Local hypoxia generated by live burial is effective in weed control within termite fungus farms

L. Katariya, P. B. Ramesh, A. Sharma & R. M. Borges

Insectes Sociaux
International Journal for the Study of Social Arthropods

ISSN 0020-1812

Insect. Soc.
DOI 10.1007/s00040-018-0644-5

Springer

Insectes Sociaux
https://doi.org/10.1007/s00040-018-0644-5

Insectes Sociaux

RESEARCH ARTICLE

Local hypoxia generated by live burial is effective in weed control within termite fungus farms

L. Katariya[1] · P. B. Ramesh[1] · A. Sharma[1] · R. M. Borges[1]

Received: 15 March 2018 / Revised: 6 June 2018 / Accepted: 27 June 2018

Abstract
Fungus-farming termites cultivate a mutualistic fungus *Termitomyces* inside their nest mounds in CO_2-rich environments. For sustainable harvests, termites must control weedy parasitic fungi such as *Pseudoxylaria* that may exploit resources meant for cultivar growth. Earlier, we discovered that termites exploit fungal scents to distinguish between crop and weedy fungi leading to weed burial that could contribute to its control. While chemical antifungals have been reported in termite farms, live burial per se as an antifungal activity has never been investigated. In this study, major and minor worker castes of termites buried the weedy fungus with soil to a significantly greater extent than the crop fungus. This live burial by worker termites led to greater decrease in the survival of the weedy fungus compared to the crop fungus, even after controlling for the differential amount of soil deposition. Such a decrease in parasite survival could result from local hypoxia generated by the burial process. Our experiments with artificial burial revealed that, in the absence of chemical factors such as fungicides, weed survival is indeed negatively affected by the resulting hypoxia alone. However, hypoxia associated with artificial burial also decreased crop survival, explaining why natural burial of crop fungi is minimal. Farmer termites may, therefore, contain weeds in their fungus farms by selectively burying weed-infested areas, resulting in antifungal activity which in part could be due to local hypoxic conditions. These results show how organisms may exploit the abiotic effects of behavioural actions as an effective defence against parasites.

Keywords Antifungal mechanism · Burial behaviour · Fungus-farming termite · Hypoxia · *Pseudoxylaria* · *Termitomyces*

Introduction

In the natural world where antagonistic interactions such as competition, parasitism and predation are rampant, mutualistic associations between organisms are also widespread. However, such mutualistic associations also need protection against antagonists. For example, myrmecophytic plants enlist the help of ants which behaviourally protect them from herbivores (Heil and McKey 2003); endophytic fungi produce metabolites which mediate host grass resistance to herbivores (Saikkonen et al. 2013), and Actinobacteria produce antifungal compounds inhibitory to the parasitic fungi of fungus-growing ant cultivars (Currie et al. 1999). Similarly, in fungus-farming termites, bacteria produce antifungal compounds (Visser et al. 2012; Um et al. 2013; Kim et al. 2014; Beemelmanns et al. 2017), many of which are non-specific in their action, and may be utilized by termites to inhibit their fungal crop parasites. Alternatively, the fungal crop could "enlist" and domesticate termites (Nobre and Aanen 2012) to behaviourally protect them from parasites.

The crop parasites commonly found in the nests of Macrotermitinae termites belong to the subgenus *Pseudoxylaria* (Ascomycota) (Visser et al. 2009; Hsieh et al. 2010; Katariya et al. 2017b). These weedy *Pseudoxylaria* fungi can compete with the mutualistic crop *Termitomyces* (Basidiomycota) for nutrition as (1) they utilize the same carbon sources, and (2) grow faster than the crop (Visser et al. 2011). Therefore, any emerging threat of *Pseudoxylaria* weeds should be speedily dealt with before the parasite overtakes the fungal farms, by using chemical compounds for example. Our previous studies showed that

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00040-018-0644-5) contains supplementary material, which is available to authorized users.

✉ R. M. Borges
renee@iisc.ac.in

[1] Centre for Ecological Sciences, Indian Institute of Science, Bangalore 560012, India

Published online: 07 July 2018

the farmer termites have the behavioural capacity to differentiate between their crops and weedy fungi (Katariya et al. 2017a). Following recognition, termites engage in live burial of *Pseudoxylaria*, raising the speculation that this behaviour may be an antifungal defence response, comparable to the behavioural defences of fungus-growing ants that groom their gardens to protect against parasitic fungi (Currie and Stuart 2001; Little et al. 2006). However, whether the burying behaviour itself can contribute to antifungal defence against weedy parasites has not been investigated in fungus-farming insects.

Burying behaviour is a common response of termites to the presence of cadavers of nestmates that have succumbed to pathogenic infections such as caused by fungi (Myles 2002; Chouvenc et al. 2008, 2012). On the other hand, burial seems to be an atypical ant behaviour (Renucci et al. 2011). Rather, waste management in ants includes dumping refuse along with cadavers in waste chambers as occurs in fungus-growing ant nests (Bot et al. 2001; Hart and Ratnieks 2001; Hart 2002). Burial may limit the negative impacts of rotting cadavers due to the presence of faecal material, saliva and glandular secretions in the depositions which have antimicrobial and fungistatic properties (Rosengaus et al. 1998, 2000, 2004; Lamberty et al. 2001; Chouvenc and Su 2012). Alternatively, burying may limit decomposition due to (1) enhanced desiccation (e.g. in sandy soil) or (2) low diffusibility of O_2 and CO_2 (e.g. in wet and clayey soil) that prevents rapid, aerobic microbial action (López-Riquelme and Fanjul-Moles 2013). Whatever the nature of the mechanism involved, burying termite corpses ultimately leads to decrease in the fungal load of the cadavers (Rosengaus et al. 1998; Chouvenc et al. 2012; Chouvenc and Su 2012). Similarly, in human agriculture, burying of seeds of weedy plants has negative effects on seedling germination (Chauhan and Johnson 2011). Factors implicated in the decreased seed germination of weeds include hypoxia and hypoxia-associated processes resulting from burial, as happens during tillage (Benvenuti and Macchia 1995).

We hypothesized that fungus-growing termites may be burying the weedy fungus (Katariya et al. 2017a) to inhibit its growth. This is because *Pseudoxylaria* grows within the termite mound environment only in the absence of contact with termites, for example in mounds abandoned by termites (Batra and Batra 1979; Katariya, pers. obs.), or when incubated within termite-proof enclosures within active mounds that allow circulation of mound air (Katariya et al. 2018). Therefore, using a locally prevalent fungus-farming termite, *Odontotermes obesus*, as our model system, we investigated if live burial is associated with antifungal activity. We estimated the decrease in *Pseudoxylaria* survival post-burial and compared it with that of the mutualistic *Termitomyces*. For the present study, we restrict ourselves to experimentally examining the effect of hypoxia alone.

Materials and methods

Termite collection and fungal cultures

Only freshly collected workers of the fungus-growing termite *Odontotermes obesus* were employed in the assays. They were collected during the day (10 a.m.–2 p.m.) from a termite mound present in the Indian Institute of Science campus (13°01′32.2″N 77°33′45.6″E) (Bangalore, India). To decrease mortality owing to desiccation, the different castes (minor and major workers) were kept (separately) in glass petri-plates containing moist tissue paper. Tested termites were never re-used.

Termitomyces and *Pseudoxylaria* fungi used in the assays were cultured on potato dextrose agar at 30 °C. For the experiments, plugs (diam. = 3.75 mm) were punched from 7- to 12-day-old *Termitomyces* cultures growing as spread plates (Visser et al. 2011) and 3- to 5-day-old *Pseudoxylaria* cultures (from an actively growing culture edge) point-inoculated in the centre of the petri-plates.

Soil assay

We used sterile garden soil (mesh size = 75 µ) (amount equivalent to ~ 3 mL) in sterile 6-well plates (Nest Biotech, flat bottom with low evaporation) for this assay (Fig. 1a). Termites utilize wet soil (a natural substrate) to make agglomerations called boluses and deposit them on the fungal plugs (Katariya et al. 2017a). To wet the soil, autoclaved MilliQ water (1.65 mL) was added and a disc (diam. = 8.8 mm) of sterile aluminium foil was positioned in the centre of the dish for placing the fungal plug. The *Pseudoxylaria* (or *Termitomyces*) plug (diam. = 3.75 mm) was placed in an inverted position inside a PCR cap (Axygen, flat 0.2 mL PCR strip caps, thin walled) to prevent direct contact of soil deposited by termites with mycelia. The PCR cap was kept over the aluminium disc and incubated in the dark at room temperature with termites (either 3 major workers or 6 minor workers). Wells without termites served as controls. After 24 h, all the soil boluses deposited on and around the inverted plugs were carefully removed (from the aluminium disc) and stored for weighing. Only those plugs that were completely covered with soil were used, i.e. the fungal plug was concealed under the soil layer and not visible to the observer's eyes. Survival of both control and test plugs of *Pseudoxylaria* (or *Termitomyces*) was quantified using a colorimetric MTT assay (see below). For this, plugs were first washed thrice with 200 µL saline (0.9% w/v) in a 96-well plate to remove any soil particles adhering to the fungal plugs, before proceeding with the assay. We used a *Termitomyces*

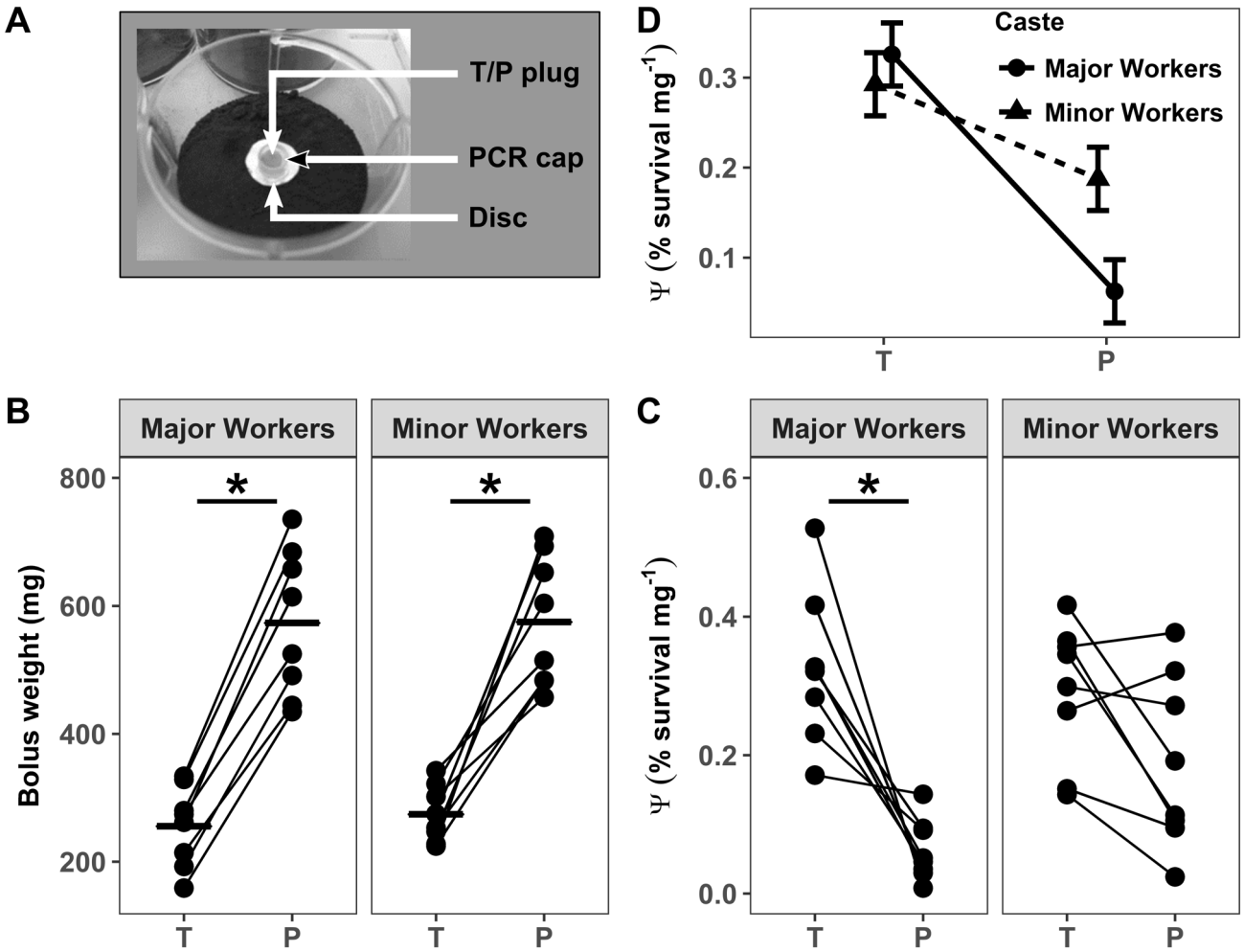

Fig. 1 Antifungal activity of soil boluses deposited by worker termites. (Anti-clockwise) **a** Assay setup showing presentation of *Termitomyces* (T) or *Pseudoxylaria* (P) fungi inside PCR caps placed over aluminium discs to termites in a 6-well plate containing soil (well diam. × depth = 35 mm × 17 mm). **b** Dot plots representing mass of soil boluses (mg) deposited on the plugs of *Termitomyces* and *Pseudoxylaria* fungi by major and minor workers in 24 h ($N = 8$). Horizontal bars represent means. Paired observations (filled circles) are joined with solid lines. **c** Dot plots representing % survival (ψ) (mg^{-1} of deposited soil) of *Termitomyces* and *Pseudoxylaria* plugs on which major or minor workers had deposited soil boluses for 24 h ($N = 8$). Paired observations (filled circles) are joined with solid lines. **d** Statistical interaction between fungus (*Termitomyces* or *Pseudoxylaria*) and worker caste (major or minor) identity. Data are adjusted means ± SEM of $N = 8$ in each group. *Significant difference ($P < 0.05$) in pairwise comparisons using Wilcoxon signed rank tests

strain (16T2) which was isolated from a termite nest in the study site (Katariya et al. 2017b). For the parasitic fungus, we used the G4X3 isolate which is a common genotype found across termite nests (the most common genotype of the most prevalent OTU) (Katariya et al. 2017b). There were 3–9 technical replicates for each of the four groups (2 Fungi × 2 Castes) in each experiment which was repeated to give eight biological replicates.

Local hypoxia assay: artificial burial

Experiments were set up in sterile 48-well plates (Nest Biotech, flat bottom with low evaporation) containing autoclaved agar (250 µL) (Fig. 2a). Fungal plugs (*Termitomyces* or *Pseudoxylaria*) (diam. = 3.75 mm) were placed in the centre of the wells and covered with PCR caps. Caps were gently pressed into the agar layer to create a closed enclosure around the plugs (i.e. artificial burial) resembling soil deposition by termites (i.e. natural burial), leading to local hypoxia. For sham burial, we used PCR caps that had four holes on the top for gaseous exchange, thus leading to the absence of local hypoxia.

To compare the effect of artificial burial on the survival of *Termitomyces* and *Pseudoxylaria* under conditions prevalent within the termite mounds, incubations were performed under two different conditions in a CO_2 incubator (Thermo Scientific Steri-Cult Model 3307): 25 °C and 95% RH for 24 h with (1) 3.2% CO_2 (high ambient CO_2; more hypoxic

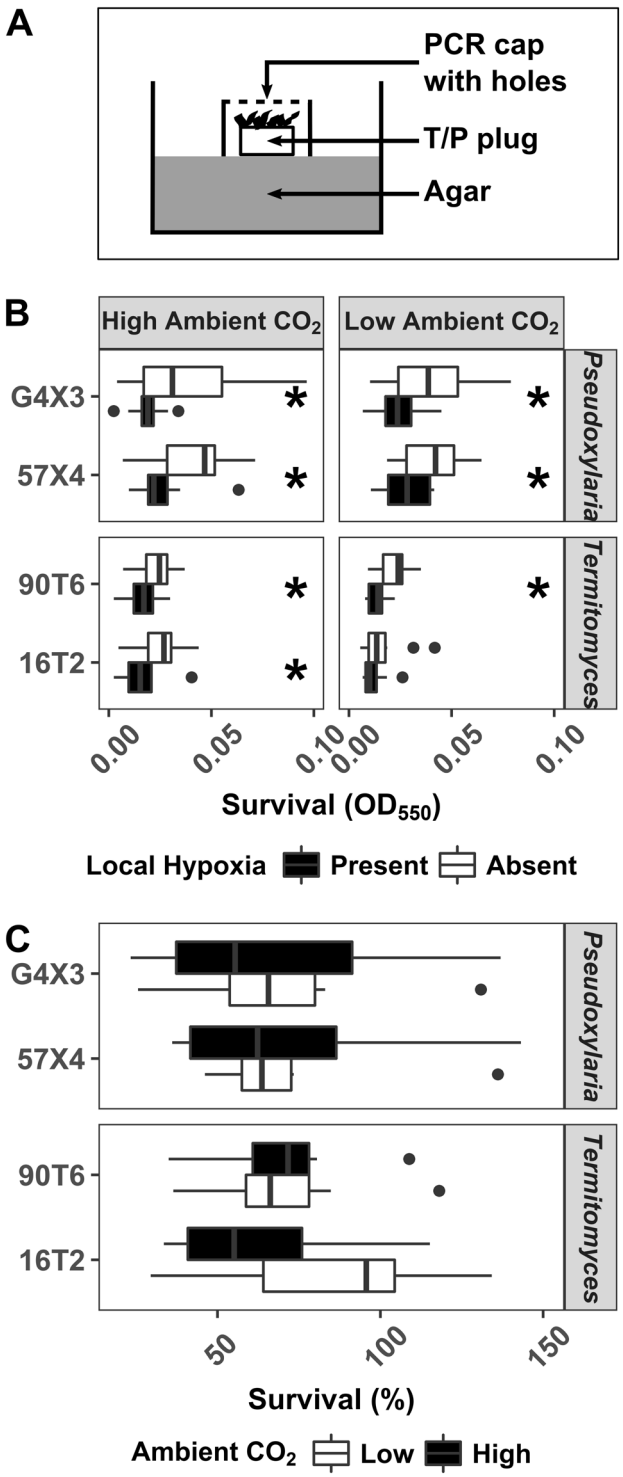

Fig. 2 Effect of hypoxic conditions on *Termitomyces* and *Pseudoxylaria* fungi. **a** Schematic of assay setup showing plugs of *Termitomyces* (T) or *Pseudoxylaria* (P) fungi artificially buried, i.e. covered inside PCR caps without holes (local hypoxia present), or sham buried, i.e. PCR caps with holes (local hypoxia absent), in a 48-well plate containing agar (well diam. × depth = 11 mm × 17 mm). **b** Box plots representing survival (OD_{550}) of *Pseudoxylaria* and *Termitomyces* fungi under the presence/absence of local hypoxic conditions for 24 h when incubated under high ($N = 10$) or low ($N = 11$) ambient CO_2 levels. **c** Box plots representing % survival of artificially buried fungi when incubated under high ($N = 10$) or low ($N = 11$) ambient CO_2 levels (using data from **b**). Horizontal lines inside boxes represent medians. Filled circles represent outliers. *Significant difference ($P < 0.05$) in **b** pairwise comparisons using Wilcoxon signed rank tests and **c** Kruskal–Wallis rank sum tests

surroundings) (Katariya et al. 2018) and (2) atmospheric CO_2 levels (low ambient CO_2; less hypoxic surroundings). In this way, artificial burial (local hypoxia present) resembles natural burial by termites inside the mound under contrasting CO_2 levels (high and low) and thus captures the combined effect of hypoxic conditions on fungi generated by burial and mound environment. Importantly, artificial burial (PCR caps) excludes the effect of any antifungal chemicals that may be deposited with soil boluses, and can thus test the effect of hypoxia alone on fungal survival. After 24-h incubation, the colorimetric MTT assay (see below) was performed to compare the survival of sham-treated (PCR cap with perforations; local hypoxia absent) and test (PCR cap without perforations; local hypoxia present) fungal plugs. Caps were moved from their positions before proceeding with the MTT assay. We used two isolates each of *Pseudoxylaria* (57X4 and G4X3) and *Termitomyces* (90T6 and 16T2) fungi, which belonged to the most prevalent OTUs, for these assays. The relative positions of four isolates (with sham treatment and test) and one blank (for subtracting the background value) in the 48-well plate were randomly changed in every experiment (number of technical replicates = 4–5). We also randomized the sequence of ambient CO_2 levels (low and high) used on different days such that there were 10 and 11 biological replicates for high and low CO_2 levels, respectively.

Colorimetric growth assay

In order to quantify the survival of *Termitomyces* or *Pseudoxylaria*, colorimetric assays were performed using MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) as described by Meletiadis et al. (2000) with necessary modifications. Fungal plugs were incubated in a 96-well plate in the dark at 30 °C with potato dextrose broth (180 µL) and MTT (20 µL, 5 mg/mL) for colour development. After ~ 6 h, all the media were decanted and 200 µL of acidic-isopropanol (1N HCl) was added to each well to lyse the cells. Plates were incubated at 25 °C at 150 rpm. After 20 min, 100 µL of coloured solution from each well was used for measuring absorbance (abs) at 550 nm (OD_{550}) in a 96-well plate.

Statistical analysis

All analyses were performed in RStudio 1.1.419 (RStudio Team 2016), user interface for R 3.4.3 (R Core Team 2017), using ggplot2 v2.2.1 (Wickham 2009) to produce figures. All figures were processed in Inkscape v0.91 (http://www.inkscape.org) and GIMP v2.8.14 (http://www.gimp.org).

Soil assay

We first calculated the % survival of individual plugs (*Termitomyces* or *Pseudoxylaria*) using the formula: % survival = $[(\text{Test}_{abs} - \text{Blank}_{abs}) \times 100]/(\mu\text{Control}_{abs})$, where $\mu\text{Control}_{abs}$ is the average of the six technical replicates ($\text{Control}_{abs} - \text{Blank}_{abs}$). Each plug was regarded as a technical replicate and % survival was normalized by dividing by the weight (mg) of the soil deposited on that particular plug. This quantity was denoted as ψ or weight normalized % survival. Finally, we calculated the mean of 3–9 technical replicates which we regarded as a biological replicate. There were eight such biological replicates for each of the four groups (2 Fungi × 2 Castes). We used Wilcoxon signed rank tests for pairwise comparisons (at $\alpha = 0.05$) between % survival (ψ) of *Termitomyces* and *Pseudoxylaria* for both major and minor workers. Pairwise comparisons between weight of soil deposited on *Termitomyces* and *Pseudoxylaria* by both major and minor workers were also analysed with Wilcoxon signed rank tests (at $\alpha = 0.05$).

We also ran an ANOVA model to analyse the effect of fungal genus and worker caste (as predictor variables) on ψ (as the response variable). Our inferences were based on the model that included both the main effects and their interaction (at $\alpha = 0.05$). MASS package v7.3.47 was used for this analysis. The interaction plot of % survival (ψ) of fungi and worker castes showing adjusted means (Fig. 1d) was drawn using the *phia* package version 0.2.1 in R. Adjusted means (least square means) are predicted values from a multiple regression equation (here ANOVA model) and thus adjusted for the imbalances arising as a result of interacting variables (here fungal genus and worker caste).

Local hypoxia assay

We calculated fungal isolate survival (OD_{550} or absorbance 550 nm) from the average of 4–5 technical replicates ($\text{Sample}_{abs} - \text{Blank}_{abs}$) and performed pairwise comparisons (Wilcoxon signed rank tests; $\alpha = 0.05$) between sham-treated (local hypoxia absent; $\text{Control}_{survival}$) and artificially buried (local hypoxia present; $\text{Test}_{survival}$) conditions. These comparisons were made for incubations done under both high ($N = 10$) as well as low ($N = 11$) ambient CO_2 levels or more hypoxic and less hypoxic surroundings, respectively. We also calculated % survival of artificially buried fungi, for each isolate using the formula: % survival = $(\text{Test}_{survival} \times 100) / (\text{Control}_{survival})$. We used Kruskal–Wallis rank sum tests (at $\alpha = 0.05$) to compare the % survival among all isolates under both high and low ambient CO_2 conditions (i.e. hypoxic and non-hypoxic surroundings).

Results

Effect of soil deposition by worker termites on *Pseudoxylaria* and *Termitomyces* survival

Both worker castes deposited significantly more amount of soil on *Pseudoxylaria* compared to *Termitomyces* (T vs. P Wilcoxon signed rank test: major workers: $N = 8$, $P = 0.008$; minor workers: $N = 8$, $P = 0.008$; Fig. 1b). The average soil deposited on weed fungi was almost twice as that on the crop (major workers: 2.25X; minor workers: 2.10X). Also, even though major workers were tested in only half the numbers than minor workers, the average amount of soil deposited by the former was similar to the latter, whether on the weed or the crop fungus (Fig. 1b). The pairwise weight normalized % survival (ψ) for *Termitomyces* was greater than for *Pseudoxylaria* but significantly different only for soil deposited by major workers (T vs. P Wilcoxon signed rank test: major workers: $N = 8$, $P = 0.008$; minor workers: $N = 8$, $P = 0.078$; Fig. 1c). Thus, a unit amount of deposited soil lowers the % survival of the weed more than the crop. Interestingly, the maximum ψ for weed fungi was still lower than minimum ψ for crop, when major workers deposited soil. Also, there was a significant interaction effect between worker caste and fungal genus on survival (ψ) (ANOVA: $F_{1,28} = 5.06$, $P = 0.032$) (Fig. 1d, Table S1). The ψ of *Pseudoxylaria* when major workers deposited soil was lower than minor workers, but the reverse was true for *Termitomyces*.

Effect of local hypoxic conditions (artificial burial) on *Pseudoxylaria* and *Termitomyces* survival

The presence of local hypoxia caused by artificial burial significantly decreased the survival (OD_{550}) of *Pseudoxylaria* and *Termitomyces* isolates, when compared to the absence of local hypoxia in sham burials. This was true for all fungal isolates incubated under high levels of ambient CO_2 (artificial vs. sham burial Wilcoxon signed rank test: G4X3: $N = 10$, $P = 0.037$; 57X4: $N = 10$, $P = 0.010$; 90T6: $N = 10$, $P = 0.004$; 16T2: $N = 10$, $P = 0.006$; Fig. 2b) and all but one isolate (16T2) incubated under low ambient CO_2 (artificial vs. sham burial Wilcoxon signed rank test: G4X3: $N = 11$, $P = 0.005$; 57X4: $N = 11$, $P = 0.007$; 90T6: $N = 11$, $P = 0.002$; 16T2: $N = 11$, $P = 0.147$; Fig. 2b). Thus, artificial burial decreased fungal survival under both less hypoxic and more hypoxic surroundings. Moreover, % survival of artificially

buried fungi with respect to sham burial did not differ significantly among the two fungi which were incubated under different ambient CO_2 concentrations (low and high levels) (Kruskal–Wallis rank sum test: $\chi^2 = 4.297$, df = 7, $P = 0.745$; Fig. 2c). Thus, the effect of local hypoxia due to artificial burial (as reflected by % survival) is similar between the contrasting high and low CO_2 levels of the surroundings and among the two fungi.

Discussion

Our results show that fungus-growing termites bury the weedy *Pseudoxylaria* to a significantly greater extent than the *Termitomyces* crop which results in relatively lowered survival (ψ) of the former compared to the latter. We also found that both the termite-associated fungi are susceptible to local hypoxia, which may occur as a result of the burying behaviour and lead to reduction in survival. Interestingly, this reduction due to local hypoxia resulting from artificial burial, not only occurred under less hypoxic surroundings (low ambient CO_2 levels) but also under more hypoxic surroundings (high ambient CO_2 levels), conditions resembling the variable CO_2 levels within the mound. Therefore, the increased burial of the fungal parasite can accrue benefits against the parasite in two ways. Firstly, since enhanced burial is specific towards the weedy fungus, burial-associated hypoxia will decrease weed survival selectively irrespective of whether the mound CO_2 levels are high or not. Secondly, lesser burial of the crop fungus will diminish the negative effect of burial-associated hypoxia on crop survival. Therefore, the burying behaviour, directed primarily at the parasitic weed, is coupled with antifungal activity which, in part, appears to be due to the effect of local hypoxic conditions.

Termites buried both fungal plugs with soil boluses when offered a natural substrate, i.e. wet soil, where the deposition was lower on the *Termitomyces*. This is interesting because in our previous study (Katariya et al. 2017a), we found workers depositing significantly more boluses on the weedy fungus when both fungi or weed and blank plugs were offered simultaneously in the same dish, but here termites were offered only one fungus at a time. This lends further support to our earlier results that the greater burial of weed is independent of crop presence (Katariya et al. 2017a). Indeed, preparation and deposition of soil boluses appear to be a constitutive activity in these termites (Zachariah et al. 2017). However, the similarity between major and minor workers in the amount of soil deposition, whether on *Pseudoxylaria* or *Termitomyces*, is suggestive of labour stoichiometry, where it appears to be 1 major:2 minor workers.

Fungal burial resulted in lower survival for *Pseudoxylaria* than *Termitomyces* and was significantly different for boluses deposited by major workers (Fig. 1c). Minor workers demonstrated a similar but weaker effect ($P = 0.078$). This decreased fungal survival associated with burial shows that termite burial behaviour is coupled with antifungal activity. Similar burial behaviour, although towards corpses, is shown by both farmer and non-farmer termites where similar positive outcomes from this sanitary behaviour are expected (Chouvenc et al. 2012; Chouvenc and Su 2012). Specifically, burial of corpses, which are potential sources of pathogens, isolates this potential threat, and if pathogens are present, it contains them locally (Cremer et al. 2007; Chouvenc and Su 2010, 2012; Chouvenc et al. 2012). Fungus-growing ants also couple behavioural defences of grooming with chemical defences where they utilize antibiotic-producing bacteria against crop parasites (Little et al. 2006). Interestingly, even though ψ is a normalized estimate of % survival which accounts for the differential amount of soil deposition between *Pseudoxylaria* and *Termitomyces*, still a difference in survival is evident between the two fungi. This shows the apparent higher specificity of the antifungal activity against *Pseudoxylaria*. In another insect–fungus symbiosis, a similar high specificity towards the parasitic fungi is seen, with the beetle's mutualistic fungi experiencing only a mild inhibition by the antifungal compounds (Scott et al. 2008; Oh et al. 2009). Such specificity may result from higher susceptibility of the parasite to the antifungal agent(s) compared to the mutualist which may be adapted to withstand antifungal exposure. It should also be noted that our conclusions based on ψ are independent of the number of termites present (e.g. 3 major or 6 minor workers per dish in this study); a unit amount of soil can be deposited by any number of termites to cause the observed lowering of fungal survival. Additionally, a significant interaction between worker caste and fungal genus (Fig. 1d) shows the higher efficiency of the major workers as a "defensive" caste. This is because major workers reduce weed survival more than what minor workers can accomplish. Equally important, major workers reduce crop survival less than the minor workers. Fungus-growing ants also show caste specialization in sanitary work with major workers specialized in weeding and minor workers in fungus grooming (Abramowski et al. 2011). Although, fungus-growing termites demonstrate age and caste polyethism in food supply and processing (Badertscher et al. 1983) and in gut bacterial microbiota (Hongoh et al. 2006), and age polyethism in queen care and foraging (Hinze and Leuthold 1999), it is not known if such age or caste polyethism exists in sanitary work. Similarly, it is unclear why termites bury the crop at all, but soil deposition on crop could be a prophylactic mechanism similar to the chemical prophylaxis in wasps (Kroiss et al. 2010) or proactive self-cleaning in farmer ants (Morelos-Juárez et al. 2010).

The decrease in fungal survival could be due not only to chemical compounds (Rosengaus et al. 1998; Chouvenc and Su 2012) present in the soil boluses but also the local

hypoxic condition generated by burial (Benvenuti and Macchia 1995; López-Riquelme and Fanjul-Moles 2013) or a combination of both mechanisms. In fact, our initial experiments indicated a strong possibility of the negative effect of hypoxia (resulting from artificial burial) on *Pseudoxylaria*, as all the four isolates with different levels of growth-rate showed decreased survival (OD_{550}) (Fig. S1). This effect is similar to the negative impact of high CO_2 levels of the ambient environment on these *Pseudoxylaria* isolates, as reported earlier (Katariya et al. 2018). In fact, any *Pseudoxylaria* sprouting inside a termite mound will experience hypoxia not only due to burying but also because of the hypoxic surroundings, since mounds are known to have high (but variable) ambient CO_2 levels (Korb and Linsenmair 1999; Katariya et al. 2018). Experiments mimicking such conditions revealed a clear negative effect of artificial burial on *Pseudoxylaria* survival (Fig. 2b), highlighting the role of local hypoxia as an antifungal mechanism. Importantly, local hypoxia decreased weed survival (OD_{550}) even when ambient CO_2 levels were low. This may be particularly significant during periods of low mound CO_2 concentrations (Korb and Linsenmair 1999; Katariya et al. 2018). Interestingly, *Termitomyces* crop showed similar results, with decreased survival (OD_{550}) due to local hypoxia under both high and low ambient CO_2 levels (except for the 16T2 isolate; low ambient CO_2). This shows the possible negative effect of burying the crop fungus and explains why termites always deposit less soil on *Termitomyces*. Also, no difference in % survival could be detected between the weed and crop fungi after artificial burial. This was in contrast to natural burial by termites which resulted in lower survival (ψ) for the weed. Thus, the non-specificity of the artificial burial-associated (local) hypoxia was in contrast to the specificity of the natural burial-associated antifungal action towards the weedy fungi. Therefore, antifungal chemicals and hypoxia must be acting together during the natural burial process.

It is important to note that the degree of local hypoxia generated by the PCR caps in these experiments is the same for both the crop and the weed, unlike the different levels of burying by termites that they experience. Similarly, the hypoxia experienced by fungi in this assay may be more adverse than the natural conditions. Therefore, in order to mimic natural deposition, an ideal experiment would have been to deposit a fixed amount of soil instead of generating hypoxia via the PCR cap enclosure. However, these experiments have proven difficult to conduct, partially owing to the inability to match the packing efficiency of soil by termites (which has important consequence for the porosity of the deposited mass) (Zachariah et al., unpub. results). Nonetheless, these results provide us important insights into the antifungal role of hypoxia.

In conclusion, our results highlight the possibility of burying behaviour per se as an antifungal mechanism deployed by farmer termites against weedy fungi like *Pseudoxylaria*. These results also show the important role of an abiotic process (local hypoxia) as an anti-parasite mechanism and indicate its role in the burying-associated decrease of weed survival. However, we cannot rule out the additional presence of antifungal chemicals in the burial materials. It is also possible that antibiotic-producing bacteria and their products are deposited along with soil on the weedy fungi. Thus, further studies using soil deposited by termite workers to isolate antifungal compounds are warranted and are underway. The relative role of hypoxia and antifungal chemicals should be investigated to determine if they act additively or synergistically. Finally, caste polyethism in antifungal defence should be investigated to uncover the role of major workers as a specialized "defensive" caste.

Acknowledgements This research was funded by the Council of Scientific and Industrial Research (37(1561)/12/EMR-II); the Ministry of Environment, Forest & Climate Change; the Department of Biotechnology; and the Department of Science and Technology-FIST, Government of India. We are grateful to C Viraktamath and Rashmi Shanbhag for termite identifications; Abdul Hakkim for the MTT assay; Tejas Murthy for providing processed soil; Yathiraj Ganesh for field work; Sunitha Murray for administrative support; E S Anupriya for help in weighing; and Thejashwini Gopalappa and Anagha Setlur for help with experiments.

Author Contributions LK and RMB conceived the study. LK designed the methodology with inputs from PBR, and LK, PBR and AS carried out the experiments. LK analysed all the data. LK wrote the first draft of manuscript and RMB and LK contributed to revision and editing. RMB acquired the funding.

References

Abramowski D, Currie CR, Poulsen M (2011) Caste specialization in behavioral defenses against fungus garden parasites in *Acromyrmex octospinosus* leaf-cutting ants. Insectes Soc 58:65–75. https://doi.org/10.1007/s00040-010-0117-y

Badertscher S, Gerber C, Leuthold RH (1983) Polyethism in food supply and processing in termite colonies of *Macrotermes subhyalinus* (Isoptera). Behav Ecol Sociobiol 12:115–119. https://doi.org/10.1007/BF00343201

Batra LR, Batra SWT (1979) Termite-fungus mutualism. In: Batra LR (ed) Insect-fungus symbiosis: nutrition, mutualism and commensalism. Allanheld and Osmun, Montclair, pp 117–163

Beemelmanns C, Ramadhar TR, Kim KH et al (2017) Macrotermycins A–D, glycosylated macrolactams from a termite-associated *Amycolatopsis* sp. M39. Org Lett. https://doi.org/10.1021/acs.orglett.6b03831

Benvenuti S, Macchia M (1995) Effect of hypoxia on buried weed seed germination. Weed Res 35:343–351. https://doi.org/10.1111/j.1365-3180.1995.tb01629.x

Bot ANM, Currie CR, Hart AG, Boomsma JJ (2001) Waste management in leaf-cutting ants. Ethol Ecol Evol 13:225–237. https://doi.org/10.1080/08927014.2001.9522772

Chauhan BS, Johnson DE (2011) Ecological studies on *Echinochloa crus-galli* and the implications for weed management in direct-seeded rice. Crop Prot 30:1385–1391. https://doi.org/10.1016/j.cropro.2011.07.013

Chouvenc T, Su N-Y (2010) Apparent synergy among defense mechanisms in subterranean termites (Rhinotermitidae) against epizootic events: limits and potential for biological control. J Econ Entomol 103:1327–1337. https://doi.org/10.1603/EC09407

Chouvenc T, Su N-Y (2012) When subterranean termites challenge the rules of fungal epizootics. PLoS ONE 7:e34484. https://doi.org/10.1371/journal.pone.0034484

Chouvenc T, Su N-Y, Elliott ML (2008) Interaction between the subterranean termite *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) and the entomopathogenic fungus *Metarhizium anisopliae* in foraging arenas. J Econ Entomol 101:885–893

Chouvenc T, Robert A, Sémon E, Bordereau C (2012) Burial behaviour by dealates of the termite *Pseudacanthotermes spiniger* (Termitidae, Macrotermitinae) induced by chemical signals from termite corpses. Insectes Soc 59:119–125. https://doi.org/10.1007/s00040-011-0197-3

Cremer S, Armitage SAO, Schmid-Hempel P (2007) Social immunity. Curr Biol 17:R693–R702. https://doi.org/10.1016/j.cub.2007.06.008

Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. Proc R Soc Lond B 268:1033–1039. https://doi.org/10.1098/rspb.2001.1605

Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701–704. https://doi.org/10.1038/19519

Hart AG (2002) Waste management in the leaf-cutting ant *Atta colombica*. Behav Ecol 13:224–231. https://doi.org/10.1093/beheco/13.2.224

Hart AG, Ratnieks FLW (2001) Task partitioning, division of labour and nest compartmentalisation collectively isolate hazardous waste in the leafcutting ant *Atta cephalotes*. Behav Ecol Sociobiol 49:387–392. https://doi.org/10.1007/s002650000312

Heil M, McKey D (2003) Protective ant-plant interactions as model systems in ecological and evolutionary research. Annu Rev Ecol Evol Syst 34:425–553. https://doi.org/10.1146/annurev.ecolsys.34.011802.132410

Hinze B, Leuthold RH (1999) Age related polyethism and activity rhythms in the nest of the termite *Macrotermes bellicosus* (Isoptera, Termitidae). Insectes Soc 46:392–397. https://doi.org/10.1007/s000400050162

Hongoh Y, Ekpornprasit L, Inoue T et al (2006) Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. Mol Ecol 15:505–516. https://doi.org/10.1111/j.1365-294X.2005.02795.x

Hsieh H-M, Lin C-R, Fang M-J et al (2010) Phylogenetic status of *Xylaria* subgenus *Pseudoxylaria* among taxa of the subfamily Xylarioideae (Xylariaceae) and phylogeny of the taxa involved in the subfamily. Mol Phylogenet Evol 54:957–969. https://doi.org/10.1016/j.ympev.2009.12.015

Katariya L, Ramesh PB, Gopalappa T et al (2017a) Fungus-farming termites selectively bury weedy fungi that smell different from crop fungi. J Chem Ecol 43:986–995. https://doi.org/10.1007/s10886-017-0902-4

Katariya L, Ramesh PB, Gopalappa T, Borges RM (2017b) Sex and diversity: the mutualistic and parasitic fungi of a fungus-growing termite differ in genetic diversity and reproductive strategy. Fungal Ecol 26:20–27. https://doi.org/10.1016/j.funeco.2016.11.003

Katariya L, Ramesh PB, Borges RM (2018) Dynamic environments of fungus-farming termite mounds exert growth-modulating effects on fungal crop parasites. Environ Microbiol 20:971–979. https://doi.org/10.1111/1462-2920.14026

Kim KH, Ramadhar TR, Beemelmanns C et al (2014) Natalamycin A, an ansamycin from a termite-associated *Streptomyces* sp. Chem Sci 5:4333–4338. https://doi.org/10.1039/C4SC01136H

Korb J, Linsenmair KE (1999) The architecture of termite mounds: a result of a trade-off between thermoregulation and gas exchange? Behav Ecol 10:312–316. https://doi.org/10.1093/beheco/10.3.312

Kroiss J, Kaltenpoth M, Schneider B et al (2010) Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nat Chem Biol 6:261–263. https://doi.org/10.1038/nchembio.331

Lamberty M, Zachary D, Lanot R et al (2001) Insect immunity: constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. J Biol Chem 276:4085–4092. https://doi.org/10.1074/jbc.M002998200

Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biol Lett 2:12–16. https://doi.org/10.1098/rsbl.2005.0371

López-Riquelme GO, Fanjul-Moles ML (2013) The funeral ways of social insects. Social strategies for corpse disposal. Trends Entomol 9:71–129

Meletiadis J, Meis JFGM, Mouton JW et al (2000) Comparison of NCCLS and 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) methods of in vitro susceptibility testing of filamentous fungi and development of a new simplified method. J Clin Microbiol 38:2949–2954

Morelos-Juárez C, Walker TN, Lopes JFS, Hughes WOH (2010) Ant farmers practice proactive personal hygiene to protect their fungus crop. Curr Biol 20:R553–R554. https://doi.org/10.1016/j.cub.2010.04.047

Myles TG (2002) Alarm, aggregation and defense by *Reticulitermes flavipes* in response to a naturally occurring isolate of *Metarhizium anisopliae*. Sociobiology 40:243–256

Nobre T, Aanen DK (2012) Fungiculture or termite husbandry? The ruminant hypothesis. Insects 3:307–323. https://doi.org/10.3390/insects3010307

Oh D-C, Scott JJ, Currie CR, Clardy J (2009) Mycangimycin, a polyene peroxide from a mutualist *Streptomyces* sp. Org Lett 11:633–636. https://doi.org/10.1021/ol802709x

R Core Team (2017) R: a language and environment for statistical computing. R Foundation for statistical Computing, Vienna, Austria. https://www.R-project.org/

Renucci M, Tirard A, Provost E (2011) Complex undertaking behavior in *Temnothorax lichtensteini* ant colonies: from corpse-burying behavior to necrophoric behavior. Insect Soc 58:9–16. https://doi.org/10.1007/s00040-010-0109-y

Rosengaus RB, Guldin MR, Traniello JF (1998) Inhibitory effect of termite fecal pellets on fungal spore germination. J Chem Ecol 24:1697–1706

Rosengaus RB, Lefebvre ML, Traniello JF (2000) Inhibition of fungal spore germination by *Nasutitermes*: evidence for a possible antiseptic role of soldier defensive secretions. J Chem Ecol 26:21–39

Rosengaus RB, Traniello JFA, Lefebvre ML, Maxmen AB (2004) Fungistatic activity of the sternal gland secretion of the dampwood termite *Zootermopsis angusticollis*. Insectes Soc 51:259–264. https://doi.org/10.1007/s00040-004-0749-x

RStudio Team (2016) RStudio: integrated development for R. RStudio, Boston, MA. https://www.rstudio.com

Saikkonen K, Gundel PE, Helander M (2013) Chemical ecology mediated by fungal endophytes in grasses. J Chem Ecol 39:962–968. https://doi.org/10.1007/s10886-013-0310-3

Scott JJ, Oh D-C, Yuceer MC et al (2008) Bacterial protection of beetle-fungus mutualism. Science 322:63–63. https://doi.org/10.1126/science.1160423

Um S, Fraimout A, Sapountzis P et al (2013) The fungus-growing termite *Macrotermes natalensis* harbors bacillaene-producing *Bacillus* sp. that inhibit potentially antagonistic fungi. Sci Rep 3:3250. https://doi.org/10.1038/srep03250

Visser AA, Ros VID, De Beer ZW et al (2009) Levels of specificity of *Xylaria* species associated with fungus-growing termites: a phylogenetic approach. Mol Ecol 18:553–567. https://doi.org/10.1111/j.1365-294X.2008.04036.x

Visser AA, Kooij PW, Debets AJM et al (2011) *Pseudoxylaria* as stowaway of the fungus-growing termite nest: interaction asymmetry between *Pseudoxylaria, Termitomyces* and free-living relatives. Fungal Ecol 4:322–332. https://doi.org/10.1016/j.funeco.2011.05.003

Visser AA, Nobre T, Currie CR et al (2012) Exploring the potential for actinobacteria as defensive symbionts in fungus-growing termites. Microb Ecol 63:975–985. https://doi.org/10.1007/s00248-011-9987-4

Wickham H (2009) Ggplot2: elegant graphics for data analysis. Springer, New York

Zachariah N, Das A, Murthy TG, Borges RM (2017) Building mud castles: a perspective from brick-laying termites. Sci Rep 7:4692. https://doi.org/10.1038/s41598-017-04295-3