Supporting Information

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SI Results and Discussion

Ethanol and Fungal Growth. To further validate the observations from the plate-based bioassays, we measured the growth of liquid cultures in the presence or absence of ethanol and in the presence or absence of 2% glucose (Fig. S1). While increasing concentrations of ethanol generally led to prolonged lag phases, these were considerably shorter for *A. grosmanniae* and *R. canadensis* (a maximum of about 20 h in the presence of 5% or 10% ethanol) than for a *Penicillium* competitor isolate (about 48 h) (Fig. S1). The growth rate was enhanced in most cases by the addition of 2% glucose, but this did not greatly affect the duration of the lag phase. While *A. roeperi* displayed extremely low overall growth rates, this symbiont grew only in the presence of ethanol in this bioassay.

Ethanol Quantification. We confirmed that biologically relevant levels of ethanol were tested as part of our current study by quantifying ethanol within samples of flood-stressed *C. florida* trees, ethanol-irrigated *C. florida* trees, and the sawdust-based substrate (Fig. S2).

C. florida trees irrigated with solutions containing 5% ethanol and the sawdust-based substrate containing 2.5% ethanol contained amounts of ethanol comparable to those present in sapwood tissues of flood-stressed *C. florida* trees. The sawdust-based substrate containing 5% ethanol contained higher levels of ethanol compared with all other treatments, and no ethanol was detected in nonflooded *C. florida* trees or in trees irrigated with 0% ethanol (Fig. S2).

Ethanol generated in flooded, anoxic roots as a byproduct of anaerobic respiration is transported via the transpiration system from the roots to stem and leaf tissues (32, 34). Our analyses confirmed the root adsorption of ethanol by *C. florida* and its movement via the transpiration stream. Ethanol was also absorbed by the roots of willow (*Salix babylonica*) trees during hydroponic studies (70). Concentrations of ethanol in sapwood tissues of ethanol-irrigated *C. florida* trees were confirmed to be within the range detected in flood-stressed *C. florida* trees that *X. germanus* would encounter under natural conditions. With the exception of the 5% ethanol-infused sawdust substrate, ethanol was also present in the sawdust rearing substrate at concentrations comparable to those ambrosia beetles would encounter in flood-stressed trees under natural conditions.

Similar to *S. babylonica* (70), leaf drying and chlorosis were observed as visible symptoms of phytotoxicity in trees irrigated with solutions containing 5% ethanol. It is unclear if ethanol phytotoxicity resulted in physiological changes within *C. florida* and *C. canadensis* trees that benefitted fungiculture by *X. germanus*; constitutive and induced host chemical defenses were not characterized as part of our current study. The impact of such defenses on ambrosia beetles and their fungal symbionts is not nearly as well understood as on the phloem-feeding bark beetles. In light of our current results, the role of host tree chemistry on the inability of *X. germanus* to colonize healthy trees devoid of ethanol warrants additional studies.

SI Methods

Ethanol and Fungal Growth. The effect of ethanol was further assessed using liquid cultures (Fig. S1). To this end, ∼2-wk-old plate cultures grown on MEA were surface-washed to collect conidia. An aliquot containing 10^6 conidia/mL was inoculated into 200-μL cultures containing 1× yeast nitrogen base (Sigma Aldrich) (pH 5.4) and 0%, 5%, or 10% ethanol (99.8%; Roth). Six replicates were run per strain and were grown for 3 d in 96-well plates at 25 °C and 200 rpm in a New Brunswick Innova 42R shaking incubator (Eppendorf). Measurements were taken in a microplate reader (Infinite M200 PRO; Tecan) at 490 nm with 5-min shaking at 432 rpm preceding each measurement.

ADH Activity. Cultures of *A. grosmanniae*, *A. roeperi*, *R. canadensis*, *A. hylecoeti*, *Penicillium* sp., and *Aspergillus* sp. were grown on cellophane discs on YEMA medium (1% malt extract, 0.3% yeast extract, 1.5% agar) for 4–12 d at 26 °C in the dark until their growth covered at least 50% of a 9-cm Petri-dish (n = 7–8 per species). Fungal tissue was fully harvested from three to four plates per fungus and was immediately frozen at −80 °C (control group, C). The four remaining plates per fungus were flooded with 1 mL of a filter-sterilized 2% ethanol solution in dH_2O, wrapped immediately with Parafilm, and stored in darkness at 26 °C. Six hours later (for the short exposure to ethanol treatment) or 93 h later (for the long exposure to ethanol treatment) the fungal growths from each remaining plate were harvested and immediately frozen at −80 °C. Samples of *A. hylecoeti* had to be discarded because of contamination with bacteria. Each fungal tissue sample (n = 56; 200–400 mg) was homogenized three times for 1 min each in 1 mL of ice-cold Tris-buffered saline (TBS) [50 mM Tris·HCl, 150 mM NaCl (pH 7.5)] using zirconium oxide beads [0.5 mm; Süd-Laborbedarf GmbH (SLG)] and a Bead Bug (no. D1030-E; Benchmark Scientific, Inc.) and was centrifuged at 13,000 × g for 10 min at 4 °C. The proteinaceous supernatant was stored on ice and was used immediately for the ADH activity assays and protein concentration measurements.

For the ADH assay, a kit (catalog no. MAK053; Sigma-Aldrich) was used according to the manufacturer's specifications with slight changes. Briefly, the ADH activity of the extracts was determined in duplicate in TBS buffer (see above) using 5 μL of sample and ethanol (133 mM) as a substrate and a brand-specific developer containing NAD^+. Incubation at 37 °C in an Infinite 200 PRO microplate reader (Tecan) resulted in a colorimetric (450 nm) product (NADH) proportional to the ADH activity present in each sample, after the colorimetric value of a control (H_2O instead of ethanol) had been subtracted. NADH generation was followed over 30 min, and ADH activity was calculated from a 3- to 5-min interval in the linear range of the activity curve. The absolute amount of NADH generated per minute was determined by comparing it with a calibration standard curve. One unit of ADH was defined as the amount of enzyme that will generate 1.0 μmol of NADH/min at pH 7.5 and 37 °C. ADH activity is therefore reported as $nmol \cdot min^{-1} \cdot mg^{-1}$ total protein = milliunits/mg. Total protein concentrations in the samples for normalization were determined using a microplate version of the Bradford assay (Roti-Quant; Roth) and a BSA calibration curve.

Ethanol Quantification. Trees were flood-stressed as described in ref. 32 or were irrigated on four occasions with ethanol solutions containing 0%, 1%, 2.5%, or 5% ethanol (vol/vol) (∼2.8 L per watering event). Trees were held in a greenhouse for 7 d. Stems were then cut at the base, and an ∼1.3-cm-thick cross-sectional disk of sapwood tissue was collected and immediately sealed within a plastic bag under a block of dry ice. Stem sections were then transferred to a −40 °C ultra-low-temperature freezer until analysis. The sawdust-based agar substrate containing 0%, 0.1%, 0.5%, 1%, 2.5%, or 5% ethanol was prepared as

previously described and held for 4 d in a laminar flood hood until analysis.

SPME-GC-MS was used to analyze ethanol (26). A comparable amount of rearing substrate was also placed in separate GC vials. Vials were suspended in a water bath at 70 °C for 5 min, removed, and then a SPME fiber [carboxen-polydimethysiloxane (CAR/PDMS); 75-μm coating; Sigma-Aldrich] was exposed to the headspace volatiles within the vial for 5 min. Then the SPME fiber was immediately thermally desorbed for 2 min at 250 °C in the injection port of an Agilent 7890B gas chromatograph (Agilent Technologies) operated in splitless mode from 0 to 2 min and then at a 1:20 split for the remainder of the run. A DB-5MS column (0.25 μm × 30 m × 0.25 μm; cross-linked/surface bonded 5% phenyl, 95% methylpolysiloxane; Agilent J&W) was used for analysis: 40 °C to 60 °C at 3 °C/min and 60°C to 230 °C at 20 °C/min. An Agilent 5977A mass spectrometer was operated in electron impact mode with a scan range of 40–415 atomic mass units. Fibers were conditioned before each analysis by exposure within the injection port for 20 min at 250 °C. The external standard method (26) was used for determining concentrations of ethanol associated with the sapwood tissue and sawdust-based agar substrate. Peak areas associated with the ethanol standards were measured, and a standard concentration curve was used to determine concentrations of ethanol associated with the samples.

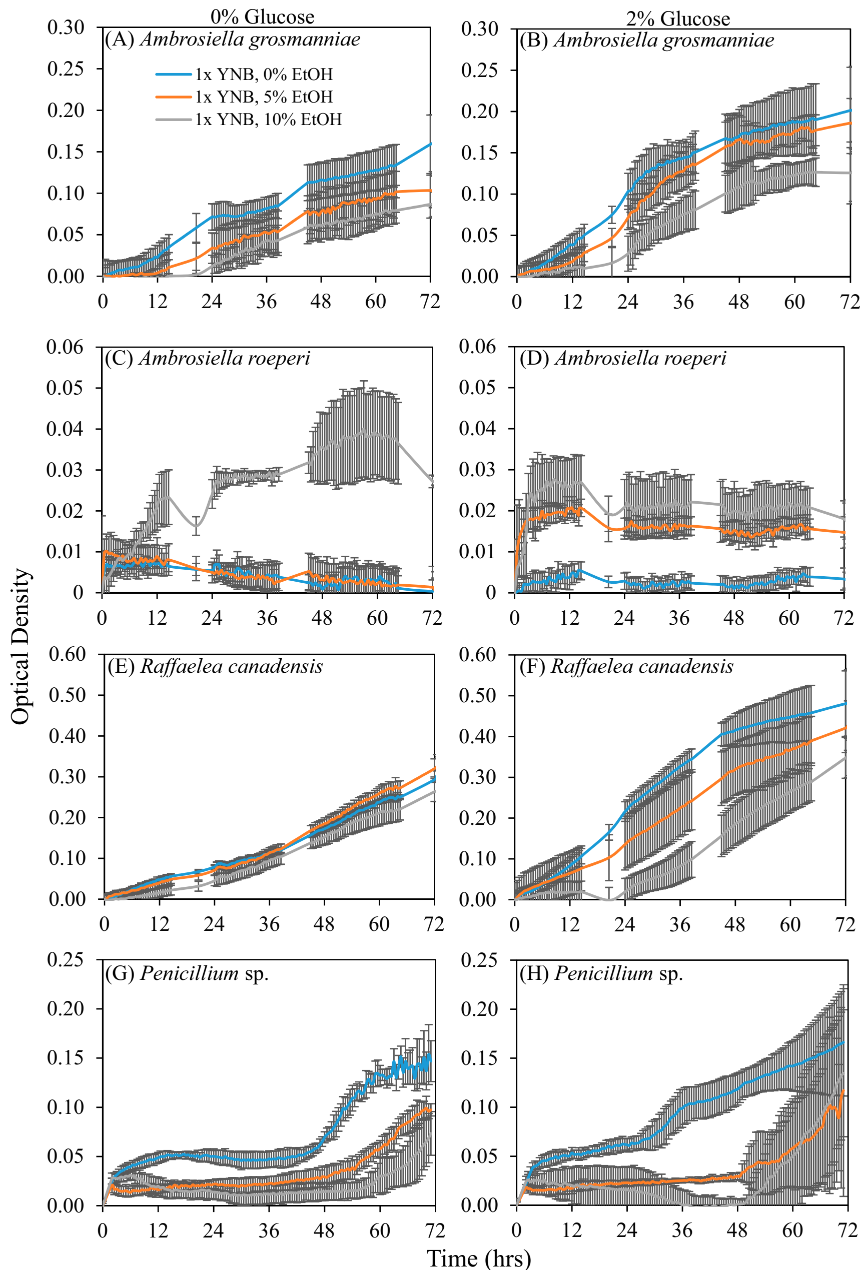

Fig. S1. Online monitoring via a microplate reader of liquid cultures characterizing the effects of ethanol in the medium at 0%, 5%, or 10% (vol/vol) on the growth rate of *A. grosmanniae* (*A* and *B*), *A. roeperi* (*C* and *D*), *R. canadensis* (*E* and *F*), and a *Penicillium* isolate as representative microbial competitor (*G* and *H*). Cultures were grown in 200 μL of yeast nitrogen base (YNB) in the presence (*B*, *D*, *F*, and *H*) or absence (*A*, *C*, *E*, and *G*) of 2% glucose (*n* = 6 per species). Error bars represent ±SD.

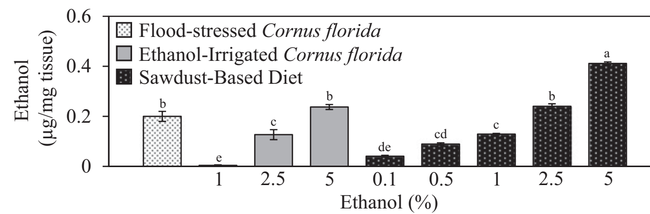

Fig. S2. Comparison of ethanol concentrations within stem sapwood tissues of *C. florida* trees that were flood-stressed or irrigated with solutions containing 0%, 1%, 2.5%, or 5% ethanol (vol/vol) and in a sawdust-based agar substrate containing ethanol at concentrations of 0%, 1%, 2.5%, or 5% ethanol (vol/wt). Analysis and quantification of ethanol were performed using SPME-GC-MS. Different letters denote significant differences in mean values using one-way ANOVA and Tukey's HSD at $P < 0.05$ ($n = 10$ flooded *C. florida* trees, $n = 5$ ethanol-irrigated *C. florida* trees per concentration, and $n = 5$ *C. florida* trees for sawdust substrates per ethanol concentration; $F_{49} = 72.22$; $P < 0.0001$). Error bars represent ±SE.

Table S1. Weighted regression equations corresponding to the influence of ethanol on the growth of selected fungi and offspring production by *X. germanus*

Species	Parameter	Figure	r^2	Equation
X. germanus	Larvae	2	0.90	$Y = 11.56 \times X - 7.18 \times X \times n(X)$
X. germanus	Pupae	2	0.96	$Y = 4.46 \times X - 2.77 \times X \times \ln(X)$
A. grosmanniae	Dry weight	3	0.99	$Y = 0.063 \times e^{(-0.5[(X - 1.369)/1.769)\text{^}2]}$
A. grosmanniae	Surface area	3	0.99	$Y = 38.4/[1 + (X/3.147)^{6.172}]$
A. grosmanniae	Density	3	0.91	$Y = (0.124 - 0.021 * \sqrt{X})/(1 - 0.353 * \sqrt{X})$
A. roeperi	Dry weight	4	0.87	$Y = 4 \times 24.11 \times {}^{(-(X + 1.365)/3.638)} \times (1 - e^{(-(X + 1.365)/3.638)})$
R. canadensis	Dry weight	4	0.99	$Y = 60.41 \times e^{(-0.5[(X - 2.363)/2.516)\text{^}2]}$
A. hylecoeti	Dry weight	4	0.72	$Y = 12.1\ 7 - 1.407 \times X$
Penicillium sp.	Dry weight	4	0.98	$Y = 5.891 + 26.768 \times e^{(-X/0.674)}$
Aspergillus sp.	Dry weight	4	0.99	$Y = 16.872 \times e^{(-0.5((X - 0.085)/2.135)\text{^}2)}$

Table S2. Origin of the different sequences used and information on beetle host

Fungus species	Culture no.	Beetle host species	Location	ITS1	ITS3	SSU
A. grosmanniae	XgK74	*X. germanus*	Bern, Switzerland	MG905632	MG905635	
A. hylecoeti	AHWK	*E. dermestoides*	Bern, Switzerland	MG913205		MG905721
Aspergillus sp.	F15	*X. saxesenii*	Bern, Switzerland			MG913203
Penicillium sp.	F4	*X. saxesenii*	Bern, Switzerland			MG905755
R. canadensis	F24	*X. saxesenii*	Bern, Switzerland		MG913206	MG905753
A. roeperi	AroeUS	*X. crassiusculus*	Pineville, LA		MG905638	

All fungal strains were isolated from adult beetles (*X. germanus*, *X. saxesenii*, *X. crassiusculus*) or their galleries (*E. dermestoides*). Internal transcribed spacer 1 (ITS1) and 2 (ITS2) of the nuclear ribosomal DNA between the rRNA genes. Small subunit (SSU) refers to ribosomal DNA transcribing for the small 18S rRNA gene.