Investigating the causes of widespread *Acacia* spp. mortality in the San Francisco Bay Area.

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for

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Introduction

In October 2020, many reports of dying Acacia were circulated, primarily *Acacia melanoxylon* (blackwood Acacia) in Oakland, Pt. Richmond, Crockett and other Bay Area locations. The observed Acacia die-off was highly unusual: groups or even entire stands of trees appeared to die-off rather rapidly, showing bright, burnt-orange crowns. Upon examination, Acacia of all conditions could be found in the vicinity of the dying trees, some green, others yellowing, some with dead branches. What appeared to be older dead Acacia were also present.
The SFPUC reported dying Acacia in the Trousdale area, near Burlingame, (San Mateo Co.) in an area scheduled for restoration in February 2021. The SPFUC is concerned that if a new, invasive pathogen was determined to be the cause of this die-off the project could be delayed, or the pathogen could spread to other species that will be planted in the area.

Goals

In order to enable management of this new tree disease concern we set out to determine its cause by answering the following questions.

Are there one or more infectious agents responsible for the reported Acacia mortality? Is it possible the same agents may also be causing mortality of other species, particularly blue gums, *Eucalyptus globolus*? Are these agents native or exotic? Whether native or non-native, can we identify or suggest their origin? Are the agents primary, aggressive pathogens, or are they pathogens thriving on plants stressed by environmental conditions? Is it possible that multiple types of

organisms may be accelerating the mortality rate of plants? Could this be just the effect of abiotic stresses or are biotic agents involved? If biotic causes are identified, can we make management recommendations based on the biology of the microbes involved?

Materials and methods

Although mortality of several tree species has been reported, we focused on two Acacia species, given they do appear to be the trees experiencing the most severe and geographically widespread symptoms. Focusing on one host increases our chances of identifying agents involved in the mortality. The main focus of the study was blackwood acacia, but occasionally silver wattle (*Acacia dealbata*) was also sampled.

The project was conducted in San Mateo, Alameda and Contra Costa Counties, with lab analysis done in Alameda County at the UC Berkeley Laboratory of Forest Pathology and Mycology. A total of 5 sites (Leona Heights, Montclair, Dimond Canyon, Carquinez strait, SFPUC) in 4 distinct locations were sampled. Eight trees per location were sampled in locations 1-3, while six trees were sampled in Location 4 (Dimond Canyon).

Samples were collected from Acacia at various stages of decline, but not yet dead, for the identification of associated fungi. Trees were evaluated for the presence of cankers or any other symptoms of ill health. Stems, twigs, roots were evaluated for the presence of pathogens by directly plating out symptomatic tissue onto several types of media (MEA, acidified PDA, Fusarium specific medium, Leptographium medium, Phytophthora PARP medium) and on washed carrot disks. Tissue containing both necrotic and healthy portions was the main focus of our sampling effort. Each sample was plated on all media and on carrots in duplicate, with one replicate bleached in 10% bleach for 30 seconds and washed for a minute in sterile water and one sample plated without surface sterilizations. All samples were collected well inside the plant tissue to minimize surface microbial contamination.

Besides sampling and direct plating of symptomatic tissue, declining trees were also tested for the presence of *Phytophthora* by baiting the soil collected near them with three different baits, namely pears, oregano stems and "Cunningham's white" rhododendron leaves. In brief, soil was dried at room temperature before being rewetted and placed at 8-10 C for 48 hours to stimulate sporangia production. Water was then added so that the soil would be submerged by one inch of water and the three baits were placed in each bag, ensuring they were only partially submerged. Baits were inspected at 3, 7 and 10 days, and any visible lesion was plated on PARP. Molecular and microscopic evaluations of any recovered fungi were made to identify the species and assess its risk to trees and shrubs. Molecular identification was based on ITS sequence and, for groups of fungi of interest, the EF-

alpha and the Histone 3 loci were additionally sequenced to obtain a more precise species ID.

Koch's postulates will be conducted to prove pathogenicity of fungi that were found repeatedly in multiple trees and/or locations. Koch's postulate will be completed by inoculating the most frequently isolated fungi on healthy potted seedlings. On March 15th 2021, we inoculated healthy trees in the SFPUC study site **using only local fungal isolates**. These trees, potentially stressed, may better allow us to infer the role played by the fungi isolated from them. A total of 12 small trees were wound inoculated using agar plugs colonized by putative pathogens, while four were mock-inoculated and will serve as controls (see below for details). We will take down the experiment in 6 – 8 weeks.

Cultures' identifications were done at the genus level using morphology and at the species level by using the DNA sequence of the barcode locus ITS for a select number of isolates. ITS sequences were used in two different ways to identify number and name of species involved: 1- Intrageneric or closely related genera sequences for fungi identified in multiple locations were aligned on Geneious and NJ trees were built using such alignments. The number of clades (branches) was used to indicate the number of species or operational taxonomic units (OTUs). 2- Sequences from each clade were compared to sequences deposited in GenBank using the BLAST function, to obtain a putative species ID. For each clade/OUT. When necessary EF-alpha or Histone 3 sequences were also BLASTed against the public database to confirm species ID.

Results

1- A total of 81 soil or tree samples were collected and/or analyzed during the study (See Table 1 for details).

Table 1. List of samples obtained and examined by culturing.

ADP#	date sampled	Site	tree	Sample Type	Spp na
1				from Igor Lacan	
2				from Igor Lacan	Na
3				from Igor Lacan	Na
4				from Igor Lacan	Na
5	12/8/20	1	1	long stem canker	silver wattle

6	12/8/20	1	1	soil	silver wattle
7	12/8/20	1	1	root flare	silver wattle
8	12/8/20	1	2	rootlets for agdia	silver wattle
9	12/8/20	1	2	root piece healthy green	silver wattle
10	12/8/20	1	2	stem above canker	silver wattle
11	12/8/20	1	2	stem below canker	silver wattle
12	12/8/20	1	2	soil	silver wattle
13	12/8/20	1	3	roots	silver wattle
14	12/8/20	1	3	stem above canker	silver wattle
15	12/8/20	1	3	stem below canker	silver wattle
16	12/8/20	1	3	soil	silver wattle
17	12/8/20	1	4	stem	black acacia
18	12/8/20	1	4	rootlets	black acacia
19	12/8/20	1	4	soil	black acacia
20	12/8/20	1	5	roots	black acacia
21	12/8/20	1	5	stem above canker	black acacia
22	12/8/20	1	5	stem below canker	black acacia
23	12/8/20	1	5	soil	black acacia
24	12/8/20	1	5	leaves with dieback	black acacia
25	12/8/20	1	6	root collar, bark	black acacia
26	12/8/20	1	6	stem below canker 5'	black acacia
27	12/8/20	1	6	stem below canker 3'	black acacia

28	12/8/20	1	6	under soil	black acacia
29	12/8/20	1	6	soil	black acacia
30	12/8/20	1	7	stem canker	black acacia
31	12/8/20	1	7	stem canker	black acacia
32	12/8/20	1	7	soil	black acacia
33	12/8/20	1	8	stem canker	black acacia
34	12/8/20	1	8	stem canker	black acacia
35	12/8/20	1	8	stem canker	black acacia
36	12/8/20	1	8	soil	black acacia
37	12/10/20	2	1	soil	black acacia
38	12/10/20	2	1	branch	black acacia
39	12/10/20	2	2	soil	black acacia
-	-	2	2	stem	out of order. It is ADP#50
40	12/10/20	2	3	soil	black acacia
41	12/10/20	2	3	stem	black acacia
42	12/10/20	2	4	soil	black acacia
43	12/10/20	2	4	side branch 1 & 2	black acacia
44	12/10/20	2	5	soil	black acacia
45	12/10/20	2	6	soil	black acacia
46	12/10/20	2	6	stem	black acacia
47	12/10/20	2	7	soil	black acacia
48	12/10/20	2	7	root collar	black acacia

49	12/10/20	2	8	soil	black acacia
50	12/10/20	2	2	stem	black acacia
51	12/14/20	3	1	soil	black acacia
52	12/14/20	3	1	root collar	black acacia
53	12/14/20	3	2	soil	black acacia
54	12/14/20	3	2	root collar nr surface	black acacia
55	12/14/20	3	2	root collar deeper in	black acacia
56	12/14/20	3	3	soil	black acacia
57	12/14/20	3	3	root collar stain	black acacia
58	12/14/20	3	4	soil	black acacia
59	12/14/20	3	4	root collar	black acacia
60	12/14/20	3	5	soil	black acacia
61	12/14/20	3	5	small side root	black acacia
62	12/14/20	3	6	soil	black acacia
63	12/14/20	3	6	side root	black acacia
64	12/14/20	3	6	root collar	black acacia
65	12/14/20	3	7	soil	black acacia
66	12/14/20	3	7	root	black acacia
67	12/14/20	3	8	soil	black acacia
68	12/14/20	3	8	side root	black acacia
69	1/13/21	4	1	root collar/base of tree	Bay
70	1/13/21	4	1	soil	Bay

71	1/13/21	4	1	branch	Bay
72	1/13/21	4	2	branch canker	Black acacia
73	1/13/21	4	2	soil	Black acacia
74	1/13/21	4	2	branch #2	Black acacia
75	1/13/21	4	3	base	Black acacia
76	1/13/21	4	3	soil	Black acacia
77	1/13/21	4	4	side root	Black acacia
78	1/13/21	4	4	soil	Black acacia
79	1/13/21	4	5	branch	Black acacia
80	1/13/21	4	5	soil	Black acacia
81	1/13/21	4	6	branch	Black acacia
82	1/13/21	4	6	soil	Black acacia

Sites:
1= Leona Heights and Montclair (Alameda Co.)
2= Carquinez (Contra Costa Co.)
3= SFPUC (San Mateo Co.)
4= Dimond Canyon (Alameda Co.)

Figure 1. Location of study sites. Note that location here is approximate, the exact location of all samples trees and all sampling locations is provided in a KMZ file below.

Figures 2 and 3. Trees dead and declining, found at the sampling sites and close ups of necrotic tree tissue.

Figure 2

Figure 3

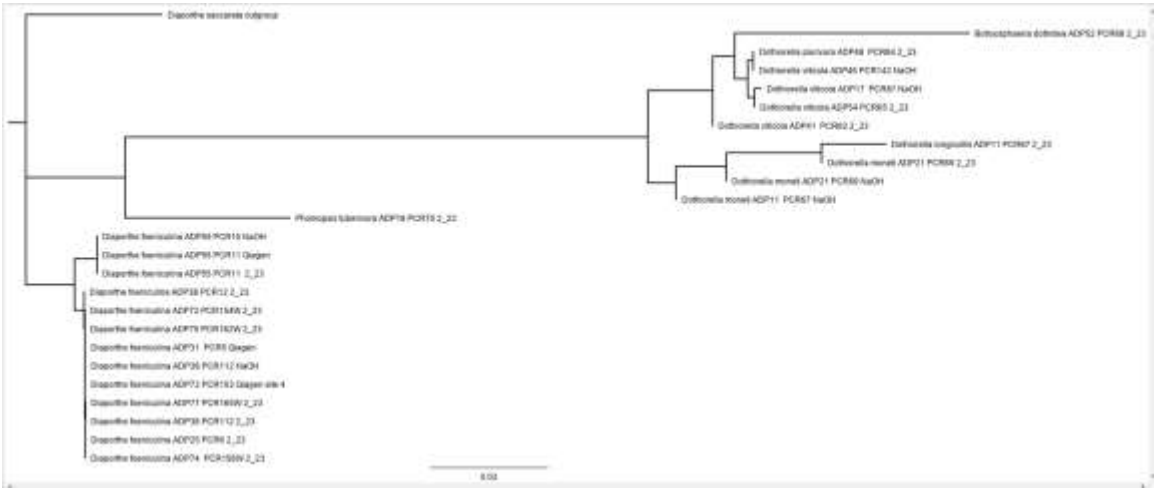
2- Approximately 200 cultures were obtained. Half of them based on the expertise of the laboratory and common knowledge were discarded as being either environmental contaminants or ubiquitous fungi with no known pathogenic action on plants. A total of 99 remaining cultures were then identified at the genus (45) and species (54) levels. Note that these 99 cultures do contain some duplicates obtained from the same sample, either by using different media or by using DNA extraction techniques.

3- **Only two groups of fungi, ecologically similar, were identified at all sites**, providing an important clue on the possible causation of the observed mortality in Acacia. The groups identified in all sites can be referred to as comprising a large number of endophytic fungi, that is fungi belonging to the genus *Botryosphaeria sensu lato* and to *Diaporthe*. The genus *Botryosphaeria sensu lato*, although still in use, is known to actually comprise multiple genera, and is currently split in 6+ genera based on the morphology of the conidia (asexual fungal spores) and on DNA sequence phylogenetic placement. On acacias in the study sites, we identified two species of *Dothiorella* and one species of *Diaporthe* (possibly split into two lineages/subspecies/OTUs). In the KMZ file available at the following URL:

https://drive.google.com/file/d/1dBkvQN4OwuwZDVmBo6Xe1iDq917hA1BA/view?usp=sharing

by zooming in it is possible to visualize results for each individual tree. All trees fit in one of four categories: negative (no fungi isolated and not visible on the map); *Diaporthe*/*Dothiorella* isolated from them; *Umbelopisis* isolated from them; only contaminants or fungi of uncertain effect isolated. A closer look shows some very important patterns: 1) trees are infected either by *Diaporthe* or by *Dothiorella* (only one tree has both); 2) *Fusarium* spp. and *Mortierella* spp. are isolated **only** from trees infected by *Diaporthe* or *Dothiorella (a Fusarium,* probably soilborne was isolated only once from a tree without *Diaporthe or Dothiorella*; 3) There are several trees infected by *Diaporthe* and *Dothiorella* that are not infected by *Fusarium, Mortierella* or *Umbelopsis.* This more detailed analysis confirms the primary role played by *Diaporthe* and *Dothiorella* in causing mortality, even if possibly on trees predisposed to infection by other factors (see below). It also appears that the two may be partitioning their respective niches, given that their co-occurrence on the same tree seems rare. The role of *Umbelopsis* remains questionable and there is absolutely no literature on the ecology and lifestyle (pathogenic vs. saprobic, endophytic or mycorrhizal) of this genus of zygomycetes. However, *Umbelopsis* was only found in the SFPUC site, suggesting it does not have a generalized role in the reported *Acacia* spp. mortality.

Figure 4. Neighbor Joining tree showing the presence of two OTUs on acacia for *Dothiorella* and one for *Diaporthe*, with the possible presence of a *Diaporthe* variant, maybe at the subspecific level or maybe a very closely related species. *Diaporthe saccarata* (sequence from GenBank) was used as an outgroup. A single *Botryosphaerya dothidea* was also identified and is of limited interested as well as a single *Phomopsis tuberivora*. Histone 3 sequences confirmed the identity of the entire *D. viticola* clade, while EF-alpha confirmed the identity of the entire *D. foeniculina* clade, including the variants. See Table 2 for species information.



Given the consistent presence of *Dothiorella* or *Diaporthe* in association with all studied tree mortality, we conclude these fungi play a significant role in the observed decline and death of acacias. However, it should be noted that the biology of both these fungi is "mixed". They all start as endophytes, without any obvious effect of tree health, they often become pathogens-some relatively aggressive- in conjunction with the onset of predisposing factors stressing trees (drought, limitation in resources due to high stand density, old age) and then survive as saprobes on the wood of the trees they killed. Some aspects of the biology of these fungi deserve attention: 1- Infection is positively correlated with abundant rainfall: thus, we expect that the record-breaking rainfall of 2017 resulted in widespread infection by these fungi; 2- The endophytic phase can last from 1 to 30+ years, meaning that disease development is almost never immediate: even in the presence of stress we expect a minimum of a 2-year lag between infection and disease expression: timing of the observed disease development is consistent with this timeline; 3- Disease is density dependent, so we expect to see more mortality where

plants are in thick clusters and monospecific stands, rather than in isolated trees: this has clear management implications ; 4- Sporulation is positively correlated with amount of dead and decaying matter: this has obvious implications for management.

Figure 5. Late stage (left) and early stage (right) of "wedge" shaped wood staining associated with *Dothiorella* infection in our study match the symptoms reported in the literature.

4- Are these *Botryosphaeria* and *Diaporthe* species native or exotic and where do they come from?

We have uncovered both native and introduced pathogens in this study. Given the complex taxonomy of the species involved, comprising many groups of multiple closely related species, this question is difficult to answer. *Diaporthe foeniculina* and *Dothiorella viticola* have a global distribution and have been reported from California multiple times, suggesting they are either native or naturalized in California. What we are observing here is most likely a host jump, given that both species are known as generalists. The host jump may have occurred in the course of several years and may have happened either directly from hosts already known in California or through an intermediate host. In either case, it is likely to have been favored by the high density of hosts (this is a simple statistical inference: the greater the pool of hosts the more likely they will be eventually infected) combined with prolonged wet conditions in 2017, followed by generalized stress conditions including high stand density/basal area as plants are growing and self-propagating, drought (2019 and 2020 both were below average for precipitation levels), smoke

(?). One notable exception is that of *Dothiorella moneti/santali:* these are highly host specific pathogens **only reported from *Acacia* spp. in Australia**. This fungus most likely arrived hitchhiking on acacia stock imported from that continent. At this point we only found it in Leona Heights, but further sequencing will indicate whether it may be present in other sites. Whether abundant or rare, **we can already state this specific pathogen to be exotic and from Australia. This is the first report of this species outside Australia.**

Table 2 below reports what we know about the three species identified in this study in terms of host and geographic range. Beware that these reports may be inaccurate due to the complex taxonomy.

Table 2. Various information about two *Dothiorella* and *Diaporthe* species isolated in 2020/2021 from declining and dying acacias in the SF Bay Area.

Species	**Reported in California**	**California hosts**	**Where else reported**	**Hosts outside California**	**Confidence in Species ID**
Dothiorella moneti/ santali	No	Na	Australia	*Acacia rostrellifera, Santalum*	Medium
Dothiorella viticola	Yes	*Vitis vinicola, Citrus sinensis*	South Africa, Australia, China, Tunisia	*Vitis, Podocarpus, Prunus, Juglans, Citrus, Vachellia*	High
*Diaporthe foeniculina** **maybe includes two very closely related species*	Yes	*Citrus latifolia, Citrus limon, Salix sp., Vitis vinifera*	Southern Europe, Germany, Serbia South Africa, Uruguay, New Zealand	*Citrus, Cupressus, Diospyrus, Foeniculum Ficus, Fuchsia, Glycine, Hemerocallis, Juglans, Lumaria, Malus, Melilotus, Microcitrus, Paraserianthes, Persea, Pyrus, Prunus, Rhus, Ribes, Rosa, Salix, Vaccinum,*	Medium/High

				Vicia, Vitis, Wisteria	

5- Are there other fungi involved in Acacia mortality? *Diaporthe foeniculina* and *Dothiorella viticola* are both present in all four sites. Both are reported as causing wood cankers on their hosts, consistent with the symptoms observed. In individual sites, we do find other fungi, taxonomically and ecologically unrelated to *Dothiorella* and *Diaporthe,* that may further accelerate tree decline. One interesting finding is that of zygomycetes found in association with necrotic roots; the interest stems from the fact these fungi are considered as being either beneficial vescicular arbuscular mycorrhizae (VAMs) or competitors of soilborne pathogens. These were found in all sites, but the OTU in each site was different. These additional fungi may in part explain the variety of symptoms observed in the cambium and xylem of branches, stems and roots of declining trees. There is a single report of *Mortierella elongata* being a pathogen of Avocado, possibly supporting this hypothesis.

The important things to note is that these fungi:

a)- Are site-specific and not widespread
b)- Have a complex taxonomy, with some taxa being reported as pathogens and others not. Is it possible that these organisms, usually beneficial may be secondary pathogens on trees with disease caused by *Diaporthe* and *Dothiorella* ?
c) With the exception of *Umbelopsis* at a single site, they are only isolated from trees infected by *Diaporthe* or *Dothiorella*: this is a pattern in agreement with a secondary role of these organisms capable of infecting trees attacked by other pathogens. On the contrary, as stated in section 3 above, *Diaporthe* or *Dothiorella* can be isolated from trees that did not yield and of these "secondary" pathogens.

Below, is a list of the fungi that may additionally contribute to tree decline. I am interested in performing Koch's postulate using *Fusarium solani, Fusarium sarcochroum, Mortierella elongata* and *Umbelopsis ramanniana*.

Table 3. Fungi that may further accelerate acacia decline

Species	**Plant part affected**	**Symptoms**	**Present in California**	**Host(s)**	**Reported as pathogen**	**ID confidence**
Fusarium oxysporum	Rootlets	n/a	Yes	Many	Yes	Low
Fusarium solani	Stem	Canker	Yes	Many	Yes	Low
Fusarium sarcochrum	Stem	Canker	No	Many	Yes	Low
Mortierella elongata	Roots/ Soil	Roots?	Yes?	Many	Once on Avocado	High

Mortierella hialina	Roots	Endophyte	?	Many	Beneficial	High
Umbelopsis ramanniana	Roots and root collar	Staining	Yes?	Tanoak, conifers	? Xylem colonization	High

6- Where other fungi isolated in the four study sites? Yes, a large number of fungi was isolated. Not including common contaminants, we isolated yeasts, entomophagic fungi, mycoparasitic fungi, saprobes, etc. Many are interesting, and may represent first reports for California, but are not likely to be involved in acacia mortality, hence they are not discussed here. The complete list of fungi isolated is presented in Table 4, below.

Site	Tree	Tree	Sample Origin	B or U	Bait	Seq Results	ID by seq or morph
3	4	Black Acacia	Root collar	Bleached		Absidia heterospora	Sequence
2	7	Black Acacia	Root collar	Unbleached		Absidia pararepens	Sequence
3	2	Black Acacia	Root collar near surface	Unbleached		Absidia pararepens	Sequence
1	4	Black Acacia	Stem	Bleached		Arthrinium kogelbergense	Sequence
1	2	Silver Wattle	Stem below canker	Unbleached		Aureobasidium pullulans	Sequence
1	2	Silver Wattle	Stem below canker	Unbleached		Aureobasidium pullulans	Sequence
1	3	Silver Wattle	Below stem canker	Bleached		Aureobasidium pullulans	Sequence
4	1	Bay laurel	Bay tree root collar base	Bleached		Cladosporium puyae	Sequence
1	1	Black Acacia	Root flare	Bleached		Clonostachys rosea/Hypomyce	Sequence
1	1	Black Acacia	Root flare	Bleached		Clonostachys solani	Sequence
1	1	Black Acacia	Root flare	Bleached		Clonostachys solani	Sequence
1	8	Black Acacia	Stem canker	Bleached		Clonostachys sp./rosea	Sequence
2	2	Black Acacia	Soil		pear	Cordyceps confragosa	Sequence
2	2	Black Acacia	Soil		pear	Cordyceps confragosa	Sequence
2	7	Black Acacia	Root collar	Bleached		Cylindrodendrum hubeiense/Ne	Sequence
1	7	Black Acacia	Stem canker 5' AGL	Unbleached		Diaporthe foeniculina	Sequence
3	2	Black Acacia	Root collar deep	Bleached		Diaporthe foeniculina	Sequence
3	2	Black Acacia	Root collar deep	Bleached		Diaporthe foeniculina	Sequence
2	1	Black Acacia	Branch	Unbleached		Diaporthe foeniculina	Sequence
4	2	Black Acacia	Branch canker	Unbleached		Diaporthe foeniculina	Sequence
1	5	Black Acacia	Stem above canker	Bleached		Dothiorella moneti	Sequence
1	2	Silver Wattle	Stem below canker	Unbleached		Dothiorella moneti/Botryosphae	Sequence
2	6	Black Acacia	Stem	Unbleached		Dothiorella viticola	Sequence
2	6	Black Acacia	Stem	Unbleached		Dothiorella viticola/Botryosphae	Sequence
1	4	Black Acacia	Stem	Unbleached		Dothiorella viticola/Spencermar	Sequence
1	6	Black Acacia	Stem below canker (oute	Unbleached		Epicoccum italicum	Sequence
3	2	Black Acacia	Root collar near surface	Unbleached		Epicoccum italicum	Sequence
1	1	Black Acacia	Long stem canker	Unbleached		Epicoccum nigrum	Sequence
4	3	Black Acacia	Base of tree	Unbleached		Epicoccum nigrum	Sequence
1	8	Black Acacia	Stem canker	Unbleached		Fusarium lateritium	Sequence
1	4	Black Acacia	Rootlets	Unbleached		Fusarium oxysporum	Sequence
1	4	Black Acacia	Rootlets	Unbleached		Fusarium oxysporum	Sequence
1	4	Black Acacia	Rootlets	Unbleached		Fusarium oxysporum	Sequence
1	7	Black Acacia	Stem canker 5' AGL	Bleached		Fusarium sarcochroum	Sequence
1	7	Black Acacia	Stem canker 5' AGL	Bleached		Fusarium sarcochroum	Sequence
1	7	Black Acacia	Stem canker 5' AGL	Bleached		Fusarium sarcochroum	Sequence
1	6	Black Acacia	Stem below canker (oute	Bleached		Fusarium solani	Sequence
1	6	Black Acacia	Stem below canker (oute	Bleached		Fusarium solani	Sequence
3	3	Black Acacia	Root collar stain	Bleached		Geomyces sp./Geomyces pann	Sequence
1	1	Black Acacia	Soil		pear	Malassezia Globosa	Sequence
1	7	Black Acacia	Soil		pear	Mortierella elongata	Sequence
1	7	Black Acacia	Soil		pear	Mortierella elongata	Sequence
1	7	Black Acacia	Soil		pear	Mortierella elongata	Sequence
1	7	Black Acacia	Soil		pear	Mortierella elongata	Sequence
1	6	Black Acacia	Stem below canker (oute	Unbleached		Mortierella hyalina	Sequence
1	6	Black Acacia	Stem below canker (oute	Unbleached		Mortierella hyalina	Sequence
1	6	Black Acacia	Stem below canker (oute	Unbleached		Mortierella hyalina	Sequence
1	6	Black Acacia	Stem below canker (oute	Unbleached		Mortierella sp./ hyalina	Sequence
1	8	Black Acacia	Soil		oregano	Mortierella sp./Mortierella elong	Sequence
3	3	Black Acacia	Root collar stain	Unbleached		Umbelopsis ramanniana	Sequence
3	3	Black Acacia	Root collar stain	Unbleached		Umbelopsis ramanniana	Sequence
4	2	Black Acacia	Branch	Bleached		Verrucoconiothyrium sp./Coniot	Sequence
4	5	Black Acacia	Branch	Unbleached		Verrucoconiothyrium sp./Coniot	Sequence
3	7	Black Acacia	Root	Unbleached		Verrucoconiothyrium sp/Coniotl	Sequence
1	6	Black Acacia	Root collar bark whitish r	Bleached		Diaporthe	Morphology
1	8	Black Acacia	Stem canker	Bleached		Diaporthe	Morphology
3	2	Black Acacia	Root collar near surface	Unbleached		Diaporthe	Morphology
3	2	Black Acacia	Root collar near surface	Unbleached		Diaporthe	Morphology
2	1	Black Acacia	Branch	Bleached		Diaporthe	Morphology
2	1	Black Acacia	Branch	Bleached		Diaporthe	Morphology
2	1	Black Acacia	Branch	Unbleached		Diaporthe	Morphology
1	7	Black Acacia	Stem canker 3' AGL	Bleached		Verrucoconiothyrium sp/Cor	Morphology
3	7	Black Acacia	Root	Bleached		Verrucoconiothyrium sp/Cor	Morphology
1	6	Black Acacia	Stem below canker (oute	Unbleached		Verrucoconiothyrium sp/Cor	Morphology
2	6	Black Acacia	Stem	Unbleached		Geomyces	Morphology
1	7	Black Acacia	Stem canker 5' AGL	Bleached		Geomyces	Morphology
1	2	Silver Wattle	Root piece healthy and g	Unbleached		Geomyces	Morphology
1	8	Black Acacia	Stem canker	Bleached		Fusarium	Morphology
1	7	Black Acacia	Stem canker 3' AGL	Unbleached		Fusarium	Morphology
3	2	Black Acacia	Root collar near surface	Bleached		Fusarium	Morphology
3	4	Black Acacia	Root collar	Bleached		Umbelopsis	Morphology
3	5	Black Acacia	Small side root	Unbleached		Umbelopsis	Morphology
3	5	Black Acacia	Small side root	Bleached		Umbelopsis	Morphology
1	6	Black Acacia	Root rhizomorphs	Bleached		Absidia	Morphology
3	8	Black Acacia	Side root	Unbleached		Absidia	Morphology
1	3	Silver Wattle	Roots	Bleached		Absidia	Morphology
1	3	Silver Wattle	Roots	Bleached		Absidia	Morphology
1	5	Black Acacia	Roots	Unbleached		Absidia	Morphology
1	5	Black Acacia	Roots	Unbleached		Absidia	Morphology
1	6	Black Acacia	Root collar bark whitish r	Unbleached		Absidia	Morphology
2	3	Black Acacia	Stem	Bleached		Dothiorella	Morphology
2	3	Black Acacia	Stem	Unbleached		Dothiorella	Morphology
2	6	Black Acacia	Stem	Bleached		Dothiorella	Morphology
3	2	Black Acacia	Root collar near surface	Unbleached		Dothiorella	Morphology
3	1	Black Acacia	Root collar	Unbleached		Dothiorella	Morphology
1	4	Black Acacia	Rootlets	Unbleached		Dothiorella	Morphology
3	1	Black Acacia	Root collar	Bleached		Dothiorella	Morphology
1	6	Black Acacia	Root rhizomorphs	Unbleached		Mortierella	Morphology
3	8	Black Acacia	Side root	Unbleached		Arthrinium	Morphology
1	1	Black Acacia	Root flare	Bleached		Arthrinium	Morphology
1	5	Black Acacia	Leaves with dieback			Verrucoconiothyrium sp/Cor	Morphology
1	5	Black Acacia	Leaves with dieback	Unbleached		Verrucoconiothyrium sp/Cor	Morphology
1	2	Silver Wattle	Root piece healthy and g	Bleached		Absidia	Morphology
1	5	Black Acacia	Roots	Unbleached		Dothiorella	Morphology
3	6	Black Acacia	Side root	Bleached		Aureobasidium	Morphology
1	2	Silver Wattle	Root piece healthy and g	Bleached		Absidia	Morphology
1	6	Black Acacia	Root rhizomorphs	Bleached		Arthrinium	Morphology
1	8	Black Acacia	Stem canker	Unbleached		Diaporthe	Morphology
3	8	Black Acacia	Side root	Bleached		Fusarium	Morphology

Table 4. Complete list of fungi isolated and identified in this study.

7- Confirming our hypotheses through controlled inoculation studies.

We have mentioned above the possibility (i.e. hypothesis) that some fungi consistently isolated from declining Acacias may be primary pathogens, responsible for the observed decline. We also had a secondary hypothesis that climate, mostly water deficiency associated with drought, higher temperatures and less fog, may be exacerbating the virulence of these, pathogens, for the most part known to co-exist "peacefully" with their plant hosts as so called "endophytes". We decided to perform controlled inoculation studies to corroborate our hypotheses.

The concept behind the approach is simple: challenge healthy plants with the "putative" culprits and see whether similar symptoms arise. This is called in jargin "Koch's postulate". If possible, double up the size of the experiment and keep half of the plants well-watered and only minimally water the other half of the plants. then compare disease symptoms in well-watered vs. poorly watered plants.

A greenhouse trial was run to fulfill Koch's postulate for fungi isolated consistently from severely symptomatic acacias in the Bay Area. Fungal isolates were all obtained from blackwood acacias and were inoculated on healthy potted plants in the hope to recreate the symptoms observed in diseased plants. Re-isolations were performed at the end of the experiment to confirm the symptoms were associated with the fungi inoculated. A total of 48 acacia trees in 5-gallon pots were used for the stem inoculations. The trees had an average height of 2.1m and average diameter of 13mm. (Fig. 6). Five fungal isolates were used:

Diaporthe foeniculina
Umbelopsis ramanniana
Dothiorella viticola
Dothiorella moneti
Mortierella elongata

Figure 6. Acacia trees used for study

Figure 7. Inoculation process

Methods

Each of the above isolates were grown on MEA for 9 days. Inoculations were placed approximately 10 cm up the stem. The inoculum area was cleaned with 70% EtOH, a 7mm corer was used to take off the bark and then a 5mm disc of

inoculum taken from the growing edge of the MEA plate was placed into the opening with the mycelium side facing the stem. The bark piece was placed back over the inoculum and the area was wrapped in parafilm and then covered with silver aluminum tape. (**Fig. 7**)

There were 2 treatments in this study, watered and unwatered (to simulate drought). All trees were thoroughly watered immediately prior to inoculations. Each isolate had 4 trees in each treatment. A mock inoculation was included using a MEA disc.

The watered treatment was watered regularly. The drought treatment was not watered from after the inoculation until the treatment trees showed visible signs of wilting (Apr 19-May 25). The trees were then watered to saturation two days in a row and not watered for the duration of the study.

The study plants were taken down on June 16 2021. Data was taken on height, diameter and foliage rating. In the lab, the inoculation area was examined, bark was removed and lesion length above and below the inoculation point was noted. Isolations were made from each end of the lesion and the lesion point using sterile technique (note - lesion point growth was not used in the data) on MEA. The plates were checked every few days for growth and subbed on to a clean MEA plate as soon as possible. In addition to morphological identification, a representative from each treatment was sequenced to confirm the pathogen.

Results

Table 5 shows the number of plants in each treatment where the pathogen was recovered. Each treatment had 4 trees. The drought treatment had more recovery of the pathogen, with 75-100% of the plants infected. The watered treatment varied greatly from 25-100%. **Figure 8** shows the typical lesions observed in inoculated plants.

Figure 8. Dark stem lesions were visible in inoculated potted blackwood acacia plants


AD 116-4

Table 5. Number of trees in each treatment with isolate recovered from inoculated stem.

isolate #	name	Recovered isolate treatment size = 4	
		drought	watered
AD11	*Diaporthe foeniculina*	4	4
AD 50	*Umbelopsis ramanniana*	4	4
AD65	*Dothiorella viticola*	4	2
AD69	*Dothiorella moneti*	3	1
AD309	*Mortierella elongata*	3	2
control	*mock*	0	0

In **Table 6**, the results are shown of the t-tests between the two treatments (watered vs. unwatered) for each pathogen species. Even if there was often no difference between the two treatments for the same pathogen, there was a strong interaction between pathogen and watering regime (abiotic stress in **Table 6A** below), so that watering regime made a substantial difference when comparing each treatment to the appropriate control.

Table 6. Results of the t-tests between the two treatments of abiotic stress (Drought and Control) for each pathogen species. CI represents the 95% Confidence Interval. The p value for *U. rammaniana* is approaching significance.

Species	Degrees of freedom	Lower CI	Upper CI	p-value
Control	3.1224	-8.003	3.003	0.249
Mortierella elongata	3.489	-1.923	10.423	0.065
Dothiorella viticola	3.4319	-8.718	2.2183	0.164
Dothiorella moneti	3.9425	-7.356	12.356	0.518
Umbelopsis ramanniana	3.0392	-13.272	50.772	0.118
Diaporthe foeniculina	3.6503	-38.179	13.179	0.239

Table 6A. Individual pairwise comparisons of lesion length between each treatment and the appropriate control. In bold are significant comparisons for which all aspects of Koch's postulate have been fulfilled. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

Watered set:

1- Control vs. *Diaporthe foeniculina* P=0.004 ***
2- Control vs. *Dothiorella moneti* P=0.006 ***
3- Control vs. *Umbelopisis ramnianana* P=0.07 (*)
4- Control vs. Mortierella elongata. P= 0.14
5- Control vs. *Dothiorella viticola* P= 0.6

Unwatered set:

1- Control vs. *Diaporthe foeniculina* P=0.0001 ***
2- Control vs. *Dothiorella moneti* P=0.26
3- Control vs. *Umbelopisis ramnianana* P=0.4
4- Control vs. *Mortierella elongata*. P= 0.01 (*Me* decreased lesion)
5- Control vs. *Dothiorella viticola* P= 0.01 **

The square root transformation was used to ensure the data came from normally distributed samples for *Mortierella elongata*. Overall, the distribution of values was barely normally distributed for all Species and Control.

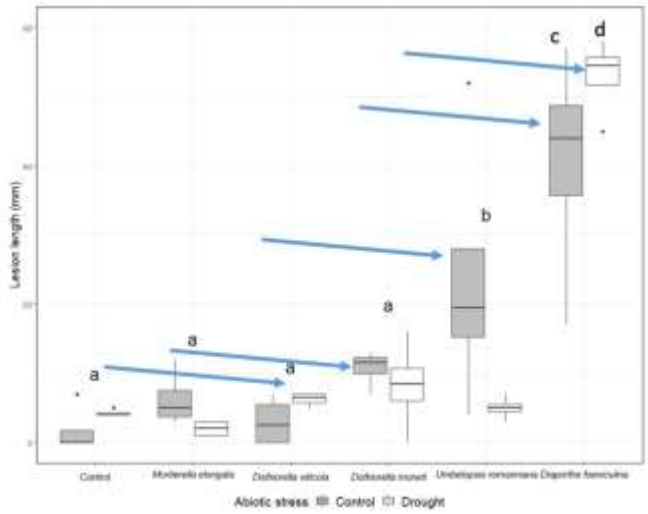
Table 7 displays the results of a Two-Way ANOVA to evaluate the effect of the different pathogens and treatments on lesion length.

Table 7. Two-Way ANOVA test results to evaluate the effect of the "Species" and "Abiotic stress" factors on the response variable "Lesion Length".

	Degrees of freedom	Sum of Squares	Mean Squares	p-value
Species	5	155.72	31.144	<0.001 ***
Abiotic stress	1	0.06	0.063	0.822
Isolate*Abiotic stress	4	23.27	4.655	0.007 **
Residuals	36	44.17	1.227	

An interaction between "Isolate" and "Abiotic Stress" was assumed for this analysis. Homoscedasticity of the dependent variable was confirmed, and the square root transformation was used to ensure the normality of the model residuals. To prove Koch's postulate, we ran *ad hoc* comparisons between the appropriate control (watered or unwatered) and each treatment. All statistical analyses were performed in R (v. 3.5.1; R Core Team 2018).

Figure 9. Lesion length of isolates and treatments (the longer the lesion the worse the disease). Dunnett's test with a confidence interval of 95% was run to test for differences between the isolates using the entire dataset. Blue arrows indicate treatments for which Koch's postulate was fully completed (significant symptoms based on ad hoc pairwise t tests between treatments and appropriate controls and reisolation was successful). Note that, in all other cases (i.e. those not identified by a blue arrow) but one, reisolation was successful, even without obvious symptoms development within the timeframe of the experiment. Nonetheless, this means infection of that host by the specific pathogen is possible.



Conclusions

The greenhouse inoculation experiment corroborated all fungi tested can be pathogens of blackwood acacia, with the exception of *Mortierella elongata*. In fact *M. elongata*, actually decreased lesion size in drought conditions. Figure 9 shows clearly there are some significant differences among the fungi tested, between watered and unwatered treatments, and some unexpected results were also obtained. The fungi tested can be placed in four groups depending on species and watered/unwatered conditions; The most aggressive treatment was that *of Diaporthe foeniculina* in unwatered plants. Lesions were extremely large in this treatment: these result support very high pathogenicity of this fungus on acacias in drought conditions. The same pathogen in watered conditions was also a treatyment that resulted in very large lesions: we conclude this pathogen is generally highly pathogenic on acacias but pathogenicity is even enhanced by drought conditions. Surprisingly, *Umbelopsis rammaniana* in watered plants had

intermediate pathogenicity, but its pathogenicity was insignificant in unwatered plants. This suggests that infection and initial disease on acacias caused by this fungus are more likely to initiate in non-drought conditions, possibly during the rainy season. *Dothiorella* spp. generated significant but smaller lesions. This may indicate that either Dothiorellas may require larger woody branches or stems to be successful, or they may be less aggressive pathogens. There was a significant difference between the native *D. viticola* and the exotic *D. moneti. D. viticola* required stress (unwatered plants) to cause significant lesions in small size plants, while *D. moneti* caused significant lesions in small size plants in the absence of stress (watered plants). Given their different ecology, the presence of both species in a site may result in overall greater disease with the exotic species being more active during the rainy season/rainy years and the native species being more active in the dry season/dry years. Overall, fungal isolation was more successful in unwatered plants, suggesting that drought conditions favor the organisms here tested.

We replicated the inoculation experiment above on trees in the SFPUC using only fungal isolates from the SFPUC. A total of 29 trees were inoculated in March 2021 and the experiment was taken down at the end of July 2022. Eight trees were inoculated with each fungus while five controls were mock-inoculated: trees were equally distributed in the two sites (4 were inoculated in each) to account for the effect environmental variation, but given the small replicate number , data from both sites were analyzed together.

Table 8. Results of the field inoculations study on adult acacias at the SFPUC

ADP SFPUC Field Sudy	Lesion Area (mm2)		
Treatment	X	sd	Reisolation (%)
Control	52.78	3.44	0
Diaporthe foeniculina	320.84	87.71	100
Dothiorella viticola	139.51	107.82	75
Umbelopsis rammaniana	190.56	79.67	87.5

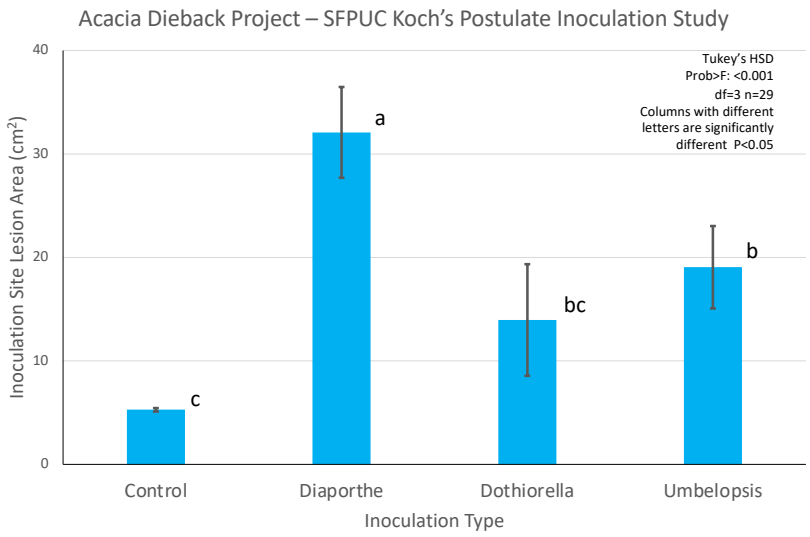


Figure 10. Statistical analysis of results of the field inoculation at the SFPUC

Results of the field inoculation on adult trees were a perfect match for those obtained on potted plants in the greenhouse and confirm the very high pathogenicity of *Diaporthe foeniculina* on blackwood acacia and the pathogenicity of *Dothiorella viticola.* Finally the large lesions caused by *Umbelopsis rammaniana* suggests it may be also a significant pathogen. Available information on this fungus is very limited, but the initial observation that declining acacias at the SFPUC were not infected by other putative primary pathogens, suggests a primary role played by this fungus, at least locally, in causing Acacia decline. One cause of concern is that disease by *U. rammaniana* did not require drought conditions. A second concern, based on the limited literature available is that it may be a generalist.
In conclusion given that *D. moneti* is an exotic, proven by our inoculation study to be more aggressive than the relatively widespread *D. viticola*, its spread should not be facilitated but rather curtailed by paying great attention to the disposal of infected, dead and dying acacias in the Oakland site where it was found. Likewise, the SFPUC should pay attention not to foster the spread of *U. rammaniana,* given that: a) it can cause disease even in the absence of drought conditions; b) it is solely responsible for the decline of some acacias in San Mateo County; c) it is likely to be a generalist capable of infecting hosts other than acacia.

8-Conclusions and management recommendations.

The only fungi consistently associated with *Acacia* spp. mortality in 5 sites across four locations and three California Counties were *Diaporthe* and *Dothiorella*. ***Diaporthe foeniculina* and *Dothiorella viticola* were present in all sites and the disease they cause is the putative major cause of the observed mortality. Thus, we can state that Diaporthe/Dothiorella canker is an emergent disease in acacias around the SF Bay Area.** Both *Diaporthe* and *Dothiorella* fungi are most likely directly involved in causing the observed large-scale mortality. These fungi are interesting because of their mixed biology: they normally initiate their relationship with the host as endophytes, they then shift to a pathogenic life-style and finally survive and sporulate as saprobes on the dead matter generated by the tree mortality they are responsible for. Although this mixed life-style has confused scientists for a long time, it is becoming increasingly accepted that identifying them as secondary pathogens would be incorrect. Secondary pathogens in fact are more properly defined as pathogens that infect a host already infected by a primary pathogen. Many Botryosphaerias and Diaporthes can be defined as pathogens, capable of causing lethal disease ***when*** hosts experience a specific predisposing physiological status. Note that this status may be: a)-unlinked to infection by primary pathogens, and, b)- caused by different reasons (limiting growing conditions in overdense stands, drought, abiotic factors, changes in soil pH and nutrients). One specific advantage these fungi have is that they are already present in their hosts as endophytes, hence, with the onset of the predisposing factors, they can rapidly cause disease, generating large scale outbreaks in a short period of time. When outbreaks are generated by infectious agents yet to infect their hosts, normally the time necessary to produce large outbreaks will be not only significantly longer, given that the infection process will take time and disease will initially manifest in scattered small clusters of mortality, due to the likelihood of escape by plants due to genetic variability (in natural populations) or microclimate variability (in invasive host populations characterized by a narrow genetic diversity). **The take home message is that some of these Botryosphaerias and Diaporthes can be aggressive pathogens, when the right conditions arise,** while secondary pathogens will always depend on primary infections by other pathogens. Even if we include in the secondary category pathogens that will infect plants stressed by abiotic factors, it is presumed those pathogens will infect their hosts after they are predisposed. In this case, the pathogens in question have already infected their hosts when they were healthy, thanks to their ability of living within plants as endophytes.

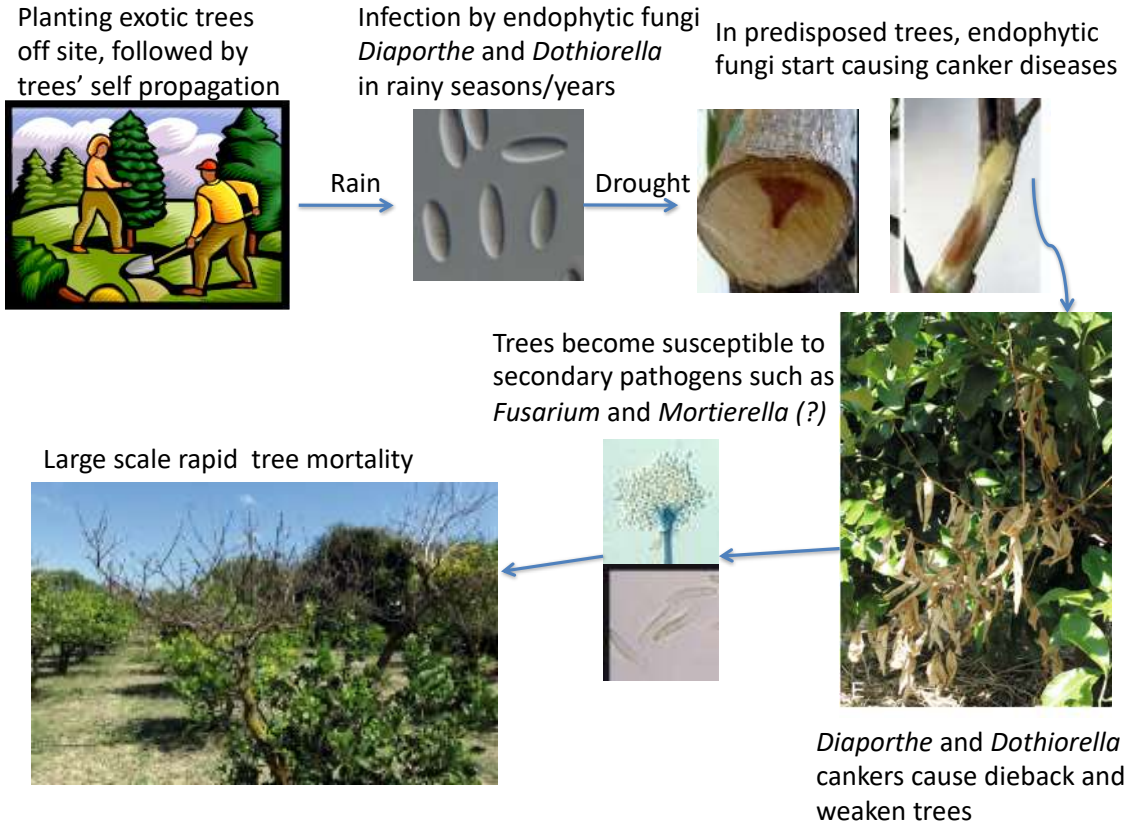
Conversely and hypothetically, some of the soilborne zygomycetes here isolated may become secondary pathogens on trees infected by *Diaporthe*/*Botryosphaeria*, further accelerating mortality. This is just a hypothesis that although in need of

substantial further work to be supported, is not in disagreement with our observations and isolation results.

Although infection occurs as part of the initial endophytic process, there is good evidence that infection rates are greatly increased in the presence of abundant rainfall. In our study, the rains of 2017 would have resulted in high infection rates. Only exceptionally do these fungi cause disease in the short term, due to the fact they mostly establish themselves as endophytes. A one to two-year lag between infection and disease is probably a minimum requirement, but in some cases the lag may last decades. If we assume infection occurred in 2017 and in wet years prior to 2017, then, mortality in 2020 matches the expectations of this scenario. It is also likely that 2017 resulted in the pervasive infection of a large percentage of individuals. We suggest that mortality is observed where infection was high in 2017, coupled with predisposing factors occurring post 2017. Certainly drought may be a major predisposing factor, but let's remember that water availability will depend on other factors as well, in particular, aspect (south and southwestern slope being more likely to dry out), frequency and abundance of coastal fog (less fog will increase evapotranspiration), soil compaction (compacted soil will absorb and retain less water), soil texture (sandier soils will retain less water; clay hardpans will prevent roots from reaching deep water during dry period), and plant density and size/age (denser stand will require more water, or in the case of equal density, stands with larger trees mat require more water), finally the effects of chemical changes in soil and leaves caused by smoke are largely unknown. It appears that these acacias are native to mesic environments in their native Australia, not a good match for the Mediterranean climate of the SF Bay Area.

Both acacias studied here are exotic and invasive, meaning they will naturally reproduce and increase rapidly their population size, without necessarily enlarging their genetic pool. Most individuals may thus be equally susceptible to predisposing factors and infection by these fungi. We believe both *Dothiorella viticola* and *Diaporthe foeniculina* to be native or long residents of California and that they may have crossed over from other hosts. The case *Dothiorella moneti/santali* is very interesting as it may have arrived from Australia as an endophyte of acacias. These two species in fact are reported exclusively from acacia

Figure 11. Proposed cycle showing the various interacting factors leading to rapid tree mortality: off-site planting of exotic invasive trees; trees propagate unchecked through self-seeding or clonal propagation; host jumps of native or naturalized fungi starts as number of invasive trees increases and is facilitated by rainfall; high rainfall leads to massive infection by such fungi that are initially endophytes; predisposing factors leading to water/resources deficiency turn endophytes into pathogens; pathogens weaken trees and become primary mortality agents; trees eventually become susceptible to secondary pathogens, multiple diseases foster quicker mortality.



What can we recommend and why?

These recommendations and the priority ratings are based on knowledge from agricultural systems. Although the biology of the pathogens here involved justifies such recommendations, their applicability in non-agricultural systems needs to be carefully evaluated through a case-by-case cost-benefit analysis. We also do not know whether the affected stands will regenerate through resprouting, a phenomenon that may compound the severity of the issue and require different prescriptions.

1- High priority: Reduce stress by thinning considerably remaining Acacia populations. Thinning has to be done in non –rainy periods, mid Summer to mid Fall

to avoid further infection associated with disturbances during thinning operations. Thinning can be done by cutting down whole trees, but also by trimming down the size of the canopy of remaining trees: these trees will require less water once their canopy is downsized.

Caveat: Due to self-propagation issues through resprouting or intensive seeding, thinning of thickets may require complex actions involving stems' removal and herbicide treatment, with uncertain outcomes. Trimming of individual high value trees, instead, may be easier to achieve and a valuable option with minimum undesirable side effects.

2- **Medium priority (only because may be hard to implement)**: Preserve only Acacia stands that are in more mesic sites (fertile deep, non sandy soils, and North or East facing slopes, good precipitation records). When possible (socially and financially), replace Acacia stands with other species, native and non-invasive, when acacias are growing in shallow or sandy soils, sites with Southern of Western Exposure, or sites that locally (because of shadow effects or mesoclimatic reasons) receive lower levels of precipitation. **These completely off-site stands will serve as a source of inoculum that may infect other hosts**: as climate changes due to global effects, native hosts may also become susceptible to disease caused by *Dothiorella* and *Diaporthe*, hence we want to minimize the sources of inoculum to avoid a domino effect. We believe that *Eucalyptus* may probably be undergoing the same types of disease due to cross-host infection by these fungi, so we already have a record of this expansion of host ranges happening in California.

3- **Medium priority (because of uncertain efficacy):** As symptoms begin to appear, prune and discard dead branches, making sure to cut at least a foot away the dead portions of the trees. This should reduce inoculum, although it is unclear whether trimmed trees will develop disease on other branches. Trimming trees will also decrease the water deficit, thus slowing down disease progression. This prescription should be preferably implemented in dry season.

Caveat: Although applicable in agricultural settings, this is a labor intensive and costly action with uncertain outcomes in self-generated Acacia stands.

4**- Very high priority:** Inoculum is produced with great abundance on dead matter resulting from tree mortality. As the host range of these fungi is broad, inoculum can infect not only acacias, but also other hosts, even native plants. Remove all dead trees and woody debris under dead and declining trees. Dispose of debris by burning, composting or by burying in a landfill (these are aerial fungi and should not fare well underground). This operation is essential, and if possible, it needs to be done before each rainy season. For 2021, it may be too late for this prescription, but for areas with abundant tree mortality I recommend delaying any operation to be done in early 2021 to later on in the year, in order to allow for this sanitation removal to occur. Any restoration done without prior removal of woody debris and dead trees may later on be compromised.

Caveat: This may be a disease-mitigating action that needs to be repeated as further dry years occur. The importance of this operation is supported by the possibility that even native trees may become massively infected due to the amount of inoculum generated by dying and dead acacias. Because of ongoing climate change, even native plant species may soon become predisposed to infection by these fungi. Although they may already be occasionally infected by these fungi, the large amount of inoculum available may lead to large scale infection events that are quantitatively different from the ones that may have been locally occurring.
Chipped wood remains infectious and hence cannot be left on site.
Please note that this is not a "all or nothing" recommendation, meaning that any level of woody debris removal will be better than no removal at all: of course, the more dead and dying woody substrate is removed, the more effective the prescription. We do not yet know the correlation between removal amount and level of efficacy.

5- High priority: Sanitize all tools and equipment used both when collecting dead matter and to prune or cut down infected trees. Sanitation will require cleaning the tools from any organic/woody debris followed by the use of chemical treatments using alcohol, bleach, or Lysol. Refer to direction for sanitation of tools by other infectious fungal diseases.

Caveat: These fungi are not currently regulated, however they are infectious and so all precautions should be taken to limit further infection. If operating in the dry season, risk of infection propagation through infected tools and machinery will be minimal.

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The truth may be in between, some Morteriella and Fusarium spp. as opportunistic pathogens

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