Advances in *Phoma macdonaldii (Leptosphaeria lindquistii)* epidemiology

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ABSTRACT

- *Phoma macdonaldii* Boerema (teleomorph *Leptosphaeria lindquistii*) is the causal agent of the black stem disease of sunflower. This disease, which appeared in the early 1990s, is currently present in the entire French sunflower cropping area. Little research on the infection stage of the disease has been carried out, and the biological cycle of *P. macdonaldii* is not completely known. Sunflower infected residues are the source of primary inoculum. Short rotations (mainly two year sunflower-wheat in south-western France) and simplified soil tillage of wheat crops increased inoculum production by leaving infected residues on the soil surface. At the end of winter, perithecia of *L. lindquistii* and pycnidia of *P. macdonaldii* appear on infected residues. In the springtime, these fructifications release some ascospores and conidia which can contaminate surrounding sunflower fields. These contaminations would be mainly due to ascospores. The aims of this study were to quantify primary inoculum production and to characterize the effects of environmental conditions on the release of *L. lindquistii* ascospores.
- In 2011, at the end of winter, sunflower residues were taken from twenty fields in south-western France. These fields were chosen to represent a wide range of environment and tillage practices. Primary inoculum production was characterized by measuring the number of perithecia per surface unit of soil. Perithecial maturation was studied during springtime on residues of two fields among the twenty. In addition, the number of ascospores per perithecium was estimated with a haemocytometer. The influence of climate on ascospore release has been studied to help predict periods of contaminations. *Burkard* ® volumetric air samplers were used to measure atmospheric concentration in *L. lindquistii* ascospores in INRA field trials (Auzeville, France), from 2008 to 2011. Infected stubble was displayed around the volumetric air samplers in order to do ease the measurement of the dynamic of primary inoculum production during the sunflower development cycle (from late spring to mid-august). Airborne particles were captured on bovine serum albumin or vaseline-coated cellophane tape wrapped around the sampler drum. The tapes were observed under a light microscope (x 400) to estimate the daily atmospheric concentration in ascospores. The obtained dynamics were plotted with respect to climatic data.
- Sunflower residues were observed in each of the 20 fields which were all contaminated by *P. macdonaldii* the previous year. The density of residues left at soil surface ranged 8 to 20 residues per square meter, with a mean value of 16 residues per square meter. The density of *L. lindquistii* perithecia ranged 33100 to 270000 m^{-2}, with a mean value of 81200 perithecia m^{-2}. Perithecial maturation had different dynamics on the two fields observed. The perithecial maturation rate was 28% on March 30th, and 80% on June 22nd for the first field whereas it was 76% on April 6th, and 100% on June 29th for the second field. The number of ascospores per perithecium ranged from 0 to 6000, with a mean value of 1676. A total of 367 days have been observed during the 4 years of spore trapping. The daily atmospheric concentration in *L. lindquistii* ascospores ranged from 0 to 176 per cubic meter. Spore emissions were observed for 80% of rainy days and for 70% of dry days. However, significant emissions were observed in 50% of rainy days whereas they were observed in only 28% of dry days. Spore discharges were thus generally triggered by rain but not necessarily. In fact, they occurred for a wide range of daily precipitations: from 0 to 39 mm day^{-1}.
- This study is the first one to quantify *L. lindquistii* primary inoculum. New experiments will be carried out to confirm these results. The main conclusions are that ascospore discharges can occur during the entire sunflower cycle and that tillage can be an efficient lever to control black stem epidemics. In addition, a high variability of emissions was observed as a function of weather variables.
- These results will be used to develop a forecast model of ascospore release to determine ascospore discharge periods and to help design control strategies against *P. macdonaldii* on sunflower.

Key words : ascospores - black stem disease – perithecium - primary inoculum.

INTRODUCTION

Black stem disease and premature ripening of sunflower (*Helianthus annuus* L.) are caused by *Phoma macdonaldii* (teleomorph *Leptosphaeria lindquistii* ; MacDonald, 1964). In France, the disease occurs each year, in every sunflower cropping areas. Disease control mainly relies on crop management (Velasquez and Formento, 2000 ; Debaeke and Peres, 2003; Seassau *et al*., 2010). Although several fungicides are registered against the disease, their epidemiological and economic efficiencies are uncertain under French conditions. Short crop rotations, mainly sunflower–wheat in south western France, and simplified tillage of wheat crops leave infected stubble at soil surface. This certainly has increased primary inoculum production (Poisson-Bammé and Pérès, 2000). Overwinter survival of the fungus on infected stubble plays an important role in disease epidemics (Poisson-Bammé and Pérès, 2000). The aim of this work is (i) to quantify the primary inoculum production from infected sunflower stubble in commercial fields and (ii) to identify the effects of weather conditions on *L. lindquistii* ascospore release.

MATERIALS AND METHODS

Primary inoculum quantification. In 2011, at the end of winter, sunflower stubble was collected from twenty fields in south-western France (Ariège, Aude, Gers, Haute-Garonne). These fields were chosen to represent a wide range of soil types (silty, silty-clay and clay soils) and tillage practices. Five fields had a following crop seeded with direct sowing (no tillage was performed after the sunflower harvest). Seven fields had simplified tillage (< 15 cm) without ploughing in the crop sequence. Eight fields had simplified tillage (< 15 cm) with ploughing in the crop sequence. The primary inoculum production was quantified at the end of winter (February) and at the beginning of spring (May). This second period plays an important role in the epidemics because it coincides with the emergence period of sunflower in surroundings fields. The density of residues per soil surface unit was counted on five samples (1 m² each) for each field and the total surface of residues was measured. The number of perithecia per soil surface unit was assessed according to the method described by Lô-Pelzer *et al*. (2009) using the perithecium size given by Maric *et al*. (1981).

The dynamics of perithecial maturation was observed on 5 residues collected in two of the twenty fields every two weeks, from March 30th to June 29th. Five perithecia per residue were sampled, observed under a binocular magnifying glass (Leica ® wild M3B) and classified as mature or immature perithecia. A perithecium was considered mature if it had differentiated asci with at least one tri-cellular ascospore (Poisson-Bammé and Pérès, 2000). Ten samples of ten perithecia coming from one infected residue (collected on June 20th in the Auzeville field, with 100% mature perithecia) were used to estimate the mean number of ascospores per perithecium. The pooled perithecia of each sample were crushed and then vortexed in 400 µl of distilled water. The ascospores content per perithecium was assessed by counting ascospores in ten samples of 10 µl of this solution under an optical microscope (Leica ® DME, magnification x 400) with a Malassez haemocytometer.

Measurement of atmospheric L lindquistii ascospore concentration. From 2008 to 2011, four experiments have been conducted at INRA (Auzeville, France) to measure the *atmospheric L lindquistii ascospore concentration* using several *Burkard*® volumetric spore samplers (Burkard Manufacturing Company Ltd., Rickmansworth, UK). In 2008 and 2009, the spore traps were operated on an open ground area (lawn) and surrounded by infected stubble showing black stem disease symptoms. In 2010 and 2011, the spore samplers were positioned in INRA sunflower field trials. Table 1 summarizes the four experiments.

The *L. lindquistii* spores were captured on a bovine serum albumin or vaseline-coated cellophane tape wrapped around the drum of the spore trap. At 7-day intervals, the exposed tape was removed from the sampler drum and cut into 7 pieces 48 mm long, each representing a 24 h period. Each piece was mounted as a microscope slide and observed with an optical microscope (Leica ® DME, magnification x 400). The daily atmospheric ascospore concentration (number of ascospores.m^{-3}) was estimated from the average number of ascospores counted over three longitudinal traverses of each piece of tape. Daily rainfall (mm), daily average temperature (°C) and daily average relative humidity (%) were recorded by the Auzeville INRA weather station.

Year	Environment	Inoculum origin	Date of inoculum supply	Sowing date	Irrigation (mm)	Spore trap functioning period
2008	Open ground area	Commercial fields close to Toulouse	May 28th	-	-	April 28th - October 10th
2009	Open ground area	Commercial fields close to Toulouse	April 3rd	-	-	April 3rd - July 7th
2010	Sunflower field trial	Sunflower field trial in 2009 at Auzeville	0.4 to 0.5 stubble.m-², July 7th	April 19th	22 – 21 – 25 (July 1st-8th-21th)	May 28th - August 26th
2011	Sunflower field trial	Sunflower field trial in 2010 at Auzeville (wheat crop in 2011)	Natural infection	April 8th	28 -43 (April 13th - May 26th)	June 11th - August 4th

Table 1. Description of the four experiments conducted to characterize the atmospheric *L lindquistii* ascospore concentration (Auzeville, from 2008 to 2011).

RESULTS

An average of 16 residues per m² was counted, ranging from 8 to 20 residues per m². In May, this density increased to 32 residues per m² (from 9 to 62, depending on the considered field), probably due to their natural rotting. No significant effect of tillage on this variable was observed. On the other hand, the total area of residues per soil surface unit remained at the same level for the two periods (mean=0.20 and 0.22 $m^2.m^{-2}$ respectively) and appeared twice higher in the case of direct sowing both at the end of winter and in May (Kruskall-Wallis test : P=0.010 and P=0.043 respectively, Figure 1).

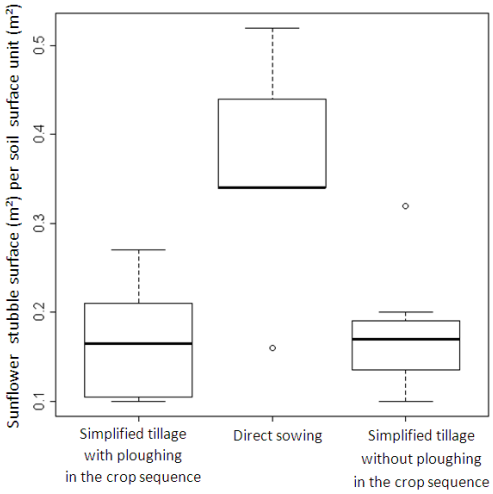

Figure 1. Total area of residues per soil surface unit as a function of tillage practices for 20 fields on May 2011.

Each of the 20 fields had experienced *P. macdonaldii* black stem disease in 2010. At the end of winter, the number of perithecia per m² of soil surface ranged from 33100 to 270000, with a mean value of 81200 m^{-2}. In May, this perithecia density decreased significantly, from 8500 to 60400, with an average of 28600 m^{-2}. Similarly, the number of perithecia per stubble unit area ranged from 15.7 to 60.3 cm^{-2} (mean value =32.9 cm^{-2}) at the end of winter. Four months later, only 11.3 perithecia per cm² of stubble surface were observed (with observed values ranging from 2.0 to 21.0 cm^{-2}). The significant discrepancies observed within the 20 fields for these two criteria were not explained by the type of soil, the type of tillage or the type of crop sown in 2011.

Under natural conditions, the dynamics of perithecial maturation was different in the two fields analysed. In the field with simplified tillage practices, the proportion of mature perithecia was 28% on the 30th March 30th, and 80% on June 22nd. In the field with direct sowing, this proportion was 76% on April 6th, and 100% on June 29th.

The daily concentration in ascospores varied from 0 to 176 ascospores per m^3, depending on the year (Figure 2).

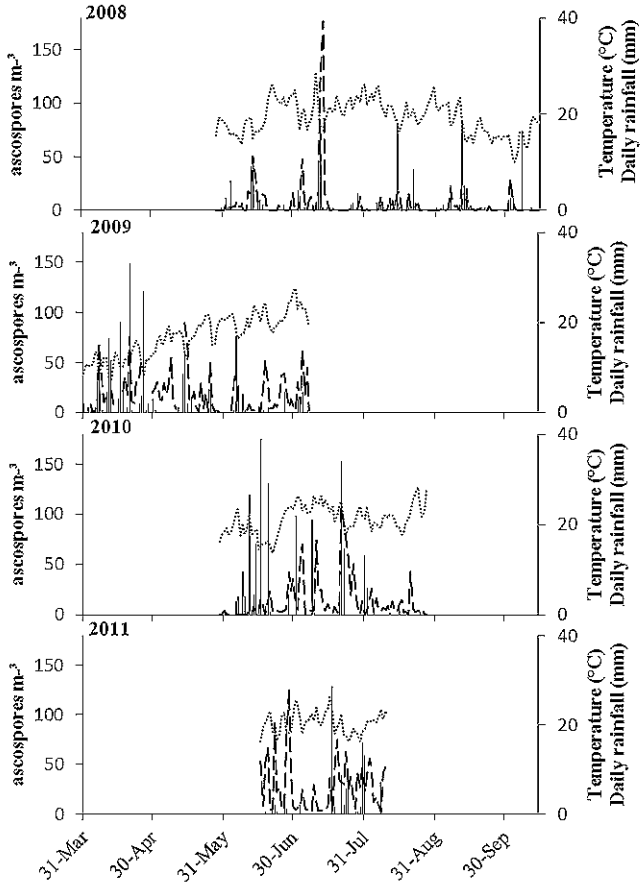

Figure 2. Atmospheric *L. lindquistii* ascospore concentration (— —) at Auzeville from 2008 to 2011, in connection to daily rainfall (▏) and daily average temperature (….).

The highest ascospore concentration was observed in July 2008. This year, this significant ascospore release was almost the only one recorded during the trapping period. In 2009, many high ascospore releases were recorded. However, only few of them reached 50 ascospores per m^3. In 2010 and 2011, several days with concentration higher than 50 ascospores per m^3 were observed. The average daily temperature during the trapping periods was 19.3 °C in 2008, 21.9 °C in 2009, 21.2 °C in 2010 and 20.3 °C in 2011. As for the rainfall regime, 48 rainy days occurred in 2008, 31 in 2009, 28 in 2010 and 19 in 2011.

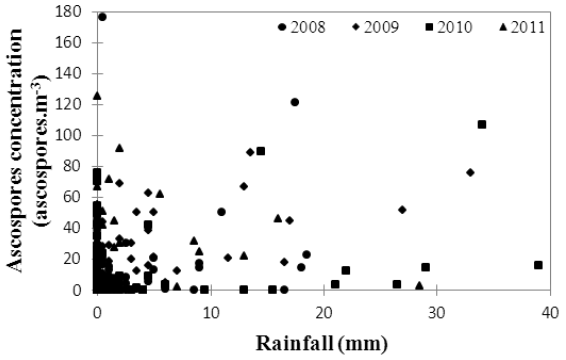

Figure 3. *L. lindquistii* ascospores concentration as a function of daily rainfall from 2008 to 2011.

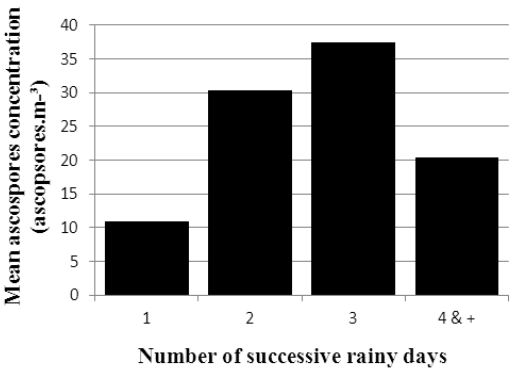

Figure 4. Average atmospheric *L. lindquistii* ascospore concentration according to the number of successive rainy days from 2008 to 2011.

Ascospore discharges generally occured during rainy days but not necessarily. In fact, emissions occurred in a large range of daily precipitations, from 0 mm to 39 mm per day. Spore emissions were observed for 80% of rainy days and for 70% of dry days. However, significant emissions were observed in 50% of rainy days whereas they were observed in only 28% of dry days. Nevertheless, 40% of the rainy days without ascospore releases were followed by ascospore emissions the day after. Moreover, 74% of the days preceding dry days with high ascospore concentration were rainy days. No linear relationship was observed between the ascospore concentration and the quantity of rainfall (Figure 3).
Average daily ascospore concentration during rainy days reached respectively 30 and 37 ascospores per m^3 after 2 and 3 succeeding rainy days while only 10 ascospores per m^3 were detected after one day of rain (Figure 4). These values decreased to 20 ascospores per m^3 for more than 4 succeeding rainy days.

DISCUSSION

A method to quantify primary inoculum of *L. lindquistii* has been developed. It allowed the first assessment of primary inoculum responsible for the *Phoma* black stem disease and premature ripening of sunflower. These first results, even if certainly not precise, give the order of magnitude of the amount of primary inoculum in the south-western French conditions. They help understand why the disease is widespread today. In our knowledge, such work has not been conducted yet on other sunflower pathogenic fungi.
Surprisingly, no difference in the number of sunflower residues has been observed according to tillage. But discrepancies on the total residue area per soil surface unit may be explained by the fact that, in the case of direct sowing, no tillage occurred between sunflower harvest and the observation period, except for the sowing operation itself. The decrease in the number of perithecia per soil surface unit or stubble surface unit between February and May can be explained by the exposure of sunflower stubble to environmental conditions (climate, soil micro-faun and micro-flora) favorable to its decomposition.
The average number of perithecia per cm² of sunflower stubble ranged from 32.9 cm^{-2} at the end of winter to 11.3 cm^{-2} in May. For *Phoma* stem canker on oilseed rape, Lô-Pelzer *et al.* (2009) had estimated that this density ranged from 1.9 to 10.8 *Leptosphaeria maculans* pseudothecia cm^{-2} as a function of the disease severity on oilseed rape. These values seem therefore to be consistent for similar pathosystems. It should be also valuable to study if the disease severity on sunflower increases the density of perithecia on infected stubble, as demonstrated for *Phoma* stem canker on oilseed rape (Lô-Pelzer *et al*., 2009).
The number of ascospores per perithecium was highly variable for the 100 counts. The mean value (1676) and the maximum value recorded (6000) are higher than those recorded by Lô-Pelzer *et al*. (2009) for *L. maculans* on oilseed rape. However, this number was certainly under-estimated since few ascospores were still observed in the perithecia after vortexing. On the other hand, as noticed by Lô-Pelzer *et al*. (2009), the estimation given by the calculation of individual volumes of a perithecium and an ascus probably over-estimated this number since the proportion of the total volume effectively occupied by asci inside a perithecium is difficult to evaluate.
Adapted tillage can improve the control of the disease through a proper stubble management that reduces the risk of contamination in the spreading area. These results have clearly demonstrated that simplified tillage does not alter significantly the quantity of primary inoculum as compared to direct sowing. In an experimental trial conducted at Auzeville, ploughing at 30 cm depth allowed the complete burial of sunflower stubble. Nevertheless, such tillage practice is not currently implemented in France due to technical constraints.

Ascospore discharges were detected during the entire sunflower cycle as mentioned by several authors (Guerin, 1997; Descorps, 2010; Frei, 2010). A high variability of ascospore concentration was observed depending on the year and the climatic conditions. Such variability has also been observed for *Leptosphaeria maculans* ascospore releases*:* Ascospores discharges are related to the occurrence of rainfall events and fluctuate in a larger range: from 0 to 800 ascospores per m³. Ascospore releases may occur if the daily rainfall is higher than 0.5 mm, for *L.lindquistii* (Delos *et al.*, 1998) and *Diaporthe helianti*. (Moinard and Eychenne, 1998). *L.lindquistii* ascospore releases are often triggered by rain but not systematically, as observed by Guerin (1997) and Descorps (2010). Some rainy days without ascospore release can occur and high concentration in ascospores can be observed during dry days. Sometimes, ascospore releases can occur within a period of one day after a rainy day. In addition, the average atmospheric ascospore concentration during rainy days increased after two and three days of rain, and then decreases for more than 4 successive rainy days. This also suggests that rain could enhance

ascospore release. The depletion of mature ascospores inside a perithecium could explain that ascospore atmospheric concentration declines after four rainy days.
This body of knowledge will be used to develop a model that predicts perithecial maturation and ascospore release. Such a model could help improve the timing of fungicide applications against *Phoma* black stem disease and premature ripening.

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