Chemical cues from beetle larvae trigger proliferation and virulence of a plant pathogen

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Abstract

Pest insects and microbial pathogens threaten agricultural crop productivity and global forest biomes, and insect-microbe interactions can increase the severity of plant decline. Whilst bacterial chemicals have been shown to influence insect behaviour, the impact of insect-derived chemistries on phytopathogens is barely understood. Here, we investigated beetle larvae-bacteria interactions in acute oak decline (AOD), a decline disease of oak characterised by inner bark necrosis caused by a polymicrobial consortium including *Brenneria goodwinii* and larval galleries of *Agrilus biguttatus*. To test the hypothesis that larval chemicals influence the activity of *B. goodwinii*, we extracted *A. biguttatus* larvae and tested the effect of extracts on the growth and gene expression of *B. goodwinii in vitro*. We demonstrate that larval chemicals elicit increased bacterial growth rate and final cell density, as well as differential expression of almost 800 genes, including the Type III Secretion System and its effectors, major virulence factors in other plant pathogens. Contrastingly, gallery woody-tissue extracts induced no such effects. Our work highlights the importance of multi-kingdom interactions in plant disease and demonstrate a novel mode-of-action for insect-derived chemical elicitors in facilitating the virulence of phytopathogens, which may be important in understanding plant diseases globally.

Introduction

Insect-microbe interactions are increasingly important in the context of plant disease due to an exponential increase of pest and disease outbreaks, driven by international trade and climate change (Coolen et al., 2022; Singh et al., 2023). Examples of insect-phytopathogen interactions include bacterial phytoplasmas causing Aster Yellows disease and the six-spotted leafhopper *Macrosteles quadrilineatus*. The insect is a vector of the bacterium and in-turn benefits from bacterial effectors, which modulate the plant environment by reducing plant defence mechanisms (Sugio et al., 2011). Similarly, *Xylella fastidiosa*, infecting a wide range of plant hosts, is transmitted by xylem-sap feeders insects (Sicard et al., 2018). Bacterial volatile organic compounds can also influence insect behaviour (Davis et al., 2013; Weisskopf et al., 2021), which in turn assist dispersion of pathogenic bacteria (Robacker et al., 2004). However, while a few studies examined the antagonist properties of insect metabolites on bacteria (Hall et al., 2011; Teh et al., 2013), the agonist effect of insect-derived chemistries on bacterial activity is barely known. This is a major knowledge gap given that bacteria use chemical signals as a primary source of information to coordinate their behaviour at the population level (Miller & Bassler, 2001).

Understanding multi-kingdom interactions in plant disease is therefore crucial, especially in decline diseases such as Acute Oak Decline (AOD), where insects could play a pivotal role. AOD is a complex decline disease affecting native British oak species (*Quercus robur* and *Q. petraea*), involving the cumulative action of multiple biotic and abiotic factors, including the larvae of bark-boring beetles and bacterial phytopathogens (Denman & Webber, 2009). The disease is characterised by inner bark tissue necrosis caused by a polymicrobial consortium composed of at least three bacterial species (*Brenneria goodwinii*, *Gibbsiella quercinecans*, and *Rahnella victoriana*), and the presence of larval galleries of the two-spotted oak buprestid, *Agrilus biguttatus*, on the phloem–sapwood interface. *B. goodwinii* is recognised as the dominant pathogen within necrotic lesions (Denman et al., 2018) and is known to express putative virulence genes in necrotic tree tissue (Doonan et al., 2020).

Transcriptome analysis of *B. goodwinii* in necrotic oak stem tissue in the presence and absence of *A. biguttatus* larvae demonstrated that expression of genes associated with biofilm formation (*chvB;* Matthysse et al., 2005), Type III secretion system effectors involved in virulence and suppression of host immunity (*srfB;* Worley et al., 2000 and

avrE; Degrave et al., 2015, respectively), and detoxification to protect against hydrogen peroxide released by host defences (*katG;* Jittawuttipoka et al., 2009), significantly increased during co-inoculation with *A. biguttatus* larvae (Doonan et al., 2020). Interestingly, the presence of *A. biguttatus* larvae also increased the area of necrotic tissue caused by bacteria (Denman et al., 2018; Doonan et al., 2020). Taken together, these findings suggest that interactions between *B. goodwinii* and *A. biguttatus* larvae in the inner bark enhances virulence and facilitates the proliferation and spread of damaging bacteria within infected trees.

Although the existence of an interaction between *B. goodwinii* and *A. biguttatus* larvae has been demonstrated *in planta* (Denman et al., 2018; Doonan et al., 2020), the mechanistic nature of this interaction remains unknown. In this study, we inoculated oak logs with *A. biguttatus* eggs and collected late-instar larvae and gallery woody-tissue from the logs. Because *A. biguttatus* larval feeding may chemically modify the woody-tissue environment and/or trigger host responses, we extracted chemical elicitors from asymptomatic as well as gallery woody-tissue and *A. biguttatus* larvae body and independently assessed their bioactivity towards *B. goodwinii* in controlled *in vitro* experiments. We tested two hypotheses: (1) *B. goodwinii* proliferation and virulence gene expression are an indirect effect of *A. biguttatus* larval activity and are triggered by gallery woody-tissue chemical elicitors; (2) *B. goodwinii* proliferation and virulence gene expression are a direct effect of *A. biguttatus* larvae and are triggered by chemical elicitors from larvae. We show that chemical elicitors from *A. biguttatus* larvae (hypothesis 2), but not gallery woody-tissue (hypothesis 1), trigger *B. goodwinii* proliferation and virulence through an increase in bacterial abundance and the upregulation of 418 genes, including expression of effectors involved in bacterial Type III secretion system. Determining the mechanisms of insect-microbiota interactions and their emergent impacts on plant disease outcomes is critical to inform integrated pest management strategies for globally important plant diseases.

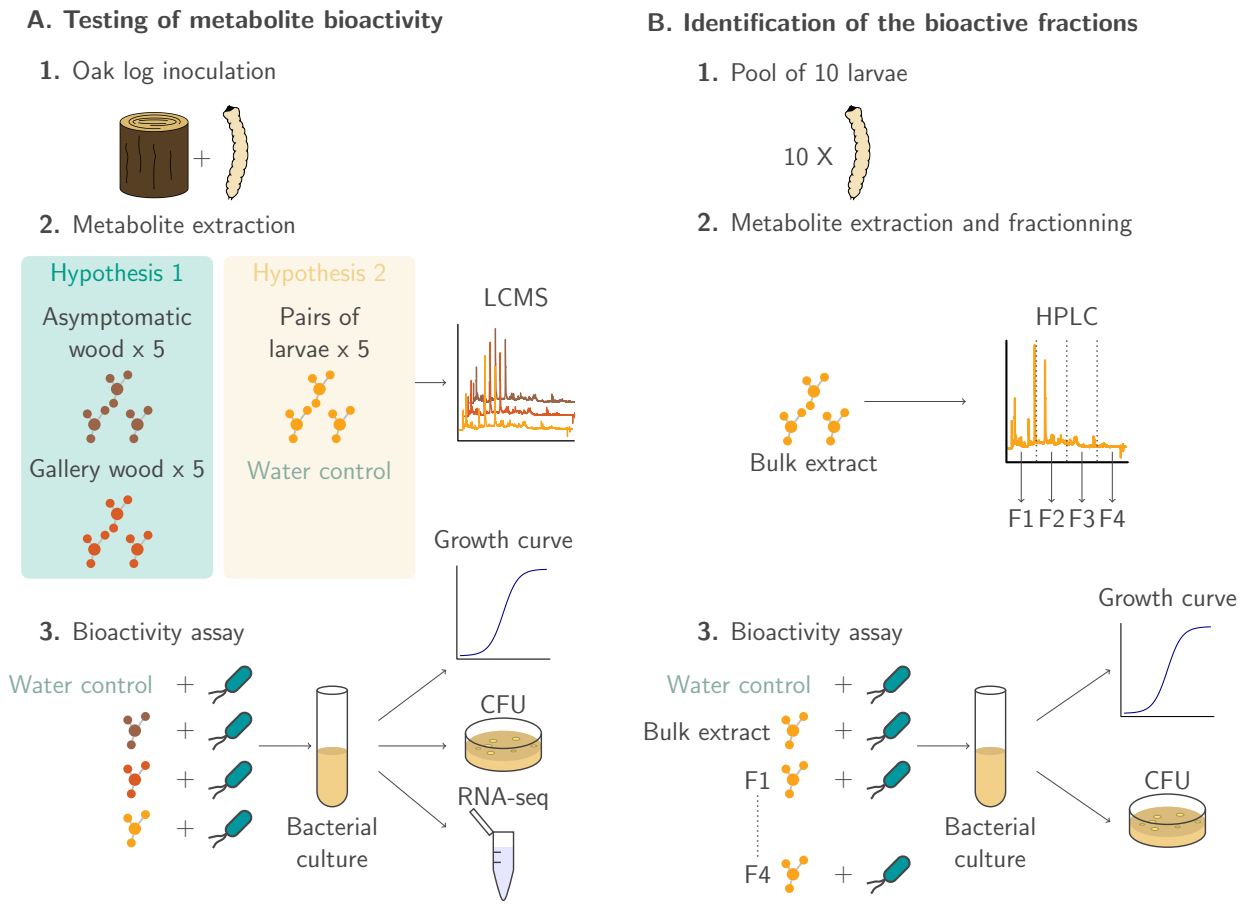

Figure 1: **Summary of the experimental design**

Results

Gallery woody-tissue extracts do not increase bacterial proliferation compared to healthy woody-tissue extracts

To test the effect of extracts from woody-tissue galleries made by *A. biguttatus* larvae on *B. goodwinii* proliferation (hypothesis 1), we compared bacterial growth in cultures supplemented with asymptomatic wood extract and gallery woody-tissue extract (Figure 1A). At 24 hpi (hours post inoculation), cultures reached an OD_{600} of 0.75 ± 0.05, 0.78 ± 0.09 and 0.9 ± 0.06 for asymptomatic wood extracts, gallery woody-tissue extracts and water control, respectively (Figure 2A). The growth rate of cultures supplemented with gallery wood extracts (0.30 ± 0.04) was significantly lower than those supplemented with asymptomatic wood extracts (0.39 ± 0.03, Figure 2B, pairwise-wilcoxon test, p-value < 0.05). The CFU density at 24 hpi did not differ significantly between gallery woody-tissue and asymptomatic wood treatments (12.69 ± 2.5 x 10^8

CFU/mL and 12.32 ± 10.0 x 10^8 CFU/mL, respectively Figure 2C). These results show that chemicals extracted from gallery woody-tissue do not cause more proliferation of *B. goodwinii* than asymptomatic wood chemicals.

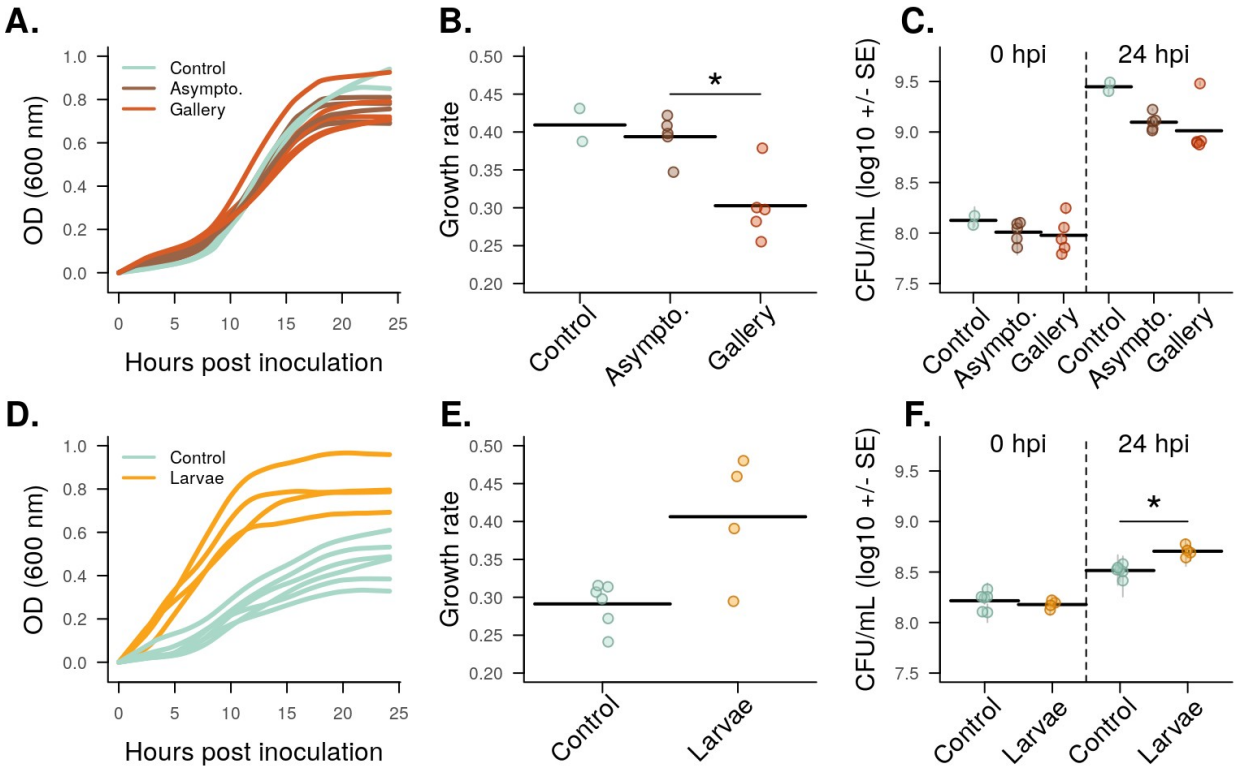

Figure 2: **Growth curves (A), growth rates (B), and cell density (C)** obtained in 3 mL liquid cultures supplemented with gallery woody-tissue (Gallery) and asymptomatic wood (Asympto.) compounds, compared to compound extraction blanks (Controls). Growth curves (D), growth rates (E), and cell density (F) obtained in 200 µL liquid cultures supplemented with beetle larvae body compounds (Larvae), compared to compound extraction blanks (Controls). The controls were the same in both experiments but the difference in growth rate observed in (A) and (D) are due to the different volumes of cultures. An additional experiment replicating the results of (A) in the same volume as (D) can be found in Figure S1. Stars indicate significant differences (pairwise-wilcoxon test (above) or t-test (below), p-value < 0.05). OD = optical density; CFU = colony forming unit; SE = standard error

A. biguttatus larval elicitors cause bacterial proliferation

To test the direct effect of *A. biguttatus* larvae chemical elicitors on *B. goodwinii* proliferation (hypothesis 2), we compared bacterial growth in cultures supplemented with larval extract or water (control). At 24 hpi, cultures in a 200 µL volume reached an OD_{600} of 0.75 ± 0.09 and 0.47 ± 0.1 for larval extracts and water control, respectively (Figure 2D). The growth rate of cultures supplemented with larvae extracts was higher

than that of control cultures (0.41 ± 0.07 and 0.30 ± 0.04, respectively; Figure 2E). The trend was confirmed by the bulk extract growth curves in Figure 4B. The CFU density at 24 hpi was also significantly higher for cultures supplemented with larval extract than for control cultures (5.1 ± 0.67 x 10^8 CFU/mL and 3.3 ± 0.4 x 10^8 CFU/mL, respectively; Figure 2F). These results indicate that chemical elicitors extracted from *A. biguttatus* larvae stimulate bacterial growth.

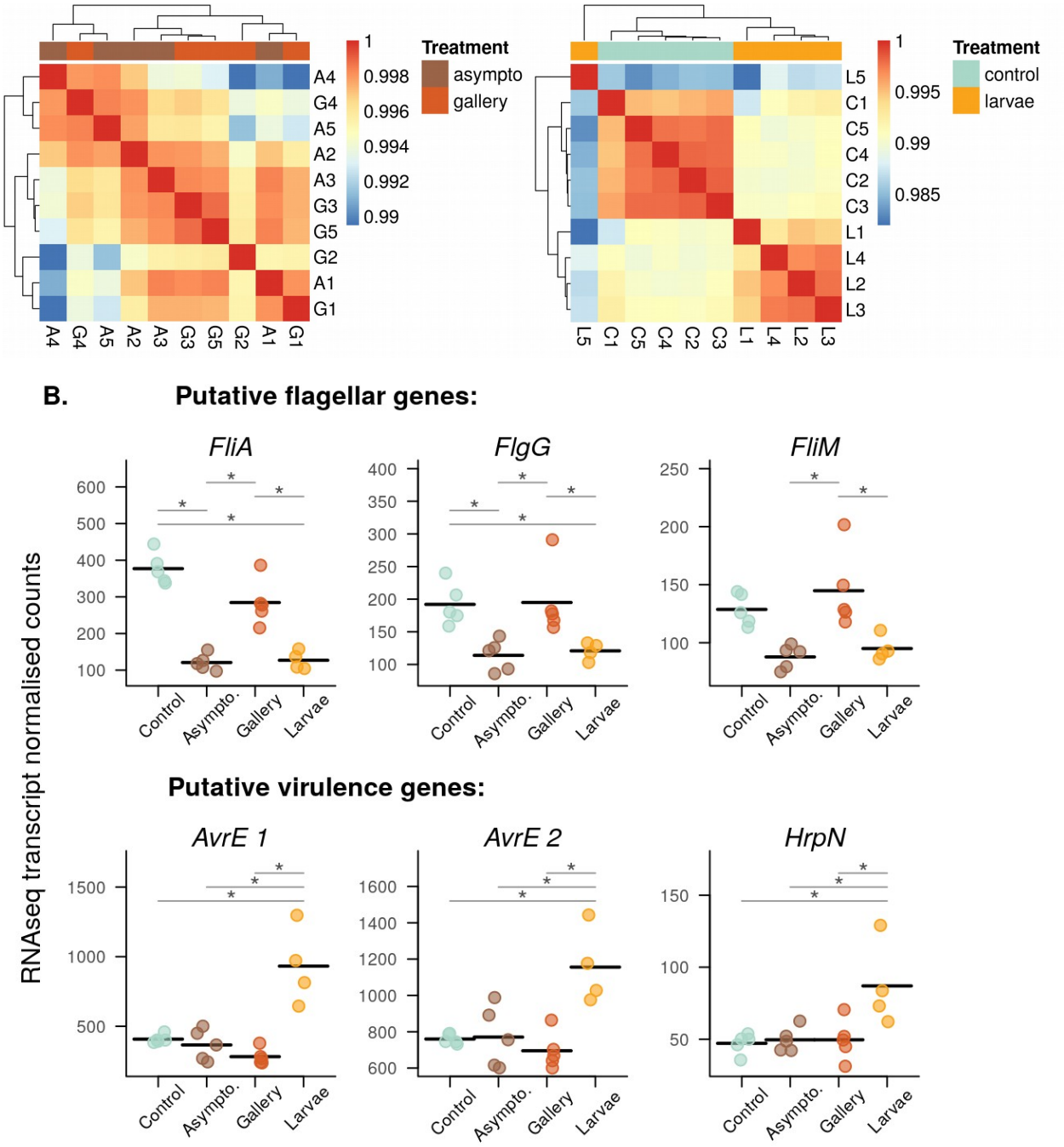

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Figure 3: **Gene expression profile and differentially expressed genes** obtained from RNAseq data of *B. goodwinii* cultures in exponential phase of groth and supplemented with chemical extracts from asymptomatic wood, gallery woody-tissue, and *A. biguttatus* larvae, or water (control). **A.** Heatmaps of the sample correlation matrix based on the transcript counts after rlog transformation. Sample L5 had a gene expression profile different from any other samples with low correlation values, and was considered as an outlier and removed from the differential expression analysis **B.** Normalised counts for genes of interest, including putative flagellar and virulence genes. Bars with stars represent significantly differentially expressed genes based on the DESeq2 analysis.

Beetle larval elicitors modify bacterial gene expression profile and increase virulence gene expression

To test for the impact of chemical elicitors on bacterial gene expression, we compared the gene expression profile of *B. goodwinii* in cultures supplemented with asymptomatic and gallery woody-tissue extracts (hypothesis 1), and *A. biguttatus* larval extracts and water control (hypothesis 2). Cultures supplemented with gallery woody-tissue or asymptomatic wood extracts did not have a distinct gene expression profile and did not cluster separately based on the correlation matrix of rlog-transformed transcript counts (Figure 3A). A total of 21 genes were differentially expressed between the gallery woody-tissue and the asymptomatic wood, with 11 up-regulated and 10 down-regulated genes in the gallery woody-tissue treatment. Seven out of the 11 up-regulated genes were directly linked to flagella production (*fliA*, *fliF*, *fliI*, *fliM*, *flgD*, *flgG*, and *flgJ*, Figure 3B). These results show that chemicals extracted from gallery woody-tissue have a small impact on the gene expression profile of *B. goodwinii* and mainly impact flagellar genes.

Contrastingly, cultures supplemented with *A. biguttatus* larval extracts had a distinct gene expression profile and formed a separate cluster from the control cultures (no larval extract) based on the correlation matrix of rlog-transformed transcript counts (Figure 3A). A total of 798 genes were differentially expressed between *B. goodwinii* cultures receiving larval extracts and the control treatment, with 418 up-regulated and 380 down-regulated genes in the larvae treatment. Up-regulated genes included Type III secretion system apparatus and effectors (*avrE* 1, *avrE* 2, *hrpN*, Figure 3B). These results show that larval elicitors have a significant impact on bacterial gene expression, and trigger the expression of bacterial virulence genes.

Bacterial proliferation is triggered by a fraction of the larval elicitors

To narrow down identification of the bioactive region(s) within the *A. biguttatus* larval extracts, we compared bacterial growth in *B. goodwinii* cultures supplemented with bulk larval extract with that of cultures supplemented with four fractions of the bulk larval extract (Fraction 1 (F1) to Fraction 4 (F4)) (Figure 1B and 4A). At 24 hpi, cultures supplemented with the bulk larval extract, F1 or F4 reached greater OD_{600} (0.50±0.04, 0.51±0.05 and 0.48±0.01, respectively; Figure 4B) compared to cultures supplemented with water (control), F2 or F3 (0.36±0.03, 0.38±0.03 and 0.40±0.05, respectively; Figure 4B). The growth rate of *B. goodwinii* in cultures supplemented with F1 was significantly higher than any other treatments, including the bulk larvae extract. The growth rate of cultures supplemented with F2, F3 and F4 did not significantly differ from the water control (Figure 4C). The cell density at 24 hpi was significantly reduced for *B. goodwinii* in cultures supplemented with F1 and F4 compared to the other treatments (Figure 4D). Altogether, these results suggest that F1 contains the chemical elicitors responsible for the proliferation (higher growth rate) of B. goodwinii, but that these compounds, when separated from the other fractions, reduce cell density in the stationary phase of growth.

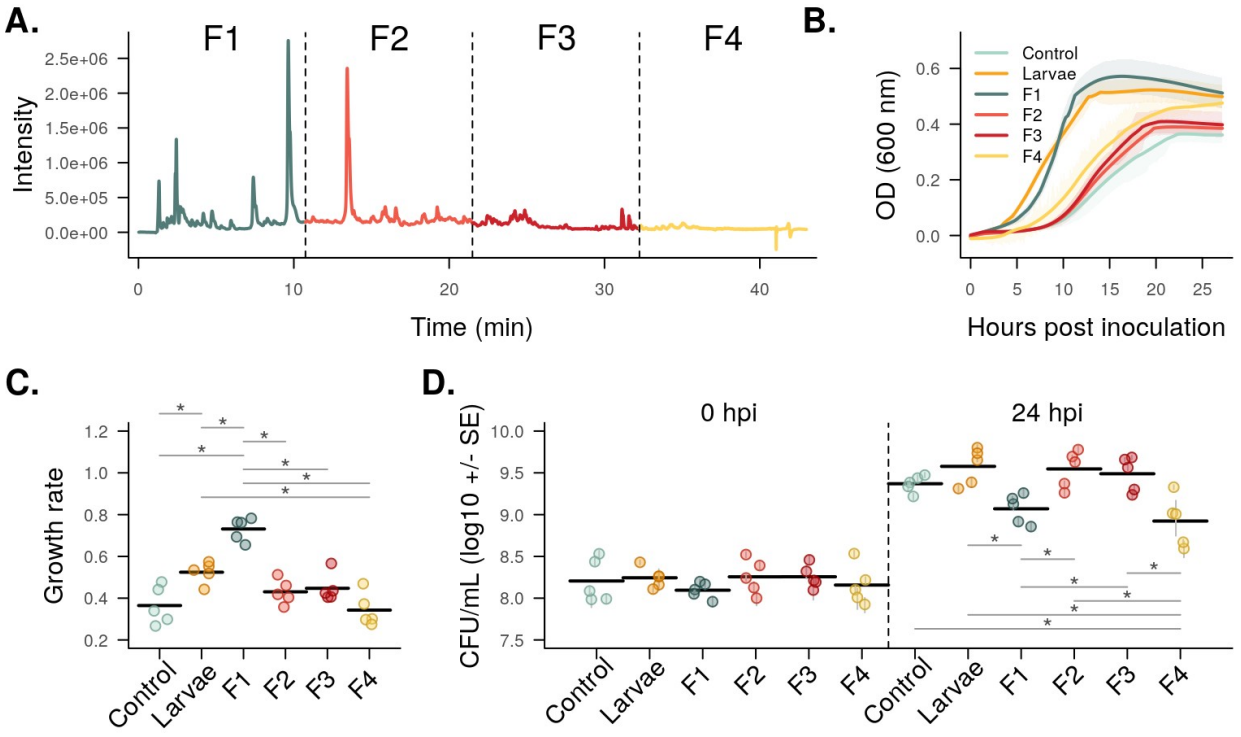

Figure 4: **Bioactivity of different fractions of the larvae extract. A.** Chromatogram/trace of the bulk larvea extract ("Larvae" treatment on bottom figures). A part of the bulk

larvae extract was then fractioned in four elution time of xx minutes (fraction F1 to F4 on the bottom figure). **B.** Growth curves, **C.** growth rates, and **D.** cell density were obtained in 200 µL cultures supplemented with the bulk larvae extract (Larvae), the four larvae extract fractions (F1 to F4) and water (Control). Bars with stars indicate significant differences (TuckeyHSD, p-value < 0.05). OD = optical density; CFU = colony forming unit; SE = standard error

Discussion

Many studies have investigated the importance of insect-bacteria interactions in plant diseases from the perspective of the role of bacterial volatile organic compounds (VOCs) in insect behaviour (Davis et al., 2013; Leroy et al., 2011; Weisskopf et al., 2021), whereas less attention has been paid on the influence of insect-derived chemical cues on bacterial growth and virulence. In this study, we investigated the impact of insect chemical elicitors on bacteria associated with Acute Oak Decline (AOD). We extracted compounds from gallery woody-tissue and *A. biguttatus* larvae and tested their activity toward the major causal pathogen in AOD lesion formation, *Brenneria goodwinii*. We found that larval elicitors, but not gallery woody-tissue compounds, increase *B. goodwinii* proliferation and virulence gene expression.

Extracts from larval gallery woody-tissue did not cause bacterial proliferation similar to healthy wood controls, suggesting that the proliferation of the bacteria around beetle gallery *in planta* (Denman et al., 2018) is not mediated by the tree host chemical compounds. The growth rate of cultures were in fact lower for gallery woody-tissue extracts compared to healthy wood extracts. This could be explained by the fact that the tree host may produce defence compounds in response larval invasion (Broberg et al., 2018), negatively affecting bacterial growth rate. However, the final cell density was not significantly different between gallery and healthy wood tissue extract treatment, suggesting that the extracted chemicals only affect the speed of proliferation but not final cell density. In addition to a reduction of growth rate, the wood gallery extracts triggered the expression of flagella-associated genes; *fliA*, *fliF*, *fliI*, *fliM*, *flgD*, *flgG*, *and flgJ*, mainly involved in bacterial motility (Thomas et al., 2001; Wilkinson et al., 2011), but also in virulence and plant wall adherence in certain plant pathogens (Jahn et al., 2008; Malamud et al., 2011). The production of flagella in the gallery environment could thus be either a way for the bacteria to move away from an unsuitable environment, or an activation of some virulence processes components. The indirect effect of *A. biguttatus* larvae on *B. goodwinii* virulence through the modification of the woody-tissue

environment is thus less powerful than the direct effect of larval extracts, but cannot be completely excluded.

Larval elicitors increased bacterial proliferation *in vitro*, showing that the interaction between insect and bacteria observed *in planta* is at least in part directly mediated by larval compounds. Previous studies conducted on artificially infected oak logs, showed that the lesion size caused by AOD bacteria significantly increased in the presence of the beetle larvae (Denman et al., 2018). In addition, these lesions are observed in close proximity to beetle galleries. In the light of our results, we can hypothesise that elicitors are released during larval activity as they progress through the woody-tissue, and that these compounds trigger the proliferation of *B. goodwinii*, which then starts degrading wood tissues. However, we cannot exclude the possibility that elicitors may be metabolised by the bacteria and may act as a food source in our experiment.

The link between larval elicitor production and bacterial lesion formation is further supported by the fact that larval extracts also triggered the expression of virulence genes in *B. goodwinii*. Among the 418 genes we found over-expressed when adding larval extracts were some potential virulence genes associated with a Type III secretion system (T3SS), which is well reported in other plant pathogens. These include the *hrp* gene encoding for the harpin constitutive of the needle of the T3SS in *Pseudomonas syringae* (Collmer et al., 2000). This T3SS belongs to the Hrc-Hrp1 family only present in plant pathogens and not in animal pathogens, and have the particularity of having a long, flexible pilus, able traverse the thick plant cell wall, suggesting that the target of this T3SS is the plant host (Troisfontaines & Cornelis, 2005). In addition, we found two *AvrE* genes up-regulated in the presence of larval elicitors, which were previously reported to be up-regulated in the presence of *A. biguttatus* larvae *in planta* (Doonan et al., 2020). The *AvrE* gene encodes for a T3SS effector, involved in the suppression of plant immunity in *Pseudomonas syringae* and other plant pathogens (Degrave et al., 2015). These results give another strong indication that beetle larvae are involved in triggering the virulence of *B. goodwinii*, and the *AvrE* gene should be knocked-out to confirm its function in the AOD system.

One fraction of the *A. biguttatus* larval extract produced the same growth curves as the bulk larvae extract when added to a culture of *B. goodwinii*. The growth rate obtained with this fraction was in fact higher than with the bulk larvae extract, while the final cell density at 24 hpi was lower. This could suggest that several chemical compounds are necessary to reproduce the full phenotype observed when supplementing cultures with the bulk larvae extract, which may be present in several of the four fractions. Identifying the bioactive compounds should be a future priority but it is hindered by the limited

availability and seasonality of *A. biguttatus* larvae for further characterisation. Despite this challenge, we successfully narrowed down the activity range of larval extracts responsible for the proliferation of *B. goodwinii*.

Our results bring further arguments in favour of the role *A. bigutattus* plays in AOD, acting as a synergistic factor with *B. goodwinii*, the major AOD lesion formation pathogen. Whether the bacteria are present in the tree in a non-pathogenic state before the beetle invasion, or if it is brought by the beetle to the tree remains to be answered, but our study clearly shows the necessity of integrating *A. bigutattus* population survey and management into programs tackling AOD.

The specificity of this insect-bacterium interaction, and the potential benefit it provides to each partner need to be further evaluated to understand their evolutionary consequences. The same bioactive compounds produced by *A. bigutattus* larvae could also be produced by other bark-boring beetle species or their associated microbiota. Our set of experiments could be repeated with other closely related species such as *A. sulcicollis* which has a similar ecology to *A. biguttatus* (Alexander, 2003; Jendek, 2016). It is also possible that this interaction provides a benefit to the beetle larvae, as the bacteria is known to express detoxification genes when infecting the tree (Denman et al., 2018; Doonan et al., 2020). This may provide protection for the larvae against plant defences as well as weakening the tree, thus increasing larval survival and overall reproductive success. For the bacteria, supporting the growth and emergence of beetle larvae could lead to facilitated multiplication and transmission between trees. On the other hand, this interaction could equally be incidental and not a result of any evolutionary relationship, with no impact on the bacteria or beetle fitness.

Although the importance of interactions between insects and bacteria is well established in the case of symbiosis (Douglas, 2009), or insect behaviour manipulation by bacterial VOCs (Davis et al., 2013), our study is the first to report the impact of chemical elicitors released in the environment by an insect that triggers life history changes in a bacterial species. The implications of this finding are of special importance in the context of recent increase in plant disease outbreaks involving pest-pathogen interactions. We show that an insect herbivore affects bacteria behaviour by triggering the expression of virulent phenotypes and facilitating lesion formation in live bark tissue, thus contributing to tree decline. Such overlooked interactions warrant future investigation to safeguard forest biomes.

Material and methods

Gallery woody-tissue and larvae sample production

A. biguttatus adult beetles were collected from hatching cages containing infested oak billets and maintained in the lab as described by (Reed et al., 2018). A maximum of 8 beetles were housed per 2L cage. Beetle eggs were laid on paper towel placed at the base of the cages, collected twice weekly and incubated in 15 mL Falcon tubes at 17.5 °C until 1 or 2 days before hatch time as predicted by the egg hatching model (Reed et al., 2018). Incubated eggs were then inserted into wounds of pretreated oak billets as described in (Reed et al., 2018). For this study oak billets were cut to a length of approximately 90-100cm and allowed to stand in water for 2 weeks prior to inoculation. Then, 10-15 eggs were placed in each wound with 4 wounds per billet. Infested billets were incubated at room temperature for 3 months before harvesting of larvae from the billets. Larvae and woody-tissue samples were collected by removing the outer bark with a wood knife to expose larval galleries. Larvae were collected in 1.5 mL tubes and frozen into liquid Nitrigen. Asymptomatic woody-tissue, and gallery woody-tissue were collected in 15 mL tubes and frozen in liquid Nitrogens. Samples were stored at -80 °C until chemical extraction.

Larval and wood compound extraction and analysis

Chemical extraction

Woody-tissue compounds were extracted from 2 cm x 2 cm (64±20 mg) sections of inner bark material (either asymptomatic or containing larval galleries) in 5 mL 100% MeOH for 2 h. To remove cellular debris, extracts were filtered through a plug of silanized glass wool held within a Pasteur pipette, into pre-weighed glass vials. Resultant extracts were dried to completion under a gentle stream of N2. Five biological replicates were performed per treatment, and an extraction blank containing only 100% methanol was also included.

Ten snap-frozen larvae collected from the oak log trial were weighed and individually extracted in 5 mL of 100 % methanol for 2 h. To remove cellular debris, extracts were filtered through a plug of silanized glass wool held within a Pasteur pipette, into pre-weighed glass vials. Extraction blanks containing only 100% methanol were also included. 400 µL of each extract were kept for LC-MS (liquid chromatography and mass

spectrometry) analysis. The remaining of the extracts were dried to completion under a gentle stream of N_2.

Dried extracts of wood and larvae were resuspended in miliQ water to a 3 mg/mL concentration and sterilised on a 0.22 µm filter (REF), for bioactivity tests. The 10 extracts were then pooled by pairs in equal volume to obtain five biological replicates and stored at -20 °C until processing.

LC-MS conditions

LCMS analysis was carried out using an Acquity ultra-high-pressure liquid chromatography (UPLC) system coupled to a Synapt G2Si Q-Tof mass spectrometer with an electrospray ionisation source (Waters, UK). The system was controlled through Masslynx 4.1 software (Waters). Chromatographic separation was carried out at a flow rate of 0.21mL min^{-1} using a UPLC BEH C18 column (2.1 x 150mm, 1.7 µm, Waters) coupled to a C18 Vanguard pre-column (2.1 x 5mm, 1.7µm, Waters). The mobile phase consisted of solvent A (0.02% formic acid v/v, water) and solvent B (0.02% formic acid v/v in methanol) with the following gradient: 0 min, 95:5 (A:B), to 85:15 at 7 min, 75:25 at 11 min, 70:30 at 13 min, 50:50 at 18 min; 25:75 at 25 min; 0:100 at 30 min, and 95:5 at 39.1 min.

An Acquity photodiode array (PDA) detector was used to monitor the UV trace (range 200-450nm), sampling rate of 10 points s-1 with resolution set to 2.4nm. The Synapt was operated in sensitivity mode and set to a mass range of 50 – 1200 Da and scan time = 0.1s, in both ionisation modes. The system was operated in MS1 mode with the following conditions: capillary voltage – 2.5KV, sample cone voltage 30V, sample offset 80V, source temperature 100°C, desolvation temperature 300°C, desolvation gas flow 800L h-1, cone gas flow 57L h -1.

Preparative HPLC conditions

To locate active fractions within the larval extracts, preparative HPLC was performed by repeated injections on a Hichrom ACE 5 C18 column (250 × 4.6 mm i.d.) connected to Shimadzu Nexera UC SFC-HPLC hybrid system. The mobile phase consisted of solvent A (water) and solvent B (methanol) with the following gradient: 0 min, 95:5 (A:B), to 85:15 at 7 min, 75:25 at 11 min, 70:30 at 13 min, 50:50 at 18 min; 25:75 at 25 min; 0:100 at 30 min, and 95:5 at 39.1 min. The injection volume was 100 µL (five successive injections), and the separation was carried out over 43 minutes with a flow rate of 1 ml/min. Fractions were collected every 10.75 min, yielding four individual fractions. The

four fractions collected using prep-HPLC were evaporated to 1 ml using a rotary evaporator, after which the fractions were dried under a gentle stream of nitrogen.

Compound bioactivity analysis

Bacterial strain and medium

Bioactivity assays were conducted on the type stain *Brenneria goodwinii* FRB141 kept in glycerol stocks at -80 °C. Before the experiment, the strain was streaked on Nutrient Agar (LAB M Limited) and incubated for 48h at 28 °C. Starter cultures were then prepared by picking a single colony and growing it overnight in 10 mL of Nutrient Broth n°2 (Oxoid) at 28 °C and 150 rpm shaking. Starter cultures were rinsed twice by pelleting the cells for 5 min at 3000 g, discarding the supernatant and adding 10 mL of Ringer 1/4 (LAB M Limited). Cells were then pelleted again, and OD (Optical Density) at 600 nm was adjusted to 0.5 in Ringer 1/4.

Subsequent growth measurements were performed in M9+glu minimal medium composed of M9 salt (Na_2HPO_4 42 mM; KH_2PO_4 22 mM; NaCl 8.55 mM; NH_4Cl 18.6 mM; $MgSO_4 \bullet 7H_2O$ 2 mM; $CaCl_2$ 0.1 mM) and 20 % glucose, with or without the addition of compounds tested for bioactivity.

Growth experiment

The bioactivity test of gallery woody-tissue compounds was performed in culture broth composed of 1 mL of gallery woody-tissue or asymptomatic woody-tissue extract (3 mg/mL), 0.66 mL of M9+glu minimum medium (4.5X), 1.04 mL of water, and 0.3 mL of starter culture (starting OD_{600} of 0.05), for a final volume of 3 mL. For controls, woody-tissue extracts were replaced by compound extraction blanks. Compounds from 5 gallery woody-tissue samples and 5 asymptomatic wood samples were tested with 1 replicate culture per sample and cultures were placed in a 12-well plate. To allow for comparison with hypothesis 2 which could not be performed in 3 mL volume, we replicated the growth experiment in 200 µL volume (Figure S1).

Because the number of larvae available for the experiment was limited, the quantity of chemical extract evalable were small. Consequently, the bioactivity test of larval compounds was performed in a smaller culture volume. The broth was composed of 60 µL of larval extract (3 mg/mL), 40 µL of M9+glu minimum medium (4.5X), 80 µL of water, and 20 µL of starter culture (starting OD_{600} of 0.05), for a final volume of 200 µL.

For controls, larval extracts were replaced by compound extraction blanks. Compounds from 4 separate larvae were tested with 6 replicate cultures per larvae to account for the higher noise in OD measurement when performed in small culture volumes and cultures were placed in a 96-well plate.

For each growth experiment, a replicate plate containing the same broth, but without inoculation was performed to get OD background correction with sterile medium and check for medium contamination. Plates were placed into the Stratus plate reader (Cerillo) for OD600 measurement every 3 minutes over the course of the experiment. The plate reader was incubated at 28 °C and shaken at 150 rpm for 24 h. A 20 µL aliquot of each culture was collected at 0 hpi (hours post inoculation) and 24 hpi for Colony Forming Unit (CFU) quantification.

The number of viable cells in the cultures was estimated at 0 hpi and 24 hpi by adding 20 µL of culture to 180 µL of sterile Ringer 1/4 and completing a serial dilution in the same way up to 10^{-8} in a 96-well microplate. Six replicates of 10 µL of each dilution were then dropped on Nutrient Agar and incubated for 24 h at 28 °C. The number of CFU was counted from the appropriate dilution, and the total CFU/mL of culture was estimated as $\frac{CFU \times 10^{dilution}}{10} \times 1000$. CFU concentration from the 6 replicate 10 µL drops were averaged and standard error was calculated.

Transcriptome analysis

To ensure sufficient biomass for RNA-seq analysis, cultures were set up in a final volume of 3 mL as described above, and larval extracts had to be pooled by 2 to obtain enough material. Extracts from 5 gallery woody-tissue samples, 5 asymptomatic woody-tissue samples and 5 pairs of larvae and 5 extraction blanks were added to culture broth and plates were incubated for 10 hours to obtain cultures in mid-exponential phase of growth. Cultures were placed into 2 mL screw cap tubes, centrifuged for 10 min at 3000 rcf to discard the supernatant, and immediately frozen into liquid nitrogen. Cell pellets were then stored at -80 °C until RNA extraction.

The total RNA was extracted from cultures using a phenol-chloroform extraction adapted from (Griffiths et al., 2000). Briefly, cells were mechanically lysed by adding to the frozen cells 0.5 g of 452-600 µm acid-washed glass beads (Sigma), 500 µL of 5 % CTAB in potassium phosphate buffer 120 mM pH 8 and 500 µL Phenol:Chloroform:Isoamyl-alcohol (25:24:1) pH 8 (REF). Tubes were then shaken for

30 s at 2500 rps with the PowerLyzer 24 (MO BIO Laboratories Inc) and centrifuged at 21952 rcf for 10 min at 4 °C. The aqueous phase was washed with 500 µL of Chloroform:Isoamyl-alcohol (24:1) and centrifuged at 21952 rcf for 10 min at 4 °C. 1 mL of 30 % Polyethylen Glycol 6000 solution was added to the aqueous phase and incubated overnight at 4 °C to precipitate nucleic acids. Tubes were then centrifuged at 21952 rcf for 30 min at 4 °C and pellets were washed with ice-cold 70 % ethanol. Pellets were dried for 20 min at room temperature and re-suspended in 50 µL water. Genomic DNA was removed using the TURBO DNAse (Invitrogen) following the manufacturer's instructions. RNA was precipitated for 2 hours at -20 °C with 25 µL of NaCl (6 M), 5 µL of Sodium Acetate (3 M) and 50 µL of ice-cold 2-propanol. Tubes were centrifuged, pellets were washed with ethanol and air-dried as previously described. RNA was re-suspended in a final volume of 20 µL of nuclease-free water. RNA integrity was checked on agarose electrophoresis and concentration and purity were assessed by spectrophotometry (ND-1000, Nanodrop) and fluorometry (Qubit RNA High Sensitivity kit and Qubit 3, Invitrogen). None of the extraction negative controls contained detectable RNA and were thus not sent to sequencing.

RNA extract Quality Control (QC) and sequencing were performed by Novogene (Cambridge). Quantity, purity, and integrity of the samples were assessed using Nanodrop (ThermoFisher), Agarose Gel Electrophoresis and Bioanalyzer 2100 (Agilent). Library preparation steps included rRNA depletion, RNA fragmentation, reverse transcription, Illumina adapter ligation, size selection and PCR amplification. The libraries were sequenced on the Illumina NovaSeq plateform with PE150 strategy.

Data analysis

Data analyses were performed on R version 4.3.2.

In vitro growth data

Raw OD600 values were averaged between technical replicates and the noise in OD measurement was reduced with a locally weighted scatterplot smoothing regression using the lowess function from the stats package v4.3.2. Growth rates were estimated from these averaged growth curves using the growthcurver package v0.3.1 (Sprouffske & Wagner, 2016). Statistical differences between treatments for growth rates and CFU number were assessed using either a t-test, with the t.test function, or a wilcoxon pairwise comparison when appropriate the condition of validity for the t-test were not met.

RNA-seq data

The bioinformatic analysis was conducted on the Supercomputing Wales cluster. The reference genome and annotations from the FRB141 strain were downloaded from Genbank under the accession GCF_002291445.1 (BioProject PRJNA308225), and the annotation file formatting was curated before downstream analysis. A transcript FASTA file was produced from the reference genome and annotations using gffread v0.11.5. The RNA-seq data were then analysed with the nf-core/rnaseq nextflow pipeline v3.10.1 (10.5281/zenodo.1400710; (Ewels et al., 2020) with raw fastq read, the reference genome, the reference annotation, and the reference transcript FASTA as input. Briefly, raw read quality was assessed with fastQC. Genome index was generated with STAR (Dobin et al., 2013) using the CDS entries from the annotation and a transcript index was generated using Salmon (Patro et al., 2017). The transcriptome reads were then aligned against the genome index using STAR and gene transcripts were quantified with Salmon and count tables were generated.

Count data were imported in R using the tximport package v1.28.01. Transcripts with less than 10 reads in total in the whole dataset were removed from the dataset. To assess the overall gene expression profile similarity between samples, and detect potential outliers, transcript counts were normalised using the median of ratio normalisation method, and regularised log (rlog) transformed using the DESeq2 package v1.40.1 (Love et al., 2014). A correlation of these rlog transformed counts was computed using the cor function and samples were represented on a clustered heatmap with hierarchical clustering using the pheatmap package v1.0.12. One culture supplemented with larvae extract clustered separately from any other cultures and was considered an outlier, and therefore excluded from the subsequent differential gene expression analysis.

Differential gene expression between treatment and controls was performed using the DESeq2 workflow from the DESeq2 package v1.40.1 (Love et al., 2014). For the first hypothesis, the treatment was gallery woody-tissue extract and the control asymptomatic wood extract. For the second hypothesis, the treatment was larvae extract, and the control was extraction negative control. p-values were computed and adjusted for multiple testing using the Benjamini-Hochberg method. Differentially expressed genes were determined considering a 5 % false rate discovery (adjusted p-value < 0.5) and at least a 1.5-fold change between treatment (log2 fold change (LFC) > 0.58).

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Authors contributions

SD, JV and JM had the original idea and secured funding for the study. MCC and GT designed the experiments. MC performed beetle collection and rearing, and set up the oak billets infestation. GT performed chemical compounds extractions and analysis, with feedback and support from JC and JV. MCC developed protocols and performed the bioactivity assays and RNA seq experiment with support and feedback from JM, JD and UH. MCC analysed the data, created the figures and wrote the first draft of the manuscript. All authors reviewed the manuscript.

Data availability

The raw RNAseq data are available on the European Nucleotide Archavie under the project accession PRJEB69286.

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Supplementary Figures

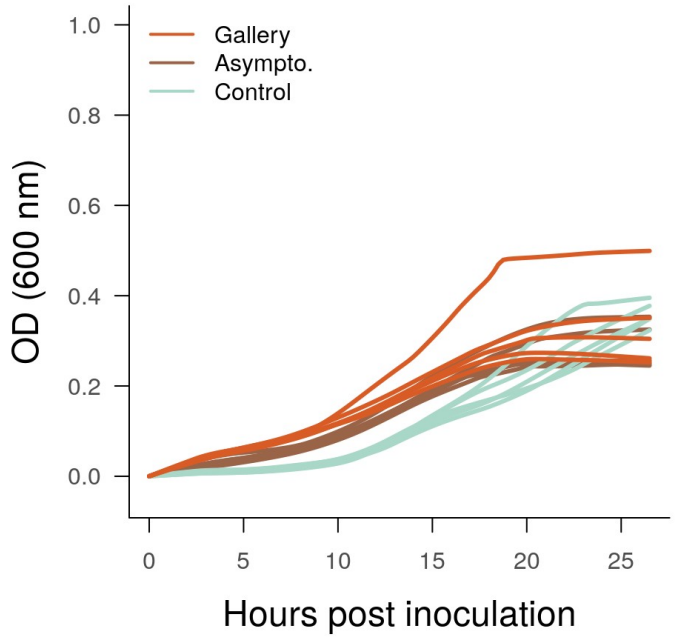

Figure S1: Growth curves obtained in 200 µL liquid cultures supplemented with gallery woody-tissue (Gallery) and asymptomatic woody-tissue (Asympto.) compounds, compared to compound extraction blanks (Controls).