Photosynthetic consequences of *Phomopsis helianthi* on two sunflower hybrids: analysis and modelling

M. Desanlis 1, P. Maury 2, J.N. Aubertot, L. Lagarrigue 1 1, P. Debaeke 1
1 INRA, AGIR, UMR 1248, F-31326 Castanet-Tolosan, France, myriam.desanlis@toulouse.inra.fr
2 Univ Toulouse, INPT, UMR AGIR, ENSAT, F-31320 Castanet Tolosan, France

ABSTRACT

- Foliar pathogens reduce the net photosynthetic rate in infected leaves by reducing green leaf area, but also through an effect on photosynthesis of the remaining green leaf tissue. Phomopsis stem canker (*Phomopsis/Diaporthe helianthi* Munt.-Cvet) produces toxin metabolites, which cause necrotic lesions on sunflower leaves, with the first signs of infection being visible at the chloroplast level. The leaf senescence and plant wilting that this fungus also induces has been documented. However, its impact on photosynthesis of asymptomatic leaf area has not been studied on sunflower. The aims of the present study were (i) to determine whether the net photosynthetic rate (NPR) of the green leaf area (green LA) was reduced by *Phomopsis*, and (ii) to characterize the relationship between disease severity and net photosynthesis in sunflower leaves.
- Two contrasting genotypes were investigated: a cultivar resistant (R) to phomopsis (cv. Bollil) and a susceptible (S) one (cv. Alhaja). A greenhouse experiment was conducted at INRA, Auzeville (France). One leaf per plant was inoculated with a monopycniospore culture of *Phomopsis* on 7 plants for each cultivar at star bud stage. Corresponding plants without leaf innoculation were used for control samples. Net CO_2 assimilation rate was measured on three areas of the leaf with a LI-COR 6400 twice a week from the star bud stage to the end of flowering. Leaf lesion sizes were measured regularly in order to calculate the rate of necrosis progression from the infection point (tip of the leaf). Lesion spread was monitored on the 3 chosen leaf areas: close to the infection point, at the middle of the leaf, and opposite to the infection point. The proportion of necrotic tissue and the surrounding chlorotic area was measured.
- On control leaves, NPR was not significantly different between the 3 leaf areas investigated, cv. Alhaja having a higher net photosynthetic rate than cv. Bollil. As leaves became older (30 days from fully leaf expansion), a 50% decrease in NPR was observed without any visible sign of senescence. The impact of *Phomopsis* on leaf photosynthesis occurred earlier and was stronger in cv. Alhaja than in cv. Bollil. The rate of necrosis progression significantly differed between sunflower cultivars: 11.5 $mm.day^{-1}$ for cv. Alhaja (susceptible), and 6.9 $mm.day^{-1}$ for cv. Bollil (resistant). At the scale of the measured areas, the decrease in NPR was greater than would have been expected from the fraction of necrotic LA, and appeared genotypic dependent. In cv. Alhaja, a fraction of necrotic LA of 0.1 reduced the relative NPR to 0.12, while fractions between 0.25 and 1 completely halted photosynthesis. In cv. Bollil, a fraction of necrotic LA of 0.1 reduced the relative NPR to 0.67, with a complete photosynthesis inhibition when the necrotic area fraction ranged between 0.78 and 1. Using the concept of virtual lesion, a mathematical model was built to represent the relationship between affected tissue areas and necrosis areas at the whole leaf scale. The distance between the visual necrosis front and the virtual necrosis front was found to be 15.7 mm for Alhaja and 10.2 mm for Bollil.
- This study quantified the relationships between phomopsis leaf injury and photosynthesis reduction. The reduction of NPR in asymptomatic leaf area may be result from stomatal and mesophyll limitations of $C0_2$ assimilation and/or an increase of respiration. The results will be integrated in an epidemiological sub-model in the SUNFLO model (Casadebaig et al., 2011).
- Until now, yield losses were calculated using the proportion of stems with a girdling spot but leaf attacks were only roughly characterized. This study improved the understanding and prediction of yield loss caused by phomopsis stem canker.

Key-words: damage mechanism - *Helianthus annuus* - modelling - *Phomopsis helianthi* - photosynthesis - sunflower genotypes

INTRODUCTION

Phomopsis stem canker, a worldwide fungal disease of sunflower, is caused by the necrotrophic fungus *Diaporthe helianthi* Munt.-Cvet. Leaves are infected by ascospores. After infection, the fungus spreads along the main veins of the leaves and grows down the petiole to the stem. It produces toxin metabolites, including phomozin, which provoke the necrotic lesions on leaves (Mazars et al., 1990), the first sign of infection being visible at the chloroplasts (Heller and Gierth, 2001). This results in premature leaf senescence, plant wilting and lodging (Masirevic and Gulya, 1992). Losses may reach up to 1.5 t ha^{-1} in yield and up to 25% in oil content (Acimovic, 1986).

Foliar pathogens generally reduce the photosynthetic activity in infected leaves by reducing green leaf area. However, Goto (1965) showed that yield loss due to leaf blast on rice exceeded yield loss caused by cutting off the same percentage of leaf area as the one covered by the fungus. This extra reduction means that a disease may influence the host plant to an extent larger than the visual lesion would do. It has been reported for *Alternaria helianthi* (Calvet et al., 2005) that photosynthesis could be affected in asymptomatic areas of diseased leaves. However, pathosystems in which the pathogen do not impair photosynthesis of remaining green leaf tissue have also been reported, e.g. *Puccinia recondita* on wheat (Spitters et al., 1990). To our knowledge, the impact of *Phomopsis helianthi* on the photosynthesis of asymptomatic leaf areas has not been studied so far on sunflower.

Bastiaans (1991) provided a relationship between the net photosynthesis and the proportion of diseased leaf area for a single disease at the leaf scale. He introduced the concept of virtual lesion, which corresponds to the leaf area in which photosynthesis is negligible (including the visual lesion). Working with *Pyricularia oryzae*, his model $P_x/P_0 = (1 - x)^{\beta}$ relates the net photosynthetic rate of diseased leaf area (P_x) relative to a healthy leaf area (P_0) and the fraction of leaf area covered by the disease (x). The parameter β quantifies the constant ratio between virtual and visual lesion. It indicates whether the effect of disease on photosynthesis is higher ($\beta > 1$), lower ($\beta < 1$) or equal ($\beta = 1$) to that explained by the observed diseased area. This concept was developed for randomly distributed, small lesions that occupy less than 20 % of the total leaf area. A different approach is required for diseases with single, expanding lesions such as *P. helianthi*. An example was reported at the whole-leaf level for *Xanthomonas campestris* pv. *oryzae* on rice (Elings et al., 1999).

In some plant-pathogen interactions, the host genotype may play a significant role in the disease impact on host photosynthesis. β-values found by Erickson *et al.* (2004) greatly varied across poplar lines indicating that *Marssonina brunnea* has different consequences on photosynthesis for different host genotypes. However, β has been found invariant across six pea cultivars for *Mycosphaerella pinodes* (Le May et al., 2005).

The aims of the present study were (i) to determine whether the net photosynthetic rate (NPR) of the green leaf area (LA) was reduced by *Phomopsis*, (ii) to characterize the relationship between disease severity and net photosynthesis in sunflower leaves. The response of the host genotype to disease was studied using two contrasting genotypes: a resistant and a susceptible one. The impact of the pathogen on photosynthesis was studied first at the scale of the measured area and then at the leaf scale.

MATERIALS AND METHODS

The two commercial hybrids were cv. Alhaja, susceptible to *P. helianthi*, and cv. Bollil, resistant. One plant was established in 10 liter pots filled with peat in the INRA greenhouse at Auzeville (Haute-Garonne, France) in spring 2010. Day/night temperature in the greenhouse was maintained at 23°C/17°C ± 2°C. Air humidity was maintained at 90% ± 5 % at night and a minimum of 60% ± 5 % in the daytime (except the 48 h after infection at near 100%). A 16 h photoperiod was applied from plant emergence to the star bud stage with 400 W High Pressure Sodium vapour lamps (SON-T AGRO, Philips). Plants were irrigated daily by a 2 $liter.h^{-1}$ emitter to maintain adequate soil moisture during the experiment. Plants were fertilized weekly with NPK solution (20.20.20, 200 ml at 2$g.liter^{-1}$).

A monopycniospore culture of *P. helianthi* was isolated from naturally infected sunflower stem residues collected in March 2010 on the experimental site. The fungus was grown in the dark at 24 ± 1°C on potato dextrose agar (PDA) amended with 150 $mg.liter^{-1}$ of streptomycin (pH 6). Inoculation followed Bertrand and Tourvieille (Bertrand and Tourvieille, 1987), using PDA disks colonized with *P. helianthi*. Infections were made at the star bud stage (CETIOM, 2004) on the tip of one leaf per plant choosing the youngest fully expanded leaf. On control plants, sterile PDA disks were used instead of mycelial explants. For each cultivar, 7 plants were inoculated and 7 other plants were used for control. Pots were randomly arranged in the greenhouse.

Lesion size was obtained by the difference between total leaf length (measured the day before inoculation) and the length of the main vein remaining green, taken on the underside of the leaf. One each leaf, 3 areas were defined: proximal (close to the infection point), median (at the middle of the leaf), and distal (at the opposite of the infection point). Gas exchange was measured on those three areas of the leaf using the 6cm² clamp-on chamber of the LI-COR 6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) between 10 am and 3 pm. Measurements of NPR were performed at light-saturation of 1500 µmol.m^{-2}.s^{-1} photosynthetic photon flux density, ambient CO_2 concentration at 400 µmol.mol^{-1}, leaf temperature of 25°C and relative humidity of 60 ± 5%. After gas exchange measurements, leaves were scanned *in situ* at 300 dpi to quantify total leaf area and disease severity. The proportion of LA affected by the disease (necrotic tissue and the surrounding chlorotic area) was estimated using Winfolia software (2005, Regent Instruments Inc., Ottawa, Canada). Leaf area of green colour was considered asymptomatic. Leaves were monitored twice a week from inoculation to 36 DPI.

Statistical analyses were performed using the R software (R Development Core Team, 2006). Parametric data were analyzed by ANOVA via the general linear model procedure. Homogeneity of variance was checked by Bartlett's test (confidence level of 0.95) and the normality of the residuals by the Shapiro-Wilks test (confidence level of 0.95). The Wilcoxon test was used for non parametric data with a significance threshold of $P < 0.05$.

RESULTS

Photosynthesis dynamic in control plants

In control plants, the NPR was homogeneous within the leaf ($P = 0.55$) and decreased with leaf aging. NPR was significantly higher for cv. Alhaja than for cv. Bollil ($P < 0.001$). A 50% decrease in NPR was observed in 30 days (from fully leaf expansion) without any visible sign of leaf senescence.

To limit NPR variability due to variations of environmental conditions, the impact of *Phomopsis* was studied relatively to a paired control plant.

Effect of phomopsis stem canker on NPR

For both cultivars, the first impacted area was the closest one to the necrosis and the last impacted area was the most remote to the necrosis (Figure 1). The ratio decreased quicker for cv. Alhaja than for cv. Bollil. The impact of Phomopsis on leaf photosynthesis occurred sooner and was more pronounced for cv. Alhaja. For median and distal areas, values of standardized relative NPR were higher than 1 for both cultivars. This phenomenon was transient and did not appear with proximal area values.

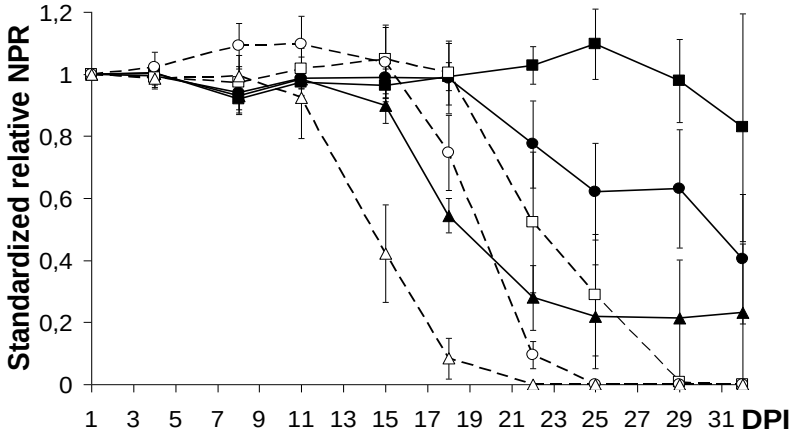

Fig 1. Relationships between standardized relative net photosynthesis rate (NPR) and number of days post-inoculation (DPI). Vertical lines represent the confidence interval at 95%. Solid symbols are for cv. Bollil and open symbols are for cv. Alhaja. The three measured areas are represented as follows: proximal area (triangles), median area (circles), distal area (squares).

At 18 DPI, standardized relative photosynthetic rate was significantly related to cultivar ($P < 0.01$) and measurement area ($P < 0.001$) but no interaction was found between cultivar and measurement area ($P = 0.07$). However, leaf size did not differ significantly between the two cultivars ($P = 0.81$, Wilcoxon test). The rate of necrosis progression was significantly related to cultivar ($P < 0.001$). For cv. Alhaja, it was 11.5 ± 1.1 mm.day^{-1} vs 6.9 ± 1.5 mm.day^{-1} for cv. Bollil. The rate of necrosis progression was

independent of the date of measurement (P = 0.09). No interaction was found between cultivar and measurement date (P = 0.07).

Short distance impact of infection on NPR

The relationship between the fraction of necrotic leaf area and the standardized relative NPR of measured areas was not linear (Figure 2): the decrease in the standardized relative NPR was greater than expected from the fraction of necrotic LA (P < 0.001, Wilcoxon test) and was genotype-dependent. For cv. Alhaja, a fraction of necrotic LA of 0.1 reduced the standardized relative NPR to 0.12. Fractions between 0.25 and 1 completely inhibited the leaf photosynthetic activity. Whereas, on cv. Bollil, a fraction of necrotic LA of 0.1 reduced the standardized relative NPR to 0.67. Fractions between 0.78 and 1 completely inhibited the photosynthetic activity of the leaf areas.

Measurements of healthy areas resulted in standardized NPR values greater than 1 (standardized NPR ratio ranged from 0.73 to 1.90).

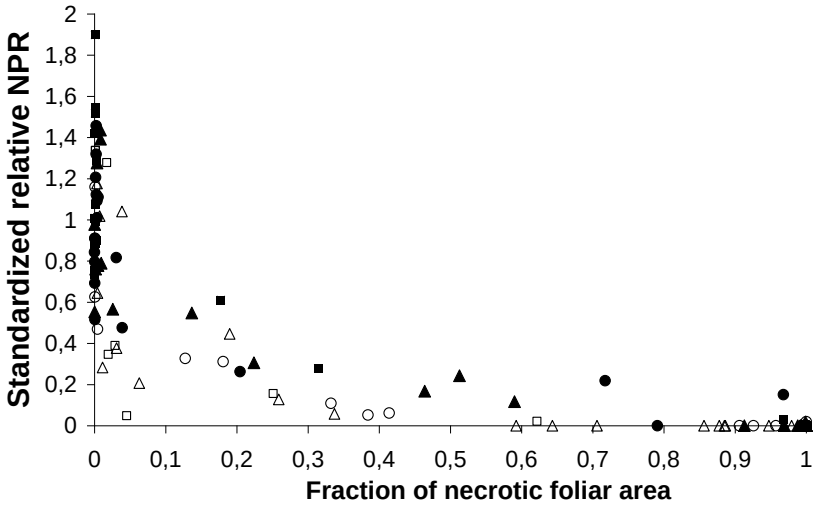

Fig 2. Standardized relative net photosynthetic rate related to the fraction of necrotic foliar area. Solid symbols are for cv. Bollil and open symbols are for cv. Alhaja. The three measured areas are represented as follows: proximal area (triangles), median area (circles), distal area (squares).

Modelling at the whole leaf scale

The necrosis caused by *Phomopsis helianthi* is characteristic of this fungus: it always spreads as a triangle on the main leaf vein from the leaf tip. Short distance impact of Phomopsis did not differ between the various measurement areas (Figure 3). The relationship between the standardized relative NPR and the fraction of necrotic LA in the measured area differed obviously between the two cultivars. We assumed that the distance between the visual and the virtual necrosis fronts remained constant for a given cultivar but differed between cultivars having different susceptibility levels.

No long distance impact of Phomopsis was evidenced: the standardized relative NPR did not decrease when the necrosis spread towards the measured area (data not shown).

Since the measurement area is parallel to the blade margin and the necrosis can be figured by a triangle on the leaf, we assumed that the necrosis front was perpendicular to the diagonal of the measurement area.

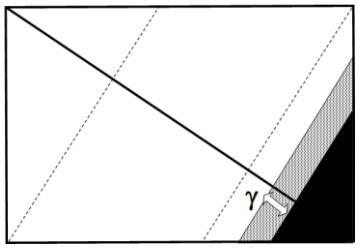

Fig 3. Representation of a lesion caused by *Phomopsis helianti* on a measurement area. The black area represents the visual lesion. The grey area represents the asymptomatic area affected by the pathogen. γ is the width of this asymptomatic but impacted area.

Four cases were considered according to the position of the necrosis on the diagonal of the measured area (Figure 3):

$$xvirt = f(l,w,x,\gamma) \quad \text{Equation 1}$$

with xvirt: fraction of impacted area in the measurement area; l: length of the measurement area (l = 30 mm); w: width of the measurement area (w = 20 mm); γ: distance between the visual necrosis front and the virtual necrosis front; x: fraction of visible necrosis area in the measurement area.

The value of parameter γ was estimated by minimizing the RMSE for xvirt. One value was calculated for each cultivar: 15.7 mm for cv. Alhaja (RMSE=0.219, bias=0.0004, Efficiency=0.55) and 10.2 mm for Bollil (RMSE=0.162, bias=-0.021, Efficiency=0.63).

The relationship between the injury and the associated damage mechanism differed between cultivars. For the same size of necrotic area, the photosynthetic damage on cv. Alhaja (susceptible) was more important than on cv. Bollil.

Photosynthesis in apparently healthy areas was affected by the pathogen. Difference in necrosis progression rate due to cultivar susceptibility was not the only difference in cultivar behaviour in regard to the pathogen: the photosynthesis was more impacted by leaf injuries for the susceptible cultivar than for the resistant one.

Until now, damages caused by Phomopsis stem canker were only characterized using the proportion of plants with a girdling spot on stem. The impact of leaf injuries had never been studied. The present study quantified the relationship between the severity of foliar Phomopsis symptoms and the reduction of leaf area photosynthetically active. This study thus improves the understanding of an important damage mechanism and will help predict yield loss caused by Phomopsis stem canker.

DISCUSSION

The proximal area was quickly affected by the pathogen on both cultivars. However, the distal area was affected later on the resistant cultivar than on the susceptible one. As the two cultivars have similar leaf sizes, this can be explained only by the difference in necrosis progression which was slower for the resistant cultivar.

The speed of the fungal growth showed significant differences between the two cultivars, with growth rates varying between 4 and 8 $mm.day^{-1}$. Degener *et al.* (1999) observed similar rates for a range of inbreeds lines of sunflower.

The transient NPR increase on median and distal areas could result from a compensation of photosynthesis in healthy areas as observed by Philip and Devadath (1981) on rice infected by bacterial blight.

Cytological observations of the infection process have revealed that *Phomopsis* kills the host cells well before hyphae colonize tissues. Hyphae colonize tissues only in the late stage of infection when tissues are already dead. This could be due to phytotoxic compounds secreted by the fungus. Indeed, several toxic metabolites were identified in Phomopsis stem canker: phomozin which provokes necrotic lesions on sunflower leaves (Mazars et al., 1990) and was suggested to be host specific, and mellein derivatives *trans*-4,6-dihydroxymellein and *cis*-4,6- dihydroxymellein (Avantaggiato et al., 1999). Mellein and its derivatives seem to have a direct inhibitory effect on the Calvin cycle or on reactions linked to it (Bethenod et al., 1982). Mellein slows down the cell cycle by expanding mitosis and has a direct effect on photosynthesis causing a decrease of the CO_2 absorption, probably due to reduced CO_2 transfer through the stomata (Bousquet et al., 1977). It was suggested that some of these biological activities could be exhibited by the two compounds isolated and may contribute to sunflower symptoms caused by *Phomopsis helianthi*. The specific mechanism underlying destruction and/or disruption of cellular constituents by phomopsis is unknown.

The cultivar difference in parameter γ clearly showed that photosynthetic impairment at a given disease severity was greater in cv. Alhaja compared with cv. Bollil. The causes for the disproportionately large metabolic effects of phomopsis are unclear, but contrasts in γ values imply that underlying mechanisms, associated either with the pathogen or host response, differed between cultivars. The basis of the leaf resistance is still largely unknown. This resistance may be influenced by the structure of the cells (Duletic-Lausevic and Mihaljcevic, 1997) as well as phenolic components (Bertrand and Tourvieille de Labrouhe, 1987).

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