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Naphthoquinone Metabolites Produced by *Monacrosporium ambrosium*, the Ectosymbiotic Fungus of Tea Shot-Hole Borer, *Euwallacea fornicatus,* in Stems of Tea, *Camellia sinensis*

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Abstract
The tea shot-hole borer beetle (TSHB, *Euwallacea fornicatus)* causes serious damage in plantations of tea, *Camellia sinensis var. assamica,* in Sri Lanka and South India. TSHB is found in symbiotic association with the ambrosia fungus, *Monacrosporium ambrosium* (syn. *Fusarium ambrosium*), in galleries located within stems of tea bushes. *M. ambrosium* is known to be the sole food source of TSHB. Six naphthoquinones produced during spore germination in a laboratory culture broth of *M. ambrosium* were isolated and identified as dihydroanhydrojavanicin, anhydrojavanicin, javanicin, 5,8-dihydroxy-2-methyl-3-(2-oxopropyl)naphthalene-1,4-dione, anhydrofusarubin and solaniol. Chloroform extracts of tea stems with red-colored galleries occupied by TSHB contained UV active compounds similar to the above naphthoquinones. Laboratory assays demonstrated that the combined ethyl acetate extracts of the fungal culture broth and mycelium inhibited the growth of endophytic fungi *Pestalotiopsis camelliae* and *Phoma multirostrata,* which were also isolated from tea stems. Thus, pigmented naphthoquinones secreted by *M. ambrosium* during spore germination may prevent other fungi from invading TSHB galleries in tea stems. The antifungal nature of the naphthoquinone extract suggests that it protects the habitat of TSHB. We propose that the TSHB fungal ectosymbiont *M. ambrosium* provides not only the food and sterol skeleton necessary for the development of the beetle during its larval stages, but also serves as a producer of fungal inhibitors that help to preserve the purity of the fungal garden of TSHB.

Keywords Ambrosia beetle · Ambrosia fungus · Ectosymbiont · Fungal metabolites · Inhibitory activity · Insect pest · Naphthoquinones

Introduction

Tea, *Camellia sinensis* L. Kuntze, is a perennial crop grown as a shrub in monoculture on large continuous areas of land in Sri Lanka and in other tropical countries. The plantations provide a stable environment for phytophagous insect pests such as the tea shot-hole borer beetle (TSHB), *Euwallacea fornicates* (Eichhoff) (Coleoptera: Scolytidae or Curculionidae: Scolytinae), which causes extensive damage to the tea plantations in Sri Lanka. This small wood-boring beetle was first described in 1868 from a specimen collected in Sri Lanka (Cranham 1966). It is an ambrosia beetle whose larvae feed on the ectosymbiotic filamentous fungus *Monacrosporium ambrosium* (Gadd and Loos 1947) syn. *Fusarium ambrosium* (Nirenberg 1990). The relationship between TSHB and the ambrosia fungus was first demonstrated by Gadd and Loos (1947) who were able to rear TSHB larvae to the adult stage in laboratory cultures of the fungus (Gadd 1947).

Euwallacea is a genus of over 40 species within the Xyleborini and is the only beetle genus known to cultivate ambrosia fusaria (Freeman et al. 2016). Different fungi are cultivated by different species of beetles and generally the symbiotic ambrosia fungi are not pathogenic. However,

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E. fornicatus Eichhoff, not only damages tea plants but is also a serious threat to the avocado industry in Israel. This beetle has also been collected from avocado in Florida, USA, where it does not cause much damage (Mendel et al. 2012). *E. fornicatus* is one of the few ambrosia beetles that can infest healthy plants and has been recorded from more than 48 plant families (Danthanarayana 1968).

The use of agriculture by humans dates from about 10,000 years ago when 'hunter-gatherers' began cultivating food crops, whereas 'insect farmers' and the capacity to cultivate fungi as a food source was evolved independently about 40–60 million years ago in attinine ants, macrotermitine termites, wood wasps and ambrosia beetles (Farrell et al. 2001; Mueller et al. 2005). Mycophagous insects that consume fungi for energy and nutrients may use behavioral strategies (Mueller et al. 2005) and small molecule control agents such as antibiotics produced with the help of bacterial symbionts (Currie et al. 1999; Ramadhar et al. 2014) for fungal crop protection. For example, TSHB is dependent on fungal food for development of both larval and adult stages (Batra 1985). Fernando (1959) reported that the newly emerging young adult female TSHB beetles transport spores in their mycangia (buccal sacs) to suitable tea stems, and inoculate the spores into the walls of galleries. Eggs are laid inside the gallery and the emerging larvae feed on the fungus. Fernando (1959) has also reported that "In a way not clearly understood, the beetles are able to suppress the development of all extraneous fungi so that the galleries contain only the ambrosia fungus growing in pure culture". Tea plants, TSHB and the ambrosia fungus form a symbiotic complex in which the TSHB are considered to be 'fungus farmers' (Farrell et al. 2001; Mueller et al. 2005).

Microorganisms including fungi may excrete substances that affect the growth and development of other species. Naphthoquinone production is widespread among fungi, particularly in the genus *Fusarium* (Tatum and Baker 1983; Tatum et al. 1987; Kimura et al. 1988; Medentsev and Akimenko 1998; Suzuki et al. 2013). Many different naphthoquinones have also been isolated as secondary metabolites from cultures of many other fungi including *Aspergillus* sp., *Penicillium* sp., and *Usnea* sp. The naphthoquinones range from simple naphthoquinones to more complex derivatives. Fungicidal properties of naphthoquinones have been reported previously (Kurobane et al. 1986; Eilenberg et al. 2010). Medentsev and Akimenko (1998) proposed that the biological activity of naphthoquinone metabolites produced by fungi impact other microorganisms, plants and other species in the ecosystem.

The objective of the current study was to identify the pigmented naphthoquinone metabolites produced by *M. ambrosium* in a laboratory culture of this fungus, and to evaluate the antifungal activity of the compounds. The antifungal properties of a crude fungal extract were also studied against two endophytic fungi isolated from tea *C. sinensis* stems. Investigating whether the red pigments secreted by the ambrosia fungus help prevent the growth of other fungi within galleries in tea stems constructed and occupied by TSHB will provide preliminary information about the ecological role of naphthoquinones.

Methods and Materials

Materials, Reagents and Solvents Silica gel [Merck Art. 7734 (70–230 mesh size) and 9385(220–440 nm mesh size), Darmstadt, Germany], Sephadex LH-20 (Fluka Art 20,100, Switzerland) and RP-18 silica (Merck, Germany, LiChroprep RP 18, 40–63 μm) were used in column chromatography. HPLC separations were carried out on a Shimadzu LC-6A apparatus equipped with a reversed phase (RP-18) STR Prep – ODS 20 × 250 mm column and UV detector (254 nm). Silica-gel-coated aluminum sheets (Merck 1.05554.0007, $60F_{254}$, Darmstadt, Germany) were used for analytical thin layer chromatography (TLC) whereas preparative thin layer chromatography (PTLC) was carried out by using silica gel coated glass plates (Merck Art. 1.05715.001, Darmstadt, Germany). Potato dextrose agar (PDA) (Dextrose-Avonchem, Oxon, UK and Agar-Meron, Cochin, India) and potato dextrose broth (PDB) were used as culture media. Czapek Dox medium (composition given in supplementary information) was used in the antifungal assay. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer (Billerica, MA, USA) in $CDCl_3$ solution.

Collection of Beetles and Isolation of Ambrosia Fungus TSHB beetles were collected from TSHB-infested TRI 2025 stems (6–10 mm diameter) from the Tea Research Institute (TRI)-Sub Station, Hantana, Kandy, surface sterilized (Hewavitharanage et al. 1999), placed on PDA and incubated at room temperature until mycelia of *M. ambrosium* were visible. *M. ambrosium* was sub-cultured until microscopic examination of the filaments and spores revealed that a pure culture was obtained.

Identification of Ambrosia Fungus from TSHB Beetles The fungus was identified as *F. ambrosium* (*syn. M.ambrosium*) by sequence analysis of the ITS region of the rDNA gene. DNA was extracted by using Promega, Wizard Genomic DNA Purification Kit (A1120) and amplification of the ITS region was carried out by using the universal eukaryotic primers of ITS1 and ITS4 (Pryce et al. 2003). BLAST search indicated that the sequence of the ITS regions had 99% similarity to that of *F. ambrosium* (GenBank Accession No. KM231801.1). Photographic evidence of *F. ambrosium* (CK-A1) is deposited at the NIFS, Kandy.

Fermentation of *M. ambrosium* and Extraction Pure cultures of *M. ambrosium* on PDA were inoculated into conical flasks

(1 L × 60), each containing PDB (400 ml), and the flasks were incubated with occasional shaking at room temperature for 28 days. The EtOAc extract of the culture broth (3.4 g) and the mycelium (0.3 g) were combined since they were similar by TLC analysis (Extract A).

Isolation of Compounds 1–6 Extract A was chromatographed over silica gel (Merck Grade 7734) with hexane, EtOAc and MeOH in increasing order of polarity to give 11 fractions (Fr. 1–11), which were further separated by SCC, PTLC and HPLC as follows. Fr. 3 was chromatographed over silica gel (Merck 9385) with hexane, CH_2Cl_2 and MeOH to give 8 fractions (Fr. 3-1–8). Fr. 3-2 was further separated by HPLC (20% water/MeOH, flow rate 5 ml/min to yield two compounds **4** (21.7 mg) as a red solid and **5** (27.0 mg) as a purple solid. Fr. 4 was chromatographed over silica gel (Merck 9385) with hexane, CH_2Cl_2 and MeOH in increasing order of polarity to collect 6 fractions (Fr. 4-1–6). Fr. 4-4 was chromatographed on a reverse-phased silica gel column with water-MeOH stepwise elution to give 10 fractions (Fr-4-4-1–10). Fr. 4-4-5 and 4-4-6 were separated by HPLC (20% water/MeOH, flow rate 5 ml/min) to isolate compound **2** (7.1 mg) as an orange solid and compound **3** (8.9 mg) as a red solid. Fr. 7 (3.7 g) was chromatographed over silica gel (Merck 9385) with hexane, CH_2Cl_2 and MeOH in increasing order of polarity. PTLC purification (developed with $CHCl_3$ in the presence of NH_3) of the fractions afforded compound **1** (8 mg) as a red solid. Fr. 9 was chromatographed over silica gel (Merck 9385) with CH_2Cl_2 and MeOH in increasing order of polarity. PTLC purification (developed with $CHCl_3$ in the presence of NH_3) of the fractions gave compound **6** (10.6 mg) as a red solid. These compounds were identified by ^1H and/or ^{13}C NMR and FAB-MS data. The spectral data are listed in Supplementary material.

Antifungal Activity of Extract A The 11 fractions separated from Extract A were tested for antifungal activity against *Cladosporium cladosporioides* by the TLC bioautographic method (Homans and Fuchs 1970).

Isolation of Endophytic Fungi from Tea Stems Pieces of TRI 2023 healthy stems (6–10 mm diameter) were washed, surface sterilized (Larran et al. 2007) and pieces from the mid region were placed on PDA for incubation at room temperature until fungal mycelia appeared. The fungi were re-inoculated on fresh PDA plates to obtain pure cultures.

Identification of Endophytic Fungi Isolated from Tea Stems The fungi were identified as *Pestalotiopsis camelliae* and *Phoma multirostrata* by sequence analysis of the ITS region of the rDNA gene (Pryce et al. 2003). BLAST search indicated that the sequence of the ITS regions had 99% similarity to that of *P. camelliae* (GenBank Accession No. KM199336.1) and 100% similarity to *P. multirostrata* (GenBank Accession No. KJ686366.1) respectively. Photographic evidence of the leaves of *C. sinensis, P. camelliae* (IFS/CCS1/2015) and *P. multirostrata* (IFS/CCS3/2015) strains is deposited at the NIFS, Kandy.

Antifungal Effects of Extract A against two Endophytic Fungi from Tea Stems: Agar Dilution Assay (Andrews 2001) The endophytic fungi, *P. camelliae* and *P. multirostrata,* and *M. ambrosium* were cultured on PDA plates containing 500 ppm or 1000 ppm concentrations of Extract A dissolved in 10% DMSO or DMSO alone (control). A 1 cm^2 piece of each endophytic fungus and 1 cm^2 piece of *M. ambrosium* was placed at the center of each plate for incubation at room temperature for seven days before measuring the diameter of each fungal colony and calculating % inhibition based on comparison to the control.

Statistical Analysis Results were calculated as mean ± standard deviation of 3 independent analyses. Data were analyzed by using the SAS statistical software version 9.1.3 (SAS Institute Inc., Cary, NC). $P < 0.05$ was considered to be significant.

Collection of TSHB-Infested Tea Stems for Extraction of Galleries TSHB infested TRI 2025 stems, which had red colored galleries (4.0 g), were collected from the TRI substation, Hantana during September–October 2016, thinly chopped, finely ground and dried in a convection oven at 40 °C for 6 h (3.2 g).

TLC Analysis of $CHCl_3$ Extract Obtained from Tea Stems with Galleries The fine wood chips (3.2 g) were subjected to Soxhlet extraction by using $CHCl_3$ (500 ml) for 4 h (de Paiva et al. 2004). The extract was evaporated to dryness (30 mg) and the dark brown residue remaining in the flask was dissolved in dichlorormethane and analyzed by TLC (100% $CHCl_3$). The R_f values of the UV active spots were compared with those of the compounds isolated from liquid cultures of *M. ambrosium*.

Results

Isolation of *M. ambrosium* from TSHB Beetles Pure cultures of white colored mycelia of ambrosia fungus were obtained from surface sterilized beetles. From the third day the mycelium at the center of the colony was pink in color and the color (Fig. S1a) deepened with time, until the agar became heavily stained with a dark reddish purple color (Fig. S1b). The mycelium of ambrosia fungus was thin, hyaline, septate and branched (Gadd and Loos 1947) whereas spores after 7 days were hyaline, clavate, non-septate with rounded apices. After 14 days the spores

were hyaline, clavate and 2–5 septate with bluntly pointed apices (Fig. S1c).

Fermentation in Liquid Cultures of *M. ambrosium* Fungal mycelia appeared in the PDB culture media after 2–3 days, and dense growth of the fungus was observed by 7 days. A reddish color appeared around the mycelial patches after 2–3 days and the color spread to the entire PDA surface by 5 days. The EtOAc extract of the liquid culture medium and the fungal mycelium as well as the MeOH extract of the mycelium were red in color. The two EtOAc extracts were combined (Extract A), and then subjected to column chromatography, preparative TLC and HPLC.

Chromatographic Separation of Extract A, Isolation and Identification of Naphthoquinones Chromatographic separation of Extract A afforded compounds **1**–**6** (Fig. 1), all of which were colored ranging from yellow to blue. The structures of the isolated compounds were identified as dihydroanhydrojavanicin(5-hydroxy-8-methoxy-2,4-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-6,9-dione) **1** (Tatum et al. 1989), anhydrojavanicin (5-hydroxy-8-methoxy-2,4-dimethylnaphtho[1,2-*b*]furan-6,9-dione) **2** (Kimura et al. 1988), javanicin (5,8-dihydroxy-2-methoxy-6-methyl-7-(2-oxopropyl)naphthalene-1,4-dione) **3** (Kimura et al. 1988; Bergeron et al. 1993), 5,8-dihydroxy-2-methyl-3-(2-oxopropyl)naphthalene-1,4-dione **4** (Kimura et al. 1988), anhydrofusarubin (5,10-dihydroxy-7-methoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione) **5** (Kimura et al. 1988; Suzuki et al. 2013) and solaniol **(**5,8-dihydroxy-7-(2-hydroxypropyl)-2-methoxy-6-methylnaphthalene-1,4-dione) **6** (Kimura et al. 1988) by comparison of the ^{1}H NMR data with literature values. For compounds **3** and **5**, ^{13}C NMR data were also compared with the reported values. The ^{13}C NMR data of compounds **1**, **2** and **4** are reported for the first time (see Supplementary material). Compounds **1**–**5** have been isolated previously from a *Fusarium* sp. as inhibitors of pollen germination of *Pinus densiflora* (Kimura et al. 1988). Compound **1** was also isolated from fungi including *F. solani* (Tatum et al. 1989) and *Phomopsis* sp. (Yang et al. 2013). Compounds **2**, **3** and **5** are rather popular fungal metabolites, which were isolated from *Fusarium* and other fungi (Medentsev and Akimenko 1998; Tatum and Baker 1983; Trisuwan et al. 2010; Trisuwan et al. 2013; Yang et al. 2013). Isolation of compound **4** was reported only in one paper (Kimura et al. 1988). Antifungal and antibacterial activities of compound **3** were described previously (Kharwar et al. 2009).

Antifungal Activity of *M. ambrosium* Extracts against *C. cladosporioides* The TLC bioautographic method using *C. cladosporioides* was used to assess the antifungal activity of the 11 fractions (Fig. S2) isolated from Extract A. Several white spots on a grey background on the TLC plate (Fig. S3) were clearly visible 3 days after spraying with a spore suspension of *C. cladosporioides*. The white spots indicated the presence of several antifungal compounds in Extract A, in fraction 1 and fractions 3–10 (Fig. S3).

The antifungal properties of Extract A were also studied against two endophytic fungi isolated from tea stems (Figs. S4–S6). In the agar dilution assay, it was found that after seven days the average diameter of *M. ambrosium* in the control medium (without Extract A) was significantly larger ($P < 0.05$) than the diameters for both low and high concentrations of Extract A (Table 1). The percentage inhibition of the growth of *M. ambrosium* colony by Extract A was significantly ($P < 0.05$) less than the inhibition of *P. camelliae* and *P. multirostrata* (Table 1). Growth of *P. multirostrata*was inhibited significantly ($P < 0.05$) less than growth of *P. camelliae* (Table 1).

Appearance of TSHB Infested Tea Stems Fresh TSHB infested tea stems with galleries that were collected were red in color, darkened, and then turned blackish purple on exposure to air (1 h). During the collection of TSHB infested tea stems, it was observed that in the early stages of TSHB gallery formation, the galleries occupied by the beetle were not red colored. At this stage the TSHB beetle was observed to be boring the stems while shoveling back a colorless powder. When the tea stems were collected 2 weeks later, the galleries contained a white powdery substance and the walls of the galleries were purple in color (Fig. 2). The galleries contained beetles as well as larval stages (white colored grubs).

TLC Comparison of $CHCl_3$ Extract of Tea Stems with TSHB Galleries and Fermentation Products of *M. ambrosium* The $CHCl_3$ solution after Soxhlet extraction (2 h) of the tea stems with red colored galleries was light orange in color. The color of the solvent after 4 h of Soxhlet extraction was also orange. The dark brown residue obtained by rotary evaporation contained several UV active spots (Fig. S7, lane 0) that had R_f values similar to those of the UV active compounds found in Extract A (Fig. S7, lanes 1, 2, 3, 7, and 9).

Discussion

The wood adjacent to TSBH beetle galleries in tea stems is usually stained dark purple (Speyer 1922). We noted that there is a time interval between the initial boring of the gallery and appearance of the purple color on the walls of the galleries. Although we found that the plated fungal cultures of *M. ambrosium* were deep purple, Gadd and Loos (1947) did not report any color for plated cultures of ambrosia spores isolated from galleries in castor bean *Ricinus communis*, consistent with lack of color in the wood around galleries in castor

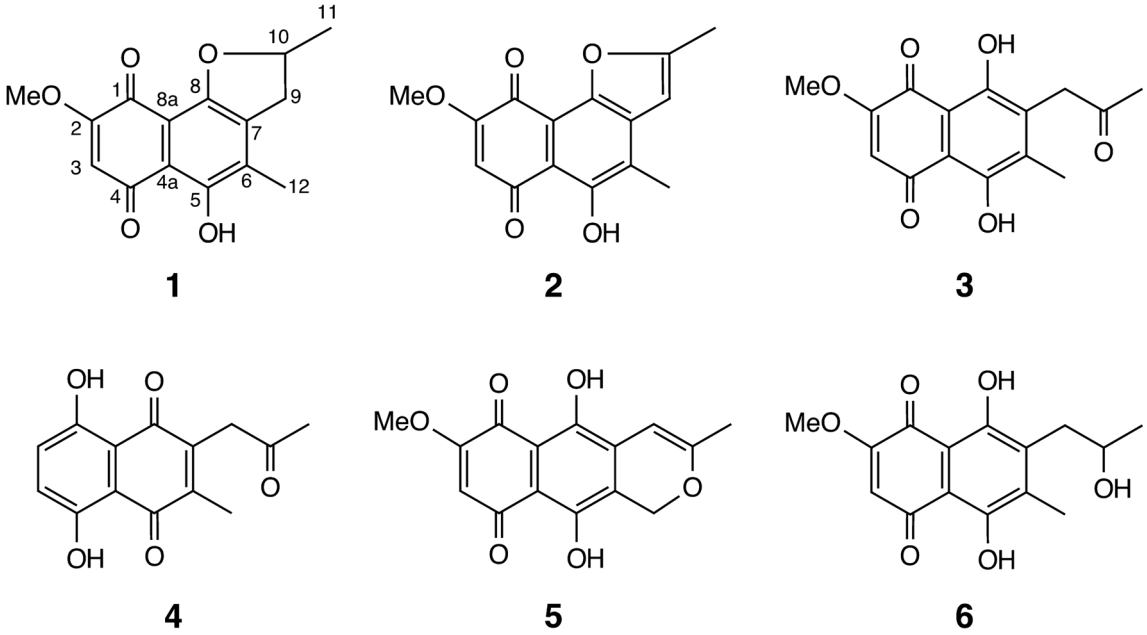

Fig. 1 Chemical structures **1**–**6**. dihydroanhydrojavanicin (5-hydroxy-8-methoxy-2,4-dimethyl-2,3-dihydronaphtho[1,2-*b*]furan-6,9-dione) **1**, anhydrojavanicin (5-hydroxy-8-methoxy-2,4-dimethylnaphtho[1,2-*b*]furan-6,9-dione) **2**, javanicin (5,8-dihydroxy-2-methoxy-2-methyl-3-(2-oxopropyl)naphthalene-1,4-dione) **3**, 5,8-dihydroxy-6-methyl-7-(2-oxopropyl)naphthalene-1,4-dione **4**, anhydrofusarubin (5,10-dihydroxy-7-methoxy-3-methyl-1H-benzo[g]isochromene-6,9-dione) **5** and solaniol **(**5,8-dihydroxy-7-(2-hydroxypropyl)-2-methoxy-6-methylnaphthalene-1,4-dione) **6**

bean. Verrall (1943) stated that the color was caused by the ambrosia fungus and deepening of the color was due to reaction of these fungi with insect secretions and other organisms. However, Gadd and Loos (1947) suggested that the color depended on the ambrosia strains carried by the beetles. It has been argued that this stain develops into wood rot, but according to King (1940), a connection has not been found between wood rot and TSHB galleries.

The deep red color of the agar medium in Fig. S1b was attributed to the presence of naphthoquinones that had been liberated into the culture medium by *M. ambrosium*. Of the six naphthoquinones isolated from Extract A (Fig. 1) of *M. ambrosium*, only compound **3** has been reported to exhibit antifungal activity (Kharwar et al. 2009). TLC of Extract A showed the presence of several UV active metabolites, some of which could be naphthoquinones (Fig. S2**).**

Antifungal activity of naphthoquinones in Extract A was detected by using the TLC bioautography method. The white spots on the dark green background of the TLC plates indicated the presence of compounds active against *C. cladosporioides* in fractions 1 and 3–10 from Extract A (Fig. S3). The TLC method was chosen to detect the presence of antifungal compounds

Table 1 Antifungal effects of extract A[#] from *Monacrosporium ambrosium* in an agar dilution assay on colony diameter of endophytic fungi isolated from tea stems

Extract A[#] Concentration/ppm	*M. ambrosium*		*Pestalotiopsis camelliae*		*Phoma multirostrata*	
	Mean Diameter (cm) of fungal colony[*]	% Inhibition[*]	Mean Diameter (cm) of fungal colony[*]	% Inhibition[*]	Mean Diameter (cm) of fungal colony[*]	% Inhibition[*]
500	3.5 ± 0.0^{a}	10.2 ± 0.0^{1}	1.1 ± 0.2^{a}	78.7 ± 3.0^{2}	2.9 ± 0.2^{a}	31.0 ± 4.1^{3}
1000	3.5 ± 0.0^{a}	10.2 ± 0.0^{1}	0.0 ± 0.0^{b}	100.0 ± 0.0^{2}	2.6 ± 0.1^{b}	38.1 ± 2.4^{3}
Control	3.9 ± 0.1^{b}		5.0 ± 0.0^{c}		4.2 ± 0.3^{c}	

[#] EtOAc extract of fungal culture, evaporated to dryness

[*] All Table entries are presented as mean ± standard deviation ($n = 3$)

Different letters within a column represent significant differences ($P < 0.05$) between the colony diameters at different concentrations of Extract A

Different numbers within a row represent significant differences ($P < 0.05$) between percentage inhibition values of different fungi at different concentrations of Extract A

[+] Control plates contained 4.5 ml of PDA and 0.5 ml of 10% DMSO in water, test plates contained PDA (4.5 ml) and Extract A in 10% DMSO (0.5 ml)

Fig. 2 A longitudinally split stem from a tea plant (cultivar TRI 2025) that was infested with tea shot-hole borer (*Euwallacea fornicates)*. The reddish-purple colored galleries in the mid region contained beetles as well as white-colored grubs

in fractions 1–11, because this method allows detection of small amounts of antifungal compounds when only limited amounts of material are available for testing.

The antifungal properties of Extract A were further investigated against *M. ambrosium* and the two endophytic fungi *P. camelliae* and *P. multirostrata* isolated from tea stems by using the agar dilution assay. Extract A (500 and 1000 ppm) had only a slight inhibitory effect on the growth of the ectosymbiont *M. ambrosium* (Fig. S4) and inhibited/prevented the growth of the two endophytic fungi *P. camelliae* and *P. multirostrata* isolated from tea stems (Figs. S5 and S6). This observation suggests that naphthoquinone metabolites liberated by *M. ambrosium* may prevent the growth of other fungi, such as endophytic fungi present in tea stems, and maintain the galleries for growth of the ambrosia fungus, which is the sole food source of TSHB beetles. Interestingly, an analogous observation has been reported in which naphthoquinone spiroketals, isolated from the mycelium of the endophytic fungus *Edenia gomezpompae*, were inhibitory against three endophytic fungi (*Colletotrichum* sp., *Phomopsis* sp., and *Guignardia manguifera*) isolated from the same plant species (*Callicarpa acuminata*, Verbenaceae) (Macias-Rubalcava et al. 2008). It has also been reported that *C. gloeosporiodes* isolated as an endophytic fungus from *C. sinensis* (Assam, N. India), inhibited the tea pathogens *Pestalotiopsis thea* and *Collectotrichum camelliae* (Rabha et al. 2014).

There are many reports of the production of pigmented naphthoquinones detected inside beetle galleries, fungal cultures and body tissues of insects. In this context it is noteworthy that the non-pathogenic fungus *Quambalaria cyanescens* (Basidiomycota: Microstomatales) associated with cankers on *Eucalyptus* and *Corymbia,* has been reported to produce antifungal naphthoquinones, which enable the formation of monocultures inside the bark beetle gallery systems. Blue to violet pigments that diffused into culture media or the phloem in galleries of bark beetles were also observed (Stodulkova et al. 2015). Deep-red colored naphthoquinones have also been produced by the ant-pathogenic fungus *Ophiocordyceps* sp. BCC 1869 in culture media (Khaokhajorn et al. 2015). *Cordyceps unilateralis*, BCC 1869, a pathogen of ants, produced naphthoquinones with anti-malarial activity (Kittakoop et al. 1999). The parasitic fungus *Ophiocordyceps unilateralis*, another pathogen of ants, produces naphthoquinones inside the body of carpenter ants *Camponotus* spp., and finally causes the death of the ant. In this example *O. unilateralis* also helps in the dispersal of fungal spores to begin a new cycle of infection in carpenter ants (Hughes et al. 2011).

Results reported here suggest that the fungal ectosymbiont, *M. ambrosium* of TSHB, is not only the food source necessary for the development of the beetle, but may also act as an inhibitor of fungi other than *M. ambrosium* by secreting naphthoquinones to prevent growth of other fungi in the TSHB galleries. In this scenario of fungus farming, the TSHB beetle is sowing its food by dispersing and depositing ambrosia spores in galleries made in suitable tea stems, while its food and nutrient source, the ectosymbiont *M. ambrosium*, also produces naphthoquinones inside the beetle gallery, to protect the habitat and fungal garden of TSHB beetles from invasion by other species of fungi.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflicts of interest.

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