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Full Length Research Paper

Survival analysis to determine the length of latent period of *Mycosphaerella pinodes* on peas (*Pisum sativum* L.)

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Blight caused by *Mycosphaerella pinodes* is one of the most devastating diseases of pea that causes yield losses of over 50% in some years and may cause total failure to the crop under epidemic conditions. During this study, a sensitive disease assessment and statistical tool was developed for *Mycosphaerella* blight on peas, the latent period was used to discriminate between different treatments. The time until appearance of the first pycnidia (latent period length) was recorded. Seedling that did not display any production of pycnidia structure during the 20 days period of observation was recorded as right censored observations. Using non parametric and semi parametric survival analysis, different hypothesis dealing with factors that might influence the latent period was tested. Survival analysis using Kaplan-Meier estimates and Cox proportional hazards were performed for data analysis. During these investigations, latent period was regressed against leaf wetness duration, pea cultivar, inoculum concentration, plant age and isolate aggressiveness. Both the Cox regression and Kaplan-Meier tests had shown the importance of leaf wetness duration, inoculum concentration and isolate aggressiveness on the survival times, thus, the median latent period length was respectively 15 and 16 for tn0203 and md0202. The median for the 3 leaf wetness was 14, 16 and 17 respectively for 06, 48 and 72 h LWD. Both the cultivar and plant age had no significant risk for the pycnidia structure appearance. Likewise, using the semi parametric Cox proportional hazard regression, the 2 covariates namely higher leaf wetness, higher inoculum dose with an aggressive isolate were all associated significantly with survival time. Hence, the hazard ratio was respectively 1.205 and 1.423 for LWD and inoculum concentration respectively.

Key words: Cox regression, survival analysis, *Mycosphaerella* blight, *Pisum sativum.*

INTRODUCTION

The anthracnosis caused by *Mycosphaerella pinodes* (Berk. et Blox.) Vestergr. is one of the most destructive pathogens of peas (Moussart et al., 1998). It is widespread throughout the major pea-growing areas worldwide (Wallen, 1965; Lawyer, 1984; Bouznad, 1988; Bretag et al., 2006; Setti et al., 2008) . The disease has caused yield losses of over 50% in Canada in some years (Wallen, 1965; Xue et al., 1997), and similar losses in Australia (Bretag, 1989). In France, Ascochyta blight causes yield losses of up to 30%. In view of its complexity and economic importance, the disease has been investigated in many studies in pea-growing regions around the world. In recent years, an increased incidence of Ascochyta blight has been seen in different production areas in Algeria and has led to increasing yield loss (Setti et al., 2008). This could be due to an increased pathogenicity of the pathogen population or a greater inoculum pressure.

Many previous studies also showed the impact on both incubation and latent period. Hence, Shaner (1981),

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Van Ginkel and Scharen (1988) suggest the exploitation of the moisture parameter for predicting aerial disease infection. Furthermore, Fitt et al. (1989) and Huber and Gillespie (1992), have already noted the impact of the free water on leaf surface on the latent period. On the other hand, Tivoli et al. (1999) and Rogger et al. (2000), Turechek (2004) considered that a parameter such as the inoculum concentration may have a great impact on the life cycle of *M. pinodes* and hence may determine all the components of disease including the latent period. On other hand, both the incubation and latent period were considered to be influenced by plant age and the isolates virulence or aggressiveness (Gibb et al., 1998).

Many previous studies have provided the latent period for different species of Ascochyta blight. This was of 5 days for *A. rabiei* on chickpea (Trapero-Casas and Kaiser, 1992), 6 days for *A. Fabae* f.sp. *lentis* (Pederson and Morrell, 1994) and 8 - 10 days for *A. Fabae* on fabae bean (Wallen and Galway, 1977).

Concerning the latent period of *M. pinodes*, setti et al. (2008, 2009) reported that the mean latent period of *M. pinodes* on peas (cv. Onward) was 14.5 ± 2.98 days (median = 15).

However, these values were calculated only for plants that were symptomatic during the study period, whereas a number of inoculated pea plants had not presented a pycnidia structure at the time when the final disease incidence assessment was performed (20 days). Such data are referred to as “censored data” because these are observations that do not fail (pycnidia formation) within the time frame of the study and therefore were not included in statistical analyses.

Survival analysis is powerful class of statistical methods especially designed to such a data. The survival analysis study the occurrence and the timing of events (such as infection) while allowing for censored observations (Hosmer and Lemeshow, 1999; Klein and Moeschberger, 2003).

The study of events involving an element of time has a long history in statistical research and practice, particularly in medical studies and insurance. Moreover, this statistical method has served also many others disciplines which are referred with others names such as event history analysis and failure time respectively in sociology and industry.

In ecological research, the use of survival analysis has become recently a widespread tool for resolving more complicated data dealing in many disciplines such biodiversity and environment toxicology (Castro et al., 2004; Vange et al., 2004).

In contrast to the medical and ecological fields, survival analysis has rarely been applied in plant pathology (Madden and Nault, 1983; Muenchow, 1986; Westra et al., 1994).

Garrett et al. (2004) and Esker et al. (2006) stated that plant pathology research data are often collected in the form of time to event data (The time until appearance of the first symptom of disease; the time until appearance of the first sexual or asexual structure; the time until germination, etc).

Survival analysis is an interesting method that enables the introduction of censored data in the analysis. In the complex pea- *M. pinodes* pathosystem, this may permit the inclusion of infected plant that did not presented pycnidia during the period of study. Hence, this method helps us to obtain a more realistic estimation of latent period. In fact, introduction of such data in the analysis might have a great importance in a epidemic study particularly for *Mycosphaerella* blight. In fact, in our previous study small differences were observed in latent period between inoculum concentration and leaf wetness duration, this might have however, important consequences on the seasonal accumulation given the polycyclic nature of the pathogen (Setti et al., 2008).

Therefore, the objectives of this study were to investigate the use of survival methods to estimate the latent period of *Mycospherella* infect peas and to assess the effect of 4 parameters on time to pycnidia formation: (i) isolates aggressiveness, (ii) leaf wetness duration, (iii) inoculum concentration, (iv) plant age and (v) host susceptibility.

MATERIALS AND METHODS

Plant material

Pea cv Onward and cv Merveille de Kelvedon”MK”, the most cultivated varieties known to differ in their level of quantitative resistant from highly susceptible to moderately resistance respectively were used in all experiments.

Seeds of each cultivar were sown in 20 cm diameter pots containing unsterilized soil/compost mixture. 10 seeds were planted per pot and seedlings were thinned to 5. The plants were maintained in glasshouse.

Fungal material and inoculum production

Two isolates of *M. pinodes* md0203 and tn0203, obtained from 2 localities in chellif region which presented respectively low and high score of aggressivity on cv Onward have been used in this study. Strains were raised on PDA medium for 10 days at 21°C. Conidia from 10 days old culture were collected by adding 10 ml of sterile distilled water to dislodge spores. The spores sus-pension was filtered through 2 layers of cheescloch to remove the mycelium and agar fragments. The concentration of spores was determined using a hemacytometer. The conidial suspension was diluted with sterile distilled water to obtain a final concentration required for each experiment.

Leaf wetness period study

Two weeks old plants of cv Onward and cv Merveille de Kelvedon were sprayed to run -off with a conidial suspension of 4×10^6 spores/ml. The pea seedlings were then subjected to a leaf wetness duration (LWD) of 24, 48 and 72 h. During the wet period, plants were covered with clear polyethylene bags sprayed inside with distilled water to allow infection. The unbagged plants

were considered as unexposed to a wet period. At the end of this period, seedlings were uncovered and kept in uncontrolled glasshouse were temperature ranged from 15 to 25°C.

Inoculum concentration and plant age study

Inoculum concentration effect was investigated on fifteen days old (three and five leaf stage) pea plants of cv Onward. Plants were inoculated by spraying to runoff with spore suspension containing 2.5×10^3, 3.5×10^5 and 5.2×10^7 spores/ml. Suspensions were applied with a spray atomizer with an adjustable nozzle to form a high density of fine droplets on the aerial parts of the plants. The plants were covered for 48 h with clear polyethylene bags immediately after inoculation and sprayed inside with distilled water to allow infection. After incubation period, the plants were uncovered and kept in uncontrolled glasshouse where temperature ranged from 15 to 25°C.

Statistical analysis and modeling *Kaplan-*

Meier estimate of survival function

The latent period which is defined as the period from host inoculation to the onset of the first pycnidia onset on leaves was referred to as survival data. To estimate the latent period (LP), plants were observed daily from the time of inoculation up to 20 days. The disease assessment of *M. pinodes* infection on the leaves was also recorded using a 0 - 5 disease severity (DS) scale according to Tivoli (1999), where 0, no lesion; 1, a few scattered flecks; 2, numerous flecks; 3, 10 - 15% leaf area necrotic and presence of flecks; 4, 50% of leaf area covered by lesions; 5, 75 - 100% of leaf area dehydrated or necrotic.

Pycnidia on leaves were recorded with the aid of a hand lens (10 x magnifications). Our survival data are censored because many individuals did not presented the event at the end of the observation, and interval-censored as survival time is only known to be between two observation times.

In this study, the data set contained multiple censored observations (that is, plant that did not presented symptoms by the end of the assessment period and those with symptoms but did not form pycnidia structure), the dependent variables is hence considered as a “survival time” (Esker et al., 2006. Scherm and Ojiambo, 2004; Garrett et al., 2004; Padovan and Gibb, 2001) . In fact, the survivor function S(t) measures the probability that an individual will survive beyond time t: S(t) = P[T > t]. Let T represent survival time. We regard T as a random variable with cumulative distribution function P(t) = Pr(T t) and probability density function p(t) = dP(t)/dt.

The more optimistic survival function S(t) is the complement of the distribution function, S(t) = Pr(T > t) = 1 − P(t). Another representation of the distribution of survival times is the hazard function, which assesses the instantaneous risk of demise at time t, conditional on survival to that time:

$$h(t) = \lim_{\Delta t \to 0} \frac{\Pr\left[(t \le T < t + \Delta t) | T \ge t\right]}{\Delta t}$$

Furthermore, the Kaplan–Meier method was used to estimate the survival rates in a group rate between 2 groups were tested for statistical significance by the log-rank test procedure. A p value 0.05 was considered as statistically significant. The estimator S(t) that was used to calculate non-parametric estimates of the survivor function is:

$$\hat{S}_{(t)} = \prod_{j=1}^{s} \left(1 - \frac{d_j}{n_j}\right)$$

Where dj is the number of individuals that experienced the event in a given interval and nj is the number at risk. Survival curves are monotone non-increasing step functions equal to 1 at time zero, and 0 as time approaches infinity. Statistical differences between survival curves were calculated using the log-rank test. Because this test cannot be calculated for interval-censored data, the mid-point of each interval was used in this case.

Modeling survival time using Cox proportional hazards

Cox regression models use the hazard function to estimate the relative risk of failure. The hazard function, h(t) is an estimate of the potential death per unit time at a particular instant, given that the case has survived until that instant (Kelinbaum, 1996).

The Cox proportional hazard model examines the influence of potential covariates on the hazard of death for an individual (Collett, 2003; Ojiambo et al., 2002; Kleinbaum, 1996; Dungan et al., 2003). The hazard at time *t* is the probability that an individual who has survived to time *t* will die in the next small period of time (Ojiambo et al. 2002, Scherm and Ojiambo, 2004, Muenchow, 1986). Both Kaplan-Meier method and Cox regression model were performed using the SPSS 8.0. and Epi Info (TM) 3.5.1.

RESULTS

Kaplan-Meier estimate of survival function

To test the hypothesis that survival time was affected by different epidemiological parameters we have compared in this study the Kaplan-Meier estimates of survival probabilities using the log rank test.

In fact, a total of 700 peas plants were infected with isolates md0202 and 740 plants were infected with tn0203. In fact, out of 1440 seedlings inoculated 1010 (70, 14%) presented events during the observation period. Moreover, 430 (29.86%) of the total seedlings failed to present event during the investigation period.

Furthermore, our survival data are right censored because many individuals did not produced pycnidia structure at the end of the observation, and interval censored as survival time is only known to be between 2 observations times.

Using the Kaplan-Meier approach, the median time to event (production of pycnidia) for md0202 and tn0203 isolates was 16 and 15 days respectively (Figure 1). Hence, the estimated survival functions were significantly different (P 0.001).

Concerning, the leaf wetness duration (LWD), the median survival was 16 days for both 48 and 72 h of LWD and was of 17 days for the LWD of 24 h (Figure 1). The differences of survival functions were also significantly different (P 0.001).

However, when we examined the pea cultivar, the estimated survival functions based on Kaplan-Meier estimates by both log-rank and Wilcoxon test no significant differences neither between cultivars nor for the 2 plant ages was observed (Table 1).

Also, Kaplan–Meier survival analysis resulted in no statistically viable differences between the plant ages of

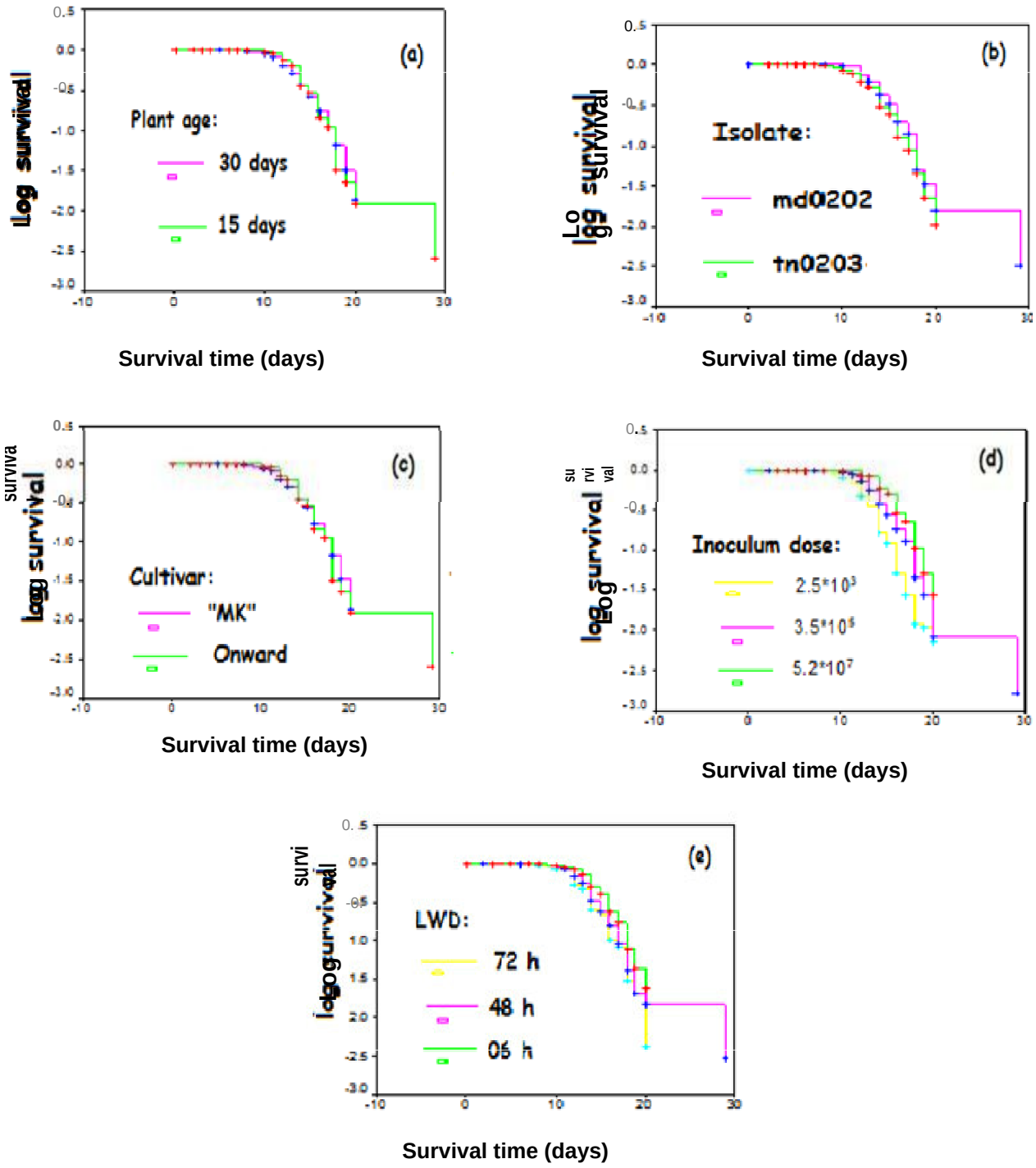

Figure 1. Kaplan-Meier survival curves of five parameters: (a) plant age, (b) isolates, (c) cultivar, (d) inoculum concentration and (e) leaf wetness duration (LWD). Survival functions were based on Kaplan-Meier estimates by log rank test.

inoculation (p 0.766) (Table 1) . Whereas, significant higher differences were observed between the inoculum doses than would be expected to occur by chance (p 0.0001). Moreover, Using the Kaplan-Meier approach, the median time to event was 18, 16 and 14 days for 2.5×10^3, 3.5×10^5 and 5.2×10^7, respectively.

Cox's proportional hazards model

The log rank test is used to test whether there is a difference between the survival times of different groups but it does not allow other explanatory variables to be taken into account. Cox's proportional hazards model is

Table 1. Survival analysis of different parameters based on Kaplan-Meier estimates by both log-rank and Wilcoxon test.

Parameters	Log-rank test		Wilcoxon test	
	Statistics	P-value	Statistics	P-value
Isolates	7.9383	0.0048	13.3705	0.0003
Cultivar	0.0882	0.766	0.0637	0.800
Inoculum dose	94.792	0.0001	138.6983	0.0001
Leaf wetness duration	27.5871	0.0001	36.3935	0.0001
Plant age	0.0658	0.7975	0.0866	0.7686

Table 2. Estimates of covariates in Cox regression.

Parameter		S. E.	Wald	Z	P-value	R	Exp( )	95% CI for Exp(B)	
								Lower	Upper
Cultivar	-1.3944	1.0042	1.9280	-1.3885	0.1650	0.0000	0.2480	0.0346	1.7751
Plant age	0.0891	0.0669	1.7720	1.3312	0.1831	0.0000	1.0932	0.9588	1.2464
Isolate	-0.1615	0.0631	6.5633	-2.5619	0.0104	-0.0186	0.8508	0.7519	0.9628
Leaf wetness duration	0.0265	0.0011	27.7490	5.2677	0.0000	0.0441	1.2056	1.303	1.0077
Inoculum concentration	0.3530	0.0393	80.7356	8.9853	0.0000	0.0772	1.4233	1.317	1.5372

analogous to a multiple regression model and enables the difference between survival times of particular groups of to be tested while allowing for other factors. Based on the examination of the effect of different covariates (isolate aggressiveness, plant age, inoculum dose, leaf wetness duration, pea cultivar) on the risk of reducing the latent period. This model had indicated that among the covariates tested, three had affected the latent period with high risk. Hence, thus risk of LWD was estimated to 1,205. Aslo, the estimated hazard of the inoculum concentration was 1,423 (Table 2).

DISCUSSION

This present study was carried out to investigate the effects of the inoculum concentrations, periods of leaf wetness and plant age and pea cultivar on latent period component. These experiments suggest that the latent period was influenced by three of the investigated factors namely the inoculum concentration and the leaf wetness. Scott et al. (1995) and Rogger et al. (1999) show that some factors must be responsible for optimal latent period in the field.

In fact, previous investigations focused on the effect of factors such as inoculum concentration (Bouznad, 1988; Maufras, 1996; Raynal, 1997; Tivoli, 1999) moisture and plant cultivars (Shaner, 1981; Van Ginkel et Scharen, 1988) on disease aggressiveness. Furthermore, Tivoli et al. (2007) suggest the exploitation of the moisture parameter for predicting aerial disease infection. Such studies were useful in explaining some of the phenomena associated with Mycosphaerella blight on peas.

However, the trend has been for investigators to consider the effect of these factors on the disease magnitude without considering the time frame during which symptoms development occurs. In fact, many authors suggested that the differences between the symptomless and symptomatic infection may be simply depend on when the plants are scored for symptoms (Bishop and Slack, 1987).

Inoculum concentration is an important factor in determining whether plants become infected and whether plants expressed symptoms after inoculation. In our previous investigations, the latent period of *Mycosphaerella* blight was correlated with the inoculum dose and the response of inoculum concentration was modulated by the cultivar and the aggressivity of the isolates (Setti et al., 2008; 2009).

Concerning, the leaf wetness duration, 6 h of LWD were sufficient to initiate the symptoms on leaves on both sensitive and the partial resistant cultivar but the reaction of the two cultivars were similar because the median for both cultivar was 16 days. Moreover, the present study suggests that the latent period is getting more decreased with an increase in the leaf wetness duration. However, in our previous study no further increase in DS was observed with a leaf wetness period beyond 48 h of LWD. In fact, most foliar fungi require the presence of leaf wetness for the infection process. However, the duration varies from one group of fungi to another (Gilles et al., 2000; Trapero-Casas and Kaiser, 1992, Pederson and Morrel, 1994). In many previous studies, latent period

was obtained with at least 48 h of LWD (Skew et al., 1988; Scott et al. 1995; Rogger et al. 1999; Davis and Fitt, 1994).

Although, these results suggest that the LP is shortest at 48 and 72 h of LWD and longest in the absence of LWD or at 6 h LWD on both the susceptible and partial resistant cultivar. Our findings support the notion of latent period. Hence, the survival times were longer in low leaf wetness condition than with higher leaf wetness. Pycnidia formation steadily decreased with reduction of period of leaf wetness. By contrast, the plant age seems to have no significant effect on the survival time.

The survival times was longer with isolate tn0203 compared to those exposed to md0202. In fact by using the survival analysis we have estimated more accurately the latent period survival times in pea *M. pinodes* pathosystem. Because in many others previous studies, the estimated value of incubation and latent period were based only on plants that presented event but ignore all others plant that did not present the event during the period of observation, the reason why this value might have been biased. In this context, Fox et al. (2001) considered that the censored data must be included in any survival study; otherwise, the survival time might not be correctly estimated.

In fact, the latent period calculated only for infect peas that presented pycnidia structure during the study was estimated to 14.5 ± 2.98 days (median = 15). Because the infectious plants that did not presented any pycnidia structure were not incorporated in this analysis, survival times were underestimated by approximatively one to two days for the md0202 and tn0203.

On the other hand, the median survival calculated using both the Kaplan Meier and Cox regression was approximately of 2 days longer than the survival times that was estimated using only the plants which did present the pycnidia structure at the end of the experiment. Moreover, the estimated hazard obtained with Cox regression model was very important and highly significant for LWD and inoculum concentration 1,205 and 1,423 respectively.

In fact, according to many authors, each survival analysis method has its own strengths and weaknesses. Hence, the Kaplan- Meier approach is an interesting and useful nonparametric exploratory analysis to investigate parameters that might be important in influencing the survival probability of an infected plant (Esker et al. 2006). On the other hand, the Cox proportional hazard models are useful when there is no prior information regarding the statistical distribution to select, or if we are interested to determine the chance of failure induced by each parameter (Esker et al., 2006; Muenchow, 1986; Scherm and Ojiambo, 2004).

Moreover, we have shown that among the risk factors that we have hypothesized, the inoclum dose, leaf wetness and isolates have influenced the survival times in *M. pinodes* infected peas. Indeed, the results of the present study highlight the importance of the environmental data on the latent period. This could be of great interest particularly in developing predictive models for *Mycosphaerella* blight on peas that can be used in any pest management program.

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