1999. Victorin induction of an apoptotic/senescence–like response in oats. *The Plant Cell* **11**: 237-250.

Pageau K., Reisdorf-Cren M., Morot-Gaudry J.-F., Masclaux-Daubresse C., 2006. The two senescence-related markers, *GS1* (cytosolic glutamine synthetase) and *GDH* (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. *Journal of Experimental Botany* **57**: 547-557.

Pontier D., Balague C., Roby, D., 1998. The hypersensitive response. A programmed cell death associated with plant resistance. *Comptes Rendus de l'Académie des Sciences. Série III, Sciences de la Vie* **321**: 721-734.

Quirino B.F., Noh Y.-S., Himelblau E., Amasino R.M., 2000. Molecular aspects of leaf senescence. *Trends in Plant Science* **5**: 278–282.

Robert C., 2003. Etude et modélisation du fonctionnement d'un couvert de blé attaqué par le complexe parasitaire *Puccinia triticina - Mycosphaerella graminicola*. Ph.D. Thesis. Institut National Agronomique Paris-Grignon, Paris, France.

Robert C., Bancal M.-O., Nicolas P., Lannou C., Ney B., 2004. Analysis and modelling of effects of leaf rust and *Septoria tritici* blotch on wheat growth. *Journal of Experimental Botany* **55**: 1-16.

Robert C., Bancal M.-O., Lannou C., Ney B., 2005. Wheat leaf photosynthesis loss due to leaf rust, with respect to lesion development and leaf nitrogen status. *New Phytologist* **165**: 227-241.

Rohel E.A., Laurent P., Fraaije B.A., Cavelier N., Hollomon D.W., 2002. Quantitative PCR monitoring of the effect of azoxystrobin treatments on *Mycosphaerella graminicola* epidemics in the field. *Pest Management Science* **58**: 248-254.

Shetty N.P., Mehrabi R., Lutken H., Haldrup A., Kema G.H.J., Collinge D.B., Jørgensen H.J.L., 2007. Role of hydrogen peroxide during the interaction between the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. *New Phytologist* **174**: 637-647.

Suffert F., Sache I., 2011. Relative importance of different ty-

pes of inoculum to the establishment of *Mycosphaerella graminicola* in wheat crops in north-west Europe. *Plant Pathology* **60**: 878-889.

Talbot N.J., McCafferty H.R.K., Ma M., Moore K., Hamer J.E., 1997. Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. *Physiological and Molecular Plant Pathology* **50**: 179-195.

Tavernier V., Cadiou S., Pageau K., Lauge R., Reisdorf-Cren M., Langin T., Masclaux-Daubresse C., 2007. The plant nitrogen mobilization promoted by *Colletotrichum lindemuthianum* in phaseolus leaves depends on fungus pathogenicity. *Journal of Experimental Botany* **58**: 3351-3360.

van Baarlen P., Staats M., van Kan J.A.L., 2004. Induction of programmed cell death in lily by the fungal pathogen *Botrytis elliptica*. *Molecular Plant Pathology* **5**: 559-574.

Wang H., Li J., Bostock R.M., Gilchrist D.G., 1996. Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *The Plant Cell* **8**: 375-391.

Received November 21, 2011
Accepted May 20, 2012