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Dynamic environments of fungus-farming termite mounds exert growth-modulating effects on fungal crop parasites

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SUMMARY

This study investigated for the first time the impact of the internal mound environment of fungus-growing termites on the growth of fungal crop parasites. Mounds of the termite *Odontotermes obesus* acted as (i) temperature and relative humidity (RH) 'stabilisers' showing dampened daily variation and (ii) 'extreme environments' exhibiting elevated RH and CO_2 levels, compared to the outside. Yet, internal temperatures exhibited seasonal dynamics as did daily and seasonal CO_2 levels. During *in situ* experiments under termite-excluded conditions within the mound, the growth of the crop parasite *Pseudoxylaria* was greater inside than outside the mound, i.e., *Pseudoxylaria* is 'termitariophilic'. Also, *ex situ* experiments on parasite isolates differing in growth rates and examined under controlled conditions in the absence of termites revealed a variable effect with fungal growth decreasing only under high CO_2 and low temperature conditions, reflecting the *in situ* parasite growth fluctuations. In essence, the parasite appears to be adapted to survive in the termite mound. Thus the mound microclimate does not inhibit the parasite but the dynamic environmental conditions of the mound affect its growth to varying extents. These results shed light on the impact of animal-engineered structures on parasite ecology, independent of any direct role of animal engineers.

Introduction

Ecosystem engineers such as termites and ants build structures that cause physical state changes in biotic or abiotic materials which result in direct and indirect effects on other organisms (Jones *et al*., 1994; Wright and Jones, 2006). These constructions are extended phenotypes as they also affect the fitness of the engineers themselves (Jones *et al*., 1994). Many positive effects of these structures are mediated by environmental factors such as the apparent benefit conferred by the optimal nest microclimate of red wood ants on the production of sexual progeny (Rosengren *et al*., 1987). The negative effects include an ideal environment for pathogen growth, e.g., the stable nest microclimate of red wood ants that facilitates a specialised decomposer community might also promote the growth of opportunistic pathogenic bacteria (Christe *et al*., 2003). Importantly, animal engineers can not only sense environmental cues, e.g., fungus-farming ants show a preference for specific CO_2 levels for fungus-rearing (Römer *et al*., 2017), but also regulate them either actively or passively (Jones and Oldroyd, 2006). However, the effect of such environmental factors on engineers and their symbionts can be compounded by genotype and environment interactions (Thomas and Blanford, 2003) which may select for specific mutualists or parasites.

Mound-building Macrotermitinae termites are major ecosystem engineers in the tropics and subtropics (Jones *et al*., 1994) and cultivate monocultures of *Termitomyces* (Basidiomycota) fungus (Aanen *et al*., 2009; Katariya *et al*., 2017a). The fungus is farmed on termite faecal matter, deposited in the form of a fungus comb inside their nest mounds, the termite's extended phenotype. The beneficial effect of the mound environment on termite crops (Korb and Linsenmair, 1999) is complemented by factors such as antibiotic-producing bacteria that can help inhibit crop parasites (Visser *et al*., 2012; Um *et al*., 2013; Kim *et al*., 2014; Beemelmanns *et al*., 2017). However, the detrimental effect of the mound environment in supressing or modulating growth of parasitic fungi has so far not been evaluated. The parasitic fungi most commonly observed belong to the subgenus *Pseudoxylaria* (Ascomycota) (Visser *et al*., 2009; Hsieh *et al*., 2010; Katariya *et al*., 2017a). Unlike the monocultured crops, multiple genotypes of *Pseudoxylaria* occur in the same mound (Visser *et al*., 2009; Katariya *et al*., 2017a) which show great diversity in

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colony appearance and growth rates (Hsieh *et al.*, 2010; L. Katariya, T. Gopalappa and R. M. Borges, pers. obs.).

Pseudoxylaria fungi are regarded as stowaway parasites that employ a sit-and-wait strategy (Visser *et al.*, 2011). In other words, they become visible only on combs that are either freshly removed from live termite mounds or mounds that have been abandoned by termites, where they completely overgrow the existing *Termitomyces* farm in a matter of days (Batra and Batra, 1979; L. Katariya, T. Gopalappa and R.M. Borges, pers. obs.). This suggests that these processes – comb removal and termites abandoning the mounds – release *Pseudoxylaria* from the inhibitory factor(s) present inside the mound. Possibly, these processes lead to changes in environmental factors that affect crop parasites such as CO_2 concentration, which is quite high inside the mounds (Korb and Linsenmair, 1999) and can inhibit *Pseudoxylaria* fungi (Batra and Batra, 1966). The crop fungus, on the other hand, appears adapted to such conditions as is evident from fungus comb sampling from mounds throughout the year (Katariya *et al.*, 2017a).

To our knowledge, no studies till now have tested the inhibitory mound microclimate hypothesis with respect to the growth of the important crop parasite *Pseudoxylaria*. Also, since multiple *Pseudoxylaria* genotypes (and OTUs), which also show physiological differences, e.g. in growth rates, are present in the same mound (Katariya *et al.*, 2017a), it is important to investigate whether mound microclimate has a differential effect on the various genotypes. It is possible that faster-growing genotypes are affected more strongly than slower-growing genotypes, since the former may have a growth disadvantage under hypoxic conditions (because of high CO_2) resulting from their high metabolic rate when compared to the latter. On the other hand, after the decline of the termite colony and the subsequent loss of the mound inhibitory effect(s), faster-growing genotypes may be able to monopolise resources (i.e., the fungal comb) more rapidly than slower-growing ones. The alternate hypothesis could be that mound microclimate does not inhibit *Pseudoxylaria* growth, rather its growth is promoted inside the termite nest. Therefore, in order to investigate the effect of mound microclimate alone on *Pseudoxylaria* growth, we (i) examined daily and monthly variation in three environmental factors of the mounds: temperature, RH and CO_2 concentration and (ii) using *Pseudoxylaria* isolates that differ in growth rates (slow to fast), investigated for the first time, (a) the comparative growth of *Pseudoxylaria* inside (*in situ*) and outside the mound and (b) the effect of various mound conditions on the *ex situ* growth of *Pseudoxylaria*.

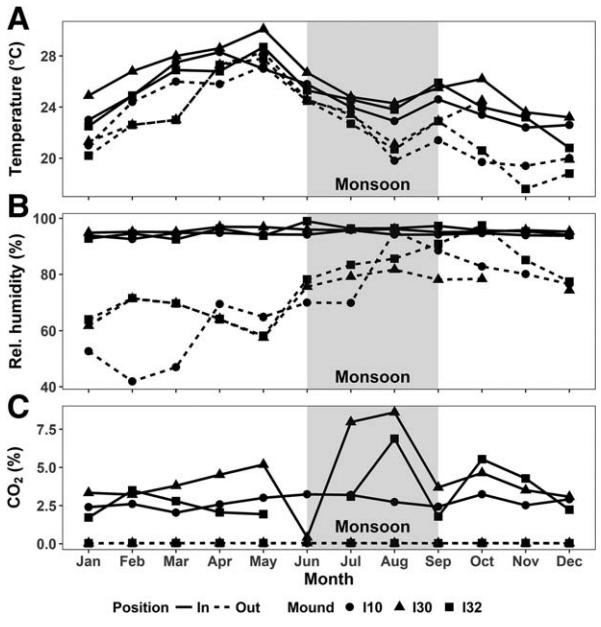

Fig. 1. Pattern of inside (In) and outside (Out) mound temperature (A) relative humidity (B) and CO_2 concentration (C) of three different mounds (I10, I30 and I32) of the fungus-growing termite *Odontotermes obesus*. Each point represents the mean of a single day's recording. Shaded region represents monsoon (rainy) season. See materials and methods for details.

Results

Mound microclimate characterisation

Air temperature and relative humidity (RH) inside and outside termite mounds. We found that at the daily level, the inside mound temperature as well as RH showed far less variation compared to the outside ($Daily^{SD}$ in Supporting Information Table S1) that was characterised by a diurnal cycle of temperature and RH fluctuations, resulting in hot days and cool nights. However, at the monthly scale, the mound temperature changed gradually from high in summer to low in winter (seasonal cycle) similar to the outside profile (Fig. 1A). Mound RH on the other hand remained stable at ~95% throughout the year as compared to the outside (Fig. 1B). The correlation (i) between the average inside temperatures of the mounds and (ii) between the average outside temperature and the corresponding inside temperature was high for all three mounds (Table 1). However, there was very low correlation between inside and outside mound RH values as well as between different mounds.

CO_2 concentration inside and outside termite mounds. The mound CO_2 concentration was extremely high ($3.4 \pm 1.7\%$) compared to the outside (only $\sim 0.05 \pm 0.00\%$). Both at the daily level as well as over the year, there was high variation in CO_2 concentration inside the mounds compared to the outside (Supporting Information Table S1, Fig. 1C). However, unlike the temperature profile, there was very low correlation between different

Table 1. Correlation matrix for temperature, relative humidity (RH) and CO_2 concentration of three mounds (I10, I30 and I32) of fungus-growing termite *Odontotermes obesus* ($N = 11$ recording days corresponding to 11 months for each mound). Values are Pearson's R, * indicates $P < 0.01$ in GLS model (after Bonferroni correction).

		Temperature			RH			CO_2		
		I10	I30	I32	I10	I30	I32	I10	I30	I32
		Inside	Inside	Inside	Inside	Inside	Inside	Inside	Inside	Inside
I10	Outside	0.9*			0.3			−0.19		
I30	Inside	0.89*			0.29			0.24		
	Outside		0.88*			−0.31			0.2	
I32	Inside	0.86*	0.91*		0.19	0.31		0.31	0.27*	
	Outside			0.9*			0.53			0.55*

mounds (Table 1). Also, the CO_2 levels peaked during the monsoon (Fig. 1C).

Effect of mound micro-climate on the growth of Pseudoxylaria *(*in situ *growth experiments).* Both faster and slower growing isolates of *Pseudoxylaria* had a higher growth rate inside than outside the mounds (LMM: 65X4 $t_{1,50} = -4.60$, $P < 0.001$; 57X3 $t_{1,47} = -5.86$, $P < 0.001$) (Fig. 2, Supporting Information Table S2a,b). Also, both isolates grew faster in the hot than the cold season (LMM: 65X4 $t_{1,45} = 6.29$, $P < 0.001$; 57X3 $t_{1,42} = 5.93$, $P < 0.001$) (Fig. 2, Supporting Information Table S2a,b).

Effect of different temperature and CO_2 conditions on the growth of Pseudoxylaria *(*ex situ *growth experiments).* There was a significant effect of CO_2 concentration ($F_{2,166} = 7.3$, $P < 0.001$) on *Pseudoxylaria* growth and a significant interaction between isolate identity and temperature ($F_{6,166} = 7.5$, $P < 0.001$) (Table 2, Fig. 3). Only high CO_2 levels showed a significant decrease in *Pseudoxylaria* growth compared to low CO_2 conditions (Table 2). A post-hoc analysis (Tukey HSD) indicated that both the slowest (3X7) as well as the fastest (57X4) isolates had a significant increase in growth at medium temperatures compared to low temperatures but the subsequent decrease in growth at high temperatures was not significantly different from medium temperatures (Supporting Information Fig. S1). Likewise, the slow (65X4) and the fast isolate (19X3) exhibited similar growth trends. There was no significant change in their growth at medium temperatures from low temperatures but both demonstrated a significant increase in growth at high temperatures (Supporting Information Fig. S1).

Discussion

Termite mounds seem to act as temperature and RH 'stabilisers', i.e., the inside mound temperature and RH remain stable and do not fluctuate with the diel cycle outside the mound. At the same time, mounds also display the gradual seasonal change in monthly temperatures similar to the outside conditions, while RH remains consistently high (~95%). Mounds are also characterised by extremely high CO_2 levels (~3.5%), compared to the outside, which fluctuate daily and monthly. Therefore, termite mounds are extreme terrestrial environments owing to the very high RH and CO_2 levels present inside. However, mound environment cannot inhibit *Pseudoxylaria* growth; rather this parasitic fungus may even be adapted to survive inside the termite nest. Nevertheless, certain factors such as high CO_2 concentration and low temperature can hamper the growth of the parasitic fungus, thereby negatively affecting its growth. Therefore, changes in mound environment

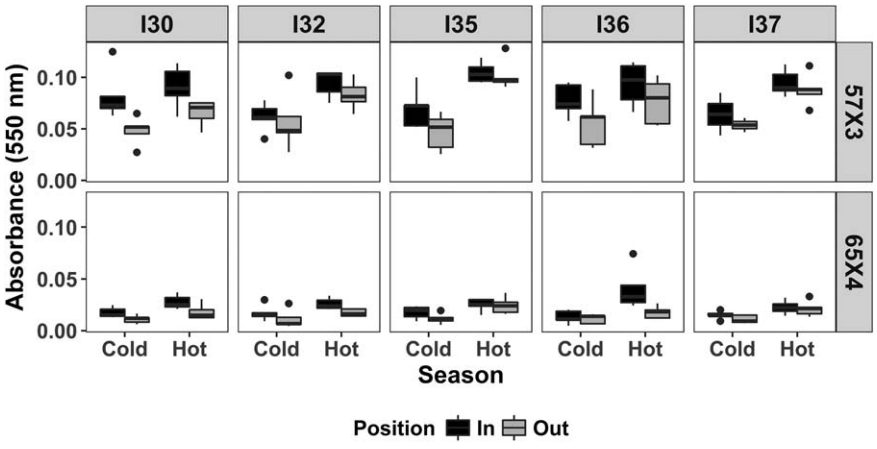

Fig. 2. Box plots of the growth pattern of slower (65X4) and faster (57X3) growing *Pseudoxylaria* isolates inside (In) and outside (Out) five different mounds (I30, I32, I35, I36 and I37) of the fungus-growing termite, *Odontotermes obesus* in hot and cold seasons. Dots represent outliers. $N = 5$ in all cases but $N = 6$ for I30 (57X3, hot season) and I37 (65X4, hot season) and $N = 2$ for I37 (57X3, cold season) where each value is a mean of three technical replicates.

Table 2. Linear model showing the effect of factors affecting *Pseudoxylaria* growth, i.e. absorbance (550 nm) under different growth conditions. $R^2 = 0.95$ for overall regression model; df = 2, 2, 3 and 6 for *F* tests for Temperature, CO_2, Isolate and Temperature x Isolate respectively (x = Interaction effect).

Term	Coefficient	95% CI	*F*	*P*
Intercept (Temp: Low, CO_2: High, Isolate: 19X3)	0.491	0.472 to 0.510		
Temperature (overall effect)			46.725	< 0.001
Temp: Medium	−0.003	−0.028 to 0.022		
Temp: High	0.079	0.053 to 0.104		
CO_2 (overall effect)			7.275	< 0.001
CO_2: Low	0.024	0.012 to 0.037		
CO_2: Medium	0.012	−0.001 to 0.024		
Isolate (overall effect)			1112.8	< 0.001
Isolate: I3X7	−0.252	−0.277 to −0.227		
Isolate: 65X4	−0.082	−0.107 to −0.056		
Isolate: 57X4	0.167	0.142 to 0.192		
Temp x Isolate (overall effect)			7.465	< 0.001
Temp: Medium x Isolate: 3X7	0.064	0.029 to 0.100		
Temp: High x Isolate: 3X7	−0.032	−0.068 to 0.003		
Temp: Medium x Isolate: 65X4	0.023	−0.013 to 0.058		
Temp: High x Isolate: 65X4	−0.004	−0.040 to 0.031		
Temp: Medium x Isolate: 57X4	0.062	0.027 to 0.098		
Temp: High x Isolate: 57X4	−0.032	−0.067 to 0.004		

modulate parasite growth that may include negative effects on the parasite.

Our finding of dampened variation in daily temperature and RH is supported by other studies of *O. obesus* mounds in India (Agarwal, 1980) and *Macrotermes bellicosus* mounds in Africa (Korb and Linsenmair, 1998; 2000). The strong correlation between the average outside temperature and the corresponding inside temperature indicates that the mound temperature closely follows the outside temperature, i.e. it is dynamic, and therefore termites probably do not control the monthly variation in the mound temperature. In fact, uninhabited but intact mounds show similar temperature characteristics (Korb and Linsenmair, 2000) confirming that the mound structure itself may indeed maintain such conditions. Therefore, there seem to be 'summers' (hot seasons) and 'winters' (cold seasons) inside the mound but no 'hot days' and 'cool nights'. Consequently, a lenticular termite mound could be likened to a 'temperature stabiliser' which reduces the variation in temperature of the 'supply', i.e., the outside environment. The constant high RH throughout the diel cycle and the seasons, similar to that observed by Agarwal (1980), indicates that termite mounds could also be likened to a 'RH stabiliser'. The RH stabilisation may be achieved by dampening humidity loss due to the thick mound walls. Such stable mound environments could not only provide suitable conditions for the termite colony development but also protect the *Termitomyces* crop from extreme fluctuations of the outside environment.

The extremely high CO_2 concentration found for the *O. obesus* lenticular mounds in the present study is comparable to other studies, e.g., 2.8% (Lüscher, 1961) and 1.2% (Korb and Linsenmair, 2000) CO_2 in *M. bellicosus* mounds and 5.2% CO_2 in other Macrotermitinae mounds

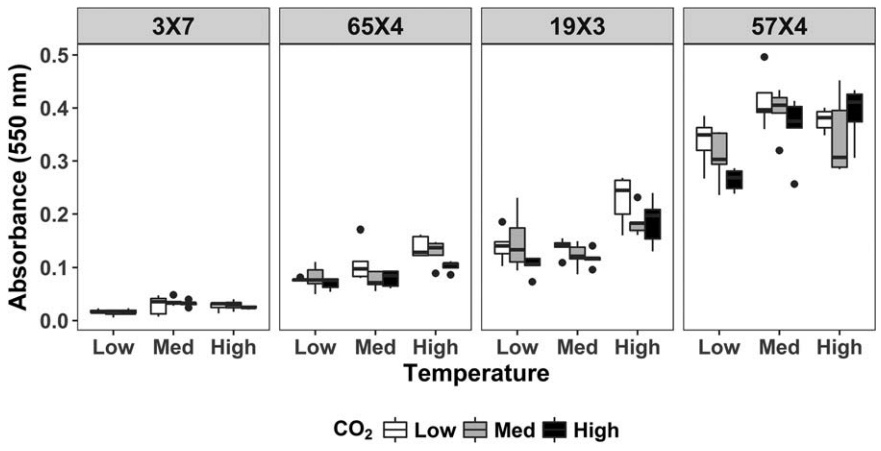

Fig. 3. Box plots of the growth pattern of four different *Pseudoxylaria* isolates (3X7, 65X4, 19X3 and 57X4) under different combinations of temperature (High, Medium and Low) and CO_2 concentration (High, Medium and Low). Dots represent outliers. $N = 5$ where each value is a mean of 5–8 technical replicates.

(Matsumoto, 1978). The high inter-mound variation as indicated by low correlations in between-mound comparisons in our study was also observed by Korb and Linsenmair (1999). This may be due to differences in termite colony size since larger colonies with a net higher respiration rate (of termites and fungi) may have higher CO_2 values compared to smaller colonies. Also, the highest CO_2 levels were recorded in the monsoon at a time when the termite colony activity may be at its peak. The only other study on *O. obesus* mounds (King *et al*., 2015) reported ~1–6% CO_2 concentration inside the mound, but this was for a cathedral, savanna-type mound. The very high CO_2 may play a role in controlling the growth of the parasitic fungus as suggested by Batra and Batra (1966). However, in a recent study on another fungus-growing insect, i.e., leaf-cutter ants, workers were found to avoid high CO_2 concentrations but chose intermediate levels when selecting places for fungus-rearing, probably because of the detrimental effect of high CO_2 on the fungi (Römer *et al*., 2017). But the termite mutualistic fungus *Termitomyces* is clearly able to grow even under such hypercarbic conditions.

Contrary to our expectation, in the absence of termite access, the growth of *Pseudoxylaria* was greater *in situ* than when placed outside the mound. Since the tested isolates were representatives of the two extremes of *Pseudoxylaria* growth rates, i.e., slowest and fastest, and these seasons represent extremes of microclimatic conditions, i.e., high to low temperature, this may mean that, in general, *Pseudoxylaria* grows better inside the mound than outside. This could be because of the moist and warmer interiors along with stable temperatures and RH inside the mound which may allow *Pseudoxylaria* to grow better than with the fluctuating temperatures and RH outside. These results indicate that the parasitic fungus is adapted to grow inside the mound (termitarium) microclimate and is thus a 'termitariophilic' parasite. These results may contribute to the mechanistic basis of *Pseudoxylaria* affinity for termite mounds. It has also been suggested that environmental factors such as temperature, soil humidity, CO_2 concentration, pH and volatiles are not the major reason for the exclusive growth of *Termitomyces* and inhibition of other fungi within the termite mound (Shinzato *et al*., 2005). However, our study is the first to examine the comparative growth of parasitic *Pseudoxylaria* fungi inside and outside the mound.

Since the growth of *Pseudoxylaria* in the winter was lower compared to the summer, the mound environment may still be able to exert a negative effect on its growth under certain conditions. With *ex situ* incubations performed for the first time to compare the growth of different isolates, we found a significant effect of CO_2 concentration on *Pseudoxylaria* growth and a significant interaction between isolate identity and temperature. But in general, *Pseudoxylaria* growth was at a minimum at high CO_2 levels and low temperatures. When we examined the dynamic patterns of temperature and CO_2 within the mound we did not find high CO_2 levels co-occurring with low temperatures but the former peaks during the monsoon while the latter dips during winters. Therefore, these environmental conditions may exert their growth-retarding effects during different seasons. These results highlight the dynamic nature of parasite growth suppression by the changing mound environment and expand the breadth of the role of the extended phenotype of the termite mounds.

Even though Batra and Batra (1966) reported inhibition of [*Pseudo*]*xylaria*-like fungi under very high CO_2 concentration, we only found a decrease in growth but no complete inhibition. But if the *Pseudoxylaria* used by these authors was a slower-growing isolate, such as 3X7 (Fig. 3), then the effect of CO_2 will erroneously manifest as inhibition, owing to the already slow growth of the isolate. This highlights the importance of using isolates with differing growth rates. Many soil-borne organisms also face hypoxic conditions, e.g., after heavy rains resulting in increased CO_2 levels. However, soil-borne fungi such as *Fusarium* seem to be adapted to grow or survive under such hypoxic conditions consistent with their resident ecological niche (Hollis, 1948; Gunner and Alexander, 1964). The parasitic *Pseudoxylaria* fungi seem to show similar characteristics as their growth is not inhibited but only decreased at higher CO_2 levels. At the same time, the lack of a significant interaction between CO_2 and isolate identity indicates that mound CO_2 concentrations affect faster- and slower-growing isolates equally, and therefore the relative success of different isolates will be dictated by other factors such as growth rate. Interestingly, the growth of entomopathogenic and soil-dwelling fungi such as *Metarhizium anisopliae* (Ouedraogo *et al*., 1997; Brooks *et al*., 2004) and *Beauveria bassiana* (Fargues *et al*., 1997) show a significant interaction between temperature and isolate identity, similar to *Pseudoxylaria* fungi (this study). However, the similarity in the growth patterns of the isolates with medium growth rates (65X4 and 19X3) compared to the fastest (57X4) or slowest growing ones (3X7) (Supporting Information Fig. S1) is interesting and it appears that being placed in the middle of the growth axis may be the best parasite strategy as evidenced by the fact that both 65X4 and 19X3 belong to the most prevalent genotype (G 7) sampled in southern India as compared to 3X7 and 57X4 which belong to rare genotypes (G 5 and G 2 respectively) (Katariya *et al*., 2017a). Probably, being too slow may mean losing out in competition with other isolates and being too fast may mean quick detection (Katariya *et al*., 2017b) and subsequent removal by termites. However, under lower temperature conditions, the growth of all *Pseudoxylaria* isolates was depressed as has been recorded for many fungi (Trinci, 1969; Miętkiewski *et al*., 1994; Davidson *et al*., 2003).

Taken together, these results indicate that not all mound microclimatic conditions are conducive for *Pseudoxylaria* growth, even though the parasitic fungus appears termitariophilic. Thus the mound, which is the extended phenotype of the termites, may serve to modulate fungal parasite growth. However, mound microclimate may contribute only partially to decreasing *Pseudoxylaria* growth, while other mechanisms such as antifungal compounds applied by termites are likely responsible for its complete inhibition in healthy nests (Visser *et al*., 2012; Um *et al*., 2013; Kim *et al*., 2014; Beemelmanns *et al*., 2017; Wyche *et al*., 2017). The parasite, if not controlled, would overtake the fungus farm since it grows faster than the mutualistic *Termitomyces* (Visser *et al*., 2011; L. Katariya, pers. obs.). Therefore, it will be interesting to compare the degree of growth inhibition of parasitic fungal isolates, which have different growth rates even under mound conditions, by the antifungal compounds discovered in the fungus-farming termite system. Even though *Termitomyces* is found growing inside the mound throughout the year, a comparative growth study between *Pseudoxylaria* and *Termitomyces* under mound conditions will further unravel the role and importance of mound conditions as an anti-parasite factor.

Experimental procedures

Mound microclimate characterisation

Temperature, RH and CO_2 were recorded in three different lenticular mounds (also called dome-shaped or gallery-forest type mounds; (Korb, 2003)) of the fungus-growing termite *O. obesus*. These mounds (labelled as I10, I30 and I32) were located in the campus of Indian Institute of Science (IISc), Bangalore, India (13°01′20.7″N 77°34′01.3″E). Air temperature and RH were recorded using USB-502+ data loggers (Measurement Computing Corporation, USA) for ~24 h once every month for a period of 1 year; readings were automatically logged at 30 s intervals. Carbon dioxide concentration was measured using a LI-820 CO_2 analyzer (LI-COR Biosciences, USA). For mound I10, readings were taken four times a day (corresponding to morning, afternoon, evening and night). However, for mounds I30 and I32, these readings were obtained only twice a day (corresponding to day and night). Recordings were taken for 1 day every month for 1 year. Every month, before data collection, the CO_2 analyzer was calibrated with a known CO_2 standard (~1% CO_2). A gap of around 6 days was maintained between CO_2 and temperature-RH measurements with the former recorded before the latter. Breaches in mounds disturb termite colonies and only intermittent breaches could be made. See Supporting Information *Experimental Procedures* for details.

Pseudoxylaria *growth: effect of mound microclimate*

Categorising Pseudoxylaria *isolates as slower or faster growers.* Fungus combs were collected from 29 different *O. obesus* mounds present in different localities of Bangalore (India) (Katariya *et al*., 2017a). *Pseudoxylaria* was isolated from these combs and cultured on potato dextrose agar (PDA) at 30°C. Eight of these isolates were chosen which visually showed different rates of growth. To confirm the relative growth rates of different isolates, quantitative colorimetric growth 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 in a 48-well plate. This experiment was repeated to give five biological replicates with the relative position of isolates in the 48-well plate randomised in every experiment. See Supporting Information *Experimental Procedures* for details.

Pseudoxylaria in situ *growth experiments within termite mounds.* From the selected eight isolates, we used two isolates which differed in their growth levels (3.6 fold difference) – a relatively slow isolate (65X4) and a relatively fast isolate (57X3) (Supporting Information Fig. S2a). These isolates were incubated inside sterile B50 tubes (TubeSpin® Bioreactor 50, TPP, Trasadingen, Switzerland) which had five openings fitted with a membrane filter for sterile exchange of gases. After one day of *in situ* incubation, the level of fungal growth was measured using the MTT colorimetric assay as described earlier. These experiments were repeated to give at least five replicates such that between each experiment there was a gap of at least 5 days. Experiments were performed in the hot (April–June, 2015) and cold season (October–December, 2015) in five lenticular mounds of *O. obesus* (labelled as I30, I32, I35, I36 and I37) located in IISc. See Supporting Information *Experimental Procedures* for details.

Pseudoxylaria *growth: effect of temperature and CO_2*

Categorising Pseudoxylaria *isolates as slower or faster growers.* Similar procedures as earlier were performed with eight different isolates of *Pseudoxylaria* and four isolates with differing growth levels were chosen for further experiments.

Pseudoxylaria ex situ *growth experiments.* Similar culture preparation procedures as earlier were used with some exceptions. Only four isolates were inoculated (number of technical replicates = 5–8/isolate) in a 48-well plate and one un-inoculated row served as a blank. These isolates were (in order of their relative growth rate): slowest 3X7, slow 65X4, fast 19X3 and fastest 57X4 (Supporting Information Fig. S2b). The 48-well plate was incubated at a variety of temperature and CO_2 combinations for 24 h, after which the MTT colorimetric assays were performed to quantify growth. We used the maximum, minimum and mean values obtained from the three mounds to set high (31.7–32.2°C, 9.2%), low (22°C, 0.4%) and medium (25.4–27.6°C, 3.2%) levels for temperature and CO_2 for these experiments which were all performed at 95% RH (Supporting Information Table S1). For each combination, there were five biological replicates. The relative positions of four isolates and one blank in the 48-well plate were randomly changed in every experiment. We randomised the sequence of CO_2 levels used on different days but could not do so completely for temperature because it was difficult to maintain stable temperatures in the CO_2 incubator (Thermo Scientific Steri-Cult Model 3307; non-cooling model) especially at low and medium temperature settings.

Data processing and statistical analysis

All analyses were performed in Rstudio 0.99.902 (RStudio Team, 2016), user interface for R 3.2.3 (R Core Team, 2015) using the ggplot package (Wickham, 2009) to produce figures.

Air temperature, RH and CO_2 concentration. For temperature and RH, we used data recorded from 4 h after placing the data logger inside the mound (for parameter stabilisation after the breach) to 1 h before removing it from the mound, for calculating various parameters such as mean, SD, minimum and maximum values such that we had one data point per month per mound for these parameters. Because of the technical failure of the outside mound data logger of I30, temperature and RH readings for November are not available. Similarly, for I32, temperature and RH readings for March and April had to be substituted with the outside mound readings for I30. Again, calculations for outside mound temperature and RH for August for I10 are based on few hours of readings because of technical issues with the data logger.

For CO_2, we first corrected all the recorded readings above 20,000 ppm (Supporting Information Fig. S3, Supporting Information *Experimental Procedures*). Only one mound reading (June for I32; $>$ 10% CO_2) was removed from the analysis. After correction, we calculated the mean of 10 min recordings such that there were four values for each month for mound I10 (since readings were taken four times a day) and two values for each month for each of the mounds – I30 and I32. We used the mean of each day for data analysis.

We used a generalised least squares (GLS) model to test for statistically significant correlations between the inside and outside of mounds with correlation structure (corAR1, autoregressive process of order 1) to account for autocorrelation in data. GLS analysis was carried out using the function gls in the nlme package (Pinheiro *et al.*, 2016).

Pseudoxylaria in situ growth experiments within termite mounds. We calculated the mean of absorbance (in the MTT growth assays) of three technical replicates from each experiment (total five/six biological replicates) such that we had five/six values for each of the two isolates for each mound in each season (except for 57X3 incubation in I37 during the winter where $N = 2$). We used a linear mixed-model (LMM) separately for the two isolates to examine the effect of position (Inside Mound/Outside Mound), season (Hot/Cold) and their interaction (all as fixed effects) on *Pseudoxylaria* growth (mean absorbance). Mound identity was used as a random effect to account for repeated measures on the same mound. Pairwise comparisons between inside and outside data for each daily experiment were made and nested within each mound as random effects. We used the nlme package (Pinheiro *et al.*, 2016) for this analysis. We carried out cautious model reduction and arrived at the final model by stepwise removal of only non-significant ($P > 0.05$) interaction terms from the maximum model to aid in interpreting relationships. In this way, our inferences were based on a final model that included all the main effects (whether significant or not) and statistically significant ($P < 0.05$) interactions.

Pseudoxylaria ex situ growth experiments. We calculated the mean of absorbance (in the MTT growth assays) of five to eight technical replicates from each experiment (total five biological replicates) such that we had five values for each isolate incubated under a particular combination of temperature and CO_2. To test for the effect of temperature and CO_2 on *Pseudoxylaria* growth, we ran an ANOVA model with amount of growth, i.e., mean absorbance, as the response variable and temperature (high, medium and low), CO_2 (high, medium and low) and isolate identity (3X7, 65X4, 19X3, 57X4) as predictor variables. For comparison, we used high CO_2 concentration and low temperature as the baseline because *in situ* experiments indicated that these conditions may hamper *Pseudoxylaria* growth. Also, isolate 19X3 was used as the baseline because its mean growth (mean absorbance) was similar to the mean growth (mean absorbance) of all isolates taken together. We included all possible interactions in our maximum model and carried out cautious model reduction as explained earlier. We used cube-root transformation of the response variable to meet assumptions of normality and linearity.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Interaction between temperature (high, medium and low) and *Pseudoxylaria* isolate identity (3X7, 65X4, 19X3, and 57X3). Significant pairwise differences between growths at different temperatures of the same isolate are shown with different alphabets (Tukey HSD; $P < 0.05$). Data are adjusted means ± SEM of $N = 15$ in each group.

Fig. S2. Box plots showing comparative growth of 8 different *Pseudoxylaria* isolates ($N = 5$).
a. Isolates 65X4 and 57X4 were used for *in situ* growth experiments.
b. Isolates 3X7, 65X4, 19X3 and 57X3 were used for *ex situ* growth experiments. The mean absorbance of six pseudo-replicates from each experiment (5 biological replicates in total) was calculated to result in a total of five values for each of the eight isolates.

Fig. S3. Standard curve of CO_2 obtained with known concentrations of 10 standards (20,200, 25,200, 30,100, 40,300, 50,000, 59,300, 67,979, 80,772, 87,958 and 99,053 ppm).

TableS1. Summary of temperature, relative humidity (RH) and CO_2 concentration of three mounds (inside and outside) of the fungus-growing termite *Odontotermes obesus* for one year. $N = 12$ in all cases but $N = 11$ for l30 (temperature and RH only) and l32 (CO_2 only).

Table S2. Linear mixed-model (LMM) for *in situ* experiments for the slow-growing isolate 65X4 (a) and fast growing isolate 57X3 (b).