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RESEARCH PAPERS - 9TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

# Effect of the inoculum dose of three grapevine trunk pathogens on the infection of artificially inoculated pruning wounds

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**Summary.** This study assessed the infection rates of different spore inoculum doses of the grapevine trunk pathogens *Diplodia seriata*, *Phaeomoniella chlamydospora* and *Eutypa lata* following artificial inoculation of pruning wounds. Potted vines of cv. Tempranillo were inoculated with doses ranging from 10 to 4000 conidia per wound of *D. seriata* and *P. chlamydospora* and led to recovery percentages of 10–100% for *D. seriata* and 16–94% for *P. chlamydospora*. *Eutypa lata*, when inoculated onto wounds of vines in a mature vineyard (cv. Shiraz) and on detached canes (cv. Cabernet Sauvignon) with a dose range of 10 to 1000 ascospores per wound, led to recovery percentages of 17–95%. In the field assay, there was no difference in recovery from wounds that were exposed to single or double inoculations with the same total spore dose, or between canes that were harvested 7 or 11 months after inoculation. The results obtained in this study showed significant variability in pathogen recovery between trials, comparable with that reported previously, which suggests that factors such as pathogen virulence, environmental parameters and experimental conditions may influence the infection process. According to this study, in order to obtain optimal recovery percentages of 50–70% for robust evaluation of pruning wound treatments, dose ranges of 100-1000 conidia of *D. seriata*, 100–2000 conidia of *P. chlamydospora*, and 100–500 ascospores of *E. lata* per wound would be required.

**Key words:** *Diplodia seriata*, *Phaeomoniella chlamydospora*, *Eutypa lata*, artificial inoculations, inoculum doses, epidemiology.

## Introduction

Botryosphaeria dieback, Esca and Eutypa dieback are three of the most serious diseases of grapevines (*Vitis vinifera* L.) worldwide. More than 20 Botryosphaeriaceae species have been associated with Botryosphaeria dieback (Úrbez-Torres, 2011). Grapevine disease symptoms caused by these fungi include leaf spots, fruit rots, shoot dieback, bud necrosis and perennial cankers (Luque *et al.*, 2009; Úrbez-Torres, 2011). *Diplodia seriata* (De Not.) is one of the species most frequently associated with dieback and decline symptoms in most wine regions around the world (Úrbez-Torres, 2011). *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams is considered one of the primary causal agents of esca and Petri diseases (Mugnai *et al.*, 1999; Edwards and Pascoe, 2004; Surico *et al.*, 2006). Esca is a disease complex where symptoms and their expression are highly variable (Mugnai *et al.*, 1999; Surico *et al.*, 2006). Most recognised foliar symptoms of esca are characterised by interveinal chlorosis or discolorations that coalesce in large necrotic areas (Surico

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*et al.*, 2006). Symptoms of Petri disease include reduced plant vigour, with retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, and dieback (Gramaje and Armengol, 2011). *Phaeomoniella chlamydospora* is associated with necrotic lesions in the wood of esca and Petri affected vines which include brown spots and streaking in the xylem vessels. Eutypa dieback is caused primarily by the fungus *Eutypa lata* (Pers.) Tul. & C. Tul., which reduces growth and yield in vineyards causing stunted growth of the shoots with short internodes, small, chlorotic and cupped leaves with marginal necrosis and V-shaped necrosis in cross-section of the wood (Sosnowski *et al.*, 2008).

Research studies evaluating pathogenicity, susceptibility of grapevine varieties and efficacy of control methods often rely on artificial inoculation with these pathogens. Different types of artificial inoculation have been routinely used; mycelial agar plugs placed in holes in the internode of stems, and spore suspensions which can be either vacuum-inoculated in grapevine canes, applied by soaking grapevine cuttings or seedlings or placed on wounded tissues. Mycelial agar plugs have been used as inoculum with *D. seriata* (Elena *et al.*, 2015), other Botryosphaeriaceae species (van Niekerk *et al.*, 2004; Amponsah *et al.*, 2011), *P. chlamydospora* and *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai (Gramaje *et al.*, 2013; Mohammadi *et al.*, 2013), *E. lata* (Sosnowski *et al.*, 2007) and a wide range of other trunk pathogens (Úrbez-Torres *et al.*, 2012). Spore suspensions which are vacuum-inoculated into grapevine canes have been used with *P. chlamydospora* and *P. aleophilum* (Gramaje *et al.*, 2009). Alternatively, spore suspensions of *Phaeoacremonium* species have been used in inoculations by soaking grapevine cuttings or seedlings (Aroca and Raposo, 2009). Other studies used spore suspensions as inocula of these fungi in inoculations on pruning wounds to better mimic natural infection by airborne fungal spores. A wide range of conidia or ascospore doses has been reported in scientific literature for inoculation of pruning wounds: lowest to highest, from 1000 to 20000 conidia per wound in studies carried out with *D. seriata* (Kotze *et al.*, 2011; Pitt *et al.*, 2012), from 4000 to 100000 conidia in the case of *P. chlamydospora* (Eskalen *et al.*, 2007; Rolshausen *et al.*, 2010) and from 10 to 20000 ascospores for *E. lata* (Kotze *et al.*, 2011; Ayres *et al.*, 2014). Given the wide range of spore doses used in previous studies, this study aimed to determine the optimal dose range for artificial inoculation of pruning wounds by the grapevine trunk pathogens *D. seriata*, *P. chlamydospora* and *E. lata* under different experimental conditions.

## Materials and methods

### Plant material

Experiments performed with *D. seriata* and *P. chlamydospora* were conducted on 5-year old potted vines of cv. Tempranillo grafted onto Richter 110 rootstock located in Cabrils, Barcelona. Vines were maintained outdoors in 50 L pots filled with a peat: perlite mixture (1:1, v:v) and watered adequately to avoid water stress.

In the case of *E. lata*, two different experiments were carried out. The first one was a field assay conducted on cv. Shiraz vines grafted in 2001 onto Sauvignon Blanc which was planted in 1985 at the Nuriootpa Research Centre in Barossa Valley, South Australia. The second experiment was a detached cane assay (DCA) using canes of cv. Cabernet Sauvignon (clone 337) collected from a 30-year old vineyard grafted onto 101-14 and located in the experimental field of the Institute National de la Recherche Agronomique (INRA), in Bordeaux area (Château Couhins, Cadaujac, France). Canes were stored in a cool room at 5ºC before they were used in the assay.

### Fungal isolates and inoculum production

*Diplodia seriata* CBS121485 and *P. chlamydospora* CBS121483 both collected in 2003 from diseased grapevines, cvs. Cabernet Sauvignon and Carignan, respectively, were used for the potted vine assay. These isolates were previously maintained as mycelial plugs contained in tubes filled with sterile distilled water (SDW) kept at 4ºC. Spore suspension of *D. seriata* was prepared by modifying the method by van Niekerk *et al.* (2005) as follows. A mycelium plug of *D. seriata* was grown for 10 days on potato dextrose agar (PDA, Difco, Becton, Dickinson and Company, Le Point de Claix, France) plates at 25ºC to generate enough mycelia for production of inocula. A mycelium plug of *D. seriata* was grown on water agar (WA, Bacto Agar, Becton, Dickinson and Company) plates with sterile 1 cm fragments of pine needles laid on the medium surface for 4 weeks at

25°C under combined near UV and white fluorescent light (Philips TL-D 18W BLB and Sylvania Standard F18W/33-640-TS cool white, respectively) with a 12 h photoperiod. One day before inoculation, fragments of pine needles (N~40) with *D. seriata* pycnidia were placed in a beaker containing 30 mL SDW. The solution was kept overnight (16 h aprox.) at 4ºC to prevent early germination of conidia and in constant agitation, with the help of a magnetic stirrer, to induce conidia release from the pycnidia. The next day, the suspension was vacuum-filtered through a 60 μm nylon mesh with a Steriflip filter (EMD Millipore Corporation, Billerica, MA, USA) in order to remove debris and produce a cleaner conidial suspension of the fungus. *Phaeomoniella chlamydospora* was grown on PDA plates for 3 weeks at 25ºC in darkness. On the day of inoculation, 10 mL of SDW was added to each plate and the mycelia gently scraped with a sterile cotton stick in order to release conidia. The conidial suspension was recovered from the plate with a pipette.

Inocula of *E. lata* were obtained from natural sources. For the field assay, dead grapevine wood with stromata of the pathogen was collected from a vineyard at the Nuriootpa Research Centre. Ascospore suspensions were obtained using a method adapted from Carter (1991) as follows: wood segments (approximately 3–4 $cm^2$) were soaked for 1 h in distilled water and then attached to polypropylene lids (70 mm diameter), which were screwed onto polycarbonate containers (300 $cm^3$) and left overnight to allow ascospores discharge. The following day, the ascospores were collected by adding approximately 5–10 mL of SDW. In the case of the DCA, *E. lata* perithecial stromata were collected from infected wood parts of 'Cabernet Sauvignon' vines growing in Bordeaux area. Three or four pieces of stroma (approximately 0.5 $cm^2$) were immersed in tubes containing 2 mL SDW and agitated for 30 min with a rotary shaker to encourage ascospore release. The resulting spore suspension was collected.

In all experiments, spore suspensions were stored at 4ºC until inoculation to prevent early spore germination.

## Inoculation procedures

Serial dilutions were performed by adding SDW and using a microscope and haemocytometer to obtain *D. seriata* and *P. chlamydospora* conidial suspensions ranging in concentration from $2.5 \times 10^2$ conidia $mL^{-1}$ to $1 \times 10^5$ conidia $mL^{-1}$. Vines were pruned in January 2011 leaving 4–5 buds per cane. For both pathogens, using a pipette, wounds were inoculated with 40 μL droplets of suspension corresponding to doses of 10, 100, 1000, 2000 and 4000 conidia per wound. A control treatment of 40 μL of SDW was included. The potted vine assay was conducted as a fully randomized design with 20 vines (replications) per pathogen with six canes per vine treated with the different inoculum doses and the control treatment. To prevent natural infection, all of the wounds were sealed with Parafilm. This experiment was repeated in January 2012.

The *E. lata* spore suspensions for the field experiment were prepared by serial dilution to make suspensions ranging in concentration from $5 \times 10^2$ ascospores $mL^{-1}$ to $5 \times 10^4$ ascospores $mL^{-1}$, corresponding to 20 μL water droplets containing 10, 50, 100, 200, 500 and 1000 ascospores, which were applied to wounds with a pipette. Prior to inoculation, 0.05% Tween 20 (BDH Laboratory Supplies, Poole, Dorset, UK) was added as a surfactant to each suspension. Additionally, double-inoculation treatments were included for 200 (100 × 2), 500 (250 × 2) and 1000 (500 × 2) ascospores per wound. On each vine, 10 canes were pruned to two buds on June 2013 with each vine assigned a treatment. The following day, wounds were moistened by spraying with SDW and inoculated. For the double-inoculation treatments, the second inoculation was performed 3 days after pruning. Non-inoculated control treatments were only sprayed with SDW. The field assay was set up as a randomised block design with 10 replications using 100 grapevines.

For the DCA, spore suspensions were prepared in a range from $5 \times 10^2$ ascospores $mL^{-1}$ to $5 \times 10^4$ ascospores $mL^{-1}$ using a microscope and haemocytometer and adding SWD, to provide 10, 50, 100, 200, 500 and 1000 ascospores per 20 μL droplet with which to inoculate the wounds. Canes of 4–5 buds were placed in pots (12.8 $dm^3$) with moistened sand (30 canes per pot). On the day of inoculation, canes were pruned leaving 2–3 buds as previously described (Lecomte *et al.*, 2004). Prior to cutting, the cane surface was sterilized with cotton wool soaked in 96% ethanol. A control treatment was inoculated with SDW. After inoculation, canes were incubated at 15ºC with a 12 h photoperiod. Canes were sprayed with SDW and sand was moistened by a watering can with tap water twice a week throughout the experiment. The DCA was designed as a completely randomized design with 30 replications using seven pots. The experiment was repeated.

In all experiments, inocula viability was assessed by counting germinated spores under a microscope after plating 100 μL of spore suspensions onto PDA for *D. seriata*, *P. chlamydospora* and *E. lata* (field assay) and Malt Agar (MA, Bacto Malt Extract, Becton, Dickinson and Company) for *E. lata* (DCA) and incubating for 24 h at 25°C.

### Isolation procedures

In all experiments, pathogens were reisolated from inoculated canes to determine the relationship between inoculum dose and infection of wounds. In the potted vine assay, reisolations of *D. seriata* and *P. chlamydospora* were performed four months after inoculation. Canes were cut about 20 cm below the pruning wounds and bark was removed with a sterile scalpel from the top 5 cm segment, including the pruning wound. The top 2 mm of the cane was discarded with sterile pruning shears and two further fragments of approximately 5 mm were cut. Fragments were surface-sterilized by soaking in 70% ethanol for 4 min and then placed onto PDA amended with streptomycin sulphate at 50 mg $L^{-1}$. Plates were incubated at 25ºC until fungal colony growth allowed for pathogen identification (Crous and Gams, 2000; Phillips *et al.*, 2007) (3–4 days for *D. seriata* and 4–7 days for *P. chlamydospora*) and recovery percentages were calculated.

For the *E. lata* field assay, treated canes from five replications were randomly selected and removed for assessment 7 months after inoculation, and the other five replications, 11 months after inoculation. In the laboratory, bark was removed from each cane using a sharp knife and then they were surface sterilized by soaking in 2.5% sodium hypochlorite for 10 min and washed two times with SDW. Secateurs were sterilized by dipping blades into ethanol and flaming, and then used to cut wood chips (*ca*. 3 × 2 × 2 mm) from each side of the margin between the stained and apparently healthy wood. For each treated cane, 10 wood chips were randomly selected and plated onto PDA amended with streptomycin sulphate (25 mg $L^{-1}$). Cultures were incubated at 25°C under fluorescent light with a 12 h photoperiod for 7 days and then assessed for the presence of *E. lata* based on culture morphology (Carter, 1991).

The DCA was assessed two weeks after inoculation. Canes were surface-sterilised by rapid flaming with 96% ethanol before and after the bark was removed with a sterile scalpel. Ten 1-mm-thick wood disks per cane were aseptically excised with the help of a cutter as used by Lecomte *et al.* (2004). Wood chips were plated onto MA supplemented with 50 μg $L^{-1}$ of chloramphenicol and placed on the medium, maintaining the order in which they were cut. Plates were incubated at 25ºC in dark conditions and assessed for the presence of *E. lata* as above.

### Statistical analysis

Mean percentage recovery was calculated for each pathogen and inoculum dose in each experiment. All data were subjected to analysis of variance (ANOVA) using statistical procedures in SAS System v 9.2 software (SAS Institute Inc.). Prior to statistical analyses, mean percentage recovery of each pathogen was checked for normality and homoscedasticity criteria and transformed if necessary. The significance of differences among treatments was tested with ANOVA and least significant difference (LSD) test was used to detect differences among the means at the 5% significance level. Regression equations were calculated for recovery percentages of each pathogen in relation to the inoculum doses.

## Results

Germination tests of each pathogen after inoculation showed greater than 90% germination in all cases (*data not shown*), indicating a similarly high viability of inocula in all experiments. Statistical analysis of data from potted vines and DCAs revealed significant differences ($P<0.05$) between repetitions so each experiment was analysed separately. For the field assay with *E. lata*, no significant differences ($P>0.05$) were found between canes removed at 7 and 11 months after inoculation, so all data were analysed together.

In the potted vine assay, neither pathogen was recovered from the non-inoculated controls. When inoculated with doses of 10 to 4000 *D. seriata* conidia per wound, pathogen recovery ranged from 44–100% and 10–100% for the two experiments, respectively (Figure 1). Recovery differed significantly ($P<0.05$) be-

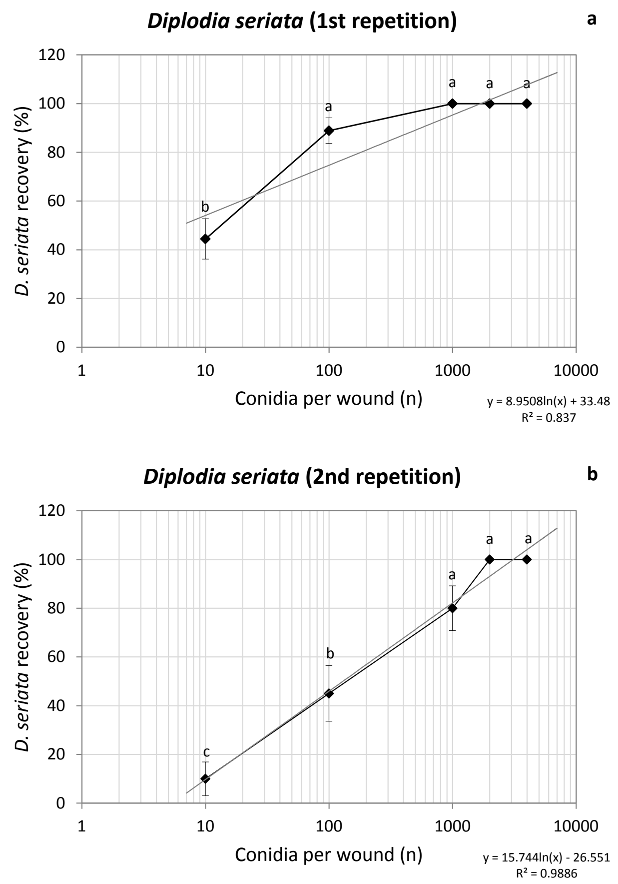


**Figure 1.** Mean percentage recovery from the two potted vine experiments (cv. Tempranillo) inoculated with *Diplodia seriata*. Twenty replications (canes) per pathogen were used for each experiment and two canes per vine allocated with each inoculum dosage of 10, 100, 1000, 2000 or 4000 conidia/wound. Significant differences among means ($P$<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

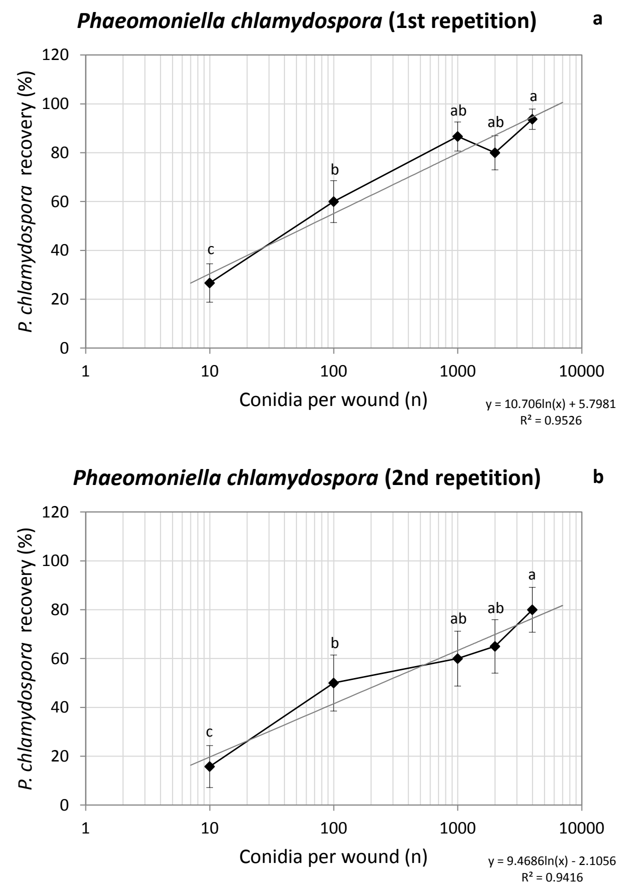


**Figure 2.** Mean percentage recovery from the two potted vine (cv. Tempranillo) experiments inoculated with *Phaeomoniella chlamydospora*. Twenty replications (canes) per pathogen were used for each experiment and two canes per vine allocated with each inoculum dosage of 10, 100, 1000, 2000 or 4000 conidia/wound. Significant differences among means ($P$<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

tween conidia doses up to 100 in the first experiment and up to 1000 in the second experiment. In the case of *P. chlamydospora*, recovery ranged from 27–94% and 16-80% for the series of inoculum doses in the two experiments, respectively (Figure 2). Recovery differed significantly ($P$<0.05) between conidia doses up to 1000 in the first experiment and up to 100, and then between 100 and 4000, in the second experiment.

In the field assay, *E. lata* was recovered from 12% of non-inoculated controls whereas pathogen recovery ranged from 27–95% when inoculation doses ranged between 10 and 1000 ascospores (Figure 3). Recovery differed significantly ($P$<0.05) between conidia doses up to 500, apart from between 100 and 200 ascospore doses. There was no significant difference ($P$>0.05) in recovery between the single and double inoculations.

In the DCA, no *E. lata* was recovered from non-inoculated controls. Recovery of *E. lata* varied from 17–87% and 23–70% in the two DCAs, respectively (Figure 4). Recovery of *E. lata* differed significantly ($P$<0.05) between inoculum doses up to 200 ascospores in the first DCA, and up to 100 ascospores in the second DCA.

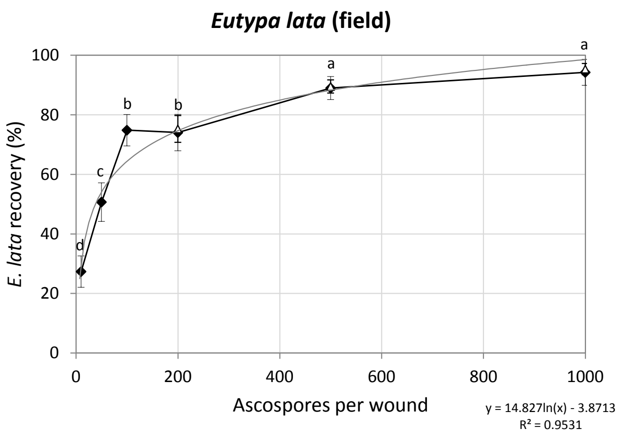


**Figure 3.** Mean percentage recovery from the field experiment (cv. Shiraz) inoculated with *Eutypa lata* at different inoculum dosages of 10, 50, 100, 200, 500 or 1000 ascospores/wound applied once (black rhombus) or twice (2 × 100, 2 × 250 and 2 × 500; white triangles). Ten replications (vines) were used with 10 canes/vine allocated to each inoculum dosage. Significant differences among means ($P$<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

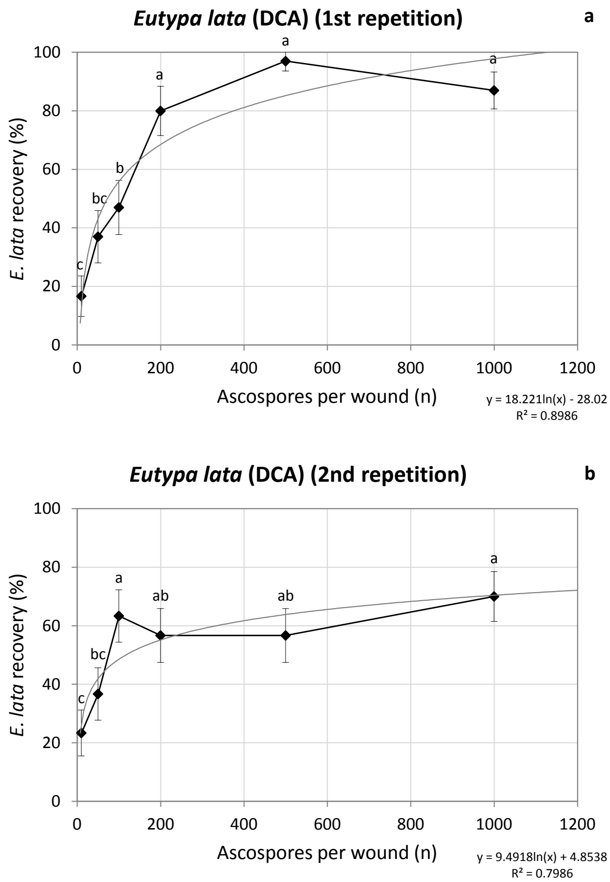


**Figure 4.** Mean percentage recovery from the two DCA experiments (cv. Cabernet Sauvignon) inoculated with *Eutypa lata*. Thirty replications (canes) were used in each experiment for each inoculum dosage of 10, 50, 100, 200, 500 or 1000 ascospores/wound. Significant differences among means ($P$<0.05) are indicated by different letters. Bars correspond to the standard error of the mean.

Regression equation analyses of mean recovery percentages of each pathogen over spore doses inoculated fitted logarithmic models with $R^2$-values between 0.80 to 0.99 (Figures 1-4).

## Discussion

In this study, the recovery percentage of the grapevine pathogens *D. seriata*, *P. chlamydospora* and *E. lata* was evaluated in artificial inoculations of pruned canes using different inoculum doses. Fungal mycelia were recovered from vines at all doses evaluated with significant logarithmic relationships between dose rate and recovery percentage for all three pathogens. Significantly variable results occurred between repeated experiments in this study, which has also been reported in other studies (Table 1).

Recovery of *D. seriata* in this study was similar to that reported in a wound susceptibility study (82%; Rolshausen *et al.*, 2010) and a fungicide evaluation (70-80%; Pitt *et al.*, 2012) when 1000 to 2500 conidia per wound were used. However, Serra *et al.* (2008) recorded a wide range of recovery (41–84%) when the same dose of conidia was used, with variability between repetitions similar to the present study. Furthermore, Bester *et al.* (2007) evaluated different fungicides by inoculating 10000 conidia per wound, and the percentage recovery of the fungus in the non-treated and inoculated wounds did not exceed 40%. However, in that study, inoculations were performed by spraying wounds with conidial suspensions, resulting in less accurate spore dosage compared with inoculating spore suspensions in a droplet. In another study, carried out to evaluate different biological control agents, Kotze *et al.* (2011) obtained 40% recovery of *D. seriata* from an inoculated control treated with 20000 conidia per wound. In this experiment, wounds were inoculated 7 days after pruning, compared with

**Table 1.** Summary of previous experiments using artificial inoculation with various spore dosages of trunk disease pathogens, percentage of recovery, type of experiment, grapevine variety, type of plant material used and location of the experiment.

| Pathogen | Dosage (spore/ wound) | % Recovery | Type of experiment | Grapevine variety | Plant material | Location | Reference |
|---|---|---|---|---|---|---|---|
| *Diplodia seriata* | 1000 | 82% | Wound Susceptibility | Chardonnay and Zinfandel | Field (vines) | California (USA) | Rolshausen *et al.* (2010) |
| | 1000 | 70-80% | Fungicide test | Semillon | Field (vines) | South Australia and New South Wales (AU) | Pitt *et al.* (2012) |
| | 2500 | 41-84% | Wound Susceptibility | Sauvignon Blanc | Field (vines) | Italy | Serra *et al.* (2008) |
| | 10000 | 39% | Fungicides test | Chenin Blanc | Greenhouse (canes) | South Africa | Bester *et al.* (2007) |
| | 20000 | 38% | Biocontrol test | Merlot and Chenin Blanc | Field (vines) | South Africa | Kotze *et al.* (2011) |
| *Phaeomoniella chlamydospora* | 1000 | 58% | Wound Susceptibility | Chardonnay and Zinfandel | Field (vines) | California (USA) | Rolshausen *et al.* (2010) |
| | 3900-4200 | 5-25% | Wound Susceptibility | Cabernet Sauvignon | Field (vines) | France | Larignon and Dubos (2000) |
| | 4000 | 15-51% | Wound Susceptibility | Sauvignon Blanc | Field (vines) | Italy | Serra *et al.* (2008) |
| | 10000 | 20-22% | Wound Susceptibility | Chenin Blanc | Field (vines) | South Africa | van Niekerk *et al.* (2011) |
| | 20000 | 58% | Wound Susceptibility | Cultivated and wild varieties | Greenhouse (potted vines) | California (USA) | Travadon *et al.* (2013) |
| | 20000 | 35% | Biocontrol test | Merlot and Chenin Blanc | Field (vines) | South Africa | Kotze *et al.* (2011) |
| | 100000 | 26-60% | Wound Susceptibility | Thompson Seedless and Cabernet Sauvignon | Field (vines) | California (USA) | Eskalen *et al.* (2007) |
| | 100000 | 94% | Pathogenicity test | Periquita | Field (vines) | South Africa | Halleen *et al.* (2007) |
| *Eutypa lata* | 10-500 | 20-80% | Inoculum dose | Shiraz | Detached cane assay | South Australia (AU) | Ayres *et al.* (2014) |
| | 100-200 | 60-72% | Fungicide test | Cabernet Franc and Cabernet Sauvignon | Field (vines) | France | Lecomte *et al.* (2004) |
| | 100-1000 | 60-90% | Fungicide test | Cabernet Sauvignon | Field (vines) | France | Lecomte and Bailey (2011) |
| | 100-1000 | 30-80% | Wound Susceptibility | Grenache | Field (vines) | California (USA) | Petzoldt *et al.* (1981) |
| | 250 | 14% | Wound Susceptibility | Concord | Field (vines) | Michigan (USA) | Trese *et al.* (1980) |
| | 500-1000 | 29-74% | Fungicide test | Cabernet Sauvignon | Field (vines) | South Australia (AU) | Sosnowski *et al.* (2008) |
| | 500-1000 | 19-79% | Fungicide test | Cabernet Sauvignon | Field (vines) | South Australia (AU) | Sosnowski *et al.* (2013) |
| | 1000 | 48% | Fungicide test | Cabernet Sauvignon | Field (vines) | South Africa | Halleen *et al.* (2010) |
| | 1000 | 28% | Wound Susceptibility | Chardonnay and Zinfandel | Field (vines) | California (USA) | Rolshausen *et al.* (2010) |
| | 1500 | 22-26% | Wound Susceptibility | Merlot | Field (vines) | France | Chapuis *et al.* (1998) |
| | 10000 | 15-20% | Wound Susceptibility | Chenin Blanc | Field (vines) | South Africa | van Niekerk *et al.* (2011) |
| | 20000 | 38% | Biocontrol test | Merlot and Chenin Blanc | Field (vines) | South Africa | Kotze *et al.* (2011) |

1 day in this study, by which time wound susceptibility may have decreased (Úrbez-Torres, 2011).

Previous studies with *P. chlamydospora* (Larignon and Dubos, 2000; Serra *et al.*, 2008; Rolshausen *et al.*, 2010) which used similar spore dose ranges to the current study, reported lower recovery percentages (5–58%) compared with 60–94% obtained in the current study. Rolshausen *et al.* (2010) observed a reduced wound colonization of *P. chlamydospora* when vines were artificially inoculated one week versus one day after pruning. Moreover, when using higher doses of $10^4$–$10^5$ spores per wound, several authors obtained a large range of recovery percentages (Eskalen *et al.*, 2007; Halleen *et al.*, 2007; Kotze *et al.*, 2011; van Niekerk *et al.*, 2011; Travadon *et al.*, 2013), but in general, they were lower than the recovery percentage obtained in the present study (Table 1).

In field and detached cane assays conducted with *E. lata*, the range of recovery rates was similar to those reported in other studies when the same amount of ascospores was inoculated per wound (Petzoldt *et al.*, 1981; Lecomte *et al.*, 2004; Sosnowski *et al.*, 2008; Lecomte and Bailey, 2011; Sosnowski *et al.*, 2013; Ayres *et al.*, 2014). In this study, when 200–500 ascospores per wound were inoculated, 57–97% recovery was obtained, whereas in other studies, infection was lower than 50% at the same or greater inoculum dosage (Trese *et al.*, 1980; Chapuis *et al.*, 1998; Halleen *et al.*, 2010; Rolshausen *et al.*, 2010; Kotze *et al.*, 2011; van Niekerk *et al.*, 2011). These differences in recovery percentages may be due to intraspecific variation in pathogenicity, which has been previously reported for *E. lata* (Sosnowski *et al.*, 2007). Moreover, Chapuis *et al.* (1998) showed that temperature was positively correlated with epiphytic contaminant fungal populations, which may reduce the ability of *E. lata* to infect the pruning wounds and, consequently, also reduce pathogen recovery. Munkvold and Marois (1995) also reported in grapevines a strong positive correlation between temperatures after pruning and the rate of colonisation of pruning wounds by naturally occurring epiphytes which may act as competitors in wound colonisation by *E. lata*.

In the current field assay, double inoculations carried out with 200, 500 and 1000 ascospores did not produce a higher percentage of pathogen recovery, thus showing that single inoculations are sufficient to produce similar infection to double inoculations. These results were consistent with those obtained by Sosnowski *et al.* (2013) where single and double inoculations were used without clear trends of improved recovery. With respect to the incubation period, no differences in *E. lata* recovery were found when canes were harvested at 7 and 11 months after inoculation, indicating that a shorter time of incubation before assessment can be considered, to obtain results earlier in the season. In this study, *E. lata* was recovered from 12% of uninoculated controls in the field experiment, representing the natural disease pressure. The same percentage of natural infection was reported by Sosnowski *et al.* (2013). Luque *et al.* (2014) observed percentages of natural infections from 0.4 to 3.2% in case of *D. seriata* and from 0.4 to 2% for *P. chlamydospora*. The aim of our experiments was to determine optimal spore dose ranges for each pathogen that will produce higher disease pressure than encountered under natural field conditions, in order to provide robust evaluation of treatments (*e.g.* wound protectants) without imposing unrealistically high disease pressure.

In the present and in other studies (Table 1), high variability in pathogen recovery was observed when the same spore doses were applied to wounds. The establishment of a pathogen in grapevines is a result of different factors including (i) environmental parameters (Serra *et al.*, 2008; Sosnowski *et al.*, 2011), (ii) susceptibility of the grapevine variety (Sosnowski *et al.*, 2007; Travadon *et al.* 2013), (iii) age of the plant tissue (Trese *et al.*, 1980; van Niekerk *et al.*, 2004), (iv) virulence and geographic origin of the isolate (Savocchia *et al.*, 2007; Sosnowski *et al.*, 2007) and (v) the experimental conditions (*e.g.* inoculation and isolation methods; Elena *et al.*, 2014). Based on the authors' experience and results from previous studies listed in Table 1, inoculated control recoveries of 50–70% are ideal for the pathogens tested here. Therefore, to achieve this range of recoveries, dose ranges of 100–1000 conidia of *D. seriata*, 100–2000 conidia of *P. chlamydospora*, and 100–500 ascospores of *E. lata* per wound would be required. Due to the high variability of recovery percentages observed in the current and previous studies discussed here, it is recommended to conduct a preliminary assessment of the optimal inoculum dosage range when planning artificial inoculations with these pathogens.

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