CONTROL OF PIERCE'S DISEASE WITH FUNGAL ENDOPHYTES OF GRAPEVINES ANTAGONISTIC TO *XYLELLA FASTIDIOSA*

Principal Investigator:
Philippe Rolshausen
Dept. of Plant Pathology & Microbiol.
University of California
Riverside, CA 92521
philrols@ucr.edu

Principal Investigator:
Caroline Roper
Dept. of Plant Pathology & Microbiol.
University of California
Riverside, CA 92521
mcroper@ucr.edu

Cooperator:
Bruce Kirkpatrick
Dept. of Plant Pathology
University of California
Davis, CA 95616

Cooperator:
Katherine Maloney
Dept. of Chemistry
Harvey Mudd College
Claremont, CA 91711

Cooperator:
James Borneman
Dept. of Plant Pathology & Microbiol.
University of California
Riverside, CA 92521

Cooperator:
Donald Cooksey
Dept. of Plant Pathology & Microbiol.
University of California
Riverside, CA 92521

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ABSTRACT
The goal of this research is to identify xylem dwelling fungi that are antagonistic to *Xylella fastidiosa* (*Xf*) that could be implemented as a preventive or curative treatment for Pierce's disease (PD). We hypothesized that some of the fungal endophytes present in PD-escaped grapevines possess anti-*Xf* properties, likely due to the production of secondary metabolites. We sampled from vineyards located in Napa and Riverside Counties that are under high disease pressure and isolated fungal endophytes living in the xylem sap and in wood spurs, shoots, petioles of these PD-escaped vines. Fungi were identified by ribosomal DNA sequencing. We have selected thus far a total of nine fungal strains that have inhibitory effects on *Xf* growth *in vitro*. We introduced several of these fungi into grapevines and then challenged them with *Xf*. Two of these fungi reduced PD progression in grapevines inoculated with *Xf* (Temecula1). In addition, we have isolated and identified the chemical structure of two fungal natural compounds produced by our candidate biocontrol fungi that inhibited *Xf* growth *in vitro*. In future experiments we will repeat the biocontrol experiment in greenhouse to confirm the prophylactic effect provided by these fungi. We will also evaluate the curative treatment potential of the fungal natural products that we have isolated in PD-infected grapevines. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

LAYPERSON SUMMARY
Several management strategies for Pierce's disease (PD) are currently being deployed, but as of today successful management largely involves vector control through the use of insecticides. Here we propose to test an alternative control strategy to complement those currently in place or being developed. Our goal was to identify fungi inhabiting grapevine that are antagonistic to *Xylella fastidiosa* (*Xf*). We hypothesized that in natural field settings grapevine escape PD because the organisms residing in the vine do not allow the establishment of *Xf*. In 2009, 2010 and 2011 we have sampled from vineyards in Napa and Riverside Counties that are under high disease pressure and identified fungi living in the xylem sap, shoots, petioles and wood spurs of diseased and PD-escaped grapevines. We have identified nine fungi that inhibit *Xf* growth in culture. Four fungi were re-introduced in grape cuttings that were inoculated with *Xf*, and two of them show a reduction in PD-disease progression. In addition, we also extracted natural compounds secreted by these fungi and identified two purified molecules inhibitory to the bacterium. In the future our goals are to; 1) screen more fungi with potential for *Xf* growth inhibition; 2) repeat the experiment with introduction of fungi in grape cuttings with additional potential biocontrols; 3) elucidate the chemical structure of additional fungal natural products antagonistic to *Xf* and test them as a curative treatment on PD-infected grapevines. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

INTRODUCTION
Current Pierce's disease (PD) management strategies largely involve vector management through the use of insecticides (3). This has contained the spread of the disease (9). However, for sustained control of PD, strategies that either target *Xylella fastidiosa* (*Xf*) or impart resistance to the plant host are required. There are several ongoing research avenues investigating the use of transgenic grapevines and rootstocks that show resistance to PD (1, 5, 10, 11). Other approaches include traditional breeding focused on introducing PD resistance into *V. vinifera* (14). Integrated control strategies are also being investigated in natural vineyard settings. These include the use of natural parasitoids to the GWSS (4) and inoculation of grapevine with mild *Xf* strains that may provide cross protection prior to infection with virulent *Xf* strains (8). However, there are no effective curative measures that can clear an infected grapevine of *Xf* besides severe pruning, assuming that the bacteria have not colonized the trunk of the grapevine resulting in a chronic infection.

Notably, control of PD with fungi or fungal metabolites is a largely unexplored research area. Fungal endophytes are receiving increasing attention by natural product chemists due to their diverse and structurally unprecedented compounds which make them interesting candidates for discovery of novel molecules and for their potential in disease control (2, 12, 15). This project focuses on identifying endophytic fungi in grapevine and evaluating their potential as biocontrol agents against *Xf*. Our objectives are to characterize the microbial diversity in grapevines that escaped PD in natural vineyard settings, and compare this population to PD-infected grapevines with the goal of identifying fungi that are unique to PD-escaped vines. We hypothesize that some of these fungal endophytes possess anti-*Xf* properties, likely due to the production of secondary metabolites. We are assessing the ability of these endophytes and their natural products (i.e. secondary metabolites) for inhibitory activity against *Xf in vitro*. Finally, we are determining in greenhouse tests if 1) fungi have potential use as prophylactic biocontrol agents for control by inoculating grapevine cuttings with endophytic, *Xf*-antagonistic fungi and 2) if treatments PD-infected grapevine with fungal natural products have curative properties. If successful, we envision that these control strategies can be implemented at the nursery level (for biocontrols) or directly in the field (for natural products).

OBJECTIVES

1. Identify fungal endophytes that are present in xylem sap and xylem tissues of PD-escaped grapevines but not in PD-symptomatic grapevines.
2. Evaluate the antagonistic properties of the fungal candidates to *Xf in vitro* and conduct a preliminary characterization of the chemical nature of the inhibitory compound(s).
3. Evaluate biological control activity of the fungal candidates *in planta*.

RESULTS AND DISCUSSION

Objective 1. Identify fungal endophytes that are present in xylem sap and xylem tissues of PD-escaped grapevines but not in PD- symptomatic grapevines.

The goal is to identify the fungal endophytic populations inhabiting grapevines infected with PD and apparently healthy grapevines adjacent to PD-infected vines (PD-escaped) (**Figure 1**) with classical isolation techniques and DNA-based methods. Plant tissues/fluids (xylem sap, green shoots, petioles, and wood spurs) were collected at bud-break and before harvest from vineyards grown in Riverside and Napa Counties (**Table 1**) and brought back to the laboratory. Culturable fungi were isolated on fungal medium (Potato Dextrose Agar, PDA), and were identified after comparing the PCR-amplified rDNA sequence to homologous sequences posted in the GenBank database.

Results in **Table 2** show showed that based on our samplings to date, *Cladosporium* sp. and *Aureobasidium* sp. are the most widespread culturable fungi inhabiting grapevine xylem. Both of these species have a high incidence in both diseased and PD-escaped grapevines (xylem sap, green shoots and wood spurs). Furthermore, these are the only culturable fungi repeatedly identified in the xylem sap of grapevine. We also found other fungal species occurring in both diseased and PD-escaped grapevines, albeit, at a lower frequency. These include *Alternaria* sp., *Cryptococcus* sp., *Penicillium* sp., and a *Geomyces* sp. Some fungi were only present in PD-escaped or diseased grapevines. The fungal species found only in diseased vines include *Epicoccum* sp., *Phomopsis* sp., *Fusarium* sp., *Biscogniauxia* sp., *Cryptosporiopsis* sp., *Ulocladium* sp., *Pezizomycete* sp, and *Didymella* sp. Most interestingly, we found several species only inhabiting PD-escaped grapevines. These include *Peyronellae* sp., *Drechslera* sp., *Discostroma* sp., *Cochliobolus* sp., *Chaetomium* sp., *Phaeosphaeria* sp., *Oidodendron* sp., and *Diplodia* sp. Identification of fungi from sampling in September/October of 2012 from vineyards 2, 3, and 4 is currently underway. Additional sampling will occur in March/April of 2012 at the same vineyards to complement these results.

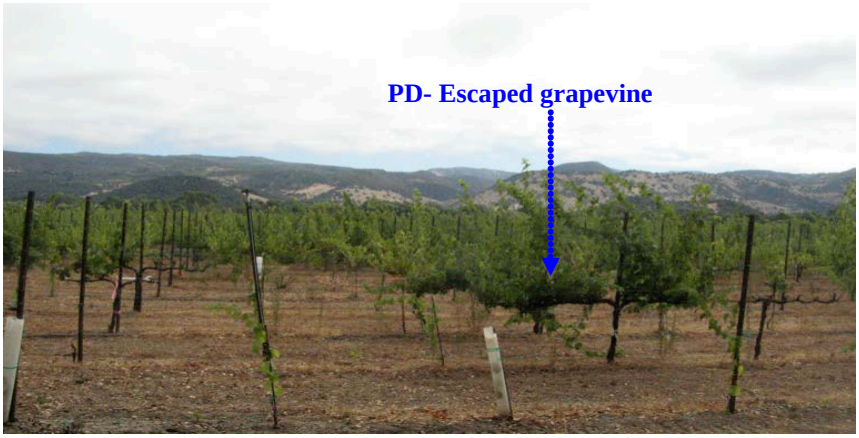

Figure 1. PD-escaped- grapevines in a Riesling block infected with Pierce's disease in Napa County.

Table 1. Location, variety and timing of the sampling of vineyards.

Location	Vineyard #	Variety	Timing of Sampling
Riverside	Vineyard 1	Cabernet Sauvignon Chardonnay	August 2009
County	Vineyard 2	Syrah	September 2010 March 2011 October 2011[a] March/April 2012[b]
Napa County	Vineyard 3	Riesling Chardonnay Merlot	August 2010 April 2011 October 2011[a] March/April 2012[b]
	Vineyard 4	Chardonnay	August 2010 April 2011 October 2011[a] March/April 2012[b]

[a:] sampling was completed and fungi are currently being identified
[b;] future sampling dates

Table 2. Identification and percent recovery of fungal taxa from PD-escaped and PD-infected grapevines. Results are based on sampling from 5 grapevine varieties (Merlot, Cabernet Sauvignon, Chardonnay, Riesling, Syrah), in 4 vineyards located in Napa and Riverside County for sampling times in August 2009, August/September 2010 and April/March 2011. Fungi were isolated from xylem sap, green shoots and wood spur.

Fungal Taxa	Percent Recovery	
	Escaped Grapevines (n=26)	Diseased Grapevines (n=19)
Cladosporium sp.	77	53
Aureobasidium sp.	81	74
Alternaria sp.	12	16
Cryptococcus sp.	12	11
Penicillium sp.	4	5
Geomyces sp.	4	5
Peyronellae sp.	8	
Drechslera sp.	4	
Discostroma sp.	4	
Cochliobolus sp.	4	
Chaetomium sp.	8	
Phaeosphaeria sp.	4	
Oidiodendron sp.	4	
Diplodia sp.	4	
Epicoccum sp.		5
Phomopsis sp.		5
Fusarium sp.		11
Biscogniauxia sp.		5
Cryptosporiopsis sp.		5
Ulocladium sp.		16
Pezizomycete sp.		11
Didymella sp.		5

Characterization of the fungal population using oligonucleotide-based fingerprinting of rRNA genes (13) is underway to characterize the total (culturable and non-culturable) fungal population inhabiting grapevines. We have extracted the total DNA from diseased and PD-escaped grapevines using Qiagen Plant DNA extraction kit, and were able to PCR amplify the total ribosomal DNA. We are currently conducting the DNA based population analysis.

Objective 2. Evaluate the antagonistic properties of the fungal candidates to *Xf in vitro* and conduct a preliminary characterization of the chemical nature of the inhibitory compound(s).

The goal of this objective is to identify fungal species and fungal natural products produced by these species that can be used as treatments for control of PD. Fungal cultures recovered from xylem sap, shoot, petioles and spur isolations (Obj. 1) were evaluated in an *in vitro* inhibition assay for antagonism against *Xf*. In brief, *Xf* liquid cultures were adjusted to OD_{600nm}=0.1 (approx. 10^7 CFU/ml); 300 µl of the *Xf* cell suspension was added to 3 ml of PD3 medium containing 0.8% agar and briefly vortexed. This mixture was overlayed onto a petri plate containing PD3 medium. A sterile circle of agar was drawn from the margin of an actively growing pure fungal culture and was placed onto the plates previously inoculated with *Xf*. Plates were incubated at 28ºC for seven days and then observed for an inhibition zone around the fungal colony (**Figure 2**). Fungal species with a halo of inhibition were considered antagonistic to *Xf*. All the fungal specimens showing inhibition are being identified to the species level using multi-gene sequencing and morphological identification.

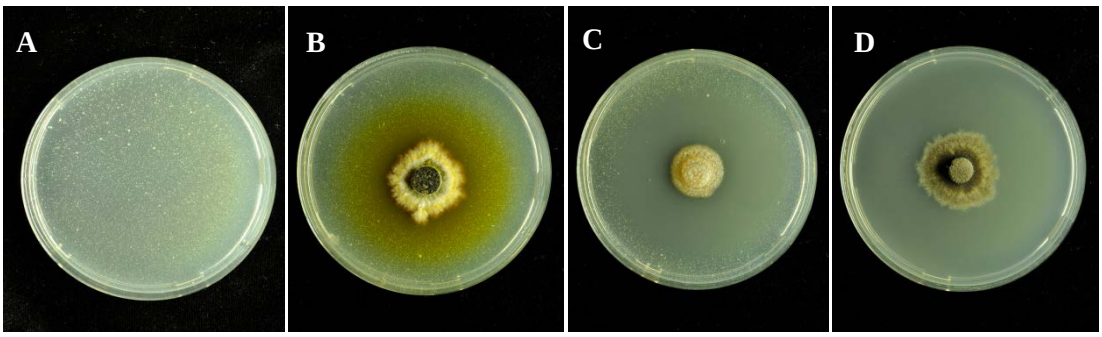

Figure 2. *In vitro* inhibition assay for fungi. Results show; (A) control; (B) no inhibition of *Xf* around fungal growth; (C) partial inhibition of *Xf* as shown with the halo around the fungal growth (D) total inhibition of *Xf*.

In addition, crude extracts collected from the fungal cultures showing inhibition towards *Xf* was collected for evaluation using the growth inhibition assay as described above. Fungal crude extracts were extracted as follows; agar plugs of 0.5 cm diameter of each fungus were used to inoculate 250 mL liquid media, and the fungi were cultivated at room temperature on a shaker. After seven days, each culture was filtered and further extracted with ethyl acetate, re-suspended in sterile methanol to an extract mass of 1mg, pipetted onto sterile paper discs and allowed to dry in a laminar flow hood. Once dry, the paper discs containing the crude extracts were placed onto the *Xf* cultures and incubated at 28ºC for seven days. Following this, plates were observed for a halo of inhibition around the paper disc and compared to control *Xf*-only plates and plates with paper discs treated with methanol only. The efficacy of fungal crude extracts was initially pre-screened in a high throughput method using three paper discs per plate (data not shown) and when inhibition of *Xf* growth was observed, the experiment was repeated using one disc per plate (**Figure 3**).

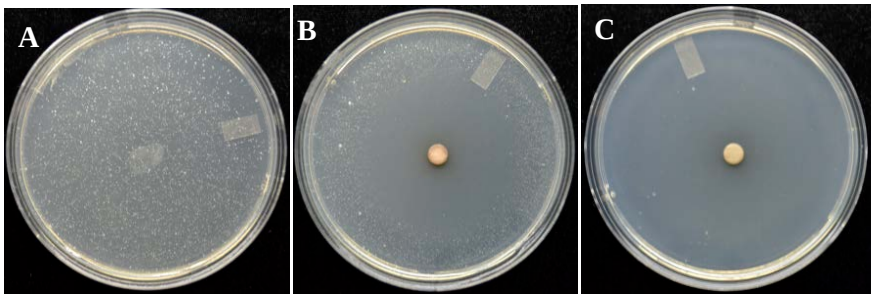

Figure 3. *In vitro* inhibition assay for fungal crude extracts. Results show; **A** Control *Xf* only; **B** intermediate inhibition of *Xf* as shown by the halo around the disc; **C** good inhibition of *Xf*, as shown by the absence of *Xf* growth around the disc. Note: no clearings were formed around the methanol-treated disc only control.

From the field sampling we have identified nine fungal taxa that inhibited *Xf* growth *in vitro* with various degrees of inhibition. We are currently testing the potential of four of these fungi as biocontrol agents *in planta* (see Objective 3). We have extracted crude extracts from six of the fungi that showed inhibition of *Xf* growth *in vitro.* We are currently fractionating the crude extracts from these six fungi in order to purify and identify the inhibitory molecule. Thus far, we have purified two individual molecules that are active against *Xf* growth *in vitro* and identified them to the chemical structural

level. These molecules and fungi are currently under review for patentability by the Executive Licensing Officer in the UC-Riverside Office of Research and, hence, their names cannot be disclosed in this report.

Objective 3. Evaluate biological control activity of the fungal candidates *in planta*.
The goal of this objective is to provide increased tolerance to PD by inoculating grapes with natural fungal endophytes that possessed anti-*Xf* properties. We have selected four fungal candidates that displayed two features; 1) they showed inhibitory effect of *Xf* in *in vitro* assays (Obj. 2); 2) they were heavily sporulating in culture. Spore formation is an important criteria. Because of the small size and shape of the spores, they are more likely to successfully infiltrate and colonize the plant xylem vessels as opposed to the larger vegetative structures, such as fungal hyphae. Spores of fungi 1 to 4 that were grown on PDA medium were harvested in sterile water and the concentration was adjusted to 10^5 to 10^6 to spores/µl. Grapes cuttings var. 'Merlot' (with 2 buds) were vacuum infiltrated (**Figure 4**) with the fungal spore suspension, planted and placed in the greenhouse. Control plants were infiltrated with sterile water only. In June of 2011, green shoots arising from these cuttings were inoculated with *Xf* (Temecula strain) by mechanical needle inoculation (7). A sub-sample of plants was left un-inoculated with *Xf* to determine if the concentration of fungal spore treatment used is detrimental itself to the grape cuttings. Plant symptoms were rated from 0 to 5 weekly (0= no symptoms; 5=Plant dead or dying) according to Guilhabert and Kirkpatrick (6). (**Figure 5**).

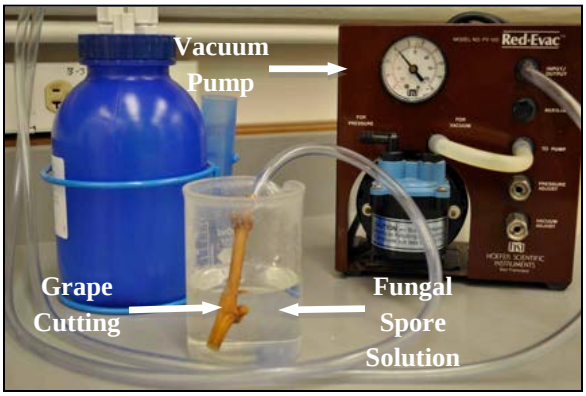

Figure 4. Technique used to vacuum infiltrate grape cuttings with spores of fungal endophytes that showed inhibitory effects in the *Xf in vitro* inhibition assay.

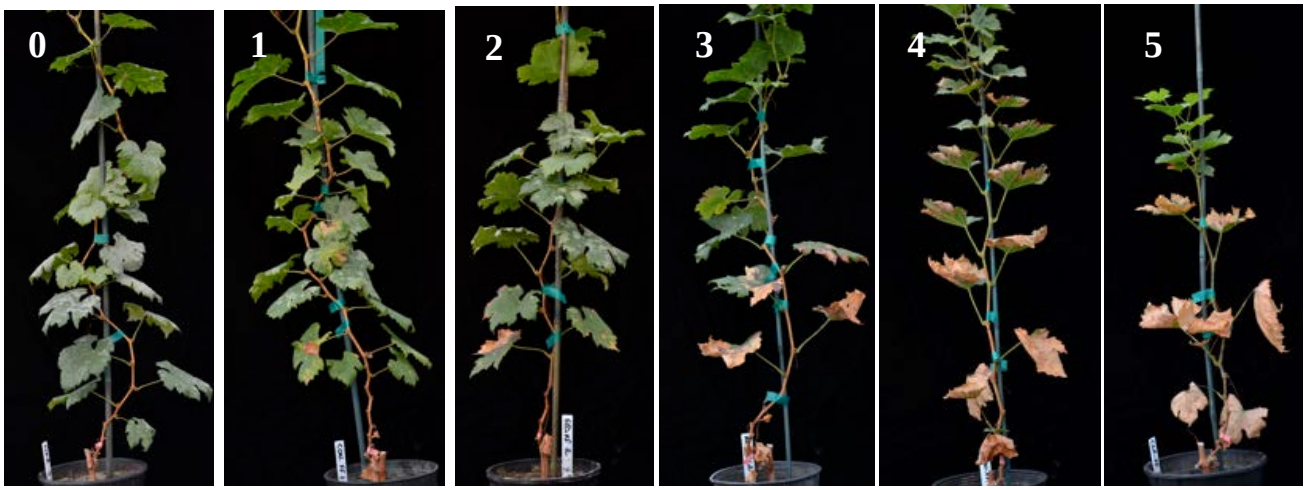

Figure 5. PD symptoms severity rating in grapevine cv. 'Merlot'; 0 = no symptoms (Mock inoculation); 1 through 5= grapes infected with the wild type strain Temecula showing an increase in the disease severity.

Our results (**Figure 6**) showed that the progression of the disease symptoms over a 14 week-period were less when grape cuttings were previously vacuum infiltrated with two fungal endophytes (fungus 1 and 3) that showed inhibition to *Xf* in the previous *in vitro* inhibition assay (Obj. 2). The two other fungal endophytes (fungus 2 and 4) show little to no reduction in disease progression. No fungi were detrimental to the plant. Plant will be rated for a total of 18 weeks post-*Xf* inoculation after which petioles will be sampled, and brought back to the laboratory to quantify *Xf* titer in the plants. Petioles will be

surface sterilized and plated on PD3 medium in order to quantify *Xf* populations per plant. We will also confirm that the inhibitory fungi were able to systemically colonize the plant by recovering them from cut petiole tissues on PDA medium. Statistical analyses will be performed at the end of the experiment to determine if the treatments are statistically different from the control. This experiment will be repeated in 2012 with additional potential biocontrols.

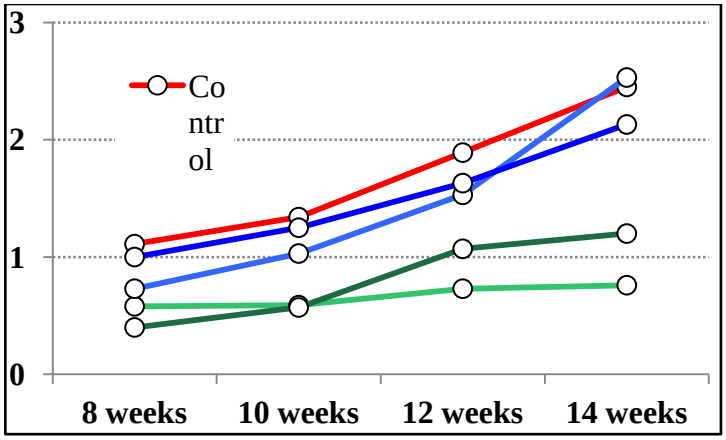

Figure 6: PD severity progression between eight and fourteen weeks post-inoculation with *Xf* 'Temecula' strain on grape cuttings cv. 'Merlot' previously vacuum infiltrated with four different fungal endophytes of grapevine showing inhibition to *Xf* in *in vitro* assays.

CONCLUSIONS

The goal of this research is to identify fungal strains or natural fungal products that have an antagonistic effect towards *Xf*. Thus far, we have isolated nine promising fungal candidates that inhibit *Xf in vitro*. Four fungi were evaluated as potential biocontrols and were vacuum-infiltrated into grape cuttings cv. 'Merlot' that were later inoculated with *Xf* Temecula strain. Our results showed that two fungi were able to reduce disease progress and severity after 14 weeks post-inoculation. The goal is to inoculate plant materials with these fungal biocontrols at the nursery level so that they can provide a prophylactic control against PD in natural vineyard settings. In addition, we are currently evaluating the antagonistic efficiency of the fungal natural products against *Xf* in *in vitro* inhibition assay and characterizing their structure and chemical properties. Thus far we have identified two molecules that inhibited *Xf in vitro*. The goal is to develop these natural products to commercial products that can be used as curative treatments for grapevines already infected with PD.

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