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Germination Pattern and Inoculum Transfer of Entomopathogenic Fungi and their Role in Disease Resistance among *Coptotermes formosanus* (Isoptera: Rhinotermitidae)

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Abstract

Laboratory bioassays were designed on the strains of *Metarhizium anisopliae* and *Beauveria bassiana* to check the feasibility of using "trap and treat" method for controlling *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). Different percentages of exposed *C. formosanus* workers 100%, 50%, 25% and 1% were then allowed to mingle with unexposed termites 0%, 50%, 75% and 99%, respectively. The mortality of combined group suggested that as the number of exposed individuals decreased, capacity of the workers to resist disease through mutual grooming was increased. Lower percentage of exposed individuals (1%), could not transfer inoculum resulting very low mortality (<3%) in any studied fungus. The strains of *M. anisopliae* were better than *B. bassiana* in producing epizootics in the experimental units because these strains produced more directly penetrating appressoria (62-67%), compared to *B. bassiana* (43-52%). These directly penetrating appressoria might be related to the virulence of the strains. We conclude that grooming ability to protect the termites from fungi is only workable when lower percentages of individuals are exposed. The workers may play pivotal role in the failure of disease epizootics by "trap and treat" method in the field through allogrooming because a colony of *C. formosanus* comprised of millions of individuals and little inoculum can not be spread among the nest-mates, resulting failure of disease transmission in the field.

Keywords: Appressorium; Allogrooming; Defense; Entomopathogenic fungi; Termites; Virulence

Introduction

Subterranean termites (Rhinotermitidae: Isoptera) construct underground nests. They forage on and below the soil surface in order to find cellulose. A single colony of *Coptotermes formosanus* Shiraki may contain several millions of termites that forage up to hundred meters in soil (King and Spink, 1969). The principal component of the termite's diet is wood, a dominant structural element of the buildings and construction industry of human society. When their needs conflict with human needs and interests, they are treated as "pests" (Pearce, 1997). Because of their colony size and foraging range, termite colonies have become serious threat to the crops and structures (Ahmed *et al*., 2006; Ahmed *et al*., 2007).

Natural enemies especially entomopathogenic fungi have long been studied for exploitation in biological control of *Coptotermes* spp. (Altson, 1947; Wright *et al.*, 2005; Yanagawa and Shimizu, 2007; Yanagawa *et al*., 2008, 2009; Ahmed *et al.*, 2009; Hussain *et al* 2010a, b; Hussain *et al.*, 2011a), in the laboratory. Most of the work focused on the possible transmission of infectious diseases in termite colonies. The authors interpreted that frequent body to body contact, an activity which facilitates the transmission of infectious diseases among healthy termite (allogrooming) (Grace and Zoberi, 1992; Wright *et al.*, 2002), increase the chances of the success of fungal epizootics in the nest. In contrast, Shimizu and Yamaji (2003); Yanagawa *et al.* (2009) and Chouvenc *et al.* (2008), demonstrated that allogrooming effectively protect the termites from fungal infection. Furthermore, they illustrated that > 90% fungal spores of *M. anisopliae* on cuticle of termites were removed by allogrooming. However, they could not find significant reduction in the spores associated with the individually reared *C. formosanus* Shiraki. In another study, Yanagawa *et al*. (2008) suggested that the termites removed the spores of *Isaria fumosoroseus* and *B. brongniartii* from their cuticles rapidly compared to *M. anisopliae*. As a result of that *M. anisopliae* spores ultimately led to their mortality.

Disease resistance in termites is socially facilitated through allogrooming, otherwise little inoculum might be spread throughout a termite nest before being detected by the insects, resulting in an epizootic (Myles, 2002; Yanagawa *et al.*, 2008). The underlying mechanism increasing disease resistance in grouped termites seems to involve the allogrooming, but the causal link between allogrooming and fungal infection has yet to be established.

The use of soil-dwelling entomopathogenic fungi is

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technically challenging due to the lifestyle and behavior of termites. It is extremely difficult to detect the termites until the sudden damage appeared. Therefore, it is not feasible to directly treat the whole colony that might comprise of millions of individuals (Su and Scheffrahn, 1998). There is an alternative trap-and-treat method, whereby the directly dusted foragers with spores were introduced into the same colony (Milner, 2001).

Here we describe the dispersion of the inoculum of *M. anisopliae* and *B. bassiana* through allogrooming among the workers, when different percentages of exposed workers allow to intermingle with the healthy individuals to determine whether allogrooming among nest-mates accelerate or impede disease transmission. Furthermore, an attempt was made to demonstrate experimentally for the first time the relationship between the virulence and directly penetrating spores of the studied strains of entomopathogenic fungi. Findings on the transmission of fungal inoculum with different germination patterns are discussed with the emphasis of controlling *C. formosanus* by "trap and treat" method in the field.

Materials and Methods

Termites

Termite traps consisted of moistened Pine wood stacks wrapped in plastic boxes buried in the ground, at Huolu Shan Forest Park, a vicinity of Guangzhou City, about 3.5 km away from the SCAU campus (South China Agricultural University), China. Traps were checked fortnightly. Trapped termites were brought to the laboratory, provided fresh moistened pine wood stacks, and held at 27±0.5ºC, in complete darkness.

Entomopathogenic Fungi

Two strains, each of *Beauveria bassiana* and *Metarhizium anisopliae* were obtained from different sources. *B. bassiana* strain EBCL 03005 was isolated from *C. formosanus* Shiraki in China and strain 200436 isolated from soil was obtained from the Department of Plant Pathology, SCAU. *M. anisopliae* strain EBCL 02049 was isolated from *C. formosanus*, while strain 406 was isolated from soil and was obtained from Department of Entomology, SCAU. All the strains were grown on potato dextrose agar. Following inoculation, the fungi were maintained in complete darkness at 25±0.5ºC.

Preparation of Fungal Spore Suspensions

The method used to prepare the spore suspensions of 24-days-old cultures at a concentration of 1×10^7 spores/mL is described in detail by Hussain *et al.* (2009). Viability of the spores was determined before each bioassay as described in our previous research (Hussain *et al.*, 2010c). The average percent viability ranged 93-99% for all the tests.

Effect of Termite Density of Exposed Individuals on the Susceptibility of Unexposed Workers

Coptotermes formosanus workers were immersed in spore suspensions in micro-centrifuge tubes. The exposed individuals were dried on filter paper (Whatman no. 1). Virulence among the workers as a result of inoculum transfer through allogrooming was sorted by providing healthy workers with different percentages of exposed workers. The ratios of the number of exposed and healthy individuals were 99:1 (1%), 25:75 (25%), 50:50 (50%) and 100:0 (100%). Each experimental unit contained 100 termite individuals and was maintained in a Petri dish (95 × 15 mm), containing dampened filter paper. Petri dishes were incubated in the dark chamber (27 ± 0.5ºC and 85 ± 5% RH). Termites treated with 0.05% aqueous solution of Tween 80, used as control. Mortality was recorded after every 24 h. Four replicates (actual replicates), each representing a different colony, were completed in this way. There were three replicates per colony (pseudo replicates). Dead termites at the end of experiment were removed and incubated till sporulation in order to confirm that termites died because of the used fungi. Mortality data were normalized by arcsine-transformed before subjecting them to analyses of variance. Tukey's test ($P < 0.05$) after one-way ANOVA was used for mean comparison (SAS Institute, 2000). LT_{50} (lethal time required to kill 50% of the treated insect population) values were only determined for the bioassay in which all the workers were exposed using probit analysis. Tukey's test ($P<0.05$) after one-way ANOVA was used for mean comparison (SAS Institute, 2000).

Scanning Electron Microscope (SEM)

Termite workers exposed with different entomopathogenic fungi (different time intervals), were fixed overnight with 2.5% (v/v) glutaraldehyde at 4ºC. All the specimens were washed thrice (10 min each) in 0.1 M phosphate buffer (pH 7). Specimens were then fixed in 1% OsO_4 for 1 h. After three rinses in 7 pH phosphate buffer (0.1M), samples were dehydrated through 50, 70-100% ethyl alcohol (10 min per step) while two changes in 100% ethyl alcohol at 25ºC. Both, the fixation and dehydration were performed in 2 mL plastic eppendorff tubes. Specimens were then dried by critical-point drying apparatus under CO_2. The termite workers were mounted on the stubs. The coating was done by using gold palladium. The samples were observed at 10 kV by XL-30 Environmental Scanning Electron Microscope (Netherlands).

Germination on Cuticle

In vivo germination and directly penetrating appressoria were observed using scanning electron microscope. To quantify germination and directly penetrating appressoria from *C. formosanus* worker's abdominal cuticle, the numbers of spores from third, fourth and fifth abdominal

segments were counted. A minimum of 100 spores were counted from each abdominal segment. Counts were made after 36 h post-inoculation. Eight insects, two from each colony were used to calculate germination and directly penetrating appressoria. Each treatment (fungal strain) was replicated seven times. Tukey's test ($P < 0.05$) after one-way ANOVA was used for mean comparison (SAS Institute, 2000). Correlation procedure was used to find possible relationship between LT_{50}, *in vivo* germination and directly penetrating appressoria (SAS Institute, 2000).

Results

Termite Workers Susceptibility to Fungal Infection

Mortality of the termites in which all the workers were treated with entomopathogenic fungi differed significantly only after 4 days (P<0.001). Although, there was non significant difference among fungi treatments after eight, twelve and sixteen days post-exposure, but they were all significantly higher than control (Fig. 1). After 4 d, the strains of *M. anisopliae* led to higher mortality (>75%) of termites compared to those of *B. bassiana* strains (<40%) (Fig. 1). Within the 16 d of experimentation, termites treated with Tween 80 showed only < 2% average mortality.

Effect of Population Density of 50% Exposed Individuals on the Susceptibility of 50% Unexposed Workers

The transfer of fungal inoculum among the healthy workers resist the infection in the experimental units of *B. bassiana* strains, resulting <20% mortality, within the first 4 d of inoculation (Fig. 2). Comparatively, workers exposed with *M. anisopliae* (2049), resulted in fungal transmission among the healthy termites and caused the highest mortality (>75%). Significant differences among the fungal strains after 4 d (P <0.001) and 8 d (P<0.001), post inoculation were observed among the experimental units treated with different fungi. There was no significant difference among fungi treatments after 12 and 16 days post-exposure, but they were all significantly higher than control (Fig. 2).

Effect of Population Density of 25% Exposed Individuals on the Susceptibility of 75% Unexposed Workers

Mortality data of *C. formosanus* workers indicated significant differences among the studied fungi after 4 d (P<0.001), 8 d (P<0.001) and 12 d (P<0.001), post inoculation. Contrarily, there was no significant difference among fungi treatments after 16 and 20 days post-exposure, but they were all significantly higher than control (Fig. 3). Within the first 4 d, 25% exposed workers in any fungus, could not transmit fungal infection among the healthy workers. At that stage<20% mortality was observed. Fungal cultures of *B. bassiana* strains caused negligible mortality, remained significantly similar with each other (Fig. 3). Both the strains of *M. anisopliae* caused 100% mortality in 16 days, while *B. bassiana* strain 200436 could not impart

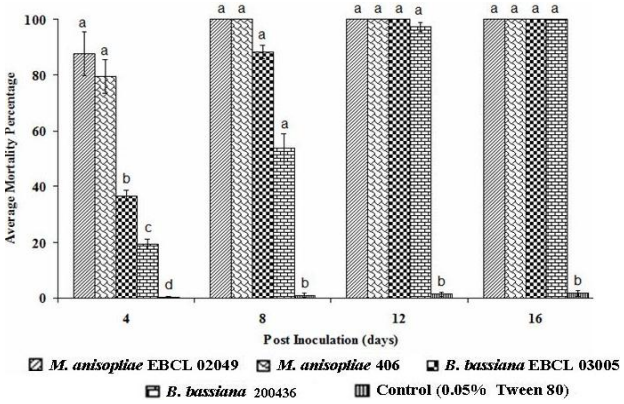

Fig. 1: Mortality of *Coptotermes formosanus* workers in the experimental units in which all the workers exposed with entomopathogenic fungi. Bars with the same letter(s) along different strains are not significantly different. (Tukey's test, p<0.05)

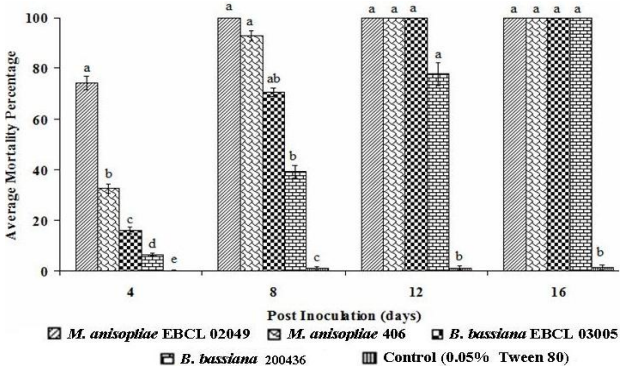

Fig. 2: Mortality of *Coptotermes formosanus* workers in the experimental units in which 50% workers exposed with entomopathogenic fungi. Bars with the same letter(s) along differentstrainsdiffernon-significantly(Tukey's test, p>0.05)

100% mortality during 20 days of experimentation.

Effect of Population Density of 1% Exposed Individuals on the Susceptibility of 99% Unexposed Workers

Coptotermes formosanus workers treated with entomopathogenic fungi were not able to transfer inoculum (Fig. 4). Non-significant differences among all the treatments were observed after 4 d (P>0.01), 8 d (P>0.01), 12 d (P>0.371) and 16 d (P>0.304), post inoculation. On the whole, less than 3% mortality was recorded in all the treatments indicated that 1% exposed individuals were not enough to cause fungal infection among the healthy workers.

Correlation between Virulence and *In vivo* Spore Germination and Penetration Pattern of Appressoria

There was a significant difference in directly penetrating appressoria depending on the fungal strain (F = 10.13; P<0.001). Directly penetrating appressoria were significantly higher among worker's cuticle immersed in *M.*

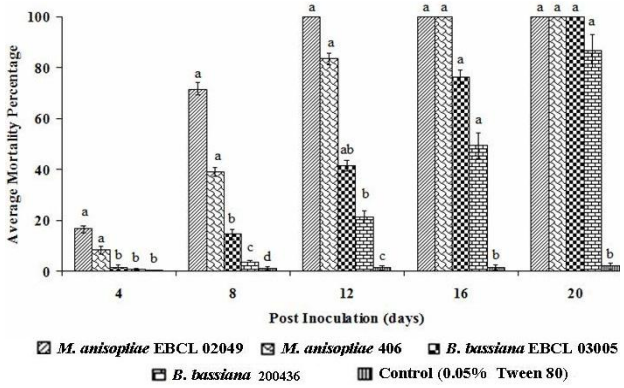

Fig. 3: Mortality of *Coptotermes formosanus* workers in the experimental units in which 25% workers exposed with entomopathogenic fungi. Bars with the same letter(s) along different strains are not significantly different. (Tukey's test, $p<0.05$)

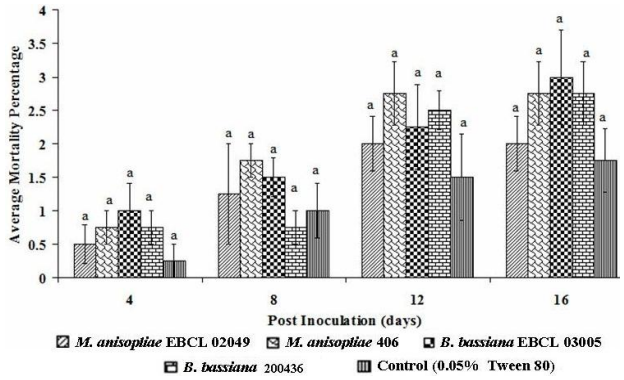

Fig. 4: Mortality of *Coptotermes formosanus* workers in the experimental units in which 1% workers exposed with entomopathogenic fungi. Bars with the same letter(s) along different strains are not significantly different. (Tukey's test, $p<0.05$)

anisopliae strains suspension compared to *B. bassiana* strains (Table 1). Furthermore, these spores showed lower LT_{50} values and significant differences were observed among the studied strains ($P<0.001$). However, spores on the cuticle of termites showed > 84% germination ($P<0.05$). Correlation analysis between LT_{50} and penetrating spores revealed a strong negative correlation between them (r = –0.978), positive correlation was observed between *in vivo* germinating spores and directly penetrating spores (r=0.834). A negative correlation effect (r = –0.865) was also observed between *in vivo* germinating spores and LT_{50} values. The highly virulent fungal strains of *M. anisopliae* had more directly penetrating appressoria; while, the strains of *B. bassiana* along with higher LT_{50} values showed lower percentages of directly penetrating appressoria (Table 1).

Discussion

The results demonstrated that the efficiency of social behavior for instance allogrooming responsible for protecting termites from fungal infection largely depends upon the percent infected individuals in the colony as well as the penetration pattern of the spores of fungal strains. As a result of that the efficacy of entomopathogenic fungi against *C. formosanus* workers varied greatly among the bioassays in which 100%, 50%, 25% and 1% exposed workers were allowed to transfer inoculum among the healthy nest mates. The spores of *M. anisopliae* were more pathogenic than those of *B. bassiana* and caused 100% mortality (<8 days) before *B. bassiana*, which took relatively more time (>12 days) to cause 100% mortality among termites in which all the *C. formosanus* workers were exposed with spore suspensions (Fig. 1). Differences in mortality among the infected individuals suggest that the strains of *M. anisopliae* kill termite workers faster. The superiority of *M. anisopliae* among the studied entomopathogenic fungi towards *C. formosanus* is consistent with previous work reported by Delate *et al.* (1995) and Hussain *et al.* (2010a, b). The mechanism underlying of faster-action *M. anisopliae* strains against *C. formosanus* workers seems to be the result of penetration pattern of the entomopathogenic fungi in order to invade the host effectively. Previously, Neves and Alves (2004) also reported that early penetrating spores are directly related with the virulence of the strain against *Cornitermes cumulans* (Kollar). In the current investigation, *M. anisopliae* showed higher (62-67%) number of directly penetrating structures (Fig. 5a-d), compared to *B. bassiana* (43-52%). These directly penetrating structures "appressoria" as shown in Fig. 5a-d are advantageous to invade termites having high ability to remove spores from the exterior surface of termites by allogrooming (Shimizu and Yamaji, 2003; Yanagawa and Shimizu, 2007). While the strains of *B. bassiana* produced fewer directly penetrating appressoria compared to *M. anisopliae* and most spores form germ tubes (Fig. 6a-d) and cover the exterior surface of the cuticle, which may easily be remove through allogrooming. On the basis of above findings, it may be

Table 1: Virulence determinant traits of entomopathogenic fungi

Fungal strain	*In vivo* spores germination (%)	Directly penetrating spores (%)	LT_{50} (days)
M. anisopliae (EBCL 02049)	93.14 ± 3.00^{ab}	67.14 ± 3.62^{a}	2.43 ± 0.13^{c}
M. anisopliae (406)	97.71 ± 1.25^{a}	62.29 ± 3.19^{ab}	2.57 ± 0.12^{c}
B. bassiana (EBCL 03005)	87.29 ± 2.89^{ab}	51.43 ± 3.58^{bc}	4.46 ± 0.20^{b}
B. bassiana (200436)	86.14 ± 3.36^{b}	43.29 ± 3.08^{c}	6.57 ± 0.25^{a}

EBCL, European Biological Control Laboratory. Mean ± SE values with the same superscript letter(s) within a column are not significantly different (Tukey's test, $p<0.05$)

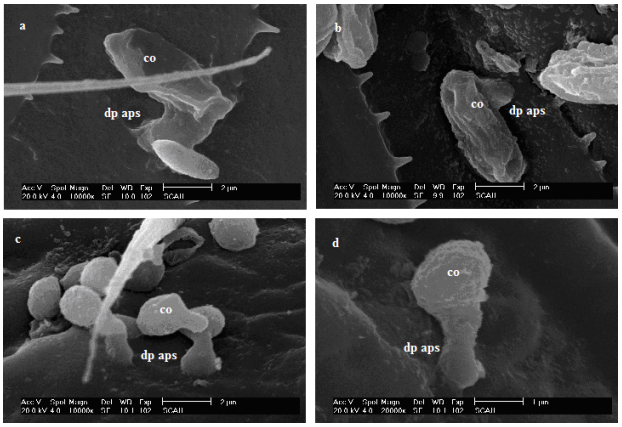

Fig. 5: SEM used for detection of *in vivo* events of directly penetrating spores of *Metarhizium anisopliae* (EBCL 02049) (a), *Metarhizium anisopliae* (406) (b), *Beauveria bassiana* (EBCL 03005) (c) and *Beauveria bassiana* (200436) (d) on the cuticle of *Coptotermes formosanus* workers. Unipolar-germinated conidium (co) directly penetrating appressorium-like structure (dp aps)

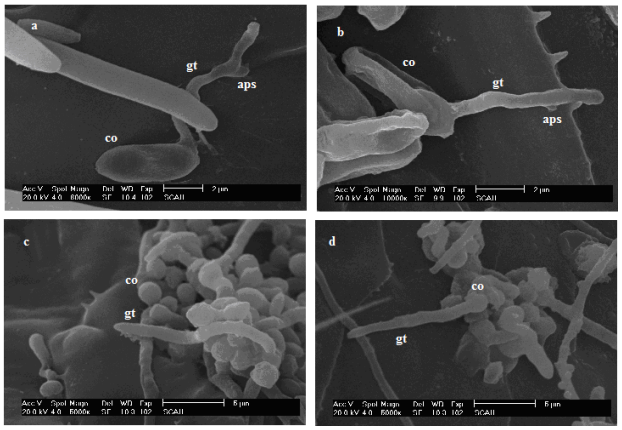

Fig. 6: SEM used for detection of *in vivo* events of germ tube of *Metarhizium anisopliae* (EBCL 02049) (a), *Metarhizium anisopliae* (406) (b), *Beauveria bassiana* (EBCL 03005) (c) and *Beauveria bassiana* (200436) (d) on the cuticle of *Coptotermes formosanus* workers. Unipolar-germinated conidium (co) with a strong germ tube (gt) having an appressorium-like structure (aps)

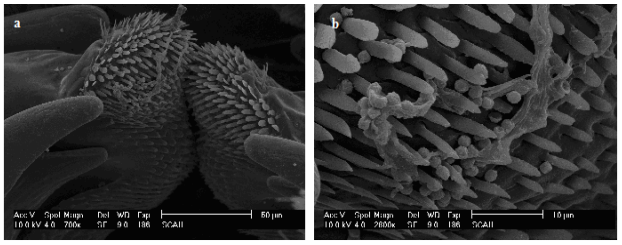

Fig. 7: SEM of Possible grooming structure (a); tooth-like maxillary extremity in detail (b). Little round structures among tooth-like maxillary extremity are spores

speculated that the directly penetrating spores through appressorium enables spore penetration and once the penetration occurs the spore firmly attached with the cuticle and cannot be removed by allogrooming. In termites, grooming is frequently use as cleaning practice, so the penetration of fungal spore through appressoria should be regarded as fungal evolutionary advantage to penetrate through the termite cuticle.

The bioassays in which 50% and 25% workers were exposed with entomopathogenic fungi resulted horizontal transmission among the unexposed workers through oral trophallaxis (mutual exchange of nutrients among colony members), allogrooming and cannibalism of dead or injured nest mates. The rapid transfer of fungal inoculum from infected to healthy termites greatly enhances the chances for inoculum to cause mortality. The results obtained from 50% and 25% of the termites being infected greatly support the idea that social behaviors play pivotal role in spores transfer (Kramm and West, 1982; Robsengaus and Traniello, 1997). In contrast, the treatment in which lower percentages of workers (1%) were exposed to fungi could not able to initiate infection among the healthy workers, which suggest that social behavior play an important role to protect *C. formosanus* workers from infection. The nature of allogrooming behavior greatly depended upon the number of exposed individuals when came into contact with one another. Thus, disease resistance was not observed when the healthy individuals contacted with the high percentages of infected workers. The same results were obtained for all the studied entomopathogenic fungi. The insects provided with 1% exposed workers greatly involved in allogrooming resulting removal of spores through maxillary extremity (Fig. 7a), that is the part of maxilla, on which tooth-like structures are present (Fig. 7b). These tooth-like structures help *C. formosanus* workers to remove spores. This structure has also been reported previously in another termite species (*Cornitermes cumulans* (Kollar) greatly involved in spore removal (Neves and Alves, 2000).

Entomopathogenic fungi are effective against termites in laboratory studies (Kramm *et al.*, 1982; Rosengaus and Traniello, 1997), but in field trials failed to cause infection (Rath, 2000). Although the soils contain fungal spores but till now there is no report regarding the natural termite colony infection. The failure might because of the fact that termite colonies comprised of millions of individuals foraging in underground complex network of galleries. Sometimes, the radius of these galleries encompass >100 m (Tamashiro *et al.*, 1980), which may cause the failure of the microbial control with single application. The failure of trap and treat strategy is because of the low number of infected individuals, because in a survey Su *et al.*, (1993) suggested that, on average, only 2.75% of a field colony was captured with field traps and this number was reduced to 0.8% for larger colonies (1 million termites). By increasing the number of captured individuals and then exposing with spores with higher directly penetrating structures might help to cause field epizootics among the colony members by trap and treat strategy. The findings of Chouvenc *et al.* (2008)

against *Reticulitermes flavipes* also strengthened our findings that lower percentage (6.25%) of infested termites in a foraging arena could not cause epizootics. Furthermore, termite defensive behaviors such as avoidance (Hussain *et al.*, 2010a), pathogen alarm behavior (Myles, 2002) and walling-off (Staples and Milner, 2000) and physiological adaptations (Chouvenc *et al.*, 2009; Hussain *et al.*, 2011b) against fungal treatments also play an important role to resist the infection among the nest mates (Zoberi, 1995). The effect of *C. formosanus* associated materials (galleries and fecal pellets), also playing an important role to inhibit the growth of entomopathogenic fungi (Hussain *et al.*, 2012). Based on these facts, the use of entomopathogenic fungi for termite management seemed unfeasible. The use of entomopathogenic fungi having most of directly penetrating appressoria in early fungal developmental cycle on termites may be an important trait for strain selection, which could be responsible for higher virulence.

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