



New findings on the effects of different factors involved in fig limb dieback caused by *Neoscytalidium dimidiatum* in California

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Abstract The San Joaquin Valley (California, USA) represents an important fig (*Ficus carica*) production area in the United States. Fig limb dieback represents a serious and emerging disease of fig caused by *Neoscytalidium dimidiatum*. In the present study we evaluated the effect of tissue age on canker development, the recovery of the fungus from fruit mummies collected in the field, the ability of *N. dimidiatum* to colonize, under laboratory condition, fig fruits, and the in vitro effects of different water potentials (Ψ s) on mycelial growth rate. Results of our study showed that the older branches (> 3-year-old) resulted in longer canker compared to the younger ages. *N. dimidiatum* was not recovered from the mummies, instead they were colonized by many other fungal saprophytes. Laboratory experiments showed the ability of this species to colonize dried fig fruits from 20 °C to 35 °C. In vitro water potentials experiment showed that the mycelial growth was reduced with

the decrease of water potential (from 1–3 -MPa), depending on salt type.

Keywords Limb dieback · Cankers · Fig · *Ficus carica* · Botryosphaeriaceae · *Neoscytalidium dimidiatum*

Introduction

The San Joaquin Valley of California represents an important fig (*Ficus carica*) production area in the United States, with the cultivation mainly concentrated in Fresno, Madera, and Kern counties. Many diseases are reported to affect figs in California and worldwide (Michailides, 2003). Among the fungal diseases affecting fig cultivation in California, *Fusarium moniliforme* (endosepsis) (Michailides & Morgan, 1998), *Aspergillus niger* (smut), *Alternaria* and *Ulocladium* (Doster et al., 1996; Michailides, 2003) are responsible for extensive losses. In addition, many tree crops in California suffer from canker diseases affecting many different organs (branches, shoots, and trunk) and blight diseases (leaves and fruit) leading to progressive yield losses over the years. Among these, species belonging to Botryosphaeriaceae family have been investigated for years, considered important pathogens in California and worldwide for many fruit crops (Bezerra et al., 2021; Díaz et al., 2022), and also nut crops (Moral et al., 2019). For *Ficus* spp., including the cultivated fig, Botryosphaeriaceae are reported as serious pathogens (Javadi & Banihashemi, 2008; Çeliker & Michailides, 2012; Mayorquin et al., 2012; El-Atta & Aref,

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2013; Mohali et al., 2017; Aiello et al., 2020; Fiorenza et al., 2022). Within this extensive group of fungi, *Neoscytalidium dimidiatum* is considered an emerging and serious pathogen of fruit trees (Gusella et al., 2021; Nouri et al., 2018). This species has been named in many different ways throughout the years, such as *Torula dimidiata*, *Hendersonula toruloidea*, *Natrassia mangiferae*, *Scytalidium dimidiatum*, *S. hyalinum*, *Fusicoccum dimidiatum*, and *Neoscytalidium hyalinum*. Crous et al. in 2006 established the new genus *Neoscytalidium* (Crous et al., 2006). Until a few years ago this genus consisted of three species: *N. dimidiatum*, *N. novaehollandiae*, and *N. orchidacearum*, now reduced to synonymous with *N. dimidiatum* (Zhang et al., 2021). This pathogen was reported in California (as *H. toruloidea*) attacking fig (Paxton et al., 1964; Warner, 1952), walnut (Wilson, 1949), citrus (Calavan & Wallace, 1954), and recently was investigated as an emerging pathogen on citrus, grape and almond (as *N. dimidiatum*) (Mayorquin et al., 2016; Nouri et al., 2018; Rolshausen et al., 2013). Moreover, *N. dimidiatum* was reported worldwide causing cankers and dieback on different *Ficus* spp., including the common fig (Al-Bedak et al., 2018; Elshafie & Ba-Omar, 2002; Giha, 1975; Güney et al., 2022; Mirzaee et al., 2002; Ray et al., 2010). Although many Botryosphaeriaceae and their interaction with different hosts have been described, no much information is available about the epidemiology and ecology of *N. dimidiatum* attacking fig or nut crops. Studies conducted on dragon fruit infected by *N. dimidiatum* revealed that this fungus behaves primarily as necrotroph, producing toxins and destroying tissues in advance of colonization (Fullerton et al., 2018). Moreover, it is known that *N. dimidiatum* produces arthroconidia (formed by the fragmentation of hyphal cells) besides the pycnidiospores (asexual spores produced within the pycnidia). This characteristic could represent an important epidemiological factor to take into account, since the arthroconidia (arthrospores) is a kind of spores that easily become airborne and can be spread to long distance. In fact, during field surveys we frequently found masses of arthroconidia under the bark of dead branches of figs and walnuts. Recent studies on the etiology of fig limb dieback revealed many aspects of its distribution in California and pathogenicity (Gusella et al., 2021). Botryosphaeriaceae spp. are considered serious pathogens for many tree crops due to their “host neutral behavior”, and the consequent ability to jump

from one host to another, especially in countries of the Mediterranean basin where fruit trees are often cultivated densely (Moral et al., 2019). Previous studies on fig limb dieback in California revealed also that injured branches are more susceptible to *Neoscytalidium* cankers; that summer pruning led to more severe cankers; and that “Kadota”, “Sierra”, and “Black Mission” cultivars are more susceptible to the disease than “Brown Turkey”, “Conadria” and “Calimyrna” (Gusella et al., 2021). This disease has caused significant yield losses in orchards in both Madera and Merced counties where growers reported an average of 20% loss, since major scaffolds and branches of fig trees are killed, and some of the trees had up to 50% reduction in productive wood (T. Michailides, *unpublished data*). Since little information on the biology and epidemiology is available about *N. dimidiatum*, in this study, we decided to further investigate other factors involved in this disease. Specifically, we determined i) the effect of tissue age on canker development based on the fact that during pruning various age shoots are cut; ii) whether fig mummies can serve as a source of inoculum, since a portion of the mummies remain in the field during the year; and iii) the *in vitro* effect of different water potential (Ψ s) on mycelial growth rate, since fig tissues are stressed for water due to severe drought conditions prevailing in California in the last several years.

Materials and methods

Effect of tissue age on canker development

In order to evaluate the effect of the tissue age on the disease development, three ages of the host tissues were considered in this experiment: 1) current shoots (non-lignified shoots), 2) 1-year-old shoots, 3) equal or older than 3-year-old shoots. According to previous results on variety susceptibility (Gusella et al., 2021), two varieties were evaluated: Sierra (more susceptible) and Calimyrna (less susceptible). Inoculations were conducted at the end of June in the KARE experimental fig orchard. In detail, a randomized block design scheme consisting of six replicates (i.e., six trees) per variety each formed by five shoots for each of the three ages was used in the experiment. To this aim, all fig shoot groups were inoculated with a 7-mm mycelial plug of *N. dimidiatum*, isolate 3G77, obtained from a 7-day-old culture and placed in a wound created

with a 7-mm cork borer by removing the bark of each shoot. Moreover, an equal number of branches/shoots inoculated with a PDA plug per wound as above served as control. The disease was recorded as average lesions length two months after the inoculations. Re-isolations were made to confirm the causal agent. Specifically, symptomatic tissues were surface disinfected with household bleach (Clorox Professional Product Company) at 10% (vol/vol) in sterile water for 3 min. Small pieces (3 to 5 by 2 to 5 mm²) from the margins of cankers were cut with a sterile scalpel and placed in Petri dishes containing 2% potato dextrose agar (PDA; Microtech Scientific, Orange, CA) acidified with lactic acid [2.5 ml of 25% (vol/vol) per liter of medium; APDA] to minimize bacterial growth. All the Petri dishes were incubated at 30 °C, for 2 to 7 days, until fungal colonies were large enough to be examined. Percentage of re-isolation was recorded for each plot.

Recovery of *Neoscytalidium dimidiatum* from fig mummies washing and inner tissues

A total of 100 mummies hanging from healthy trees were randomly collected from the KARE experimental fig orchard during May of 2020. Half of the mummies (50 fruit) were soaked in 300 mL of sterile distilled water (SDW) containing Tween 20 at 1% and shaken for 20 min on an orbital shaker. Total volume of 100 µL of the 1% (vol/vol) mummy washing solution was streaked onto Petri dishes containing 2% APDA to minimize bacterial growth and incubated at 25 °C until fungal colonies were large enough to be examined and identified. The remaining 50 fruit mummies were cut in small pieces (5 mm²) with a sterile scalpel to expose the inner tissues, surface disinfected with household bleach (Clorox Professional Product Company) at 10% (vol/vol) in sterile water for 3 min, placed in APDA. Petri dishes, and the diseases were incubated at 25 ± 2 °C for 3 to 7 days until fungal colonies were large enough to be examined.

Ability to colonize dried fig fruits at different temperatures under laboratory conditions

In order to investigate the ability of *N. dimidiatum* to colonize the fruit tissues, a total of 84 dried figs were used to simulate the fruit left in the field to become mummies. Dried figs were firstly soaked in

SDW for 20 min to allow a partial rehydration of the tissues. After the rehydration, fruits were airdried on top of clean paper towels in a laboratory laminar hood. Fruits were then sterilized twice by dipping in a 70% ethanol solution for 1 min and airdried again in a laboratory laminar hood. Once completely dried, seven fruit per treatment were placed in plastic containers (30×23×10 cm) onto a plastic mesh (10×10×10 mm) to avoid the direct contact with water, and then inoculated by pipetting 500 µL of spore suspension (5×10⁴ arthrospores/mL) of *N. dimidiatum* isolate 2D3 from a 7-days-old culture grown on PDA, on the entire fruit surface. The bottom of each container was filled with 200 mL water to increase humidity. Control consisted of fruits inoculated only with SDW. Six different incubation temperatures were used in this experiment: 10, 15, 20, 25, 30, and 35 °C. Percentage (%) of fruits with *N. dimidiatum* mycelium was recorded for each temperature after 7 days of incubation. The experiment was repeated once.

In vitro effect of water potential (Ψs) on mycelial growth rate

The effect of Ψs on mycelial growth rate of *N. dimidiatum* in culture was determined on PDA. A 5-mm mycelial plug from the growing edges of a 5-days-old colony of isolate 2D3 was transferred to the center of PDA plates. To obtain six different Ψs values, PDA was amended with KCl or NaCl prior to sterilization, according to Robinson and Stokes (1959) for the following values: 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 -MPa. Non-amended PDA (0.3 -MPa) was used as control. Following inoculation, the plates were incubated at 30 °C. Five plates were used as replicates for each Ψs value of each salt. Two perpendicular diameters of the colonies were measured after 3 days of incubation. Means were converted to radial growth rate. The experiment was repeated once. Mycelial growth response to Ψs was analyzed among detected values to ascertain any growth reduction or increase.

Data analysis

Data from *in vitro* assays and in field experiments were analyzed by using the Statistica package software (version 10; Statsoft Inc., Tulsa, OK, USA). Therefore, the means of radial growth, growth rates and fig lesion of *N. dimidiatum*, were calculated, averaging the values

of single replicates for each treatment/ Ψ s concentration. ANOVA analyses of shoot lesion length and mycelial growth rate were performed by calculating F and P values associated to evaluate whether the effects of each tested factor (age, variety, salt, and water potential) and age \times variety and salt \times water potential interactions were significant. In the post-hoc analyses, treatment effects were evaluated, and the mean data were subsequently separated by the Tukey's significant difference test ($\alpha=0.05$).

Results

Effect of tissue age on canker development

Analysis of variance (ANOVA) on age and variety effects on disease revealed that only shoot age showed a significant effect against fig limb canker lesion, whereas age \times variety interactions and variety effects were both not significant (Table 1). Post-hoc analysis data on shoot age effects on disease are reported for single varieties in Table 2, although ranking of effects and disease pressures were similar on Sierra and Calimyrna varieties. Based on these data, the number of canker lesions caused by *N. dimidiatum* on ≥ 3 -year-old shoots were significantly higher than those on current and 1-year-old shoots. On the other hand, disease lesion levels detected for these latter shoots did not differ between them (Table 2). In current shoots, characterized by no-lignified tissues, black rounded lesions were observable on the bark (Fig. 1a) and in all inoculated shoots internal cankers extended acropetally and basipetally. Observations in the field revealed

Table 1 Analysis of variance for limb canker and dieback lesion of fig caused by *Neoscytalidium dimidiatum* inoculated on three ages (current, 1-year old and ≥ 3 -year-old) shoots of Sierra and Calimyrna varieties

Factor(s)	Average lesion (cm) by <i>Neoscytalidium dimidiatum</i> ^y		
	df	F	P value
Age	2	40.8502	<0.0001
Variety	1	0.3572	0.554531 ns
Age \times Variety	2	0.2726	0.763262 ns

^y df=degrees of freedom, F test of fixed effects, and P value associated to F; ns=not significant data

Table 2 Post-hoc analysis of shoot age effects on average dieback of three age shoots of Sierra and Calimyrna cultivars inoculated with *Neoscytalidium dimidiatum*

Treatment (age)	Variety ^y	
	Sierra	Calimyrna
Current shoots (<1-year-old)	3.52 \pm 0.30 b ^z	3.04 \pm 0.19 b
1-year-old shoots	4.06 \pm 0.42 b	4.27 \pm 0.99 b
≥ 3 -year-old shoots	18.01 \pm 2.76 a	15.80 \pm 2.86 a

^y Data are the means of 30 shoots for each age, i.e., 5 shoots per 6 trees (6 replicates) on two different cultivars followed by the standard error of the means (SEM). ^z Values [cm] followed by the different letters within each column are significantly different according to Tukey's honestly significance difference test ($\alpha=0.05$)

that sometimes the inoculated shoots wilted and died (Fig. 1b). Re-isolations showed that *N. dimidiatum* was consistently present in all the inoculated branches or shoots, but it was not found on the controls. Occasionally fungi belonging to the genera of *Alternaria*, *Aspergillus*, *Mucor*, and *Penicillium* were isolated as well.

Recovery of *Neoscytalidium dimidiatum* from fig mummies

Because a portion of the mummies hang on to trees during the season, we determined whether these mummified fruit would serve as inoculum source. Results of both assays showed the presence of different fungi belonging to the genera of *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Mucor*, *Neurospora*, *Penicillium* and *Rhizopus* (Fig. 1c). *N. dimidiatum* was not recovered either from mummy surfaces or inner tissue. In the mummy washings experiment, *Aspergillus niger* was the most prevalent species (100%), and isolations of inner fruit tissues revealed that *Cladosporium* spp. was the most prevalent (98.75%). Details of the fungal colony frequency (%) are shown in Table 3. These fungi were identified morphologically on the basis of culture and spore characteristics.

Ability to colonize dried fig fruits at different temperatures under laboratory conditions

The presence of *N. dimidiatum* mycelium, evaluated as percentage of fruits colonized by the fungus, was

Fig. 1 **a)** Current shoot inoculated with *Neoscytalidium dimidiatum*. Red arrow indicates the inoculation point. Two leaves close to the inoculation point are completely dead. **b)** 1-year-old shoot wilted and dead. Red arrow indicates the inoculation point. **c)** Fig mummy hanging from the tree completely covered by fungal saprophytes (spring 2020). **d)** *Neoscytalidium dimidiatum* growing from the peduncle to a dried fig fruit



found from 20 °C to 35 °C exposures as follows: 42% at 20 °C, 57% at 25 °C, 92% at 30 and 35 °C. Fruits incubated for 7 days at 10 and 15 °C did not develop mycelium of *N. dimidiatum*. Small amounts of mycelium were observed on fruits incubated at 20 °C compared to results at higher temperatures for which the mycelium was more abundant and dark gray. Interestingly, the mycelium of *N. dimidiatum* developed mainly on the peduncle of fruits, probably due to the lignification of the peduncle tissues (Fig. 1d).

Table 3 Fungi recovered from fig mummies collected from an experimental orchard at the Kearney Agricultural Research and Extension Center in Parlier, CA

Fungal genera/species	Mummies water suspension %	Mummies isolation %
<i>Alternaria</i>	0	5
<i>Aspergillus niger</i>	100	0
<i>Cladosporium</i>	0	99
<i>Fusarium</i>	33	0
<i>Mucor</i>	37	4
<i>Neoscytalidium dimidiatum</i>	0	0
<i>Neurospora</i>	0	2
<i>Penicillium</i>	23	0
<i>Rhizopus</i>	63	20

In vitro effect of different water potential (Ψ_s) on mycelial growth

The effect of salt, water potential (Ψ_s) and salt \times water potential interaction on *N. dimidiatum* growth rate were significant (Table 4). Thus, the data were presented and analyzed to compare effects of salts (NaCl and KCl) and within each salt considering effects of water potential (Ψ_s) values (0.3, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 -MPa) on mycelial growth rate (Fig. 2). These data showed that as *N. dimidiatum* growth rate decreased as water potential (Ψ_s) increased. These reductions were significant for NaCl salt from Ψ_s value of 1.0 -MPa, whereas for KCl salt, significant growth rate reductions

Table 4 Analysis of variance for average mycelial growth of *Neoscytalidium dimidiatum* on potato-dextrose agar amended with two salts (NaCl and KCl) at different water potential rate

Factor(s)	Mycelial growth of <i>Neoscytalidium dimidiatum</i> ^y		
	df	F	P value
Salt	1	740.90	<0.0001
Water potential (Ψ_s)	6	467.39	<0.0001
Salt \times Water potential	6	64.83	<0.0001

^y df = degrees of freedom, F test of fixed effects, and P value associated to F; ns = not significant data

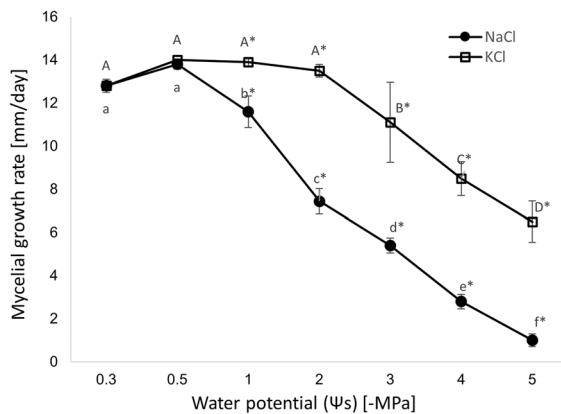


Fig. 2 Effects of water potential (Ψ_s) on the mycelial growth of *Neoscytalidium dimidiatum* on PDA amended with NaCl and KCl at various concentrations. Values followed by the same letter along salt line are not significantly different according to Tukey's honestly significance difference test ($\alpha = 0.05$), whereas * denotes significant point-by-point differences between two salts for each water potential (Ψ_s) value

were detected from Ψ_s value of 3.0 -MPa. Comprehensively, Ψ_s point-by-point differences showed as NaCl salt significantly reduced *N. dimidiatum* growth rate more than KCl salt from Ψ_s value of 1.0 -MPa (Fig. 2).

Discussion

Results of our study revealed new aspects of an emerging fig disease in California. Results obtained in this study represent a first starting point for the understanding of the biology and ecology of this fungus on fig. Regarding the ability to induce lesions on different age tissues, *N. dimidiatum*, infected all different tissues tested in the field but lesions on older branches (≥ 3 -year-old) were significantly greater than those developed in the shoots of lower age. Other studies regarding the effect of tissue age on Bot canker revealed similar results. In fact, Amponsah et al. (2014) showed that lesion development by *Neofusicoccum luteum* on grapevine was more rapid in older tissues than in younger tissues. Balci et al. (2008) demonstrated that lesions on trunks of 20-year-old oak trees infected by *Phytophthora* spp. were larger than those on 1–2-year-old seedlings. On the other hand, other studies showed no differences in lesion length based on tissue age (Morales et al., 2012; Úrbez-Torres & Gubler, 2011). In contrast, in the case

of walnut shoots inoculated with *N. mediterraneum*, one- to two-year-old shoots were more susceptible than 3- to 4-year-old shoots (Agustí-Brisach et al., 2019). Experimental results seem to do not agree, and this is probably due to different factors like the pathogen species, the host, the cultivar etc. In our case, presence of longer lesions on older branches could be explained by the massive presence of arthrospores commonly found on the old limbs compared to the young shoots during our field surveys.

Neoscytalidium dimidiatum was not recovered from the mummies collected in the orchard. As expected, other fungal species, mostly *Aspergillus niger*, *Cladosporium* spp., *Penicillium* spp., and *Rhizopus* spp., were isolated from mummy surface and from inner tissues as well. Mummified fruit represent an important source of inoculum for some fungal diseases, like the brown rot of stone fruit (Casals et al., 2015; Holtz et al., 1998; Ogawa & English, 1991) and olive anthracnose (Moral & Trapero, 2012). In the case of brown rot, winter survival of the spores and adequate climatic conditions are essential to allow mummies to be the source of primary inoculum (Casals et al., 2015). The carpo-stromosphere represents an important ecological niche for many microorganisms. Recent investigations on mummified peach fruits revealed the presence of 197 fungal species and 283 bacteria inhabiting the mummies (Jo et al., 2020). Previous studies conducted in California on the mycoflora of stone fruit mummies revealed the presence of many species belonging to the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botrytis*, *Cladosporium*, *Fusarium*, *Gilbertella*, *Monilinia*, *Mucor*, *Penicillium*, *Rhizopus* and *Trichoderma* (Hong et al., 2000). For brown rot disease, other colonizing fungi on the mummy surface can inhibit the primary inoculum due to different mechanisms such as substrate competition, niche exclusion, and antibiosis (Blakeman & Fokkema, 1982; Ogawa & English, 1991). As demonstrated by Hong et al. (2000), lower recovery of *M. fructicola* on mummies was associated with the presence of other inhabiting fungi such as *Mucor*, *Penicillium*, *Rhizopus* and *Trichoderma*. Regarding Botryosphaeriaceae, species belonging to this family usually overwinter also on fruit mummies, differentiating their asexual structures (pycnidia) on mummified surface (Moral et al., 2019). In our experiment and in field survey observations, the presence of other inhabiting fungi on the mummies during the

fall and winter probably inhibited the growth of *N. dimidiatum* which is considered a warm temperature pathogen. Moreover, although it was able to grow under a range of temperature (20–35 °C) on dried figs in the laboratory, we confirmed that mummies do not represent the ideal substrate to serve as the primary inoculum source of this pathogen in the field. On the other hand, as demonstrated in a previous study (Gusella et al., 2021) and confirmed after new field surveys, presence of black masses of arthroconidia under the naturally peeled off bark can be considered the most relevant source of primary inoculum in the field.

The experiment of fruit colonization under a range of temperatures revealed that the highest percentage of colonization was at 30 °C, confirming the fact that this species prefers high temperature, as demonstrated also by its geographical distribution in regards to the annual temperature means (Batista et al., 2021). In vitro responses to different Ψ s revealed that *N. dimidiatum* mycelial growth rate decreased with lower Ψ s, showing differences for the two osmotica tested, probably due to higher toxicity of NaCl than KCl. Results of our experiments of the decrease of mycelial growth rate with lower values of Ψ s are in accordance with previous studies (Boddy, 1983; Kaiser & Bruehl, 1996; Whiting & Rizzo, 1999; Wilson & Griffin, 1979). Regarding *Botryosphaeriaceae* species, drought is one of the most important stress factors for disease development, especially in causing nutritional deficiencies and impeding plant defenses (Mehl et al., 2013). Studies conducted in vitro as well as in vivo in California regarding the relationship between drought stress and the *Botryosphaeria* panicle and shoot blight of pistachio, showed that the mycelial growth of *B. dothidea* increased as Ψ s decreased from 0 to 2.0 -MPa and declined as Ψ s decreased below 2.0 -Mpa in laboratory conditions, and the severity of the disease increased under water stress conditions (Ma et al., 2001). Similarly, diseases caused by *Botryosphaeriaceae* have been reported to increase under severe water stress in other hosts, such as apple, sweetgum, European white birch, peach, dogwood, and almond (Hutton, 1958; Neely, 1968; Crist & Schoeneweiss, 1975; Pusey, 1989; Mullen et al., 1991; Agustí-Brisach et al., 2020). Our laboratory results are not in accordance with what observations of Ma et al. (2001) for *B. dothidea*, but we do not exclude at all the possibility that *N. dimidiatum* infections in the fields could be seriously aggravated by drought stress. As

observed in a previous study (Gusella et al., 2021), different stress factors severely influence infections by *N. dimidiatum*. Moreover, studies conducted by Agustí-Brisach et al. (2020) on almond branch dieback and decline revealed that symptoms were observed mainly in the experimental plots of trees irrigated with less amount of water, with consistency of fungal isolation (mainly *Botryosphaeriaceae*) increasing with the disease severity.

More studies need to be carried out in order to better understand the dynamics of diffusion and development of *N. dimidiatum* in fig orchards. Understanding the epidemiology of this fungus will be crucial to develop precise management strategies in the orchard. Our results represent another step in building our knowledge on this important disease of fig in California.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval The authors declare that ethical standards have been followed and that no human participants or animals were involved in this research.

Consent to participate Not applicable.

Consent for publication Consent for publication was obtained from all co-authors.

Conflicts of interest/competing interests The authors declare that no conflicts of interest exist.

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