Effects of plant density on sunflower premature ripening caused by *Phoma macdonaldii*

C. Seassau[a], G. Dechamp-Guillaume[b], E. Mestries[c] and P. Debaeke[d]

[a] INP-EIPURPAN, UMR AGIR, BP57611, F-31076 Toulouse cedex, France, celia.seassau@purpan.fr
[b] INP-ENSAT, UMR AGIR, BP 32607, F-31326 Castanet-Tolosan cedex, France, dechamp@ensat.fr
[c] CETIOM, ENSAT, BP 32607, F-31326 Castanet-Tolosan cedex, France, mestries@cetiom.fr
[d] INRA, UMR AGIR, BP 52627, F-31326 Castanet-Tolosan cedex, France, debaeke@toulouse.inra.fr

ABSTRACT

- Premature ripening (PR) is one of the most important fungal diseases in France, responsible for high yield losses in sunflower crop in the main regions of production. Previous results indicated that girdling canker of the stem base, caused by *Phoma macdonaldii*, was its primary cause. Previous studies have reported the influence of nitrogen and water supply on the incidence and severity of PR but an additional study was required to analyse the effect of plant density on the level of attack for a more comprehensive cultural control of PR.
- In a 2-year field study (2008 and 2009) in Toulouse (France), a susceptible cultivar artificially inoculated with *P. macdonaldii* was grown under three contrasted plant densities (4, 6.5 and 9 plants/m²) arranged with two N-fertilization rates (0 and 150 kg $N.ha^{-1}$) and two water regimes (irrigated and rainfed). Disease attacks were scored weekly to calculate percentage of PR plants. Microclimatic conditions were monitored using thermo-hygrometers within the crop. The fraction of fPARi intercepted by the canopy was regularly measured. Plant water and N status were characterized by crop simulation (SUNFLO) and measurements (e.g. N content and N Nutrition Index).
- Increasing plant density resulted in a greater proportion of PR plants. This finding was amplified when N was fully applied and the crop not irrigated. Despite contrasted canopy development, differences in microclimatic conditions were not sufficiently discriminating between density levels to explain PR expression. Conversely, N measurement and the plant diameter at stem base were closely related to PR incidence. Thin plants (grown under high density) with non-limiting N supply were more susceptible to premature ripening.
- This study supplements the known effects of crop management on sunflower PR and emphasizes the key role of crop canopy in understanding the processes involved in the development of the disease. This morphology could be manipulated through crop management (plant density, N fertilization) and probably breeding.

Keywords: Crop management, *Leptosphaeria lindquistii*, Plant density, Plant morphology, Premature ripening

INTRODUCTION

Phoma macdonaldii Boerema (McDonald, 1964) is one of the most widespread sunflower (*Helianthus annuus* L.) pathogen, reported worldwide (Gulya et al., 1997). The disease is mainly characterized by the appearance of black lesions on the stem, at the foliar nodes. A girdling canker at the base of the stem appears to be particularly damaging and can induce a wilt and stalk rot called premature ripening (PR) syndrome. From mid- to late summer, leaves become wilted and necrotic, the stalk turns dark brown to black leading to plant death a few weeks before physiological maturity (Donald et al., 1987).

Recent studies aimed at a better understanding of the etiology of sunflower PR and the identification of agronomic factors that promote the disease. Although different genotypic susceptibilities to Phoma black stem have been observed (Darvishzadeh and Sarrafi, 2007), environmental factors were suggested to play a key role in disease development and PR occurrence. Residues left on the soil surface are the main source of *P. macdonaldii* inoculum. Thus, Seassau et al. (2010b) demonstrated that sunflower PR was primarily due to aerial infections of the stem base and not to direct root attacks. Beyond that, field studies concluded to a significant effect of crop management on PR. A combination of good nitrogen nutrition and post-anthesis water shortage resulted in high PR pressure especially with susceptible cultivars Seassau et al. (2010a). However, the effects of plant density on PR have never been reported so far. Therefore there was a need for a more detailed knowledge of the influence of different agronomic factors in interaction on PR. This paper reports the conclusions of a 2-year experiment where contrasted plant density, N fertilization and irrigation levels were applied to a cultivar susceptible to PR.

MATERIALS AND METHODS

Experimental design and crop management system: The field experiment was conducted in 2008 and 2009 at INRA station in Auzeville (south-western France, 43°36N, 1°26E). The lay-out was a split-plot design. A susceptible cultivar to *P. macdonaldii* (cv. Heliasol RM) was either artificially inoculated (AI) or naturally infected (NI). Each trial was subdivided into 2 water regimes (rainfed vs irrigated), then 2 levels of nitrogen (0 vs 150 kg/ha) and 3 plant densities (4 (D1), 6.5 (D2) and 9 (D3) plants m^{-2}). N-fertilization was applied at sowing and at early flower bud stage. To satisfy the water requirements, 120 mm (2008) and 70 mm (2009) were applied on the irrigated treatment; the rainfed treatment received no more than 20 mm for N incorporation.

Phoma isolates and plant inoculation: A single conidium culture of *P. macdonaldii* (MPH2 strain), selected for its aggressiveness, was used for artificial inoculation. The fungus was plated on Petri dishes containing potato dextrose agar (39 g l^{-1}, 150 mg of streptomycin, pH 6) and grown at 25 ± 1 °C for 10 days in the dark. Inoculation of the AI plots with mycelium of *P. macdonaldii* was carried out at star bud stage on 25 uniform plants tagged within the two central rows. A 6 mm diameter disk of PDA with mycelium was placed at the stem base of each plant and left for five days. Drying of the disk was avoided by applying a moist cotton wool plug covered with aluminum foil around the stem base.

Disease assessment: Development of necrotic areas at the stem base induced by *P. macdonaldii* was assessed weekly from 7 and 16 days after AI, and 30 and 23 days post-inoculation (DPI) in 2008 and 2009 respectively. The disease was scored using a 0-4 scale: 0 = healthy plant, 1 = less than ¾ of the stem base circumference black, 2 = spots circling the stem base, 3 = all leaves wilted but the stem green, 4 = plant completely dead. A PR plant (scale 4) was thus defined as one completely dry before physiological maturity with necrosis circling the stem base. Over the experiments, 100 % of AI and NI tagged plants were infected by *P. macdonaldii*. Disease assessment was assessed by calculating the percent of PR plants from AI up to 81 DPI in 2008 and 77 DPI in 2009, *i.e*. ten days before the onset of normal senescence. At least 11 recordings were taken in 2008 and 10 in 2009. In NI, the progression of the fungus and PR 9 and 8 recording were taken in 2008 and 2009 respectively.

Environment, microclimate and plants measurements: Mean air temperature (°C, 2m height), relative air humidity (%) and precipitations (mm) were recorded daily. Temperature and relative humidity (RH) within the canopy (at 0.4 m above the soil) were recorded every 30 min using thermohygrometers Rotronic MP100A (Campbell Scientific Ltd., Les Ulis, France). Selected management treatments representing a range of sunflower canopies were equipped with the sensors in irrigated and rainfed plots: D1-N0, D3-N0 and D3-N150. The fraction of photosynthetically active radiation intercepted (fPARi) was measured per plot at flowering on both central inter-row using a hand-held Picqhelios (Aeric, Balma, France) apparatus. Leaf area index was measured with a LICOR-2000 on two replicates per unit plot.

Two indicators of the Nitrogen nutrition status of the plant were measured: the shoot N content and the Nitrogen Nutrition Index (NNI). At flowering, five plants were cut at soil surface on each unit plot. Plant

N concentration was determined using Dumas method then NNI was calculated as follows (Debaeke et al., submitted):

$NNI = Nm/Nc \quad (Nc = 4.53 \times ADM^{-0,42})$

where Nm is the total N concentration measured for all the aerial parts and Nc is the critical total N concentration calculated for the weight of aerial dry matter (ADM) measured *in situ*. A value of NNI ≥ 1 indicates a crop with ample N supply (N non-limiting), NNI = 1 is optimal N nutrition and NNI < 1 reveals N deficiency.
From inoculation to physiological maturity, water satisfaction index (WSI) was simulated using SUNFLO crop model (Casadebaig et al, 2011) The influence of N, plant density and irrigation on soil water depletion was thus simulated The output variable used was the ratio of actual to potential evapotranspiration (Et_a/ET_p).
The stem base diameter (SBD) was measured with a 150mm Stainless Steel Digital Caliper (in/mm LCD) on a sample of 10 plants for each replicate of the 18 "water regime x nitrogen x plant density" combinations. STD was measured at the end of flowering when stem growth in diameter was completed.

Statistical analyses: The final percentage of PR plants was subjected to analysis of variance (ANOVA) *via* the general linear model procedure of Statgraphics Plus 5.1 statistical software (Rockville, MA, USA). Prior to ANOVA, square-root transformations were applied to SBD values, and arcsine transformations to PR. When significant differences were found at $P \leq 0.05$, means were compared using Fisher's protected least significant difference test (95 % LSD).
Linear correlations (Pearson's coefficient of correlation) were attempted between the final percentage of PR plants and the canopy variables (Nm, NNI, WSI, LAI, fPARi and SBD).
A stepwise regression analysis was computed to describe the relationship between PR and the different previous canopy variables determined on 36 cultural situations (year x water regime x N-fertilization x plant density). A probability of $P \leq 0.05$ was used for inclusion in the stepwise analyses.

RESULTS
Weather conditions: Mean temperature from May to September was higher in 2009 (20.6°C) than in 2008 (18.1°C). More rainfall was recorded throughout the cropping season in 2008 (296 mm) than in 2009 (148 mm). However, the relative air humidity was less contrasted between the 2 seasons (72 % in 2008 *vs* 67 % in 2009).

Characterisation of crop canopy development for contrasted crop management
1. Nitrogen content, fPARi and water stress index: As irrigation was generally applied just before or after anthesis, no significant differences were observed between rainfed and irrigated plots for N content and fPARi at anthesis. However, WSI was significantly higher ($P < 0.0001$) with irrigation. By manipulating crop canopy by management (N available, plant density and water regime), it was possible to generate contrasted values of N content, fPARi and WSI. Nitrogen content was significantly ($P < 0.005$) contrasted between N-fertilization treatments, Nm in N150 being twice higher than in N0 (Table 1). Although the N content of plants grown at low plant density was 7 to 19% higher than Nm in high density, the differences were not significant. fPARi values were higher in 2009 than in 2008 and significant ($P < 0.0001$) differences were observed with N availability, fPARi increasing up to 18 % in 150N for D1 (Table 1). fPARi increased with plant density whatever N inputs and year. WSI changed with irrigation, nitrogen and density management and growing season. In irrigated plots, WSI was above 0.8 and always below 0.7 in rainfed plots. Simulated water stress was significantly ($P < 0.05$) higher at high plant density, especially under rainfed management and in 2009.

Table 1 - Nitrogen content (%) and fraction of PAR intercepted by the canopy (%) at flowering for plants infected with *P. macdonaldii*, and water satisfaction index (WSI) under two levels of N fertilization (N0, N 150) and three plant densities (4, 6.5 and 9 plants/m²) in 2008 and 2009.

	Nitrogen content (%)[a]				fPARi (%)				WSI ($ET_{actual}/ET_{potential}$)[a]			
	2008		2009		2008		2009		2008		2009	
	N0	N150	N0	N150	N0	N150	N0	N150	Irrigated	Rainfed	Irrigated	Rainfed
Plant density												
D1 (4 plants/m²)	1.26 ns b	2.36 ns	1.45 ns	2.42 ns	76.9 ns	85.8 a	77.6 ns	94.3 a	0.87 a	0.74 a	0.84 a	0.63 a
D2 (6.5 plants/m²)	1.14 ns	2.10 ns	1.29 ns	1.62 ns	78.3 ns	87.3 b	83.6 ns	96.1 ab	0.84 b	0.69 b	0.80 b	0.59 b
D3 (9 plants/m²)	1.13 ns	2.10 ns	1.17 ns	2.13 ns	80.3 ns	88.6 b	85.7 ns	97.8 b	0.81 c	0.66 c	0.78 b	0.56 b

[a] High values of ET_a / ET_p indicated an adequate water supply and water stress occurred for ETa / ET_p below 1.

[b] For each given agronomic factor (N-fertilisation and plant density), means values followed by different letters for each year (2008 and 2009) are significantly different from one another based on $LSD_{0.05}$ and ns– not significant at 5% level.

2. Stem base diameter: SBD increased significantly for low plant density (+ 6 mm in D1 in 2008, + 4.3 mm in 2009) and high N (+ 6 mm in N150 in 2008, + 4.8 mm in 2009) compared respectively to D3 and N0. In 2008, SBD was more than 3 mm greater than in 2009 ($P < 0.0001$) (Table 2).

Table 2. Mean stem base diameter (SBD, mm) at flowering for the three plant densities (4, 6.5 and 9 plants/m²) for 2 levels of N-fertilisation (N0 and N150) in 2008 and 2009.

	Stem base diameter (mm)					
	2008			2009		
	N0	N150	Mean	N0	N150	Mean
Plant density						
D1 (4 plants/m²)	23.3 a[a]	26.2 a	26.2 a	19.4 a	22.5 a	21.1 a
D2 (6,5 plants/m²)	20.3 b	22.2 b	22.2 b	17.6 b	18.8 b	18.1 b
D3 (9 plants/m²)	18.1 c	20.2 c	20.2 c	15.7 c	17.7 c	16.8 c

[a] Within each column, mean values followed by different letters are significantly different from one another based on $LSD_{0.05}$.

3. Characterization of the microclimate under the cover: The microclimate was marked by 2009, dryer than 2008. In July and August 2009, 9 days where the temperature was above 25 °C and 11 days were RH was above 80 % against 0 and 31 days for these variables in 2008. The difference in microclimate between D1 and D3 was very low. Temperatures were slightly higher in D1 than D3 with maximum differences per day of 1.5°C in 2008 and 1.2°C in 2009. Throughout the disease development period, RH was significantly ($P = 0.003$) higher in D1 than in D3 but only in 2008. Mean RH in D1 was 80.2 % and 78.9 % in D3 in 2008. Corresponding values in 2009 were 74.8 % and 75.0 %.

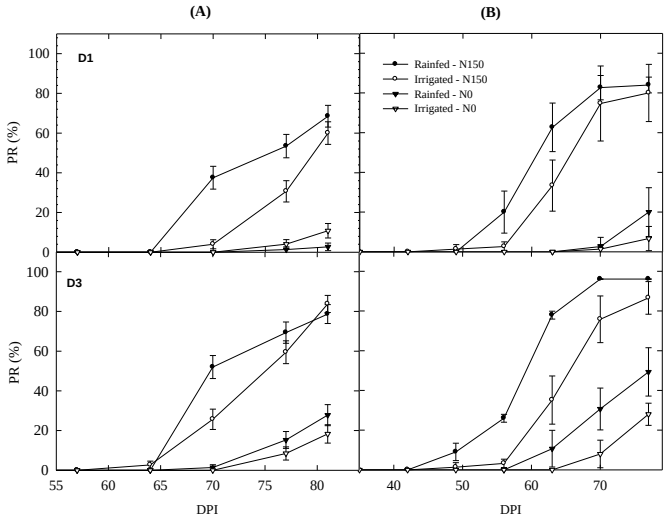

Fig. 1. Annual disease progress curve of premature ripening (PR) plants after artificial inoculation with *P. macdonaldii* for two plant densities D1 and D3 (4 and 9 plants/m²) and four water-nitrogen regimes:
(A) 2008 ; (B) 2009.

Effects of plant density on disease incidence: At 30 DPI in 2008, 100 % of AI plants exhibited girdling black necrosis at the stem base, against 90 % in NI, whatever their plant density. At 35 DPI in 2009, only 27% (D1) and 47 % (D2) of NI plants presented girding necrosis and 72 % (D1) and 79 % (D2) of AI plants. AI and high plant density both increased the proportion of PR plants.
Annual disease progress curves of PR in AI clearly showed significant effects of crop management (Fig.1). For both years, the time-course of PR differed between plant densities. First PR plants were observed in D2/D3 and a seven-day delay was observed in D1. Both N-fertilization and limiting water supply significantly ($P < 0.001$) enhanced PR. PR expression was earlier in D2 and D3 for N150 x rainfed plots than in D1. Globally, high plant density (D3) increased PR by 26 to 29 % compared with low density (D1).

Statistical relationships between PR and canopy variables: Final percentage of PR plants was significantly and positively correlated to fPARi, LAI, plant Nm and NNI. PR was negatively correlated to SBD and WSI. Moreover, Nm, SBD, NNI, WSI, fPARi and LAI were positively correlated among themselves, while LAI, fPARi were negatively correlated to WSI. The stepwise regression model for PR pointed out two canopy variables that were associated with an increased risk of premature ripening: PR increased with high fPARi and NNI.
PS = -270.483 + 3.37*fPARi + 38.19*NNI ($R^2 = 0.852$)

DISCUSSION

Accounting for crop management (including plant density) helps in predicting or understanding the performance of sunflowers inoculated with *Phoma macdonaldii*. To correctly assess the effect of plant density in interaction with N rate and water regime on PR, a thorough characterization of crop canopy was necessary.
The incidence of the disease at stem base was earlier and higher with AI than NI for all crop densities. Diversity for aggressiveness among *P. macdonaldii* strains could partially explain differences between NI and AI for percent PR. In 2008, collar black necrosis appeared earlier and the progression of Phoma around the collar was faster than in 2009. In 2008, climatic conditions (low air temperature and high rainfall) were probably more favourable to spore emission. If the influence of temperature on spore emission has not been shown (Bordat et al., 2011), pathogen growth rate is largely dependent on temperature and relative humidity (RH), with optimum growth between 20 to 30 °C and RH above 80 % (Weeraratne and Priyantha, 2003). Then microclimate under cover may have constituted a major climatic parameter in disease epidemiology in order to understand successful infection and development of the pathogen at stem base (Huber and Gillespie, 1992). Plants grown at high density (D3) presented a

necrosis more important than at low density (D1). Despite contrasted canopy cover (through fPARi) stronger in D3 than in D1, the lack of significant microclimatic differences between the two densities suggests that this factor is not mainly involved in fungus spread and by the way on PR expression.
A previous report on sunflower PR showed that in conditions where water satisfaction was limiting, heaviest attacks were observed (Seassau et al., 2010a). Stepwise linear regression showed that WSI influenced negatively PR; however, this variable is not considered as inducing PR but as enhancing the syndrome whereas N-fertilisation and plant density have a more direct action on phoma development at collar ad stem level (Gulya et al., 1997; Debaeke and Pérès, 2003; Seassau et al., 2011a). In the present study, the enhancing effect of N on PR was once more confirmed without ambiguity. The severity of PR appears more likely to be determined by physiological and anatomical changes that could affect host receptivity to the pathogen. This hypothesis could be reinforced considering the effect of plant density on PR modified by nitrogen supply.
Moreover, high plant densities enhanced PR as the result of physiological and anatomical changes (thinner plants more susceptible to girdling attacks). The higher LAI in dense canopies may have increased the drought risk (low WSI) as it was observed in rice (Haefele et al., 2008).
To understand how plant development and morphology could be involved in the harmful expression of the disease, the authors go on to a previous study that discussed some assumptions on the underlying causes of water deficit and high N content on premature ripening (Seassau et al., 2010a). Under water stress, the diameters of the widest sunflower xylem vessels are narrowed as an adaptation to the risk of occurrence of vascular embolisms (Hacke et al., 2001). Hyphae of *P. macdonaldii* in the xylem of PR plants (Seassau et al., 2010b) could be involved in vessel blockage leading to the death of the plant (Put and Clerkx, 1988). This phenomenon could be increased with high nitrogen content in the plant, where nitrogen could be trophic for the fungus, enhancing his development in the plant (Walters and Bingham, 2007). This situation could be much more damaging for plants presenting thin stems notably at high plant density. The rapid progression of the fungus in those plants would induce a strong vessel-plugging which agrees with the higher proportion of PR plants in D3 than in D1 for both years.
To reduce the expression of the disease, control canopy development (biomass development, plant morphology and plant water satisfaction) could be exploited more instead of developing fungicide protection. Promising cultivars, with large stems, should therefore be screened at high density and plethoric N supplies under water-limited conditions, a procedure which could be used in resistance tests during breeding programs.

ACKNOWLEDGEMENTS

This study was supported by CETIOM, Midi-Pyrénées government and PROMOSOL association. We are grateful to the technical staff of INRA (D. Raffaillac, M. Labarrère, E. Bazerthe) for their assistance in experimental procedures. We would like also to thank C. Jourda and A. Ribero for their collaboration in disease scoring.

REFERENCES

Bordat, A., P. Debaeke, G. Dechamp-Guillaume, E. Mestries, C. Seassau, and P. Vincourt. 2011. Phoma et dessèchement précoce du tournesol. In : Cetiom (ed.), Point technique du CETIOM, Paris, France

Casadebaig, P., L. Guilioni, J. Lecoeur, A. Christophe, L. Champolivier, and P. Debaeke P. 2011. SUNFLO, a model to simulate genotype-specific performance of the sunflower crop in contrasting environments. Agricultural and Forest Meteorology 151:163-178.

Darvishzadeh, R., and A. Sarrafi. 2007. Genetic analysis of partial resistance to black stem (*P. macdonaldii*) in sunflower. Plant Breed. 126:334-336.

Debaeke, P., and A. Pérès. 2003. Influence of sunflower (*Helianthus annuus* L.) crop management on Phoma black stem (*Phoma macdonaldii* Boerema). Crop Prot. 22:741-752.

Debaeke, P., E. van Oosterom, E. Justes, L. Champolivier, A. Merrien, L.A.N. Aguirrezabal, V. Gonzalez-Dugo, A.M. Massignam , and F. Montemurro. A species-specific critical nitrogen dilution curve for sunflower *(Helianthus annuus L.).* Field Crops Res. (submitted).

Donald, P., C. Hartman,, J. Venette, and G. Secor.1987. Response of sunflowers to culture filtrates from *Phoma macdonaldii*. Phytopathology 75:466-468.

Gulya, T., K.Y. Rashid, and S.M. Masirevic. 1997. Sunflower diseases: Phoma black stem. P 319-322. In: A.A. Schneiter (ed.), Sunflower Production and Technology. Agronomy Monograph 35. ASA, CSSA and SSSA, Madison, WI, USA.

Hacke, U.G., V. Stiller, J.S. Sperry, J. Pittermann, and K.A. McCulloh. 2001. Cavitation fatigue : embolism and refilling cycles can weaken the cavitation resistance of xylem. Plant Physiol. 125:779–786.

Haefele, S.M., S.M.A. Jabbar, J.D.L.C. Siopongco, A. Tirol-Padre, S.T. Amarante, P.C.S. Cruz, and W.C. Cosico. 2008. Nitrogen use efficiency in selected rice (*Oryza sativa* L.) genotypes under different water regimes and nitrogen levels. Field Crops Res. 107:137-146.

Huber, D.M., and T.J. Gillespie. 1992. Modelling leaf wetness in relation to plant disease epidemiology. Ann. Rev. Phytopathol 30:553-577.

McDonald, W.C. 1964. Phoma black stem of sunflower. Phytopathology 54:492-493.

Put, H.M.C., and A.C.M. Clerkx. 1988. The infiltration ability of microorganisms *Bacillus*, *Fusarium*, *Kluyveromyces* and *Pseudomonas* spp into xylem vessels of *Gerbera* cv. "Fleur" and *Rosa* cv. "Sonia" cut flowers: a scanning electron microscope study. Journal of Applied Bacteriology 64:515-530.
Seassau, C., G. Dechamp-Guillaume, E. Mestries, and P. Debaeke. 2010a. Nitrogen and water management can limit premature ripening of sunflower induced by *Phoma macdonaldii*. Field Crops Res. 115:99-106.
Seassau, C., P. Debaeke, E. Mestries, and G. Dechamp-Guillaume. 2010b. Evaluation of inoculation methods to reproduce sunflower premature ripening caused by *Phoma macdonaldii*. Plant Dis. 94:1398-1404.
Walters, D.R., and I.J. Bingham. 2007. Influence of nutrition on disease development caused by fungal pathogens: implications for plant disease control. Ann. Appl. Biology 151:307-324.
Weeraratne, W., and M. Priyantha. 2003. First report of Phoma black stem of sunflower in Sri Lanka and its management. Annals of the Sri Lanka Department of Agriculture 5:263-270.