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Survival analysis for estimating risk factors in incubation period of *Didymella pinodes* on pea (*Pisum sativum* L.)

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Ascochyta blight caused by *Didymella pinodes* (Berk. et Blox.) Vestergr. is one of the most devastating diseases, causing severe damage in pea. A new statistical approach based on factor risk using non-parametric and semi parametric survival analysis was used in this study. Different hypotheses dealing with factors that might influence the incubation period were tested. Survival analysis using Kaplan-Meier estimates and Coxproportional hazards was performed for data analysis. During these investigations, incubation period was regressed against leaf wetness duration (LWD), inoculum concentration, plant age and isolate's aggressiveness. The non-parametric Kaplan-Meier test had shown the importance of leaf wetness duration, inoculum concentration and plant age in the survival curve for the incubation period. Thus, the lowest median incubation period was obtained under the LWD of 72 h. This was 9.0 days (95% CI 8,402-9,598 days). On the other hand, the highest inoculum concentration induced the shortest incubation period with a median value of 9.0 days (95% CI 7,772-9,531 days). Likewise, using the semi parametric Cox proportional hazard regression, only two covariates (leaf wetness, inoculum dose) were associated with survival time with an hazard ratio of 1.144 p=0.03) and 1.015 (p<0.0001). Moreover, neither the plant age inoculation nor the isolate presented a significant hazard ratio for the best fit of the model.

Key words: *Pisum sativum, Ascochyta blight*, Cox regression, *Didymella pinodes.*

INTRODUCTION

The Ascochyta blight caused by *Didymella pinodes* (Berk. et Blox.) Vestergr. is one of the most destructive pathogens of pea (Moussart et al., 1998; Chilvers et al., 2009; Le May et al., 2012). It is wide spread throughout the major pea-growing areas worldwide (Wallen, 1965; Lawyer, 1984; Bouznad, 1988; Bretag et al., 2006).

In recent years, the incidence of Ascochyta blight was observed in different production areas in Algeria, which has led to increased yield loss (Setti et al., 2008). This could be due to an increased pathogenicity of the

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pathogen population or a greater inoculum pressure. Numerous previous studies have shown the key role of the incubation period on epidemics of Ascochyta blight (Tivoli et al., 1999; Roger et al., 1999; Turechek, 2004; Tivoli and Banniza, 2007). On the other hand, Shaner (1981), and Van Ginkel and Scharen (1988) suggested the use of moisture parameters for predicting aerial disease infection. Similarly, Fitt et al. (1998) and Huber et Gillespie (1992) already noted the impact of free water on leaf surface on the incubation and latent period. Furthermore, Tivoli et al. (1999), Roger et al. (1999) and Turechek (2004) considered that a parameter such as the inoculum concentration may have a great impact on the life cycle of *Didymella pinodes* (*D. pinodes*) and hence may determine all the components of disease, including the incubation period. Gibb et al. (1998) suggested that both the incubation and latent period might be influenced by plant age and the isolate's virulence. Many previous studies determined and quantified the latent period in different plant pathogens' interaction (Trapero-Casas and Kaiser, 1992; Pederson and Morrall, 1994; Wallen and Galway, 1977) but little is known about the duration of the incubation period for *D. pinodes* in pea. In our previous study, the incubation period values were calculated only for plants that were symptomatic during the study period, whereas a number of inoculated pea plants had not presented any symptoms at the time when the final disease symptom was recorded. The plants that did not present symptoms during the frame of the study are referred to as "censored data". Survival analysis is a powerful class of nonparametric and semi parametrics tool, especially designed for such data. Survival analysis involves the timing of events (such as infection, germination, pycnidia production, symptoms appareance) while allowing censored observations (Hosmer and Lemeshow, 1999; Klein and Moeschberger, 2003). This type of analysis has a long history in statistical research and practice, particularly in medical studies. Moreover, this statistical method is also used in several others disciplines, and referred to with others names such as event history analysis and failure time in sociology and industry, respectively. Recently, the use of the survival analysis has become a widespread tool for resolving more complicated data in ecological studies such as biodiversity and environmental 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 (until the appearance of the first symptom of disease; appearance ofpycnidia structure and spore germination, etc.). Survival analysis is an interesting method that enables the introduction of censored data in the analysis. In the pea-*D.pinodes* pathosystem, this may permit the inclusion of infected plant that did not present symptoms during the period of study. Hence, this method will help us to obtain a more realistic estimation of incubation period. Such data might have a great importance in the estimation of cultivar resistance, and also in each biotic factor in any plant pathogen's interaction. This information may play a key role for the comprehension of the epidemic structure of the pathogen. In fact, in our previous study, the influence of abiotic parameters was approached, taking into account only the mean and variance analysis (Setti et al., 2008). This is the reason why we tried in this study to introduce a survival analysis approach, which is considered as a robut statistical tooltodeterminate accurately the incubation time length. In fact, the comprehension of this period could have an important consequences on the epidemic development due to the seasonal spore accumulation, given the polycyclicnature of the pathogen (Motisi et al., 2013 ; Leclerc et al., 2014). Moreover, empirical data on the incubation period of numerous plant pathogens are rarely available (*Motisi* et al., 2013). Therefore, the objectives of this study were to investigate the use of survival methods to estimate the incubation period of the *Mycospherella* spp. that infects pea and to assess the effect of four factors on time for the appearance of the first symptom of disease using the Cox semi parametric analysis: (i) isolates' aggressiveness, (ii) leafwetness duration, (iii) inoculum concentration, (iv) and plant age.

MATERIALS AND METHODS

Plant and fungal material

Two *Didymella pinodes* isolates (md0203 and tn0203) that present different degree of aggressivness were used in this study. Isolates were grown on PDA medium for 10 days at 21°C. Conidia from 10 days old culture were collected by adding 10 ml of sterile deionised water to dislodge spores. The concentration of spores was determined using a haemocytometer. The conidial suspension was diluted with sterile deionised water to obtain a final concentration required for each experiment. The cv 'Merveille de Kelvedon', one of the most cultivated cultivars which is considered as moderately resistant to Asochyta blight was used in this study. Seedswere sown in pots containing unsterilized soil/compost mixture. Fifteen seeds were planted per pot and seedlings were thinned to ten. The plants were maintained in glasshouse.

Effect of inoculum concentration, leaf wetness, and plant age on incubation period

Inoculum concentration effect was investigated on 15- and 30- day old plants of cv 'Merveille de Kelvedon'. Plants were inoculated by spraying to runoff with spore suspension. Three inoculum concentrations (IC) were assessed namely $3x10^3$, $5x10^5$, $7x10^7$ 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. For the investigation of the leaf wetness (LWD) effect, the pea seedlings weresubjected to LWD of 6, 48 and 72 h. Plants were covered with clear polyethylene bags immediately after inoculation and sprayed inside with deionised water to facilitate infection. The plants were thenuncoveredat each LWD, and kept in uncontrolled glasshouse where temperature ranged from 15 to 25°C.

Risk factor analysis methods

Univariate analysis using Kaplan-Meier estimator

Incubation period is defined as the period from the host inoculation to the onset of the first symptoms referred to as survival data. To estimate the incubation period (IP), plants were observed daily from the time of inoculation up to 10 days, when the experiment was terminated. The Kaplan-Meier method of survival analysis (Kleinbaum, 1996) was used to generate and adjust survival curves using preoperative variables that differed among the treatment groups. The censored observations are plants that did not develop symptoms by the end of the assessment period. 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 dependent variable is hence considered as a "survival time" (Esker et al., 2006. Scherm and Ojiambo, 2004; Garrett et al., 2004; Padovan and Gibb, 2001). Another representation of the distribution of survival times is the hazard function, which assesses the instantaneous risk at time t:

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

Overall survival rates were calculated by the Kaplan–Meier method and the log-rank test was used for differences between survival curves. A P-value of < 0.05 was accepted as statistically significant. Variables were subjected to univariate analysis. The estimator S(t) that was used to calculate non-parametric estimates of the survivor function is:

$$\hat{S}(t) = \prod_{j:t_j<t} \left(\frac{n_j - d_j}{n_j}\right) = \prod_{j:t_j<t} \left(1 - \frac{d_j}{n_j}\right)$$

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 Mantel–Haenszel log-rank test (Rothman and Greenland, 1998).

Multivariate analysis using semi parametric 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).Cox (1972) first introduced his proportional hazards approach as a way to incorporate covariate information into a survival model without having to assume an underlying distributional form for the data. The model is defined in terms of the hazard function as:

$$h(t, \mathbf{X}) = h_0(t)\exp\left(\sum_{i=1}^{p} \beta_i X_i\right)$$

Where, X is the explanatory/predictor variable, and $h_0(t)$ is the unspecified baseline hazard function (that is when X = 0). Here β is a p-vector of parameters. The Cox proportional hazard model examines the influence of potential covariates on the hazard of event 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 the Kaplan-Meier and Cox regression model analyses were performed using the SPSS 17.0.

RESULTS

Kaplan-Meir analysis

Concerning the inoculum concentration, the shortest incubation period (IP) value was seen with an IC of $7x10^7$ with a median value of 8 days (Table 1). The Kaplan–Meier survival curve was statistically significant between the different IC(log-rank = 5,674, P= 0,045). The shortest IP length occurred with the isolate tn0203 at 72 h of LWD. The median value was 8 days (Table 1). Moreover, the IP increased with the decreasing of LWD. The highest IP value was seen at 6 hours of LWD. The IP ranged from 8 to 12.5 days. The Kaplan–Meier survival curve showing a statistically longer differences between the three LWD would be expected to occur by chance (log-rank = 24,88, P < 0,001). Mean and median difference was observed between the isolates for incubation period (Table 2). The median incubation period was estimated using Kaplan-Meier probabilities of developing disease (Figure 1). The median values were respectively12.0 (sd: 0,384) (95% CI 9,939–12,442 days) and 10.39 days (sd: 0,203) (95% (CI 9,531–10,469) for tn0203 and md0202 (Table 1, Figure 1). However, the Kaplan–Meier survival curve showed a statistically non significant survival time between the two isolates (log-rank = 1,163, P = 0,064).

The survival analysis of the incubation time revealed that IP increased with age of the inoculated plants. Hence, the median value of the lowest IP was obtained with the inoculated 15 days old plants. This was 9.780 days (SE:0,420) (95% CI (8,329 – 9,671 days). The estimation of the survival function with the Kaplan Meier estimator has revealed however non significant differences between the IP of the two inoculated plants' ages (Log-rank =24. 88, P= 0,055). However, compared with the Wilcoxon test, the IP has revealed differences between the two plants' ages (P=0. 043) (Table 2).

The Cox's proportional hazards model

The survival analysis estimators such as the log rank test and the Wilcoxon test are used to compare between groups (Figure 1) for one parameter without taking into account the other explanatory variables. This is the reason why it is of great importance for such analysis to apply the semi parametric model known as the Cox's

Table 1. Medians for survival time of incubation period of *Didymella pionodes.*

Parameters		Median		
		Estimate	SD	95CI
Isolate	md0203	12,000	0.384	(9,939; 12,442)
	tn0203	10,397	0.203	(9,531; 10,469)
Inoculum concentration (spores/ml)	$03*10^3$	10,000	0.467	(9,635; 10,915)
	$5*10^5$	10,000	0.239	(9,085; 10,102)
	$7*10^7$	8,000	0.439	(7,772; 9,531)
Plant age (days)	15	9,780	0.420	(8,329; 9,671)
	30	10,000	0.239	(9,531; 10,469)
LWD (h)	6	12,551	0.305	(9,125; 10,002)
	48	10,117	0.322	(9,349; 10,902)
	72	8,000	0.305	(7,402; 9,598)

SE, Standard error; CI, 95% Confidence interval.

Table 2. Kaplan Meier survival estimator of the four parameters.

Parameters	Log rank test		Wilcoxon test	
	Statistics	P value	Statistics	P value
Isolate	1.163	0.064	0.075	0.510
Inoculum concentration	5.674	0.045	6.703	0.035
Plant age	3.831	0.055	4.114	0.043
Leaf wetness duration (LWD)	24.88	0.000	19.01	0.000

proportional hazards that takes into account all the parameters at the same time. Based on the examination of the effect of different covariates on the risk of reducing the IP length, the hazard was estimated for plant age (β= -0,091), IC(β=0,13), LWD(β=0,015), and isolate (β=0,152). This model had indicated that among the covariates tested, two had affected the incubation period with high risk (Table 3).

The overall best fit for the influences of abiotic parameters on the IP time length was provided by a model that included only the inoculum concentration and the leaf wetness duration. Neither the plant age inoculation nor isolates' aggressiveness was significant in this model (Table 3).The IC had an estimated hazard ratio of 1,144 (P=0.034), indicating their influence on the appearance of disease symptoms. On the other hand, the LWD had an estimated hazard ratio of 1,015 (P<0.0001), indicating the importance of this explanatory variable in the Cox regression model.

DISCUSSION

The present investigation examined the incubation period using survival functions for isolates' aggressiveness, plant age inoculation, LWD and inoculum concentration. They were estimated by non-parametric method of Kaplan-Meier and compared by the logrank test and semi parametric techniques, using the Cox regression model. The infection cycle is mainly the period of infection during which the pathogen enters and infects the host, the period of incubation that follows infection, and ends with the appearance of symptoms. In this study, survival analysis of the data has shown that three of the parameters tested were associated with the incubation period lengths when the nonparametric survival analysis was performed. This is in agreement with other studies which suggested that short latent and incubation period length were observed with increase in both inoculum concentration and leaf wetness duration (Scott et al., 1985; Roger et al., 1999).

The Cox semi-parametric model permits evaluation of the effects of all the studied parameters at the same time. The best fit was obtained with only two parameters, LWD and IC when estimated usingthe Log rank test and Wilcoxon test. This confirms the importance of LWD in the infection process due to the estimated hazard ratio which was relatively high (1,015). The LWD in general is an important factor that enables the numerous fungal

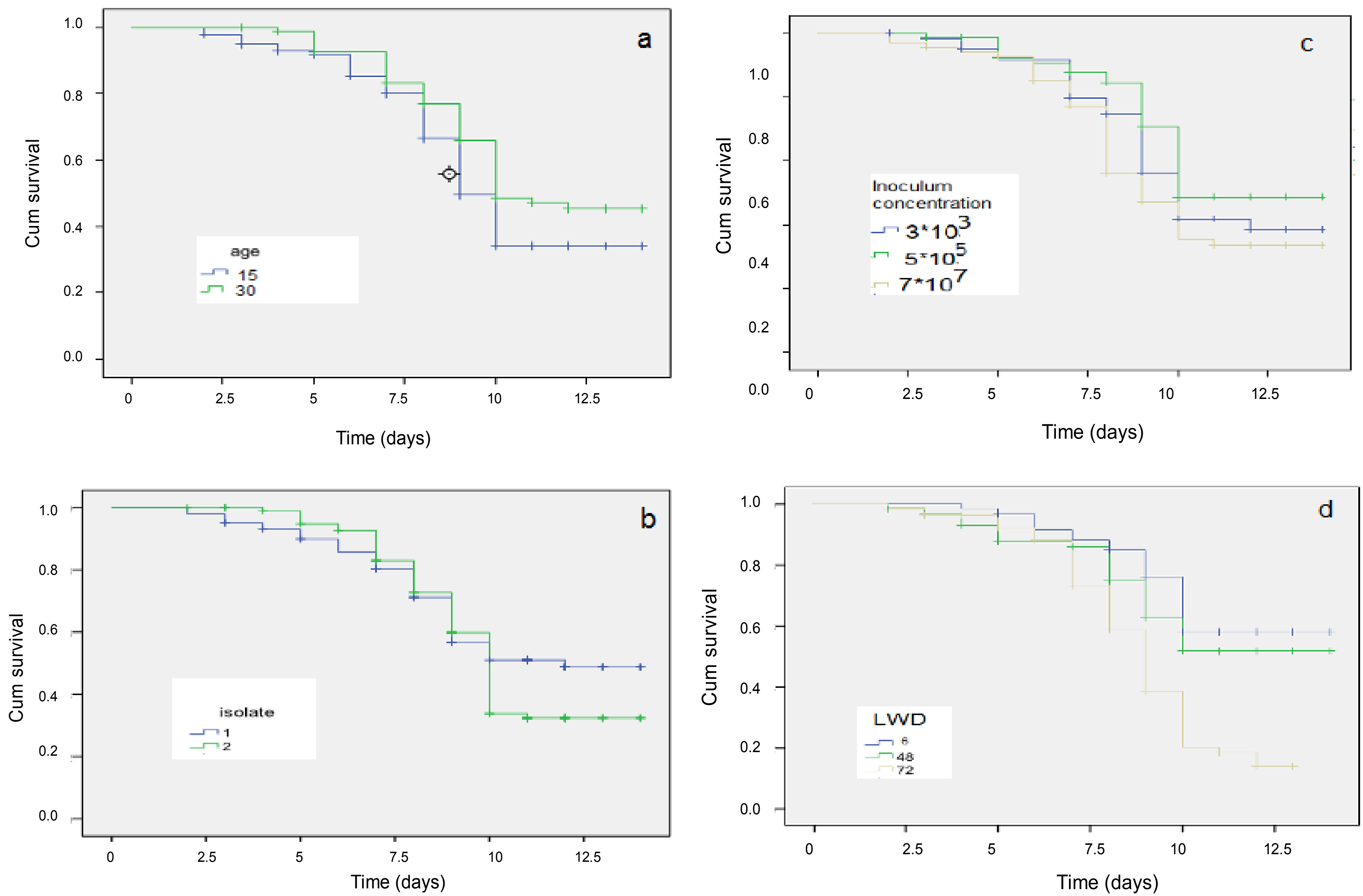

Figure 1. Kaplan-Meier survival for the incubation period of *D. pinodes* on cv 'Merveille de Kelvedon'. Effect of a) plant age, b) isolates, c) inoculum concentration and d) LWD.

Table 3. Results of Cox models for the incubation period length of Ascochyta blight on pea caused by *Didymella pinodes*.

Explanotory variable	β	Exp(β)	SE	Sig.	95.0% CI	
					Lower	Upper
Age	-0.091	0.913	0.041	0.78	0.843	0.989
Inoculum concentration (IC)	0.13	1.144	0.130	0.03	0.887	1.474
Leaf wetness duration(LWD)	0.015	1.015	0.004	0.000	1.008	1.023
isolate	0.152	1.164	0.202	0.65	0.784	1.729

plant pathogens, particularly those infecting F the aerial parts of plants. Weather moisture is frequently used as an indicator of the likelihood of an epidemic (Royle and Butler, 1986). Most foliar fungi can infect the leaves of a plant only while the leaves are wet. The optimal wetness, however, varies depending on the specific pathogen (Trapero-Casas and Kaiser, 1992; Pederson and Morrall, 1994; Gilles et al., 2000). Many previous studies have reported that severe disease was obtained with a LWD of at least 48 h (Shew et al., 1988; Davis and Fitt, 1994; Scott et al., 1985; Roger et al., 1999). Roger et al. (1999) and Setti et al. (2008; 2009) have observed a positive correlation between IC and incubation and latent period and also between the IC and the disease severity for *D. pinodes*. In our experiment, the estimated hazard ration for the IC was 1,144. Such a positive correlation between IC and disease severity was also demonstrated for other Didymella spp. (Scott et al., 1985; Shew et al., 1988; Setti et al., 2010). According to Pederson and Morrall (1994), both the incubation period and the latent period are strongly affected by the IC.

Concerning the isolate effect, non significant differences were observed between the two isolates on the cv 'Merveille de Kelvedon'. The lack of differences could be explained partly by the behavior of this cultivar towards the Ascochyta blight. In fact, the cv 'Merveille de Kelvedon' is considered as moderately resistant against the Ascochyta blight and consequently the effect could be reduced. On the other hand, using the Kaplan Meier estimator namely the Wilcoxon test, our study has also determined the influence of the plant age and the survival curve of the incubation period; however, no evidence of this influence was seen and consequently, the plant age does not appear to best fit the cox regression model. The present study highlights the importance of the incubation period as one of the components of plant disease resistance that can reduce the rate at which disease epidemics develop. Moreover, other important components of resistance must be studied to limit the epidemic propagation of the pathogen such as germination and infection efficiency, and rate and duration of spore production. The integration of the incubation period and latent period associated with other epidemic components is of great importance in disease forecasting systems, especially in systems.

Conflict of interest

The authors did not declare any conflict of interest.

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