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Studies on the infestation by *Eutypa lata* of grapevine spring wounds

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Summary

Natural infestation and controlled inoculations of grapevine (mostly on the cultivar 'Cabernet Sauvignon'), were conducted in Bordeaux vineyards from 1998 to 2004 to evaluate the susceptibility to *E. lata* of spring wounds caused by the removal of either excess buds or excess suckers. Natural infestation was assessed across a range of sites to quantify and compare the relative risk of spring and winter pruning wounds to contamination by *E. lata*. Infestation caused by inoculation of wounds was examined in one site using either 100 (lower inoculum density) or 1000 (higher inoculum density) ascospores per wound. Wounds were allowed to incubate at the wound site for either two weeks or one year before isolations. For natural colonisation of wounds, a low level of infestation of spring wounds (average 2.1 %) was detected, less than those for winter pruning wounds (average 13 %). A similar trend was detected in trials involving inoculation of spring and winter pruning wounds despite infestation with identical levels of inoculum. No difference in recovery rates of *E. lata* was found between spring wounds caused by the removal of buds or suckers. A longer incubation period significantly increased the mean efficiency of recovery. We conclude that spring wounds may pose a significant risk to the colonisation of grapevine by the pathogen *E. lata*, albeit less than that of winter pruning wounds, suggesting a secondary role in the epidemiology of Eutypa dieback.

K e y w o r d s : *Botryosphaeria*, Eutypa dieback, incubation, inoculum dose, pruning wound, susceptibility.

Introduction

Eutypa lata (Pers.: Fr.) Tul. & C. Tul. (synonym *E. armeniacae* Hansf. and M. V. Carter; anamorph *Libertella blepharis* A. L. Smith) is a fungal wound pathogen that causes Eutypa dieback on grapevine (CARTER 1991, PAILLASSA *et al.* 1992, PÉROS 1995, DUBOS 1996, LECOMTE *et al.* 2003, LECOMTE *et al.* 2004, 2005) and infects a wide range of perennial plants (BOLAY and CARTER 1985, CARTER 1991). This wood disease, also known as "dying arm disease", leads to significant decline or yield reductions in the temperate and humid areas devoted to grapevine production in both hemispheres (CARTER 1991, MUNKVOLD *et al.* 1994, CREASER and WICKS 2000). Vineyard longevity is reduced and wine quality may also be spoiled due to uneven berry maturation (WICKS and DAVIES 1999) or by affecting aroma (DUBOURDIEU and DARRIET 1994).

As for all epidemics, the risk of disease depends on seasonal changes in the abundance of susceptible hosts, the presence of inoculum and the conduciveness of the environment. For grapevine, wood is particularly susceptible after wounding or pruning, when the xylem is exposed to infection by ascospores (ENGLISH and DAVIS 1978, MOLLER and KASIMATIS 1978). Pruning occurs mainly in the winter when canes are removed, and in the spring to remove suckers from the trunk and excess shoots. Pruning is occasionally practiced in summer to remove secondary, lateral shoots or some grape bunches (vendanges vertes). For *E. lata*, inoculum takes the form of ascospores released from mature perithecia following periods of rain exceeding 0.5 mm per day (CARTER 1957, RAMOS *et al.* 1975, PETZOLDT *et al.* 1983, PAILLASSA 1992). Because of the frequency of rainfall and the availability of mature perithecia, spore release is highest during the winter period (RAMOS *et al.* 1975, PETZOLDT *et al.* 1982). However, ascospore release is still possible in the spring (PAILLASSA 1992). *E. lata* (formerly *E. armeniacae*) was first diagnosed as the pathogen responsible for dieback of apricot. Pruning wound susceptibility to infection by *E. lata* under various climatic conditions has been studied in apricot (CARTER 1957, 1960, CARTER and MOLLER 1970, RAMOS *et al.* 1975, CARTER 1991), as well as in grapevine (MOLLER and KASIMATIS 1980, TRESE *et al.* 1980, 1980, PETZOLDT *et al.* 1981, 1982, TRESE *et al.* 1982, MUNKVOLD and MAROIS 1995, CHAPUIS *et al.* 1998). To date, work has focused almost exclusively on the infection of wounds caused by winter pruning. Moreover, whilst differences in the densities of spores released during the winter and spring are known to be a key factor determining differences in pruning wound infection by *E. lata* (Ramos *et al.* 1975, Trese *et al.* 1980, Carter 1991), the role of host susceptibility during these periods has not been examined. In particular, the risk of infection by *E. lata* via wounds caused by spring pruning (removal of suckers, buds or lateral shoots) has been little assessed. Some preliminary trials involving the inoculation of young plants of 'Cabernet Sauvignon' grown in sheltered conditions suggest that this risk does exist (LECOMTE *et al.* 2001).

Wood samples were collected in Bordeaux vineyards between 1999 and 2004 to monitor the *E. lata* colonisation of wounds resulting from either natural infestation or controlled inoculation. Partial results obtained in the vine-

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yard from wounds caused by spring pruning (removal of suckers, buds or lateral shoots) were previously reported (LECOMTE *et al.* 2004, 2005). In this paper, whole data are presented and the putative risk of infection by *E. lata* of spring wounds is compared to that of wounds caused by winter pruning.

Material and Methods

Natural infestation: To examine and compare the risk of spring and winter pruning wounds, naturally infected plants were monitored in the Bordeaux region of France during the period 1999-2004. Previous climatic records, indicated that annual rainfall in this region varied from 740 mm to 1250 mm with a mean value of 975 mm per year. For instance, during May and June, the mean rainfall during the 30 years 1960-1990 was 71.6 mm and 47.6 mm respectively, suggesting spring conditions favourable for the release of ascospores of *E. lata*. Natural colonisation occurring on spring wounds and winter pruning wounds was assessed on a total of 713 and 330 samples respectively, removed at random from grape varieties located at four and three sites respectively selected for having a range of disease severity or inoculum pressure (Tab. 1). In particular, the site at Ladaux (Entre-Deux-Mers) was severely affected by Eutypa dieback. In this vineyard, an assessment made in 2004 indicated that 45 % of vines expressed foliar or wood symptoms and 27 % of vines had already been replaced as a result of the disease.

Controlled inoculations: Inoculation of grapevine wounds was conducted using mature vines (*i.e.* older that 20 years) grown in an experimental vineyard near Bordeaux (Latresne), France, selected for having low levels of natural disease (Tab. 2). Controlled inoculation has the advantage of minimising the effects of variable spore production between the winter and spring periods on the infection process. The cultivar *Vitis vinifera* 'Cabernet Sauvignon', known to be susceptible to *E. lata* (PÉROS 1995), was mostly used throughout the study. One inoculation trial was carried out on the cultivar 'Cabernet Franc' (Experiment 1 - February 1998), a cultivar also known to be susceptible to *E. lata* (DUBOS 1996). In all vineyards, vines had been trained to a bilateral cordon. Trials were conducted on healthy vines with no dead arms or foliar symptoms. The susceptibility of winter pruning wounds was assessed twice (Tab. 2, Experiment 1). In 1998 and 2001, 60 and 25 one-year-old canes respectively from mature vine plants were labelled, pruned and inoculated. During the same period, the relative susceptibility of two types of spring wound, caused by the removal of either suckers (Tab. 2, Experiment 2) or buds (Tab. 2, Experiment 3), was examined and compared to the susceptibility of winter pruning wounds. Wounds were achieved by removing either suckers that emerged directly from the trunk (originating mostly from the base) and arms (Experiment 2) or excess buds growing on one-year-old canes (Experiment 3). An example of a spring wound, resulting from sucker removal, is provided in Fig. 1. To follow the progress of infestation, spring wounds were examined after

Table 1

Details and results of a field survey to assess the risk (% recovery) to grapevine of spring and winter pruning wounds naturally infested by *E. lata*

Cause of wound	Site	Location, cultivar	Crop year	No. of wounds examined	% of *E. lata* recovery
Winter pruning	1	Latresne, Cabernet Franc	1999	60	18,5
	2	Latresne, Cabernet Sauvignon	2001	20	0
		Latresne, Cabernet Sauvignon	2002	25	4
		Latresne, Cabernet Sauvignon	2003	30	3.33
		Latresne, Cabernet Sauvignon	2004	150	9.33
	3	Ladaux, Cabernet Sauvignon	2004	30	43.33
				Mean :	13.03
Spring pruning	2	Latresne, Cabernet Sauvignon	1999	80	3.75
			2000	100	0
			2001	120	2.50
			2002	78	1.28
		Latresne, Cabernet Sauvignon	2002	123	3.25
			2003	47	0
	3	Ladaux, Cabernet Sauvignon	2004	96	4.16
	4	Latresne, Merlot	2002	28	0
			2003	43	0
				Mean :	2.10

Table 2

Inoculation dates of experiments carried out from 1998 to 2001 to assess the susceptibility of spring and winter pruning wounds under vineyard conditions

Experiment			Inoculation date
No.	Wounding	Incubation	
1	Winter pruning wound	1 year	Feb 17, 1998 Feb 6, 2001
2a	Spring removal of suckers	2 weeks	May 19, 1999 June 2, 1999 May 23, 2000 June 6, 2000
2b	Spring removal of suckers	1 year	May 29, 2000 June 16, 2000 May 18, 2001
3a	Spring removal of buds	2 weeks	June 8, 1999 June 9, 2000 May 18, 2001
3b	Spring removal of buds	1 year	May 25, 2000 June 16, 2000 May 18, 2001 June 8, 2001

Fig. 1: Example of spring wound on the trunk of a 'Cabernet. Sauvignon' vine after sucker removal.

two weeks (Experiments 2a and 3a) or after one year (Experiments 2b and 3b). Between 20 and 30 spring wounds were assessed per treatment.

Culture medium: All isolations were made onto malt-agar medium (MA) composed of 15 g of Cristomalt (Materne, Fruibourg, France) and 20 g of agar-agar (Algorene®, Univar, France) per liter of deionised water. After autoclaving, the medium was supplemented with 50 $\mu g \cdot L^{-1}$ of chloramphenicol to limit the development of bacteria.

Inoculum: Perithecial stroma were collected from 'Cabernet Sauvignon' vines growing in the Bordeaux area. Pieces of stroma (approximately 0.5 cm^2) were immersed in 5 mL of sterile water for 30 min. A 1 mL aliquot of this suspension was collected and diluted in a further 5 mL of sterile water. Spore concentration was estimated using a haemocytometer. Suspensions were diluted with distilled water to provide either 5 or 50 spores per µl and stored at 4 °C prior to use. A high level (greater than 90 %) of ascospore germination rate was confirmed by microscope examination after plating 200 µl of spore suspension onto MA medium and incubating for 1-2 d at 22 °C.

Inoculation: Prior to shoot removal, the area surrounding the wounding zone was disinfected with alcohol. Each wound received a 20-25 µl droplet containing 100 or 1000 ascospores whereas non-inoculated wounds received sterile water.

Sampling and isolations: Wounded material was sampled after one year for natural infestation or after either two weeks Chapuis (1998) or one year (Carter 1991) for controlled inoculations. Spring wound material was removed using a drill and transferred immediately to the laboratory in a plastic bag for assessment. For spring wounds assessed two weeks after inoculation, wound tissues were surface-sterilised by rapid flaming. Ten to twenty wood chips measuring approximately 3 mm x 3mm x 2 mm were aseptically excised from each wound using shears or scissors. For spring wounds assessed after one year incubation, 20-30 wood chips, of approximately the same size, were cut using shears. Before plating, and to limit the development of rapidly growing contaminant or saprophytic fungi, wood chips were disinfected with calcium hypochlorite (1.8 % active chlorine for 10s) and rinsed in sterile distilled water. Plates were incubated at 22 °C with a 12 h-photoperiod and inspected visually for the presence of *E. lata* after 4 to 8 d. Diagnosis was based on morphological comparison of subcultures with a range of reference strains of *E. lata* (Bx1-10, 8D and 8F) used previously (Lecomte *et al.* 2000). For winter pruning wounds, labelled spurs, 10-15 cm in length, were collected the following winter. Bark located under the pruning wound (3-4 cm) was stripped off using a scalpel. A single wood block (about 2 cm long) was cut with shears and surface sterilized by a rapid flaming. The isolation procedure thereafter was identical to that described above for spring wounds.

Data analyses: Proportions of samples naturally infested by *E. lata* were analysed using contingency tables with a chi-squared statistic. Percentages of *E. lata* recovery resulting from inoculation experiments were transformed to normalize distributions (arcsin of percent recovery) and analysed by ANOVA using StatboxPro (version 5.0, GrimmerSoft). Means were compared using the Newman-Keuls range test.

Results

Natural infestation: Natural infestation of grapevine by *E. lata* was highly variable according to the year and the site ranging from 0 % on Merlot in 'Cabernet Sauvignon' in 2001 to 43.3 % on 'Cabernet Sauvignon' in Ladaux in 2003 (Tab. 1). Natural infestation of

spring wounds by *E.lata* was detected although the overall efficiency of recovery of *E. lata* was significantly lower ($\chi^2 = 44.5$, $p < 0.05$) than that for winter pruning wounds with mean rates of recovery of 2.1 % and 13.0 % respectively. The highest rate of recovery from spring wounds (4.2 %) was observed in samples from the Ladaux site, the vineyard with the highest disease incidence (Tab.1). In this vineyard, the highest recovery rate (43.3 %) for winter pruning wounds was recorded. Furthermore, analyses of wounded tissues also revealed the presence of a range of saprotrophic fungi including species commonly associated with grapevine (e.g. *Botryosphaeria* spp., *Alternaria* spp., *Epicoccum* spp., *Penicillium* spp., *Rhizopus* spp.). Among them, *Botryosphaeria* species, described also as pathogens, and specifically *B. obtusa,* were the most common, found at varying levels in 96 % of wounds.

Controlled inoculations: For controlled inoculations (Figs 2 and 3), rates of recovery were generally higher than for natural infestation. Differences due to inoculum level were not always significant. Recovery after two weeks incubation from wounds caused by the removal of suckers (Fig. 2, Exp. 2 a) varied from 4.0 % to 16.0 % at low inoculum density and from 0 % to 20.0 % at high inoculum density with means of 9.8 and 9.6 % respectively. Increasing the incubation period to one year, increased the rates of recovery to 29.7 % and 36.0 % on average (Fig. 2, Exp. 2 b). In this experiment, recovery from wounds varied from 15.0 % to 46.0 % at low inoculum density and from 26.0 % to 52.0 % at high inoculum density. For wounds caused by the removal of buds and examined after two weeks incubation, recovery was only achieved from inoculations involving a high density of spores and only with an average rate of 3.0 % (Fig. 2; Exp 3a). When the incubation period was increased to one year, the percentage of recovery was 16.1 % and 39 % on average for low and high inoculum density respectively (Fig. 2; Exp. 3b). In this experiment, recovery varied from 12.0 % to 31.0 % at low inoculum density and from 23.0 % to 52.0 % at high inoculum density. There was no significant difference in recovery rates of *E. lata* between spring wounds caused by the removal of buds or suckers, but the incubation period significantly affected the mean efficiency of recovery ($\chi^2 = 81.4$ on $1_{d.f.}$, $p < 0.01$). Recovery rates from winter pruning wounds ranged from 35.0 % to 84.0 % at low inoculum density and 87.0 % to 92 % at high inoculum density. The mean infestation of winter pruning wounds ranged from 59.5 % at the lower inoculum density to 89.5 % at the higher inoculum density (Fig. 3, Exp. 1) and was significantly ($\chi^2 = 100.1$, $p < 0.01$) higher than comparable studies of the infestation across both types of spring wounds despite the high level of natural infestation in 1998. Overall, levels of recovery from spring wounds were still approximately half that obtained from winter pruning wounds.

Discussion

In this paper, a survey of naturally infested grapevine wounds, carried out in the Bordeaux area between 1998 and 2004, was combined with controlled inoculations to assess the risk of infestation of spring wounds by the dieback pathogen *E. lata*. Significant levels of infestation of spring wounds, albeit less than that of winter pruning wounds, were detected, confirming that spring wounds

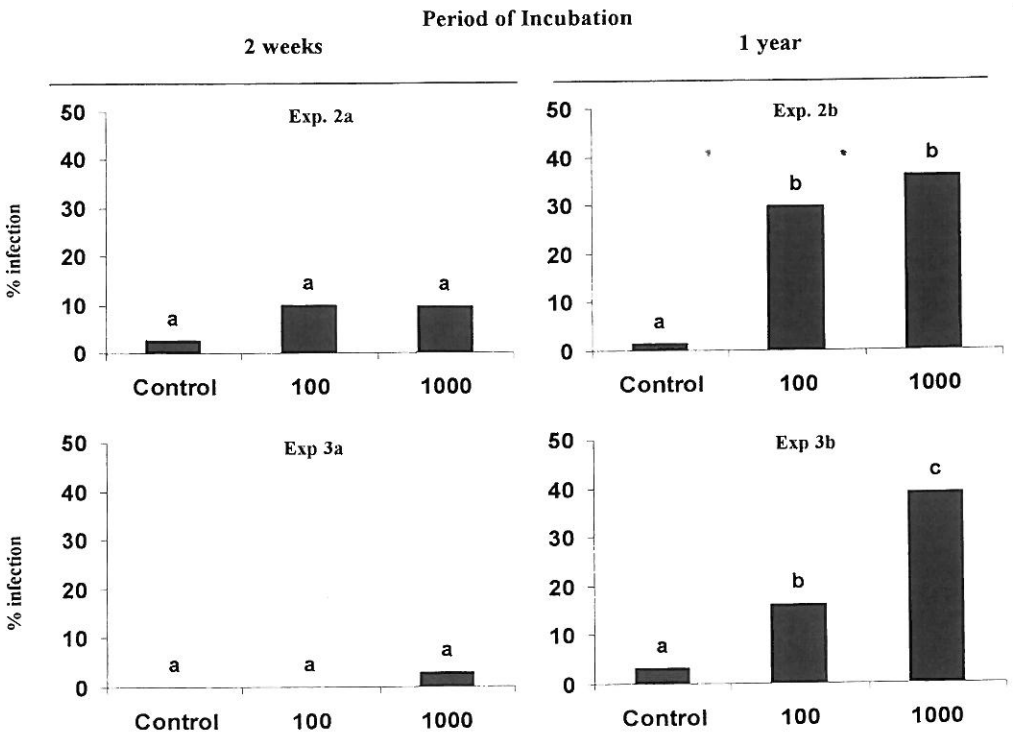

Fig. 2: Efficiency (%) of *E. lata* recovery from grapevine spring wounds inoculated in the vineyard with **0**, **100** and **1000** ascospores (see Tab. 2 for experiment details). Bars with different letters represent means that are significantly different ($P \leq 0.05$).

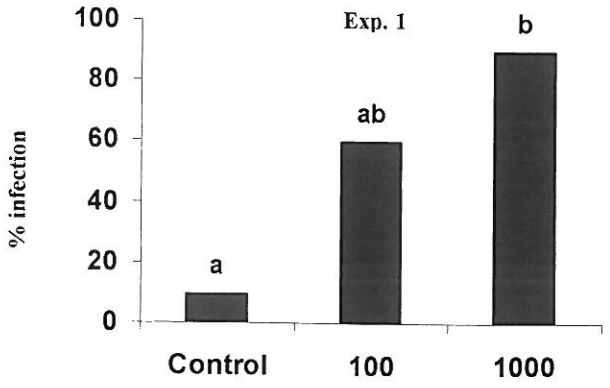

Fig. 3: Efficiency (%) of *E. lata* recovery from grapevine pruning wounds inoculated in the vineyard with **0**, **100** and **1000** ascospores after one year of incubation (see Tab. 2 for experiment details). Bars with different letters represents means that are significantly different ($P \leq 0.05$).

may be sites for infection as reported previously (LECOMTE *et al.* 2004 and 2005).

The difference in susceptibility between spring and winter pruning wounds could be due mostly to differences in environmental conditions that affect (i) the release of ascospores and thus the probability that a spore lands on a wound, (ii) the germinability of spores once they reach the wound which is conditioned by the local environment and (iii) the susceptibility of the wound site to infestation by *E. lata*, conditioned by the host response to wounding and the presence of competing microorganisms.

Considering changes in the presence of inoculum, ascospores can be released during each period of rainfall throughout the year (MOLLER and CARTER 1965, PEARSON 1980, PAILLASSA *et al.* 1992) although seasonal patterns of ascospore production were revealed in California (RAMOS *et al.* 1975) and in Michigan (TRESE *et al.*, 1980). In the Bordeaux area, climatic conditions are generally wetter in winter than in late spring. Consequently, spring wounds may be less exposed to inoculum than winter pruning wounds. Moreover, a concomitant change in the susceptibility of pruning wounds has been shown, decreasing from fall pruning to spring pruning (MUNKVOLD and MAROIS 1995, CHAPUIS *et al.* 1998). For this reason, many authors advise delaying the grapevine pruning until late winter. At this time, sap flow can also limit spore deposition and wounds are less likely to coincide with the release of ascospores (TRESE *et al.* 1980, PETZOLDT *et al.* 1981, DUBOS 2002, DUMOT 2003).

For natural infestation, *E. lata* was only rarely recovered from the wounds examined after either two weeks or at least one year of exposure, suggesting a minor role in the epidemiology of *E. lata*. However, the lack of spring wounds protected by a physical barrier as a control in our study did not allow the distinction between new infections from already established infections either. Therefore, further studies would be necessary to better assess the susceptibility of spring wounds under natural conditions and to examine necrosis development from spring wounds (for example by cutting vine trunks longitudinally).

For controlled inoculations, significant levels of infestation of spring wounds from vines grown under vineyard conditions were detected but again far less than that found for the controlled inoculation of winter pruning wounds (Tab. 2 and Fig. 2). This difference confirms experimentally that, in addition to changes in inoculum level, wound susceptibility plays an important role in the success of *E. lata* as a pathogen of grapevine. This notable change in wound susceptibility may be attributed either directly to the hosts physiology or to the local environment, the latter comprising the local micro-environment at the wound site as well as the mesoclimatic conditions that differ during winter and spring. The declining susceptibility of spring wounds might be related to vessel healing, which may reduce the period of wound exposure, or to exudation following sap flow (MUNKVOLD and MAROIS 1995) which may limit spore deposition thus reducing the likelihood of spores of *E. lata* making direct contact with fresh wound sites. Moreover, in contrast to the senescent status of wood beneath wounds resulting from winter pruning, the wood exposed by spring cutting is physiologically active and most likely able to elicit a strong resistance reaction post-colonisation leading to reduced and perhaps latent infection. Furthermore, spring wounds may also be less susceptible because of the presence of more active competing micro-organisms at the wound site (CHAPUIS *et al.* 1995). Whilst this work was restricted to studying the susceptibility and the early colonisation of grapevine wounds by *E. lata* and not to the fate of this colonisation, it was noted, where mycelia had developed, that the necrotic tissues of spring wounds were relatively superficial, perhaps a consequence of the colonisation of a physiologically active host. Therefore, further studies will be necessary to assess the development of typical Eutypa sector-shaped necrosis from spring wounds (e.g. wood sections to observe initial infections from spring wounds) and to assess their exact role in terms of disease incidence.

The effect of competing microbes, either within the wound site or after the plating of samples onto growth media, may partly explain the differences in the efficiency of recovery from wounds following different incubation periods. Others have also reported that the presence of saprophytic fungi may reduce the success rate of isolating *E. lata* from grapevine (TRESE *et al.* 1980, LECOMTE *et al.* 2003). For plants growing in the vineyard, with mature, thick and moist bark, we would expect a well-developed microflora surrounding the wound. This microflora may be capable of rapid reinvasion of a wound and may represent a significant barrier to early colonisation by the slow growing *E. lata*. The higher rates of recovery following one year of incubation may then be attributed to the extended time required for colonies of *E. lata* to establish itselves.

Among the additional species detected in wound tissues, the presence of *Botryosphaeria* species was noted. These fungi are associated with different wood lesions (PHILLIPS 2002) on many perennial crops. Their high incidence here was attributed to their presence in the bark close to the wound. The exact role of these wood parasites, mostly considered either as saprophytes or endophytes,

and very frequently isolated from necrotic wood, remains to be studied (BERRAF and PÉROS 2005, LECOMTE *et al.* 2006, Péros *et al.* 2008).

Whilst we detected a trend towards an increased efficiency of colonisation of wounds by *E. lata* from a higher inoculum density, the differences were not significant. A similar result was also reported by PETZOLDT *et al.* (1981) and may be due to the very high levels of inoculum we used in this study (perhaps near to the asymptotic levels of maximal infection). The lack of a clearly detectable trend in the rate of recovery of *E. lata* linked to inoculum density may also reflect the inherent variability in successful colonisation by this pathogen. The effect of inoculum dose of *E. lata* on the success of infection has been studied previously on apricot (CARTER and MOLLER 1971, RAMOS *et al.* 1975) as well as for winter pruning wounds of grapevine (TRESE *et al.* 1980, PETZOLDT *et al.* 1981). In most cases, higher rates of infection resulted when wounds were inoculated with higher quantities of ascospores. On apricot, it is assumed that natural deposition in the field is unlikely to exceed about 10 ascospores per wound (CARTER 1991). Hence, it is evident that the density of spores used in our work, either 100 or 1000 per wound as previously used by others, were above that of natural infection and that the levels of susceptibility of grapevine spring wounds detected here by artificial inoculation represent a worst-case scenario.

We conclude that spring wounds pose a risk to grapevine of colonisation by the pathogen *E. lata*, albeit lower than that of winter pruning wounds, indicating a probable secondary role in the epidemiology of *Eutypa* dieback. Although ascospore release has been reported as higher during the winter compared to the spring months, this work suggests that differences in wound susceptibility during these periods, attributed either directly to the host or to the local environment at the wound site, also play an important role in the risk to infection by *E. lata*. These results also suggest the need for a careful strategy of protection during both the winter and spring when pruning should be timed to coincide with minimum release of ascospores. Multivalent fungicide sprays against powdery mildew or downy mildew (that also have activity against *E. lata*) might be also applied shortly after (and not before) spring shoot removal.

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