Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Spore dispersal of *Eutypella* species under desert grape-growing conditions of southern California

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OPEN ACCESS

Citation: J. R. Úrbez-Torres, C. Gispert, F. P. Trouillas (2020) Spore dispersal of *Eutypella* species under desert grape-growing conditions of southern California. *Phytopathologia Mediterranea* 59(3): 485-501. doi: 10.14601/Phyto-11807

Accepted: July 24, 2020

Published: December 30, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Fontaine F., Université de Reims Champagne-Ardenne, Reims, France.

Summary. The seasonal abundance of *Diatrypaceae* spores was studied in southern California's desert table grape-growing region of Coachella Valley. Glass microscope slides covered with petroleum jelly were placed in a mature cv. Mid-Night Beauty® vineyard, and collected weekly from September 2006 to May 2009 (1400 samples for 140 consecutive weeks). Overall, *Diatrypaceae*-like colonies were recorded from Petri plates after processing spore traps in 93 (66%) of 140 weeks. Phylogenetic analyses showed *Eutypella citricola* and *Eutypella microtheca* to be the *Diatrypaceae* spp. captured from the spore traps. Though spores were captured throughout each year, their incidence varied among the different seasons. The greatest number of *Eutypella* spores were captured in autumn (38.7% of the total) followed by winter (30.6%), summer (19.7%), and spring (11%). The greatest numbers of spores were captured in October each year (15.7%) and least in June (1%). *Eutypella* spore release was correlated with rainfall only in 26 (28%) of the 93 weeks that spores were captured during the study. Analysis of diseased samples collected from the cv. Mid-Night Beauty® vineyard showed that *E. citricola*, *E. microtheca* and *E. scoparia* were the most prevalent fungi isolated from cankers. A pathogenicity study showed that *E. citricola* and *E. microtheca* isolates collected from spore traps caused larger vascular necrosis than the non-inoculated controls, indicating their role as pathogens on grapevines. This study has demonstrated *Eutypella* spp. to play an important role in grapevine health under desert growing conditions of southern California. In addition, and contrary to what it is largely accepted, results from this research suggest that *Diatrypaceae* spore release can occur in the absence of precipitation. This study expands current knowledge on epidemiology of *Diatrypaceae* spp. other than *Eutypa lata*, and provides important information for enhancing control strategies against grapevine trunk diseases under desert growing conditions.

Keywords. Canker, *Diatrypaceae*, epidemiology, *Eutypa*, *Eutypella*, grapevine trunk diseases, spore trap, *Vitis vinifera*.

INTRODUCTION

Eutypa dieback (ED) is an economically important grapevine trunk disease (GTD) with a cosmopolitan distribution, and is responsible for important yield losses and the shortening of vineyard lifespans (Kaplan *et al*., 2016; Munkvold *et al*., 1994; Siebert, 2001; Wicks and Davies, 1999). Grapevine symptoms associated with ED are characterized by stunted shoots, which often present chlorotic cupped leaves with necrotic margins. Symptomatic shoots may present clusters with poor fruit set early in the growing season, which develop into small bunches that ripen unevenly leading to berry shrivel before harvest. The main vascular symptoms associated with ED are cankers, usually in the form of a wedge-shape (Rolshausen *et al*., 2015). Infections start at pruning wounds and cankers develop through spurs, cordons and trunks. Eutypa dieback affects mature vineyards and symptoms of the disease can take up to 3 years to appear after infection (Gramaje *et al*., 2018). Once symptoms appear, infected vines start declining, and progressive death of spurs and cordons occurs in following years with eventual death of affected vines.

More than 20 species of *Diatrypaceae* have been associated with grapevine cankers and consequent dieback (Luque *et al*., 2006; 2012; Moyo *et al.* 2018a; Trouillas *et al*., 2010; 2011). However, *Eutypa lata* is considered the most virulent, and the only species proven to cause ED foliar symptoms (Pitt *et al*., 2013; Sosnowski *et al*., 2007; Trouillas and Gubler, 2010a). These symptoms probably result from secondary metabolites produced by the fungus and translocated to aerial parts of infected plants (Andolfi *et al*., 2011; Rolshausen *et al*., 2008; Rolshausen *et al*., 2015). *Diatrypaceae* species associated with grapevine dieback have also been reported to cause cankers and decline in a wide range of other woody perennial crops, and in native and/or introduced forest tree species (Carter 1991; Trouillas *et al*., 2011; Úrbez-Torres *et al*., 2013; Trouillas and Gubler, 2016; Moyo *et al*., 2018b).

Eutypa dieback has long been known to occur in grapevines in California (Moller and Kasimatis, 1978). This disease can be found in most grape-growing regions throughout California, with greatest incidence in vineyards in the Northern San Joaquin Valley, the Sacramento Valley and the North Coast (Úrbez-Torres *et al*., 2006, Trouillas and Gubler, 2010b). For many years, *E. lata* was thought to be the sole diatrypaceous fungus associated with cankers and dieback in California; however, studies conducted since mid-2000 have isolated and identified up to 11 *Diatrypaceae* species from grapevine cankers, including *Cryptosphaeria pullmanensis*, *Cryptovalsa ampelina*, *Diatrype oregonensis*, *D. stigma*, *D. whitmanensis*, *Diatrypella verrucaeformis*, *Eutypa leptoplaca*, *Eutypella citricola*, *E. scoparia*, *E. leprosa*, and *E. microtheca* (Trouillas and Gubler, 2004; Rolshausen *et al*., 2006; Trouillas *et al*., 2010; Trouillas *et al*., 2011). Pathogenicity studies showed some species, including *C. ampelina*, *D. stigma*, and *E. leptoplaca* capable of colonizing dormant canes and causing vascular necroses similar to those caused by *E. lata*. However, other species such as *D. oregonensis* and *D. verrucaeformis* did not produce significant lesions in shoots and canes, so they were suggested to be saprophytic rather than pathogenic to grapevine (Trouillas and Gubler, 2010a).

The epidemiology of *E. lata* in grapevines has been well-studied and there is good understanding of the pathogen life cycle (Rolshausen *et al*., 2015). Ascospores of *E. lata* are released from perithecia formed in old infected parts of vines such as spurs, cordons and/or trunks (Trouillas and Gubler, 2010b). Under favorable environmental conditions, ascospores are discharged and spread by rain droplets and/or wind to short or long distances. Ascospores land on susceptible exposed xylem tissues (pruning wounds and cuts) of vines, where they germinate and start new infections (Rolshausen *et al*., 2015; Gramaje *et al*., 2018). The presence of *E. lata* is restricted to geographical locations with at least 350 mm of annual rainfall, and release of ascospores occurs when temperatures are above freezing and with as little as 0.2 mm of rainfall (Trouillas and Gubler, 2010b; Rolshausen *et al*., 2015; Billones-Baaijens *et al*., 2017). Accordingly, high risk infection periods in vineyards may vary throughout the year and geographical regions, but primarily coincide with the dormant pruning season (Gramaje *et al*., 2018).

Eutypa dieback spore trapping studies have primarily been conducted in grape-growing regions with temperate climates, where most viticulture takes place in the Northern and Southern Hemispheres (Gramaje *et al*., 2018). Discharge of *E. lata* ascospores from perithecia is primarily correlated with rainfall events. In regions with moderate winters, such as Australia, California and South Africa, ascospores are primarily released from late autumn to late winter (Ramos *et al*., 1975; Petzoldt *et al*., 1983a; 1983b; Trouillas *et al*., 2009; van Niekerk *et al*., 2010; Billones-Baaijens *et al*., 2017). In regions where temperatures below 0°C are common throughout the winter, as in New York and Michigan in the United States of America or British Columbia in Canada, ascospores discharge primarily occurs from late winter to late spring when temperatures above freezing are reached (Pearson, 1980; Trese *et al*., 1980; Úrbez-Torres *et al*., 2017). However, grapevine production, primarily table grapes, also occurs under tropical, sub-tropical

and desert climates (Winkler *et al*., 1974). In California, the third largest table grape production area is located in the Coachella Valley (Riverside County) in southern California. This region has a desert climate, classified as BWh under the Köppen climate classification and characterized by warm winters (14.7°C average temperature), hot summers (31.5°C), and annual precipitation of only 2.7 mm. Though this area is a desert, abundant underground water and aqueducts built from the Colorado River make production possible of a broad range of economically important agriculture commodities, including table grapes (USDA-NASS, 2018).

Field surveys conducted in California between 2004 and 2007 to determine the main fungal pathogens associated with grapevine cankers and dieback throughout California did not find *E. lata*, but other diatrypaceous taxa were associated with grapevine dieback in southern California, particularly in table grapes grown in the Coachella Valley (Trouillas *et al*., 2010; Úrbez-Torres *et al*., 2006). In addition, spore trapping studies conducted in different grape growing regions throughout California to determine the epidemiology of *Botryosphaeriaceae* spp. also yielded *Diatrypaceae* colonies from spore traps from different regions, including the Coachella Valley (Úrbez-Torres *et al*., 2010). Some of these colonies were later identified as *E. citricola* and *E. microtheca*, highlighting the important role that diatrypaceous fungi other than *E. lata* may play in grapevine dieback in this desert region (Trouillas *et al*., 2010; 2011).

The main objectives of the present study were to; (i) further investigate the epidemiology of *Diatrypaceae* spp. under desert climate conditions using spore trapping, (ii) identify the main *Diatrypaceae* spp. present in spore traps by morphological and molecular assessments, and (iii) complete pathogenicity assays to determine the roles that these diatrypaceous fungi play in grapevine health.

MATERIAL AND METHODS

Study site

The study was conducted in a *Vitis vinifera* cv. Mid-Night Beauty® commercial block located near Indio, Coachella Valley, Riverside County, in California. At the start of the study, the vineyard was 16 years old, with vines spaced 1.5 m apart and rows oriented north-south. Vines were trained on an open gable trellis system with up to four spur-pruned cordons in each vine (Figure 1). Vines were drip irrigated and fertilized as required, based on standard practices for the cultivar and the region. To increase the chilling hours, overhead sprinkler irrigation was applied for hydro-cooling the vineyard for 12 h each day from mid October to early December. The vineyard was selected based on previous studies indicating high incidence of vines showing dieback symptoms (Úrbez-Torres *et al*., 2006).

Glass microscope slide spore trapping

Glass microscope slides were used as spore traps to capture *Diatrypaceae* spores in the selected vineyard, as previously described (Eskalen and Gubler, 2001; Úrbez-Torres *et al*., 2006). Glass microscope slides (25 × 76 mm) each coated on both sides with a thin layer of white petroleum jelly were placed on grapevine cordons at approx. 80 cm above the soil surface (Figure 1). One microscope glass slide was placed on each of the cordons of ten different vines. Vines containing a spore trap were separated from each other by four vines, and all vines were selected in the centre of the block. Spore traps were replaced each week and individually collected into sterile 50 mL capacity screw-cap tubes (Sarstedt, Inc.) from 6 September, 2006 to 27 May, 2009. The tubes containing microscope slides were sent each week to the Plant Pathology laboratory at the University of California, Davis for processing. Screw-caps of the tubes were open inside a laminar flow cabinet, and 10 mL of autoclaved distilled water previously warmed to 20°C were added into each tube containing a microscope slide. The tubes were closed with the screw-caps and shaken by hand for approx. 60 sec. The microscope slides were then removed and two aliquots of 200 µL per tube were collected and plated onto two different 85 mm diam. Petri dishes (20 Petri dishes per week) containing potato dextrose agar (PDA: Difco Laboratories) amended with tetracycline hydrochloride (0.01%) (Sigma-Aldrich) (PDA-tet). The 200 uL aliquot was spread over the agar medium in each Petri plate using a curved glass rod, which was sterilized between plates. The inoculated Petri plates were air-dried for 10 min. inside the laminar flow hood, and were then all placed on a laboratory bench and incubated in room light and temperature (25°C ± 1°C) conditions. *Diatrypaceae*-like fungal colonies were recorded after 7 d (Figure 1) based on described taxonomic characters (Trouillas and Gubler, 2004), and were subcultured onto fresh PDA for morphological and molecular identifications.

Individual *Diatrypaceae* colonies growing from the isolation plates were counted as single spores captured from the spore trap, and were recorded each week during the study period. Total numbers of spores per week were calculated as the sum of all colonies observed from the 20 isolation plates, and multiplying the total spore

Figure 1. Spore trap study. **A** and **B**. Cv. Mid-Night Beauty® vineyard where the study was conducted in the Coachella Valley in southern California. **C.** Microscope glass spore trap coated with petroleum jelly and placed on the cordon of a grapevine. **D.** Petri dishes showing fungal colonies obtained from spore traps. Black arrows indicate characteristic *Diatrypella*-like colonies. **E** and **F.** Wedge-shape cankers observed and collected from symptomatic grapevines from the studied vineyard.

count by 25 to account for the subsampling factor. Total number of spores per week were superimposed with weekly environmental data, including average temperature and total precipitation accumulated per week. Meteorological data were collected from a California Irrigation Management Information System (CIMIS, Oasis, Imperial, Station 136) weather station located in the proximity of the vineyard.

Diatrypaceae *species identification*

Diatrypaceae isolates collected from spore traps in different weeks during the study were selected for molecular identification (Table 1). In addition, seven *Diatrypaceae* isolates identified in a previous spore trapping study conducted in California by Úrbez-Torres *et al.* (2010) were included in phylogenetic analyses for comparison and identification purposes (Table 1). A field survey was also conducted at the vineyard site on 15 July 2007 to determine *Diatrypaceae* spp. associated with wood cankers. In total, 20 symptomatic samples from 20 different vines in the vineyard block were collected. Cordons showing dieback symptoms, including dead spurs and wedge-shaped cankers in cordon cross sections were collected (Figure 1). Fungal pathogen isolation from cankers was conducted as described by Úrbez-Torres *et al.*

Table 1. *Diatrypaceae* isolates from spore traps and cankers identified in this study, and isolates retrieved from GenBank included in phylogenetic analyses.

Species	Isolate	Source - Week Collected	ITS[b]	*TUB*[c]
Cryptovalsa ampelina	A001	*Vitis vinifera*	GQ293901	GQ293972
C. ampelina	UCD2Mo	Spore trap	**MT845608**	**MT857226**
C. ampelina	UCD1Na	Spore trap	**MT845609**	**MT857227**
C. ampelina	UCD2Na	Spore trap	**MT845610**	**MT857228**
C. ampelina	UCD3Na	Spore trap	**MT845611**	**MT857229**
C. ampelina	UCD1SLO	Spore trap	**MT845612**	**MT857230**
Cryptovalsa rabenhorstii	WA08CB	*V. vinifera*	HQ692619	HQ692523
Eutypa lata	DCA900	*V. vinifera*	GQ293948	GQ294007
Eutypella australiensis	CNP03	*Acacia longifolia*	HM581945	HQ692479
Eutypella citricola	T3R2S2	*V. vinifera*	HQ692576	HQ692519
E. citricola	HVIT08	*V. vinifera*	HQ692583	HQ692513
E. citricola	UCRDC83	*Citrus limon*	KF620372	KF620408
E. citricola	STEU 8103	*V. vinifera*	KY111639	KY111592
E. citricola	UCD5Co [a]	Spore trap - Jan/30/07	**MT845613**	**MT857231**
E. citricola	UCD6Co [a]	Spore trap - Jan/30/07	*GQ293959*	*GQ294023*
E. citricola	UCD7Co [a]	Spore trap - Jan/30/07	*GQ293961*	*GQ294024*
E. citricola	UCD8Co [a]	Spore trap - May/15/07	*GQ293960*	*GQ294025*
E. citricola	UCD9Co	Spore trap - May/15/07	**MT845614**	**MT857232**
E. citricola	UCD11Co	Spore trap - Jul/24/07	**MT845615**	**MT857233**
E. citricola	UCD12Co	Spore trap - Sep/18/07	**MT845616**	**MT857234**
E. citricola	UCD18Co	Spore trap - Jul/14/08	**MT845617**	**MT857235**
E. citricola	UCD19Co	Spore trap - Dec/22/08	**MT845618**	**MT857236**
E. citricola	UCD20Co	Spore trap - Dec/22/08	**MT845619**	**MT857237**
E. citricola	UCD21Co	Spore trap - Dec/22/08	**MT845620**	**MT857238**
E. citricola	UCD22Co	Spore trap - Jan/13/09	**MT845621**	**MT857239**
E. citricola	UCD25Co	Spore trap - Apr/15/09	**MT845622**	**MT857240**
E. citricola	UCD1SJ	Spore trap	**MT845623**	**MT857241**
E. citricola	UCD2339Co	Canker in cordon - Jul/15/07	**MT845624**	**MT857242**
E. citricola	UCD2342Co	Canker in cordon - Jul/15/07	*GQ293968*	*GQ294022*
E. citricola	UCD2343Co	Canker in cordon - Jul/15/07	**MT845625**	**MT857243**
E. citricola	UCD2348Co	Canker in cordon - Jul/15/07	**MT845626**	**MT857244**
E. citricola	UCD2349Co	Canker in cordon - Jul/15/07	*GQ293969*	*GQ294021*
E. citricola	UCD2350Co	Canker in cordon - Jul/15/07	*GQ293970*	**MT857245**

(Continued)

Table 1. (Continued).

Species	Isolate	Source - Week Collected	ITS[b]	*TUB*[c]
E. citricola	UCD2353Co	Canker in cordon - Jul/15/07	*GQ293971*	*GQ294020*
Eutypella cryptovalsoidea	HVFIG05	*Ficus carica*	HQ692574	HQ692525
Eutypella leprosa	UCD713SJ	*V. vinifera*	GQ293955	GQ294016
Eutypella microtheca	UCRDC103	*Citrus paradisi*	KF620387	KF620423
E. microtheca	BCMX01	*V. vinifera*	KC405563	KC405560
E. microtheca	BCMX02	*V. vinifera*	KC405562	KC405561
E. microtheca	YC16	*V. vinifera*	HQ692561	HQ692529
E. microtheca	STEU-8107	*V. vinifera*	KY111629	KY111608
E. microtheca	UCD1Co [a]	Spore trap - Sep/20/06	**MT845627**	**MT857246**
E. microtheca	UCD2Co [a]	Spore trap - Sep/20/06	*GQ293958*	*GQ294018*
E. microtheca	UCD3Co [a]	Spore trap - Nov/18/06	*GQ293957*	*GQ294019*
E. microtheca	UCD4Co [a]	Spore trap - Jan/11/07	**MT845628**	**MT857247**
E. microtheca	UCD10Co	Spore trap - Jul/24/07	**MT845629**	**MT857248**
E. microtheca	UCD13Co	Spore trap - Jan/21/08	**MT845630**	**MT857249**
E. microtheca	UCD14Co	Spore trap - Feb/19/08	**MT845631**	**MT857250**
E. microtheca	UCD15Co	Spore trap - Feb/19/08	**MT845632**	**MT857251**
E. microtheca	UCD16Co	Spore trap - Feb/19/08	**MT845633**	**MT857252**
E. microtheca	UCD17Co	Spore trap - Jul/14/08	**MT845634**	**MT857253**
E. microtheca	UCD23Co	Spore trap - Jan/13/09	**MT845635**	**MT857254**
E. microtheca	UCD24Co	Spore trap - Apr/15/09	**MT845636**	**MT857255**
E. microtheca	UCD2345Co	Canker in cordon - Jul/15/07	**MT845637**	**MT862687**
E. microtheca	UCD2346Co	Canker in cordon - Jul/15/07	**MT845638**	**MT862688**
E. microtheca	UCD2352Co	Canker in cordon - Jul/15/07	**MT845639**	**MT857256**
E. microtheca	UCD2354Co	Canker in cordon - Jul/15/07	**MT845640**	**MT857257**
E. microtheca	UCD2355Co	Canker in cordon - Jul/15/07	**MT845641**	**MT857258**
E. microtheca	UCD2SJ	Spore trap	**MT845642**	**MT857259**
Eutypella scoparia	DFMAL100	*Robinia pseudoacacia*	GQ293962	GQ294029
E. scoparia	UCRDC142	*C. limon*	KF620391	KF620427
E. scoparia	UCRDC210	*C. paradisi*	KF620393	KF620429
E. scoparia	UCD2331Co	Canker in cordon - Jul/15/07	**MT845643**	**MT857260**
E. scoparia	UCD2332Co	Canker in cordon - Jul/15/07	**MT845644**	**MT857261**
E. scoparia	UCD2333Co	Canker in cordon - Jul/15/07	**MT845645**	**MT857262**
E. scoparia	UCD2334Co	Canker in cordon - Jul/15/07	*GQ293963*	*GQ294027*
E. scoparia	UCD2335Co	Canker in cordon - Jul/15/07	*GQ293964*	*GQ294028*
E. scoparia	UCD2336Co	Canker in cordon - Jul/15/07	*GQ293965*	*GQ294027*
E. scoparia	UCD2344Co	Canker in cordon - Jul/15/07	**MT845646**	**MT857263**
E. scoparia	UCD2347Co	Canker in cordon - Jul/15/07	**MT845647**	**MT857264**
Eutypella vitis	MSUELM13	*V. vinifera*	DQ006943	DQ006999
E. vitis	UCD2428TX	*V. vinifera*	FJ790851	GU294726
Lasiodiplodia theobromae	UCD2337aCo	Canker in cordon - Jul/15/07	**MT845648**	n/a
L. theobromae	UCD2337bCo	Canker in cordon - Jul/15/07	**MT845649**	n/a
L. theobromae	UCD2341aCo	Canker in cordon - Jul/15/07	**MT845650**	n/a
L. theobromae	UCD2341bCo	Canker in cordon - Jul/15/07	**MT845651**	n/a
Phaeoacremonium parasiticum	UCD2351Co	Canker in cordon - Jul/15/07	**MT845652**	n/a

[a] *Eutypella* isolates used in the pathogenicity study.
[b] ITS: Internal Transcribed Spacer GenBank Accession Numbers.
[c] *TUB:* beta-tubulin GenBank Accession Numbers.
GenBank Accession Numbers in bold were generated in this study.
GenBank Accession Numbers in italics were reported by Trouillas *et al.* (2010).
GenBank Accession Numbers in underlined italics were reported by Trouillas *et al.* (2011).
n/a: Not available.

(2006). Pure isolates of *Diatrypaceae*-like colonies selected from spore traps and from grapevine cordon cankers were obtained by hyphal tip isolations from PDA cultures, and were incubated in Parafilm-sealed Petri plates at room light and temperature conditions for 7 to 10 d. Total genomic DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN Inc). Oligonucleotide primers ITS1 and ITS4 (White *et al*., 1990) and T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995) were used to amplify the nuclear rDNA ITS1-5.8S-ITS2 region (ITS) and part of the beta-tubulin (*TUB2*), in a thermal cycler (PTC-100TM, MJ Research), following the PCR temperature profiles described by Trouillas *et al*. (2010). Amplified products were purified using the QIAquick PCR purification Kit (QIAGEN Inc), and forward and reverse ITS and *TUB2* sequences were obtained using a ABI Prism 377 DNA Sequencer (Perkin-Elmer), at the Division of Biological Sciences sequencing facility at the University of California, Davis.

Sequences were edited and assembled using Sequencher™ version 4.1 (Gene Codes). Consensus sequences were aligned using the ClustalW multiple alignment program (Thompson *et al*., 1994) in BioEdit Sequence Alignment Editor Version 7.1.3.0 (Hall, 1999), and manually adjusted. *Diatrypaceae* spp. sequences from GenBank were selected based on their high similarity with the query sequences using MegaBLAST. Combined ITS and *TUB2* phylogenetic analyses were conducted first using the Neighbor-Joining (NJ) with the Maximum Composite Likelihood method and 1000 replicates to assess branch robustness and the Tamura-Nei model. The tree with the greatest log likelihood value was selected. In addition, the combined dataset was further analyzed using Maximum Parsimony (MP) with the bootstrap test (1000 random additional sequence replicates) and the Tree-Bisection-Regrafting (TBR) algorithm in MEGA-X (Kumar *et al*., 2018). *Diatrypaceae* sequences from this study were deposited into GenBank, and isolates were stored in the Plant Pathology Department fungal collection at the University of California, Davis.

Pathogenicity study

Four different isolates from the two species identified from the spore traps were used in the pathogenicity test (Table 1). The trial was conducted in a 7-year-old double cordon and spur pruned and trained cv. Red Globe vineyard at the University of California Field Station in Davis, California. Green shoots of the new vegetative growth were inoculated with mycelium plugs obtained from 7-d-old active growing colonies of fungus isolates, by wounding between the 4th and 5th internodes of each shoot, in June 2007 as described by Úrbez-Torres and Gubler (2009). One shoot per grapevine, on each of five grapevines per fungus treatment, was inoculated. The same number of negative controls were inoculated using non-colonized agar plugs. Shoots were collected 5 months after inoculation and brought to the laboratory for processing. The shoots were surface disinfected by submerging them on 4% sodium hypochlorite for 5 min. Samples were then air-dried and each sectioned in half longitudinally through the point of inoculation. Upward and downward vascular discolouration was measured from the point of inoculation, and results are presented as the mean of both measurements from each sample. In order to fulfill Koch's postulates, inoculated fungi were re-isolated as described by Úrbez-Torres and Gubler (2009). Data from the pathogenicity test were analyzed using SAS (Version 9.1.3; SAS Institute). Differences in length of vascular discolouration caused by each *Diatrypaceae* isolate were determined by one-way analyses of variance. Treatment means were compared using Fisher's least significant difference (LSD) test at the 5% significance level.

RESULTS

Spore trapping

A total of 1400 spore traps, corresponding to 140 consecutive weeks, were collected and processed from 6 September 2006 to 27 May, 2009 (Figure 2). *Diatrypaceae* fungi were recorded from 93 (66%) of the 140 weeks monitored. *Diatrypaceae* spores were captured throughout each year, but their incidence varied among the different seasons. The greatest number of *Diatrypaceae* spores were captured in autumn (38.7% of the total), followed by winter (30.6%), summer (19.7%), and spring (11%). The greatest numbers of *Diatrypaceae* spores were captured each year in October (15.7% of the total), followed by December (14.1%) and January (13.9%) (Figure 2). The month with fourth greatest numbers of spores captured was August (11.6%), which with July were the hottest and driest months of each year. The least numbers of spores were detected in June (1% of the total), followed by May (5.5%) and April and November (all 5.5%).

No rainfall was recorded from the start of the study on 6 September 2006 to 2 January 2008, and again from 2 February 2008 to 18 November 2008 (Figure 2). *Diatrypaceae* spore release was correlated with rainfall only in 26 (28%) of the 93 weeks when spores were captured (Figure 3). Although rain was not the main factor contributing to spore discharge, results showed that the

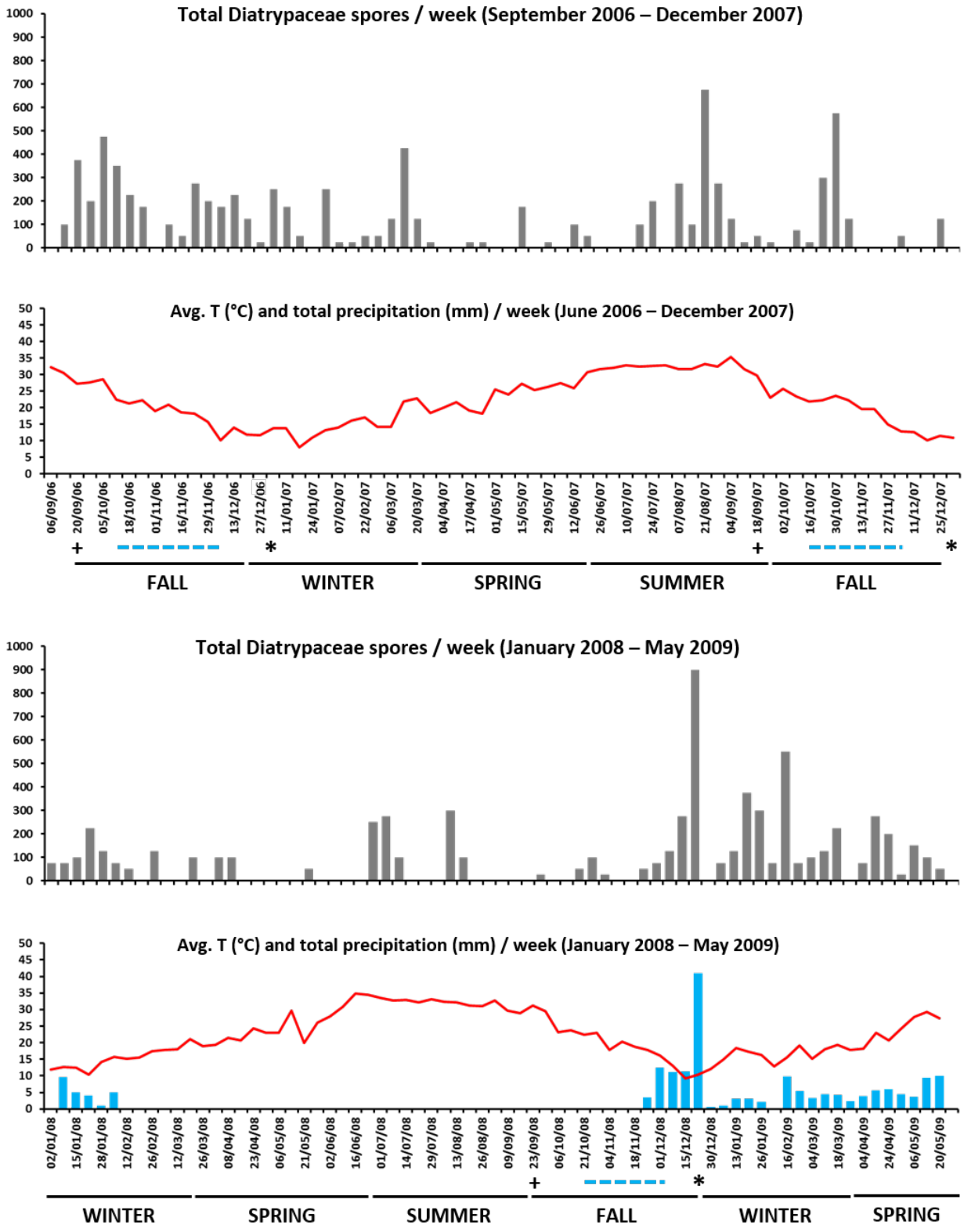

Figure 2. Total numbers of *Eutypella* spores trapped per week using glass microscope slides in the cv. Mid-Night Beauty® vineyard, average temperature and accumulated rainfall per week. Dashed blue line under the x axis shows overhead sprinkler irrigation period at the vineyard. + indicates week that overhead sprinklers were tested; * indicates pruning dates.

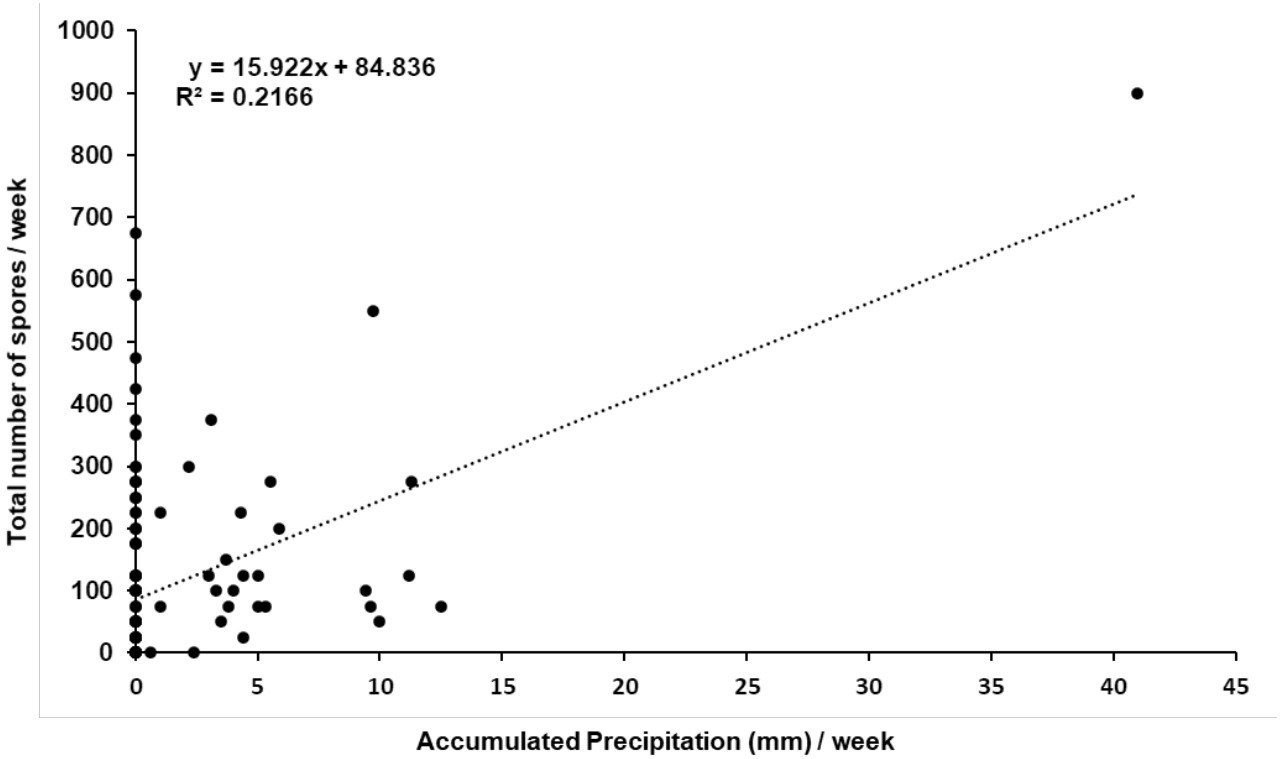

Figure 3. Scatter plot of the relationship between total spores trapped per week using glass microscope slides and precipitation. Linear regression line and equation are also shown.

largest numbers of spores were released during the week of 12 December 2008, when the greatest amount of precipitation was recorded (41 mm) during the course of this study (Figure 3). Overhead sprinkler irrigation was turned on in the studied vineyard from 18 October to 29 November in 2006, from 23 October to 27 November in 2007, and from 21 October to 25 November in 2008. *Diatrypaceae* spores were captured in six of seven weeks of overhead irrigation in 2006, three of six weeks in 2007, and four of six weeks in 2008. However, the total number of spores recorded during the weeks irrigation was not significantly greater when compared to the numbers of spores captured in weeks without irrigation (Figure 2). *Diatrypaceae* spore release showed no obvious correlation in this study with temperature, and spore release occurred under average temperatures ranging from 8°C to 35°C (Figure 4).

Diatrypaceae *species identifications*

PCR amplifications of ITS and *TUB2* regions gave respective products of approx. 500 and 700 bp. To study the phylogenetic relationships among *Diatrypaceae* isolates obtained in this study, ITS and *TUB2* sequences were BLASTed to select closely related sequences for the phylogenetic analysis (Table 1). In total, 72 isolates were included in the combined phylogenetic analysis with 1391 positions in the final dataset, including gaps. The greatest log likelihood Neighbor-Join phylogenetic tree of the combined ITS and *TUB2* sequences is shown in Figure 4. The MP evolutionary history generated seven most parsimonious trees (length = 1088; consistency index = 0.781855; retention index = 0.970641; composite index = 0.779724). The MP analysis resulted in trees with similar topology to the NJ tree. Based on this phylogenetic study, *Diatrypaceae* isolates obtained from spore traps placed in the Mid-Night Beauty® vineyard in Coachella Valley grouped in two well-supported clades with previously identified isolates of *E. citricola* (99% bootstrap for both NJ and MP) and *E. microtheca* (99% bootstrap for both NJ and MP), from different hosts from California and other countries (Figure 5). Additional *Diatrypaceae* isolates obtained from spore traps placed in vineyards in Monterrey, Napa, and San Luis Obispo, California, from a previous study (Úrbez-Torres *et al.*, 2010), grouped with *C. ampelina* isolate A001 from *V. vinifera* from Australia in a well-supported separate clade (99% bootstrap for both NJ and MP). In addition,

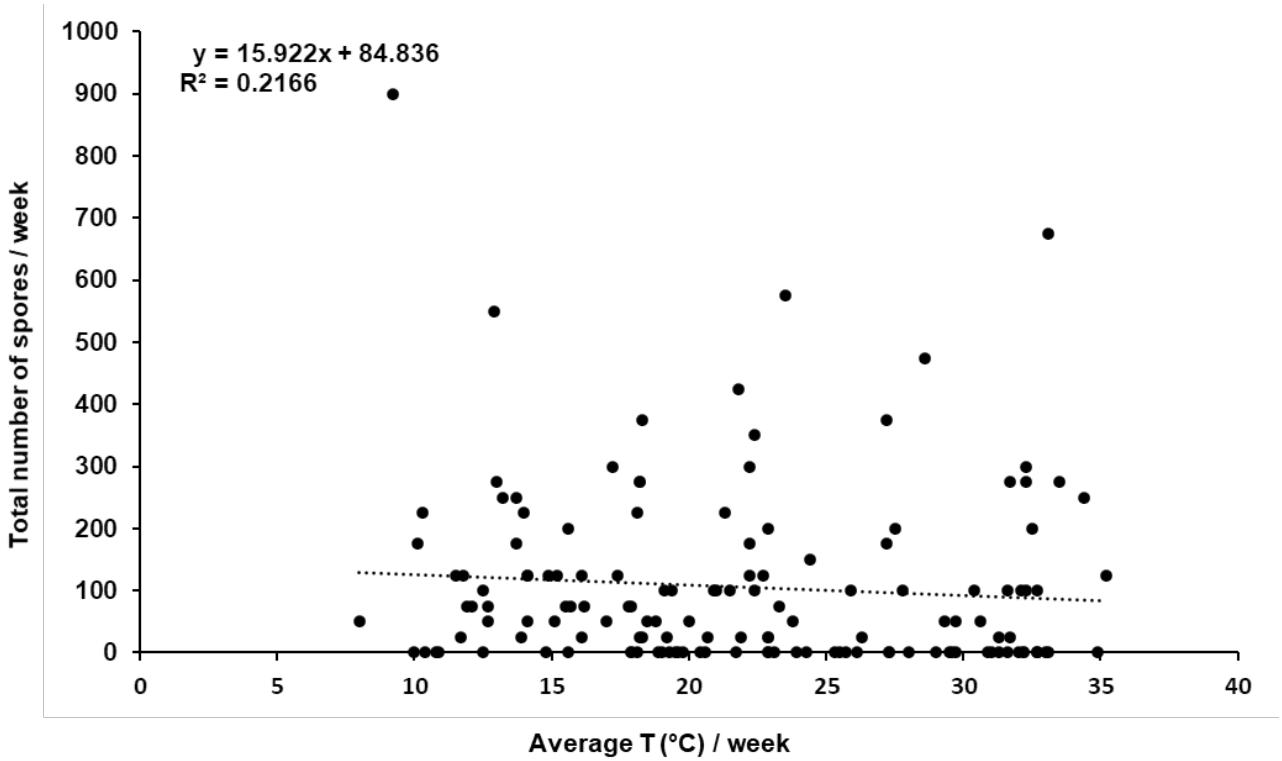

Figure 4. Scatter plot of the relationship between total spores trapped per week using glass microscope slides and average temperature. Linear regression line and equation are also shown.

two isolates from spore traps collected from San Joaquin County, California, (Úrbez-Torres *et al.*, 2010) grouped with *E. citricola* isolates.

A total of 25 fungal colonies were isolated and identified from 20 symptomatic cordon samples collected in a field survey at the spore trap block on 15 July 2007. BLAST results along with the combined ITS and *TUB2* phylogenetic analyses identified eight isolates as *E. scoparia* (including three isolates previously identified by Trouillas *et al.*, 2010; 2011), seven isolates as *E. citricola* (including three isolates previously identified by Trouillas *et al.*, 2010; 2011), five isolates as *E. microtheca*, four isolates as *Lasiodiplodia theobromae*, and one isolate as *Phaeoacremonium parasiticum* (Table 1; Figure 5).

Pathogenicity test

Results of the pathogenicity tests are summarized in Figure 6. Mean lesion lengths varied from 15.5 to 21.2 mm for *E. citricola* isolates, and from 13.5 to 16.9 for *E. microtheca* isolates (Figure 6). *Eutypella citricola* isolate UCD6Co caused the greatest mean lesion length, and while this was not significantly different from the mean lesion length from *E. citricola* isolate UCD8Co, it was significantly different from the mean lesion lengths from the other isolates tested (Figure 6). Mean lesion lengths obtained from inoculated canes were all longer than the negative controls ($P < 0.05$). Five months after inoculation, vascular necroses upwards and downwards from the inoculation sites were observed in longitudinal sections made from fungus inoculated canes (Figure 6B). Negative controls had significantly less discolouration extending from the inoculation sites (mean = 3.5 mm). *Eutypella citricola* and *E. microtheca* were re-isolated from lesions from all the inoculated canes, confirming that these fungi were the same as the inoculated isolates based, on morphology of the colonies. No fungal pathogens were isolated from the negative controls.

DISCUSSION

Eutypa lata has been known as the main causal agent of Eutypa dieback (ED) of grapevines since the early 1970s. However, few epidemiological studies have been conducted to understand spore dispersal patterns of this pathogen in vineyards (Pearson, 1980; Trese *et al.*, 1980; Trouillas and Gubler, 2010; van Niekerk *et al.*, 2010;

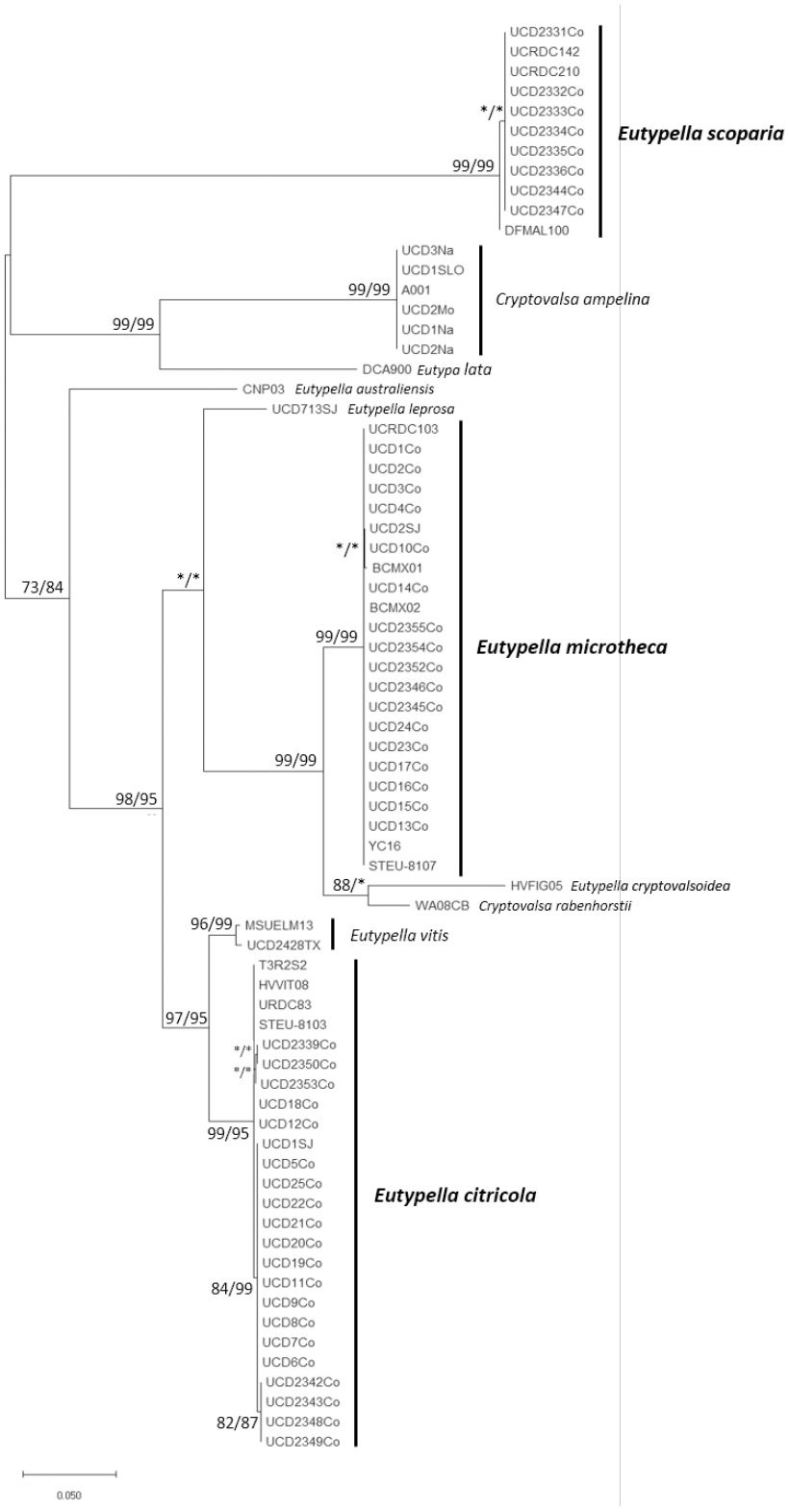

Figure 5. Greatest log likelihood Neighbor-Joining phylogenetic tree of the combined ITS and *TUB2* tree generated with 72 *Diatrypaceae* nucleotide sequences. Numbers in front and after the slashes represent, respectively, likelihood and parsimony bootstrap values from 1000 replicates. Values accompanied by an asterisk were less than 70% bootstrap. *Eutypella* isolates from spore traps from this study are indicated in bold font.

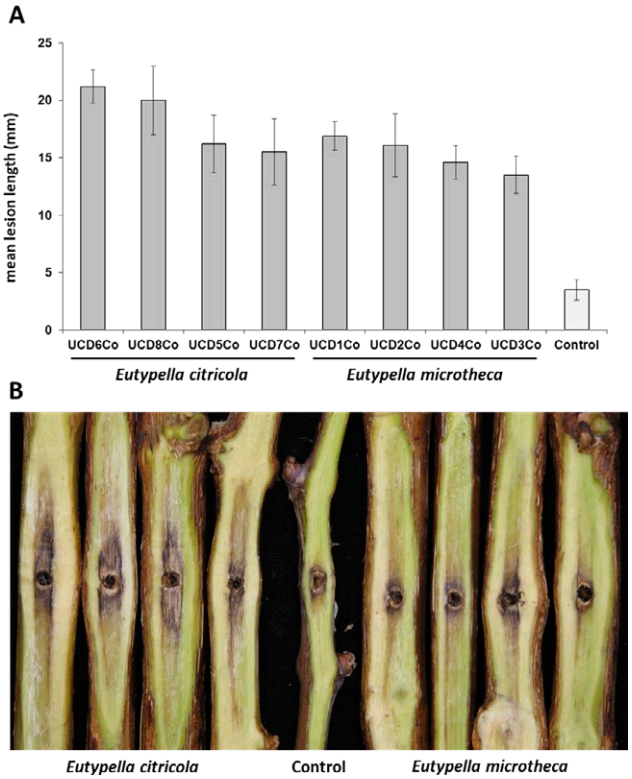

Figure 6. Pathogenicity test on cv. Red Globe grapevine plants inoculated with *Eutypella citricola* and *E. microtheca* isolates from vineyard spore traps. **A.** Mean lesion lengths caused by *E. citricola* and *E. microtheca* 5 months after inoculation. **B.** Vascular necrosis caused by *E. citricola* and *E. microtheca* in cv. Red Globe dormant canes compared to non-inoculated control.

Billones-Baaijens *et al.*, 2017; Úrbez-Torres *et al.*, 2017). Only recently, the use of DNA molecular identification has allowed the discovery of other diatrypaceous fungi associated with grapevine dieback, though their biology and epidemiology are still not fully understood (Trouillas and Gubler, 2004; Luque *et al.*, 2006; 2012; Trouillas *et al.*, 2010; 2011; Moyo *et al.*, 2018a). Only recent studies completed in South Australia (Billones-Baaijens *et al.*, 2017) and British Columbia, Canada (Úrbez-Torres *et al.*, 2017) have investigated spore dispersal patterns of *E. lata* and *Diatrypaceae* spp., using multi-species primers in quantitative PCR (qPCR) or droplet digital™ PCR (ddPCR™) assays. The present study adds to these latter studies investigating the role that diatrypaceous fungi may play in vineyards, and reports for the first time the seasonal spore release patterns of *Diatrypaceae* spp. other than *E. lata* under the desert conditions of California.

Based on previous spore trapping studies conducted in vineyards, it is well-accepted that spores of *E. lata* are released from perithecia within a few hours after the onset of rainfall, and release continues throughout each rain event and can last for up to 36 h after rain ends and until each stroma dries (Pearson, 1980; Trese *et al.*, 1980; Petzoldt *et al.*, 1983b; Trouillas and Gubler, 2010b). Similar conclusions can be reached from *E. lata* spore trapping studies conducted on apricots (Moller and Carter, 1965; Petzoldt *et al.*, 1983a; 1983b; Ramos *et al.*, 1975). It has also been suggested that a minimum of 2 mm of rainfall is required for stroma to start discharging *E. lata* ascospores (Trese *et al.*, 1980). However, a study conducted in South Africa showed a minimum of 1 mm of rainfall was enough for spores of *E. lata* to be discharged from perithecia in the Stellenbosch grape-growing region (van Niekerk *et al.*, 2010). More recent studies conducted in Australia and Canada have shown that spores of *Diatrypaceae* spp. were captured after as little as 0.5 mm of rain (Billones-Baaijens *et al.*, 2017; Úrbez-Torres *et al.*, 2017). Therefore, a strong correlation between rain events and *E. lata* spore release has been suggested, primarily during the grapevine dormant period (late autumn to early spring).

Most of the above-mentioned studies only investigated *E. lata* and did not consider other diatrypaceous fungi, although these have been commonly recognized as grapevine pathogens. However, based on morphological and taxonomic similarities between *E. lata* and other *Diatrypaceae* spp. in the genera *Cryptosphaeria*, *Cryptovalsa*, *Diatrype*, *Diatrypella*, and *Eutypella*, it is likely that these pathogens require similar environmental conditions for spore release, infection and perithecium formation to those for *E. lata* (Trouillas *et al.*, 2010; 2011). Nevertheless, the present study conducted under the desert conditions of the Coachella Valley in southern California showed rainfall was only responsible for 28% of the spore release events in the study vineyard, while overhead sprinkler irrigation contributed to another 22%. The greatest amount of rain recorded during this study occurred between the last week of November 2008 and the last week of May 2009 (89.4 mm), and this corresponded also with the greatest number of spores captured (4325). During this period, 900 spores were also captured in a single week, which also correlated with the greatest rainfall within a single week (41 mm). These results indicate a strong correlation between rainfall and *Eutypella* spp. spore release in the Coachella Valley. However, if we compare the same period from the last week of November of 2006 to the last week of May of 2007, from which no rainfall was recorded, the total number of spores captured was only slightly less (3000). Accordingly, if rainfall is suggested as the main factor responsible for spore release of *E. lata*, over 70% of *Eutypella* spp. spores captured in this study occurred without the

occurrence of rainfall events. These results are similar to those reported by Úrbez-Torres *et al.* (2017) from spore trapping studies conducted in the Okanagan Valley, a semi-arid grape-growing region located in the southern interior of British Columbia. In this study, Úrbez-Torres *et al.* (2017) showed that a significant number of *Diatrypaceae* spp. spore release events did not always correlate with rainfall. In addition, recent spore trapping studies conducted in Australia have also reported diatrypaceous spores to be captured during dry periods (Billones-Baaijens *et al.*, 2017). Based on these results, and as suggested by Billones-Baaijens et al. (2017), we hypothesize that environmental conditions other than rainfall (e.g. temperature, relative humidity, dew and/or wind) may be involved with spore release of *Diatrypaceae* spp., particularly under desert conditions where rainfall is scarce or limited to few days throughout each year.

Previous studies have correlated spore release of *E. lata* with high RH values (Trese *et al.*, 1980; Pearson, 1980; van Niekerk *et al.*, 2010). In the present study, relative humidity for most of the duration of the study were below 30% (data not shown), and only during rain events reached greater than 70%. Therefore, relative humidity probably did not play a role on *Eutypella* spp. spore release. Studies conducted in Australia (Moller and Carter, 1965) and California (Ramos *et al.*, 1975) suggested that wind currents can disseminate spores of *E. lata* long distances (50 and 160 km) from inoculum sources, but this always follows rain events. It is possible that some of the *Eutypella* spores captured in the present study did not originate in the monitored vineyard, but originated from the large number of citrus orchards planted in the region, where high incidence of *Eutypella* spp. infections has been reported (Mayorquin *et al.*, 2016). However, the desert climate under which the study was conducted occupies a large area of southern California, so even if spores came from surrounding vineyards, orchards and/or other native or introduced hosts, similar environmental conditions would have occurred in those locations, including the lack of precipitation. It is also possible that spores can be disseminated from distances greater than those previously reported, but further research is required to support this hypothesis. It is difficult to explain the high inoculum of *E. citricola* and *E. microtheca* spores present in this desert region during long periods without rain. Therefore, further research is required determine which other environmental conditions play roles in spore release and perithecium formation of *Eutypella* spp. under desert climatic conditions.

Eutypella spore captures were most common in winter months, followed by autumn. However, greatest spore quantities were captured during autumn (38.7% of the total), with almost half of spores captured in October. A large proportion (30.7%) of spores were also captured in winter with 50% of these captured in January. These results agree with previous studies conducted in California on *E. lata*. Ramos et al. (1975) and Petzoldt et al. (1983b) reported *E. lata* spore release from perithecia on apricots in the Salinas and Sacramento valleys to be high in mid- and late-autumn, medium in winter, high again in mid- and late-spring and low or nil in summer. Similar results were reported by Trouillas and Gubler (2009) for data from volumetric spore traps placed in vineyards in Sonoma, Napa and San Joaquin counties. Different results were reported by Moller and Carter (1965) based on *E. lata* spore trapping studies conducted on apricot in South Australia, where high spore release was detected from mid-autumn to early-winter, low release from mid-winter to early-spring, and high to medium release from mid-spring to mid-summer. The periods of high, medium and low discharge are partly explained by the more than 12 d required for released asci to be replenished with new mature ascospores in the perithecia under optimum environmental conditions (Petzoldt *et al.,* 1983b; Rolshausen *et al.*, 2015). The present study recorded similar total numbers of spore capture events occurring in spring and summer in the Coachella Valley. However, much greater numbers of spores were captured in summer (19.7% of the total) compare to spring (11%). In addition, August was the month with the greatest number of *Eutypella* spores captured after October, December and January. Though Moller and Carter (1965) reported some of the greatest numbers of *E. lata* spores captured during summer months, these releases were always correlated with rainfall. In contrast, July and August are among the hottest and driest months in the Coachella Valley, with average temperatures reaching up to 39°C. Úrbez-Torres *et al.* (2017) also reported large numbers of *Diatrypaceae* spores captured during July and August in British Columbia during dry periods. This is further evidence that environmental factors other than moisture favour spore release of *Eutypella* spp., and more specifically, release of *Eutypella* spores under desert climatic conditions.

Only *E. citricola* and *E. microtheca* were identified from spore traps in the present study, and both fungi were also isolated from symptomatic samples in the studied vineyard, indicating their roles as pathogens causing grapevine cankers. *Eutypella scoparia*, the third *Diatrypaceae* spp. isolated from cankers, was not identified from spore trap samples. Considering the difficulty to discriminate among *Diatrypaceae* spp. using colony morphology (Trouillas *et al.*, 2010; 2011), along with the

small number of samples selected each week to complete molecular identifications, it is possible that *E. scoparia* was missed from all of the spore samples collected. A much larger number of colony samples observed per week would need to be selected for molecular identification to confirm the presence of *E. scoparia* from spore traps. In addition, this would also allow more precise spore dispersal patterns for each *Eutypella* spp. found in this study. Trouillas *et al.* (2011) showed common occurrence of the perithecia of *E. citricola* and *E. microtheca* on diseased grapevine wood while perithecia of *E. scoparia* have not yet been found on this host.

To date, *E. lata* is the sole diatrypaceous fungus known to cause the characteristic ED foliar symptoms. In this study, the absence of *E. lata* from spore traps and cankers correlates with the lack of ED symptoms observed in the vineyard. This result is similar to those from surveys conducted in mid-2000s, in which ED symptoms along with *E. lata* isolated from cankers were mostly recorded from grape-growing regions in the Northern San Joaquin Valley, Sacramento Valley and North California (Úrbez-Torres *et al.*, 2006), with much greater precipitation than the present study area. *Eutypa lata* is restricted to geographical locations with at least 350 mm of annual rainfall, and perithecia are rarely found in areas with less than 250 mm (Ramos *et al.*, 1975; Trouillas and Gubler, 2010b; Rolshausen *et al.*, 2015). The historical average annual precipitation accumulated in the region of study (Indio, Coachella Valley) is about 200 mm, which may explain the absence of *E. lata*. These results agree with previous studies in which no *E. lata* but other *Diatrypaceae* spp. were found associated with dieback of perennial woody crops grown under desert conditions. Paolinelli-Alfonso *et al.* (2015) found *E. microtheca* but not *E. lata* as the cause of grapevine cankers in the grape-growing region of Baja California, Mexico. Similarly, Mayorquin *et al.* (2016) identified three *Diatrypaceae* spp., including *E. citricola*, *E. microtheca* and a *Eutypella* sp., to be involved in branch dieback of citrus in the southern California desert regions of Riverside (including Coachella Valley), Imperial and San Diego counties. The phylogenetic analyses conducted in the present study showed the unidentified *Eutypella* sp. from citrus in southern California to share 99% similarity with *E. scoparia* isolates from grapevine cankers, confirming that *E. scoparia* also occurs in citrus in California. Similarly, a much greater prevalence of *Diatrypaceae* spp. other than *E. lata* was found associated with grapevine cankers in the Okanagan Valley semi-arid region of British Columbia (Úrbez-Torres and O'Gorman, 2016). In addition, Trouillas *et al.* (2011) found *Eutypella* spp. to be the only diatrypaceous taxa occurring in vineyards in Western Australia. Based on these results *Diatrypaceae* spp., primarily in the genus *Eutypella*, are probably well-adapted to regions with desert or semi-arid climates. This information is important and deserves further investigation to determine the most prevalent and important fungal species involved on grapevine dieback under desert conditions, so that appropriate control strategies can be developed. Furthermore, molecular identification conducted in this study from spore trap samples previously collected from different regions in California (Úrbez-Torres et al., 2010) and thought to be *E. lata* resulted in colonies being identified as *C. ampelina*, *E. citricola* and *E. microtheca*. This highlights the difficulty to discriminate among *Diatrypaceae* spp. based solely on spore and colony morphology. To avoid fungal species misidentification in spore trapping studies, it is imperative to include molecular identification and quantification assays in future studies investigating fungal spore dispersal patterns (Billones-Baaijens *et al.*, 2017; Úrbez-Torres *et al.*, 2017; González-Domínguez *et al.*, 2020).

This study has confirmed the pathogenicity of *E. citricola* and *E. microtheca* isolates obtained from spore traps when artificially inoculated onto healthy grapevine shoots. Average necroses lengths recorded from both species after 5 months were small (18.2 mm for *E. citricola* and 15.2 mm for *E. microtheca*), but were greater than necroses from non-inoculated controls. These results agree with those published by Pitt *et al.* (2013), where *E. citricola* and *E. microtheca* isolates from grapevines in Australia resulted in average lesion lengths slightly greater than 20 mm after 9 months incubation on cv. Cabernet Sauvignon potted vines. In contrast, Paolinelli-Alfonso *et al.* (2015) showed that *E. microtheca* caused grapevine necrotic lesions of lengths 5 to 7 mm, which can be explained by the 3 month incubation period used. Necrotic lesions measured in the present study from both *E. citricola* and *E. microtheca* on grapevines were similar to those measured by Mayorquin *et al.* (2016) on inoculated lemon tree branches. In contrast, Moyo *et al.* (2018b) showed *E. citricola* and *E. microtheca* isolates from dieback on apricot and plum trees in South Africa caused larger lesions when inoculated onto these hosts under similar experimental conditions. Based on these results, fruit trees are probably more susceptible to these species than grapevines, but further studies are required to confirm this suggestion.

Mayorquin *et al.* (2016) showed a *Eutypella* sp., renamed in the present study as *E. scoparia*, to be highly virulent when inoculated onto branches of lemon trees, causing necrosis lengths greater than 80 mm after 8 months. Although *E. scoparia* was isolated from grape-

vine cankers in the present study, this fungus was not included in the pathogenicity assay. To the best of our knowledge, the pathogenicity of *E. scoparia* has not been evaluated on grapevines, although the results obtained by Mayorquin *et al.* (2016) on citrus indicate that this species could also be pathogenic to grapevine, and possibly more virulent than *E. citricola* and *E. microtheca*. Studies to determine the role that *E. scoparia* has in grapevine health are required, since this species is probably involved in dieback of economically important woody perennial crops in desert regions of southern California. Virulence of *E. citricola* and *E. microtheca* on grapevines is comparable to other *Diatrypaceae* spp., such as *C. ampelina*, *D. stigma*, *D. whitmanensis*, *Diatrypella* sp., and *E. leptoplaca* but less overall than *E. lata* (Luque *et al.*, 2006; Trouillas and Gubler, 2010; Pitt *et al.*, 2013). In addition, no foliar symptoms similar to those caused by *E. lata* were observed from any of the vines inoculated with *E. citricola* and *E. microtheca* in the present study.

Eutypa lata has long been known to be the most important diatrypaceous fungus causing dieback of a wide range of woody perennial hosts, including grapevines (Carter 1991). In general, *Diatrypaceae* spp. have been considered as saprophytes on decaying wood of angiosperms (de Almeida *et al.*, 2016), and the importance of some of these species as grapevine pathogens has only recently been demonstrated. The present study has shown that pruning dates in the studied block (29 December 2006; 27 December 2007; 22 December 2008) coincided with some of the greatest amounts of *Eutypella* spores in the vineyard. Since pruning wound infection has been suggested to occur after spores are discharge from perithecia during rain events, it is possible that the lack of precipitation in this region persuaded growers to not treat and protect pruning wounds to prevent pathogen infections. The present study demonstrated the importance of protecting pruning wounds soon after pruning under desert conditions, even if no rainfall occurs. In addition, further studies are required to better understand length of pruning wound susceptibility to *Eutypella* spp. under desert conditions. This information will assist development of appropriate fungicide spray programmes to ensure that pruning wounds are protected while susceptible. Results from this study are relevant, since they add to knowledge of the aetiology and epidemiology of GTD, especially under desert grape-growing conditions, which have not been extensively investigated. In other regions, large areas of table grapes are produced in desert climates. For instance, Coachella Valley represents about 7% of the total table grapes produced in California, with a farm gate value of $US118 million (USDA-NASS, 2018). Understanding the epidemiology of these pathogens in vineyards of southern California provides valuable information for improving disease control strategies under desert growing conditions.

ACKNOWLEDGEMENTS

This research was funded by the California Competitive Grant Program for Research in Viticulture and Enology, American Vineyard Foundation, Viticulture Consortium West, and the California Table Grape Commission.

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