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ORIGINAL ARTICLE

Predicting interactions of the frass-associated yeast *Hyphopichia heimii* with *Olea europaea* subsp. *cuspidata* and twig-boring bark beetles

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Abstract
Bark beetles are destructive insect pests known to form symbioses with different fungal taxa, including yeasts. The aim of this study was to (1) determine the prevalence of the rare yeast *Hyphopichia heimii* in bark beetle frass from wild olive trees in South Africa and to (2) predict the potential interaction of this yeast with trees and bark beetles. Twenty-eight culturable yeast species were isolated from frass in 35 bark beetle galleries, including representatives of *H. heimii* from nine samples. Physiological characterization of *H. heimii* isolates revealed that none was able to degrade complex polymers present in hemicellulose; however, all were able to assimilate sucrose and cellobiose, sugars associated with an arboreal habitat. All isolates were able to produce the auxin indole acetic acid, indicative of a potential symbiosis with the tree. Sterol analysis revealed that the isolates possessed ergosterol quantities ranging from 3.644 ± 0.119 to 13.920 ± 1.230 mg/g dry cell weight, which suggested that *H. heimii* could serve as a source of sterols in bark beetle diets, as is known for other bark beetle–associated fungi. In addition, gas chromatography–mass spectrometry demonstrated that at least one of the isolates, *Hyphopichia heimii* CAB 1614, was able to convert the insect pheromone *cis*-verbenol to the anti-aggregation pheromone verbenone. This indicated that *H. heimii* could potentially influence beetle behaviour. These results support the contention of a tripartite symbiosis between *H. heimii*, olive trees, and bark beetles.

Keywords *Hyphopichia heimii* · Bark beetle · Olive trees · Symbiosis

Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are infamous for their ability to weaken and kill some economically important trees, such as Norway spruce (*Picea abies*) and olives (*Olea europaea*) (Harrington 2005; Kreutz et al. 2004; Ruano et al. 2010; Six and Bentz 2003; White et al. 1980). Bark- and wood-boring beetles known to be associated with olive trees in Southern Africa, including the Stellenbosch region, are *Lanurgus oleae* and *Xyleborinus aemulus* (Jordal 2021; Wood and Bright 1992; F. Roets, personal communication). Beetle-induced tree death is commonly a result of a girdled circulatory system that occurs when the beetles feed on the subcortical tissues (Christiansen and Ericsson 1986; Valiev et al. 2009; White et al. 1980). However, vascular tissues, such as phloem, are relatively poor in nutrients and beetles overcome this obstacle by forming symbioses with different bacteria, filamentous fungi, and yeasts (Dowd and Shen 1990; Hernández-Martínez et al. 2016; Postma et al. 2012; Six 2013).

Yeasts are associated with all ontogenetic stages of bark beetles (Callaham and Shifrine 1960). Similar to many mycangial fungal associates, it is believed that yeasts aid beetle nutrition by either increasing nitrogen concentrations in the phloem of host trees or by providing beetles with a source of sterols (Ayres et al. 2000; Bentz and Six 2006; Klepzig and Six 2004; Durand et al. 2018). Dietary sterol sources are crucial for beetles, as these insects are unable to synthesize their own sterols, which play important roles in the biology of insects (Behmer et al. 2013; Bentz and

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Six 2006; Kok and Norris 1973). For example, sterols are important for membrane function, serve as precursors for hormones, act as signalling molecules, and are essential for ontogenetic development. The major sterol component of fungal cell membranes, ergosterol (Nes et al. 1978), is highly nutritious for beetles (Kok and Norris 1973; Klepzig and Six 2004) and higher concentrations of ergosterol in phloem colonized by fungi are thought to be more beneficial to beetle success compared to uncolonized phloem deficient in this sterol (Bentz and Six 2006).

In response to microbial invasion, plants elicit various defence reactions, including the increased release of starch into vascular tissues. However, fungi (including yeasts) are known to produce enzymes such as amylases (Linardi and Machado 1990), cellulases (Sulman and Rehman 2013), and xylanases (Scorzetti et al. 2000), all of which may weaken a trees' resistance against beetle attack. In addition, yeasts may potentially influence the behaviour of beetles as was demonstrated for certain ascomycetous yeast species, such as *Hansenula capsulata* (*Kuraishia capsulata*) and *Hansenula holstii* (*Pichia holstii*) that are associated with the bark beetles *Dendroctonus ponderosae* and *Ips typographus* (Hunt and Borden 1990; Leufvén et al. 1984). These studies revealed that the yeasts were capable of efficiently converting the beetle aggregation pheromones, *cis*- and *trans*-verbenol, to the anti-aggregation pheromone, verbenone.

Interestingly, it was contended that in addition to the above-mentioned yeast-insect interactions, arboreal endophytic yeasts may exert a positive effect on tree health (Moller et al. 2016b). Some arboreal yeasts are incapable of degrading plant polymers such as cellulose, starch, and xylan, but can produce plant growth–promoting hormones like the auxin, indole acetic acid (IAA). This implies that a tripartite symbiosis could potentially exist between arboreal yeasts, trees, and beetles.

A number of yeast species, including *Candida kashinagacola*, *Candida pseudovanderkliftii*, *Candida vanderkliftii*, *Ogataea pini*, and the rare ascomycetous yeast *Hyphopichia heimii*, have thus far only been isolated from beetle-associated habitats (Davis et al. 2011; Endoh et al. 2008; Pignal 1970). The latter was discovered by Pignal (1970) who isolated it from an insect-gallery in wood from Equatorial Africa; however, the author neglected to specify to which tree species the wood belonged. Following the discovery of *H. heimii*, few strains of this yeast have been reported in subsequent studies (Olatinwo et al. 2013; Ali et al. 2019). Considering that *H. heimii* was isolated from wood-associated insects, it is possible that this yeast occurs within the gut and/or mycangia of bark beetles. It also suggests that *H. heimii* may not be associated with a particular beetle species, but could rather be associated with multiple insect taxa that interact with certain tree species, or it may even be an arboreal endophyte (Kurtzman 1987, 2011a; Moller et al. 2016b; Pignal 1970, Rodriguez et al. 2009). Interestingly, while prospecting for basidiomycetous yeasts in dry beetle frass from wild olive trees in Stellenbosch, South Africa, we isolated a representative of *H. heimii* using benomyl-dichloran (BDS) agar (Worrall 1991) without dichloran, but supplemented with chloramphenicol. This suggested that the perceived scarcity of culturable representatives of *H. heimii* in the natural environment may have been the result of inadequate selective procedures during the isolation process. These findings also indicated that the modified BDS medium used to obtain basidiomycetous yeasts from woody environments can also be used to isolate *H. heimii* from these habitats.

Thus far, aside from the taxonomical description available for *H. heimii* (Pignal 1970; Kurtzman 2011a), this yeast remains highly understudied, with nothing known about the potential association between *H. heimii* and either bark beetles or olive trees. The aim of this study was therefore to evaluate the potential interaction of this yeast with wild olive trees and bark beetles. The first objective was to employ above-mentioned modified BDS medium to determine the prevalence of *H. heimii* in beetle frass from wild olive trees located in the greater Stellenbosch region, South Africa, the area from where our first isolate originated. The second objective was to predict the potential interaction of *H. heimii* with wild olive trees and bark beetles based on its physiological characteristics, a first step in generation of hypotheses on the complex interactions between bark beetles and these microbes (Hulcr et al. 2020). To achieve these objectives, isolates were tested for the production of wood-degrading enzymes, plant growth–promoting factors, and the ability to convert insect pheromones.

Materials and methods

Sample collection

Frass samples were obtained from wild olive trees (*Olea europaea* subsp. *cuspidata*) growing in a Mediterranean climatic region at the southern tip of Africa. A total of nine sampling events were undertaken from March 2017 to February 2018. The sampling sites were situated in Stellenbosch, South Africa, at the gardens of the Stellenbosch Institute for Advanced Study (STIAS; 33°56′06.4″S 18°52′26.4″E), along the banks of the Eerste River (33°56′21.7″S 18°51′57.4″E), as well as in the upper parts of the Pappegaaiberg area (33°56′24.0″S 18°50′27.0″E). At each sampling site, dead and dry twigs that were still attached to living wild olive trees were examined for insect borer damage. Those twigs that were presumably previously infested by Scolytinae beetles (as indicated by the presence of holes of less than 5 mm in diameter and that contained

galleries with frass and often also remnants of beetles) were collected and stored in paper bags at room temperature until further processing. We focused on dry frass samples from Scolytinae as this was the source of previous isolates of our focal yeast, *H. heimii*, in an earlier study.

Isolation of yeasts from frass

To isolate culturable yeasts from frass, a sampled twig from each tree was first surface sterilized via sequential submersion (10 to 15 s) in 70% (v/v) ethanol, distilled water (dH_2O), and 1% (v/v) sodium hypochlorite. Thereafter, a scalpel was used to aseptically transfer frass (approximately one loopful) from inside the borer tunnels of each twig to glass test tubes containing 9 mL sterile physiological saline solution. A tenfold dilution series was then prepared and aliquots of each dilution (100 µL) were plated onto a modified selective medium for basidiomycetous yeasts, namely benomyl-dichloran (BDS) agar without dichloran (Worrall 1991), supplemented with 0.2 g/L chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA). We used this medium as this proved successful for isolation of our focal yeast, *H. heimii*, in a previous study. The inoculated plates were incubated for 72 to 96 h at 26 °C, after which yeast colonies were randomly selected using Harrison's disc method (Harrigan and McCance 1976) and transferred to yeast malt extract agar (Kurtzman et al. 2011b), supplemented with 0.2 g/L chloramphenicol (YMc). The latter medium was used for the purification of yeast isolates by successive inoculation and incubation at 26 °C.

Classification and identification of yeast isolates

Yeast isolates were maintained on YM agar at 26 °C and classified using restriction fragment length polymorphism (RFLP) analyses of the internal transcribed spacer (ITS) region (Moller et al. 2016a). The identities of the yeasts were confirmed using sequence analysis of the D1/D2 region of the large-subunit ribosomal DNA (26S rDNA) (Fell et al. 2000; Scorzetti et al. 2002; Vreulink et al. 2010). To achieve this, yeast isolates were first cultured for 24 h to 48 h in 10 mL YM broth at 26 °C on a TC-7 tissue culture roller drum (New Brunswick Scientific Co., Edison, NJ, USA) set to 60 revolutions per minute (rpm).

Yeast genomic DNA (gDNA) extraction and PCR amplification of the ITS region

The extraction protocol described by Vreulink et al. (2010) was used to obtain the gDNA of each culture. PCR amplification of the ITS region was performed using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Inqaba Biotechnological Industries, Pretoria, South Africa; White et al. 1990). Each reaction had a final volume of 25 µL, which consisted of 12.5 µL of 2 × Taq ready mastermix (New England Biolabs, Ipswich, MA, USA), 7.5 µL of nuclease-free distilled water (ThermoFisher, Waltham, MA, USA), 1.5 µL of each primer (10 µmol/L; Inqaba Biotechnical Industries), and 2 µL gDNA. Thereafter, amplification was conducted using an Applied Biosystems 2720 thermal cycler under the following conditions: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, elongation at 72 °C for 1 min, and a final extension step at 72 °C for 4 min (Vreulink et al. 2010). The PCR products were separated on a 0.8% (w/v) agarose gel containing 1% (w/v) ethidium bromide (Sigma-Aldrich) and visualised under UV light.

RFLP analyses

To obtain RFLP profiles, the amplified ITS regions of the yeast isolates were digested with the restriction endonucleases *Hin*6I, *Hin*fI, and *Msp*I according to the manufacturer's specifications (ThermoFisher) (Moller et al. 2016a). The resulting fragments were separated at 90 V for 3 h on a 2% (w/v) agarose gel, containing 1% (w/v) ethidium bromide (Sigma-Aldrich) and the banding patterns formed were visualised under UV light (GeneFlash Syngene Bioimaging Unit). Banding pattern fragment sizes were estimated with the GeneRuler 100-bp Plus DNA ladder (0.1 µg/µL, Thermo Scientific) and measured using the GeneTools analysis software from Syngene. In addition, RFLP profiles of the isolates were compared and those showing identical banding patterns were grouped together.

Identification using ribosomal gene sequence analysis

At least one representative yeast isolate from each RFLP profile was selected to be identified using sequence analyses of the D1/D2 region of the 26S large subunit ribosomal DNA (26S rDNA). This included PCR amplification with the forward primer F63 (5′-GCATATACAATAAGC GGAGGAAAAG-3′) and the reverse primer LR3 (5′-GGT CCGTGTTTCAAGACGG-3′) (Inqaba Biotechnological Industries, Pretoria, South Africa; Fell et al. 2000). Each reaction had a final volume of 25 µL, which consisted of 12.5 µL of 2 × Taq ready mastermix (New England Biolabs, Ipswich, MA, USA), 7.5 µL of nuclease-free distilled water (ThermoFisher, Waltham, MA, USA), 1.5 µL of each primer (10 µmol/L; Inqaba Biotechnical Industries), and 2 µL gDNA. Thereafter, amplification was conducted using an Applied Biosystems 2720 thermal cycler employing the following conditions: an initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C

for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final extension step at 72 °C for 7 min (Vreulink et al. 2010). The sequences of the resulting PCR products were determined using an Applied Biosystems ABI3130xl genetic analyser, whereafter the yeasts were identified by comparing their representative sequences with known sequences available on GenBank via a BLAST search (http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analysis of the D1/D2 region using MEGA X was used to confirm the identities of the yeast isolates (Figs. S1 to S8) by verifying the phylogenetic distance between the isolates and the type strains of the species they represent (Kumar et al. 2018). For this purpose, sequences were first aligned using MUSCLE (Edgar 2004), trimmed, and the evolutionary history inferred using the maximum-likelihood or neighbour-joining methods. A phylogeny with the highest log-likelihood regarding tree topology was constructed for sequenced representatives of *H. heimii*. Confidence limits were determined by bootstrap iterations for 100 pseudoreplicates.

Enzyme assays and carbon source assimilation tests

Isolates of *H. heimii* were assessed for the ability to degrade five different polymeric carbon sources associated with woody material. Screening was conducted on agar plates (pH 5) containing 6.7 g/L yeast nitrogen base (YNB; Difco), 20 g/L agar, and either 10 g/L Avicel cellulose (Sigma-Aldrich), 5 g/L carboxymethyl cellulose (CMC, Sigma-Aldrich), 20 g/L corn starch (Sigma-Aldrich), 10 g/L locust bean gum (β-mannan, Sigma-Aldrich), or 2 g/L remazole brilliant blue Xylan (RBB-Xylan, Sigma-Aldrich). Isolates were spot inoculated onto the different media mentioned above and incubated at 26 °C for 7 days. After incubation, all plates, except those supplemented with RBB-Xylan, were stained with Gram's iodine solution (Sigma-Aldrich) for 5 min and examined for the formation of clearance zones around the yeast colonies (Kasana et al. 2008). The presence of xylanase activity was indicated by pale clearing zones surrounding yeast colonies. The yeast-like fungus *Coniochaeta pulveracea* CAB 683 served as the positive control for the production of cellulases, cellobiohydrolases, and β-mannanases while *Lipomyces starkeyi* CAB 1927 was used to verify the presence of amylases via starch hydrolysis. *Saccharomyces cerevisiae* CAB 2714 served as a negative control in all enzyme plate assays. In addition, representatives of *H. heimii*, that were tested for enzyme production, were also tested for their ability to assimilate sucrose (Saarchem, Univar, Merck) and cellobiose (Sigma-Aldrich) according to standard methods described by Kurtzman et al. (2011b).

Indole acetic acid (IAA) assay

The ability of *H. heimii* to produce the plant growth–promoting factor, IAA, in vitro was assessed according to a modified method described by Moller et al. (2016a). Briefly, yeast strains were inoculated into 50 mL YM broth contained in conical flasks and incubated for 24 h at 26 °C under constant agitation (100 rpm, Excella E10 platform shaker, Eppendorf, Hamburg, Germany). Hereafter, cells were washed twice in physiological saline solution (PSS) by centrifugation (8161 × *g*, 22 °C, 2 min) and the concentration of the resulting yeast suspension was adjusted to log 6 cells/mL. An aliquot (100 µL) of this suspension was then used to inoculate test tubes containing either 10 mL of Dworkin and Foster (DF) minimal medium (control) or 10 mL DF minimal medium containing 0.1% (w/v) tryptophan (DF_{trp}; Sigma-Aldrich). Inoculated tubes were placed on a TC-7 tissue culture roller drum (60 rpm) for 4 days at 26 °C and IAA production was quantified daily. To achieve this, 1.2 mL of each culture was centrifuged (12,000 × *g*, 5 min, 4 °C) and 1 mL of the resulting supernatant was mixed with 2 mL of ferric chloride-perchloric acid ($FeCl_3$-$HClO_4$) reagent. These mixtures were incubated in the dark for 25 min, after which their absorbances were measured at 530 nm using a SmartSpec Plus spectrophotometer (BioRad, Laboratories Ltd., Johannesburg, South Africa). To quantify IAA production, a calibration curve was prepared under the same conditions mentioned above by mixing a range of IAA concentrations with $FeCl_3$-$HClO_4$ reagent. The yeast *Papiliotrema laurentii* CAB 91 was included as a positive control.

Ergosterol quantification

The total intracellular ergosterol was extracted from yeast strains using the method of Arthington-Skaggs et al. (2000). Each *H. heimii* isolate was cultured on Sabouraud glucose agar for 48 h at 26 °C, whereafter the culture was used to prepare a ca. 1 McFarland cell suspension in sterile PSS. Subsequently, 100 µL of the cell suspension was inoculated into each of six replicate conical flasks that contained 50 mL YCB media supplemented with 0.01 g ammonium chloride (NH_4Cl; Merck). After incubation at 26 °C for 48 h on a rotary shaker (120 rpm; New Brunswick Scientific Co. Inc.), stationary phase cells (see Fig. S9) from each of the six replicate cultures were harvested by centrifugation (5000 rpm for 5 min at 22 °C) using a Biofuge Stratos high-speed benchtop centrifuge (Heraeus, Hanau, Germany). Resultant cell pellets were washed in sterile dH_2O and again centrifuged (5000 rpm for 5 min at 22 °C) to determine the wet weight of yeast cells.

The dry/wet weight ratio of the pellets originating from each isolate was determined by drying three out of the six pellets in an oven maintained at roughly 80 °C to constant weight. The other three pellets were used to determine the

sterol content of the cells by resuspending each pellet in 3 mL of a 25% alcoholic potassium hydroxide (KOH) solution (25 g KOH in 36 mL dH_2O brought to 100 mL with 100% ethanol) and vortexing for 1 min. The resulting cell suspensions were transferred to acid-washed glass test tubes and incubated for 1 h in a water bath at approximately 80 to 85 °C. The tubes were cooled to room temperature (20 to 23 °C) and sterols were extracted by adding a mixture of 1 mL sterile dH_2O and 3 mL n-heptane (Merck) to each tube. The suspensions were vortexed for 3 min and heptane layers that were formed in each case were transferred to clean glass test tubes. The quantities of sterols in the heptane layer were determined by measuring the absorbance at 281 nm and 230 nm (Breivik and Owades 1957) using a Spectroquant Pharo 300 spectrophotometer (Merck). The ergosterol content per dry cell weight was subsequently calculated for each yeast isolate.

Interconversion of insect pheromones

The interconversion of the insect pheromones, verbenol and verbenone, by *H. heimii* CAB 1614 was tested using the methods of Hunt and Borden (1990). A loopful of yeast cells from cultures grown for 48 h on YMc agar plates at 26 °C served as inoculum for 50 mL Sabouraud glucose broth (SGB) contained in 250-mL conical flasks. Each flask also received 250 µL of an ethanolic stock solution of either verbenol or verbenone prepared by dissolving 3 mg/mL of either *cis*-verbenol (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol; Sigma-Aldrich) or verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one; Sigma-Aldrich) in 95% ethanol (Merck). Resultant culture suspensions that contained the respective pheromones were incubated at 26 °C under constant agitation (100 rpm, Excella E10 platform shaker, Eppendorf, Hamburg, Germany). After 24 h, a further 250 µL of the ethanolic solutions was added to the medium, resulting in a final concentration of 1% (v/v). Yeast cells were incubated for another 24 h at 26 °C, after which 10-mL aliquots of culture fluid were transferred into acid-washed glass McCartney bottles and flushed with nitrogen gas. The sparged culture samples were sealed and stored on ice for later analysis using gas chromatography-mass spectrometry (GC–MS) methods.

GC–MS analysis of pheromones

The relative quantities of either *cis*-verbenol or verbenone present in the above-mentioned sampled culture aliquots were determined by separating the volatile compounds on a gas chromatograph (Agilent 6890 N; Agilent, Palo Alto, CA, USA) coupled with an Agilent mass spectrometer detector (Agilent 5975 MS; Agilent, Palo Alto, CA). For the separation, 5 mL of each sample was mixed with an equal volume of 30% (w/v) sodium chloride, to reduce enzymatic degradation and facilitate the diffusion of volatiles into the vial headspace. The volatiles, trapped in the vial headspace, were subsequently extracted via solid phase micro-extraction (HS-SPME) by first placing vials in the incubator of the CTC autosampler attached to the gas chromatograph. Vial contents were equilibrated for 5 min at 50 °C. Thereafter, volatiles were extracted by exposure to a 50/30 µm divinylbenzene/-carboxen/-polydimethylsiloxane-coated fibre in the headspace for 15 min at 50 °C.

After the extraction process was complete, desorption of the volatile compounds from the fibre coating was carried out for 10 min in the injection port (maintained at 240 °C) of the GC–MS operated in splitless mode. This was followed by chromatographic separation on a DB-WAX (60-m length, 0.25-mm inner diameter, and 0.25-µm film thickness) capillary column from Agilent Technologies. The analyses were conducted using helium (He) as carrier gas at a constant flow rate of 1 mL/min. The oven temperature was as follows: 40 °C for 1 min; then ramped up to 250 °C at 5 °C/min and held for 2 min. The mass selective detector (MSD) was operated in full scan mode and the temperatures of the ion source (240 °C) and quadropole (150 °C) were kept constant. The transfer line temperature was maintained at 250 °C, with a total run time of ca. 28 min. Authentic standards were available for both compounds of interest, which were used to identify the presence of verbenol or verbenone in samples by comparison of retention times and by making use of data available on mass spectral libraries (NIST, version 2.0). Andisole-d8 (Sigma-Aldrich) was used as the internal standard for all samples. The values that were calculated represent the relative abundances and were expressed as a percentage.

Statistical analyses of data

To determine if significant differences existed between *H. heimii* isolates regarding their ergosterol content and IAA production, statistical analyses of data were performed using Statistica v.13 software (StatSoft, Tulsa, OK, USA). The data residuals were tested for normality of distribution using the Shapiro–Wilk *W* test and Kolmogorov–Smirnov and Lilliefors test. Hereafter, data were analysed using the non-parametric Kruskal–Wallis test with post hoc Dunn's test for multiple comparisons. The significance level was set at $P < 0.05$ for all analyses that were performed.

Results

Yeast species isolated and identified from frass samples

During this study, more than 300 yeasts culturable on the isolation medium were purified from frass samples that were

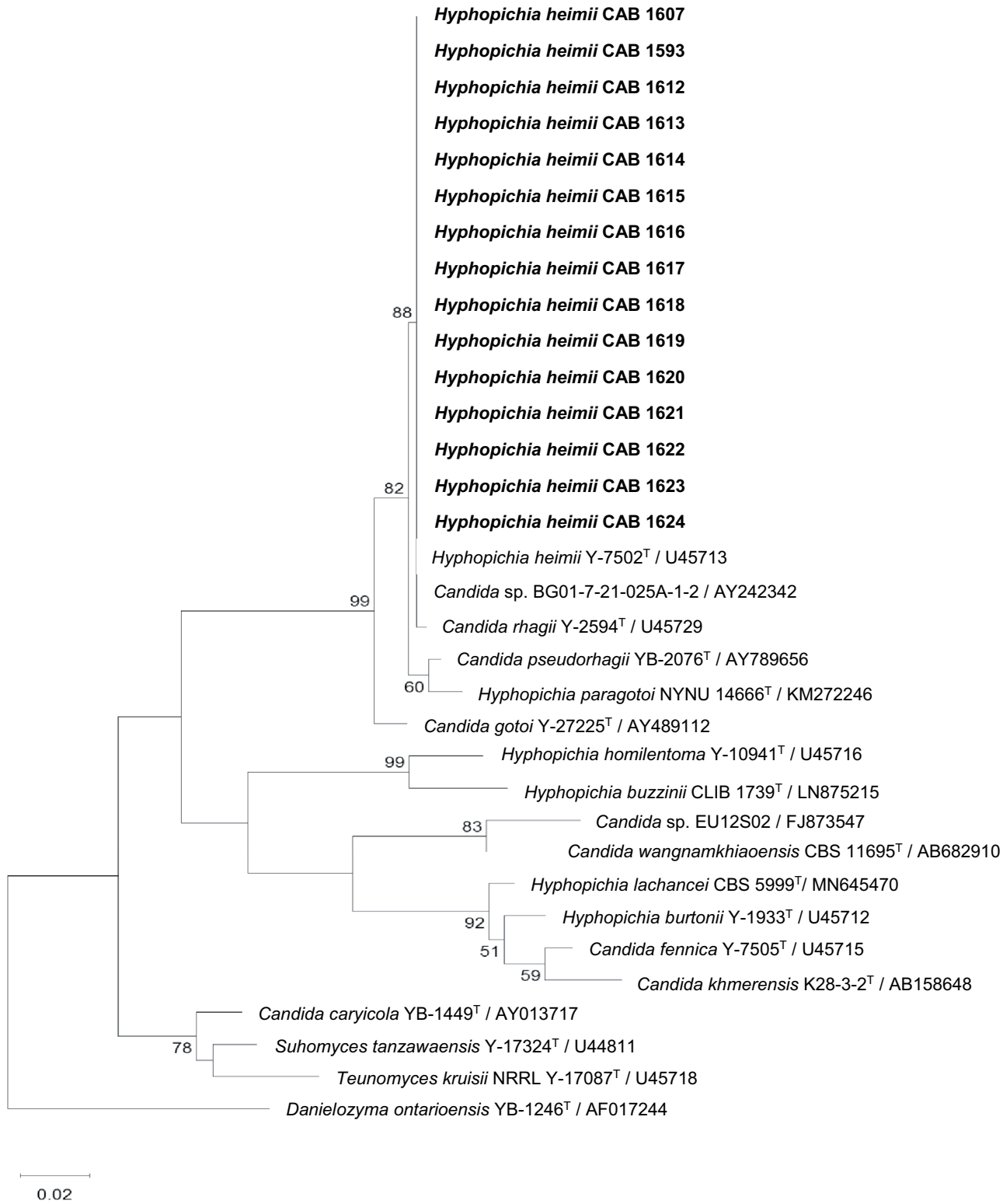

Fig. 1 Phylogeny depicting the placement of yeast strains denoted by "CAB-" suffix obtained from environmental sampling of insect frass of wild olive trees in the genus *Hyphopichia* (bold type) and closely related species based on D1/D2 sequences of the LSU rRNA gene. The tree was constructed by maximum-likelihood analysis of 299 aligned positions based on the General Time Reversible substitution model. Bootstrap values were determined from 100 pseudoreplicates. *Danielozyma ontarioensis* was used as the outgroup for the analysis. Bar indicates 0.05 substitutions per site

collected from 35 olive trees in Stellenbosch (Figs. S1 to S8). The samples yielded both ascomycetous and basidiomycetous yeast species (Table S1). Yeast isolates represented 28 species, of which 29% were ascomycetes, while the majority (71%) were basidiomycetes. This was not surprising since the selected isolation medium was originally designed to isolate basidiomycetous fungi (Worrall 1991). The most dominant yeasts belonged to the genera *Colacogloea* (Fig. S6) and *Cryptococcus* (Fig. S7) and the rare ascomycetous yeast, *H. heimii* (Table S1, Fig. 1).

Hyphopichia heimii was present on nine out of 35 trees that were sampled. Interestingly, the sequenced representatives of *H. heimii* differed regarding their nucleotide composition from *H. heimii* NG_054830 (type strain) by either less than 1% (≤5 bp divergence), or by 6 bp (98.8% identity) in the case of strains CAB 1613 and CAB 1915 (Table S1). In accordance with their phylogenetic position (Fig. 1), however, both these groups were treated as *H. heimii.*

Hydrolytic enzyme production and assimilation of selected carbohydrates

The 121 *H. heimii* isolates were screened for the presence of enzymes capable of degrading complex polysaccharides present in woody material. It should be noted that only 9 of the 121 isolates were screened for xylanase activity. Overall, extracellular hydrolytic enzymes (cellulases, cellobiohydrolases, β-mannanases, amylases, and xylanases) were not detected in the isolates, which indicated that representatives of this species were incapable of degrading (hemi)cellulosic material. When representatives of *H. heimii* were tested for the ability to assimilate sucrose and cellobiose, all isolates were able to assimilate both sugars as sole carbon sources (Table 1).

Table 1 Ability of representatives of *Hyphopichia heimii* to assimilate sucrose and cellobiose as sole carbon sources

Isolate number	Carbon source[a]	
	Sucrose	Cellobiose
CAB 1845	+ + +	+ + +
CAB 1317	+ + +	+ + +
CAB 1621	+ + +	+ + +
CAB 1914	+ + +	+ + +
CAB 1624	+ + +	+ + +
CAB 1593	+ + +	+ + +
CAB 1795	+ + +	+ + +
CAB 1620	+ + +	+ + +
CAB 1614	+ + +	+ + +

[a]Degree of growth read and scored after 2 weeks according to standard procedures (Kurtzman et al. 2011b). All experiments were conducted in triplicate

+ + +, positive with lines completely obscured

+ +, positive with lines appearing as diffuse bands

+, positive with distinguishable lines blurred at the edges

IAA production and ergosterol content

All nine tested isolates of *H. heimii* were found to produce the phytohormone IAA in vitro and had a total intracellular ergosterol content that ranged from 3.644 ± 0.119 to 13.920 ± 1.230 mg/g dry cell weight (Table 2).

Pheromone conversion

To determine whether *H. heimii* has the ability to convert insect pheromones (Hunt and Borden 1990), the isolate *H. heimii* CAB 1614 was incubated in SGB media supplemented with *cis*-verbenol or verbenone, respectively. Controls, prepared with uninoculated SGB medium, supplemented with 1% (v/v) of either *cis*-verbenol or verbenone, were included in the experimentation (Figs. S10, S11, S12, and S13). GC/MS analyses revealed that verbenol eluted at ca. 20.2 min, using fragment ion *m*/*z* 94 Da in the alcohol's MS spectrum as a marker, while verbenone was detected after a retention time of ca. 21.2 min, using the base peak (*m*/*z* 107 Da) in the molecules' MS spectrum as a marker (Figs. S10, S11, S12, and S13). The results indicated that the yeast was unable to convert verbenone to verbenol since negligible verbenol concentrations were detected in both the verbenone controls and samples treated with verbenone only (Figs. 3 and S14). The yeast, however, was able to successfully convert *cis*-verbenol to verbenone (Figs. 2, 3, and S15).

Discussion

Using the modified BDS agar medium, we showed that *H. heimii* is associated with beetle frass on wild olive trees located in the Stellenbosch region, South Africa. In addition, it was one of the dominant species of which representatives were able to grow on a benomyl-containing medium, originally used to isolate basidiomycetous fungi (Worrall 1991), the latter of which made up the majority of yeasts growing on the medium. It must be noted that since some *H. heimii* isolates were found to differ in their base pair composition according to their D1/D2 sequence data, it is expedient that future studies should include sequence analyses of the ITS regions of those isolates most divergent from the type strain. The results of such analyses will indicate whether the variability observed, among the D1/D2 sequences of the different isolates, was because of intra- or interspecific variation. Nevertheless, all representatives of *H. heimii*, as identified in the current study, showed no enzyme activity regarding the degradation of (hemi)cellulosic material, indicating that this

Table 2 Indole acetic acid (IAA) production and ergosterol content of the *Hyphopichia heimii* isolates. Results are represented as the mean ± standard error of the mean of at least three biological replicates*

Yeast strain ID	Ergosterol content (mg/g dry cell weight)	IAA production	
		µg/log CFU	µg/mL
CAB 1845	13.920 ± 1.230^{c}	2.458 ± 0.122^{bc}	17.739 ± 0.903^{c}
CAB 1317	4.210 ± 0.202^{ab}	0.614 ± 0.015^{a}	4.499 ± 0.185^{a}
CAB 1621	4.642 ± 0.486^{ab}	0.989 ± 0.057^{a}	7.312 ± 0.428^{a}
CAB 1914	3.817 ± 0.155^{a}	1.748 ± 0.540^{abc}	12.811 ± 4.191^{abc}
CAB 1624	9.025 ± 1.310^{bc}	1.098 ± 0.103^{ab}	8.088 ± 0.656^{ab}
CAB 1593	9.968 ± 0.098^{bc}	3.062 ± 0.250^{c}	21.496 ± 1.813^{c}
CAB 1795	6.068 ± 1.337^{abc}	1.316 ± 0.299^{abc}	9.215 ± 1.978^{abc}
CAB 1620	3.644 ± 0.119^{a}	1.433 ± 0.103^{abc}	9.829 ± 0.752^{abc}
CAB 1614	4.622 ± 0.081^{abc}	2.912 ± 0.507^{c}	20.887 ± 4.029^{c}
CAB 91	-	2.774 ± 0.048^{c}	16.412 ± 0.421^{bc}

*Superscript letters within the same column are comparable and denote significant differences between strains

species does not directly interact with its natural woody substrate. The representatives of *H. heimii* were, however, able to assimilate sucrose and cellobiose, both carbohydrates that would typically be available to fungi while in a symbiotic relationship. Sucrose is a readily available carbohydrate in phloem (Bongi 2002; Conde et al. 2008) and it is known that many plant-associated fungi can utilise this disaccharide (Lam et al. 1995; Harman 2011), while cellobiose is known to be utilised by yeasts that are in a syntrophic relationship with lignicolous fungi growing on trees (van Heerden et al. 2011).

Our study also showed that representatives of *H. heimii* were able to produce the plant growth–promoting hormone, IAA. This auxin, known to be produced by some yeasts, was suggested to promote growth of several plant species

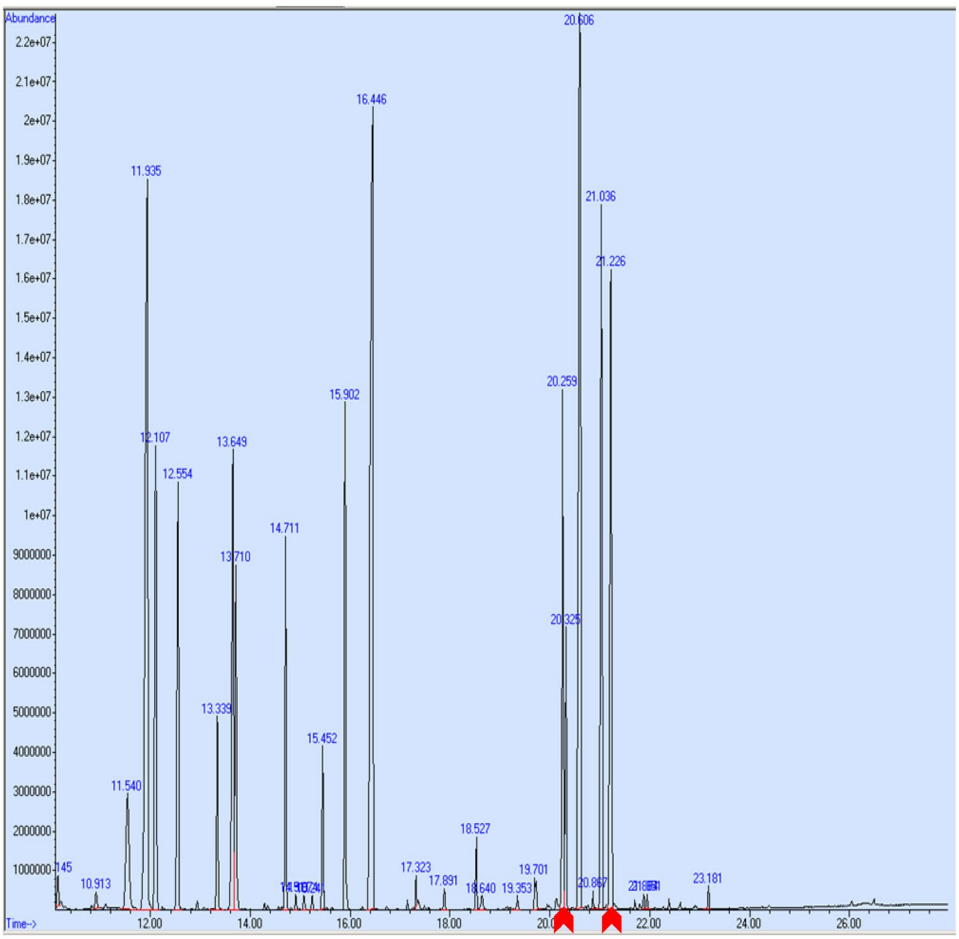

Fig. 2 GC/MS chromatogram of an aliquot from a liquid culture of *H. heimii* CAB 1614 grown in SGB medium supplemented with verbenol. The *y*-axis depicts the intensity or concentration of the different compounds in the culture fluid, while the *x*-axis depicts the retention time (min) of a specific compound. The peak selected for the detection of verbenol (*m*/*z* 94 Da, Fig. S10) showed a retention time of 20.259 min, while the base peak of the verbenone (*m*/*z* 107 Da, Fig. S12) produced during cultivation had a retention time of 21.226 min (peaks at these retention times are indicated on the *x*-axis by triangular arrows)

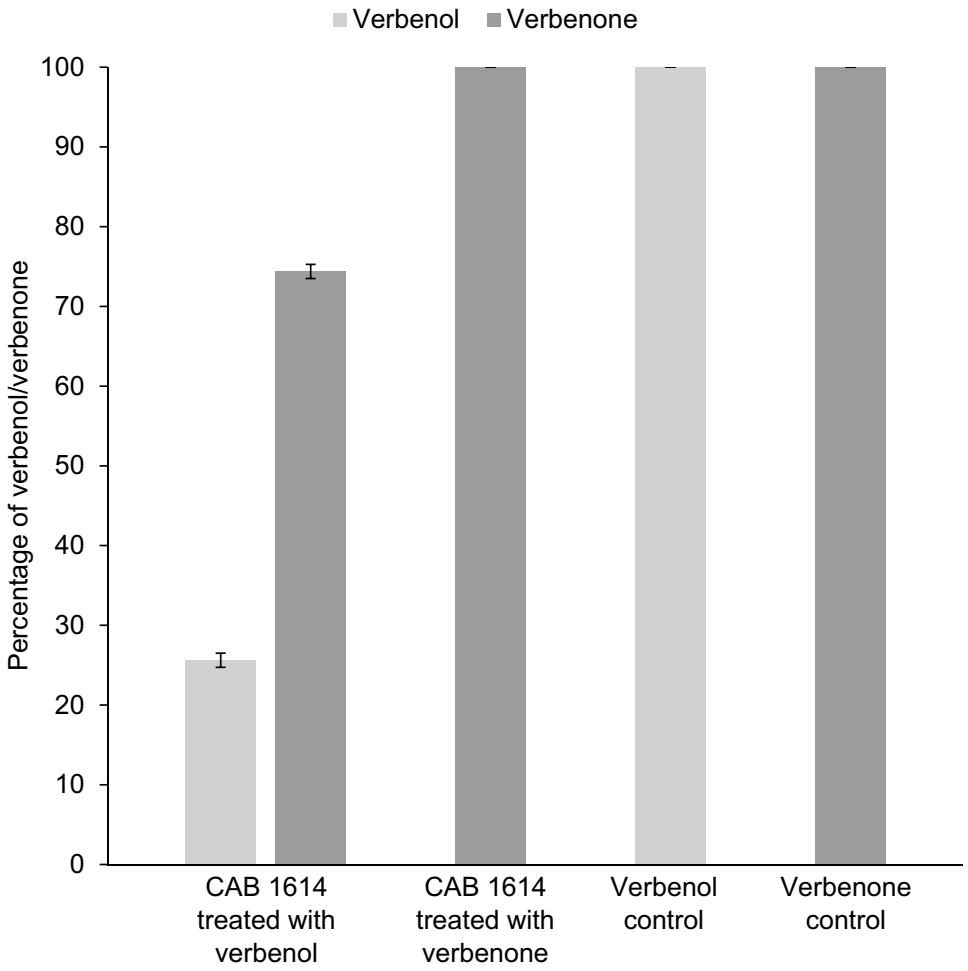

Fig. 3 Percentages of verbenol and verbenone in the culture fluid of *H. heimii* CAB 1614 and the uninoculated SGB medium controls after 48 h of incubation. The relative quantities of the two compounds were determined by comparing the area of their respective peaks on GC/MS chromatograms to that of an internal standard (Andisole-d8), which was included in all samples. Values are the means of two repeats. Error bars indicate standard error of the mean

(Lachance et al. 2001; Moller et al. 2016a, b). Observed differences in IAA production of the *H. heimii* isolates in our study are suggestive of intraspecies diversity in the metabolism of this yeast (Limtong et al. 2014; Limtong and Koowadjanakul 2012). Nonetheless, the concentrations of IAA produced by *H. heimii* were similar to those produced by the known plant growth–promoting yeast *Papiliotrema laurentii* (syn. *Cryptococcus laurentii)* (25.03 ± 1.70 µg/mL), as was found by Moller et al. (2016a).

The fact that all isolates of *H. heimii* produced IAA but showed no hemicellulolytic enzymatic activity is indicative of a symbiosis with wild olive trees that promotes tree growth. In this instance, *H. heimii* most closely resembles the description of nonclavicipitaceous (NC) class 3 endophytes as this yeast may be vectored by beetles, was isolated from aboveground parts of olive trees, and could potentially improve plant growth (Rodriguez et al. 2009). However, this is purely speculative as IAA production was shown to occur frequently among phylloplane yeasts (Limtong and Koowadjanakul 2012).

Another potential interaction of *H. heimii* could be with bark beetles, as was revealed when this yeast was found to produce the sterol ergosterol at concentrations ranging from 3.6 ± 0.12 to 14.0 ± 1.2 mg/g dry cell weight (Table 2). These concentrations were found to be similar to ascomycetous fungal ergosterol levels recorded by others using GC–MS, which ranged from 2.5 to 14.3 mg/g dry cell weight (Axelsson et al. 1995) and 2.6 to 14.0 mg/g dry weight (Pasanen et al. 1999). Unable to synthesise their own sterols, bark beetles rely on external dietary sources of sterols, such as the tissues of their host plants (Bentz and Six 2006; Kok and Norris 1973). These tissues contain phytosterols and cholesterol (Behmer et al. 2013). Studies revealed that cholesterol was the main sterol recovered from phloem sap of bean and tobacco plants (Behmer et al. 2013). Except for phytosterols, another source of sterols for insects is ergosterol, which is abundant in fungal symbionts (Grieneisen 1994; Kok and Norris 1973; Bentz and Six 2006). It must be noted however that cholesterol, not ergosterol, was shown to be the most frequently recovered sterol in phytophagous insects (Behmer et al. 2013; Behmer and Nes 2003; Grieneisen 1994). Also, the ecdysteroid derivatives of cholesterol were found to be involved in regulating different developmental processes, such as growth, maturation, and moulting. It seems that

beetles, acquiring sterols from phloem, would need to be able to convert carbohydrate (glycosylated)- or lipid (acylated)-bound forms of cholesterol, phytosterols, or ergosterol to cholesterol derivatives that can be metabolised (Behmer et al. 2013; Grieneisen 1994; Kok and Norris 1973; Bentz and Six 2006). Interestingly, some ascomycetous yeasts, such as *S. cerevisiae*, were shown to perform both acetylation and deacetylation modifications of cholesterol (Tiwari et al. 2007). Thus, it is possible that yeasts associated with the beetle frass may not only act as a dietary source of sterol for the insects but may also be able to convert phytosterols into derivatives that play a pivotal role in the insect's sterol metabolism.

Apart from potential nutritional benefits conferred on beetles by yeasts, some yeast species were shown to convert pheromones produced by these insects (Hunt and Borden 1990; Leufvén et al. 1984; Rottava et al. 2010). We found that a representative of *H. heimii*, originating from beetle frass, was able to convert *cis*-verbenol to verbenone. Similar observations were reported by Hunt and Borden (1990) when representatives of *Kuraishia capsulata* (syn. *Hansenula capsulata*) and *Ogataea pini* (syn. *Pichia pinus*), known to be associated with *D. ponderosae* pine beetles, were incubated with α-pinene, *cis*/*trans*-verbenol, and verbenone. Both yeasts were able to interconvert *cis*- and *trans*-verbenol; however, *K. capsulata* was shown to be more efficient at converting *trans*-verbenol to verbenone. The authors suggested that this yeast may be responsible for terminating aggregation on an infested host tree through the conversion of *trans*-verbenol to the anti-aggregation pheromone verbenone, which may also signal beetles to attack adjacent trees. Another study proposed the same repellent function for verbenone produced by yeasts associated with *Ips typographus*, as verbenone concentrations in gallery walls were higher in late attack phases, which were characterised by increased total yeast counts originating from beetles (Leufvén and Nehls 1986).

It is contended that beetle-produced pheromonal verbenol is derived from host tree α-pinene (Blomquist et al. 2010; Raffa et al. 2015). Interestingly, α-pinene is known to be one of the major volatile compounds produced by olive trees (Anastasaki et al. 2021; Jurišić Grubešić et al. 2021; Vural and Akay 2021). Consequently, it is likely that bark beetles occupying these trees may be able to hydroxylate this α-pinene to verbenol, which in turn could be converted to verbenol via either the beetles' or the yeasts' metabolisms (Blomquist et al. 2010; Raffa et al. 2015). Potentially, this verbenol could have a repellent effect on different beetle species visiting the olive trees, since it is known that verbenol not only acts on conifer-associated beetles (Agnello et al. 2021; Martini et al. 2020; Staffan Lindgren and Miller 2002). It is therefore tempting to speculate that *H. heimii* within the beetle frass, that was collected from aboveground portions of olive trees, may be able to carry out a similar function to *K. capsulata* and could affect the behaviour of beetles that respond to verbenone in a manner similar to that of *D. ponderosae* and *I. typographus* (Hunt and Borden 1990; Leufvén et al. 1984). Considering *H. heimii* was also able to convert *cis*-verbenol, it could provide further support for an association of this yeast with beetles.

Overall, this study provided conclusive evidence that *H. heimii* is associated with beetle frass on wild olive trees located in the Stellenbosch region, South Africa. Upon studying the eco-physiology of isolates representing this species, it was found that although the yeast is not capable of degrading and utilising the hemicellulosic components of wood, it does assimilate cellobiose. The latter characteristic indicates that it can potentially form a syntrophic relationship with lignicolous fungi growing on wood. The ability of *H. heimii* to assimilate sucrose and produce IAA on the other hand is indicative of a symbiosis with olive trees. It may also be that the IAA produced by *H. heimii*, as well as the ability of this yeast to convert the insect hormone *cis*-verbenol to verbenone, is suggestive of a symbiosis with bark beetles. To conclude, this study has provided tentative evidence of a tripartite symbiosis between the ascomycetous yeast, *H. heimii*, olive trees, and bark beetles. The ability of *H. heimii* to convert monoterpene alcohols into bioactive insect pheromones and serve as a sufficient dietary sterol source for beetle associates should also be explored in greater depth, which should include the use of an olive wood–enriched medium to better replicate the conditions of the olive frass as well as the use of additional terpenoids, to determine the range of compounds that could be converted by *H. heimii*. Subsequent experiments involving beetles should be conducted as this yeast could serve as a biological control agent in pest management.

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Code availability Not applicable.

Declarations

Ethics approval Not applicable.

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Consent for publication All authors consent to the publication of this article.

Conflict of interest The authors declare no competing interests.

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